Bacteriophages and Antibiotic Resistance Genes in Enterococci

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This thesis is dedicated to my family and my teachers

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Glossary and Table of Abbreviations

A

A = Acid

aa = Amino acid

AAC = Aminoglycoside acetyltransferases

AAD = Aminoglycoside adenyltransferases

ABR = Antibiotic resistance

AFLP = Amplified fragment length polymorphism

AGISAR = WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance

AME = Aminoglycoside modifying enzymes

AMP = Ampicillin

AMR = Antimicrobial resistance

APC = Acid-polyamine-organocation family

APH = Aminoglycoside phosphotransferases

APVMA = Australian Pesticides and Veterinary Medicines Authority

ARA, GYL, RAF, SUC, TRE, XYL, MAN, SOR, SBL, MGP = Acid formation in broth containing 1% arabinose, glycerol, raffinose, sucrose, trehalose, xylose, manitol, sorbose, sorbitol and methyl-α-D-glucopyranoside, respectively

ARBAO-II = Antibiotic Resistance in Bacteria of Animal Origin – II Group

ARG = Arginine

AVI = Avilamycin

AVO = Avoparcin

B

BAC = Bacitracin

BAC = Bacterial artificial chromosome

bp = Base pair

C

°C = Degree Celsius

Ca = Calcium

CDC = Centers for Disease Control and Prevention

CGH = Comparative genomic hybridization

CHMP/CVMP = Committee for Human Medicinal Products/Committee for Veterinary Medicinal Products

CIJIG = Commonwealth Interdepartmental JETACAR (Joint Expert Technical Advisory Committee on Antibiotic Resistance) Implementation Group

CIPARS = The Canadian Integrated Program for Antimicrobial Resistance Surveillance

Cl = Chloride

CLSI = The Clinical and Laboratory Standards Institute

CRAB = The National Reference Centre for Resistance to Antibiotics

CRISPR = Clustered, regularly interspaced, short palindromic repeats

CTn = Conjugative transposon

Cu = Copper

D

Da = Dalton

DAFF = Department of Agriculture, Fisheries and Forestry

DANMAP = The Danish Integrated Antimicrobial Resistance Monitoring and Research

DNA = Deoxyribonucleic acid

dsDNA = Double-stranded DNA

dNTP = Deoxyribonucleotide triphosphate

DOP-PCR = Degenerate oligonucleotide primed amplification

E

EAGAR/JETACAR = Expert Advisory Group on Antimicrobial Resistance/the Joint Expert Technical Advisory Committee on Antibiotic Resistance

EARSS = The European Antimicrobial Resistance Surveillance System

EMEA = The European Medicines Agency

ESCMID = European Society of Clinical Microbiology and Infectious Diseases

ESGARS = ESCMID Study Group for Antimicrobial resistance Surveillance

Enterococcus group = *Enterococcus* spp., *Vagococcus* spp. and some *Lactococcus* spp.

Enterococci = *Enterococcus* spp.

F

FLA = Flavophospholipol

G

g = Gram

g = G-force

GEN = Gentamicin

GRE = Gentamicin resistant enterococci

H

HEM = Hemolytic activity on tryptone soy 5% sheep blood agar

HGT = Horizontal gene transfer

HIP = Hippurate hydrolysis

HLAR = High-level resistance to aminoglycosides

HLGR = High-level resistance to gentamicin

HLPR = High-level resistance to penicillin

I

I = Intermediate

ICE = Integrative conjugative element

ICTV = International Committee on Taxonomy of Viruses

ICU = Intensive Care Unit

IMVS = Institute for Medical and Veterinary Science

J

JETACAR = Joint Expert Technical Advisory Committee on Antibiotic Resistance

L

LAB = Lactic acid bacteria

LGT = Lateral gene transfer

LM = Litmus milk

L-PCR = Long range PCR

LTR = Long terminal repeat

L/W = Length to width ratio

M

MARAN = Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands

MCP = Major capsid proteins

Mg = Magnesium

mg = Milligram

MGE = Mobile genetic element

MIC = Minimum inhibitory concentration

mL = Millilitre

μL = Microlitre

MLS_B = Macrolide, lincosamide and streptogramin B

MLST = Multilocus sequence typing

MLVA = Multiple-locus variable number tandem repeat analysis

mM = Millimole

MOT = Motility

M-PCR = Multiplex PCR

MRE = Multi resistant enterococci

MRSA = Methicillin resistant *Staphylococcus aureus*

MTP = Major tail protein

N

N/A = Not announced or applicable

NAR = Narasin

N/D = Not determined or differentiated

NDPSC = National Drugs and Poisons Schedule Committee

NNIS = National Nosocomial Infections Surveillance

NORM/NORM-VET = Norwegian Monitoring Program for Resistance in Microbes

NRA = The National Registration Authority for Agricultural and Veterinary Chemicals

NSF = The National Science Foundation

O

OMP = Outer membrane protein

ORF = Open reading frame

P

PAI = Pathogenicity islands

PBP = penicillin binding protein

PCR = Polymerase chain reaction

PEG = Polyethylene glycol

PFGE = Pulsed field gel electrophoresis

PFP = Polyhedral, filamentous and pleomorphic

PIG = Pigment

pmol = Picomole

PYU = Pyruvate utilization

R

R = Resistant

RAPD = Random amplification of polymorphic DNA

RFLP = Restriction fragment length polymorphism

ROS = Reactive oxygen species

S

S = Sensitive

SBH = Sequencing by hybridization

SBS = Sequencing by synthesis

SCOPE = Surveillance and Control of Pathogens of Epidemiologic Importance

SEM = Scanning electron microscopy

SNP = Single nucleotide polymorphisms

sp. = Species (singular)

S-PCR = Single PCR

spp. = Species (plural)

SVARM/SWEDRES = Swedish Veterinary Antimicrobial Resistance Monitoring

T

TBE = Tris/Borate/EDTA

TE = Transposable elements

TEL = Tolerate to 0.04% tellurite

TEM = Transmission electron microscope

TET = Tetracycline

TIA = Tiamulin

TMP = Tape measure proteins

TSA = Tryptone soy agar

TYL = Tylosin

U

U = Unit

V

V = Variable

V = Volt

VAN = Vancomycin

VIR = Virginiamycin

VRE = Vancomycin resistant enterococci

W

WCH = Women's and Children's Hospital

WGA = Whole genome amplification

WGS = Whole-genome shotgun

WGSA = Whole genome shotgun approach

WHO = World Health Organization

Z

Zn = Zinc

Summary

In recent decades, bacterial antimicrobial resistance has become a significant problem for human and veterinary medicine. One of these bacterial groups, enterococci, is an important group of bacteria which are inherently resistant to some antimicrobial drugs. They can also readily acquire antimicrobial resistance. Furthermore, enterococci have an important role in transferring determinants of antibacterial resistance to members of intestinal microflora. The responsible genes are usually transferred on mobile genetic elements such as plasmids sometimes carried by bacteriophages. The aim of this study is to investigate antibiotic resistance in enterococci and the bacteriophages responsible for transferring resistance genes. This was achieved in four major steps: 1) Assessment of antimicrobial resistance; 2) Assessment of antimicrobial resistance genes; 3) Detection and characterization of bacteriophages; and 4) Determination of the role of bacteriophages in the transfer of resistance genes.

The antimicrobials (ampicillin, avilamycin, avoparcin, bacitracin, gentamicin, flavophospholipol, narasin, tetracycline, tiamulin, tylosin, vancomycin, virginiamycin, copper and zinc) used in this study were chosen due to their importance in animal husbandry and human and veterinary medicine. Of 230 enterococci isolated from pigs (207 isolates including 80 *E. faecalis*, 71 *E. faecium*, 13 *E. casseliflavus*, 21 *E. gallinarum*, 17 *E. hirae/durans*, two *E. hirae*, one undefferintiated, two *E. raffinosus*), chickens (15 isolates including one *E. faecalis* and 14 *E. faecium*) and humans (eight isolates including three *E. faecalis*, three *E. faecium*, one *E. casseliflavus* and one *E. gallinarum*), the vast majority were found sensitive to ampicillin, avilamycin, gentamicin and narasin. Similarly nearly all isolates were resistant to tetracycline and tylosin and intermediately susceptible to copper. However, most of the isolates were sensitive to avoparcin, vancomycin, virginiamycin and zinc but resistant to bacitracin, tiamulin and flavophospholipol.

Most of the isolates contained *erm*B, *tet*L, *tcr*B and *tet*M genes while the spread of other genes among isolates was relatively low. No *tet*O (except one), *rpl*C, *rpl*D, *van*B, *vat*A and *vat*D (except one) genes were found. The results show that antimicrobial resistance is widespread in enterococci as all isolates (except one) were multiple resistant.

Different lytic and lysogenic bacteriophages were found in this study. These bacteriophages were induced from enterococcal isolates or isolated from environmental

samples. Most of the bacteriophages were isometric with single tail while others were polyhedral, filamentous, pleomorphic or droplet in shape. Assessing the transduction activity of bacteriophages, tetracycline (*tet*M) and gentamicin (*ant*2-I) resistance genes were shown to be transferred between the same and different enterococcal species. This study is one of the first reports on transduction in enterococci, especially interspecies transduction. Moreover, non-tailed bacteriophages (e.g. droplet-shaped phages) were isolated for the first time in enterococci of non-human origin. Genome sequencing of a selected bacteriophage showed a significant identity to another enterococcal bacteriophage EFAP-1.

This study has presented a comprehensive study of the genetic pattern of antibiotic resistance in enterococci and has contributed to an understanding of the role of bacteriophages in transferring antimicrobial resistance genes in enterococci. However, antimicrobial resistance in bacteria needs more investigation. Therefore, further research on antimicrobial resistance and bacteriophages in enterococci is recommended.

« Declaration »

This thesis presents work carried out by myself and does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university. To the best of my knowledge, it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, are clearly acknowledged.

Ramin Mazaheri Nezhad Fard

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Chapter 1

Introduction

Enterococci

Enterococcus spp. or enterococci (formerly called streptococci Group D) (Figures 1-1 and 1-2) are predominantly facultatively anaerobic, Gram-positive, coccal form (0.5−1 μM diameter), non-motile (except strains of *E. gallinarum* and *E. casseliflavus*) non-spore-forming bacteria with a low GC content (Merquior et al., 2008; Karmarkar et al., 2004; Portenier et al., 2004; Murray, 1990). This genus was first proposed by Schleifer and Kilpper-Balz in 1984. Enterococci belong to the Division Firmicutes (Gram-positive bacteria), Class Bacillus, Order Lactobacillales and the Family Enterococcaceae (Donadio et al., 2007; Pollacka et al., 2005). The genus consists of 35 species as at 2008 (Table 1-1) (Felis et al., 2009). The genera most closely related to enterococci are *Vagococcus*, *Tetragenococcus* and *Carnobacterium* (Vancanneyt et al., 2001). *Enterococcus* spp. belongs to the group of lactic acid bacteria (LAB), which produce lactic acid as the final metabolic product. This group includes *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Bifidobacterium* (Mathur & Singh, 2005; Moreira et al., 2005). Some of the LAB bacteria such as *L. plantarum*, *L. acidophilus*, *L. casei* and some species of *Bifidobacterium* and *Propionibacterium* are currently used as probiotic food supplements as well as are some *Enterococcus* spp. (Pelicioli Riboldi et al., 2009; Chukeatirote, 2003; Holzapfel et al., 2001). *E. faecium* SF68 has recently been used in mice as a probiotic organism to enhance the immune response to *Giardia intestinalis* (Benyacoub et al., 2005).

Enterococci do not produce catalase, oxidase or gas (Gilmore, 2002). Whether they produce alpha- or beta-hemolysin is dependent on the type of blood and on the bacterial strain. Enterococci form biofilms by expressing *esp* genes located on a pathogenicity island within the genome (Mohamed & Huang, 2007; Ramadhan & Hegedus, 2005). The most prevalent *Enterococcus* spp. are *E. faecalis* and *E. faecium* (Prakash et al., 2005; Ruoff et al., 1990). The term "enterococci" possibly originated with the discovery of the first cocci-shaped bacteria of this group (entero, gut and coccus, spherical organism) (Rakieten & Tiffany, 1938). They are an important group of bacteria in terms of their interaction with humans (Marothi et al., 2005; Murray, 2000). Some strains are used for

the manufacture of foods while others are pathogens (Bhardwaj et al., 2008; Huys et al., 2004; Lukasova & Sustackova, 2003). Although enterococci are not recognised as primary pathogens, they are a frequent cause of a wide variety of infections in humans such as nosocomial and superinfections (Leavis et al., 2006; Antony, 1998). For example, they have been the second most common causative agent of nosocomial infections within the US hospitals (Hidron et al., 2008). These bacteria most commonly infect the urogenital tract, bloodstream, endocardium, abdomen, pelvis, biliary tract, burn wounds and in-dwelling foreign devices (such as intravascular catheters) (Wisell et al., 2008; Patel et al., 2001; Emori & Gaynes, 1993). Less commonly, enterococci can infect the central nervous system, lungs, soft tissues, paranasal sinuses, ears, eyes and periodontal tissue (Iaria et al., 2005; Ostrowsky & Eliopoulos, 1999; Murray, 1990). Enterococci are reported to cause enteritis and food poisoning in experimental animals (Lapointe et al., 2000; Etheridge et al., 1988). Eighty to ninety percent of human enterococcal infections are caused by *E. faecalis* strains while another species, *E. faecium,* causes the remaining 10–15% (Woo et al., 2008; Jett et al., 1994). Other enterococcal species—including *E. avium*, *E. casseliflavus*, *E. cecorum*, *E. columbae*, *E. dispar*, *E. durans*, *E. flavescens*, *E. gallinarum*, *E. hirae*, *E. malodoratus*, *E. mundtii*, *E. pseudoavium*, *E. raffinosus*, *E. saccharolyticus*, *E. seriolicida*, *E. solitaries* and *E. sulfurous*—are infrequent causes of human infections (Sood et al., 2008; Pillar & Gilmore, 2004; Mundy et al., 2000).

Enterococci are ubiquitous and encountered in nearly everything in the human environment. They are commonly found in human and animal alimentary tract, female genital tract, soil, water and foods (Table 1-2) (Koluman et al., 2009; Jett et al., 1994; Swenson et al., 1992). The enterococci are resistant bacteria that grow at 10–45°C (optimum 35°C) or even higher for some species (Ahmad et al., 2002). They can survive in high concentrations of salt and variable pH such as 6.5% NaCl and pH 9.6 (Lukasova & Sustackova, 2003; Paulsen et al., 2003). This genus is a strict fermenter due to the lack of a Kreb's cycle and respiratory chain, except for *E. faecalis* (Ritchey & Seeley, 1976). Enterococci, particularly *E. faecium* and *E. faecalis*, are inherently resistant to many antimicrobials compared to other clinically important bacteria (Tang & Moxon, 2001; Morris et al., 1995) but the reason for this phenomenon is not still clear (Huycke et al., 1998; Murray, 1990). Enterococcal virulence factors include cytolysin (hemolysin), hyaluronidase, proteinase (gelatinase), bacteriocin (enterocin), aggregation substance, sex pheromones, extracellular superoxide, surface protein (Esp) and autoinducing peptide (AIP) of quorum sensing system (Kayaoglu & Ørstavik, 2004; Kayser, 2003; Foulquie

Moreno et al., 2006; Tendolkar et al., 2003). A polysaccharide capsule has been identified in some enterococcal species (Hufnagel et al., 2004). Analysis of the bacterial 16S rRNA sequences has revealed that there is a relationship between *Enterococcus*, *Streptococcus* and *Lactococcus* species (Fisher & Phillips, 2009). Enterococcal isolates can be typed by different molecular techniques such as Amplified Fragment Length Polymorphism (AFLP), Multilocus Sequence Typing (MLST), Multiple-locus Variable Number Tandem Repeat Analysis (MLVA), Pulsed-field Gel Electrophoresis (PFGE), Random Amplification of Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphism (RFLP) (Li et al., 2009; McBride et al., 2009; Ogier & Serror, 2008; Werner et al., 2007). The enterococci are categorized into five major Groups, I to V, based on acid formation in mannitol and sorbose broths and hydrolysis of arginine (Tables 1-3 and 1-4). This categorization is only based on conventional biochemical tests and does not correlate to grouping by 16S rRNA sequencing or other molecular methods. This grouping method could be conveniently used by most diagnostic laboratories. However, *E. faecalis* has characterizations in common with several of the phenotypically determined groups.

Antimicrobials

Since the first experiments by Fleming in 1929, many people have owed their survival to antibiotics (Wheat, 2001). An antibiotic is a substance produced by a microorganism (mostly bacteria and fungi) and inhibits or kills other microorganisms at low concentrations (Yim et al., 2006). A broader drug category, antimicrobials, includes any natural, semi synthetic or synthetic substance that kills or inhibits the growth of microorganisms (Gao & Cranston, 2008). Antibiotics, sulfonamides, fluoroquinolones, some heavy metals and dyes are included in this category. Since the 1940s, antibiotics have been used in animal husbandry, gardening, aquaculture and horticulture besides their treatment purposes (Aminov, 2009; Salyers et al., 2007). In animal husbandry, antibiotics are added to the food of healthy livestock and poultry to promote feed effectiveness and weight gain, namely as growth promoters, production enhancers, digestive enhancers or feed additives (Table 1-5) (Feighner & Dashkevicz, 1987). Antibacterials improve growth performance by unknown mechanisms but several hypotheses have been proposed for this effect as the result of selective loss in microflora following the use of antimicrobials, including: (I) efficiency of food absorption, (II) better protection of nutrients, (III) elimination of pathogens causing subclinical infections, and (IV) reduction of growth-suppressing toxins and metabolites (Feighner & Dashkevicz, 1987). In general, they have

significant effects on the composition of intestinal microflora (Sørum & Sunde, 2001). In addition, some antimicrobials are used in cancer therapy (e.g. mitomycin).

Microorganisms including enterococci are resistant to antibiotics by several mechanisms including alteration of drug target, changes in target accessibility, degradation of drug by enzymes and active expulsion of the drug using efflux pumps (Figure 1-3) (Wright, 2003). In general, resistance of enterococci to antimicrobials can be defined in two ways, inherent/intrinsic and acquired resistance (Marothi et al., 2005; Murray, 1990). Intrinsic resistance is almost always chromosomally mediated. This resistance includes resistance to penicillinase susceptible penicillin (low level), penicillinase-resistant penicillins, cephalosporins, lincosamides, nalidixic acid, low level aminoglycoside resistance and low level clindamycin resistance. Acquired resistance results from either mutation in DNA or acquisition of new DNA. Examples of acquired resistance include resistance to penicillins, high-levels of aminoglycosides (HLAR), vancomycin, chloramphenicol, erythromycin, high-level clindamycin, tetracycline and fluroquinolone (Marothi et al., 2005).

Other antimicrobials, heavy metals, include some essentially toxic chemical elements and their derivate chemical compounds (Antunes et al., 2003). Heavy metals frequently generate strong reactive oxygen species (ROS), directly/indirectly causing gene mutations, damaging cell proteins and competing with essential elements required by the cell. As a result, they are deleterious to mammalian cells and bacteria. However, some heavy metals such as copper and zinc have dual roles, essential for life in low concentrations but toxic in high concentration (Nies, 1999). In order to survive in the wild, bacteria need to develop mechanisms to provide resistance to heavy metals. These mechanisms include the formation and sequestration of heavy metals in complexes, the reduction of a metal to a less toxic species (generally redox chemistry to convert to a less toxic metal-ion species) and direct efflux of the metal ion out of the cell (Altug & Balkis, 2008; Hernandez et al. 1998; Rasmussen & Sorensen, 1998). Heavy metal-resistance genes are found on plasmids in many bacteria (Gupta et al., 2001; Permina et al., 2006). Bacteria develop the heavy metal resistance mostly for their survival, in the environment. Environmental strains can be resistant to heavy metals with or without the presence of heavy metals. Studies have shown that resistance to heavy metals is directly influenced by pollution (Wright et al., 2006; Berg et al., 2005). The correlation between heavy metal levels and heavy metal-resistant strains shows that the latter might occur in heavy metal-polluted areas. This implies that plasmid transfer of resistance can occur in these strains in the natural environment. Alternatively, it is suggested that bacterial heavy-metal resistance is due to

the use of medical/veterinary antibiotics. For example, bacterial antibiotic resistance-encoding plasmids existed in bacteria before the widespread use of antibiotics by humans, but they were co-selected by the therapeutic use of antibiotics, which selected heavy metal resistance from antibiotic resistant strains. These plasmids carry a variety of genes (e.g. heavy metal resistance genes) rather than just those encoding for antibiotic resistance (Van Houdt et al., 2009; Nemergut et al., 2004). Subsequently, the bacteria harboring these plasmids are selected for by the presence of heavy metals in the environment. Therefore, selective pressure imposed by a heavy metal may indirectly select for resistance to antibiotics as well (Stepanauskas et al., 2005, 2006; Aminov & Mackie, 2007). For example, Tn*5385* is a composite transposon including several smaller transposable elements such as Tn*5384* (Bonafede et al., 1997). This transposon encodes resistance to erythromycin, gentamicin and mercuric chloride and it is suggested it has evolved from the cointegration of enterococcal and staphylococcal broad-host-range plasmids. However, it should be stressed that antibiotic and metal resistance genes share many functional characteristics (Baker-Austin et al., 2006). Although most of this resistance is linked to plasmids, some have chromosomal origins (Canton, 2009). However, it is not known why bacterial heavy metal resistance is frequently associated with bacterial antibiotic resistance. The next section provides an explanation and the mechanisms of action and resistance for 14 antimicrobials used in this study. These antimicrobials are briefly described in Tables 1-6 and 1-7.

Ampicillin

Ampicillin (aminobenzylpenicillin) is an intermediately broad spectrum bactericidal antibiotic used in eradicating Gram-positive and Gram-negative bacteria (Ashnagar & Gharib Naseri, 2007). Beta-lactam antibiotics, to which ampicillin belongs, prevent bacterial cell wall construction by interfering with the final stage of peptidoglycan synthesis.

Resistance to ampicillin is due to the production of β-lactamases in many Gram-positive and Gram-negative bacteria but not in enterococci (Dzidic et al., 2008; Poeta et al., 2007). Enterococci, especially strains of *E. faecium* and *E. raffinosus*, are intrinsically resistant to most β-lactam antibiotics because of low affinity penicillin binding proteins (PBPs) (*php*5 gene) which give enterococci high-level resistance to penicillin (HLPR) and ampicillin (MIC $\geq$ 256 μg/mL) (Poeta et al., 2007; Marothi et al., 2005; Jureen et al., 2003; Jacoby,

1996). There is a phenotypic association of ampicillin and vancomycin resistance in some instances (Rice, 2001).

Ampicillin is sometimes administrated orally in poultry for the prophylaxis or treatment of salmonellosis and *Escherichia coli* or *Staphylococcus aureus* septicemia. It is also used in pigs and other animals to treat infections (Barton & Wilkins, 2001).

Avilamycin

Avilamycin is a bactericidal oligosaccharide (orthosomycin) antibiotic (Weitnauer et al., 2004). Avilamycin is mainly active against Gram-positive bacteria such as enterococci, staphylococci and streptococci (Aarestrup & Jensen, 2000). It acts through binding with the 30S subunit (and possibly 50S subunit) of the ribosome and interferes with the polypeptide-synthesizing function by affecting the attachment of aminoacyl-tRNA to the ribosome (Eichhorn et al., 2004; McNicholas et al., 2000).

Resistance to avilamycin is associated with mutations in the L16 50S subunit ribosomal protein in *E. faecalis* and *E. faecium* (Aarestrup & Jensen, 2000). However, another resistance mechanism found in animal enterococci is mediated by a methyltransferase encoded by the *emt* gene, which is located on a plasmid-borne transposable element (Mann et al., 2001).

Avilamycin is approved for nutritional use in livestock in some countries (Lukasova & Sustackova, 2003).

Glycopeptides

Avoparcin

Avoparcin is a glycopeptide antibiotic with a Gram-positive spectrum of bacteriostatic activity. This antibiotic inhibits synthesis of cell wall peptidoglycan by forming bonds with the D-alanyl-D-alanine terminal of muramyl dipeptide (Cooper, 2000; Collignon, 1999).

The mechanism of resistance to avoparcin is similar to vancomycin resistance and is encoded by *van* genes (Lu et al., 2004).

Prior to 1997 (Collignon, 1999), avoparcin had been used in Australian livestock feeds for growth promotion and improved feed efficiency in broiler chickens, growing pigs, calves

and beef cattle (Barton & Wilkins, 2001). Avoparcin was withdrawn from the market in 2000 (APVMA, 2001).

Vancomycin

Vancomycin is a bactericidal antibiotic belonging to the glycopeptides (Wilhelm & Estes, 1999), active against most Gram-positive bacteria, including *Enterococcus*, *Streptococcus*, *Staphylococcus*, *Corynebacterium*, *Clostridium*, *Listeria* and *Bacillus* species (McNamara & Steckelberg, 2005). Vancomycin inhibits the biosynthesis of the major structural cell wall polymer, peptidoglycan. In addition, vancomycin may impair RNA synthesis (Mercier et al., 2002) and alter the permeability of cytoplasmic membranes (Cetinkaya et al., 2000). Vancomycin cannot cross the outer cell membrane of Gram-negative bacteria because of its large size and is therefore not effective against these bacteria, except for some isolates of *Neisseria gonorrhoeae* (Wilhelm & Estes, 1999).

Resistance to glycopeptide antibiotics was described first in enterococci and is preferentially associated with the *E. faecium* related enterococci including *E. hirae* (Leclercq et al., 1988; Uttley et al., 1988). Six types of vancomycin resistance genes have been described in enterococci that are found on MGEs such as plasmids and transposons (Khan et al., 2008). These genes include *van*A, *van*B, *van*C, *van*D, *van*E and *van*G (Domingo et al., 2007; Fraimow et al., 2005; Garnier et al., 2004; Patel et al., 2000). These genes mediate synthesis of abnormal precursors in peptidoglycan of bacterial cell walls, reducing the affinity of vancomycin to bind to this target site (Scheffers & Pinho, 2005; Lowy, 2003). The *van*A and *van*B genes are are the most prevalent and predominant type of vancomycin resistance usually associated with *E. faecalis* and *E. faecium*, whereas *van*C gene is found in *E. gallinarum* and *E. casseliflavus* strains (Berktas et al., 2008). The genes *van*D and *van*E are phenotypically similar to *van*C and are sometimes reported in *E. faecium* and *E. faecalis* isolates respectively (Sood et al., 2008; Werner et al., 2008). The isolation of *van*G gene has only been reported from Australia and Canada (Boyd et al., 2006; McKessar et al., 2000). All *van*G genes have been identified within *E. faecalis* isolates of human origin. Interestingly, some VanA- and VanB-type VRE are vancomycin dependent and require its presence for growth (Sood et al., 2008). These enterococci can grow in the presence of substitute dipeptides. Interestingly Bell et al. (1998) reported *E. faecalis* strains from Australia that were resistant to vancomycin but did not carry any vancomycin resistance genes. This suggests that some unknown genes rather that those described in the literature may encode resistance to vancomycin in bacteria.

Bacitracin

Bacitracin is a narrow spectrum antibiotic, which is bactericidal against Gram-positive bacteria (Butaye et al., 2003). It inhibits the formation of bacterial cell wall peptidoglycan by forming a complex with a pyrophosphate carrier and inhibiting the dephosphorylation reaction required for its regeneration.

Bacitracin resistance has been reported in different bacterial genera such as *Escherichia*, *Bacillus*, *Streptococcus*, *Staphylococcus* and *Enterococcus* (Matos et al., 2009; Tsuda et al., 2002). However the resistance mechanism in enterococci remains largely unclear (Manson et al., 2004a); resistance mediated by a unidirectional pump has recently been reported. Bacitracin resistance genes *bcr* are thought to be regulated by *bcr*R, but no association with MGEs has been demonstrated (Thibodeau et al., 2008; Mathers, 2008). No cross resistance with other antibiotics has been reported for bacitracin (Mathers, 2008).

Bacitracin is used in human medicine and approved as a growth promotion additive in cattle, swine and poultry food in some countries (Barton & Wilkins, 2001; Rossiter et al., 2001; Manson et al., 2004a). Although the clinical use of topical bacitracin in humans is not critical and alternative compounds are available, bacitracin can be used in the eradication of resistant enterococci from the human intestinal flora (Phillips, 1999).

Flavophospholipol (Moenomycin)

Flavophospholipol (synonyms: moenomycin, bambermycin and flavomycin) is a phosphoglycolipid antibiotic (Zehl et al., 2006). It inhibits peptidoglycan synthesis by inhibiting peptidoglycan polymerases through the impairment of transglycolase activities of penicillin-binding proteins (PBPs). Differences in PBPs might explain different susceptibilities to flavophospholipol between *Enterococcus* species (Butaye et al., 2000).

Some species of the *E. gallinarum* group (*E. gallinarum* and *E. casseliflavus*) and most species from the *E. faecium* group (*E. faecium, E. mundtii* and *E. hirae*) show natural resistance to flavophospholipol (Dutta & Devriese, 1982; Butaye et al, 1998; Butaye et al, 2000; van den Bogaard et al., 2002b). Similarly to ionophores and bacitracin, flavophospholipol inhibits the transfer of a wide variety of plasmids belong to different incompatibility groups, containing a variety of resistance determinants *in vitro*. In a recent study, bambermycin has been described as inhibiting the transfer of the plasmids containing vancomycin resistance genes in *E. faecium* (Riedl et al., 2000).

This antibiotic is approved for use in Australia in animal husbandry to increase production efficiency (Barton & Wilkins, 2001; Zehl et al., 2006).

Gentamicin

Gentamicin belongs to aminoglycoside antibiotics (such as gentamicin, streptomycin, kanamycin, tobramycin and amikacin). It binds to the 30S subunit of the ribosome, which results in misreading of the genetic code on the messenger ribonucleic acid (mRNA) template (Zingman et al., 2007). This antibiotic belongs to a narrow spectrum group of bactericidal antibiotics useful in treatment of infections due to aerobic Gram-positive bacteria including staphylococci, Gram-negative bacteria and mycoplasmas, but not against anaerobes and *Rickettsia* (Shakil et al., 2008).

Three mechanisms of resistance to aminoglycosides have been recognized: ribosome alteration, decreased permeability and drug inactivation by modifying enzymes (Wright, 2003; Collard, 1999). The first mechanism involves the changes in the binding sites within the ribosomal proteins (Zaman et al., 2003). Aminoglycoside modifying enzymes (AME) is the second mechanism of resistance. At least seven enzymes have been identified which inactivate gentamicin. There are R plasmid-encoded enzymes—classified as phosphotransferases (APH), acetyltransferases (AAC) and adenyltransferases (AAD)—which cause the most clinically important resistance to aminoglycosides by modifying their hydroxyl or amino groups to prevent ribosomal binding. A bifunctional enzyme encoding gene, *aac*6-*aph*2, is responsible for resistance to high-level gentamicin (> 500 μg/mL) and is mostly carried by MGEs such as plasmids (Simjee et al., 2002a; Chow, 2000). Strains, which harbor this gene, show cross-resistance to all other antibiotics within the group except streptomycin. This mechanism is clinically important because the responsible genes can be disseminated by MGEs (Collard, 1999; Simjee & Gill, 1997). The last mechanism, chromosomal mutation, is relatively unimportant. However, other mechanisms of resistance to gentamicin may be involved (Simjee & Gill, 1997). Roughly 1/3 of VRE strains produce multiple enzymes and are therefore resistant to all aminoglycosides (Delisle & Perl, 2003).

Ionophores (Narasin)

Ionophore antibiotics (such as narasin, monensin, lasalocid and salinomycin) alter the bacterial cell permeability (Butaye et al., 2003). They form a complex with sodium in the cell membrane to allow passive transport of potassium ions out of the cell where are

replaced by hydrogen ions, resulting in cell death by lowering intracellular pH. Since the cell walls of most Gram-negative bacteria are not permeable to hydrophobic molecules with molecular weights of 600 Da and above, these bacteria are not susceptible to ionophores (Callaway et al., 2003). Additionally, ionophore degradation by aerobes and eukaryotes is oxygen dependent, and hence is not efficient in anaerobic environments such as the rumen (Russell & Houlihan, 2003). Although ruminal Gram-positive bacteria are more sensitive to ionophores than Gram-negative bacteria due to the reason above, some Gram-negative ruminal bacteria are ionophore sensitive while some Gram-positive bacteria are resistant. Ionophores are categorized into three major classes based on their transport modes: neutral, carboxylic (including those used in animal feeds) and channel-forming quasi ionophores (Butaye et al., 2003).

Resistance to ionophores seems to be mediated by bacterial extracellular glycocalyx polysaccharides, which exclude ionophores from the cell membrane (Russell & Houlihan, 2003). No resistance genes for ionophores have been detected in ruminal bacteria. However, the vertical transfer of ionophore resistance between the bacteria has been suggested (Russell & Houlihan, 2003). There is no complete cross-resistance between ionophore antibiotics.

Ionophores are used in veterinary medicine or animal husbandry to control coccidiosis in livestock. Ionophores are not used in human medicine (Summers, 2002) and are largely used only in agriculture as a growth promoter by improving the efficiency of ruminal fermentation. These antibiotics are approved in use in poultry in Australia for their nutritional and anticoccidial activity (Barton & Wilkins, 2001; Callaway et al., 2003). Narasin was chosen as the ionophore antibiotic for study in this project.

Tetracyclines

Tetracyclines are broad-spectrum bacteriostatic antibiotics effective against Gram-positive and Gram-negative bacteria, mycoplasmas, rickettsias, chlamydiae and protozoan parasites (Chopra & Roberts, 2001). Tetracyclines irreversibly bind to the 30S subunit of the bacterial ribosome, interfering with the binding of the aminoacyltransfer RNA to the acceptor site on the messenger RNA ribosome complex. This reaction prevents the addition of amino acids to the elongating peptide chain, therefore inhibiting protein synthesis.

Resistance to tetracyclines is the most common form of bacterial antibiotic resistance in nature (Patterson et al., 2007). Up to 68% of enterococci have been found resistant to tetracycline (Stine et al., 2007). Bacteria usually mediate tetracycline resistance by four different mechanisms (efflux, ribosomal protection, enzymatic inactivation and target modification) mediated by *tet* genes (Patterson et al., 2007). Efflux pump mechanisms appear to be more common in Gram-negative bacteria while ribosomal protection is more common in Gram-positive bacteria (Bryan et al., 2004). Enzymatic inactivation and target modification are considered minor mechanisms of tetracycline resistance. Additional mechanisms have been described in a few bacterial genera. More than 30 *tet* genes have been identified in bacteria, of which tetracycline determinants K, L, M, N, O, P, Q, S, T, U, W and Z are usually found in Gram-positive bacteria (Chopra & Roberts, 2001; Murray, 1990). Moreover, the *tet*K, L, M, O, S, T, U genes are most commonly found in enterococci (Li et al., 2006; Fairchild et al., 2005; Nishimoto et al., 2005). The *tet*M gene is, however, the most common Tc^R determinant. Interestingly, the expression of extra chromosomal *tet* genes not only confers tetracycline resistance, but also may increase the susceptibility of Gram-negative bacteria to commonly used aminoglycoside antibiotics and heavy metals (Griffith et al. 1982, 1988; Merlin et al. 1989). Importantly, the association of resistance to tetracycline and integrons has not been proven (Sunde, 2005).

Tetracyclines were previously for growth promotion and prophylaxis in poultry and pigs in many countries (Barton & Wilkins, 2001; Lukasova & Sustackova, 2003). Since the mechanisms of drug action and resistance are similar within the tetracyclines, tetracycline was chosen as a prototype cycline antibiotic in this study.

Tiamulin

Tiamulin is a semisynthetic derivative of the diterpene bacteriostatic antibiotic pleuromutilin (Bøsling et al., 2003). Pleuromutilins have outstanding activity against anaerobic bacteria and mycoplasma and are exclusively used in animals, largely in swine. The mechanism of action is similar to mechanism of tylosin.

As with macrolides, chromosomal mutation—resulting in alteration of L3 ribosomal protein—facilitates bacterial resistance in the presence of the antibiotic (Bøsling et al., 2003). The rate of emergence is significantly lower than that observed for tylosin.

Tiamulin is used as a growth promoter in swine, rabbit and poultry in some countries. In Australia, it is approved for use in pigs to treat swine dysentery and chronic peunomonia, and to treat mycoplasmal infections in poultry (CVMP, 2008).

Tylosin

Tylosin is a macrolide (Liu & Douthwaite, 2002). Macrolides are a large group of related antibiotics with narrow spectrum bacteriostatic activity against Gram-positive and Gram-negative bacteria (except Enterobacteriaceae), mycoplasmas and chlamydiae. Tylosin inhibits protein synthesis by binding to subunits of the 50S ribosome and inhibiting the translocation step (Liu & Douthwaite, 2002).

Resistance mechanisms are similar to those of erythromycin that is encoded by *erm*B genes (van den Bogaard et al., 2002a; Zalacain & Cundlife, 1989). Resistance to tylosin is due to target site methylation, and is usually mediated by plasmids (Bonnier et al., 2006; Carsenti-Dellamonica et al., 2005; Douthwaite et al., 2004; Liu & Douthwaite, 2002). As with most macrolides, resistance due to chromosomal mutation develops rapidly *in vitro* but is relatively unstable (Alekshun & Levy, 2007).

Tylosin is approved as a feed additive and therapeutic antibiotic in Australia (Barton et al., 2003). It was previously used in pigs in many countries to promote growth, improve weight gain and treat infections (Lukasova & Sustackova, 2003; Bauer et al., 1988). In poultry, it is used to improve pigmentation, growth rate, feed efficiency and egg production.

Virginiamycin

Virginiamycin is a streptogramin bacteriostatic antibiotic registered for use in animals (Butaye et al., 2003). Virginiamycin consists of two different types of streptogramins, virginiamycin M (streptogramin A) and virginiamycin S (streptogramin B), working synergistically together (Thal & Zervos, 1999). Virginiamycin interferes with protein synthesis by deforming the ribosomal A site to inhibit binding of aminoacyl tRNA and the peptidyl transferase reaction, resulting in bacterial cell death. However, each component individually is a bacteriostatic agent.

Resistance to streptogramins is mediated by target site alteration (*erm* genes), inactivation of the antibiotic (*vat* genes), or active efflux of the antibiotic (*vga*, *vgb* and *mrs* genes) (Wright, 2003; Roberts et al., 1999; Thal & Zervosa, 1999).

This antibiotic was previously widely used as a growth promotant in growing pigs, broilers, layer hens and turkeys; and is currently registered in Australia for reducing lactic acidosis in sheep and cattle; or for preventing necrotic enteritis in broiler chickens (APVMA, 2004; Barton et al., 2003). Virginiamycin-containing feed is also used to reduce the risk of laminitis in horses (APVMA, 2004). Use of virginiamycin, as a feed additive, may result in the selection of resistant faecal enterococci with cross-resistance to the related streptogramin antibiotic quinupristin-dalfopristin (Synercid) (Kieke et al., 2006; Turnidge, 2001; Thal & Zervos, 1999). This antibiotic was introduced into human medicine for the treatment of vancomycin resistant enterococci and other infections.

Heavy Metals

Copper

Copper sulphate is one of the most commonly used disinfectants in veterinary medicine, for example for disinfecting the claws of cattle (Aarestrup & Hasman, 2004). Copper is an essential trace element involved in many biological functions of all living cells (Nies, 1999). It is extensively used as a growth promoter in pig production. The concentrations of added copper to animal food are higher than the standard nutritional requirement. This accelerates the emergence of resistance to copper in animal bacterial flora (Hasman et al., 2006).

Many bacteria have developed copper resistance to survive the toxicity of high concentrations of copper (Hasman & Aarestrup, 2002). Acquired copper resistance is mediated by the *tcr*B gene and has been described for human and animal *E. faecium* and *E. faecalis* isolates (Aarestrup & Hasman, 2004), as well as some other Gram-positive and Gram-negative bacteria in different countries (Hasman & Aarestrup, 2002). The *tcr*B gene is a part of *tcr*YAZB operon, consisting of four genes similar to *cop*YZAB copper homeostasis operon from *E. hirae* (Hasman, 2005). Bacteria carrying the plasmid borne *tcr*YAZB operon can grow in the presence of up to 28mM copper sulfate *in vitro*, whereas bacteria lacking this gene can tolerate copper concentrations of only up to 8mM (Hasman et al., 2006).

Zinc

Zinc is an essential component of many enzymes and DNA-binding proteins, such as zinc-finger proteins (Chou et al. 1998). Currently, antimicrobial agents such as heavy metals are used more for disinfection than for treatment of animals (Gant et al, 2007).

Resistance to heavy metals used in animal husbandry can be seen in bacteria (Mullapudi et al., 2008; Aarestrup & Hasman, 2004; Levy, 1998). For example, the *czc*D gene encodes an important Zn^{2+}-resistance determinant in human Gram-positive pathogens (Kloosterman et al., 2007).

Animal feed is supplemented with zinc compounds and/or copper sulphate (Aarestrup & Hasman, 2004). The effectiveness of zinc chloride and copper sulphate is probably due to the inhibition of some bacterial genera in the animal intestinal tract that results in the improvement of feed utilization in animals.

Molecular microbiology of antibiotic resistance

Antimicrobial agents such as antibiotics have been used to control microorganisms for many years (Aarestrup, 2005). In recent decades, antimicrobial (antibiotic) resistance has become a significant problem for patients, and for medical and veterinary practitioners. Many bacteria have become less responsive to antibiotics (Larson, 2007; Levy & Marshall, 2004). These resistant bacteria can infect humans causing an infectious disease that is more difficult to treat and then results in substantial costs to the healthcare system. One report, including unpublished data from the Centers for Disease Control and Prevention (CDC), estimates that costs of antimicrobial resistance in the early 1990s were 100 to 200 million dollars, with related medical costs exceeding four billion dollars in USA (Cassell, 1997). It is estimated that the total economic effect of antimicrobial resistance on Australia's health care budget may be in excess of 250 million dollars per year, as resistance rises in an exponential manner (Webber, 2006). The number of acquired infections in Australian hospitals is estimated to be nearly 150,000 each year, resulting in about 7,000 deaths annually. As an example, treatment of every patient with methicillin resistant *S. aureus* (MRSA) costs 22,400 dollars and is estimated to cost more for patients infected by vancomycin resistant bacteria (National Summit on Antibiotic Resistance, 2001). One of these resistant bacteria, enterococci, are found amongst multiple-resistant opportunistic pathogens isolated from long term hospitalized patients (Murray, 2000). These enterococci may infect humans via direct contact, waste and surface water, or consumption of meat products (Silva et al., 2006; Ellerbroek et al., 2004). The problem of resistance relates both the antimicrobial drug and the genetic determinants (genes) of resistance (Rice, 2000; Levy, 1994, 2002). These genetic determinants can be transferred to offspring bacteria or other bacteria in environment via inherent (vertical) or acquired (horizontal) routes, respectively (Courvalin, 2008).

Horizontal transfer of these antimicrobial resistance determinants occurs through three major mechanisms (Figure 1-4). One method is transformation or direct uptake of short naked DNA between closely related bacteria, mediated by cellular proteins (Rattanachaikunsopon & Phumkhachorn, 2009). An example of a gene transferred by this mechanism is the vancomycin resistance *van* gene from *E. faecalis* strains (Choi et al., 2004; Viera et al., 2001; Friesenegger et al., 1991). A second method of horizontal transfer is conjugation or bacterial mating mediated by close cell to cell contact allowing transfer of conjugative plasmids and chromosomally integrative conjugative elements (ICEs), e.g. conjugative transposons (CTns). Conjugation is well established and has been known as the major route of gene transfer in enterococci, involving both pheromone-responsive plasmids restricted to *E. faecalis* and non-pheromone-responsive conjugative plasmids in different species as well as conjugative transposons that transfer only *tet*M genes (Rice et al., 2007; Kozlowicz et al., 2006; Franz et al., 2003; Clewell & Dunny, 2002; Rice, 1998). Some antimicrobial resistance genes, hemolysin and bacteriocin genes are transferred by conjugation in enterococci (Rice, 2000; Clewell, 2005). The third method of horizontal gene transfer is transduction which is bacteriophage mediated and usually transfers MGEs (Thomas & Nielsen, 2005). Transduction in enterococci has not been addressed in the literature, except in a report by Zighelboim-Daum et al. (1988) on transfer of gentamicin resistance from staphylococcal isolates to enterococci by bacteriophages.

Many factors contribute to the spread of antimicrobial resistance, including widespread inappropriate use of antimicrobials in human and veterinary medicine, agriculture and animal husbandry (Orzech & Nichter, 2008; Aarestrup, 2005; Thal & Zervos, 1999) (Figure 1-5). Another important factor relates to the ubiquitous nature of genetic determinants such as MGEs, which are involved in horizontal transfer of resistance (Salyers et al., 2007). Horizontal transfer or lateral gene transfer (LGT) is assumed to be the major cause of the widespread prevalence of resistance, whereas vertical transfer which takes a longer time to occur is considered a minor component. An overview of biochemical and molecular aspects of antibiotic resistance mechanisms is shown in Figure 1-6.

Mobile genetic elements (MGEs)

Antimicrobials exert a selective pressure and select for resistant clones within the bacterial populations (Frost et al., 2005; Bennett, 2004; Klare et al., 2003; Baquero et al., 2002). The resistance genes are present in bacterial genomes or plasmids and pre date the

introduction of antimicrobials but are only expressed when the bacteria are exposed to the particular antimicrobial. The rapid emergence of bacterial resistance following the introduction of a new antibiotic shows the ability of microbial populations to adapt rapidly to environmental changes (Perron et al., 2008). The analysis of bacterial genomes has revealed that antibiotic resistance is associated with underlying genetic determinants (Hegreness et al., 2008). Initially antibiotic resistance was believed to develop by point mutation and selection, as can be demonstrated *in vitro* (Frost et al., 2005). Antimicrobials can select for resistance in bacteria which is mediated by random mutations (Woo et al., 2003). The frequency of mutation depends on factors such as the number of target genes and the number of gene positions which can be mutated (Adam et al., 2008). Furthermore, a higher mutation rate could be induced due to a triggered SOS response by the presence of some antimicrobial agents such as fluoroquinolones as well as other DNA damaging agents such as ultraviolet light, reactive oxygen intermediates or salicylic acid (Baharoglu et al., 2010; Kohanski et al., 2010; Lopez et al., 2007; Gillespie et al., 2005). However, the acquisition of new characters without making any change in bacterial survival is predicted to occur over a long time (Martinez & Baquero, 2002). From an evolutionary viewpoint, chromosomal mutations alone have failed as the sole explanation for the presence of multiresistant bacteria (Eberhard, 1989). This failure was confirmed by the discovery of gene transfer and the acquisition of additional genetic material in the form of extrachromosomal DNA. This DNA can be transferred by bacteriophages, plasmids or transposons (Levy & Marshall, 2004; Novick, 2003; Osborn & Boltner, 2002; Boyd & Brussow, 2002). These MGE-encoded genes assist organisms to survive in challenging environments (Eberhard, 1989; Canchaya et al., 2003). This assistance includes resistance to antibiotics or heavy metals, bacteriocin activity, metabolism of unusual substrates and virulence factors. As previously described, bacterial heavy metal resistance is important due to the associated risk of promoting antibiotic resistance, as heavy metal resistance genes are usually carried on the mobile genetic elements which also carry antibiotic resistance genes at the same time (Baker-Austin et al., 2006). Therefore, activation of such MGEs in bacterial hosts in response to environmental conditions may result in the expression of antibiotic resistance genes as well. Sometimes however, these MGEs may result in gene deletion and hence loss of resistance in bacteria (Rice, 2000).

Enterococci have the ability to exchange genes and MGEs between themselves and other genera including species of Enterobacteriaceae, *Neisseria, Clostridium, Bacillus, Staphylococcus, Streptococcus, Campylobacter* and *Mycoplasma* (Silbergeld et al., 2008;

Clewell, 1990; Schaberg & Zervos, 1986). Furthermore, enterococci have a key role in transfer of antibacterial resistance determinants within the members of intestinal microflora (Sørum & Sunde, 2001). Trieu-Cuot et al. (1988) described this genetic element transfer between the distinct bacteria as "heterogramic" transfer. Molecular analysis of the genetic elements encoding antibacterial resistance in disparate bacterial groups has demonstrated that intergenic spread has occurred between commensal and clinical strains such as *Listeria* spp., *S. aureus* and especially enterococci (Courvalin, 2005; Dahms et al., 1998). For example, the *tet*M gene determining resistance to tetracycline has been detected in Gram-positive, Gram-negative and cell wall-free bacteria (Speer et al., 1992). Some of these bacteria such as *E. faecalis* are members of intestinal microflora. Observation of these facts provides evidence of transfer of antibacterial resistance determinants within members of human normal microflora (Sørum & L'Abee-Lund, 2002; Martinez & Baquero, 2002). Since *tet*M has also been detected in pathogenic bacteria, transfer of DNA between members of the normal microflora and pathogens is strongly suggested (Figure 1-7) (Speer et al., 1992; Dzidic et al., 2008). This transfer has been reported between *Enterococcus* and different genera including *Bacillus*, *Campylobacter*, *Clostridium*, *Mycoplasma*, *Neisseria*, *Staphylococcus*, *Streptococcus* and genera within Enterobacteriaceae. Antibiotic resistance (ABR) determinants have been shown to transfer from Gram-positive to Gram-negative bacteria whereas the reverse is uncommon (Trieu-Cuot et al., 1985). Although evidence of bacterial transfer from large animals such as cattle to humans and other animals—including insects, rodents and birds—has already been found (Nichols, 2005; Cole et al., 2005; Henzler & Opitz, 1992), transfer of virulence and antimicrobial resistance (AMR) genes between the animal and human bacterial species is not thoroughly documented (Marshall et al., 2009).

Enterococci naturally transfer these genetic elements by at least three conjugative systems. First, narrow host range pheromone-responsive plasmids that are unique to enterococci. These plasmids transfer at high frequencies on solid surfaces, in broth and *in vivo* (Clewell, 1993). Second, many plasmids with a broad host range (e.g. pAMB1) transfer at low frequency within *Enterococcus* spp., *Streptococcus* spp., *S. aureus*, *Lactobacillus* spp., *Bacillus subtilis* and other species (Clewell, 1990; Schaberg & Zervos, 1986). Transfer requires contact between donor and recipient cells on a solid surface or *in vivo* (Morelli et al., 1988). Finally, conjugative gene exchange occurs through transposons found in Gram-positive and Gram-negative bacteria (Scott, 1992). One transposon, Tn*1545*, has been shown to transfer at low frequency from *E. faecalis* to *L. monocytogenes*

in the intestinal tracts of gnotobiotic mice (Doucet-Populaire et al., 1991). Another transposon, Tn*916*—the most comprehensively characterized conjugative transposon, has been used extensively in molecular genetic studies (Scott, 1992). Composite transposons such as Tn*5385*, encoding resistance to vancomycin and ampicillin in *E. faecalis*, can also be involved in other genetic exchange mechanisms such as chromosome-to-chromosome gene exchange mechanism (Rice, 2002; Rice & Carias, 1998). However, conjugation is known as the most important route for the spread of antibiotic resistance elements (Marraffini & Sontheimer, 2008). Other mechanisms of natural gene exchange in enterococci include uptake of short naked DNA (transformation) (Rattanachaikunsopon & Phumkhachorn, 2009) and infection with transducing bacteriophages (Nigutova et al., 2008).

The use of some growth-promoting antibiotics is known to contribute to the horizontal transfer of antimicrobial resistance genes between animal bacteria (Kohler et al., 2000). A possible explanation is the release of bacteriophages containing those resistance genes from the bacteria directly into the animal intestines following the death of bacteria due to the action of the antibiotics (Kohler et al., 2000). One example is the spread of Shiga toxin-encoding bacteriophages in the intestines of animals following the use of olaquindox and carbadox (Schmidt, 2001). However, studies have shown that some other antibiotics such as tylosin and monensin had an inhibitory effect on the bacteriophage induction (Kohler et al., 2000).

Bacteriophages

Bacteriophages are viruses that are able to infect bacteria (Canchaya et al., 2003). They are more abundant than any other biological entities on earth (approximate population of 10^{31}) (Canchaya et al. 2004; Pedulla et al., 2003) and the bacteriophage population is estimated as being ten times greater than the bacterial population (Hendrix, 2002). Bacteriophages destroy a significant portion (1/5) of bacterial populations every two days and are more important in adoption of pathogens to new hosts more any other mechanism, with new strains emerging and epidemics occurring (Brussow et al., 2004; Boyd & Brussow, 2002). Therefore, host-pathogen interaction is redefined by a novel concept "host-pathogen-phage interaction" (Brussow et al., 2004; Canchaya et al., 2004). Bacteriophages can play a role in carbon cycling, bacterial pathogenicity and evolution. Practically they can be used for developing DNA and protein vaccines, gene therapy, finding new antibiotics and antiviral drugs, bioremediation, and phage therapy (Serwer et al., 2007). Bacteriophages

can be inactivated by ultraviolet (UVB) irradiation; some are sensitive to chemicals such as ether and chloroform (Fuhrman, 1999; Kokjohn & Miller, 1994; Kokjohn & Miller, 1987).

Currently, the International Committee on Taxonomy of Viruses (ICTV, http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/) classifies bacteriophages in one order, 13 families, and 34 genera plus one unassigned genus (*Salterprovirus*) (Lavigne et al., 2008) (Table 1-8). Four main families seem to be more widespread in nature, namely *Podoviridae* (short tail stub), *Siphoviridae* (long flexible non-contractile tail), *Myoviridae* (contractile tail) and *Inoviridae* (filamentous) (Nelson, 2004; Lawrence et al., 2002). A typical structure of tailed bacteriophages consists of an isocohedral head containing the virus capsid and genome, a neck or collar, a tail (some contain multiple tails [Cummings & Bolin, 1975]), and a baseplate (Figure 1-8). The tail includes a core covered by a helical sheath that ends to the base plate, which bears long tail fibers and short tail fibers or tail pins (Ackermann, 1998). The genome of bacteriophages can be composed of either single- or double-stranded DNA or RNA and can range in size from a few to several hundred kb (Frost et al., 2005).

Bacteriophages were first discovered in the late 1800s. Hankin inactivated *Vibrio cholerae* in 1896 using filtrates of water from the Ganges and Jumna rivers. This was repeated later by Gamaleya (1898) while working with *B. subtilis*. In 1915, Twort suggested this causative agent was a "bacterial virus". In 1917, d'Herelle used a plaque method and reported the first bacterial lysis, and he named the causative agent a bacteriophage. Clark and Clark (1927) made the first successful isolation of a streptococcal bacteriophage. Enterococcal bacteriophages were firstly isolated from sewage by Evans (1934) and later by Rakieten and Tiffany (1938), Tiffany and Rakieten (1939), and Evans (1941). Kjems (1955) found evidence of lysogeny and isolated four strains of bacteriophages from Group D streptococci, including *S. faecalis* and *S. faecium*. More isolations were carried out by Bleiweis and Zimmerman (1961), Brock et al. (1963) and Brock (1964). Later, Rogers and Sarles isolated and characterized enterococcal bacteriophages from the small intestine of rats in 1963. Pleceas and Brandis (1974) developed a rapid identification of enterococci by means of bacteriophages. Similarly, Caprioli et al. (1975) reported a phage-typing scheme for urogenital Group D streptococci, using bacteriophages specifically active against these isolates. This was later continued by other researchers such as Ramirez et al. (2006).

Bacteriophages infect bacterial hosts in three major steps: 1) Binding of the bacteriophage to a bacterial specific receptor such as surface protein, carbohydrate, or lipopolysaccharide molecule; 2) Injection of the bacteriophage genome into the bacterial cell; and 3) Synthesis of the bacteriophage genome proteins using bacterial replication machinery (often as fast as half an hour) (Heineman & Bull, 2007). The bacterial phage receptors can be located in the outer membrane of Gram-negative bacteria, in the cell wall of Gram-positive bacteria, in the capsular or slime layer, and in association with flagella or pili (Lindberg, 1973). Bacteria can resist bacteriophage infections by blocking each of above steps. Furthermore, some bacterial genera resist bacteriophage infections using clustered, regularly interspaced, short palindromic repeats (CRISPR) within their genomes, which contain sequences similar to those of bacteriophages (Hegstad et al., 2010). Additionally, bacteriophages are functionally categorized into two groups: lysogenic and lytic bacteriophages (Canchaya et al., 2003). The former group (alternatively called temperate or mild bacteriophages) infects bacteria and resides inside them without any disruption in the bacterial cell, while the latter multiplies at the expense of bacterial life. In lysogeny, in which the viral genome is integrated into the bacterial DNA and known as a "prophage", the bacteriophage uses its host for replication of its genome and expression of some proteins (Brussow et al., 2004). Prophages are found in two-thirds of all gammaproteobacteria and low-GC Gram-positive bacteria mostly share in conjugative plasmids and related proteins (Weaver et al., 2009; Brussow et al., 2004). However, lysogenic bacteriophages can shift to a lytic phase (lysogenic induction) by sublethal doses of ultraviolet (UVC) irradiation or subtherapeutic concentrations of some antibiotics such as mitomycin C and norfloxacin (Herold et al., 2004; Yang et al., 2002; Kohler et al., 2000; Miller & Kokjohn, 1990). This results in replication and production of progeny phages.

Transduction can be either generalized or specialized (Kelly et al., 2009). Generalized transduction occurs when the bacteriophage packages a segment of the donor chromosome by chance during its replication in the cell. Specialized transduction occurs when a specific fragment of the chromosome near the prophage integration site is packaged. This DNA and encoded genes are then transferred to a new bacterial host when the bacteriophage infects the new host. This process plays an important role in bacterial evolution as many virulence factors can be transferred between different bacteria (Canchaya et al., 2003). This is important because some changes in bacterial virulence and

fitness such as acquisition of antimicrobial resistance can also occur (Brussow et al., 2004).

Transduction is one of the most widespread forms of indirect horizontal gene transfer (HGT) and is reported as a significant mechanism in microbial ecology (Weinbauer, 2004; Weinbauer & Rassoulzadegan, 2004). Sometimes, bacteriophage transduction results in the transfer of multiple resistance R plasmids between Gram-positive bacteria (e.g. *S. aureus* and *S. pyogenes*); however, cellular conjugation is recognized as the major route of transferring genetic elements within bacteria (Mathur & Singh, 2005). In both Gram-positive and Gram-negative bacterial pathogens, inserted bacteriophage genomes—prophages—can encode and express virulence factors such as toxins (e.g. Shiga toxins of *E. coli*, leukocidins, and superantigens of *S. aureus* and *S. pyogenes*) (Maiques et al, 2007; Ubeda et al., 2007; Baba et al., 2002; Acheson et al., 1998). Lysogeny can rapidly drive genetic change and evolution by processes such as gene duplication and mutation in contrast to vertical gene transfer. In fact, horizontal gene transfer replaces a sexual life cycle in bacteria. The DNA transferred horizontally ranges in size from less than 1 kb to more than 100 kb (Canchaya et al., 2003) and can encode metabolic pathways, surface structures, toxins, and mobile pathogenicity islands (Brussow et al., 2004; Ruzin et al., 2001). For example, toxins of *Corynebacterium diphtheria* (diphtheria), *Clostridium botulinum* (botulism), *Bordetella pertussis* (pertussis or whooping cough), *Yersinia enterocolitica* (yersiniosis), spirochetes (spirochetosis), *S. pyogenes* (scarlet fever), *S. aureus* (food poisoning) and *E. coli* (Shiga toxins) are all bacteriophage encoded (Novick, 2003; Krylov, 2003; Boyd & Brussow, 2002). Moreover, some outer membrane proteins (OMPs) of *E. coli* and some antibiotic resistance genes (e.g. *van* genes) are encoded by genes carried by bacteriophages. Examples of other proteins that are encoded by the genes carried by the bacteriophages include ADP-ribosyl transferase toxins, superantigens, lipopolysaccharide-modifying enzymes, type III effector proteins, detoxifying enzymes, hydrolytic enzymes and serum resistance proteins. Many prophage genes linked to potential virulence factors have been identified in bacterial pathogens, but their role in bacterial pathogenicity is still unknown (Brussow et al., 2004). However, the products of these genes may have significant effects on the host bacterium, which can have its phenotype modified by expression of genes encoded by the prophage. These changes range from protection against further bacteriophage infection to increasing the virulence of a pathogenic host. For example, in some cases, regulatory proteins expressed

by prophages are known to alter the pathogenesis of the host bacteria (Serra-Moreno et al., 2006).

However, prophages may not be necessary for the evolution of every pathogen. Additionally, acquisition of virulence genes is not a unique evolutionary mechanism in bacteria. Pathogenic bacteria also develop from commensal bacteria by loss of genes, which may accidentally occur by excision of temperate bacteriophages. In contrast to temperate bacteriophages, the mechanism of lytic bacteriophages (also known as virulent or killer bacteriophages) is quite different. In the lytic phase, the virus destroys bacteria as a natural consequence of multiplication (Deresinski, 2009). Bacteriophages (usually dsDNA bacteriophages) use murein-degrading enzymes (endolysins or lysins) and small hydrophobic pore-forming proteins (holins), which assist the endolysin, in the destruction of bacterial cell wall and release of newly synthesized bacteriophages (Fischetti, 2008; Wang, 2006). These enzymes work synergically with each other and also with antibiotics as well. Up to 10−100 virions/cell can be released. The relationship between the activity of lytic bacteriophages and the decrease in number of enteropathogens has been studied (Mizoguchi et al., 2003). Lysogenic bacteriophages, however, only undergo the lytic phase under special usually stressful conditions (Shkilnyj & Koudelka, 2007; McCabe et al., 2005).

The recent public interest in bacteriophages relates to their relevance in: 1) genome and evolution research, 2) horizontal gene transfer, 3) therapeutic use, and 4) bacterial genetics and as molecular biology tools (Kiljunen et al., 2005). Bacteriophages have been used to treat different infections such as dysentery, salmonellosis and gastroenteritis (Sarkar, 2002; Sulakvelidze et al., 2001). In addition, they are used to treat skin, mucosa and wound infections (Taylor et al., 2002; Sulakvelidze et al., 2001).

In enterococci, bacteriophages are involved in natural gene exchange (Torres Viera et al., 2001; Simjee & Gill, 1997). Enterococcal bacteriophages have been isolated from stool samples and other sources and found to have a narrow host range (Ramirez et al., 2006; Paisano et al., 2004; Kuhnen et al., 1988). However, the ability of enterococcal bacteriophages to spread virulence genes and antibiotic resistance determinants is largely unknown.

Plasmids

Plasmids were first described by Lederberg (1952) as an extrachromosomal hereditary determinant. Plasmids are extra-chromosomal DNA molecules, mostly circular and double

stranded DNA, capable of replicating independently or integrating into the host chromosome and are found in many bacterial species (Bennett, 2008; Frost et al., 2005). A group of plasmids with linear DNA has been found in some species such as *Borrelia*, *Streptomyces*, *Nocardia*, *Rhodococcus* and *Mycobacterium* (Ravel et al., 2000). The transfer mechanisms of plasmids depend upon the class of these plasmids. In Gram-positive bacteria, these mechanisms are divided into three main categories: pheromone-responsive plasmids described primarily in *E. faecalis* and apparently restricted to this species, nonpheromone-responsive conjugative plasmids and nonconjugative mobilizable plasmids (Rice, 2000). Conjugative plasmids are important in enterococci because many putative virulence factors are located on plasmids (Shiojima et al., 1997; Fujimoto et al., 1995; Clewell, 1993, 1981). In enterococci, toxins (e.g. hemolysin and bacteriocin) are encoded by the genes usually transferred via conjugative systems rather than other mechanisms such as transduction (Ruiz-Barba et al., 2007; Cox et al., 2005; Shankar et al., 2004). Haas and Gilmore (1999) and later Coburn and Gilmore (2003) described a cytolysin from *E. faecalis* which is active against eukaryotic and prokaryotic cells. The operon of this cytolysin is found on pheromone-responsive, self-transmissible plasmids and also in pathogenicity islands (PAI) (Coburn et al., 2004). Additionally, antibiotic resistance in enterococci is mostly transferred by plasmids (Rice et al., 2007).

The existence of resistance plasmids was first suggested by the observation of transfer of resistance between bacterial strains (Watanabe, 1963). During the last two decades, evidence has indicated that in many cases bacterial resistance to antimicrobials is due to the presence of plasmids carrying the resistance determinants (Hughes & Datta, 1983). Several factors are involved in the ubiquitous nature of plasmid encoded antibiotic resistance (Lyon et al., 1987). Some bacteria such as human and animal intestinal enterococci may carry several different plasmids, most of them with unknown function (cryptic plasmids) (Martinez-Bueno et al., 2000, Wyckoff et al., 1996). Cryptic plasmids are small stable MGEs that code for proteins only involved in their replication and mobilization (Zaleski et al., 2006). However, some plasmids are not transferable (Seol et al., 2006; Poirel et al., 2000). Plasmids can contribute to the development of chromosomal resistance in two ways. First, the plasmid partially or completely integrates into the bacterial chromosome. In Gram-negative bacteria, a plasmid may bear short segments of DNA or insertion sequence (IS) that resides on the plasmid or chromosome and provides limited sequence homology for recombination. Second, the plasmid is not inserted in the bacterial chromosome and is carried in extra chromosomal form (Lyon et al., 1987). In

fact, plasmids can be transferred between the *Enterococcus* spp. and other genera such as *Lactococcus* and vice versa. An example of the former transfer route is the transfer of multiresistance pK214 plasmid from *Lactococcus* to *E. faecalis* (Perreten et al., 1997) and example of the latter way is the transfer of *E. faecalis* conjugative pRE25 plasmid to *Lactococcus lactis* (Teuber et al., 2003).

Transposons

Transposons (colloquially named jumping genes) are MGEs that can copy themselves to different locations on the bacterial chromosome and extrachromosomal plasmids and can be transferred to other bacteria via mechanisms such as conjugation (Bennett, 2008; Ferguson, 1999). These transposable elements (TEs) have been identified in more than 50 different bacterial genera, including Gram-positive and Gram-negative bacteria, playing a major role in the spread of antibiotic resistance determinants between the genera (Jones et al., 2006). Transposons can be categorized into two major classes: DNA transposons and retrotransposons (Bao et al., 2010; Novikova & Blinov, 2009). In eukaryotes, retrotransposons can be divided into non-long terminal repeat (LTR) retrotransposons and LTR retrotransposons. Transposons and insertion sequences appear to be the most common mechanisms for spreading resistance genes in Gram-positive bacteria (Rice, 2000). For example, most conjugative transposons in enterococci transfer *tet*M genes, except for transposons Tn*1546* and Tn*5382* in *E. faecium* that transfer *van*A and *van*B genes respectively (Takeuchi et al., 2005; Manson et al., 2003). Genetic exchange is possible on a large scale within the microbial population of human and animal gastrointestinal tracts (Shoemaker et al., 2001; Roberts et al., 1999). Gastrointestinal anaerobic bacteria are shown to contain both novel antibiotic resistance genes and conjugative transposons (Scott, 2002). These conjugative transposons contain genes encoding resistance to aromatic compounds (e.g. toluene and phenols), heavy metals (e.g. copper, zinc and mercury) and degrading xenobiotic compounds (e.g. toluene) (Scott et al., 2008; Roy, 1999; Bonafede et al., 1997). One of these conjugative transposons—Tn*916*—was originally discovered in *E. faecalis*, encoding resistance to tetracycline and minocycline by the *tet*M gene and resistance as well as to β-lactams (Agersø et al., 2006; Rice et al, 1992; Franke & Clewell, 1981). Another prototype transposon, Tn*917*, encodes erythromycin and clindamycin resistance in enterococci and streptococci by the expression of the *erm*AM gene (Tomich et al., 1980). These conjugative transposons can be transferred to many commensal bacteria both in *vitro* and *in vivo* (Scott, 2002).

Good examples of composite transposons involving in antimicrobial resistance transfer between the enterococci are Tn*5252,* Tn*5253*, Tn*5281*, Tn*5381*, Tn*5382*, Tn*5383*, Tn*5384*, Tn*5385* and Tn*5386* (Rice, 2000). Composite transposons are genetic elements, which can move because of their insertion elements (Rice et al., 1995). Tn*5252* encodes tetracycline resistance while Tn*5253* encodes chloramphenicol resistance in addition to tetracycline resistance (Rice et al., 2005). Another transposon, Tn*5281*, is flanked by IS*256* element confering resistance to high levels of aminoglycosides such as gentamicin (Hodel-Christian & Murray, 1991). Transposon Tn*5281* is functionally similar to transposons Tn*4001* and Tn*4031*. Tn*4001*-like elements involve in dissemination of high-level aminoglycoside resistance within enterococci and streptococci (Kaufhold et al., 1992). This is mediated by a bifunctional enzyme 6'-acetyltransferase-2"-phosphotransferase (*aac*6'-*aph*2") (Sahm & Gilmore, 1994). Vancomycin resistance transfer between *E. faecium* strains is generally associated with co-transfer of PBP5-mediated ampicillin resistance in a larger MGE (Rice, 2002). Tn*5381* and Tn*5383* confer resistance to tetracycline and minocycline (Rice et al., 1992), and Tn*5385*—a composite MGE including Tn*5384*—encodes resistance to erythromycin, gentamicin, mercuric chloride, streptomycin, tetracycline and vancomycin (Bonafede et al., 1997). Tn*5385* is found in some strains of *E. faecalis* flanked by directly repeated copies of IS*1216* (Willems et al., 1999). Tn*5386*, a novel Tn*916*-like MGE, is found in tetracycline- and ampicillin-resistant *E. faecium* (Rice et al., 2005). Other transposons such as Tn*1546* and Tn*1547* are flanked by copies of IS*1216* and IS*256* respectively and have an established role in transferring glycopeptide (vancomycin) resistance within enterococci (Rice, 2002).

Integrons

Integrons are specialized genetic elements integrating or mobilizing gene cassettes by site-specific recombination (Bennett, 2008; Chang et al., 2000). Investigation has shown that many resistant bacteria contain integrons (Sunde, 2005). Of several types of integron, classes 1, 2, 3 and 4 are associated with resistance gene cassettes while classes 1, 2 and 3 are the most prevalent in clinical isolates (Boucher et al., 2007; Hsu et al., 2006; Su et al., 2006). In enterococci, an integron sequence containing the *aad*A gene from *E. faecalis* W4770 has been reported by Clark et al. in 1999. Furthermore, integron-like structures have been described in enterococci (Holmes et al., 2003; Rice, 2000). However, although class 1 integrons have been detected in variety of Gram-positive bacteria including *Staphylococcus* spp. and *Corynebacterium* spp., (Xu et al., 2008; Shi et al., 2006; Nandi et

al., 2004), integrons are unlikely to play an important role in the evolution of antimicrobial resistance in Gram-positive bacteria (Diaz-Mejia et al., 2008).

Aims of the thesis

The global problem of antimicrobial resistance in enterococci has become of high concern in human and veterinary medicine by researchers and the decision makers (Rahangdale et al., 2008). These resistant isolates can be transferred to humans via direct contact, food consumption, nosocomial or community acquisition (Hayes et al., 2003; Mathur et al., 2003). Moreover, the resistance can be transferred from animal bacteria to human bacterial population by MGEs such as plasmids and transposons (Kapoor et al., 2005; Hudson et al., 2002). Recent studies have shown that the use of antibiotics in veterinary medicine or animal husbandry contributes significantly to the spread of antimicrobial resistance across the world (Pelicioli Riboldi, 2009; Petersen & Dalsgaard, 2003). In Australia, studies reveal that resistance in clinical isolates of enterococci, especially *E. faecium* isolates, to some antibiotics such as vancomycin and aminoglycoside high-level has been increased over ten years from 1995 to 2005 (Christiansen et al., 2007). A wide range of antibiotics is used in farming, human and veterinary medicine in large quantities in Australia (Turnidge, 2001). Since there is no formal system for monitoring or surveillance of antibiotic resistance in animal bacteria in Australia (Barton et al., 2003), research in this field is therefore important. Although some investigations were conducted in the late 1970s and 1980s and also a pilot study by DAFF in 2007, it is difficult to compare this historical data with current data due to the poor standardization of sensitivity testing methods.

Since the incidence of antibiotic resistance is increasing due to both legitimate and inappropriate use of antibiotics (Tennstedt et al., 2003; Teale, 2003), it is necessary to develop new approaches to detect and identify antibiotic resistance genes and MGEs responsible for transferring those genes in bacterial populations. In addition, understanding of prevalence of antibiotic resistant bacteria increases the ability to prevent the hazards and negative consequences. In addition, an improved knowledge of the molecular mechanisms of antibiotic resistance, their origins, evolution, and distribution through bacterial genomes is needed to understand the resistance (Dzidic et al., 2008; Harbarth & Samore, 2005; Wright, 2003). In this thesis these aims were achieved in four major steps: 1) Assessment of antimicrobial resistance in enterococci, using minimum inhibitory concentration method; 2) Detection of antimicrobial resistance genes, using methods such as polymerase chain reaction and sequencing of the resistance genes; 3)

Investigation and characterization of bacteriophages, including lysogenic induction of temperate bacteriophages, detection and purification of lytic bacteriophages from environmental samples and characterization of the bacteriophages by electron microscopy and DNA sequencing; and 4) Determination of the role of bacteriophages in the transfer of resistance genes, including transduction of the resistance genes by exposing donor bacteriophages to recipient enterococci and then assessing the transferred antimicrobial resistance by minimum inhibitory concentration, polymerase chain reaction and sequencing.

Table 1-1. *Enterococcus* species

No.	***Enterococcus* species**	No.	***Enterococcus* species**	No.	***Enterococcus* species**
1	*E. aquimarinus*	13	*E. durans*	25	*E. pallens*
2	*E. asini*	14	*E. faecalis*	26	*E. phoeniculicola*
3	*E. avium*	15	*E. faecium*	27	*E. pseudoavium*
4	*E. caccae*	16	*E. gallinarum*	28	*E. raffinosus*
5	*E.scamelliae*	17	*E. gilvus*	29	*E. ratti*
6	*E. canintestini*	18	*E. haemoperoxidus*	30	*E. saccharolyticus*
7	*E. canis*	19	*E. hermanniensis*	31	*E. silesiacus*
8	*E. casseliflavus*	20	*E. hirae*	32	*E. sulfurous*
9	*E. cecorum*	21	*E. italicus*	33	*E. termitis*
10	*E. columbae*	22	*E. malodoratus*	34	*E. thailandicus*
11	*E. devriesei*	23	*E. moraviensis*	35	*E. villorum*
12	*E. dispar*	24	*E. mundtii*		

From Felis et al., 2009

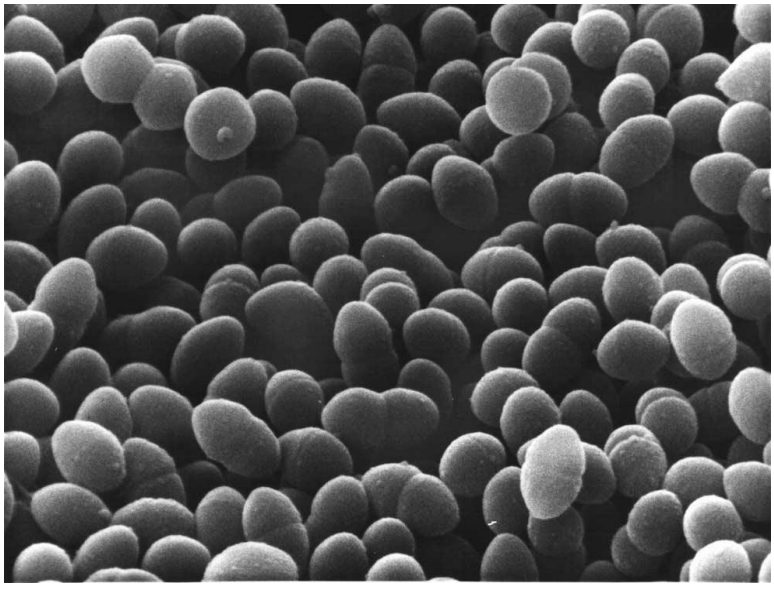


Figure 1-1. SEM of *E. faecalis* (×17,160)

From Phillips, 1995

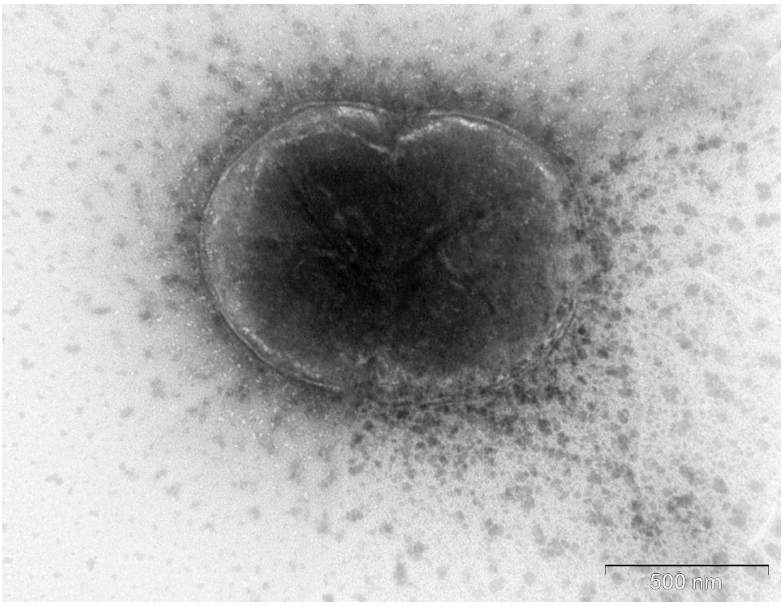


Figure 1-2. TEM of *E. faecalis* (×54,000)

From this study

Table 1-2. Natural hosts and environments of enterococci

Enterococci	**Host/environment**					
E. faecalis	Human	Animals		Plants	Water	Food
E. faecium	Human	Chicken	Pig	Plants	Water	Food
E. casseliflavus				Plants	Soil	Food
E. mundtii				Plants		
E. durans	Human	Poultry				Food
E. gallinarum		Poultry				Food
E. avium		Poultry				
E. hirae		Farm animals				

From Portenier et al., 2004; Klare et al., 2001

Table 1-3. Phenotypic characteristics used for identification of *Enterococcus* species

Enterococci	MAN	SOR	ARG	ARA	SBL	RAF	TEL	MOT	PIG	SUC	PYU	MGP
Group I												
E. avium	+	+	-	+	+	-	-	-	-	+	+	V
E. malodoratus	+	+	-	-	+	+	-	-	-	+	+	V
E. raffinosus	+	+	-	+	+	+	-	-	-	+	+	V
E. pseudoavium	+	+	-	-	+	-	-	-	-	+	+	+
E. saccharolyticus	+	+	-	-	+	+	-	-	-	+	-	+
E. pallens	+	+	-	-	+	+	-	-	+	+	-	+
E. gilvus	+	+	-	-	+	+	-	-	+	+	+	-
Group II												
E. faecalis	+	-	+	-	+	-	+	-	-	+	+	-
E. faecium	+	-	+	+	V	V	-	-	-	+	-	-
E. casseliflavus	+	-	+	+	V	+	-	-	+	+	V	+
E. mundtii	+	-	+	+	V	+	-	-	+	+	-	-
E. gallinarum	+	-	+	+	-	+	-	-	-	+	-	+
Group III												
See Table#	-	-	+	V	-	V	V	-	-	V	V	V
Group IV												
E. asini	-	-	-	-	-	-	-	-	-	+	-	-
E. sulfureus	-	-	-	-	-	+	-	-	+	+	-	+
E. cecorum	-	-	-	-	+	+	-	-	-	+	+	-
Group V												
E. casseliflavus	+	-	-	+	V	+	V	+	+	+	V	+
E. gallinarum	+	-	-	+	-	+	-	+	-	+	-	+
E. faecalis	+	-	-	-	+	-	+	-	-	+	+	-
E. columbae	+	-	-	+	+	+	-	-	-	+	+	-

V, variable; ARA, RAF, SUC, MAN, SOR, SBL, MGP, acid formation in broth containing 1% arabinose, raffinose, sucrose, manitol, sorbose, sorbitol and methyl-α-D-glucopyranoside, respectively; ARG, arginine; MOT, motility; PIG, pigment; PYU, pyruvate utilization; TEL, tolerate to 0.04% tellurite

From Gilmore, 2002

Table 1-4. Phenotypic characteristics of Group III *Enterococcus* species

Enterococcus	LM	PYU	HIP	TEL	ARA	GYL	RAF	SUC	TRE	XYL	MGP
E. durans	A/C	-/0	+/82	-/0	-/0	-/0	-/0	-/0	+/100	-/	-/0
E. porcinus	A/ -	-/0	-/0	-/0	-/0	-/0	-/0	-/0	+/100	+/100	-/0
E. ratti	-/-	-/0	V/60	-/0	-/0	-/0	-/0	-/0	-/20	-/	-/0
E. hirae	A/-	-/6	-/3	-/0	-/0	-/5	+/100	+/100	+/100	-/	-/0
E. dispar	A/-	+/100	+/100	-/0	-/0	+/100	+/100	+/100	+/100	-/	+/100
E. faecalis	A/C	+/76	-/13	+/88	-/0	-/12	-/0	-/12	-/12	-/	-/0
E. faecium	A/V	-/0	V/56	-/6	+/100	-/6	-/13	V/38	+/75	-/	-/0

V, variable; ARA, GYL, RAF, SUC, TRE, XYL, MGP, acid formation in broth containing 1% arabinose, glycerol, raffinose, sucrose, trehalose, xylose and methyl-α-D-glucopyranoside, respectively; HIP, hippurate hydrolysis; LM, litmus milk; PYU, pyruvate utilization; TEL, tolerate to 0.04% tellurite

From Gilmore, 2002

Table 1-5. Antibiotics used in the feed of Australian livestock

Group/Class	Antimicrobial	Usage
Arsenicals	3-nitro-arsonic acid	Pigs, poultry
Glycopeptides	Avoparcin*	Pigs, meat poultry, cattle
Macrolides	Kitasamycin	Pigs
	Oleandomycin	Cattle
	Tylosin	Pigs
Polyethers (ionophores)	Lasalocid	Cattle
	Monensin	Cattle
	Narasin	Cattle
	Salinomycin	Pigs, cattle
Polypeptides	Bacitracin	Meat poultry
Quinoxalines	Olaquindox	Pigs
Streptogramins	Virginiamycin	Pigs, meat poultry
Flavophospholipols	Moenomycin	Pigs, poultry, cattle

*Avoparcin has been removed from the market since 2000

From Silbergeld et al., 2008; Barton, 2000

Table 1-6. Characterization of antimicrobials used in this study

Antimicrobial	Family	Human medicine	Veterinary medicine	Animal husbandry
Ampicillin	Beta-lactam	Yes	Yes	Yes
Avilamycin	Oligosaccharide	No	Yes	Yes
Avoparcin	Glycopeptide	No	Yes	Yes
Bacitracin	Cyclic polypeptide	Yes	Yes	Yes
Flavophospholipol	phosphoglycolipid	No	No	Yes
Gentamicin	Aminoglycoside	Yes	Yes	No
Narasin	Ionophore	No	No	Yes
Tetracycline	Cycline	Yes	Yes	Yes
Tiamulin	Pleuromutilin	No	Yes	Yes
Tylosin	Macrolide	No	Yes	Yes
Vancomycin	Glycopeptide	Yes	No	No
Virginiamycin	Streptogramin	No	Yes	Yes
Copper	Heavy metal	Yes	Yes	Yes
Zinc	Heavy metal	Yes	Yes	Yes

From Courvalin et al., 2010; Guardabassi et al., 2008; WHO, 2007; Walsh, 2003; Lewis et al., 2002; Prescott et al., 2000; Pratt & Fekety, 1986

Table 1-7. Importance of antimicrobials used in this study

Drug	M	V	Feed	Comment
AMP	No	Yes	-	WHO rating: High, EAGAR rating: Low, JETACAR rating: High
AVI	No	No	+	-
AVO	Yes	Yes	-	Cross resistance to VAN, JETACAR rating: High
BAC	No	Yes	+	WHO rating: Low, EAGAR rating: Low, JETACAR rating: High
FLA	No	No	+	-
GEN	Yes	Yes	-	WHO rating: High, EAGAR rating: Medium
NAR	-	No	+	JETACAR rating: Medium
TET	No	Yes	-	WHO rating: High, JETACAR rating: High
TIA	Yes	Yes	+	Similar to macrolides
TYL	Yes	Yes	+	JETACAR rating: High
VAN	Yes	Yes	-	WHO rating: High
VIR	Yes	Yes	+	VRE (cross ABR to synercid, WHO and JETACAR rating: High)
Cu	No	No	+	AMR importance is still unclear
Zn	No	No	+	AMR importance is still unclear

M, importance in medicine; V, importance in veterinary medicine; Feed, Animal feed; VRE, vancomycin resistant enterococci; AMR, antimicrobial resistance; ABR, antibiotic resistance; AMP, ampicillin; AVI, avilamycin; AVO, avoparcin; BAC, bacitracin; FLA, flavophospholipol; GEN, gentamicin; NAR, narasin; TET, tetracycline; TIA, tiamulin; TYL, tylosin; VAN, vancomycin; VIR, virginiamycin; Cu, copper; Zn, zinc; EAGAR, Expert Advisory Group on Antimicrobial Resistance; JETACAR, The Joint Expert Technical Advisory Committee on Antibiotic Resistance; WHO, World Health Organization
From WHO, 2007; EAGAR, 2006; JETACAR, 2000

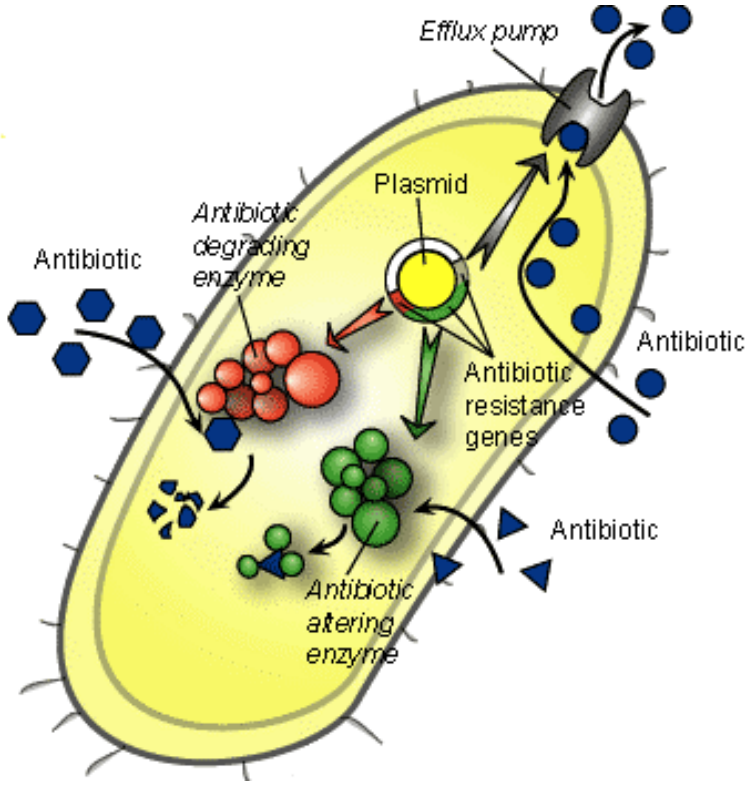


Figure 1-3. Mechanisms of antimicrobial resistance in bacteria

From Todar, 2008

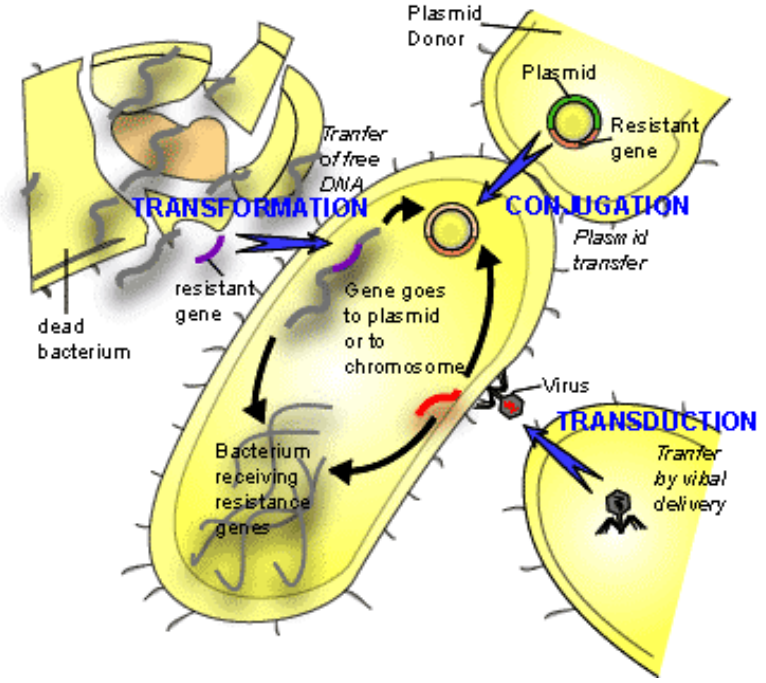


Figure 1-4. Mechanisms of horizontal gene transfer in bacteria

From Todar, 2008

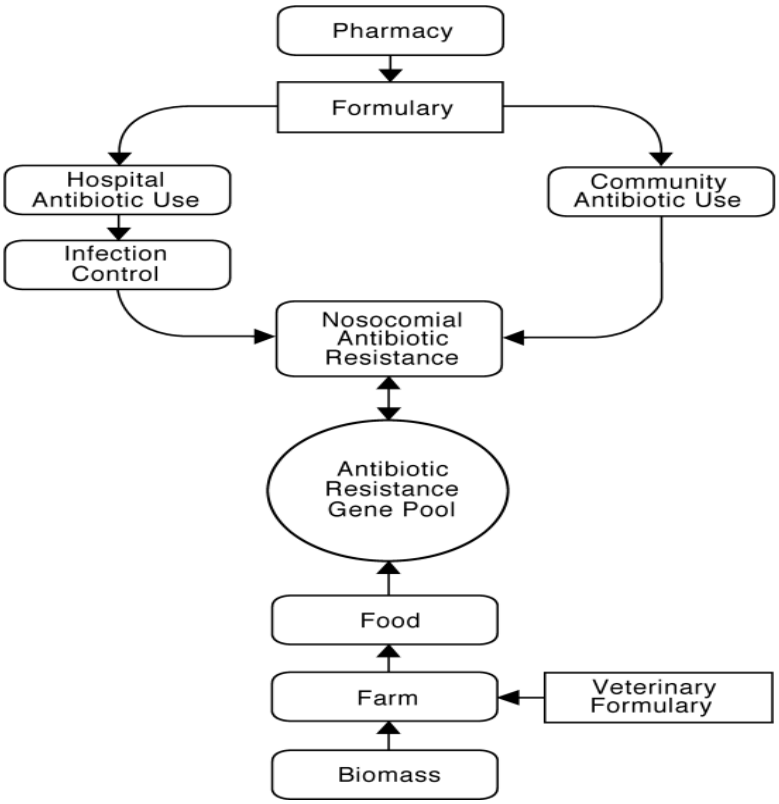


Figure 1-5. Epidemiology of antimicrobial resistance

From Gelone, 2002

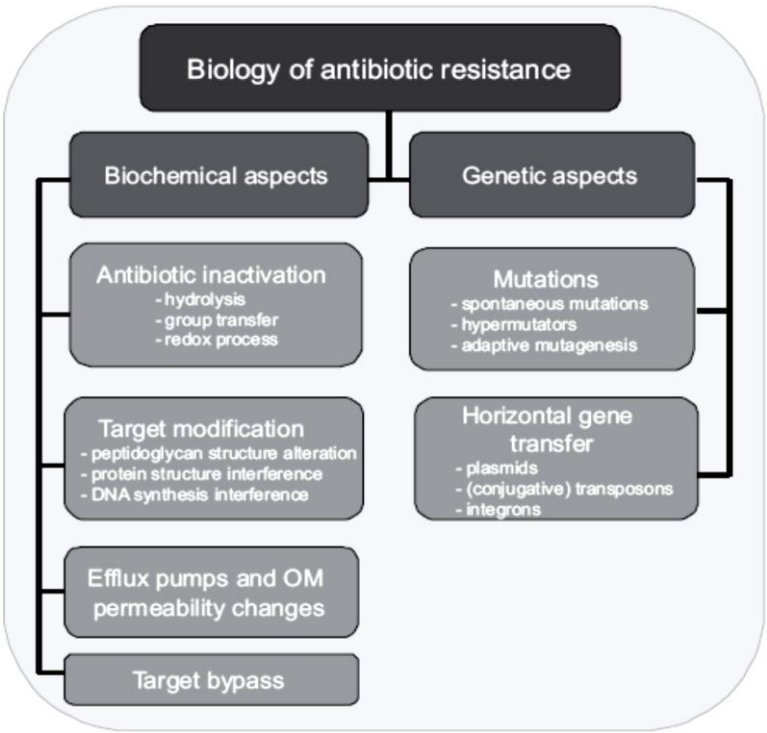


Figure 1-6. Biology of antibiotic resistance

From Dzidic et al., 2008

Table 1-8. Taxonomy and classification of bacteriophages

Order	Family	Specifications
No order	*Corticiviridae*	Non-enveloped, icosahedral capsid with lipid layer, circular supercoiled dsDNA
No order	*Cystoviridae*	Enveloped, icosahedral capsid, lipids, three molecules of linear dsRNA
No order	*Guttaviridae*	Pleomorphic, envelope, protrusions, beard of long fibers, circular dsDNA
No order	*Fuselloviridae*	Pleomorphic, envelope, lipids, capsid, protrusions, short tail-like fibers, circular supercoiled dsDNA
No order	*Inoviridae,* genus *Inovirus*	Non-enveloped, long filaments with helical symmetry, circular ssDNA
No order	*Inoviridae,* genus *Plectrovirus*	Non-enveloped, short rods with helical symmetry, circular ssDNA
No order	*Leviviridae*	Non-enveloped, quasi-icosahedral capsid, one molecule of linear ssRNA
No order	*Lipothrixviridae*	Enveloped filaments, lipids, linear dsDNA
No order	*Microviridae*	Non-enveloped, icosahedral capsid, circular ssDNA
Caudovirales	*Myoviridae,* A1*	Non-enveloped, tail contractile, head isometric, linear dsDNA
Caudovirales	*Myoviridae,* A2	Non-enveloped, tail contractile, head elongated (length/width ratio = 1.3−1.8), linear dsDNA
Caudovirales	*Myoviridae,* A3	Non-enveloped, tail contractile, head elongated (length/width ratio ≥ 2), linear dsDNA
No order	*Plasmaviridae*	Pleomorphic, envelope, lipids, no capsid, circular supercoiled dsDNA
Caudovirales	*Podoviridae,* C1*	Non-enveloped, tail short and noncontractile, head isometric, linear dsDNA
Caudovirales	*Podoviridae,* C2	Non-enveloped, tail short and noncontractile, head elongated (length/width ratio = 1.4), linear dsDNA
Caudovirales	*Podoviridae,* C3*	Non-enveloped, tail short and noncontractile, head elongated (length/width ratio ≥ 2.5), linear dsDNA
No order	*Rudiviridae*	Non-enveloped, helical rods, linear dsDNA
Caudovirales	*Siphoviridae,* B1*	Non-enveloped, tail long and noncontractile, head isometric, linear dsDNA
Caudovirales	*Siphoviridae,* B2*	Non-enveloped, tail long and noncontractile, head elongated (L/W ratio = 1.2−2), linear dsDNA
Caudovirales	*Siphoviridae,* B3*	Non-enveloped, tail long and noncontractile, head elongated (length/width ratio ≥ 2.5), linear dsDNA
No order	*Tectiviridae*	Non-enveloped, icosahedral capsid with inner lipoprotein vesicle, linear dsDNA, tail produced for DNA injection, linear dsDNA
Unassigned	*Salterprovirus*	Pleomorphic, envelope, capsid, short tail-like fibers, linear dsDNA

*Identified in enterococci

From ICTV, 2008

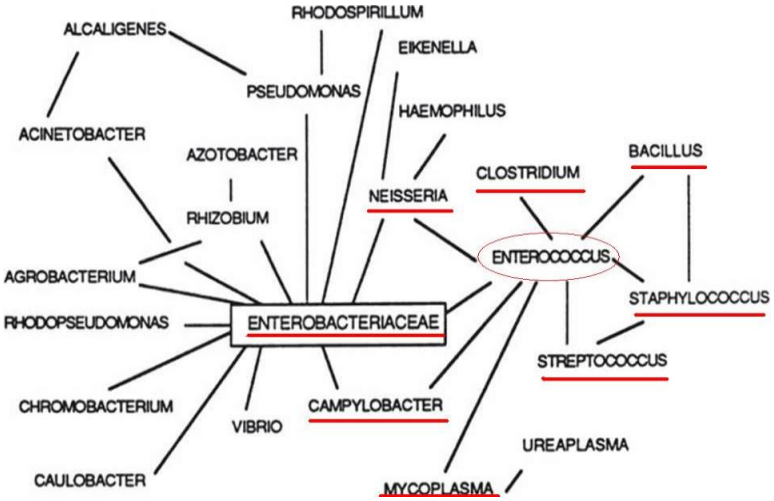


Figure 1-7. The genetic exchange between bacteria

From Silbergeld et al., 2008

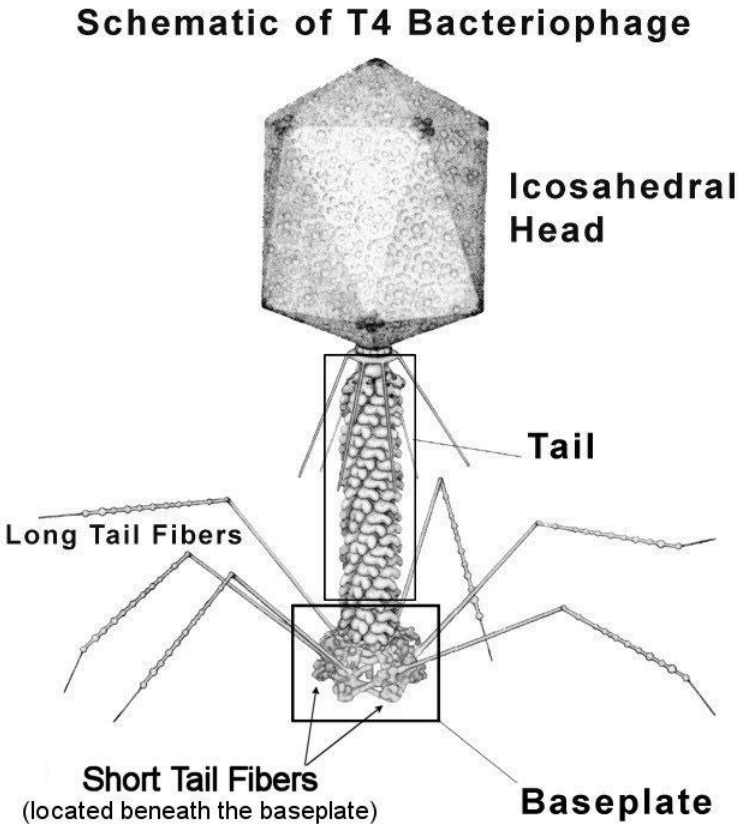


Figure 1-8. Schematic of a tailed bacteriophage

From National Science Foundation website

http://www.nsf.gov/od/lpa/news/02/pr0207.htm

Chapter 2

General Materials and Methods

Maintenance of isolates

Bacterial cultures were transferred into glycerol broth and stored in a -80°C freezer for long-term maintenance. These bacteria were recovered by culturing on Blood Columbia Agar or Tryptone Soy Agar (TSA) plates and overnight incubation at 37°C with 5% CO_2. The media and the recipes used in this study are shown in Appendix 2.

Molecular identification

Isolates were typed to genus level by PCR, using genus-specific primers. Bacterial DNAs were extracted by a boiling method. A few colonies of each isolate cultivated overnight on Blood Columbia Agar were suspended in 200µL of sterile distilled water. Bacterial cells were heated at 95°C for 20 minutes. The mixture was then centrifuged at 7500 g for five minutes and the supernatant containing DNA was used as a template for PCR. Extractions were stored at -20°C until used. To prepare PCR Master Mix, 5 µL of 5× PCR buffer, 1 µL of 25 mM $MgCl_2$, 0.2 µL of 25 mM dNTPs, 1 µL of each primer in a concentration of 10 pmol and 0.2 µL of 5 U/µL Taq DNA polymerase were mixed. A sufficient amount of sterile distilled water was added to the mixture to make a total volume of 23 µL. Then, 2 µL of extracted DNA were added as a template (modified from Hartman et al., 2003). Following an initial denaturation at 95°C for three minutes, products were amplified by 30 cycles of denaturation at 95°C for 30 seconds, annealing at different temperatures for 30 seconds and extension at 72°C for one minute. Amplification was followed by a final extension at 72°C for five minutes (Jackson et al., 2004a). Negative and positive controls were performed for each PCR experiment. PCR products were detected by electrophoresis in 1 µg/mL ethidium bromide-stained 1% agarose gels in 0.5× TBE buffer at 100 V for 90 minutes and then by visualizing under the UV light.

Antimicrobial susceptibility testing

All enterococcal isolates were phenotypically tested for susceptibility to ampicillin, avilamycin, avoparcin, bacitracin, gentamicin, flavophospholipol, narasin, tetracycline, tiamulin, tylosin, vancomycin, virginiamycin, copper and zinc by minimum inhibitory concentration (MIC), according to the approved standard procedure of Clinical and

Laboratory Standards Institute (CLSI, 2008). This was carried out using an agar dilution method. The results were interpreted on the basis of the breakpoints published by CLSI and other authorities including DANMAP (2005), NARMS (2004) and NORM/NORM-VET (2004). These well-established references were chosen when isolates from animal origin were tested since CLSI mostly publishes breakpoints for human isolates. Furthermore, the decision was made to use these widely-accepted breakpoints rather than developing own breakpoints. These selected studies used isolates of the same genera obtained from the same animal species. Briefly, different concentrations of antimicrobials were added to Mueller-Hinton agar media and poured into petri dishes. Then, a single colony of a pure culture on Tryptone Soy Agar (TSA) was selected and suspended in sterile normal saline to a turbidity equivalent to 0.5 McFarland. This suspension was diluted again 1:10 in sterile saline to make a final concentration of 10^7 cfu/mL and inoculated onto the Mueller-Hinton plates by a replicator. The inoculated plates were incubated at 35–37°C for 16–24 hours and then were read.

PCR amplification of resistance genes

Polymerase chain reaction (Single and Multiplex PCR) was used for the detection of antibiotic resistant genes in enterococci, using specific primers. These primers were categorized into six groups based on their annealing temperatures and sequence characteristics. The PCR protocol was modified and optimized for antibiotic resistant genes in each group. The general protocol used for PCR is given as follows: A few fresh enterococcal colonies on Blood Columbia Agar were suspended in 200 µL of sterile distilled water. Bacterial cells were heated at 95°C for 20minutes for DNA extraction. The mixture was then centrifuged at 7500 g for five minutes and the supernatant was collected. To prepare 25 µL of Master Mix for each sample, 5 µL of 5× PCR buffer, 1 µL of 25 mM $MgCl_2$, 0.2 µL of 25 mM dNTPs, 1 µL of each primer in 10 pmol concentration and 0.2 µL of 5 U/µL Taq DNA polymerase were mixed in a sterile microtube. A sufficient amount of sterile distilled water was added to this mixture to reach the total volume of 23 µL and then 2 µL of extracted DNA was added (modified from Hartman et al., 2003). The thermocycler was run with an initial denaturation for five minutes at 95°C, then followed by 35 cycles; each cycle included denaturation at 95°C for 30 seconds, 30-second annealing at various temperatures based on the primer melting temperature, and extension at 72°C for one minute. Final extension was carried out at 72°C for five minutes. Negative and positive (if applicable) controls were included in each gel electrophoresis. Amplicons were detected by electrophoresis in 1 µg/mL ethidium

bromide-stained 1% agarose gels in 0.5× TBE buffer at 100 V for 90 minutes and then by visualizing under the UV light.

Gene sequencing

Amplified DNA of some isolates, representing each antibiotic resistance gene, was sequenced and the results were compared with GenBank data. DNA of bacteria was purified according to the following protocol before sequencing. Five volumes of bind solution (UltraClean PCR Cleanup Kit, Mo Bio Laboratories Inc, USA) was added to the PCR product and transferred to a spin filter unit. The mixture was centrifuged at 10,000 g for 10−30 seconds then the column was removed and the liquid was discarded. The column was replaced and 300 µL of clean buffer was added to the filter. The filter was centrifuged at 10,000 g for 10−30 seconds then the column was removed and the liquid was discarded. The column was replaced and centrifuged at 10,000 g for 10−30 seconds. Then, the column was transferred to a new tube and 50 µL of elution buffer (10 mM Tris) solution were added. This was left for five minutes and then was centrifuged at 10,000 g for 10−30 seconds and the DNA was collected.

Chapter 3

Antimicrobial Resistance in Enterococci

Introduction

Antimicrobial resistance of enterococci is a significant medical problem due to its prevalence, costs and consequences for the patients and the societies (van den Bogaard et al., 2002a; Yee, 1997). Prevalence of antimicrobial resistance is also increasing in animal isolates probably as a result of extensive use of antimicrobial agents in past decades in addition to intrinsic bacterial resistance (Hershberger et al., 2005; Sørensen et al., 2001). Multiple resistant isolates are commonly found among enterococci (Mundy et al., 2000; Pepper et al., 1987). They are not only inherently resistant to many antibiotics, but also spread the antibiotic resistance by transferring genetic elements (acquired resistance) within the genus members (Nallapareddy et al., 2005; Sillanpaa et al., 2004; Huycke et al., 1998). Examples of intrinsic antibiotic resistance include resistance to penicillinase-susceptible penicillin (low level), penicillinase-resistant penicillins, cephalosporins, lincosamides, nalidixic acid, aminoglycosides (low level) and clindamycin (low level). Examples of acquired antibiotic resistance include resistance to some penicillins, vancomycin, chloramphenicol, erythromycin, tetracycline, fluroquinolone, aminoglycosides (HLAR) (high level) and clindamycin (high level) (Marothi et al., 2005). Enterococci have been recognized as emerging pathogens in recent years (Tendolkar et al., 2003). They are repeatedly reported as the cause of different human and animal infections (Nicklas et al., 2010; Canalejo et al., 2008; Raja et al., 2006; Klare et al., 2003). Examples of increased animal infections in recent decades include enteroadherent bacterial infections in pre-weaning-age kittens and diarrhoea in calves, foals, piglets and puppies (Nicklas et al., 2010; Cheon & Chae, 1996; Rogers et al., 1992; Collins et al., 1988; Tzipori et al., 1984). Treatment of resistant enterococcal infections is considered to impose a significant impact on a country's health budget (Donovan, 2001). These facts highlight the importance of monitoring antimicrobial resistance in enterococci. In this chapter, the antimicrobial resistance profile of 230 enterococcal isolates to twelve antibiotics and two heavy metals was assessed using MIC and PCR of relevant resistance genes.

Materials and Methods

Bacterial strains

Two-hundred and thirty enterococcal isolates from pigs (*n* = 207), chickens (*n* = 15) and human faecal specimens (*n* = 8) collected by University of South Australia and the Women's and Children's Hospital (WCH), Adelaide, were used in this study (Appendix 1). These included 84 *E. faecalis* (36.5%), 88 *E. faecium* (38.3%), 22 *E. gallinarum* (9.6%), 14 *E. casseliflavus* (6.1%), 17 *E. hirae*/durans (undifferentiated) (7.4%), two *E. hirae* (0.9%), two *E. raffinosus* (0.9%) and one unassigned (0.3%) isolates. The enterococcal isolates used in this study are listed in Table 3-3.

The pig isolates had been obtained in 2002–2005 from caecal contents of pigs slaughtered at a South Australian pig abattoir (Barton, 2005). The chicken isolates were obtained in 1999–2000 from chicken carcass rinse samples from a major chicken company with producers in many states of Australia (Barton & Wilkins, 2001). Human isolates were collected from healthy human volunteers in 2001 (Hart & Barton, 2002).

All isolates had previously been identified to the species level using differential culture media, biochemical tests and in the case of *E. faecalis* and *E. faecium*, by species-specific PCR primers.

Maintenance of cultures

Bacterial cultures were transferred into glycerol broth and stored in -80°C freezer for long-term storage. These frozen bacteria were recovered when needed by culturing on blood enriched Columbia Agar (Oxoid, Australia) or Tryptone Soy Agar (TSA) (Oxoid, Australia) plates and overnight incubation at 37°C with 5% CO_2 atmosphere. The culture media and buffers used in this study are described in Appendix 2.

Molecular identification

To confirm the identification of the bacterial isolates to be used in the study, all isolates were typed to genus level using genus-specific PCR primers. Bacterial DNA was extracted by heating. Each isolate was subcultured overnight on Blood Columbia Agar and a few colonies suspended in 200 μL of sterile distilled water. Bacterial suspensions were heated at 95°C for 20 minutes. The mixture was then centrifuged at 7500 g for five minutes and the supernatant containing DNA was used as a template for PCR. Extractions were stored at -20°C until used. Isolates were typed to genus level by PCR, using genus-specific

primers (Ke et al., 1999). This included the forward primer Ent1: 5'-TACTGACAAACCATTCATGATG-3' and the reverse primer Ent2: 5'-AACTTCGTCACCAACGCGAAC-3' targeting the *tuf* gene and producing an 112-bp band. To prepare a final volume of 25 µL PCR Master Mix for each sample, 5 µL of 5× PCR buffer, 1 µL of 25 mM $MgCl_2$, 0.2 µL of 25 mM dNTPs, 1 µL of each primer in a total concentration of 10 pmol and 0.2 µL of 5 U/µL Taq DNA polymerase (Promega, USA) were mixed in a sterile microtube. Sterile distilled water was added to the mixture to make a total volume of 23 µL. Then, 2 µL of extracted DNA were added as a template (Table 3-1) (modified from Hartman et al., 2003). Following an initial denaturation at 95°C for three minutes, products were amplified by 30 cycles of denaturation at 95°C for 30 seconds, annealing at different temperatures for 30 seconds and extension at 72°C for one minute. Amplification was followed by a final extension at 72°C for five minutes (Jackson et al., 2004a) (Table 3-1). One sample without DNA and when applicable one sample containing control enterococcal DNA were respectively used as negative and positive controls for each PCR. PCR products were detected by electrophoresis in 1 µg/mL ethidium bromide-stained 1% agarose gels in 0.5× TBE buffer at 100 V for 90 minutes and then by visualizing under the UV light (Figure 3-1). Two microlitres of 100 bp DNA ladder were loaded as marker for each gel electrophoresis. Weak putative amplified bands were rechecked by repeating PCR.

Principles for PCR assay design

The following issues were considered in designing the PCRs.

1) Usually 5 µL of 5× buffer were used,

2) 1 to 4 mM (usually 1) of $MgCl_2$ were used,

3) Usually 0.2 mM of dNTPs was used,

4) Usually 0.4 pmol of each primer was used,

5) 0.5 to 1.25 U(s) (usually one) of Taq DNA polymerase were used,

6) 10 pg to 1 µg of purified DNA were used,

7) Initial denaturation was set between one to three minutes (up to ten minutes for high GC content) at 95°C,

8) 25 to 30 cycles (40 cycles for high GC content) were set,

9) Denaturation was set 30 seconds to 2 minutes at 94 or 95°C,

10) Annealing was usually set 5°C below the lowest melting temperature (Tm) for 30 seconds to two minutes,

11) Extension was set one minute at 70 to 75°C (usually 72),

12) Final extension was set five to 15 minutes at 72°C,

13) 0.7 to 2−3% (usually one) agarose gels were used,

14) 0.5% TBE or 1% TAE (preferably TBE) was used for gel electrophoresis,

15) 0.5 to 1 μg/mL (usually 1) ethidium bromide was used for gel staining.

Antimicrobial susceptibility testing

In determining enterococci antimicrobial resistance patterns, all enterococcal isolates were tested against ampicillin, avilamycin, avoparcin, bacitracin, gentamicin, flavophospholipol, narasin, tetracycline, tiamulin, tylosin, vancomycin, virginiamycin, copper and zinc by minimum inhibitory concentration (MIC) methodology, according to the approved standard procedure of Clinical and Laboratory Standards Institute (CLSI, 2008). Antibiotics were obtained from Sigma-Aldrich, USA, except those exclusively used in veterinary medicine or animal husbandry, including avilamycin, narasin and virginiamycin. Flavophospholipol was provided by International Animal Health Products, Sydney, Australia.

Agar dilution method

The agar dilution method for determining antimicrobial susceptibility is a well-established technique. To make a working solution, an appropriate amount of each antibiotic and heavy metal was calculated using this formula.

$$\text{Weight (mg)} = \frac{\text{Volume (mL)} \times \text{Concentration (μg/mL)}}{\text{Assay Potency (μg/mg)}}$$

Or

$$\text{Volume (mL)} = \frac{\text{Weight (mg)} \times \text{Assay Potency (μg/mg)}}{\text{Concentration (μg/mL)}}$$

Appropriate concentrations of working solutions were made and added to 20 mL of molten Mueller-Hinton Agar (Oxoid, Australia) and then poured into petri dishes to make plates containing serial dilutions of the antimicrobial agents (Table 3-5). The number of concentrations tested for a particular antimicrobial agent encompassed the interpretive breakpoints described in CLSI guidelines. Then, a single colony of pure culture on Tryptone Soy Agar (TSA) was selected and resuspended in sterile saline to make the desired turbidity, which was compared with a 0.5 McFarland Standard. Generally, the 0.5 McFarland suspensions were diluted 1:10 in sterile saline to make a final concentration of 10^7 cfu/mL and then were inoculated onto the prepared Mueller-Hinton Agar plates, using a replication device. Optimally, the final inocula on the agar would then contain approximately 10^4 cfu per spot. The inoculated plates were incubated at 35–37^{o}C for 16–24 hours and then were read (Lorian, 2005; Wentworth, 1987) (Figure 3-2). The end points were determined and MIC results reported based on CLSI (2008), DANMAP (2005), NARMS (2004) and NORM/NORM-VET (2004) reports (Table 3-2). *E. coli* ATCC 25929, *E. faecalis* ATCC 29212, *E. faecium* ATCC 19434, *S. aureus* ATCC 25923 and *S. aureus* ATCC 29213 were used in this study as control bacteria.

The resistance of isolates to copper was tested on Mueller-Hinton Agar plates containing serial dilutions of copper sulfate ($CuSO_4.5H_2O$) (2, 4, 5, 6, 7, 8, 12, 16, 20, 24, 28 and 32 mM/mL) adjusted to pH 7 with 1 M NaOH. The resistance breakpoints for copper sulphate were considered ≤ 2 to ≥ 28 mM/mL. The plates were then incubated at 37°C for 16–20 hours and growth was observed (Hasman et al., 2006). Susceptibility to zinc was tested on Mueller-Hinton Agar plates containing serial dilutions of zinc chloride (2, 4, 8, 12, 16 mM/mL) with the pH adjusted to 5.5 (Aarestrup & Hasman, 2004). The resistance breakpoints for zinc chloride were considered ≤ 4 to ≥ 12 mM/mL.

Results

Detection of phenotypic antimicrobial resistance

Overall, the enterococcal isolates (except one *E. hirae* of pig origin) were multiple resistant to two or more antimicrobials as enterococci are naturally resistant to many antimicrobials, although patterns differ between the various species. All isolates (100%) were sensitive to ampicillin, avilamycin, gentamicin (except one *E. faecium* isolate of human origin) and narasin, but resistant to tetracycline (except one *E. hirae* isolate of pig origin) and tylosin (except one *E. hirae* isolate of pig origin). All enterococcal isolates (except one *E. gallinarum* isolate of human origin) were intermediately resistant to copper.

Most of the isolates were sensitive to avoparcin (including one *E. faecalis* and 12 *E. faecium*), vancomycin (including 12 *E. casseliflavus* and 12 *E. faecium*), virginiamycin (including one *E. casseliflavus*, 53 *E. faecalis*, 16 *E. faecium*, one *E. hirae* and seven *E. hirae/durans*) and zinc (including 78 *E. faecalis*, four *E. faecium* and two *E. raffinosus*) but resistant to bacitracin (including 12 *E. casseliflavus*, 29 *E. faecalis*, 76 *E. faecium*, 21 *E. gallinarum*, 14 *E. hirae/durans* and two *E.raffinosus*) and tiamulin (including four *E. casseliflavus*, 80 *E. faecalis*, 59 *E. faecium*, 21 *E. gallinarum*, nine *E. hirae/durans* and two *E. raffinosus*). All avoparcin resistant isolates (except one *E. faecalis* and one *E. faecium* isolates of human origin) were from chickens. The least resistance to bacitracin was detected in human isolates ($P < 0.05$). No resistance to vancomycin was seen in human isolates. Resistance to flavophospholipol was detected in half of the isolates (including ten *E. casseliflavus*, two *E. faecalis*, 73 *E. faecium*, 15 *E. gallinarum*, one *E. hirae*, 13 *E. hirae/durans* and one unassigned isolates), while fewer chicken isolates were resistant to flavophospholipol ($P < 0.05$). Results are shown in Appendices 3 and 5–18, Tables 3-4 and 3-5, and Figure 3-3.

The raw data were statistically analyzed using a Chi-Square Test and *P* values included.

Discussion

In general, all enterococcal isolates (except one *E. hirae* of pig origin) were multiple resistant to two or more antimicrobials, from which 100% were found sensitive to ampicillin, avilamycin and narasin. In Australia, ampicillin is registered for use in poultry, narasin in cattle and poultry and avilamycin in poultry and swine. Furthermore, ampicillin is used in human and veterinary medicine and avilamycin is used only in veterinary medicine. However, narasin is not used in human medicine because of high toxicity for eukaryotic cells (Butaye et al., 2003). Hence, the lack of resistance to narasin may be due to the limited use of these antibiotics in restricted target groups. These antibiotics are not used or important in human medicine.

Resistance to ampicillin

No resistance to ampicillin was found in isolates, which is similar to findings by other researchers. Hart et al. (2004) reported that no ampicillin resistance was seen in enterococcal isolates associated with pigs in South Australia in contrast to *E. coli* isolates (Hart et al., 2004). Furthermore, the prevalence of ampicillin resistance has also been limited in other studies from elsewhere such as Europe and New Zealand (de Jong et al.,

2009; Nulsen et al., 2008). In Australia, a survey by DAFF (2007) showed that ampicillin resistance was not broadly prevalent in enterococcal isolates of animal origin. Only 3.3% of pig *E. faecium*, 2.6% of a combination of pig *E. hirae* and *E. casseliflavus* isolates and 4.9% of chicken *E. faecium* were resistant to ampicillin.

Resistance to glycopeptides

Only a low percentage (6%) of resistance to avoparcin was detected; all in chicken *E. faecium* (100%), except one *E. faecalis* of human origin. Avoparcin was used in Australian livestock (Collignon, 1999), but has been banned since 2000 (NDPSC, 2003). The resistance to avoparcin in chicken isolates was probably due to the previous use of avoparcin in animal husbandry (Cooper, 2000; Collignon, 1999) and the fact that these isolates had been collected before the ban on use of avoparcin. Avoparcin resistance seen in one human isolate may be due to use of vancomycin in human medicine. Differences in results for avoparcin and vancomycin were found. This is not surprising because a similar difference has been reported in variety of papers. For example, a study by Cormican et al. (1997) has shown that although cross-resistance between avoparcin and other glycopeptides existed, a perfect correlation was not observed. Avoparcin was significantly less active than vancomycin or teicoplanin against human isolates of staphylococci. This might be seen due to a second mechanism for vancomycin resistance. Another reason for this difference could be the difference between MIC breakpoints, which are ≥ 16 and < 16 μg/mL for avoparcin, and ≥ 8 and ≤ 4 μg/mL for vancomycin and also assessment of resistance/susceptibility of organisms with MICs close to breakpoint values. Fourty-five isolates, including 12 *E. casseliflavus*, 12 *E. faecium* and 21 *E. gallinarum*, with vancomycin resistance were investigated, of which 12 (100%) were isolated from chickens; however, *E. casseliflavus* and *E. gallinarum* species are known to be naturally resistant to vancomycin. Vancomycin has never been used in animal feed, therefore all resistance to this antibiotic in chickens must be due to cross-resistance with avoparcin; both of which belong to the glycopeptide class of antibiotics (Lu et al., 2004). However, vancomycin resistance was detected more frequently in *E. faecium* than *E. faecalis* (12 instead of zero) ($P < 0.05$) as reported by other researchers (Tenover & McDonald, 2005). Furthermore, studies have shown a significant decrease in the prevalence of vancomycin resistance in *Enterococcus* spp. when the effectiveness of avoparcin ban on the prevalence of vancomycin resistance in enterococci was evaluated (Lauderdale et al., 2007; van den Bogaard et al., 2000). In Australia, avoparcin was used in animal husbandry as a growth promoter (Collignon, 1999) but was withdrawn from the market in 2000 (Turnidge, 2001).

However, other reports reveal that vancomycin resistance can persist in enterococci years after the ban of avoparcin use in animals (Lim et al., 2006; Johnsen et al., 2005; Butaye et al., 1999) and in some cases, may relate to co-selection from the use of tetracycline or tylosin (Hammerum et al., 2004). All vancomycin resistant isolates within this study were resistant to tetracycline and tylosin as well. However, there are a few reports on vancomycin-resistant enterococcal isolates of animal origin. One study carried out by Hart et al. (2004) showed no resistance to avoparcin or vancomycin in pig-related enterococci two years after the ban of avoparcin use in Australian livestock. In a similar study carried out by DAFF (2007), no resistance to vancomycin was found in enterococci isolated from either pigs or chickens (except one chicken *E. faecalis* isolate with low-level resistance encoding the *van*C gene) three years after the ban of avoparcin use in animal husbandry in Australia. Vancomycin is of high importance to human medicine.

Resistance to bacitracin

Resistance to bacitracin was found in 153 isolates, including all species (*E. faecalis*, *E. faecium*, *E. hirae/durans*, *E. casseliflavus*, *E. gallinarum* and *E. raffinosus*). This resistance was always seen in conjunction with other antimicrobial resistance (mostly flavophospholipol, tetracycline, tiamulin and tylosin); nearly all chicken isolates (*E. faecium*) were resistant. Bacitracin resistance has been reported previously in enterococci isolated from Australian broiler chickens (Barton & Wilkins, 2001). Bywater et al. (2005) investigated the prevalence of resistance in 1742 *E. faecium* isolates from pigs in four European countries. Resistance to bacitracin was common in all countries. Interestingly, the resistance rate to bacitracin has not decreased after the removal of this antibiotic from the market in Europe in contrast to other antibiotics. A high rate (up to 100%) of resistance to bacitracin was seen in both *E. faecalis* and *E. faecium* (Bywater et al., 2005). Further, the bacitracin MIC for *E. faecium* usually shows a rather extended range compared to *E. faecalis* (Butaye et al., 2001). This indicates that different resistance mechanisms might be in place. Relatively, the lowest level of bacitracin resistance was detected in *E. faecalis* isolates. Only one resistant isolate (*E. faecalis*) was found within human isolates that might reflect the fact that bacitracin is not used extensively in human medicine.

Resistance to gentamicin

No enterococci (except one *E. faecium* isolate of human origin) showed high-level (1024 µg/mL) resistance to gentamicin. This is in contrast with other studies, which mostly

reveal the prevalence of high-level gentamicin resistance. The prevalence of high-level gentamicin resistance (HLGR) strains varies from location to location, but is generally high (Brown et al., 2008; Jeljaszewicz et al., 2000). van den Bogaard et al. (2002a) reported a significant percentage of high-level resistance in broilers and laying-hens. Similarly, Novais et al. (2005) found that 34% of enterococci isolated from poultry feed samples in Portugal were highly resistant to gentamicin. US studies have reported a high prevalence of high-level gentamicin resistance in enterococci from pigs (Jackson et al., 2005; Donabedian et al., 2003a). However, a report by Fracalanzza et al. (2007) showed a very low percentage (1.8%) of HGR in different enterococcal species isolated from poultry. However, use of gentamicin in livestock in Australia is banned. This has probably influenced the absence of prevalence of high-level gentamicin resistance in the current study. In the DAFF study in 2007, a high-level resistance to gentamicin was not detected in enterococci isolated from pigs or in those isolated from chickens. The occurrence of gentamicin resistant enterococci (GRE) in food animals may also be associated with the use of the glycopeptide antibiotic avoparcin (Butaye et al., 2003) due to co-selection of resistance determinants. In the current study, avoparcin resistance was found predominantly in chicken isolates and less in human isolates ($P < 0.05$). Furthermore, the spread of GRE in food animals may occur due to the use of apramycin as isolates resistant to apramycin are also resistant to gentamicin and always mediated by *aac*(3)-IV genes (Jensen et al., 2006). Apramycin is registered in Australia for use in pigs. Gentamicin is of medium importance to human medicine.

Resistance to tetracycline

All enterococci (except one *E. hirae* isolate of human origin) were found resistant to tetracycline, which is the most prevalent form of bacterial resistance to antimicrobials in nature (Patterson et al., 2007). Tetracycline has been widely used in animal feeds but is not as important as in human medicine as previously. The widespread (> 99%) tetracycline resistance seen in isolates maybe related to past use of tetracycline in animals for prophylaxis (Lukasova & Sustackova, 2003; Barton & Wilkins, 2001) and the use of some cycline antibiotics such as minocycline and doxycycline in humans (Ross, 2003). However, the tetracycline resistant isolates were often resistant to other antimicrobial agents, especially tiamulin, tylosin, vancomycin and copper.

Resistance to tiamulin

A high percentage of enterococcal isolates, including all species (*E. faecalis*, *E. faecium*, *E. hirae/durans*, *E. casseliflavus*, *E. gallinarum* and *E. raffinosus*), were found to be resistant to tiamulin—most of the sensitive isolates were *E. faecium* ($P < 0.05$). This antibiotic is exclusively used in veterinary medicine, largely in swine (CVMP, 2008). Tiamulin has a similar action to macrolides. Therefore, the mechanism of resistance to both antibiotics is similar (Bøsling et al., 2003). Most tiamulin resistant isolates were also resistant to tylosin. Hart et al. (2004) found that a proportion of tiamulin resistant enterococcal isolates associated with pigs were resistant to tylosin as well.

Resistance to tylosin

All enterococci (except one *E. hirae* isolate of pig origin) were resistant to tylosin. Resistance to macrolides is widespread in isolates from swine fed diets containing tylosin. Tylosin is fully cross-resistant with erythromycin (Manson et al., 2004b; van den Bogaard et al., 2002a; Aarestrup & Carstensen, 1998). Martel et al. (2003) found resistance to both tylosin and erythromycin in nearly all enterococci isolated from 33 pigs from 33 farms. Similar results were found within streptococcal isolates. In another study, Jackson et al. (2004b) reported that a proportion of enterococcal strains isolated from the farm using tylosin for growth promotion or treatment were resistant to erythromycin as well. About 95% of resistance within the isolates was encoded by *erm*B genes. A study conducted by Boerlin et al. (2001) showed a significant drop in resistance to macrolides in enterococci isolated from pigs after the complete ban of antimicrobial growth promoters in Switzerland. A genetic linkage has also been found between the glycopeptide and macrolide resistance amongst enterococci from pigs (Aarestrup, 2000). However, antimicrobial susceptibility results have showed that enterococcal isolates from cattle fed with monensin and tylosin had a greater level of resistance to macrolides (Jacob et al., 2008).

Resistance to virginiamycin

Seventy-eight enterococci were resistant to virginiamycin including one *E. casseliflavus*, 53 *E. faecalis*, 16 *E. faecium*, one *E. hirae* and seven *E. hirae/durans*, with one being a human isolate (*E. faecalis*). The relatively low prevalence (34%) of virginiamycin resistance was not surprising as this antibiotic is rarely used in pigs. However, the resistance rates to virginiamycin vary in different reports depending on the region and

species (de Jong et al., 2009; Bywater et al., 2005). *E. faecalis* has intrinsic reduced sensitivity to virginiamycin (natural insensitivity) (Bouanchaud, 1997) and in this study, only one third of isolates were sensitive. All of the virginiamycin sensitive isolates were resistant (two intermediately resistant) to tylosin since cross-resistance to MLS_B antibiotics is seen in bacteria. In a study on the resistance profile of *E. faecium* isolates from pigs and chickens to growth-promoting antibiotics (e.g. virginiamycin) in different European countries, Bywater et al. (2005) found a small percentage of isolates with virginiamycin resistance. They also found a significant (40%) decrease in the resistance rate one year after the ban of virginiamycin in animal food. The most common cross-resistance was seen in association with macrolides (tylosin). In another study, Aarestrup et al. (2001) investigated a decrease in resistance to virginiamycin within *E. faecium* isolates from broilers and pigs after the ban of this antibiotic and a cross-resistant antibiotic family, macrolides (tylosin and erythromycin), in animal husbandry. In the DAFF (2007) report, resistance to virginiamycin was common in pig *E. faecalis* (97.9%), pig *E. faecium* (43.3%), a combination of pig *E. hirae* and *E. casseliflavus* (21%), chicken *E. faecalis* (92.8%), chicken *E. faecium* (26.2%) and a combination of chicken *E. hirae* and *E. casseliflavus* (33.4%). Use of virginiamycin, as a feed additive, may result in the selection of resistant faecal enterococci with cross-resistance to a related streptogramin antibiotic quinupristin-dalfopristin (Synercid®), which is important in human medicine (Turnidge, 2001; Thal & Zervos, 1999).

Resistance to heavy metals (copper)

All enterococci (except one *E. gallinarum* isolate of human origin) were intermediately resistant to copper. Copper is extensively used in pigs as a growth promoter (Hasman et al., 2006). As a natural response, many bacterial species have developed resistance mechanisms to overcome copper toxicity (Silver & Phung, 1996). This copper resistance has been reported in human and animal *E. faecium* and *E. faecalis* as well as other bacteria (Hasman & Aarestrup, 2002). Hasman and Aarestrup (2002) found a strong correlation between the copper resistance on one hand, and macrolide and glycopeptide resistance within the pig isolates on the other hand. Although the link between the macrolide and glycopeptide resistance genes has previously been shown in *E. faecium* isolates, it is now clear that the *tcr*B gene is connected to these resistance genes as well. Since the heavy metal (including copper) resistance genes are usually transferred on the common MGEs (mostly plasmids) with the antibiotic resistance genes (Van Houdt et al., 2009), the high copper resistance of human isolates can be explained.

Resistance to heavy metals (zinc)

Eighty-eight isolates showed resistance (some intermediately resistant) to zinc, mostly in pig *E. faecalis*. Animal feed is supplemented with zinc compounds in different concentrations, mostly in pigs (Aarestrup & Hasman, 2004). The difference between the resistance in pig and chicken isolates to zinc could be explained by the difference in the amount of zinc added to their feed. As discussed for copper resistance, the linkage of heavy metal and antibiotic resistance genes carried on the same MGEs has been reported (Nemergut et al., 2004) and contributes to the spread of zinc resistance in bacterial species isolated from animals or humans. In the current research, the resistance to zinc in other species—including four *E. faecium*, one *E. hirae* and two *E. raffinosus*—was relatively low.

Conclusions

In summary, all isolates (except one *E. hirae* of pig origin) showed multi-resistance, generally to more than three antimicrobials. This high rate of resistance in animal isolates is probably related to a combination of intrinsic resistance and the extensive use of antimicrobials in animals. In human isolates, resistance could be a consequence of antibiotic use or there may be a link to use of antibiotics in animals. As there is increasing evidence for transfer of resistant bacteria (including enterococci) from animals to humans, use of antibiotics in animals should be reduced as far as possible.

Table 3-1. Master Mix ingredients and thermal cycling conditions for PCR of *tuf* genes

Master Mix (25 µL)		**Thermal cycling**		
Reagent	**Amount (µL)**	**Step**	**Temp. (°C)**	**Time**
Distilled water	14.6	● Initial denaturation	95	3 min
5× PCR buffer	5	● 30 cycles		
25 mM $MgCl_2$	1	– denaturation	95	30 sec
25 mM dNTPs	0.2	– annealing	Variable	30 sec
10 pmol primer (each)	1 (× 2)	– extension	72	1 min
5 U/µL Taq polymerase	0.2	● Final extension	72	5 min

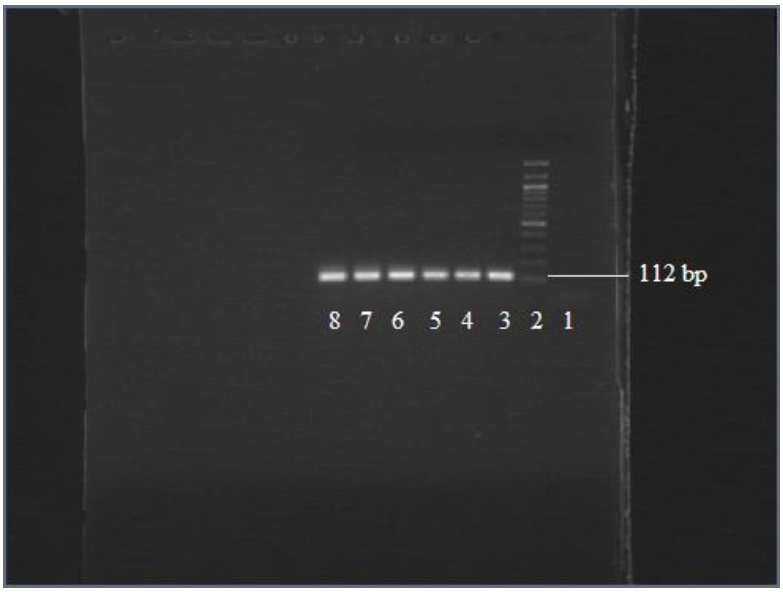


Figure 3-1. Molecular typing of enterococcal isolates using genus-specific *tuf* gene. Lane 1, negative control; Lane 2, 100 bp marker; Lanes 3, 4, 5, 6, 7 & 8, 112 bp bands of *Enterococcus* spp. isolates nos. 179, 180, 181, 182, 183 & 184

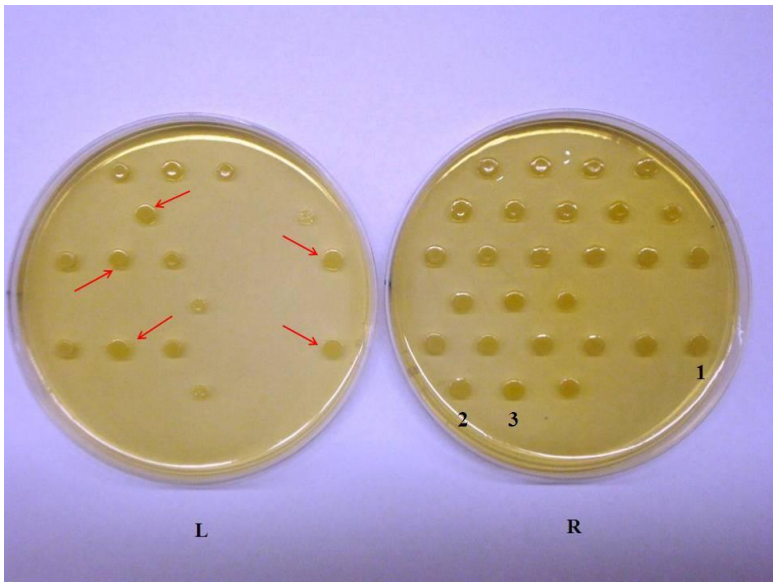


Figure 3-2. Antimicrobial sensitivity test plates for flavophospholipol. L, isolate plate containing 16 µg/mL flavophospholipol; R, control plate excluding flavophospholipol. Control isolates: no. 1, *E. coli* ATCC 25929; no. 2, *E. faecalis* ATCC 29212 and no. 3, *S. aureus* ATCC 29213. Many isolates demonstrate resistance to 16 µg/mL flavophospholipol (arrows)

Table 3-2. MIC breakpoints (µg/mL) for antibiotics

	AMP[1]	AVI[2]	AVO	BAC*[3]	FLA[2]	GEN[2]	NAR[4]	TET[1]	TIA	TYL[3]	VAN[1]	VIR[4]
R	≥ 16	≥ 16	≥ 16	≥ 64	≥ 16	≥ 1024	≥ 2	≥ 16	≥ 32	≥ 8	≥ 8	≥ 8
S	≤ 8	< 16	< 16	≤ 32	≤ 8	< 1024	< 2	≤ 4	≤ 4	≤ 4	≤ 4	< 8

R, resistant; S, sensitive; AMP, ampicillin; AVI, avilamycin; AVO, avoparcin; *BAC, bacitracin (IU/mL); FLA, flavophospholipol; GEN, gentamicin; NAR, narasin; TET, tetracycline; TIA, tiamulin; TYL, tylosin; VAN, vancomycin; VIR, virginiamycin

Ref.: [1]CLSI, 2008; [2]DANMAP, 2005; [3]NARMS, 2004; [4]NORM/NORM-VET, 2004

Table 3-3. Enterococcal isolates used in this study

Enterococci	Chicken	Pig	Human
E. faecalis	1	80	3
E. faecium	14	71	3
E. hirae	0	2	0
E. hirae/durans	0	17	0
E. casseliflavus	0	13	1
E. gallinarum	0	21	1
E. raffinosus	0	2	0
Unassigned	0	1	0
Total	15	207	8

Table 3-4. Overall phenotypic antimicrobial resistance in enterococcal isolates

Drug	Total			Pigs			Chickens			Humans		
	R	I	S	R	I	S	R	I	S	R	I	S
AMP	0 (*0%*)	0 (*0%*)	230 (*100%*)	0 (*0%*)	0 (*0%*)	207 (*100%*)	0 (*0%*)	0 (*0%*)	15 (*100%*)	0 (*0%*)	0 (*0%*)	8 (*100%*)
AVI	0 (*0%*)	0 (*0%*)	230 (*100%*)	0 (*0%*)	0 (*0%*)	207 (*100%*)	0 (*0%*)	0 (*0%*)	15 (*100%*)	0 (*0%*)	0 (*0%*)	8 (*100%*)
AVO	14 (*6%*)	0 (*0%*)	216 (*94%*)	0 (*0%*)	0 (*0%*)	207 (*100%*)	12 (*80%*)	0 (*0%*)	3 (*20%*)	2 (*25%*)	0 (*0%*)	6 (*75%*)
BAC	154 (*67%*)	0 (*0%*)	76 (*33%*)	140 (*68%*)	0 (*0%*)	67 (*32%*)	13 (*87%*)	0 (*0%*)	2 (*13%*)	1 (*12%*)	0 (*0%*)	7 (*88%*)
FLA	115 (*50%*)	0 (*0%*)	115 (*50%*)	108 (*52%*)	0 (*0%*)	99 (*48%*)	2 (*13%*)	0 (*0%*)	13 (*87%*)	5 (*62%*)	0 (*0%*)	3 (*38%*)
GEN	1 (*1%*)	0 (*0%*)	229 (*99%*)	0 (*0%*)	0 (*0%*)	207 (*100%*)	0 (*0%*)	0 (*0%*)	15 (*100%*)	1 (*12%*)	0 (*0%*)	7 (*88%*)
NAR	0 (*0%*)	0 (*0%*)	230 (*100%*)	0 (*0%*)	0 (*0%*)	207 (*100%*)	0 (*0%*)	0 (*0%*)	15 (*100%*)	0 (*0%*)	0 (*0%*)	8 (*100%*)
TET	229 (*99%*)	0 (*0%*)	1 (*1%*)	206 (*99%*)	0 (*0%*)	1 (*1%*)	15 (*100%*)	0 (*0%*)	0 (*0%*)	8 (*100%*)	0 (*0%*)	0 (*0%*)
TIA	175 (*76%*)	42 (*18%*)	13 (*6%*)	165 (*80%*)	35 (*17%*)	7 (*3%*)	7 (*47%*)	7 (*47%*)	1 (*6%*)	3 (*37%*)	0 (*0%*)	5 (*63%*)
TYL	229 (*99%*)	0 (*0%*)	1 (*1%*)	206 (*99%*)	0 (*0%*)	1 (*1%*)	15 (*100%*)	0 (*0%*)	0 (*0%*)	8 (*100%*)	0 (*0%*)	0 (*0%*)
VAN	45 (*19%*)	1 (*1%*)	184 (*80%*)	33 (*16%*)	1 (*0%*)	173 (*84%*)	12 (*80%*)	0 (*0%*)	3 (*20%*)	0 (*0%*)	0 (*0%*)	8 (*100%*)
VIR	78 (*34%*)	0 (*0%*)	152 (*66%*)	71 (*34%*)	0 (*0%*)	136 (*66%*)	6 (*40%*)	0 (*0%*)	9 (*60%*)	1 (*12%*)	0 (*0%*)	7 (*88%*)
Cu	0 (*0%*)	229 (*99%*)	1 (*1%*)	0 (*0%*)	207 (*100%*)	0 (*0%*)	0 (*0%*)	15 (*100%*)	0 (*0%*)	0 (*0%*)	7 (*88%*)	1 (*12%*)
Zn	84 (*36%*)	4 (*2%*)	142 (*62%*)	79 (*38%*)	4 (*2%*)	124 (*60%*)	2 (*13%*)	0 (*0%*)	13 (*87%*)	3 (*37%*)	0 (*0%*)	5 (*63%*)

R, resistant; S, sensitive; I, intermediate; AMP, ampicillin; AVI, avilamycin; AVO, avoparcin; BAC, bacitracin; FLA, flavophospholipol; GEN, gentamicin; NAR, narasin; TET, tetracycline; TIA, tiamulin; TYL, tylosin; VAN, vancomycin; VIR, virginiamycin; Cu, copper; Zn, zinc

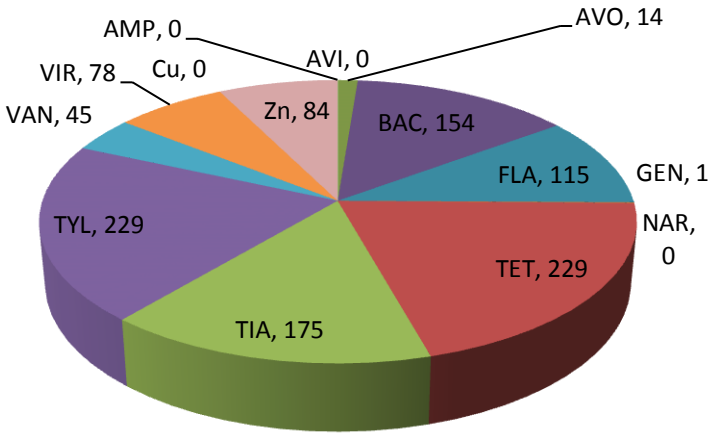


Figure 3-3. Summary of phenotypic antimicrobial resistance. AMP, ampicillin; AVI, avilamycin; AVO, avoparcin; BAC, bacitracin; FLA, flavophospholipol; GEN, gentamicin; NAR, narasin; TET, tetracycline; TIA, tiamulin; TYL, tylosin; VAN, vancomycin; VIR, virginiamycin; Cu, copper; Zn, zinc

Table 3-5. Minimum inhibitory concentrations (MICs) of *Enterococcus* spp. to 12 antibiotics

Drug	**No. of isolates with MIC (µg/mL)**												**Resistance**	
Isolate (no.)	**1**	**2**	**4**	**8**	**12**	**16**	**20**	**32**	**64**	**128**	**512**	**1024**	***n***	**%**
Ampicillin														
E. faecalis (84)			55	29									0	0
E. faecium (88)			3	85									0	0
E. gallinarum (22)	13	7	1	1									0	0
E. casseliflavus (14)	10	1	1	2									0	0
E. hirae/durans (19)			1	18									0	0
E. raffinosus (2)				2									0	0
N/D (1)				1									0	0
Avilamycin														
E. faecalis (84)				84									0	0
E. faecium (88)				68									0	0
E. gallinarum (22)				22									0	0
E. casseliflavus (14)				14									0	0
E. hirae/durans (19)				19									0	0
E. raffinosus (2)				2									0	0
N/D (1)				1									0	0
Avoparcin														
E. faecalis (84)				83		1							1	1
E. faecium (88)				79		1		8					9	10
E. gallinarum (22)				22									0	0
E. casseliflavus (14)				14									0	0
E. hirae/durans (19)				16									0	0
E. raffinosus (2)				2									0	0
N/D (1)				1									0	0
Bacitracin*														
E. faecalis (84)						55			20	9			29	35
E. faecium (88)						10		3	71	4			75	85
E. gallinarum (22)						1			21				21	95
E. casseliflavus (14)						2			12				12	86
E. hirae/durans (19)						5			14				14	74
E. raffinosus (2)									2				2	100
N/D (1)						1							0	0
Flavophospholipol														
E. faecalis (84)			81	1				2					2	2
E. faecium (88)			13	1	1			73					73	83
E. gallinarum (22)				7				15					15	68
E. casseliflavus (14)				4				10					10	71
E. hirae/durans (19)			4	1				14					14	74
E. raffinosus (2)			2										0	0
N/D (1)								1					1	100
Gentamicin														
E. faecalis (84)											84		0	0
E. faecium (88)											87	1	1	1
E. gallinarum (22)											22		0	0
E. casseliflavus (14)											14		0	0
E. hirae/durans (19)											19		0	0
E. raffinosus (2)											2		0	0
N/D (1)											1		0	0
Narasin														
E. faecalis (84)	84												0	0
E. faecium (88)	88												0	0
E. gallinarum (22)	22												0	0
E. casseliflavus (14)	14												0	0

Drug	No. of isolates with MIC (µg/mL)												Resistance	
Isolate (no.)	**1**	**2**	**4**	**8**	**12**	**16**	**20**	**32**	**64**	**128**	**512**	**1024**	***n***	**%**
E. hirae/durans (19)	19												0	0
E. raffinosus (2)	2												0	0
N/D (1)	1												0	0
Tetracycline														
E. faecalis (84)						84							84	100
E. faecium (88)						88							88	100
E. gallinarum (22)						2		17	3				22	100
E. casseliflavus (14)						2		7	5				14	100
E. hirae/durans (19)			1			18							18	95
E. raffinosus (2)						2							2	100
N/D (1)						1							1	100
Tiamulin														
E. faecalis (84)			1			2	1		80				80	95
E. faecium (88)		8	2	1		18			59				59	67
E. gallinarum (22)						1			21				21	95
E. casseliflavus (14)			1			9			4				4	29
E. hirae/durans (19)						8	2		9				9	47
E. raffinosus (2)									2				2	100
N/D (1)						1							0	0
Tylosin														
E. faecalis (84)				9			20	55					84	100
E. faecium (88)				73				3	12				88	100
E. gallinarum (22)				22									22	100
E. casseliflavus (14)				13				1					14	100
E. hirae/durans (19)			1	18									18	95
E. raffinosus (2)				2									2	100
N/D (1)				1									1	100
Vancomycin														
E. faecalis (84)			84										0	0
E. faecium (88)			76					12					12	14
E. gallinarum (22)			1	21									21	95
E. casseliflavus (14)			2	12									12	86
E. hirae/durans (19)			19										0	0
E. raffinosus (2)			2										0	0
N/D (1)			1										0	0
Virginiamycin														
E. faecalis (84)			32	51		1							52	62
E. faecium (88)			70	10		8							18	20
E. gallinarum (22)			22										0	0
E. casseliflavus (14)			13	1									1	7
E. hirae/durans (19)			12	1		6							7	37
E. raffinosus (2)			2										0	0
N/D (1)			1										0	0

N/D, not determined; *IU/mL

Concentrations tested:

Ampicillin: 1, 2, 4, 8 & 16 µg/mL
Avilamycin: 8 & 16 µg/mL
Avoparcin: 8, 16 & 32 µg/mL
Bacitracin: 16, 32, 64 & 128 IU/mL
Flavophospholipol: 4, 8, 16 & 32 µg/mL
Gentamicin: 512 & 1024 µg/mL
Narasin: 1 & 2 µg/mL
Tetracycline: 4, 16, 32 & 64 µg/mL
Tiamulin: 2, 4, 8, 16, 32 & 64 µg/mL
Tylosin: 0.25, 4, 8 & 32 µg/mL
Vancomycin: 4, 8 & 32 µg/mL
Virginiamycin: 4, 8 & 16 µg/mL

Table 3-6. Minimum inhibitory concentrations (MICs) of *Enterococcus* spp. to 2 heavy metals

Drug	**No. of isolates with MIC (mM/mL)**												**Resistance**	
Isolate (no.)	**2**	**4**	**5**	**6**	**7**	**8**	**12**	**16**	**20**	**24**	**28**	**32**	***n***	**%**
Copper														
E. faecalis (84)			2	50	32								0	0
E. faecium (88)			15	71	2								0	0
E. gallinarum (22)		1	1	12									0	0
E. casseliflavus (14)			5	9									0	0
E. hirae/durans (19)			11	8									0	0
E. raffinosus (2)				2									0	0
N/D (1)				1									0	0
Zinc														
E. faecalis (84)						6		78					78	93
E. faecium (88)	1					83		4					4	5
E. gallinarum (22)						22							0	0
E. casseliflavus (14)						9							0	0
E. hirae/durans (19)						19							0	0
E. raffinosus (2)								2					2	100
N/D (1)						1							0	0

N/D, not determined

Concentrations tested:

Copper: 2, 4, 5, 6, 7, 8, 12, 16, 20, 24, 28 & 32 mM/mL

Zinc: 2, 4, 8, 12 & 16 mM/mL

Chapter 4

Antibiotic Resistance Genes in Enterococci

Introduction

Bacterial antimicrobial (antibiotic) resistance has become an important problem for human and veterinary medicine (Gilmore & Ferretti, 2003). Due to evolution, bacteria have developed mechanisms to protect themselves against antimicrobials. Four general mechanisms of resistance to antimicrobials have been recognized, namely target alteration, decreased influx/increased efflux, alternative methabolic pathway and inactivation of the drugs by modifying enzymes (Wright, 2005; Hooper, 2005). The latter mechanism is clinically important since the genes encoding modifying enzymes can be disseminated by MGEs such as plasmids and transposons. This horizontal route of gene transfer contributes to the extensive prevalence of antimicrobial resistance within most pathogenic and non-pathogenic bacteria such as enterococci (Wright, 2007; Mundy et al., 2000). Additionally, a relative insensitivity to several antimicrobials such as fluoroquinolones, macrolides and aminoglycosides has been described in enterococci (Fisher & Phillips, 2009; Hayes et al., 2003; Gold & Moellering, 1996). In recent decades, the emergence of antimicrobial resistance, especially in enterococci, has forced medical and veterinary experts to investigate and monitor resistance genes in bacterial populations (Birgand, 2009; Huycke et al., 1998). The current chapter assesses the genes encoding antibiotic resistance to 14 antimicrobials, previously tested and described in Chapter 3.

Materials and Methods

PCR amplification of resistance genes

Polymerase chain reaction (Single and Multiplex PCR) was used for detecting antibiotic resistant genes in enterococci. An overview of primers and the multiplex gene groups in this study is given in Table 4-1. However, primers for *lsa* genes were adopted from primers designed originally to amplify tiamulin resistance genes in *S. aureus* due to lack of published specific sequences for *lsa* genes in enterococci. The PCR protocol was modified and optimized for the amplification of antibiotic resistant genes in the functional groups in which genes were categorized. The gene groups and protocols are listed in Table 4-2. The general protocol used for PCR in this study was as follows: A few colonies of each isolate

were cultivated overnight on Blood Columbia Agar (Oxoid, Australia) and suspended in 200 µL of sterile distilled water. Bacterial cells were then heated at 95°C for 20 minutes. The mixture was then centrifuged at 7500 g for five minutes and the supernatant containing DNA was used as a template for PCR. Extractions were stored at -20°C until used. To prepare a final volume of 25 µL PCR Master Mix for each sample, 5 µL of 5× PCR buffer, 1 µL of 25 mM $MgCl_2$, 0.2 µL of the 25 mM dNTPs, 1 µL of each primer (10 pmol), and 0.2 µL of 5 U/µL Taq DNA polymerase (Promega, USA) were mixed in a sterile microtube. Sterile distilled water was added to the mixture to make a total volume of 23 µL, finaly 2 µL of extracted template DNA was added to the mix (modified from Hartman et al., 2003) (Table 4-3). The thermalcycler (Bio-Rad MyCycler, USA) conditions are as follows: Initial denaturation was five minutes at 95°C, then followed by 35 cycles, each cycle consisted of denaturation at 95°C for 30 seconds, 30-second annealing at various temperatures optimized for different primers, and extension at 72°C for one minute. Final extension was carried out at 72°C for five minutes (Table 4-3). One sample without DNA template and template DNA from strains known to harbour the target genes (if available) were used as negative and positive controls. PCR products were detected by electrophoresis in 1 µg/mL ethidium bromide-stained 1% agarose gels in 0.5× TBE buffer at 100 V for 90 minutes and then by visualizing under the UV light (Figures 4-1, 4-2 and 4-3). Two microlitres of 100 bp DNA ladder were loaded as a size marker for each gel electrophoresis. Results were confirmed by repeating each gel electrophoresis.

Gene sequencing

Amplified DNA products from isolates with putative *aac*6-*aph*2, *erm*B, *lsa*A, *lsa*B, *tet*K, *tet*L, *tet*M, *tet*O, *tet*S, *tcr*B, *van*A, *van*C1, *van*C2, *vat*D and *vat*E antimicrobial resistance genes were sequenced and the results were compared with GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ExPASy (http://au.expasy.org/links.html) reference databases. The sequencing was carried out at the SouthPath Sequencing Facility (Flinders University, Adelaide) using a fluorescence-based capillary electrophoresis system (Applied Biosystems3130*xl*, USA). Briefly, a cycle sequencing PCR protocol using BigDye Terminator (Applied Biosystems) was perform to generate samples for the instrument. The products of this sequencing reaction were treated to remove excess dNTPs etc using the Cleanseq magnetic bead based method (Beckman Coulter). Then, the products were injected electrokinetically into machine capillaries filled with polymer. The negatively charged DNA fragments moved through the polymer in the capillaries toward the positive electrode due to the high voltage. The separated fluorescent-labeled DNA

fragments (BigDye Terminator v3.1) moved through the path of a laser beam and were detected by an optical detection device. The fluorescence signals were converted to digital data and the data were analysed by the software.

Bacterial DNA samples were prepared for sequencing as follows: DNA samples were extracted and amplified according to the methods specified in Chapter 3. Amplified DNA was purified from any contamination with primers, linkers, enzymes, salts and dNTPs using the following protocol. Five volumes of bind solution (UltraClean PCR Clean-up Kit, Mo Bio Laboratories Inc, USA) were added to PCR product and transferred to a spin column unit. The mixture was centrifuged at 10,000 g for 10−30 seconds then the column was removed and the liquid was discarded. The column was placed in the same tube and 300 μL of clean buffer were added to the spin column. The column was then centrifuged at 10,000 g for 10−30 seconds and the column was removed and the liquid was discarded. The column was replaced and the tube was centrifuged at 10,000 g for 10−30 seconds. Then, the column was transferred to a new tube and 50 μL of elution buffer (10 mM Tris) solution were added. This was left at the room temperature for five minutes then was centrifuged at 10,000 g for 10−30 seconds. Ten microlitres of the purified DNA were electrophoresed to check purity before discarding the column and collecting the DNA.

Results

Detection of resistance genes by PCR

In summary, all enterococcal isolates contained multiple genes encoding antimicrobial resistance. Most of the isolates contained *erm*B (including ten *E. casseliflavus*, 65 *E. faecalis*, 75 *E. faecium*, 19 *E. gallinarum*, one *E. hirae*, 13 *E. hirae/durans* and one *E. raffinosus*), *tet*L (including two *E. casseliflavus*, 53 *E. faecalis*, 76 *E. faecium*, eight *E. gallinarum*, one *E. hirae*, 15 *E. hirae/durans*, one unassigned and two *E. raffinosus*), *tcr*B (including 12 *E. casseliflavus*, 46 *E. faecalis*, 54 *E. faecium*, 14 *E. gallinarum*, one *E. hirae/durans* and one unassigned) and *tet*M (including four *E. casseliflavus*, 50 *E. faecalis*, 40 *E. faecium*, ten *E. gallinarum*, ten *E. hirae/durans* and two *E. raffinosus*) while the incidence of other genes was lower (less than 1/3 of all isolates). No *tet*O (except one *E. gallinarum* isolate of human origin), *tet*K (except one *E. gallinarum* isolate of pig origin) or *rpl*C, *rpl*D, *van*B, *vat*A, and *vat*D (except one *E. faecalis* isolate of human origin) were found. Other genes detected were *aac*6-*aph*2 (four *E. faecalis* and two *E. faecium*), *lsa*A (including five *E. faecalis* and one *E. raffinosus*), *lsa*B (including 61 *E. faecalis*, two *E. faecium* and two *E. raffinosus*), *tet*S (including ten *E. casseliflavus*, one

E. faecalis, one *E. faecium* and 12 *E. gallinarum*), *van*A (one *E. faecalis* and 12 *E. faecium*), *van*C1 (including three *E. casseliflavus*, one *E. faecalis* and 18 *E. gallinarum*), *van*C2 (including ten *E. casseliflavus*, one *E. faecium* and two *E. gallinarum*), and *vat*E (including one *E. faecalis*, 14 *E. faecium* and seven *E. hirae/durans*). Although less than half of the genes (eight out of 19) were found in the chicken isolates, the percentage of isolates carrying the detected genes was high. All chicken isolates contained *erm*B. In contrast, no *aac*6-*aph*2 or *van*C1 genes were found in chicken isolates but one *van*C2 was found in an *E. faecium* of chicken origin. The lowest number of *tet*S genes was found in chicken isolates. No *van*A genes were detected in enterococci isolated from pigs but one *van*C1 was found in an *E. faecalis* of pig origin. No *van*C2, *lsa*A, *vat*E or *tcr*B were found in enterococcal isolates of human origins. The results of resistance gene assessment are shown in Tables 4-4, Figure 4-4, and Appendices 4 and 5−18. Distribution of antimicrobial resistance genes and phenotypic characteristics as defined by PCR are shown in Table 4-5.

Sequencing to confirm resistance gene targets

To confirm amplification resulted in the correct target gene, gene sequencing was undertaken. Gene sequencing results for all genes detected showed at least 98% identity with GenBank reference genes (Table 4-6 and Appendix 19). Original accession numbers: *aac*6-*aph*2, AB206333.1; *erm*B, AB247327.1; *lsa*A, AY737526.1; *lsa*B, AY737526.1; *tet*K, AJ888003.1; *tet*L, AY081910.1; *tet*M, DQ223244.1; *tet*O, AY660532.1; *tet*S, DQ295784.1; *tcr*B, EU869871.1; *van*A, AM932524.1; *van*C1, EU151772.1; *van*C2, EU151757.1; *vat*D, AF368302.1; and *vat*E, AY043212.1.

Discussion

In summary, a significant proportion of isolates contained resistance genes (usually more than two) as determined by PCR. No genes encoding resistance to ampicillin, avilamycin, bacitracin, narasin and zinc were included in this study.

Resistance to glycopeptides

Only a low number of genes (13 *van*A) encoding resistance to avoparcin was detected; all bar two in chicken isolates. In Australia, avoparcin was used to a limited extent in pigs prior to 1997 (Hart and Barton, 2002) but widely used in chickens) up to 2000; use in livestock was also common in Europe (Collignon, 1999). Use of avoparcin has been reported to be largely responsible for the amplification of *van*A vancomycin resistant

enterococci (VRE) in animals (Ferguson, 1999). In the current study, twelve of *van*A genes were found in *E. faecium* of chickens. It is worthy of mention that these isolates had been collected before avoparcin was banned from the Australian market. *E. faecium* isolates bearing *van*A genes have been reported in studies as the most prevalent avoparcin resistant member of the enterococci isolated from chickens (Borgen et al., 2001; Yoshimura et al., 1998). Two *van*A genes (one in *E. faecalis* and one in *E. faecium*) were also found in human isolates. This gene also mediates resistance to all glycopeptide antibiotics, including vancomycin. Not surprisingly, vancomycin resistance was also seen in all chicken isolates resistant to avoparcin, carrying *van*A. This phenomenon was seen in some countries such as Japan (Sakai etal., 2003), Sweden, Denmark, Germany, the Netherlands, Norway, Belgium, France, Finland and UK (van den Bogaard et al., 2000; Devriese et al., 1996) where avoparcin was extensively used in animal husbandry as a growth promoter. This resistance has not been found in animal isolates in other countries (e.g. USA) with no use of this antibiotic in animals (Wegener et al., 1999a). Apart from the *van*A genes mentioned previously, one *van*C1 gene in *E. gallinarum* was found in human isolates. The number of human isolates included in this study is too small to allow any comments on the significance of these findings. In Australia, Padiglione et al. (2000, 2003) used routine and molecular assays to assess the prevalence of vancomycin resistant enterococci (VRE). These studies suggested that a faecal colonization with VRE was present but uncommon in Australia. Although avoparcin was used in chickens and to a lesser extent pigs before 2000, *van*A gene was either not identified in *E. faecium* in their study or was reported in low numbers. *E. faecium* carrying *van*B gene, the most commonly clinical VRE species in Australia, has been identified in healthy Australians (Padiglione et al**.,** 2000, 2003; Schooneveldt et al., 2000). Similar findings were reported by Bell et al. (1998) and Burrell et al. (2005) in Adelaide and Melbourne, respectively. In contrast, no *van*B isolates were found in the current study. The fact that *van*B-type vancomycin resistance is not associated to the use of avoparcin in animals (Barton et al., 2003) may explain the difference between the clinical and livestock isolates. Xavier et al. (2006) found no *van*A or *van*B genes in enterococci of poultry in Brazil. Similarly, Lemcke and Bulte (2000) did not find *van*B genes in VRE enterococcal strains isolated from poultry and pork in Germany; however, they reported *van*A genes in nearly half of the isolates. In the current study, all *van*C1 and *van*C2 (except one from chicken origin) were found in pig isolates. However, these genes were found in intrinsically resistant *E. casseliflavus* and *E. gallinarum*, in accordance with previous studies. Seo et al. (2005) compared vancomycin resistance in 67 enterococcal isolates from poultry and pigs in Korea and

found that *van*C1 or *van*C2/3 was associated with low level resistance to vancomycin in *E. gallinaum*, *E. casseliflavus* and *E. flavescens* isolates. Reports by Xavier et al. (2006), and Lemcke and Bulte (2000) indicate the prevalence of *van*C1 and *van*C2/3 genes in enterococcal samples of pig and chicken origin, mostly *van*C1 in *E. gallinarum* and *van*C2/3 in *E. casseliflavus*. In the current research, one *van*C1 was found in an *E. faecalis* of pig origin and one *van*C2 in an *E. faecium* of chicken origin. However, *van*C encoded resistance to vancomycin has been reported as a feature of *E. gallinarum*, *E. casseliflavus* and *E. flavescens* species (Haenni et al., 2009; Cetinkaya et al., 2000; Lemcke & Bulte, 2000; Mendez-Alvarez et al., 2000; Dutka-Malen et al., 1995), there are other reports on the detection of *van*C genes from enterococcal species other than those listed above; mostly from clinical isolates. For example, Patel et al. (1997) detected *van*C1 from one *E. faecalis* and one *E. faecium* strains clinically isolated from humans.

Resistance to gentamicin

Only a few isolates (four *E. faecalis* of pigs and two *E. faecium* of humans) contained *aac*6-*aph*2 genes encoding resistance to gentamicin although none of them were phenotyplcally resistant. The *aac*6-*aph*2 gene is reported as the most prevalent resistance gene encoding high-level gentamicin resistance and resistance to other aminoglycosides except streptomycin (Chow, 2000). Other studies have shown that *aac*6-*aph*2 is prevalent in gentamicin resistant enterococci. del Campo et al. (2000) investigated aminoglycoside modifying enzymes in enterococci with high-level streptomycin and gentamicin resistance. The most prevalent modifying enzymes were APH(3') (67.1%) and AAC(6')-APH(2") (64.7%) encoded by *aac*6-*aph*2 and *aph*3 genes, respectively. Gentamicin is not registered for use in livestock in Australia. This may have influenced the low prevalence of genes encoding high-level resistance to gentamicin in this study. Although few reports on the association of resistance to gentamicin and vancomycin have been published (Poole et al., 2005; Simjee et al., 2002a), no *van* genes (except a *van*A from *E. faecium* of human origin) were found within the isolates containing *aac*6-*aph*2 in this study.

Resistance to tetracycline

All isolates (except one *E. hirae* of pig origin) were resistant to tetracycline and contained recognised *tet* genes, except 26 isolates of different species in which none of the *tet* genes investigated in this study were detected. Most of the *tet* genes found in this study were *tet*L and *tet*M; no *tet*K (except one *E. gallinarum* isolate of pig origin) or *tet*O (except one *E. gallinarum* isolate of human origin) genes were detected and the prevalence of *tet*S was

low (less than 10%). Tetracycline resistance is the most prevalent form of bacterial resistance in nature, mediated by *tet* genes (Patterson et al., 2007). Of different *tet* genes, the *tet*M gene is the most prevalent in enterococci, often carried on conjugative transposons such as Tn*1545* and encodes ribosomal protection factors (Jones et al., 2006, Chu et al., 1996). Other studies have reported *tet*M and *tet*L (another transposon-transferred resistance gene encoding synthesis of efflux proteins) as the most prevalent *tet* genes in enterococcal isolates associated with animals and humans (Rizzotti et al., 2009; Wilcks et al., 2005; De Leener et al., 2004; Huys et al., 2004; Aarestrup et al., 2000). The proportion of *tet*L and *tet*M positive isolates appeared to be increased by tetracycline treatment (Fairchild et al., 2005). In contrast it was found that only one *Enterococcus* isolate from his research flock containted *tet*O gene and no isolate with *tet*K was found, whereas 41.3% and 1.3% of the *Enterococcus* isolates from commercial farms contained *tet*O and *tet*K, respectively. Both groups included a significant percentage of *tet*M and *tet*L. In this study, no *tet* gene could be detected in 26 tetracycline resistant isolates, suggesting that the resistance is encoded by genes other than those tested. No difference was seen in isolates with different *tet* genes that all were resistant to the same concentration (16 μg/mL) of tetracycline. Tetracycline resistance was generally associated with resistance to other antimicrobials, especially tiamulin, tylosin, vancomycin and copper in this study. Merlin et al. (1989) found that the *tet* expression increased bacterial susceptibility to heavy metals as well as aminoglycosides. Other studies relate this association to integrity of genes encoding resistance to antibiotics and heavy metals on the same MGEs such as plasmids (Heinemann et al., 2000; Valenzuela et al., 1996).

Resistance to tiamulin

No *rpl*C and *rpl*D genes were found in resistant or intermediately resistant isolates to tiamulin. However, primers used in this study were original designed for the investigation of *rpl* genes in *S. aureus*. The reason for choosing those primers is that there is no published information on equivalent primer sequences for enterococci and that staphylococci are the closest Gram-positive bacteria to enterococci after LAB bacteria. Although these protein synthesis-inhibitor genes are mostly reported in the literature as being responsible for resistance to tiamulin (Locke et al., 2009; Miller et al., 2008), these results suggest that other mechanisms including non-ribosomal mechanisms of resistance may be present. A novel gene encoding resistance to tiamulin is *cfr*, encoding a methyltransferase that modifies 23S rRNA at A2503 has been reported in *S. aureus* and *E. coli* (Long et al., 2006).

Resistance to tylosin

All enterococci (except one *E. hirae* isolate of pig origin) were resistant to tylosin, but the *erm*B gene was not found in nearly 20% of these isolates. The resistance mechanism may relate to other *erm* genes such as *erm*A and *erm*C (Jackson et al., 2004b) or erythromycin resistance-encoding genes because tylosin is fully cross-resistant with erythromycin (van den Bogaard et al., 2002a). In another study, antimicrobial susceptibility results have shown that enterococcal isolates from cattle fed with monensin and tylosin had a greater level of resistance to macrolides (Jacob et al., 2008). Hasman and Aarestrup (2005) found that copper resistance (*tcr*B) is associated with macrolide resistance to tylosin (*erm*B) in pig *E. faecium*. Similarly, all pig *E. faecium* in this study were found resistant to both tylosin and copper. Furthermore, the prevalence of *erm*B genes in isolates may be high in animals fed virginiamycin-containing diets (Kieke et al., 2006).

Resistance to virginiamycin

All virginiamycin resistant enterococci harbored resistance genes *erm*B, *lsa*A, *lsa*B and/or *vat*E while more than half of the sensitive isolates carried resistance genes, predominantly *erm*B and *lsa*B. No *vat*A or *vat*D (except one *E. faecalis* isolate of human origin) genes were found. In the current study, *lsa* genes were detected in most of the *E. faecalis* isolates (about 73%). *lsa* genes, encoding a chromosomal ABC transporter, cause intrinsic resistance to lincosamides and streptogramins in *E. faecalis*. The widespread prevalence of *lsa*A, *lsa*B, *vat*E and especially *erm*B in enterococci of farm animals such as turkey, chicken, swine and cattle has been reported previously (Graham et al., 2009; De Graef et al., 2007; Jackson et al., 2007; Donabedian et al., 2006; Simjee et al., 2002b), while the prevalence of other *vat* genes—including *vat*A, *vat*B, *vat*C and *vat*D—is less (Christensen et al., 2008; Simjee et al., 2002b). The *erm* genes mediate resistance to streptogramin B and *vat*E to streptogramin A compounds (McDermott et al., 2005; Solway et al., 2003). Accordingly, the *erm*B gene commonly confers resistance to the streptogramin B component of virginiamycin (Thal & Zervosa, 1999) and it can be transferred by transposons such as Tn*917* and Tn*1545* (Leclercq & Courvalin, 1991). Resistance to both streptogramin A and B components of virginiamycin is usually produced in high-level form while low-level resistance may exist in the absence of these genes, suggesting other mechanisms of resistance. Virginiamycin has beenwidely used in animals for prophylaxis and growth promotion (Barton et al., 2003). Hence, virginiamycin resistance genes are expected to be distributed in animal isolates. The high percentage of resistance genes in

sensitive isolates suggests lack of expression of these genes following tighter control on the use of virginiamycin. Furthermore, the *erm*B gene confers resistance to macrolides and lincosamide as well as streptogramins (MLS_B) (Martel et al., 2003). Cross-resistance to MLS_B antibiotics is mediated by methylation of 23S rRNA and is encoded by *erm* genes (Min et al., 2003). Therefore, the presence of resistance genes in phenotypically sensitive isolates may relate to the use of macrolide antibiotics such as erythromycin, tylosin or lincosamide. All of the virginiamycin sensitive isolates were resistant (two intermediately resistant) to tylosin.

Resistance to heavy metals (copper)

All enterococci were intermediately resistant to copper, and nearly 55% contained the *tcr*B gene. Copper is used in animal industries as a growth promoter and a disinfectant agent (Hasman et al., 2006; Aarestrup & Hasman, 2004; Hasman & Aarestrup, 2002). As a natural response, many bacterial species have developed resistance mechanisms to overcome copper toxicity (Silver & Phung, 1996). Copper resistance, which is usually mediated by a plasmid-born *tcr*B gene, has been reported in *E. faecium* and *E. faecalis* isolated from humans and animals. It has also been detected in other bacterial species (Hasman & Aarestrup, 2002). The *tcr*B gene was recently shown to be part of an operon structure *tcr*YAZB (Hasman, 2005). Hasman et al. (2006), Hasman (2005) and Leelawatcharamas et al. (1997) have confirmed the relationship between copper-containing animal feeds and the existence of copper resistance in bacteria. In contrast, very few cases of copper resistance in Gram-positive bacteria have been reported from wild-type isolates (Hasman & Aarestrup, 2002; Aarestrup & McNicholas, 2002). Therefore, it can be concluded that although *tcr*B genes are widespread in nature, they probably encode low-level resistance or are silent. Generally, all isolates within the current study were intermediately resistant to copper (up to 6 mM/mL). Moreover, Hasman and Aarestrup (2002, 2005) and later Garcia-Migura et al. (2007) found a significant relationship between the copper resistance (*tcr*B), glycopeptide (avoparcin) resistance (*van*A) (Tn*1546*) and macrolide (tylosin) resistance (*erm*B) genes in *E. faecium* isolated from pigs. The *tcr*B gene was located closely upstream of the Tn*1546* element on a conjugative plasmid. However, the continued use of copper sulfate in their study failed to maintain high levels of macrolide and glycopeptide resistance. Subsequent experiments showed that the high levels of copper given to piglets as a feed supplement in pig production selected for copper resistant *E. faecium* and macrolide- and glycopeptide-resistant bacteria. This suggests that the co-selection had been observed in the study (Hasman et al., 2006). This

co-selection was highly likely in the current study as all isolates that contained either *erm*B or *van*A gene were resistant to tylosin (macrolide) or vancomycin and avoparcin (glycopeptides) antibiotics.

Conclusions

In summary, antimicrobial resistance genes were widely spread in enterococcal isolates. Results from this study confirm previous findings that healthy livestock animals can act as reservoirs of antimicrobial resistant enterococci. Enterococci from non-human resources have the potential to play a role in the dispersal of antibiotic resistance determinants in the gastrointestinal tract of humans. The high prevalence of the detected genes in the isolates may relate to current and previous extensive use of antimicrobials in animals including growth promoters, prophylactic and therapeutic use and the role of MGEs in bacteria in addition to intrinsic resistance of enterococci to some antimicrobial drugs (Leavis et al., 2006).

In general, the high levels of resistance to bacitracin, tiamulin and tylosin reflect the widespread usage of these antibiotics in the Australian pig industry. It is not surprising that no *van*A genes were found in pig isolates in this study as avoparcin was not used in pigs after 1997. The *van*C genes were found in enterococcal species other than *E. gallinarum* and *E. casseliflavus*. The widespread nature of *tet* genes is in concert with other reports, while the low prevalence of gentamicin resistance gene is in contrast to other studies. The high prevalence of virginiamycin resistance genes was surprising as this antibiotic is rarely used in pigs. This observation may relate to cross-resistance to MLBs antibiotics. The large prevalence of copper resistance genes in isolates may be due to the widespread use of copper and zinc in piggeries. However, like virginiamycin, co-resistance with other factors has been also described for heavy metals and may provide a partial explanation.

Table 4-1. Overview of target genes and PCR primers used in this study

Drug	Gene		Sequences (5'→3')	bp	Reference
GEN	*aac*6-*aph*2	F	CCAAGAGCAATAAGGGCATA	220	Rouch et al. (1987)
		R	CACTATCATAACCACTACCG		
TET	*tet*K	F	TTAGGTGAAGGGTTAGGTCC	718	Aarestrup et al. (2000)
		R	GCAAACTCATTCCAGAAGCA		
TET	*tet*L	F	CATTTGGTCTTATTGGATCG	488	Aarestrup et al. (2000)
		R	ATTACACTTCCGATTTCGG		
TET	*tet*M	F	GTTAAATAGTGTTCTTGGAG	657	Aarestrup et al. (2000)
		R	CTAAGATATGGCTCTAACAA		
TET	*tet*O	F	GATGGCATACAGGCACAGAC	614	Aarestrup et al. (2000)
		R	CAATATCACCAGAGCAGGCT		
TET	*tet*S	F	TGGAACGCCAGAGAGGTATT	660	Aarestrup et al. (2000)
		R	ACATAGACAAGCCGTTGACC		
TIA	*rpl*C	F	AACAGTTGACGCTTTAATGGGC	N/A	Gentry et al. (2007)
		R	GGCTCAATACCGAATACTGCATCGC		
TIA	*rpl*D	F	CACAGAAAACAAAGTTATCTTAGTAA	N/A	Gentry et al. (2007)
		R	GGTTTGTAATTCATGATATTAACACTT		
AVO, VAN	*van*A	F	GGGAAAACGACAATTGC	732	Dutka-Malen et al. (1995)
		R	GTACAATGCGGCCGTTA		
VAN	*van*B	F	ATGGGAAGCCGATAGTC	635	Dutka-Malen et al. (1995)
		R	GATTTCGTTCCTCGACC		
VAN	*van*C1	F	GGTATCAAGGAAACCTC	822	Dutka-Malen et al. (1995)
		R	CTTCCGCCATCATAGCT		
VAN	*van*C2	F	CTCCTACGATTCTCTTG	439	Dutka-Malen et al. (1995)
		R	CGAGCAAGACCTTTAAG		
TYL, VIR	*erm*B	F	CATTTAACGACGAAACTGGC	738	Jensen et al. (1999)
		R	GGAACATCTGTGGTATGGCG		
VIR	*lsa*A	F	CGCTCCAGCTGTATGAGAACTGC	916−9	Dina et al. (2003)
		R	TCAAGCGATTGACTTCTTTTTTG		
VIR	*lsa*B	F	CAAGTGGCTGAATATTTGAAG	842−4	Dina et al. (2003)
		R	TCCCTTTACTTTTTCAAGATG		
VIR	*vat*A	F	TGGAGTGTGACAAGATAGGC	144–7	Soltani et al. (2000)
		R	GTGACAACAGCTTCTGCAGC		
VIR	*vat*D	F	GCTCAATAGGACCAGGTGTA	144–7	Soltani et al. (2000)
		R	TCCAGCTAACATGTATGGCG		
VIR	*vat*E	F	ACTATACCTGACGCAAATGC	904	Soltani et al. (2000)
		R	GGTTCAAATCTTGGTCCG		
Cu	*tcr*B	F	CATCACGGTAGCTTTAAGGAGATTTTC	663	Hasman et al. (2006)
		R	ATAGAGGACTCCGCCACCATTG		

N/A, not announced; AVO, avoparcin; GEN, gentamicin; TET, tetracycline; TIA, tiamulin; TYL, tylosin; VAN, vancomycin; VIR, virginiamycin; Cu, copper

Table 4-2. Gene target groups and annealing temp. for Multiplex and Single PCRs

Group	Gene	Temperature (°C)
1	*van*A, *van*B, *van*C1, *van*C2, *tet*M (*erm*B)	47
2	*vat*A, *tet*K, *tet*O, *vat*D, *rpl*D, *vat*E, *aac*6-*aph*2	52
3	*tet*S, *tcr*B, *rpl*C, *lsa*A	58
4	*tet*L	47
5	*lsa*B	47
6	*erm*B	45−47

Table 4-3. Master Mix ingredients and thermal cycling conditions

Master Mix (25 µL)		Thermal cycling		
Reagent	**Amount (µL)**	**Step**	**Temp. (°C)**	**Time**
Distilled water	Variable	● Initial denaturation	95	5 min
5× PCR buffer	5	● 35 cycles		
25 mM $MgCl_2$	1	– denaturation	95	30 sec
25 mM dNTPs	0.2	– annealing	Variable	30 sec
10 pmol primer (each)	1	– extension	72	1 min
5 U/µL Taq polymerase	0.2	● Final extension	72	5 min

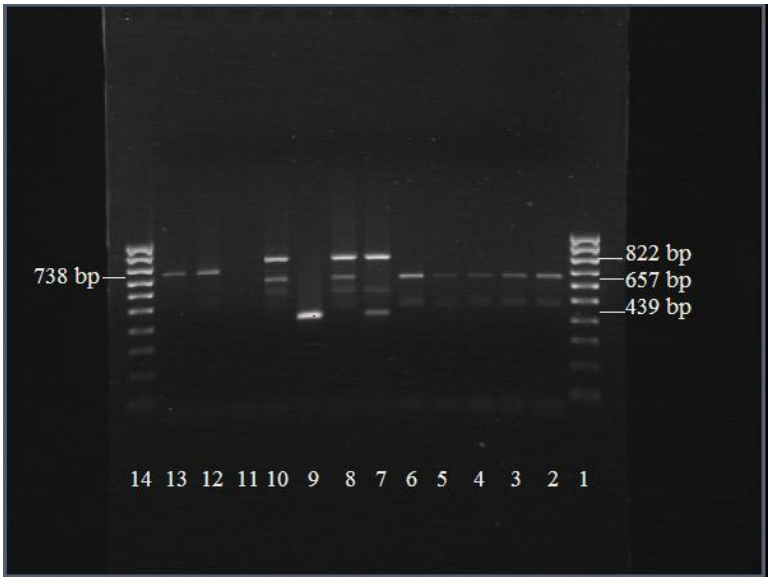


Figure 4-1. Multiplex-PCR of antibiotic resistance genes Group 1 (*van*A, *van*B, *van*C1, *van*C2, *tet*M, *erm*B). Lanes 1 & 14, 100 bp marker; Lanes 2, 3, 4, 5, 6, 8 & 10, *tet*M (657 bp); Lanes 7, 8 & 10, *van*C1 (822 bp); Lanes 7 & 9, *van*C2 (439 bp); Lanes 12 & 13, *erm*B (738 bp)

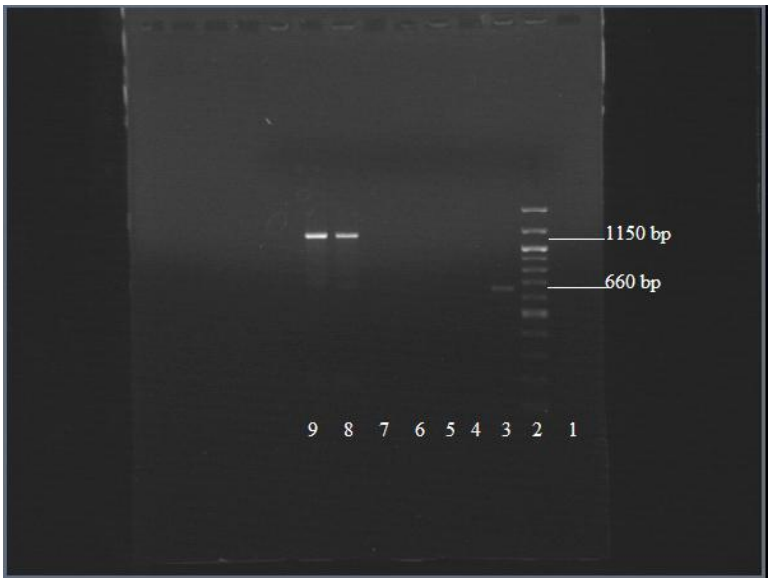


Figure 4-2. Multiplex-PCR of antibiotic resistance genes Group 3 (*tet*S, *tcr*B, *rpl*C, *lsa*A). Lane 1, negative control; Lane 2, 100 bp marker; Lane 3, *tet*S (660 bp); Lanes 8 & 9, *lsa*A (916−9 bp)

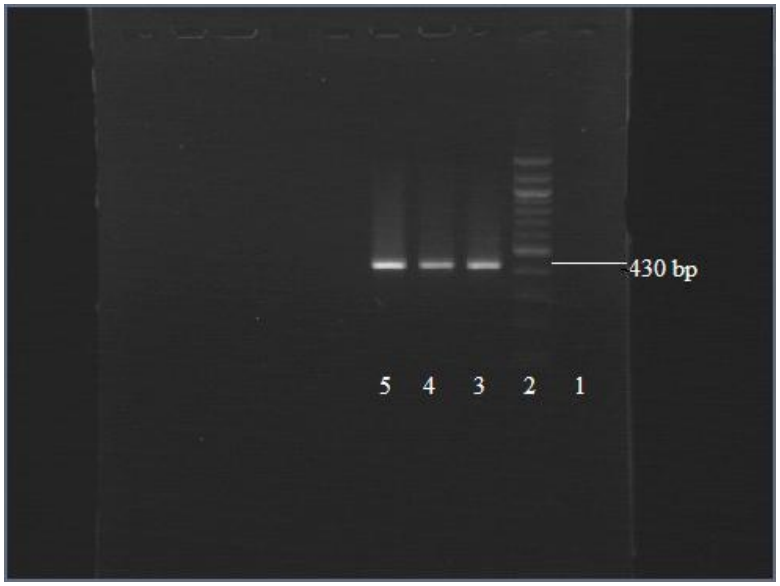


Figure 4-3. Single-PCR of antibiotic resistance genes. Lane 1, negative control; Lane 2, 100 bp marker; Lane 3, 4 & 5, *erm*B (430 bp)

Table 4-4. Numbers of genes detected by PCR assay

Gene	Total (*n* = 230)	Pigs (*n* = 207)	Chickens (*n* = 15)	Humans (*n* = 8)
*aac*6-*aph*2	6 (*3%*)	4 (*2%*)	0 (*0%*)	2 (*25%*)
*tet*K	1 (*1%*)	1 (*1%*)	0 (*0%*)	0 (*0%*)
*tet*L	158 (*69%*)	141 (*68%*)	12 (*80%*)	5 (*62%*)
*tet*M	116 (*50%*)	105 (*51%*)	7 (*47%*)	4 (*50%*)
*tet*O	1 (*1%*)	0 (*0%*)	0 (*0%*)	1 (*12%*)
*tet*S	24 (*10%*)	22 (*11%*)	1 (*7%*)	1 (*12%*)
*rpl*C	0 (*0%*)	0 (*0%*)	0 (*0%*)	0 (*0%*)
*rpl*D	0 (*0%*)	0 (*0%*)	0 (*0%*)	0 (*0%*)
*erm*B	185 (*80%*)	167 (*81%*)	15 (*100%*)	3 (*37%*)
*van*A	13 (*6%*)	0 (*0%*)	11 (*73%*)	2 (*25%*)
*van*B	0 (*0%*)	0 (*0%*)	0 (*0%*)	0 (*0%*)
*van*C1	22 (*10%*)	21 (*10%*)	0 (*0%*)	1 (*12%*)
*van*C2	13 (*6%*)	12 (*6%*)	1 (*7%*)	0 (*0%*)
*lsa*A	6 (*3%*)	5 (*3%*)	1 (*7%*)	0 (*0%*)
*lsa*B	65 (*28%*)	60 (*29%*)	2 (*13%*)	3 (*37%*)
*vat*A	0 (*0%*)	0 (*0%*)	0 (*0%*)	0 (*0%*)
*vat*D	1 (*1%*)	0 (*0%*)	0 (*0%*)	1 (*12%*)
*vat*E	22 (*10%*)	13 (*6%*)	9 (*60%*)	0 (*0%*)
*tcr*B	128 (*56%*)	121 (*58%*)	7 (*47%*)	0 (*0%*)

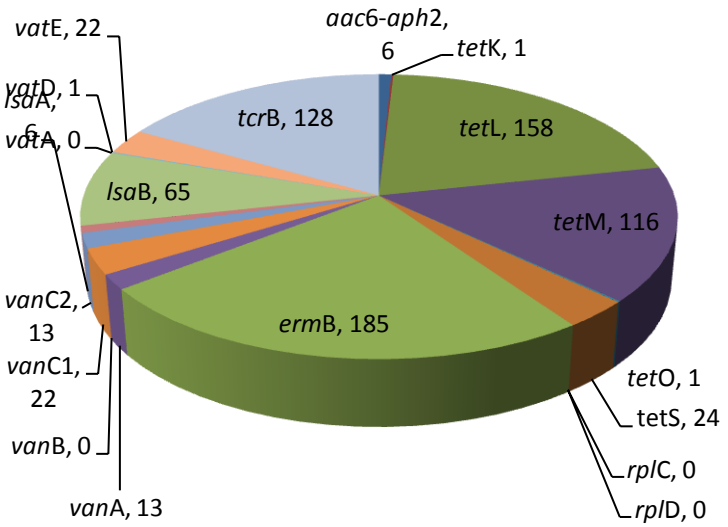


Figure 4-4. Overview of antimicrobial resistance gene PCR assay

Table 4-5. Distribution of antimicrobial resistance genes identified as relating to phenotype

No.	Phenotype	Genes Identified*
Pig *E. faecalis* (*n* = 80)		
3	TET, TIA, TYL, Cu (I), Zn (1: I)	*aac*6-*aph*2, *tet*M, *tet*L, *erm*B, *lsa*A, *lsa*B, *tcr*B
1	BAC, TET, TIA, TYL, Cu (I), Zn	*tet*M, *tet*L, *erm*B, *lsa*B, *tcr*B
1	FLA, TET, TIA, TYL, Cu (I)	*tet*M, *tet*L, *erm*B, *van*C1, *tcr*B
25	BAC	Not tested
1	FLA	Not tested
73	Zn (2: I)	Not tested
3	GEN (3: S)	*aac*6-*aph*2
75	TET, TIA (2: I), TYL, Cu (75: I)	*tet*M, *tet*L, *erm*B, *tcr*B
52	VIR	*erm*B, *lsa*A, *lsa*B, *vat*E
Pig *E. faecium* (*n* = 71)		
3	FLA, TET, TIA, TYL, Cu (I)	*tet*L, *erm*B, *tcr*B
64	BAC	Not tested
65	FLA	Not tested
3	Zn	Not tested
68	TET, TYL, Cu (68: I)	*tet*M, *tet*L, *erm*B, *tcr*B
61	TIA (12: I)	Negative
57	VIR (47: S)	*erm*B, *lsa*B, *vat*E
Pig *E. gallinarum* (*n* = 21)		
14	FLA	Not tested
21	BAC, TET, TIA (1: I), TYL, VAN, Cu (21: I)	*tet*S, *tet*M, *tet*L, *tet*K, *erm*B, *van*C1, *van*C2, *tcr*B
19	VIR (19: S)	*erm*B
Pig *E. casseliflavus* (*n* = 13)		
12	BAC	Not tested
9	FLA	Not tested
13	TET, TIA (9: I), TYL,VAN (1: I), Cu (13: I)	*tet*S, *tet*M, *tet*L, *erm*B, *tcr*B, *van*C1, *van*C2
1	VIR	*erm*B
9	VIR (9: S)	*erm*B
Pig *E. hirae/durans* (*n* = 19)**		
1	FLA, TIA (I), Cu (I)	Negative
2	FLA, TET, TIA (1: I), TYL, Cu (I)	*tet*M, *tet*L, *erm*B
14	BAC	Not tested
11	FLA	Not tested
1	Zn (1: I)	Not tested
16	TET, TIA (8: I), TYL, Cu (16: I)	*tet*M, *tet*L, *erm*B, *tcr*B
13	VIR (7: S)	*erm*B, *lsa*A, *lsa*B, *vat*E
Pig *E. raffinosus* (*n* = 2)		
2	BAC, TET, TIA, TYL, VIR (2: S), Cu (I), Zn	*tet*M, *tet*L, *erm*B, *lsa*A, *lsa*B
Pig N/D (*n* = 1)		
1	FLA, TET, TYL, TIA (I), Cu (I)	*tet*L, *tcr*B
Chicken *E. faecalis* (*n* = 1)		
1	BAC, TET, TIA (I), TYL, Cu (I), Zn	*tet*M, *tet*L, *erm*B, *lsa*A, *lsa*B
Chicken *E. faecium* (*n* = 14)		
1	TET, TIA, TYL, VIR, Cu (I)	*tet*M, *erm*B, *vat*E
1	BAC, TET, TIA, TYL, Cu (I), Zn	*tet*M, *tet*L, *erm*B, *lsa*B
12	TET, TYL, VAN, VIR (7: S), Cu (12: I)	*tet*S, *tet*M, *tet*L, *erm*B, *van*A, *van*C2, *tcr*B, *erm*B,*vat*E
11	AVO, BAC, TIA (5: I)	Not tested or negative
Human *E. faecalis* (*n* = 3)		
1	TET, TYL, Cu (I), Zn	*tet*L, *lsa*B
1	AVO, BAC, TET, TIA, TYL, VIR, Cu (I), Zn	*tet*M, *tet*L, *tet*S, *erm*B, *van*A, *lsa*B, *vat*D
1	TET, TIA, TYL, Cu (I), Zn	*erm*B, *lsa*B
Human *E. faecium* (*n* = 3)		
1	FLA, TET, TYL, Cu (I)	Negative
1	FLA, GEN, TET, TYL, Cu (I)	*aac*6-*aph*2, *tet*M, *erm*B, *van*A
1	AVO, FLA, TET, TYL, Cu (I)	*aac*6-*aph*2, *tet*M, *tet*L
Human *E. gallinarum* (*n* = 1)		
1	FLA, TET, TIA, TYL	*tet*M, *tet*L, *tet*O, *van*C1
Human *E. casseliflavus* (*n* = 1)		
1	FLA, TET, TYL, Cu (I)	*tet*L

*Some genes were not detected in phenotypically resistant isolates; S, sensitive; I, intermediate; **Not differentiated; N/D, not determined; AVO, avoparcin; BAC, bacitracin; FLA, flavophospholipol; GEN, gentamicin; TET, tetracycline; TIA, tiamulin; TYL, tylosin; VAN, vancomycin; VIR, virginiamycin; Cu, copper; Zn, zinc

Table 4-6. Sequenced isolates used as positive controls in this study. Strains had at least 98% identity to the reference data

No.	Bacteria	Origin	Gene	Identities (%)	Ref. accession no.
18	*E. faecium*	Chicken	*van*A	100	AM932524.1
55	*E. casseliflavus*	Pig	*van*C1	100	EU151772.1
176	*E. casseliflavus*	Pig	*van*C2	100	EU151757.1
1	*E. faecalis*	Pig	*tet*M	100	DQ223244.1
49	*E. faecium*	Chicken	*tet*L	100	AY081910.1
93	*E. faecalis*	Pig	*lsa*B	98	AY737526.1
188	*E. casseliflavus*	Pig	*erm*B	99	AB247327.1
185	*E. gallinarum*	Pig	*tet*K	100	AJ888003.1
P11	*E. faecalis*	Human	*vat*D	100	AF368302.1
50	*E. faecium*	Chicken	*vat*E	99	AY043212.1
58	*E. faecalis*	Pig	*aac*6-*aph*2	100	AB206333.1
P16	*E. gallinarum*	Human	*tet*O	99	AY660532.1
188	*E. casseliflavus*	Pig	*tet*S	100	DQ295784.1
188	*E. casseliflavus*	Pig	*tcr*B	98	EU869871.1
P1	*E. faecalis*	Chicken	*lsa*A	99	AY737526.1

Chapter 5

Detection and Characterization of Bacteriophages in Enterococci

Introduction

Bacteriophages are viruses that infect bacteria (Canchaya et al., 2003) and are the most prevalent (estimated population of 10^{31}) biological entities on earth (Pedulla et al., 2003). Bacteriophages are morphologically categorized into tailed and non-tailed bacteriophages. Tailed bacteriophages include *Podoviridae* (short tail stub) (Figure 5-1), *Siphoviridae* (long flexible non-contractile tail) (Figure 5-2) and *Myoviridae* (contractile tail) (Figure 5-3) Families (Table 5-1) (Nelson, 2004; Lawrence et al., 2002) and non-tailed bacteriophages include filamentous (e.g. *Inoviridae*) (Figure 5-4), polyhedral (e.g. *Leviviridae*) (Figure 5-5) and pleomorphic (*Guttaviridae* and *Fusselloviridae*) (Figures 5-6 and 5-7) morphologies (Kyle et al., 2008; Ackermann, 2003). The morphology of the enterococcal bacteriophages investigated up to date is described in Table 5-2.

Bacteriophages specifically transfer genetic information from one bacterium to another by a process called "transduction" (Wagner & Waldor, 2002). Generally, the effect of bacteriophages on bacterial communities depends on whether the bacteriophage is lytic or temperate (Ortmann et al., 2002). Bacterial infection by lytic bacteriophages results in cell lysis and production of bacteriophage particles (Shao & Wang, 2008). In contrast, the genome of temperate bacteriophages can be incorporated into the host genome and result in bacteriophage replication only following an induction event (Canchaya et al., 2003). However, although much research has been conducted since early detection of bacteriophages in enterococci (then classified as *Streptococcus* sp.), many bacteriophages in *Enterococcus* spp. remain to be investigated (Stevens et al., 2009). Therefore, this chapter describes the methods of isolation, identification and characterization of enterococcal bacteriophages.

Materials and Methods

Induction of bacteriophages

To release temperate bacteriophages from putative lysogens, an overnight culture of the suspected lysogen was added 1/10 to Brain Heart Infusion (BHI) Broth (Oxoid, Australia)

with shaking for three hours at 37°C. Mitomycin C (Sigma-Aldrich, USA) was added at a final concentration of 1 µg/mL and incubated with vigorous shaking (Prigent et al., 2005; Yee et al., 1993). Putative bacteriophages were harvested by centrifugation at 5400 g for 20 minutes at 4°C. Then, the putative bacteriophage lysate was sterilized using a 0.45 µM filter (Millipore, USA) (Figure 5-8). The crude lysates were stored at 4°C until use.

Detection of bacteriophages

Detection of environmental bacteriophages was carried out by a "Cross Test" method (modified from Rakieten, 1932 and Hansen et al., 2007). Most of the isolates used in this study were highly resistant to antimicrobials phenotypically and genotypically. This included all enterococcal species. Two hundred microlitres of log-phase bacterial culture, as indicator, were added to 3 mL of 45°C top agar (0.75% TSA). The mixture was poured on top of a TSA plate. This was left to set for 15 minutes and then dried in a 37°C incubator for 30 minutes. Five microlitres of the sample (effluent, sewage or water) filtrate were pipetted onto the indicator strain lawn. The plate was left to dry for one hour before overnight incubation at 37°C. The plates were then examined for plaques the next day (Figure 5-9).

Plaque purification and propagation

A solid purification process was applied to make "pure line" (Rakieten & Rakieten, 1937b) bacteriophages after the primary isolation. A single plaque was picked using 1 mL glass Pasteur pipette and resuspended in 200 µL of SM buffer (modified from Carey-Smith et al., 2006). The mixture was mixed gently and filtered using a 0.45 µm pore filter. The suspension was then added to a molten overlay (0.75% TSA) containing 200 µL of exponential phase host culture (Hancock et al., 2003; Shepard & Gilmore, 2002; Bourbeau et al., 1989). The overlays were then poured onto agar base plates and incubated overnight. The procedure was repeated three times then a plate containing well isolated plaques was chosen and 5 mL SM buffer were added to the plate. The top agar was scraped off and transferred to a container. The agar mixture was incubated 30 minutes at 37°C. After incubation, the mixture was centrifuged at 5400 g for 20 minutes and filtered by a 0.45 µm pore filter. The bacteriophage filtrate was stored at 4°C until use. This solid purification process was repeated successively until the bacteriophage titre reached a minimum of 1×10^8 pfu/mL.

Bacteriophage titration

To titre bacteriophages, a 10-fold serial dilution of each crude bacteriophage filtrate was made in SM buffer. The order of dilution was as follows: 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}. 100 μL of each dilution and 200 μL of the log-phase bacterial indicator were added to 5 mL of 0.75% TSA and overlaid on a TSA plate. An alternative technique was to spot the bacteriophage dilutions on top agar mixed with bacterial indicator. After overnight incubation at 37°C, the plaques were counted and the titre of bacteriophages was calculated through the standard formula.

$$C\ (\text{pfu/mL}) = \frac{n}{d \times V\ (\text{mL})}$$

C = bacteriophage titre
n = plaque count
d = dilution factor
V = volume of dilution added

Bacteriophage genome extraction

The following protocol modified from the original protocol from Liao et al. (2008) was used for the bacteriophage genome extraction. RNase A and DNase I (EPICENTRE Biotechnologies, USA) were added into 10 mL of bacteriophage lysate (at least 10^{9} pfu/mL) in a final concentration of 1 μg/mL (1 U/mL DNase I) each. The lysate was incubated with the endonucleases at 37°C for 30 minutes. Four millilitres of the precipitant solution (20% PEG 6000 plus 2 M NaCl) was added to the mixture and mixed well. The mixture was incubated on ice for one hour and centrifuged at 17,000 g for 20 minutes at 4°C. The supernatant was discarded and the resulting pellet was dissolved in 600 μL of SM buffer then transferred to a microfuge tube. An equal volume of the organic solution (phenol:chloroform:isoamyl alcohol 25:24:1) was added to the mixture and vortexed. The mixture was centrifuged at 10,000 g for 20 minutes at room temperature. The upper aqueous layer was transferred to a new tube. An equal volume of chloroform was added to the solution and mixed well. The mixture was centrifuged at 10,000 g for 20 minutes at room temperature. The upper aqueous layer was transferred to a new tube then an equal volume of isopropyl alcohol was added to the solution and mixed well. This mixture was incubated at -80°C for 12 minutes then was centrifuged at 10,000 g for 20 minutes at room temperature. The supernatant was discarded and 700 μL of 70% ethyl alcohol was added to the tube. The tube was centrifuged at 10,000 g for 20 minutes at room temperature. The supernatant was discarded and the resulting pellet was dried in vacuum for ten minutes then dissolved in 50 μL of TE buffer.

Bacteriophage genome typing

The type of bacteriophage genomes (dsDNA, ssDNA, dsRNA or ssRNA) was determined using the following protocol. The purity of extracted genomes was evaluated at A230/260 nm and A260/280 nm. One unit of DNase I, 1 μg/U of RNase A, a combination of both endonucleases, and 1 U of nuclease S1 (EPICENTRE Biotechnologies, USA) were separately added to 1 μg of each extraction and incubated for two to three hours at 37°C (modified to Shirako & Ehara, 1985) (Table 5-3). Ten microlitres of each reaction were electrophoresed on a 1% agarose gel then visualized under UV light (Figure 5-10).

Bacteriophage genome fingerprinting

Only bacteriophages (EFRM8, EFRM11, EFRM31, EFRM42, EFRM54 and EGRM195) that were used in transduction experiments were fingerprinted. This selection was based on the ability of bacteriophages to form plaques on the host and recipient cultures together with antimicrobial resistance profiles of the bacterial hosts and bacterial recipients. One microgram of the genome extract were mixed with 2 μL of 10× restriction enzyme buffer and 1 U of digestion enzymes including *Bam*HI, *Eco*RI, *Hind*III, *Nde*I, *Pst*I, *Rsa*I, *Sau*3AI, *Sma*I, *Spe*I, *Xba*I (NEB, USA) and a combination of two or three enzymes. A sufficient amount of sterile distilled water was added to the mixture to reach the final volume of 20 μL then incubated overnight at 37°C. This digest was loaded on a 1% agarose gel and electrophoresed. However, bacteriophage genomes were also diluted and treated with protinase K to decrease the inhibitory effect of possible contaminants such as proteins. The results were analyzed by comparision of the restriction fragment bands. The properties of the bacteriophages used in fingerprinting are shown in Table 5-4.

Electron microscopy

Bacteriophages were prepared for EM using a modified protocol originally proposed by Deveau et al. (2006). The bacteriophage samples were prepared in high ($\geq 1 \times 10^8$ pfu/mL) concentration without enrichment or preservation. One drop of bacteriophage suspension was deposited directly onto a colladium coated 200-mesh copper grid (2 mm diameter; Graticules, England), stabilized with a thin layer of carbon. This suspension was allowed to adsorb for 60 seconds then excess liquid was removed using a piece of filter paper. The sample was stained by 1% aqueous phosphotungstic acid (BDH AnalaR, UK) for 60 seconds and dried in air before being examined by a transmission electron microscope (TEM) (Philips CM 100, The Netherlands) at 80 KV.

Alternatively, a concentration method was applied when needed (Deveau et al., 2006). Five millilitres of bacteriophage suspension was centrifuged at 24,000 g for 60 minutes at 4°C. The supernatant was discarded and the resulting pellet was resuspended in 500 µL of 0.1 M ammonium acetate (pH 7.5) or TM buffer. This method was repeated and the concentrated phage suspension was used in electron microscopy as described above.

Results

Detection of enterococcal bacteriophages

Different types of plaques were detected after testing bacteriophage sources including piggery effluent and beach sewage samples on enterococci strains. These bacteriophages were isolated by testing a piggery effluent sample (Roseworthy piggery, SA) on pig enterococcal isolates including *E. faecalis* (*n* = 7), *E. faecium* (*n* = 7) and *E. gallinarum* (*n* = 1). Additionally, four bacteriophages were isolated from *E. faecalis* induced by mitomycin C. The bacteriophage properties are recorded in Table 5-4.

Characterization of the bacteriophages

Genome typing

Genome typing showed that most of the bacteriophage genomes consisted of dsDNA, with a few ssDNA or ssRNA (Table 5-4).

Attempts to fingerprint bacteriophages to determine the ancestral relationships between the bacteriophages by restriction enzyme cleavage failed to yield usable results. This technique usually generated only one or two bands, using commonly available restriction enzymes (Figure 5-10).

Electron microscopy

In the EM study, ten bacteriophages were detected that were isometric in shape with either a long flexible (nine *Siphoviridae* including bacteriophages EFRM8, EFRM31, EFRM 31M, EFRM42, EFRM54, EFRM54M, EFRM106, EFRM110 and EFRM111) tails (Figures 5-11, 5-12, 5-13 and 5-14) or non-flexible tail (*Myoviridae* EFRM11) (Figure 5-15) while one had an elongated head with long flexible tail (*Siphoviridae* EGRM195) (Figure 5-16) and one had an isometric head with a short non-flexible tail (*Podoviridae* EFRM8) (Figure 5-17). Of non-tailed bacteriophages, one was polyhedral (*Leviviridae* EFRM8), four filamentous (mostly *Inoviridae* including bacteriophages EFRM31M, EFRM54, EFRM70 and EGRM195) (Figures 5-18 and 5-19) and two pleomorphic (one

Guttaviridae-like EFRM42 and one *Fuselloviridae* EFRM54) (Figures 5-20 and 5-21) in morphology. All tailed bacteriophages contained double-stranded DNA (dsDNA) while non-tailed bacteriophages contained a DNA (ss) or RNA genome. The tailed bacteriophages varied (from 79 to 293 nm) and had tails in variable length (Table 5-5). PFP bacteriophages ranged from 41 to about 1628 nm in length depending on the shape (Table 5-6).

Discussion

Morphology of detected bacteriophages

Although most of the bacteriophages found in this study belonged to the Order *Caudovirales* (dsDNA tailed bacteriophages), PFP bacteriophages were observed for the first time in association with enterococci of non-human origin. Thousands of bacteriophages (at least 5568 including viruses of eubacteria and archaea) have been examined by electron microscope (EM) since 1959 (Ackermann, 2007). Previous papers indicate that approximately 96% of all bacteriophages have a tail, of which up to 90% feature a single tail while aminority are polyhedral, filamentous or pleomorphic (PFP) (Ackermann, 1996, 2001, 2007). Most (61%) tailed bacteriophages feature long, noncontractile tails and are categorized in the *Siphoviridae* Family whereas filamentous bacteriophages are categorized in three Families: *Inoviridae*, *Lipothrixviridae* and *Rudiviridae* (Ackermann, 2003). The current literature has not reported the isolation of PFP bacteriophages from enterococci of non-human origin and indeed only a limited number of tailed enterococcal bacteriophages have been characterized (Ackermann & Kropinski, 2007; Ackermann, 2007). The only report of isolation of PFP bacteriophages (undifferentiated) from enterococci is by Bachrach et al. (2003). Therefore, the present study broadens the range of bacteriophages associated with enterococci.

Bacterial host isolates

Relatively little is known about enterococcal bacteriophages (Stevens et al., 2009; Nigutova et al., 2008). In this study, most of the bacteriophages were isolated or induced from pig strains of *E. faecalis* and fewer from *E. faecium* and *E. gallinarum*. Enterococcal bacteriophages have previously been isolated from *E. faecalis* and *E. faecium* strains. Pleceas and Brandis (1974) conducted an experiment for rapid identification of enterococci by means of bacteriophages including those from *E. faecalis* and *E. faecium*. Similarly, Caprioli et al. (1975) described a phage-typing scheme for urogenital Group D

streptococci, using bacteriophages specifically active against these isolates. Later, Ramirez et al. (2006) isolated and characterized lytic bacteriophages from enterococci including strains of *E. faecalis* and *E. faecium*.

Tailed bacteriophages

Siphoviridae bacteriophages

All tailed bacteriophages detected in the current study, except two (*Myoviridae* EFRM11 and *Podoviridae* EFRM8), were typical of *Siphoviridae* Family. By 2007, only 36 tailed bacteriophages have been identified in *Enterococcus* spp.; mostly belong to the *Siphoviridae* (Ackermann, 2007). The *Siphoviridae* Family belongs to the *Caudovirales* Order (dsDNA-tailed bacteriophages of both Gram-positive and Gram-negative bacteria) of bacteriophages and consists of isometric or elongated capsids with long noncontractile tails (Labrie et al., 2008). Ramirez et al. (2006) isolated and characterized lytic-tailed bacteriophages (*Siphoviridae*) of *E. faecalis* and *E. faecium* strains from clinical samples; one included broad-range hosts.

Myoviridae bacteriophages

Another bacteriophage found in the current study belonged to *Myoviridae*. The *Myoviridae* Family (Order *Caudovirales*) features isometric heads with contractile tails (Said et al., 2009; O'Flaherty et al., 2005). In 1993, Jarvis et al. propagated *Myoviridae* bacteriophages on an *Enterococcus* sp. The bacteriophages had isometric heads, contractile tails and complex base plates. The bacteriophage proteins were identical to proteins of other isolated *Myoviridae* bacteriophages *B. subtilis, B. thuringiensis, L. plantarum* and *S. aureus.*

Podoviridae bacteriophages

A *Podoviridae* bacteriophage (EFRM8) was identified in this study. The *Podoviridae* Family is classified in the *Caudovirales* Order and contains isometric heads with short tails (Ackermann, 2003). Up-to-date, only a few (n = 3) bacteriophages of *Podoviridae* have been isolated from enterococci (Ackermann, 2007). However, a proportion of bacteriophages in nature do not contain tails and are placed in the polyhedral, filamentous or pleomorphic category.

PFP bacteriophages

Inoviridae bacteriophages

In the current study, filamentous bacteriophages (*Inoviridae* EFRM31M, EFRM54, EFRM70 and EGRM195) were found more often than polyhedral and pleomorphic bacteriophages (ratio 4:3). Filamentous bacteriophages were isolated from *E. faecalis* (n = 3) and *E. gallinarum* (n = 3) strains of pig origin. These bacteriophages are found in both eubacteria (*Inoviridae*) (Campos et al., 2003) and archaea (*Lipothrixviridae* and *Rudiviridae*) (Geslin et al., 2003). *Inoviridae* (ICTVdB Virus Code: 00.035) are either long slender tube or short rod bacteriophages with a maximum size of 2 μm and contain a single-stranded circular DNA genome (Klieve et al., 2004). They are distributed worldwide and can be isolated from a variety of bacteria such as Enterobacteria, *Vibrio* spp., *Pseudomonas* spp., *Xanthomonas* spp., *Acholeplasma* spp. and *Spiroplasma* spp. (Fauquet & Pringle, 2000). Yu et al. (2006) isolated and characterized *Thermus* bacteriophages from alkaline hot springs in different locations including Iceland, New Zealand, Russia and the USA. They suggested that these bacteriophages contribute to horizontal gene transfer (HGT).

Leviviridae bacteriophages

Another PFP bacteriophage with spherical shape (poliovirus-like EFRM8) was isolated on an *E. faecium* strain of pig origin in the current study. Poliovirus-like bacteriophage group (e.g. MS2) belongs to *Leviviridae* Family (ICTVdB Virus Code: 00.037) of bacteriophages. These polyhedral bacteriophages contain a single-stranded linear molecule of RNA (Vinje et al., 2004). Single-stranded RNA bacteriophages are widely found in bacterial isolates from sewage and feces of mammals but unlikely to be found in avian bacterial species (Bollback & Huelsenbeck, 2001). *Leviviridae* are small bacteriophages with a reported size of 23 to 30 nm (Love et al., 2008; Klovins et al., 2002). However, the *Leviviridae*-like particles found in the current study ranged from 41 to 43 nm in size.

Guttaviridae bacteriophages

Furthermore, a few pleomorphic bacteriophages (droplet shape EFRM54 and lemon shape EFRM42) were detected from *E. faecalis* isolates. Droplet-shaped bacteriophages are classified in the *Guttaviridae* Family (ICTVdb Virus Code: 00.095); firstly investigated by Zillig et al. (1998) and described by Arnold et al. (2000) in archaebacteria and have never been previously reported in eubacteria (Liu et al., 2009; Lipps, 2006; Vestergaard et al., 2005). These bacteriophages bear long thin filaments (beard) at one end and contain a

dsDNA genome (Prangishvili et al., 2001; Prangishvili et al., 1999). The genome consists of single segment containing a circular dsDNA molecule (approx. size 20 kb) (Rice et al., 2001). This segment forms a covalently closed circle, which is extensively methylated (Sau & Deb, 2008; Prangishvili & Garrett, 2004). However, the droplet-shaped bacteriophage (EFRM54) isolated in this study seems to lack monopolar filaments.

Fuselloviridae bacteriophages

Another pleomorphic bacteriophage (EFRM42) found in the current study is more likely to belong to the Family *Fuselloviridae* (ICTVdb Virus Code: 00.028). *Fuselloviridae* represents lemon/spindle-shaped bacteriophages having peripheral projections and short tail-like fiber(s) attached to one pole (Kraft et al., 2004; Prangishvili et al., 2001). The bacteriophages contain a single molecule of circular, supercoiled dsDNA (Wiedenheft et al., 2004). As with *Guttaviridae*, *Fuselloviridae* have been isolated only on or from archaebacteria (Xiang et al., 2005; Rice et al., 2004; Stedman et al., 2003). However, the detection of possible archaeal bacteriophages in eubacteria cannot be completely ruled out as other bacteriophage families (*Siphoviridae* and *Myoviridae*) are found in both bacterial kingdoms (Geslin et al., 2003). In conclusion, the diversity of the bacteriophages found in the current study is significant. One probable reason relates to the source of the bacteriophages which was piggery effluent rather than human material, as most enterococcal bacteriophages have previously been isolated from enterococci of human, pet or zoo origins. Novel bacteriophages are likely to be found in enterococci of other livestock or environmental samples as well. More studies are needed to determine the role of these novel bacteriophages in the genetic modification of enterococci.

Bacteriophage genome fingerprinting

Fingerprinting of the genome of microorganisms depends on multiple factors such as the yield of amplification, the coverage of the genome and contamination of the sample (Barbaux et al., 2001). The bacteriophage genome usually contains highly modified nucleotides such as 5-hydroxymethyl cytosine and 5-hydroxymethyl uracil, which are resistant to the restriction enzymes (Uchiyama et al., 2008a). In the current study, some of the most commonly used restriction enzymes were chosen to map the bacteriophage genomes. Furthermore, the possible restriction sites within the genome of bacteriophage EFRM31 were identified using Kodon® software and online tools such as NEBcutter V2.0, Webcutter 2.0 and RestrictionMapper version 3. This included 52 cutting sites for *Rsa*I restriction enzyme. Cutting sites for other enzymes included one for either *Sau*3AI or *Pst*I,

three for *Spe*I and eight for either *Hind*III or *Nde*I. No cutting sites were found for *Bam*HI, *Eco*RI, *Sma*I and *Xba*I. However, bacteriophage genome fingerprinting by the digestion method failed to produce multiple segments of restriction digested genome probably due to: DNA protection by bound proteins, methylation, or other site-specific DNA modifications; bad quality DNA (Magrini et al., 1997; McClelland et al., 1994; Vogelsang-Wenke & Oesterhelt, 1988). Other digestions with different non-sequenced phage genomes (EFRM8, EFRM11, EFRM42, EFRM54 and EGRM195) failed to produce more than one band, probably due to the problems discussed above. Therefore, this study was unable to determine the possible relationship or common ancestors of the bacteriophages in this study.

Conclusions

In the few studies previously reported, the enterococcal bacteriophages largely belonged to the tailed bacteriophage family (Family *Siphoviridae*). Up to now, filamentous and pleomorphic bacteriophages have not been reported in enterococci and enterococcal polyhedral bacteriophages have only been reported from isolates of human origin. This study is the first to report filamentous and pleomorphic bacteriophages from enterococci or enterococcal-associated material and polyhedral bacteriophages from enterococci of non-human origin. In conclusion, the diversity of the bacteriophages found in the current study is significant. This diversity might be related to the fact that the bacteriophages were isolated from piggery effluent rather than from human, pet or zoo samples. More research is needed to determine the role of these novel bacteriophages play in the transfer of genetic information between enterococci.

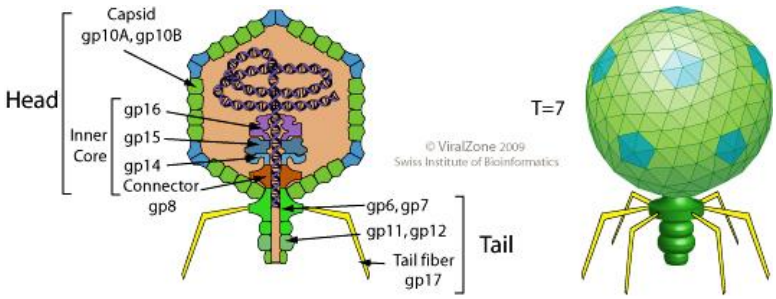


Figure 5-1. Schematic of *Podoviridae*

From Viral Zone—ExPASy website

http://www.expasy.ch/viralzone/all_by_species/141.html

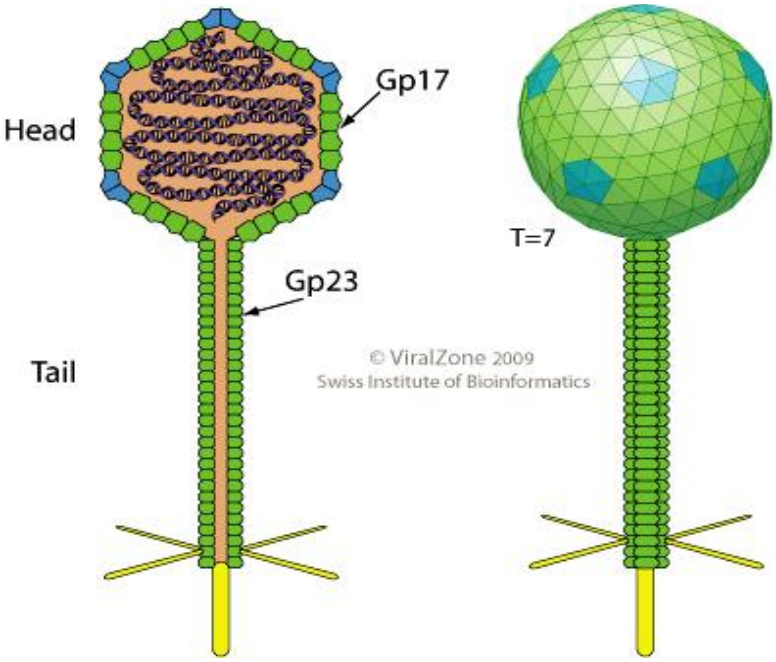


Figure 5-2. Schematic of *Siphoviridae*

From Viral Zone—ExPASy website

http://www.expasy.ch/viralzone/all_by_species/142.html

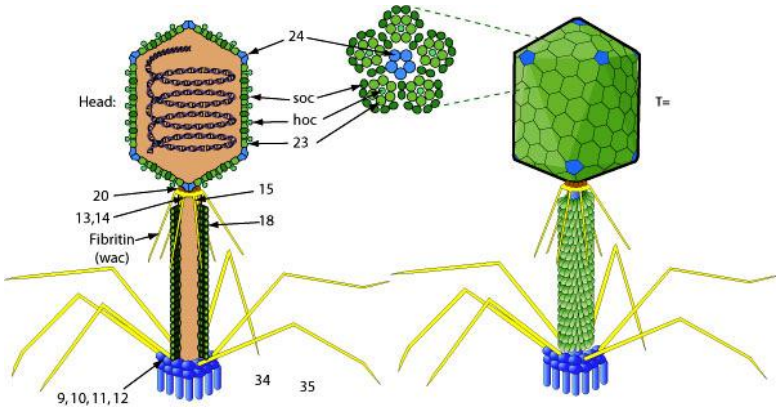


Figure 5-3. Schematic of *Myoviridae*

From Viral Zone—ExPASy website

http://www.expasy.ch/viralzone/all_by_species/140.html

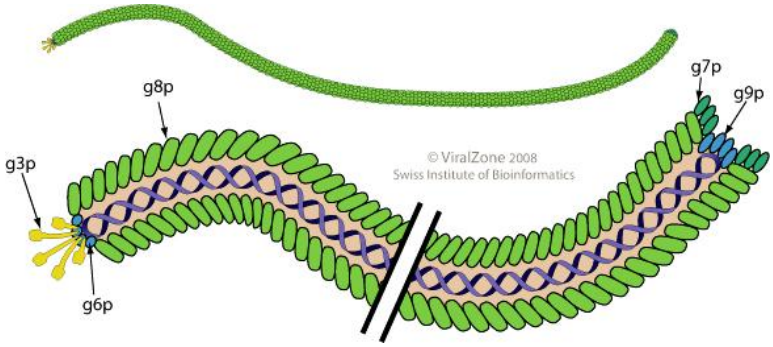


Figure 5-4. Schematic of *Inoviridae*

From Viral Zone—ExPASy website

http://www.expasy.ch/viralzone/all_by_species/113.html

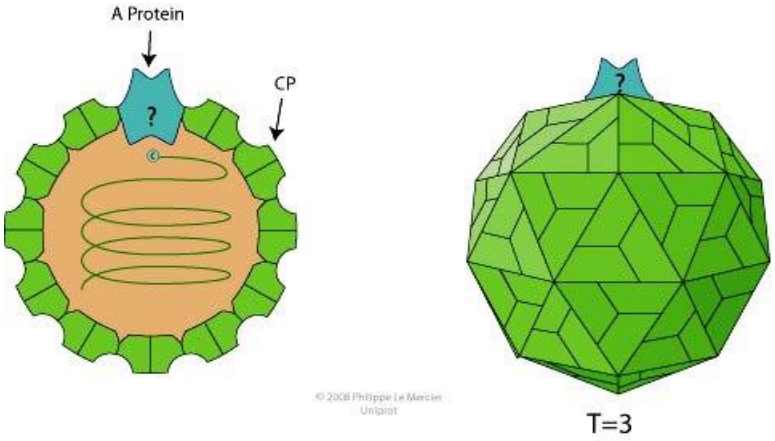


Figure 5-5. Schematic of *Leviviridae*

From Viral Zone—ExPASy website

http://www.expasy.ch/viralzone/all_by_species/163.html

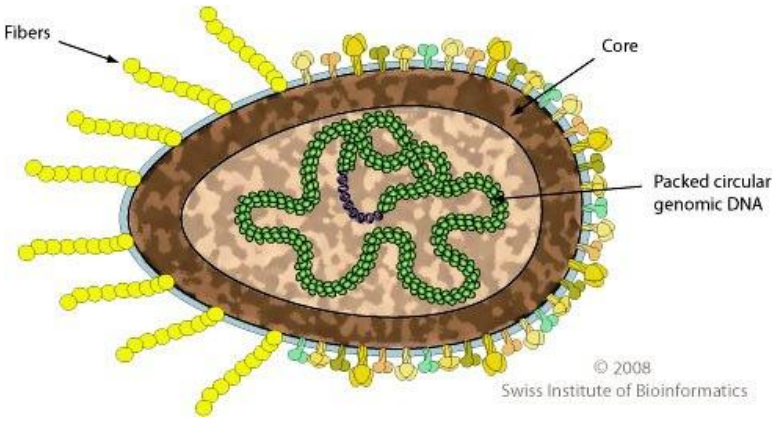


Figure 5-6. Schematic of *Guttaviridae*

From Viral Zone—ExPASy website

http://www.expasy.ch/viralzone/all_by_species/234.html

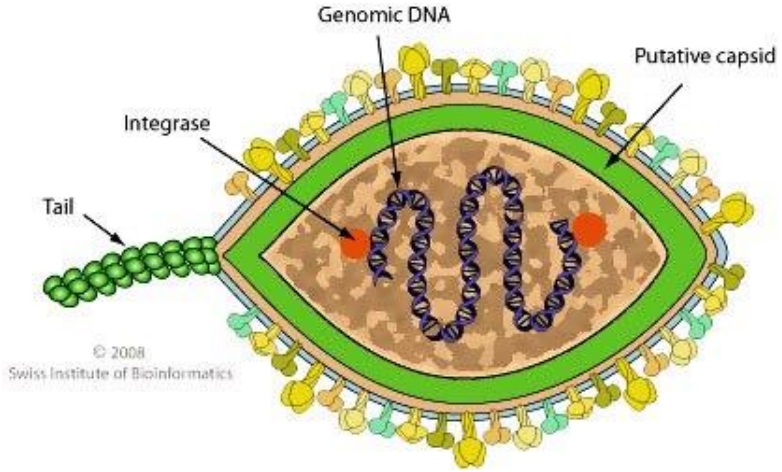


Figure 5-7. Schematic of *Fuselloviridae*

From Viral Zone—ExPASy website

http://www.expasy.ch/viralzone/all_by_species/20.html

Table 5-1. Morphology of tailed bacteriophages

Family	Head	Tail
Siphoviridae	Single, isometric or long	Single or multiple, long, noncontractile
Myoviridae	Single, isometric	Single or multiple, long, contractile
Podoviridae	Single, isometric	Single or double, short, noncontractile

From Ackermann, 2003

Table 5-2. Enterococcal bacteriophage morphology (Order *Caudovirales*)

Family	Bacteriophage name	Specifications
Myoviridae, A1	DF_{78}, F1, F2, 1, 2, 4, 14, 41, 867	Non-enveloped, tail contractile, head isometric, Linear dsDNA
Podoviridae, C1	D1, SB24, 2BV, 182, 225	Non-enveloped, tail short and noncontractile, head isometric, Linear dsDNA
Podoviridae, C3	C2, C2F, E3, E62	Non-enveloped, tail short and noncontractile, head elongated (L/W ≥ 2.5), Linear dsDNA
Siphoviridae, B1	DS96, H24, M35, P3, P9, SB101, S2, 2BII, 5, 182a, 705, 873, 881, 940, 1051, 1057, 21096C, NN-*Enterococcus*	Non-enveloped, tail long and noncontractile, head isometric, Linear dsDNA
Siphoviridae, B2	PE1	Non-enveloped, tail long and noncontractile, head elongated (L/W = 1.2−2), Linear dsDNA
Siphoviridae, B3	F1, F3, F4, VD13, 1, 200, 235, 341	Non-enveloped, tail long and noncontractile, head elongated (L/W ≥ 2.5), Linear dsDNA

L/W, lenth to width ratio

From Ackermann & Abedon, 2000

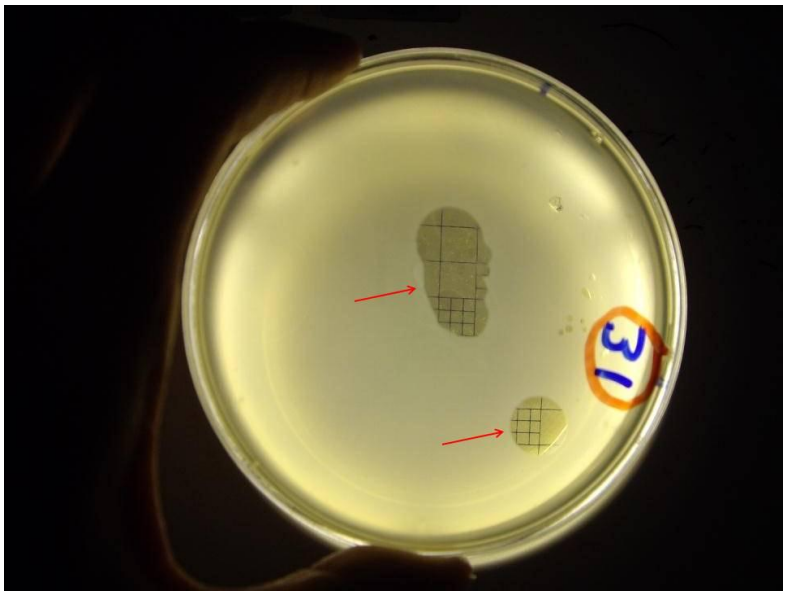


Figure 5-8. Enterococcal bacteriophage plaques induced by mitomycin C

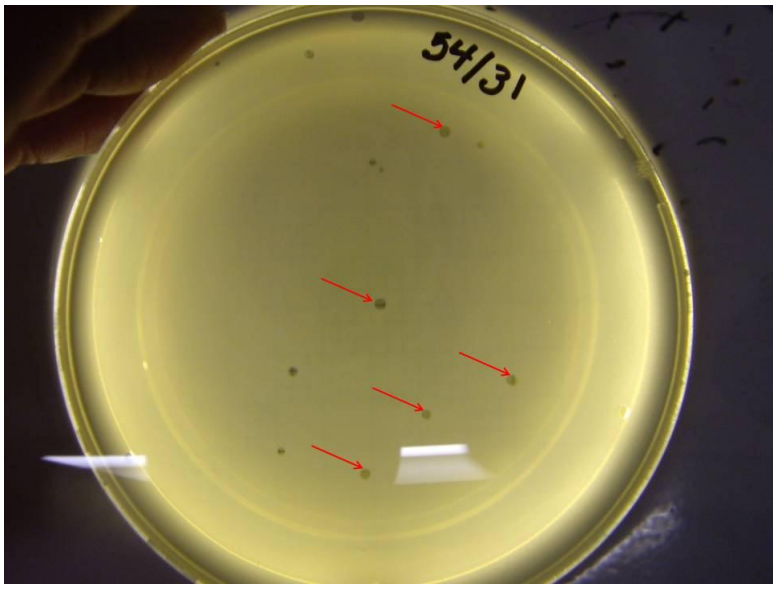


Figure 5-9. Enterococcal bacteriophage plaques on TSA plate. Bacteriophages were isolated from a piggery effluent sample

Table 5-3. Rationale of bacteriophage genome characterization

DNase		RNase		DNase & RNase		Nuclease S1	
+	-	+	-	+	-	+	-
ssDNA dsDNA	ssRNA dsRNA	ssRNA	ssDNA dsDNA dsRNA	ssDNA dsDNA ssRNA	dsRNA	ssDNA ssRNA	dsDNA dsRNA

+, digested; -, undigested



Figure 5-10. Bacteriophage genome typing of EFRM31. Lane 1, 1 kb marker; Lane 2, DNase; Lane 3, RNase; Lane 4, DNase & RNase; Lane 5, nuclease S1

Table 5-4. The bacteriophage specifications

Phage no.	Source	Host (no.)	Ori^	Indicator (no.)	Ori^	Titre (pfu/mL)	Genome
EFRM31M*	Isolate	*E. faecalis* (31)	Pig	*E. faecalis* (31)	Pig	6.5×10^7	dsDNA
EFRM54M*	Isolate	*E. faecalis* (31)	Pig	*E. faecalis* (54)	Pig	1.9×10^7	dsDNA
EFRM31M*	Isolate	*E. faecalis* (54)	Pig	*E. faecalis* (31)	Pig	1.1×10^7	dsDNA
EFRM54M*	Isolate	*E. faecalis* (54)	Pig	*E. faecalis* (54)	Pig	4×10^6	dsDNA
EFRM31**	Sample#	-	-	*E. faecalis* (31)	Pig	7×10^9	dsDNA
EFRM54**	Sample#	-	-	*E. faecalis* (54)	Pig	6×10^{10}	dsDNA/ssDNA
EFRM148	Sample#	-	-	*E. faecium* (148)	Pig	N/A	N/A
EGRM195**	Sample#	-	-	*E. gallinarum* (195)	Pig	4×10^9	dsDNA/DNA
EFRM8**	Sample#	-	-	*E. faecium* (8)	Pig	1.6×10^{11}	dsDNA/ssRNA
EFRM11**	Sample#	-	-	*E. faecium* (11)	Pig	8×10^9	dsDNA
EFRM42**	Sample#	-	-	*E. faecalis* (42)	Pig	4.2×10^{10}	dsDNA
EFRM70	Sample#	-	-	*E. faecalis* (70)	Pig	3×10^9	DNA
EFRM94	Sample#	-	-	*E. faecium* (94)	Pig	N/A	N/A
EFRM95	Sample#	-	-	*E. faecalis* (95)	Pig	N/A	N/A
EFRM101	Sample#	-	-	*E. faecalis* (101)	Pig	N/A	N/A
EFRM102	Sample#	-	-	*E. faecalis* (102)	Pig	N/A	N/A
EFRM106	Sample#	-	-	*E. faecium* (106)	Pig	1.5×10^8	dsDNA
EFRM 110	Sample#	-	-	*E. faecium* (110)	Pig	2.4×10^8	dsDNA
EFRM111	Sample#	-	-	*E. faecium* (111)	Pig	4.4×10^8	dsDNA

*Induction by mitomycin C; **used in fingerprinting; ^Origin; #piggery effluent (Roseworthy, SA); N/A, not applicable

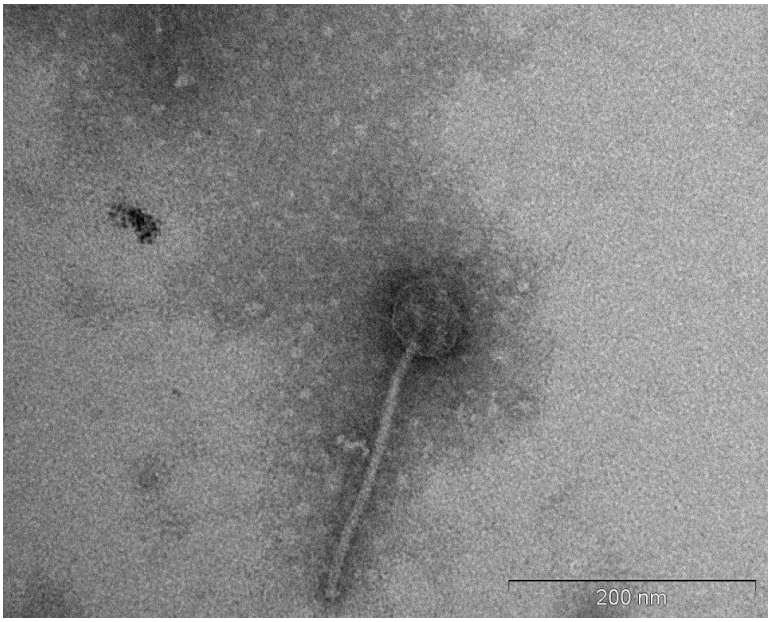


Figure 5-11. An isometric head, long tailed bacteriophage—EFRM42 (130,000×)

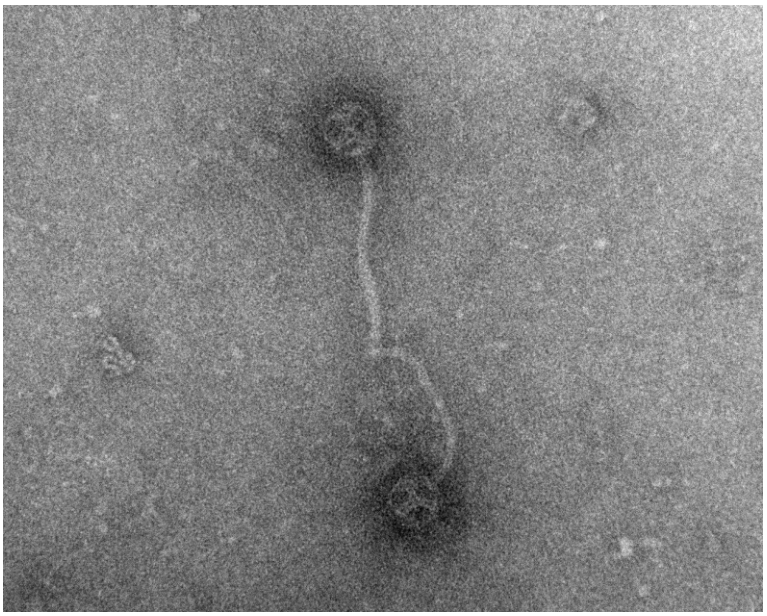


Figure 5-12. Isometric head, long tailed bacteriophages—EFRM31 (92,000×)

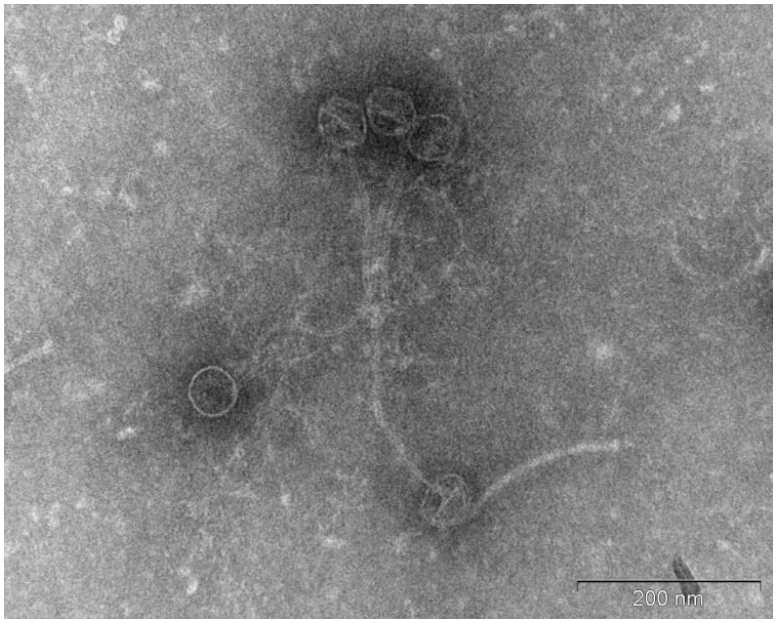


Figure 5-13. Isometric head, long tailed bacteriophages—EFRM110 (92,000×)

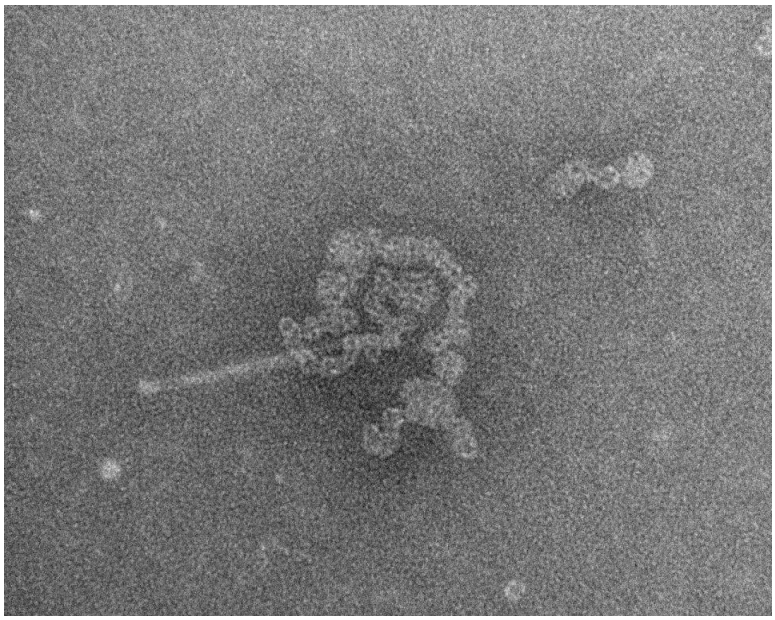


Figure 5-14. A tailed bacteriophage—EFRM54 (130,000×). Circular double-stranded genome is removed from the bacteriophage

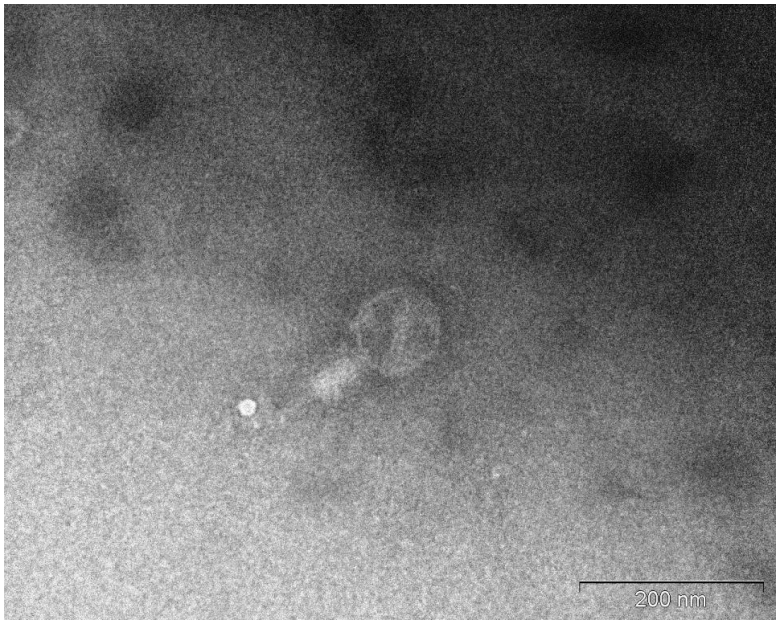


Figure 5-15. An isometric head, long tailed bacteriophage—EFRM11 (92,000×)

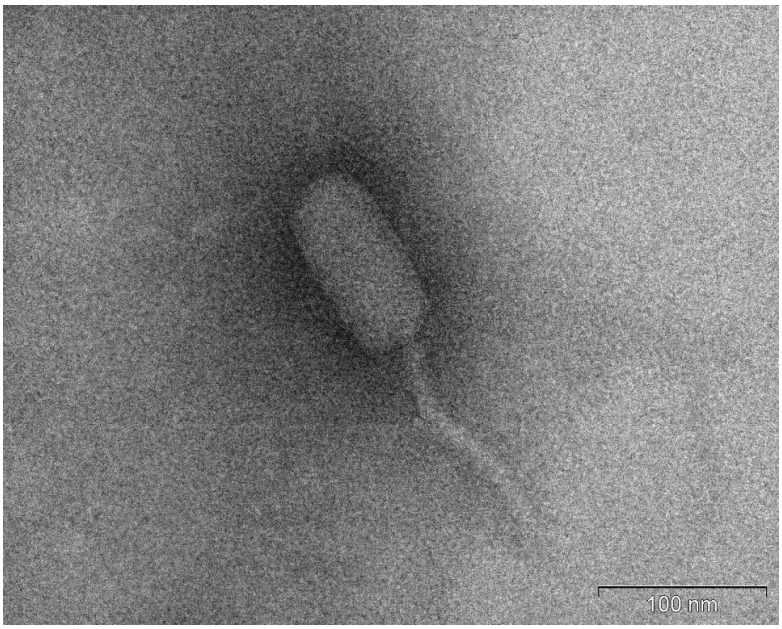


Figure 5-16. A long head, long tailed bacteriophage—EGRM195 (180,000×)

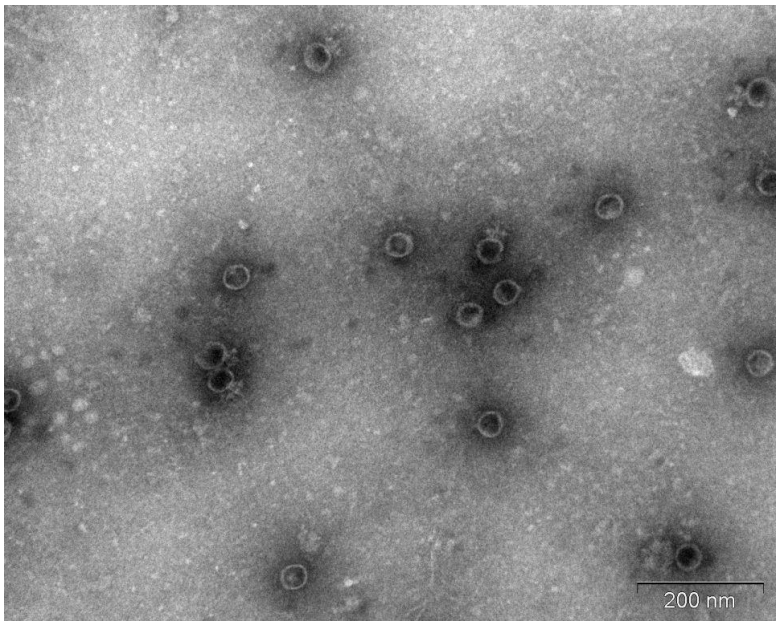


Figure 5-17. Isometric head, short tailed bacteriophages—EFRM8 (92,000×)

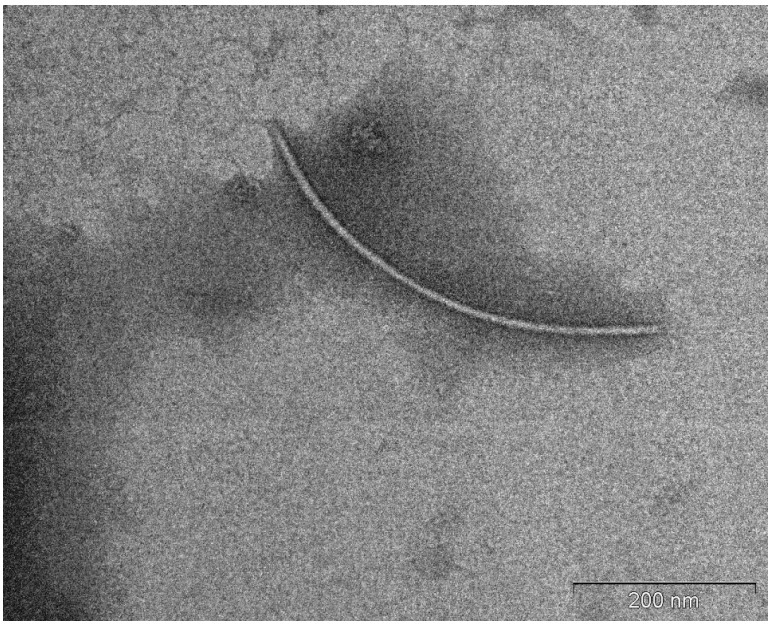


Figure 5-18. A filamentous bacteriophage—EFRM70 (92,000×)

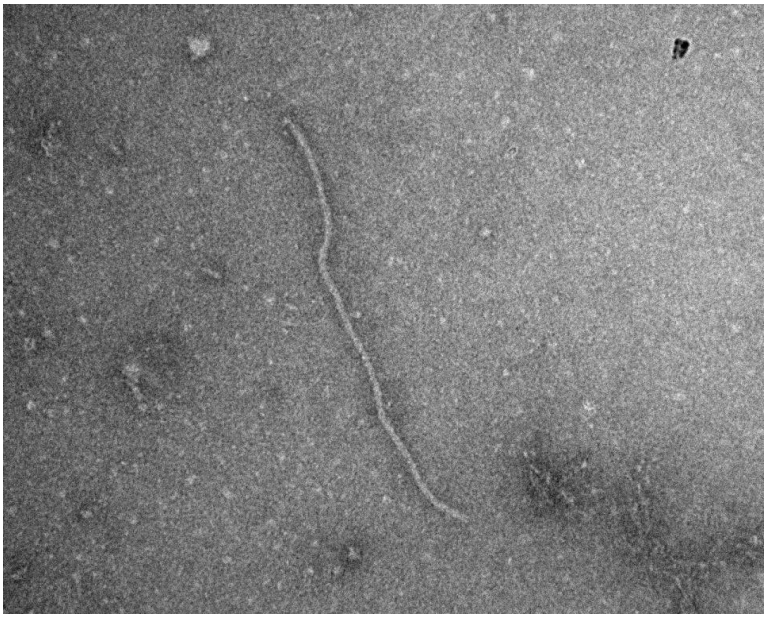


Figure 5-19. A filamentous bacteriophage—EFRM54 (64,000×)

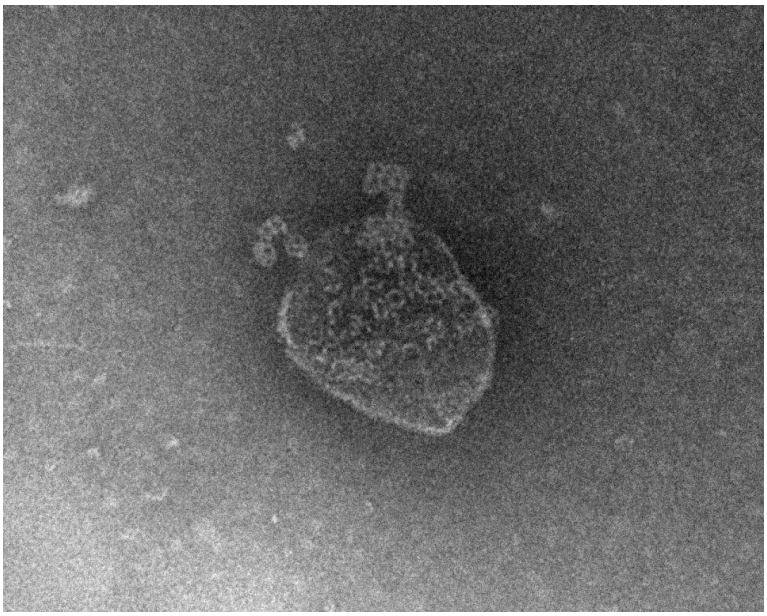


Figure 5-20. A lemon shaped (pleomorphic) bacteriophage—EFRM42 (130,000×)

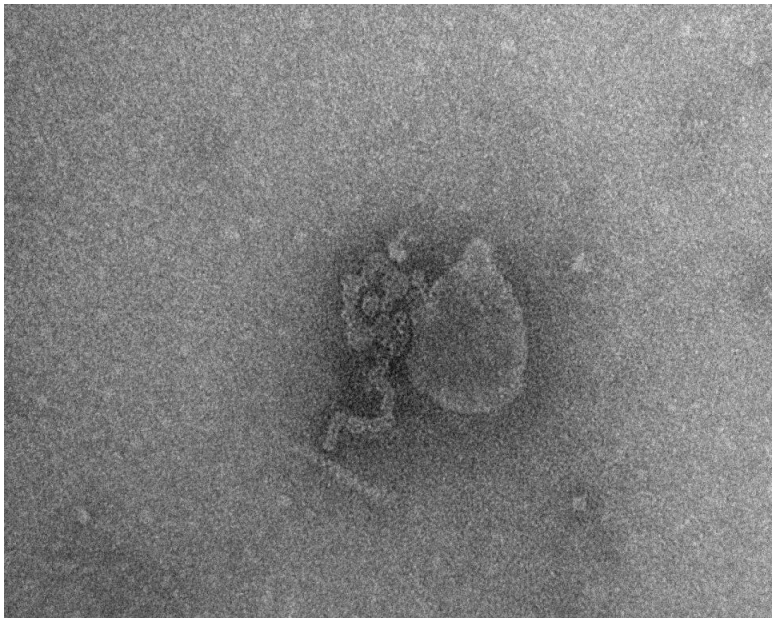


Figure 5-21. A droplet shaped (pleomorphic) bacteriophage—EFRM54 (92,000×). Circular double-stranded genome is leaking from the bacteriophage

Table 5-5. Properties of enterococcal tailed bacteriophages

No.	Morphology		Mean dimensions (nm)					Genome	*	Indicator
	Head	Tail	Head (*l*)	Head (*w*)	Tail (*l*)	Tail (*w*)	Overall			
EFRM8	I	S.L.F.NC	65	63	222	10	287	dsDNA	PE	*E. faecium*
EFRM8	I	S.S*.NF.NC	50	48	29	43	79	dsDNA	PE	*E. faecium*
EFRM11	I	S.L.NF.C	105	93	122	14[†]–30[‡]	227	dsDNA	PE	*E. faecium*
EFRM31	I	S.L.F.NC	62	55	206	11	268	dsDNA	PE	*E. faecalis*
EFRM31M	I	S.L.F.NC	59	54	211	13	270	dsDNA	PE	*E. faecalis*
EFRM42	I	S.L.F.NC	63	59	230	9	293	dsDNA	PE	*E. faecalis*
EFRM54	I	S.L.F.NC	50	49	189	12	239	dsDNA	PE	*E. faecalis*
EFRM54M	I	S.L.F.NC	52	50	231	9	283	dsDNA	PE	*E. faecalis*
EFRM106	I	S.L.F.NC	69	68	156	10	225	dsDNA	PE	*E. faecium*
EFRM110	I	S.L.F.NC	60	59	196	10	256	dsDNA	PE	*E. faecium*
EFRM111	I	S.L.F.NC	57	56	201	10	258	dsDNA	PE	*E. faecium*
EGRM195	L	S.L.F.NC	103	58	134	11	237	dsDNA	PE	*E. gallinarum*

Source; [†]Sheath; [‡]Core; M, mitomycin C induced; I, isometric; S, single; L, long; S, short; F, flexible; NF, non-flexible; C, contractile; NC, non-contractile; PE, piggery effluent

Table 5-6. Properties of enterococcal PFP bacteriophages

No.	Morphology	Mean dimensions (nm)		Genome	Source	Indicator
		Length	Width			
EFRM8	Spherical	41−43	4.5 ± 0.1[†]	ssRNA	PE	*E. faecium*
EFRM31M	Filamentous	532	22	DNA	PE	*E. faecalis*
EFRM42	Lemon shape	141[‡]	107	dsDNA	PE	*E. faecalis*
EFRM54	Droplet shape	143	104	dsDNA	PE	*E. faecalis*
EFRM54	Filamentous	1599−1628	20	ssDNA	PE	*E. faecalis*
EFRM70	Filamentous	540	9	DNA	PE	*E. faecalis*
EGRM195	Filamentous	745	13	DNA	PE	*E. gallinarum*

[†]Cell wall diameter; [‡]Tail-like fibers varied; M, mitomycin C induced; PE, piggery effluent

Chapter 6

Role of Bacteriophages in Transfer of Resistance Genes

Introduction

Bacteriophages can play a role in transferring genes such as those that are responsible for antibiotic resistance via transduction (Ortmann et al., 2002). Gene transfer via transduction occurs when bacteriophages package host bacterial DNA rather than bacteriophage DNA during bacteriophage replication. Transduction is divided into two major types, generalized and specialized, based on the number and location of genes transferred (Kelly et al., 2009). In generalized transduction, potentially any bacterial gene can be transferred into the recipient cell. The bacterial host DNA is broken down into smaller fragments and randomly packed into the replicating bacteriophage (lytic) particles (Brussow et al., 2004). The size of packed DNA is small enough to fit into a bacteriophage head (Canchaya et al., 2003). This genome transfer may result in recombination. In specialized transduction, in contrast, only certain genes can be transferred from the donor bacteria to the recipient cell, meaning that only the host DNA on either side of the insertion site of prophage can be transferred (Tanyashin et al., 2003; Mazodier & Davies, 1991). The release of inserted prophages is controlled by several factors as environmental (e.g. radiation, humidity, temperature), nutritional and chemical (e.g. antibiotics) conditions (Morrison, 1996). Possible recombination between the donor and recipient genes may occur.

Lysogenic conversion is naturally seen in both Gram-positive and Gram-negative bacteria (Boyd et al., 2001). Examples of virulence genes transferred between Gram-positive bacteria by lysogenic conversion include neurotoxin genes in *C. botulinum* (Hauser et al., 1994; Barksdale & Arden, 1974), enterotoxin, staphylokinase and toxic shock syndrome toxin-1 genes in *S. aureus* (Fitzgerald et al., 2001; Lindsay et al., 1998; Coleman et al., 1989; Betley & Mekalanos, 1985), erythrogenic toxin genes in *S. pyogenes* (McShan & Ferretti, 1997; Weeks & Ferretti, 1984) and diphtheria toxin genes in *C. diphtheriae* (Freeman, 1951). Examples of virulence genes transferred between Gram-negative bacteria by lysogenic conversion include Shiga toxins, enterohemolysin, serum resistance and host cell envelope protein genes in *E. coli* O157:H7 (Neely & Friedman, 1998; Beutin et al., 1993; Barondess & Beckwith, 1990; Newland et al., 1985), O-antigen acetylase and glucosyl transferase genes in *S. flexneri* (Mavris et al., 1997; Huan et al.,

1997a, 1997b; Clark et al., 1991; Verma et al., 1991), type III effector, superoxide dismutase, neuraminidase, insertion element and glucosylation genes in *S. enterica* (Stanley et al., 2000; Mirold et al., 1999; Figueroa-Bossi & Bossi, 1999; Hardt et al., 1998; Wright, 1971), cholera toxin, G-protein like and TCP pilin genes in *V. cholera* (Karaolis et al., 1999; Waldor & Mekalanos, 1996; Reidl & Mekalanos, 1995) and cytotoxin genes in *P. aeruginosa* (Hayashi et al., 1993). In contrast, most of the antimicrobial resistance genes are transferred by the mechanisms other than transduction such as the conjugation. Conjugation is the major mechanism of acquired gene (e.g. antibiotic resistance and virulence) transfer in Gram-positive bacteria including enterococci (Dunny et al., 1995).

In conjugation or bacterial mating, MGEs are transferred from a donor to the recipient bacteria, which is controlled by intercellular signalling (peptide pheromones) (Dunny, 2007). For example, *van* genes are transferred via conjugation in *Enterococcus* spp. (Lund et al., 2006). The *van*A gene, which encodes for high-level resistance to vancomycin, has been shown to be transferred by conjugative transposons such as Tn*1546* (Courvalin, 2006). Other conjugative transposons like Tn*1547* and Tn*1549*-Tn*5382* in *E. faecium* are responsible for transferring *van*B genes that encode variable levels of resistance to vancomycin. Furthermore, Tn*1545* carries kanamycin and erythromycin resistance genes in *E. faecalis* as well as the *tet*M gene (Courvalin & Carlier, 1986). The *tet*M gene, which confers resistance to tetracycline and minocycline, is usually carried on a conjugative transposon Tn*916* and also a related element Tn*5386* in *E. faecalis* (Rice et al., 2007). Another gene transfer route in enterococci is chromosome-to-chromosome gene exchange, as shown for Tn*5385* in *E. faecalis* for vancomycin and ampicillin transfer mechanisms (Rice, 2000). However, some antimicrobial resistance encoding genes have been shown to transfer between the members of Gram-positive and Gram-negative bacteria by transduction (Zhang & LeJeune, 2008; Westwater et al., 2002; Blahova et al., 1999; Willi et al., 1997; Cerquetti & Hooke, 1993; Zeph et al., 1988; Piepersberg et al., 1983; Landen et al., 1981).

Since the first report on the successful isolation of bacteriophages from a Group D *Streptococcus* sp. (later classified as enterococci) by Clark and Clark in 1927, some valuable research for the identification and characterization of enterococcal bacteriophages has been conducted, but few studies report on the molecular biology, epidemiology and role of bacteriophages in transferring antibiotic resistance genes. Most of the contemporary research on enterococcal bacteriophages seems to focus on the use of

bacteriophages to treat enterococcal infections (phage therapy) (Paisano et al., 2004; Fischetti et al, 2004; Thiel, 2004). Therefore, studies on the role of bacteriophages in transferring antimicrobial resistance genes are important, since antimicrobial resistance transfer is an important problem in human health. This may help us to understand the antimicrobial resistance phenomenon better and to uncover some secrets about the resistance transfer mechanisms. This chapter includes the protocols used for the assessment of the role of enterococcal bacteriophages in transfer of resistance genes.

Materials and Methods

Transduction conditions

The selected bacteriophages, phage hosts and the bacterial recipients were chosen on the basis of their ability to form plaques on the host and recipient cultures together with antimicrobial resistance profiles of the host and recipient bacteria. Only enterococcal isolates were used in this experiment. All bacteriophage host strains where phenotypically and/or genotypically resistant to antimicrobials tested in the experiment, which were absent in recipient bacterial strains. The resistance profiles of putative transductants were determined by a combination of MIC, PCR and sequencing methods. One millilitre of a high-titre bacteriophage with at least 10^8 pfu/mL was treated with nucleases and added to 9 mL of an overnight-culture bacterial recipient in TSB (Oxoid, Australia) and incubated 30 minutes at 37^{o}C. The bacterial cells were washed with TSB then resuspended in 1 mL TSB and incubated one hour at 37^{o}C. After incubation, this mixture was plated on ten antimicrobial plates (100 µL each) containing the highest breakpoint concentration of antimicrobials (resistance breakpoint). These antimicrobials were chosen depended on the isolate resistance pattern and included gentamicin (32, 64 and 1024 µg/mL), tetracycline (16 and 64 µg/mL), bacitracin (64 IU/mL), flavophospholipol (16 µg/mL), vancomycin (32 µg/mL) and copper (28 mM/mL). The plates were incubated at 37^{o}C for 24 hours and then examined for growth. Negative controls were plated on which no bacteria inoculated were incubated for more 24 hours under the same conditions. Control organisms *E. coli* ATCC 25929, *E. faecalis* ATCC 29212, *E. faecium* ATCC 19434, *S. aureus* ATCC 25923 and *S. aureus* ATCC 29213 were used in each set. The methods for preparing antimicrobial stock and working solutions are described in Chapter 3. An overview of the bacteriophages and the bacterial recipients is available in Tables 6-1 and 6-2. If the recipient bacteria grew on the antimicrobial plates after the transduction experiment, the transduction was confirmed by PCR and gene sequencing, identifying the transferred

resistance genes. Table 6-3 contains specifications of the primers used for the PCR. The protocols for extracting DNA, preparing PCR Master Mix, thermal cycling conditions and visualizing amplified products are explained in Chapter 4.

Gene cassette detection

Gene cassette transfer was investigated within the transductants. This was carried out by testing the transductants to different concentrations of other antimicrobials, including 64 IU/mL bacitracin, 16 μg/mL flavophospholipol, 32 μg/mL vancomycin and 256 μg/mL sulfamethoxazole, and assessing resistance genes such as *aph*(3)-IIIa and *ant*(4)-Ia which encode resistance to aminoglycosides other than gentamicin (Vakulenko et al., 2003). Antibiotic susceptibility tests and gene assay protocols are respectively explained in Chapters 3 and 4.

Results

Transduction of tetracycline resistance

Transduction of tetracycline resistance to sensitive recipients was demonstrated. Resistance to 64 μg/mL tetracycline was transferred by bacteriophage EGRM195 propagated on *E. gallinarum* to bacterial recipients nos 31 (*E. faecalis*), 42 (*E. faecalis*) and 54 (*E. faecalis*) (Table 6-2). Transduction occurred with frequencies between 5×10^{-8} and 5×10^{-9} transductants/pfu. In all cases, the transducing bacteriophages were members of the *Siphoviridae* Family and had dsDNA genomes. In all transduction experiments, host control and phage-only control failed to grow on selective plates.

Transduction of gentamicin resistance

Gentamicin resistance could be transferred by the bacteriophage EFRM31 propagated on *E. faecalis* to bacterial recipients nos 13 (*E. hirae/durans*), 70 (*E. faecalis*), 94 (*E. faecium*), 95 (*E. faecalis*), 101 (*E. faecalis*) and 102 (*E. faecalis*). Gentamicin resistance could also be transferred by bacteriophage EFRM42 propagated on *E. faecalis* to bacterial recipients nos 13 (*E. hirae/durans*), 55 (*E. casseliflavus*) and 70 (*E. faecalis*) (Table 6-2). Transduction occurred with frequencies between 1×10^{-7} and 8×10^{-9} transductants/pfu with no significant difference between interspecies transfer and the same species transfer rates ($P < 0.05$). In all cases, the transducing bacteriophages were members of the *Siphoviridae* Family and had dsDNA genomes. In all transduction

experiments, host control and phage-only control failed to grow colonies on selective plates.

Detecting of transduced resistance genes by PCR

All three recipients of tetracycline resistance were shown to have received the *tet*M gene after transduction and that the *tet*M gene was responsible for resistance to 64 μg/mL tetracycline. Three transductants (95, 101 and 102) were obtained that had received *ant*(2)-I genes via transduction from *E. faecalis*. *ant*(2)-I genes were detected by PCR in the recipients. This gene was responsible for the phenotypic resistance to 32 or 64 μg/mL gentamicin in the three recipients.

Sequencing to confirm resistance gene type

Sequencing of the transferred genes showed 100% and 98% identities with GenBank reference genes for *tet*M (original accession number DQ223244.1) and *ant*(2)-I (original accession number AE016830.1), respectively.

Other resistance phenotypes not transferred

No bacitracin, flavophospholipol, vancomycin, sulfamethoxazole or copper resistance phenotype was transferred via transduction. In searching for transferred genes by cotransduction, no additional transferred resistance gene was detected.

Discussion

Transduction of tetracycline resistance

In the current study, bacteriophage mediated transduction was demonstrated for tetracycline and gentamicin. Tetracycline resistance was transferred in three transduction experiments by EGRM195 mediated by *tet*M genes. *tet*M encompasses ribosomal protection proteins and is found as the most prevalent gene encoding resistance to tetracyclines in both Gram-positive and Gram-negative bacteria (Zhang et al., 2009). Rizzotti et al. (2009) studied the transfer of tetracycline resistance gene, *tet*M, within different species of swine enterococci. They found that most of the tetracycline resistance (up to 256 μg/mL) was encoded by *tet*M genes, mostly carried on Tn*916-1545* family transposons. These MGEs were able to transfer the resistance to other genera such as *L. innocua*. The transfer of *tet*M genes between enterococcal isolates (*E. faecalis*) has been

assumed to occur *in vivo* as well by Akhtar et al. (2009). This transfer was studied in house fly gut (*Musca domestica*), and was mediated by a plasmid pCF10.

Transduction of gentamicin resistance

In the current study, the transfer of gentamicin resistance (up to 1024 μg/mL) between enterococcal isolates was investigated as well. The transfer of gentamicin resistance from staphylococci to enterococci by transduction has previously been shown by Zighelboim-Daum et al. (1988). They suggested this mechanism as the major transfer mechanism of gentamicin resistance between those isolates. However, conjugation has been frequently stated as the main route of gentamicin resistance transfer in enterococci (Simjee et al., 2002a; Casetta et al., 1998). Fourteen aminoglycoside resistance genes were investigated to identify the gene responsible for the gentamicin resistance transferred by the bacteriophages in this study. One of these genes, *ant*(2)-I, is widespread in all Gram-negative bacteria but is found less often in enterococcal isolates (Feizabadi et al., 2006; Jackson et al., 2004c; Vakulenko et al., 2003). *ant*(2)-I subcategory members—*ant*(2)-Ia, *ant*(2)-Ib and *ant*(2)-Ic—encode resistance to gentamicin by adenylylation (Shaw et al., 1993). However, gentamicin resistance transferred in this transduction experiment seems to be encoded by the genes other than those tested. A similar suggestion has been made by Jackson et al. (2005) while studying high-level aminoglycoside resistant enterococci. None of eight genes tested known to encode for resistance to gentamicin, kanamycin and streptomycin found in nearly 26% of swine resistant isolates.

Bacterial host isolates

In the current study, most of the bacteriophages have been isolated on pig *E. faecalis* (Chapter 5, Table 5-5), which is the most prevalent enterococcal species (with *E. faecium*) in nature (Behnia et al., 2002; Joyanes et al., 2000). Similarly, literature review shows that most of the enterococcal lytic tailed bacteriophages have been isolated on *E. faecalis* strains. Uchiyama et al. (2008) isolated 30 *E. faecalis* bacteriophages from the environment. Later, Santiago-Rodriguez et al. (2010) isolated enterophages from different sources such as raw sewage and water samples on *E. faecalis* strains recovered from cattle feces. Furthermore, Bonilla et al. (2010) have developed a method for the isolation of environmental enterophages on *E. faecalis* strains.

Interspecies transduction

Some of the bacteriophages (EFRM31, EFRM42 and EGRM195) found in this study are not species specific as they transferred resistance genes between different enterococcal species. Those interspecies transfers were carried out from *E. gallinarum* to *E. faecalis*, and from *E. faecalis* to *E. faecium*, *E. hirae/durans* (undifferentiated) and *E. casseliflavus.* Transduction can be specific to bacterial species or genera, or can occur in broad-host-ranges (Keese, 2008). Many experiments have been conducted on interspecies transduction in bacteria. Rakieten and Rakieten (1937a), and Rakieten and Tiffany (1938) showed for the first time that staphylophages adsorbed into other genera such as enterococcic streptococci. They also demonstrated adsorption of enterococcal bacteriophages on staphylococci strains. Later, the transfer of plasmids from *S. thermophilus* to *L. lactis* via transduction was reported by Ammann et al. (2008). In 2009, Chen and Novick transferred superantigens to *L. monocytogenes* by staphylococcal bacteriophages. Furthermore, Villion et al. (2009) sequenced the complete genome of a lactococcal *Siphoviridae* bacteriophage, P087, and found significant identities between this bacteriophage and the gene structure of a prophage from a clinical isolate of *E. faecalis* V583. This finding supports the hypothesis proposing the genetic exchange between low GC bacteria (Lepage et al., 2006). It is estimated that most of the myoviruses which infect the different genera of *Listeria*, *Staphylococcus*, *Bacillus* and *Enterococcus* belong to broad-host-ranges, virulent SPO1-like bacteriophages such as A511 (Klumpp et al., 2008). Moreover, interspecies transduction has been confirmed in Gram-negative bacteria as well (Brabban et al., 2005). Affinity of bacteriophages to mammalian cells was shown in the late 90s (Larocca et al., 1998). However, the importance of bacteriophages in gene transfer is underestimated, compared to other transfer routes such as transformation and conjugation. Contrary to this heterospecific transfer evidence, literature reviews commonly mention that most of the bacteriophages are highly species specific, having a strong affinity to a particular group of bacteria (Parisien et al., 2008; Harcombe & Bull, 2005; Hagens & Loessner, Langley et al., 2003; Weber-Dabrowska et al., 2000; Chibani-Chennoufi et al., 2004). This specific host range—which is used in the typing of bacteria such as *Escherichia, Salmonella* and *Shigella*—is probably due to the production of specific lysing enzymes by the bacteriophages (Fischetti, 2008). However, these enzymes sometimes act more flexibly and lyse a broader spectrum of bacterial genera, such as the effect of an enterococcal bacteriophage lysin on other Gram-positive pathogens such as *S. pyogenes*, Group B streptococci, and *S. aureus* (Yoong et al., 2004). O'Flaherty et al.

(2005) cloned and expressed a staphylococcal lysin (LysK) in *L. lactis*. They found LysK had an antimicrobial potency against staphylococci. Moreover, some researchers argue that the bacteriophage infection mechanism is established on a strain-specific basis (Bradbury, 2004). For example, vibriophages have successfully been applied for typing of 1000 *Vibrio* strains (Sarkar, 2002).

Conclusions

This study has demonstrated transduction in enterococci including interspecies transduction (from *E. gallinarum* to *E. faecalis*, and from *E. faecalis* to *E. faecium*, *E. hirae/durans* (undifferentiated) and *E. casseliflavus.*). Previously, there had been little research on enterococcal bacteriophages (Nigutova et al, 2008) and fewer reports on the transfer of virulence or antibiotic resistance (gentamicin) genes by transduction in enterococci have been published (Yasmin et al., 2010; Garcia-Migura et al., 2009). However, transduction has been studied broadly in related Gram-positive bacteria such as streptococci, *Listeria* spp., *Bacillus* spp., *Lactobacillus* spp. and staphylococci (Chandry et al., 2002; Hodgson, 2000; Storrs et al., 1988). These studies of transduction included different functions such as mutagenesis, packaging and replicating activities of lytic and temperate bacteriophages. For example, transduction is supposed to be the most common horizontal transfer mechanism in *S. aureus* (Lindsay & Holden, 2006). However in the current study, only enterococcal bacteriophages, hosts and recipients were used that had the potential to transfer/receive antimicrobial resistance. The selection was based on the presence of phenotypic and/or genotypic resistance to antimicrobials in bacteriophage host strains and the absence of those characteristics in recipient bacterial strains. The resistance profiles were previously determined by PCR methodology. In summary, transduction has been demonstrated to have a role in transfer of antimicrobial resistance genes in enterococci. More studies are needed to investigate the impact of transduction in life fitness and evolution of enterococci.

Table 6-1. Summary of the bacteriophages and bacterial hosts used in transduction experiments

Phage no.	Source	Indicator bacteria (no.)	Origin	Titre (pfu/mL)
EFRM31	Piggery effluent	*E. faecalis* (31)	Pig	2×10^8
EFRM42	Piggery effluent	*E. faecalis* (42)	Pig	1.6×10^{10}
EGRM195	Piggery effluent	*E. gallinarum* (195)	Pig	1×10^{10}

Table 6-2. Transduction experiment scheme

Phage (host) genotype	Recipient (strain)	Drug[†]	Result[‡]
EGRM195 (*E. gallinarum*): *tet*L, *tet*M, *tet*S	31 (*E. faecalis*)	TET	TET (64), *tet*M*
EGRM195 (*E. gallinarum*): *tet*L, *tet*M, *tet*S	42 (*E. faecalis*)	TET	TET (64), *tet*M*
EGRM195 (*E. gallinarum*): *tet*L, *tet*M, *tet*S	54 (*E. faecalis*)	TET	TET (64), *tet*M*
EFRM31 (*E. faecalis*): *aac*6-*aph*2, *ant*(2)-I	13 (*E. hirae/durans*)	GEN	GEN (64)
EFRM31 (*E. faecalis*): *aac*6-*aph*2, *ant*(2)-I	70 (*E. faecalis*)	GEN	GEN (64)
EFRM31 (*E. faecalis*): *aac*6-*aph*2, *ant*(2)-I	94 (*E. faecium*)	GEN	GEN (32)
EFRM31 (*E. faecalis*): *aac*6-*aph*2, *ant*(2)-I	95 (*E. faecalis*)	GEN	GEN (32), *ant*(2)-I*
EFRM31 (*E. faecalis*): *aac*6-*aph*2, *ant*(2)-I	101 (*E. faecalis*)	GEN	GEN (32), *ant*(2)-I*
EFRM31 (*E. faecalis*): *aac*6-*aph*2, *ant*(2)-I	102 (*E. faecalis*)	GEN	GEN (64), *ant*(2)-I*
EFRM42 (*E. faecalis*): *aac*6-*aph*2, *ant*(2)-I	13 (*E. hirae/durans*)	GEN	GEN (64)
EFRM42 (*E. faecalis*): *aac*6-*aph*2, *ant*(2)-I	55 (*E. casseliflavus*)	GEN	GEN (32)
EFRM42 (*E. faecalis*): *aac*6-*aph*2, *ant*(2)-I	70 (*E. faecalis*)	GEN	GEN (64)
EFRM31 (*E. faecalis*): *aac*6-*aph*2, *ant*(2)-I	54 (*E. faecalis*)	GEN	No transfer
EFRM31 (*E. faecalis*): *aac*6-*aph*2, *ant*(2)-I	55 (*E. casseliflavus*)	GEN	No transfer
EFRM42 (*E. faecalis*): *aac*6-*aph*2, *ant*(2)-I	94 (*E. faecium*)	GEN	No transfer
EFRM42 (*E. faecalis*): *aac*6-*aph*2, *ant*(2)-I	95 (*E. faecalis*)	GEN	No transfer
EFRM42 (*E. faecalis*): *aac*6-*aph*2, *ant*(2)-I	101 (*E. faecalis*)	GEN	No transfer
EFRM42 (*E. faecalis*): *aac*6-*aph*2, *ant*(2)-I	102 (*E. faecalis*)	GEN	No transfer

*Genes encoding resistance phenotype detected by PCR; [†]Selective drug (16 and 64 μg/mL tetracycline; 32, 64 and 1024 μg/mL gentamicin); [‡]Transferred phenotype (μg/mL) and resistance genes; GEN, gentamicin; TET, tetracycline

Table 6-3. Specifications of the primers used in transduction experiments

Drug	Gene		Primer sequences (5'→3')	bp	Reference
TET	*tet*L	F	CATTTGGTCTTATTGGATCG	488	Aarestrup et al.
		R	ATTACACTTCCGATTTCGG		(2000)
TET	*tet*M	F	GTTAAATAGTGTTCTTGGAG	657	Aarestrup et al.
		R	CTAAGATATGGCTCTAACAA		(2000)
TET	*tet*S	F	TGGAACGCCAGAGAGGTATT	660	Aarestrup et al.
		R	ACATAGACAAGCCGTTGACC		(2000)
GEN	*aac*6-*aph*2	F	CCAAGAGCAATAAGGGCATA	220	Rouch et al.
		R	CACTATCATAACCACTACCG		(1987)
GEN	*aph*(2)-Ib	F	CTTGGACGCTGAGATATATGAGCAC	867	Vakulenko et al.
		R	GTTTGTAGCAATTCAGAAACACCCTT		(2003)
GEN	*aph*(2)-Ic	F	CCACAATGATAATGACTCAGTTCCC	444	Vakulenko et al.
		R	CCACAGCTTCCGATAGCAAGAG		(2003)
GEN	*aph*(2)-Id	F	GTGGTTTTTACAGGAATGCCATC	641	Vakulenko et al.
		R	CCCTCTTCATACCAATCCATATAACC		(2003)
GEN	*aph*(3)-IIIa	F	GGCTAAAATGAGAATATCACCGG	523	Vakulenko et al.
		R	CTTTAAAAAATCATACAGCTCGCG		(2003)
GEN	*ant*(4)-Ia	F	CAAACTGCTAAATCGGTAGAAGCC	294	Vakulenko et al.
		R	GGAAAGTTGACCAGACATTACGAACT		(2003)
GEN	*aac*(3)-I	F	ACCTACTCCCAACATCAGCC	169	Saenz et al.
		R	ATATAGATCTCACTACGCGC		(2004)
GEN	*aac*(3)-II	F	ACTGTGATGGGATACGCGTC	237	Saenz et al.
		R	CTCCGTCAGCGTTTCAGCTA		(2004)
GEN	*aac*(3)-III	F	CACAAGAACGTGGTCCGCTA	185	Saenz et al.
		R	AACAGGTAAGCATCCGCATC		(2004)
GEN	*aac*(3)-IV	F	CTTCAGGATGGCAAGTTGGT	286/250	Saenz et al.
		R	TCATCTCGTTCTCCGCTCAT		(2004)
GEN	*ant*(2)-I	F	ATGTTACGCAGCAGGGCAGTCG	187	Saenz et al.
		R	CGTCAGATCAATATCATCGTGC		(2004)

TET, tetracycline; GEN, gentamicin

Chapter 7

Bacteriophage Genome Sequencing

Introduction

Recently, comparative genomic hybridization (CGH) studies have shown a significant variation in genomes of different *Enterococcus* spp., especially *E. faecalis* strains (van Schaik & Willems, 2010). A variety of MGEs such as bacteriophages are presumed to contribute greatly to this variation. Around 500 bacteriophage genomes have been secuenced at the time of writing, that is considerably lower than the number of bacterial genomes sequenced with nearly 750 sequenced despite having a genome 100 times larger (Hatfull, 2008). Although some identities are seen within the genomes of different bacteriophages of the same bacterial host, there is a significant diversity between the different bacteriophage clusters. The dsDNA tailed bacteriophages constitute a substantial proportion of bacteriophage population; some are completely sequenced (Hatfull et al., 2006). Most of these sequenced bacteriophages infect enteric and dairy bacteria as well as *Mycobacterium* spp., *Pseudomomas* spp., *Staphylococcus* spp. and marine bacteria. A variety of techniques are used for preparing microbial genomes for sequencing in prokaryotes. Degenerate Oligonucleotide Primed Amplification (DOP-PCR) is an example of these techniques used for the Whole Genome Amplification (WGA) of microbial DNA. Other techniques include Multiple Displacement Amplification (MDA), Primer Extension Preamplification (PEP), Improved Primer Extension Preamplification (iPEP) and Long Products from Low DNA Quantities Degenerate-Oligonucleotide-Primed PCR (LL-DOP-PCR). These methods, capable of generating high-quality amplicons from the entire genome (Lasken & Egholm, 2003), were first described by Telenius et al. in 1992. The species-independent method, DOP-PCR, is applied for fingerprinting and genome mapping of microorganisms even with low quantity or poor quality genomic DNA template (Barbaux et al., 2001). In general, the whole genome amplification of genomic DNA is carried out by different methods such as the Sanger method and the "Next Generation" sequencing method. The Sanger technique, introduced by Sanger et al. in 1977, is the premier method for *de novo* whole genome sequencing (Amaral et al., 2009). This technique includes synthesis of a complementary DNA template using natural 2-deoxynucleotides (dNTPs) and termination of synthesis using 2,3-dideoxynucleotides (ddNTPs) by DNA polymerase (Sanger et al., 1977). However, this method is relatively

expensive and time consuming (Amaral et al., 2009). Other methods for high-throughput DNA sequencing have recently been developed that are relatively cheaper and much faster. Some of these methods include Sequencing by Hybridization (SBH), Nanopore Sequencing and Sequencing by Synthesis (SBS) (Metzker, 2005). For examples, the new Illumina 1 G Genome Analyzer (ILLUMINA, USA), which uses a "sequencing by synthesis" method, sequences millions of short fragments (generally 25–50 bp) in parallel (Amaral et al., 2009). This method is not only very accurate, but also does not require extra cost, incurring steps such as cloning. This novel method also allows identification of single nucleotide polymorphisms (SNPs).

Material and Methods

Whole genome sequencing

A head-tail bacteriophage, EFRM31, was selected for the sequencing. Bacteriophage EFRM31 belonged to *Siphoviridae* Family (dsDNA) of bacteriophages and had been isolated on a pig *E. faecalis* isolate. This bacteriophage had successfully been used in transduction experiments (described in the previous chapter).

DOP PCR

The DOP-PCR method is modified from the original protocol drafted by Arthur et al. (2009). DOP-PCR was performed using a general primer (5'-AGCAGCGACTGTCGACACNNNNNNGTGG-3') described previosely (Hirose et al., 2001; Cheung & Nelson, 1996), in which N could be replaced by either A, C, G, or T nucleotides. To prepare a final volume of 50 μL Master Mix, 21.5 μL of sterile distilled water, 10 μL of 5× PCR buffer, 5 μL of 25 mM $MgCl_2$, 4 μL of 2.5 mM dNTPs, 5 μL of primer in a total concentration of 10 pmol, and 0.5 μL of 5 U/μL Taq DNA polymerase (Promega, USA) were mixed in a sterile microtube. Four microlitres of DNA in 1:200 dilution were added as a template. The thermal cycling conditions were set up as follows (Table 7-1): Initial denaturation was carried out at 94^{o}C for ten minutes followed by ten cycles of denaturation at 94^{o}C for 30 seconds, annealing at 30^{o}C for one minute, and extension at 72^{o}C for five minutes. The annealing temperature was ramped to extension temperature in six steps with 6^{o}C intervals; each step was held for one minute. The PCR was continued with 30 cycles, each cycle consisted of denaturation at 94^{o}C for 30 seconds, annealing at 50^{o}C for one minute, and extension at 72^{o}C for five minutes. The annealing temperature was ramped to extension temperature in six steps with 3^{o}C intervals; each step was held for 30 seconds. Temperature ramping was adapted to be compatible with the

thermocycler. The DOP-PCR finished with a final extension at 72^{o}C for five minutes. PCR products were detected by electrophoresis in 1 μg/mL ethidium bromide-stained 1% agarose gels in 0.5× TBE buffer at 100 V for two hours and then by visualizing under the UV light (Figure 7-1). Two microlitres of 1 kb DNA ladder were loaded as marker for each gel electrophoresis.

DOP-PCR bands run on agarose gels were used in direct amplification methods, in addition to use in cloning. This was carried out by cutting pieces of gel containing few bands then extracting the DNA from the gel by means of clean up kits. An alternative technique was to transfer DNA within the gel by a toothpick and using the DNA as a template for other amplification methods.

Principles for primer design

To design the primers properly, the following principles were considered.

1) Primers ended with C or G at 3′,

2) Primers start with A at 5′,

3) No more than 3 Cs or Gs were used in the five bases closest to the 3′ end,

4) No four or more similar bases were repeated consequently,

5) All four nucleotide types (A, C, G, T) were used in primer design,

6) The melting temperature (Tm) of primers was calculated around 50^{o}C (57−59^{o}C for the DOP-PCR primer) using Oligo Calculator at http://www.pitt.edu/~rsup/OligoCalc.html,

7) It was ensured that the forward and the reverse primers did not form dimers,

8) No more than one G or C was used at the end of primers,

9) GC content was adjusted between 40 to 60%,

10) Primers consisted of 20 to 36 bases (usually 18 to 24).

Semi DOP-PCR

A Semi-DOP PCR was used to amplify segments for filling the gaps within the genome sequence. The following strategy was adopted: The primers forward F3: 5′-GCTAACAATTGGTGATAACC-3′ and reverse R3: 5′-GTTCACGTGTTCAAGAATTGAC-3′ were separately used in combination with

DOP primer to amplify the bacteriophage DNA. Then, each of the products was used as a template for amplification by the primers forward F4: 5′-GTAGGCATCAAACGTCTGTGG-3′ and reverse R4: 5′-GCCGTATTATCTGATTTTGTGCG-3′ in combination with the tag primer in separate reactions. The PCR amplicons were used in separate sequencing PCR reactions with each of the primers forward F5: 5′-CCATGATTACGCCAAGCTCAG-3′ or reverse R5: 5′-GCTGAACAATTAAGAGAGATAC-3′ and the products were sent for the nucleotide sequencing. The following protocol was used for the first step. Two and half microlitres of 5× PCR buffer, 2 μL of 25 mM $MgCl_2$, 2 μL of 2.5 mM dNTPs, 2.5 μL of each primer (F3 or R3 and DOP) in a total concentration of 10 pmol, and 0.5 μL of 5 U/μL DNA polymerase were mixed in a sterile microtube then 11.5 μL of sterile distilled water were added to make 25 μL Master Mix. Two microlitres of DNA in 1:10 dilution were added as a template. The thermal cycling conditions were set up as follows: Initial denaturation at 94^{o}C for ten minutes followed by ten cycles of denaturation at 94^{o}C for 30 seconds, annealing at 30^{o}C for one minute, and extension at 72^{o}C for five minutes. The annealing temperature was ramped to extension temperature in six steps of 6^{o}C intervals; each step was held for one minute. The PCR was continued with 30 cycles, each cycle consisted of denaturation at 94^{o}C for 30 seconds, annealing at 50^{o}C for one minute, and extension at 72^{o}C for five minutes. The annealing temperature was ramped to extension temperature in six steps of 3^{o}C intervals; each step was held for 30 seconds. Temperature ramping was adapted to be compatible with the thermocycler. The DOP-PCR finished with a final extension at 72^{o}C for five minutes.

The following protocol was used for the second step. Five microlitres of 5× PCR buffer, 2.5 μL of 25 mM $MgCl_2$, 2 μL of 2.5 mM dNTPs, 1.5 μL of each primer (F4 or R4 and Tag) in a total concentration of 10 pmol, and 0.5 μL of 5 U/μL DNA polymerase were mixed in a sterile microtube then 10 μL of sterile distilled water were added to make 25 μL Master Mix. Two microlitres DNA of the previous step were added as a template. The thermal cycling conditions were set up as follows: Initial denaturation at 94°C for five minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 45 seconds, and extension at 72°C for 2.5 minutes. Final extension was carried out at 72°C for five minutes. The following protocol was used for the sequencing PCR. For a 10-μL sequencing PCR reaction, 6 μL of sterile distilled water, 1.5 μL of buffer, 0.5 μL of BigDye 3, 1 μL of 10 pmol primer (F5 or R5), and 1 μL of DNA from the previous step were mixed and run using the following thermal cycling program. Initial denaturation at

96°C for one minute followed by 30 cycles, each cycle consisted of denaturation at 96°C for ten seconds, annealing at 50°C for ten seconds, and extension at 60°C for four minutes. The PCR products were cleaned up using a Qiagen Cleanup Kit before sending for the sequencing. PCR products were detected by electrophoresis in 1 μg/mL ethidium bromide-stained 1% agarose gels in 0.5× TBE buffer at 100 V for two hours and then by visualizing under the UV light. Two microlitres of 1 kb DNA ladder were loaded as marker for each gel electrophoresis.

Cloning

Two different concentrations of the DOP-PCR product were used for cloning (modified from Guilfoyle & Smith, 1994). DOP-PCR products were cleaned up before the cloning, using a Qiagen Cleanup Kit. Reagents were mixed as: 1 μL of 1:2 diluted plasmid vector (Invitrogen TOPO TA Cloning Kit for Sequencing), 1 μL of salt solution (1.2 M NaCl, 0.06 M $MgCl_2$), 1 μL of DNA for the low and 4 μL for the high concentrations, and 3 μL of γ-radiated distilled water for the low concentration. These mixtures were left at room temperature for two hours for ligation between the plasmid vector and the DOP-PCR product to occur. Then, 2 μL of each ligation were mixed with 50 μL of the competant host *E. coli* and incubated on ice for 30 minutes. Transformation of plasmid vector into the bacterial host was completed using a heat shock at 42^{o}C for 45 seconds and then the bacteria were placed on ice for two minutes. Two hundred and fifty microlitres of Super Optimal broth with catabolite reoression (SOC broth) were added to each tube and incubated with vigorous shaking at 225 rpm. After 90 minutes incubation at 37^{o}C, 100 μL of the bacteria were spread on 1.5% LB agar (containing 50 μL/mL kanamycin and 50 μL/mL X-gal) plates (two plates per concentration) and incubated overnight at 37^{o}C. White colonies (indicating an insert) were chosen for the amplification.

M13 PCR

M13 PCR was applied to amplify cloned bacteriophage genome. The forward primer was 5′-GTAAAACGACGGCCAG-3′ and the reverse primer was 5′-CAGGAAACAGCTATGAC-3′. For 25 μL of Master Mix, 17.65 of distilled water, 2.5 μL of 5× PCR buffer, 2.5 μL of 25 mM MgCl2, 2 μL of 2.5 mM dNTPs, 0.05 μL of each primer in a total concentration of 100 μmol and 0.25 μL of 5 U/μL Taq DNA polymerase were mixed in a sterile microtube (Table 7-2). Then, a tiny cloned colony was picked up using a toothpick and mixed into the PCR reaction as a template. The thermal cycling conditions were set up as follows: Initial denaturation at 94°C for ten minutes

followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for two minutes. Final extension was carried out at 72°C for ten minutes (Table 7-2). PCR products were detected by electrophoresis in 1 μg/mL ethidium bromide-stained 1% agarose gels in 0.5× TBE buffer at 100 V for two hours and then by visualizing under the UV light. Two microlitres of 1 kb DNA ladder were loaded as marker for each gel electrophoresis.

Single PCR

A Single PCR was designed based on the sequencing results of the DOP-PCR fragments. These primers were designed to amplify the bacteriophage genome when needed to fill the possible gaps in the genome sequence. The forward primer was F1: 5′-GTGTACTCTTGTTTGTGG-3′ and the reverse primer was R1: 5′-GCCAAAGTGAACATACC-3′. For 50 μL Master Mix, 24 μL of sterile distilled water, 10 μL of 5× PCR buffer, 7 μL of 25 mM $MgCl_2$, 0.5 μL of 25 mM dNTPs, 2 μL of each primer having a total concentration of 10 pmol and 0.5 μL of 5 U/μL Taq DNA polymerase were mixed in a sterile microtube. Then, 4 μL of the bacteriophage template DNA extraction was added to the reaction (Table 7-3). The thermal cycling conditions were as follows: Initial denaturation at 94°C for five minutes followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for one minute, and extension at 72°C for four minutes. Final extension was carried out at 72°C for five minutes (Table 7-3). PCR products were detected by electrophoresis in 1 μg/mL ethidium bromide-stained 1% agarose gels in 0.5× TBE buffer at 100 V for two hours and then by visualizing under the UV light. Two microlitres of 1 kb DNA ladder were loaded as marker for each gel electrophoresis. An alternative (Tag) primer was used to amplify the bacteriophage genome with the same protocol. The Tag primer consisted of following sequences: 5′-AGCAGCGACTGTCGACAC-3′.

More primers were designed and applied to fill the gaps between the amplified segments of the bacteriophage genome. These pairs of primers were designed as forward GF1: 5′-TCAAGTGTCATAGGAATACC-3′ and reverse GR1: 5′-TTGTGAAGCCGTATTATCTG-3′ generating 2440 bp amlicons; forward GF2: 5′-ATCACCGTCTGTACTTATAAC-3′ and reverse GR2: 5′-TTCTGTGGTTTAGTTGACGG-3′ generating 1491 bp amlicons; and forward GF4: 5′-ACGTTTAATGAAGCTGTTAACTC-3′ and reverse GR4: 5′-AACAGAGTACCAGCAACAAG-3′ generating 1498 bp amlicons. For preparing

25 µL Master Mix, 10.75 of sterile distilled water, 5 µL of 5× PCR buffer, 2.5 µL of 25 mM $MgCl_2$, 2 µL of 25 mM dNTPs, 1.5 µL of each primer in a total concentration of 10 pmol and 0.25 µL of 5 U/µL Taq DNA polymerase were mixed in a sterile microtube. Then, 2 µL of 1:10 eluted bacteriophage DNA extraction was added to the reaction as a template (Table 7-4). The thermal cycling conditions were set up as follows: Initial denaturation at 94°C for five minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 45 seconds, and extension at 72°C for two and half minutes. Final extension was carried out at 72°C for five minutes (Table 7-4). PCR products were detected by electrophoresis in 1 µg/mL ethidium bromide-stained 1% agarose gels in 0.5× TBE buffer at 100 V for two hours and then by visualizing under the UV light. Two microlitres of 1 kb DNA ladder were loaded as marker for each gel electrophoresis.

Long range PCR

Long range PCR is a rapid and reliable technique used to amplify long segments of DNA genome which may or may not be in poor quality or in low concentration (Kurosaki et al., 2008). The following protocol was applied to amplify long segments of the bacteriophage genome with thousands of base pairs. The designed primers were forward GF3: 5′-AGGTATCATTGCATTACCATC-3′ and reverse GR3: 5′-ATATTAATCCATTGCCCTAGTAC-3′ generating 4057 bp amlicons, and forward GF5: 5′-GTGTTAAAGTGATATATTTACCAG-3′ and reverse GR5: 5′-GGGAACATAGGTTGGATATAG-3′ generating 6480 bp amlicons. More primers were used with the similar protocol (Table 7-5). For preparing 25 µL Master Mix, 10.25 µL of sterile distilled water, 5 µL of 5× PCR buffer, 2.5 µL of 25 mM $MgCl_2$, 2 µL of 2.5 mM dNTPs, 1.5 µL of each primer in a total concentration of 10 pmol and 0.25 µL of 5 U/µL Taq DNA polymerase (QIAGEN, USA) were mixed in a sterile microtube. Then, 2 µL of the bacteriophage DNA extraction diluted 1:10 was added to the reaction as a template (Table 7-6). The thermal cycling conditions were set up as follows: Initial denaturation at 94°C for five minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 45 seconds, and extension at 72°C for 150 seconds. Final extension was carried out at 72°C for five minutes (Table 7-6). For the very long amplicons with more than 4000 bp, the amount of Taq DNA polymerase was increased to 0.5 µL and the thermal cycling conditions were modified as follows: Initial denaturation at 94°C for three minutes followed by 40 cycles of denaturation at 94°C for 15 seconds, annealing at 50°C for 30 seconds, and extension at 68°C for ten minutes. Final extension

was carried out at 68°C for seven minutes (Table 7-7). An alternative protocol was used when a Qiagen Long Range PCR kit was used. For preparing 25 µL Master Mix, 11 µL of sterile distilled water, 2.5 µL of 5× PCR buffer, 5 µL of Q solution, 1.25 µL of 10 mM dNTPs, 1.5 µL of each primer in a total concentration of 10 pmol and 0.25 µL of 5 U/µL Taq DNA polymerase were mixed in a sterile microtube. Then, 2 µL of the bacteriophage DNA extraction diluted 1:10 was added to the reaction as a template (Table 7-8). The thermal cycling conditions were set up as follows: Initial denaturation at 94°C for two minutes followed by 35 cycles of denaturation at 94°C for 15 seconds, annealing at 50°C for 30 seconds, and extension at 68°C for 10 minutes. Final extension was carried out at 68°C for seven minutes (Table 7-8). PCR products were detected by electrophoresis in 1 µg/mL ethidium bromide-stained 1% agarose gels in 0.5× TBE buffer at 100 V for two hours and then by visualizing under the UV light. Two microlitres of 1 kb DNA ladder were loaded as marker for each gel electrophoresis.

Sequencing

The M13 PCR products were cleaned up using a Qiagen Cleanup Kit. For a 10-µL sequencing PCR reaction, 6 µL of sterile distilled water, 1.5 µL of buffer, 0.5 µL of BigDye 3, 1 µL of 1.6 pmol primer (F or R), and 1 µL of DNA were mixed and run using the following thermal cycling program. Initial denaturation at 96°C for one minute followed by 30 cycles, each cycle consisted of denaturation at 96°C for ten seconds, annealing at 50°C for ten seconds, and extension at 60°C for four minutes (Table 7-9). The PCR products were cleaned up using a Qiagen Cleanup Kit before the sequencing. The sequencing was carried out at the Molecular Pathology Sequencing Centre (IMVS, Adelaide) using a capillary electrophoresis-based genetic analyzer (Applied Biosystems-Hitachi 3730, USA). The following paragraph simply describes the methodology used for the sequencing (http://www3.appliedbiosystems.com/AB_Home/applicationstechnologies/DNASequencingbyCapillaryElectrophoresis/index.htm). Samples were ready for analysis after performing the post cycle-sequencing reaction (Dye Terminator) purification. The products of the cycle sequencing reaction were then injected electrokinetically into capillaries filled with polymer. The negatively charged DNA fragments moved through the polymer in the capillaries toward the positive electrode due to applying high voltage. Capillary electrophoresis could resolve DNA molecules by molecular weight. These separated fluorescent-labeled DNA fragments (BigDye® Terminator) moved through the path of a laser beam and were detected by an optical detection device. The data collection software

converted the fluorescence signal to digital data then recorded these data. After electrophoresis, data were analyzed by collection software (primary analysis) and downstream software (secondary analysis).

Analysis of sequencing results

The sequencing results were analysed using the Kodon® Software and bioinformatics database such as GenBank and ExPASy.

Complementary sequencing

In addition to above-mentioned technique, a commercial shotgun sequencing method was used to sequence the bacteriophage genome (GeneWorks® Technologies, Australia). The 35 b contigs from sequencing were assembled using NGen® assembler software (DNASTAR®, http://www.dnastar.com/). These reads could be assembled using a closely related genome sequence (template assembly), an attempt at *de novo* assembly made or a combination of the two methods.

Results

Genome sequencing characteristics

A high percentage of genome identity to the reference bacteriophage EFAP-1 (NCBI Reference Sequence: NC_012419/FJ792813) (Figure 7-2 and Table 7-10) was found when sequencing the bacteriophage EFRM31 (Figures 7-3 and 7-4, Table 7-11, and Appendix 20). The putative EFRM31 genome (NCBI Reference Sequence: GU815339) was a low GC-content (34.5%) DNA molecule which consisted of 16945 readable nucleotides including 37.9% A, 27.6% T, 14.9% C and 19.6% G. No CpG Islands were found. The whole genome consisted of 82 putative ORFs from 102 to 1725 bp in length and 33 to 574 amino acids (aa) (Table 7-12). The analysis of sequencing results showed that the bacteriophage genome consisted of 17 genes with high identities (up to 99%) to genes of EFAP-1 bacteriophage and some eubacteria (mostly Gram-positive bacteria) and six genes encoding hypothetical proteins with unknown function (Appendix 21). The genes found in EFRM31 included those encoding one head-tail adaptor protein, three head-tail joining proteins, two HNH endonucleases, one major capsid protein, one major tail protein, one portal protein, one prohead protease, one terminase large subunit, one terminase small subunit and one tape measure protein. Sequences similar to those encoding a zinc protease, a DNA primase, an antibiotic resistance transmembrane protein and an APC amino acid-polyamine-organocation transporter were also found. Furthermore, ORFs with no

published identity were identified. However, no MGEs were found in the bacteriophage genome.

Discussion

Whole genome sequencing

In genome sequencing, many factors such as DNA length, secondary structure and GC content affect genome amplification and may result in variations in findings (Lasken & Egholm, 2003). DOP-PCR is a rapid, efficient technique for DNA amplification of the abnormal microorganisms or eukaryotic cell genome used for genetic linkage analysis and genetic diversity studies, regardless of DNA quality and quantity (Cheung & Nelson, 1996; Telenius et al., 1992). Unlike most PCRs, this technique uses one primer (instead of one pair of primers), usually consisting of 22 bases (22-mer primer). In the current study, DOP-PCR products were randomly cloned and amplified to make short segments (mostly less than 1000 bp in length) of the bacteriophage genome. These short segments then were sequenced using the chain termination method. Moreover, a chromosome walking technique was used for filling the gaps between the sequenced segments of the genome, in which the sequencing of the genome is progressed through the entire strand, piece by piece. Therefore, these reliable techniques were applied as appropriate tools to produce proper segments of the bacteriophage genome, securing the possible interpretation of the result. However, due to the presence of gaps within the sequenced genome using the above-mentioned technique and failure of the technique to amplify the bacteriophage whole genome, a commercial shotgun method was also used in this study. Contrary to a top-down sequencing method, in which a large source clone is firstly mapped by a BAC (bacterial artificial chromosome) to BAC approach and then is broken up into smaller sub-clones, the whole-genome shotgun (WGS) method produces a random mixture and a random sub-clone instead of a physical map (Kae, 2003). Therefore, this WGS method is much faster and cheaper but assembly of random sequenced contigs is more difficult and more likely to fail to produce the complete genome. However, whole genome shotgun approach (WGSA) using DNA library is reported as the most efficient method to sequence the genome of bacteria and dsDNA bacteriophages (Kaiser et al., 2003).

Genome sequencing characteristics

The sequencing results for EFRM31 showed a partial identity with enterococcal bacteriophage phi-EF24C chromosome complete sequence, Pubmed ID: 18456848,

http://www.ncbi.nlm.nih.gov/nuccore/NC_009904 (Uchiyama et al., 2008b) and a significant identity with enterococcal bacteriophage EFAP-1 complete genome sequence, Pubmed ID: FJ792813, http://www.ncbi.nlm.nih.gov/nuccore/NC_012419 (Son et al., 2009). The former bacteriophage, *E. faecalis* phi-EF24C, is a member of broad-host-range SPO1-like group of bacteriophages including *Listeria* bacteriophage A511 and *L. plantarum* bacteriophage LP65 (Alemayehu et al., 2009). Bacteriophage, EFAP-1, (described above) is a tailed bacteriophage isolated by co-cultivation of pooled-environmental samples of a cattle farm on *E. faecalis* strains (Son et al., 2009). This bacteriophage contains a dsDNA genome of 21,115 bp that consists of 24 putative genes namely gp01 to gp24. In general, these genes can be classified into two major groups: housekeeping and regulatory genes. Housekeeping genes encode proteins such as head-tail joining proteins, head-tail adaptor proteins, host specificity proteins, portal proteins, prohead protease, tail component, tail major proteins, tape measure proteins, terminase large subunit and terminase small subunit. Regulatory genes encode proteins such as amidase, holin and HNH endonuclease. Other gene products include hypothetical proteins. In the current study, many such similar genes were found in bacteriophage EFRM31. The genes found in EFRM31 and their functions are discussed as follows:

A head-tail adaptor protein gene (gp20, Table 7-11) consisting of 336 bp (111 aa) was found. This gene was located between head-tail joining protein genes and showed a high identity (84%) to the similar gene of EFAP-1 bacteriophage. Head-tail adaptor proteins mediate the interaction between the head and tail proteins (Edmonds et al., 2005). These proteins allow the assembly of pieces of head and tail of bacteriophages that may be encoded by different cassettes (Nikolaichik & Donachie, 2000). Ventura et al. (2006) found a partial identity between the Sal1 prophage of *Lactobacillus* spp. and an *E. faecium* bacteriophage.

Three head-tail joining protein genes (gp19, gp21, gp22, Table 7-11) (303, 408 and 366 bp respectively encoding 100, 135 and 121 aa) were detected next to each other with 83 to 94% identities with head-tail joining protein genes of EFAP-1 bacteriophage. The set of genes also was closest to a major tail protein gene and a head-tail adaptor protein gene. Head-tail joining proteins are needed for the last step of head and tail attachment (Maxwell et al., 2002). The encoding genes are usually found in superfamily of genes.

Two 372 and 522 bp (123 and 173 aa) HNH endonuclease genes (gp1, gp11, Table 7-11) were located adjacent to hypothetical protein genes. The identity of these genes were 94

and 49% compared to that of EFAP-1 bacteriophage. HNH endonuclease genes encode homing endonuclease enzymes which cleave dsDNA, ssDNA and ssRNA (Pommer et al., 2001). Homing endonucleases are classified into four groups LAGLIDADG, GIY-YIG, His-Cys box and HNH (Mate & Kleanthous, 2004). The HNH family of bacteriocins includes colicins of *E. coli* and pyocins of *P. aeruginosa*. Homing endonucleases are found in enterococci as well.

A major capsid protein (gp17, Table 7-11) of 862 bp (418 aa) with high identity (96%) to a major capsid protein of EFAP-1 bacteriophage was found. The locus was surrounded by two hypothetical protein genes. The majority of the bacteriophage proteins include the major capsid proteins (MCPs) (Chibani Azaiez et al., 1998). They are highly conserved in the structure of bacteriophages and used for the bacteriophage classification (Uchiyama et al., 2008a). In the current study, the MCP gene of EFRM31was found to be similar to the gene of the EFAP-1 bacteriophage. Therefore, it can be concluded that both bacteriophages share a significant identity. MCPs have been found to be produced during bacterial infections and bind to the host transcriptional factors (Bingham et al, 2000; Moineau et al., 1993). Hence, these proteins can be used for the bacteriophage detection.

A major tail protein gene (gp23, Table 7-11) (567 bp and 188 aa) with high identity (83%) to a major tail protein of EFAP-1 bacteriophage was located downstream of a head-tail joining protein gene. Noncontractile tails of bacteriophages consist of multiple copies of the major tail protein (MTP) (Pell et al., 2009). The highest identity to enterococcal MTP is usually found in lactococcal and staphylococcal bacteriophages. Recently, Uchiyama et al. (2008a) characterized and sequenced a novel *E. faecalis* bacteriophage phi-EF24C and reported that the most identity to MTP of this bacteriophage was found in bacteriophages of *Staphylococcus*, *Listeria* and *Lactobacillus* origins.

A portal protein gene (gp15, Table 7-11) with 1152 bp (383 aa) length was detected on the bacteriophage genome upstream of a prohead protease gene. Portal proteins form a DNA channel in dsDNA viruses such as head-tail bacteriophages (Zheng et al., 2008). The bacteriophages inject their genome into the host cells throughout this channel. Genome packaging is terminated when the portal channel is closed due to the change in the portal protein structure (Lhuillier et al., 2009). Although it is stated in literature reviews that portal proteins of different bacteriophages have a low sequence identity (Wang & Zhang, 2008), the portal protein gene found in the current study features a high percentage (90%) of identity to the portal protein gene of EFAP-1 bacteriophage.

A prohead protease gene (gp16, Table 7-11) was also found of 588 bp (195 aa) located downstream of a portal protein gene. Prohead (procapsid) protease is an essential enzyme for the maturation of capsid in dsDNA bacteriophages through its proteolytic cleavage activity (Liu & Mushegian, 2004). This activity is the second step of bacteriophage head assembly (Cheng et al., 2004). The encoding gene cluster is usually located adjacent to the gene encoding capsid proteins. In the current study, the gene locus was located closest a gene encoding major capsid proteins.

The loci of terminase small subunit (gp12, Table 7-11) and terminase large subunit (gp13, Table 7-11) were found close to each other, similar to those of EFAP-1 bacteriophage. Terminase small subunit and terminase large subunit genes were 474 bp (157 aa) and 1725 bp (574 aa) in size, respectively. Terminase small- and large-subunit genes are DNA packaging genes (Tuohimaa et al., 2006). Terminase proteins cleave concatemers within the bacteriophage genome so the genome can be packed up into the capsid (Govind et al., 2006). The gene loci of terminase small and large subunits are genetically linked and located next to each other (Stanley et al., 1997).

An ORF (gp3, Table 7-11) with partial identity (37%) to a daunorubicin resistance transmembrane protein gene of *V. fischeri* was identified. The ORF had a 333 bp length that encoded 110 aa. The daunorubicin resistance transmembrane protein is encoded by *drr* gene cluster and confers daunorubicin and doxorubicin (anticancer drugs) resistance by an export mechanism (Parajuli et al., 2003). This mechanism has been described in a range of Gram-positive and Gram-negative bacteria such as *S. pyogenes*, *S. peucetius* and *M. tuberculosis* (Chang et al., 2008; Vlaminckx et al., 2007; Parajuli et al., 2002). The gene product is similar to ABC-2 integral membrane protein family and ABC transmembrane type-2 domain. In 2001, Oggioni and Pozzi compared genome sequences of a serotype 3 *S. pneumonia* strain to sequences of a type 4 *S. pneumonia* strain and found a partial identity between daunorubicin resistance transmembrane proteins of these two distinct genomes.

An ORF (gp4, Table 7-11) with 378 bp that encodes 125 aa with partial identity (30%) to a tape measure protein of a *S. aureus* bacteriophage was characterized. Tape measure proteins (TMPs) are usually the longest proteins encoded by the bacteriophages and are involved in the assembly of bacteriophage tail and tail length determination (Crutz-Le Coq et al., 2002; Pedersen et al., 2000). Mutation in the TMP gene results in variation in tail length (Deveau et al., 2006).

An ORF (gp5, Table 7-11) with partial identity (38%) to an amino acid-polyamine-organocation (APC family) transporter protein gene of *L. fermentum* was identified. The ORF had a 189 bp length that encoded 62 aa. The APC superfamily is one of the largest transporter superfamilies found in both bacteria and archaea (Jack et al., 2000; Saier et al., 1999). The family has many members, which transfer solute-solute or solute-cation in prokaryotes. This allows the bacteria to accumulate nutrients using these energy-coupled transporters. This ORF also had an identity to an arginine-ornithine antiporter encoding gene of *Lactobacillus fermentum*. The arginine-ornithine antiporter transports arginine in LAB bacteria such as *Lactobacillus* spp. as well as other bacteria including *E. faecalis* (Arena et al., 2002; Driessen et al., 1988; Poolman et al., 1987).

An ORF (gp6, Table 7-11) with 405 bp that encodes a 134 aa with partial identity (57%) to the hypothetical protein EFP_gp188 of enterococcal bacteriophage phi-EF24C was identified. This domain also shared partial identity (48%) to a cytidine deaminase of the same bacteriophage. Cytidine deaminase, encoded by *cdd* genes, comprises a family of zinc-dependent enzymes that catalyze the hydrolytic deamination of cytidine to uridine and deoxycytidine to deoxyuridine (Vincenzetti et al., 1999; Josephsen et al., 1983). Cytidine deaminase deficiency in the bacterial host has been shown to affect the replication of bacteriophages considerably (Doskocil & Sorm, 1971). The enzyme has been identified in different bacteriophages including lambda bacteriophage (Yang et al., 1992). Recently, Uchiyama et al. (2008b) conducted whole genome sequencing of a *Myoviridae* bacteriophage isolated from an *E. faecalis* strain. The gene locus was located some distance (20 kb) from other bacteriophage housekeeping genes. In the current study, however, the encoding gene was located between housekeeping and antibiotic resistance genes—including APC family Amino acid/polyamine/organocation transporter, daunorubicin resistance transmembrane protein, zinc protease and DNA primase—usually found in eubacteria. This aggregation suggests the probable uptake and incorporation of a part of a bacterial genome by the bacteriophage.

An ORF (gp7, Table 7-11) with partial identity (38%) to a bacterial zinc protease gene was identified close to another bacterial gene DNA primase. The ORF was 237 bp in length that encoded 78 aa. Zinc proteases are digestive metalloenzymes that hydrolyze bonds within proteins and polypeptides (Kairys et al., 2006).

A large ORF (gp8, Table 7-11) of 1587 bp that encodes 528 aa with partial identity (32%) to DNA primase of *E. gallinarum* was characterized. The DNA primase is a polymerase

enzyme that synthesizes small RNA primers needed for the chromosomal DNA synthesis (Lee et al., 2006). This gene plays an important role in bacteriophage replication and propagation (Yother et al., 2002) and is found in different DNA bacteriophages (Alemayehu et al., 2009; Shaburova et al., 2009; Uchiyama et al., 2008b).

Six hypothetical protein-encoding genes (gp2, gp6, gp9, gp10, gp14, gp18, Table 7-11) with unclear functions were also identified. These hypothetical protein genes had different lengths and different loci on the genome; some shared identities to hypothetical genes of enterococcal bacteriophage EFAP-1 (gp15, gp19, gp24). However, the functions of these hypothetical proteins remain to be elucidated.

Moreover, clusters of ORF including sequences with no identity published in databases were identified. These sequences could belong to introns (interrupted genes), dispersed repetitious DNA or unclassified spacer DNA which have been reported in eubacteria and their bacteriophages (Deveau et al., 2008; Rajashekara et al., 1998; Mikkonen & Alatossava; 1995; Versalovic et al., 1991; Innis et al., 1986; Horiuchi, 1980). These short non-coding sequences, known as selfish DNA, are less affected by evolutionary selective procedure over the time than those sequences encoding proteins. However, the genome of prokaryotes is composed of less interrupted DNA compared to the genome of eukaryotes due their comparatively small size (Lederberg, 1987).

Interestingly, the following cluster of packaging and structural genes were found in both EFRM31 (gp12 to gp23) and EFAP-1 (gp21 to gp10) bacteriophages with the same order but in different directions. The gene cluster consisted of those encoding terminase small subunit, terminase large subunit, hypothetical protein, portal protein, prohead protease, major capsid protein, hypothetical protein, head-tail joining protein, head-tail adaptor protein, head-tail joining protein, head-tail joining protein and major tail protein. This suggests a close relationship or a common ancestor for these two bacteriophages.

Conclusions

In summary, most genes found in EFRM31 shared a high identity to genes from other enterococcal bacteriophages especially *E. faecalis* dsDNA-tailed bacteriophage EFAP-1. Most dsDNA-tailed bacteriophages seem to have common ancestors and share functional genetic elements (Hendrix et al., 1999). In bacteriophages from low-GC Gram-positive bacteria—including dsDNA-tailed bacteriophages—genes encoding small and large terminases, portal protein, prohead protease and major head protein mostly share common

sequences (Fouts, 2006; Zimmer et al., 2002). In the current study, this sequence similarity included the proteins above and others including a major tail protein, three head-tail joining proteins, a head-tail adaptor protein, a HNH endonuclease and three hypothetical proteins with 62–99% identity to those of EFAP-1, which suggests a similarity between EFRM31 and EFAP-1 bacteriophages.

Table 7-1. Thermal cycling conditions for DOP-PCR

	● Initial denaturation	94°C/10 min
10 cycles	– Denaturation	94°C/30 sec
	– Annealing	30°C/1 min
	– Ramping 30 → 72°C/7 min, or	36°C/1 min 42°C/1 min 48°C/1 min 54°C/1 min 60°C/1 min 66°C/1 min
	– Extension	72°C/5 min
30 cycles	– Denaturation	94°C/30 sec
	– Annealing	50°C/1 min
	– Ramping 50 → 72°C/220 sec, or	53°C/30 sec 56°C/30 sec 59°C/30 sec 62°C/30 sec 65°C/30 sec 68.5°C/35 sec
	– Extension	72°C/5 min
	● Final extension	72°C/5 min

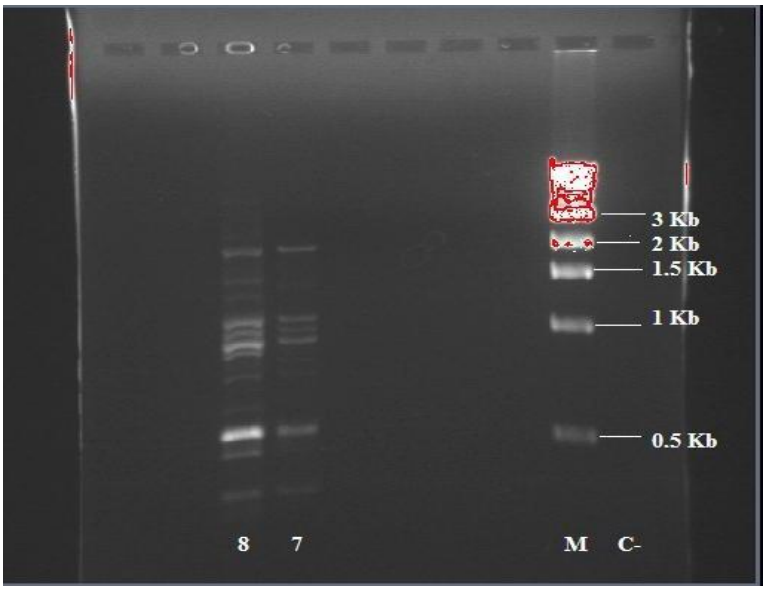


Figure 7-1. DOP-PCR of enterococcal bacteriophage genome. Lane 1, negative control; Lane 2, 1 kb marker; Lane 7, bacteriophage DNA in 0.05 dilution; Lane 8, bacteriophage DNA in 0.005 dilution

Table 7-2. Master Mix ingredients and thermal cycling conditions for M13 PCR

Master Mix (25 µL)		Thermal cycling		
Reagent	**Amount (µL)**	**Step**	**Temp. (°C)**	**Time**
Distilled water	17.65	● Initial denaturation	94	10 min
5× PCR buffer	2.5	● 30 cycles		
25 mM $MgCl_2$	2.5	– denaturation	94	30 sec
2.5 mM dNTPs	2	– annealing	56	30 sec
100 µmol primer (each)	0.05 (× 2)	– extension	72	2 min
5 U/µL Taq polymerase	0.25	● Final extension	72	10 min

Table 7-3. Master Mix ingredients and thermal cycling conditions for S-PCR

Master Mix (50 µL)		Thermal cycling		
Reagent	**Amount (µL)**	**Step**	**Temp. (°C)**	**Time**
Distilled water	24	● Initial denaturation	94	5 min
5× PCR buffer	10	● 40 cycles		
25 mM $MgCl_2$	7	– denaturation	94	30 sec
25 mM dNTPs	0.5	– annealing	45	1 min
10 pmol primer (each)	2 (× 2)	– extension	72	4 min
5 U/µL Taq polymerase	0.5	● Final extension	72	5 min

Table 7-4. Master Mix ingredients and thermal cycling conditions for S-PCR

Master Mix (25 µL)		Thermal cycling		
Reagent	**Amount (µL)**	**Step**	**Temp. (°C)**	**Time**
Distilled water	10.75	● Initial denaturation	94	5 min
5× PCR buffer	5	● 35 cycles		
25 mM $MgCl_2$	2.5	– denaturation	94	30 sec
25 mM dNTPs	2	– annealing	50	45 sec
10 pmol primer (each)	1.5 (× 2)	– extension	72	150 sec
5 U/µL Taq polymerase	0.25	● Final extension	72	5 min

Table 7-5. The list of primers used in Long-range PCR

Primer		Sequences (5'→3')
G1-F	F	ACTGGGTTTGACGTATTTAAATC
G2-R	R	AGGAAATACTACGTTTGATATTTATG
G2-F	F	AAATGATTCAGGCAATGCACG
G3-R	R	ACGTTTAATGAAGCTGTTAACTC
G3-F	F	ACCAAACTTGGCTAGTTGTTC
G4-R	R	ATGATGCAATCATCATGCAACG
G4-F	F	AGGGTGTAGATAATACCATTAAAAC
G5-R	R	AGTTGCTCAAATAATGCGTGAATC
G5-F	F	ACCTAAAGCTTCTGCTAACTC
G6-R	R	ATCCTTTTGAGGGTAATGATGG
G6-F	F	ACGGAGCGCAAATAATCTTG
G7-R	R	ATCAGGAAAGTAGGTGAAACC
G7-F	F	AGTGACAAATTATATTGTTGACATTG
G8-R	R	ATGATGAAATAACAGTTGGTGAC
G8-F	F	ACTTATAAATGATTGTAGCCTTTAAC
G9-R	R	AAAGGTTTTGAAGATATTCGAGG
G9-F	F	ACTTATGAATGCCTTCTTGCG
G10-R	R	AGAACAATCAGCCAACAACG
G10-F	F	AGCTGGTGGGTATAGAGC
G1-F	R	ACTGGGTTTGACGTATTTAAATC
G6-F	F	ACGGAGCGCAAATAATCTTG
G6-RR	R	ACGGAGCGCAAATAATCTTG
G9-F	F	ACTTATGAATGCCTTCTTGCG
G9-RR	R	ACTTATGAATGCCTTCTTGCG
G10-F	F	AGCTGGTGGGTATAGAGC
G10-RR	R	AGCTGGTGGGTATAGAGC

Table 7-6. Master Mix ingredients and thermal cycling conditions for L-PCR

Master Mix (25 µL)		Thermal cycling		
Reagent	**Amount (µL)**	**Step**	**Temp. (°C)**	**Time**
Distilled water	10.25	● Initial denaturation	94	5 min
5× PCR buffer	5	● 35 cycles		
25 mM $MgCl_2$	2.5	– denaturation	94	30 sec
2.5 mM dNTPs	2	– annealing	50	45 sec
10 pmol primer (each)	1.5 (× 2)	– extension	72	150 sec
5 U/µL Taq polymerase	0.25	● Final extension	72	5 min

Table 7-7. Master Mix ingredients and thermal cycling conditions for L-PCR

Master Mix (25 µL)		Thermal cycling		
Reagent	**Amount (µL)**	**Step**	**Temp. (°C)**	**Time**
Distilled water	10	● Initial denaturation	94	3 min
5× PCR buffer	5	● 40 cycles		
25 mM $MgCl_2$	2.5	– denaturation	94	15 sec
2.5 mM dNTPs	2	– annealing	50	30 sec
10 pmol primer (each)	1.5 (× 2)	– extension	68	10 min
5 U/µL Taq polymerase	0.5	● Final extension	68	7 min

Table 7-8. Master Mix ingredients and thermal cycling conditions for L-PCR

Master Mix (25 µL)		Thermal cycling		
Reagent	**Amount (µL)**	**Step**	**Temp. (°C)**	**Time**
Distilled water	11	● Initial denaturation	94	2 min
5× PCR buffer	2.5	● 35 cycles		
Q solution	5	– denaturation	94	15 sec
10 mM dNTPs	1.25	– annealing	50	30 sec
10 pmol primer (each)	1.5 (× 2)	– extension	68	10 min
5 U/µL Taq polymerase	0.25	● Final extension	68	7 min

Table 7-9. Master Mix ingredients and thermal cycling conditions for sequencing PCR

Master Mix (10 µL)		Thermal cycling		
Reagent	**Amount (µL)**	**Step**	**Temp. (°C)**	**Time**
Distilled water	6	● Initial denaturation	96	1 min
Buffer	1.5	● 30 cycles		
BigDye 3	0.5	– denaturation	96	10 sec
Primer (1.6 pmol)	1	– annealing	50	10 sec
DNA	1	– extension	60	4 min

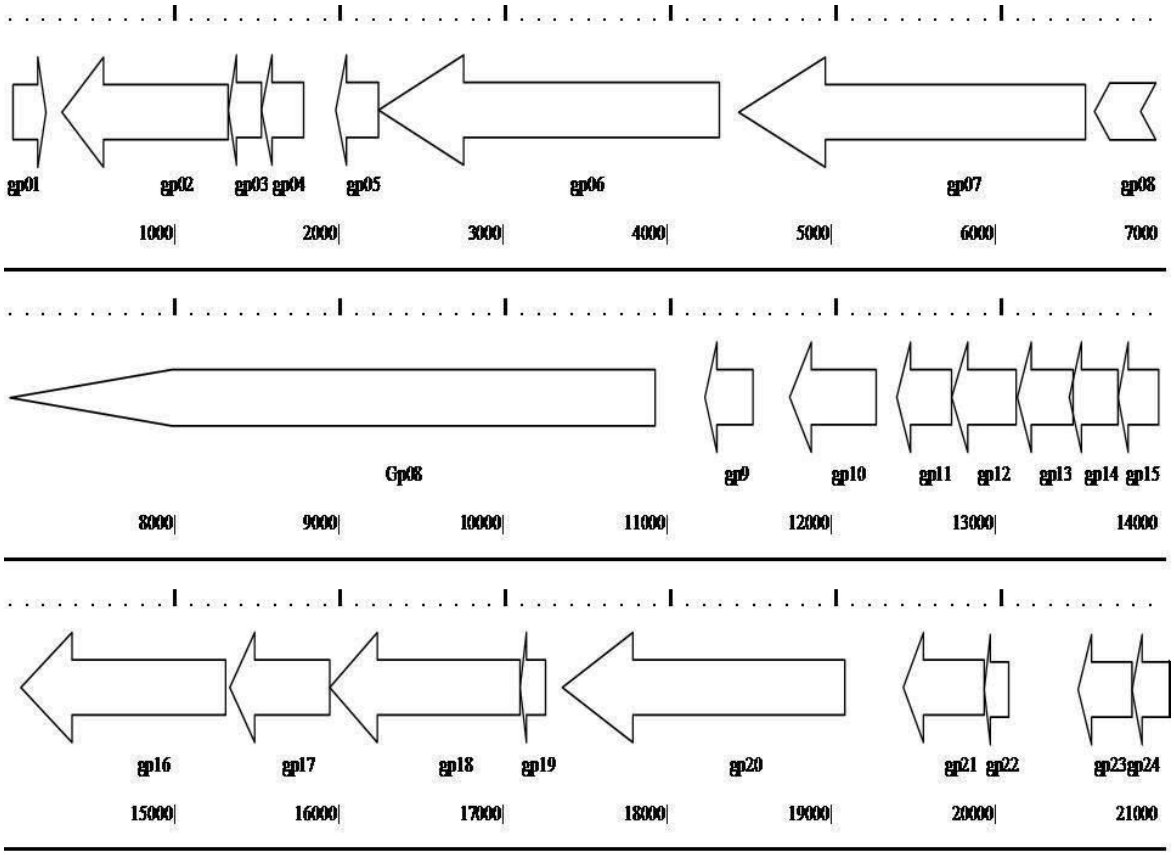


Figure 7-2. *E. faecalis* bacteriophage EFAP-1 gene map

Table 7-10. Gene specifications of bacteriophage EFAP-1

Gene	Protein	Group	Locus	Direction	Protein ID
gp01	Hypothetical protein	N/A	28−213	→	ACN86315.1
gp02	Amidase	L	297−1283	←	ACN86316.1
gp03	Holin	L	1280−1516	←	ACN86317.1
gp04	Hypothetical protein	N/A	1531−1776	←	ACN86318.1
gp05	Hypothetical protein	N/A	1955−2242	←	ACN86319.1
gp06	Host specificity protein	HS	2258−4327	←	ACN86320.1
gp07	Tail component	PS	4397−6478	←	ACN86321.1
gp08	Tape measure protein	PS	6561−10931	←	ACN86322.1
gp09	Hypothetical protein	N/A	11188−11499	←	ACN86323.1
gp10	Major tail protein	PS	11691−12260	←	ACN86324.1
gp11	Head-tail joining protein	PS	12336−12701	←	ACN86325.1
gp12	Head-tail joining protein	PS	12698−13105	←	ACN86326.1
gp13	Head-tail adaptor protein	PS	13102−13437	←	ACN86327.1
gp14	Head-tail joining protein	PS	13409−13708	←	ACN86328.1
gp15	Hypothetical protein	N/A	13724−13942	←	ACN86329.1
gp16	Major capsid protein	PS	14062−15318	←	ACN86330.1
gp17	Prohead protease	PS	15389−15952	←	ACN86331.1
gp18	Portal protein	PS	15939−17090	←	ACN86332.1
gp19	Hypothetical protein	N/A	17095−17259	←	ACN86333.1
gp20	Terminase large subunit	PS	17329−19053	←	ACN86334.1
gp21	Terminase small subunit	PS	19425−19898	←	ACN86335.1
gp22	Hypothetical protein	N/A	19913−20065	←	ACN86336.1
gp23	HNH endonuclease	L	20458−20829	←	ACN86337.1
gp24	Hypothetical protein	N/A	20829−21035	←	ACN86338.1

N/A, not applicable; HS, host specificity; L, lysis; RR, replication and regulation; PS, packaging and structural

http://www.ncbi.nlm.nih.gov/nuccore/225346548

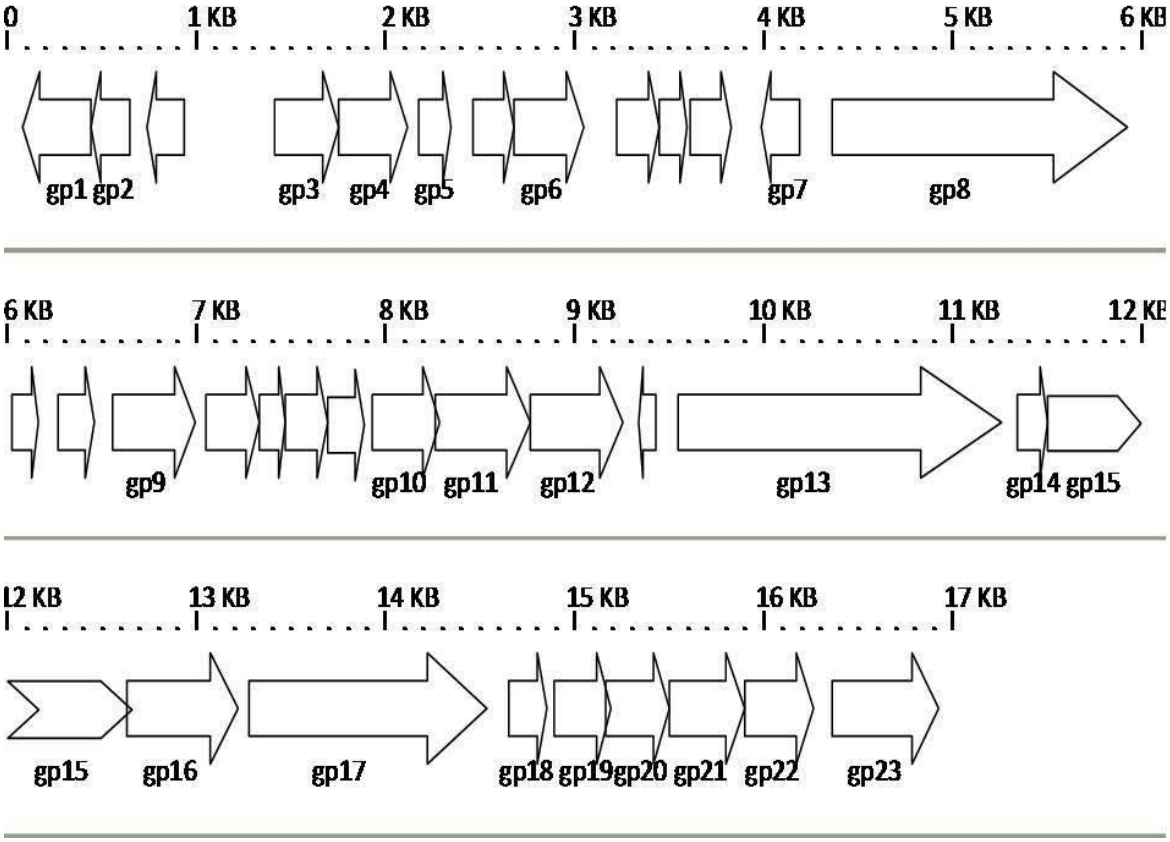


Figure 7-3. *E. faecalis* bacteriophage EFRM31 gene map

Table 7-11. Gene specifications of bacteriophage EFRM31

ORF	Gene	Protein	Group	Locus	bp	aa	*	GenBank	E.Value	%
1	gp1	HNH endonuclease	L	80–451	372	123	←	YP_002727895.1	8e-65	94
2	gp2	Hypothetical protein	N/A	451–654	204	67	←	YP_002727896.1	1e-20	89
3	-	Unknown	-	737–940	204	67	←	-	-	-
4	gp3	Antibiotic resistance	AB	1418–1750	333	110	→	YP_002158147.1	5.6	37
5	gp4	Tape measure protein	PS	1747–2124	378	125	→	ZP_06317090.1	4.7	30
6	gp5	APC transporter	H	2165–2353	189	62	→	ZP_03945177.1	6.6	38
6	gp5	Arginine-ornithine antiporter	H	2165–2353	189	62	→	YP_001843737.1	6.7	38
7	-	Unknown	-	2447–2662	216	71	→	-	-	-
8	gp6	Hypothetical protein	N/A	2663–3067	405	134	→	YP_001504297.1	3e-16	57
8	gp6	Putative cytidine deaminase	H	2663–3067	405	134	→	YP_001504212.1	4e-07	48
9	-	Unknown	-	3222–3446	225	74	→	-	-	-
10	-	Unknown	-	3446–3601	156	51	→	-	-	-
11	-	Unknown	-	3613–3834	22	73	→	-	-	-
12	gp7	Zinc protease	L	3981–4217	237	78	←	NP_661599.1	9.0	38
13	gp8	DNA primase	RR	4343–5929	1587	528	→	ZP_05650419.1	8e-42	32
14	-	Unknown	-	6019–6186	168	55	→	-	-	-
15	-	Unknown	-	6283–6471	189	62	→	-	-	-
16	gp9	Hypothetical protein	N/A	6544–7005	462	153	→	YP_238560.1	4e-04	30
17	-	Unknown	-	7054–7326	273	90	→	-	-	-
18	-	Unknown	-	7328–7489	162	53	→	-	-	-
19	-	Unknown	-	7491–7700	210	69	→	-	-	-
20	-	Unknown	-	7703–7894	192	63	→	-	-	-
21	gp10	Hypothetical protein	N/A	7920–8294	375	124	→	ZP_04797562.1	6.7	26
22	gp11	HNH endonuclease	L	8266–8787	522	173	→	ZP_03212364.1	9e-35	49
23	gp12	Terminase small subunit	PS	8788–9261	474	157	→	YP_002727893.1	4e-49	62
24	-	Unknown	-	9335–9439	105	34	←	-	-	-
25	gp13	Terminase large subunit	PS	9556–11280	1725	574	→	YP_002727892.1	0.0	97
26	gp14	Hypothetical protein	N/A	11326–11514	189	62	→	YP_002727891.1	2e-18	94
27	gp15	Portal protein	PS	11519–12670	1152	383	→	YP_002727890.1	0.0	90
28	gp16	Prohead protease	PS	12633–13220	588	195	→	YP_002727889.1	2e-103	99
29	gp17	Major capsid protein	PS	13290–14537	1248	415	→	YP_002727888.1	0.0	96
30	gp18	Hypothetical protein	N/A	14663–14863	201	66	→	YP_002727887.1	8e-15	62
31	gp19	Head-tail joining protein	PS	14901–15203	303	100	→	YP_002727886.1	4e-41	83
32	gp20	Head-tail adaptor protein	PS	15175–15510	336	111	→	YP_002727885.1	6e-51	84
33	gp21	Head-tail joining protein	PS	15507–15914	408	135	→	YP_002727884.1	1e-64	85
34	gp22	Head-tail joining protein	PS	15911–16276	366	121	→	YP_002727883.1	8e-63	94
35	gp23	Major tail protein	PS	16355–16921	567	188	→	YP_002727882.1	2e-82	83

†Antibiotic resistance, daunorubicin resistance transmembrane protein; ‡APC transporter, APC family amino acid/polyamine/organocation transporter; *Direction; bp, base pair; aa, amino acid; %, maximum identities; GenBank, NCBI GenBank original accession number; N/A, not applicable; L, lysis; RR, replication and regulation; PS, packaging and structural; AB, antibiotic resistance; H, housekeeping

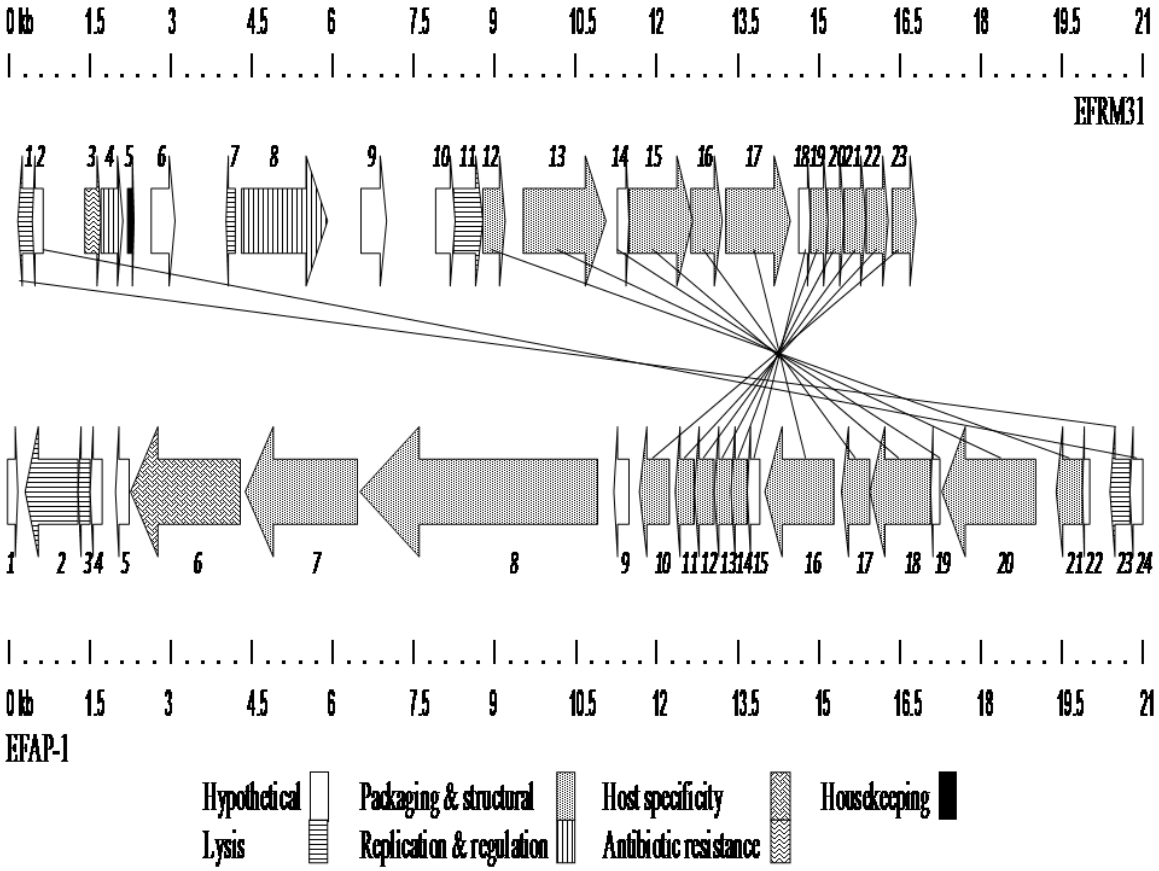


Figure 7-4. Comparative gene maps of EFRM31 and EFAP-1. Connective lines represent the maximum identity between the genes; dot lines represent genome length in kilo bases; Italic numbers refer to protein-encoding genes

Table 7-12. ORFs and their specifications within the genome of bacteriophage EFRM31

No.	From	To	bp	aa	Protein	%
1	9556	11280	1725	574	Terminase large subunit	97
2	4343	5929	1587	528	DNA primase	32
3	13290	14537	1248	415	Major capsid protein	96
4	11519	12670	1152	383	Portal protein	90
5	12633	13220	588	195	Prohead protease	99
6	16355	16921	567	188	Major tail protein	83
7	8266	8787	522	173	HNH endonuclease family protein	49
8	13274	13783	510	169	-	-
9	8788	9261	474	157	Terminase small subunit	62
10	6544	7005	462	153	ORF083	30
11	15507	15914	408	135	Head-tail joining protein	85
12	2663	3067	405	134	Hypothetical protein EFP_gp188	57
					Putative cytidine deaminase	48
13	1747	2124	378	125	Tape measure protein	30
14	7920	8294	375	124	Conserved hypothetical protein	28
					Hypothetical protein LSL_0755	26
15	80	451	372	123	HNH endonuclease	94
16	15911	16276	366	121	Head-tail joining protein	94
17	9954	10304	351	116	Putative regulatory protein	29
18	15175	15510	336	111	Head-tail adaptor protein	84
19	1418	1750	333	110	Daunorubicin resistance transmembrane protein	37
20	14901	15203	303	100	Head-tail joining protein	83
21	5530	5826	297	98	ABC superfamily ATP binding cassette transporter	33
					Glucose-methanol-choline oxidoreductase	51
22	7054	7326	273	90	Non bacterial genome	54
23	10356	10622	267	88	Molybdenum cofactor synthesis domain-containing protein	30
24	3981	4217	237	78	Zinc protease	38
25	3222	3446	225	74	-	-
26	13387	13608	222	73	-	-
27	3613	3834	222	73	-	-
28	3827	4045	219	72	-	-
29	2447	2662	216	71	-	-
30	7491	7700	210	69	-	-
31	4042	4248	207	68	Non bacterial genome	46
32	737	940	204	67	-	-
33	451	654	204	67	Hypothetical protein EFAP1_gp24	89
34	14663	14863	201	66	Hypothetical protein EFAP1_gp15	62
35	15686	15880	195	64	Non bacterial genome	38
36	1762	1956	195	64	Non bacterial genome	35
37	7703	7894	192	63	-	-
38	1365	1556	192	63	-	-
39	11326	11514	189	62	Hypothetical protein EFAP1_gp19	94
40	6283	6471	189	62	Non bacterial genome	33
41	2165	2353	189	62	APC family amino acid-polyamine-organocation transporter	38
					Arginine-ornithine antiporter	38
42	11764	11949	186	61	Non bacterial genome	51
43	16663	16845	183	60	Non bacterial genome	34
44	12722	12898	177	58	-	-
45	6909	7076	168	55	-	-
46	6019	6186	168	55	-	-
47	4157	4321	165	54	-	-
48	7328	7489	162	53	-	-
49	4737	4898	162	53	-	-
50	3242	3403	162	53	-	-
51	4071	4229	159	52	-	-
52	3446	3601	156	51	-	-
53	9502	9651	150	49	-	-
54	298	447	150	49	-	-
55	7645	7791	147	48	-	-

No.	From	To	bp	aa	Protein	%
56	6276	6422	147	48	-	-
57	2025	2171	147	48	-	-
58	16737	16868	132	43	-	-
59	15652	15780	129	52	-	-
60	8470	8595	126	41	-	-
61	3424	3546	123	40	-	-
62	3271	3393	123	40	-	-
63	7913	8032	120	39	-	-
64	335	454	120	39	-	-
65	15630	15746	117	38	Non bacterial genome	37
66	14275	14463	189	62	Non bacterial genome	42
67	8125	8235	111	43	-	-
68	6012	6122	111	36	-	-
69	1569	1679	111	36	-	-
70	15037	15144	108	35	-	-
71	12775	12882	108	35	-	-
72	12283	12390	108	35	-	-
73	15133	15237	105	34	-	-
74	9335	9439	105	34	-	-
75	5526	5630	105	34	-	-
76	2581	2685	105	46	-	-
77	14966	15067	102	42	Non bacterial genome	34
78	7465	7566	102	43	-	-
79	6606	6707	102	33	-	-
80	5571	5672	102	33	-	-
81	5347	5448	102	60	-	-
82	702	803	102	33	-	-

%, maximum identity to GenBank reference data; bp, base pair; aa, amino acid

Chapter 8

General Discussion and Conclusions

Multiple resistant strains are common among some enterococcal populations due to the use of antibiotics in human and veterinary medicine and livestock production, in addition to intrinsic resistance to some antibiotics in many enterococci. These enterococcal strains are postulated to be important reservoirs of antibiotic resistance genes. Enterococci which can sometimes cause infections in humans and animals include many species such as *E. avium, E. casseliflavus, E. cecorum, E. columbae, E. dispar, E. durans, E. flavescens, E. gallinarum, E. hirae, E. malodoratus, E. mundtii, E. pseudoavium, E. raffinosus, E. saccharolyticus, E. seriolicida, E. solitaries* and *E. sulfurous* (Sood et al., 2008; Pillar & Gilmore, 2004; Mundy et al., 2000) in addition to *E. faecalis* and *E. faecium* which are the most common enterococcal species involved in human infections (Woo et al., 2008; Prakash et al., 2005).

Furthermore, bacteriophages are found in enterococci that could be responsible for transferring those antibiotic resistance genes usually carried on mobile genetic elements such as plasmids and transposons to other bacteria. Based upon these hypotheses, the following methodology was set as the basis of the current study.

To study the range of antibiotic resistance in enterococci, 14 antibiotics and heavy metals were used. These antibiotics are associated with the most common resistance phenotypes in enterococci, especially those isolated from chickens and pigs. This study is a comparative study because the focus is on the range of antibiotics used in veterinary medicine and the livestock industries. The enterococcal bacteriophages and their role in transferring antimicrobial resistance genes in enterococci have rarely been studied. Therefore, the purposes of this study were determined as: 1) assessment of antimicrobial resistance phenotypes in enterococci with emphasis on species other than *E. faecalis* and *E. faecium*; 2) detection of genes responsible for encoding the resistances; 3) isolation and characterization of enterococcal bacteriophages; and 4) determination of the role of bacteriophages in the transfer of resistance genes between enterococci.

Results from this study showed that multiple resistant enterococci are widespread in healthy livestock as almost all isolates were multiple resistant. Ruzauskas et al. (2008) investigated antimicrobial resistance in enterococci isolated from healthy and sick animals.

Resistance was found more often in enterococci isolated from pigs and poultry. In a survey on the prevalence of resistance to 11 antimicrobials including avilamycin, gentamicin, tetracycline, vancomycin and copper in swine enterococci in three European countries, Aarestrup and McNicholas (2002) found multiple resistance in both *E. faecalis* and *E. faecium* with some differences probably due to the different uses of antimicrobial agents in some countries. Similar results were found by Poeta et al. (2006). Indeed, Emborg et al. (2003) showed that the resistance rate in *E. faecium* isolated from broilers and broiler meat to four antibiotics—including avilamycin, erythromycin, vancomycin and virginiamycin—used in animal husbandry was significantly decreased after the withdrawal of those antibiotics from the market. In general, results from the current study were in agreement with other studies with some exceptions. The lack of high-level gentamicin resistance in this study contrasts with findings from other studies which have reported a high prevalence of high-level gentamicin resistance in enterococci from pigs (Jackson et al., 2005; Donabedian 2003). However, gentamicin is not registered for use in livestock in Australia. The *van*C1 and *van*C2 genes were not unexpectedly found in *E. casseliflavus* and *E. gallinarum* in accordance with previous studies (Seo et al., 2005). Interestingly, one *van*C1 was found in an *E. faecalis* of pig origin and one *van*C2 in an *E. faecium* of chicken origin. There are rare reports on the detection of *van*C genes in isolates other than *E. casseliflavus* and *E. gallinarum* (Patel et al., 1997). The high levels of resistance to tetracycline, bacitracin, tiamulin and tylosin reflect the widespread usage of these antibiotics in the Australian pig industry.

In the current study, some problems emerged. For example, there was sometimes a difference between the phenotype and molecular gene detection results. Generally, it should be said that although PCR is useful for accurately detecting the presence of antimicrobial resistant genes, it cannot determine whether or not the genes are being expressed. This could be confirmed by detecting the predicted encoded proteins associated with resistance or detection of specific mRNA. PCR is a very sensitive assay to detect and characterize even few copies of bacterial genes. There are several PCR techniques such as Single PCR, Multiplex PCR, Real-time PCR (Quantitative PCR), Nested PCR, Arbitrary PCR and RT PCR applied for different types of research. PCR varies in sensitivity and specificity according to the primers and protocols used. Multiplex PCRinvolves use of two or more pairs of primers to detect multiple genes in a single sample, whereas Single PCR uses one set of primers. Multiplex PCR is therefore more difficult to set up and design compared with routine Single PCR. The reasons for this include formation of primer

dimers due to homologous sequences, difficulty in optimizing the annealing temperatures because of different melting temperatures, difficulty in adjusting the Master Mix composition ($MgCl_2$ and buffer) to suit the multiple primers. An example relates to *erm*B gene, which codes the resistance to macrolides (e.g. tylosin and erythromycin). PCR failed to detect *erm*B in all intermediately resistant isolates when *erm*B primer pairs were applied in combination with other primers (Multiplex). The PCR (Single) was repeated for *erm*B only resulting in detection of *erm*B genes. An explanation is that *erm*B primer nucleotides hybridised other primer nucleotides, which prevented the binding of the primers to the bacterial DNA template in the annealing step. However, although molecular assays for the detection of a variety of resistance genes are generally highly reliable, these assays sometimes seem to fail to detect all genes (Fluit et al., 2001).

Enterococci have had limited attention from medical and veterinary experts as potential pathogens as most research focuses on more serious pathogens such as *E. coli* and *S. enterica*. Although enterococci have not been considered as primary pathogens and are ubiquitous in soil, water, foods, and animal and human guts, they have increasingly emerged as opportunist bacteria causing serious nosocomial and community infections such as bacteremia and endocarditis (Bender et al., 2009; Fracalanzza et al., 2007). A significant proportion of the bacteria causing these infections are found to be resistant to clinically important antimicrobials such as vancomycin. For example, the prevalence of VRE in enterococcal isolates from hospitalized patients increased from < 1% to approximately 15% from 1990 to 1997. Furthermore, VRE isolates are included in about 25% of enterococci in the National Nosocomial Infections Surveillance (NNIS) Intensive Care Units (ICUs) in 1999 and 28.5% in 2003 (Siegel et al., 2006). Enterococci are able to become resistant to a variety of antimicrobials via acquired gene transfer besides their natural resistance to many antimicrobials (Leavis et al., 2006). Thus in the last two decades, enterococci have emerged with an increasing frequency of multidrug resistance including high-level resistance to aminoglycosides, vancomycin, ampicillin and linezolid (Sood et al., 2008; Thapa et al., 2007; Shaghaghi et al., 2007; Matsumoto et al., 2004; Livermore, 2003). Therefore, more researchers have become interested in studying enterococci and infections related to them.

As mentioned before, enterococcal bacteriophages are poorly studied and limited numbers have been characterized. Moreover, the role of bacteriophage lysogeny on the interactions of bacteriophages and animal bacterial hosts is still insufficiently understood (Letarov & Kulikov, 2009). Recently, most research has focused on the isolation of bacteriophages

and their effects on enterococci as an antimicrobial treatment (phage therapy) (Thiel, 2004; Bachrach et al., 2003; Duckworth & Gulig, 2002). In 2002, Biswas et al. treated bacteremic mice using enterococcal bacteriophages. Yoong et al. (2004) identified a broadly active lytic enzyme from an *E. faecalis* bacteriophage with lytic activity against antibiotic resistant strains of *E. faecalis* and *E. faecium*. Paisano et al. (2004) assessed antimicrobial effects of bacteriophages on human dentine infected with *E. faecalis*. More recently, Letkiewicz et al. (2009) successfully used bacteriophages for the elimination of resistant strains of *E. faecalis* in patients with prostate infections. In summary, less attention has been paid to molecular biology, epidemiology and the role of bacteriophages in transferring genes responsible for the antibiotic resistance in enterococci. Although outside the scope of this thesis, the possibility of the use of bacteriophages isolated in this study in the treatment of enterococcal infections (and perhaps other Gram-positive bacteria) should be considered as possible topic for further study.

The current study has expanded the range of bacteriophages known to be associated withenterococci. Eleven bacteriophages were of isometric shape with long flexible or non-flexible tails or short tail while one had a long head with a long flexible tail; all contained double-stranded DNA molecules. Seven polyhedral, filamentous and pleomorphic shaped bacteriophages containing DNA or RNA were also observed. The pleomorphic bacteriophages were droplet or lemon-shaped in morphology. This study is the first report on non-tailed bacteriophages in enterococci of non-human origin. Furthermore, most of the enterococcal bacteriophages were isolated on *E. faecalis*, the most prevalent enterococcal species in nature (Behnia et al., 2002; Joyanes et al., 2000). As these bacteriophages usually carry MGEs which may include virulence genes, it may be concluded that bacteriophages can mediate transfer of those MGEs to *E. faecalis* strains to enhance the survival of this species over other species (Brockhurst et al., 2006; Pai & Kim, 1998). However, other factors may contribute to the broader prevalence of *E. faecalis*, such as a cytochrome-like respiration system and virulence genes (Poeta et al., 2005; Shepard & Gilmore, 2002; Ritchey & Seeley, 1976).

Another outcome from this study was demonstration of the role of bacteriophages play in antibiotic resistence gene transfer between enterococci for the first time. The importance of these experiments is highlighted when one considers that interspecies transduction was demonstrated. This study has investigated the best methods for the bacteriophage propagation. The results suggest that propagation of enterococcal bacteriophages in liquid culture media may not be optimal for obtaining high titres. Although the liquid phase is

described in many studies as an appropriate technique for propagating high-titres of bacteriophages (Shirasaki et al., 2009; Pajunen et al., 2000; Trouwborst et al., 1974), the solid-phase method was found more useful in this study. The liquid-phase propagation method is known to depend on several factors such as the amount of culture media used for propagating, and supplementary salt concentration such as $Ca(NO_3)_2$ and $MgSO_4$ (Konthur & Walter, 2002; Caso et al., 1995).

Another significance of this study is the whole genome sequencing of a selected bacteriophage EFRM31. Benefits of doing whole genome sequencing include identification and characterization of virulence and antimicrobial resistance genes, and also mobile genetic elements such as prophages, plasmids, transposons and integrons which are involved in transferring those genes. Furthermore, gene locations and arrangements can be detected on the genome. Another feature of the whole genome sequencing method includes the opportunity of studying non-coding sequences. In the current study, two different methods were used for the genome sequencing. Firsty, a whole genome amplification based DOP-PCR method followed by a genome walking method was applied in the laboratory. This method generates a highly pure DNA from the entire genome even with low quantity or bad quality genomic DNA input (Lasken & Egholm, 2003; Barbaux et al., 2001). However, this method failed to amplify the complete genome of the bacteriophage. Therefore, the second method, a shotgun sequencing method (GeneWorks® Technologies, Australia), was used to sequence the bacteriophage genome and complement the DOP-PCR data. Shotgun sequencing method is reported as the most efficient method to sequence the genome of bacteria and dsDNA bacteriophages (Kaiser et al., 2003). The genome sequencing demonstrated a significant identity to another previously characterized enterococcal bacteriophage EFAP-1.

In summary, enterococci from nonhuman sources appear to play a critical role in the acquisition and transfer of antibiotic resistance determinants. The investigation and control of use of antimicrobial agents in human and veterinary medicine have been an important medical finding in the twentieth century. However, after utilizing antimicrobial agents as growth promoters, feed additives or treatments, bacteria have acquired resistance to many antibiotics. This phenomenon has been spread with such a speed that it has forced governments and decision makers to restrict the use of antibiotics in animal husbandry. The most effective way to limit the spread of antimicrobial resistance is to limit the use of antimicrobials in routine medicine and to ban the use of those, which are used in human medicine, as growth promoters in food animal production (Turnidge, 2004). To limit the

emergence and spread of antimicrobial resistance and its consequences, it is necessary to collect data on factors involved in this phenomenon (Gilmore, 2002). Accordingly, work groups and antimicrobial resistance surveillance programs such as AGISAR, APVMA, ARBAO-II, CHMP/VVMP, CIPARS, CRAB, DANMAP, EAGAR/JETACAR, EARSS, ESGARS, MARAN, NARMS, NORM/NORM-VET, NRA, SCOPE, SENTRY and SVARM/SWEDRES have been formed to prospectively monitor changes in antimicrobial susceptibility of pathogens. One recommendation is that antibiotic susceptibility testing of isolates from various sources, especially for enterococci, should be considered in more countries in order to select the best choices in the case of infections. Another recommendation is that periodically well-organized monitoring and surveying of antimicrobial resistance, on local or international scale (such as Codex), seem to be necessary to decrease the expenditure of public funds on the treatment of resistant microbial infections as a consequence of spreading antimicrobial resistance. Finally, now that the molecular characterization and analysis of enterococcal bacteriophages, including the determination of encoded proteins by the bacteriophage genes, mobile genetic elements and the study of the predicted role of bacteriophages in the evolutionary ecology of the bacterial populations, will help scientists and medical experts to have a better understanding of the nature of resistance mechanisms and assist in planning new strategies for decreasing the losses and socioeconomic efforts of the antimicrobial resistance phenomenon. The potential role of enterococcal bacteriophages in the treatment of severe and persistant infections caused by *Enterococcus* spp. should also be considered, especially in those cases when the causative bacteria show extensive resistance to the available antimicrobial agents. In fact some researchers are currently investigating the effect of bacteriophages on the eradication and treatment of microbial infections because of the prevalence of microbial resistance to conventional drugs. Therefore, molecular study of enterococcal bacteriophagescould aid progress in this field.

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Appendices

Appendix 1. The isolate collection used in this thesis

No.	Batch	*Enterococcus*	Origin	Collection
1	40E	*faecalis*	Pig	UniSA Collection
2	55E	*faecalis*	Pig	UniSA Collection
3	36W	*faecalis*	Pig	UniSA Collection
4	38W	*faecalis*	Pig	UniSA Collection
5	35G	*faecium*	Pig	UniSA Collection
6	37G	*faecium*	Pig	UniSA Collection
7	38G	*faecium*	Pig	UniSA Collection
8	44G	*faecium*	Pig	UniSA Collection
9	53G	*faecium*	Pig	UniSA Collection
10	54G	*faecium*	Pig	UniSA Collection
11	56G	*faecium*	Pig	UniSA Collection
12	58G	*faecium*	Pig	UniSA Collection
13	92G	*hirae/durans*	Pig	UniSA Collection
14	99G	*faecium*	Pig	UniSA Collection
15	100G	*faecalis*	Pig	UniSA Collection
16	101G	*hirae/durans*	Pig	UniSA Collection
17	121G	*faecalis*	Pig	UniSA Collection
18	99438-3	*faecium*	Chicken	UniSA Collection
19	133G	*faecium*	Pig	UniSA Collection
20	134G	*faecium*	Pig	UniSA Collection
21	135G	*faecium*	Pig	UniSA Collection
22	136G	*faecium*	Pig	UniSA Collection
23	137G	*faecium*	Pig	UniSA Collection
24	138G	*faecium*	Pig	UniSA Collection
25	139G	*faecium*	Pig	UniSA Collection
26	140G	*faecium*	Pig	UniSA Collection
27	143G	*faecium*	Pig	UniSA Collection
28	147G	*faecium*	Pig	UniSA Collection
29	149G	*faecium*	Pig	UniSA Collection
30	4	*faecalis*	Pig	UniSA Collection
31	7	*faecalis*	Pig	UniSA Collection
32	6	*faecalis*	Pig	UniSA Collection
33	7	*faecalis*	Pig	UniSA Collection
34	13	*faecalis*	Pig	UniSA Collection
35	11	*faecalis*	Pig	UniSA Collection
36	99427-5	*faecium*	Chicken	UniSA Collection
37	15E	*faecium*	Pig	UniSA Collection
38	8	*faecalis*	Pig	UniSA Collection
39	9ery	*faecium*	Pig	UniSA Collection
40	12	*faecalis*	Pig	UniSA Collection
41	14	*faecalis*	Pig	UniSA Collection
42	10	*faecalis*	Pig	UniSA Collection
43	4	*faecalis*	Pig	UniSA Collection
44	6	*hirae*	Pig	UniSA Collection
45	2	*faecalis*	Pig	UniSA Collection
46	4	*faecalis*	Pig	UniSA Collection
47	99453-3	*faecium*	Chicken	UniSA Collection
48	19E	*faecalis*	Pig	UniSA Collection
49	99453-4	*faecium*	Chicken	UniSA Collection

50	99463-5	*faecium*	Chicken	UniSA Collection
51	31	*faecalis*	Pig	UniSA Collection
52	32	*faecalis*	Pig	UniSA Collection
53	34	*faecalis*	Pig	UniSA Collection
54	3	*faecalis*	Pig	UniSA Collection
55	13	*casseliflavus*	Pig	UniSA Collection
56	14	*faecalis*	Pig	UniSA Collection
57	13	*faecalis*	Pig	UniSA Collection
58	24	*faecalis*	Pig	UniSA Collection
59	40	*faecalis*	Pig	UniSA Collection
60	73	*faecalis*	Pig	UniSA Collection
61	9	*faecalis*	Pig	UniSA Collection
62	18	*faecalis*	Pig	UniSA Collection
63	26	*faecalis*	Pig	UniSA Collection
64	14Ery	*faecalis*	Pig	UniSA Collection
65	29	*faecalis*	Pig	UniSA Collection
66	23Ery	*faecalis*	Pig	UniSA Collection
67	34	*faecalis*	Pig	UniSA Collection
68	6	*faecalis*	Pig	UniSA Collection
69	20	*faecalis*	Pig	UniSA Collection
70	26	*faecalis*	Pig	UniSA Collection
71	28	*faecalis*	Pig	UniSA Collection
72	45	*faecalis*	Pig	UniSA Collection
73	38Ery	*faecalis*	Pig	UniSA Collection
74	43	*faecalis*	Pig	UniSA Collection
75	32Ery	*faecium*	Pig	UniSA Collection
76	16	*faecalis*	Pig	UniSA Collection
77	38	*faecalis*	Pig	UniSA Collection
78	36	*faecalis*	Pig	UniSA Collection
79	37	*faecalis*	Pig	UniSA Collection
80	40	*faecalis*	Pig	UniSA Collection
81	41	*faecalis*	Pig	UniSA Collection
82	42	*faecalis*	Pig	UniSA Collection
83	43	*faecalis*	Pig	UniSA Collection
84	44	*faecalis*	Pig	UniSA Collection
85	45	*faecalis*	Pig	UniSA Collection
86	39	*faecalis*	Pig	UniSA Collection
87	2	*faecalis*	Pig	UniSA Collection
88	4	*faecalis*	Pig	UniSA Collection
89	5	*faecalis*	Pig	UniSA Collection
90	14	*faecalis*	Pig	UniSA Collection
91	11	*faecalis*	Pig	UniSA Collection
92	33	*faecalis*	Pig	UniSA Collection
93	35	*faecalis*	Pig	UniSA Collection
94	120F	*faecium*	Pig	UniSA Collection
95	131F	*faecalis*	Pig	UniSA Collection
96	247G	*faecium*	Pig	UniSA Collection
97	98F	*faecium*	Pig	UniSA Collection
98	110F	*faecium*	Pig	UniSA Collection
99	112F	*faecium*	Pig	UniSA Collection
100	80F	*faecium*	Pig	UniSA Collection
101	79F	*faecalis*	Pig	UniSA Collection
102	92F	*faecalis*	Pig	UniSA Collection
103	72F	*faecium*	Pig	UniSA Collection
104	75F	*faecium*	Pig	UniSA Collection
105	71F	*faecium*	Pig	UniSA Collection

106	202G	*faecium*	Pig	UniSA Collection
107	81G	*hirae/durans*	Pig	UniSA Collection
108	195G	*faecium*	Pig	UniSA Collection
109	204G	*faecium*	Pig	UniSA Collection
110	206G	*faecium*	Pig	UniSA Collection
111	203G	*faecium*	Pig	UniSA Collection
112	99498-3	*faecium*	Chicken	UniSA Collection
113	160F	*hirae/durans*	Pig	UniSA Collection
114	223G	*hirae/durans*	Pig	UniSA Collection
115	226G	*faecalis*	Pig	UniSA Collection
116	230G	*faecium*	Pig	UniSA Collection
117	248G	*faecium*	Pig	UniSA Collection
118	65F	*faecium*	Pig	UniSA Collection
119	66F	*faecium*	Pig	UniSA Collection
120	69F	*faecium*	Pig	UniSA Collection
121	75G	*hirae/durans*	Pig	UniSA Collection
122	76G	*hirae/durans*	Pig	UniSA Collection
123	77G	*faecium*	Pig	UniSA Collection
124	99510-2	*faecium*	Chicken	UniSA Collection
125	232G	*faecium*	Pig	UniSA Collection
126	99521-1	*faecium*	Chicken	UniSA Collection
127	132D	*hirae/durans*	Pig	UniSA Collection
128	133D	*hirae/durans*	Pig	UniSA Collection
129	322G	*raffinosus*	Pig	UniSA Collection
130	264G	*faecium*	Pig	UniSA Collection
131	267G	*faecium*	Pig	UniSA Collection
132	272G	*faecium*	Pig	UniSA Collection
133	273G	*faecalis*	Pig	UniSA Collection
134	274G	*faecium*	Pig	UniSA Collection
135	275G	*faecalis*	Pig	UniSA Collection
136	281G	*faecium*	Pig	UniSA Collection
137	66FR	*faecium*	Pig	UniSA Collection
138	69FR	*faecium*	Pig	UniSA Collection
139	24G	*raffinosus*	Pig	UniSA Collection
140	61E	*faecium*	Pig	UniSA Collection
141	76E	*faecium*	Pig	UniSA Collection
142	83E	*faecium*	Pig	UniSA Collection
143	94E	*faecium*	Pig	UniSA Collection
144	41D	*hirae/durans*	Pig	UniSA Collection
145	255G	*faecium*	Pig	UniSA Collection
146	251G	*faecium*	Pig	UniSA Collection
147	252G	*faecium*	Pig	UniSA Collection
148	111D	*faecium*	Pig	UniSA Collection
149	68D	*faecium*	Pig	UniSA Collection
150	59D	*hirae/durans*	Pig	UniSA Collection
151	122D	*hirae/durans*	Pig	UniSA Collection
152	117D	*hirae/durans*	Pig	UniSA Collection
153	120D	*hirae/durans*	Pig	UniSA Collection
154	85F	*faecium*	Pig	UniSA Collection
155	98FR	*faecium*	Pig	UniSA Collection
156	99FR	*faecium*	Pig	UniSA Collection
157	10FR	*faecalis*	Pig	UniSA Collection
158	2FR	*faecium*	Pig	UniSA Collection
159	6FR	*faecium*	Pig	UniSA Collection
160	20FR	*faecium*	Pig	UniSA Collection
161	11W	*faecalis*	Pig	UniSA Collection

162	12W	*faecalis*	Pig	UniSA Collection
163	18W	*faecalis*	Pig	UniSA Collection
164	13W	*faecalis*	Pig	UniSA Collection
165	218W	*faecalis*	Pig	UniSA Collection
166	30W	*faecalis*	Pig	UniSA Collection
167	23W	*faecium*	Pig	UniSA Collection
168	27W	*faecalis*	Pig	UniSA Collection
169	43F	*faecium*	Pig	UniSA Collection
170	140G	*gallinarum*	Pig	UniSA Collection
171	138G	*casseliflavus*	Pig	UniSA Collection
172	139G	*gallinarum*	Pig	UniSA Collection
173	137G	*gallinarum*	Pig	UniSA Collection
174	127G	*gallinarum*	Pig	UniSA Collection
175	136G	*gallinarum*	Pig	UniSA Collection
176	104G	*casseliflavus*	Pig	UniSA Collection
177	101G	*gallinarum*	Pig	UniSA Collection
178	22D	*gallinarum*	Pig	UniSA Collection
179	63F	*gallinarum*	Pig	UniSA Collection
180	54F	*casseliflavus*	Pig	UniSA Collection
181	61F	*gallinarum*	Pig	UniSA Collection
182	49G	*gallinarum*	Pig	UniSA Collection
183	1F	*casseliflavus*	Pig	UniSA Collection
184	20D	*gallinarum*	Pig	UniSA Collection
185	142G	*gallinarum*	Pig	UniSA Collection
186	170G	*gallinarum*	Pig	UniSA Collection
187	163G	*casseliflavus*	Pig	UniSA Collection
188	169F	*casseliflavus*	Pig	UniSA Collection
189	66D	*gallinarum*	Pig	UniSA Collection
190	63W	*casseliflavus*	Pig	UniSA Collection
191	24FR	*casseliflavus*	Pig	UniSA Collection
192	26FR	*gallinarum*	Pig	UniSA Collection
193	28FR	*casseliflavus*	Pig	UniSA Collection
194	206G	*gallinarum*	Pig	UniSA Collection
195	183G	*gallinarum*	Pig	UniSA Collection
196	188G	*gallinarum*	Pig	UniSA Collection
197	76E	*gallinarum*	Pig	UniSA Collection
198	99438-4	*faecium*	Chicken	UniSA Collection
199	61E	*gallinarum*	Pig	UniSA Collection
200	66W	*casseliflavus*	Pig	UniSA Collection
201	53FR	*gallinarum*	Pig	UniSA Collection
202	290G	*casseliflavus*	Pig	UniSA Collection
203	83D	*casseliflavus*	Pig	UniSA Collection
204	99426-4	*faecium*	Chicken	UniSA Collection
205	99427-4	*faecium*	Chicken	UniSA Collection
206	99426-2	*faecium*	Chicken	UniSA Collection
1C	99364-3	*faecalis*	Chicken	UniSA Collection
2C	99363-1	*faecium*	Chicken	UniSA Collection
3C	99363-4	*faecium*	Chicken	UniSA Collection
4C	3	N/D	Pig	UniSA Collection
5C	4	*hirae*	Pig	UniSA Collection
6C	23	*faecalis*	Pig	UniSA Collection
7C	15 Ery	*faecalis*	Pig	UniSA Collection
8C	29	*faecalis*	Pig	UniSA Collection
9C	*E. casseliflavus*	*casseliflavus*	Human	WCH
10C	*E. faecalis*	*faecalis*	Human	WCH
11C	*E. faecalis*	*faecalis*	Human	WCH

12C	*E. faecalis*	*faecalis*	Human	WCH
13C	*E. faecium*	*faecium*	Human	WCH
14C	*E. faecium*	*faecium*	Human	WCH
15C	*E. faecium*	*faecium*	Human	WCH
16C	*E. gallinarum*	*gallinarum*	Human	WCH
17C	3D Rachel	*hirae/durans*	Pig	UniSA Collection
18C	4D Rachel	*faecium*	Pig	UniSA Collection
19C	13D Rachel	*hirae/durans*	Pig	UniSA Collection
20C	14D Rachel	*faecium*	Pig	UniSA Collection
21C	36G Rachel	*faecalis*	Pig	UniSA Collection
22C	68G Rachel	*faecium*	Pig	UniSA Collection
23C	54F Rachel	*faecalis*	Pig	UniSA Collection
24C	55F Rachel	*hirae/durans*	Pig	UniSA Collection
No.	**Batch**	***Enterococcus***	**Origin**	**Collection**

N/D, not determined

Appendix 2. Media and Buffer Recipes

Tryptone Soy Agar (Oxoid, USA)

Typical formula (g/L)

Pancreatic digest of casein: 15

Enzymatic digest of soya bean: 5

Sodium chloride: 5

Agar: 15

Method

Suspend 40 grams in one litre of RO water. Bring to the boil with shaking to dissolve completely. Sterilize by autoclaving at 121^{o}C for 15 minutes. Store at 18^{o}C.

Brain Heart Infusion Broth (Oxoid, USA)

Typical formula (g/L)

Calf brain infusion solids: 12.5

Beef heart infusion solids: 5

Proteose peptone: 10

Glucose: 2

Sodium chloride: 5

Di-sodium phosphate: 2.5

Method

Add 37 grams to one litre of RO water. Mix well and sterilize by autoclaving at 121^{o}C for 15 minutes. Store at 18^{o}C.

Tryptone Soy Broth (Oxoid, USA)

Typical formula (g/L)

Pancreatic digest of casein: 15

Enzymatic digest of soya bean: 5

Sodium chloride: 5

Di-potassium hydrogen phosphate: 2.5

Glucose: 2.5

Method

Dissolve 30 grams in one litre of RO water. Sterilize by autoclaving at 121^{o}C for 15 minutes. Store at 18^{o}C.

Mueller-Hinton Agar (Oxoid, USA)

Typical formula (g/L)

Beef, dehydrate infusion from: 300

Casein hydrolysate: 17.5

Starch: 1.5

Agar: 17

Method

Suspend 38 grams in one litre of RO water. Bring to the boil with shaking to dissolve completely. Sterilize by autoclaving at 121^{o}C for 15 minutes. Store at 18^{o}C.

Blood Columbia Agar (Oxoid, USA)

Typical formula (g/L)

Special peptone: 23

Starch: 1

Sodium chloride: 5

Agar: 10

Method

Suspend 39 grams in one litre of RO water. Bring to the boil with shaking to dissolve completely. Sterilize by autoclaving at 121^{o}C for 15 minutes. Cool to 50 and add 5% sterile defibrinated blood. Store at 18^{o}C.

Luria-Bertani Agar

Typical formula (g/L)

Bacto-tryptone: 10

Yeast extract: 5

Sodium chloride: 10

Agar: 15

Method

Suspend 35 grams in one litre of RO water (final pH 7.5). Bring to the boil with shaking to dissolve completely. Sterilize by autoclaving at 121^{o}C for 15 minutes. Store at 18^{o}C.

Glycerol Broth

Typical formula (g/L)

Calf brain infusion solids: 12.5

Beef heart infusion solids: 5

Proteose peptone: 10

Glucose: 2

Sodium chloride: 5

Di-sodium phosphate: 2.5

Glycerol: 10%

Method

Add 37 grams to one litre of RO water. Add 10% glycerol. Mix well and sterilize by autoclaving at 121^{o}C for 15 minutes. Store at 18^{o}C.

SM Buffer

Typical formula

NaCl: 5.8 g

$MgSO_4 \bullet 7H_2O$: 2 g

Tris-HCl (1 M, pH 7.5): 50 mL

Gelatin (2%, w/v): 5 mL

Method

Dissolve the substances in one litre of RO water. Melt 5 ml of gelatin (2%, w/v) and add to the solution. Mix well and sterilize by autoclaving at 121^{o}C for 20 minutes. Store at 18^{o}C.

TM Buffer

Typical formula

Tris-HCl (1 M, pH 8.1): 100 mL

$MgCl_2$ (1 M): 100 mL

Method

Mix the solutions and add RO water to make a final volume of one litre. Sterilize by autoclaving at 121^{o}C for 20 minutes. Store at 18^{o}C.

TE Buffer

Typical formula

Tris-HCl (1 M, pH 7.5): 10 mL

EDTA (500 mM, pH 8.0): 2 mL

Method

Mix the solutions and add RO water to make a final volume of one litre. Sterilize by autoclaving at 121^{o}C for 20 minutes. Store at 18^{o}C.

10× TBE Buffer

Typical formula

Tris base: 108 g

Boric acid: 55 g

EDTA (0.5 M, pH 8.0): 40 mL

Method

Add RO water to substances to make a final volume of one litre and mix well. Sterilize by autoclaving at 121^{o}C for 20 minutes. Store at 18^{o}C. Dilute 1:20 with RO water before using.

Appendix 3. Antimicrobial sensitivity test results

	Enterococcus	AMP	AVI	AVO	BAC	FLA	GEN	NAR	TET	TIA	TYL	VAN	VIR	Cu	Zn
1	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
2	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
3	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
4	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
5	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
6	*faecium*	S	S	S	R	R	S	S	R	S	R	S	S	I	S
7	*faecium*	S	S	S	R	R	S	S	R	I	R	S	S	I	S
8	*faecium*	S	S	S	R	R	S	S	R	I	R	S	S	I	S
9	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
10	*faecium*	S	S	S	R	R	S	S	R	I	R	S	S	I	R
11	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
12	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
13	*hirae/durans*	S	S	S	R	R	S	S	R	I	R	S	S	I	S
14	*faecium*	S	S	S	R	S	S	S	R	R	R	S	S	I	S
15	*faecalis*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
16	*hirae/durans*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
17	*faecalis*	S	S	S	R	S	S	S	R	I	R	S	S	I	S
18	*faecium*	S	S	S	R	S	S	S	R	I	R	R	S	I	S
19	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
20	*faecium*	S	S	S	R	R	S	S	R	S	R	S	S	I	S
21	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
22	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
23	*faecium*	S	S	S	R	R	S	S	R	I	R	S	S	I	S
24	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
25	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
26	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
27	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
28	*faecium*	S	S	S	R	R	S	S	R	I	R	S	S	I	S
29	*faecium*	S	S	S	R	R	S	S	R	R	R	S	R	I	S
30	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	S	I	R
31	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	S	I	R
32	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	S	I	R
33	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
34	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
35	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
36	*faecium*	S	S	R	S	S	S	S	R	R	R	R	R	I	S
37	*faecium*	S	S	S	S	R	S	S	R	R	R	S	R	I	S
38	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
39	*faecium*	S	S	S	S	R	S	S	R	I	R	S	R	I	S
40	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
41	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
42	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
43	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
44	*hirae*	S	S	S	S	S	S	S	R	I	R	S	R	I	I
45	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	R	I	I
46	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
47	*faecium*	S	S	R	R	S	S	S	R	I	R	R	R	I	S
48	*faecalis*	S	S	S	R	S	S	S	R	I	R	S	R	I	R
49	*faecium*	S	S	R	R	S	S	S	R	I	R	R	R	I	S
50	*faecium*	S	S	R	R	S	S	S	R	S	R	R	R	I	S
51	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
52	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
53	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
54	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R

55	*casseliflavus*	S	S	S	S	R	S	S	R	R	R	I	R	I	S
56	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	R	I	R
57	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
58	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
59	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
60	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
61	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	R	I	R
62	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
63	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
64	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	R	I	R
65	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
66	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
67	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
68	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
69	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
70	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
71	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
72	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
73	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
74	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
75	*faecium*	S	S	S	S	R	S	S	R	R	R	S	R	I	S
76	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
77	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
78	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
79	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
80	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
81	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
82	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
83	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
84	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
85	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
86	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
87	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
88	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
89	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
90	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	R	I	R
91	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
92	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
93	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	R	I	R
94	*faecium*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
95	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
96	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
97	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
98	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
99	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
100	*faecium*	S	S	S	R	S	S	S	R	I	R	S	S	I	R
101	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
102	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
103	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
104	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
105	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
106	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
107	*hirae/durans*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
108	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
109	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
110	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S

111	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
112	*faecium*	S	S	R	R	S	S	S	R	I	R	R	S	I	S
113	*hirae/durans*	S	S	S	R	R	S	S	R	I	R	S	S	I	S
114	*hirae/durans*	S	S	S	R	R	S	S	R	I	R	S	S	I	S
115	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	I
116	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
117	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
118	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
119	*faecium*	S	S	S	R	R	S	S	R	I	R	S	S	I	S
120	*faecium*	S	S	S	S	R	S	S	R	I	R	S	S	I	S
121	*hirae/durans*	S	S	S	R	S	S	S	R	I	R	S	S	I	S
122	*hirae/durans*	S	S	S	R	S	S	S	R	I	R	S	S	I	S
123	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
124	*faecium*	S	S	R	R	S	S	S	R	R	R	R	S	I	S
125	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
126	*faecium*	S	S	R	R	S	S	S	R	I	R	R	R	I	S
127	*hirae/durans*	S	S	S	R	R	S	S	R	I	R	S	R	I	S
128	*hirae/durans*	S	S	S	R	R	S	S	R	R	R	S	R	I	S
129	*raffinosus*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
130	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
131	*faecium*	S	S	S	R	R	S	S	R	S	R	S	S	I	S
132	*faecium*	S	S	S	R	R	S	S	R	S	R	S	S	I	S
133	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
134	*faecium*	S	S	S	R	R	S	S	R	I	R	S	S	I	S
135	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
136	*faecium*	S	S	S	R	R	S	S	R	I	R	S	S	I	S
137	*faecium*	S	S	S	R	R	S	S	R	R	R	S	R	I	S
138	*faecium*	S	S	S	R	R	S	S	R	R	R	S	R	I	S
139	*raffinosus*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
140	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
141	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
142	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
143	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
144	*hirae/durans*	S	S	S	R	S	S	S	R	R	R	S	R	I	S
145	*faecium*	S	S	S	R	R	S	S	R	S	R	S	S	I	S
146	*faecium*	S	S	S	R	R	S	S	R	S	R	S	S	I	S
147	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
148	*faecium*	S	S	S	R	R	S	S	R	R	R	S	R	I	S
149	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
150	*hirae/durans*	S	S	S	R	R	S	S	R	R	R	S	R	I	S
151	*hirae/durans*	S	S	S	R	R	S	S	R	R	R	S	R	I	S
152	*hirae/durans*	S	S	S	R	R	S	S	R	R	R	S	R	I	S
153	*hirae/durans*	S	S	S	R	R	S	S	R	R	R	S	R	I	S
154	*faecium*	S	S	S	R	R	S	S	R	R	R	S	R	I	S
155	*faecium*	S	S	S	R	R	S	S	R	R	R	S	R	I	S
156	*faecium*	S	S	S	R	R	S	S	R	R	R	S	R	I	S
157	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
158	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
159	*faecium*	S	S	S	R	R	S	S	R	S	R	S	S	I	S
160	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
161	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
162	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
163	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
164	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
165	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
166	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	R

167	*faecium*	S	S	S	R	R	S	S	R	I	R	S	S	I	S
168	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
169	*faecium*	S	S	S	R	R	S	S	R	R	R	S	S	I	S
170	*gallinarum*	S	S	S	R	S	S	S	R	R	R	R	S	I	S
171	*casseliflavus*	S	S	S	R	S	S	S	R	R	R	R	S	I	S
172	*gallinarum*	S	S	S	R	S	S	S	R	R	R	R	S	I	S
173	*gallinarum*	S	S	S	R	S	S	S	R	R	R	R	S	I	S
174	*gallinarum*	S	S	S	R	R	S	S	R	I	R	R	S	I	S
175	*gallinarum*	S	S	S	R	S	S	S	R	R	R	R	S	I	S
176	*casseliflavus*	S	S	S	R	S	S	S	R	I	R	R	S	I	S
177	*gallinarum*	S	S	S	R	R	S	S	R	R	R	R	S	I	S
178	*gallinarum*	S	S	S	R	R	S	S	R	R	R	R	S	I	S
179	*gallinarum*	S	S	S	R	R	S	S	R	R	R	R	S	I	S
180	*casseliflavus*	S	S	S	R	R	S	S	R	R	R	R	S	I	S
181	*gallinarum*	S	S	S	R	R	S	S	R	R	R	R	S	I	S
182	*gallinarum*	S	S	S	R	S	S	S	R	R	R	R	S	I	S
183	*casseliflavus*	S	S	S	R	R	S	S	R	I	R	R	S	I	S
184	*gallinarum*	S	S	S	R	R	S	S	R	R	R	R	S	I	S
185	*gallinarum*	S	S	S	R	S	S	S	R	R	R	R	S	I	S
186	*gallinarum*	S	S	S	R	R	S	S	R	R	R	R	S	I	S
187	*casseliflavus*	S	S	S	R	R	S	S	R	I	R	R	S	I	S
188	*casseliflavus*	S	S	S	R	S	S	S	R	I	R	R	S	I	S
189	*gallinarum*	S	S	S	R	R	S	S	R	R	R	R	S	I	S
190	*casseliflavus*	S	S	S	R	R	S	S	R	I	R	R	S	I	S
191	*casseliflavus*	S	S	S	R	R	S	S	R	I	R	R	S	I	S
192	*gallinarum*	S	S	S	R	R	S	S	R	R	R	R	S	I	S
193	*casseliflavus*	S	S	S	R	R	S	S	R	I	R	R	S	I	S
194	*gallinarum*	S	S	S	R	S	S	S	R	R	R	R	S	I	S
195	*gallinarum*	S	S	S	R	R	S	S	R	R	R	R	S	I	S
196	*gallinarum*	S	S	S	R	R	S	S	R	R	R	R	S	I	S
197	*gallinarum*	S	S	S	R	R	S	S	R	R	R	R	S	I	S
198	*faecium*	S	S	R	R	S	S	S	R	R	R	R	S	I	S
199	*gallinarum*	S	S	S	R	R	S	S	R	R	R	R	S	I	S
200	*casseliflavus*	S	S	S	R	S	S	S	R	I	R	R	S	I	S
201	*gallinarum*	S	S	S	R	R	S	S	R	R	R	R	S	I	S
202	*casseliflavus*	S	S	S	R	R	S	S	R	I	R	R	S	I	S
203	*casseliflavus*	S	S	S	R	R	S	S	R	R	R	R	S	I	S
204	*faecium*	S	S	R	R	R	S	S	R	R	R	R	S	I	S
205	*faecium*	S	S	R	R	S	S	S	R	I	R	R	S	I	S
206	*faecium*	S	S	R	R	R	S	S	R	R	R	R	S	I	S
1C	*faecalis*	S	S	S	R	S	S	S	R	I	R	S	S	I	R
2C	*faecium*	S	S	S	S	S	S	S	R	R	R	S	R	I	S
3C	*faecium*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
4C	N/D	S	S	S	S	R	S	S	R	I	R	S	S	I	S
5C	*hirae*	S	S	S	S	R	S	S	S	I	S	S	S	I	S
6C	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	S	I	R
7C	*faecalis*	S	S	S	R	S	S	S	R	R	R	S	S	I	R
8C	*faecalis*	S	S	S	S	R	S	S	R	R	R	S	S	I	S
9C	*casseliflavus*	S	S	S	S	R	S	S	R	S	R	S	S	I	S
10C	*faecalis*	S	S	S	S	S	S	S	R	S	R	S	S	I	R
11C	*faecalis*	S	S	R	R	S	S	S	R	R	R	S	R	I	R
12C	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	S	I	R
13C	*faecium*	S	S	S	S	R	S	S	R	S	R	S	S	I	S
14C	*faecium*	S	S	S	S	R	R	S	R	S	R	S	S	I	S
15C	*faecium*	S	S	R	S	R	S	S	R	S	R	S	S	I	S
16C	*gallinarum*	S	S	S	S	R	S	S	R	R	R	S	S	S	S

17C	*hirae/durans*	S	S	S	S	R	S	S	R	R	R	S	S	I	S
18C	*faecium*	S	S	S	S	R	S	S	R	R	R	S	S	I	S
19C	*hirae/durans*	S	S	S	S	R	S	S	R	I	R	S	S	I	S
20C	*faecium*	S	S	S	S	R	S	S	R	R	R	S	S	I	S
21C	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	S	I	R
22C	*faecium*	S	S	S	S	R	S	S	R	R	R	S	S	I	S
23C	*faecalis*	S	S	S	S	S	S	S	R	R	R	S	S	I	I
24C	*hirae/durans*	S	S	S	S	S	S	S	R	I	R	S	S	I	S
	Enterococcus	AMP	AVI	AVO	BAC	FLA	GEN	NAR	TET	TIA	TYL	VAN	VIR	Cu	Zn

R, resistant; S, sensitive; I, intermediate; N/D, not determined; AMP, ampicillin; AVI, avilamycin; AVO, avoparcin; BAC, bacitracin; FLA, flavophospholipol; GEN, gentamicin; NAR, narasin; TET, tetracycline; TIA, tiamulin; TYL, tylosin; VAN, vancomycin; VIR, virginiamycin; Cu, copper; Zn, zinc

Appendix 4. PCR results for antimicrobial resistance gene assessment

No.	*Enterococcus*	*aac6-aph2*	*tetK*	*tetL*	*tetM*	*tetO*	*tetS*	*rplC*	*rplD*	*ermB*	*vanA*	*vanB*	*vanC1*	*vanC2*	*lsaA*	*lsaB*	*vatA*	*vatD*	*vatE*	*tcrB*
1	*faecalis*	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
2	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
3	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
4	*faecalis*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
5	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
6	*faecium*	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
7	*faecium*	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
8	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
9	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
10	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
11	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
12	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
13	*hirae/durans*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
14	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
15	*faecalis*	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	*hirae/durans*	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	*faecalis*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
18	*faecium*	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	+
19	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
20	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
21	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
22	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
23	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
24	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
25	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
26	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
27	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
28	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
29	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
30	*faecalis*	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
31	*faecalis*	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
32	*faecalis*	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
33	*faecalis*	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
34	*faecalis*	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
35	*faecalis*	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
36	*faecium*	-	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	+	+
37	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
38	*faecalis*	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
39	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
40	*faecalis*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
41	*faecalis*	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
42	*faecalis*	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
43	*faecalis*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
44	*hirae*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
45	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
46	*faecalis*	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
47	*faecium*	-	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+
48	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
49	*faecium*	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+
50	*faecium*	-	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-
51	*faecalis*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
52	*faecalis*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
53	*faecalis*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
54	*faecalis*	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
55	*casseliflavus*	-	-	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	+

56	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
57	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
58	*faecalis*	+	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
59	*faecalis*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
60	*faecalis*	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
61	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
62	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
63	*faecalis*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
64	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
65	*faecalis*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
66	*faecalis*	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
67	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
68	*faecalis*	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
69	*faecalis*	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
70	*faecalis*	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
71	*faecalis*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
72	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
73	*faecalis*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
74	*faecalis*	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
75	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
76	*faecalis*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
77	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
78	*faecalis*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
79	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
80	*faecalis*	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
81	*faecalis*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
82	*faecalis*	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
83	*faecalis*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
84	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
85	*faecalis*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
86	*faecalis*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
87	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
88	*faecalis*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	+	+
89	*faecalis*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
90	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
91	*faecalis*	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
92	*faecalis*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
93	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
94	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
95	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
96	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
97	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
98	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
99	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
100	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
101	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
102	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
103	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
104	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
105	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
106	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
107	*hirae/durans*	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
108	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
109	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
110	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
111	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
112	*faecium*	-	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
113	*hirae/durans*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
114	*hirae/durans*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
115	*faecalis*	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
116	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
117	*faecium*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

118	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
119	*faecium*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
120	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
121	*hirae/durans*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
122	*hirae/durans*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
123	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
124	*faecium*	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	+
125	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
126	*faecium*	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	+
127	*hirae/durans*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
128	*hirae/durans*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
129	*raffinosus*	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
130	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
131	*faecium*	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
132	*faecium*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
133	*faecalis*	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
134	*faecium*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
135	*faecalis*	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
136	*faecium*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
137	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+
138	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
139	*raffinosus*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
140	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
141	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
142	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
143	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
144	*hirae/durans*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
145	*faecium*	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
146	*faecium*	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
147	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
148	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+
149	*faecium*	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
150	*hirae/durans*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
151	*hirae/durans*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
152	*hirae/durans*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
153	*hirae/durans*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
154	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
155	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
156	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
157	*faecalis*	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
158	*faecium*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
159	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
160	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
161	*faecalis*	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
162	*faecalis*	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
163	*faecalis*	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
164	*faecalis*	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
165	*faecalis*	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
166	*faecalis*	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
167	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
168	*faecalis*	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
169	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
170	*gallinarum*	-	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	+
171	*casseliflavus*	-	-	-	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	+
172	*gallinarum*	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	+
173	*gallinarum*	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	+
174	*gallinarum*	-	-	-	+	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+
175	*gallinarum*	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	+
176	*casseliflavus*	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	+
177	*gallinarum*	-	-	+	+	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+
178	*gallinarum*	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
179	*gallinarum*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-

180	*casseliflavus*	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
181	*gallinarum*	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
182	*gallinarum*	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	+
183	*casseliflavus*	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+
184	*gallinarum*	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-
185	*gallinarum*	-	+	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	+
186	*gallinarum*	-	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	+
187	*casseliflavus*	-	-	+	-	-	+	-	-	+	-	-	-	+	-	-	-	-	-	+
188	*casseliflavus*	-	-	-	+	-	+	-	-	+	-	-	-	+	-	-	-	-	-	+
189	*gallinarum*	-	-	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-
190	*casseliflavus*	-	-	-	-	-	+	-	-	+	-	-	-	+	-	-	-	-	-	+
191	*casseliflavus*	-	-	-	-	-	+	-	-	+	-	-	-	+	-	-	-	-	-	+
192	*gallinarum*	-	-	+	+	-	+	-	-	+	-	-	+	-	-	-	-	-	-	+
193	*casseliflavus*	-	-	-	-	-	+	-	-	+	-	-	-	+	-	-	-	-	-	+
194	*gallinarum*	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-
195	*gallinarum*	-	-	+	+	-	+	-	-	+	-	-	+	-	-	-	-	-	-	+
196	*gallinarum*	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	+
197	*gallinarum*	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	+
198	*faecium*	-	-	-	-	-	+	-	-	+	-	-	-	+	-	-	-	-	-	+
199	*gallinarum*	-	-	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-
200	*casseliflavus*	-	-	-	-	-	+	-	-	+	-	-	-	+	-	-	-	-	-	+
201	*gallinarum*	-	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	+
202	*casseliflavus*	-	-	-	+	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+
203	*casseliflavus*	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+
204	*faecium*	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-
205	*faecium*	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-
206	*faecium*	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-
1C	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-
2C	*faecium*	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
3C	*faecium*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
4C	N/D	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
5C	*hirae*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6C	*faecalis*	+	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
7C	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
8C	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	+
9C	*casseliflavus*	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10C	*faecalis*	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
11C	*faecalis*	-	-	+	+	-	+	-	-	+	+	-	-	-	-	+	-	+	-	-
12C	*faecalis*	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
13C	*faecium*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14C	*faecium*	+	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
15C	*faecium*	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16C	*gallinarum*	-	-	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-
17C	*hirae/durans*	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18C	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
19C	*hirae/durans*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
20C	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
21C	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+
22C	*faecium*	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
23C	*faecalis*	-	-	+	+	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-
24C	*hirae/durans*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

No.	***Enterococcus***	***aac6-aph2***	***tet*K**	***tet*L**	***tet*M**	***tet*O**	***tet*S**	***rpl*C**	***rpl*D**	***erm*B**	***van*A**	***van*B**	***van*C1**	***van*C2**	***lsa*A**	***lsa*B**	***vat*A**	***vat*D**	***vat*E**	***tcr*B**

N/D, not determined

Appendix 5. Antimicrobial sensitivity test result for ampicillin (μg/mL)

No.	*Enteroccocus*	1	2	4	8	16	MIC	S/R
1	*faecalis*				-	-	≤ 8	S
2	*faecalis*				-	-	≤ 8	S
3	*faecalis*				-	-	≤ 8	S
4	*faecalis*				-	-	≤ 8	S
5	*faecium*				-	-	≤ 8	S
6	*faecium*				-	-	≤ 8	S
7	*faecium*				-	-	≤ 8	S
8	*faecium*				-	-	≤ 8	S
9	*faecium*				-	-	≤ 8	S
10	*faecium*				-	-	≤ 8	S
11	*faecium*				-	-	≤ 8	S
12	*faecium*				-	-	≤ 8	S
13	*hirae/durans*				-	-	≤ 8	S
14	*faecium*				-	-	≤ 8	S
15	*faecalis*				-	-	≤ 8	S
16	*hirae/durans*				-	-	≤ 8	S
17	*faecalis*				-	-	≤ 8	S
18	*faecium*				-	-	≤ 8	S
19	*faecium*				-	-	≤ 8	S
20	*faecium*				-	-	≤ 8	S
21	*faecium*				-	-	≤ 8	S
22	*faecium*				-	-	≤ 8	S
23	*faecium*				-	-	≤ 8	S
24	*faecium*				-	-	≤ 8	S
25	*faecium*				-	-	≤ 8	S
26	*faecium*				-	-	≤ 8	S
27	*faecium*				-	-	≤ 8	S
28	*faecium*				-	-	≤ 8	S
29	*faecium*				-	-	≤ 8	S
30	*faecalis*			-	-	-	≤ 4	S
31	*faecalis*			-	-	-	≤ 4	S
32	*faecalis*			-	-	-	≤ 4	S
33	*faecalis*			-	-	-	≤ 4	S
34	*faecalis*			-	-	-	≤ 4	S
35	*faecalis*			-	-	-	≤ 4	S
36	*faecium*			+	+	-	16	S
37	*faecium*			-	-	-	≤ 4	S
38	*faecalis*			-	-	-	≤ 4	S
39	*faecium*			-	-	-	≤ 4	S
40	*faecalis*			-	-	-	≤ 4	S
41	*faecalis*			-	-	-	≤ 4	S
42	*faecalis*			-	-	-	≤ 4	S
43	*faecalis*			-	-	-	≤ 4	S

44	*hirae*	-	-	-	≤ 4	S
45	*faecalis*	-	-	-	≤ 4	S
46	*faecalis*	-	-	-	≤ 4	S
47	*faecium*		-	-	≤ 8	S
48	*faecalis*	-	-	-	≤ 4	S
49	*faecium*		-	-	≤ 8	S
50	*faecium*		-	-	≤ 8	S
51	*faecalis*	-	-	-	≤ 4	S
52	*faecalis*	-	-	-	≤ 4	S
53	*faecalis*	-	-	-	≤ 4	S
54	*faecalis*	-	-	-	≤ 4	S
55	*casseliflavus*	-	-	-	≤ 4	S
56	*faecalis*	-	-	-	≤ 4	S
57	*faecalis*	-	-	-	≤ 4	S
58	*faecalis*	-	-	-	≤ 4	S
59	*faecalis*	-	-	-	≤ 4	S
60	*faecalis*	-	-	-	≤ 4	S
61	*faecalis*	-	-	-	≤ 4	S
62	*faecalis*	-	-	-	≤ 4	S
63	*faecalis*	-	-	-	≤ 4	S
64	*faecalis*	-	-	-	≤ 4	S
65	*faecalis*	-	-	-	≤ 4	S
66	*faecalis*	-	-	-	≤ 4	S
67	*faecalis*	-	-	-	≤ 4	S
68	*faecalis*	-	-	-	≤ 4	S
69	*faecalis*	-	-	-	≤ 4	S
70	*faecalis*	-	-	-	≤ 4	S
71	*faecalis*	-	-	-	≤ 4	S
72	*faecalis*	-	-	-	≤ 4	S
73	*faecalis*	-	-	-	≤ 4	S
74	*faecalis*	-	-	-	≤ 4	S
75	*faecium*	-	-	-	≤ 4	S
76	*faecalis*	-	-	-	≤ 4	S
77	*faecalis*	-	-	-	≤ 4	S
78	*faecalis*	-	-	-	≤ 4	S
79	*faecalis*	-	-	-	≤ 4	S
80	*faecalis*	-	-	-	≤ 4	S
81	*faecalis*	-	-	-	≤ 4	S
82	*faecalis*	-	-	-	≤ 4	S
83	*faecalis*	-	-	-	≤ 4	S
84	*faecalis*	-	-	-	≤ 4	S
85	*faecalis*	-	-	-	≤ 4	S
86	*faecalis*	-	-	-	≤ 4	S
87	*faecalis*	-	-	-	≤ 4	S
88	*faecalis*	-	-	-	≤ 4	S

89	*faecalis*	-	-	-	≤ 4	S
90	*faecalis*	-	-	-	≤ 4	S
91	*faecalis*	-	-	-	≤ 4	S
92	*faecalis*	-	-	-	≤ 4	S
93	*faecalis*	-	-	-	≤ 4	S
94	*faecium*		-	-	≤ 8	S
95	*faecalis*		-	-	≤ 8	S
96	*faecium*		-	-	≤ 8	S
97	*faecium*		-	-	≤ 8	S
98	*faecium*		-	-	≤ 8	S
99	*faecium*		-	-	≤ 8	S
100	*faecium*		-	-	≤ 8	S
101	*faecalis*		-	-	≤ 8	S
102	*faecalis*		-	-	≤ 8	S
103	*faecium*		-	-	≤ 8	S
104	*faecium*		-	-	≤ 8	S
105	*faecium*		-	-	≤ 8	S
106	*faecium*		-	-	≤ 8	S
107	*hirae/durans*		-	-	≤ 8	S
108	*faecium*		-	-	≤ 8	S
109	*faecium*		-	-	≤ 8	S
110	*faecium*		-	-	≤ 8	S
111	*faecium*		-	-	≤ 8	S
112	*faecium*		-	-	≤ 8	S
113	*hirae/durans*		-	-	≤ 8	S
114	*hirae/durans*		-	-	≤ 8	S
115	*faecalis*		-	-	≤ 8	S
116	*faecium*		-	-	≤ 8	S
117	*faecium*		-	-	≤ 8	S
118	*faecium*		-	-	≤ 8	S
119	*faecium*		-	-	≤ 8	S
120	*faecium*		-	-	≤ 8	S
121	*hirae/durans*		-	-	≤ 8	S
122	*hirae/durans*		-	-	≤ 8	S
123	*faecium*		-	-	≤ 8	S
124	*faecium*		-	-	≤ 8	S
125	*faecium*		-	-	≤ 8	S
126	*faecium*		-	-	≤ 8	S
127	*hirae/durans*		-	-	≤ 8	S
128	*hirae/durans*		-	-	≤ 8	S
129	*raffinosus*		-	-	≤ 8	S
130	*faecium*		-	-	≤ 8	S
131	*faecium*		-	-	≤ 8	S
132	*faecium*		-	-	≤ 8	S
133	*faecalis*		-	-	≤ 8	S

134	*faecium*				-	-	≤ 8	S
135	*faecalis*				-	-	≤ 8	S
136	*faecium*				-	-	≤ 8	S
137	*faecium*				-	-	≤ 8	S
138	*faecium*				-	-	≤ 8	S
139	*raffinosus*				-	-	≤ 8	S
140	*faecium*				-	-	≤ 8	S
141	*faecium*				-	-	≤ 8	S
142	*faecium*				-	-	≤ 8	S
143	*faecium*				-	-	≤ 8	S
144	*hirae/durans*				-	-	≤ 8	S
145	*faecium*				-	-	≤ 8	S
146	*faecium*				-	-	≤ 8	S
147	*faecium*				-	-	≤ 8	S
148	*faecium*				-	-	≤ 8	S
149	*faecium*				-	-	≤ 8	S
150	*hirae/durans*				-	-	≤ 8	S
151	*hirae/durans*				-	-	≤ 8	S
152	*hirae/durans*				-	-	≤ 8	S
153	*hirae/durans*				-	-	≤ 8	S
154	*faecium*				-	-	≤ 8	S
155	*faecium*				-	-	≤ 8	S
156	*faecium*				-	-	≤ 8	S
157	*faecalis*				-	-	≤ 8	S
158	*faecium*				-	-	≤ 8	S
159	*faecium*				-	-	≤ 8	S
160	*faecium*				-	-	≤ 8	S
161	*faecalis*				-	-	≤ 8	S
162	*faecalis*				-	-	≤ 8	S
163	*faecalis*				-	-	≤ 8	S
164	*faecalis*				-	-	≤ 8	S
165	*faecalis*				-	-	≤ 8	S
166	*faecalis*				-	-	≤ 8	S
167	*faecium*				-	-	≤ 8	S
168	*faecalis*				-	-	≤ 8	S
169	*faecium*				-	-	≤ 8	S
170	*gallinarum*	+	-	-	-	-	2	S
171	*casseliflavus*	+	-	-	-	-	2	S
172	*gallinarum*	+	-	-	-	-	2	S
173	*gallinarum*	-	-	-	-	-	≤ 1	S
174	*gallinarum*	-	-	-	-	-	≤ 1	S
175	*gallinarum*	-	-	-	-	-	≤ 1	S
176	*casseliflavus*	-	-	-	-	-	≤ 1	S
177	*gallinarum*	-	-	-	-	-	≤ 1	S
178	*gallinarum*	+	-	-	-	-	2	S

179	*gallinarum*	-	-	-	-	-	≤ 1	S
180	*casseliflavus*	-	-	-	-	-	≤ 1	S
181	*gallinarum*	-	-	-	-	-	≤ 1	S
182	*gallinarum*	-	-	-	-	-	≤ 1	S
183	*casseliflavus*	-	-	-	-	-	≤ 1	S
184	*gallinarum*	-	-	-	-	-	≤ 1	S
185	*gallinarum*	-	-	-	-	-	≤ 1	S
186	*gallinarum*	+	-	-	-	-	2	S
187	*casseliflavus*	-	-	-	-	-	≤ 1	S
188	*casseliflavus*	-	-	-	-	-	≤ 1	S
189	*gallinarum*	-	-	-	-	-	≤ 1	S
190	*casseliflavus*	-	-	-	-	-	≤ 1	S
191	*casseliflavus*	-	-	-	-	-	≤ 1	S
192	*gallinarum*	+	+	-	-	-	4	S
193	*casseliflavus*	+	+	+	-	-	8	S
194	*gallinarum*	-	-	-	-	-	≤ 1	S
195	*gallinarum*	-	-	-	-	-	≤ 1	S
196	*gallinarum*	+	-	-	-	-	2	S
197	*gallinarum*	+	-	-	-	-	2	S
198	*faecium*				-	-	≤ 8	S
199	*gallinarum*		-	-	-	-	≤ 2	S
200	*casseliflavus*	-	-	-	-	-	≤ 1	S
201	*gallinarum*	-	-	-	-	-	≤ 1	S
202	*casseliflavus*	-	-	-	-	-	≤ 1	S
203	*casseliflavus*	-	-	-	-	-	≤ 1	S
204	*faecium*				-	-	≤ 8	S
205	*faecium*				+	-	16	S
206	*faecium*				-	-	≤ 8	S
1C	*faecalis*				-	-	≤ 8	S
2C	*faecium*				-	-	≤ 8	S
3C	*faecium*				-	-	≤ 8	S
4C	N/D				-	-	≤ 8	S
5C	*hirae*				-	-	≤ 8	S
6C	*faecalis*				-	-	≤ 8	S
7C	*faecalis*				-	-	≤ 8	S
8C	*faecalis*				-	-	≤ 8	S
9C	*casseliflavus*				-	-	≤ 8	S
10C	*faecalis*				-	-	≤ 8	S
11C	*faecalis*				-	-	≤ 8	S
12C	*faecalis*				-	-	≤ 8	S
13C	*faecium*				-	-	≤ 8	S
14C	*faecium*				-	-	≤ 8	S
15C	*faecium*				-	-	≤ 8	S
16C	*gallinarum*				-	-	≤ 8	S
17C	*hirae/durans*				-	-	≤ 8	S

18C	*faecium*				-	-	≤ 8	S
19C	*hirae/durans*				-	-	≤ 8	S
20C	*faecium*				-	-	≤ 8	S
21C	*faecalis*				-	-	≤ 8	S
22C	*faecium*				-	-	≤ 8	S
23C	*faecalis*				-	-	≤ 8	S
24C	*hirae/durans*				-	-	≤ 8	S
No.	***Enteroccocus***	**1**	**2**	**4**	**8**	**16**	**MIC**	**S/R**

N/D, not determined

Appendix 6. Antimicrobial sensitivity test result for avilamycin (μg/mL)

No.	*Enteroccocus*	8	16	MIC	S/R
1	*faecalis*	-	-	≤ 8	S
2	*faecalis*	-	-	≤ 8	S
3	*faecalis*	-	-	≤ 8	S
4	*faecalis*	-	-	≤ 8	S
5	*faecium*	-	-	≤ 8	S
6	*faecium*	-	-	≤ 8	S
7	*faecium*	-	-	≤ 8	S
8	*faecium*	-	-	≤ 8	S
9	*faecium*	-	-	≤ 8	S
10	*faecium*	-	-	≤ 8	S
11	*faecium*	-	-	≤ 8	S
12	*faecium*	-	-	≤ 8	S
13	*hirae/durans*	-	-	≤ 8	S
14	*faecium*	-	-	≤ 8	S
15	*faecalis*	-	-	≤ 8	S
16	*hirae/durans*	-	-	≤ 8	S
17	*faecalis*	-	-	≤ 8	S
18	*faecium*	-	-	≤ 8	S
19	*faecium*	-	-	≤ 8	S
20	*faecium*	-	-	≤ 8	S
21	*faecium*	-	-	≤ 8	S
22	*faecium*	-	-	≤ 8	S
23	*faecium*	-	-	≤ 8	S
24	*faecium*	-	-	≤ 8	S
25	*faecium*	-	-	≤ 8	S
26	*faecium*	-	-	≤ 8	S
27	*faecium*	-	-	≤ 8	S
28	*faecium*	-	-	≤ 8	S
29	*faecium*	-	-	≤ 8	S
30	*faecalis*	-	-	≤ 8	S
31	*faecalis*	-	-	≤ 8	S
32	*faecalis*	-	-	≤ 8	S
33	*faecalis*	-	-	≤ 8	S
34	*faecalis*	-	-	≤ 8	S
35	*faecalis*	-	-	≤ 8	S
36	*faecium*	-	-	≤ 8	S
37	*faecium*	-	-	≤ 8	S
38	*faecalis*	-	-	≤ 8	S
39	*faecium*	-	-	≤ 8	S
40	*faecalis*	-	-	≤ 8	S
41	*faecalis*	-	-	≤ 8	S
42	*faecalis*	-	-	≤ 8	S
43	*faecalis*	-	-	≤ 8	S

44	*hirae*	-	-	≤ 8	S
45	*faecalis*	-	-	≤ 8	S
46	*faecalis*	-	-	≤ 8	S
47	*faecium*	-	-	≤ 8	S
48	*faecalis*	-	-	≤ 8	S
49	*faecium*	-	-	≤ 8	S
50	*faecium*	-	-	≤ 8	S
51	*faecalis*	-	-	≤ 8	S
52	*faecalis*	-	-	≤ 8	S
53	*faecalis*	-	-	≤ 8	S
54	*faecalis*	-	-	≤ 8	S
55	*casseliflavus*	-	-	≤ 8	S
56	*faecalis*	-	-	≤ 8	S
57	*faecalis*	-	-	≤ 8	S
58	*faecalis*	-	-	≤ 8	S
59	*faecalis*	-	-	≤ 8	S
60	*faecalis*	-	-	≤ 8	S
61	*faecalis*	-	-	≤ 8	S
62	*faecalis*	-	-	≤ 8	S
63	*faecalis*	-	-	≤ 8	S
64	*faecalis*	-	-	≤ 8	S
65	*faecalis*	-	-	≤ 8	S
66	*faecalis*	-	-	≤ 8	S
67	*faecalis*	-	-	≤ 8	S
68	*faecalis*	-	-	≤ 8	S
69	*faecalis*	-	-	≤ 8	S
70	*faecalis*	-	-	≤ 8	S
71	*faecalis*	-	-	≤ 8	S
72	*faecalis*	-	-	≤ 8	S
73	*faecalis*	-	-	≤ 8	S
74	*faecalis*	-	-	≤ 8	S
75	*faecium*	-	-	≤ 8	S
76	*faecalis*	-	-	≤ 8	S
77	*faecalis*	-	-	≤ 8	S
78	*faecalis*	-	-	≤ 8	S
79	*faecalis*	-	-	≤ 8	S
80	*faecalis*	-	-	≤ 8	S
81	*faecalis*	-	-	≤ 8	S
82	*faecalis*	-	-	≤ 8	S
83	*faecalis*	-	-	≤ 8	S
84	*faecalis*	-	-	≤ 8	S
85	*faecalis*	-	-	≤ 8	S
86	*faecalis*	-	-	≤ 8	S
87	*faecalis*	-	-	≤ 8	S
88	*faecalis*	-	-	≤ 8	S

89	*faecalis*	-	-	≤ 8	S
90	*faecalis*	-	-	≤ 8	S
91	*faecalis*	-	-	≤ 8	S
92	*faecalis*	-	-	≤ 8	S
93	*faecalis*	-	-	≤ 8	S
94	*faecium*	-	-	≤ 8	S
95	*faecalis*	-	-	≤ 8	S
96	*faecium*	-	-	≤ 8	S
97	*faecium*	-	-	≤ 8	S
98	*faecium*	-	-	≤ 8	S
99	*faecium*	-	-	≤ 8	S
100	*faecium*	-	-	≤ 8	S
101	*faecalis*	-	-	≤ 8	S
102	*faecalis*	-	-	≤ 8	S
103	*faecium*	-	-	≤ 8	S
104	*faecium*	-	-	≤ 8	S
105	*faecium*	-	-	≤ 8	S
106	*faecium*	-	-	≤ 8	S
107	*hirae/durans*	-	-	≤ 8	S
108	*faecium*	-	-	≤ 8	S
109	*faecium*	-	-	≤ 8	S
110	*faecium*	-	-	≤ 8	S
111	*faecium*	-	-	≤ 8	S
112	*faecium*	-	-	≤ 8	S
113	*hirae/durans*	-	-	≤ 8	S
114	*hirae/durans*	-	-	≤ 8	S
115	*faecalis*	-	-	≤ 8	S
116	*faecium*	-	-	≤ 8	S
117	*faecium*	-	-	≤ 8	S
118	*faecium*	-	-	≤ 8	S
119	*faecium*	-	-	≤ 8	S
120	*faecium*	-	-	≤ 8	S
121	*hirae/durans*	-	-	≤ 8	S
122	*hirae/durans*	-	-	≤ 8	S
123	*faecium*	-	-	≤ 8	S
124	*faecium*	-	-	≤ 8	S
125	*faecium*	-	-	≤ 8	S
126	*faecium*	-	-	≤ 8	S
127	*hirae/durans*	-	-	≤ 8	S
128	*hirae/durans*	-	-	≤ 8	S
129	*raffinosus*	-	-	≤ 8	S
130	*faecium*	-	-	≤ 8	S
131	*faecium*	-	-	≤ 8	S
132	*faecium*	-	-	≤ 8	S
133	*faecalis*	-	-	≤ 8	S

134	*faecium*	-	-	≤ 8	S
135	*faecalis*	-	-	≤ 8	S
136	*faecium*	-	-	≤ 8	S
137	*faecium*	-	-	≤ 8	S
138	*faecium*	-	-	≤ 8	S
139	*raffinosus*	-	-	≤ 8	S
140	*faecium*	-	-	≤ 8	S
141	*faecium*	-	-	≤ 8	S
142	*faecium*	-	-	≤ 8	S
143	*faecium*	-	-	≤ 8	S
144	*hirae/durans*	-	-	≤ 8	S
145	*faecium*	-	-	≤ 8	S
146	*faecium*	-	-	≤ 8	S
147	*faecium*	-	-	≤ 8	S
148	*faecium*	-	-	≤ 8	S
149	*faecium*	-	-	≤ 8	S
150	*hirae/durans*	-	-	≤ 8	S
151	*hirae/durans*	-	-	≤ 8	S
152	*hirae/durans*	-	-	≤ 8	S
153	*hirae/durans*	-	-	≤ 8	S
154	*faecium*	-	-	≤ 8	S
155	*faecium*	-	-	≤ 8	S
156	*faecium*	-	-	≤ 8	S
157	*faecalis*	-	-	≤ 8	S
158	*faecium*	-	-	≤ 8	S
159	*faecium*	-	-	≤ 8	S
160	*faecium*	-	-	≤ 8	S
161	*faecalis*	-	-	≤ 8	S
162	*faecalis*	-	-	≤ 8	S
163	*faecalis*	-	-	≤ 8	S
164	*faecalis*	-	-	≤ 8	S
165	*faecalis*	-	-	≤ 8	S
166	*faecalis*	-	-	≤ 8	S
167	*faecium*	-	-	≤ 8	S
168	*faecalis*	-	-	≤ 8	S
169	*faecium*	-	-	≤ 8	S
170	*gallinarum*	-	-	≤ 8	S
171	*casseliflavus*	-	-	≤ 8	S
172	*gallinarum*	-	-	≤ 8	S
173	*gallinarum*	-	-	≤ 8	S
174	*gallinarum*	-	-	≤ 8	S
175	*gallinarum*	-	-	≤ 8	S
176	*casseliflavus*	-	-	≤ 8	S
177	*gallinarum*	-	-	≤ 8	S
178	*gallinarum*	-	-	≤ 8	S

179	*gallinarum*	-	-	≤ 8	S
180	*casseliflavus*	-	-	≤ 8	S
181	*gallinarum*	-	-	≤ 8	S
182	*gallinarum*	-	-	≤ 8	S
183	*casseliflavus*	-	-	≤ 8	S
184	*gallinarum*	-	-	≤ 8	S
185	*gallinarum*	-	-	≤ 8	S
186	*gallinarum*	-	-	≤ 8	S
187	*casseliflavus*	-	-	≤ 8	S
188	*casseliflavus*	-	-	≤ 8	S
189	*gallinarum*	-	-	≤ 8	S
190	*casseliflavus*	-	-	≤ 8	S
191	*casseliflavus*	-	-	≤ 8	S
192	*gallinarum*	-	-	≤ 8	S
193	*casseliflavus*	-	-	≤ 8	S
194	*gallinarum*	-	-	≤ 8	S
195	*gallinarum*	+	-	16	S
196	*gallinarum*	+	-	16	S
197	*gallinarum*	+	-	16	S
198	*faecium*	+	-	16	S
199	*gallinarum*	-	-	≤ 8	S
200	*casseliflavus*	-	-	≤ 8	S
201	*gallinarum*	-	-	≤ 8	S
202	*casseliflavus*	-	-	≤ 8	S
203	*casseliflavus*	-	-	≤ 8	S
204	*faecium*	+	-	16	S
205	*faecium*	+	-	16	S
206	*faecium*	+	-	16	S
1C	*faecalis*	-	-	≤ 8	S
2C	*faecium*	-	-	≤ 8	S
3C	*faecium*	-	-	≤ 8	S
4C	N/D	-	-	≤ 8	S
5C	*hirae*	-	-	≤ 8	S
6C	*faecalis*	-	-	≤ 8	S
7C	*faecalis*	-	-	≤ 8	S
8C	*faecalis*	-	-	≤ 8	S
9C	*casseliflavus*	-	-	≤ 8	S
10C	*faecalis*	-	-	≤ 8	S
11C	*faecalis*	-	-	≤ 8	S
12C	*faecalis*	-	-	≤ 8	S
13C	*faecium*	-	-	≤ 8	S
14C	*faecium*	-	-	≤ 8	S
15C	*faecium*	-	-	≤ 8	S
16C	*gallinarum*	-	-	≤ 8	S
17C	*hirae/durans*	-	-	≤ 8	S

18C	*faecium*	-	-	≤ 8	S
19C	*hirae/durans*	-	-	≤ 8	S
20C	*faecium*	-	-	≤ 8	S
21C	*faecalis*	-	-	≤ 8	S
22C	*faecium*	-	-	≤ 8	S
23C	*faecalis*	-	-	≤ 8	S
24C	*hirae/durans*	-	-	≤ 8	S
No.	***Enteroccocus***	**8**	**16**	**MIC**	**S/R**

N/D, not determined

Appendix 7. Antimicrobial sensitivity test result for avoparcin (μg/mL)

No.	*Enteroccocus*	8	16	32	MIC	S/R	*van*A
1	*faecalis*	-	-	-	≤ 8	S	-
2	*faecalis*	-	-	-	≤ 8	S	-
3	*faecalis*	-	-	-	≤ 8	S	-
4	*faecalis*	-	-	-	≤ 8	S	-
5	*faecium*	-	-	-	≤ 8	S	-
6	*faecium*	-	-	-	≤ 8	S	-
7	*faecium*	-	-	-	≤ 8	S	-
8	*faecium*	-	-	-	≤ 8	S	-
9	*faecium*	-	-	-	≤ 8	S	-
10	*faecium*	-	-	-	≤ 8	S	-
11	*faecium*	-	-	-	≤ 8	S	-
12	*faecium*	-	-	-	≤ 8	S	-
13	*hirae/durans*	-	-	-	≤ 8	S	-
14	*faecium*	-	-	-	≤ 8	S	-
15	*faecalis*	-	-	-	≤ 8	S	-
16	*hirae/durans*	-	-	-	≤ 8	S	-
17	*faecalis*	-	-	-	≤ 8	S	-
18	*faecium*	-	-	-	≤ 8	S	+
19	*faecium*	-	-	-	≤ 8	S	-
20	*faecium*	-	-	-	≤ 8	S	-
21	*faecium*	-	-	-	≤ 8	S	-
22	*faecium*	-	-	-	≤ 8	S	-
23	*faecium*	-	-	-	≤ 8	S	-
24	*faecium*	-	-	-	≤ 8	S	-
25	*faecium*	-	-	-	≤ 8	S	-
26	*faecium*	-	-	-	≤ 8	S	-
27	*faecium*	-	-	-	≤ 8	S	-
28	*faecium*	-	-	-	≤ 8	S	-
29	*faecium*	-	-	-	≤ 8	S	-
30	*faecalis*	-	-	-	≤ 8	S	-
31	*faecalis*	-	-	-	≤ 8	S	-
32	*faecalis*	-	-	-	≤ 8	S	-
33	*faecalis*	-	-	-	≤ 8	S	-
34	*faecalis*	-	-	-	≤ 8	S	-
35	*faecalis*	-	-	-	≤ 8	S	-
36	*faecium*	+	+	+	> 32	R	+
37	*faecium*	-	-	-	≤ 8	S	-
38	*faecalis*	-	-	-	≤ 8	S	-
39	*faecium*	-	-	-	≤ 8	S	-
40	*faecalis*	-	-	-	≤ 8	S	-
41	*faecalis*	-	-	-	≤ 8	S	-
42	*faecalis*	-	-	-	≤ 8	S	-
43	*faecalis*	-	-	-	≤ 8	S	-

44	*hirae*	-	-	-	≤ 8	S	-
45	*faecalis*	-	-	-	≤ 8	S	-
46	*faecalis*	-	-	-	≤ 8	S	-
47	*faecium*	+	+	+	> 32	R	+
48	*faecalis*	-	-	-	≤ 8	S	-
49	*faecium*	+	+	-	32	R	+
50	*faecium*	+	+	-	32	R	+
51	*faecalis*	-	-	-	≤ 8	S	-
52	*faecalis*	-	-	-	≤ 8	S	-
53	*faecalis*	-	-	-	≤ 8	S	-
54	*faecalis*	-	-	-	≤ 8	S	-
55	*casseliflavus*	-	-	-	≤ 8	S	-
56	*faecalis*	-	-	-	≤ 8	S	-
57	*faecalis*	-	-	-	≤ 8	S	-
58	*faecalis*	-	-	-	≤ 8	S	-
59	*faecalis*	-	-	-	≤ 8	S	-
60	*faecalis*	-	-	-	≤ 8	S	-
61	*faecalis*	-	-	-	≤ 8	S	-
62	*faecalis*	-	-	-	≤ 8	S	-
63	*faecalis*	-	-	-	≤ 8	S	-
64	*faecalis*	-	-	-	≤ 8	S	-
65	*faecalis*	-	-	-	≤ 8	S	-
66	*faecalis*	-	-	-	≤ 8	S	-
67	*faecalis*	-	-	-	≤ 8	S	-
68	*faecalis*	-	-	-	≤ 8	S	-
69	*faecalis*	-	-	-	≤ 8	S	-
70	*faecalis*	-	-	-	≤ 8	S	-
71	*faecalis*	-	-	-	≤ 8	S	-
72	*faecalis*	-	-	-	≤ 8	S	-
73	*faecalis*	-	-	-	≤ 8	S	-
74	*faecalis*	-	-	-	≤ 8	S	-
75	*faecium*	-	-	-	≤ 8	S	-
76	*faecalis*	-	-	-	≤ 8	S	-
77	*faecalis*	-	-	-	≤ 8	S	-
78	*faecalis*	-	-	-	≤ 8	S	-
79	*faecalis*	-	-	-	≤ 8	S	-
80	*faecalis*	-	-	-	≤ 8	S	-
81	*faecalis*	-	-	-	≤ 8	S	-
82	*faecalis*	-	-	-	≤ 8	S	-
83	*faecalis*	-	-	-	≤ 8	S	-
84	*faecalis*	-	-	-	≤ 8	S	-
85	*faecalis*	-	-	-	≤ 8	S	-
86	*faecalis*	-	-	-	≤ 8	S	-
87	*faecalis*	-	-	-	≤ 8	S	-
88	*faecalis*	-	-	-	≤ 8	S	-

89	*faecalis*	-	-	-	≤ 8	S	-
90	*faecalis*	-	-	-	≤ 8	S	-
91	*faecalis*	-	-	-	≤ 8	S	-
92	*faecalis*	-	-	-	≤ 8	S	-
93	*faecalis*	-	-	-	≤ 8	S	-
94	*faecium*	-	-	-	≤ 8	S	-
95	*faecalis*	-	-	-	≤ 8	S	-
96	*faecium*	-	-	-	≤ 8	S	-
97	*faecium*	-	-	-	≤ 8	S	-
98	*faecium*	-	-	-	≤ 8	S	-
99	*faecium*	-	-	-	≤ 8	S	-
100	*faecium*	-	-	-	≤ 8	S	-
101	*faecalis*	-	-	-	≤ 8	S	-
102	*faecalis*	-	-	-	≤ 8	S	-
103	*faecium*	-	-	-	≤ 8	S	-
104	*faecium*	-	-	-	≤ 8	S	-
105	*faecium*	-	-	-	≤ 8	S	-
106	*faecium*	-	-	-	≤ 8	S	-
107	*hirae/durans*	-	-	-	≤ 8	S	-
108	*faecium*	-	-	-	≤ 8	S	-
109	*faecium*	-	-	-	≤ 8	S	-
110	*faecium*	-	-	-	≤ 8	S	-
111	*faecium*	-	-	-	≤ 8	S	-
112	*faecium*	+	+	+	> 32	R	+
113	*hirae/durans*	-	-	-	≤ 8	S	-
114	*hirae/durans*	-	-	-	≤ 8	S	-
115	*faecalis*	-	-	-	≤ 8	S	-
116	*faecium*	-	-	-	≤ 8	S	-
117	*faecium*	-	-	-	≤ 8	S	-
118	*faecium*	-	-	-	≤ 8	S	-
119	*faecium*	-	-	-	≤ 8	S	-
120	*faecium*	-	-	-	≤ 8	S	-
121	*hirae/durans*	-	-	-	≤ 8	S	-
122	*hirae/durans*	-	-	-	≤ 8	S	-
123	*faecium*	-	-	-	≤ 8	S	-
124	*faecium*	+	+	+	> 32	R	+
125	*faecium*	-	-	-	≤ 8	S	-
126	*faecium*	+	+	+	> 32	R	+
127	*hirae/durans*	-	-	-	≤ 8	S	-
128	*hirae/durans*	-	-	-	≤ 8	S	-
129	*raffinosus*	-	-	-	≤ 8	S	-
130	*faecium*	-	-	-	≤ 8	S	-
131	*faecium*	-	-	-	≤ 8	S	-
132	*faecium*	-	-	-	≤ 8	S	-
133	*faecalis*	-	-	-	≤ 8	S	-

134	*faecium*	-	-	-	≤ 8	S	-
135	*faecalis*	-	-	-	≤ 8	S	-
136	*faecium*	-	-	-	≤ 8	S	-
137	*faecium*	-	-	-	≤ 8	S	-
138	*faecium*	-	-	-	≤ 8	S	-
139	*raffinosus*	-	-	-	≤ 8	S	-
140	*faecium*	-	-	-	≤ 8	S	-
141	*faecium*	-	-	-	≤ 8	S	-
142	*faecium*	-	-	-	≤ 8	S	-
143	*faecium*	-	-	-	≤ 8	S	-
144	*hirae/durans*	-	-	-	≤ 8	S	-
145	*faecium*	-	-	-	≤ 8	S	-
146	*faecium*	-	-	-	≤ 8	S	-
147	*faecium*	-	-	-	≤ 8	S	-
148	*faecium*	-	-	-	≤ 8	S	-
149	*faecium*	-	-	-	≤ 8	S	-
150	*hirae/durans*	-	-	-	≤ 8	S	-
151	*hirae/durans*	-	-	-	≤ 8	S	-
152	*hirae/durans*	-	-	-	≤ 8	S	-
153	*hirae/durans*	-	-	-	≤ 8	S	-
154	*faecium*	-	-	-	≤ 8	S	-
155	*faecium*	-	-	-	≤ 8	S	-
156	*faecium*	-	-	-	≤ 8	S	-
157	*faecalis*	-	-	-	≤ 8	S	-
158	*faecium*	-	-	-	≤ 8	S	-
159	*faecium*	-	-	-	≤ 8	S	-
160	*faecium*	-	-	-	≤ 8	S	-
161	*faecalis*	-	-	-	≤ 8	S	-
162	*faecalis*	-	-	-	≤ 8	S	-
163	*faecalis*	-	-	-	≤ 8	S	-
164	*faecalis*	-	-	-	≤ 8	S	-
165	*faecalis*	-	-	-	≤ 8	S	-
166	*faecalis*	-	-	-	≤ 8	S	-
167	*faecium*	-	-	-	≤ 8	S	-
168	*faecalis*	-	-	-	≤ 8	S	-
169	*faecium*	-	-	-	≤ 8	S	-
170	*gallinarum*	-	-	-	≤ 8	S	-
171	*casseliflavus*	-	-	-	≤ 8	S	-
172	*gallinarum*	-	-	-	≤ 8	S	-
173	*gallinarum*	-	-	-	≤ 8	S	-
174	*gallinarum*	-	-	-	≤ 8	S	-
175	*gallinarum*	-	-	-	≤ 8	S	-
176	*casseliflavus*	-	-	-	≤ 8	S	-
177	*gallinarum*	+	-	-	16	S	-
178	*gallinarum*	+	-	-	16	S	-

179	*gallinarum*	+	-	-	16	S	-
180	*casseliflavus*	+	-	-	16	S	-
181	*gallinarum*	+	-	-	16	S	-
182	*gallinarum*	+	-	-	16	S	-
183	*casseliflavus*	-	-	-	≤ 8	S	-
184	*gallinarum*	+	-	-	16	S	-
185	*gallinarum*	+	-	-	16	S	-
186	*gallinarum*	-	-	-	≤ 8	S	-
187	*casseliflavus*	-	-	-	≤ 8	S	-
188	*casseliflavus*	-	-	-	≤ 8	S	-
189	*gallinarum*	+	-	-	16	S	-
190	*casseliflavus*	-	-	-	≤ 8	S	-
191	*casseliflavus*	-	-	-	≤ 8	S	-
192	*gallinarum*	+	-	-	16	S	-
193	*casseliflavus*	-	-	-	≤ 8	S	-
194	*gallinarum*	-	-	-	≤ 8	S	-
195	*gallinarum*	-	-	-	≤ 8	S	-
196	*gallinarum*	-	-	-	≤ 8	S	-
197	*gallinarum*	-	-	-	≤ 8	S	-
198	*faecium*	+	+	+	> 32	R	-
199	*gallinarum*	-	-	-	≤ 8	S	-
200	*casseliflavus*	-	-	-	≤ 8	S	-
201	*gallinarum*	-	-	-	≤ 8	S	-
202	*casseliflavus*	-	-	-	≤ 8	S	-
203	*casseliflavus*	-	-	-	≤ 8	S	-
204	*faecium*	+	+	-	32	R	+
205	*faecium*	+	+	+	> 32	R	+
206	*faecium*	+	+	-	32	R	+
1C	*faecalis*	-	-		≤ 8	S	-
2C	*faecium*	-	-		≤ 8	S	-
3C	*faecium*	-	-		≤ 8	S	-
4C	N/D	-	-		≤ 8	S	-
5C	*hirae*	-	-		≤ 8	S	-
6C	*faecalis*	-	-		≤ 8	S	-
7C	*faecalis*	-	-		≤ 8	S	-
8C	*faecalis*	-	-		≤ 8	S	-
9C	*casseliflavus*	-	-		≤ 8	S	-
10C	*faecalis*	-	-		≤ 8	S	-
11C	*faecalis*	+	+		≥ 16	R	+
12C	*faecalis*	-	-		≤ 8	S	-
13C	*faecium*	-	-		≤ 8	S	-
14C	*faecium*	-	-		≤ 8	S	+
15C	*faecium*	+	+		≥ 16	R	-
16C	*gallinarum*	-	-		≤ 8	S	-
17C	*hirae/durans*	-	-		≤ 8	S	-

18C	*faecium*	-	-		≤ 8	S	-
19C	*hirae/durans*	-	-		≤ 8	S	-
20C	*faecium*	-	-		≤ 8	S	-
21C	*faecalis*	-	-		≤ 8	S	-
22C	*faecium*	-	-		≤ 8	S	-
23C	*faecalis*	-	-		≤ 8	S	-
24C	*hirae/durans*	-	-		≤ 8	S	-
No.	***Enteroccocus***	**8**	**16**	**32**	**MIC**	**S/R**	***van*A**

N/D, not determined

Appendix 8. Antimicrobial sensitivity test result for bacitracin (IU/mL)

No.	*Enteroccocus*	16	32	64	128	MIC	S/R
1	*faecalis*	+	+	+		> 64	R
2	*faecalis*	+	+	+		> 64	R
3	*faecalis*	+	+	+		> 64	R
4	*faecalis*	+	+	+		> 64	R
5	*faecium*	+	+	+		> 64	R
6	*faecium*	+	+	+		> 64	R
7	*faecium*	+	+	+		> 64	R
8	*faecium*	+	+	+		> 64	R
9	*faecium*	+	+	+		> 64	R
10	*faecium*	+	+	+		> 64	R
11	*faecium*	+	+	+		> 64	R
12	*faecium*	+	+	+		> 64	R
13	*hirae/durans*	+	+	+		> 64	R
14	*faecium*	+	+	+		> 64	R
15	*faecalis*	+	+	+		> 64	R
16	*hirae/durans*	+	+	+		> 64	R
17	*faecalis*	+	+	+		> 64	R
18	*faecium*	+	+	+		> 64	R
19	*faecium*	+	+	+		> 64	R
20	*faecium*	+	+	+		> 64	R
21	*faecium*	+	+	+		> 64	R
22	*faecium*	+	+	+		> 64	R
23	*faecium*	+	+	+		> 64	R
24	*faecium*	+	+	+		> 64	R
25	*faecium*	+	+	+		> 64	R
26	*faecium*	+	+	+		> 64	R
27	*faecium*	+	+	+		> 64	R
28	*faecium*	+	+	+		> 64	R
29	*faecium*	+	+	+		> 64	R
30	*faecalis*	-	-	-	-	≤ 16	S
31	*faecalis*	-	-	-	-	≤ 16	S
32	*faecalis*	-	-	-	-	≤ 16	S
33	*faecalis*	-	-	-	-	≤ 16	S
34	*faecalis*	-	-	-	-	≤ 16	S
35	*faecalis*	-	-	-	-	≤ 16	S
36	*faecium*	+	+	-	-	64	S
37	*faecium*	-	-	-	-	≤ 16	S
38	*faecalis*	-	-	-	-	≤ 16	S
39	*faecium*	-	-	-	-	≤ 16	S
40	*faecalis*	-	-	-	-	≤ 16	S
41	*faecalis*	-	-	-	-	≤ 16	S
42	*faecalis*	-	-	-	-	≤ 16	S
43	*faecalis*	-	-	-	-	≤ 16	S

44	*hirae*	-	-	-	-	≤ 16	S
45	*faecalis*	+	+	+	+	> 128	R
46	*faecalis*	-	-	-	-	≤ 16	S
47	*faecium*	+	+	+	+	> 128	R
48	*faecalis*	+	+	+	+	> 128	R
49	*faecium*	+	+	+	+	> 128	R
50	*faecium*	+	+	+	+	> 128	R
51	*faecalis*	-	-	-	-	≤ 16	S
52	*faecalis*	-	-	-	-	≤ 16	S
53	*faecalis*	-	-	-	-	≤ 16	S
54	*faecalis*	-	-	-	-	≤ 16	S
55	*casseliflavus*	-	-	-	-	≤ 16	S
56	*faecalis*	+	+	+	+	> 128	R
57	*faecalis*	-	-	-	-	≤ 16	S
58	*faecalis*	-	-	-	-	≤ 16	S
59	*faecalis*	-	-	-	-	≤ 16	S
60	*faecalis*	-	-	-	-	≤ 16	S
61	*faecalis*	+	+	+	+	> 128	R
62	*faecalis*	-	-	-	-	≤ 16	S
63	*faecalis*	-	-	-	-	≤ 16	S
64	*faecalis*	+	+	+	-	128	R
65	*faecalis*	-	-	-	-	≤ 16	S
66	*faecalis*	-	-	-	-	≤ 16	S
67	*faecalis*	-	-	-	-	≤ 16	S
68	*faecalis*	-	-	-	-	≤ 16	S
69	*faecalis*	-	-	-	-	≤ 16	S
70	*faecalis*	-	-	-	-	≤ 16	S
71	*faecalis*	-	-	-	-	≤ 16	S
72	*faecalis*	-	-	-	-	≤ 16	S
73	*faecalis*	-	-	-	-	≤ 16	S
74	*faecalis*	-	-	-	-	≤ 16	S
75	*faecium*	-	-	-	-	≤ 16	S
76	*faecalis*	-	-	-	-	≤ 16	S
77	*faecalis*	-	-	-	-	≤ 16	S
78	*faecalis*	-	-	-	-	≤ 16	S
79	*faecalis*	-	-	-	-	≤ 16	S
80	*faecalis*	-	-	-	-	≤ 16	S
81	*faecalis*	-	-	-	-	≤ 16	S
82	*faecalis*	-	-	-	-	≤ 16	S
83	*faecalis*	-	-	-	-	≤ 16	S
84	*faecalis*	-	-	-	-	≤ 16	S
85	*faecalis*	-	-	-	-	≤ 16	S
86	*faecalis*	-	-	-	-	≤ 16	S
87	*faecalis*	-	-	-	-	≤ 16	S
88	*faecalis*	-	-	-	-	≤ 16	S

89	*faecalis*	-	-	-	-	≤ 16	S
90	*faecalis*	+	+	+	+	> 128	R
91	*faecalis*	-	-	-	-	≤ 16	S
92	*faecalis*	-	-	-	-	≤ 16	S
93	*faecalis*	-	-	-	-	≤ 16	S
94	*faecium*	+	+	+		> 64	R
95	*faecalis*	+	+	+		> 64	R
96	*faecium*	+	+	+		> 64	R
97	*faecium*	+	+	+		> 64	R
98	*faecium*	+	+	+		> 64	R
99	*faecium*	+	+	+		> 64	R
100	*faecium*	+	+	+		> 64	R
101	*faecalis*	+	+	+		> 64	R
102	*faecalis*	+	+	+		> 64	R
103	*faecium*	+	+	+		> 64	R
104	*faecium*	+	+	+		> 64	R
105	*faecium*	+	+	+		> 64	R
106	*faecium*	+	+	+		> 64	R
107	*hirae/durans*	+	+	+		> 64	R
108	*faecium*	+	+	+		> 64	R
109	*faecium*	+	+	+		> 64	R
110	*faecium*	+	+	+		> 64	R
111	*faecium*	+	+	+		> 64	R
112	*faecium*	+	+	+		> 64	R
113	*hirae/durans*	+	+	+		> 64	R
114	*hirae/durans*	+	+	+		> 64	R
115	*faecalis*	+	+	+		> 64	R
116	*faecium*	+	+	+		> 64	R
117	*faecium*	+	+	+		> 64	R
118	*faecium*	+	+	+		> 64	R
119	*faecium*	+	+	+		> 64	R
120	*faecium*	-	-	-	-	≤ 16	S
121	*hirae/durans*	+	+	+		> 64	R
122	*hirae/durans*	+	+	+		> 64	R
123	*faecium*	+	+	+		> 64	R
124	*faecium*	+	+	+		> 64	R
125	*faecium*	+	+	+		> 64	R
126	*faecium*	+	+	+		> 64	R
127	*hirae/durans*	+	+	+		> 64	R
128	*hirae/durans*	+	+	+		> 64	R
129	*raffinosus*	+	+	+		> 64	R
130	*faecium*	+	+	+		> 64	R
131	*faecium*	+	+	+		> 64	R
132	*faecium*	+	+	+		> 64	R
133	*faecalis*	+	+	+		> 64	R

134	*faecium*	+	+	+	> 64	R
135	*faecalis*	+	+	+	> 64	R
136	*faecium*	+	+	+	> 64	R
137	*faecium*	+	+	+	> 64	R
138	*faecium*	+	+	+	> 64	R
139	*raffinosus*	+	+	+	> 64	R
140	*faecium*	+	+	+	> 64	R
141	*faecium*	+	+	+	> 64	R
142	*faecium*	+	+	+	> 64	R
143	*faecium*	+	+	+	> 64	R
144	*hirae/durans*	+	+	+	> 64	R
145	*faecium*	+	+	+	> 64	R
146	*faecium*	+	+	+	> 64	R
147	*faecium*	+	+	+	> 64	R
148	*faecium*	+	+	+	> 64	R
149	*faecium*	+	+	+	> 64	R
150	*hirae/durans*	+	+	+	> 64	R
151	*hirae/durans*	+	+	+	> 64	R
152	*hirae/durans*	+	+	+	> 64	R
153	*hirae/durans*	+	+	+	> 64	R
154	*faecium*	+	+	+	> 64	R
155	*faecium*	+	+	+	> 64	R
156	*faecium*	+	+	+	> 64	R
157	*faecalis*	+	+	+	> 64	R
158	*faecium*	+	+	+	> 64	R
159	*faecium*	+	+	+	> 64	R
160	*faecium*	+	+	+	> 64	R
161	*faecalis*	+	+	+	> 64	R
162	*faecalis*	+	+	+	> 64	R
163	*faecalis*	+	+	+	> 64	R
164	*faecalis*	+	+	+	> 64	R
165	*faecalis*	+	+	+	> 64	R
166	*faecalis*	+	+	+	> 64	R
167	*faecium*	+	+	+	> 64	R
168	*faecalis*	+	+	+	> 64	R
169	*faecium*	+	+	+	> 64	R
170	*gallinarum*	+	+	+	> 64	R
171	*casseliflavus*	+	+	+	> 64	R
172	*gallinarum*	+	+	+	> 64	R
173	*gallinarum*	+	+	+	> 64	R
174	*gallinarum*	+	+	+	> 64	R
175	*gallinarum*	+	+	+	> 64	R
176	*casseliflavus*	+	+	+	> 64	R
177	*gallinarum*	+	+	+	> 64	R
178	*gallinarum*	+	+	+	> 64	R

179	*gallinarum*	+	+	+		> 64	R
180	*casseliflavus*	+	+	+		> 64	R
181	*gallinarum*	+	+	+		> 64	R
182	*gallinarum*	+	+	+		> 64	R
183	*casseliflavus*	+	+	+		> 64	R
184	*gallinarum*	+	+	+		> 64	R
185	*gallinarum*	+	+	+		> 64	R
186	*gallinarum*	+	+	+		> 64	R
187	*casseliflavus*	+	+	+		> 64	R
188	*casseliflavus*	+	+	+		> 64	R
189	*gallinarum*	+	+	+		> 64	R
190	*casseliflavus*	+	+	+		> 64	R
191	*casseliflavus*	+	+	+		> 64	R
192	*gallinarum*	+	+	+		> 64	R
193	*casseliflavus*	+	+	+		> 64	R
194	*gallinarum*	+	+	+		> 64	R
195	*gallinarum*	+	+	+		> 64	R
196	*gallinarum*	+	+	+		> 64	R
197	*gallinarum*	+	+	+		> 64	R
198	*faecium*	+	+	+		> 64	R
199	*gallinarum*	+	+	+		> 64	R
200	*casseliflavus*	+	+	+		> 64	R
201	*gallinarum*	+	+	+		> 64	R
202	*casseliflavus*	+	+	+		> 64	R
203	*casseliflavus*	+	+	+		> 64	R
204	*faecium*	+	+	+		> 64	R
205	*faecium*	+	+	+		> 64	R
206	*faecium*	+	+	+		> 64	R
1C	*faecalis*	+	+	+	+	128	R
2C	*faecium*	+	-	-	-	32	S
3C	*faecium*	+	+	+	+	128	R
4C	N/D	-	-	-	-	≤ 16	S
5C	*hirae*	-	-	-	-	≤ 16	S
6C	*faecalis*	-	-	-	-	≤ 16	S
7C	*faecalis*	+	+	+	+	128	R
8C	*faecalis*	-	-	-	-	≤ 16	S
9C	*casseliflavus*	-	-	-	-	≤ 16	S
10C	*faecalis*	-	-	-	-	≤ 16	S
11C	*faecalis*	+	+	+	+	128	R
12C	*faecalis*	-	-	-	-	≤ 16	S
13C	*faecium*	-	-	-	-	≤ 16	S
14C	*faecium*	-	-	-	-	≤ 16	S

15C	*faecium*	-	-	-	-	≤ 16	S
16C	*gallinarum*	-	-	-	-	≤ 16	S
17C	*hirae/durans*	-	-	-	-	≤ 16	S
18C	*faecium*	-	-	-	-	≤ 16	S
19C	*hirae/durans*	-	-	-	-	≤ 16	S
20C	*faecium*	-	-	-	-	≤ 16	S
21C	*faecalis*	-	-	-	-	≤ 16	S
22C	*faecium*	-	-	-	-	≤ 16	S
23C	*faecalis*	-	-	-	-	≤ 16	S
24C	*hirae/durans*	-	-	-	-	≤ 16	S
No.	***Enteroccocus***	**16**	**32**	**64**	**128**	**MIC**	**S/R**

N/D, not determined

Appendix 9. Antimicrobial sensitivity test result for flavophospholipol (μg/mL)

No.	*Enteroccocus*	4	8	16	32	MIC	S/R	*van*A
1	*faecalis*	-	-	-	-	≤ 4	S	-
2	*faecalis*	-	-	-	-	≤ 4	S	-
3	*faecalis*	-	-	-	-	≤ 4	S	-
4	*faecalis*	-	-	-	-	≤ 4	S	-
5	*faecium*	+	+	+	+	> 32	R	-
6	*faecium*	+	+	+	+	> 32	R	-
7	*faecium*	+	+	+	+	> 32	R	-
8	*faecium*	+	+	+	+	> 32	R	-
9	*faecium*	+	+	+	+	> 32	R	-
10	*faecium*	+	+	+	+	> 32	R	-
11	*faecium*	+	+	+	+	> 32	R	-
12	*faecium*	+	+	+	+	> 32	R	-
13	*hirae/durans*	+	+	+	+	> 32	R	-
14	*faecium*	-	-	-	-	≤ 4	S	-
15	*faecalis*	+	+	+	+	> 32	R	-
16	*hirae/durans*	+	+	+	+	> 32	R	-
17	*faecalis*	-	-	-	-	≤ 4	S	-
18	*faecium*	-	-	-	-	≤ 4	S	+
19	*faecium*	+	+	+	+	> 32	R	-
20	*faecium*	+	+	+	+	> 32	R	-
21	*faecium*	+	+	+	+	> 32	R	-
22	*faecium*	+	+	+	+	> 32	R	-
23	*faecium*	+	+	+	+	> 32	R	-
24	*faecium*	+	+	+	+	> 32	R	-
25	*faecium*	+	+	+	+	> 32	R	-
26	*faecium*	+	+	+	+	> 32	R	-
27	*faecium*	+	+	+	+	> 32	R	-
28	*faecium*	+	+	+	+	> 32	R	-
29	*faecium*	+	+	+	+	> 32	R	-
30	*faecalis*	-	-	-	-	≤ 4	S	-
31	*faecalis*	-	-	-	-	≤ 4	S	-
32	*faecalis*	-	-	-	-	≤ 4	S	-
33	*faecalis*	-	-	-	-	≤ 4	S	-
34	*faecalis*	-	-	-	-	≤ 4	S	-
35	*faecalis*	-	-	-	-	≤ 4	S	-
36	*faecium*	-	-	-	-	≤ 4	S	+
37	*faecium*	+	+	+	+	> 32	R	-
38	*faecalis*	-	-	-	-	≤ 4	S	-
39	*faecium*	+	+	+	+	> 32	R	-
40	*faecalis*	-	-	-	-	≤ 4	S	-
41	*faecalis*	-	-	-	-	≤ 4	S	-
42	*faecalis*	-	-	-	-	≤ 4	S	-
43	*faecalis*	-	-	-	-	≤ 4	S	-

44	*hirae*	-	-	-	-	≤ 4	S	-
45	*faecalis*	-	-	-	-	≤ 4	S	-
46	*faecalis*	-	-	-	-	≤ 4	S	-
47	*faecium*	-	-	-	-	≤ 4	S	+
48	*faecalis*	-	-	-	-	≤ 4	S	-
49	*faecium*	-	-	-	-	≤ 4	S	+
50	*faecium*	-	-	-	-	≤ 4	S	+
51	*faecalis*	-	-	-	-	≤ 4	S	-
52	*faecalis*	-	-	-	-	≤ 4	S	-
53	*faecalis*	-	-	-	-	≤ 4	S	-
54	*faecalis*	-	-	-	-	≤ 4	S	-
55	*casseliflavus*	+	+	+	+	> 32	R	-
56	*faecalis*	-	-	-	-	≤ 4	S	-
57	*faecalis*	-	-	-	-	≤ 4	S	-
58	*faecalis*	-	-	-	-	≤ 4	S	-
59	*faecalis*	-	-	-	-	≤ 4	S	-
60	*faecalis*	-	-	-	-	≤ 4	S	-
61	*faecalis*	-	-	-	-	≤ 4	S	-
62	*faecalis*	-	-	-	-	≤ 4	S	-
63	*faecalis*	-	-	-	-	≤ 4	S	-
64	*faecalis*	-	-	-	-	≤ 4	S	-
65	*faecalis*	-	-	-	-	≤ 4	S	-
66	*faecalis*	-	-	-	-	≤ 4	S	-
67	*faecalis*	-	-	-	-	≤ 4	S	-
68	*faecalis*	-	-	-	-	≤ 4	S	-
69	*faecalis*	-	-	-	-	≤ 4	S	-
70	*faecalis*	-	-	-	-	≤ 4	S	-
71	*faecalis*	-	-	-	-	≤ 4	S	-
72	*faecalis*	-	-	-	-	≤ 4	S	-
73	*faecalis*	-	-	-	-	≤ 4	S	-
74	*faecalis*	-	-	-	-	≤ 4	S	-
75	*faecium*	+	+	+	+	> 32	R	-
76	*faecalis*	-	-	-	-	≤ 4	S	-
77	*faecalis*	-	-	-	-	≤ 4	S	-
78	*faecalis*	-	-	-	-	≤ 4	S	-
79	*faecalis*	-	-	-	-	≤ 4	S	-
80	*faecalis*	-	-	-	-	≤ 4	S	-
81	*faecalis*	-	-	-	-	≤ 4	S	-
82	*faecalis*	-	-	-	-	≤ 4	S	-
83	*faecalis*	-	-	-	-	≤ 4	S	-
84	*faecalis*	-	-	-	-	≤ 4	S	-
85	*faecalis*	-	-	-	-	≤ 4	S	-
86	*faecalis*	-	-	-	-	≤ 4	S	-
87	*faecalis*	-	-	-	-	≤ 4	S	-
88	*faecalis*	-	-	-	-	≤ 4	S	-

89	*faecalis*	-	-	-	-	≤ 4	S	-
90	*faecalis*	-	-	-	-	≤ 4	S	-
91	*faecalis*	-	-	-	-	≤ 4	S	-
92	*faecalis*	-	-	-	-	≤ 4	S	-
93	*faecalis*	-	-	-	-	≤ 4	S	-
94	*faecium*	-	-	-	-	≤ 4	S	-
95	*faecalis*	-	-	-	-	≤ 4	S	-
96	*faecium*	+	+	+	+	> 32	R	-
97	*faecium*	+	+	+	+	> 32	R	-
98	*faecium*	+	+	+	+	> 32	R	-
99	*faecium*	+	+	+	+	> 32	R	-
100	*faecium*	+	+	-	-	8	S	-
101	*faecalis*	-	-	-	-	≤ 4	S	-
102	*faecalis*	-	-	-	-	≤ 4	S	-
103	*faecium*	+	+	+	+	> 32	R	-
104	*faecium*	+	+	+	+	> 32	R	-
105	*faecium*	+	+	+	+	> 32	R	-
106	*faecium*	+	+	+	+	> 32	R	-
107	*hirae/durans*	+	+	+	+	> 32	R	-
108	*faecium*	+	+	+	+	> 32	R	-
109	*faecium*	+	+	+	+	> 32	R	-
110	*faecium*	+	+	+	+	> 32	R	-
111	*faecium*	+	+	+	+	> 32	R	-
112	*faecium*	-	-	-	-	≤ 4	S	+
113	*hirae/durans*	+	+	+	+	> 32	R	-
114	*hirae/durans*	+	+	+	+	> 32	R	-
115	*faecalis*	-	-	-	-	≤ 4	S	-
116	*faecium*	+	+	+	+	> 32	R	-
117	*faecium*	+	+	+	+	> 32	R	-
118	*faecium*	+	+	+	+	> 32	R	-
119	*faecium*	+	+	+	+	> 32	R	-
120	*faecium*	+	+	+	+	> 32	R	-
121	*hirae/durans*	-	-	-	-	≤ 4	S	-
122	*hirae/durans*	-	-	-	-	≤ 4	S	-
123	*faecium*	+	+	+	+	> 32	R	-
124	*faecium*	-	-	-	-	≤ 4	S	+
125	*faecium*	+	+	+	+	> 32	R	-
126	*faecium*	-	-	-	-	≤ 4	S	+
127	*hirae/durans*	+	+	+	+	> 32	R	-
128	*hirae/durans*	+	+	+	-	> 32	R	-
129	*raffinosus*	-	-	-	-	≤ 4	S	-
130	*faecium*	+	+	+	+	> 32	R	-
131	*faecium*	+	+	+	+	> 32	R	-
132	*faecium*	+	+	+	+	> 32	R	-
133	*faecalis*	-	-	-	-	≤ 4	S	-

134	*faecium*	+	+	+	+	> 32	R	-
135	*faecalis*	-	-	-	-	≤ 4	S	-
136	*faecium*	+	+	+	+	> 32	R	-
137	*faecium*	+	+	+	+	> 32	R	-
138	*faecium*	+	+	+	+	> 32	R	-
139	*raffinosus*	-	-	-	-	≤ 4	S	-
140	*faecium*	+	+	+	+	> 32	R	-
141	*faecium*	+	+	+	+	> 32	R	-
142	*faecium*	+	+	+	+	> 32	R	-
143	*faecium*	+	+	+	+	> 32	R	-
144	*hirae/durans*	+	+	-	-	16	S	-
145	*faecium*	+	+	+	+	> 32	R	-
146	*faecium*	+	+	+	+	> 32	R	-
147	*faecium*	+	+	+	+	> 32	R	-
148	*faecium*	+	+	+	+	> 32	R	-
149	*faecium*	+	+	+	+	> 32	R	-
150	*hirae/durans*	+	+	+	-	32	R	-
151	*hirae/durans*	+	+	+	+	> 32	R	-
152	*hirae/durans*	+	+	+	+	> 32	R	-
153	*hirae/durans*	+	+	+	+	> 32	R	-
154	*faecium*	+	+	+	-	32	R	-
155	*faecium*	+	+	+	-	32	R	-
156	*faecium*	+	+	+	-	32	R	-
157	*faecalis*	-	-	-	-	≤ 4	S	-
158	*faecium*	+	+	+	+	> 32	R	-
159	*faecium*	+	+	+	+	> 32	R	-
160	*faecium*	+	+	+	+	> 32	R	-
161	*faecalis*	-	-	-	-	≤ 4	S	-
162	*faecalis*	-	-	-	-	≤ 4	S	-
163	*faecalis*	-	-	-	-	≤ 4	S	-
164	*faecalis*	-	-	-	-	≤ 4	S	-
165	*faecalis*	-	-	-	-	≤ 4	S	-
166	*faecalis*	-	-	-	-	≤ 4	S	-
167	*faecium*	+	+	+	+	> 32	R	-
168	*faecalis*	-	-	-	-	≤ 4	S	-
169	*faecium*	+	+	+	+	> 32	R	-
170	*gallinarum*	+	+	-	-	16	S	-
171	*casseliflavus*	+	+	-	-	16	S	-
172	*gallinarum*	+	+	-	-	16	S	-
173	*gallinarum*	+	+	-	-	16	S	-
174	*gallinarum*	+	+	+	+	> 32	R	-
175	*gallinarum*	+	+	-	-	16	S	-
176	*casseliflavus*	+	+	-	-	16	S	-
177	*gallinarum*	+	+	+	+	> 32	R	-
178	*gallinarum*	+	+	+	+	> 32	R	-

179	*gallinarum*	+	+	+	+	> 32	R	-
180	*casseliflavus*	+	+	+	+	> 32	R	-
181	*gallinarum*	+	+	+	+	> 32	R	-
182	*gallinarum*	+	-	-	-	8	S	-
183	*casseliflavus*	+	+	+	-	32	R	-
184	*gallinarum*	+	+	+	+	> 32	R	-
185	*gallinarum*	+	-	-	-	8	S	-
186	*gallinarum*	+	+	+	+	> 32	R	-
187	*casseliflavus*	+	+	+	+	> 32	R	-
188	*casseliflavus*	+	-	-	-	8	S	-
189	*gallinarum*	+	+	+	+	> 32	R	-
190	*casseliflavus*	+	+	+	+	> 32	R	-
191	*casseliflavus*	+	+	+	+	> 32	R	-
192	*gallinarum*	+	+	+	+	> 32	R	-
193	*casseliflavus*	+	+	+	+	> 32	R	-
194	*gallinarum*	+	+	-	-	16	S	-
195	*gallinarum*	+	+	+	+	> 32	R	-
196	*gallinarum*	+	+	+	+	> 32	R	-
197	*gallinarum*	+	+	+	+	> 32	R	-
198	*faecium*	-	-	-	-	≤ 4	S	-
199	*gallinarum*	+	+	+	+	> 32	R	-
200	*casseliflavus*	+	-	-	-	8	S	-
201	*gallinarum*	+	+	+	+	> 32	R	-
202	*casseliflavus*	+	+	+	+	> 32	R	-
203	*casseliflavus*	+	+	+	+	> 32	R	-
204	*faecium*	+	+	+	+	> 32	R	+
205	*faecium*	-	-	-	-	≤ 4	S	+
206	*faecium*	+	+	+	+	> 32	R	+
1C	*faecalis*	-	-	-	-	≤ 4	S	-
2C	*faecium*	+	+	-	-	16	S	-
3C	*faecium*	-	-	-	-	≤ 4	S	-
4C	N/D	+	+	+	+	≥ 32	R	-
5C	*hirae*	+	+	+	+	≥ 32	R	-
6C	*faecalis*	-	-	-	-	≤ 4	S	-
7C	*faecalis*	-	-	-	-	≤ 4	S	-
8C	*faecalis*	+	+	+	+	≥ 32	R	-
9C	*casseliflavus*	+	+	+	+	≥ 32	R	-
10C	*faecalis*	-	-	-	-	≤ 4	S	-
11C	*faecalis*	-	-	-	-	≤ 4	S	+
12C	*faecalis*	-	-	-	-	≤ 4	S	-
13C	*faecium*	+	+	+	+	≥ 32	R	-
14C	*faecium*	+	+	+	+	≥ 32	R	+
15C	*faecium*	+	+	+	+	≥ 32	R	-
16C	*gallinarum*	+	+	+	+	≥ 32	R	-
17C	*hirae/durans*	+	+	+	+	≥ 32	R	-

18C	*faecium*	+	+	+	+	≥ 32	R	-
19C	*hirae/durans*	+	+	+	+	≥ 32	R	-
20C	*faecium*	+	+	+	+	≥ 32	R	-
21C	*faecalis*	+	-	-	-	8	S	-
22C	*faecium*	+	+	+	+	≥ 32	R	-
23C	*faecalis*	-	-	-	-	≤ 4	S	-
24C	*hirae/durans*	-	-	-	-	≤ 4	S	-
No.	***Enteroccocus***	**4**	**8**	**16**	**32**	**MIC**	**S/R**	***van*A**

N/D, not determined

Appendix 10. Antimicrobial sensitivity test result for gentamicin (μg/mL)

No.	*Enteroccocus*	512	1024	MIC	S/R	*aac*6-*aph*2
1	*faecalis*	-	-	≤ 512	S	-
2	*faecalis*	-	-	≤ 512	S	-
3	*faecalis*	-	-	≤ 512	S	-
4	*faecalis*	-	-	≤ 512	S	-
5	*faecium*	-	-	≤ 512	S	-
6	*faecium*	-	-	≤ 512	S	-
7	*faecium*	-	-	≤ 512	S	-
8	*faecium*	-	-	≤ 512	S	-
9	*faecium*	-	-	≤ 512	S	-
10	*faecium*	-	-	≤ 512	S	-
11	*faecium*	-	-	≤ 512	S	-
12	*faecium*	-	-	≤ 512	S	-
13	*hirae/durans*	-	-	≤ 512	S	-
14	*faecium*	-	-	≤ 512	S	-
15	*faecalis*	-	-	≤ 512	S	-
16	*hirae/durans*	-	-	≤ 512	S	-
17	*faecalis*	-	-	≤ 512	S	-
18	*faecium*	-	-	≤ 512	S	-
19	*faecium*	-	-	≤ 512	S	-
20	*faecium*	-	-	≤ 512	S	-
21	*faecium*	-	-	≤ 512	S	-
22	*faecium*	-	-	≤ 512	S	-
23	*faecium*	-	-	≤ 512	S	-
24	*faecium*	-	-	≤ 512	S	-
25	*faecium*	-	-	≤ 512	S	-
26	*faecium*	-	-	≤ 512	S	-
27	*faecium*	-	-	≤ 512	S	-
28	*faecium*	-	-	≤ 512	S	-
29	*faecium*	-	-	≤ 512	S	-
30	*faecalis*	-	-	≤ 512	S	-
31	*faecalis*	-	-	≤ 512	S	+
32	*faecalis*	-	-	≤ 512	S	-
33	*faecalis*	-	-	≤ 512	S	-
34	*faecalis*	-	-	≤ 512	S	-
35	*faecalis*	-	-	≤ 512	S	-
36	*faecium*	-	-	≤ 512	S	-
37	*faecium*	-	-	≤ 512	S	-
38	*faecalis*	-	-	≤ 512	S	-
39	*faecium*	-	-	≤ 512	S	-
40	*faecalis*	-	-	≤ 512	S	-
41	*faecalis*	-	-	≤ 512	S	-
42	*faecalis*	-	-	≤ 512	S	+
43	*faecalis*	-	-	≤ 512	S	-

44	*hirae*	-	-	≤ 512	S	-
45	*faecalis*	-	-	≤ 512	S	-
46	*faecalis*	-	-	≤ 512	S	-
47	*faecium*	-	-	≤ 512	S	-
48	*faecalis*	-	-	≤ 512	S	-
49	*faecium*	-	-	≤ 512	S	-
50	*faecium*	-	-	≤ 512	S	-
51	*faecalis*	-	-	≤ 512	S	-
52	*faecalis*	-	-	≤ 512	S	-
53	*faecalis*	-	-	≤ 512	S	-
54	*faecalis*	-	-	≤ 512	S	-
55	*casseliflavus*	-	-	≤ 512	S	-
56	*faecalis*	-	-	≤ 512	S	-
57	*faecalis*	-	-	≤ 512	S	-
58	*faecalis*	-	-	≤ 512	S	+
59	*faecalis*	-	-	≤ 512	S	-
60	*faecalis*	-	-	≤ 512	S	-
61	*faecalis*	-	-	≤ 512	S	-
62	*faecalis*	-	-	≤ 512	S	-
63	*faecalis*	-	-	≤ 512	S	-
64	*faecalis*	-	-	≤ 512	S	-
65	*faecalis*	-	-	≤ 512	S	-
66	*faecalis*	-	-	≤ 512	S	-
67	*faecalis*	-	-	≤ 512	S	-
68	*faecalis*	-	-	≤ 512	S	-
69	*faecalis*	-	-	≤ 512	S	-
70	*faecalis*	-	-	≤ 512	S	-
71	*faecalis*	-	-	≤ 512	S	-
72	*faecalis*	-	-	≤ 512	S	-
73	*faecalis*	-	-	≤ 512	S	-
74	*faecalis*	-	-	≤ 512	S	-
75	*faecium*	-	-	≤ 512	S	-
76	*faecalis*	-	-	≤ 512	S	-
77	*faecalis*	-	-	≤ 512	S	-
78	*faecalis*	-	-	≤ 512	S	-
79	*faecalis*	-	-	≤ 512	S	-
80	*faecalis*	-	-	≤ 512	S	-
81	*faecalis*	-	-	≤ 512	S	-
82	*faecalis*	-	-	≤ 512	S	-
83	*faecalis*	-	-	≤ 512	S	-
84	*faecalis*	-	-	≤ 512	S	-
85	*faecalis*	-	-	≤ 512	S	-
86	*faecalis*	-	-	≤ 512	S	-
87	*faecalis*	-	-	≤ 512	S	-
88	*faecalis*	-	-	≤ 512	S	-

89	*faecalis*	-	-	≤ 512	S	-
90	*faecalis*	-	-	≤ 512	S	-
91	*faecalis*	-	-	≤ 512	S	-
92	*faecalis*	-	-	≤ 512	S	-
93	*faecalis*	-	-	≤ 512	S	-
94	*faecium*	-	-	≤ 512	S	-
95	*faecalis*	-	-	≤ 512	S	-
96	*faecium*	-	-	≤ 512	S	-
97	*faecium*	-	-	≤ 512	S	-
98	*faecium*	-	-	≤ 512	S	-
99	*faecium*	-	-	≤ 512	S	-
100	*faecium*	-	-	≤ 512	S	-
101	*faecalis*	-	-	≤ 512	S	-
102	*faecalis*	-	-	≤ 512	S	-
103	*faecium*	-	-	≤ 512	S	-
104	*faecium*	-	-	≤ 512	S	-
105	*faecium*	-	-	≤ 512	S	-
106	*faecium*	-	-	≤ 512	S	-
107	*hirae/durans*	-	-	≤ 512	S	-
108	*faecium*	-	-	≤ 512	S	-
109	*faecium*	-	-	≤ 512	S	-
110	*faecium*	-	-	≤ 512	S	-
111	*faecium*	-	-	≤ 512	S	-
112	*faecium*	-	-	≤ 512	S	-
113	*hirae/durans*	-	-	≤ 512	S	-
114	*hirae/durans*	-	-	≤ 512	S	-
115	*faecalis*	-	-	≤ 512	S	-
116	*faecium*	-	-	≤ 512	S	-
117	*faecium*	-	-	≤ 512	S	-
118	*faecium*	-	-	≤ 512	S	-
119	*faecium*	-	-	≤ 512	S	-
120	*faecium*	-	-	≤ 512	S	-
121	*hirae/durans*	-	-	≤ 512	S	-
122	*hirae/durans*	-	-	≤ 512	S	-
123	*faecium*	-	-	≤ 512	S	-
124	*faecium*	-	-	≤ 512	S	-
125	*faecium*	-	-	≤ 512	S	-
126	*faecium*	-	-	≤ 512	S	-
127	*hirae/durans*	-	-	≤ 512	S	-
128	*hirae/durans*	-	-	≤ 512	S	-
129	*raffinosus*	-	-	≤ 512	S	-
130	*faecium*	-	-	≤ 512	S	-
131	*faecium*	-	-	≤ 512	S	-
132	*faecium*	-	-	≤ 512	S	-
133	*faecalis*	-	-	≤ 512	S	-

134	*faecium*	-	-	≤ 512	S	-
135	*faecalis*	-	-	≤ 512	S	-
136	*faecium*	-	-	≤ 512	S	-
137	*faecium*	-	-	≤ 512	S	-
138	*faecium*	-	-	≤ 512	S	-
139	*raffinosus*	-	-	≤ 512	S	-
140	*faecium*	-	-	≤ 512	S	-
141	*faecium*	-	-	≤ 512	S	-
142	*faecium*	-	-	≤ 512	S	-
143	*faecium*	-	-	≤ 512	S	-
144	*hirae/durans*	-	-	≤ 512	S	-
145	*faecium*	-	-	≤ 512	S	-
146	*faecium*	-	-	≤ 512	S	-
147	*faecium*	-	-	≤ 512	S	-
148	*faecium*	-	-	≤ 512	S	-
149	*faecium*	-	-	≤ 512	S	-
150	*hirae/durans*	-	-	≤ 512	S	-
151	*hirae/durans*	-	-	≤ 512	S	-
152	*hirae/durans*	-	-	≤ 512	S	-
153	*hirae/durans*	-	-	≤ 512	S	-
154	*faecium*	-	-	≤ 512	S	-
155	*faecium*	-	-	≤ 512	S	-
156	*faecium*	-	-	≤ 512	S	-
157	*faecalis*	-	-	≤ 512	S	-
158	*faecium*	-	-	≤ 512	S	-
159	*faecium*	-	-	≤ 512	S	-
160	*faecium*	-	-	≤ 512	S	-
161	*faecalis*	-	-	≤ 512	S	-
162	*faecalis*	-	-	≤ 512	S	-
163	*faecalis*	-	-	≤ 512	S	-
164	*faecalis*	-	-	≤ 512	S	-
165	*faecalis*	-	-	≤ 512	S	-
166	*faecalis*	-	-	≤ 512	S	-
167	*faecium*	-	-	≤ 512	S	-
168	*faecalis*	-	-	≤ 512	S	-
169	*faecium*	-	-	≤ 512	S	-
170	*gallinarum*	-	-	≤ 512	S	-
171	*casseliflavus*	-	-	≤ 512	S	-
172	*gallinarum*	-	-	≤ 512	S	-
173	*gallinarum*	-	-	≤ 512	S	-
174	*gallinarum*	-	-	≤ 512	S	-
175	*gallinarum*	-	-	≤ 512	S	-
176	*casseliflavus*	-	-	≤ 512	S	-
177	*gallinarum*	-	-	≤ 512	S	-
178	*gallinarum*	-	-	≤ 512	S	-

179	*gallinarum*	-	-	≤ 512	S	-
180	*casseliflavus*	-	-	≤ 512	S	-
181	*gallinarum*	-	-	≤ 512	S	-
182	*gallinarum*	-	-	≤ 512	S	-
183	*casseliflavus*	-	-	≤ 512	S	-
184	*gallinarum*	-	-	≤ 512	S	-
185	*gallinarum*	-	-	≤ 512	S	-
186	*gallinarum*	-	-	≤ 512	S	-
187	*casseliflavus*	-	-	≤ 512	S	-
188	*casseliflavus*	-	-	≤ 512	S	-
189	*gallinarum*	-	-	≤ 512	S	-
190	*casseliflavus*	-	-	≤ 512	S	-
191	*casseliflavus*	-	-	≤ 512	S	-
192	*gallinarum*	-	-	≤ 512	S	-
193	*casseliflavus*	-	-	≤ 512	S	-
194	*gallinarum*	-	-	≤ 512	S	-
195	*gallinarum*	-	-	≤ 512	S	-
196	*gallinarum*	-	-	≤ 512	S	-
197	*gallinarum*	-	-	≤ 512	S	-
198	*faecium*	-	-	≤ 512	S	-
199	*gallinarum*	-	-	≤ 512	S	-
200	*casseliflavus*	-	-	≤ 512	S	-
201	*gallinarum*	-	-	≤ 512	S	-
202	*casseliflavus*	-	-	≤ 512	S	-
203	*casseliflavus*	-	-	≤ 512	S	-
204	*faecium*	-	-	≤ 512	S	-
205	*faecium*	-	-	≤ 512	S	-
206	*faecium*	-	-	≤ 512	S	-
1C	*faecalis*	-	-	≤ 512	S	-
2C	*faecium*	-	-	≤ 512	S	-
3C	*faecium*	-	-	≤ 512	S	-
4C	N/D	-	-	≤ 512	S	-
5C	*hirae*	-	-	≤ 512	S	-
6C	*faecalis*	-	-	≤ 512	S	+
7C	*faecalis*	-	-	≤ 512	S	-
8C	*faecalis*	-	-	≤ 512	S	-
9C	*casseliflavus*	-	-	≤ 512	S	-
10C	*faecalis*	-	-	≤ 512	S	-
11C	*faecalis*	-	-	≤ 512	S	-
12C	*faecalis*	-	-	≤ 512	S	-
13C	*faecium*	-	-	≤ 512	S	-
14C	*faecium*	+	+	≥ 1024	R	+
15C	*faecium*	-	-	≤ 512	S	+
16C	*gallinarum*	-	-	≤ 512	S	-
17C	*hirae/durans*	-	-	≤ 512	S	-
18C	*faecium*	-	-	≤ 512	S	-
19C	*hirae/durans*	-	-	≤ 512	S	-
20C	*faecium*	-	-	≤ 512	S	-
21C	*faecalis*	-	-	≤ 512	S	-
22C	*faecium*	-	-	≤ 512	S	-

23C	*faecalis*	-	-	≤ 512	S	-
24C	*hirae/durans*	-	-	≤ 512	S	-
No.	***Enteroccocus***	**512**	**1024**	**MIC**	**S/R**	***aac*6-*aph*2**

N/D, not determined

Appendix 11. Antimicrobial sensitivity test result for narasin (μg/mL)

No.	*Enteroccocus*	1	2	MIC	S/R
1	*faecalis*	-	-	≤ 1	S
2	*faecalis*	-	-	≤ 1	S
3	*faecalis*	-	-	≤ 1	S
4	*faecalis*	-	-	≤ 1	S
5	*faecium*	-	-	≤ 1	S
6	*faecium*	-	-	≤ 1	S
7	*faecium*	-	-	≤ 1	S
8	*faecium*	-	-	≤ 1	S
9	*faecium*	-	-	≤ 1	S
10	*faecium*	-	-	≤ 1	S
11	*faecium*	-	-	≤ 1	S
12	*faecium*	-	-	≤ 1	S
13	*hirae/durans*	-	-	≤ 1	S
14	*faecium*	-	-	≤ 1	S
15	*faecalis*	-	-	≤ 1	S
16	*hirae/durans*	-	-	≤ 1	S
17	*faecalis*	-	-	≤ 1	S
18	*faecium*	-	-	≤ 1	S
19	*faecium*	-	-	≤ 1	S
20	*faecium*	-	-	≤ 1	S
21	*faecium*	-	-	≤ 1	S
22	*faecium*	-	-	≤ 1	S
23	*faecium*	-	-	≤ 1	S
24	*faecium*	-	-	≤ 1	S
25	*faecium*	-	-	≤ 1	S
26	*faecium*	-	-	≤ 1	S
27	*faecium*	-	-	≤ 1	S
28	*faecium*	-	-	≤ 1	S
29	*faecium*	-	-	≤ 1	S
30	*faecalis*	-	-	≤ 1	S
31	*faecalis*	-	-	≤ 1	S
32	*faecalis*	-	-	≤ 1	S
33	*faecalis*	-	-	≤ 1	S
34	*faecalis*	-	-	≤ 1	S
35	*faecalis*	-	-	≤ 1	S
36	*faecium*	-	-	≤ 1	S
37	*faecium*	-	-	≤ 1	S
38	*faecalis*	-	-	≤ 1	S
39	*faecium*	-	-	≤ 1	S
40	*faecalis*	-	-	≤ 1	S
41	*faecalis*	-	-	≤ 1	S
42	*faecalis*	-	-	≤ 1	S
43	*faecalis*	-	-	≤ 1	S

44	*hirae*	-	-	≤ 1	S
45	*faecalis*	-	-	≤ 1	S
46	*faecalis*	-	-	≤ 1	S
47	*faecium*	-	-	≤ 1	S
48	*faecalis*	-	-	≤ 1	S
49	*faecium*	-	-	≤ 1	S
50	*faecium*	-	-	≤ 1	S
51	*faecalis*	-	-	≤ 1	S
52	*faecalis*	-	-	≤ 1	S
53	*faecalis*	-	-	≤ 1	S
54	*faecalis*	-	-	≤ 1	S
55	*casseliflavus*	-	-	≤ 1	S
56	*faecalis*	-	-	≤ 1	S
57	*faecalis*	-	-	≤ 1	S
58	*faecalis*	-	-	≤ 1	S
59	*faecalis*	-	-	≤ 1	S
60	*faecalis*	-	-	≤ 1	S
61	*faecalis*	-	-	≤ 1	S
62	*faecalis*	-	-	≤ 1	S
63	*faecalis*	-	-	≤ 1	S
64	*faecalis*	-	-	≤ 1	S
65	*faecalis*	-	-	≤ 1	S
66	*faecalis*	-	-	≤ 1	S
67	*faecalis*	-	-	≤ 1	S
68	*faecalis*	-	-	≤ 1	S
69	*faecalis*	-	-	≤ 1	S
70	*faecalis*	-	-	≤ 1	S
71	*faecalis*	-	-	≤ 1	S
72	*faecalis*	-	-	≤ 1	S
73	*faecalis*	-	-	≤ 1	S
74	*faecalis*	-	-	≤ 1	S
75	*faecium*	-	-	≤ 1	S
76	*faecalis*	-	-	≤ 1	S
77	*faecalis*	-	-	≤ 1	S
78	*faecalis*	-	-	≤ 1	S
79	*faecalis*	-	-	≤ 1	S
80	*faecalis*	-	-	≤ 1	S
81	*faecalis*	-	-	≤ 1	S
82	*faecalis*	-	-	≤ 1	S
83	*faecalis*	-	-	≤ 1	S
84	*faecalis*	-	-	≤ 1	S
85	*faecalis*	-	-	≤ 1	S
86	*faecalis*	-	-	≤ 1	S
87	*faecalis*	-	-	≤ 1	S
88	*faecalis*	-	-	≤ 1	S

89	*faecalis*	-	-	≤ 1	S
90	*faecalis*	-	-	≤ 1	S
91	*faecalis*	-	-	≤ 1	S
92	*faecalis*	-	-	≤ 1	S
93	*faecalis*	-	-	≤ 1	S
94	*faecium*	-	-	≤ 1	S
95	*faecalis*	-	-	≤ 1	S
96	*faecium*	-	-	≤ 1	S
97	*faecium*	-	-	≤ 1	S
98	*faecium*	-	-	≤ 1	S
99	*faecium*	-	-	≤ 1	S
100	*faecium*	-	-	≤ 1	S
101	*faecalis*	-	-	≤ 1	S
102	*faecalis*	-	-	≤ 1	S
103	*faecium*	-	-	≤ 1	S
104	*faecium*	-	-	≤ 1	S
105	*faecium*	-	-	≤ 1	S
106	*faecium*	-	-	≤ 1	S
107	*hirae/durans*	-	-	≤ 1	S
108	*faecium*	-	-	≤ 1	S
109	*faecium*	-	-	≤ 1	S
110	*faecium*	-	-	≤ 1	S
111	*faecium*	-	-	≤ 1	S
112	*faecium*	-	-	≤ 1	S
113	*hirae/durans*	-	-	≤ 1	S
114	*hirae/durans*	-	-	≤ 1	S
115	*faecalis*	-	-	≤ 1	S
116	*faecium*	-	-	≤ 1	S
117	*faecium*	-	-	≤ 1	S
118	*faecium*	-	-	≤ 1	S
119	*faecium*	-	-	≤ 1	S
120	*faecium*	-	-	≤ 1	S
121	*hirae/durans*	-	-	≤ 1	S
122	*hirae/durans*	-	-	≤ 1	S
123	*faecium*	-	-	≤ 1	S
124	*faecium*	-	-	≤ 1	S
125	*faecium*	-	-	≤ 1	S
126	*faecium*	-	-	≤ 1	S
127	*hirae/durans*	-	-	≤ 1	S
128	*hirae/durans*	-	-	≤ 1	S
129	*raffinosus*	-	-	≤ 1	S
130	*faecium*	-	-	≤ 1	S
131	*faecium*	-	-	≤ 1	S
132	*faecium*	-	-	≤ 1	S
133	*faecalis*	-	-	≤ 1	S

134	*faecium*	-	-	≤ 1	S
135	*faecalis*	-	-	≤ 1	S
136	*faecium*	-	-	≤ 1	S
137	*faecium*	-	-	≤ 1	S
138	*faecium*	-	-	≤ 1	S
139	*raffinosus*	-	-	≤ 1	S
140	*faecium*	-	-	≤ 1	S
141	*faecium*	-	-	≤ 1	S
142	*faecium*	-	-	≤ 1	S
143	*faecium*	-	-	≤ 1	S
144	*hirae/durans*	-	-	≤ 1	S
145	*faecium*	-	-	≤ 1	S
146	*faecium*	-	-	≤ 1	S
147	*faecium*	-	-	≤ 1	S
148	*faecium*	-	-	≤ 1	S
149	*faecium*	-	-	≤ 1	S
150	*hirae/durans*	-	-	≤ 1	S
151	*hirae/durans*	-	-	≤ 1	S
152	*hirae/durans*	-	-	≤ 1	S
153	*hirae/durans*	-	-	≤ 1	S
154	*faecium*	-	-	≤ 1	S
155	*faecium*	-	-	≤ 1	S
156	*faecium*	-	-	≤ 1	S
157	*faecalis*	-	-	≤ 1	S
158	*faecium*	-	-	≤ 1	S
159	*faecium*	-	-	≤ 1	S
160	*faecium*	-	-	≤ 1	S
161	*faecalis*	-	-	≤ 1	S
162	*faecalis*	-	-	≤ 1	S
163	*faecalis*	-	-	≤ 1	S
164	*faecalis*	-	-	≤ 1	S
165	*faecalis*	-	-	≤ 1	S
166	*faecalis*	-	-	≤ 1	S
167	*faecium*	-	-	≤ 1	S
168	*faecalis*	-	-	≤ 1	S
169	*faecium*	-	-	≤ 1	S
170	*gallinarum*	-	-	≤ 1	S
171	*casseliflavus*	-	-	≤ 1	S
172	*gallinarum*	-	-	≤ 1	S
173	*gallinarum*	-	-	≤ 1	S
174	*gallinarum*	-	-	≤ 1	S
175	*gallinarum*	-	-	≤ 1	S
176	*casseliflavus*	-	-	≤ 1	S
177	*gallinarum*	-	-	≤ 1	S
178	*gallinarum*	-	-	≤ 1	S

179	*gallinarum*	-	-	≤ 1	S
180	*casseliflavus*	-	-	≤ 1	S
181	*gallinarum*	-	-	≤ 1	S
182	*gallinarum*	-	-	≤ 1	S
183	*casseliflavus*	-	-	≤ 1	S
184	*gallinarum*	-	-	≤ 1	S
185	*gallinarum*	-	-	≤ 1	S
186	*gallinarum*	-	-	≤ 1	S
187	*casseliflavus*	-	-	≤ 1	S
188	*casseliflavus*	-	-	≤ 1	S
189	*gallinarum*	-	-	≤ 1	S
190	*casseliflavus*	-	-	≤ 1	S
191	*casseliflavus*	-	-	≤ 1	S
192	*gallinarum*	-	-	≤ 1	S
193	*casseliflavus*	-	-	≤ 1	S
194	*gallinarum*	-	-	≤ 1	S
195	*gallinarum*	-	-	≤ 1	S
196	*gallinarum*	-	-	≤ 1	S
197	*gallinarum*	-	-	≤ 1	S
198	*faecium*	-	-	≤ 1	S
199	*gallinarum*	-	-	≤ 1	S
200	*casseliflavus*	-	-	≤ 1	S
201	*gallinarum*	-	-	≤ 1	S
202	*casseliflavus*	-	-	≤ 1	S
203	*casseliflavus*	-	-	≤ 1	S
204	*faecium*	-	-	≤ 1	S
205	*faecium*	-	-	≤ 1	S
206	*faecium*	-	-	≤ 1	S
1C	*faecalis*	-	-	≤ 1	S
2C	*faecium*	-	-	≤ 1	S
3C	*faecium*	-	-	≤ 1	S
4C	N/D	-	-	≤ 1	S
5C	*hirae*	-	-	≤ 1	S
6C	*faecalis*	-	-	≤ 1	S
7C	*faecalis*	-	-	≤ 1	S
8C	*faecalis*	-	-	≤ 1	S
9C	*casseliflavus*	-	-	≤ 1	S
10C	*faecalis*	-	-	≤ 1	S
11C	*faecalis*	-	-	≤ 1	S
12C	*faecalis*	-	-	≤ 1	S
13C	*faecium*	-	-	≤ 1	S
14C	*faecium*	-	-	≤ 1	S
15C	*faecium*	-	-	≤ 1	S
16C	*gallinarum*	-	-	≤ 1	S
17C	*hirae/durans*	-	-	≤ 1	S

18C	*faecium*	-	-	≤ 1	S
19C	*hirae/durans*	-	-	≤ 1	S
20C	*faecium*	-	-	≤ 1	S
21C	*faecalis*	-	-	≤ 1	S
22C	*faecium*	-	-	≤ 1	S
23C	*faecalis*	-	-	≤ 1	S
24C	*hirae/durans*	-	-	≤ 1	S
No.	***Enteroccocus***	**1**	**2**	**MIC**	**S/R**

N/D, not determined

Appendix 12. Antimicrobial sensitivity test result for tetracycline (μg/mL)

No.	*Enteroccocus*	4	16	32	64	MIC	S/R	*tet*K	*tet*L	*tet*M	*tet*O	*tet*S
1	*faecalis*	+	+			> 16	R	-	+	+	-	-
2	*faecalis*	+	+			> 16	R	-	+	+	-	-
3	*faecalis*	+	+			> 16	R	-	+	+	-	-
4	*faecalis*	+	+			> 16	R	-	+	-	-	-
5	*faecium*	+	+			> 16	R	-	+	-	-	-
6	*faecium*	+	+			> 16	R	-	+	-	-	-
7	*faecium*	+	+			> 16	R	-	+	-	-	-
8	*faecium*	+	+			> 16	R	-	+	-	-	-
9	*faecium*	+	+			> 16	R	-	+	-	-	-
10	*faecium*	+	+			> 16	R	-	+	-	-	-
11	*faecium*	+	+			> 16	R	-	+	-	-	-
12	*faecium*	+	+			> 16	R	-	+	-	-	-
13	*hirae/durans*	+	+			> 16	R	-	+	-	-	-
14	*faecium*	+	+			> 16	R	-	+	-	-	-
15	*faecalis*	+	+			> 16	R	-	+	-	-	-
16	*hirae/durans*	+	+			> 16	R	-	+	-	-	-
17	*faecalis*	+	+			> 16	R	-	+	-	-	-
18	*faecium*	+	+			> 16	R	-	+	-	-	-
19	*faecium*	+	+			> 16	R	-	+	+	-	-
20	*faecium*	+	+			> 16	R	-	+	-	-	-
21	*faecium*	+	+			> 16	R	-	+	+	-	-
22	*faecium*	+	+			> 16	R	-	+	+	-	-
23	*faecium*	+	+			> 16	R	-	+	-	-	-
24	*faecium*	+	+			> 16	R	-	+	-	-	-
25	*faecium*	+	+			> 16	R	-	+	+	-	-
26	*faecium*	+	+			> 16	R	-	+	-	-	-
27	*faecium*	+	+			> 16	R	-	+	-	-	-
28	*faecium*	+	+			> 16	R	-	+	-	-	-
29	*faecium*	+	+			> 16	R	-	+	+	-	-
30	*faecalis*	+	+			> 16	R	-	-	-	-	-
31	*faecalis*	+	+			> 16	R	-	-	-	-	-
32	*faecalis*	+	+			> 16	R	-	-	-	-	-
33	*faecalis*	+	+			> 16	R	-	-	+	-	-
34	*faecalis*	+	+			> 16	R	-	+	+	-	-
35	*faecalis*	+	+			> 16	R	-	-	+	-	-
36	*faecium*	+	+			> 16	R	-	+	+	-	-
37	*faecium*	+	+			> 16	R	-	+	-	-	-
38	*faecalis*	+	+			> 16	R	-	-	+	-	-
39	*faecium*	+	+			> 16	R	-	+	-	-	-
40	*faecalis*	+	+			> 16	R	-	-	-	-	-
41	*faecalis*	+	+			> 16	R	-	-	+	-	-
42	*faecalis*	+	+			> 16	R	-	-	-	-	-
43	*faecalis*	+	+			> 16	R	-	-	-	-	-

44	*hirae*	+	+	> 16	R	-	+	-	-	-
45	*faecalis*	+	+	> 16	R	-	+	+	-	-
46	*faecalis*	+	+	> 16	R	-	+	+	-	-
47	*faecium*	+	+	> 16	R	-	+	+	-	-
48	*faecalis*	+	+	> 16	R	-	+	+	-	-
49	*faecium*	+	+	> 16	R	-	+	-	-	-
50	*faecium*	+	+	> 16	R	-	+	+	-	-
51	*faecalis*	+	+	> 16	R	-	+	-	-	-
52	*faecalis*	+	+	> 16	R	-	+	-	-	-
53	*faecalis*	+	+	> 16	R	-	+	-	-	-
54	*faecalis*	+	+	> 16	R	-	-	-	-	-
55	*casseliflavus*	+	+	> 16	R	-	-	+	-	-
56	*faecalis*	+	+	> 16	R	-	+	+	-	-
57	*faecalis*	+	+	> 16	R	-	+	+	-	-
58	*faecalis*	+	+	> 16	R	-	+	+	-	-
59	*faecalis*	+	+	> 16	R	-	+	-	-	-
60	*faecalis*	+	+	> 16	R	-	+	+	-	-
61	*faecalis*	+	+	> 16	R	-	+	+	-	-
62	*faecalis*	+	+	> 16	R	-	+	+	-	-
63	*faecalis*	+	+	> 16	R	-	+	-	-	-
64	*faecalis*	+	+	> 16	R	-	+	+	-	-
65	*faecalis*	+	+	> 16	R	-	+	-	-	-
66	*faecalis*	+	+	> 16	R	-	-	+	-	-
67	*faecalis*	+	+	> 16	R	-	+	+	-	-
68	*faecalis*	+	+	> 16	R	-	-	+	-	-
69	*faecalis*	+	+	> 16	R	-	-	-	-	-
70	*faecalis*	+	+	> 16	R	-	-	+	-	-
71	*faecalis*	+	+	> 16	R	-	-	-	-	-
72	*faecalis*	+	+	> 16	R	-	+	+	-	-
73	*faecalis*	+	+	> 16	R	-	+	-	-	-
74	*faecalis*	+	+	> 16	R	-	-	+	-	-
75	*faecium*	+	+	> 16	R	-	+	+	-	-
76	*faecalis*	+	+	> 16	R	-	-	-	-	-
77	*faecalis*	+	+	> 16	R	-	+	+	-	-
78	*faecalis*	+	+	> 16	R	-	+	-	-	-
79	*faecalis*	+	+	> 16	R	-	+	+	-	-
80	*faecalis*	+	+	> 16	R	-	-	+	-	-
81	*faecalis*	+	+	> 16	R	-	+	-	-	-
82	*faecalis*	+	+	> 16	R	-	-	-	-	-
83	*faecalis*	+	+	> 16	R	-	+	-	-	-
84	*faecalis*	+	+	> 16	R	-	+	+	-	-
85	*faecalis*	+	+	> 16	R	-	+	-	-	-
86	*faecalis*	+	+	> 16	R	-	+	-	-	-
87	*faecalis*	+	+	> 16	R	-	+	+	-	-
88	*faecalis*	+	+	> 16	R	-	+	-	-	-

89	*faecalis*	+	+	> 16	R	-	+	-	-	-
90	*faecalis*	+	+	> 16	R	-	+	+	-	-
91	*faecalis*	+	+	> 16	R	-	-	+	-	-
92	*faecalis*	+	+	> 16	R	-	+	-	-	-
93	*faecalis*	+	+	> 16	R	-	+	+	-	-
94	*faecium*	+	+	> 16	R	-	+	+	-	-
95	*faecalis*	+	+	> 16	R	-	+	+	-	-
96	*faecium*	+	+	> 16	R	-	+	-	-	-
97	*faecium*	+	+	> 16	R	-	+	-	-	-
98	*faecium*	+	+	> 16	R	-	+	-	-	-
99	*faecium*	+	+	> 16	R	-	+	-	-	-
100	*faecium*	+	+	> 16	R	-	+	-	-	-
101	*faecalis*	+	+	> 16	R	-	+	+	-	-
102	*faecalis*	+	+	> 16	R	-	+	+	-	-
103	*faecium*	+	+	> 16	R	-	+	+	-	-
104	*faecium*	+	+	> 16	R	-	+	+	-	-
105	*faecium*	+	+	> 16	R	-	+	-	-	-
106	*faecium*	+	+	> 16	R	-	+	-	-	-
107	*hirae/durans*	+	+	> 16	R	-	+	+	-	-
108	*faecium*	+	+	> 16	R	-	+	+	-	-
109	*faecium*	+	+	> 16	R	-	+	+	-	-
110	*faecium*	+	+	> 16	R	-	+	+	-	-
111	*faecium*	+	+	> 16	R	-	+	+	-	-
112	*faecium*	+	+	> 16	R	-	+	+	-	-
113	*hirae/durans*	+	+	> 16	R	-	+	-	-	-
114	*hirae/durans*	+	+	> 16	R	-	+	-	-	-
115	*faecalis*	+	+	> 16	R	-	-	+	-	-
116	*faecium*	+	+	> 16	R	-	+	+	-	-
117	*faecium*	+	+	> 16	R	-	-	-	-	-
118	*faecium*	+	+	> 16	R	-	+	+	-	-
119	*faecium*	+	+	> 16	R	-	-	-	-	-
120	*faecium*	+	+	> 16	R	-	+	-	-	-
121	*hirae/durans*	+	+	> 16	R	-	+	+	-	-
122	*hirae/durans*	+	+	> 16	R	-	+	+	-	-
123	*faecium*	+	+	> 16	R	-	+	+	-	-
124	*faecium*	+	+	> 16	R	-	+	-	-	-
125	*faecium*	+	+	> 16	R	-	+	+	-	-
126	*faecium*	+	+	> 16	R	-	+	-	-	-
127	*hirae/durans*	+	+	> 16	R	-	+	+	-	-
128	*hirae/durans*	+	+	> 16	R	-	+	-	-	-
129	*raffinosus*	+	+	> 16	R	-	+	+	-	-
130	*faecium*	+	+	> 16	R	-	+	+	-	-
131	*faecium*	+	+	> 16	R	-	+	+	-	-
132	*faecium*	+	+	> 16	R	-	-	-	-	-
133	*faecalis*	+	+	> 16	R	-	+	+	-	-

134	*faecium*	+	+		> 16	R	-	-	-	-	-
135	*faecalis*	+	+		> 16	R	-	+	+	-	-
136	*faecium*	+	+		> 16	R	-	-	-	-	-
137	*faecium*	+	+		> 16	R	-	+	+	-	-
138	*faecium*	+	+		> 16	R	-	+	+	-	-
139	*raffinosus*	+	+		> 16	R	-	+	+	-	-
140	*faecium*	+	+		> 16	R	-	+	+	-	-
141	*faecium*	+	+		> 16	R	-	+	+	-	-
142	*faecium*	+	+		> 16	R	-	+	+	-	-
143	*faecium*	+	+		> 16	R	-	+	+	-	-
144	*hirae/durans*	+	+		> 16	R	-	+	-	-	-
145	*faecium*	+	+		> 16	R	-	+	-	-	-
146	*faecium*	+	+		> 16	R	-	+	+	-	-
147	*faecium*	+	+		> 16	R	-	+	+	-	-
148	*faecium*	+	+		> 16	R	-	+	+	-	-
149	*faecium*	+	+		> 16	R	-	-	-	-	-
150	*hirae/durans*	+	+		> 16	R	-	+	+	-	-
151	*hirae/durans*	+	+		> 16	R	-	+	+	-	-
152	*hirae/durans*	+	+		> 16	R	-	+	+	-	-
153	*hirae/durans*	+	+		> 16	R	-	+	+	-	-
154	*faecium*	+	+		> 16	R	-	+	-	-	-
155	*faecium*	+	+		> 16	R	-	+	-	-	-
156	*faecium*	+	+		> 16	R	-	+	-	-	-
157	*faecalis*	+	+		> 16	R	-	+	+	-	-
158	*faecium*	+	+		> 16	R	-	-	-	-	-
159	*faecium*	+	+		> 16	R	-	+	+	-	-
160	*faecium*	+	+		> 16	R	-	+	+	-	-
161	*faecalis*	+	+		> 16	R	-	-	+	-	-
162	*faecalis*	+	+		> 16	R	-	-	-	-	-
163	*faecalis*	+	+		> 16	R	-	-	+	-	-
164	*faecalis*	+	+		> 16	R	-	-	+	-	-
165	*faecalis*	+	+		> 16	R	-	-	-	-	-
166	*faecalis*	+	+		> 16	R	-	-	-	-	-
167	*faecium*	+	+		> 16	R	-	+	+	-	-
168	*faecalis*	+	+		> 16	R	-	-	+	-	-
169	*faecium*	+	+		> 16	R	-	+	+	-	-
170	*gallinarum*	+	+	+	> 32	R	-	+	-	-	+
171	*casseliflavus*	+	+	+	> 32	R	-	-	-	-	+
172	*gallinarum*	+	+	+	> 32	R	-	-	-	-	+
173	*gallinarum*	+	+	+	> 32	R	-	-	-	-	+
174	*gallinarum*	+	+	+	> 32	R	-	-	+	-	-
175	*gallinarum*	+	+	+	> 32	R	-	-	-	-	+
176	*casseliflavus*	+	+	+	> 32	R	-	-	-	-	+
177	*gallinarum*	+	+	+	> 32	R	-	+	+	-	-
178	*gallinarum*	+	+	+	> 32	R	-	-	+	-	-

179	*gallinarum*	+	+	+		> 32	R	-	+	+	-	-
180	*casseliflavus*	+	+	+		> 32	R	-	-	+	-	-
181	*gallinarum*	+	+	+		> 32	R	-	-	+	-	-
182	*gallinarum*	+	+	+		> 32	R	-	-	-	-	+
183	*casseliflavus*	+	+	+		> 32	R	-	-	-	-	+
184	*gallinarum*	+	+	+		> 32	R	-	-	-	-	+
185	*gallinarum*	+	+	+		> 32	R	+	-	-	-	+
186	*gallinarum*	+	+	+		> 32	R	-	+	-	-	+
187	*casseliflavus*	+	+	+		> 32	R	-	+	-	-	+
188	*casseliflavus*	+	+	+	+	> 64	R	-	-	+	-	+
189	*gallinarum*	+	+			> 16	R	-	-	+	-	-
190	*casseliflavus*	+	+	+	+	> 64	R	-	-	-	-	+
191	*casseliflavus*	+	+	+		> 32	R	-	-	-	-	+
192	*gallinarum*	+	+	+		> 32	R	-	+	+	-	+
193	*casseliflavus*	+	+	+		> 32	R	-	-	-	-	+
194	*gallinarum*	+	+	+		> 32	R	-	-	-	-	-
195	*gallinarum*	+	+	+		> 32	R	-	+	+	-	+
196	*gallinarum*	+	+	+		> 32	R	-	-	-	-	+
197	*gallinarum*	+	+	+	+	> 64	R	-	-	-	-	-
198	*faecium*	+	+			> 16	R	-	-	-	-	+
199	*gallinarum*	+	+	+	+	> 64	R	-	-	+	-	-
200	*casseliflavus*	+	+	+	+	> 64	R	-	-	-	-	+
201	*gallinarum*	+	+	+	+	> 64	R	-	+	-	-	+
202	*casseliflavus*	+	+	+	+	> 64	R	-	-	+	-	-
203	*casseliflavus*	+	+	+	+	> 64	R	-	-	-	-	+
204	*faecium*	+	+			> 16	R	-	+	-	-	-
205	*faecium*	+	+			> 16	R	-	-	-	-	-
206	*faecium*	+	+			> 16	R	-	+	-	-	-
1C	*faecalis*	+	+			≥ 16	R	-	+	+	-	-
2C	*faecium*	+	+			≥ 16	R	-	-	+	-	-
3C	*faecium*	+	+			≥ 16	R	-	+	+	-	-
4C	N/D	+	+			≥ 16	R	-	+	-	-	-
5C	*hirae*	-	-			≤ 4	S	-	-	-	-	-
6C	*faecalis*	+	+			≥ 16	R	-	-	+	-	-
7C	*faecalis*	+	+			≥ 16	R	-	+	+	-	-
8C	*faecalis*	+	+			≥ 16	R	-	+	+	-	-
9C	*casseliflavus*	+	+			≥ 16	R	-	+	-	-	-
10C	*faecalis*	+	+			≥ 16	R	-	+	-	-	-
11C	*faecalis*	+	+			≥ 16	R	-	+	+	-	+
12C	*faecalis*	+	+			≥ 16	R	-	-	-	-	-
13C	*faecium*	+	+			≥ 16	R	-	-	-	-	-
14C	*faecium*	+	+			≥ 16	R	-	-	+	-	-
15C	*faecium*	+	+			≥ 16	R	-	+	+	-	-
16C	*gallinarum*	+	+			≥ 16	R	-	+	+	+	-
17C	*hirae/durans*	+	+			≥ 16	R	-	-	+	-	-

18C	*faecium*	+	+			≥ 16	R	-	+	-	-	-
19C	*hirae/durans*	+	+			≥ 16	R	-	+	+	-	-
20C	*faecium*	+	+			≥ 16	R	-	+	-	-	-
21C	*faecalis*	+	+			≥ 16	R	-	+	+	-	-
22C	*faecium*	+	+			≥ 16	R	-	+	-	-	-
23C	*faecalis*	+	+			≥ 16	R	-	+	+	-	-
24C	*hirae/durans*	+	+			≥ 16	R	-	-	-	-	-
No.	***Enteroccocus***	**4**	**16**	**32**	**64**	**MIC**	**S/R**	***tet*K**	***tet*L**	***tet*M**	***tet*O**	***tet*S**

N/D, not determined

Appendix 13. Antimicrobial sensitivity test result for tiamulin (μg/mL)

No.	*Enteroccocus*	2	4	8	16	32	64	MIC	S/R	*rplC*	*rplD*
1	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
2	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
3	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
4	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
5	*faecium*	+	+	+	+	-	-	32	R	-	-
6	*faecium*	+	+	-	-	-	-	8	S	-	-
7	*faecium*	+	+	+	+	-	-	32	I	-	-
8	*faecium*	+	+	+	-	-	-	16	I	-	-
9	*faecium*	+	+	+	+	+	+	> 64	R	-	-
10	*faecium*	+	+	+	+	-	-	32	I	-	-
11	*faecium*	+	+	+	+	+	-	64	R	-	-
12	*faecium*	+	+	+	+	+	+	> 64	R	-	-
13	*hirae/durans*	+	+	+	+	-	-	32	I	-	-
14	*faecium*	+	+	+	+	+	+	> 64	R	-	-
15	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
16	*hirae/durans*	+	+	+	+	+	+	> 64	R	-	-
17	*faecalis*	+	+	+	+	-	-	32	I	-	-
18	*faecium*	+	+	+	+	-	-	32	I	-	-
19	*faecium*	+	+	+	+	+	-	64	R	-	-
20	*faecium*	+	-	-	-	-	-	4	S	-	-
21	*faecium*	+	+	+	+	+	-	64	R	-	-
22	*faecium*	+	+	+	+	+	-	64	R	-	-
23	*faecium*	+	+	+	+	-	-	32	I	-	-
24	*faecium*	+	+	+	+	+	-	64	R	-	-
25	*faecium*	+	+	+	+	+	-	64	R	-	-
26	*faecium*	+	+	+	+	+	-	64	R	-	-
27	*faecium*	+	+	+	+	+	-	64	R	-	-
28	*faecium*	+	+	+	+	-	-	32	I	-	-
29	*faecium*	+	+	+	+	+	-	64	R	-	-
30	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
31	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
32	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
33	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
34	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
35	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
36	*faecium*	+	+	+	+	+	+	> 64	R	-	-
37	*faecium*	+	+	+	+	+	+	> 64	R	-	-
38	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
39	*faecium*	+	+	+	+	-	-	32	I	-	-
40	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
41	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
42	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
43	*faecalis*	+	+	+	+	+	+	> 64	R	-	-

44	*hirae*	+	+	+	+	-	-	32	I	-	-
45	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
46	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
47	*faecium*	+	+	+	+	-	-	32	I	-	-
48	*faecalis*	+	+	+	+	-	-	32	I	-	-
49	*faecium*	+	+	+	+	-	-	32	I	-	-
50	*faecium*	+	-	-	-	-	-	4	S	-	-
51	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
52	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
53	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
54	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
55	*casseliflavus*	+	+	+	+	+	-	64	R	-	-
56	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
57	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
58	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
59	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
60	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
61	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
62	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
63	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
64	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
65	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
66	*faecalis*	+	+	+	+	+	-	64	R	-	-
67	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
68	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
69	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
70	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
71	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
72	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
73	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
74	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
75	*faecium*	+	+	+	+	+	+	> 64	R	-	-
76	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
77	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
78	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
79	*faecalis*	+	+	+	+	+	-	64	R	-	-
80	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
81	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
82	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
83	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
84	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
85	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
86	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
87	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
88	*faecalis*	+	+	+	+	+	+	> 64	R	-	-

89	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
90	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
91	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
92	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
93	*faecalis*	+	+	+	+	+	-	64	R	-	-
94	*faecium*	+	+	+	+	+	+	> 64	R	-	-
95	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
96	*faecium*	+	+	+	+	+	+	> 64	R	-	-
97	*faecium*	+	+	+	+	+	+	> 64	R	-	-
98	*faecium*	+	+	+	+	+	-	64	R	-	-
99	*faecium*	+	+	+	+	+	-	64	R	-	-
100	*faecium*	+	+	+	+	-	-	32	I	-	-
101	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
102	*faecalis*	+	+	+	+	+	+	> 64	R	-	-
103	*faecium*	+	+	+	+	+	+	> 64	R	-	-
104	*faecium*	+	+	+	+	+	-	64	R	-	-
105	*faecium*	+	+	+	+	+	-	64	R	-	-
106	*faecium*	+	+	+	+	+	-	64	R	-	-
107	*hirae/durans*	+	+	+	+	+	-	64	R	-	-
108	*faecium*	+	+	+	+	+	-	64	R	-	-
109	*faecium*	+	+	+	+	+	-	64	R	-	-
110	*faecium*	+	+	+	+	+	-	64	R	-	-
111	*faecium*	+	+	+	+	+	-	64	R	-	-
112	*faecium*	+	+	+	+	-	-	32	I	-	-
113	*hirae/durans*	+	+	+	+	-	-	32	I	-	-
114	*hirae/durans*	+	+	+	+	-	-	32	I	-	-
115	*faecalis*	+	+	+	+	+	-	64	R	-	-
116	*faecium*	+	+	+	+	+	-	64	R	-	-
117	*faecium*	+	+	+	+	+	-	64	R	-	-
118	*faecium*	+	+	+	+	+	-	64	R	-	-
119	*faecium*	+	+	+	+	-	-	32	I	-	-
120	*faecium*	+	+	+	-	-	-	16	I	-	-
121	*hirae/durans*	+	+	+	+	-	-	32	I	-	-
122	*hirae/durans*	+	+	+	+	-	-	32	I	-	-
123	*faecium*	+	+	+	+	+	-	64	R	-	-
124	*faecium*	+	+	+	+	+	-	64	R	-	-
125	*faecium*	+	+	+	+	+	-	64	R	-	-
126	*faecium*	+	+	+	+	-	-	32	I	-	-
127	*hirae/durans*	+	+	+	+	-	-	32	I	-	-
128	*hirae/durans*	+	+	+	+	+	-	64	R	-	-
129	*raffinosus*	+	+	+	+	+	-	64	R	-	-
130	*faecium*	+	+	+	+	+	-	64	R	-	-
131	*faecium*	-	-	-	-	-	-	≤ 2	S	-	-
132	*faecium*	-	-	-	-	-	-	≤ 2	S	-	-
133	*faecalis*	+	+	+	+	+	-	64	R	-	-

134	*faecium*	+	+	+	+	-	-	32	I	-	-
135	*faecalis*	+	+	+	+	+	-	64	R	-	-
136	*faecium*	+	+	+	+	-	-	32	I	-	-
137	*faecium*	+	+	+	+	+	-	64	R	-	-
138	*faecium*	+	+	+	+	+	-	64	R	-	-
139	*raffinosus*	+	+	+	+	+	-	64	R	-	-
140	*faecium*	+	+	+	+	+	-	64	R	-	-
141	*faecium*	+	+	+	+	+	-	64	R	-	-
142	*faecium*	+	+	+	+	+	-	64	R	-	-
143	*faecium*	+	+	+	+	+	-	64	R	-	-
144	*hirae/durans*	+	+	+	+	+	-	64	R	-	-
145	*faecium*	-	-	-	-	-	-	≤ 2	S	-	-
146	*faecium*	-	-	-	-	-	-	≤ 2	S	-	-
147	*faecium*	+	+	+	+	+	-	64	R	-	-
148	*faecium*	+	+	+	+	+	-	64	R	-	-
149	*faecium*	+	+	+	+	+	-	64	R	-	-
150	*hirae/durans*	+	+	+	+	+	-	64	R	-	-
151	*hirae/durans*	+	+	+	+	+	-	64	R	-	-
152	*hirae/durans*	+	+	+	+	+	-	64	R	-	-
153	*hirae/durans*	+	+	+	+	+	-	64	R	-	-
154	*faecium*	+	+	+	+	+	-	64	R	-	-
155	*faecium*	+	+	+	+	+	-	64	R	-	-
156	*faecium*	+	+	+	+	+	-	64	R	-	-
157	*faecalis*	+	+	+	+	+	-	64	R	-	-
158	*faecium*	+	+	+	+	+	-	64	R	-	-
159	*faecium*	-	-	-	-	-	-	≤ 2	S	-	-
160	*faecium*	+	+	+	+	+	-	64	R	-	-
161	*faecalis*	+	+	+	+	+	-	64	R	-	-
162	*faecalis*	+	+	+	+	+	-	64	R	-	-
163	*faecalis*	+	+	+	+	+	-	64	R	-	-
164	*faecalis*	+	+	+	+	+	-	64	R	-	-
165	*faecalis*	+	+	+	+	+	-	64	R	-	-
166	*faecalis*	+	+	+	+	+	-	64	R	-	-
167	*faecium*	+	+	+	+	-	-	32	I	-	-
168	*faecalis*	+	+	+	+	+	-	64	R	-	-
169	*faecium*	+	+	+	+	+	-	64	R	-	-
170	*gallinarum*	+	+	+	+	+	-	64	R	-	-
171	*casseliflavus*	+	+	+	+	+	-	64	R	-	-
172	*gallinarum*	+	+	+	+	+	-	64	R	-	-
173	*gallinarum*	+	+	+	+	+	-	64	R	-	-
174	*gallinarum*	+	+	+	+	-	-	32	I	-	-
175	*gallinarum*	+	+	+	+	+	-	64	R	-	-
176	*casseliflavus*	+	+	+	+	-	-	32	I	-	-
177	*gallinarum*	+	+	+	+	+	-	64	R	-	-
178	*gallinarum*	+	+	+	+	+	-	64	R	-	-

179	*gallinarum*	+	+	+	+	+	-	64	R	-	-
180	*casseliflavus*	+	+	+	+	+	-	64	R	-	-
181	*gallinarum*	+	+	+	+	+	-	64	R	-	-
182	*gallinarum*	+	+	+	+	+	-	64	R	-	-
183	*casseliflavus*	+	+	+	-	-	-	16	I	-	-
184	*gallinarum*	+	+	+	+	+	-	64	R	-	-
185	*gallinarum*	+	+	+	+	+	-	64	R	-	-
186	*gallinarum*	+	+	+	+	+	-	64	R	-	-
187	*casseliflavus*	+	+	+	+	-	-	32	I	-	-
188	*casseliflavus*	+	+	+	+	-	-	32	I	-	-
189	*gallinarum*	+	+	+	+	+	-	64	R	-	-
190	*casseliflavus*	+	+	+	+	-	-	32	I	-	-
191	*casseliflavus*	+	+	+	+	-	-	32	I	-	-
192	*gallinarum*	+	+	+	+	+	-	64	R	-	-
193	*casseliflavus*	+	+	+	+	-	-	32	I	-	-
194	*gallinarum*	+	+	+	+	+	+	> 64	R	-	-
195	*gallinarum*	+	+	+	+	+	-	64	R	-	-
196	*gallinarum*	+	+	+	+	+	-	64	R	-	-
197	*gallinarum*	+	+	+	+	+	-	64	R	-	-
198	*faecium*	+	+	+	+	+	-	64	R	-	-
199	*gallinarum*	+	+	+	+	+	-	64	R	-	-
200	*casseliflavus*	+	+	+	+	-	-	32	I	-	-
201	*gallinarum*	+	+	+	+	+	-	64	R	-	-
202	*casseliflavus*	+	+	+	+	-	-	32	I	-	-
203	*casseliflavus*	+	+	+	+	+	-	64	R	-	-
204	*faecium*	+	+	+	+	+	-	64	R	-	-
205	*faecium*	+	+	+	-	-	-	16	I	-	-
206	*faecium*	+	+	+	+	+	-	64	R	-	-
1C	*faecalis*	+	+	+	+	-	-	32	I	-	-
2C	*faecium*	+	+	+	+	+	-	64	R	-	-
3C	*faecium*	+	+	+	+	+	-	64	R	-	-
4C	N/D	+	+	+	-	-	-	16	I	-	-
5C	*hirae*	+	+	+	+	-	-	32	I	-	-
6C	*faecalis*	+	+	+	+	+	+	≥ 64	R	-	-
7C	*faecalis*	+	+	+	+	+	+	≥ 64	R	-	-
8C	*faecalis*	+	+	+	+	+	+	≥ 64	R	-	-
9C	*casseliflavus*	+	-	-	-	-	-	4	S	-	-
10C	*faecalis*	+	-	-	-	-	-	4	S	-	-
11C	*faecalis*	+	+	+	+	+	+	≥ 64	R	-	-
12C	*faecalis*	+	+	+	+	+	+	≥ 64	R	-	-
13C	*faecium*	-	-	-	-	-	-	≤ 2	S	-	-
14C	*faecium*	-	-	-	-	-	-	≤ 2	S	-	-
15C	*faecium*	-	-	-	-	-	-	≤ 2	S	-	-
16C	*gallinarum*	+	+	+	+	+	-	64	R	-	-
17C	*hirae/durans*	+	+	+	+	+	-	64	R	-	-

18C	*faecium*	+	+	+	+	+	-	64	R	-	-
19C	*hirae/durans*	+	+	+	-	-	-	16	I	-	-
20C	*faecium*	+	+	+	+	+	-	64	R	-	-
21C	*faecalis*	+	+	+	+	+	+	≥ 64	R	-	-
22C	*faecium*	+	+	+	+	+	+	≥ 64	R	-	-
23C	*faecalis*	+	+	+	+	+	+	≥ 64	R	-	-
24C	*hirae/durans*	+	+	+	+	-	-	32	I	-	-
No.	***Enteroccocus***	**2**	**4**	**8**	**16**	**32**	**64**	**MIC**	**S/R**	***rplC***	***rplD***

N/D, not determined

Appendix 14. Antimicrobial sensitivity test result for tylosin (μg/mL)

No.	*Enteroccocus*	0.25	4	8	32	MIC	S/R	*erm*B
1	*faecalis*	+	+	+		> 8	R	-
2	*faecalis*	+	+	+		> 8	R	+
3	*faecalis*	+	+	+		> 8	R	+
4	*faecalis*	+	+	+		> 8	R	+
5	*faecium*	+	+	+		> 8	R	+
6	*faecium*	+	+	+		> 8	R	-
7	*faecium*	+	+	+		> 8	R	-
8	*faecium*	+	+	+		> 8	R	+
9	*faecium*	+	+	+		> 8	R	+
10	*faecium*	+	+	+		> 8	R	+
11	*faecium*	+	+	+		> 8	R	+
12	*faecium*	+	+	+		> 8	R	+
13	*hirae/durans*	+	+	+		> 8	R	+
14	*faecium*	+	+	+		> 8	R	+
15	*faecalis*	+	+	+		> 8	R	-
16	*hirae/durans*	+	+	+		> 8	R	-
17	*faecalis*	+	+	+		> 8	R	+
18	*faecium*	+	+	+	+	64	R	+
19	*faecium*	+	+	+		> 8	R	+
20	*faecium*	+	+	+		> 8	R	+
21	*faecium*	+	+	+		> 8	R	+
22	*faecium*	+	+	+		> 8	R	+
23	*faecium*	+	+	+		> 8	R	+
24	*faecium*	+	+	+		> 8	R	+
25	*faecium*	+	+	+		> 8	R	+
26	*faecium*	+	+	+		> 8	R	+
27	*faecium*	+	+	+		> 8	R	+
28	*faecium*	+	+	+		> 8	R	+
29	*faecium*	+	+	+		> 8	R	+
30	*faecalis*	+	+	+	+	> 32	R	+
31	*faecalis*	+	+	+	+	> 32	R	+
32	*faecalis*	+	+	+	+	> 32	R	+
33	*faecalis*	+	+	+	+	> 32	R	-
34	*faecalis*	+	+	+	+	> 32	R	-
35	*faecalis*	+	+	+	+	> 32	R	-
36	*faecium*	+	+	+	+	64	R	+
37	*faecium*	+	+	+	+	> 32	R	+
38	*faecalis*	+	+	+	+	> 32	R	-
39	*faecium*	+	+	+	+	> 32	R	+
40	*faecalis*	+	+	+	+	> 32	R	-
41	*faecalis*	+	+	+	+	> 32	R	-
42	*faecalis*	+	+	+	+	> 32	R	+
43	*faecalis*	+	+	+	+	> 32	R	-

44	*hirae*	+	+	+	+	> 32	R	+
45	*faecalis*	+	+	+	+	> 32	R	+
46	*faecalis*	+	+	+	+	> 32	R	-
47	*faecium*	+	+	+	+	64	R	+
48	*faecalis*	+	+	+	+	> 32	R	+
49	*faecium*	+	+	+	+	64	R	+
50	*faecium*	+	+	+	+	64	R	+
51	*faecalis*	+	+	+	+	> 32	R	+
52	*faecalis*	+	+	+	+	> 32	R	+
53	*faecalis*	+	+	+	+	> 32	R	+
54	*faecalis*	+	+	+	+	> 32	R	+
55	*casseliflavus*	+	+	+	+	> 32	R	+
56	*faecalis*	+	+	+	+	> 32	R	+
57	*faecalis*	+	+	+	+	> 32	R	+
58	*faecalis*	+	+	+	+	> 32	R	+
59	*faecalis*	+	+	+	+	> 32	R	+
60	*faecalis*	+	+	+	+	> 32	R	-
61	*faecalis*	+	+	+	+	> 32	R	+
62	*faecalis*	+	+	+	+	> 32	R	+
63	*faecalis*	+	+	+	+	> 32	R	+
64	*faecalis*	+	+	+	+	> 32	R	+
65	*faecalis*	+	+	+	+	> 32	R	+
66	*faecalis*	+	+	+	+	> 32	R	+
67	*faecalis*	+	+	+	+	> 32	R	+
68	*faecalis*	+	+	+	+	> 32	R	+
69	*faecalis*	+	+	+	+	> 32	R	+
70	*faecalis*	+	+	+	+	> 32	R	+
71	*faecalis*	+	+	+	+	> 32	R	-
72	*faecalis*	+	+	+	+	> 32	R	+
73	*faecalis*	+	+	+	+	> 32	R	+
74	*faecalis*	+	+	+	+	> 32	R	-
75	*faecium*	+	+	+	+	> 32	R	+
76	*faecalis*	+	+	+	+	> 32	R	-
77	*faecalis*	+	+	+	+	> 32	R	+
78	*faecalis*	+	+	+	+	> 32	R	+
79	*faecalis*	+	+	+	+	> 32	R	+
80	*faecalis*	+	+	+	+	> 32	R	-
81	*faecalis*	+	+	+	+	> 32	R	+
82	*faecalis*	+	+	+	+	> 32	R	+
83	*faecalis*	+	+	+	+	> 32	R	+
84	*faecalis*	+	+	+	+	> 32	R	+
85	*faecalis*	+	+	+	+	> 32	R	+
86	*faecalis*	+	+	+	+	> 32	R	+
87	*faecalis*	+	+	+	+	> 32	R	+
88	*faecalis*	+	+	+	+	> 32	R	+

89	*faecalis*	+	+	+	+	> 32	R	+
90	*faecalis*	+	+	+	+	> 32	R	+
91	*faecalis*	+	+	+	+	> 32	R	+
92	*faecalis*	+	+	+	+	> 32	R	+
93	*faecalis*	+	+	+	+	> 32	R	+
94	*faecium*	+	+	+		> 8	R	+
95	*faecalis*	+	+	+		> 8	R	+
96	*faecium*	+	+	+		> 8	R	+
97	*faecium*	+	+	+		> 8	R	+
98	*faecium*	+	+	+		> 8	R	+
99	*faecium*	+	+	+		> 8	R	+
100	*faecium*	+	+	+		> 8	R	+
101	*faecalis*	+	+	+		> 8	R	+
102	*faecalis*	+	+	+		> 8	R	+
103	*faecium*	+	+	+		> 8	R	+
104	*faecium*	+	+	+		> 8	R	+
105	*faecium*	+	+	+		> 8	R	+
106	*faecium*	+	+	+		> 8	R	+
107	*hirae/durans*	+	+	+		> 8	R	-
108	*faecium*	+	+	+		> 8	R	+
109	*faecium*	+	+	+		> 8	R	+
110	*faecium*	+	+	+		> 8	R	+
111	*faecium*	+	+	+		> 8	R	+
112	*faecium*	+	+	+	+	64	R	+
113	*hirae/durans*	+	+	+		> 8	R	+
114	*hirae/durans*	+	+	+		> 8	R	+
115	*faecalis*	+	+	+		> 8	R	+
116	*faecium*	+	+	+		> 8	R	+
117	*faecium*	+	+	+		> 8	R	-
118	*faecium*	+	+	+		> 8	R	+
119	*faecium*	+	+	+		> 8	R	-
120	*faecium*	+	+	+		> 8	R	+
121	*hirae/durans*	+	+	+		> 8	R	+
122	*hirae/durans*	+	+	+		> 8	R	+
123	*faecium*	+	+	+		> 8	R	+
124	*faecium*	+	+	+	+	64	R	+
125	*faecium*	+	+	+		> 8	R	+
126	*faecium*	+	+	+	+	64	R	+
127	*hirae/durans*	+	+	+		> 8	R	+
128	*hirae/durans*	+	+	+		> 8	R	+
129	*raffinosus*	+	+	+		> 8	R	-
130	*faecium*	+	+	+		> 8	R	+
131	*faecium*	+	+	+		> 8	R	-
132	*faecium*	+	+	+		> 8	R	-
133	*faecalis*	+	+	+		> 8	R	-

134	*faecium*	+	+	+	> 8	R	-
135	*faecalis*	+	+	+	> 8	R	-
136	*faecium*	+	+	+	> 8	R	-
137	*faecium*	+	+	+	> 8	R	+
138	*faecium*	+	+	+	> 8	R	+
139	*raffinosus*	+	+	+	> 8	R	+
140	*faecium*	+	+	+	> 8	R	+
141	*faecium*	+	+	+	> 8	R	+
142	*faecium*	+	+	+	> 8	R	+
143	*faecium*	+	+	+	> 8	R	+
144	*hirae/durans*	+	+	+	> 8	R	+
145	*faecium*	+	+	+	> 8	R	-
146	*faecium*	+	+	+	> 8	R	-
147	*faecium*	+	+	+	> 8	R	+
148	*faecium*	+	+	+	> 8	R	+
149	*faecium*	+	+	+	> 8	R	+
150	*hirae/durans*	+	+	+	> 8	R	+
151	*hirae/durans*	+	+	+	> 8	R	+
152	*hirae/durans*	+	+	+	> 8	R	+
153	*hirae/durans*	+	+	+	> 8	R	+
154	*faecium*	+	+	+	> 8	R	+
155	*faecium*	+	+	+	> 8	R	+
156	*faecium*	+	+	+	> 8	R	+
157	*faecalis*	+	+	+	> 8	R	-
158	*faecium*	+	+	+	> 8	R	-
159	*faecium*	+	+	+	> 8	R	+
160	*faecium*	+	+	+	> 8	R	+
161	*faecalis*	+	+	+	> 8	R	+
162	*faecalis*	+	+	+	> 8	R	+
163	*faecalis*	+	+	+	> 8	R	+
164	*faecalis*	+	+	+	> 8	R	+
165	*faecalis*	+	+	+	> 8	R	+
166	*faecalis*	+	+	+	> 8	R	+
167	*faecium*	+	+	+	> 8	R	+
168	*faecalis*	+	+	+	> 8	R	+
169	*faecium*	+	+	+	> 8	R	+
170	*gallinarum*	+	+	+	> 8	R	+
171	*casseliflavus*	+	+	+	> 8	R	+
172	*gallinarum*	+	+	+	> 8	R	+
173	*gallinarum*	+	+	+	> 8	R	+
174	*gallinarum*	+	+	+	> 8	R	+
175	*gallinarum*	+	+	+	> 8	R	+
176	*casseliflavus*	+	+	+	> 8	R	+
177	*gallinarum*	+	+	+	> 8	R	+
178	*gallinarum*	+	+	+	> 8	R	-

179	*gallinarum*	+	+	+		> 8	R	+
180	*casseliflavus*	+	+	+		> 8	R	-
181	*gallinarum*	+	+	+		> 8	R	-
182	*gallinarum*	+	+	+		> 8	R	+
183	*casseliflavus*	+	+	+		> 8	R	-
184	*gallinarum*	+	+	+		> 8	R	+
185	*gallinarum*	+	+	+		> 8	R	+
186	*gallinarum*	+	+	+		> 8	R	+
187	*casseliflavus*	+	+	+		> 8	R	+
188	*casseliflavus*	+	+	+		> 8	R	+
189	*gallinarum*	+	+	+		> 8	R	+
190	*casseliflavus*	+	+	+		> 8	R	+
191	*casseliflavus*	+	+	+		> 8	R	+
192	*gallinarum*	+	+	+		> 8	R	+
193	*casseliflavus*	+	+	+		> 8	R	+
194	*gallinarum*	+	+	+		> 8	R	+
195	*gallinarum*	+	+	+		> 8	R	+
196	*gallinarum*	+	+	+		> 8	R	+
197	*gallinarum*	+	+	+		> 8	R	+
198	*faecium*	+	+	+	+	64	R	+
199	*gallinarum*	+	+	+		> 8	R	+
200	*casseliflavus*	+	+	+		> 8	R	+
201	*gallinarum*	+	+	+		> 8	R	+
202	*casseliflavus*	+	+	+		> 8	R	+
203	*casseliflavus*	+	+	+		> 8	R	-
204	*faecium*	+	+	+	+	64	R	+
205	*faecium*	+	+	+	+	64	R	+
206	*faecium*	+	+	+	+	64	R	+
1C	*faecalis*	+	+	+		≥ 8	R	+
2C	*faecium*	+	+	+		≥ 8	R	+
3C	*faecium*	+	+	+		≥ 8	R	+
4C	N/D	+	+	+		≥ 8	R	-
5C	*hirae*	+	+	-		8	S	-
6C	*faecalis*	+	+	+		≥ 8	R	+
7C	*faecalis*	+	+	+		≥ 8	R	+
8C	*faecalis*	+	+	+		≥ 8	R	+
9C	*casseliflavus*	+	+	+		≥ 8	R	-
10C	*faecalis*	+	+	+		≥ 8	R	-
11C	*faecalis*	+	+	+		≥ 8	R	+
12C	*faecalis*	+	+	+		≥ 8	R	+
13C	*faecium*	+	+	+		≥ 8	R	-
14C	*faecium*	+	+	+		≥ 8	R	+

15C	*faecium*	+	+	+		≥ 8	R	-
16C	*gallinarum*	+	+	+		≥ 8	R	-
17C	*hirae/durans*	+	+	+		≥ 8	R	-
18C	*faecium*	+	+	+		≥ 8	R	+
19C	*hirae/durans*	+	+	+		≥ 8	R	+
20C	*faecium*	+	+	+		≥ 8	R	+
21C	*faecalis*	+	+	+		≥ 8	R	+
22C	*faecium*	+	+	+		≥ 8	R	+
23C	*faecalis*	+	+	+		≥ 8	R	+
24C	*hirae/durans*	+	+	+		≥ 8	R	-
No.	***Enteroccocus***	**0.25**	**4**	**8**	**32**	**MIC**	**S/R**	***erm*B**

N/D, not determined

Appendix 15. Antimicrobial sensitivity test result for vancomycin (μg/mL)

No.	*Enteroccocus*	4	8	32	MIC	S/R	*van*A	*van*B	*van*C1	*van*C2
1	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
2	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
3	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
4	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
5	*faecium*	-	-	-	≤ 4	S	-	-	-	-
6	*faecium*	-	-	-	≤ 4	S	-	-	-	-
7	*faecium*	-	-	-	≤ 4	S	-	-	-	-
8	*faecium*	-	-	-	≤ 4	S	-	-	-	-
9	*faecium*	-	-	-	≤ 4	S	-	-	-	-
10	*faecium*	-	-	-	≤ 4	S	-	-	-	-
11	*faecium*	-	-	-	≤ 4	S	-	-	-	-
12	*faecium*	-	-	-	≤ 4	S	-	-	-	-
13	*hirae/durans*	-	-	-	≤ 4	S	-	-	-	-
14	*faecium*	-	-	-	≤ 4	S	-	-	-	-
15	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
16	*hirae/durans*	-	-	-	≤ 4	S	-	-	-	-
17	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
18	*faecium*	+	+	+	> 32	R	+	-	-	-
19	*faecium*	-	-	-	≤ 4	S	-	-	-	-
20	*faecium*	-	-	-	≤ 4	S	-	-	-	-
21	*faecium*	-	-	-	≤ 4	S	-	-	-	-
22	*faecium*	-	-	-	≤ 4	S	-	-	-	-
23	*faecium*	-	-	-	≤ 4	S	-	-	-	-
24	*faecium*	-	-	-	≤ 4	S	-	-	-	-
25	*faecium*	-	-	-	≤ 4	S	-	-	-	-
26	*faecium*	-	-	-	≤ 4	S	-	-	-	-
27	*faecium*	-	-	-	≤ 4	S	-	-	-	-
28	*faecium*	-	-	-	≤ 4	S	-	-	-	-
29	*faecium*	-	-	-	≤ 4	S	-	-	-	-
30	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
31	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
32	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
33	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
34	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
35	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
36	*faecium*	+	+	+	> 32	R	+	-	-	-
37	*faecium*	-	-	-	≤ 4	S	-	-	-	-
38	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
39	*faecium*	-	-	-	≤ 4	S	-	-	-	-
40	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
41	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
42	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
43	*faecalis*	-	-	-	≤ 4	S	-	-	-	-

44	*hirae*	-	-	-	≤ 4	S	-	-	-	-
45	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
46	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
47	*faecium*	+	+	+	> 32	R	+	-	-	-
48	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
49	*faecium*	+	+	+	> 32	R	+	-	-	-
50	*faecium*	+	+	+	> 32	R	+	-	-	-
51	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
52	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
53	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
54	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
55	*casseliflavus*	+	+	-	32	I	-	-	+	-
56	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
57	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
58	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
59	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
60	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
61	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
62	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
63	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
64	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
65	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
66	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
67	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
68	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
69	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
70	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
71	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
72	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
73	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
74	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
75	*faecium*	-	-	-	≤ 4	S	-	-	-	-
76	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
77	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
78	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
79	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
80	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
81	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
82	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
83	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
84	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
85	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
86	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
87	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
88	*faecalis*	-	-	-	≤ 4	S	-	-	-	-

89	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
90	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
91	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
92	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
93	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
94	*faecium*	-	-	-	≤ 4	S	-	-	-	-
95	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
96	*faecium*	-	-	-	≤ 4	S	-	-	-	-
97	*faecium*	-	-	-	≤ 4	S	-	-	-	-
98	*faecium*	-	-	-	≤ 4	S	-	-	-	-
99	*faecium*	-	-	-	≤ 4	S	-	-	-	-
100	*faecium*	-	-	-	≤ 4	S	-	-	-	-
101	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
102	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
103	*faecium*	-	-	-	≤ 4	S	-	-	-	-
104	*faecium*	-	-	-	≤ 4	S	-	-	-	-
105	*faecium*	-	-	-	≤ 4	S	-	-	-	-
106	*faecium*	-	-	-	≤ 4	S	-	-	-	-
107	*hirae/durans*	-	-	-	≤ 4	S	-	-	-	-
108	*faecium*	-	-	-	≤ 4	S	-	-	-	-
109	*faecium*	-	-	-	≤ 4	S	-	-	-	-
110	*faecium*	-	-	-	≤ 4	S	-	-	-	-
111	*faecium*	-	-	-	≤ 4	S	-	-	-	-
112	*faecium*	+	+	+	> 32	R	+	-	-	-
113	*hirae/durans*	-	-	-	≤ 4	S	-	-	-	-
114	*hirae/durans*	-	-	-	≤ 4	S	-	-	-	-
115	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
116	*faecium*	-	-	-	≤ 4	S	-	-	-	-
117	*faecium*	-	-	-	≤ 4	S	-	-	-	-
118	*faecium*	-	-	-	≤ 4	S	-	-	-	-
119	*faecium*	-	-	-	≤ 4	S	-	-	-	-
120	*faecium*	-	-	-	≤ 4	S	-	-	-	-
121	*hirae/durans*	-	-	-	≤ 4	S	-	-	-	-
122	*hirae/durans*	-	-	-	≤ 4	S	-	-	-	-
123	*faecium*	-	-	-	≤ 4	S	-	-	-	-
124	*faecium*	+	+	+	> 32	R	+	-	-	-
125	*faecium*	-	-	-	≤ 4	S	-	-	-	-
126	*faecium*	+	+	+	> 32	R	+	-	-	-
127	*hirae/durans*	-	-	-	≤ 4	S	-	-	-	-
128	*hirae/durans*	-	-	-	≤ 4	S	-	-	-	-
129	*raffinosus*	-	-	-	≤ 4	S	-	-	-	-
130	*faecium*	-	-	-	≤ 4	S	-	-	-	-
131	*faecium*	-	-	-	≤ 4	S	-	-	-	-
132	*faecium*	-	-	-	≤ 4	S	-	-	-	-
133	*faecalis*	-	-	-	≤ 4	S	-	-	-	-

134	*faecium*	-	-	-	≤ 4	S	-	-	-	-
135	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
136	*faecium*	-	-	-	≤ 4	S	-	-	-	-
137	*faecium*	-	-	-	≤ 4	S	-	-	-	-
138	*faecium*	-	-	-	≤ 4	S	-	-	-	-
139	*raffinosus*	-	-	-	≤ 4	S	-	-	-	-
140	*faecium*	-	-	-	≤ 4	S	-	-	-	-
141	*faecium*	-	-	-	≤ 4	S	-	-	-	-
142	*faecium*	-	-	-	≤ 4	S	-	-	-	-
143	*faecium*	-	-	-	≤ 4	S	-	-	-	-
144	*hirae/durans*	-	-	-	≤ 4	S	-	-	-	-
145	*faecium*	-	-	-	≤ 4	S	-	-	-	-
146	*faecium*	-	-	-	≤ 4	S	-	-	-	-
147	*faecium*	-	-	-	≤ 4	S	-	-	-	-
148	*faecium*	-	-	-	≤ 4	S	-	-	-	-
149	*faecium*	-	-	-	≤ 4	S	-	-	-	-
150	*hirae/durans*	-	-	-	≤ 4	S	-	-	-	-
151	*hirae/durans*	-	-	-	≤ 4	S	-	-	-	-
152	*hirae/durans*	-	-	-	≤ 4	S	-	-	-	-
153	*hirae/durans*	-	-	-	≤ 4	S	-	-	-	-
154	*faecium*	-	-	-	≤ 4	S	-	-	-	-
155	*faecium*	-	-	-	≤ 4	S	-	-	-	-
156	*faecium*	-	-	-	≤ 4	S	-	-	-	-
157	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
158	*faecium*	-	-	-	≤ 4	S	-	-	-	-
159	*faecium*	-	-	-	≤ 4	S	-	-	-	-
160	*faecium*	-	-	-	≤ 4	S	-	-	-	-
161	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
162	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
163	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
164	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
165	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
166	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
167	*faecium*	-	-	-	≤ 4	S	-	-	-	-
168	*faecalis*	-	-	-	≤ 4	S	-	-	-	-
169	*faecium*	-	-	-	≤ 4	S	-	-	-	-
170	*gallinarum*	+	+		> 8	R	-	-	+	-
171	*casseliflavus*	+	+		> 8	R	-	-	+	+
172	*gallinarum*	+	+		> 8	R	-	-	+	-
173	*gallinarum*	+	+		> 8	R	-	-	+	-
174	*gallinarum*	+	+		> 8	R	-	-	-	+
175	*gallinarum*	+	+		> 8	R	-	-	+	-
176	*casseliflavus*	+	+		> 8	R	-	-	-	-
177	*gallinarum*	+	+		> 8	R	-	-	-	+
178	*gallinarum*	+	+		> 8	R	-	-	-	-

179	*gallinarum*	+	+		> 8	R	-	-	-	-
180	*casseliflavus*	+	+		> 8	R	-	-	+	-
181	*gallinarum*	+	+		> 8	R	-	-	+	-
182	*gallinarum*	+	+		> 8	R	-	-	+	-
183	*casseliflavus*	+	+		> 8	R	-	-	-	+
184	*gallinarum*	+	+		> 8	R	-	-	+	-
185	*gallinarum*	+	+		> 8	R	-	-	+	-
186	*gallinarum*	+	+		> 8	R	-	-	+	-
187	*casseliflavus*	+	+		> 8	R	-	-	-	+
188	*casseliflavus*	+	+		> 8	R	-	-	-	+
189	*gallinarum*	+	+		> 8	R	-	-	+	-
190	*casseliflavus*	+	+		> 8	R	-	-	-	+
191	*casseliflavus*	+	+		> 8	R	-	-	-	+
192	*gallinarum*	+	+		> 8	R	-	-	+	-
193	*casseliflavus*	+	+		> 8	R	-	-	-	+
194	*gallinarum*	+	+		> 8	R	-	-	+	-
195	*gallinarum*	+	+		> 8	R	-	-	+	-
196	*gallinarum*	+	+		> 8	R	-	-	+	-
197	*gallinarum*	+	+		> 8	R	-	-	+	-
198	*faecium*	+	+	+	> 32	R	-	-	-	+
199	*gallinarum*	+	+		> 8	R	-	-	+	-
200	*casseliflavus*	+	+		> 8	R	-	-	-	+
201	*gallinarum*	+	+		> 8	R	-	-	+	-
202	*casseliflavus*	+	+		> 8	R	-	-	-	+
203	*casseliflavus*	+	+		> 8	R	-	-	-	+
204	*faecium*	+	+	+	> 32	R	+	-	-	-
205	*faecium*	+	+	+	> 32	R	+	-	-	-
206	*faecium*	+	+	+	> 32	R	+	-	-	-
1C	*faecalis*	-	-		≤ 4	S	-	-	-	-
2C	*faecium*	-	-		≤ 4	S	-	-	-	-
3C	*faecium*	-	-		≤ 4	S	-	-	-	-
4C	N/D	-	-		≤ 4	S	-	-	-	-
5C	*hirae*	-	-		≤ 4	S	-	-	-	-
6C	*faecalis*	-	-		≤ 4	S	-	-	-	-
7C	*faecalis*	-	-		≤ 4	S	-	-	-	-
8C	*faecalis*	-	-		≤ 4	S	-	-	+	-
9C	*casseliflavus*	-	-		≤ 4	S	-	-	-	-
10C	*faecalis*	-	-		≤ 4	S	-	-	-	-
11C	*faecalis*	-	-		≤ 4	S	+	-	-	-
12C	*faecalis*	-	-		≤ 4	S	-	-	-	-
13C	*faecium*	-	-		≤ 4	S	-	-	-	-
14C	*faecium*	-	-		≤ 4	S	+	-	-	-
15C	*faecium*	-	-		≤ 4	S	-	-	-	-
16C	*gallinarum*	-	-		≤ 4	S	-	-	+	-
17C	*hirae/durans*	-	-		≤ 4	S	-	-	-	-

18C	*faecium*	-	-		≤ 4	S	-	-	-	-
19C	*hirae/durans*	-	-		≤ 4	S	-	-	-	-
20C	*faecium*	-	-		≤ 4	S	-	-	-	-
21C	*faecalis*	-	-		≤ 4	S	-	-	-	-
22C	*faecium*	-	-		≤ 4	S	-	-	-	-
23C	*faecalis*	-	-		≤ 4	S	-	-	-	-
24C	*hirae/durans*	-	-		≤ 4	S	-	-	-	-
No.	***Enteroccocus***	**4**	**8**	**32**	**MIC**	**S/R**	***van*A**	***van*B**	***van*C1**	***van*C2**

N/D, not determined

Appendix 16. Antimicrobial sensitivity test result for virginiamycin (μg/mL)

No.	*Enteroccocus*	4	8	16	MIC	S/R	*erm*B	*lsa*A	*lsa*B	*vat*A	*vat*D	*vat*E
1	*faecalis*	-	-	-	≤ 4	S	-	-	+	-	-	-
2	*faecalis*	-	-	-	≤ 4	S	+	-	+	-	-	-
3	*faecalis*	-	-	-	≤ 4	S	+	-	+	-	-	-
4	*faecalis*	-	-	-	≤ 4	S	+	-	-	-	-	-
5	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
6	*faecium*	-	-	-	≤ 4	S	-	-	-	-	-	-
7	*faecium*	-	-	-	≤ 4	S	-	-	-	-	-	-
8	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
9	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
10	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
11	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
12	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
13	*hirae/durans*	-	-	-	≤ 4	S	+	-	-	-	-	-
14	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
15	*faecalis*	-	-	-	≤ 4	S	-	-	-	-	-	-
16	*hirae/durans*	-	-	-	≤ 4	S	-	-	-	-	-	-
17	*faecalis*	-	-	-	≤ 4	S	+	-	-	-	-	-
18	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	+
19	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
20	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
21	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
22	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
23	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
24	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
25	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
26	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
27	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
28	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
29	*faecium*	+	+	-	16	R	+	-	-	-	-	+
30	*faecalis*	-	-	-	≤ 4	S	+	-	-	-	-	-
31	*faecalis*	-	-	-	≤ 4	S	+	-	-	-	-	-
32	*faecalis*	-	-	-	≤ 4	S	+	-	+	-	-	-
33	*faecalis*	+	+		> 8	R	-	-	+	-	-	-
34	*faecalis*	+	+		> 8	R	-	-	+	-	-	-
35	*faecalis*	+	+		> 8	R	-	-	+	-	-	-
36	*faecium*	+	+		> 8	R	+	-	-	-	-	+
37	*faecium*	+	+		> 8	R	+	-	-	-	-	-
38	*faecalis*	+	+		> 8	R	-	-	+	-	-	-
39	*faecium*	+	+		> 8	R	+	-	-	-	-	-
40	*faecalis*	+	+		> 8	R	-	-	+	-	-	-
41	*faecalis*	+	+		> 8	R	-	+	+	-	-	-
42	*faecalis*	+	+		> 8	R	+	-	-	-	-	-
43	*faecalis*	+	+		> 8	R	-	-	+	-	-	-

44	*hirae*	+	+	> 8	R	+	-	-	-	-	-
45	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
46	*faecalis*	+	+	> 8	R	-	-	+	-	-	-
47	*faecium*	+	+	> 8	R	+	-	-	-	-	-
48	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
49	*faecium*	+	+	> 8	R	+	-	-	-	-	-
50	*faecium*	+	+	> 8	R	+	-	-	-	-	+
51	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
52	*faecalis*	+	+	> 8	R	+	-	-	-	-	-
53	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
54	*faecalis*	+	+	> 8	R	+	-	-	-	-	-
55	*casseliflavus*	+	+	> 8	R	+	-	-	-	-	-
56	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
57	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
58	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
59	*faecalis*	+	+	> 8	R	+	-	-	-	-	-
60	*faecalis*	+	+	> 8	R	-	-	+	-	-	-
61	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
62	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
63	*faecalis*	+	+	> 8	R	+	-	-	-	-	-
64	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
65	*faecalis*	+	+	> 8	R	+	-	-	-	-	-
66	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
67	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
68	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
69	*faecalis*	+	+	> 8	R	+	-	-	-	-	-
70	*faecalis*	+	+	> 8	R	+	-	-	-	-	-
71	*faecalis*	+	+	> 8	R	-	-	+	-	-	-
72	*faecalis*	+	+	> 8	R	+	-	-	-	-	-
73	*faecalis*	+	+	> 8	R	+	-	-	-	-	-
74	*faecalis*	+	+	> 8	R	-	-	+	-	-	-
75	*faecium*	+	+	> 8	R	+	-	-	-	-	-
76	*faecalis*	+	+	> 8	R	-	-	-	-	-	-
77	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
78	*faecalis*	+	+	> 8	R	+	-	-	-	-	-
79	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
80	*faecalis*	+	+	> 8	R	-	-	+	-	-	-
81	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
82	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
83	*faecalis*	+	+	> 8	R	+	-	-	-	-	-
84	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
85	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
86	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
87	*faecalis*	+	+	> 8	R	+	-	+	-	-	-
88	*faecalis*	+	+	> 8	R	+	-	+	-	-	+

89	*faecalis*	+	+		> 8	R	+	-	+	-	-	-
90	*faecalis*	+	+		> 8	R	+	-	+	-	-	-
91	*faecalis*	+	+		> 8	R	+	-	+	-	-	-
92	*faecalis*	+	+		> 8	R	+	-	-	-	-	-
93	*faecalis*	+	+		> 8	R	+	-	+	-	-	-
94	*faecium*	-	-	-	≤ 4	S	+	-	+	-	-	-
95	*faecalis*	-	-	-	≤ 4	S	+	-	+	-	-	-
96	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
97	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
98	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
99	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
100	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
101	*faecalis*	-	-	-	≤ 4	S	+	-	-	-	-	-
102	*faecalis*	-	-	-	≤ 4	S	+	-	-	-	-	-
103	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
104	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
105	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
106	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
107	*hirae/durans*	-	-	-	≤ 4	S	-	-	-	-	-	-
108	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
109	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
110	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
111	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
112	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
113	*hirae/durans*	-	-	-	≤ 4	S	+	-	-	-	-	-
114	*hirae/durans*	-	-	-	≤ 4	S	+	-	-	-	-	-
115	*faecalis*	-	-	-	≤ 4	S	+	-	+	-	-	-
116	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
117	*faecium*	-	-	-	≤ 4	S	-	-	-	-	-	-
118	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
119	*faecium*	-	-	-	≤ 4	S	-	-	-	-	-	-
120	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
121	*hirae/durans*	-	-	-	≤ 4	S	+	-	-	-	-	-
122	*hirae/durans*	-	-	-	≤ 4	S	+	-	-	-	-	-
123	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
124	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	+
125	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
126	*faecium*	+	+	-	16	R	+	-	-	-	-	+
127	*hirae/durans*	+	+	-	16	R	+	-	-	-	-	+
128	*hirae/durans*	+	+	+	> 16	R	+	-	-	-	-	+
129	*raffinosus*	-	-	-	≤ 4	S	-	+	+	-	-	-
130	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
131	*faecium*	-	-	-	≤ 4	S	-	-	-	-	-	-
132	*faecium*	-	-	-	≤ 4	S	-	-	-	-	-	-
133	*faecalis*	-	-	-	≤ 4	S	-	+	+	-	-	-

134	*faecium*	-	-	-	≤ 4	S	-	-	-	-	-	-
135	*faecalis*	-	-	-	≤ 4	S	-	+	+	-	-	-
136	*faecium*	-	-	-	≤ 4	S	-	-	-	-	-	-
137	*faecium*	+	+	-	16	R	+	-	-	-	-	+
138	*faecium*	+	+	-	16	R	+	-	-	-	-	+
139	*raffinosus*	-	-	-	≤ 4	S	+	-	+	-	-	-
140	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
141	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
142	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
143	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
144	*hirae/durans*	+	+	+	> 16	R	+	-	-	-	-	+
145	*faecium*	-	-	-	≤ 4	S	-	-	-	-	-	-
146	*faecium*	-	-	-	≤ 4	S	-	-	-	-	-	-
147	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
148	*faecium*	+	+		8	R	+	-	-	-	-	+
149	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
150	*hirae/durans*	+	+	+	> 16	R	+	-	-	-	-	+
151	*hirae/durans*	+	+	+	> 16	R	+	-	-	-	-	+
152	*hirae/durans*	+	+	+	> 16	R	+	-	-	-	-	+
153	*hirae/durans*	+	+	+	> 16	R	+	-	-	-	-	+
154	*faecium*	+	+	+	> 16	R	+	-	-	-	-	-
155	*faecium*	+	+	+	> 16	R	+	-	-	-	-	-
156	*faecium*	+	+	+	> 16	R	+	-	-	-	-	+
157	*faecalis*	-	-	-	≤ 4	S	-	-	+	-	-	-
158	*faecium*	-	-	-	≤ 4	S	-	-	-	-	-	-
159	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
160	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
161	*faecalis*	-	-	-	≤ 4	S	+	-	+	-	-	-
162	*faecalis*	-	-	-	≤ 4	S	+	-	+	-	-	-
163	*faecalis*	-	-	-	≤ 4	S	+	-	+	-	-	-
164	*faecalis*	-	-	-	≤ 4	S	+	-	-	-	-	-
165	*faecalis*	-	-	-	≤ 4	S	+	-	+	-	-	-
166	*faecalis*	-	-	-	≤ 4	S	+	-	+	-	-	-
167	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
168	*faecalis*	-	-	-	≤ 4	S	+	-	+	-	-	-
169	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
170	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
171	*casseliflavus*	-	-	-	≤ 4	S	+	-	-	-	-	-
172	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
173	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
174	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
175	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
176	*casseliflavus*	-	-	-	≤ 4	S	+	-	-	-	-	-
177	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
178	*gallinarum*	-	-	-	≤ 4	S	-	-	-	-	-	-

179	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
180	*casseliflavus*	-	-	-	≤ 4	S	-	-	-	-	-	-
181	*gallinarum*	-	-	-	≤ 4	S	-	-	-	-	-	-
182	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
183	*casseliflavus*	-	-	-	≤ 4	S	-	-	-	-	-	-
184	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
185	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
186	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
187	*casseliflavus*	-	-	-	≤ 4	S	+	-	-	-	-	-
188	*casseliflavus*	-	-	-	≤ 4	S	+	-	-	-	-	-
189	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
190	*casseliflavus*	-	-	-	≤ 4	S	+	-	-	-	-	-
191	*casseliflavus*	-	-	-	≤ 4	S	+	-	-	-	-	-
192	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
193	*casseliflavus*	-	-	-	≤ 4	S	+	-	-	-	-	-
194	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
195	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
196	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
197	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
198	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
199	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
200	*casseliflavus*	-	-	-	≤ 4	S	+	-	-	-	-	-
201	*gallinarum*	-	-	-	≤ 4	S	+	-	-	-	-	-
202	*casseliflavus*	-	-	-	≤ 4	S	+	-	-	-	-	-
203	*casseliflavus*	-	-	-	≤ 4	S	-	-	-	-	-	-
204	*faecium*	+	-	-	8	S	+	-	-	-	-	+
205	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	+
206	*faecium*	+	-	-	8	S	+	-	-	-	-	+
1C	*faecalis*	-	-	-	≤ 4	S	+	+	+	-	-	-
2C	*faecium*	+	+	-	16	R	+	-	-	-	-	+
3C	*faecium*	-	-	-	≤ 4	S	+	-	+	-	-	-
4C	N/D	-	-	-	≤ 4	S	-	-	-	-	-	-
5C	*hirae*	-	-	-	≤ 4	S	-	-	-	-	-	-
6C	*faecalis*	-	-	-	≤ 4	S	+	-	+	-	-	-
7C	*faecalis*	-	-	-	≤ 4	S	+	-	+	-	-	-
8C	*faecalis*	-	-	-	≤ 4	S	+	-	-	-	-	-
9C	*casseliflavus*	-	-	-	≤ 4	S	-	-	-	-	-	-
10C	*faecalis*	-	-	-	≤ 4	S	-	-	+	-	-	-
11C	*faecalis*	+	+	+	≥ 16	R	+	-	+	-	+	-
12C	*faecalis*	-	-	-	≤ 4	S	+	-	+	-	-	-
13C	*faecium*	-	-	-	≤ 4	S	-	-	-	-	-	-
14C	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
15C	*faecium*	-	-	-	≤ 4	S	-	-	-	-	-	-
16C	*gallinarum*	-	-	-	≤ 4	S	-	-	-	-	-	-
17C	*hirae/durans*	-	-	-	≤ 4	S	-	-	-	-	-	-

18C	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
19C	*hirae/durans*	-	-	-	≤ 4	S	+	-	-	-	-	-
20C	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
21C	*faecalis*	-	-	-	≤ 4	S	+	-	+	-	-	-
22C	*faecium*	-	-	-	≤ 4	S	+	-	-	-	-	-
23C	*faecalis*	-	-	-	≤ 4	S	+	+	+	-	-	-
24C	*hirae/durans*	-	-	-	≤ 4	S	-	-	-	-	-	-
No.	***Enteroccocus***	**4**	**8**	**16**	**MIC**	**S/R**	***erm*B**	***lsa*A**	***lsa*B**	***vat*A**	***vat*D**	***vat*E**

N/D, not determined

Appendix 17. Antimicrobial sensitivity test result for copper (mM/mL)

No.	*Enteroccocus*	2	4	5	6	7	8	12	16	20	24	28	32	MIC	S/R	*tcr*B
1	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
2	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
3	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
4	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
5	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
6	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
7	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
8	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
9	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
10	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
11	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
12	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
13	*hirae/durans*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
14	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
15	*faecalis*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
16	*hirae/durans*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
17	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
18	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
19	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
20	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
21	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
22	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
23	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
24	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
25	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
26	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
27	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
28	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
29	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
30	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
31	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
32	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
33	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
34	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
35	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
36	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
37	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
38	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
39	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
40	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
41	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
42	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
43	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-

44	*hirae*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
45	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
46	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
47	*faecium*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
48	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
49	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
50	*faecium*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
51	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
52	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
53	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
54	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
55	*casseliflavus*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
56	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
57	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
58	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
59	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
60	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
61	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
62	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
63	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
64	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
65	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
66	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
67	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
68	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
69	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
70	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
71	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
72	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
73	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
74	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
75	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
76	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
77	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
78	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
79	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
80	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
81	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
82	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
83	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	-
84	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
85	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
86	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
87	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
88	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+

89	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
90	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
91	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	-
92	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
93	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
94	*faecium*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
95	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
96	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
97	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
98	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
99	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
100	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
101	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
102	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
103	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
104	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
105	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
106	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
107	*hirae/durans*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
108	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
109	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
110	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
111	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
112	*faecium*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
113	*hirae/durans*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
114	*hirae/durans*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
115	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
116	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
117	*faecium*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	+
118	*faecium*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
119	*faecium*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
120	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
121	*hirae/durans*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
122	*hirae/durans*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
123	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
124	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
125	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
126	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
127	*hirae/durans*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
128	*hirae/durans*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
129	*raffinosus*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
130	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
131	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
132	*faecium*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
133	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-

134	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
135	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
136	*faecium*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
137	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
138	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
139	*raffinosus*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
140	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
141	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
142	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
143	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
144	*hirae/durans*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
145	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
146	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
147	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
148	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
149	*faecium*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
150	*hirae/durans*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
151	*hirae/durans*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
152	*hirae/durans*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
153	*hirae/durans*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
154	*faecium*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
155	*faecium*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
156	*faecium*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
157	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
158	*faecium*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
159	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
160	*faecium*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	+
161	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
162	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
163	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
164	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
165	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
166	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
167	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
168	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
169	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
170	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
171	*casseliflavus*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
172	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
173	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
174	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
175	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
176	*casseliflavus*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
177	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
178	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-

179	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
180	*casseliflavus*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
181	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
182	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
183	*casseliflavus*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
184	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
185	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
186	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
187	*casseliflavus*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	+
188	*casseliflavus*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	+
189	*gallinarum*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
190	*casseliflavus*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
191	*casseliflavus*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
192	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
193	*casseliflavus*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
194	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
195	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
196	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
197	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
198	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
199	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
200	*casseliflavus*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
201	*gallinarum*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
202	*casseliflavus*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	+
203	*casseliflavus*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	+
204	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
205	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
206	*faecium*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
1C	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
2C	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
3C	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
4C	N/D	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
5C	*hirae*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
6C	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
7C	*faecalis*	+	+	+	+	-	-	-	-	-	-	-	-	7	I	+
8C	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
9C	*casseliflavus*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
10C	*faecalis*	+	+	-	-	-	-	-	-	-	-	-	-	5	I	-
11C	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
12C	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
13C	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
14C	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
15C	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	5	I	-
16C	*gallinarum*	+	-	-	-	-	-	-	-	-	-	-	-	4	S	-
17C	*hirae/durans*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-

18C	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
19C	*hirae/durans*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
20C	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
21C	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
22C	*faecium*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	+
23C	*faecalis*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
24C	*hirae/durans*	+	+	+	-	-	-	-	-	-	-	-	-	6	I	-
No.	***Enteroccocus***	**2**	**4**	**5**	**6**	**7**	**8**	**12**	**16**	**20**	**24**	**28**	**32**	**MIC**	**S/R**	***tcr*B**

N/D, not determined

Appendix 18. Antimicrobial sensitivity test result for zinc (mM/mL)

No.	*Enteroccocus*	2	4	8	12	16	MIC	S/R
1	*faecalis*	+	+	+	+	+	> 16	R
2	*faecalis*	+	+	+	+	+	> 16	R
3	*faecalis*	+	+	+	+	-	16	R
4	*faecalis*	+	+	+	+	-	16	R
5	*faecium*	+	+	-	-	-	8	S
6	*faecium*	+	+	-	-	-	8	S
7	*faecium*	+	+	-	-	-	8	S
8	*faecium*	+	+	-	-	-	8	S
9	*faecium*	+	+	-	-	-	8	S
10	*faecium*	+	+	+	+	-	16	R
11	*faecium*	+	+	-	-	-	8	S
12	*faecium*	+	+	-	-	-	8	S
13	*hirae/durans*	+	+	-	-	-	8	S
14	*faecium*	+	+	-	-	-	8	S
15	*faecalis*	+	+	-	-	-	8	S
16	*hirae/durans*	+	+	-	-	-	8	S
17	*faecalis*	+	+	-	-	-	8	S
18	*faecium*	+	+	-	-	-	8	S
19	*faecium*	-	-	-	-	-	≥ 2	S
20	*faecium*	+	+	-	-	-	8	S
21	*faecium*	+	+	-	-	-	8	S
22	*faecium*	+	+	-	-	-	8	S
23	*faecium*	+	+	-	-	-	8	S
24	*faecium*	+	+	-	-	-	8	S
25	*faecium*	+	+	-	-	-	8	S
26	*faecium*	+	+	-	-	-	8	S
27	*faecium*	+	+	-	-	-	8	S
28	*faecium*	+	+	-	-	-	8	S
29	*faecium*	+	+	-	-	-	8	S
30	*faecalis*	+	+	+	+	+	> 16	R
31	*faecalis*	+	+	+	+	+	> 16	R
32	*faecalis*	+	+	+	+	+	> 16	R
33	*faecalis*	+	+	+	+	+	> 16	R
34	*faecalis*	+	+	+	+	+	> 16	R
35	*faecalis*	+	+	+	+	+	> 16	R
36	*faecium*	+	+	-	-	-	8	S
37	*faecium*	+	+	-	-	-	8	S
38	*faecalis*	+	+	+	+	+	> 16	R
39	*faecium*	+	+	-	-	-	8	S
40	*faecalis*	+	+	+	+	+	> 16	R
41	*faecalis*	+	+	+	+	+	> 16	R
42	*faecalis*	+	+	+	+	+	> 16	R
43	*faecalis*	+	+	+	+	+	> 16	R

44	*hirae*	+	+	+	-	-	12	I
45	*faecalis*	+	+	+	-	-	12	I
46	*faecalis*	+	+	+	+	+	> 16	R
47	*faecium*	+	+	-	-	-	8	S
48	*faecalis*	+	+	+	+	+	> 16	R
49	*faecium*	+	+	-	-	-	8	S
50	*faecium*	+	+	-	-	-	8	S
51	*faecalis*	+	+	+	+	+	> 16	R
52	*faecalis*	+	+	+	+	+	> 16	R
53	*faecalis*	+	+	+	+	-	16	R
54	*faecalis*	+	+	+	+	+	> 16	R
55	*casseliflavus*	+	+	-	-	-	8	S
56	*faecalis*	+	+	+	+	+	> 16	R
57	*faecalis*	+	+	+	+	+	> 16	R
58	*faecalis*	+	+	+	+	+	> 16	R
59	*faecalis*	+	+	+	+	+	> 16	R
60	*faecalis*	+	+	+	+	+	> 16	R
61	*faecalis*	+	+	+	+	+	> 16	R
62	*faecalis*	+	+	+	+	+	> 16	R
63	*faecalis*	+	+	+	+	+	> 16	R
64	*faecalis*	+	+	+	+	+	> 16	R
65	*faecalis*	+	+	+	+	+	> 16	R
66	*faecalis*	+	+	+	+	+	> 16	R
67	*faecalis*	+	+	+	+	+	> 16	R
68	*faecalis*	+	+	+	+	+	> 16	R
69	*faecalis*	+	+	+	+	+	> 16	R
70	*faecalis*	+	+	+	+	+	> 16	R
71	*faecalis*	+	+	+	+	+	> 16	R
72	*faecalis*	+	+	+	+	+	> 16	R
73	*faecalis*	+	+	+	+	+	> 16	R
74	*faecalis*	+	+	+	+	+	> 16	R
75	*faecium*	+	+	-	-	-	8	S
76	*faecalis*	+	+	+	+	+	> 16	R
77	*faecalis*	+	+	+	+	+	> 16	R
78	*faecalis*	+	+	+	+	+	> 16	R
79	*faecalis*	+	+	+	+	+	> 16	R
80	*faecalis*	+	+	+	+	+	> 16	R
81	*faecalis*	+	+	+	+	+	> 16	R
82	*faecalis*	+	+	+	+	+	> 16	R
83	*faecalis*	+	+	+	+	+	> 16	R
84	*faecalis*	+	+	+	+	+	> 16	R
85	*faecalis*	+	+	+	+	+	> 16	R
86	*faecalis*	+	+	+	+	+	> 16	R
87	*faecalis*	+	+	+	+	+	> 16	R
88	*faecalis*	+	+	+	+	+	> 16	R

89	*faecalis*	+	+	+	+	+	> 16	R
90	*faecalis*	+	+	+	+	+	> 16	R
91	*faecalis*	+	+	+	+	+	> 16	R
92	*faecalis*	+	+	+	+	+	> 16	R
93	*faecalis*	+	+	+	+	+	> 16	R
94	*faecium*	+	+	+	+	+	> 16	R
95	*faecalis*	+	+	+	+	+	> 16	R
96	*faecium*	+	+	-	-	-	8	S
97	*faecium*	+	+	-	-	-	8	S
98	*faecium*	+	+	-	-	-	8	S
99	*faecium*	+	+	-	-	-	8	S
100	*faecium*	+	+	+	+	+	> 16	R
101	*faecalis*	+	+	+	+	+	> 16	R
102	*faecalis*	+	+	+	+		16	R
103	*faecium*	+	+	-	-	-	8	S
104	*faecium*	+	+	-	-	-	8	S
105	*faecium*	+	+	-	-	-	8	S
106	*faecium*	+	+	-	-	-	8	S
107	*hirae/durans*	+	+	-	-	-	8	S
108	*faecium*	+	+	-	-	-	8	S
109	*faecium*	+	+	-	-	-	8	S
110	*faecium*	+	+	-	-	-	8	S
111	*faecium*	+	+	-	-	-	8	S
112	*faecium*	+	+	-	-	-	8	S
113	*hirae/durans*	+	+	-	-	-	8	S
114	*hirae/durans*	+	+	-	-	-	8	S
115	*faecalis*	+	+	+	-	-	12	I
116	*faecium*	+	+	-	-	-	8	S
117	*faecium*	+	+	-	-	-	8	S
118	*faecium*	+	+	-	-	-	8	S
119	*faecium*	+	+	-	-	-	8	S
120	*faecium*	+	+	-	-	-	8	S
121	*hirae/durans*	+	+	-	-	-	8	S
122	*hirae/durans*	+	+	-	-	-	8	S
123	*faecium*	+	+	-	-	-	8	S
124	*faecium*	+	+	-	-	-	8	S
125	*faecium*	+	+	-	-	-	8	S
126	*faecium*	+	+	-	-	-	8	S
127	*hirae/durans*	+	+	-	-	-	8	S
128	*hirae/durans*	+	+	-	-	-	8	S
129	*raffinosus*	+	+	+	+	+	> 16	R
130	*faecium*	+	+	-	-	-	8	S
131	*faecium*	+	+	-	-	-	8	S
132	*faecium*	+	+	-	-	-	8	S
133	*faecalis*	+	+	+	+	+	> 16	R

134	*faecium*	+	+	-	-	-	8	S
135	*faecalis*	+	+	+	+	+	> 16	R
136	*faecium*	+	+	-	-	-	8	S
137	*faecium*	+	+	-	-	-	8	S
138	*faecium*	+	+	-	-	-	8	S
139	*raffinosus*	+	+	+	+	+	> 16	R
140	*faecium*	+	+	-	-	-	8	S
141	*faecium*	+	+	-	-	-	8	S
142	*faecium*	+	+	-	-	-	8	S
143	*faecium*	+	+	-	-	-	8	S
144	*hirae/durans*	+	+	-	-	-	8	S
145	*faecium*	+	+	-	-	-	8	S
146	*faecium*	+	+	-	-	-	8	S
147	*faecium*	+	+	-	-	-	8	S
148	*faecium*	+	+	-	-	-	8	S
149	*faecium*	+	+	-	-	-	8	S
150	*hirae/durans*	+	+	-	-	-	8	S
151	*hirae/durans*	+	+	-	-	-	8	S
152	*hirae/durans*	+	+	-	-	-	8	S
153	*hirae/durans*	+	+	-	-	-	8	S
154	*faecium*	+	+	-	-	-	8	S
155	*faecium*	+	+	-	-	-	8	S
156	*faecium*	+	+	-	-	-	8	S
157	*faecalis*	+	+	+	+	+	> 16	R
158	*faecium*	+	+	-	-	-	8	S
159	*faecium*	+	+	-	-	-	8	S
160	*faecium*	+	+	-	-	-	8	S
161	*faecalis*	+	+	+	+	+	> 16	R
162	*faecalis*	+	+	+	+	+	> 16	R
163	*faecalis*	+	+	+	+	+	> 16	R
164	*faecalis*	+	+	+	+	+	> 16	R
165	*faecalis*	+	+	+	+	+	> 16	R
166	*faecalis*	+	+	+	+	+	> 16	R
167	*faecium*	+	+	-	-	-	8	S
168	*faecalis*	+	+	+	+	+	> 16	R
169	*faecium*	+	+	-	-	-	8	S
170	*gallinarum*	+	+	-	-	-	8	S
171	*casseliflavus*	+	+	-	-	-	8	S
172	*gallinarum*	+	+	-	-	-	8	S
173	*gallinarum*	+	+	-	-	-	8	S
174	*gallinarum*	+	+	-	-	-	8	S
175	*gallinarum*	+	+	-	-	-	8	S
176	*casseliflavus*	+	+	-	-	-	8	S
177	*gallinarum*	+	+	-	-	-	8	S
178	*gallinarum*	+	+	-	-	-	8	S

179	*gallinarum*	+	+	-	-	-	8	S
180	*casseliflavus*	+	+	-	-	-	8	S
181	*gallinarum*	+	+	-	-	-	8	S
182	*gallinarum*	+	+	-	-	-	8	S
183	*casseliflavus*	+	+	-	-	-	8	S
184	*gallinarum*	+	+	-	-	-	8	S
185	*gallinarum*	+	+	-	-	-	8	S
186	*gallinarum*	+	+	-	-	-	8	S
187	*casseliflavus*	+	+	-	-	-	8	S
188	*casseliflavus*	+	+	-	-	-	8	S
189	*gallinarum*	+	+	-	-	-	8	S
190	*casseliflavus*	+	+	-	-	-	8	S
191	*casseliflavus*	+	+	-	-	-	8	S
192	*gallinarum*	+	+	-	-	-	8	S
193	*casseliflavus*	+	+	-	-	-	8	S
194	*gallinarum*	+	+	-	-	-	8	S
195	*gallinarum*	+	+	-	-	-	8	S
196	*gallinarum*	+	+	-	-	-	8	S
197	*gallinarum*	+	+	-	-	-	8	S
198	*faecium*	+	+	-	-	-	8	S
199	*gallinarum*	+	+	-	-	-	8	S
200	*casseliflavus*	+	+	-	-	-	8	S
201	*gallinarum*	+	+	-	-	-	8	S
202	*casseliflavus*	+	+	-	-	-	8	S
203	*casseliflavus*	+	+	-	-	-	8	S
204	*faecium*	+	+	-	-	-	8	S
205	*faecium*	+	+	-	-	-	8	S
206	*faecium*	+	+	-	-	-	8	S
1C	*faecalis*	+	+	+	+	+	≥ 16	R
2C	*faecium*	+	+	-	-	-	8	S
3C	*faecium*	+	+	+	+	+	≥ 16	R
4C	N/D	+	+	-	-	-	8	S
5C	*hirae*	+	+	-	-	-	8	S
6C	*faecalis*	+	+	+	+	+	≥ 16	R
7C	*faecalis*	+	+	+	+	+	≥ 16	R
8C	*faecalis*	+	+	-	-	-	8	S
9C	*casseliflavus*	+	+	-	-	-	8	S
10C	*faecalis*	+	+	+	+	-	16	R
11C	*faecalis*	+	+	+	+	+	≥ 16	R
12C	*faecalis*	+	+	+	+	+	≥ 16	R
13C	*faecium*	+	+	-	-	-	8	S
14C	*faecium*	+	+	-	-	-	8	S
15C	*faecium*	+	+	-	-	-	8	S
16C	*gallinarum*	+	+	-	-	-	8	S
17C	*hirae/durans*	+	+	-	-	-	8	S

18C	*faecium*	+	+	-	-	-	8	S
19C	*hirae/durans*	+	+	-	-	-	8	S
20C	*faecium*	+	+	-	-	-	8	S
21C	*faecalis*	+	+	+	+	-	16	R
22C	*faecium*	+	+	-	-	-	8	S
23C	*faecalis*	+	+	+	-	-	12	I
24C	*hirae/durans*	+	+	-	-	-	8	S
No.	***Enteroccocus***	**2**	**4**	**8**	**12**	**16**	**MIC**	**S/R**

N/D, not determined

Appendix 19. Comparission of the sequenced resistance genes with reference database

NCBI BLAST ref. number: AM932524.1

Primer seq. query: *van*A-F

Score = 1168 bits (632), Expect = 0.0, Identities = 632/632 (100%), Gaps = 0/632 (0%), Strand = Plus/Plus

```
Query  1     GCACGGATTACTTGTTAAAAAGAACCATGAATATGAAATCAACCATGTTGATGTAGCATT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7203  GCACGGATTACTTGTTAAAAAGAACCATGAATATGAAATCAACCATGTTGATGTAGCATT
                                    7262

Query  61    TTCAGCTTTGCATGGCAAGTCAGGTGAAGATGGATCCATACAAGGTCTGTTTGAATTGTC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7263  TTCAGCTTTGCATGGCAAGTCAGGTGAAGATGGATCCATACAAGGTCTGTTTGAATTGTC  7322

Query  121   CGGTATCCCTTTTGTAGGCTGCGATATTCAAAGCTCAGCAATTTGTATGGACAAATCGTT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7323  CGGTATCCCTTTTGTAGGCTGCGATATTCAAAGCTCAGCAATTTGTATGGACAAATCGTT  7382

Query  181   GACATACATCGTTGCGAAAAATGCTGGGATAGCTACTCCCGCCTTTTGGGTTATTAATAA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7383  GACATACATCGTTGCGAAAAATGCTGGGATAGCTACTCCCGCCTTTTGGGTTATTAATAA  7442

Query  241   AGATGATAGGCCGGTGGCAGCTACGTTTACCTATCCTGTTTTTGTTAAGCCGGCGCGTTC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7443  AGATGATAGGCCGGTGGCAGCTACGTTTACCTATCCTGTTTTTGTTAAGCCGGCGCGTTC  7502

Query  301   AGGCTCATCCTTCGGTGTGAAAAAAGTCAATAGCGCGGACGAATTGGACTACGCAATTGA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7503  AGGCTCATCCTTCGGTGTGAAAAAAGTCAATAGCGCGGACGAATTGGACTACGCAATTGA  7562

Query  361   ATCGGCAAGACAATATGACAGCAAAATCTTAATTGAGCAGGCTGTTTCGGGCTGTGAGGT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7563  ATCGGCAAGACAATATGACAGCAAAATCTTAATTGAGCAGGCTGTTTCGGGCTGTGAGGT  7622

Query  421   CGGTTGTGCGGTATTGGGAAACAGTGCCGCGTTAGTTGTTGGCGAGGTGGACCAAATCAG  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7623  CGGTTGTGCGGTATTGGGAAACAGTGCCGCGTTAGTTGTTGGCGAGGTGGACCAAATCAG  7682

Query  481   GCTGCAGTACGGAATCTTTCGTATTCATCAGGAAGTCGAGCCGGAAAAAGGCTCTGAAAA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7683  GCTGCAGTACGGAATCTTTCGTATTCATCAGGAAGTCGAGCCGGAAAAAGGCTCTGAAAA  7742

Query  541   CGCAGTTATAACCGTTCCCGCAGACCTTTCAGCAGAGGAGCGAGGACGGATACAGGAAAC  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7743  CGCAGTTATAACCGTTCCCGCAGACCTTTCAGCAGAGGAGCGAGGACGGATACAGGAAAC  7802

Query  601   GGCaaaaaaaaTATATAAAGCGCTCGGCTGTA  632
              |||||||||||||||||||||||||||||||
Sbjct  7803  GGCAAAAAAAATATATAAAGCGCTCGGCTGTA  7834
```

Primer seq. query: *van*A-R

Score = 1181 bits (639), Expect = 0.0, Identities = 639/639 (100%), Gaps = 0/639 (0%), Strand = Plus/Minus

```
Query  1     AGACCTCTACAGCCGAGCGCTTTATATAttttttttGCCGTTTCCTGTATCCGTCCTCGC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7841  AGACCTCTACAGCCGAGCGCTTTATATATTTTTTTTGCCGTTTCCTGTATCCGTCCTCGC  7782

Query  61    TCCTCTGCTGAAAGGTCTGCGGGAACGGTTATAACTGCGTTTTCAGAGCCTTTTTCCGGC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7781  TCCTCTGCTGAAAGGTCTGCGGGAACGGTTATAACTGCGTTTTCAGAGCCTTTTTCCGGC  7722

Query  121   TCGACTTCCTGATGAATACGAAAGATTCCGTACTGCAGCCTGATTTGGTCCACCTCGCCA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7721  TCGACTTCCTGATGAATACGAAAGATTCCGTACTGCAGCCTGATTTGGTCCACCTCGCCA  7662

Query  181   ACAACTAACGCGGCACTGTTTCCCAATACCGCACAACCGACCTCACAGCCCGAAACAGCC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7661  ACAACTAACGCGGCACTGTTTCCCAATACCGCACAACCGACCTCACAGCCCGAAACAGCC  7602

Query  241   TGCTCAATTAAGATTTTGCTGTCATATTGTCTTGCCGATTCAATTGCGTAGTCCAATTCG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7601  TGCTCAATTAAGATTTTGCTGTCATATTGTCTTGCCGATTCAATTGCGTAGTCCAATTCG  7542

Query  301   TCCGCGCTATTGACTTTTTTCACACCGAAGGATGAGCCTGAACGCGCCGGCTTAACAAAA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7541  TCCGCGCTATTGACTTTTTTCACACCGAAGGATGAGCCTGAACGCGCCGGCTTAACAAAA  7482

Query  361   ACAGGATAGGTAAACGTAGCTGCCACCGGCCTATCATCTTTATTAATAACCCAAAAGGCG  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7481  ACAGGATAGGTAAACGTAGCTGCCACCGGCCTATCATCTTTATTAATAACCCAAAAGGCG  7422

Query  421   GGAGTAGCTATCCCAGCATTTTTCGCAACGATGTATGTCAACGATTTGTCCATACAAATT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7421  GGAGTAGCTATCCCAGCATTTTTCGCAACGATGTATGTCAACGATTTGTCCATACAAATT  7362

Query  481   GCTGAGCTTTGAATATCGCAGCCTACAAAAGGGATACCGGACAATTCAAACAGACCTTGT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7361  GCTGAGCTTTGAATATCGCAGCCTACAAAAGGGATACCGGACAATTCAAACAGACCTTGT  7302

Query  541   ATGGATCCATCTTCACCTGACTTGCCATGCAAAGCTGAAAATGCTACATCAACATGGTTG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7301  ATGGATCCATCTTCACCTGACTTGCCATGCAAAGCTGAAAATGCTACATCAACATGGTTG  7242

Query  601   ATTTCATATTCATGGTTCTTTTTAACAAGTAATCCGTGC  639
              |||||||||||||||||||||||||||||||||||||
Sbjct  7241  ATTTCATATTCATGGTTCTTTTTAACAAGTAATCCGTGC  7203
```

NCBI BLAST ref. number: AJ888003.1

Primer seq. query: *tet*K-F

Score = 1157 bits (626), Expect = 0.0, Identities = 626/626 (100%), Gaps = 0/626 (0%), Strand = Plus/Minus

```
Query  1    TTGGTCTTACCTACTTATACTTCCTATGATTACAATAGTAACTATACCTTTTCTTATTAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  993  TTGGTCTTACCTACTTATACTTCCTATGATTACAATAGTAACTATACCTTTTCTTATTAA  934

Query  61   AGTAATGGTACCTGGTAAATCAACAAAAAATACATTAGATATCGTAGGTATTGTTTTAAT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  933  AGTAATGGTACCTGGTAAATCAACAAAAAATACATTAGATATCGTAGGTATTGTTTTAAT  874

Query  121  GTCTATAAGTATTATATGTTTTATGTTATTTACGACAAATTATAATTGGACTTTTTTAAT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  873  GTCTATAAGTATTATATGTTTTATGTTATTTACGACAAATTATAATTGGACTTTTTTAAT  814

Query  181  ACTCTTCACAATCTTTTTTGTGATTTTTATTAAACATATTTCAAGAGTTTCTAACCCTTT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  813  ACTCTTCACAATCTTTTTTGTGATTTTTATTAAACATATTTCAAGAGTTTCTAACCCTTT  754

Query  241  TATTAATCCTAAACTAGGGAAAAACATTCCGTTTATGCTTGGTTTGTTTTCTGGTGGGCT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  753  TATTAATCCTAAACTAGGGAAAAACATTCCGTTTATGCTTGGTTTGTTTTCTGGTGGGCT  694

Query  301  AATATTTTCTATAGTAGCTGGTTTTATATCAATGGTGCCTTATATGATGAAAACTATTTA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  693  AATATTTTCTATAGTAGCTGGTTTTATATCAATGGTGCCTTATATGATGAAAACTATTTA  634

Query  361  TCATGTAAATGTAGCGACAATAGGTAATAGTGTTATTTTTCCTGGAACCATGAGTgttat  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  633  TCATGTAAATGTAGCGACAATAGGTAATAGTGTTATTTTTCCTGGAACCATGAGTGTTAT  574

Query  421  tgtttttggttattttggtggtttttttagtggatagaaaaggatcattatttgtttttat  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  573  TGTTTTTGGTTATTTTGGTGGTTTTTTAGTGGATAGAAAAGGATCATTATTTGTTTTTAT  514

Query  481  tttaggatcattgtctatctctataagttttttaactattgcatttttttgttgAGTTTAG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  513  TTTAGGATCATTGTCTATCTCTATAAGTTTTTTAACTATTGCATTTTTTGTTGAGTTTAG  454

Query  541  TATGTGGTTGACTACTTTTATGTTTATATTTGTTATGGGCGGATTATCTTTTACTAAAAC  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  453  TATGTGGTTGACTACTTTTATGTTTATATTTGTTATGGGCGGATTATCTTTTACTAAAAC  394

Query  601  AGTTATATCAAAAATAGTATCAAGTA  626
             |||||||||||||||||||||||||
Sbjct  393  AGTTATATCAAAAATAGTATCAAGTA  368
```

Primer seq. query: *tet*K-R

Score = 1205 bits (652), Expect = 0.0, Identities = 652/652 (100%), Gaps = 0/652 (0%), Strand = Plus/Plus

```
Query  1     CTTGATACTATTTTTGATATAACTGTTTTAGTAAAAGATAATCCGCCCATAACAAATATA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  370   CTTGATACTATTTTTGATATAACTGTTTTAGTAAAAGATAATCCGCCCATAACAAATATA  429

Query  61    AACATAAAAGTAGTCAACCACATACTAAACTcaacaaaaaatgcaatagttaaaaaactt  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  430   AACATAAAAGTAGTCAACCACATACTAAACTCAACAAAAAATGCAATAGTTAAAAAACTT  489

Query  121   atagagatagacaatgatcctaaaataaaaacaaataatgatccttttctatccactaaa  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  490   ATAGAGATAGACAATGATCCTAAAATAAAAACAAATAATGATCCTTTTCTATCCACTAAA  549

Query  181   aaaccaccaaaataaccaaaaacaataacACTCATGGTTCCAGGAAAAATAACACTATTA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  550   AAACCACCAAAATAACCAAAAACAATAACACTCATGGTTCCAGGAAAAATAACACTATTA  609

Query  241   CCTATTGTCGCTACATTTACATGATAAATAGTTTTCATCATATAAGGCACCATTGATATA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  610   CCTATTGTCGCTACATTTACATGATAAATAGTTTTCATCATATAAGGCACCATTGATATA  669

Query  301   AAACCAGCTACTATAGAAAATATTAGCCCACCAGAAAACAAACCAAGCATAAACGGAATG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  670   AAACCAGCTACTATAGAAAATATTAGCCCACCAGAAAACAAACCAAGCATAAACGGAATG  729

Query  361   TTTTTCCCTAGTTTAGGATTAATAAAAGGGTTAGAAACTCTTGAAATATGTTTAATAAAA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  730   TTTTTCCCTAGTTTAGGATTAATAAAAGGGTTAGAAACTCTTGAAATATGTTTAATAAAA  789

Query  421   ATCACAAAAAAGATTGTGAAGAGTATTAAAAAAGTCCAATTATAATTTGTCGTAAATAAC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  790   ATCACAAAAAAGATTGTGAAGAGTATTAAAAAAGTCCAATTATAATTTGTCGTAAATAAC  849

Query  481   ATAAAACATATAATACTTATAGACATTAAAACAATACCTACGATATCTAATGTATTTTTT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  850   ATAAAACATATAATACTTATAGACATTAAAACAATACCTACGATATCTAATGTATTTTTT  909

Query  541   GTTGATTTACCAGGTACCATTACTTTAATAAGAAAAGGTATAGTTACTATTGTAATCATA  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  910   GTTGATTTACCAGGTACCATTACTTTAATAAGAAAAGGTATAGTTACTATTGTAATCATA  969

Query  601   GGAAGTATAAGTAGGTAAGACCAATGAATATAATGTGCTATTATTCCCCCTA  652
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  970   GGAAGTATAAGTAGGTAAGACCAATGAATATAATGTGCTATTATTCCCCCTA  1021
```

NCBI BLAST ref. number: EU151772.1

Primer seq. query: *van*C1-F

Score = 1280 bits (693), Expect = 0.0, Identities = 693/693 (100%), Gaps = 0/693 (0%), Strand = Plus/Plus

```
Query  1    AAACTGTCACCAGCTGACTTTTTCTAGCCAAGGATTTATATTAGGAGAAAAACGAATCGT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  231  AAACTGTCACCAGCTGACTTTTTCTAGCCAAGGATTTATATTAGGAGAAAAACGAATCGT  290

Query  61   CCCTGATGTCCTCTTTCCAGTCTTGCATGGGAAGTATGGCGAGGATGGCTGTATCCAAGG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  291  CCCTGATGTCCTCTTTCCAGTCTTGCATGGGAAGTATGGCGAGGATGGCTGTATCCAAGG  350

Query  121  ACTGCTTGAACTAATGAACCTGCCTTATGTTGGTTGCCATGTCGCTGCCTCCGCATTATG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  351  ACTGCTTGAACTAATGAACCTGCCTTATGTTGGTTGCCATGTCGCTGCCTCCGCATTATG  410

Query  181  TATGAACAAATGGCTCTTGCATCAACTTGCTGATACCATGGGAATCGCTAGTGCTCCCAC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  411  TATGAACAAATGGCTCTTGCATCAACTTGCTGATACCATGGGAATCGCTAGTGCTCCCAC  470

Query  241  TTTGCTTTTATCCCGCTATGAAAACGATCCTGCCACAATCGATCGTTTTATTCAAGACCA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  471  TTTGCTTTTATCCCGCTATGAAAACGATCCTGCCACAATCGATCGTTTTATTCAAGACCA  530

Query  301  TGGATTCCCGATCTTTATCAAGCCGAATGAAGCCGGTTCTTCAAAAGGGATCACAAAAGT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  531  TGGATTCCCGATCTTTATCAAGCCGAATGAAGCCGGTTCTTCAAAAGGGATCACAAAAGT  590

Query  361  AACTGACAAAACAGCGCTCCAATCTGCATTAACGACTGCTTTTGCTTACGGTTCTACTGT  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  591  AACTGACAAAACAGCGCTCCAATCTGCATTAACGACTGCTTTTGCTTACGGTTCTACTGT  650

Query  421  GTTGATCCAAAAGGCGATAGCGGGTATTGAAATTGGCTGCGGCATCTTAGGAAATGAGCA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  651  GTTGATCCAAAAGGCGATAGCGGGTATTGAAATTGGCTGCGGCATCTTAGGAAATGAGCA  710

Query  481  ATTGACGATTGGTGCTTGTGATGCGATTTCTCTTGTCGACGGttttttttGATTTTGAAGA  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  711  ATTGACGATTGGTGCTTGTGATGCGATTTCTCTTGTCGACGGTTTTTTTGATTTTGAAGA  770

Query  541  GAAATACCAATTAATCAGCGCCACGATCACTGTCCCAGCACCATTGCCTCTCGCGCTTGA  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  771  GAAATACCAATTAATCAGCGCCACGATCACTGTCCCAGCACCATTGCCTCTCGCGCTTGA  830

Query  601  ATCACAGATCAAGGAGCAGGCACAGCTGCTTTATCGAAACTTGGGATTGACGGGTCTGGC  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  831  ATCACAGATCAAGGAGCAGGCACAGCTGCTTTATCGAAACTTGGGATTGACGGGTCTGGC  890

Query  661  TCGAATCGATTTTTTCGTCACCAATCAAGGAGC  693
            ||||||||||||||||||||||||||||||||
Sbjct  891  TCGAATCGATTTTTTCGTCACCAATCAAGGAGC  923
```

Primer seq. query: *van*C1-R

Score = 1323 bits (716), Expect = 0.0, Identities = 716/716 (100%), Gaps = 0/716 (0%), Strand = Plus/Minus

```
Query  1    ATAAATCGCTCCTTGATTGGTGACGAAAAAATCGATTCGAGCCAGACCCGTCAATCCCAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  930  ATAAATCGCTCCTTGATTGGTGACGAAAAAATCGATTCGAGCCAGACCCGTCAATCCCAA  871

Query  61   GTTTCGATAAAGCAGCTGTGCCTGCTCCTTGATCTGTGATTCAAGCGCGAGAGGCAATGG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  870  GTTTCGATAAAGCAGCTGTGCCTGCTCCTTGATCTGTGATTCAAGCGCGAGAGGCAATGG  811

Query  121  TGCTGGGACAGTGATCGTGGCGCTGATTAATTGGTATTTCTCTTCAAAATCaaaaaaaCC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  810  TGCTGGGACAGTGATCGTGGCGCTGATTAATTGGTATTTCTCTTCAAAATCAAAAAAACC  751

Query  181  GTCGACAAGAGAAATCGCATCACAAGCACCAATCGTCAATTGCTCATTTCCTAAGATGCC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  750  GTCGACAAGAGAAATCGCATCACAAGCACCAATCGTCAATTGCTCATTTCCTAAGATGCC  691

Query  241  GCAGCCAATTTCAATACCCGCTATCGCCTTTTGGATCAACACAGTAGAACCGTAAGCAAA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  690  GCAGCCAATTTCAATACCCGCTATCGCCTTTTGGATCAACACAGTAGAACCGTAAGCAAA  631

Query  301  AGCAGTCGTTAATGCAGATTGGAGCGCTGTTTTGTCAGTTACTTTTGTGATCCCTTTTGA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  630  AGCAGTCGTTAATGCAGATTGGAGCGCTGTTTTGTCAGTTACTTTTGTGATCCCTTTTGA  571

Query  361  AGAACCGGCTTCATTCGGCTTGATAAAGATCGGGAATCCATGGTCTTGAATAAAACGATC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  570  AGAACCGGCTTCATTCGGCTTGATAAAGATCGGGAATCCATGGTCTTGAATAAAACGATC  511

Query  421  GATTGTGGCAGGATCGTTTTCATAGCGGGATAAAAGCAAAGTGGGAGCACTAGCGATTCC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  510  GATTGTGGCAGGATCGTTTTCATAGCGGGATAAAAGCAAAGTGGGAGCACTAGCGATTCC  451

Query  481  CATGGTATCAGCAAGTTGATGCAAGAGCCATTTGTTCATACATAATGCGGAGGCAGCGAC  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  450  CATGGTATCAGCAAGTTGATGCAAGAGCCATTTGTTCATACATAATGCGGAGGCAGCGAC  391

Query  541  ATGGCAACCAACATAAGGCAGGTTCATTAGTTCAAGCAGTCCTTGGATACAGCCATCCTC  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  390  ATGGCAACCAACATAAGGCAGGTTCATTAGTTCAAGCAGTCCTTGGATACAGCCATCCTC  331

Query  601  GCCATACTTCCCATGCAAGACTGGAAAGAGGACATCAGGGACGATTCGTTTTTCTCCTAA  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  330  GCCATACTTCCCATGCAAGACTGGAAAGAGGACATCAGGGACGATTCGTTTTTCTCCTAA  271

  Query  661  TATAAATCCTTGGCTAGAAAAAGTCAGCTGGTGACAGTTTTTGTGATCTTCTAGCC  716
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||
  Sbjct  270  TATAAATCCTTGGCTAGAAAAAGTCAGCTGGTGACAGTTTTTGTGATCTTCTAGCC  215
```

NCBI BLAST ref. number: AF368302.1

Primer seq. query: *vat*D-F

Score = 339 bits (183), Expect = 5e-91, Identities = 183/183 (100%), Gaps = 0/183 (0%), Strand = Plus/Plus

```
Query  1     TTTAATTTATTTGGTAATGGATGGGAGAAACATATGCCAAAATTAGATCAACTACCTATT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2972  TTTAATTTATTTGGTAATGGATGGGAGAAACATATGCCAAAATTAGATCAACTACCTATT  3031

Query  61    AAGGGGGATACAATAATAGGTAATGATGTATGGATAGGAAAAGATGTTGTAATTATGCCA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3032  AAGGGGGATACAATAATAGGTAATGATGTATGGATAGGAAAAGATGTTGTAATTATGCCA  3091

Query  121   GGAGTAAAAATCGGGGATGGTGCAATAGTAGCTGCTAATTCTGTTGTTGTAAAAGATATA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3092  GGAGTAAAAATCGGGGATGGTGCAATAGTAGCTGCTAATTCTGTTGTTGTAAAAGATATA  3151

Query  181   GCG  183
              |||
Sbjct  3152  GCG  3154
```

Primer seq. query: *vat*D-R

Score = 396 bits (214), Expect = 4e-108, Identities = 214/214 (100%), Gaps = 0/214 (0%), Strand = Plus/Minus

```
Query  1     AATTAGCAGCTACTATTGCACCATCCCCGATTTTTACTCCTGGCATAATTACAACATCTT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3131  AATTAGCAGCTACTATTGCACCATCCCCGATTTTTACTCCTGGCATAATTACAACATCTT  3072

Query  61    TTCCTATCCATACATCATTACCTATTATTGTATCCCCCTTAATAGGTAGTTGATCTAATT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3071  TTCCTATCCATACATCATTACCTATTATTGTATCCCCCTTAATAGGTAGTTGATCTAATT  3012

Query  121   TTGGCATATGTTTCTCCCATCCATTACCAAATAAATTAAATGGATATGTTGAGCCATCCA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3011  TTGGCATATGTTTCTCCCATCCATTACCAAATAAATTAAATGGATATGTTGAGCCATCCA  2952

Query  181   TTCTATGATTTGCTCCATTCATAATAATAGTTAC  214
              |||||||||||||||||||||||||||||||||
Sbjct  2951  TTCTATGATTTGCTCCATTCATAATAATAGTTAC  2918
```

NCBI BLAST ref. number: EU151757.1

Primer seq. query: *van*C2-F

Score = 630 bits (341), Expect = 2e-178, Identities = 341/341 (100%), Gaps = 0/341 (0%), Strand = Plus/Plus

```
Query  1    GTTTTCTTTAAGCCTAATGAAGCGGGCTCCTCAAAAGGGATCACTAAAGTCACCTGCGTT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  560  GTTTTCTTTAAGCCTAATGAAGCGGGCTCCTCAAAAGGGATCACTAAAGTCACCTGCGTT  619

Query  61   GAAGAAATCGCTTCTGCCTTAAAAGAAGCCTTTACTTATTGTTCCGCAGTGCTCCTACaa  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  620  GAAGAAATCGCTTCTGCCTTAAAAGAAGCCTTTACTTATTGTTCCGCAGTGCTCCTACAA  679

Query  121  aaaaaTATTGTCGGTGTTGAGATCGGTTGCGGTATTTTGGGCAACGACTCTTTGACTGTC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  680  AAAAATATTGTCGGTGTTGAGATCGGTTGCGGTATTTTGGGCAACGACTCTTTGACTGTC  739

Query  181  GGTGCTTGTGACGCCATTTCATTAGAAGACGGCTTTTTCGATTTTGAAGAAAAGTACCAG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  740  GGTGCTTGTGACGCCATTTCATTAGAAGACGGCTTTTTCGATTTTGAAGAAAAGTACCAG  799

Query  241  CTGATCAGCGCCAAAATCACCGTCCCTGCGCCATTGCCTGAAACGATTGAAACCAAGGTC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  800  CTGATCAGCGCCAAAATCACCGTCCCTGCGCCATTGCCTGAAACGATTGAAACCAAGGTC  859

Query  301  AAAGAACAAGCTCAGCTGCTCTATCGTAGTCTTGGTCTTAA  341
            |||||||||||||||||||||||||||||||||||||||||
Sbjct  860  AAAGAACAAGCTCAGCTGCTCTATCGTAGTCTTGGTCTTAA  900
```

Primer seq. query: *van*C2-R

Score = 688 bits (372), Expect = 0.0, Identities = 375/376 (99%), Gaps = 1/376 (0%), Strand = Plus/Minus

```
Query  1    GCTGAGCTTGTTCTTTGACCTTGGTTTC-ATCGTTTCAGGCAATGGCGCAGGGACGGTGA  59
            |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  875  GCTGAGCTTGTTCTTTGACCTTGGTTTCAATCGTTTCAGGCAATGGCGCAGGGACGGTGA  816

Query  60   TTTTGGCGCTGATCAGCTGGTACTTTTCTTCAAAATCGAAAAAGCCGTCTTCTAATGAAA  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  815  TTTTGGCGCTGATCAGCTGGTACTTTTCTTCAAAATCGAAAAAGCCGTCTTCTAATGAAA  756

Query  120  TGGCGTCACAAGCACCGACAGTCAAAGAGTCGTTGCCCAAAATACCGCAACCGATCTCAA  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  755  TGGCGTCACAAGCACCGACAGTCAAAGAGTCGTTGCCCAAAATACCGCAACCGATCTCAA  696

Query  180  CACCGACAATAttttttttGTAGGAGCACTGCGGAACAATAAGTAAAGGCTTCTTTTAAGG  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  695  CACCGACAATATTTTTTTTGTAGGAGCACTGCGGAACAATAAGTAAAGGCTTCTTTTAAGG  636

Query  240  CAGAAGCGATTTCTTCAACGCAGGTGACTTTAGTGATCCCTTTTGAGGAGCCCGCTTCAT  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  635  CAGAAGCGATTTCTTCAACGCAGGTGACTTTAGTGATCCCTTTTGAGGAGCCCGCTTCAT  576

Query  300  TAGGCTTAAAGAAAACTGGAAAGCCATGGGTCTGGATAAAAGCTTCGATTTGTTCTTGCT  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  575  TAGGCTTAAAGAAAACTGGAAAGCCATGGGTCTGGATAAAAGCTTCGATTTGTTCTTGCT  516

                    Query  360  GGTTGGCTTGATTTGT  375
                                 ||||||||||||||||
                    Sbjct  515  GGTTGGCTTGATTTGT  500
```

NCBI BLAST ref. number: AY043212.1

Primer seq. query: *vat*E-F

Score = 800 bits (433), Expect = 0.0, Identities = 433/433 (100%), Gaps = 0/433 (0%), Strand = Plus/Plus

```
Query  1    AACGTGATCAAAAGTCCCAATATTGAAATTGGGGACTACACCTATTATGATGACCCAGTA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67   AACGTGATCAAAAGTCCCAATATTGAAATTGGGGACTACACCTATTATGATGACCCAGTA  126

Query  61   AATCCCACCGATTTTGAGAAACACGTTACCCATCACTATGAATTTCTAGGCGACAAATTA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  127  AATCCCACCGATTTTGAGAAACACGTTACCCATCACTATGAATTTCTAGGCGACAAATTA  186

Query  121  ATCATCGGTAAATTTTGTTCTATCGCCAGTGGCATTGAATTTATCATGAACGGTGCCAAC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  187  ATCATCGGTAAATTTTGTTCTATCGCCAGTGGCATTGAATTTATCATGAACGGTGCCAAC  246

Query  181  CACGTAATGAAAGGTATTTCGACTTATCCATTTAATATTTTAGGTGGCGATTGGCAACAA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  247  CACGTAATGAAAGGTATTTCGACTTATCCATTTAATATTTTAGGTGGCGATTGGCAACAA  306

Query  241  TACACTCCTGAACTGACTGATTTGCCGTTGAAAGGTGATACTGTAGTCGGAAATGACGTG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  307  TACACTCCTGAACTGACTGATTTGCCGTTGAAAGGTGATACTGTAGTCGGAAATGACGTG  366

Query  301  TGGTTTGGGCAAAATGTGACCGTCCTACCAGGCGTAAAAATAGGTGACGGTGCCATTATC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  367  TGGTTTGGGCAAAATGTGACCGTCCTACCAGGCGTAAAAATAGGTGACGGTGCCATTATC  426

Query  361  GGAGCAAATAGTGTTGTAACAAAAGACGTCGCTCCATATACAATTGTCGGTGGCAATCCA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427  GGAGCAAATAGTGTTGTAACAAAAGACGTCGCTCCATATACAATTGTCGGTGGCAATCCA  486

Query  421  ATTCAACTCATCG  433
            |||||||||||||
Sbjct  487  ATTCAACTCATCG  499
```

Primer seq. query: *vat*E-R

Score = 845 bits (457), Expect = 0.0, Identities = 464/467 (99%), Gaps = 2/467 (0%), Strand = Plus/Minus

```
 Query  1    TGCC-CCGAC-ATTGTATATGGAGCGACGTCTTTTGTTACAACACTATTTGCTCCGATAA  58
             |||| ||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  TGCCACCGACAATTGTATATGGAGCGACGTCTTTTGTTACAACACTATTTGCTCCGATAA  422

Query  59   TGGCACCGTCACCTATTTTTACGCCTGGTAGGACGGTCACATTTTGCCCAAACCACACGT  118
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  TGGCACCGTCACCTATTTTTACGCCTGGTAGGACGGTCACATTTTGCCCAAACCACACGT  362

Query  119  CATTTCCGACTACAGTATCACCTTTCAACGGCAAATCAGTCAGTTCAGGAGTGTATTGTT  178
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  CATTTCCGACTACAGTATCACCTTTCAACGGCAAATCAGTCAGTTCAGGAGTGTATTGTT  302

Query  179  GCCAATCGCCACCTAAAATATTAAATGGATAAGTCGAAATACCTTTCATTACGTGGTTGG  238
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GCCAATCGCCACCTAAAATATTAAATGGATAAGTCGAAATACCTTTCATTACGTGGTTGG  242

Query  239  CACCGTTCATGATAAATTCAATGCCACTGGCGATAGAACAAAATTTACCGATGATTAATT  298
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CACCGTTCATGATAAATTCAATGCCACTGGCGATAGAACAAAATTTACCGATGATTAATT  182

Query  299  TGTCGCCTAGAAATTCATAGTGATGGGTAACGTGTTTCTCAAAATCGGTGGGATTTACTG  358
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  TGTCGCCTAGAAATTCATAGTGATGGGTAACGTGTTTCTCAAAATCGGTGGGATTTACTG  122

Query  359  GGTCATCATAATAGGTGTAGTCCCCAATTTCAATATTGGGACTTTTGATCACGTTCTTGA  418
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 Sbjct  121 GGTCATCATAATAGGTGTAGTCCCCAATTTCAATATTGGGACTTTTGATCACGTTCTTGA  62

       Query  419  TAAAGACAACCTCTTTGATGGCTGAGTTAGGATAGATTGCATTTGCG  465
                    |||||||||||||| |||||||||||||||||||||||||||||
        Sbjct  61  TAAAGACAACCTCTATGATGGCTGAGTTAGGATAGATTGCATTTGCG  15
```

NCBI BLAST ref. number: DQ223244.1

Primer seq. query: *tet*M-F

Score = 1048 bits (567), Expect = 0.0, Identities = 567/567 (100%), Gaps = 0/567 (0%), Strand = Plus/Plus

```
Query  1     TTGAAAATCCGCTCCCTCTGCTGCAAACAACTGTTGAACCGAGCAAACCTCAACAAAGGG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1016  TTGAAAATCCGCTCCCTCTGCTGCAAACAACTGTTGAACCGAGCAAACCTCAACAAAGGG  1075

Query  61    AAATGTTACTTGATGCACTTTTAGAAATCTCCGACAGTGACCCGCTTCTACAATATTATG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1076  AAATGTTACTTGATGCACTTTTAGAAATCTCCGACAGTGACCCGCTTCTACAATATTATG  1135

Query  121   TGGATTCTACGACACATGAAATTATACTTTCTTTCTTAGGGAAAGTACAAATGGAAGTGA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1136  TGGATTCTACGACACATGAAATTATACTTTCTTTCTTAGGGAAAGTACAAATGGAAGTGA  1195

Query  181   TTAGTGCACTGTTGCAAGAAAAGTATCATGTGGAGATAGAACTAAAAGAGCCTACAGTCA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1196  TTAGTGCACTGTTGCAAGAAAAGTATCATGTGGAGATAGAACTAAAAGAGCCTACAGTCA  1255

Query  241   TTTATATGGAGAGACCGTTAAAAAATGCAGAATATACCATTCACATCGAAGTGCCGCCAA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1256  TTTATATGGAGAGACCGTTAAAAAATGCAGAATATACCATTCACATCGAAGTGCCGCCAA  1315

Query  301   ATCCTTTCTGGGCTTCCATTGGTTTATCTGTATCACCGCTTCCGTTGGGAAGTGGAATGC  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1316  ATCCTTTCTGGGCTTCCATTGGTTTATCTGTATCACCGCTTCCGTTGGGAAGTGGAATGC  1375

Query  361   AGTATGAGAGCTCGGTTTCTCTTGGATACTTAAATCAATCATTTCAAAATGCAGTTATGG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1376  AGTATGAGAGCTCGGTTTCTCTTGGATACTTAAATCAATCATTTCAAAATGCAGTTATGG  1435

Query  421   AAGGGATACGCTATGGTTGCGAACAAGGATTATATGGTTGGAATGTGACGGATTGTAAAA  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1436  AAGGGATACGCTATGGTTGCGAACAAGGATTATATGGTTGGAATGTGACGGATTGTAAAA  1495

Query  481   TCTGTTTTAAGTATGGCTTATACTATAGCCCTGTTAGTACCCCAGCAGATTTTCGGATGC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1496  TCTGTTTTAAGTATGGCTTATACTATAGCCCTGTTAGTACCCCAGCAGATTTTCGGATGC  1555

Query  541   TTGCTCCTATTGTATTGGAACAAGTCT  567
             |||||||||||||||||||||||||||
Sbjct  1556  TTGCTCCTATTGTATTGGAACAAGTCT  1582
```

Primer seq. query: *tet*M-R

Score = 1057 bits (572), Expect = 0.0, Identities = 572/572 (100%), Gaps = 0/572 (0%), Strand = Plus/Minus

```
 Query  1     CAATAGGAGCAAGCATCCGAAAATCTGCTGGGGTACTAACAGGGCTATAGTATAAGCCAT  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1567  CAATAGGAGCAAGCATCCGAAAATCTGCTGGGGTACTAACAGGGCTATAGTATAAGCCAT  1508

 Query  61    ACTTAAAACAGATTTTACAATCCGTCACATTCCAACCATATAATCCTTGTTCGCAACCAT  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1507  ACTTAAAACAGATTTTACAATCCGTCACATTCCAACCATATAATCCTTGTTCGCAACCAT  1448

 Query  121   AGCGTATCCCTTCCATAACTGCATTTTGAAATGATTGATTTAAGTATCCAAGAGAAACCG  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1447  AGCGTATCCCTTCCATAACTGCATTTTGAAATGATTGATTTAAGTATCCAAGAGAAACCG  1388

 Query  181   AGCTCTCATACTGCATTCCACTTCCCAACGGAAGCGGTGATACAGATAAACCAATGGAAG  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1387  AGCTCTCATACTGCATTCCACTTCCCAACGGAAGCGGTGATACAGATAAACCAATGGAAG  1328

 Query  241   CCCAGAAAGGATTTGGCGGCACTTCGATGTGAATGGTATATTCTGCATTTTTTAACGGTC  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1327  CCCAGAAAGGATTTGGCGGCACTTCGATGTGAATGGTATATTCTGCATTTTTTAACGGTC  1268

 Query  301   TCTCCATATAAATGACTGTAGGCTCTTTTAGTTCTATCTCCACATGATACTTTTCTTGCA  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1267  TCTCCATATAAATGACTGTAGGCTCTTTTAGTTCTATCTCCACATGATACTTTTCTTGCA  1208

 Query  361   ACAGTGCACTAATCACTTCCATTTGTACTTTCCCTAAGAAAGAAAGTATAATTTCATGTG  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1207  ACAGTGCACTAATCACTTCCATTTGTACTTTCCCTAAGAAAGAAAGTATAATTTCATGTG  1148

 Query  421   TCGTAGAATCCACATAATATTGTAGAAGCGGGTCACTGTCGGAGATTTCTAAAAGTGCAT  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1147  TCGTAGAATCCACATAATATTGTAGAAGCGGGTCACTGTCGGAGATTTCTAAAAGTGCAT  1088

 Query  481   CAAGTAACATTTCCCTTTGTTGAGGTTTGCTCGGTTCAACAGTTGTTTGCAGCAGAGGGA  540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1087  CAAGTAACATTTCCCTTTGTTGAGGTTTGCTCGGTTCAACAGTTGTTTGCAGCAGAGGGA  1028

 Query  541   GCGGATTTTCAATTCTCTCTCTCTGTGGCAAT  572
              ||||||||||||||||||||||||||||||||
Sbjct  1027  GCGGATTTTCAATTCTCTCTCTCTGTGGCAAT  996
```

NCBI BLAST ref. number: AY081910.1

Primer seq. query: *tet*L-F

Score = 754 bits (408), Expect = 0.0, Identities = 408/408 (100%), Gaps = 0/408 (0%), Strand = Plus/Plus

```
Query  1    GCGATTGGTGGAATGATAGCCCATTATATTCATTGGTCCTATCTTCTACTCATTCCTATG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  489  GCGATTGGTGGAATGATAGCCCATTATATTCATTGGTCCTATCTTCTACTCATTCCTATG  548

Query  61   ATAACAATTATCACTGTTCCGTTTCTTATGAAATTATTAAAGAAAGAAGTAAGGATAAAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  549  ATAACAATTATCACTGTTCCGTTTCTTATGAAATTATTAAAGAAAGAAGTAAGGATAAAA  608

Query  121  GGTCATTTTGATATCAAAGGAATTATACTAATGTCTGTAGGCATTGTATTTTTTATGTTG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  609  GGTCATTTTGATATCAAAGGAATTATACTAATGTCTGTAGGCATTGTATTTTTTATGTTG  668

Query  181  TTTACAACATCATATAGCATTTCTTTTCTTATCGTTAGCGTGCTGTCATTCCTGATATTT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  669  TTTACAACATCATATAGCATTTCTTTTCTTATCGTTAGCGTGCTGTCATTCCTGATATTT  728

Query  241  GTAAAACATATCAGGAAAGTAACAGATCCTTTTGTTGATCCCGGATTAGGGAAAAATATA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  729  GTAAAACATATCAGGAAAGTAACAGATCCTTTTGTTGATCCCGGATTAGGGAAAAATATA  788

Query  301  CCTTTTATGATTGGAGTTCTTTGTGGGGGAATTATATTTGGAACAGTAGCAGGGTTTGTC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  789  CCTTTTATGATTGGAGTTCTTTGTGGGGGAATTATATTTGGAACAGTAGCAGGGTTTGTC  848

Query  361  TCTATGGTTCCTTATATGATGAAAGATGTTCACCAGCTAAGTACTGCC  408
            ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  849  TCTATGGTTCCTTATATGATGAAAGATGTTCACCAGCTAAGTACTGCC  896
```

NCBI BLAST ref. number: AY660532.1

Primer seq. query: *tet*O-F

Score = 972 bits (526), Expect = 0.0, Identities = 530/532 (99%), Gaps = 0/532 (0%), Strand = Plus/Plus

```
Query  1    TCAATAAAATTGACCAAGAGGGGATTGATTTGCCAATGGTATATCGGGAAATGAAAGCAA  60
            |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  393  TCAATAAAATTGACCAAGAGGGGATTGATTTGCCAATGGTATATCGAGAAATGAAAGCAA  452

Query  61   AGCTTTCTTCGGAAATTATAGTGAAGCAAAAGGTTGGGCAGCATCCCCATATAAATGTAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  453  AGCTTTCTTCGGAAATTATAGTGAAGCAAAAGGTTGGGCAGCATCCCCATATAAATGTAA  512

Query  121  CGGACAATGACGATATGGAACAGTGGGATGCGGTAATTATGGGAAACGATGAACTATTAG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  513  CGGACAATGACGATATGGAACAGTGGGATGCGGTAATTATGGGAAACGATGAACTATTAG  572

Query  181  AGAAATATATGTCAGGGAAACCGTTTAAAATGTCAGAACTGGAACAGGAAGAAAACAGGA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  573  AGAAATATATGTCAGGGAAACCGTTTAAAATGTCAGAACTGGAACAGGAAGAAAACAGGA  632

Query  241  GATTCCAAAACGGAACGTTATTTCCCGTTTATCACGGAAGCGCTAAAAACAATCTGGGGA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  633  GATTCCAAAACGGAACGTTATTTCCCGTTTATCACGGAAGCGCTAAAAACAATCTGGGGA  692

Query  301  TTCGGCAGCTTATAGAAGTAATTGCCAGTAAATTTTATTCATCAACGCCTGAAGGTCAAT  360
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  693  TTCGGCAGCTTATAGAAGTGATTGCCAGTAAATTTTATTCATCAACGCCTGAAGGTCAAT  752

Query  361  CTGAACTATGCGGGCAGGTTTTTAAGATTGAATATTCAGAGAAAAGGCGGCGTTTTGTTT  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  753  CTGAACTATGCGGGCAGGTTTTTAAGATTGAATATTCAGAGAAAAGGCGGCGTTTTGTTT  812

Query  421  ATGTGCGTATATATAGCGGAACATTGCATTTGAGGGATGTTATTAGAATATCTGAAAAAG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  813  ATGTGCGTATATATAGCGGAACATTGCATTTGAGGGATGTTATTAGAATATCTGAAAAAG  872

Query  481  AGAAAATAAAAATCACAGAGATGTGTGTTCCGACAAACGGTGAATTATATTC  532
            ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  873  AGAAAATAAAAATCACAGAGATGTGTGTTCCGACAAACGGTGAATTATATTC  924
```

Primer seq. query: *tet*O-R

Score = 1048 bits (567), Expect = 0.0, Identities = 573/576 (99%), Gaps = 0/576 (0%), Strand = Plus/Minus

```
Query  1    CCGTTTGTCGGAACACACATCTCTGTGATTTTTATTTTCTCTTTTTCAGATATTCTAATA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  912  CCGTTTGTCGGAACACACATCTCTGTGATTTTTATTTTCTCTTTTTCAGATATTCTAATA  853

Query  61   ACATCCCTCAAATGCAATGTTCCGCTATATATACGCACATAAACAAAACGCCGCCTTTTC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  852  ACATCCCTCAAATGCAATGTTCCGCTATATATACGCACATAAACAAAACGCCGCCTTTTC  793

Query  121  TCTGAATATTCAATCTTAAAAACCTGCCCGCATAGTTCAGATTGACCTTCAGGCGTTGAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  792  TCTGAATATTCAATCTTAAAAACCTGCCCGCATAGTTCAGATTGACCTTCAGGCGTTGAT  733

Query  181  GAATAAAATTTACTGGCAATTACTTCTATAAGCTGCCGAATCCCCAGATTGTTTTTAGCG  240
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  732  GAATAAAATTTACTGGCAATCACTTCTATAAGCTGCCGAATCCCCAGATTGTTTTTAGCG  673

Query  241  CTTCCGTGATAAACGGGAAATAACGTTCCGTTTTGGAATCTCCTGTTTTCTTCCTGTTCC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  672  CTTCCGTGATAAACGGGAAATAACGTTCCGTTTTGGAATCTCCTGTTTTCTTCCTGTTCC  613

Query  301  AGTTCTGACATTTTAAACGGTTTCCCTGACATATATTTCTCTAATAGTTCATCGTTTCCC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  612  AGTTCTGACATTTTAAACGGTTTCCCTGACATATATTTCTCTAATAGTTCATCGTTTCCC  553

Query  361  ATAATTACCGCATCCCACTGTTCCATATCGTCATTGTCCGTTACATTTATATGGGGATGC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  552  ATAATTACCGCATCCCACTGTTCCATATCGTCATTGTCCGTTACATTTATATGGGGATGC  493

Query  421  TGCCCAACCTTTTGCTTCACTATAATTTCCGAAGAAAGCTTTGCTTTCATTTCCCGATAT  480
            |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  492  TGCCCAACCTTTTGCTTCACTATAATTTCCGAAGAAAGCTTTGCTTTCATTTCTCGATAT  433

Query  481  ACCATTGGCAAATCAATCCCCTCTTGGTCAATTTTATTGATGaaaaaaaTTGTCGGAATC  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  432  ACCATTGGCAAATCAATCCCCTCTTGGTCAATTTTATTGATGAAAAAAATTGTCGGAATC  373

             Query  541  TTCATTATCTGTAGTGCATGAAACAGTATACGGGTC  576
                          |||||| ||||||||||||||||||||||||||||
             Sbjct  372  TTCATTGTCTGTAGTGCATGAAACAGTATACGGGTC  337
```

NCBI BLAST ref. number: AY737526.1

Primer seq. query: *lsa*B-F

Score = 1042 bits (564), Expect = 0.0, Identities = 578/585 (98%), Gaps = 0/585 (0%), Strand = Plus/Plus

```
Query  1     GATCGGGCATTTGTTGATGAAGTGGTTGATCATATTTTGGCGATTGAAAAAAGTCAATTG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  825   GATCGGGCATTTGTTGATGAAGTGGTTGATCATATTTTGGCGATTGAAAAAAGTCAATTG  884

Query  61    ACGCTGTATCAAGGGAATTTTTCTATTTATGAAGAGCaaaaaaaaTTAAGAGATGCTTTT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  885   ACGCTGTATCAAGGGAATTTTTCTATTTATGAAGAGCAAAAAAAATTAAGAGATGCTTTT  944

Query  121   GAACTAGCAGaaaatgaaaaaatcaaaaaaGAAGTCAATCGCTTGAAAGAAACCGCTCGT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  945   GAACTAGCAGAAAATGAAAAAATCAAAAAAGAAGTCAATCGCTTGAAAGAAACCGCTCGT  1004

Query  181   AAAAAAGCGGAATGGTCGATGAACCGTGAAGGTGATAAGTACGGCAATGCTAAAGAAAAA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1005  AAAAAAGCGGAATGGTCGATGAACCGTGAAGGTGATAAGTACGGCAATGCTAAAGAAAAA  1064

Query  241   GGGAGCGGGGCGATTTTTGATACAGGAGCCATTGGTGCCCGGGCAGCGCGCGTAATGAAG  300
             || |||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  1065  GGAAGCGGGGCGATTTTTGATACAGGAGCCATTGGTGCCCGGGCAACGCGCGTAATGAAG  1124

Query  301   CGCTCGAAACACATTCAACAACGCGCCGAAACACAATTAGCAGAAAAAGAAAAACTATTA  360
             |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  1125  CGCTCGAAACACATTCAACAACGTGCCGAAACACAATTAGCAGAAAAAGAAAAACTATTA  1184

Query  361   AAAGATATTGAGTATATTGATTCTTTGTCAATGGATTATCAGCCAACGCATCACAAAACA  420
             |||||| ||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  1185  AAAGATCTTGAGTATATTGATCCTTTGTCAATGGATTATCAGCCAACGCATCACAAAACA  1244

Query  421   TTATTGACGGTGGAAGAGCTTCGTCTGGGCTACGAGAAAAAATTGGCTATTTGCGCCAATT  480
             |||||||||||||||||||||||||| |||||||||||||||||| ||||||||||||
Sbjct  1245  TTATTGACGGTGGAAGAGCTTCGTCTAGGCTACGAGAAAAATTGGCTGTTTGCGCCAATT  1304

Query  481   TCTTTTTCAATAAACGCGGGAGAAATTGTTGGAATAACAGGAAAAAATGGCTCAGGAAAA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1305  TCTTTTTCAATAAACGCGGGAGAAATTGTTGGAATAACAGGAAAAAATGGCTCAGGAAAA  1364

Query  541   TCGAGCTTGATTCAGTATTTGTTGGGGGATTTTTCTGGAGATTCA  585
             |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1365  TCGAGCTTGATTCAGTATTTGTTGGGGGATTTTTCTGGAGATTCA  1409
```

Primer seq. query: *lsa*B-R

Score = 300 bits (162), Expect = 2e-79, Identities = 164/165 (99%), Gaps = 0/165 (0%), Strand = Plus/Minus

```
Query  1     ATTGCAGGCCTCACAGATAAGATTAGCGCTTCTAATTGTTGATGATTAAACACATCCAAG  60
             ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738  ATTGCAGGCTTCACAGATAAGATTAGCGCTTCTAATTGTTGATGATTAAACACATCCAAG  1679

Query  61    TAATTAAGGGGTTCATCCCAAATATAAAGTTCAGCTGATTGAGACAATGATTTGGCTACT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1678  TAATTAAGGGGTTCATCCCAAATATAAAGTTCAGCTGATTGAGACAATGATTTGGCTACT  1619

Query  121   TCGACTTTTTTCCGTTGCCCCATACTCATTTGTTCAATTCGATTA  165
              |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1618  TCGACTTTTTTCCGTTGCCCCATACTCATTTGTTCAATTCGATTA  1574
```

NCBI BLAST ref. number: DQ295784.1

Primer seq. query: *tet*S-F

Score = 1068 bits (578), Expect = 0.0, Identities = 578/578 (100%), Gaps = 0/578 (0%), Strand = Plus/Plus

```
Query  1    ATGTTAAAGTAAATATTGTAGATACTCCTGGACACATGGATTTTTTGGCAGATGTATACC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  360  ATGTTAAAGTAAATATTGTAGATACTCCTGGACACATGGATTTTTTGGCAGATGTATACC  419

Query  61   GTTCATTATCTGTTTTGGATGGAGCTATTTTGCTAATCTCTGCAAAAGATGGAGTACAGT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  420  GTTCATTATCTGTTTTGGATGGAGCTATTTTGCTAATCTCTGCAAAAGATGGAGTACAGT  479

Query  121  CACAAACTCGTATACTATTCCATGCACTTAGAAAGATGAACATACCTATAATATTTTTTA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  480  CACAAACTCGTATACTATTCCATGCACTTAGAAAGATGAACATACCTATAATATTTTTTA  539

Query  181  TTAACAAAATTGATCAAAATGGAATAAATTTGCCAGATGTTTATCAAGATATTAAGGACA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  540  TTAACAAAATTGATCAAAATGGAATAAATTTGCCAGATGTTTATCAAGATATTAAGGACA  599

Query  241  AACTTTCTGACGACATCATAATTAAGCAGACTGTGAATCTAAATTTGAAACCTTATGTAA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  600  AACTTTCTGACGACATCATAATTAAGCAGACTGTGAATCTAAATTTGAAACCTTATGTAA  659

Query  301  TAGATTATACTGAACCAGAACAATGGGAGACAGTAATTGTGGGAAATGATTATTTATTAG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  660  TAGATTATACTGAACCAGAACAATGGGAGACAGTAATTGTGGGAAATGATTATTTATTAG  719

Query  361  AAAAATATACCATTGGGAAAACATTGAATATTGCAGAACTTGAAAAGGAGGAAAACGAAA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  720  AAAAATATACCATTGGGAAAACATTGAATATTGCAGAACTTGAAAAGGAGGAAAACGAAA  779

Query  421  GAATTCAAAGTTGCTCCTTATATCCTGTTTATCACGGAAGTGCAAAGAATAATATTGGAA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  780  GAATTCAAAGTTGCTCCTTATATCCTGTTTATCACGGAAGTGCAAAGAATAATATTGGAA  839

Query  481  TTAAACAACTTATAGAGGTAATTACTAGCAAATTATTTTCACCCACACAACTCAATTCAG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  840  TTAAACAACTTATAGAGGTAATTACTAGCAAATTATTTTCACCCACACAACTCAATTCAG  899

Query  541  ATAAACTTTGTGGAAATGTTTTTAAAGTAGAATATTCA  578
             |||||||||||||||||||||||||||||||||||||
Sbjct  900  ATAAACTTTGTGGAAATGTTTTTAAAGTAGAATATTCA  937
```

Primer seq. query: *tet*S-R

Score = 1061 bits (574), Expect = 0.0, Identities = 574/574 (100%), Gaps = 0/574 (0%), Strand = Plus/Minus

```
Query  1    AAGTTTATCTGAATTGAGTTGTGTGGGTGAAAATAATTTGCTAGTAATTACCTCTATAAG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  907  AAGTTTATCTGAATTGAGTTGTGTGGGTGAAAATAATTTGCTAGTAATTACCTCTATAAG  848

Query  61   TTGTTTAATTCCAATATTATTCTTTGCACTTCCGTGATAAACAGGATATAAGGAGCAACT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  847  TTGTTTAATTCCAATATTATTCTTTGCACTTCCGTGATAAACAGGATATAAGGAGCAACT  788

Query  121  TTGAATTCTTTCGTTTTCCTCCTTTTCAAGTTCTGCAATATTCAATGTTTTCCCAATGGT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  787  TTGAATTCTTTCGTTTTCCTCCTTTTCAAGTTCTGCAATATTCAATGTTTTCCCAATGGT  728

Query  181  ATATTTTTCTAATAAATAATCATTTCCCACAATTACTGTCTCCCATTGTTCTGGTTCAGT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  727  ATATTTTTCTAATAAATAATCATTTCCCACAATTACTGTCTCCCATTGTTCTGGTTCAGT  668

Query  241  ATAATCTATTACATAAGGTTTCAAATTTAGATTCACAGTCTGCTTAATTATGATGTCGTC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  667  ATAATCTATTACATAAGGTTTCAAATTTAGATTCACAGTCTGCTTAATTATGATGTCGTC  608

Query  301  AGAAAGTTTGTCCTTAATATCTTGATAAACATCTGGCAAATTTATTCCATTTTGATCAAT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  607  AGAAAGTTTGTCCTTAATATCTTGATAAACATCTGGCAAATTTATTCCATTTTGATCAAT  548

Query  361  TTTGTTAATAAAAAATATTATAGGTATGTTCATCTTTCTAAGTGCATGGAATAGTATACG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547  TTTGTTAATAAAAAATATTATAGGTATGTTCATCTTTCTAAGTGCATGGAATAGTATACG  488

Query  421  AGTTTGTGACTGTACTCCATCTTTTGCAGAGATTAGCAAAATAGCTCCATCCAAAACAGA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  487  AGTTTGTGACTGTACTCCATCTTTTGCAGAGATTAGCAAAATAGCTCCATCCAAAACAGA  428

Query  481  TAATGAACGGTATACATCTGCCAAAAAATCCATGTGTCCAGGAGTATCTACAATATTTAC  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427  TAATGAACGGTATACATCTGCCAAAAAATCCATGTGTCCAGGAGTATCTACAATATTTAC  368

Query  541  TTTAACATTTTCCCGTTGAAAAGATGTTATTGCG  574
             ||||||||||||||||||||||||||||||||
Sbjct  367  TTTAACATTTTCCCGTTGAAAAGATGTTATTGCG  334
```

NCBI BLAST ref. number: AB247327.1

Primer seq. query: *erm*B-F

Score = 579 bits (313), Expect = 5e-163, Identities = 313/313 (100%), Gaps = 0/313 (0%), Strand = Plus/Plus

```
Query  1     TTCGTGTCACTTTAATTCACCAAGATATTCTACAGTTTCAATTCCCTAACAAACAGAGGT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4864  TTCGTGTCACTTTAATTCACCAAGATATTCTACAGTTTCAATTCCCTAACAAACAGAGGT  4923

Query  61    ATAAAATTGTTGGGAATATTCCTTACCATTTAAGCACACAAATTATTAAAAAAGTGGTTT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4924  ATAAAATTGTTGGGAATATTCCTTACCATTTAAGCACACAAATTATTAAAAAAGTGGTTT  4983

Query  121   TTGAAAGCCATGCGTCTGACATCTATCTGATTGTTGAAGAAGGATTCTACAAGCGTACCT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4984  TTGAAAGCCATGCGTCTGACATCTATCTGATTGTTGAAGAAGGATTCTACAAGCGTACCT  5043

Query  181   TGGATATTCACCGAACACTAGGGTTGCTCTTGCACACTCAAGTCTCGATTCAGCAATTGC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5044  TGGATATTCACCGAACACTAGGGTTGCTCTTGCACACTCAAGTCTCGATTCAGCAATTGC  5103

Query  241   TTAAGCTGCCAGCGGAATGCTTTCATCCTAAACCAAAAGTAAACAGTGTCTTAATAAAAC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5104  TTAAGCTGCCAGCGGAATGCTTTCATCCTAAACCAAAAGTAAACAGTGTCTTAATAAAAC  5163

Query  301   TTACCCGCCATAC  313
             |||||||||||||
Sbjct  5164  TTACCCGCCATAC  5176
```

Primer seq. query: *erm*B-R

Score = 689 bits (373), Expect = 0.0, Identities = 378/380 (99%), Gaps = 2/380 (0%), Strand = Plus/Minus

```
 Query  1     TTACTTTTGGTTTAGGATGAAAGCATTCCGCTGGCAGCTTAAGCAATTGCTGAATCG-GA  59
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  5145  TTACTTTTGGTTTAGGATGAAAGCATTCCGCTGGCAGCTTAAGCAATTGCTGAATCGAGA  5086

 Query  60    CTTGAGTGTGCAAGAGCAACCCTAGTGTTCGGTGAATATCCAAGGTACGCTTGTAGAATC  119
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5085  CTTGAGTGTGCAAGAGCAACCCTAGTGTTCGGTGAATATCCAAGGTACGCTTGTAGAATC  5026

 Query  120   CTTCTTCAACAATCAGATAGATGTCAGACGCATGGCTTTCAAAAACCACTTTTTTAATAA  179
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5025  CTTCTTCAACAATCAGATAGATGTCAGACGCATGGCTTTCAAAAACCACTTTTTTAATAA  4966

 Query  180   TTTGTGTGCTTAAATGGTAAGGAATATTCCCAACAATTTTATACCTCTGTTTGTTAGGGA  239
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4965  TTTGTGTGCTTAAATGGTAAGGAATATTCCCAACAATTTTATACCTCTGTTTGTTAGGGA  4906

 Query  240   ATTGAAACTGTAGAATATCTTGGTGAATTAAAGTGACACGAATGTTCAGTTTTAATTTTT  299
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4905  ATTGAAACTGTAGAATATCTTGGTGAATTAAAGTGACACGAATGTTCAGTTTTAATTTTT  4846

 Query  300   CTGACGATAAGTTGAATAGATGACTGTCTAATTCAATAGACGTTACCTGTTTACTTATTT  359
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4845  CTGACGATAAGTTGAATAGATGACTGTCTAATTCAATAGACGTTACCTGTTTACTTATTT  4786

              Query  360   TAGCCAGTTTCGGTCGTTAA  379
                            |||||||||||| |||||||
              Sbjct  4785   TAGCCAGTTTCG-TCGTTAA  4767
```

NCBI BLAST ref. number: EU869871.1

Primer seq. query: *tcr*B-F

Score = 760 bits (411), Expect = 0.0, Identities = 425/432 (98%), Gaps = 0/432 (0%), Strand = Plus/Plus

```
Query  1    CTTACTCATTACTCCTATGATGGATATTCAGTTGCCTTTCCAAATTATTTTTCCTTATGC  60
            |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  19   CTTACTCATTACTCCTATGATGGATATTCAGTTGCCTTTCCAAATTATCTTTCCTTATGC  78

Query  61   AGACGTTGTAGCGGCTGTATTGGCAACAATTCTATACATTTATGGCGGAAAACCATTCTA  120
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79   AGACGTTGTAGCAGCTGTATTGGCAACAATTCTATACATTTATGGCGGAAAACCATTCTA  138

Query  121  CATGGGTGCGAAAGATGAGTTTAATTCAAAAGCTCCAGGCATGATGTCCTTGATTACTTT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  139  CATGGGTGCGAAAGATGAGTTTAATTCAAAAGCTCCAGGCATGATGTCCTTGATTACTTT  198

Query  181  GGGAATAACGGTTTCTTATGCCTATAGTGTTTACGCAGTGGCCGCTCGATATGTGACCGG  240
            |||||||||||||||||||||||||||||||||||||||||||||||||| || ||
Sbjct  199  GGGAATAACGGTTTCTTATGCCTATAGTGTTTACGCAGTGGCCGCTCGATATGTAACTGG  258

Query  241  AGAACATGTCATGGACTTCTTCTTTGAGTTTACAACGTTAATTTTAATCATGTTATTAGG  300
            |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  259  AGAACATGTCATGGACTTCTTCTTTGAGTTTGCAACGTTAATTTTAATCATGTTATTAGG  318

Query  301  ACACTGGATTGAAATGAAGGCATTGGGTGAAGCAGGGGACGCGCAAAAAGCATTGGCTGA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  319  ACACTGGATTGAAATGAAGGCATTGGGTGAAGCAGGGGACGCGCAAAAAGCATTGGCTGA  378

Query  361  GTTGGTGCCGAAAGATGCTCATGTTGTTTTAGAAGACGATTCGATTGAGACTCGTCCTGT  420
            |||||||||||||||||||||||||||||||||||||||||||||||| || ||||||
Sbjct  379  GTTGGTGCCGAAAGATGCTCATGTTGTTTTAGAAGACGATTCGATTGAGACGCGCCCTGT  438

                      Query  421  GTCTGAACTTCA  432
                             ||||||||||||
                      Sbjct  439  GTCTGAACTTCA  450
```

Primer seq. query: *tcr*B-R

Score = 989 bits (535), Expect = 0.0, Identities = 553/562 (98%), Gaps = 0/562 (0%), Strand = Plus/Minus

```
 Query  1    TTGGTTTCGATTCCCCAGTCACAAGCGCTTCGTTTACACGTGATTCTCCGCGAATAATGA  60
             |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  563   TTGGTTTCGATTCCCCAGTCACAAGCGCTTCGTTTACACGGGATTCTCCGCGAATAATGA  504

Query  61   TACCATCAGCTGGAACATTTTCTCCTGCTTGAACACGGATAACATCTCCAATCTGAAGTT  120
             |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  503  TACCATCAGCTGGAACATTTTCTCCTGCTTGAACACGGATAACATCTCCAACCTGAAGTT  444

Query  121  CAGACACAGGACGAGTCTCAATCGAATCGTCTTCTAAAACAACATGAGCATCTTTCGGCA  180
             |||||||||| || ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  443  CAGACACAGGGCGCGTCTCAATCGAATCGTCTTCTAAAACAACATGAGCATCTTTCGGCA  384

Query  181  CCAACTCAGCCAATGCTTTTTGCGCGTCCCCTGCTTCACCCAATGCCTTCATTTCAATCC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  383  CCAACTCAGCCAATGCTTTTTGCGCGTCCCCTGCTTCACCCAATGCCTTCATTTCAATCC  324

Query  241  AGTGTCCTAATAACATGATTAAAATTAACGTTGTAAACTCAAAGAAGAAGTCCATGACAT  300
             |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  323  AGTGTCCTAATAACATGATTAAAATTAACGTTGCAAACTCAAAGAAGAAGTCCATGACAT  264

Query  301  GTTCTCCGGTCACATATCGAGCGGCCACTGCGTAAACACTATAGGCATAAGAAACCGTTA  360
             ||||||| || ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  263  GTTCTCCAGTTACATATCGAGCGGCCACTGCGTAAACACTATAGGCATAAGAAACCGTTA  204

Query  361  TTCCCAAAGTAATCAAGGACATCATGCCTGGAGCTTTTGAATTAAACTCATCTTTCGCAC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  203  TTCCCAAAGTAATCAAGGACATCATGCCTGGAGCTTTTGAATTAAACTCATCTTTCGCAC  144

Query  421  CCATGTAGAATGGTTTTCCGCCATAAATGTATAGAATTGTTGCCAATACAGCCGCTACAA  480
             |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
 Sbjct  143 CCATGTAGAATGGTTTTCCGCCATAAATGTATAGAATTGTTGCCAATACAGCTGCTACAA  84

Query  481  CGTCTGCATAAGGAAAAATAATTTGGAAAGGCAACTGAATATCCATCATAGGAGTAATGA  540
             ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
 Sbjct  83  CGTCTGCATAAGGAAAGATAATTTGGAAAGGCAACTGAATATCCATCATAGGAGTAATGA  24

                  Query  541  GTAAGATTGCAATTCCTAATGG  562
                               ||||||||||||||||||||||
                    Sbjct  23 GTAAGATTGCAATTCCTAATGG  2
```

NCBI BLAST ref. number: AY737526.1

Primer seq. query: *lsa*A-F

Score = 1061 bits (574), Expect = 0.0, Identities = 583/587 (99%), Gaps = 2/587 (0%), Strand = Plus/Plus

```
Query  1    TCGCTTGTGTTTTAGCGAATAATTTAAATTTTTATCAGAATGAAAGCAAGGGGCCTTCGT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6    TCGCTTGTGTTTTAGCGAATAATTTAAATTTTTATCAGAATGAAAGCAAGGGGCCTTCGT  65

Query  61   TGACAAATCAGTGAAGAGATGCTAAATTCTAGATAATAAAAGACGAAAAGAGGGATTCGT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  66   TGACAAATCAGTGAAGAGATGCTAAATTCTAGATAATAAAAGACGAAAAGAGGGATTCGT  125

Query  121  GCGTTGAAAATTTAAGTTAATTTTTTAGAGAAACTGTATAACTTTGCCTGAATATAACGG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  126  GCGTTGAAAATTTAAGTTAATTTTTTAGAGAAACTGTATAACTTTGCCTGAATATAACGG  185

Query  181  GTGGAGTGTGCGGATTTTCGGAAATTAGCTTAAAGAATTCAACCACGGGTCTTTTTGTAT  240
            |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  186  GTGGAGTGTGCGGATTTTCGGAAATTAGCTTAAAGAATTCAGCCACGGGTCTTTTTGTAT  245

Query  241  ACCTTGGATTGATGCCGCTGATTTCCGTAAAGCTGCATCAATTTTGCTTTATGGAG-G-T  298
            |||||||||||||||||||||||||||||||||||||||||||||||||||||| | |
Sbjct  246  ACCTTGGATTGATGCCGCTGATTTCCGTAAAGCTGCATCAATTTTGCTTTATGGAGAGGT  305

Query  299  ATATTTATGTCGAAAATTGTACTAAAACAACTATCTTTTGCCTATGATAATCAAGAAGCG  358
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  306  ATATTTATGTCGAAAATTGAACTAAAACAACTATCTTTTGCCTATGATAATCAAGAAGCG  365

Query  359  TTGCTTTTTGATCAGGCAAATATCACGATGGATACCAATTGGAAATTAGGATTGATTGGC  418
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  366  TTGCTTTTTGATCAGGCAAATATCACGATGGATACCAATTGGAAATTAGGATTGATTGGC  425

Query  419  CGCAATGGCCGTGGGAAAACAACCTTATTAAGATTGTTACAAAAGCAGTTGGATTACCAA  478
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  426  CGCAATGGCCGTGGGAAAACAACCTTATTAAGATTGTTACAAAAGCAGTTGGATTACCAA  485

Query  479  GGAGAGATTCTTCATCAAGTCGATTTCGTCTATTTTCCACAAACAGTTGCAGAAGAACAA  538
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  486  GGAGAGATTCTTCATCAAGTCGATTTCGTCTATTTTCCACAAACAGTTGCAGAAGAACAA  545

Query  539  CAGCTCACTTATTATGTCTTACAAGAGGTGACTTCTTTTGAACAGTG  585
            |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  546  CAGCTCACTTATTATGTCTTACAAGAGGTGACTTCTTTTGAACAGTG  592
```

Primer seq. query: *lsa*A-R

Score = 1048 bits (567), Expect = 0.0, Identities = 578/583 (99%), Gaps = 2/583 (0%), Strand = Plus/Minus

```
Query  1    TGCCCGATCGTGGCTGACTAAAATAAACCCGTGTTTCTTTTTCTTCAAATATTCAGCCAC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  833  TGCCCGATCGTGGCTGACTAAAATAAACCCGTGTTTCTTTTTCTTCAAATATTCAGCCAC  774

Query  61   TTGTTGTCTGCCAGCTAGATCTAAATGATTTGTTGGCTCGTCAATTAAAGGAAAGGCATT  120
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  773  TTGTTGTCTGCCAGCTAGATCTAAATGATTTGTTGGCTCGTCAATTAAAGGAAAGGCCTT  714

Query  121  TTCTTCAATAAAAAGAAGACCTAATAAAACTTTCGTCTTTTCGCCGCCTGATAAAGAAGA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  713  TTCTTCAATAAAAAGAAGACCTAATAAAACTTTCGTCTTTTCGCCGCCTGATAAAGAAGA  654

Query  181  AAAGGGCCGCCATAAAACTTCAGGATCAACGTTTAAAAGCGTTAATTCTCGTTCTAATTT  240
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  653  AAAGGGCCGCCATAAAACTTCAGGATCAACGTTTAAAAGCGTTAATTCTCGTTCTAATTC  594

Query  241  CCACTGTTCAAAAGAAGTCACCTCTTGTAAGACATAATAAGTGAGCTGTTGTTCTTCTGC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  593  CCACTGTTCAAAAGAAGTCACCTCTTGTAAGACATAATAAGTGAGCTGTTGTTCTTCTGC  534

Query  301  AACTGTTTGTGGAAAATAGACGAAATCGACTTGATGAAGAATCTCTCCTTGGTAATCCAA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  533  AACTGTTTGTGGAAAATAGACGAAATCGACTTGATGAAGAATCTCTCCTTGGTAATCCAA  474

Query  361  CTGCTTTTGTAACAATCTTAATAAGGTTGTTTTCCCACGGCCATTGCGGCCAATCAATCC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  473  CTGCTTTTGTAACAATCTTAATAAGGTTGTTTTCCCACGGCCATTGCGGCCAATCAATCC  414

Query  421  TAATTTCCAATTGGTATCCATCGTGATATTTGCCTGATCAAAAAGCAACGCTTCTTGATT  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  413  TAATTTCCAATTGGTATCCATCGTGATATTTGCCTGATCAAAAAGCAACGCTTCTTGATT  354

Query  481  ATCATAGGCAAAAGATAGTTGTTTTAGTACAATTTTCGACATAAATATACCTC-C-ATAA  538
            |||||||||||||||||||||||||| |||||||||||||||||||||| | ||||
Sbjct  353  ATCATAGGCAAAAGATAGTTGTTTTAGTTCAATTTTCGACATAAATATACCTCTCCATAA  294

Query  539  AGCAAAATTGATGCAGCTTTACGGAAATCAGCGGCATCAATCC  581
            |||||||||||||||||||||||||||||||||||||||||||
Sbjct  293  AGCAAAATTGATGCAGCTTTACGGAAATCAGCGGCATCAATCC  251
```

NCBI BLAST ref. number: AE016830.1

Primer seq. query: *ant*2-I-F

Score = 451 bits (244), Expect = 1e-124, Identities = 252/256 (98%), Gaps = 0/256 (0%), Strand = Plus/Plus

```
Query  1        ATGTGATTGCCGAAGAAGCAACTTTATCTGGTACAATTCGCACATTGACAGCAGAAACTA  60
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  1106503 ATGTGATTGCCGAAGAAGCAACTTTATCTGGTACAATTCGCACATTGACAGCAGAAACGA
1106562

Query  61       ATGAACAAACGCAGCGAAGAATTCGCGAAATTAGTGAAGGTATTGCGCAATCTTTTCAAT 120
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1106563  ATGAACAAACGCAGCGAAGAATTCGCGAAATTAGTGAAGGTATTGCGCAATCTTTTCAAT
1106622

Query  121      GTGAAGTAACTGTTCATCTTGATCAGAAAGGGTATTTACCTGTAGTGAATGAGCCCGCAT 180
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  1106623  GTGAAGTAACTGTTCATCTTGATCAGAAAGGGTATTTACCTGTAGTGAATGAGCCAGCAT
1106682

Query  181      GTACCACGAATTTTATTGAATATATAAGCAAACAAGCAACAGTTCAATTTCAACAAGCAC 240
                |||||||||||||||||||||| || ||||||||||||||||||||||||||||||||||
Sbjct  1106683  GTACCACGAATTTTATTGAATACATGAGCAAACAAGCAACAGTTCAATTTCAACAAGCAC
1106742

Query  241      CGGTTGCAATGACAGG  256
                ||||||||||||||||
Sbjct  1106743  CGGTTGCAATGACAGG  1106758
```

Primer seq. query: *ant*2-I-R

Score = 375 bits (203), Expect = 5e-102, Identities = 211/215 (98%), Gaps = 0/215 (0%), Strand = Plus/Minus

```
Query  1        CCGGTGCTTGTTGAAATTGAACTGTTGCTTGTTTGCTTATATATTCAATAAAATTCGTGG  60
                |||||||||||||||||||||||||||||||||||| || ||||||||||||||||||
Sbjct  1106745  CCGGTGCTTGTTGAAATTGAACTGTTGCTTGTTTGCTCATGTATTCAATAAAATTCGTGG
1106686

Query  61       TACATGCGGGCTCATTCACTACAGGTAAATACCCTTTCTGATCAAGATGAACAGTTACTT 120
                ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1106685  TACATGCTGGCTCATTCACTACAGGTAAATACCCTTTCTGATCAAGATGAACAGTTACTT
1106626

Query  121      CACATTGAAAAGATTGCGCAATACCTTCACTAATTTCGCGAATTCTTCGCTGCGTTTGTT 180
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1106625  CACATTGAAAAGATTGCGCAATACCTTCACTAATTTCGCGAATTCTTCGCTGCGTTTGTT
1106566

            Query  181      CATTAGTTTCTGCTGTCAATGTGCGAATTGTACCA  215
                             |||| ||||||||||||||||||||||||||||||
        Sbjct  1106565      CATTCGTTTCTGCTGTCAATGTGCGAATTGTACCA  1106531
```

Appendix 20. Comparission of EFRM31 genome with BLAST reference genomes

gb|FJ792813.1| *Enterococcus* phage EFAP-1, complete genome, Length = 21115, Score = 9664 bits (5233), Expect = 0.0, Identities = 6863/7626 (89%), Gaps = 208/7626 (2%), Strand = Plus/Minus

```
Query  9379   AAGTGTTGTGAGGAACG-CCGTATACAGAAGGTCCGCTAACTGTATAACAACACTTTAGC  9437
              ||||||||||||| | | |||||||   || |||||||| |||   |||||||||||| | |
Sbjct  19229  AAGTGTTGTGAGGCA-GTCCGTATGTGGACGGTCCGCCAACCACATAACAACACTTAATC  19171

Query  9438   ATTAAAGAATGGTTGCTGTGCGACCAATTGACTTAGGTAAGTTTGTTATCCCCTTTGGAT  9497
              ||| || || ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19170  ATTTAATAACGGTTGCTGTGCGACCAATTGACTTAGGTAAGTTTGTTATCCCCTTTGGAT  19111

Query  9498   AAACTCATGACCTAAGTCTTTTTATTTGTGCTATAATA-ATGATAAGGAGGTAATACATA  9556
              |||||||||||||||||||||||||||||||||||| |   | |||||||| |||||||
Sbjct  19110  AAACTCATGACCTAAGTCTTTTTATTTGTGCTATAATAGA-AACAAGGAGGT-ATACATA  19053

Query  9557   TGAATAATATTGATTATGTA-CAAGACTATAT-AGACTACGTAAAAG-AAAATCAAATAC  9613
              |||||||||| |||| |||| |  || || || | | ||||| || | | ||||||||||
Sbjct  19052  TGAATAATATAGATTTTGTAGC-TGATTACATCA-ATTACGT-AAGGTATAATCAAATAC  18996

Query  9614   CAATAGGTAATAAAATCAAACAAGCTATTAGACGACATGAAAAAGATTTAGAGAAGTCAA  9673
              |||||||||||| || |||||||| |||||||| ||||| || || ||||||  ||||
Sbjct  18995  CAATAGGTAATAAGATTAAACAAGCCATTAGACGTCATGAGAAGGACTTAGAGCGGTCAC  18936

Query  9674   AAGACCCAGACTATCCATATTACTATGACCCTAAAGAAGCATTAGAACCAGTGGTTTTTA  9733
               ||||||||| |||||||| |||||||||||||||||||| |||||||||| |  ||||
Sbjct  18935  TAGACCCAGATTATCCATACTACTATGACCCTAAAGAAGCACTAGAACCAGTTGCATTTA  18876

Query  9734   TTGAAATGCTACCAGACCCAAAGACACGCAAAACAAATAAGCTAGCTAAGTTCCAGAAGT  9793
              |||||||||||||||||||||||||| || |||||||| ||||||||||||||||||||
Sbjct  18875  TTGAAATGCTACCAGACCCAAAGACACGTAAGACAAATAAACTAGCTAAGTTCCAGAAGT  18816

Query  9794   TTATTATTGCAATGATATATGGGTGGCGCAAAAAGAAAAACGGA-ATGCGCAGATTCAGA  9852
              |||||||||| |||||||||| |||||||||||||||||  | ||||| |||||||||
Sbjct  18815  TTATTATTGCTATGATATATGGTTGGCGCAAAAAGAAAAAC-AAGATGCGTAGATTCAGA  18757

Query  9853   AAAGTATATATCAGTTTAGCACGTAAGAACGGAAAATCTATTCTGGTTGCTGGTATCTCA  9912
              ||||||||||||||  |||||||||| ||||| || || ||||||||||||||||||||
Sbjct  18756  AAAGTATATATCAGCCTAGCACGTAAAAACGGTAAGTCAATTCTGGTTGCTGGTATCTCA  18697

Query  9913   CTATACGAGTTTC-TATTAGGTCAATATCCACAGGCAAGC-CGTCAGATAGTCGCCGCCG  9970
               |||| ||| ||| |||||||||| |||||| | ||    |  | ||||| || || || |
Sbjct  18696  TTATATGAG-TTCTTATTAGGTCAGTATCCAAACGC-TTCAAGACAGATTGTAGCGGCGG  18639

Query  9971   CCAACACCAAAGACCAAGCAGGTATTGTATTCAACATGTTAAAATCACAGCTAAAGGCAT  10030
              | ||||| |||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  18638  CTAACACTAAAGACCAAGCTGGTATTGTATTCAACATGTTAAAATCACAGCTAAAGGCAT  18579

Query  10031  TGCGTGCAGTAAGTGATGGAACAAGGAAAGTAACCAAAGTTAATAAGAAAGACATTGAAC  10090
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  18578  TGCGTGCAGTAAGTGATGGAACAAGGAAAGTAACCAAAGTTAATAAGAAAGACATTGAAC  18519

Query  10091  ACTTAGAAGATGAATCAACGGTTAAGCCACTATCAAGTGATGCAGATAGCTTAGACGGAC  10150
              |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  18518  ACTTAGAAGATGAATCAACGGTTAAGCCACTATCAAGTGATGTAGATAGCTTAGACGGAC  18459

Query  10151  TAGACGTGCTATGTGGTGTACTTGATGAGTATGGAGAAGCTAAAAGCACAGCCATGATTG  10210
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  18458  TAGACGTGCTATGTGGTGTACTTGATGAGTATGGAGAAGCTAAAAGCACAGCCATGATTG  18399

Query  10211  AGGTATTAGAAAGTTCACAGTCACAACAATTACAAGGTTTAATTTTGATTATTAGTACAA  10270
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Sbjct  18398  AGGTATTAGAAAGTTCACAGTCACAACAATTACAAGGTTTAATTTTGATTATTAGTACAA  18339

Query  10271  CAACCAAAAAACTTGAATGGACCAATGCACAGCATAGAATACCCATTTATAACTAAGCTAC  10330
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Sbjct  18338  CAACCAAAAACTTGAATGGACCAATGCACAGCATAGAATACCCATTTATAACTAAGCTAC  18279

Query  10331  TTAATGAGGAAGTGGAAGCTGATGCTTACCTTGCATTGTGTTGGGAAATGGATAGCCTAA  10390
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Sbjct  18278  TTAATGAGGAAGTGGAAGCTGATGCATACCTTGCCTTGTGTTGGGAAATGGATAGCCTAA  18219
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Query  10391  GTGAAGTAGATGATGAAGCTAACTGGATTAAGAGTAACCCATTATTTGAGAATGCACAGT  10450
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Sbjct  18218  GTGAAGTAGATGATGAAGCTAACTGGATTAAGAGCAACCCATTATTTGAGAATGCACAGT  18159

Query  10451  TACATGAAACAATGTACGAACATAAGGTTAACTCATTAGCAGAGTATAAGGCTAAGGGTG  10510
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Sbjct  18158  TACATGAAACAATGTACGAACATAAGGTTAACTCATTAGCAGAGTATAAGGCTAAGGGTG  18099

Query  10511  ACATGAGTGGTTGGCTAACTAAAGAGATGAACTTCTGGGTACAATCATCACAAGACAGTT  10570
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Sbjct  18098  ACATGAGTGGTTGGCTAACTAAAGAGATGAACTTCTGGGTACAATCATCACAAGACAGTT  18039

Query  10571  TCATTGATAAAGAATCTTGGGAAGCTGTTAAACAAACACAACCGTATGACATAAAAGGTA  10630
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Query  10631  GACCAGTTTATATAGGGCTGGATTTGGCACGTACAGGCGATATGACGGCTGTTAGTTGGG  10690
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Query  10691  TGATACCTATTGCAGAGGAACGCAAGCTACTTCTTGACAGTCACGCTTTTATAGCCTCTG  10750
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Sbjct  17918  TGATACCTATTGCAGAGGAACGCAAGCTACTTCTTGACAGTCACGCTTTTATAGCTTCTG  17859

Query  10751  TGGGCGGTATTGAAGCTAAACAGATAAAAGATAAGATACCGTATAGACAGTATGAGAACT  10810
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Query  10811  TAGGACTAGTAAACATCAGTCAGCGACCAGATGGTTTGATAGACCATGAAGATATGTGCG  10870
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Sbjct  17798  TAGGACTAGTAAACATCAGTCAGCGACCAGATGGTTTGATAGACCATGAAGATATGTGCG  17739

Query  10871  AATGGATAAGAGAGTTCATAACAGAGTATGACTTAGACCTACAAGGTATTTACTATGACG  10930
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Sbjct  17738  AATGGATAAGAGAGTTCATAACAGAGTATGACTTAGACCTACAAGGTATTTACTATGACG  17679

Query  10931  GACACCAAGCAACACCATCTGTAATCAACTTAAGCAAGGACTTTCCAGACAAACTAATTG  10990
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Query  10991  AAGTACCACAGCGCATCCAGTATTTAAATGCACCTACAAAGTACTTACGTGATGCAATTA  11050
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Sbjct  17618  AAGTACCACAGCGCATCCAGTATTTAAATGCACCTACAAAGTACTTACGTGATGCAATTA  17559

Query  11051  TCAAGGGTGAAGTGATTCAAACAGATAACCCACTACTAACTAGAGCGGTATATAATGCTA  11110
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Sbjct  17558  TCAAGGGTGAAGTGATTCAAACAGATAACCCACTACTAACTAGAGCGGTATATAATGCTA  17499

Query  11111  TTATGCGTGAGTTTGCGGACAATATAGCTATTGAAAAGGCTATGAATCGTAACAAAATTG  11170
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Sbjct  17498  TTATGCGTGAGTTTGCGGACAATATAGCTATTGAAAAGGCTATGAATCGTAACAAAATTG  17439

Query  11171  ACCCTATAGATGCCTTAATTAATGCAATGAGTGAAGCAATGTACTTTGATTATGAGTATA  11230
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Query  11231  TTAGCTTTAAAGAACTGATTGAAGAAGGTAAATTTGGATTTGGTGTATAAAGAGTGCTTT  11290
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Query  11291  AACTAGCACTCTTTTTTCTATTGTTTTGTGGTAAAATGGGGTATATAGGAGGGGTAAATA  11350
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Sbjct  17318  AACTAGCACTCTTTTTTCTATTGTTTTGTGGTAAAATGAGGTATATAGGAGGGGTAAATA  17259

Query  11351  TGCAAAATCCGGTATTGTTAGTAGTAATTTTCTACGTTTTAGGTATGCTATCACTAGTCA  11410
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Sbjct  17258  TGCAAAATGCGGTATGGTTAGTAGTAATTTTCTACGTTTTAGGTATGCTATCACTAGTCA  17199

Query  11411  CAGCAATGTTTTTCACTGGTTTAGTAGCTGGTTTAGTAGCCTTAGCAGTAGCATTATTAA  11470
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Sbjct  17198  CATCAATGTTTTTCACTGGTTTAGTAGCTGGTTTAGTAGCCTTAGCAGTAGCATTATTAA  17139

Query  11471  TTCCATCAGTAATCTTATACAAAGAATTGGGAGAAGGTGAATAATTAAATGGGAGTTTTT  11530
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Sbjct  17138  TTCCATCAGTAATCTTATACAAAGAATTGGGAGAAGGTGAGTAATTAAATGGGAGTTTTT  17079

Query  11531  GTCGCACCTAGAAGCACAACAAAGCAGAACTTCATTGACTTTATCGAAAGCAGTGACATT  11590
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Sbjct  17078  GTAGCACCTAGAAGTACAACAAAGCAGAACTTCATTGACTTTATCGAAAGCAGTGACATT  17019

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Sbjct  17018  TATTCAACAGAAATCACAGGTGAAAAAGCATTACGCAATTCAGACATTTTCACAGGTATC  16959

Query  11651  AACATTATTAGTGGTGACTTAGGACAGTCAAAATTTAGACCA-ACTAAGAATAGTGAAGC  11709
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Sbjct  16958  AACATTATTAGTGGTGACTTAGGACAGTCAAGCTTTAGACCAGTCAAAG-ATATTCCAGT  16900

Query  11710  TGATGAACAATTTC-TAAAAATGATTAACAAACGACCAAACAATAAACAAAGTCATTACA  11768
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Sbjct  16899  TGATGAA-GAGTTCTTGAAGATGATTAACAAACGACCAAACGACAAACAAAGTCATTACA  16841

Query  11769  TGTTCATGTACGCAACAGTAGCAAACTTGATTCTAAGCGGTAACTCATATGCATTAATCC  11828
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Sbjct  16840  TGTTCATGTATGCAACAGTAGCAAACTTGATTCTAAGCGGTAACTCATACGCATTGATTC  16781

Query  11829  ACAGAGATA-AATTAGG-AGTAGTTGAGAGCTTGGAGTTTGTAAAACCTGAACAAGTAAA  11886
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Query  11887  TGTAATCCAAAACGTTACAACAGGCGAATGGTCGTATGATGTAACAATGGAGTATGGTAG  11946
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Sbjct  16722  TGTAATCCAAAATGTAACGACTGGTGAGTGGTCATATGATGTAACAATGGATTATGGTAG  16663

Query  11947  CATTATGTACAAATGTACACCAGAAGATATTTTGCACTTTAGAATTACAACTGTAGATGG  12006
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Sbjct  16662  TATCATGTACAAGTGTAAACCAGAAGATATTTTACACTTTAGAATTACAACCGTAGATGG  16603

Query  12007  TTTCCTTGGTAGAAGTCCTTTACTATCATTGAAAGATGAAGTAGCTATGCAGTCAAACGG  12066
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Sbjct  16602  TTTCCTTGGAAGAAGTCCTTTATTATCATTAAAAGATGAAGTAGCTATGCAGTCAAACGG  16543

Query  12067  TAGTAAGATTTTAAGCAAGTTCTTTGCCAACGGTGTATTTGGAGGAGGTATCTTAAAACT  12126
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Sbjct  16542  TAGTAAGATTTTAAGTAAGTTCTTTGCCAACGGAGTATTCGGCGGAGGTATCTTAAAACT  16483

Query  12127  AAAAGGCGGTTACGTTGACAACGAAACAAAAGCAAAAATCAGACAGGACTTTGAAAAGGC  12186
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Sbjct  16482  AAAAGGCGCTTACGTTGATAACGATACTAAAGCTAAAATCAGAAAAGACTTTGAAAAGGC  16423

Query  12187  AAACGGTGGTAGCACTAACAGTAATGGCGTTATTGTCTTAGACGAATCAACAGAGTTCCA  12246
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Query  12247  AGAATACAAAATGAATACTGATATTCTTAAACTGATTCAAGGTAACAAGTTTAGTACACA  12306
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Query  12307  ACAGATTGCAAAAGTATTAGGTATTCCATTGAACCGCTTTGGTATGGAATTAGTAAACTC  12366
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Sbjct  16302  ACAGATTGCAAAAGTATTAGGTATTCCATTGAACCGCTTTGGTATGGAGTTAGTAAACTC  16243

Query  12367  TACAGATAGTGGTGCAAATGACATCTATATTGCTTCAACTATTAGCCAATATGAAAGAGC  12426
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Sbjct  16242  TACAGATAGTGGAGCGAACGACATCTACATTGCATCAACTATTAGCCAGTATGAAAGAGC  16183

Query  12427  TATCTGTGATGAAATTGAAATTAAAACAGGTAACG-AGTTAGAATTAGACCTCACAACAT  12485
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Sbjct  16182  CATCTGTGATGAAATTGAAATTAAAACAGGTAACACA-TTAGAGTTAGACTTGACAACAT  16124

Query  12486  TATTAAATGATACCTATGAAGATAGACGTAAACGAGTTTTTGAAGGTAAA-CAGAATAAT  12544
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Sbjct  16123  TATTGAATGATACTTATGAAGATAGACGGAAACGAGTATTTGAAGGTAAGTCAGG-TAAA  16065

Query  12545  GAGTTGCTAAAAGCAATT-AAGACTAACGAAATTCGTGACTATTTAGGTTATGCAGA-TG  12602
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Sbjct  16064  GAACTATTGGGTGCTATCCAAGTG-AATGAAATCCGTGACTATTTAGGTTATG-AGAAT-  16008

Query  12603  TTGA--AGAAGGTGA-A-GAATTAATGAAAGTAGATGAAAAAGAAGGAGTGTCAGCAAGT  12658
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Sbjct  16007  TT-ACCAGATGGCGATACGCAC-A-TCCAAGTAAAAGGACAAGAAGGAGTGTCAGCAAGT  15951

Query  12659  GAAGAATCTTGAAATTAGACAACTACAACAGGTTGAAACAGTAACAGACAATATTGTTGA  12718
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Sbjct  15950  GAAGAATCTTGAAATTAGACAACTACAGCAAGTTGAAACAGTAACAGACAACATTGTTGA  15891

Query  12719  AGGCTACGCTTTAAAGTTTGATTCACCTAGTGAAAACTTAGGTGGATTCATTGAGTTCAT  12778
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Sbjct  15890  GGGGTACGCTTTAAAGTTTGATTCACCTAGTGAGAACTTAGGTGGATTCATTGAATTCAT  15831

Query  12779  AGATAAGCGTGCATTAGATGGTGTTGATATGAGTGACGTTCGTATGTTCGTTGACCATGA  12838
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Sbjct  15830  TGATAAGCGTGCTTTAGATGGTGTTGATATGAGTGACGTTCGTATGTTCGTTGACCATGA  15771

Query  12839  TTCAAGTAAGTTATTAGGACGGACAAAGAGTGGAACATTAACATTAGAGGTTGACGACAT  12898
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Sbjct  15770  TTCAAGTAAGTTATTAGGACGGACAAAGAGTGGAACATTAACATTAGAGGTTGACGACAT  15711

Query  12899  TGGTTTAAAATTTAGAGCATTACTTCCTGATACATCAGTTGGACGTGATGCAATGGAACT  12958
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Sbjct  15710  TGGGTTAAAATTTAGAGCATTACTTCCTGATACATCAGTTGGACGTGATGCAATGGAACT  15651

Query  12959  AGTTAAACGCGGTGACCTTAGTCAGTGTTCGTTTGGATTCACAGTTGCTAAAGATGAATG  13018
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Sbjct  15650  AGTTAAACGCGGTGACCTTAGTCAGTGTTCGTTTGGATTCACAGTTGCTAAAGATGAATG  15591

Query  13019  GCGCAAAGCTGATGGAATAAATCACAGAACTATCAAACAAATCAAGTCACTATTTGAAAT  13078
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Query  13079  CTCACTGGTTTCAATTCCAGCATATGCAGACACAGATGTTAGTGTGGCTAAACGTTCATT  13138
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Sbjct  15530  CTCACTGGTTTCAATTCCAGCATATGCAGACACAGATGTTAGTGTGGCTAAACGTTCATT  15471

Query  13139  AGAACAAATGGAAGAGGAAGCAAAACAATTTCAAAAGCGTAAACTAGAATTAGAATTAAA  13198
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Sbjct  15470  AGAACAAATGGAAGAGGAAGCAAAACAATTTCAAAAGCGTAAACTAGAATTAGAATTAAA  15411

Query  13199  CCTATTAGGGTTATACAAGTAACACCCACAAGTTCCCTTGTTCGTGATATACTATCTTTA  13258
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Sbjct  15410  CCTATTAGGGTTATACAAGTAACACCCACAAGTTCCCTTGTTCGTGATATACTATCTTTA  15351

Query  13259  GTAAGAAAAATAATAT--TAAAGGGGTGTGCGTATGTCACGGCAAGAACTTATGGAACAA  13316
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Sbjct  15350  GTAAGAAAAATAAAAAACTAA-GGGGTGTGCGTATGTCACGGCAAGAACTTATGGAACAA  15292

Query  13317  GCACAAACTTTATTATCAGAAGGTAAACTTGATGAAGCTGAAAAAGTAATGCAACAAATT  13376
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Query  13377  AAAGCTTTAGATGAGGAAAAACCTAAAGAAGAAGAACGTGCAGTTGATAATAAAGAAGAG  13436
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Query  13437  GAGAAACCAGAGGAAGAACCTAAAGCagaagatgctaaggacgaaccaaaggaagaacct  13496
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Query  13497  aaagaagataaggaagaaacaaaagaagaacGTTCAGCAAAAACTGAACAAGATTTTGAA  13556
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Query  13557  GAACCTAAAGAGGAACCAAAGGAAGAACCTAAAACAGAAAAACGCTCACTAGAGCaaaaa  13616
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Sbjct  15051  GAACCTAAAGAGGAACCAAAGGAAGAACCTAAAACAGAAAAACGCTCACTAGAGCAAAAA  14992

Query  13617  ggagaagaaaacatggagaaagttattttagatggaaaagaagtagaaaataaagaagTG  13676
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Query  13677  CGTGGATTCTTAGAATACTTACGCTCAAAAGAAACACGTGCATTGCCTGAATCATTTGAA  13736
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Query  13737  GGTGTTAAGTCTGCGGATGCATCAGCAATTATCCCAGAAGAAATCATCACAAAAGCTAAA  13796
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Query  13797  ATGTTACCTGAAACAGTTGTTGACTTACGTAATATGGTTACACGTCAAAAAGTAACAAAT  13856
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Query  13857  GCTATGGGTAAATATCCAATCTTGAAAGCTAATGAAGCCGTGTTGGCATCAGTTGAAGAA  13916
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Sbjct  14751  GCAATGGGTAAATATCCAATCTTGAAAGCTAATGAAGCAGTATTGGCAACAGTTGAAGAA  14692
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Query  13917  CTTAAAAAGAACCCTGATTTAGAAGGACCAGCTTTCGAAGAAGTCAAATATGAAGTTGAA  13976
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Query  13977  ACTTACCGTGGTCAAATTGCAGTAGCAGAAGAAGCTTTACAAGATTCTGATGATGACTTA  14036
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Sbjct  14631  ACTTACCGTGGTCAAATTGCAGTAGCAGAAGAAGCTTTACAAGATTCTGATGATGACTTA  14572

Query  14037  TCTGGAATCATTGCACGTCACATTCAACGTCAAGGTTTGAACACAGCTAACAAAGCTATC  14096
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Sbjct  14571  TCTGGAATCATTGCACGTCACATTCAACGTCAAGGTTTGAACACAGCTAACAAAGCTATT  14512

Query  14097  GTTACTAAATTAAAA-ACAGCAACAGCAGTAGCCGC-AACATCAATTGATGACTTGAAAA  14154
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Sbjct  14511  GTTGCTAAATTAAAAGA-AGCTACAGCAGTAGCTGCGAA-ATCTATTGATGACTTGAAAA  14454

Query  14155  CACAAGTTAACACTGGTTTTGACCCAGCTTACAACTTAGAGTTTATTGTTTCTCAATCAT  14214
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Sbjct  14453  CGCAAGTTAACACTGGTTTTGACCCAGCTTACAACTTAGAATTTATCGTTTCACAATCAT  14394

Query  14215  TCTTTAACGCTTTAGACCAAATGAAAGACGCTAACGGTCGTTACTTACTAGAAGATGACA  14274
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Query  14275  TCAAAGCACCATCTGGTAAATCTCTATTAGGACGTAAAGTAACTGTTTTAGCTGATAAAT  14334
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Query  14335  TAATTGGTA-C-AGTAGACGGTGACAAAGTAGCATTCTTAGGACAACCTGATGCATTCGC  14392
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Sbjct  14273  TAATTGG-AGCTAA-AGATGGTGACATGGTAGCATTCTTAGGACAACCTGACGCGTTCGC  14216

Query  14393  AGTCTTCTTCGACCGCGTAGACACAACTGTACGTTGGGTTGAGCATCAATACTTCGGTCA  14452
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Sbjct  14215  GGTATTCTTTGACCGTGTAGACACTACTGTCCGTTGGGTAGAACACCAATACTACGGTCA  14156

Query  14453  AGTATTAGCAGTAGCTATGCGCTTTGACTGTGAAGTCGTTGATAAAAACGCTGGTAAATA  14512
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Sbjct  14155  AGTATTAGCAGTAGCAATGCGCTTTGACTGTGAAATCGTTGATAAAAACGCTGGTAAGT-  14097

Query  14513  T-ATCACTTTAACACC-AGCACC--C--T-AGTAC-GCCACCTG-TGGTAGG-GCAGGTG  14562
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Sbjct  14096  TCATCACTTTGACACCCAGTGTCGGCGGTGAGTAAAGC-ACC-GCTAGTTGGTG-AGATA  14040

Query  14563  ACACC-AGCTGAAACATCAGCAACAATTGAATT-ATCGTAAACACTAAG-AGG-TGGGGC  14618
              ||| | | |||||||||||||||||||||||| |  || ||| | ||| ||| | ||||
Sbjct  14039  ACAACTA-CTGAAACATCAGCAACAATTGAATTGA--GTTAAC-C-AAGGAGGGTAGGGC  13985

Query  14619  ATT-CGC-CT-TACCTCTTTTTAAACTATAAAAGGAGAGTGTAA-ATTATGGCTAAAGTA  14674
               || ||| || | |||||| ||| | |||||| ||||| |  || |  |||| |||||||
Sbjct  13984  -TTGCGCTCTAT-CCTCTTATTATATTATAAA-GGAGA-T--AACACAATGGTTAAAGTA  13931

Query  14675  TTTAACGTATACAAGAAAACA-GGTAA-AAAG-ATTGTGACTGGACA-ACCCTCACCAGT  14730
              |||||  | ||||| |||| | |||   |||| |||   |||||    ||  |||||||
Sbjct  13930  TTTAATATCTACAA-AAAAGATGGTTCTAAAGTATTA--ACTGGGGTTACA-TCACCAGC  13875

Query  14731  AACAATCA-CTGGTTTAACAGCAGAA-ACACCATATAGTAAAGGTGATTTTCAAGTAACA  14788
              || ||| | | ||  ||||| |  || |||| |||| | |||||||| | ||||| ||||
Sbjct  13874  AA-AATTAACAGGACTAACACCT-AATACACAATATGGAAAAGGTGACTATCAAGCAACA  13817

Query  14789  GCTATTG-AAGATGGAAAACCCGAGTCTAGTAAA-GTAGATGTACCAGCATTCACTACAA  14846
              || | || |||||||||| |  || ||| | ||| |||||| ||||||||||||  ||||
Sbjct  13816  GC-AGTGGAAGATGGAAAGCTAGAATCT-GAAAAAGTAGATTTACCAGCATTCAAAACAA  13759

Query  14847  CTGCAACAGCTGGATAATAAAAGAGGGTTAAGCCCTCTTTTTAACTAAGGAGGTA-TGAT  14905
                | ||||| |  | | |  || |   | |  ||     | |||  ||||||||| || |
Sbjct  13758  AAGAAACAG-T--A-AGTCCAACA---TCA--CCAGAAGTATAA--AAGGAGGTAATG-T  13711

Query  14906  AA-TG---TTAACA-TTAGCAGAAGTAAAAAACAATCTAAGA-TTAGACTATGATTTTGA  14959
              || ||   || ||| || | | |||| ||||||||| ||||| || ||| || |||||||
Sbjct  13710  AAATGACATTGACACTT-GAACAAGTTAAAAACAATTTAAGACTT-GACCATAATTTTGA  13653

Query  14960  TGAT-ACCTACCT-ACAAGGACTGATTGATA-CGAGTGAACTATTCAT-CTTAGGAGCTA  15015
              |||| |  ||  | ||| ||||| ||| | | | |||||| | || || ||| || ||||
Sbjct  13652  TGATGAA-TATATTACA-GGACTAATT-ACAACTAGTGAAATGTTTATACTT-GGTGCTA  13597

Query  15016  TTGAGTTAA-AGACCGCACCAGATGATAAACGATTCAAGACATTACAGTTCATGTTGGTT  15074
              |||| |||| |||  || ||||||||| |||| || || ||  ||||||||||||||||
Sbjct  13596  TTGAATTAACAGAA-GCGCCAGATGATGAACGCTTTAACACCCTACAGTTCATGTTGGTT  13538
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Query  15075  TCATTATGGTATGAAAACAGAGTACCAGCAACAAGCGCA-TTACAACAACAAGTACCCTT  15133
              ||| | |||||||||||||||||||||||||| |||||| || |||||||||||||| ||
Sbjct  13537  TCACTCTGGTATGAAAACAGAGTACCAGCAACTAGCGCACTT-CAACAACAAGTACCATT  13479

Query  15134  TACAATCGTAGCAATGATTCATCAATTAAGGGGGTTACAAGATGCCACTGATACCAACAC  15193
              ||| || ||||| |||||||||||| |||||||||||||||||| ||| | || ||||||
Sbjct  13478  TACTATTGTAGCTATGATTCATCAACTAAGGGGGTTACAAGATGGCACAGGTAGCAACAC  13419

Query  15194  AACGACTTGACAGAAAG-ATA-AGTATCCAACATAAAACCACTCATAAGAACGAATATTA  15251
              |||||||||| | || | ||  |  || ||| | ||||||||||||||||||||||||||
Sbjct  13418  AACGACTTGATA-AACGTATTGAC-ATTCAAGAGAAAACCACTCATAAGAACGAATATTA  13361

Query  15252  TGAGTGGGTCACTGATTGGGAAACCAAGGCCACTATATGGTGTTCAGTAAAACAACAGTA  15311
              |||||||||||| |||||||||| ||  ||| ||| |||||||||| ||| |||| ||
Sbjct  13360  TGAGTGGGTCACAGATTGGGAAACAAAATCCAGTATCTGGTGTTCAGTGAAAGAACAATA  13301

Query  15312  -TTTCAAAGACTATAAAGAATCATTAGGTACTGTTTTAGAGGATACAACAAACTTCATTA  15370
               ||| |||||||||||||||| |||||||| || |||||||||||||||||||| ||||
Sbjct  13300  CTTT-AAAGACTATAAAGAATCACTAGGTACAGTGTTAGAGGATACAACAAACTTTATTA  13242

Query  15371  TCAGATACGAACAACAGATTCATATTGACAATACTATGCGTGTTGTATACAACGGTGTTA  15430
              ||||||| ||||||||  |||| |||||||||| ||||||||||||||| ||||| ||||
Sbjct  13241  TCAGATATGAACAACATTTTCAAATTGACAATAGTATGCGTGTTGTATATAACGGAGTTA  13182

Query  15431  ACTATGACATCATACAAGTATTAGAAGGTAGCTTCCAACGTGACTTTACAACGCTTGTAT  15490
              ||||||| ||  | ||||| |||||||||| |||||||||||||||||||| |||||||
Sbjct  13181  ACTATGATATTGTTCAAGTGTTAGAAGGTAGTTTCCAACGTGACTTTACAACCCTTGTAT  13122

Query  15491  GTAAGAGGGTGAAGGAA-TGAGTAGTCCA-AACTACGTTGACTTTTCACAAGTC-TACAA  15547
              |||||||||||||| || ||||| ||  | || || ||||| |||||||| | | || ||
Sbjct  13121  GTAAGAGGGTGAAG-AAGTGAGTGGTA-AGAATTATGTTGATTTTTCACAGG-CATATAA  13065

Query  15548  GCGATTAGAAAAAAGC-GGTAAA-AAGGCTGACCTA-ATTGTTAGTAAGGC-AGTGAATA  15603
              ||| || | ||| || || ||| || || |||| | ||||| ||||| || | | || |
Sbjct  13064  ACGACTACAGAAA-GCTGG-AAAGAATGCGGACC-AGATTGTAAGTAAAGCTA-TAAACA  13009

Query  15604  CAGCTGGTGAAAA-AGCAATG-AATGAACTAAAGCAAAATACACCACGTTTTAACGGCAC  15661
              ||||||||||   || | || || ||  |||| |  |||||||||||||||||||| ||
Sbjct  13008  CAGCTGGTGAAGCTAGTA-TGCAA-GAGTTAAAACGTAATACACCACGTTTTAACGGTAC  12951

Query  15662  AAAGTATAGTAGGGATGGTCAGGACTACAAAAAAGAACATATGCGTGACCATGTTGTAAT  15721
              ||||||||||  |||| |||||| ||||| |||||||| ||||| |||||| | |||||
Sbjct  12950  AAAGTATAGTAAAGATGCTCAGGATTACAAGAAAGAACACATGCGGGACCATATAGTAAT  12891

Query  15722  GAGTAAAGCAAATAAGAATAACCATATAGCAGAAGTTGGCTTTGATGATGATGTGGCATG  15781
              ||||||||||  ||||| || ||| | || || ||||||||||||||||||||||||||
Sbjct  12890  GAGTAAAGCAAGCAAGAACAAGCATGTTGCGGAGGTTGGCTTTGATGATGATGTGGCATG  12831

Query  15782  GATGGCACACTTTCCAGAGTTAGGAACAATCAAACAACGACCACAAGGGTTTATACAAAA  15841
              |||||||| |||||||| ||||| ||||| |||||| |||||||||| ||||||||||
Sbjct  12830  GATGGCACATTTTCCAGAATTAGGCACAATTAAACAAAGACCACAAGGTTTTATACAAAA  12771

Query  15842  AACAATAAACAGTATTGA-GAGTGAAGTTGCATCAATCATTCAAAAAGCATTACAGGAGG  15900
              ||| || || ||||| || || ||||||||||||||||||||||||||||||||||||||
Sbjct  12770  AACCATGAATAGTATAGAAGA-TGAAGTTGCATCAATCATTCAAAAAGCATTACAGGAGG  12712

Query  15901  CGTTTCTAAAATGAGACTACCCA-TATTACAAGTAGCAGAAGCTTTAGAGCAAGCACACC  15959
              ||||| |||||||||| |||| | |||||||||||||| ||| |  |||||||| ||||||
Sbjct  12711  CGTTTTTAAAATGAGATTACC-AGTATTACAAGTAGCTGAAACACTAGAGCAATCACACC  12653

Query  15960  CAGAAGTCAACTGGTTTACTAATGAAGTACCCACTGAATTTCAAACATTACC-ACAATTA  16018
              |||||||||||||||||||||||||||| || || ||||| |||||| |||| | || ||
Sbjct  12652  CAGAAGTCAACTGGTTTACTAATGAAGTGCCAACAGAATTCCAAACACTACCTA-AACTA  12594

Query  16019  CCAGTAGGTAGAATTGTAGAACTTGACGGACGGTACACAGAATATGCCAGTGCAGACCCT  16078
              ||||| |||||| | |||||||||||||||||||||||||||||||| || |||||||||
Sbjct  12593  CCAGTTGGTAGAGTGGTAGAACTTGACGGACGGTACACAGAATATGCAAGCGCAGACCCT  12534

Query  16079  AATTATTTTGTAACACATGTTCAGGTGGATTTGTGGTGTGAGGACTTGAAAGAAGTTGAA  16138
              |||||||||||||||||||| ||||| || ||||||||||||||||||||||||||||||
Sbjct  12533  AATTATTTTGTAACACATGTACAGGTAGACTTGTGGTGTGAGGACTTGAAAGAAGTTGAA  12474

Query  16139  AAGTATTACTTTGAAATTGACAAGACCATGCGTGCAGATAATGTGCAGTGCGTATTATCA  16198
              |||||||||||||| |||||||||| |||||||||||||||| ||||||||||||||||
Sbjct  12473  AAGTATTACTTTGATATTGACAAGACAATGCGTGCAGATAATGTTCAGTGCGTATTATCA  12414

Query  16199  CAACAAT-CGTACGACCCAGACCTAGAGGGTGCACGTAGGGTTATCAAACGTTACACAAT  16257
              ||||||  | |||||||||||||||| |  || |||||||||||||||||| ||||||
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Sbjct  12413  CAACAAAGC-TACGACCCAGACCTAGAAGACGCTCGTAGGGTTATCAAACGTTATACAAT  12355

Query  16258  CAGTCAAAGAGTTGTCTAAGTGACACCCTAAAACACGTTGATTAATGATATAATTATTCT  16317
               ||||||||| |||||||||||||||||||||||| |||||||||||||||||||||||
Sbjct  12354  CAGTCAAAGGGTTGTCTAAGTGACACCCTAAAACAGGTTGATTAATGATATAATTATTCT  12295

Query  16318  AGTAAGAAAATATAAACACTAAAGGAGAGAAACAATTATGGCAGTAGTAGGTTTTAAGAA  16377
               |||||||||||||||| || ||||||||     ||| |||||||||||||| ||||| ||
Sbjct  12294  AGTAAGAAAATATAAAAAC-AAAGGAGATTTT-AAT-ATGGCAGTAGTAGGGTTTAA-AA  12239

Query  16378  AG-CTATTAT--CAGCGTACCTAAAG-C-A-GAAGGTTCTGGCG-TTGATAAATACAC--  16428
               || |||| ||  |||  ||| |   | | | || ||       |  |||| ||| |||
Sbjct  12238  AGGCTATCATTTCAG--TACGTGGTGGCGAAGATGGAAAAA-CAATTGA-AAA-ACATGT  12184

Query  16429  AATTGATAAAAGC-GGTGGCGGTACTATTGAAGCGTCAATTAAAGGAATTTCAGCAGAAC  16487
               |||||||||||| | |||||||||||||||||||| || |||  ||||||||||||||||
Sbjct  12183  AATTGATAAAA-CAGGTGGCGGTACTATTGAAGCTTCTATTTCAGGAATTTCAGCAGAAC  12125

Query  16488  AAACAACCGTTTATGCGTCAAACGTTCCAATTTGGGTATCAGCTAAAGGGGTTGGAGAGT  16547
               ||||||| |||||||| |||||||||||||||||||||| |||||||| ||||| ||||
Sbjct  12124  AAACAACTGTTTATGCTTCAAACGTTCCAATTTGGGTATCTGCTAAAGGTGTTGGTGAGT  12065

Query  16548  TAACAGCTTCATTAAACGTATTTGACTTGTATAAAGACA-ACGTTTATGAGAAAATTTTA  16606
               || ||||| |||||||||| |||||||| || ||||    | || ||||| ||||| ||
Sbjct  12064  TATCAGCTACATTAAACGTCTTTGACTTATACAAAGGTGGA-GTATATGAAAAAATCTTG  12006

Query  16607  GGTATTGA-ACGTGGTGAAGATGGTATTGCAATC-GTTGGTGAAGATACAGAAGCACCTT  16664
               |||||| | ||||| |||||| || |||||| || ||||| ||||||||||||||||| |
Sbjct  12005  GGTATT-ACACGTGATGAAGAAGGCATTGCA-TCAGTTGGCGAAGATACAGAAGCACCAT  11948

Query  16665  ACGTTTCAGC-AGTATTTGTTGCAGACGGTGCAGACGGTAAAGAAATGTACTTTGGTTTA  16723
               |||| ||||  |  ||| || |||||||| ||||||||||||||||||||||||||||||
Sbjct  11947  ACGTATCAGTTAC-ATTCGTAGCAGACGGCGCAGACGGTAAAGAAATGTACTTTGGTTTA  11889

Query  16724  GTTAAAGGACGTTTCAGCCACCCAGAAATCGCATTGAATACAACAGAATCTGGTGGTACA  16783
                 |||||| ||||||||||||||||||| ||||| || ||||| |||||||| || |||
Sbjct  11888  ACTAAAGGTCGTTTCAGCCACCCAGAAATTGCATTAAACACAACCGAATCTGGCGGAACA  11829

Query  16784  GAACCAAACACAGAAACAATTGAAGGTTCATTTGTAACAGATTCAC-GTGGCT-TTGCAT  16841
               ||||||||||||||||||||||||||||||||||||||||||||  || |||| | |||| |
Sbjct  11828  GAACCAAACACAGAAACAATTGAAGGTTCATTTGTAACAGATG-ACCGTGG-TATTGCTT  11771

Query  16842  ACATG--AGTGCTGTTTCAAGCGA-AACATTAACACTT-GATAAGTTTGTTGACAAAGT-  16896
               |||||  || || || ||  | || || ||||||| || ||||| |||||||||| ||
Sbjct  11770  ACATGTCAG-GC-GTATCTGGTGAGAA-ATTAACA-TTAGATAAATTTGTTGACAA-GTT  11716

Query  16897  AAACAA  16902
               ||||||
Sbjct  11715  AAACAA  11710
```

Score = 1110 bits (601), Expect = 0.0, Identities = 698/743 (93%), Gaps = 14/743 (1%), Strand = Plus/Plus

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Query  1      AAAATTTAGCT-CTCCCTCCCATTTTATTTTGGTAGTCCCTCCCACTTTTTAAAATCTGA  59
               ||||||||| | ||||||||| |||||||||||| ||||||||  ||||||||||||||
Sbjct  20378  AAAATTTAGGTACTCCCTCCCGTTTTATTTTGGTACTCCCTCCC-GTTTTTAAAATCTGA  20436

Query  60     TAGCCC-C-AACCTACCTGCAATCACCCCTTGCGGTGGCGGAAGCTGGCTGAATGCTCTT  117
                | ||| | || |||||||||| |||||||||||||||||||| ||||| ||||
Sbjct  20437  CACCCCTCTAA-CTACCTGCAATTACCCCTTGCGGTGGCGGAAGCTGGCAGAATGTTCTT  20495

Query  118    CTTTATTGTGACACTCTTGACAGATGCTTTCTAGGTTATCTATATCCAACCTATGTTCCC  177
               ||||||||||||||||||||| || ||||||||||||||||||||||||||||||||||
Sbjct  20496  CTTTATTGTGACACTCTTGACATATACTTTCTAGGTTATCTATATCCAACCTATGTTCCC  20555

Query  178    AACCCATTGTGGTCCGTACCTCTTGCCTATGATGCACTATGGTTGCTATGCTTGTATTAC  237
               |||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  20556  AACCCATTGTGGTCCGTACCTCTTGCCTATGATGCACTATGGTTGCTATGCTTGCATTAC  20615

Query  238    ATACTTCACACATTGGATTCATTGATAGCTTTCTTGCTCTAACGTTTCGCCACTGTGTTG  297
               |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  20616  ATACTTCACACATTGGATTCATTGATAGCTTTCTTGCTCTCACGTTTCGCCACTGTGTTG  20675

Query  298    ATGCATAGAAGTCCGCATACTTCTTGTTATCTGGTGAATGTCTTACTTGCTTGTTGTATG  357
               |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
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Sbjct  20676  ATGCATAGAAGTCCGCATACTTCTTGTTGTCTGGTGAATGTCTTACTTGCTTGTTGTATG  20735

Query  358    T-CTTTGCT-TGTTGTCCTCTGTGTTGTTCACAGTATACTTCTGGTATGTCTACATACTC  415
              | | ||| | | ||  ||| |||||| ||||||||||||||||||||||||||||||||
Sbjct  20736  TTCGTTG-TGTATTA-CCTTTGTGTTCTTCACAGTATACTTCTGGTATGTCTACATACTC  20793

Query  416    CCTGCAGTGTGCAACCTTACACCGTCGCTTAGGCATTAGAAGCCCCACATATCTTTGTTA  475
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20794  CCTGCAGTGTGCAACCTTACACCGTCGCTTAGGCATTAGAAGCCCCACATATCTTTGTT-  20852

Query  476    GT-ATTACTA-T--TAAAGATGTCATAAGATACTTTAggtttggttgttggttctgttgt  531
              || |||  |  |  | ||||||||||||||||||||||||| ||||||||||||||||
Sbjct  20853  GTTATTGGTGGTGCTTAAGATGTCATAAGATACTTTAGGTTTGCTTGTTGGTTCTGTTGT  20912

Query  532    tggttgttctgttgtgtctgatgtgttggtAACCATTGCTTGCTCTAACTGTCTGATGTA  591
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20913  TGGTTGTTCTGTTGTGTCTGATGTGTTGGTAACCATTGCTTGCTCTAACTGTCTGATGTA  20972

Query  592    TGATACTAACTCTAACTTAGTATGAGATAGTAGTTGCTTGTCTGTCATGCCCTCCATGTT  651
              |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||| |
Sbjct  20973  TGATACTAACTCTAACTTAGTATGAGATAGTAGTTGCTTGTCTGTCATACCCTCCATGGT  21032

Query  652    CATATAGTATACCCTCCTCTATGTTGTGTTGTCAAGGGTACAGTATTAAACTATACCCTC  711
              |||||||||||||||||||||||||| || ||||||||||||||||||||||||||||||
Sbjct  21033  CATATAGTATACCCTCCTCTATGTTGTATTCTCAAGGGTACAGTATTAAACTATACCCTC  21092

Query  712    CATTCTATATTGTATATGGTGGT  734
              || ||| | ||||||||||||||
Sbjct  21093  CACTCTCTGTTGTATATGGTGGT  21115
```

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gb|AY954970.1| *Staphylococcus* phage Twort, complete genome, Length = 130706

Score = 62.1 bits (33), Expect = 1e-04, Identities = 35/36 (97%), Gaps = 0/36 (0%), Strand = Plus/Minus

```
Query  7011    atCATTTATACTAAGTATATAATCAATTAGGAGGAA  7046
               ||| ||||||||||||||||||||||||||||||||
Sbjct  107113  ATCCTTTATACTAAGTATATAATCAATTAGGAGGAA  107078
```

Appendix 21. Comparision of proteins encoded by EFRM31 with GenBank database

ref|YP_002727892.1| terminase large subunit [*Enterococcus* phage EFAP-1]
gb|ACN86334.1| terminase large subunit [*Enterococcus* phage EFAP-1], Length = 574
GENE ID: 7668753 EFAP1_gp20 | terminase large subunit [*Enterococcus* phage EFAP-1]
Score = 1132 bits (2929), Expect = 0.0, Identities = 562/574 (97%),
Positives = 567/574 (98%), Gaps = 0/574 (0%)

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Query  1    MNNIDYVQDYIDYVKENQIPIGNKIKQAIRRHEKDLEKSKDPDYPYYYDPKEALEPVVFI  60
            MNNID+V DYI+YV+ NQIPIGNKIKQAIRRHEKDLE+S DPDYPYYYDPKEALEPV FI
Sbjct  1    MNNIDFVADYINYVRYNQIPIGNKIKQAIRRHEKDLERSLDPDYPYYYDPKEALEPVAFI  60

Query  61   EMLPDPKTRKTNKLAKFQKFIIAMIYGWRKKKNGMRRFRKVYISLARKNGKSILVAGISL  120
            EMLPDPKTRKTNKLAKFQKFIIAMIYGWRKKKN MRRFRKVYISLARKNGKSILVAGISL
Sbjct  61   EMLPDPKTRKTNKLAKFQKFIIAMIYGWRKKKNKMRRFRKVYISLARKNGKSILVAGISL  120

Query  121  YEFLLGQYPQASRQIVAAANTKDQAGIVFNMLKSQLKALRAVSDGTRKVTKVNKKDIEHL  180
            YEFLLGQYP ASRQIVAAANTKDQAGIVFNMLKSQLKALRAVSDGTRKVTKVNKKDIEHL
Sbjct  121  YEFLLGQYPNASRQIVAAANTKDQAGIVFNMLKSQLKALRAVSDGTRKVTKVNKKDIEHL  180

Query  181  EDESTVKPLSSDADSLDGLDVLCGVLDEYGEAKSTAMIEVLESSQSQQLQGLILIISTTT  240
            EDESTVKPLSSD DSLDGLDVLCGVLDEYGEAKSTAMIEVLESSQSQQLQGLILIISTTT
Sbjct  181  EDESTVKPLSSDVDSLDGLDVLCGVLDEYGEAKSTAMIEVLESSQSQQLQGLILIISTTT  240

Query  241  KNLNGPMHSIEYPFITKLLNEEVEADAYLALCWEMDSLSEVDDEANWIKSNPLFENAQLH  300
            KNLNGPMHSIEYPFITKLLNEEVEADAYLALCWEMDSLSEVDDEANWIKSNPLFENAQLH
Sbjct  241  KNLNGPMHSIEYPFITKLLNEEVEADAYLALCWEMDSLSEVDDEANWIKSNPLFENAQLH  300

Query  301  ETMYEHKVNSLAEYKAKGDMSGWLTKEMNFWVQSSQDSFIDKESWEAVKQTQPYDIKGRP  360
            ETMYEHKVNSLAEYKAKGDMSGWLTKEMNFWVQSSQDSFIDKESWEAVKQT+PYDIKGRP
Sbjct  301  ETMYEHKVNSLAEYKAKGDMSGWLTKEMNFWVQSSQDSFIDKESWEAVKQTKPYDIKGRP  360

Query  361  VYIGLDLARTGDMTAVSWVIPIAEERKLLLDSHAFIASVGGIEAKQIKDKIPYRQYENLG  420
            VYIGLDLARTGDMTAVSWVIPIAEERKLLLDSHAFIASVGGIEAKQIKDKIPYRQYENLG
Sbjct  361  VYIGLDLARTGDMTAVSWVIPIAEERKLLLDSHAFIASVGGIEAKQIKDKIPYRQYENLG  420

Query  421  LVNISQRPDGLIDHEDMCEWIREFITEYDLDLQGIYYDGHQATPSVINLSKDFPDKLIEV  480
            LVNISQRPDGLIDHEDMCEWIREFITEYDLDLQGIYYDGHQATPSVINLSKDFPDKLIEV
Sbjct  421  LVNISQRPDGLIDHEDMCEWIREFITEYDLDLQGIYYDGHQATPSVINLSKDFPDKLIEV  480

Query  481  PQRIQYLNAPTKYLRDAIIKGEVIQTDNPLLTRAVYNAIMREFADNIAIEKAMNRNKIDP  540
            PQRIQYLNAPTKYLRDAIIKGEVIQTDNPLLTRAVYNAIMREFADNIAIEKAMNRNKIDP
Sbjct  481  PQRIQYLNAPTKYLRDAIIKGEVIQTDNPLLTRAVYNAIMREFADNIAIEKAMNRNKIDP  540

Query  541  IDALINAMSEAMYFDYEYISFKELIEEGKFGFGV  574
            IDALINAMSEAMYFDYEYISFKELIEEGKFGFGV
Sbjct  541  IDALINAMSEAMYFDYEYISFKELIEEGKFGFGV  574
```

ref|ZP_05650419.1| DNA primase [*Enterococcus gallinarum* EG2]

gb|EEV33752.1| DNA primase [*Enterococcus gallinarum* EG2], Length = 477

Score = 176 bits (445), Expect = 8e-42, Identities = 108/335 (32%),

Positives = 177/335 (52%), Gaps = 22/335 (6%)

```
Query  161  PFNDKMDPNKIAFKNGTYRFKEDTLKPTVKEDYQTTRIEYDYIDNPKHNI----VAEWIE  216
            PFN+   P    FKNGTY  K   LKP   +DY      +DY+ +PK N+      EW+
Sbjct  117  PFNNS-KPYLANFKNGTYNIKTGELKPHDTKDYILQ--SHDYVVDPKSNLKPEKAIEWLR  173

Query  217  YIL--EEDAKTLFQLIGRIFYRNQDP-QAMVFATGEGSNGKSHVMSFIEDLVGKSNTSHA  273
             +   E+ A+ L ++IG  FYR+  P Q +    G G NGKS  + F+  ++GK NTS+
Sbjct  174  DLTGDEKSAQHLMEIIGYCFYRSYAPFQTITILQGSGENGKSTFLDFLTKVIGKDNTSNM  233

Query  274  TLASLSGNNDKFASSQLFGKMVNIETDMPAQHIKQTGTLKTLSGNDVMSAEYKGINKFTF  333
            TL  L    ++FA + LF K  N+  D+ ++ +K TG LK L+G D +SAE+KG + F F
Sbjct  234  TLQELGNKQNRFAGANLFQKEANLFADVDSEFLKSTGLLKALTGGDRLSAEFKGKDHFMF  293

Query  334  TNYALMIFTTNNMPTFSDTSHGFLRRIITLPFNKTMGRDNPTDSMWLERSKNFTYEEKSE  393
             N+A +IF+ N +P F+D +HGF RR+  +PF+  +      +S   +   N   +E
Sbjct  294  VNFAKLIFSANELPAFNDFTHGFERRLYVVPFDCVI-----DESFKTKHDLNAIEKEIPL  348

Query  394  FISYCLQQYRNVLFGLNGETKGQFWTSDNANKLRDAFIQGNDTMANFIELNELEFTNNID  453
            F +YC+  ++  L       + +   SD  N+ +D +++ ++ +  FIE  +
Sbjct  349  FATYCISMFKEAL------DRKELTVSDKMNQAKDKWLKESNHIMRFIE-EKCSIDMESS  401

Query  454  DVIPNTELLEAYNTMLREENLMEVSAKKFIPELKR  488
            +   + ++ E Y     +E+L E+S  KF  +L++
Sbjct  402  EGDSSKKIYEEYQKFCYQESLRELSQPKFSKQLEK  436
```

ref|YP_002727888.1| major capsid protein [*Enterococcus* phage EFAP-1]

gb|ACN86330.1| major capsid protein [*Enterococcus* phage EFAP-1], Length = 418

GENE ID: 7668749 EFAP1_gp16 | major capsid protein [*Enterococcus* phage EFAP-1]

Score = 702 bits (1813), Expect = 0.0, Identities = 398/414 (96%),

Positives = 406/414 (98%), Gaps = 0/414 (0%)

```
Query  1    MSRQELMEQAQTLLSEGKLDEAEKVMQQIKALDEEKPKEEERAVDNKEEEKPEEEPKAED  60
            MSRQELMEQAQTLLSEGKLDEAEKVMQQIKALDEEKPKEEERAVDNKEEEKPEEEPKAED
Sbjct  1    MSRQELMEQAQTLLSEGKLDEAEKVMQQIKALDEEKPKEEERAVDNKEEEKPEEEPKAED  60

Query  61   AKDEPKEEPKEDKEETKEERSAKTEQDFEEPKEEPKEEPKTEKRSLEQKGEENMEKVILD  120
            AKDEPKEEPKEDKEETKEERSAKTEQD EEPKEEPKEEPKTEKRSLEQKGEENMEKVILD
Sbjct  61   AKDEPKEEPKEDKEETKEERSAKTEQDVEEPKEEPKEEPKTEKRSLEQKGEENMEKVILD  120

Query  121  GKEVENKEVRGFLEYLRSKETRALPESFEGVKSADASAIIPEEIITKAKMLPETVVDLRN  180
            GKEVENKEVRGFLEYLRSKETRALPESFEGVKSADASAIIPEEIITKAKMLPETVVDLRN
Sbjct  121  GKEVENKEVRGFLEYLRSKETRALPESFEGVKSADASAIIPEEIITKAKMLPETVVDLRN  180

Query  181  MVTRQKVTNAMGKYPILKANEAVLASVEELKKNPDLEGPAFEEVKYEVETYRGQIAVAEE  240
            M+TRQKVT+AMGKYPILKANEAVLA+VEELKKNPDLEGPAFEEVKYEVETYRGQIAVAEE
Sbjct  181  MITRQKVTHAMGKYPILKANEAVLATVEELKKNPDLEGPAFEEVKYEVETYRGQIAVAEE  240

Query  241  ALQDSDDDLSGIIARHIQRQGLNTANKAIVTKLKTATAVAATSIDDLKTQVNTGFDPAYN  300
            ALQDSDDDLSGIIARHIQRQGLNTANKAIV KLK ATAVAA SIDDLKTQVNTGFDPAYN
Sbjct  241  ALQDSDDDLSGIIARHIQRQGLNTANKAIVAKLKEATAVAAKSIDDLKTQVNTGFDPAYN  300

Query  301  LEFIVSQSFFNALDQMKDANGRYLLEDDIKAPSGKSLLGRKVTVLADKLIGTVDGDKVAF  360
            LEFIVSQSFFNALDQMKDANGRYLLEDDIKA SGK+LLGRKVTVLADKLIG  DGD VAF
Sbjct  301  LEFIVSQSFFNALDQMKDANGRYLLEDDIKAQSGKALLGRKVTVLADKLIGAKDGDMVAF  360

Query  361  LGQPDAFAVFFDRVDTTVRWVEHQYFGQVLAVAMRFDCEVVDKNAGKYITLTPA  414
            LGQPDAFAVFFDRVDTTVRWVEHQY+GQVLAVAMRFDCE+VDKNAGK+ITLTP+
Sbjct  361  LGQPDAFAVFFDRVDTTVRWVEHQYYGQVLAVAMRFDCEIVDKNAGKFITLTPS  414
```

ref|YP_002727890.1| portal protein [*Enterococcus* phage EFAP-1]

gb|ACN86332.1| portal protein [*Enterococcus* phage EFAP-1], Length = 383

GENE ID: 7668751 EFAP1_gp18 | portal protein [*Enterococcus* phage EFAP-1]

Score = 689 bits (1779), Expect = 0.0, Identities = 345/383 (90%),

Positives = 363/383 (94%), Gaps = 0/383 (0%)

```
Query  1    MGVFVAPRSTTKQNFIDFIESSDIYSTEITGEKALRNSDIFTGINIISGDLGQSKFRPTK  60
            MGVFVAPRSTTKQNFIDFIESSDIYSTEITGEKALRNSDIFTGINIISGDLGQS FRP K
Sbjct  1    MGVFVAPRSTTKQNFIDFIESSDIYSTEITGEKALRNSDIFTGINIISGDLGQSSFRPVK  60

Query  61   NSEADEQFLKMINKRPNNKQSHYMFMYATVANLILSGNSYALIHRDKLGVVESLEFVKPE  120
            +   DE+FLKMINKRPN+KQSHYMFMYATVANLILSGNSYALIHRD++G +ESLEFV+PE
Sbjct  61   DIPVDEEFLKMINKRPNDKQSHYMFMYATVANLILSGNSYALIHRDRMGGIESLEFVRPE  120

Query  121  QVNVIQNVTTGEWSYDVTMEYGSIMYKCTPEDILHFRITTVDGFLGRSPLLSLKDEVAMQ  180
            QVNVIQNVTTGEWSYDVTM+YGSIMYKC PEDILHFRITTVDGFLGRSPLLSLKDEVAMQ
Sbjct  121  QVNVIQNVTTGEWSYDVTMDYGSIMYKCKPEDILHFRITTVDGFLGRSPLLSLKDEVAMQ  180

Query  181  SNGSKILSKFFANGVFGGGILKLKGGYVDNETKAKIRQDFEKANGGSTNSNGVIVLDEST  240
            SNGSKILSKFFANGVFGGGILKLKG YVDN+TKAKIR+DFEKANGGSTNSNGVIVLDEST
Sbjct  181  SNGSKILSKFFANGVFGGGILKLKGAYVDNDTKAKIRKDFEKANGGSTNSNGVIVLDEST  240

Query  241  EFQEYKMNTDILKLIQGNKFSTQQIAKVLGIPLNRFGMELVNSTDSGANDIYIASTISQY  300
            EFQEYKMNTDILKLIQGNKFSTQQIAKVLGIPLNRFGMELVNSTDSGANDIYIASTISQY
Sbjct  241  EFQEYKMNTDILKLIQGNKFSTQQIAKVLGIPLNRFGMELVNSTDSGANDIYIASTISQY  300

Query  301  ERAICDEIEIKTGNELELDLTTLLNDTYEDRRKRVFEGKQNNELLKAIKTNEIRDYLGYA  360
            ERAICDEIEIKTGN LELDLTTLLNDTYEDRRKRVFEGK   ELL AI+ NEIRDYLGY
Sbjct  301  ERAICDEIEIKTGNTLELDLTTLLNDTYEDRRKRVFEGKSGKELLGAIQVNEIRDYLGYE  360

Query  361  DVEEGEELMKVDEKEGVSASEES  383
            ++ +G+  ++V  +EGVSASEES
```

ref|YP_002727889.1| prohead protease [*Enterococcus* phage EFAP-1]

gb|ACN86331.1| prohead protease [*Enterococcus* phage EFAP-1], Length = 187

GENE ID: 7668750 EFAP1_gp17 | prohead protease [*Enterococcus* phage EFAP-1]

Score = 378 bits (970), Expect = 2e-103, Identities = 186/187 (99%),

Positives = 187/187 (100%), Gaps = 0/187 (0%)

```
Query  9    VKNLEIRQLQQVETVTDNIVEGYALKFDSPSENLGGFIEFIDKRALDGVDMSDVRMFVDH  68
            +KNLEIRQLQQVETVTDNIVEGYALKFDSPSENLGGFIEFIDKRALDGVDMSDVRMFVDH
Sbjct  1    MKNLEIRQLQQVETVTDNIVEGYALKFDSPSENLGGFIEFIDKRALDGVDMSDVRMFVDH  60

Query  69   DSSKLLGRTKSGTLTLEVDDIGLKFRALLPDTSVGRDAMELVKRGDLSQCSFGFTVAKDE  128
            DSSKLLGRTKSGTLTLEVDDIGLKFRALLPDTSVGRDAMELVKRGDLSQCSFGFTVAKDE
Sbjct  61   DSSKLLGRTKSGTLTLEVDDIGLKFRALLPDTSVGRDAMELVKRGDLSQCSFGFTVAKDE  120

Query  129  WRKADGINHRTIKQIKSLFEISLVSIPAYADTDVSVAKRSLEQMEEEAKQFQKRKLELEL  188
            WRKADGINHRTIKQIKSLFEISLVSIPAYADTDVSVAKRSLEQMEEEAKQFQKRKLELEL
Sbjct  121  WRKADGINHRTIKQIKSLFEISLVSIPAYADTDVSVAKRSLEQMEEEAKQFQKRKLELEL  180

Query  189  NLLGLYK  195
            NLLGLYK
Sbjct  181  NLLGLYK  187
```

ref|YP_002727882.1| major tail protein [*Enterococcus* phage EFAP-1]

gb|ACN86324.1| major tail protein [*Enterococcus* phage EFAP-1], Length = 189

GENE ID: 7668744 EFAP1_gp10 | major tail protein [*Enterococcus* phage EFAP-1]

Score = 308 bits (788), Expect = 2e-82, Identities = 156/187 (83%),

Positives = 166/187 (88%), Gaps = 1/187 (0%)

```
Query  1    MAVVGFKKAIISVPKAE-GSGVDKYTIDKSGGGTIEASIKGISAEQTTVYASNVPIWVSA  59
            MAVVGFKKAIISV   E G  ++K+ IDK+GGGTIEASI GISAEQTTVYASNVPIWVSA
Sbjct  1    MAVVGFKKAIISVRGGEDGKTIEKHVIDKTGGGTIEASISGISAEQTTVYASNVPIWVSA  60

Query  60   KGVGELTASLNVFDLYKDNVYEKILGIERGEDGIAIVGEDTEAPYVSAVFVADGADGKEM  119
            KGVGEL+A+LNVFDLYK  VYEKILGI R E+GIA VGEDTEAPYVS  FVADGADGKEM
Sbjct  61   KGVGELSATLNVFDLYKGGVYEKILGITRDEEGIASVGEDTEAPYVSVTFVADGADGKEM  120

Query  120  YFGLVKGRFSHPEIALNTTESGGTEPNTETIEGSFVTDSRGFAYMSAVSSETLTLDKFVD  179
            YFGL KGRFSHPEIALNTTESGGTEPNTETIEGSFVTD RG AYMS VS E LTLDKFVD
Sbjct  121  YFGLTKGRFSHPEIALNTTESGGTEPNTETIEGSFVTDDRGIAYMSGVSGEKLTLDKFVD  180

Query  180  KVNNINP  186
            K+NN++P
Sbjct  181  KLNNVSP  187
```

ref|ZP_03212364.1| prophage LambdaSa2, HNH endonuclease family protein [*Lactobacillus rhamnosus* HN001]

gb|EDY98252.1| prophage LambdaSa2, HNH endonuclease family protein [*Lactobacillus rhamnosus* HN001], Length = 176, Score = 149 bits (377), Expect = 9e-35, Identities = 84/169 (49%), Positives = 111/169 (65%), Gaps = 10/169 (5%)

```
Query  10   EEWKDIKGYEGLYQVSNLGRVKSL-------KFGKERILKTGVDRYGYISVNLYKNNKRK  62
            E WKDIK YEGLYQ+SNLGRV+SL       +  K  +L + + + GY  V L ++ KRK
Sbjct  5    EIWKDIKDYEGLYQISNLGRVRSLDRVDGNGRGWKGIMLSSKLRKNGYREVILCRDGKRK  64

Query  63   LCKVHRLVAQAFIPNHENKPQVNHIDEDKTNNMVSNLEWVTAKENVNHGTRNDRVSKA--  120
               VHRLVA+AF+PN +N PQVNH DEDK NN V NLEW  +K N N+G    RV++A
Sbjct  65   YMLVHRLVAEAFLPNQDNLPQVNHKDEDKGNNRVENLEWCDSKYNNNYGNHIKRVAEARE  124

Query  121  VKVIDIANGEYNYYNS-INECVRQLGLHQGNVVSCLKGRRKTCGGYTFK  168
             ++  + N  + YY S +++  R LGLH  N+ +CL GR+KTC GYTF+
Sbjct  125  KQIYVVTNSGHRYYFSGVSKAARLLGLHYQNITACLHGRQKTCHGYTFE  173
```

ref|YP_002727893.1| terminase small subunit [*Enterococcus* phage EFAP-1]

gb|ACN86335.1| terminase small subunit [*Enterococcus* phage EFAP-1], Length = 157

GENE ID: 7668754 EFAP1_gp21 | terminase small subunit [*Enterococcus* phage EFAP-1]

Score = 197 bits (500), Expect = 4e-49, Identities = 98/157 (62%),

Positives = 124/157 (78%), Gaps = 0/157 (0%)

```
Query  1    MARPRKLINATNKHYTEEERIQKQKEEKKLYSYEPLDFSYFPMGLLRGAYAEWERIGNFV  60
            M RPRKLINA   + T E++ +++KEE+ LY+YE LDFSY+P GLL+GA+ EWERI +F+
Sbjct  1    MGRPRKLINAQVGNLTTEQQEERKKEEEALYNYEKLDFSYYPQGLLQGAFPEWERISHFI  60

Query  61   GDLPLSELDSQTLVSYCNYVYLYSKLAEEVSIEGEVTEDGKLNPKVTAMMGYSKQIKSAT  120
            GDLP+SELD QT+V YCNY YLYS+  E +  EGE+T DGK NP+V  M  YSK++KSAT
Sbjct  61   GDLPISELDQQTMVRYCNYTYLYSEATERLMEEGEITPDGKKNPRVDIMNSYSKELKSAT  120

Query  121  NDLGLTINSRLKLVAPKEVEDESKDPLGQLLKMRTQA  157
             DLGLTIN+RLK+VAP E   ES DPLGQL+K+R Q+
Sbjct  121  ADLGLTINARLKIVAPAEKTKESNDPLGQLIKLRQQS  157
```

ref|YP_238560.1| ORF083 [*Staphylococcus* phage Twort]

gb|AAX92377.1| ORF083 [*Staphylococcus* phage Twort], Length = 151

GENE ID: 5130306 TwortORF083 | hypothetical protein [*Staphylococcus* phage Twort]

Score = 47.4 bits (111), Expect = 4e-04, Identities = 45/147 (30%),

Positives = 71/147 (48%), Gaps = 21/147 (14%)

```
Query  12   QGQVKKHITQCTTQEKEQLNKAKDRVKDLWLKRGIKTGYHLQDKIRNGETKFSYLRAMEA  71
            + Q KKH +Q T QEK+        V +    +  K   H ++K     T+  +
Sbjct  12   ERQRKKHYSQMTLQEKKDCVSLLQNVFEQC--KFFKMTPHFKEK---SLTRIDFDLLKNF  66

Query  72   MIHGE-----IIEYNET----GADKRVLIRSGIGHVGESIDDIVVQCI--VVSLFSGKVI  120
            ++H       IIEYNET       KR++++       E +    + C   +V   +GK++
Sbjct  67   ILHNNYAFLNIIEYNETKTRYNTQKRIVLK-----YPEIVKVKGIPCFQYLVITNNGKLV  121

Query  121  TSYLNKASDRHKTLDPRRYNKNLKINL  147
            T+Y NK +D HKTLD   Y+KNLKI++
Sbjct  122  TTYYNKINDVHKTLDLTYYDKNLKISV  148
```

ref|YP_002727884.1| head-tail joining protein [*Enterococcus* phage EFAP-1]

gb|ACN86326.1| head-tail joining protein [*Enterococcus* phage EFAP-1], Length = 135

GENE ID: 7668746 EFAP1_gp12 | head-tail joining protein [*Enterococcus* phage EFAP-1]

Score = 248 bits (634), Expect = 1e-64, Identities = 115/135 (85%),

Positives = 125/135 (92%), Gaps = 0/135 (0%)

```
Query  1    MSSPNYVDFSQVYKRLEKSGKKADLIVSKAVNTAGEKAMNELKQNTPRFNGTKYSRDGQD  60
            MS  NYVDFSQ YKRL+K+GK AD IVSKA+NTAGE +M ELK+NTPRFNGTKYS+D QD
Sbjct  1    MSGKNYVDFSQAYKRLQKAGKNADQIVSKAINTAGEASMQELKRNTPRFNGTKYSKDAQD  60

Query  61   YKKEHMRDHVVMSKANKNNHIAEVGFDDDVAWMAHFPELGTIKQRPQGFIQKTINSIESE  120
            YKKEHMRDH+VMSKA+KN H+AEVGFDDDVAWMAHFPELGTIKQRPQGFIQKT+NSIE E
Sbjct  61   YKKEHMRDHIVMSKASKNKHVAEVGFDDDVAWMAHFPELGTIKQRPQGFIQKTMNSIEDE  120

Query  121  VASIIQKALQEAFLK  135
            VASIIQKALQEAFLK
Sbjct  121  VASIIQKALQEAFLK  135
```

ref|YP_001504297.1| hypothetical protein EFP_gp188 [*Enterococcus* phage phiEF24C]

dbj|BAF81456.1| hypothetical protein [*Enterococcus* phage phiEF24C], Length = 296

GENE ID: 5666565 EFP_gp188 | hypothetical protein [*Enterococcus* phage phiEF24C]

Score = 87.8 bits (216), Expect = 3e-16, Identities = 42/73 (57%),

Positives = 53/73 (72%), Gaps = 1/73 (1%)

```
Query  1   MKYTEKDIKEGTKLICTKGNIIWWTVGKLYEVKLTKDDSLSIKDDHGHDAYTDYMLDCLN  60
           M+YTE D+K GTKL CTK   +WWTVGK+Y+V L++D  L I DD+G  AY DYML CL
Sbjct  11  MRYTENDLKVGTKLRCTKAKPVWWTVGKVYDVSLSEDGMLIIIDDNGDYAYADYMLFCLE  70

Query  61  GDH-IVKFEIVEQ  72
           G H  V FEI+++
Sbjct  71  GKHNPVAFEIIKE  83
```

Score = 49.3 bits (116), Expect = 1e-04, Identities = 31/82 (37%),

Positives = 47/82 (57%), Gaps = 10/82 (12%)

```
Query  1    MKYTEKDIKEGTKLICTKGNII-WWTVGKLYEV-----KLTKD--DSLSIKDDHG--HDA  50
            +KYTE+D+ EG KL C   +++ WWT+GK YE+     KL  D   +  I DD G  HD
Sbjct  87   IKYTEEDLYEGMKLRCVDNSVVKWWTIGKEYEIVKSVSKLDVDKMGAYYILDDEGDKHDT  146

Query  51   YTDYMLDCLNGDHIVKFEIVEQ  72
                +    + + I++FE+VE+
Sbjct  147  EVSILRGLNDKNAIIRFEVVEE  168
```

Score = 43.1 bits (100), Expect = 0.009, Identities = 34/114 (29%),

Positives = 63/114 (55%), Gaps = 17/114 (14%)

```
Query  26   VGKLYEVKLTKDDSLSIKDD---HGHDAYTDYMLDCLNGDHIVKFEIVEQ--PTATITVD  80
            +GK Y++ ++ D++LS  DD   + +D +  Y +         ++E+VE+  P A    D
Sbjct  195  IGKTYKI-VSYDNALS--DDCYIYLNDKHPRYFISE------AQYELVEKEYPVADAKAD  245

Query  81   VEQRLNDKIESLRSERENILSKMDRMEAQQLKLRDKINKLSEAKKALEILKEFK  134
            V++ +  KI++L +E E + +K DR+E   + L  K  +L E    +E +KEF+
Sbjct  246  VKEAIQAKIDALTTEAERLFTKRDRLEEHAINLNAKARRLEE---VVETIKEFE  296
```

ref|YP_001504212.1| putative cytidine deaminase [*Enterococcus* phage phiEF24C]

dbj|BAF81371.1| putative cytidine deaminase [*Enterococcus* phage phiEF24C], Length = 199

GENE ID: 5666411 EFP_gp103 | putative cytidine deaminase [*Enterococcus* phage phiEF24C]

Score = 57.4 bits (137), Expect = 4e-07, Identities = 36/74 (48%),

Positives = 46/74 (62%), Gaps = 3/74 (4%)

```
Query  2   KYTEKDIKEGTKLICTKGN-IIWWTVGKLYEVKLTKDDSLSIKD-DHGHDAYTDYMLDCL  59
           KYTEKDIKEG KL CT  N  + WT G +YEVK T +  L I+D + G  +Y D +L  L
Sbjct  3   KYTEKDIKEGMKLRCTDNNGYVRWTTGGMYEVKKTDEGVLYIEDVEGGCRSYIDAILFRL  62

Query  60  N-GDHIVKFEIVEQ  72
           N  D    FE++E+
Sbjct  63  NHNDESAIFEVIEE  76
```

Score = 40.8 bits (94), Expect = 0.042, Identities = 27/81 (33%), Positives = 46/81 (56%),

Gaps = 11/81 (13%)

```
Query  61   GDHIV---KFEIVEQPTA-----TITVDVEQRLNDKIESLRSERENILSKMDRMEAQQLK  112
            G+H++   +F  V++P+         +DVE ++   IE L++E E + +K DR+  Q L
Sbjct  121  GNHVLLPDQFVFVDEPSNDGEKDVEELDVEAKILADIEQLKTEAECLFAKRDRVNEQALN  180

Query  113  LRDKINKLSEAKKALEILKEF  133
            L  K  KL E   +LE+L+E+
Sbjct  181  LNAKARKLEE---SLEVLREY  198
```

---

emb|CAC67535.1| hypothetical transcriptional regulator [*Streptococcus* thermophilus], Length = 232, Score = 35.0 bits (79), Expect = 2.6, Identities = 20/55 (36%), Positives = 29/55 (52%), Gaps = 5/55 (9%)

```
Query  32  VVDSLGTHYIITETEEPETQEPENE-IQKLEQLIKSTQDHFTE----DLYNCVDN  81
           + D LG  Y      E   +EP  E + +LEQL+K ++ +FTE     LYN + N
Sbjct  21  IADQLGISYQAYSAWERGIKEPSKEKVNRLEQLLKVSKGYFTEIEIVRLYNTLSN  75
```

ref|ZP_06317090.1| phage tail tape measure protein [*Staphylococcus aureus* subsp. aureus WW2703/97]

gb|EFB57139.1| phage tail tape measure protein [*Staphylococcus aureus* subsp. aureus WW2703/97]

gb|EFC28577.1| putative membrane protein [*Staphylococcus aureus* subsp. aureus A017934/97], Length = 1509, Score = 34.3 bits (77), Expect = 4.7,

Identities = 13/42 (30%), Positives = 25/42 (59%), Gaps = 0/42 (0%)

```
Query  83   NFYYSQGIMTDYREYVKQYDGIMKRYGERLKDLNKKLEKLKQ  124
            NF Y++     Y++ +K+ DG +  Y + + DL K+ +K+ Q
Sbjct  43   NFKYTEKSTDSYKQRIKELDGTIAGYKKNIDDLAKQYDKVSQ  84
```

ref|ZP_04797562.1| conserved hypothetical protein [*Staphylococcus epidermidis* W23144]
gb|EES35777.1| conserved hypothetical protein [*Staphylococcus epidermidis* W23144],
Length = 103, Score = 37.0 bits (84), Expect = 0.64, Identities = 25/88 (28%),
Positives = 40/88 (45%), Gaps = 4/88 (4%)

```
Query  4   EANFSREVVKYLKSRGALVNVN--TANIYDRVGRADIEACYKGYYIALELKTGNYKADKL  61
           EA F  + +++LK       V     N +   G  D+ AC  G +  +ELKT       +
Sbjct  13  EAAFQNDFMQFLKHNPNAFAVKYWAGNKFTVNGIPDVLACINGVFHGIELKTNTGVVRGI  72

Query  62  QIRYLQEIRCAGGYGLLLRDTLDDLEDL  89
           Q   +++I+ AGG   +LR    D E+
Sbjct  73  QKERMKQIKLAGGEAYVLRP--KDFEEW  98
```

ref|YP_535648.1| hypothetical protein LSL_0755 [*Lactobacillus* phage Sal1]

gb|ABD99565.1| Hypothetical protein, phage associated [*Lactobacillus* phage Sal1], Length = 137

GENE ID: 3976834 LSL_0755 | hypothetical protein [*Lactobacillus salivarius* UCC118]

Score = 33.5 bits (75), Expect = 6.7, Identities = 30/112 (26%), Positives = 49/112 (43%), Gaps = 22/112 (19%)

```
Query  1    MGREANFSREVVKYLKSRGALVNVNT-----------------ANIYDRVGRADIEACYK  43
            M  E  F ++V KYL+S+G   N NT                   +   G  D+
Sbjct  1    MAGEKRFEKKVEKYLESQGIYQN-NTKKQDKTIKDNGWFFKVWGGGFQSGGIPDLICNIN  59

Query  44   GYYIALELKTGNYKADKLQIRYLQEIRCAGGYGLLL----RDTLDDLEDLLL  91
            G+++++ELK    +  +LQIR  + I    G GL+L     DT   + +++L
Sbjct  60   GFFLSIELKDIRGQPSELQIRNTKLINQTNGIGLILYPQGFDTFKKIVEVML  111
```

ref|YP_002727895.1| HNH endonuclease [*Enterococcus* phage EFAP-1]

gb|ACN86337.1| HNH endonuclease [*Enterococcus* phage EFAP-1], Length = 123

GENE ID: 7668756 EFAP1_gp23 | HNH endonuclease [*Enterococcus* phage EFAP-1]

Score = 249 bits (635), Expect = 8e-65, Identities = 116/123 (94%),

Positives = 119/123 (96%), Gaps = 0/123 (0%)

```
Query  1    MPKRRCKVAHCREYVDIPEVYCEQHRGQQAKTYNKQVRHSPDNKKYADFYASTQWRNVRA  60
            MPKRRCKVAHCREYVDIPEVYCE+H+G   +TYNKQVRHSPDNKKYADFYASTQWRNVRA
Sbjct  1    MPKRRCKVAHCREYVDIPEVYCEEHKGNTQRTYNKQVRHSPDNKKYADFYASTQWRNVRA  60

Query  61   RKLSMNPMCEVCNTSIATIVHHRQEVRTTMGWEHRLDIDNLESICQECHNKEEHSASFRH  120
            RKLSMNPMCEVCN SIATIVHHRQEVRTTMGWEHRLDIDNLESICQECHNKEEHSASFRH
Sbjct  61   RKLSMNPMCEVCNASIATIVHHRQEVRTTMGWEHRLDIDNLESICQECHNKEEHSASFRH  120

Query  121  RKG  123
            RKG
Sbjct  121  RKG  123
```

ref|YP_002727883.1| head-tail joining protein [*Enterococcus* phage EFAP-1]

gb|ACN86325.1| head-tail joining protein [*Enterococcus* phage EFAP-1], Length = 121

GENE ID: 7668745 EFAP1_gp11 | head-tail joining protein [*Enterococcus* phage EFAP-1]

Score = 242 bits (618), Expect = 8e-63, Identities = 114/121 (94%),

Positives = 119/121 (98%), Gaps = 0/121 (0%)

```
Query  1    MRLPILQVAEALEQAHPEVNWFTNEVPTEFQTLPQLPVGRIVELDGRYTEYASADPNYFV  60
            MRLP+LQVAE LEQ+HPEVNWFTNEVPTEFQTLP+LPVGR+VELDGRYTEYASADPNYFV
Sbjct  1    MRLPVLQVAETLEQSHPEVNWFTNEVPTEFQTLPKLPVGRVVELDGRYTEYASADPNYFV  60

Query  61   THVQVDLWCEDLKEVEKYYFEIDKTMRADNVQCVLSQQSYDPDLEGARRVIKRYTISQRV  120
            THVQVDLWCEDLKEVEKYYF+IDKTMRADNVQCVLSQQSYDPDLE ARRVIKRYTISQRV
Sbjct  61   THVQVDLWCEDLKEVEKYYFDIDKTMRADNVQCVLSQQSYDPDLEDARRVIKRYTISQRV  120

Query  121  V  121
            V
Sbjct  121  V  121
```

ref|NP_460347.1| putative regulatory protein [*Salmonella typhimurium* LT2]

emb|CAB37417.1| ORF 408 [*Salmonella typhimurium*]

gb|AAG31760.1| ribokinase-like protein [*Salmonella enterica* subsp. Enteric serovar Typhimurium]

gb|AAL20306.1| putative regulatory protein, deoR family [*Salmonella enteric* subsp. enterica serovar Typhimurium str. LT2]

emb|CBG24398.1| putative ribokinase/regulatory protein [*Salmonella enterica* subsp. enterica serovar Typhimurium str. D23580]

gb|ACY88154.1| putative regulatory protein [*Salmonella enterica* subsp. Enteric serovar Typhimurium str. 14028S], Length = 408

GENE ID: 1252900 orf408 | putative regulatory protein [*Salmonella typhimurium* LT2]

Score = 37.0 bits (84), Expect = 0.67, Identities = 26/89 (29%), Positives = 43/89 (48%), Gaps = 8/89 (8%)

```
Query  34   MAVLLASPYSSSTPHSTS-----SPSKLSASLDSGLTV---DSSSKCSMSFLLTLVTFLV  85
            M +L  +PY+    H  S     +P++  AS  SG+T+   + + K     L + V  ++
Sbjct  255  MIMLNPAPYTKQVTHLLSDIDIITPNETEASFLSGVTITDINDAKKAGNIILQSGVKKVI  314

Query  86   PSLTARNAFSCDFNMLNTIPAWSLVLAAA  114
             +L AR +  C+      IPAWS V+  A
Sbjct  315  ITLGARGSLICEHARTLYIPAWSAVVKDA  343
```

ref|YP_216390.1| DeoR family transcriptional regulator [*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SC-B67]

ref|ZP_02685763.1| putative regulatory protein, DeoR family [*Salmonella enteric* subsp. enterica serovar Hadar str. RI_05P066]

ref|YP_002146658.1| putative regulatory protein, DeoR family [*Salmonella enteric* subsp. enterica serovar Agona str. SL483]

ref|YP_002637905.1| putative regulatory protein, deoR family [*Salmonella enteric* subsp. enterica serovar Paratyphi C strain RKS4594]

gb|AAX65309.1| putative regulatory protein, deoR family [*Salmonella enteric* subsp. enterica serovar Choleraesuis str. SC-B67]

gb|ACH52445.1| putative regulatory protein, DeoR family [*Salmonella enteric* subsp. enterica serovar Agona str. SL483]

gb|EDZ34156.1| putative regulatory protein, DeoR family [*Salmonella enteric* subsp. enterica serovar Hadar str. RI_05P066]

gb|ACN46464.1| putative regulatory protein, deoR family [*Salmonella enteric* subsp. enterica serovar Paratyphi C strain RKS4594], Length = 408

GENE ID: 3333859 rbsk | DeoR family transcriptional regulator [*Salmonella enterica* subsp. enterica serovar Choleraesuis str. SC-B67], Score = 37.0 bits (84), Expect = 0.68, Identities = 26/89 (29%), Positives = 43/89 (48%), Gaps = 8/89 (8%)

```
Query  34   MAVLLASPYSSSTPHSTS-----SPSKLSASLDSGLTV---DSSSKCSMSFLLTLVTFLV  85
            M +L  +PY+    H  S     +P++  AS  SG+T+   + + K     L + V  ++
Sbjct  255  MIMLNPAPYTKQVTHLLSDIDIITPNETEASFLSGVTITDINDAKKAGNIILQSGVKKVI  314

Query  86   PSLTARNAFSCDFNMLNTIPAWSLVLAAA  114
             +L AR +  C+      IPAWS V+  A
Sbjct  315  ITLGARGSLLCEHARTLYIPAWSAVVKDA  343
```

ref|YP_002727885.1| head-tail adaptor protein [*Enterococcus* phage EFAP-1]

gb|ACN86327.1| head-tail adaptor protein [*Enterococcus* phage EFAP-1], Length = 111

GENE ID: 7668747 EFAP1_gp13 | head-tail adaptor protein [*Enterococcus* phage EFAP-1]

Score = 202 bits (515), Expect = 6e-51, Identities = 94/111 (84%),

Positives = 103/111 (92%), Gaps = 0/111 (0%)

```
Query  1    MPLIPTQRLDRKISIQHKTTHKNEYYEWVTDWETKATIWCSVKQQYFKDYKESLGTVLED  60
            M  + TQRLD++I IQ KTTHKNEYYEWVTDWETK++IWCSVK+QYFKDYKESLGTVLED
Sbjct  1    MAQVATQRLDKRIDIQEKTTHKNEYYEWVTDWETKSSIWCSVKEQYFKDYKESLGTVLED  60

Query  61   TTNFIIRYEQQIHIDNTMRVVYNGVNYDIIQVLEGSFQRDFTTLVCKRVKE  111
            TTNFIIRYEQ   IDN+MRVVYNGVNYDI+QVLEGSFQRDFTTLVCKRVK+
Sbjct  61   TTNFIIRYEQHFQIDNSMRVVYNGVNYDIVQVLEGSFQRDFTTLVCKRVKK  111
```

ref|YP_002158147.1| daunorubicin resistance transmembrane protein [*Vibrio fischeri* MJ11]
gb|ACH64068.1| daunorubicin resistance transmembrane protein [*Vibrio fischeri* MJ11], Length = 374
GENE ID: 6808186 VFMJ11_A0598 | daunorubicin resistance transmembrane protein [*Vibrio fischeri* MJ11], Score = 33.9 bits (76), Expect = 5.6, Identities = 11/29 (37%), Positives = 18/29 (62%), Gaps = 0/29 (0%)

```
Query  46   ACKHFNEWSDTEERITVFDFTYALFIIAI  74
             C    EW D ++R+++F+FT   F IA+
Sbjct  213  GCNSLREWFDKQDRLSIFNFTLVYFCIAM  241
```

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ref|YP_002727886.1| head-tail joining protein [*Enterococcus* phage EFAP-1]
gb|ACN86328.1| head-tail joining protein [*Enterococcus* phage EFAP-1], Length = 99
GENE ID: 7668735 EFAP1_gp14 | head-tail joining protein [*Enterococcus* phage EFAP-1]
Score = 170 bits (431), Expect = 4e-41, Identities = 81/97 (83%), Positives = 88/97 (90%), Gaps = 0/97 (0%)

```
Query  4   LTLAEVKNNLRLDYDFDDTYLQGLIDTSELFILGAIELKTAPDDKRFKTLQFMLVSLWYE  63
           LTL +VKNNLRLD++FDD Y+ GLI TSE+FILGAIEL  APDD+RF TLQFMLVSLWYE
Sbjct  3   LTLEQVKNNLRLDHNFDDEYITGLITTSEMFILGAIELTEAPDDERFNTLQFMLVSLWYE  62

Query  64  NRVPATSALQQQVPFTIVAMIHQLRGLQDATDTNTTT  100
           NRVPATSALQQQVPFTIVAMIHQLRGLQD T +NTTT
Sbjct  63  NRVPATSALQQQVPFTIVAMIHQLRGLQDGTGSNTTT  99
```

ref|ZP_05736772.1| ABC superfamily ATP binding cassette transporter, membrane protein [*Granulicatella adiacens* ATCC 49175]

gb|EEW38263.1| ABC superfamily ATP binding cassette transporter, membrane protein [*Granulicatella adiacens* ATCC 49175], Length = 458, Score = 37.7 bits (86), Expect = 0.39, Identities = 22/65 (33%), Positives = 36/65 (55%), Gaps = 1/65 (1%)

```
Query  12   GMNFLADTSIRFSSRNIVLYASNNSVFGITSSILFVNSSSFNSIKLAIVSFPWINASRNL  71
            G+N+ A T ++ S     L  S N V+G+T+  LF+ S  F++I L ++ F W N+ R
Sbjct  271  GINWKAYTLVKSSQNYPTLQKSINLVYGLTNK-LFIGSLIFSAIILVLILFLWTNSRRKE  329

Query  72   FALSL  76
              + L
Sbjct  330  IGIRL  334
```

ref|YP_662252.1| glucose-methanol-choline oxidoreductase [*Pseudoalteromonas atlantica*]

gb|ABG41198.1| glucose-methanol-choline oxidoreductase [*Pseudoalteromonas atlantica* T6c], Length = 561

GENE ID: 4174446 Patl_2686 | glucose-methanol-choline oxidoreductase [*Pseudoalteromonas atlantica* T6c], Score = 33.5 bits (75), Expect = 6.8,

Identities = 17/33 (51%), Positives = 21/33 (63%), Gaps = 0/33 (0%)

```
Query  29   VLYASNNSVFGITSSILFVNSSSFNSIKLAIVS  61
            V+ A  N  +  TS ILFVN+SSFNS  L + S
Sbjct  279  VIDAETNETYEFTSKILFVNASSFNSTWLLMNS  311
```

ref|YP_001791054.1| molybdenum cofactor synthesis domain-containing protein [*Leptothrix cholodnii* SP-6]

gb|ACB34289.1| molybdenum cofactor synthesis domain protein [*Leptothrix cholodnii* SP-6], Length = 436

GENE ID: 6163571 Lcho_2022 | molybdenum cofactor synthesis domain-containing protein [*Leptothrix cholodnii* SP-6], Score = 33.1 bits (74), Expect = 9.5,

Identities = 16/53 (30%), Positives = 32/53 (60%), Gaps = 1/53 (1%)

```
Query  34   QPLMSPLALYSANELTLCSYIVSCNCAFSNNGLLLIQLASSSTSLRLSISQHN  86
            +PL   P A+Y++N  TLC+ + S  C  ++ G++  +L ++  +LR +   H+
Sbjct  216  EPL-KPGAIYNSNRYTLCALLQSLGCEVTDLGIVPDRLDATRAALRDAAHGHD  267
```

ref|NP_661599.1| zinc protease, putative [*Chlorobium tepidum* TLS]

gb|AAM71941.1| zinc protease, putative [*Chlorobium tepidum* TLS], Length = 248

GENE ID: 1006333 CT0704 | zinc protease, putative [*Chlorobium tepidum* TLS]

Score = 33.1 bits (74), Expect = 9.0, Identities = 16/42 (38%), Positives = 28/42 (66%), Gaps = 1/42 (2%)

```
Query  37   KQPNKTTNATAKHNASNLLILLILLPPQIVVFVLLLLLCHTL  78
            KQ ++  + +++ N  NL + LI LPP +V ++++  LCHTL
Sbjct  167  KQKSRWGSCSSRGNI-NLNLKLIFLPPLLVRYIMIHELCHTL  207
```

ref|YP_002727896.1| hypothetical protein EFAP1_gp24 [*Enterococcus* phage EFAP-1]
gb|ACN86338.1| hypothetical protein EFAP1_gp24 [*Enterococcus* phage EFAP-1],
Length = 68

GENE ID: 7668757 EFAP1_gp24 | hypothetical protein [*Enterococcus* phage EFAP-1]

Score = 102 bits (254), Expect = 1e-20, Identities = 61/68 (89%), Positives = 64/68 (94%), Gaps = 1/68 (1%)

```
Query  1   MNMEGMTDKQLLSHTKLELVSYIRQLEQAMVTNTSDTTEQPTTEPTTKPKVSYDIFN-SN  59
           M MEGMTDKQLLSHTKLELVSYIRQLEQAMVTNTSDTTEQPTTEPT+KPKVSYDI + +N
Sbjct  1   MTMEGMTDKQLLSHTKLELVSYIRQLEQAMVTNTSDTTEQPTTEPTSKPKVSYDILSTTN  60

Query  60  TNKDMWGF  67
            NKDMWGF
Sbjct  61  NNKDMWGF  68
```

ref|YP_002727887.1| hypothetical protein EFAP1_gp15 [*Enterococcus* phage EFAP-1]
gb|ACN86329.1| hypothetical protein EFAP1_gp15 [*Enterococcus* phage EFAP-1],
Length = 72
GENE ID: 7668748 EFAP1_gp15 | hypothetical protein [*Enterococcus* phage EFAP-1]
Score = 83.2 bits (204), Expect = 8e-15, Identities = 40/64 (62%),
Positives = 47/64 (73%), Gaps = 0/64 (0%)

```
Query  1   MAKVFNVYKKTGKKIVTGQPSPVTITGLTAETPYSKGDFQVTAIEDGKPESSKVDVPAFT  60
           M KVFN+YKK G K++TG  SP  +TGLT  T Y KGD+Q TA+EDGK ES KVD+PAF
Sbjct  1   MVKVFNIYKKDGSKVLTGVTSPAKLTGLTPNTQYGKGDYQATAVEDGKLESEKVDLPAFK  60

Query  61  TTAT  64
           T  T
Sbjct  61  TKET  64
```

ref|YP_002727891.1| hypothetical protein EFAP1_gp19 [*Enterococcus* phage EFAP-1]

gb|ACN86333.1| hypothetical protein EFAP1_gp19 [*Enterococcus* phage EFAP-1],

Length = 54

GENE ID: 7668752 EFAP1_gp19 | hypothetical protein [*Enterococcus* phage EFAP-1]

Score = 95.1 bits (235), Expect = 2e-18, Identities = 51/54 (94%),

Positives = 52/54 (96%), Gaps = 0/54 (0%)

```
Query  9   MQNPVLLVVIFYVLGMLSLVTAMFFTGLVAGLVALAVALLIPSVILYKELGEGE  62
           MQN V LVVIFYVLGMLSLVT+MFFTGLVAGLVALAVALLIPSVILYKELGEGE
Sbjct  1   MQNAVWLVVIFYVLGMLSLVTSMFFTGLVAGLVALAVALLIPSVILYKELGEGE  54
```

ref|ZP_03945177.1| APC family amino acid-polyamine-organocation transporter [*Lactobacillus fermentum* ATCC 14931]

gb|EEI21772.1| APC family amino acid-polyamine-organocation transporter [*Lactobacillus fermentum* ATCC 14931], Length = 492, Score = 33.5 bits (75), Expect = 6.6, Identities = 15/39 (38%), Positives = 23/39 (58%), Gaps=0/39 (0%)

```
Query  9    VKYTDSFWFDELGKIEGEQPNTIVFTVEWASETVLRLAG  47
            + +   FWF   GK E ++P TIV  + W + T++ LAG
Sbjct  443  IVWETGFWFFYQGKKEQQRPFTIVEKITWLAITLMALAG  481
```

ref|YP_001843737.1| arginine-ornithine antiporter [*Lactobacillus fermentum* IFO 3956]
dbj|BAG27257.1| arginine-ornithine antiporter [*Lactobacillus fermentum* IFO 3956],
Length = 485

GENE ID: 6232894 LAF_0921 | arginine-ornithine antiporter [*Lactobacillus fermentum* IFO 3956], Score = 33.5 bits (75), Expect = 6.7, Identities = 15/39 (38%), Positives = 23/39 (58%), Gaps = 0/39 (0%)

```
Query  9    VKYTDSFWFDELGKIEGEQPNTIVFTVEWASETVLRLAG  47
            + +   FWF   GK E ++P TIV  + W + T++ LAG
Sbjct  436  IVWETGFWFFYQGKKEQQRPFTIVEKITWLAITLMALAG  474
```