

Biodegradation of Triclosan as a Representative of Pharmaceuticals and Personal Care Products (PPCPs) in the Wastewater Environment

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For all those who helped me

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List of Acronyms

ACN	Acetonitrile
ACP	Acyl carrier protein
AHLs	Acylated homoserine lactones
AI	Auto Inducer
BH broth	Bushnell-Haas broth
BHL	N-butyryl-$_L$-homoserine lactone
BLAST	Basic Local Alignment Search Tool
BOD	Biological Oxygen Demand
CAM	Camphor
CBP	Chloro-biphenol
DAD	Diode array detection
DCM	Dichloromethane
DGGE	Denaturing Gradient Gel Electrophoresis
DMSO	Dimethyl sulfoxide
Fab/FAB	Fatty acid biosynthesis
FCM	Flow Cytometry
GC-MS	Gas Chromatography-Mass Spectroscopy
HPLC	High Performance Liquid Chromatography
HPLC-UV	High Performance Liquid Chromatography-Ultraviolet
HRT	Hydraulic retention time
IM	Inner membrane
IPTG	Isopropylthio-β-D-galactoside
LB broth	Luria Bertani broth
MCS	Multiple cloning site
MDR	Multidrug resistance
MFP	Membrane fusion protein
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
NADP	Nicotinamide adenine dinucleotide phosphate
NAH	Naphthalene
NCBI	National Centre for Biotechnology Information

ORFs	Open Reading Frames
PAH	Polycyclic aromatic hydrocarbons
PCB	Polychlorbiphenyls
PCR	Polymer Chain Reaction
PPCPs	Pharmaceuticals and Personal Care Products
PVC	Polyvinyl chloride
RB	Rich Broth
RND	Resistance Nodulation Division
SAM	S-adenosylmethionine
SCV	Small Colony Variants
SOC medium	Derivative of SOB (Super Optimal Broth), C stands for Catobolite
SRT	Sludge Retention Time
TCS	Triclosan
TCSM medium	Triclosan minimal medium
TFB 1	Transformation Broth 1
TFB 2	Transformation Broth 2
TOL	Toluene
TSS	Total suspended solids
UV	Ultraviolet (detection)
VB1	Vitamin B1, also known as Thiamin
WWTP	Waste Water Treatment Plant
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

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Summary

Triclosan is a synthetic broad-spectrum antimicrobial agent that has been widely used in Pharmaceuticals and Personal Care Products (PPCPs) since its introduction in 1972. Recent concern has arisen that the increased use of triclosan in the PPCPs and the presence of this compound in the water and wastewater environment may contribute to the emergence of bacterial antibiotic resistance. Efficient removal of triclosan during the wastewater treatment process becomes important but challenging in limiting the development of cross resistance in bacteria, due to the wide presence of triclosan in the wastewater environment and the recalcitrant property of this compound.

Mechanisms involved in triclosan removal in conventional wastewater treatment processes include sorption and biodegradation. Achieving efficient biodegradation of triclosan through wastewater treatment processes is critical in ensuring the reduction of this compound present in the effluent of wastewater treatment plants as well as the natural water environment. This study investigates the mechanisms of triclosan biodegradation using general microbiology, molecular microbiology and analytical chemistry techniques, with the aim of broadening the knowledge base required to develop engineering options in improving triclosan removal efficiency of wastewater treatment processes.

Triclosan degradative isolates were obtained by gradually adapting triclosan resistant isolates from the wastewater environment to grow in triclosan minimal medium, which contains triclosan as sole carbon source. A triclosan degradation related plasmid, namely pF12, was obtained from a *Pseudomonas citronellolis* degradative isolate and was proved in the laboratory to be involved in triclosan biodegradation using an electroporation technique. Restriction digested linear fragments of this plasmid were cloned into Promega™ pGEM®-5Zf(+) cloning vector and replicated in rubidium chloride processed chemically competent DH5α *E. coli* cells. Infected cells carrying recombinant plasmid DNA were selected by a combination of antibiotic marker and blue and white colony screening method.

The cloned DNA fragments were then sequenced using T7 universal and SP6 promoter primers. The entire DNA sequence of this plasmid was obtained by gradual primer walking. This plasmid contains 17,770bp DNA and 29 ORFs likely to encode gene functions.

Further bioinformatics results revealed that plasmid pF12 has a similar modular organisation to some characterized organic degradative plasmids, including a plasmid

house-keeping function module and a triclosan degradation related module. The primary functions of the plasmid house-keeping module include plasmid replication/maintenance, mobilization, and stabilization. The triclosan degradative related module, which is carried in one putative transposon element, contains Open Reading Frames (ORFs) that have low similarity to gene cassettes encoding known chloroaromatic degradative pathways. Despite the size constraints of this putative functional module, *det*ABCDEFG was proposed to compose the putative gene cassette responsible for triclosan biodegradation. These enzymes include a TPP binding domain containing protein, unknown protein, dehydrogenase, hydrolase, oxidase, transcriptional regulator and a tentative mandelate racemase/muconate enzyme. Most of these proteins belong to a family of oxidoreductases, and encode candidate genes for involvement in triclosan biodegradation.

Triclosan biodegradation intermediates and by-products were analysed using LC/MS and GC/MS/MS. Key degradation products were identified as $C_{12}H_7Cl_3O_3$ (tentatively named as 2',4'-dichlorophenyl 4-chloro-6-oxo-2,4-hexadienoate), proto-anemonin, dichloro-phenol and an unknown compound having close mass spectrum to triclorobenzene. A possible triclosan biodegradation pathway was tentatively predicted based on the findings in the genetic study and chemical analysis.

Intermediate 2',4'-dichlorophenyl 4-chloro-6-oxo-2,4-hexadienoate identified in this study is a novel compound that has not been reported in any literature. In addition, triclosan degradation by-product proto-anemonin and dichloro-phenol, the latter of which has now been confirmed to be triclosan biodegradation product by a most recent study, do not show identity to published triclosan degradation metabolites at the time of thesis submission.

This study also attempted to explore the potential for chromosome encoded genes that could be responsible for triclosan biodegradation, but none were found. The effect of exposure to high concentration of triclosan in the wastewater bacterial community was assessed using Denatured Gradient Gel Electrophoresis (DGGE), and a significant shift of bacterial community profile was observed after both short term and long term exposure.

This study is the first to describe preliminary analysis of genes involved in triclosan degradation and provides a basis for more detailed study to be performed on the genes involved, their regulation and the enzymology of the degradative pathway.

Declaration

I declare that:

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, is clearly acknowledged.

Signature _____ Date _____

Xiaoxia Qiu

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Chapter 1: Literature Review

1.1. *Triclosan and its characteristics*

1.1.1. General properties of triclosan

Triclosan (*TCS, $C_{12}H_7Cl_3O_2$, Irgasan*) is a synthetic broad spectral antimicrobial compound with a molecular weight of 289.54. Figure 1 below shows the molecular structure of this compound. It belongs to a group of 2-hydroxydiphenyl ethers that is a class of compounds showing antimicrobial activity.

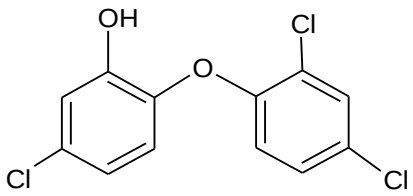


Figure 1 Molecular structure of triclosan

Triclosan was originally introduced to health care settings as a surgical scrub in 1972, and was first included in toothpaste as an additive in Europe in 1985. Since then, triclosan has been used as an active antimicrobial component in many Pharmaceuticals and Personal Care Products (PPCPs), including detergents, soaps, deodorants, cosmetics, lotions, toothpastes, mouthwashes and plastics for hospital and household use. In recent years, there has been a remarkable increase in the popularity of the addition of antimicrobial chemicals to consumer products, and triclosan is commonly used in products ranging from chopping boards, pizza-cutters, mop handles and mattresses to bowling ball finger inserts and baby toys (Russell, 2004, Saleh et al., 2011).

Triclosan exhibits a broad spectrum and clinically significant antimicrobial activity (Regos et al., 1979, Vischer & Regos, 1974). It is acutely or chronically toxic to aquatic organisms (Nassef et al., 2010, Price et al., 2010, Ricart et al., 2010, Riva et al., 2012, Tatarazako et al., 2004), but has limited antiviral and antifungal properties (Ricart et al., 2010, Samsøe-Petersen et al., 2003). The viability of Diatoms, a major group of algae, was found to be affected by increasing concentrations of triclosan (Ricart et al., 2010). Its potential biochemical and genetic toxicity has been demonstrated as having sub-lethal effects on non-target soil organisms, such as earth worms (Lin et al., 2010a) .

The solubility of triclosan in water is poor, i.e. 0.001g/100g ($1x10^{-5}$ g /mL) at 20°C (NICNAS, 2009), under acidic and neutral conditions, and this poor solubility significantly complicates the study of triclosan. However, triclosan is fat-soluble, i.e. soluble in most organic solvents, and it is also water soluble in alkaline conditions (pH>7). Triclosan is chemically stable, and has a decomposition temperature of 280°C (NICNAS, 2009).

1.1.2. Antimicrobial properties and mechanisms of action of triclosan on bacteria

A recent review of environmental concentrations of biocides (in contrast with toxicity threshold values of susceptible organisms) has summarised a toxicity threshold concentration span of more than six orders of magnitude (Chalew & Halden, 2009). Crustacea and algae were identified as the most sensitive species, susceptible to adverse effects from biocide exposures in the ng/L range (Capdevielle et al., 2008, Chalew & Halden, 2009).

Triclosan is active against many, but not all, types of Gram-positive and Gram-negative bacteria. It is bacteriostatic at low concentrations, but at higher concentration it is bactericidal. Many studies have been done to evaluate the effectiveness of triclosan as a disinfectant for dentifrices and hand-wash products as well as its use in clinical antibacterial settings (Barry et al., 1984, Cullinan et al., 2011, Faoagali et al., 1999, Fine et al., 1998, Lin et al., 2010b, Stephen et al., 1990, Walker et al., 1994, Webster, 1992, Wicht et al., 2005). Triclosan was found to be an efficient active component in toothpaste to prevent the development of plaque on teeth and in hand washes and scrubs for general hygiene (Aiello et al., 2004, Cullinan et al., 2011, McBain et al., 2003a). One of the most significant findings about the biocidal efficiency of triclosan has been its effectiveness in the control of outbreaks of methicillin-resistant *Staphylococcus aureus* (MRSA) (Rashid et al., 2006, Webster et al., 1994, Zafar et al., 1995), although resistance to triclosan in MRSA has been recorded as a result of extended use of this compound in clinical settings (Bamber & Neal, 1999, Brenwald & Fraise, 2003, Cookson et al., 1991, Fan et al., 2002, Suller & Russell, 2000)

The efficiency of the antimicrobial activity of triclosan is dependent on several factors. A bacterial population kinetic study (Gomez Escalada et al., 2005a) showed that generally bacterial growth was delayed under triclosan exposure and the extent of delay corresponded to the concentrations to which bacteria were exposed. Interestingly, instead of showing a longer delay effect at higher concentrations, the study showed a longer delay

at lower concentrations. This is probably due to the low solubility of triclosan. It was observed in this study that triclosan tends to crystallize from solution at high concentrations (i.e. higher than saturation concentration; triclosan solubility in water 0.01g/L at 20°C). It is possible that once the crystallization process is initiated, the formed crystal will become a nucleus to promote further crystallization even in an unsaturated solution. This will result in soluble triclosan in the solution present at a concentration lower than the saturated level. This explains the reason for the longer delay at low concentrations, where less crystal nuclei are formed and more soluble triclosan is present in the solution. Only soluble triclosan is bio-available and this accounts for the more efficient biocidal effect.

Bacterial growth phase is another key factor that influences the efficacy of triclosan action. Bacteria in the log phase of growth are more susceptible to triclosan compared to bacteria in stationary phase, and washed cells are least affected (Tabak et al., 2007).

Concentration (soluble component) is another very important factor in determining the lethality of triclosan. At high concentrations, triclosan was found to be lethal regardless of the growth phase of the bacterial population and this lethal activity could be effective within a time period as short as 15 seconds (Gomez Escalada et al., 2005b). The effect of triclosan at high concentration is positively related to contact time and triclosan concentration. However, this effective triclosan concentration (represented by Minimal Inhibitory Concentration (MICs)) varies among different isolates (Braoudaki & Hilton, 2004a, Gomez Escalada et al., 2005a, McBain et al., 2004, Slayden et al., 2000, Suller & Russell, 2000).

These facts indicate that the mechanisms of triclosan bactericidal activity are complicated and concentration related. Until a specific target was identified in 1998, triclosan had long been thought to act as a non-specific biocide by attacking the bacterial cell envelope and rendering it too porous to retain nutrients, which leads to the death of cells. The publication of McMurry and coworkers' study on the genetic target of triclosan (McMurry et al., 1998a), namely *fabI*, brought the study of mechanisms of triclosan resistance into a new era. This study showed that triclosan acts on a specific target in the bacterial fatty acid biosynthetic pathway, on the NADH-dependent enoyl ACP (acyl carrier protein) reductase (FabI). Subsequent studies investigated various areas of composition, kinetics and genetics of this pathway (El-Attar et al., 2011, Fabiane et al., 1998, Galleni et al., 2001, Heath et al., 2000a, Heath & Rock, 2004, Heath et al., 1999, Heath et al., 2000b, Hoang & Schweizer,

1999, Jiten Singh et al., 2010, Levy et al., 1999, McMurry et al., 1999, McMurry et al., 1998a, Roujeinikova et al., 1999a, Seefeld et al., 2003, Sivaraman, 2002, Sivaraman et al., 2004, Tabak et al., 2007).

1.1.3. Triclosan inhibition mechanism at low concentrations - targeting fatty acid biosynthesis pathway FabI

Bacterial FAS (fatty acid synthesis) is carried out by a collection of enzymes each encoded by distinct genes known as *fab* genes (Sivaraman, 2002). There are four basic chemical reactions that constitute a single round of elongation in the final reaction of which the product of the *fabI* gene, enoyl-ACP reductase, catalyzes the reaction and reduces the trans-2-enoyl ACP substrate into the saturated acyl-ACP product utilizing NADH (Sivaraman, 2002).

Triclosan's inhibition of the fatty acid biosynthetic pathway was first described in *E. coli*, when five independent triclosan-resistant mutants of *E. coli* were isolated on LB agar plates containing 0.2 μg/mL triclosan and used to map the resistance function to the *fabI* gene encoding the NADH-dependent enoyl-acyl carrier protein (ACP) reductase (*fab*I gene) (McMurry et al., 1998b). Later it was shown that triclosan is also responsible for the inhibition of FabI in other pathogenic bacteria such as *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Heath et al., 1999, Russell, 2004, Sivaraman et al., 2004).

It is now known that triclosan binds tightly to the enoyl reductase active site at a region adjacent to the nicotinamide ring of the nucleoside co-factor (Kapoor et al., 2004b, Roujeinikova et al., 1999b). Triclosan's phenol ring forms a face-to-face interaction with the nicotinamide ring, allowing intensive multiple points of interactions. The determination of the crystal structure confirmed that triclosan forms non-covalent high-affinity ternary complexes with the active site residues and $NAD(P)^+$, thus preventing the enzyme from participating in the biosynthetic pathway (Stewart et al., 1999). This finding was supported by a report that triclosan acts as an inhibitor, at picomolar concentrations, of enoyl-ACP reductase by mimicking its natural substrate to interact with amino acid residues of the enzyme active site (Roujeinikova et al., 1999a, Roujeinikova et al., 1999b). At the same time, biological chemical studies also corroborated that triclosan acts as a slow binding picomolar inhibitor of FabI and requires NAD+ for potent inhibition (Heath et al., 1999). The binding was reversible and promoted by NAD^+ binding, suggesting that triclosan was uncompetitive with respect to NAD^+ and competed with the fatty acyl substrate (Kuo,

2003). The original non-specific effects on the outer membrane can therefore be explained as secondary ones arising from specific inhibition of the fatty acid biosynthetic pathway, which in turn affects the many processes dependent on lipid synthesis.

Following the identification of *fabI*, other homologue genes have been identified in other microorganisms, such as *fabK* in *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* (Seefeld et al., 2003), *fabL* in *Bacillus subtilis* (Heath et al., 2000), and *inhA* in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* (McMurry et al., 1999, Parikh et al., 2000, Rawat et al., 2003, Slayden et al., 2000). Such inhibition of fatty acid biosynthesis by triclosan was also reported in higher organisms such as *Plasmodium falciparum* (Kapoor et al., 2004a, Kapoor et al., 2004b, Muench et al., 2003) and *Toxoplasma gondii* (Roberts et al., 2003). Furthermore, this activity is also found in mammalian breast cancer cells resulting in an inhibition of the type I fatty acid synthase in mammals which differs from the "dissociated" fatty acid biosynthesis pathways (Li & Hou, 2003, Liu et al., 2002).

In addition to providing fatty acid intermediates for a multitude of cellular constituents, the FAS pathway has recently been proposed to provide the acyl groups for the synthesis of acylated homoserine lactones (AHLs) that are the signaling molecules termed autoinducers (AI) in the bacterial quorum sensing system (Hoang & Schweizer, 1999). In this study, these researchers discovered by a site-directed mutagenesis method that when coupled to a mutant FabI that retained the reductase activity and was highly resistant to triclosan, purified *P. aeruginosa* N-butyryl-$_L$-homoserine lactone (BHL) synthase, RhlI (counterpart as LasI in 3-oxo-C_{12}-HSL (OdDHL) synthase), was able to synthesize BHL from crotonyl-ACP and S-adenosylmethionine (SAM).

1.1.4. Other mechanisms involved in triclosan biocidal activity

Although the capacity of triclosan to target the fatty acid biosynthesis pathway has been well established in recent years, triclosan biocidal action by targeting FabI in bacterial fatty acid synthesis pathway can only form part of the story of triclosan activity. This mechanism applies only when the concentration of triclosan is not high enough to kill the bacteria immediately, as the inhibition in bacterial fatty acid synthesis results in the inhibition of bacterial proliferation. At this low triclosan concentration even when a complete inhibition of fatty acid synthesis is achieved, triclosan will only inhibit the

growth of organisms, but will not kill them (Gomez Escalada et al., 2005a, Gomez Escalada et al., 2005b). Recent studies have shown that the inhibitory factor is actually a ternary complex of triclosan, NAD+ and InhA; therefore, triclosan can only act as a slow binding inhibitor of InhA due to the nature of its requirement to form a complex with NAD+ (Kapoor et al., 2004b, Kuo, 2003). However, we can speculate that with the bacteriostatic effect, if lipid synthesis was prevented from taking place over a long time, and no cell membranes could be renewed, the bacteria would eventually die. Thus the bacteriostatic activity of triclosan at lower concentration can still be used as a strategy in hygiene or bacterial growth control, yet it is not enough to achieve an immediate lethal effect at this concentration range.

Obviously there are some other more efficient lethal actions of interest, which might be independent or related to fatty acid biosynthesis that remain to be elucidated.

1.1.5. Use of triclosan and resulting concerns

Triclosan is widely used as a constituent in pharmaceuticals & personal care products, and so is commonly discharged into the water and wastewater environment via sewage and discharge of waste. Triclosan, at various concentrations, has been detected worldwide in the water and wastewater environments (Boyd et al., 2003, Jackson & Sutton, 2008, Kuster et al., 2008). As discussed later in Section 1.3.1 triclosan concentrations vary from 0.02 µg/L to 0.21 µg/L in surface waters, and much higher levels of 0.1-21.9 µg/L have been detected in the sewage plant influents worldwide (Chau et al., 2008, Lishman et al., 2006, McAvoy et al., 2002, Miege et al., 2008, Okumura & Nishikawa, 1996). Although these concentrations may not be significant enough to select triclosan resistance, the concentration of triclosan in the digested sludge from wastewater treatment plants ranges from 0.09 to 16.79 mg/kg (dry wt) (Kookana et al., 2011, McAvoy et al., 2002), which may be close to the MICs of some bacteria. In addition, much a higher concentration of triclosan is expected to be present in the sewer immediately downstream of households as well as major triclosan users, such as medical clinics. Concerns have thus arisen in the water and wastewater management area that the biocidal property of triclosan against many bacteria and the acute toxicity to algae and other aquatic organisms will significantly influence the balance of the natural ecology in water environments. Another key concern arising because of the presence of triclosan at sub-lethal levels is the potential for selection

of bacterial resistance to this bis-phenol (Saleh et al., 2011, Thompson et al., 2005, Xia et al., 2005, Yazdankhah et al., 2006).

In studies demonstrating the effective eradication of MRSA by triclosan in a clinical environment, it was found that MRSA showed reduced triclosan susceptibility (Bamber & Neal, 1999, Brenwald & Fraise, 2003, Suller & Russell, 1999, Suller & Russell, 2000), probably due to the use of triclosan as a disinfectant in an attempt to control the MRSA. Concerns arose that continuous exposure to low (sub-MIC) concentrations of triclosan might exert pressure on an organism and give rise to strains with reduced susceptibility to this biocide (Brenwald & Fraise, 2003, Fan et al., 2002, Gomez Escalada et al., 2005b, Saleh et al., 2011, Yazdankhah et al., 2006).

Furthermore, a suspicion of cross-resistance to some antibiotics currently used in clinical settings has arisen (Aiello & Larson, 2003, Dann & Hontela, 2011, McBain & Gilbert, 2001, Randall et al., 2004, Saleh et al., 2011, Schweizer, 2001, Yazdankhah et al., 2006). In fact, problems associated with the use of triclosan were reported during the evaluation of triclosan's impact on a bacterial community, indicating an induced change in microbial composition, leading to the emergence of bacteria with low susceptibility to triclosan and other antibiotics as well (Bamber & Neal, 1999, Barry et al., 1984, Christensen et al., 2011, Cookson et al., 1991, D'Arezzo S et al., 2012, Fan et al., 2002, Suller & Russell, 2000, Svenningsen et al., 2011, Webster et al., 1994, Zambon et al., 1995).

1.2. Bacterial resistance to triclosan

1.2.1. Triclosan resistance and adapted resistance in bacteria

Many bacteria, such as *Neisseria subflava, Prevotella nigrescens, Porphyromonas gingivalis* and *E. coli, Salmonella*, and *Staphylococcus*, are intrinsically susceptible to triclosan at low concentrations (0.1–3.9 mg/L) (Copitch et al., 2010, Randall et al., 2004), whereas *Lactobacillus* and *Streptococcus mutans* were less susceptible (MIC range 15.6–20.8 mg/L) (Randall et al., 2004). Most *Pseudomonas* strains are found to be resistant to triclosan even at high concentrations (Chuanchuen et al., 2002, Ellison et al., 2007).

Acquired resistance to triclosan has also been widely observed across bacterial genera (Ledder et al., 2006)., and the trait varies in different species. A highly significant reduction (400-fold) in triclosan susceptibility was achieved in a laboratory *E. coli* strain when exposed to triclosan, whereas only minor (two-fold) decreases in triclosan susceptibility occurred for *Prevotella nigrescens* and *Streptococcus* strains (Randall et al., 2004).

In a recent study, forty environmental and human isolates were tested for effects of chronic triclosan exposure on antimicrobial susceptibility; however only 5 isolates gained acquired resistance (Ledder et al., 2006).

The inconsistency seen in studies on acquired resistance in bacteria under prolonged exposure of triclosan suggests that this adaption is not through a common pathway shared by all bacteria, but it is confined to certain bacterial species that share similar specific pathways.

1.2.2. Mechanisms of resistance to triclosan

A recent study investigated the prevalence of decreased susceptibility to triclosan and a panel of antibiotics in over 400 human and animal isolates of *Salmonella enterica*. Seventy four percent of the isolates were shown to be susceptible to triclosan at a concentration of 0.12 mg/L. Of the 111 isolates that grew on media containing 0.12 mg/L, only 16 strains showed consistent triclosan resistance, but mostly at a low level (MIC 0.25-0.5 mg/L). Only 1 isolate had MIC of 4 mg/L. Of these strains showing reduced triclosan susceptibility, 56% were also multidrug-resistant, and expressed higher efflux activity compared to the strains showing reduced susceptibility only to triclosan. No specific mutations within *fab*I was detected for these multi-drug resistant (MDR) strains (Copitch et al., 2010). This indicates that triclosan resistance may be a result of generic resistance mechanisms that can be

promoted by a multidrug efflux system. However, this investigation was restricted to *Salmonella* strains and mechanisms in other bacteria could be significantly different.

A structural analysis revealed minimal overall structural change of enoyl-ACP carrier protein reductase (ENR) in a triclosan highly resistant *E. coli* mutant, except that a flexible α-helical turn around triclosan is slightly pushed away. The weakening of some edges to face aromatic interactions around triclosan in the mutant results in significant resistance to triclosan (Jiten Singh et al., 2010).

Pseudomonas is intrinsically resistant to triclosan, and the mechanisms involved in triclosan resistance as well as cross resistance to other antibiotics have been studied extensively. So far, the most commonly accepted mechanisms of reduced susceptibility to triclosan and cross-resistance to antibiotics in *Pseudomonas* are mutation of *fabI* and over-expression of Resistance Nodulation Division (RND) efflux system with more emphasis on the latter.

1.2.2.1. Mutation of the targeted fabI gene

As described in the above section, FabI or homologs are the specific targets for triclosan in many bacteria. Thus, a mutation of the *fabI* gene may lead potentially to resistance to this antimicrobial agent as demonstrated in the original work by McMurry to identify FabI as the molecular target of triclosan (McMurry et al., 1998b). Further studies have also confirmed this assumption (Hoang & Schweizer, 1999, Jiten Singh et al., 2010, Kapoor et al., 2004a, Sivaraman, 2002, Sivaraman et al., 2004).

1.2.2.2. Multidrug efflux pump systems in Pseudomonas

Pseudomonas strains intrinsically resistant to triclosan have been isolated and increased triclosan resistance was observed after exposure to triclosan. Induced cross resistance to some other antimicrobial and antibiotics in *Pseudomonas* was also related to the exposure (Chuanchuen et al., 2001).

Active efflux is increasingly recognized as a major mechanism for antibiotic resistance, especially for multidrug resistance. Efflux pumps in bacteria are proteinaceous transporters localized in the cytoplasmic membrane, where toxic substances or antibiotics are

transported out of the cell via an energy-dependent mechanism. Some efflux systems are substrate-specific, whereas others may accommodate multiple drugs, such as multidrug resistance (MDR) pumps. The observation that RND efflux pump systems in *P. aeruginosa* are effective against both biocides and clinically important antibiotics has added weight to the debate on the biocide/antibiotic cross-resistance and the possible selection of multiple antibiotic-resistant strains by biocides (Chuanchuen et al., 2001). Due to the diversity of substrates accommodated by these efflux systems, it is likely that many novel or yet to be discovered antimicrobials will themselves be efflux substrates and, as such, efflux inhibitors may become an important component of Gram-negative antimicrobial therapy (Poole, 2004).

Chromosomally-encoded multidrug efflux pumps such as the Mex groups in *P. aeruginosa* are key factors in determining the intrinsic resistance of Gram-negative bacteria to both biocides and antibiotics; also they play an important role in the development of acquired multidrug resistance. The most important multidrug efflux systems in *P. aeruginosa* are members of the RND family, to which the Mex groups belong. The targets of this type of efflux pump system include a broad range of structurally unrelated molecules that can include most classes of antibiotics, as well as biocides, dyes, detergents, metabolic inhibitors, aromatic hydrocarbons, cationic antimicrobial peptides, toxic fatty acids, bile salts, and acetylated homoserine lactones (AHLs). The latter are associated with quorum-sensing, which plays a role in virulence factors, biofilm formation and swarming phenomena (Evans & Passador, 1998, Pearson & Van Delden, 1999).

The *P. aeruginosa* strain PAO1 genome (Stover et al., 2000) encodes 12 proposed RND pumps, and to date, triclosan is shown to be a substrate for four of the seven experimentally identified RND pumps (shown in Table 1). In fact, triclosan is a good substrate in inducing the expression of multidrug efflux pumps in *P. aeruginosa*.

Table 1 RND efflux pumps encoded in the *P. aeruginosa* genome

(Chuanchuen et al., 2001, Chuanchuen et al., 2002, Evans & Passador, 1998, Morita et al., 2001, Morita et al., 2003, Pearson & Van Delden, 1999, Poole, 2004, Poole et al., 1996, Schweizer, 2003)

NO	MFP (Membrane fusion protein)	IM (Inner membrane)	OMP (Outer membrane protein	regulator	function	+/- encoding	inducer/signal	triclosan involved in PA
1	*MexA*	*MexB*	*OprM*	*MexR*	repressor	+	unknown	+
				(nalB loci)	target mutation triggers expression of efflux			
2	*MexC*	*MexD*	*OprJ*	*nfxB*	repressor	--	unknown	+
3	*MexE*	*MexF*	*OprN*	*MexS/T*	repressor		unknown	+
4	*MexX*	*MexY*		*MexZ*	repressor	--	selected pump substrates	+
5	*MexJ*	*MexK*		*MexL*	repressor	--	unknown	--
6	*MexGH*	*MexI*	*OpmD*	*?LasR/RrhlR*	unknown		cell density	--
	MexV	*MexW*						
7	*PA3523*	*PA3522*	*OpmE*	unknown	unknown	unknown	unknown	unknown
8	*PA1435*	*PA1436*		unknown	unknown	unknown	unknown	unknown
9	*PA4374*	*PA4375*		unknown	unknown	unknown	unknown	unknown
10	*PA2528*	*PA2527/6*	*OpmB*	unknown	unknown	unknown	unknown	unknown
11	*CzrB*	*CzrA*	*OpmN*	CzrR/S	sensor/kinase		Cd/Zn	unknown
12	*PA157/6*	*PA158*		unknown	unknown	unknown	unknown	unknown

It is generally believed that of the four efflux pumps of *P. aeruginosa* active against triclosan, only MexAB-OprM is expressed in wild-type strains; the other pumps remain inactive until mutations have been acquired in repressors of these systems. However, a low level of MexC was found to be expressed in PAO1 under normal laboratory conditions. Earlier studies found that the *mexCD-oprJ* operon is not silent in wild-type PAO1; in fact, the CD efflux pump is an inducible pump in wild type PAO1 cells (Morita et al., 2001, Morita et al., 2003). Wild-type NfxB, which had been identified as the repressor of the operon, represses the transcription of the *mexCD-oprJ* operon in constructed efflux pump strains (Chuanchuen et al., 2001, Poole et al., 1996). These authors proposed that the inducers of the *mexCD-oprJ* operon bind to the wild-type NfxB protein and prevent the repression of the *CD* operon leading to the expression of the pump. A later study also proved that disinfectants could induce the overexpression of the MexCD-OprJ efflux system (Morita et al., 2003). As such, we can see that the *mexCD-oprJ* operon together with *mexAB-oprM* operon could play an important role in intrinsic resistance to triclosan and other antibacterial agents in wild-type *P. aeruginosa*.

Other possible mechanisms related to triclosan resistance

Biofilms formed by bacteria can contribute to antibiotic resistance. The biofilm is an effective protective shield to assist bacteria to survive an environment containing antibiotics (McBain & Gilbert, 2001). *Pseudomonas* species use quorum sensing, which generally is dependent on two AHL dependent systems, namely the *las* system and *rhl* system (Greenberg, 2000, Juhas et al., 2005, Whitehead et al., 2001). They are both homologues of the *lux* system, first discovered in *Vibrio fischeri*, and consist of a transcriptional activator LasR/RhlR, and the AI synthase enzyme LasI/RhlI, respectively. This system has been extensively studied in recent years as a mechanism that regulates virulence factor gene expression. In addition, it could also function as a mechanism in biofilm-related antibiotic resistance, since quorum sensing is a cell-to-cell signaling system that controls the population density and growth of microorganisms, which leads to the development of biofilm (De Kievit et al., 2001, Parsek & Greenberg, 2005, Van Houdt et al., 2005).

Two recent studies, (Bayston et al., 2007, Seaman et al., 2007) reported a novel triclosan resistance mechanism in methicillin-resistant *Staphylococcus aureus* expressed as small

colony variants (SCV), in which higher triclosan MICs of 8- to 67-fold were obtained compared to parental triclosan susceptible strains. No further information has been reported to date about the mechanisms at the molecular level involved in this phenomenon.

Due to the immediate biocidal effect of triclosan, another possible resistance mechanism, biodegradation may only work synergistically with other mechanisms (Saleh et al., 2011, Schweizer, 2001). However, it is worth noting that the intermediates formed during the biodegradation process could also be toxic and so may demonstrate inhibition to the growth of the degraders (Aranami & Readman, 2007, Canosa et al., 2005). In this situation, triclosan biodegradation would actually function as an inhibitory mechanism for these bacteria.

1.2.3. Cross resistance

Due to the broad substrate specificity of some resistance determinants or the sharing of common targets by antimicrobial drugs, triclosan resistance determinants may also select for resistance to other biocides, as well as some currently used clinical antibiotics. Hence, the risk of bacterial cross resistance to antibiotics induced by triclosan is of increasing concern (Aiello & Larson, 2003, Randall et al., 2004, Schweizer, 2001).

The ability to acquire cross resistance to other antimicrobials or antibiotics by exposure to triclosan is not universal and the level of adaption varies in different bacterial species (McBain et al., 2004). A10-100 fold increase in the mean frequency of mutation resulting in resistance to antibiotics or cyclohexane in salmonellae was observed after exposure to triclosan (Randall et al., 2004). *E. coli* O157 strain acquired high levels of resistance to triclosan after only two exposures under a sub-lethal concentration and, when adapted, repeatedly demonstrated decreased susceptibilities to various antibiotics, including chloramphenicol, erythromycin, imipenem, tetracycline, and trimethoprim, as well as to a number of biocides (Braoudaki & Hilton, 2004a). Adaptive resistance was also achievable for *Salmonella enterica*, although more passages were required compared to *E. coli*, and the cross-resistance varied with serotype (Braoudaki & Hilton, 2004a). Also the adapted cross-resistance does not show reciprocal effect between antibiotics studied. *Salmonella enterica* serovar Enteritidis expressed cross-resistance to chloramphenicol, whereas *Salmonella enterica* serovar Typhimurium expressed cross-resistance to chlorhexidine after exposure to triclosan. Benzalkonium chloride-resistant *Salmonella enterica* serovar Virchow showed elevated resistance to chlorhexidine; however, chlorhexidine-resistant

Salmonella serovar Virchow did not demonstrate reciprocal cross-resistance to benzalkonium chloride (Braoudaki & Hilton, 2004a, Braoudaki & Hilton, 2005).

Despite the fact that many laboratory studies demonstrated the significance of cross-resistance induced by triclosan exposure (Braoudaki & Hilton, 2004b, Chuanchuen et al., 2001), most of the field studies carried out in clinical, community or industrial settings on triclosan resistance have provided no clear evidence that the presence of residual biocide concentrations in the environment promotes the emergence of bacterial tolerance to the biocides or the cross-resistance to other antibiotics (Cottell et al., 2009, Lear et al., 2006, Suller & Russell, 2000). Instead, it was noted that triclosan-tolerant strains of *E. coli* were more susceptible to gentamicin than the wild-type strains (Cottell et al., 2009). However, there are a small number of field studies under similar conditions, which found the opposite and consequently renewed fears that the use of triclosan may lead to an increased selective pressure towards antibiotic resistance (Aiello & Larson, 2003, Aiello et al., 2004). Aiello (Aiello et al., 2004) observed a positive trend between increasing triclosan MICs and resistance to antibiotics in bacteria isolated from hands after a year of exposure to triclosan at a general usage level in the community, although no statistically significant association could be confirmed. A most recent study reported the sub-lethal exposure of *Listeria monocytogenes* to triclosan resulting in decreased resistance to gentamicin and other amino-glycosides (Christensen et al., 2011). Another recent report also highlighted an apparent link between triclosan resistance and antibiotic resistance in a clinical setting, where *Pseudomonas aeruginosa* strains were isolated during an hospital epidemic and were shown to be of identical genotypes and resistance patterns, i.e. resistant to fluoroquinolones, amonoglycosides as well as high level resistance to triclosan. The source of infection was found to be a soap dispenser containing triclosan soap (D'Arezzo S et al., 2012).

The major reasons for the view casting doubt on cross resistance could include related factors such as the concentration and nature of the biocides present in the environment, the stability of the acquired resistance, and more importantly, the approach and methods used to assess resistance in these studies.

However, ecological studies have shown an obvious change in bacterial profile after long term exposure to triclosan, which indicates an impact of chronic exposure of triclosan on the bacterial species distribution in water and soil consortium (Aspray et al., 2005, Hay et al., 2001).

1.2.4. Mechanisms involved in cross resistance

In McBain's study (Ledder et al., 2006), a conclusion was made that "change in triclosan susceptibility did not affect susceptibility towards chemically unrelated antimicrobials", which indicated that substrate structure specific mechanisms are involved in cross resistance; whereas in Braoudaki's and Hilton's studies, the authors successfully associated the reduction in bacterial susceptibility to chemically unrelated antibiotics with adaptive triclosan resistance in *E. coli* and various *Salmonellae* (Braoudaki & Hilton, 2004a, Braoudaki & Hilton, 2004b, Braoudaki & Hilton, 2005). The conflicting results are partly because of the different concentrations of triclosan used in these studies and different species of bacteria investigated which gave varying responses; however, this may also indicate that the cross resistance mechanisms involved are complicated.

Theoretically, triclosan-induced resistance to antibiotics could involve all four generally recognized resistance mechanisms, including selection of gene mutations, enhanced expression of target genes, active efflux from the cell, or enzymatic inactivation/degradation.

Cross resistance induced by triclosan was addressed soon after the mechanism of triclosan targeting ENR (Enoyl-acyl carrier protein reductase) was discovered (McMurry et al., 1999, McMurry et al., 1998b). As discussed earlier, this enzyme, namely FabI, is an essential enzyme in the last step of the fatty acid biosynthesis pathway. Mutation of the *fabI* gene will lead to triclosan resistance, which will also result in antibiotic resistance to other antibiotics that share the same target in the fatty acid biosynthesis pathway.

However, the attribution of cross resistance to the mutation of the triclosan targeting gene *fabI* cannot explain the reason of the cross resistance to some other non-*fabI* targeting antibiotics. Even for *fabI* targeting antibiotics, concerns about cross resistance remained theoretical, since no antibiotic with this mechanism had been identified. However, it was subsequently found that the anti-tuberculosis drug isoniazid targets the same step in the fatty acid biosynthesis pathway as triclosan (Heath & Rock, 2004). In addition, an early study showed that the diazaborine family of antibacterial agents also acts on enoyl reductase in *E coli* (Roujeinikova et al., 1999a, Roujeinikova et al., 1999b). The enoyl reductases of *E. coli* are also a target of the biocide hexachlorophene (Heath et al., 2000). Because of the shared targets, it is possible to propose that triclosan might be able to select cross resistance to these compounds. It should be noted that fatty acid biosynthesis together

with isoprenoid biosynthesis have already been identified as potential targets for new generation antibiotics (Heath & Rock, 2004, Wiesner & Seeber, 2005). Thus the proposed antibiotic resistance induced by triclosan would possibly have significant potential impact on the efficiency of new generation antibiotics.

As with the mutation of a target gene, if *fabI* is over-expressed in the cells, then even under exposure to triclosan, there will still be adequate FabI to function effectively in the biosynthesis pathway. As a result of this enhanced expression of target gene, the cells will become resistant to triclosan and potentially cross resistant to antibiotics that share the same target.

On the other hand, at a molecular level, studies have already attributed the cross resistance between triclosan and antibiotics to multidrug efflux pumps (Chuanchuen et al., 2001, Chuanchuen et al., 2003, Copitch et al., 2010, Hernández et al., 2011, McMurry et al., 1998a). High levels of triclosan exposure could initiate the over-expression of a number of multidrug efflux pumps in some species, such as *Pseudomonas* and *MRSA* (Chuanchuen et al., 2001, Chuanchuen et al., 2003, Copitch et al., 2010). Efflux pumps are associated with resistance to a diverse range of antimicrobials including fluoroquinolones, tetracyclines and glycylcyclines, chloramphenicol and florfenicol, macrolides, lincosamides and ketolides, β-lactams, aminoglycosides and oxazolinidones (Poole, 2005) Although these have been investigated (Levy, 2002), the correlation between adaptive triclosan resistance and antibiotic resistance resulting from the multidrug efflux pumps induced as a response to triclosan exposure remains debatable. Although resistance to quinolones was found to be induced in *Stenotrophomonas maltophilia* by use of triclosan and the up-regulation of efflux pumps (Hernández et al., 2011), Cottell et al (2009) did not find any increased resistance in triclosan-tolerant strains of *E coli*, *Staphylococcus aureus* or *Acinetobacter johnsonii*. In fact they noted that triclosan-tolerant strains of *E coli* were more susceptible to gentamicin than wild-type strains. Similarly Suller and Russell (2000) were unable to demonstrate any link between triclosan resistance and resistance to mupirocin or other antimicrobials. Other workers have reported similar findings with *S aureus* and MRSA (Yazdankhah et al., 2006). More work is required because of the number of the efflux pumps already identified, and yet to be identified, and also the complexity of each multidrug efflux pump system.

Despite the focus on the effect of efflux pumps on triclosan resistance and its cross resistance to other antimicrobial agents, several studies have linked triclosan resistance and

cross-resistance with the alteration of cell membrane structures (Champlin et al., 2005, Tkachenko et al., 2007). The reduced uptake and efflux is most evident in the synergistic action of the outer membrane permeability barrier and active efflux, which results in high-level intrinsic and/or acquired resistance in many clinically important Gram-negative bacteria (Kumar & Schweizer, 2005).

Quorum sensing is another possible mechanism of cross-resistance, but even more complicated system that might be involved in triclosan resistance and cross resistance to antibiotics. Some of the quorum sensing pathways might collaborate with multidrug efflux pumps, compounding the difficulties in studying the mechanisms of triclosan resistance. In the quorum sensing in *P. aeruginosa*, a fatty acid derived signaling messenger is employed (Mattmann et al., 2008), which indicates a possible relationship between triclosan resistance and its cell-to-cell signaling system. Furthermore; it is worth noting that phenotypic profiles of mutant strains that over-express multidrug efflux pump systems appear to be similar to these of strains listed in the study of quorum sensing, where bacterial properties of cell fitness, virulence, growth phase, attachment ability and biofilm formation were investigated (De Kievit et al., 2001, McBain & Gilbert, 2001, Parsek & Greenberg, 2005, Van Houdt et al., 2005). Therefore, it seems illogical to neglect the involvement of cell-to-cell signaling systems in the investigation of triclosan resistance in *P. aeruginosa*.

If efflux pump systems in *P. aeruginosa* are associated with production, degradation or diffusion of small quorum sensing signals, then it is perhaps easy to understand the reason why many more studies of triclosan resistance in *P. aeruginosa* have investigated efflux pumps rather than *fabI* mutations, although it has been accepted by many researchers that *fabI* is a target of triclosan activity and that mutations in this site lead to triclosan resistance (Fan et al., 2002, Heath et al., 1999, McMurry et al., 1999). In addition, the association between efflux pumps and quorum sensing will help to explain the complexity of intrinsic and inducible high-level triclosan resistance in *P. aeruginosa* and the cross resistance to biocides and antibiotics. Furthermore, the primary function of efflux pumps in the active efflux of signaling molecules will help to explain the broad substrate specificity of these pumps.

Biodegradation is also the possible mechanism of cross resistance; triclosan degraders may be able to degrade other antimicrobials that share a similar biodegradation pathway with triclosan, and thus determine cross resistance to other antimicrobials.

1.3. Triclosan in the wastewater environment

1.3.1. Detection of triclosan and its effects on the water and wastewater environment

The wastewater environmental is clearly a contributing factor to bacterial antibiotic resistance. Antibiotic resistant bacteria and antibiotic residues have been found in waste water (Baquero et al., 2008). Faecal organisms, such as enterococci, were found to be resistant to tetracyclines and fluoroquinolones, and faecal coliforms were found to be resistant to amoxicillin, tetracyclines and ciprofloxacin (Da Silva et al., 2006, Ferreira Da Silva et al., 2007). Lefkowitz and Duran (2009) reported resistant strains of *E. coli* at different locations of the wastewater treatment process train, with significant resistance detected to tetracyclines, streptomycin and β-lactams. Increased percentages of isolates with multiple resistance was noted through the treatment process. Nagulapally et al. (2009) detected faecal coliforms resistant to fluoroquinolones and trimethoprim-sulphamethoxazole and enterococci (principally *E. faecalis*) resistant to vancomycin. Enterococci showing a similar resistance profile, i.e. vancomycin resistant, have also been detected in sewage sludge (Sahlström et al., 2009). Studies of hospital sewage treatment plants have yielded extended-spectrum β-lactamase producing organisms (Prado et al., 2008, Uyaguari et al., 2011) and gentamicin resistant strains of *E. coli* (Jakobsen et al., 2008). Similar to the results for faecal organisms, studies of environmental organisms in wastewater have also revealed microbial resistance. An increase in the percentage of isolates resistant to clinically important antibiotics such as amoxicillin/clavulanate, chloramphenicol and rifampicin (and multiple resistance) through treatment process has also been reported in *Acinetobacter* spp (Zhang et al., 2009). Ticarcillin and quinolone resistant *Aeromonas* spp. and fluoroquinolone resistant *Pseudomonas* spp. have been detected in the wastewater streams (Figueira et al., 2011, Schwartz et al., 2006).

The presence of triclosan in water environments has been reported worldwide (Boyd et al., 2003, Chau et al., 2008, Federle et al., 2002, Jackson & Sutton, 2008, Lishman et al., 2006, McAvoy et al., 2002, NICNAS, 2009, Ricart et al., 2010, Sabaliunas et al., 2003, Samsøe-Petersen et al., 2003, *pp.* 7-42, Singer et al., 2002, Spongberg et al., 2011, Xia et al., 2005). The concentrations of triclosan reported in Australia ranged from 23 to 434 ng/L (median 108 ng/L) and in 17 biosolids from 0.09 to 16.79 mg/kg on dry weight basis (median 2.32 mg/kg) (Kookana et al., 2011). Triclosan at concentrations of up to 75 ng/L was detected in receiving waters from five creeks affected by effluent discharge from WWTPs (Kookana et al., 2011). Table 2 below summarizes the levels of triclosan reported

recently in the water and wastewater environments. A recent review summarised that triclosan concentrations in WWTP effluents were in the range of 0.027-2.7 µg/L, with the maximum level detected in rivers being 2.3 µg/L (Chalew & Halden, 2009, Ricart et al., 2010).

Table 2 Occurrence of triclosan in wastewater environment as reported in the literature

	Surface Water (µg/L)	**Sewage Plant**		
		Influent (µg/L)	effluent (µg/L)	
Europe	0.04-0.21	0.6-21.9	0.11-1.1	(Miege et al., 2008)
USA	0.021	3.8-16.6	0.2-2.7	(McAvoy et al., 2002)
Japan	0.05-0.15	NA	NA	(Okumura & Nishikawa, 1996)
China	0.004-0.117	NA	NA	(Chau et al., 2008)
Canada		<4.01	<0.324	(Lishman et al., 2006)
France			0.09-0.43	(Miege et al., 2008)
Australia	0.023-0.434	0.573-0.845	<0.1-0.74	(Kookana et al., 2011, NICNAS, 2009, Ying & Kookana, 2007)

It can be seen that triclosan is present in the wastewater environment and is removed during wastewater treatment processes, which is indicated by the significant drop of triclosan concentrations between the influent and effluent of sewage plants. Furthermore, when discharged into a natural water body, the triclosan-containing effluent from sewage plants is also diluted in the surface water by natural dilution and the self-purification capacity of the natural water body. The resulting concentrations of triclosan in surface water after the wastewater treatment process and the natural process thus fall far below inhibitory concentration levels and thus will not have a lethal effect on environmental microorganisms and other ecosystems.

However, the predicted no effect concentration (PNEC) of triclosan for algae is as low as 50 ng/L (Singer et al., 2002). Therefore, even after all the natural and artificial removal processes, the triclosan levels listed above are still significantly above this PNEC level. Therefore, the presence of triclosan in the natural water environment as well as in the sewage plants may potentially have an impact on the ecological environment (Tatarazako

et al., 2004). A recent study (Thompson et al., 2005) highlighted the necessity for further investigations into the effect of residuals, at concentrations far below inhibitory concentration, on bacterial populations and their role, if any, in the continued problem of antibiotic resistance.

Concentrations of triclosan in local sewage collection systems leading to the sewage plant could be much higher than those listed as influent concentrations of triclosan in Table 2 due to the well documented adsorptive property of this substrate (Federle et al., 2002, Heidler & Halden, 2007, Singer et al., 2002). The concentration of triclosan measured in sewage plant influent may therefore be lower than that in the local sewer system, because of the sorption of triclosan onto the surface of organic material, such as polyvinyl chloride (PVC) pipes. Therefore, the sub-lethal concentration of triclosan immediately or shortly after discharge from homes could act as an inducer of the expression of bacterial multidrug resistance determinants, such as multidrug efflux. As discussed previously there could be some other factors, such as biofilm formation or disinfectant residual, that could facilitate the selection of resistance determinants. The potential for triclosan to select for antibiotic resistant bacteria has been addressed by scientists all over the world. Various studies have reported an increase of triclosan resistance and a possible reduction in susceptibilities to antibiotics after exposure to triclosan (Braoudaki & Hilton, 2004b, Chuanchuen et al., 2001, Schweizer, 2001).

In the natural water environment, apart from the potential selection of antibiotic resistant bacteria, the presence of triclosan also raised various other concerns about the impact that triclosan could have on the ecological environment. For example, triclosan is acutely or chronically toxic to some aquatic organisms, as in the case of acute toxicity to algae at trace levels, i.e. PNEC of 50 ng/L (Tatarazako et al., 2004).

Triclosan is also found to have a high potential for bio-accumulation (Coogan et al., 2007). Snail bioaccumulation of triclosan was found in a stream affected by a wastewater treatment plant runoff in US (Coogan & La Point, 2008). In a Japanese study, a range of 0.89-2.5 μg/kg of triclosan was detected in fish tissues (Okumura & Nishikawa, 1996), and a range of 0 to 2100 μg/kg lipid was found in human breast milk samples (Dayan, 2007). Despite the lack of knowledge on how this accumulation of triclosan will affect human or environmental health, it is significant enough to cause an alert on public health grounds.

The formation of dioxin has been reported in a natural water body as a result of

chlorination of triclosan in the presence of ultraviolet light (UV) (Aranami & Readman, 2007, Chung et al., 2007, Lores et al., 2005, Son et al., 2007, Yu et al., 2006). These much more toxic triclosan derivatives will have greater impacts on the aquatic fauna (Dann & Hontela, 2011).

The presence of triclosan in the wastewater environment may have similar impacts as that in the natural water body as described above. In addition, it may also have significant impact on the performance of the wastewater treatment plant. The effects of triclosan on microbial communities and degradation of domestic xenobiotics were studied in simulated sewage-drain-field soil, where cultivable microbial populations were found to be significantly inhibited and triclosan-resistant *Pseudomonas* strains were strongly enriched (Svenningsen et al., 2011). Triclosan degradation was found to be slow at all concentrations tested (0.33-81 mg/kg) during 50-days of incubation.

The wastewater treatment processes in the sewage plants represent artificial enhancement of biological processes that occur spontaneously in the natural water environment. It employs a biological process as the principal treatment process, where the behavior of the microbiological community dictates the performance of the processes. In particular, for conventional wastewater treatment process, the nutrient removal efficiency relies largely on the activity of microorganisms in the activated sludge bio-reactors. Therefore, a selective pressure from the presence of the biocidal effect of triclosan will potentially provide an inhibitory impact on the biological environment and affect the bacterial community profile as well as the viability of the microorganisms (Svenningsen et al., 2011). This will affect the efficiency of the treatment process, particularly when triclosan enters the process as a matter of plug flow, when a high level of triclosan enters within a short period of time.

There are limited studies investigating the effect of triclosan on the performance of the wastewater treatment process. A bench-top study showed that the load of triclosan at levels expected in household and manufacturing wastewaters is unlikely to upset sewage treatment processes (Federle et al., 2002). However, it is noteworthy that in this study, the sewage treatment process tested was a triclosan pre-acclimated system (dosed with additional triclosan), which was evaluated after the adaptation to triclosan had already occurred. Therefore, the result does not reflect the real situation.

A recent study (Heidler & Halden, 2007) revealed that a large proportion of particle-active

triclosan (composed of 80±22% on average of the total triclosan inflow) was bound to particulates and eventually sequestered and accumulated in excess sludge in the wastewater treatment process. This study therefore raised concerns about the practice of applying large quantities of triclosan-containing sludge to soils used for animal husbandry and crop production, and consequent concerns over the ecological, environmental and health impacts. However, in this study, no information on sludge digestion was given; therefore it is hard to determine if the concern is valid. In another study (McAvoy et al., 2002) investigating triclosan in digested sludge, it was revealed that triclosan was adequately removed from digested sludge prior to disposal for land application.

However, a change of bacterial community composition was observed in simulated sewage-drains soil as a result of triclosan exposure and the inhibition of xenobiotic degradation was also reported (Svenningsen et al., 2011), which highlights the concerns that environmentally realistic concentrations of triclosan in the wastewater may affect the efficiency of biodegradation in percolation systems.

To conclude, the presence of triclosan in water and wastewater environment represents a potential risk to public health and the natural environment (Akiyama et al., 2010, Ferreira Da Silva et al., 2006, Nagulapally et al., 2009, Sahlström et al., 2009, Zhang et al., 2009). The removal through sewage treatment plants is thus critical to mitigate the risk.

1.3.2. Wastewater treatment process and triclosan removal efficiency

A conventional wastewater treatment process train includes preliminary treatment, primary treatment, secondary treatment and sludge treatment (Wiesmann et al., 2007, *pp.* 25-42). The preliminary treatment normally includes screens and a grit removal tank, which removes large objects and a proportion of the suspended solids. Most of the suspended solids are removed through the primary sedimentation basin, where a proportion of organics that attached to the suspended solid are also removed.

Secondary treatment is considered as the main process for removal of organics and other nutrients. Conventional secondary treatment includes aeration and sedimentation. During the startup of a new system, the indigenous microorganisms in the source water are concentrated; the microorganisms that are capable of utilizing organics and other nutrients in the source water proliferate and outgrow other microorganisms. By working

synergistically, the dominant microorganisms gradually evolve the ability to utilize the organic compounds in the source water as carbon sources and thus treat the water.

In the aeration basin, aerobic microorganisms utilize oxygen, organics and other nutrients in the water to proliferate. As a result, biomass increases in amount and the water is treated. Downstream from the aeration basin, biomass is settled in the secondary sedimentation basin. A proportion of the settled biomass is removed from the water treatment system before further treatment in the sludge treatment process prior to disposal, while another portion of the settled sludge from the secondary sedimentation basin is recycled into the aeration basin as inoculum to facilitate the biological process in the aeration basin.

Excess sludge from the secondary sedimentation tank is digested using aerobic and/or anaerobic processes to remove organics and nutrients through nitrification.

Triclosan may be removed by wastewater treatment processes together with other organic compounds in the water. Therefore its removal efficiency is related to the overall organics removal efficiency of the treatment process. Table 3 lists a brief summary of reports on triclosan removal from wastewater treatment plants in different geographic locations. It is assumed that these wastewater treatment plants accepted influent streams composed of mostly domestic wastewater and they all operated well. In all processes, Biological Oxygen Demand (BOD) and Total Suspended Solids (TSS) removal were steady and efficient.

From data listed above, a good removal rate (over 90%) of triclosan may be achieved by most secondary biological treatment processes (Heidler & Halden, 2007). Removal of hydrophobic compounds was reported to be consistently above 85% (Tadkaew et al., 2011), which supports the high removal efficiency of triclosan. Sorption was thought to be the primary mechanism involved in this removal process (Kookana et al., 2011), although other studies attribute high removal efficiency to other mechanism such as biological degradation .

Of the commonly used wastewater treatment processes, conventional activated sludge treatment demonstrated reliable performance for triclosan removal with an effluent concentration of around 1µg/L even at a high triclosan load in the influent. A high removal percentage of 95% can be achieved in most activated sludge processes. This is probably due to the high Hydraulic Retention Time (HRT) and Sludge Retention Time (SRT), commonly seen in most conventional activated sludge processes. In some activated sludge

processes designed for enhanced removal of phosphorus and nitrogen, the HRT can be maintained up to 12-18 hours and the SRT up to 15-30 days. Trickling filters and biological contactors normally have less HRT, and therefore are slightly less efficient in triclosan removal. Other parameters of operational conditions like temperature at the WWTP will also influence the efficiency of the treatment processes. Nevertheless, efficient removal of triclosan from wastewater can be achieved in most secondary treatment processes (as shown in Table 3).

However, we can also see from this table that downstream of a successful triclosan removal treatment process, there are still hundreds or thousands of nano-grams of triclosan per litre of effluent, and this concentration will still cast an influence on the natural water environment (Coogan & La Point, 2008, McAvoy et al., 2002, Miege et al., 2008, Zhang et al., 2009). Up to 75 ng/L of triclosan was detected at WWTPs receiving environment in South Australia (Kookana et al., 2011). Investigations into triclosan removal mechanisms in these wastewater treatment processes are necessary in order to develop strategies for an enhanced triclosan removal during these processes or to identify some advanced supplementary treatment process to ensure that the residual concentration of triclosan in the wastewater environment has minimum adverse impact on the natural environment.

The effect of triclosan transformation by advanced oxidation, such as ozonation process, has recently been investigated in a bench scale, and was found to be effective (Chen et al., 2012). Reduced genotoxic effects were observed after ozonation, though the resultant chlorophenol was harmful to aquatic organisms (Chen et al., 2012). Other oxidation processes using free chlorine, potassium permanganate ($KMnO_4$), manganese oxides (MnO_2), Fe(III) saturated montmorillonite have also been investigated for triclosan degradation (Inoue et al., 2010, Jiang et al., 2009, Liyanapatirana et al.). However, most of these methods have resulted in only partial degradation or generation of toxic end products like chlorinated phenoxy-phenols, chlorinated phenols, trihalomethanes and toxins, which are known to be carcinogenic. Biological treatment may be a useful approach to eliminate triclosan from contaminated materials (Kim et al., 2011), although biodegradation might become limited due to the bacterial inhibitory effect of triclosan on a wide range of bacteria (Saleh et al., 2011, Yazdankhah et al., 2006).

A most recent study evaluated the effect of Fe-Pd bimetallic nano-particles on diphenyl ether degrading bacterial strain *Sphingomonas* sp. PH-07 as well as a sequential nano-bio hybrid process with Fe–Pd as catalytic reductant and PH-07 as biocatalyst for degradation

of triclosan (Murugesan et al., 2011). This study revealed this hybrid treatment as a potential future strategy for the degradation of triclosan.

Table 3 Triclosan Removal Efficiency from sewage by wastewater treatment processes

(Bester, 2003, Heidler & Halden, 2007, McAvoy et al., 2002, Miege et al., 2008, Sabaliunas et al., 2003, Thompson et al., 2005, Ying & Kookana, 2007)

Type of Works	Percentage removal	Influent conc.(µg/L)	Effluent conc.(µg/L)	Source
Activated sludge	95%	21.9	1.1	UK
Activated sludge	83%	5.4	0.9	UK
Activated sludge	98%	4.7	0.07	USA
Activated sludge	96%	1.2	0.05	Germany
Activated sludge	95%	5.2	0.2	USA
Activated sludge	96%	10.7	0.41	USA
Activated sludge with stabilisation lagoons	92%	0.85	0.06	Australia
Activated sludge with sand filtration	72%	0.58	0.17	Australia
Trickling filter	58%	3.8	1.6	USA
Trickling filter	86%	16.6	2.1	USA
Trickling filter	83%	15.4	2.7	USA
Trickling filter	79%	2.8	0.6	UK
Trickling filter	95%	7.5	0.34	UK
Bioreactors	89%	0.8	0.09	Australia
Stabilisation lagoons	85%	0.59	0.09	Australia
Oxidation ditches	92%	0.79	0.6	Australia

1.3.3. Mechanisms involved in triclosan removal from activated sludge wastewater processes

Flow of triclosan in activated sludge wastewater treatment process

Triclosan enters wastewater systems in forms of soluble triclosan in the aquatic phase and also particulate-active mode. A molecular level of sorption and desorption process occurs at all times, and the ratio of soluble and particulate triclosan remains kinetically balanced according to the property of the carrying water and the performance of the treatment during the whole process.

Triclosan in wastewater will undergo a biological treatment process as described previously, where physical adsorption and biological degradation work synergistically for the removal of triclosan. At the biological treatment process, primary wastewater is mixed with activated sludge at the beginning of the process, where soluble triclosan is adsorbed on activated sludge. This adsorption is expected to be effective due to the hydrophobicity of triclosan (Tadkaew et al., 2011). At the same time, the large percentage of triclosan in particle form will be mixed and trapped into activated sludge. The active component of activated sludge is the consortium of microorganisms that are able to utilize the contaminants in water as nutrients (Carr et al., 2011a, Gangadharan Puthiya Veetil et al., 2012, Kim et al., 2011, Tadkaew et al., 2011, Thompson et al., 2005). Under optimal conditions provided by the bioreactor, these microorganisms will utilize triclosan and other organics to metabolize and proliferate. During this process, triclosan is biodegraded, and the remaining triclosan will be separated and sediment to the bottom of the reactor and be disposed of as excessive sludge at the end of the water treatment process. The excessive sludge will then undergo an aerobic and anaerobic digestion, where contaminants in the sludge are further degraded prior to disposal (Kookana et al., 2011, Thompson et al., 2005).

Variable contribution of different triclosan removal mechanisms

Despite the fact that the triclosan removal rate in most activated sludge treatment processes remains consistently high (95-98%) (Sabaliunas et al., 2003, Bester, 2003, McAvoy et al., 2002)(Bester, 2003, Kookana et al., 2011, McAvoy et al., 2002, Sabaliunas et al., 2003, Tadkaew et al., 2011), the mechanisms of this removal have been subject to debate. In a recent mass balance study on triclosan removal from conventional wastewater treatment processes (Heidler & Halden, 2007), an overall 50±19% of the triclosan entering the plant

was found to remain in the excessive sludge after the whole treatment process, and only 48±16% of the triclosan intake was biodegraded or transformed through other mechanisms. However, in a bench-top based study (Federle et al., 2002) on continuous activated-sludge systems, biodegradation and removal of triclosan radio-labeled with C^{14} was monitored, and the results showed that, over a series of triclosan concentrations tested, 94-99.3% of triclosan in this system underwent primary biodegradation, and only 1.5-4.5% was adsorbed to the waste solids. The conflicts in these literature suggest that although the mechanisms involved in triclosan removal are similar, the dominant mechanism may vary in different systems, and they may to some extent influence the removal efficiency.

For an existing wastewater treatment plant, the efficiency of treatment depends on lots of factors such as the properties of wastewater influent, properties of activated sludge and operational conditions including aeration rate and temperature. The adaptation of activated sludge to a specific wastewater influent is very important for a successful treatment of this wastewater. As described previously, hydraulic retention time (HRT) will substantially influence the efficiency of secondary wastewater treatment.

Biodegradation as a primary mechanism to be promoted for triclosan removal

All studies on the efficiency of removal of triclosan from wastewater treatment systems are based on the concentration of triclosan residual in the water phase of the effluent after solids/liquid separation occurs in the secondary sedimentation process. Because of its polarity, triclosan is readily absorbed into activated sludge, which guarantees a good removal rate of triclosan (over 90%) (Sabaliunas et al., 2003, Bester, 2003, McAvoy et al., 2002) seen in most conventional activated sludge processes.

However, better or complete removal of triclosan requires faster biodegradation rate and longer HRT. Fast biodegradation also leads to a rapid and sustained decrease of triclosan concentration in the excess sludge, where biodegradation also occurs. As discussed before, a kinetic balance of triclosan exists between the water phase and the sludge phase, so more triclosan from water will be absorbed into the sludge phase, if the degradation of triclosan in the sludge phase takes place at a higher rate. This will consequently reduce the triclosan concentration in the treated water.

This theory is consistent with published experimental results (Federle et al., 2002), which described that adaptation of activated sludge to triclosan favored its removal and it is related to the overall treatment efficiency of the system.

Based on the above theory, variable efficiency in the removal of triclosan in treatment processes described in the previous section could be largely attributed to the properties of activated sludge and variable HRTs in different systems. These two factors determine the extent of biodegradation that occurs in the sludge. The better acclimatised sludge has more degradation capacity, and decomposes triclosan more quickly, thus will achieve a better removal in a system running at a specific HRT. For one system with pre-established activated sludge composition, the longer the HRT, the more biodegradation in sludge will occur, and a higher removal rate will be achieved.

This theory also explains the conflicting results of studies on the efficiency of triclosan removal from excess sludge in the sludge treatment process. A recent mass balance assessment of triclosan removal from the wastewater treatment process (Heidler & Halden, 2007) revealed that despite the good removal rate of triclosan from the liquid phase, a large proportion of particle-active triclosan (composed of 80±22% on average of the total triclosan inflow) was bound to particulates and eventually sequestered and accumulated in excess sludge in the process. In contrast, in another study (McAvoy et al., 2002) investigating triclosan in digested sludge, it was revealed that triclosan was adequately removed from digested sludge particularly when using an aerobic digestion process. Removal of triclosan from digested sludge under anaerobic conditions was much less efficient.

The above comparison indicates that an aerobic biodegradation of triclosan took place in the McAvoy study, where a good triclosan removal was achieved in the sludge treatment; whereas no or minimum biodegradation of triclosan occurred in the Heidler investigation and triclosan accumulated in the excess sludge. It becomes obvious that biodegradation is a key mechanism for assured triclosan removal efficiency in the wastewater and sludge treatment process. Additionally, it is also a more environmentally friendly and sustainable mechanism that should be promoted in the triclosan treatment process.

As described above, triclosan biodegradation occurs naturally as well as in the wastewater treatment plants. However, the performance varies between plants. Understanding of the mechanisms involved in triclosan biodegradation will assist in providing information for process optimization, improve triclosan removal efficiency, and consequently reduce the total quantity of triclosan present in the environment.

1.4. Triclosan biodegradation in Pseudomonas

1.4.1. Pseudomonas and aromatic metabolism theories

Studies on the genus *Pseudomonas* date back to 1895. The organisms have a complex taxomonical history with investigations searching for natural relationships (Gunsalus, 1996b, Ramette et al., 2003). The miscellany of names used for these bacteria include *Pseudomonas* (known as "true pseudomonads"), *Acidovorax*, *Burkholderia*, *Ralstonia*, *Brevundimonas* and *Stenotrophomonas* (Holloway, 1996, *pp*. 22). Pseudomonads are able to utilize organic compounds such as benzene, toluene, xylenes, ethylbenzene and styrene as the sole carbon source. These organic compounds are the most common products in the chemical production industry and are widely used in the organics manufacturing industry. As a consequence, large amounts of these compounds have been released into the environment and are present in water and soil as problematic components. This has motivated many investigations into the biodegradation of these compounds by microorganisms. In most cases, *Pseudomonas* spp were used as a bacterial model for these investigations (Favaro et al., 1996b, Fong et al., 2000, Galli, 1996, pp49) The knowledge of the degradative pathways of naturally occurring aromatic compounds has also contributed greatly toward the understanding of xenobiotic biodegradation (Galli, 1996, pp49). *Pseudomonas spp* consequently became the bacterial model that has been commonly used in the last three decades for characterisation of degradation pathways of many synthesized organic compounds (Afzal Khan et al., 2006, Boronin et al., 1993, Campbell et al., 1995, Di Gioia et al., 1998, Fong et al., 2000, Haro & de Lorenzo, 2001, Khan et al., 1997, Kitayama et al., 1996, Monticello et al., 1985, Saint & Ribbons, 1990, Sangodkar et al., 1988, Short et al., 1990, Tomasek et al., 1989).

Studies in enzymes, reactions, structures and later chemistry and physics have increased since the 1940s. The isolation of mandelate racemases (MR), dehydrogenases, decarboxylases and benzoate dehydrogenases from strain *P. putida* ATCC 12366 set a milestone in 1953 (Gunsalus et al., 1953a, Gunsalus et al., 1953b, Stanier et al., 1953), and the identification of enzymes and pathways for mineralization of D and L-mandelate were subsequently explored (Gunsalus, 1996b, Ramette et al., 2003). These discoveries led to the early but solid advances in biochemical and microbiological aspects of theories of metabolism of aromatic compounds. Based on tertiary structure studies, MR together with analogous mucono-lactonizing enzyme (MLE) and galactose dehydratase (GalD) and other alpha-hydroxyl acid enzymes, 12 in total so far, are categorised together under the term "the enolase superfamily" (Gunsalus, 1996a). New insights continue to be added on

hydrogen biocatalysis.

Different perspectives of *Pseudomonas* in biological, chemical and physical activities, such as oxygenases, plasmid-borne metabolisms, heme and sulphur iron, are advanced under the detailed structural and dynamic analysis of the preferred heme model, p-450cam (Di Primo et al., 1992, Gunsalus, 1996b, Heimbrook & Sligar, 1981, Kellner et al., 1997). Many catabolic oxygenase-positive Pseudomonades were isolated and characterized, and are still widely in use in different aspects of pseudomonad research (Gunsalus, 1996b). The chemical structures of degradation intermediates have been identified and metabolic pathways proposed (Gunsalus et al., 1953a, Gunsalus, 1996a). Stringent or relaxed substrate specific plasmid encoding genes for degradation of aromatic or chloroaromatic compounds such as chloro-biphenol (CBP), toluene (TOL), camphor (CAM), and Naphthalene (NAH) have also been discovered, characterized to some extent, and are still under intensive investigations today (Aldrich et al., 1987, Barriault & Sylvestre, 1999, Brusa et al., 2001, Chatterjee & Chakrabarty, 1984, Chu et al., 1991, Darzins et al., 1986, Deretic et al., 1987, Di Primo et al., 1992, Favaro et al., 1996, Frantz et al., 1987, Fujiwara & Chakrabarty, 1994, Haugland et al., 1991, Heimbrook & Sligar, 1981, Herrero et al., 1993, Izmalkova et al., 2006, Kato et al., 1989, Kellner et al., 1997, Kim et al., 1994, Li et al., 2004, Maharaj et al., 1993, Masai et al., 1997, McFall et al., 1998, Moon et al., 1995, Mylroie et al., 1978, Parsek et al., 1995, Sachiye et al., 1988, Saint & Ribbons, 1990, Sangodkar et al., 1988, Sanseverino et al., 1993, Shields et al., 1985, Takizawa et al., 1999, Wubbolts et al., 1994).

1.4.2. Biodegradation of environmentally significant aromatic compounds by Pseudomonas

The study on biodegradation of environmentally significant aromatic compounds has been driven by the need for bioremediation of soils contaminated with natural or synthetic compounds (Heipieper, 2007, *pp.* 1-4). Various bacteria as well as fungi have been used in studies on biodegradation of aromatic compounds in water and soil (Fulthorpe & Wyndham, 1991, Galushko et al., 1999, Holm et al., 1986, Jonas et al., 2000, Li et al., 2010b, Mori & Kondo, 2003, Reddy et al., 2010).

Atrazine degradation

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a herbicide that has been widely used all over the world. The intensive usage has resulted in atrazine being detected at high levels in soils as well as in ground water and natural surface water. Mixed cultures of natural or bioprocessed materials (Ghosh & Philip, 2004, Kadian et al., 2008, Satsuma, 2009, Siripattanakul et al., 2009b), as well as pure cultures of a variety of microorganisms, including *Trametes versicolor*, *Pleurotus pulmonarius, Arthrobacter* sp., *Agrobacterium radiobacter*, and *Nocadioides* sp. were found to be capable of degrading atrazine through various pathways (Bastos & Magan, 2009, Masaphy et al., Piutti et al., 2003, Siripattanakul et al., 2009a, Vaishampayan et al., 2007). Atrazine is most often used as a nitrogen source, since full oxidation of the carbon atoms in the s-triazine ring renders them unusable as an energy source (García-González et al., 2007, *pp.* 31-40). However, a few bacterial strains have been identified capable of utilizing atrazine as carbon source (Piutti et al., 2003, Vaishampayan et al., 2007).

Pseudomonas sp. ADP-1 has been used as a model strain in many studies on atrazine biodegradation (Clausen et al., 2002, Katz et al., 2000), and the degradation pathways have been well characterized in this model. Six genes, namely *atz*A, *atz*B, *atz*C, *atz*D, *atz*E, *atz*F, are found to be responsible for atrazine degradation and are encoded on a 108 Kb plasmid pADP-1. Genes *atz*A-C, encoding atrazine chlorohydrolase, hydroxyatrazine ehylaminohydrolase and N-isopropylammelide isopropylaminohydrolase respectively, are located within an unstable region of pADP-1 (García-González et al., 2007, *pp.* 31-40). The deletion of one or more of these genes was found to occur at high frequency when the host strain of the plasmid was cultured on a non-selective medium, and this deletion often resulted in the loss of the atrazine biodegradation phenotype. These three genes and homologues have also been found to be widely spread on other plasmids carried by other atrazine degradative bacteria (García-González et al., 2007, *pp.* 31-40). These findings may indicate that the three genes are carried on plasmid-borne mobile elements such as transposons. In contrast, genes *atz*D-F were found to be located at close proximity within the operon and they are also more conserved to specific host strains (García-González et al., 2007, *pp.* 31-40). A later study on the regulation of the atrazine degradative pathway in *Pseudomonas* discovered that the presence of preferential nitrogen sources has an inhibitory effect on the degradation of atrazine in soil and results in low degradation rates (García-González et al., 2007, *pp.* 31-40). This study also identified that the inhibitory effect of nitrogen operates at the level of gene expression, and one of the targets of this

regulation is the cyanuric acid degradative operon *atzDEF*, which is activated both by nitrogen limitation and the presence of cyanuric acid (García-González et al., 2007, *pp.* 31-40) .

PCB degradation

Polychlorbiphenyls (PCB) are structurally closely related to triclosan, and they are highly toxic, carcinogenic, and bio-accumulative in the humans (Fortin et al., 2005, Schofield & Dinovo, 2010). The biodegradation of this group of compounds was found to be very difficult due to the toxicity of this chemical as well as the possible toxic degradation by-products (Dann & Hontela, 2011, Fortin et al., 2005, Frenzel et al., 2010, Schofield & Dinovo, 2010). *Pseudomonas* species capable of degrading 3-chlorobenzoate showed poor growth in liquid 3-chlorobenzoate minimal medium but could degrade this compound in continuous cultures dosed with micromolar levels of the chemical (Fulthorpe & Wyndham, 1991), which indicated the potential inhibition by the accumulation of toxic degradation by-products.

A later study identified *Comamonas testosterone* (later referred to as *Pandoraea pnomenusa* (Gomez-Gil et al., 2007)), *Rhodococcus rhodochrous*, and a strain of *Pseudomonas putida* obtained by conventional enrichment techniques as being capable of degrading Aroclor 1242 significantly (Boyle et al., 1992). A wild type *Pseudomonas* strain, *Alcaligenes latus* together with a *Hansenulla californica* strain were also isolated recently and proved to be capable of reducing PCB concentrations in soil when applied directly in upper soil layers (*in situ*) and can be used for bioremediation (Zharikov et al., 2007, *pp.* 93-104). The optimal factors for improving degradation speed are high humidity and temperature of soil (Zharikov et al., 2007, *pp.* 93-104).

Two strains of *Pseudomonas sp*. strain KKS102 and *Psedomonas fluorescens* KKL101 were found to degrade biphenyl and PCBs in a mutualistic way, with *Pseudomonas sp.* KKS102 as the major degrader (Kikuchi et al., 1994b, Kimbara et al., 1988). Other PCB-degrading bacteria include *Achromobacter, Moraxella, Corynebacterium* and *Authrobacter* (Asturias & Timmis, 1993, Furukawa et al., 1989).

While it was found that Gram-negative isolates are generally limited in the range of PCBs they are able to break down, a group of Gram-positive bacteria, such as *Rhodococcus globerulus* were found to be capable of attacking a wide range of PCBs (McKay et al., 1997).

The genetic studies of aerobic PCB degradation, focused on cloning and characterization of the degradative pathway genes and have been carried out in a variety of bacteria (Springael et al., 1996). The catabolic genes are found to be encoded by chromosomal genes in many PCB-degrading bacterial isolates (Ahmad et al., 1990, Asturias & Timmis, 1993, Erickson & Mondello, 1992, Fortin et al., 2005, Furukawa et al., 1989, Hayase et al., 1990, Kikuchi et al., 1994a, Kikuchi et al., 1994b, Labbé et al., 1997, McKay et al., 1997, Sondossi et al., 1992) .

Chromosomal DNA fragments encoding biphenyl degradation, *bph* genes, were mobilized by pRP4 derivative vector from *Burkholderia sp.* into *Alcaligenes entrophus* CH34 (Springael et al., 1996). When integrated into the recipient chromosome and recovered as catabolic prime plasmids, the *bph* fragments allowed the metabolism of biphenyl, 2-, 3- and 4-chlorobiphenyl, and diphenylmethane (Springael et al., 1996). Bph prime plasmids were also able to express in hosts when transferred to *Burkholderia cepacia*, *Pseudomonas aeruginosa*, *Comamonas testosterone* and *A. eutrophus* (Springael et al., 1996). Transfer of the plasmid to the 3-chlorobenzoate-degrading bacterium *Pseudomonas sp.* B13 allowed the recipient to be able to mineralize 3-chlorobiphenyl (Springael et al., 1996).

The *bphA1A2A3A4* gene cluster was isolated from a Gram-positive degrader of a wide spectrum of polychlorobiphenyls *Rhodococcus globerulus* P6 strain (Asturias & Timmis, 1993, McKay et al., 1997). The biphenyl degradation pathway was characterised as a four-step catabolic pathway (McKay et al., 1997):

a. (Chloro)biphenyl (CB) is oxidised to (chloro)biphenyl-2,3-dihydrodiol (CBDHD) under the activity of ring-hydroxylating biphenyl dioxygenase (BDO) encoded by *bphA1A2A3A4* gene cluster;
b. CBDHD is converted to chloro 2,3-dihydroxybiphenyl (CDHB) by enzyme encoded by *bphB* gene;
c. CDHB is oxidised to chloro 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (CHOPDA) by enzyme encoded by *bphC* gene;
d. CHOPDA is converted to chlorobenzoic acid by enzyme encoded by *bphD* gene;

A similar pathway is also found to be shared by Gram-negative bacteria (Boyle et al., 1992), including *Pseudomonas putida* KF715 (Hayase et al., 1990). Significant homology was also found among *bphABC* gene clusters present in different species of bacteria,

indicating a mechanism for transferring the gene from one strain to another (Ahmad et al., 1990, Furukawa et al., 1989).

The 4-step pathway was further extended in *Pseudomonas sp.* strain KKS102, and 12 genes in the order of *bphEGF(ORF4)A1A2A3BCD(ORF1)A4* were characterised (Kikuchi et al., 1994b), where gene *bphEGF* showed homology with *dmpE, dmpF* and *dmpG* from *Pseudomonas sp*. strain CF600 (Kikuchi et al., 1994b, Shingler et al., 1992).

A number of studies have reported the involvement of plasmids in PCB metabolism (Carrington et al., 1994, Lloyd-Jones et al., 1994, Springael et al., 1993a, Springael et al., 1994, Springael et al., 1993b, Warren et al., 2004).

Although clusters of genes related to catabolism were identified, characterization of plasmids from PCB-degrading *Rhodococcus sp.* RHA1 strain did not reveal genes homologous to characteristic *bph* genes (Warren et al., 2004). However, this plasmid is still of significant interest, as the presence of aromatic compound degradation related oxygenase- and dehydrogenase-encoding genes, together with the structural and regulatory organization strongly suggested that this plasmid plays a role in the assimilation of PCB (Warren et al., 2004).

An endogenous IncP1 plasmid pSS50 carried by *Alcaligenes eutrophus* A5 was characterized and was found to catalyse the degradation of biphenyl and 4-chlorobiphenyl to benzoate and 4-chlorobenzoate in the PCB biodegradation pathway (Springael et al., 1993b). The catabolic genes were found to reside on a transposon Tn*4371*; however, the transfer of this transposon into other hosts does not always confer PCB degradation capability to the recipient strain, and only *A. eutrohpus* and an *Acinetobacter sp*. strain was found to acquire the degradation ability through this transposon (Springael et al., 1993b).

Biodegradation of other environmentally significant aromatic compounds in *Pseudomonas*

Biodegradation of other environmentally significant aromatic compounds has also been studied extensively in *Pseudomonas* strains, from simple aromatic hydrocarbon, such as phenol, benzene, and biphenyl, to more complicated polycyclic aromatic hydrocarbons (PAH), including naphthalene and phenanthrene (Demir et al., 2000, Li et al., 2010b, Resnick et al., 1996, Suenaga et al., 2007).

Pseudomonas putida F1 is one of the best studied degraders of aromatic compounds at

both molecular and biochemical level (Keane et al., 2007, *pp.* 105-124). The toluene degradation pathway (*tod*) has been characterised using this model. A multi-component toluene dioxygenase enzyme system was attributed to convert toluene to *cis*-toluene dihydrodiol via a dioxygenation pathway (Zylstra & Gibson, 1989), which is followed by the traditional catechol cleavage pathway. A hybrid histidine kinase (TodS) was found to be involved in the regulation of the *tod* pathway via a two-component signal transduction system (Tod ST*)* (Lau et al., 1997). A membrane protein (TodX) may also be involved in transportation of toluene into cells or as part of the TodST signal transduction process (Keane et al., 2007, *pp.* 105-124). TOL plasmid has been found to be present in a number of *Pseudomonas* isolates. In *Pseudomonas sp.* MT14, it co-exists with another catabolic plasmid encoding for phenylacetate catabolism and mercury resistance in this host (Pickup et al., 1983).

Pseudomonas spp. are able to metabolize a number of toxic aromatic compounds (Esteve-Nunez et al., 2001, Silvia et al., 1999). The biodegradative abilities are sometimes plasmid-encoded, as in the case of mobilisable TOL plasmid pWWO of *P. putida*, which harbours the genes of the toluene catabolic pathway (Lau et al., 1997, Wubbolts et al., 1994, Zylstra & Gibson, 1989). The horizontal gene transfer via plasmids among *Pseudomonas* populations has been highlighted (Espinosa-Urgel, 2004), and this may contribute to the wide spectrum of biodegradative ability of *Pseudomonas* strains.

A plasmid encoded catechol *meta*-pathway was characterised in *Sphingomonas* sp. strain HV3 (formerly *Pseudomonas* sp. HV3), which degrades aromatics and chloroaromatics. This meta operon consists of a *cmpF* gene encoding a 2-hydroxymuconic semialdehyde hydrolase, a *cmpE* encoding a ring-cleavage catechol 2,3-dioxygenase, which has a 6 bp overlap with *cmpF*, a 429 bp ORF of unknown function and a *cmpC* gene encoding a 2-hydroxymuconic semialdehyde dehydrogenase. (Yrjälä et al., 1997).

Ralstonia sp. strain PS12, capable of using 2,4-, 2,5-, and 3,4-dichlorotoluene as growth substrates was found to form dichloromethylcatechols as central intermediates (Pollmann et al., 2002). Dichloromethylcatechol was formed by *tecA* tetrachlorobenzene dioxygenase-mediated activation at two adjacent unsubstituted carbon atoms followed by *TecB* chlorobenzene dihydrodiol dehydrogenase-catalyzed rearomatization and then channelling into a chlorocatechol ortho cleavage pathway involving a chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, and dienelactone hydrolase (Pollmann et al., 2002).

Biodegradation enzymes

Enzymes involved in biological oxidation processes can be classified into two major categories. Dehydrogenases and oxidases transfer electrons and are involved mainly, if not exclusively, in ATP synthesis. Oxygenases, on the other hand, transfer molecular oxygen and play important roles in the metabolism of essential nutrients and foreign compounds (Hayaishi, 1996).

Catechol dioxygenases were found to play an important role in the metabolism of environmentally significant aromatic compounds (Asturias & Timmis, 1993, Jencova et al., 2008, Köiv et al., 1996, Kukor & Olsen, 1991, Lee et al., 1994, Suenaga et al., 2007).

Chlorocatechol 1,2-dioxygenase (CC120) encoding gene *tfdC* was found to be located on a 4.2-kb DNA fragment cloned from the 2,4-dichlorophenoxyacetic acid (2,4D) degrading plasmid pEST4011 (Köiv et al., 1996). The *bphE* gene encoding catechol 2,3-dioxygenase was identified in a *Pseudomonas* sp. strain IC able to grow on biphenyl, 3-methylbiphenyl and 4-methylbiphenyl (Carrington et al., 1994). The enzyme catechol 2,3-dioxygenase (C230) encoded by the *nahH* gene of plasmid NAH7 converts catechol to [alpha]-hydroxymuconic [epsilon]-semialdehyde in *Pseudomonas putida* (Ghosal et al., 1987). Both catechol 1,2- and 2,3-dioxygenase activities were found to be active in a simultaneous induction test on *Rhodococcus rhodochrous* NCIMB 13259 growing on styrene, toluene, ethylbenzene, and benzene as sole carbon sources (Warhurst et al., 1994). The crude extracts from the isolate *Pseudomonas sp.* obtained from nitrobenzene-contaminated soil and groundwater utilizing 2-nitrotoluene as the sole carbon source, contained catechol 2,3-dioxygenase (Haigler et al., 1994). Catabolism of 3-methylcatechol as an intermediate in the toluene degradation pathway was found to proceed via catechol-2,3-dioxygenase (Shields et al., 1989). A ring-hydroxylating oxygenase ortho-halobenzoate oxygenase was identified in a modified plasmid pA81 encoded ortho-cleavage pathway for the degradation of chlorocatechol as an intermediate in the haloaromatic acid-degrading bacterium *Achromobacter xylosoxidans* A8 (Jencova et al., 2008).

A functional screening of the presence of extra-dioxygenases (EDO, catechol *meta*-cleavage pathway) using a meta-genomic approach in the activated sludge used to treat coke plant wastewater resulted in 91 EDO-positive clones and a total of 43 EDO genes were identified (Suenaga et al., 2007). The prevalence of EDO in wastewater activated sludge indicated a significant role of EDO genes in the environmental aromatic degradation.

In most studies where homologues of catechol dioxygenases were isolated, hydrolase activity was also observed concurrently, such as *bphD* in *Pseudomonas* sp. strain IC (Carrington et al., 1994), *tfdD* in plasmid pEST4011 (Köiv et al., 1996), the 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase activity observed in nitrobenzene degradation *Pseudomonas sp.* (Haigler et al., 1994), and the 2-hydroxymuconic acid semialdehyde hydrolase activity observed in *Rhodococcus rhodochrous* NCIMB 13259 (Warhurst et al., 1994). Hydroxymuconic semialdehyde hydrolase was found to be involved in the catabolism of 3-methyl catechol as an intermediate in the toluene degradation pathway in the trichloroethylene-degrading bacterium G4 (Shields et al., 1989). Salicylate 5-hydroxylase was also encoded on plasmid pA81 carried by *Achromobacter xylosoxidans* A8 (Jencova et al., 2008).

Recent research has discovered novel ring cleavage pathways different to the traditional catechol cleavage pathway in bacteria (Gescher et al., 2005, Gescher et al., 2006, Gescher et al., 2002, Rather et al., 2010, Zaar et al., 2001, Zaar et al., 2004). It was found that in the bacterium *Azoarcus evansii*, benzoate was converted to benzoyl-CoA, followed by an oxygenase reaction and a nonoxygenolytic ring cleavage (Gescher et al., 2005, Gescher et al., 2006, Gescher et al., 2002, Zaar et al., 2001, Zaar et al., 2004). Benzoyl-CoA transformation is catalyzed by the iron-containing benzoyl-CoA oxygenase (BoxB) in conjunction with FAD and iron-sulphur centres containing reductase (BoxA), which donates electrons from NADPH (Gescher et al., 2005, Gescher et al., 2006, Gescher et al., 2002, Rather et al., 2010). Instead of 2, 3-dihydrodiol, 2, 3-epoxide was formed as a result of benzoyl-Co A transformation (Rather et al., 2010). An enoyl-CoA hydratase (BoxC) uses two molecules of water to first hydrolytically open the ring of 2,3-epoxybenzoyl-CoA, which may proceed via its tautomeric seven-membered oxepin ring form (Rather et al., 2010). Then ring C2 is hydrolyzed off as formic acid, yielding 3, 4-dehydroadipyl-CoA semialdehyde (Rather et al., 2010). The semialdehyde is oxidized by a NADP+-dependent aldehyde dehydrogenase (BoxD) to 3, 4-dehydroadipyl- CoA (Gescher et al., 2006, Rather et al., 2010, Zaar et al., 2001). Final products of the pathway are formic acid, acetyl-CoA, and succinyl-CoA (Rather et al., 2010).

Laccase-mediated triclosan transformation and detoxification was also studied using laccase in the presence and absence of redox mediates. Under the presence nof 1-hydroxybenzotriazole and syringaldehyde, 2,4-dichlorophenol and its dechlorinated forms were detected as transformation products (Murugesan et al., 2009) .

1.4.3. Biodegradation of halogenated compounds in Pseudomonas

Biodegradation of halogenated compounds, particularly chlorinated compounds, has been of particular interest for environmental scientists. Low molecular weight chlorinated compounds, such as some environmentally important chemicals including chloroform, trichloroethylene, and 1,1,1-trichloroethane were found to be recalcitrant mainly due to the lack of critical enzymes in catabolic pathways (Janssen et al., 2005). The low molecular weight and the simple structure of these compounds demand particularly specific enzymes, which can only target a particular compound in a short catabolic pathway, and are therefore less likely to be shared in the bacterial community. On the other hand, some other halogenated compounds, like chloro-toluene or chloro-acetic acids with more complicated structures are readily degradable in the natural bacterial community. In addition, others can only be degraded after gradual adaptation of bacteria by pre-exposure to these chemicals or corresponding structurally related chemicals, in a co-metabolic process.

The degradability or recalcitrance of chlorinated organic compounds was reviewed by Janssen (Janssen et al., 2005), where chlorinated compounds were classified by their degradability as illustrated in Figure **2**.

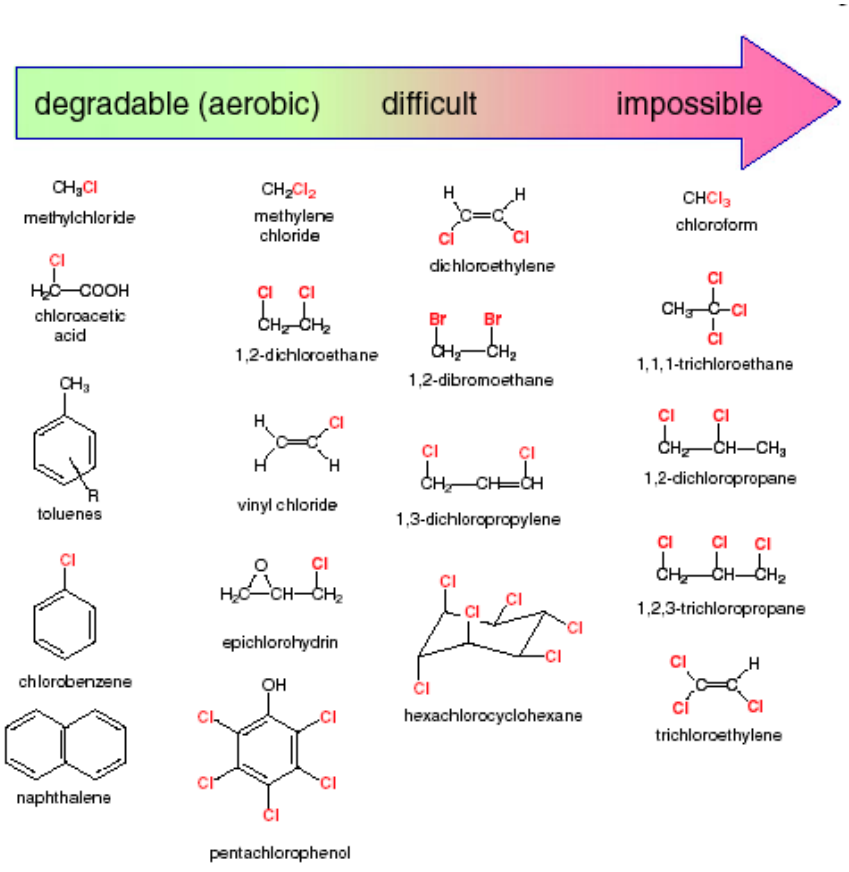


Figure 2 Degradability of chlorinated organic compounds by biological metabolism (Janssen et al., 2005)

Based on these theories and studies, triclosan could be considered as a moderately difficult compound that could be selectively degraded by bacteria after prolonged exposure.

Dehalogenation, irrespective of the mechanism (hydrolysis, oxidation or reduction) is an important step in the biodegradation pathway of a halogenated compound. Often the loss of halogen from a halogenated compound as a result of dehalogenase activity initiates a potential catabolic pathway for this compound, although the toxicity of intermediates of this pathway could also be key determinants in the maturity of this pathway, particularly for compounds carrying multiple halogen groups (Dann & Hontela, 2011, van Hylckama Vlieg et al., 2000). Chlorinated aliphatic compounds can be utilized by both aerobic bacteria and strict anaerobic bacteria as growth substrates (Altenschmidt & Fuchs, 1992, Biegert & Fuchs, 1995, Caldwell et al., 1999, Leutwein & Heider, 1999, Li et al., 2010a, Lovley & Lonergan, 1990, Pries et al., 1994). Some compounds, such as chloroacetate and 2-chloropropionate can be directly utilized by bacteria in pure cultures, while others, like di-chlorinated allyl alcohol and tri-chloro allyl alcohol were found to be involved in a co-metabolisation pathway (van der Waarde et al., 1994). Chloroacetate was found to be only co-metabolized by one *Pseudomonas* strain, which can only degrade this compound in the presence of another readily metabolisable carbon source. As comparison, one proteobacteria from r-subgroup was isolated and was successfully adapted to live on trichloroacetate as the only carbon and energy source (Laturnus et al., 2005, Yu & Welander, 1995). Interestingly, this bacterium cannot utilize monochloroacetate or dichloroacetate as the sole carbon source (Yu & Welander, 1995). Fewer data are available in the studies on degradation of chlorinated compounds by anaerobic bacteria partly due to the difficulty in culturing and studying anaerobes. An inducible enzyme was found to be responsible for transferring the methyl group of chloromethane onto tetrahydrofolate and therefore provide methyl-tetrahydrofolate as a substrate in the acetate synthesis pathway in strict anaerobes (Meßmer et al., 1996). Some reductive dehalogenases were isolated and identified in anaerobic bacteria, such as *Dehalospirillum multivorans*, and *Desulfomonile tiedjei* (Louie et al., 1997, Neumann et al., 1995).

Dehalogenases

PCBs were found to be degraded by a hybrid bioinorganic catalyst to three dichlorobiphenyls (33.9%), two monochlorobiphenyls (12%), and biphenyl (30.7%), and the same catalyst was also found to degrade polybrominated diphenyl (Harrad et al., 2007).

In bacteria, a broad range of dehalogenases, both for aliphatic and aromatic halogenated compounds, have been studied and characterised so far (de Jong & Dijkstra, 2003, Hill & Weightman, 2003, Meßmer et al., 1996, Neumann et al., 1995, Thomas et al., 1992). A methyl chloride dehalogenase was found and characterised in an early study (Meßmer et al., 1996). Five groups of aliphatic compound dehalogenases have been systematically reviewed (Janssen et al., 2005).

X-ray crystallography studies have shown that most dehalogenases not only react with a certain natural halogenated compound, but also catalyse dehalogenation reactions in both natural and synthetic compounds that share similar carbon-halogen bonds (de Jong & Dijkstra, 2003). The same study also revealed that dehalogenases always structurally belong to a protein superfamily with some other proteins that catalyse completely different reactions (de Jong & Dijkstra, 2003).

Methyl chloride dehalogenase was isolated and identified as a methyl transfer protein (Meßmer et al., 1996). A reductive dehalogenase, consisting of a single polypeptide of 57kDa, was successfully purified and characterised in *Dehalospirillum multivorans* that was able to catalyse an oxidation reaction using tetrachloroethene or trichloroethene as an electron acceptor (Neumann et al., 1995). Another inducible reductive dechlorinase was isolated from the cytoplasmic membrane of a sulphate reducing bacterium, and the protein sequence was not found to have substantial similarity to any other sequence in GenBank (Louie et al., 1997). Instead of converting 3-chloro-*cis,cis*-muconate to the bacteriotoxic protoanemonin under the activity of usual muconate cycloisomerases, chloromuconate cycloisomerases of bacteria utilizing chloroaromatic compounds were found to convert 3-chloro-*cis,cis*-muconate to *cis*-dienelactone (*cis*-4-carboxymethylenebut-2-en-4-olide). The formation of *cis*-dienelactone, in contrast, could occur either by dehydrohalogenation of 4-chloromuconolactone or, more directly, by chloride elimination from the enol/enolate intermediate (Kaulmann et al., 2001).

In summary, instead of being a group of unique enzymes sharing commonality in structure, function and activities, dehalogenases are enzymes belonging to several families of enzymes that catalyse completely different reactions. They may be encoded in the chromosome or on a plasmid or a transposon (Krajmalnik-Brown et al., 2007, Sota et al., 2002, Wyndham et al., 1994). Evidence of plasmid encoded dechlorinases was observed in the early 1990s (Johri et al., 1991) and more plasmid borne dehalogenases have been identified and characterised since then (Hill & Weightman, 2003, Wyndham et al., 1994).

Plasmid encoded group I and group II dehalogenase genes (*deh* genes) were present in primary, secondary and tertiary enrichment cultures from a polluted soil (Hill & Weightman, 2003). One of the dehalogenases *dehI* isolated from *Pseudomonas putida* PP3, capable of utilizing halogenated alkanoic acids was found to be carried on a mobile genetic element, namely DEH, located on the chromosome of strain PP3 (Thomas et al., 1992). Tn4651/Tn4653 and Tn4655 were found to be responsible for toluene and naphthalene degradation (Genka et al., 2002, Sota et al., 2006). Chlorobenzoate catabolic transposon Tn5271 and chlorobenzene catabolic transposon Tn5280 were found to encode aromatic ring dioxygenases (Nakatsu et al., 1997). Tn4371 was characterised as catabolic transposon for the catabolism of halogenated alkanoic acids and chlorobiphenyl (Kim et al., 2004, Springael et al., 2001, Toussaint et al., 2003).

1.4.4. Triclosan biodegradation and possible pathways

It is well known that aromatic hydrocarbons and their substituted derivatives must be modified into o-diphenols through different peripheral reactions before ring cleavage can take place (Galli, 1996, pp49). Aromatic ring cleavage occurs through intradiol or extradiol ring cleavage pathways and a few homologous catechol 2,3-dioxygenases in different bacteria isolates have been cloned and characterised (Arensdorf & Focht, 1994, Bartilson & Shingler, 1989, Di Gioia et al., 1998, Kitayama et al., 1996, Moon et al., 1995).

Some *Pseudomonas* strains have demonstrated the ability to degrade *meta*- and *para*-xylene through the formation of methylbenzoates as a result of oxidation or dehydrogenation (Favaro et al., 1996b, Wubbolts et al., 1994). Aromatic rings without substitute groups are generally more recalcitrant, and the introduction of one or more substituent groups onto the aromatic nucleus offers the possibility of alternative modes of microbial attack (Galli, 1996, pp49). Microbial attack on substituent group, i.e. modification and/or removal of the substituent group, may occur before or after ring cleavage, depending on the chemical structure, the position of the substituent and the selected microorganism (Smith, 1990).

Direct modification on the aromatic ring is not commonly seen; however, direct hydroxylation of the aromatic ring of methylbenzene has been demonstrated with a *Pseudomonas putida* strain and a *Nocardia corallina* strain (Galli et al., 1992, pp268). The genetic determinant and enzymology of this hydroxylation process has not been

characterised fully. Direct hydroxylation of two carbons in the *ortho*-position on styrene was also observed in a *Rhodococcus rhodochrous* strain. The 3-vinylcatechol was produced as an intermediate compound, which then undergoes a complete *meta*-cleavage pathway accompanied by the loss of acrylic acid (Galli, 1996, pp49).

Transformation of triclosan in the natural water environment involves photolytic degradation (Aranami & Readman, 2007, Ozaki et al., 2011), chlorination (Canosa et al., 2005), and methylation (Bester, 2005, Coogan et al., 2007, Leiker et al., 2009). Apart from these degradation pathways that occur naturally in the water environment, a small scale laboratory based investigation was carried out on sonochemical degradation of triclosan in various environmental samples including seawater, urban runoff and influent domeatic wastewater as well as pure and saline water (Sanchez-Prado et al., 2008). A complete removal of triclosan was achieved in 120 minutes of reaction from most samples spiked with 5 µg/L of triclosan, except the influent domestic wastewater for which only 60% of removal was achieved within 180 minutes. No chlorinated and other toxic by-products were detected under the conditions studied (Sanchez-Prado et al., 2008). A recent study revealed that triclosan reacts rapidly with commercial grade aqueous ferrate (Fe(VI)), and a mechanism for the oxidation of triclosan by the scission of ether bond and phenoxy radical addition reaction was proposed (Yang et al., 2011).

Recently, triclosan biodegradation in soil has been reported (Carr et al., 2011b, Kookana et al., 2011, Kwon & Xia, 2012, Langdon et al., 2011, Lozano et al., 2012, Svenningsen et al., 2011, Waria et al., 2011, Ying et al., 2007). In Ying's study (2007), a gradual decrease of triclosan concentration in non-sterile soil was observed from 1.01mg/kg at the beginning of the experiment to 0.08 mg/kg over 70 days of the investigation period. Interestingly, a consistently higher dehydrogenase activity was also observed in the triclosan incorporated soil compared to the triclocarban incorporated soil, which showed much poorer removal of triclocarban.

A most recent study used two biosolids treatments to examine the dissipation of triclosan, under field conditions in South Australia, but the concentration of triclosan showed no marked decrease (Langdon et al., 2012). Similarly, a study in the United States observed only small percentages of the transformed triclosan after a 101-d column study, in the form of methyl-triclosan. This product was not detectable in the leachates but was detected in the top 5 cm soil layer, with more appearing in the biosolids-applied soil, which suggested that this transformation may be related to biological activity, and either the investigated

transformation pathways were minor pathways or further rapid transformation of this product had occurred (Kwon & Xia, 2012). Similarly, TCS disappearance corresponding with MeTCS appearance was observed in a land application of biosolids to an experimental agricultural plot. Soil incorporation and degradation processes were thought to take place simultaneously and the triclosan background levels were achieved within two years. The half life of triclosan was determined to be 104 days (Lozano et al., 2012), which is consistent to the result of a similar study by Waria et al (2011). However, biodegradation in a clay loam soil was noted with a half life of only 18 days, which supported that the half-lives of triclosan under field conditions are expected to be very different (Kookana et al., 2011).

Aerobic triclosan biodegradation was also discussed in a study on the triclosan removal during the wastewater treatment processes (McAvoy et al., 2002). Much higher triclosan contents in the anaerobically digested sludge than that in aerobic sludge indicated a much more efficient triclosan biodegradation in aerobic conditions.

A wastewater consortium was found to be able to degrade triclosan (Hay et al., 2001). Two bacterial strains *Pseudomonas putida* and *Alcaligenes xylosoxdans* subsp. *denitrificans* were isolated from soil and they were demonstrated to inactivate triclosan in liquid and on solid substrates and to use triclosan as a sole carbon source (Meade et al., 2001). Biotransformation of triclosan by fungi is also reported. The fungus *Pycnoporus cinnabarinus* was found to methylate the hydroxyl group of triclosan during cultivation, and the fungus *Trametes versicolor* metabolized triclosan and produced three metabolites, namely 2-O-(2,4,4'-tichlorodiphenyl ether)-β-D-xylopyranoside, 2-O-(2,4,4'-trichlorodiphenyl ether)-β-D-glucopyranoside, and 2,4-dichlorophenol (Hundt et al., 2000).

A recent study has evaluated the susceptibility and biodegradation potential of triclosan by three *Sphingomonas* strains that were able to degrade aromatic pollutants with structural similarities to triclosan, and has found one strain PH-07 was able to catabolise triclosan to intermediates that included hydroxylated compounds and the ether bond cleavage products (Kim et al., 2011). A most recent study, published after this thesis was submitted, reported a triclosan-degrading wastewater bacterial isolate, *Sphingopyxis* strain KCY1, which was unable to grow on media with triclosan as a sole carbon source, however, was found to retain its degradation ability toward triclosan when grown with complex nutrient medium containing a trace amount of triclosan as low as 5 µg/L (Lee et al., 2012). This study also identified five metabolites and detected the activity of catechol 2,3-dioxygenase in the cell

extract. Inhibition of 3-fluorocatechol was observed and a triclosan degradation *meta*-cleavage pathway was proposed.

In conclusion, aerobic triclosan degradation may occur both spontaneously in the natural water environment as well as in the human monitored wastewater treatment processes. The biodegradation pathway involved would vary in different strains, where different metabolism by-products may be produced. There is clearly a lack of information concerning the catabolism of triclosan by pure bacterial cultures, as only two bacterial strains were demonstrated recently to be triclosan degraders (Kim et al., 2011, Lee et al., 2012). It is worth noting that both these strains were unable to utilize triclosan as sole carbon source, but retain the degradation ability through co-metabolism. There are no reports so far on the mechanisms of triclosan biodegradation at a genetic molecular level.

1.5. This study

In order to minimise the impact of triclosan on the selection of bacterial biocidal resistance as well as the potential bacterial cross resistance to antibiotics currently in use, it is important to reduce the overall triclosan content and minimise the selective pressure present in the natural environment. Due to the high concentration of triclosan detected recently in wastewater streams, it is logical to seek WWTP performance improvement on triclosan removal efficiency.

Biodegradation of organic compounds is the predominant mechanisms seen in the wastewater treatment processes for most organic compounds removal. This study is aimed at exploring the potential of triclosan biodegradation by the indigenous bacteria present in the wastewater environment. If the bacterial triclosan biodegradation occurs naturally or can be induced in the bacteria population under long term exposure of this compound, this naturally occurring or adapted triclosan degradation activity of indigenous bacteria in the activated sludge may be enhanced by process optimisation during conventional wastewater treatment, thus improving the triclosan removal efficiency of the wastewater treatment processes. Activated sludge containing active triclosan degradative bacteria may also be inoculated into other wastewater treatment systems to enhance triclosan removal efficiency of these systems.

Understanding the mechanisms involved in bacterial triclosan biodegradation would

provide critical information as the basis for process enhancement. Investigation into the genetic determinants of this biodegradation activity at a genetic molecular level would help provide information for the potential construction of engineering bacterial strains capable of degrading triclosan at a higher efficiency. In combination with the outcome of molecular microbiology study, chemical analysis on biodegradation intermediate or by product would help characterise the triclosan biodegradation pathway.

The following studies were proposed in order to approach the above goals:

- Isolation and identification of putative triclosan degraders from the wastewater environment;
- Chemical analysis to identify potential biodegradation by-products or intermediates;
- Genetic study to identify triclosan degradation related genes;

Chapter 2: General materials and methods

2.1 Bacterial strains

Putative triclosan resistant isolates and degraders were isolated from Bolivar sewage water. A list of strains, isolation methods and identification are described in Chapter 3 and Chapter 4 of this thesis. *E. coli* donor or candidate donor strains used in transposon mutagenesis study (details in Chapter 4) were accessed from a collection at the microbiology laboratory, Australian Water Quality Centre, Bolivar.

Wild type triclosan degradative strain *Pseudomonas citronellolis* F12, containing a triclosan degradation related plasmid, was isolated from Bolivar sewage water. Isolation method and identification of this strain are described in Chapter 3. Derivatives of this strain C2 and C6, without plasmid, were obtained by temperature curing of F12. Wild type triclosan resistant isolate, non-degrader, *Pseudomonas aeruginosa* 10R2A06 was isolated from Bolivar sewage water. Isolation and identification of this strain are described in Chapter 3. Its derivative strain *Pseudomonas aeruginosa* RT10R2A06 was obtained by electroporation of this strain with wild type plasmid pF12, which was isolated from *Pseudomonas citronellolis* F12.

Laboratory *E. coli* DH5α strain used in electroporation, cloning and sequencing experiments was accessed from microbiology laboratory, Institute of Medical and Veterinary Science, Adelaide.

All bacterial isolates were stored on inverted agar plates at 4°C for short term storage or at -70˚C using freezing media for maintenance of all bacterial stocks.

2.2 Chemicals and reagents

The following analytical grade chemicals were used. Triclosan (≥97.0%, HPLC) was supplied from Fluka/Sigma-Aldrich, Castle Hill, Australia. Triclosan stock of 100 mg/mL was made by dissolving in 0.1M KOH. A diluted stock solution of 1 mg/mL was made by diluting the 100 mg/mL stock in MilliQ® water. Triclosan was added into media from the 1 mg/mL solution. Triclosan stocks were kept in glass vials, wrapped in foil and stored at 4 ˚C for a maximum of 1 week. Potassium phosphates K_2HPO_4 and KH_2PO_4 were accessed from microbiology laboratory, University of South Australia, originally purchased from Ajax Chemicals, NSW, Australia. Other inorganic chemicals were supplied from BDH

Chemicals (Victoria, Australia) or Sigma-Aldrich (Australia).

All reagents used for PCR were supplied from Applied Biosystems. DNA markers and primers were purchased from Geneworks, SA, Australia.

Antibiotics were accessed from microbiology laboratory, Australian Water Quality Centre or University of South Australia, and were originally supplied from NBC or Sigma-Aldrich. Ampicillin sodium salt (Sigma-Aldrich) was dissolved in milliQ® water at a concentration of 100mg/ml. Tetracycline hydrochloride (Sigma-Aldrich) was dissolved in ethanol at a concentration of 1mg/ml. Kanamycin sulphate (NBC) was dissolved in milliQ® water at a concentration of 2mg/ml. Rifampicin (Sigma) was dissolved in 0.1mM HCl at a concentration of 10mg/ml. Streptomycin sulphate (Sigma) was dissolved in milliQ® water at a concentration of 10mg/ml. All antibiotic stock solutions were filter sterilized through a 0.22µm polyethersulfone membrane (Millipore Express®) and stored in Eppendorf® tubes, wrapped in foil, at -20 ˚C for future use.

2.3 Culture medium and conditions

2.3.1 Liquid media

The following liquid media were used in this study for bacterial cultivation. Nutrient broth (Oxoid) was used for the enrichment of wild type wastewater isolates. Luria-Bertani broth, containing 1% (w/v) tryptone (Oxoid), 0.5% (w/v) yeast extract (Oxoid), 1% (w/v) sodium chloride was used for the enrichment of *E. coli* and wild type isolates. Mueller-Hinton III broth (Oxoid) was used for the cultivation of *Pseudomonas* isolates in plasmid extraction experiments. Psi broth, containing 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) magnesium sulphate, was used for preparation of chemical competent cells. Bushnell-Haas broth, containing 0.1% (w/v) NH_4NO_3, 0.1% (w/v) K_2HPO_4, 0.1% (w/v) KH_2PO_4, 0.01% (w/v) $MgSO_4 \cdot 7H_2O$, 0.01% (w/v) $FeCl_3 \cdot 6H_2O$, 0.001% (w/v) $CaCl_2 \cdot 2H_2O$ was used in triclosan degradation experiments, and 0.2% (w/v) triclosan was added, where required, as the sole carbon source in the triclosan incorporated Bushnell-Haas broth. Triclosan minimal media, made from three stock solutions (as shown in Appendix 1) containing various mineral salts, was also used for triclosan degradation experiments. SOC medium, containing 20g Bacto® Tryptone, 5g Bacto® Yeast Extract, 2mL of 5M NaCl, 2.5mL of 1M KCl, 10mL of 1M $MgCl_2$, 10mL of 1M $MgSO_4$, 20mL of 1M glucose in 1L, was used for the recovery of electroporated cells in the electroporation experiments.

All powdered broth media were dissolved in Milli Q water before pH was adjusted to 7.5

using 0.1M KOH solution. Medium was sterilized by autoclaving at 120 ˚C for 15 minutes before use.

Microbank® Bacterial and Fungal Preservation System or glycerol broth (LB broth with 30% glycerol) was used for the long term storage of bacterial cells.

2.3.2 Solid media

R2A agar (Oxoid) was used for the cultivation of wastewater isolates. Powdered TSA (Oxoid) was used in the enrichment of wastewater isolates. Agar bacterial No1 (Oxoid) at concentration of 1.2%, were added in in nutrient broth (Oxoid), LB broth, triclosan minimal medium to make agar plates. Agar was melted before being sterilized by autoclaving at 120 ˚C for 15 minutes.

X-gal/IPTG agar plates were used in cloning experiments for blue-white screening. Method of making these plates is shown in Appendix 1.

2.3.3 Culture conditions

All plates were inverted and incubated aerobically at 35˚C overnight and all broth cultures were incubated at 35˚C overnight with shaking at 100 rpm, unless otherwise indicated.

2.4 Bacterial cell counting

2.4.1 Plate counting

Spread plate bacterial cell counting was performed in duplicate on pre-poured agar plates. Ten-fold dilutions of each sample, when necessary, were prepared in sterile 0.85% saline, and 100µl of sample or dilution (in duplicate or triplicate) were spread over the surface of agar plate using a sterile spreader. Plates were then incubated aerobically at 35°C for 24h or 48h when applicable. After incubation, colonies were counted manually and the mean of duplicates was recorded.

2.4.2 Flow Cytometry

Flow Cytometry assays were performed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an air-cooled 15 mW argon ion laser,

emitting at a fixed wavelength of 488nm (Hoefel, 2004, pp 26-103). Detector voltages and threshold settings were adjusted for each individual sample based on blanks (unstained samples) and negative controls (inactivated samples stained with vital dyes, described below) (Hoefel, 2004, pp 26-103). A low flow setting (12±3 µl/min) of the sheath fluid consisting of Milli-Q water (Millipore Ultra-Pure Water System) was used for this study.

Cells were stained using the LIVE/DEAD® Baclight™ Bacterial Viability Kit (Molecular Probes Inc, Eugene, OR, USA). Equal volumes of SYTO-9 (3.34mM in DMSO) and PI (20mM in DMSO) were combined and vortexed prior to use. One µL of this stain mixture was added to 1 mL of sample and incubated in the dark for 15 minutes at the room temperature.

One mL of pre-stained samples in D75 mm polystyrene tubes was weighed out and then loaded onto the sample injection port soon after the data acquisition started. Data acquisition was started before loading the sample and stopped after the sample was removed from the sample injection port so that there was no loss of sample while data acquisition was not being performed. The whole data acquisition process was performed over a minimum of a 2 minute period depending on the concentration of samples so as to ensure enough events were detected. Data were recorded and analysed using the FACStation Data Management System consisting of a Power Macintosh® G3 computer with CellQuest™ software (Becton Dickinson) (Hoefel, 2004, pp 26-103). After data acquisition, suspensions were then weighed out and the mass loss attributed to the volume of sample analysed. The count of bacteria was the number of bacteria detected divided by the sample volume analysed (Hoefel, 2004, pp 26-103).

Instrument preparation, before and after use, was performed according to the instruction from the manufacturer's manual.

2.5 Bacterial identification

2.5.1 Microbact ™ GNB24E test strips

Gram-negative enterobacteria identification was performed, when necessary, using a GNB 24E test strips (Microbact™, USA). Sample preparation, incubation conditions and result reading were carried out according to manufacturer's manual. Data were recorded and analysed using Microbact software.

2.5.2 Api 20NE test strips

Miscellaneous Gram-negative isolates from wastewater were initially identified using *Api* 20NE identification kit (bioMérieux SA, France). Sample preparation, incubation and result reading were carried out according to manufacturer's manual. Data were recorded and analysed using online *Api* 20NE data analysis system apiweb®.

2.5.3 16S rDNA sequencing

16S rDNA sequencing was used to identify the origin of derivative strains in molecular studies. DNA was extracted as described below and nested-PCR was performed to obtain sequencing template. Samples were then visualised on 1% agarose gel and purified using the commercial QIAquick® PCR purification kit (QIAGEN Pty Ltd, Victoria, Australia) to remove contaminating RNA where necessary (based on the gel picture). Purified DNA samples were then prepared according to Australian Genome Research Facility (AGRF) instructions for preparation of purified DNA for sequencing. DNA concentrations were assessed using a spectrophotometer; the amount of each primer was then calculated and added into samples according to the instructions. After analysis by AGRF, data were downloaded from the AGRF website and analysed using DNASTAR Lasergene 7 package.

2.6 PCR and gel electrophoresis

2.6.1 PCR

For 16S rDNA sequencing and Denaturing Gradient Gel Electroporation (DGGE), nearly the full-length of 16S rDNA of the target bacteria was amplified by PCR using universal primers (Muyzer et al., 1993) 27f (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1942r (5'-TAC GH TAC CTT GTT ACG ACT T-3') under the following conditions: initial denaturation at 95 ºC for 5 min followed by 10 cycles of denaturation at 95ºC for 45s, annealing at 65 ºC for 15s, and extension at 70 ºC for 60s. For touchdown PCR, the annealing temperature was decreased by 1ºC each cycle from 55ºC to 45ºC for the first 10 cycles, followed by 20 cycles of annealing at 45 ºC. A 3 min extension at 70 ºC was included after completion of the 30 cycles of touchdown amplification.

In each 50 µl PCR reaction, containing 2.5mM $MaCl_2$, 1 × PCR buffer, 1 µM of forward and reverse primers, 200 µM of each dNTPs and 5% of DMSO, 5µl of appropriately diluted DNA sample was used, and 0.5 U of Ampli *Taq* Gold DNA polymerase (Applied Biosystems, USA) was added immediately before the thermal cycle.

Purified DNA of three identified bacterial isolates was used as positive control and nuclease free water was used instead of a DNA sample as negative control for each PCR reaction.

2.6.2 Nested PCR for Denaturing Gradient Gel Electrophoresis (DGGE)

Nested-PCR, using the same reaction mix with the first round PCR, was used to amplify a small section of 16S rDNA of bacterial mixture with the eubacterium-specific primers 341Muyzer F-GC (5'-CCT ACG GGA GGC AGC AG-3') and Muyzer R (5'-ATT ACC GCG GCT GCT GG-3')(Muyzer et al., 1993) under the following conditions: 10 min of initial denaturation at 95ºC followed by 4 cycles of denaturation at 94ºC for 1 min, annealing at 65ºC for 1 min, and extension at 72ºC for 2 min. For touchdown PCR, the annealing temperature was decreased by 2ºC each 4 cycles from 63ºC to 57ºC for the first 16 cycles, followed by 13 cycles of annealing at 55ºC. Two periods of 10 min extension at 72ºC were included after completion of the 29 cycles of the touchdown amplifications.

2.6.3 Agarose gel electrophoresis

Agarose gel (0.5-1%) was used in electrophoresis. TAE buffer (40mM Tris-base, 0.12% [v/v] acetic acid (glacial), 1.0 mM EDTA, pH 8.0) was used as running buffer. Running voltage ranging from 60-100 mV for 0.5-4 hours was used for electrophoresis. The gel was stained by 0.5µg/mL Ethidium Bromide and visualized by Quantity One® Gel Doc photo systems (Bio-Rad) under UV light.

2.6.4 Denaturing gradient gel electrophoresis

Nested-PCR product was cleaned up to remove primer dimers and residual reactants using a Montage® PCR centrifugal filter device (Millipore) following the manufacturer's instructions. DGGE was used to analyse the community composition of the wastewater consortium exposed to triclosan by the D GENE^{TM} Denaturing Gel Electrophoresis System (Bio-Rad). A polyacrylamide (8.0%) gel of 16 by 10 cm was run with TAE buffer. A denaturation gradient from 40% (6% [wt/vol] acrylamide-bisacrylamide [37.5:1], 8% formamide, 1.4 M urea, 2% glycerol) to 70% was selected for this community analysis. Electrophoresis was carried out at 60V at 56ºC for 16 hours. The gel was then stained with SYBR Green I (Applied Biosystems) for at least 2 hours. DNA was visualized with an Electrophoresis Documentation and Analysis System using Kodak Image Analysis Software (Eastman Kodak, NY, USA).

2.7 Bacterial conjugation

Overnight cultures of a donor and a recipient were diluted 1:100 in culture media without antibiotic and grown at 35°C to an optical density at 600 nm (OD_{600}) of 0.3 (Le Roux et al., 2007). Filter mating procedures (Biskri et al., 2005, Le Roux et al., 2007) were used for bacterial conjugation between a wild type triclosan degrading bacterium and host *E coli.* carrying *Tn5* transposon and pNJ5000 as conjugative plasmid. Transconjugants were selected on LB agar plate containing 5µg/mL of kanamycin, and then checked and maintained on LB agar plate containing 10µg/mL of tetracycline.

2.8 DNA extraction

2.8.1 Chromosomal DNA extraction

Chromosomal DNA extraction from a single colony

A single colony from a fresh overnight culture was resuspended into 100µl InstaGene® Matrix (Bio-Rad, USA), and DNA was extracted according to the manufacturer's instructions.

Chromosomal DNA extraction from liquid culture

Samples (0.5ml) were taken from the wastewater consortium flask incorporated with triclosan at monthly intervals. DNA was extracted using a boiling method. Samples were heated at 100 ºC for 15 min, and cooled to 56 ºC for 10min, with this cycle repeated for one more time. Mixture was then centrifuged on a bench top spinner for 1 min. Supernatant DNA was removed into a fresh Eppendorf tube and stored under -20 ºC. A one in ten dilution was made when necessary.

2.8.2 Plasmid DNA extraction

Generally, alkaline lysis-phenol extraction methods are widely used in preparation of plasmid DNA. With some variations in materials, they all have three main steps: lysis of bacterial cells, selective release of plasmid DNA from cell matrices and removal of contaminants to recover plasmid DNA (Adachi & Iyer, 1980, Hogrefe & Friedrich, 1984, Singh & Banerjee, 2006). However, this method requires the use of highly alkaline SDS solution in order to minimise the presence of chromosomal DNA, but this is not always

successful in removing all the chromosomal DNA (Cannon & Dunican, 1970, Mukhopadhyay & Mandal, 1983).

Qiagen QIAprep Spin Miniprep Kit is regarded by users as one of the best commercially available plasmid preparation kits compared to similar products from other manufacturers. However this kit has limitations when applied to a low copy number plasmids or plasmids with size over 10kb (QIAGEN users' guide). The QIAGEN 15mL prep kit has a similar limitation.

INSTA-MINI-PREP™ is a protocol for direct extraction of plasmid DNA, using a two-phase solution consisting of phenol-chloroform-isoamyl alcohol and buffer. The separation of the two phases is achieved by centrifugation in the presence of the INSTA-PREP gel barrier material. This method is straight forward, and is considerably faster than the standard mini-prep procedures, however it is not suitable for extraction of plasmid DNA from bacteria such as *Ps. aeruginosa* that synthesize large quantities of EPS (Ferrus, 1999).

The QIAGEN Large-construct kit was improved by using a patented resin to protect the plasmid from cell lysis and according to the manufacturer's information sheet; this resin has made it easier to elute the large plasmid from the resin than membrane filters used in most other kits. The use of this improved kit resulted in a better yield for low copy number large plasmid DNA. Another advantage of this method compared to others mentioned above is that the shearing of plasmid is minimised by using a large volume of solutions in large containers. Many processing steps in the extraction, purification, and precipitation of plasmid can impart significant shear stress that can fracture the phosphodiester backbone of the polynucleotides, and result in poor quality plasmid preparation (Lengsfeld & Anchordoquy, 2002). The disadvantages of this large construct kit are that it consumes more time to finish one preparation and requires the use of a high-speed centrifuge instead of a bench-top microcentrifuge commonly available in biology laboratories.

Using the Mini prep kit

Plasmids with size smaller than 10kb were extracted using QIAGEN mini prep kit (QIAGEN, Australia) according to the manufacture's instructions. Ten mL of overnight LB culture was used for each column. DNA was eluted in 50µl of pre-warmed (55°C) elution buffer.

Using the QIAGIN® large construct kit

Plasmids of size greater than 10kb were extracted using QIAGEN large construct kit (QIAGEN, Australia) following the manufacturer's instructions. Muller Hinton III broth was used instead of LB broth for the culture medium. A total volume of 500mL, as recommended maximum volume, overnight culture was used for each preparation. DNA was eluted in 3mL Tris-HCl 8.0.

2.9 Bacterial transformation

Bacterial transformation techniques, including chemical transformation and electroporation, were used at different stages of the experiments in this study. Recipient microorganisms in these experiments included *E coli* and various pseudomonad strains. The methods used for different strains or for different purposes were slightly modified to optimise transformation efficiency. The final optimised methods are summarised hereafter.

2.9.1 Chemical transformation

E. coli

Chemical transformation of plasmid DNA or ligation reaction into *E. coli* DH5α cells was carried out according to the following procedures based on an online protocol (http://stanxterm.aecom.yu.edu/wiki/index.php?page=Competent_cells):

- A single colony of the target *E. coli* strain from an overnight culture in LB broth was inoculated into 2 mL of Psi broth and incubated overnight at 37°C with shaking.
- A volume of 330 µl of the above overnight culture was subcultured into 10mL of fresh Psi broth and incubated with vigorous shaking for 1.5-3h at 37°C until OD_{600}=0.6.
- A 5mL aliquot of the above culture was subcultured again into 100mL of Psi broth (pre-warmed to 37°C) and incubated with vigorous shaking for 1.5h at 37°C until optimal OD_{600}=0.6.
- The above culture was placed on ice for 5 min and centrifuged in a Sorvall HB-4 rotor at 2520 ×g for 5 min at 4 ˚C.
- The pellet was gently resuspended in 40mL of ice cold transformation broth 1

(TFB1 as described in Appendix 1) and incubated on ice for another 5 min.

- All disposables used, including pippete tips, tubes and flasks etc. were ice-chilled from this point on.
- The above suspension was centrifuged again in a Sorvall HB-4 rotor at 2520 ×g for 5 min at 4 ˚C.
- The pellet was resuspended in 4mL of ice cold transformation broth 2 (TFB2 as described in Appendix 1) and incubated on ice for 16-60 min.
- The above suspension was dispensed as 200µL aliquots into top-labelled 0.2mL pre-chilled Eppendorf tubes on ice and was snap frozen in a dry ice-ethanol bath for 30 sec.
- The prepared competent cells were stored at -70 ˚C.
- To transform, an aliquot of competent cells was thawed on ice, and 1 ng of circular plasmid DNA was added into 25µL cells for plasmid transformation;
- Or a 5-10 µL (depending on the concentration for each reaction) of ligation reaction (as described in Chapter 5) was added into 100µl prepared competent cells;
- The mixture was then incubated for 1 h on ice, heat shocked for 45 seconds at 37°C, incubated on ice for further 2 min, diluted 15-fold into SOC broth with no antibiotics;
- For phenotypic expression, the mixture was incubated with vigorous shaking at 37°C for 20 min before being plated on selective medium.

Pseudomonas strains

Chemically competent *Pseudomonas* strains were prepared using the same procedure as described above. However, *Pseudomonas* isolates need to be cultured in MH broth and competent cells prepared freshly each time for efficient transformation. The incubation time of 40 min was used for phenotypic expression for *Pseudomonas* strains after transformation.

2.9.2 Electroporation

The transfer of large plasmid into *Pseudomonas* strains, including *Pseudomonas aeruginos*a, *Pseudomonas fluorescens* and *Pseudomonas citronellolis*, was undertaken using electroporation.

Electro-competent pseudomonas strains was prepared according to Choi's method (Choi et

al., 2006). Electro-competent *E. coli* strains were prepared according to Dower's method (Dower et al., 1988).

Appropriate amount, 100 µl for *Pseudomonas* or 70 µL for *E. coli*, of ice-thawed electro-competent cells were added to the bottom of a chilled sterile 0.2 cm electroporation cuvette (Bio Rad, Australia). Ten µl of purified plasmid was added and immediately mixed by gentle tapping. Electroporation was then carried out immediately on the Bio Rad Gene Pulser with capacitance extender (Model 1652078). Settings used in electroporation in this study were 25 µFD capacitance, 200Ω resistance, and 2.5 kV pulse strength. One mL of SOC medium was added promptly after electric shock into the cuvette to suspend cells from narrow bottom of cuvette to allow transfer using sterile glass pipette, and then transferred into a sterile universal container (Disposable Products Co., SA Australia) and incubated at 35°C for 1-3 hours to allow plasmid expression.

2.9.3 Selection of transformants

Transformants from chemical transformation or electroporation were both selected on selective plates containing appropriate antibiotics or triclosan. Plates were then incubated at 35°C for 24 hours or until colonies appeared.

2.10 Restriction digestion

Restriction enzymes at a concentration of 10-12 unit/µL from Promega Corporation® were used in restriction digest reactions. Total volumes of 20µL restriction digest reaction, consisting of 7.3 µL of nuclease free water, 0.2 µL of acetylated BSA (10 µg/ µL), and 10 µl of purified plasmid DNA (at concentration of approximately 100 ng/µL) was assembled in order in a sterile 1.5 mL Eppendorf tube. The mixture was then mixed by flicking before 0.5 µL of restriction enzyme (10 unit/ µL) was added.

The 20 µl mixture was gently mixed by flicking and then spun in a bench centrifuge briefly for 3 seconds to bring down all the liquid to the bottom of tube. Reactions were then incubated at the optimum temperature (for individual enzyme) overnight for digestion. After digestion, four µL of 6× loading buffer was added before proceeding to gel electrophoresis.

2.11 Plasmid fragments cloning

After restriction enzyme digestion, the following ligation reaction was assembled in a sterile Eppendorf tube:

2× Rapid Ligation Buffer	5 µL
pGEM®-5Zf(+) or pWKS130 vector	0.1 µL
RE reaction	2 µL
T4 DNA Ligase	1 µL
Nuclease Free Water	1.9 µL
Final volume	10 µL

The content in the tube was mixed by gentle flicking and was incubated at room temperature for 5 minutes before proceeding for transformation.

Cells transformed with ligation reactions were plated on X-Gal/IPTG agar plate (as described in Appendix 1) containing 100 µg/mL of ampicillin. Plates were incubated at 37˚C overnight. White colonies appeared were selected and potential transformants were purified on LB+Amp100 agar plates.

2.12 DNA sequencing of Plasmid inserts

Cloned inserts were sequenced at the sequencing facility, Flinders Medical Centre using BigDye Terminator 3 sequencing kit (Applied Biosystems, Australia). Data were analysed using DNASTAR Lasergene 7 package (DNASTAR, USA). Primers used in the primer walking method to obtain the complete sequence of the DNA molecule (pF12) were designed using Seqman in this package and were synthesised at Geneworks™, Adelaide SA, Australia. A list of primers used in primer walking is included in Section 6.2.6.

2.13 Triclosan detection and quantification using HPLC-UV

Triclosan in aqueous phase was detected using reversed phase HPLC-UV.

The separation of triclosan was performed using an Alltech C_8 analytical column at flow rate of 1 ml/min using a binary gradient eluant composed of 20 mM NH_4Ac and 100% ACN at a gradient of 40% ACN to 100% ACN in 30 min. The detection was carried out using a Diode Array Detector (DAD), which detects UV absorbance of a sample at multi-wavelength ranging from 200 nm to 300 nm. Absorbance spectra were scanned in full scan mode. A UV spectrum at a wavelength of 210nm was used initially to establish

the detection of triclosan in pure aquous sample, and a UV absorbance at a wavelength of 270 nm was used for the quantification.

Solute identification was based on the retention time of triclosan and by comparison between the UV spectrum of the standard in the library and the UV spectrum of the detected peak in the sample. A match equal was used to confirm identification between both spectra for triclosan.

Quantification was calibrated using external standard method. Calibration curve standards were constructed by dissolving triclosan (Fluka/Sigma-Aldrich, Castle Hill, Australia) in methanol and diluted to a series of standards at concentrations of 0.05, 0.10, 0.50 and 1.00 µg/mL using acetonitrile (ACN). Standards were analysed using a fully automated Varian on-line HPLC system (Varian, Australia). Data analysis was carried out automatically using the Varian Star Workstation Software (Varian, Australia). Recovery and accuracy of the method was assessed.

2.14 Triclosan degradation intermediate analysis

Intermediate analysis was carried out using HPLC-ESI-MS and GC-MS.

2.14.1 HPLC-ESI-MS

An Agilent 1100 Liquid Chromatograph coupled with an atmospheric pressure (AP)-electrospray ionization (ESI) mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) was used to detect putative triclosan biodegradation by-products.

The separation process was performed on a C_8 column (Agilent). The samples, diluted in 80% methanol, were injected using an 1100 auto-sampler at an injection volume of 10 µL. The mobile phase flow-rate was 0.8 mL/min and was operated at 25 °C. The instrument was operated in the negative ionization mode. The operating conditions for ESI were nebulizer gas (nitrogen) 40 psi; drying gas (nitrogen) flow rate 12 L/min; capillary voltage 3500 V and gas temperature 350 ^{0}C. The fragmentor voltage was set at 70V. Data were collected using full scan. The system was controlled and the data were processed using Agilent's Chemstation software package (Agilent Technologies, Australia).

2.14.2 GC-MS

Triclosan degradation intermediate analysis was also undertaken using gas

chromatography-mass spectrometry (GC-MS) coupled with a purge-and-trap (P&T) system for the extraction, separation and identification of analytes from bacterial culture samples. Organic compounds were extracted using hexane and dichloromethane (DCM) for comparison, and DCM was chosen as the more efficient extraction solvent.

Tekmar 3100 Purge and Trap system (Teledyne Tekmar, USA) with a Vocarb-3000 trap and a 5 mL frit sparger was used. An Aquatek 70 Liquid Autosampler (Teledyne Tekmar, USA) was programmed to automatically dispense 5 mL sample aliquots into a 15 mL purging device. The sample was purged with a stream of Helium gas at 40 mL/min for 12 minutes at ambient temperature. After sample loading, the trapped sample components were desorbed by heating the Vocarb-3000 cartridges at 250 °C and passing the Helium gas at 3 mL/min for 2 min. The sample then underwent the baking procedure under 270 °C for 4 minutes.

HP 6890 gas chromatograph (Agilent Technologies, USA) equipped with an HP 5973 Mass-selective Detector (Agilent Technologies, USA) was used for the separation and detection of triclosan degradation intermediates. A DB-1ms, 0.25mm*30m*0.25µm fused-silica capillary column (Agilent 122-0132) was used. Helium was used as carrier gas at 54ml/min. The oven was set at 55 °C (for 1 min) initially and was raised to 170 °C (for 1min) at 12°C/min and then raised again to 300 °C at 16°C/min. The final temperature was maintained for 10 min and the total run time was 29.71 min. Electron ionization was carried out at 70 eV, the source temperature and GC interface temperature were set at 200 and 250 °C, respectively. The MS system was operated in the full scan mode, m/z 50–550. The identification of interest peaks were carried out by searching in the MS library database. Best match was used for identification.

Chapter 3 Selection & identification of triclosan degraders from a wastewater environment

3.1 Introduction

The occurrence of triclosan in water and wastewater resources has been reported worldwide. A key concern arising from the wide presence of triclosan in the water environment is the potential for selection of bacterial resistance to this bis-phenol as well as to some antibiotics currently used in clinical settings (Randall et al., 2004).

The removal of triclosan from the water environment is thus becoming an important task for water researchers and engineers, and has been under extensive investigation recently. The major way to remove triclosan from the water system is through wastewater treatment processes, where triclosan is removed by biological processes together with other organic contaminants in the water.

Triclosan can be effectively removed from wastewater in most secondary biological treatment processes, where a removal rate of greater than 95% can be achieved. Despite the high efficiency of the wastewater treatment processes, the concentration in the effluent of Wastewater Treatment Plants (WWTPs) is still far above the PNEC (Predicted Non Effective Concentration). Even after taking into account the natural dilution effect by the water body, the amount of triclosan in the water environment is still high enough to arouse concerns among environmental scientists, due to the toxicological effect of triclosan itself as well as the transformed triclosan derivatives, such as dioxin.

Therefore, it is necessary to develop some wastewater treatment processes efficient in removing triclosan or enhance the triclosan removal of the existing treatment processes simply by modification of the operational conditions. This identifies the need for a study on the mechanisms of triclosan removal in the secondary biological treatment processes.

The major mechanisms involved in triclosan removal in the wastewater treatment processes include physical sorption and biodegradation. The proportion removed through each of these mechanisms varies according to the process and the operating conditions. Since biodegradation is the key to complete triclosan removal, the focus of this study was on the mechanisms of triclosan biodegradation.

A close to complete removal relies mostly on the success and complete biodegradation process, to which the establishment of the activated sludge and the hydraulic retention time

are two essential factors. Longer hydraulic retention time allows more biodegradation to occur, and therefore achieve better removal rate. Studies have shown that a much higher removal rate is always achieved in processes under aerobic conditions than those under anaerobic condition (Carr et al., 2011a, Gangadharan Puthiya Veetil et al., 2012). To sum up, the above observations indicate that triclosan biodegradation happens mostly in the aerobic bacterial community resident in the activated sludge, and the biodegradation process is always relatively slow.

In this part of the study, putative triclosan degraders were isolated from the wastewater environment by exposing a wastewater consortium of microorganisms to triclosan. Bacterial community change during this exposure was monitored using Denaturing Gradient Gel Electrophoresis (DGGE) analysis. Putative degraders were isolated and purified. Growth of purified culture in triclosan minimal medium was monitored by Flow CytoMetry (FCM) and triclosan removal was assessed by triclosan quantification by HPLC-UV in parallel with the increase in bacteria numbers monitored by quantitative FCM assay. These putative degraders were then further identified using the Api 20NE Gram-negative bacteria identification system. The ID of some interesting strains was further confirmed by 16S rDNA sequencing and three *Pseudomonas* spp. strains were then chosen for the further genetic studies.

3.2 Methods

3.2.1. Bacteriological media

Isolation of triclosan resistant strains was carried out in R2A medium. Triclosan (Fluka), at concentrations of 0, 0.05, 0.1, 0.5, 1, 2 and 10 mg/L, respectively, was incorporated into this agar. Single colonies were subcultured on TSA agar plates containing 20 or 100 mg/L of triclosan. Higher concentration of triclosan was used to test the resistance level and also to ensure triclosan resistance was maintained in these isolates.

High concentration of up to 2g/L triclosan was also incorporated into the R2A plates which resulted in turbid plates for use in the agar plate clearance test.

Induction/adaptation of triclosan degradation capacity was done in Bushnell-Haas (BH) Broth (Atlas, 1993). Triclosan, at a concentration of 2g/L, was incorporated into this medium. Commercial Tryptone Soy Broth (TSB, Oxoid) was used as enrichment medium for subculture.

3.2.2. Selection of triclosan degraders from the wastewater environment

Primary sewage from the Bolivar Wastewater Treatment Plant (Bolivar, SA) was used as a source of inoculum for isolation of triclosan resistant strains. Wastewater was serially diluted in 0.9% Phosphate Buffered Saline (PBS) and plated out on R2A agar plates containing triclosan at concentrations of 0, 0.05, 0.1, 0.5, 1, 2 and 10 mg/L, respectively. Colonies arising from R2A agar plates containing triclosan at concentrations of 2 and 10 mg/L were further purified and enriched on TSA agar plates containing 20 and 100 mg/L of triclosan. Purified isolates were stored on inverted agar plates at 4 ˚C for short term or at -80 ˚C using the Microbank® Bacterial and Fungal Preservation System for long term storage.

3.2.3. Adaptation of triclosan resistant bacteria

To assess the adaptation of the bacteria to the growth on triclosan, a single colony of each isolate was inoculated into TSB broth and incubated at 35ºC overnight. Cells were then washed twice with BH broth, and the cell pellets were resuspended into 3 mL of BH broth. One mL of the cell suspension was then inoculated into triclosan-enriched BH broth with added trace elements ($CaSO_4$ 2mg/L, $FeSO_4 \cdot 7H_2O$ 2mg/L, H_3BO_3 50µg/L, $MnCl_2$ 0.2 mg/L, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 1mg/L, $CuSO_4 \cdot 5H_2O$ 0.2mg/L, $CoCl_2 \cdot 6H_2O$ 0.1mg/L) in sterile 35mL clear flat bottom glass vials. Control vials inoculated with autoclaved cell suspensions in triclosan-incorporated medium and blank vials inoculated with a live cell suspension but in plain BH broth without triclosan were also included in this experiment.

All samples and controls were incubated at 35ºC with shaking at 160 rpm.

3.2.4. Live/dead bacteria cell count

Viable cell counts were carried out on a weekly basis by a culture-based serial dilution method on TSA plates incubated at 35ºC overnight before counting. Cell counts were also carried out by FCM assay. Details of method and procedures are discussed in Chapter 2.

3.2.5. Triclosan quantification

The triclosan concentration in broth cultures was assayed by HPLC-UV using a diode array detector (see Chapter 2 for details). Sample preparation procedure is described in Chapter 4. Samples were assayed in duplicates and average value was presented.

3.2.6. Monitoring of bacterial community composition change under triclosan exposure

Bacterial DNA of the wastewater consortium incubated with triclosan was extracted and nested Muyzer PCR was performed to amplify a small section of 16S rDNA of the DNA mixture. Amplified DNA was then cleaned up using Montage™ PCR Centrifugal Filter Devices or UltraClean® PCR Clean-Up Kit to remove undesirable reaction by-products, such as primer dimers, unused primer, dNTPs, and reaction residuals. DGGE was used to analyze the community composition change of the wastewater consortium exposed to triclosan. Details of method and procedures are discussed in Chapter 2.

3.3 Results

3.3.1. Selection of putative triclosan degraders from the wastewater consortium by adaptation

3.3.1.1. Triclosan resistant bacterial profile in wastewater

The bacterial triclosan resistance profile is illustrated in Table 4. The viable count on R2A agar plates, containing concentrations of triclosan ranging from 0.05 to 10 mg/L, was shown to be closely related to the concentration of triclosan, which indicates the susceptibility of the dominant wastewater bacterial population to triclosan. Triclosan activity was first seen against this wastewater bacterial community at a concentration of 0.5 mg/L. Ten mg/L inactivated most of these wastewater bacteria so that they could not be recovered after overnight incubation on nutrient agar plates.

Table 4 Bacterial triclosan resistance profile

Triclosan concentration (mg/L)	0	0.05	0.1	0.5	1	2	10
Cell count (10^5 cu/ml)	72	73	74	55	31	16	2

3.3.1.2. Isolation of triclosan resistant strains from wastewater

Triclosan resistant isolates growing on R2A agar plates containing concentrations of triclosan at 2 and 10 mg/L were purified and enriched on TSA agar plates containing 20 and 100 mg/L of triclosan. One of the two colonies arising from R2A plate containing 10

mg/L triclosan could not grow on TSA plate containing a higher concentration of triclosan, whereas the other isolate (labelled as 10R2A01) grew well on TSA plates containing triclosan at 20 and even 100 mg/L concentrations. Seven out of 16 isolates from R2A plates containing 2 mg/L of triclosan could not be cultured on TSA plates containing higher concentrations of triclosan, whereas the rest (labelled as 2R2A01-09) grew well on TSA plates containing 20 mg/L of triclosan.

The isolation of triclosan resistant bacteria from a wastewater consortium was repeated at a lower dilution (10^{-2}) on R2A agar containing 10 mg/L of triclosan, and another 2 isolates (labelled as 10R2A 06, 10R2A07) of different morphology capable of growing on TSA plates containing 100 mg/L of triclosan were isolated.

The above 12 strains were randomly isolated from the same wastewater consortium, so it was expected that some of the isolates may actually be the same organism. Bacterial 16S rDNA signature of these 12 isolates was monitored by DGGE. Bacterial chromosomal DNA was extracted from the above 12 isolates, and amplified by PCR using 72f/1492r primers.

PCR products were then amplified by nested PCR using Muyzer F-GC clamp and Muyzer R primers. The nested PCR products were separated using DGGE, as shown in Figure 3. It can be observed that isolate 2R2A02, 2R2A04 and 2R2A09 have similar DGGE signatures, and isolates 2R2A01, 2R2A06 and 2R2A07 have similar DGGE signatures. Therefore, eight different isolates were characterised as triclosan resistant strains, listed as A to H in Figure 3.

3.3.1.3. Identification of triclosan resistant strains

Triclosan resistant isolates were identified using *Api* 20NE identification kit (bioMérieux SA, France) and a summary of results is shown in Table 5.

All of these triclosan resistant isolates belong to the *Pseudomonas* family with *Pseudomonas aeruginosa* as the most common species.

1	2	3	4	5	6	7	8	9	10	11	12	13	14
	(C)	(B)	(A)	(D)	(A)	(E)	(B)	(B)	(F)	(A)	(G)	(H)	

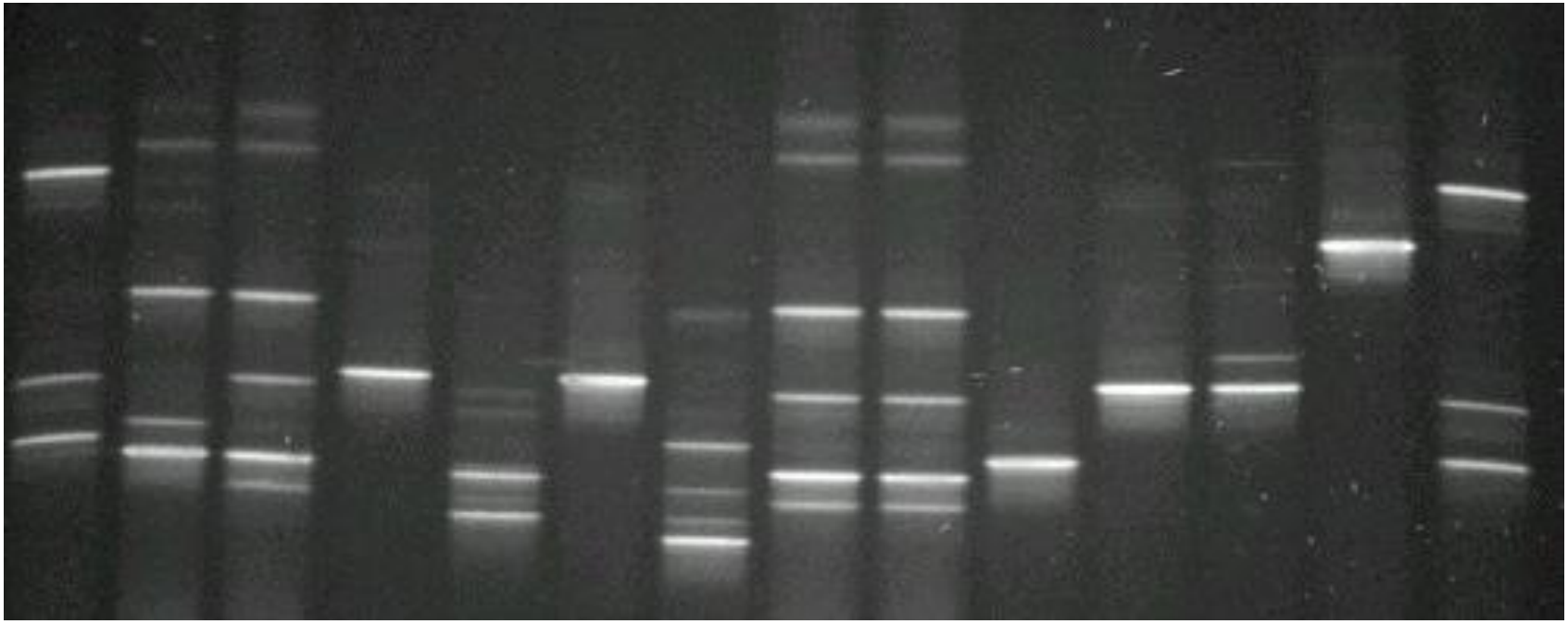


Figure 3 Bacterial 16S rDNA signature of triclosan resistant isolates by DGGE

Lane 1-14: +ve control using known DNA of H1/10, 10R2A01 (C), 2R2A01 (B), 2R2A02 (A), 2R2A03 (D), 2R2A04 (A), 2R2A05 (E), 2R2A06 (B), 2R2A07 (B), 2R2A08 (F), 2R2A09 (A), 10R2A06 (G), 10R2A07(H), +ve control

Table 5 Identification of Triclosan resistant isolates

Label	ID	Rarity (Likelihood of matching assimilation profile in bacterial population, as result from API)	Identity (as result from API)
2R2A01	*Pseudomonas fluorescens-25*	1/28,666,481	56.00%
2R2A02	*Pseudomonas aeruginosa*	1/23,078,654	55.32%
2R2A03	*Alcaligenes xylosoxidans ss.xylos*	1/46,704	88.10%
2R2A05	*Moraxella sp.*	1/73,956,568	42.81%
2R2A08	*Pseudomonas stutzeri*	1/2,542	58.26%
10R2A01	*Alcaligenes faecalis*	1/15,760	63.38%
10R2A06	*Pseudomonas aeruginosa*	1/27,047,041	82.03%
10R2A07	*Pseudomonas aeruginosa*	1/3,342,893	97.75%

3.3.1.4. Adaptation of triclosan resistant isolates for growth in triclosan minimal medium monitored by FCM

Triclosan resistant isolates were then adapted to BH broth with triclosan as the sole carbon source at concentration of approximately 2 mg/L. Bacterial growth in the sample vials as well as blank control (BH broth without triclosan inoculated with similar amount of bacteria sample) were monitored over a 2 week period.

After the initial 2 weeks incubation and subsequently every fortnightly thereafter, cells were washed and sub-cultured into fresh medium of the same composition for approximately another 4 weeks. An aliquot of each sample was taken every two to three days during the exposure period of approximately 6 weeks, after vigorous shaking by hand, for cell count by plate counting or flow cytometry (FCM) assay.

Of all the eight triclosan resistant isolates, only 1 isolate (2R2A08) adapted to growth in BH broth with triclosan. As shown in FCM image in Figure 4, isolate 2R2A08 was inoculated on Day 0, reduced bacterial cells were observed after 8 hours of inoculation. This reduction continued until day 4, which indicated that the biocidal activity of triclosan in the medium inhibited the growth of the organism. However, from Day 7 an increase in viable cell numbers were observed in the sample, which indicated the proliferation of bacteria in the BH broth containing triclosan after the initial adaption period of 4 days. This growth continued as indicated by the increasing viable cell counts at the end of the monitoring period (Day14). In contrast, the FCM monitoring of bacterial growth in the control vial containing BH broth without triclosan inoculated with similar bacteria cells, as shown in Figure 5, does not show the inhibitory effect of triclosan and no proliferation of bacteria in the medium was observed over the monitoring period either.

This result indicated that isolate 2R2A08 underwent an initial adaptation period and was then able to grow in BH broth containing a high concentration of triclosan and utilise triclosan as sole carbon source.

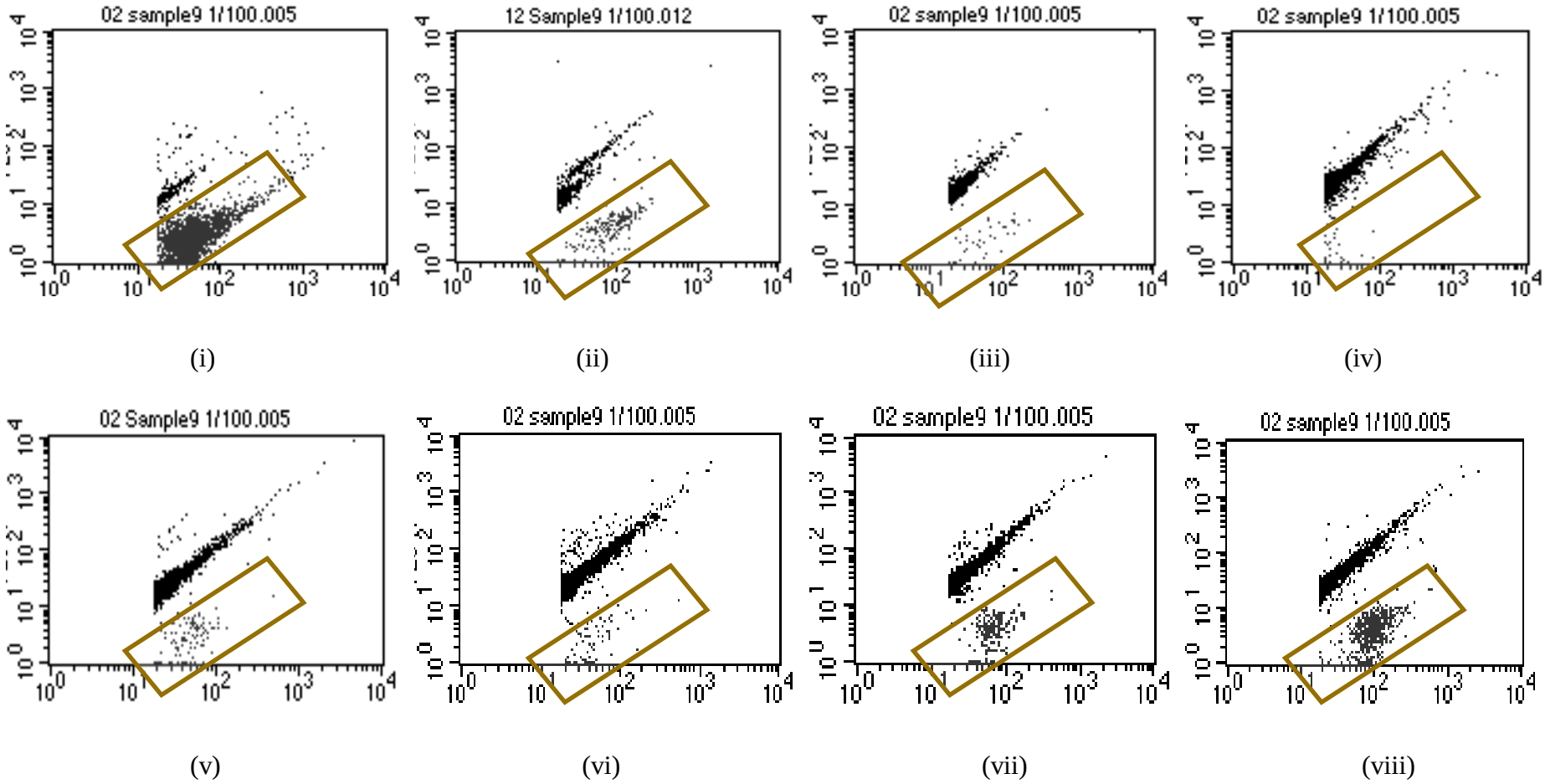


Figure 4 FCM analysis of 2R2A08 isolate growing in BH broth with 2mg/L triclosan over a 2 week period, stained with the BacLight kit
Y-axis indicates Red Fluorescence; X-axis indicates Green Fluorescence; Gated regions represent live cells
(i) Day0, (ii) 8hr, (iii) Day2, (iv) Day4, (v) Day7, (vi) Day9, (vii) Day11, (viii) Day14

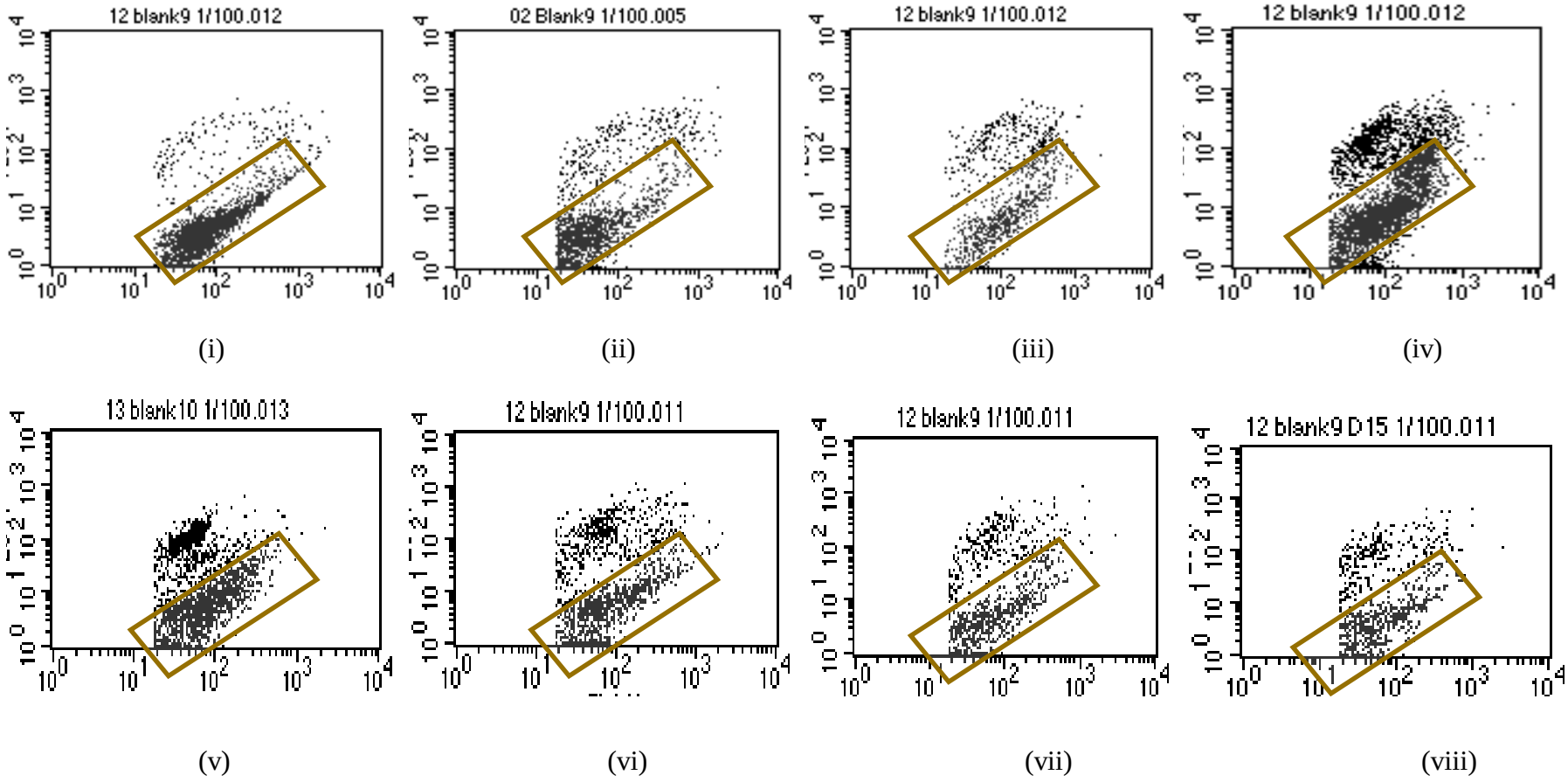


Figure 5 FCM analysis of 2R2A08 isolate growing in BH broth without triclosan over a 2 week period, stained with the BacLight kit
Y-axis: Red Fluorescence; X-axis: Green Fluorescence; Gated region: live cells
(i) Day0, (ii) 8hr, (iii) Day2, (iv) Day4, (v) Day7, (vi) Day9, (vii) Day11, (viii) Day14

Other isolates all showed no detectable adaptation (data not shown) and were not able to grow in BH broth containing 2 mg/L of triclosan. While these isolates were able to grow on TSA plates containing higher concentrations of triclosan up to 100 mg/L, they were not able to grow in BH broth with lower triclosan concentrations. This indicated that these isolates, although resistant to triclosan at a high level, they were not able to utilize triclosan as carbon source for proliferation. It is also worth noting that although some bacteria showed similar susceptibility in broth culture and agar plate (Di Bonaventura et al., 2002, Domig et al., 2007), some bacteria demonstrated higher MIC on agar plate than in broth culture (Hussain et al., 1987, Ramotar et al., 2001, Waites et al., 1991).

The FCM image of isolate 10R2A06 sample vials and control vials showing no adaptation are pictured in Figure 6. An extended sampling period was used for these samples in order to amplify the signal to ensure any growth in BH broth containing triclosan was detected, however no evidence of growth was observed in sample vials as shown in Figure 6. This has resulted in more events count in FCM images for the blank vials for isolate 10R2A06 than that for the blank vial for isolate 2R2A06 (shown in Figure 5).

FCM images of isolate 10R2A07 sample vials and control vials showing no adaptation process are pictured in Figure 7. The live cell count in the sample vial reduced gradually over the 2 week monitoring period, and followed a similar pattern to the blank vial with no triclosan in the medium. This indicated that 10R2A07 strain is highly resistant to triclosan, therefore no obvious inhibitory effect of triclosan at 2 mg/L of concentration was observed. However, because the medium does not contain any carbon source other than triclosan, and this isolate was not capable of growing in this medium, this indicates that, although it may be resistant by one of the triclosan resistance mechanisms described previously, this isolate could not degrade triclosan and also did not acquire the ability to utilise triclosan as a carbon source during the 2 week monitoring period.

For samples showing no obvious adaptation to triclosan degradation, at the end of the two weeks monitoring period, the culture in sample vials was re-inoculated into fresh BH broth containing the same concentration of triclosan, and the FCM monitoring was repeated. However, no adaptation was observed in any samples even after the 2nd round of adaptation/monitoring period (data not shown).

Isolate 2R2A08 was tentatively characterised as a triclosan degrader.

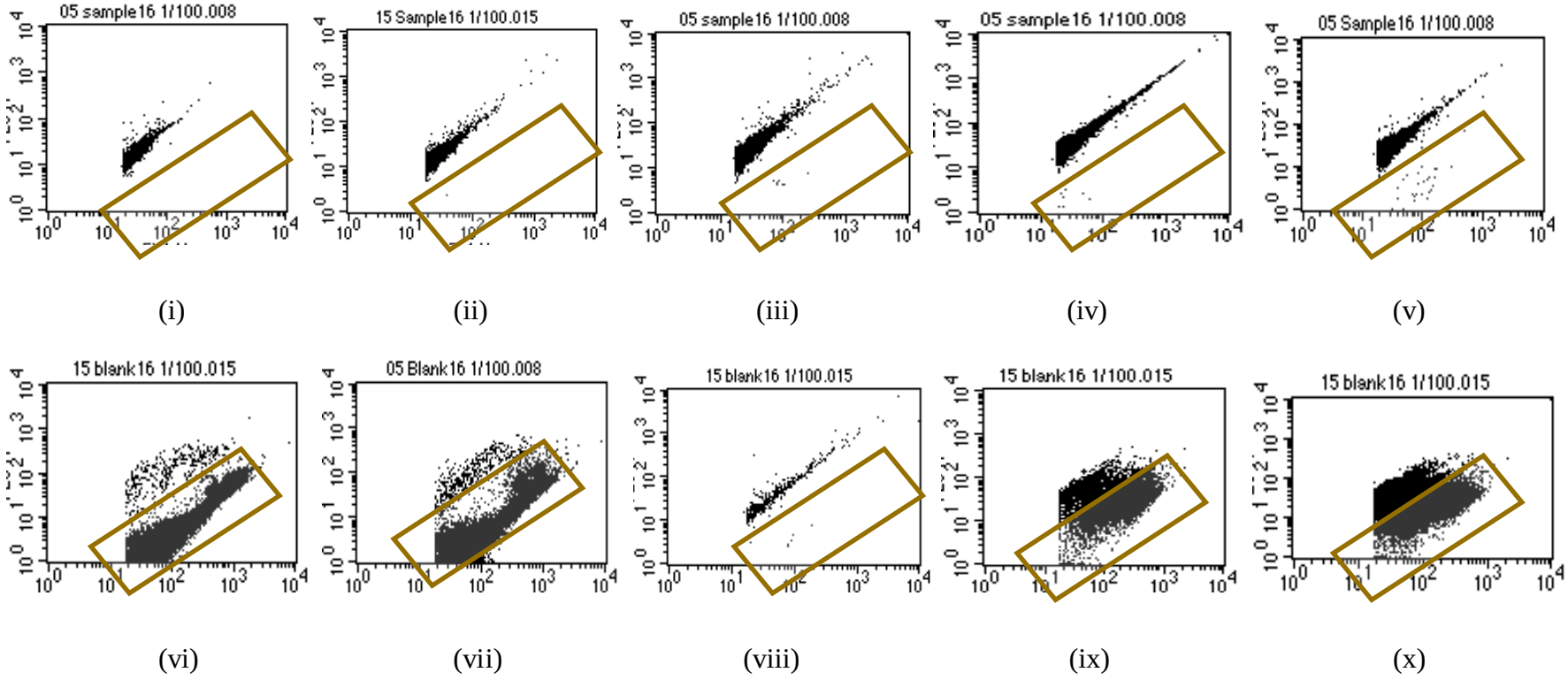


Figure 6 FCM analysis of 10R2A06 isolate growing in BH broth with 2mg/L triclosan (i-v) and without triclosan (vi-x) over 2 weeks period, stained with the BacLight kit

Y-axis: Red Fluorescence; X-axis: Green Fluorescence; Gated region: live cells

(i, vi) Day0, (ii, vii) Day4, (iii,viii) Day7, (iv,ix) Day11, (v,x) Day14

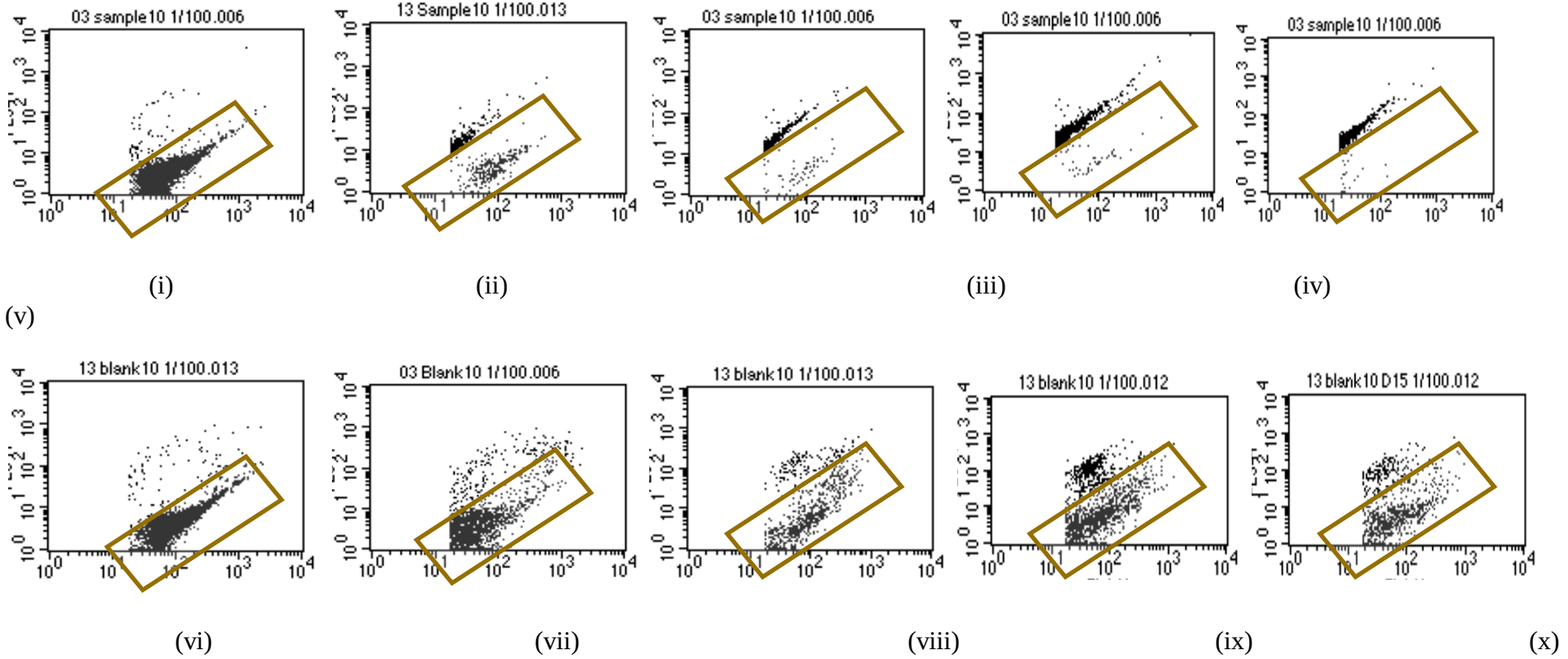


Figure 7 FCM analysis of 10R2A07 isolate growing in BH broth with 2mg/L triclosan (i-v) and without triclosan (vi-x) over 2 weeks period, stained with the BacLight kit

Y-axis: Red Fluorescence; X-axis: Green Fluorescence; Gated region: live cells

(i, vi) Day0, (ii, vii) Day4, (iii,viii) Day7, (iv,ix) Day11, (v,x) Day14

3.3.2. Selection of putative triclosan degraders from a wastewater consortium by incubating the wastewater consortium with a high concentration of triclosan

The selection of triclosan degraders was also carried out directly from a wastewater consortium by incubating wastewater sludge with a high concentration of triclosan.

3.3.2.1. Bacterial community composition change monitored by DGGE during the selection of triclosan degraders

Activated sludge from the Bolivar Wastewater Treatment Plant (Bolivar, SA) was used as the consortium for selection of triclosan degraders from wastewater resources. One gram of triclosan powder was initially added into a 1L flask containing 500mL of activated sludge, incubated at 35ºC with shaking at 160 rpm.

The concentration of triclosan in this wastewater consortium was monitored using HPLC-UV every week. Another 1g of triclosan powder was added once the concentration of triclosan in the flask decreased to below 200 mg/L.

PCR amplification and DGGE was carried out to monitor the change in composition of the bacterial community exposed to triclosan. Figure 8 shows the DGGE profile over the monitoring period.

The DGGE profile was assessed over an 8 week period, and after 8 weeks incubation in the presence of triclosan, the bacterial community composition was significantly simpler when compared to the initial Day 0-1 DGGE signature. Some new bands were detected after one day of selection (as shown in Figure 8), which could represent the signature of triclosan resistance strains. After long term incubation under triclosan selective pressure, some of the original bands were lost and some new bands (indicated by arrows on the right hand of the photo in Figure 8) appeared indicating that triclosan degraders outgrew the non-degraders under the exposure to a high concentration of triclosan.

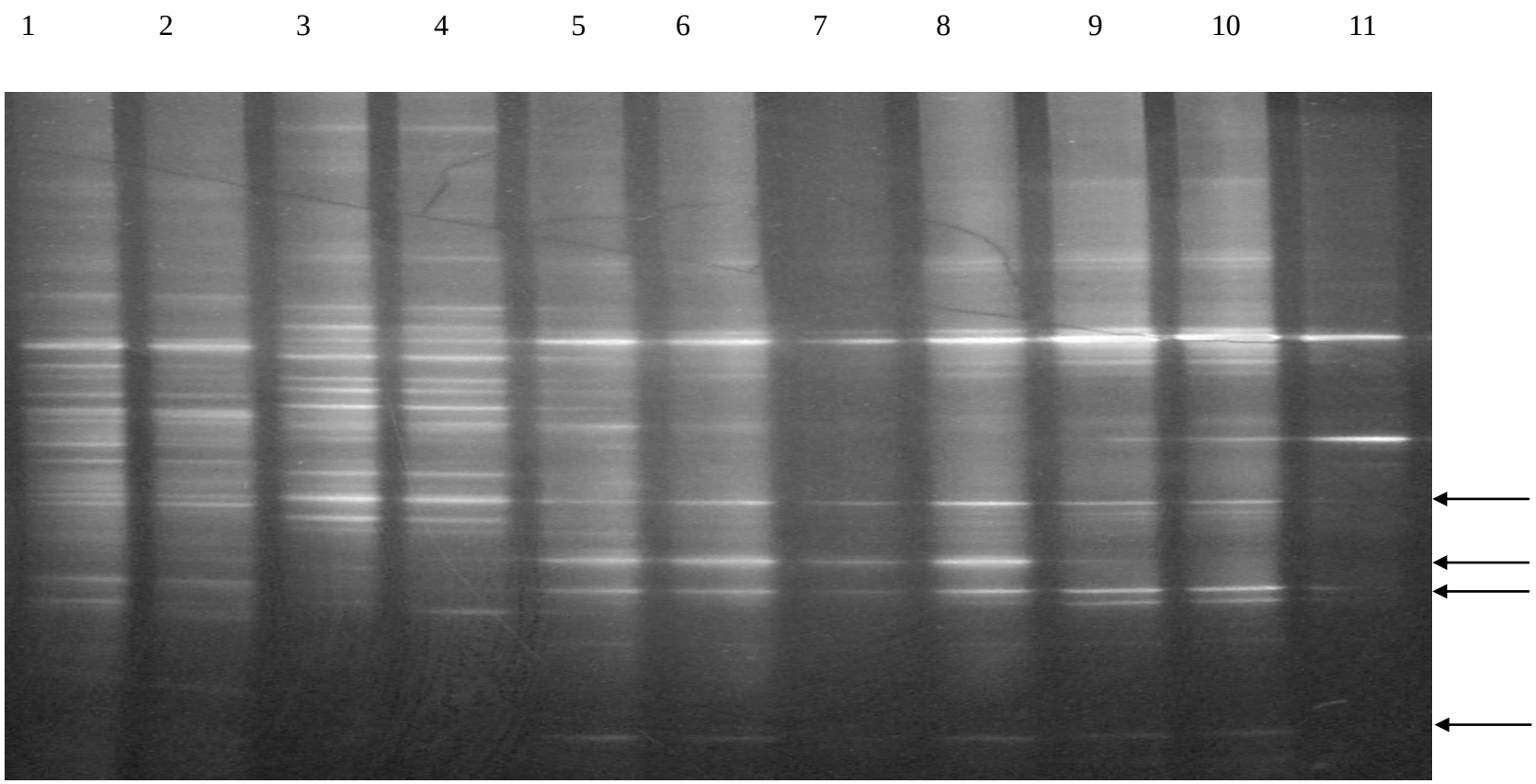
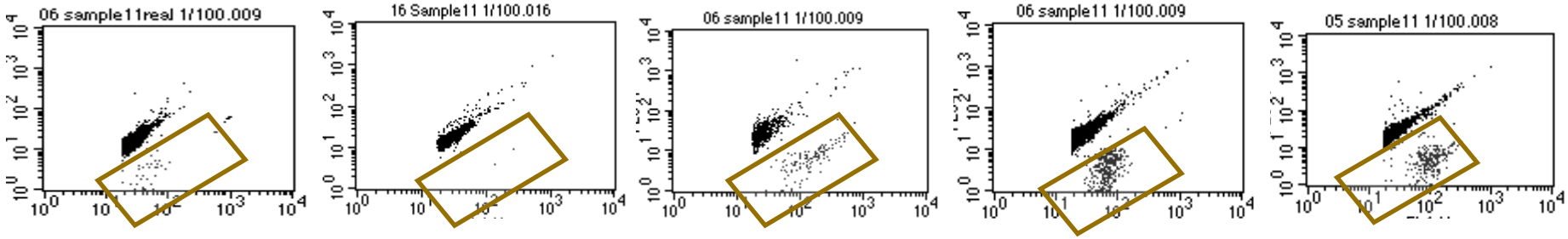


Figure 8 Bacterial community composition change monitored by DGGE during selection of triclosan degraders from wastewater.
Lane 1, 2 Day 0 (prior to exposure under triclosan); Lane 3,4after short term selection (1 day); Lane 5,6 under long term selective pressure (2 weeks); Lane 7,8 after 4 weeks exposure; Lane 9,10 after 8 weeks exposure; Lane 11 –ve control (no sample DNA was added in PCR reactions, i.e. bands shown as primer dimers)

3.3.2.2. Growth of putative triclosan degraders in BH broth containing triclosan monitored by FCM

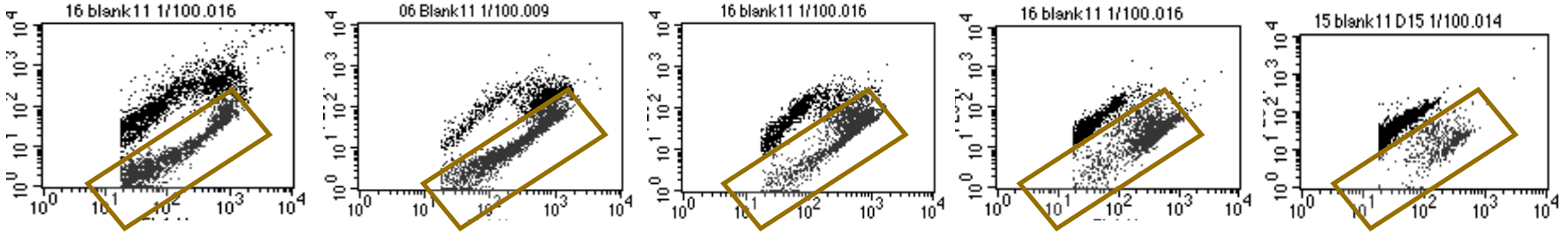
After three months of exposure to a high concentration (0.2-1g/L) of triclosan, two isolates were obtained when plated out onto minimal agar plate containing 1g/L of triclosan and casamino acids as supplement, namely F11 and F12. These 2 strains were then subjected to the growth adaptation in BH broth with triclosan as sole carbon source.

Similar to isolate 2R2A08, F11 and F12 isolates showed an initial adaptation period during the first few days of inoculation in BH broth with triclosan (shown in Figure 9 and Figure 10, respectively). Bacterial growth was observed following this adaptation period. However, subsequent growth of bacteria was noticeably slow, which was indicated by the steady number of live cells in the sample vials.



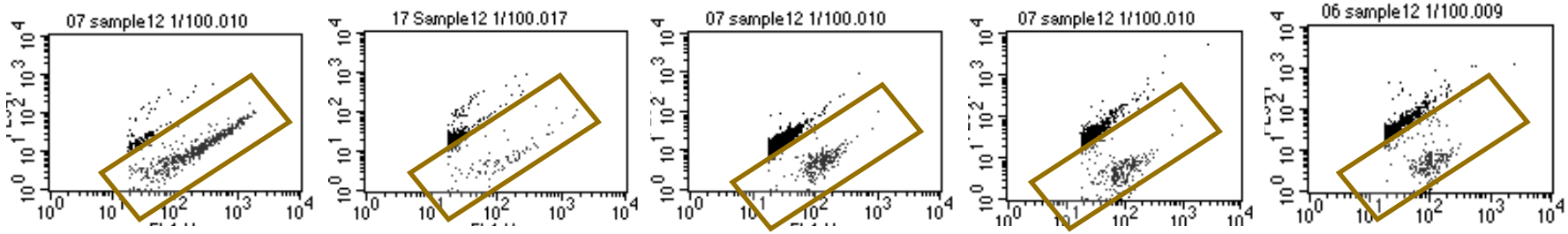
(i) (ii) (iii) (iv)

(v)



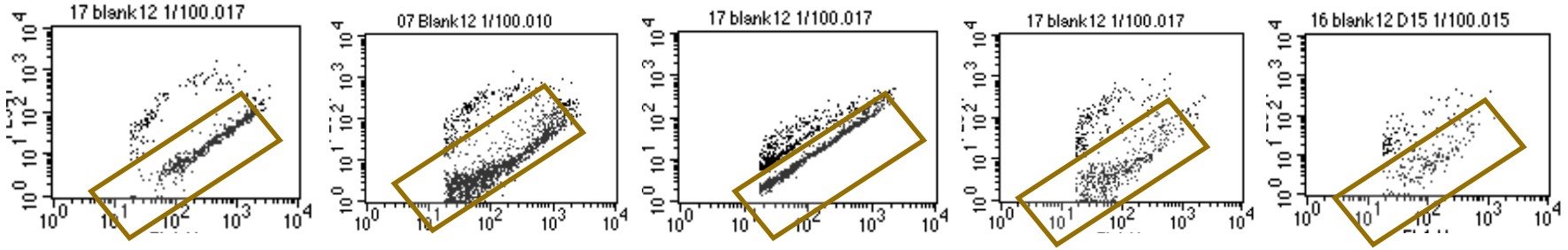
(vi) (vii) (viii) (ix) (x)

Figure 9 FCM analysis of isolate F11growing in BH broth with 2mg/L triclosan (i-v) and without triclosan (vi-x) over 2 weeks period, stained with the BacLight kit
Y-axis: Red Fluorescence; X-axis: Green Fluorescence; Gated region: live cells
(i, vi) Day0, (ii, vii) 8 hrs, (iii,viii) Day4, (iv,ix) Day11, (v,x) Day14



(i) (ii) (iii) (iv)

(v)



(vi) (vii) (viii) (ix) (x)

Figure 10 FCM analysis of isolate F12 growing in BH broth with 2mg/L triclosan (i-v) and without triclosan (vi-x) over 2 weeks period, stained with the BacLight kit
Y-axis: Red Fluorescence; X-axis: Green Fluorescence; Gated region: live cells
(i, vi) Day0, (ii, vii) 8 hrs, (iii,viii) Day4, (iv,ix) Day11, (v,x) Day14

3.3.3. Characterisation and identification of putative triclosan degraders

3.3.3.1. Confirmation of bacterial triclosan degradation by HPLC-UV/FCM

Bacterial triclosan degradation was characterised by monitoring the increase of bacterial cell count using FCM and the corresponding decrease of triclosan concentration in the culture using HPLC-UV.

F12, F11 and 2R2A08, were tentatively identified as triclosan degraders, as they are capable of growing in BH broth containing 2 mg/L of triclosan. The growth curve was also monitored when the strain was inoculated into minimal medium broth containing 300 mg/L of triclosan.

Figure 11 shows the growth curve for strain F12 in triclosan minimal broth. An increase in bacterial cell count and a corresponding decrease of triclosan concentration in the culture medium were observed. For the two control series, bacterial growth in minimal medium without triclosan gave a constant cell count background and no detection of triclosan in the medium; and dead bacterial cells inoculated into triclosan minimal medium showed a zero cell count and a constantly high concentration of triclosan in the culture medium. This indicated that the decrease of triclosan in the culture was due to the growth of bacteria. A slow but steady growth was achieved by these putative degraders, indicating triclosan utilization had occurred in the culture.

The degradation of triclosan on minimal agar plates was also confirmed by the production of the zone of clearance around the colonies growing on the minimal agar plates containing 2 mg/L of triclosan (as shown in Figure 12).

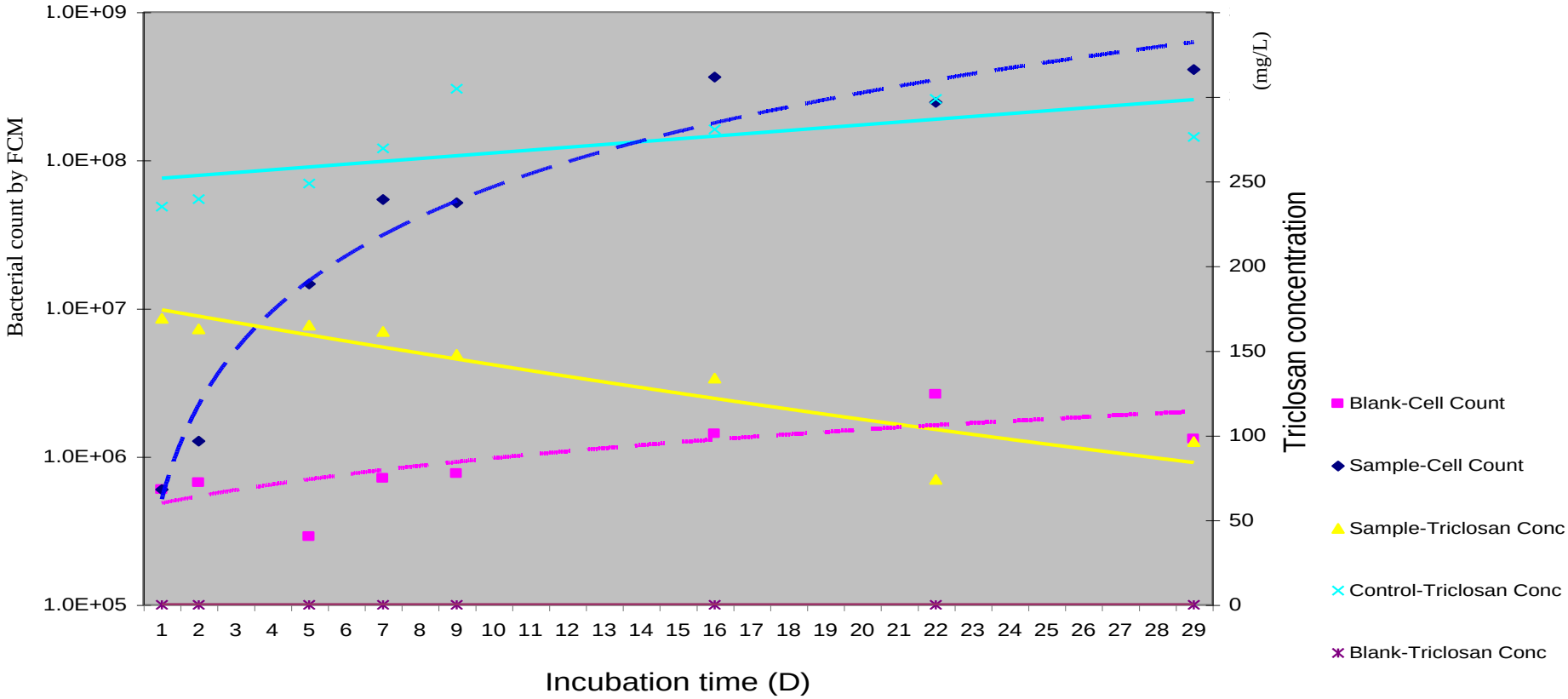


Figure 11 Growth of F12 in Triclosan minimal medium containing 300mg/L of triclosan
(Sample series: Live bacteria inoculated in triclosan minimal medium;
Blank series: live bacteria inoculated in minimal medium without triclosan;
Control series: autoclaved bacteria (dead) inoculated in triclosan minimal medium)

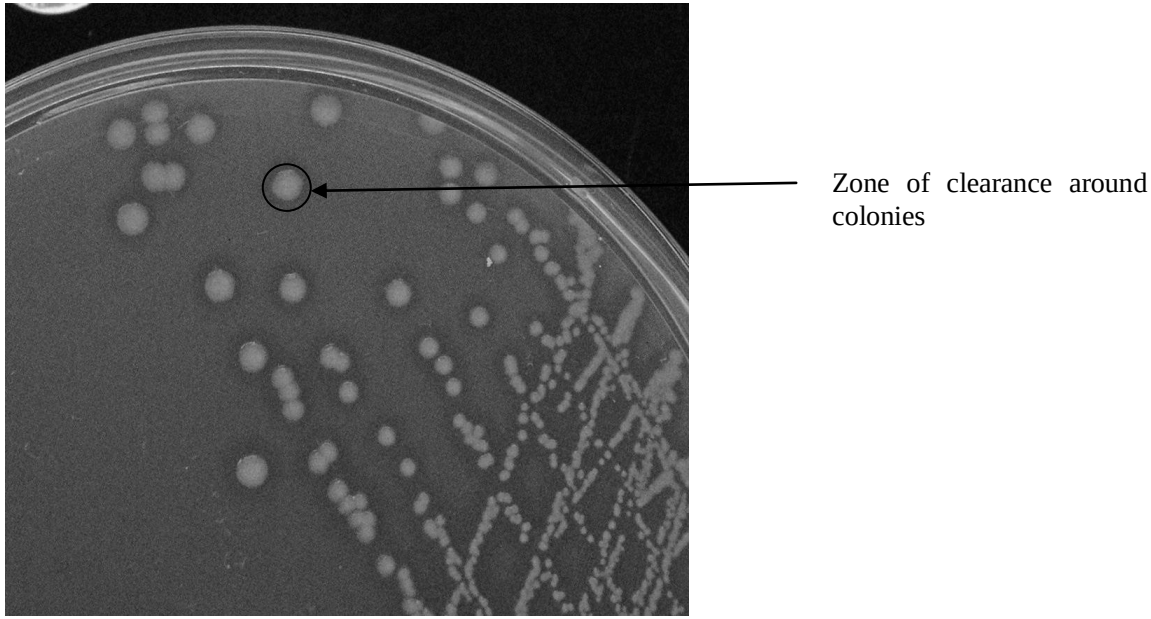


Figure 12 Clearance of triclosan around colonies growing on triclosan plates

3.3.3.2. ID by 16s rDNA sequencing

Interesting putative degraders isolated from wastewater consortium, F11 and F12, were further identified by 16S rDNA sequencing. The detail of the sequencing chromatograph is attached in Appendix 1. Isolate F11 was identified as *Pseudomonas aeruginosa,* and F12 was identified as *Pseudomonas citronellolis.*

3.4 Discussion

As precursors of triclosan degraders, eight pure cultures of triclosan resistant isolates were obtained from wastewater, which were identified mainly as *Pseudomonas* strains, such as *Pseudomonas fluorescens-25*, *Pseudomonas aeruginosa*, *Alcaligenes xylosoxidans ss.xylos*, *Moraxella sp.*, *Pseudomonas stutzeri*, and *Alcaligenes faecalis,* with *Pseudomonas aeruginosa* most commonly isolated (shown in Table 5).

Pseudomonads are widely distributed in natural water and soil, as well as in wastewater treatment plants (Cho & Tiedje, 2000, Misko & Germida, 2002, Ramette et al., 2003). Pseudomonads are known to be intrinsically resistant to triclosan at various concentration levels (Beinlich et al., 2001, Champlin et al., 2005, Chuanchuen et al., 2003, McBain & Gilbert, 2001). *Pseudomonas aeruginosa* is widely used as a model of triclosan resistance in investigations of triclosan resistance in bacteria. A recent study explored the effect of outer membrane permeabilisation on the intrinsic resistance to low triclosan levels in *Pseudomonas aeruginosa*, and found that the outer membrane exclusionary properties of *P. aeruginosa* for non-polar molecules confers intrinsic resistance to low concentrations of triclosan (Champlin et al., 2005). The same study also suggested the potential contribution of this mechanism to bacterial resistance to high triclosan concentrations *in-vitro* (Champlin et al., 2005). However, multidrug efflux pump systems have been widely cited to be responsible for high levels of triclosan resistance in *Pseudomonas aeruginosa* (Beinlich et al., 2001, Chuanchuen et al., 2003), as well as other microorganisms, such as *Salmonella enterica* and *Escherichia coli* (Braoudaki & Hilton, 2005, Copitch et al., 2010, McMurry et al., 1998b). Changes in outer membrane or loss of plasmids resulting in less burden to the strain have also been speculated recently (Cottell et al., 2009) as triclosan resistance mechanisms, however no experimental

evidence was provided to support this assumption. A recent study restated the important role of FabI and homologues in triclosan resistance in *Pseudomonas aeruginosa PAO1 (Zhu et al., 2010)*. Zhu's study demonstrated that FabI in PAO1 is not responsible for triclosan resistance, whereas its homologue FabV, a triclosan-resistant enoyl-ACP reductase recently demonstrated in *Vibrio chlorerae,* confers triclosan resistance on *P. aeruginosa*, and the deletion of the *fab*V gene resulted in the mutant strain becoming over 2000-fold more sensitive than the wild-type strain.

Three confirmed triclosan degraders, capable of growing in BH broth with triclosan as sole carbon source, were obtained using various methods discussed in this chapter, and these isolates were identified as *Pseudomonas stutzeri, Pseudomonas aeruginosa* and *Pseudomonas citronellolis* respectively. The growth curve of *Pseudomonas citronellolis* strain in triclosan minimal broth containing 300 mg/L of triclosan further demonstrated its ability to utilize triclosan as sole carbon source, albeit slowly.

Studies on triclosan are mainly focused on antimicrobial effects and resistance (Jones et al., 2000), cross-resistance to other antimicrobial agents including antibiotics, and the mechanisms involved in drug resistance, but the studies on biodegradation of triclosan by pure cultures of bacteria are not extensive (Federle et al., 2002, Singer et al., 2002, Thompson et al., 2005). Photolytic degradation (Aranami & Readman, 2007), chlorination (Canosa et al., 2005), and methylation (Coogan et al., 2007) of triclosan has been observed in the natural water environment as well as in activated sludge under aerobic conditions (Chen et al., 2011). Sonochemical degradation of triclosan in various environmental samples including seawater, urban runoff and influent domestic wastewater as well as pure and saline water has been reported recently and a rapid reduction in triclosan concentration was achieved by a micro-organism consortium (Sanchez-Prado et al., 2008). The white rot fungus *Pycnoporus cinnabarinus* and *Trametes versicolor* were found to metabolize triclosan (Hundt et al., 2000, Inoue et al., 2010). An auxotrophic *Sphingomonas*-like organism, which was able to partially mineralize triclosan when grown on complex media, was also isolated from a triclosan degrading bacterial consortium (Hay et al., 2001).

The genus of *Pseudomonas* are fast-growing nutritionally versatile bacteria that are capable of utilizing a wide variety of carbon sources (Lloyd-Jones et al., 2005), and are capable of the degradation of environmentaly important organic compounds (Wackett, 2003). However, there

have been no records of *Pseudomonas citronellolis* and *Pseudomonas stutzeri* showing a capability to degrade triclosan or utilise triclosan as carbon source. **S**oil strains of *Pseudomonas putida* and *Alcaligenes xylosoxidans subsp. denitrificans*, were observed to be able to use triclosan as a sole carbon source and clear particulate triclosan from agar (Meade et al., 2001). The rate of triclosan degradation by bacteria varies between different isolates. For example, a decrease in triclosan concentration was observed within 6 hours of inoculation with a *Pseudomonas putida* strain, whereas 24 h was required with *Alcaligenes xylosoxidans* strain (Meade et al., 2001). The slow degradation rate observed in the triclosan minimal broth in this study corresponds to the slow rate of bioremediation in soil contaminated with triclosan at a rate of 1mg/Kg over 70 days (Al-Rajab et al., 2009, Lozano et al., 2010, Roh et al., 2009). Slow biodegradation of triclosan by indigenous micro-organisms in the wastewater treatment plant has been observed (McAvoy et al., 2002, Ying et al., 2007). Two *Psedomonas* isolates isolated from the waters and sediments of flowthrough mesocosms dosed with various concentrations of 3-chlorobenzoate showed the ability to produce chlorocatechol when exposed to 2-chlorobenzoate at millmolar concentrations in the presence of yeast extract (Fulthorpe & Wyndham, 1991). These species were found to be able to grow only slowly in liquid chlorobenzoate minimal medium but could degrade 3Cba in continuous cultures dosed with micromolar levels of the chemical (Fulthorpe & Wyndham, 1991), potentially due to the toxicity of the degradation product chlorocatechol (Dann & Hontela, 2011).

Of other pseudomonad strains isolated in this study, no *Pseudomonas putida* was obtained through the isolation methods described in this chapter. An *Alcaligenes xylosoxidans* strain (*subsp. xylos)* was isolated as triclosan resistant strain in this study but was not found to be able to utilise triclosan as a sole carbon source.

The successful selection of triclosan degraders from sewage water by simply incubating a wastewater consortium with a high concentration of triclosan indicated that triclosan degraders were originally present in the wastewater bacterial community, and were obtainable under triclosan selective pressure. However, only one out of the eight triclosan resistant strains demonstrated the ability to adapt to growth in BH broth containing triclosan as a sole carbon source. In addition, the repeated attempts to induce other resistant strains to utilize triclosan were unsuccessful. This may indicate that although triclosan degrading bacteria are present in the wastewater environment, non-degraders cannot easily adapt to acquire the capability of

utilizing triclosan as a carbon source even after repeated exposure to this compound.

Horizontal transfer of catabolic genes, including catabolic plasmids, has been documented previously (Hill & Weightman, 2003, Madsen, 2002, Sorensen et al., 2005, Stuart-Keil et al., 1998). 3-Chlorobenzoate degrading bacteria isolated from sediments dosed with 3-chlorobenzoate were found to carry a plasmid introduced with *Alcaligenes* sp. coding a transposable element and 3-chlorobenzoate degradation (Fulthorpe & Wyndham, 1991). The sharing of common catabolic genes was also found in different bacterial species having similar origin (Sorensen et al., 2005). In this project, the lack of shared metabolism traits within the bacterial community obtained from the same wastewater consortium indicated that the triclosan catabolism determinants are not easily transferable among species by horizontal gene transfer. The fact that the triclosan degradative plasmid identified in this study (as discussed in Chapter 5) is a non-conjugative plasmid, i.e. not easily transferred, coincides with this observation.

The significant progressive changes in the DGGE profile demonstrated the process of the selection, which started with a minority of triclosan resistant bacteria in the water that was capable of surviving in high concentrations of triclosan. As the selection process continued, reduced amounts of other organic compounds originally present in the water were available due to utilization by micro-organisms. As a result of competition for nutrients, the triclosan resistant bacteria that could not utilize triclosan as carbon source gradually died off, whereas triclosan degraders could utilize triclosan as a carbon source and eventually outgrew other non-degraders.

Similar findings were observed most recently on the micro scale analysis of the effects of triclosan and trichlocarban on the structure and function of river biofilm communities (Lawrence et al., 2009). Confocal laser scanning microscopy (CLSM) showed different community architecture and composition compared to the control experiment. The use of carbon utilization assays based on growth indicated a suppressing effect of triclosan, and the community, cultivated with 10 µg/L triclosan, was altered from one dominated by autotrophic processes to one dominated by heterotrophic processes. DGGE analyses indicated a significant change occurred in the bacterial community as a consequence of triclosan treatments (Lawrence et al., 2009).

In this chapter, it was demonstrated that bacterial triclosan degraders were present in the wastewater environment, and may outgrow other micro-organisms under triclosan selective pressure. Induction of triclosan degradation in pure cultures was shown to be difficult. Triclosan degrading isolates obtained using isolation methods described in this chapter are mainly *Pseudomonas* sp. The degraders are able to grow on triclosan minimal medium, although at a low rate. Three bacterial triclosan degraders were obtained and identified. The following 3 chapters are aimed at exploring the biochemical and genetical explanation for the bacterial biodegradation of triclosan.

Chapter 4 Triclosan detection and biodegradation pathway study based on intermediate analysis

4.1 Introduction

In order to isolate and identify any triclosan degrading bacteria from the wastewater environment, a quick and convenient method for detection of triclosan is a critical step in the methodology. Failure to detect triclosan in triclosan-containing medium after the growth of a bacterial culture could indicate the utilization of triclosan by this bacterium. This reasoning was used in this study as an initial screening method to isolate any potential triclosan degraders from the large variety of bacteria in the wastewater environment.

The detection of triclosan in water is also a basis for the development of an efficient and accurate triclosan quantification method used to measure the concentrations of triclosan in culture media and to quantitatively monitor the decrease of triclosan concentration as a result of utilization by the triclosan degraders. The characterisation of metabolic kinetics also relies on efficient and accurate quantification of triclosan in a bacterial culture. This chapter also forms the basis for the triclosan quantification in minimal medium culture used to confirm the triclosan reduction seen in the culture of the putative triclosan degraders (See Chapter 3 for details).

This chapter reports the analysis used to

- detect the presence of triclosan in water solution by HPLC-UV;
- detect the presence of triclosan in bacterial culture by HPLC-UV;
- quantify the concentration of triclosan in bacterial culture by HPLC-UV;
- tentatively characterise the potential triclosan biodegradation intermediates or by-products by LC-MS and GC-MS;

UV detection can be achieved by single spectra UV detector, multi spectra UV detector or a Diode Array Detector (DAD), which detects UV absorbance of a sample at multi-wavelength ranging from 200nm to 300nm. When a substance is eluted from the sample to the sensor cell, all the outputs from the array can be acquired and the result can be used to construct

absorption spectra for this unknown substance. By comparing these spectra with standard spectra of a known chemical, the tested substance can be identified. Furthermore, by selecting the appropriate diode, at which there is maximum absorption of the target compound and least interference from other components in the sample, the quantification can be achieved at the maximum detection sensitivity for that substance. DAD was used in this study for triclosan detection and quantification.

Triclosan shows two characteristic UV spectra at 210nm and 280nm as well as a shoulder at 235nm. These characteristic spectra can be used for the detection of triclosan after separation using LC. The strongest absorbance at 210nm was used initially to establish the detection of triclosan in pure aquous sample. However, for complex samples containing a matrix of organic compounds, this is not appropriate as many other organic compounds in the sample may also show strong absorbance at this wavelength thus interfering with the detection and quantification of triclosan. A wavelength of 270nm was shown to have the least interference from other components in the sample and was therefore used for the quantification.

LC-ESI-MS and GC-MS can both be used to characterise the potential triclosan metabolic intermediates present in the culture of putative triclosan degraders (Ferrer & Thurman, 2003, pp 1-13).

Although the sample preparation is much more complicated and range of chemicals that can be assayed by GC-MS is limited and some of the polar by-products cannot be captured by GC/MS without any prior derivatization steps (Ferrer & Mezcua, 2004); GC-MS has a full analytes library, against which unknown compounds can be characterised by comparing the mass spectrographs with these of the known chemicals in the MS library. One degradation by-product has previously been characterised directly by GC/MS as methylated-triclosan (Bester, 2005).

LC-MS is capable of analysing a wide range of chemicals with various polarities, and has been used widely as a powerful analytical tool for the identification of metabolites and unknown compounds in environmental samples (Ferrer & Thurman, 2003, pp 1-13). However, due to the complexity of growth culture used in this study, and the lack of a full chemical library, high-resolution techniques are needed to provide additional structural information (Ferrer & Mezcua, 2004, Ferrer & Thurman, 2003).

Mass measurements by mass spectrometry are predominantly used to determine the chemical composition of organic compounds. The standard practice uses mono-isotopic peak as signature of the compound for identification as the accurate mass can be conveniently calculated from the chemical formula. However, this standard analysis does not seem to be effective for isotopically complex molecules, in which the mono isotopic mass peak may be too weak to be useful (Rockwood et al., 1995). Isotope analysis is the identification of isotopic signature, the distribution of certain stable isotopes and chemical elements within chemical compounds. A complex compound may be characterised by its isotopic pattern, which may be constructed based on calculation of accurate masses using isotopes of primary elements, such as carbon, oxygen, halogen, and metals etc. Chemometric methods using isotope patterns and computer assistance is considered as a promising way to analyse mass spectra (Szymura et al.), and has been applied for elemental analysis of typical organic compounds, such as tribromo-trichloro-ethane, tetra-alkyltins, phenylalanine and tyrosin etc (Goni & Eglinton, 1996, Meesters et al., 2009, Rockwood et al., 2004, Szymura et al., 2010). Unknown oxidation products, such as lignin-derived CuO oxidation by-products were also analysed using isotope ratio monitoring GC-MS (Goni & Eglinton, 1996).

An isotopic pattern of a compound is a ratio of masses resulting from the isotopes of elements in the composition of an investigated material. Isotopic patterns are information-rich and practically independent of spectrometer type and ionization technique (Szymura et al.). There are algorithms published to calculate isotopic distributions that have been used to elucidate fragmentation pathways, elemental composition, and chemical structure of high molecular weight compounds (Rockwood et al., 1995, Rockwood et al., 2004, Szymura et al., 2010, Tenhosaari, 1988). Isotopic signature analysis of triclosan and the potential triclosan degradation intermediates in bacterial cultures was undertaken using high resolution RP-HPLC coupled with ESI-MS.

During data analysis, only the ion chromatogram of a particular molecular mass is taken into account and extracted as EIC (Extracted Ion Current) chromatogram, thus specific information (EIC) of a particular compound of interest is extracted mathematically out of the crude data (Total ion current or TIC) (Verzele et al., 2007). This chemometric method enhances MS analysis of unknown compounds by separating individual compounds mathematically using molecular masses, which are then compounded, during data analysis, with the separation

achieved by HPLC in the form of peaks shown at different retention time on the EIC chromatogram. Therefore, by analysis of isotopic pattern, an unknown organic compound showing a particular molecular mass can be characterised and identified as a compound having matching isotope pattern.

In this study, triclosan was confirmed using this method, and putative degradation intermediates were also characterised and tentatively predicted using isotopic pattern method.

4.2 Methods

4.2.1 Glassware and consumables

Due to the low solubility of triclosan in water, the concentrations used in microbiological studies are normally low, in the order of µg/L. Therefore a sensitive detection method was required in order to efficiently quantify the amount of triclosan in culture media. It was found in this study that triclosan is prone to attach onto any plastic item, including medium containers, centrifuge tubes, culture flasks, petri dishes, pipette tips, and this property led to significant difficulties in this project. Plastic consumables were avoided in every step of the experiments, and recyclable glassware was used for triclosan containing cultures and solutions.

For similar reasons, triclosan is prone to attach onto parts of the analytical instrument, an appropriate solvent able to dissolve triclosan in order to prevent the attachment onto parts of the analytical instrument is therefore critical for an accurate quantitative analysis of this compound. Various solvents were tested in order to achieve the most reliable results during the initial method establishment stage. Similarly, various solvents were investigated for extraction of residual triclosan and degradative intermediates or by-products from the culture medium for the identification of degradation intermediates or by-products using LC-MS or GC-MS.

4.2.2 Chemicals and solutions

All reagents used in the study obtained from Aldrich-Sigma (Sydney, Australia), were of analytical grade and used without further purification. LC grade acetonitrile (ACN) was obtained from BDH (Poole, England). The mobile phase required for chromatography was prepared by dissolution of 20mM NH_4Ac and 100% ACN in Milli-Q® water. The mobile phase eluent was filtered through a 0.45 µm membrane filter (Millipore, Australia) and degassed in an ultrasonic bath (Bransonic, B-220, USA) prior to use. Milli-Q® water was filtered prior to use through a 0.45 µm disposable nylon membrane (Millipore, Australia).

A triclosan stock solution of 1000 µg/mL was prepared by weighing 10mg of the powdered triclosan into a 10mL volumetric flask and making up to the mark with methanol. This solution was used for preparation of the standard solution. The stock solution was stored at -20 °C and prepared freshly every month. A 10 µg/mL triclosan standard solution was

prepared by diluting 1 mL of the 1000 µg/mL stock solution into 100mL of ACN. Calibration standard solutions for HPLC-UV containing 0.05, 0.10, 0.50 and 1.00 µg/mL of triclosan were prepared by transferring 5, 10, 50 and 100 µL of the 10ug/mL triclosan standard into 995, 990, 950 and 900 µL of ACN.

4.2.3 Sampling and preparation

The putative triclosan degrader was inoculated in triclosan minimal broth medium supplemented with casamino acids, as described previously and incubated with shaking in the dark until turbidity was noticeable. Two controls for each sample, one with no bacterial inoculum in the same medium and one using medium containing no triclosan inoculated with the same bacterium, were included for comparison.

Cultures were transferred to 40 mL Tekmar® amber glass vials with no headspace volume left to prevent air bubbles from passing through the samples. Samples were transported on ice in an esky to the chemical laboratory on the same day and stored in the fridge. All samples were analysed within 2 to 3 days after collection to avoid storage loss.

Samples were diluted using methanol at 1:4 (sample/methanol vol/vol) ratio to contain 80% methanol when transferred to 1 mL Agilent glass loading vials (Agilent Technologies, USA) for HPLC analysis. Duplicates for unknown samples and triplicates for standard curve and recovery assessment were employed for quantification analysis.

Samples undergoing GC analysis were extracted using hexane and dichloromethane (DCM) for comparison. The sample was mixed with the same volume of extraction solvent by manual shaking until homogenized and let stand for 30 min for separation. Aqueous phase after extraction was loaded for LC analysis, and the more efficient extraction solvent, which showed disappearance of most of the peaks in total ion chromatograms after extraction process, was used for GC analysis.

4.2.4 Instrumentation

All HPLC-UV analysis was performed on a fully automated Varian on-line HPLC system (Varian, Australia), including an automatic sample processor from Varian (Varian 9200 Prospect), a solvent delivery system (Varian 9012, Australia), a 10 µl Injector loop and a diode

array detector (DAD) (Varian 9065, Australia). The system was controlled and data analyzed using Varian Star Workstation software (Varian, Australia).

An Agilent 1100 Liquid Chromatograph coupled with an atmospheric pressure (AP)-electrospray ionization (ESI) mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) was used to detect any putative triclosan biodegradation by-products.

HP 6890 gas chromatograph (Agilent Technologies, USA) equipped with HP 5973 mass-selective detector was also used for the separation and detection of any putative triclosan biodegradation by-products.

4.3 Results

4.3.1 Triclosan detection in standard solution and quantification in bacterial culture by HPLC-UV

In this experiment, the HPLC was equipped with a Diode Array Detector (DAD), which allowed a range of wavelengths, in this case 200-400 nm, to be detected simultaneously at any point of time during separation process by HPLC.

Triclosan was detected by HPLC-UV at T=20.260 min as shown in Appendix 3. The identity was confirmed based on the characteristics of triclosan UV absorbance at 280 nm and 236nm, represented by a sharp peak at 280 nm and a shoulder at 236 nm (as shown in Appendix 2).

Triclosan concentration was determined by the area under the curve of the representative peak of this chemical on the absorbance spectrum.

The HPLC-UV method developed for the quantification of triclosan in bacterial culture was calibrated using a series of external standards spiked with known concentration of triclosan. A standard curve using these external standards was run and plotted with every assay of serially diluted triclosan bacterial culture samples. Triclosan concentrations of unknown samples were then calculated based on the standard curve run in the same batch. Individual recovery was assessed using external standards for each run, and every batch of assay was validated by acceptable recovery of above 80% with relative standard deviation below 10%. Unsatisfactory data were discarded.

During triclosan quantification in biological samples, all samples were serially diluted before assay, and the readings that fell within the middle range of the calibration curve obtained using external standards tested in the same batch were then chosen for the calculation of the triclosan concentration for this sample, taking into account the dilution factor. This increased the accuracy and consistency of the test results.

Signal suppression was observed during the method establishment phase without sample dilution, so 1 in 4 (sample/methanol vol/vol) dilution using methanol was incorporated in the sample preparation procedure. The powerful equipment and solvent with optimised gradient in the method establishment phase was found to have effectively minimised signal suppression by allowing interfering compounds to be separated as a front peak in the chromatograph as shown in Appendix 3. Additional dilution applied for quality control, as described in Section

4.2 also assisted to rectify signal suppression problem. A blank as well as a control series of bacterial culture, as discussed in Section 4.3.2, was also included as a quality control measure.

Standard curve for triclosan quantification

Standard solutions containing 0.05, 0.10, 0.50 and 1.00 µg/mL of triclosan in ACN were prepared. Three replicate injections for each concentration were performed. The absolute recoveries were calculated.

Table 6 Data used for standard curve for triclosan quantification

Triclosan (µg/mL)	Mean peak area	%RSD Relative standard deviation
0.05	12.182	4.8
0.1	29.010	19.4
0.5	135.825	1.6
1	268.267	1.8

Table 6 shows the calculated mean peak areas of triclosan for each of the four standard solutions. The calibration curve is a plot of triclosan concentration versus the mean peak area of the same.

Triclosan standard calibration curve determined by HPLC-UV was linear from 0.05 to 1µg/mL with a coefficient of 0.9998. External standards containing triclosan at similar concentration of the tested samples were used as a quality control for the quantification of triclosan. A standard curve with suitable concentration external standard was repeated with every run of triclosan detection to minimize system errors and to produce more reliable and consistent data. Standard curves obtained in each assay were analysed and data were discarded if the standard curve R^2 was less than 0.995.

Recovery for triclosan quantification

The concentration of triclosan in standard solutions was calculated from the mean peak area of

three replicate extractions and the recovery analysis was carried out at concentrations of 0.1 and 0.2 µg/mL with triplicates for each concentration. An example of the recovery obtained for one of the assays is presented in Table 7.

Table 7 Triclosan recovery in standard solutions

Triclosan level (ug/mL)	**% Recovery**	**% RSD**
0.1	83.02	6.0
0.2	87.83	3.6
Mean	**85.42**	**4.8**

The recovery for both concentrations are above 80% with a mean RSD of 4.8%, which is considered acceptable, given the tendency of triclosan to attach onto the surface of plastic parts of the instrument.

4.3.2 Triclosan biodegradation intermediates/by-products analysis using LC-ESI-MS

A putative triclosan degrader was inoculated into modified minimal medium containing 100 mg/L triclosan and incubated overnight. Samples were then tested using Liquid chromatography. Results for samples using F11 and F12 as inoculum are shown in Figure 15 and Figure 16.

A series of controls was included to identify any natural degradation of triclosan in minimal medium and potential signal suppression or enhancement. The same triclosan minimal medium and minimal medium without triclosan were used separately as controls, but no bacteria were inoculated into the media. It was found that after overnight incubation, no significant degradation of triclosan had occurred (shown in Figure 13), represented by the presence of triclosan peak and the absence of obvious additional peaks in the sample containing triclosan but no inoculum.

Potential non-triclosan degraders, unidentified but isolated from the wastewater lagoon samples, were also inoculated into the same medium with and without triclosan as another control series to observe any potential metabolites that may be produced by bacterial growth on other potential contaminants. No significant peak was detected as shown in Figure 14.

For each sample undergoing HPLC for biodegradation intermediate testing, a blank series was also included for comparison to demonstrate that the emerging peaks represented the degradation intermediates from triclosan as distinguished from other metabolites from potential contaminants. The liquid chromatography of sample F11 and the blank overlaps in Figure 15, and additional peaks can be observed in sample F11 but not in the blank (Figure 15) or control series (Figure 13). This indicates the emerging peaks (as shown in Figure 15), which are highlighted by green arrows, represent the metabolites of triclosan biodegradation as a result of bacterial growth of F11 in triclosan minimal medium.

Similar TIC pattern is also seen for samples inoculated with F12 strain (shown in Figure 16), although there seems to be some carry-over of elutes for certain peaks during the separation process by HPLC, as highlighted by black arrows in Figure 16 for the blank series. It is worth noting that the carry-over observed here was not due to cross-contamination, but due to the sequence of analysis. The assay was carried out with 20-40 samples with blanks and controls each time, and it was difficult to give longer elution time to minimise this carry-over. In fact

40 mins were used, but due to the extreme hydrophobic property of the compound, the attempt to eliminate the carry-over was unsuccessful. However, the assay was developed for intermediate characterisation only not for any quantitative analysis; therefore this carry-over should not be of significant concern and is considered acceptable and will not affect the interpretation of the results.

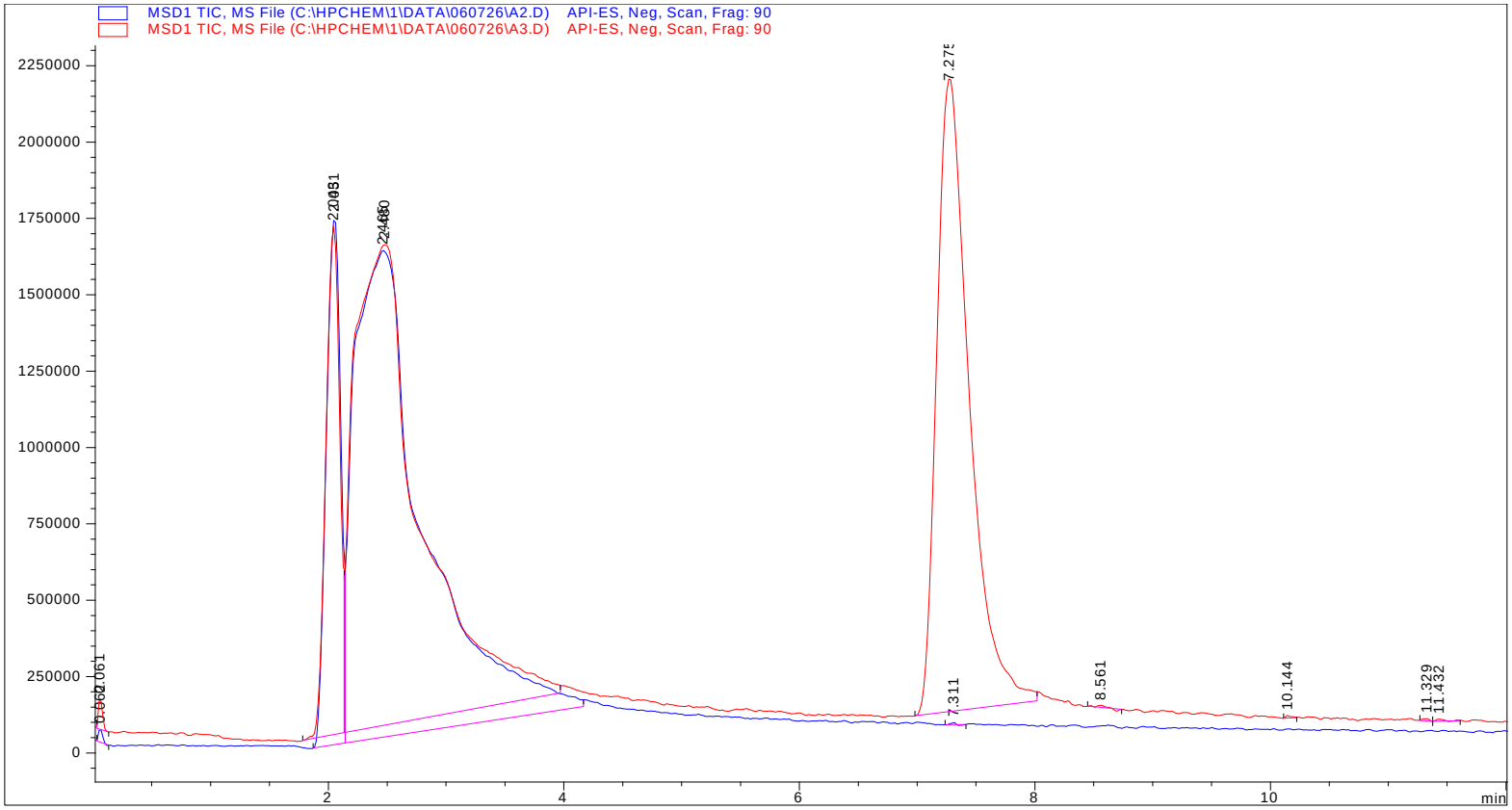


Figure 13 Total ion chromatogram of system controls
(Obtained using Alltech C8 analytical column at flow rate of 1 ml/min of a binary gradient eluant composed of 20 mM NH_4Ac and 100% ACN at a gradient of 0% ACN to 100% ACN in 30 min, where the samples were diluted at 1:4 ratios with methanol; Controls with no bacteria inoculated into the triclosan minimal medium and minimal medium without triclosan)

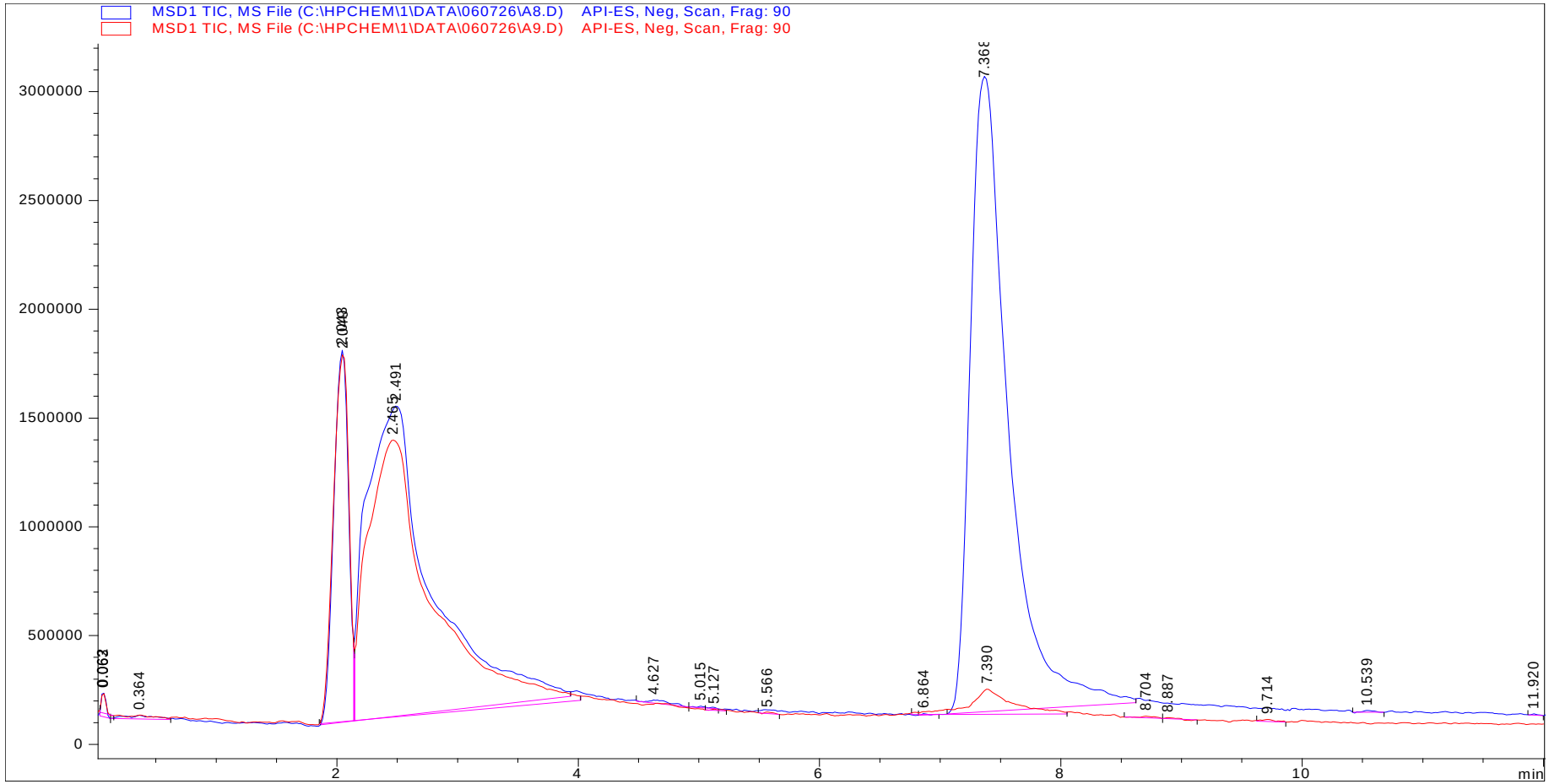


Figure 14 Total ion chromatograms for samples inoculated with non-degraders

(Obtained using Alltech C8 analytical column at flow rate of 1 ml/min of a binary gradient eluant composed of 20 mM NH4Ac and 100% ACN at a gradient of 0% ACN to 100% ACN in 30 min, where the samples were diluted at 1:4 ratios with methanol)

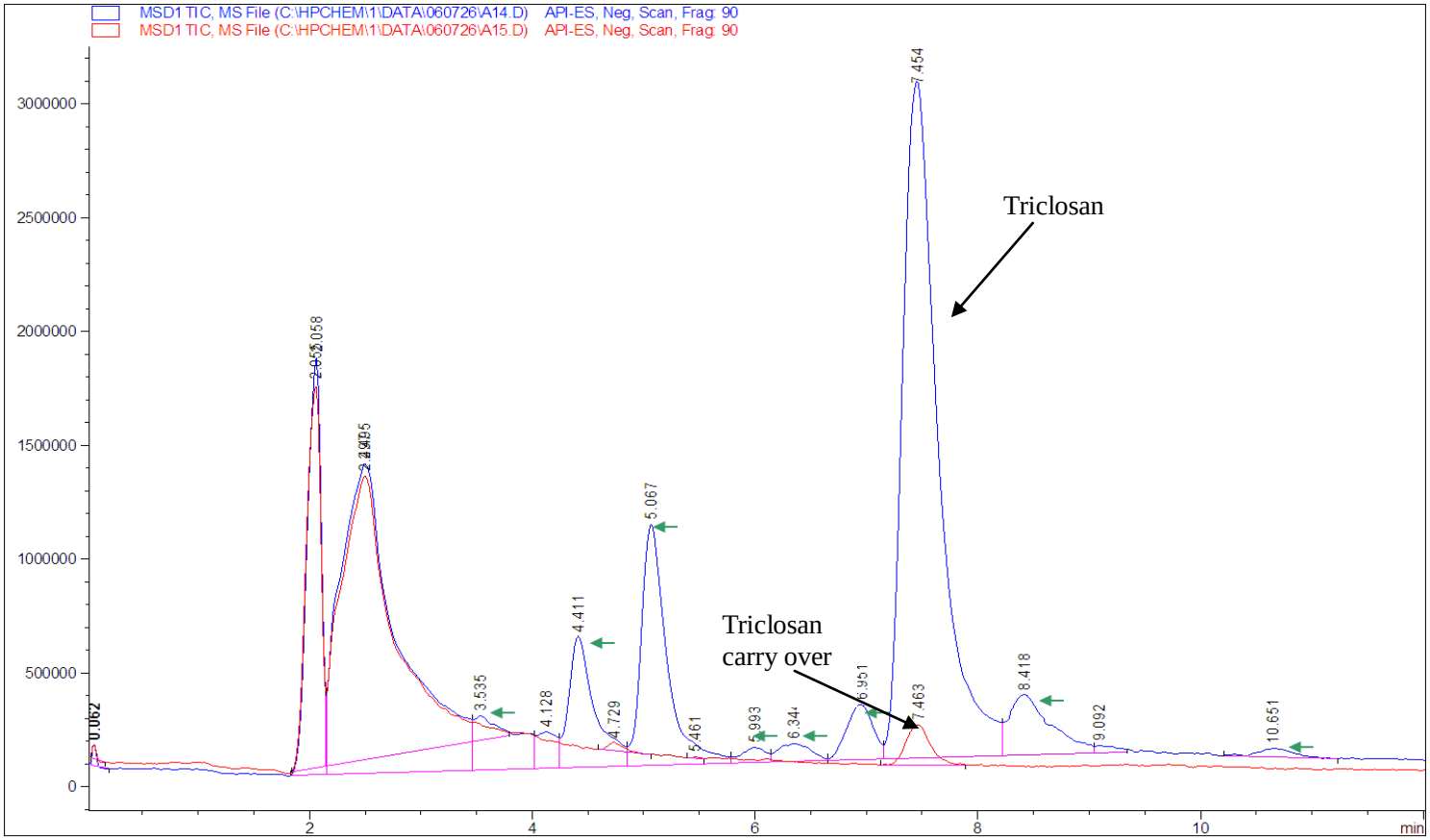


Figure 15 Total ion chromatograms showing the production of biodegradation intermediates as a result of bacterial growth (F11) in triclosan medium

(Obtained using Alltech C8 analytical column at flow rate of 1 ml/min of a binary gradient eluant composed of 20 mM NH4Ac and 100% ACN at a gradient of 0% ACN to 100% ACN in 30 min, where the samples were diluted at 1:4 ratios with methanol; Blue: inoculated with F11 strain in triclosan minimal medium; Pink: inoculated with the same culture but no triclosan in medium; Green arrows: Potential biodegradation intermediates)

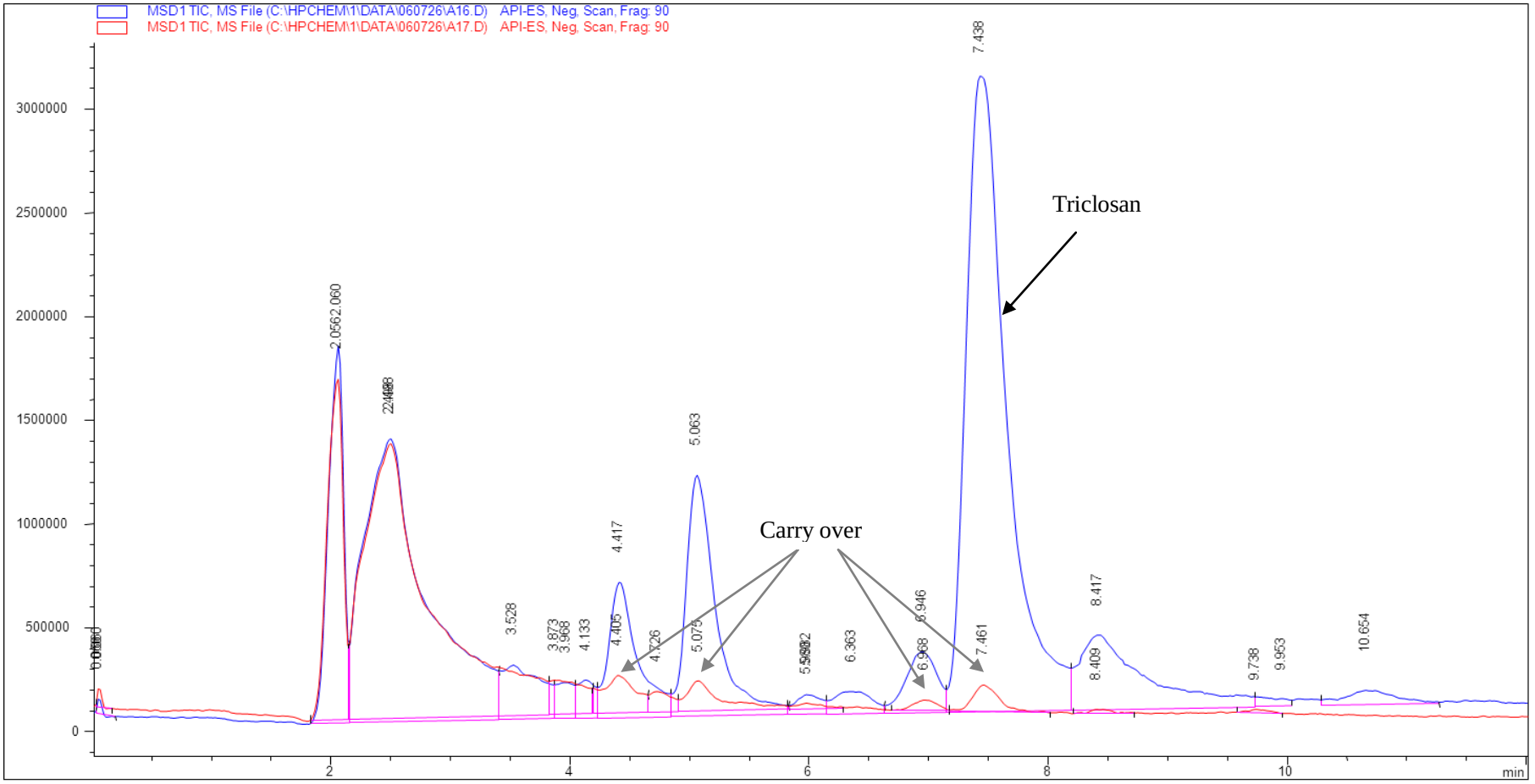


Figure 16 Total ion chromatograms showing the production of biodegradation intermediates as a result of bacterial growth (F12) in triclosan medium

(Obtained using Alltech C8 analytical column at flow rate of 1 mL/min of a binary gradient eluant composed of 20 mM NH4Ac and 100% ACN at a gradient of 0% ACN to 100% ACN in 30 min, where the samples were diluted at 1:4 ratios with methanol; Blue: inoculated with F12 strain in triclosan minimal medium; Pink: inoculated with the same culture but no triclosan in medium)

As described above, when bacterial strains F11 and F12 were inoculated into triclosan minimal medium, the growth of these isolates resulted in the typical chromatogram as shown in Figure 15. This further confirmed that these isolates were potential triclosan degraders and were included in the subsequent analyses.

Mass and DAD graphs were obtained for new peaks in the typical chromatogram shown in Figure 15, but triclosan degradation by-products could not be identified immediately due to the lack of LC-MS library. EIC was extracted, and putative intermediates were tentatively predicted using molecular isotope pattern of potential intermediates of a certain molecular mass. The isotope pattern was calculated using an online software Isotope Pattern Calculator v4.0 (http://www.geocities.com/junhuayan/pattern.htm).

The largest peak in Figure 15 was predicted to be triclosan. As shown in Figure 17 the isotope pattern of triclosan has a profile of mass 288 100%, 290 97.1%, 291 12.7%, 292 31.8%, which corresponds to the mass spectrum and the DAD profile at the corresponding mass channel. There is an overall 1 *m/z* difference in mass between the isotope (at mass 288, 290, 291 and 292) and mass spectrum (at mass 287, 289, 290 and 291) for this peak, which is expected as a result of the negative ionisation mode used in the instrument settings.

Similarly, as shown in Figure 18, the peak at 7.39 min was tentatively predicted to be a putative intermediate having a molecular structure shown in the isotope pattern graph of this peak. This is the first level tentative intermediate as illustrated in the triclosan degradation pathway proposed (as discussed in the later chapter). This molecule has molecular formula $C_{12}H_7Cl_3O_3$ with a molecular weight of 305.5 and exact mass of 303.9 as predicted using the same Isotope Pattern Calculator. It was named as 2',4'-dichlorophenyl 4-chloro-6-oxo-2,4-hexadienoate based on general chemical nomenclature rules.

Similarly, as shown in Figure 19, the peak at 2.491min was tentatively predicted as a putative degradation by-product having a molecular structure shown in the isotope pattern graph of this peak (known as 4-methylene but-2-en-4-olide or Protoanemonin). This is likely to be the end product as illustrated in the triclosan degradation pathway proposed (as discussed later in the chapter). This molecule has molecular formula as $C_5H_4O_2$ with molecular weight of 96.1 and exact mass of 96.0 as predicted using the same Isotope Pattern Calculator.

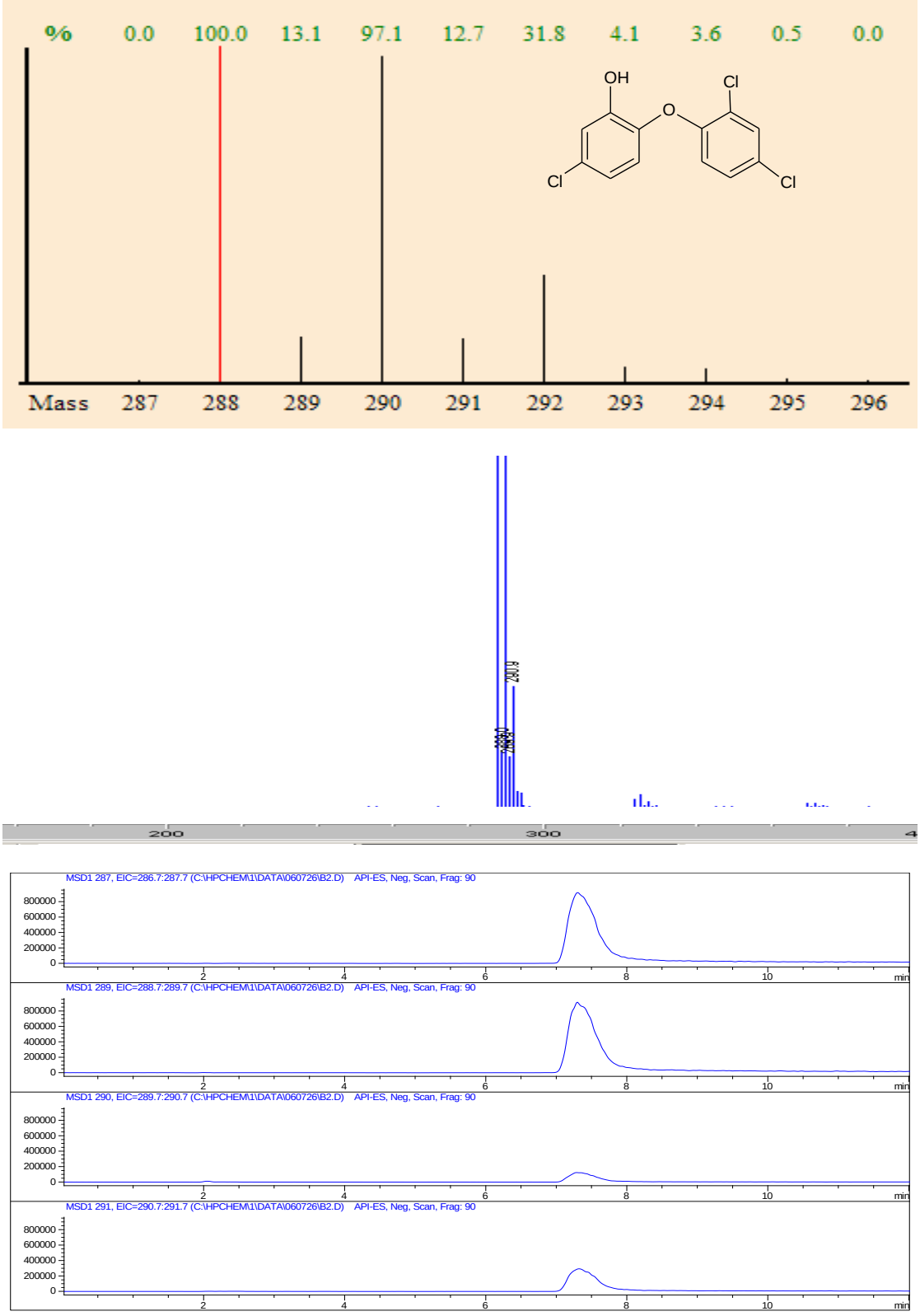
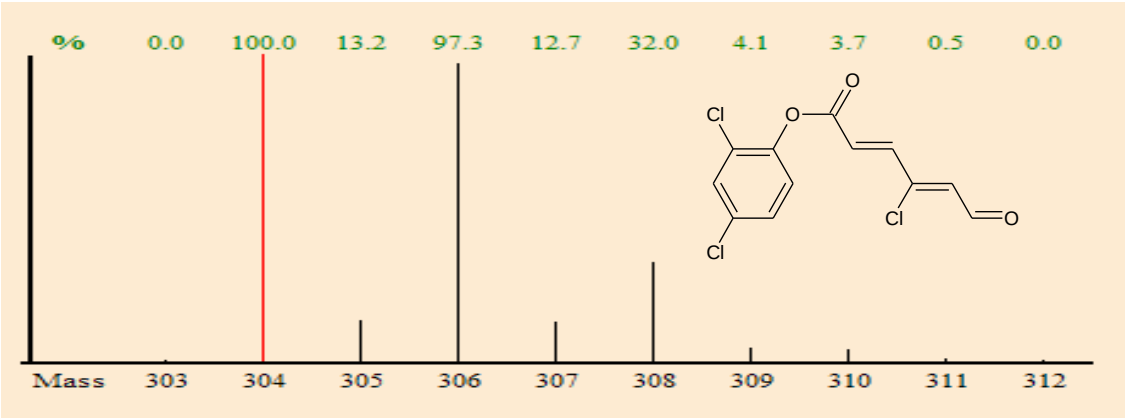
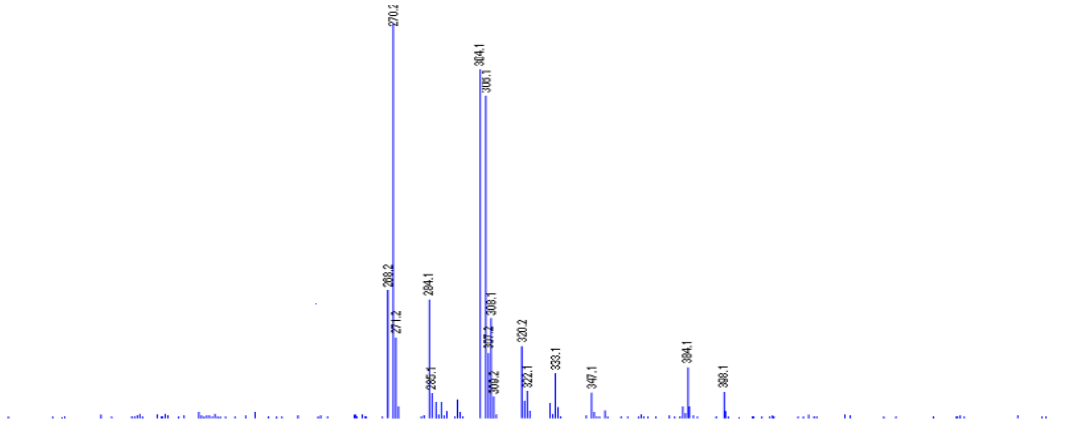


Figure 17 Prediction of triclosan (peak at 7.390min) using isotope pattern, mass spectrum and DAD pattern





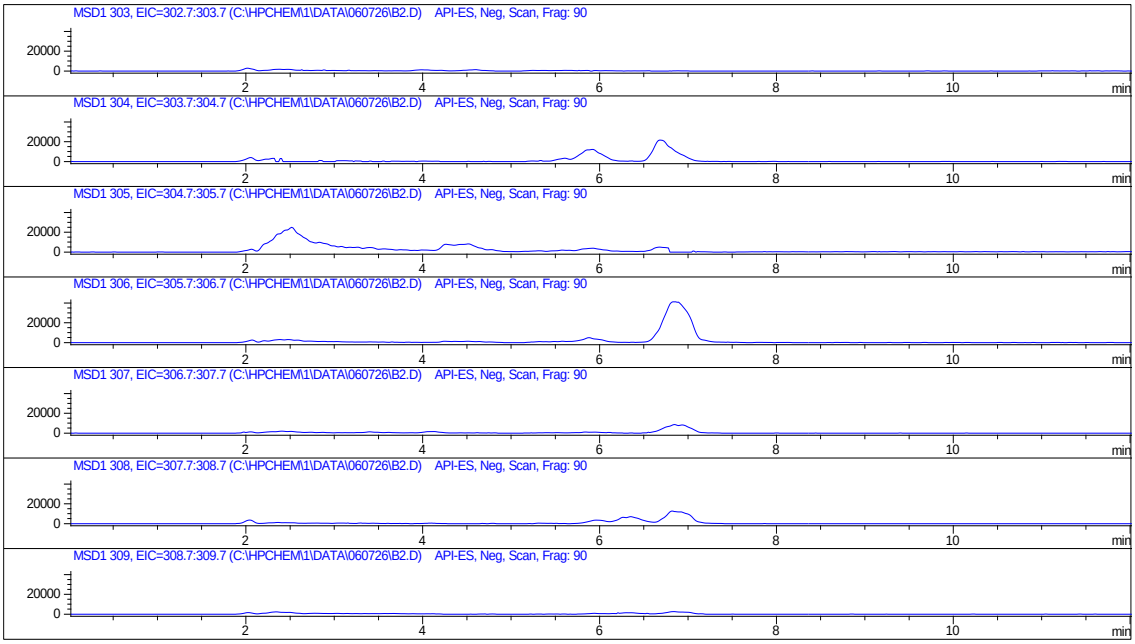
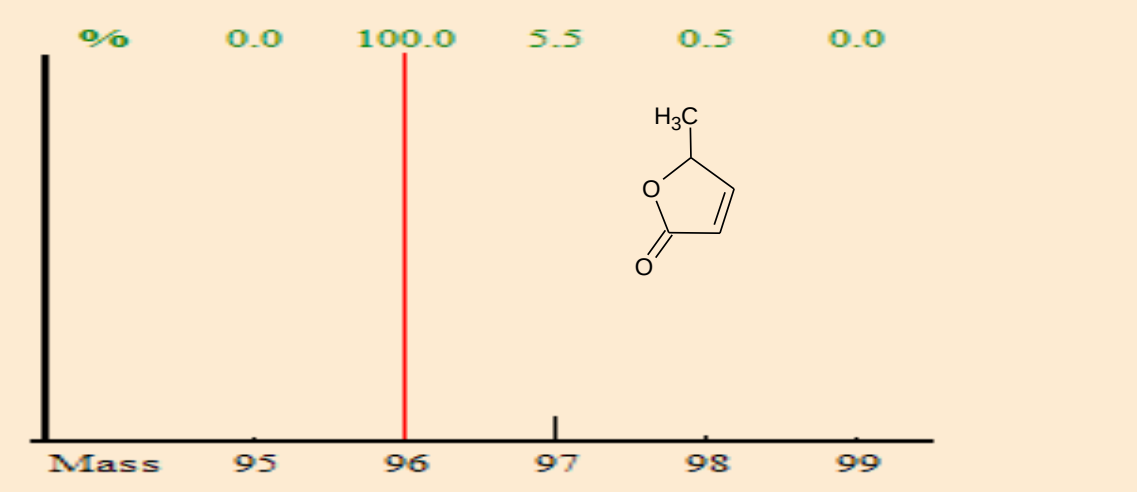


Figure 18 Prediction of peak at 6.951min using isotope pattern, mass spectrum and DAD pattern



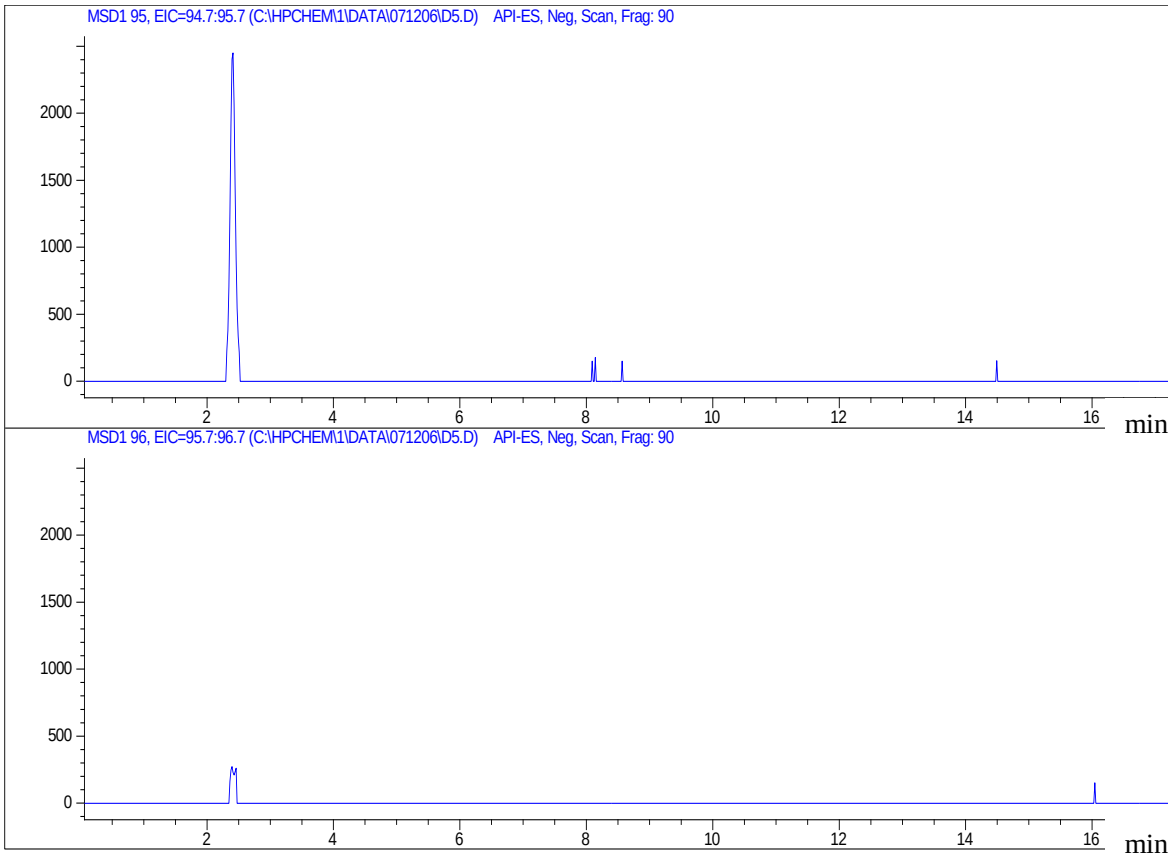


Figure 19 Prediction of peak at2.491min using isotope pattern and mass spectrum

4.3.3 *Triclosan biodegradation by-products analysis using GC-MS*

As described previously, the HPLC analysis further confirmed the degradative isolates, and identified triclosan as well as two putative degradation intermediates. The liquid chromatograph also showed some additional peaks that might potentially represent triclosan biodegradation intermediates or by-products. However, the identification of these compounds was limited with this method due to the lack of a full library. Attempts were therefore made to identify more biodegradation by-products/intermediates using GC-MS.

GC is capable of analysing smaller molecules than LC, provided the target molecules evaporate under high temperature. Therefore, the use of GC-MS has allowed the identification of triclosan degradation by-products, which are likely to be smaller molecules than the intermediates.

The remaining culture samples from previous HPLC analysis, which showed typical chromatograph as shown in Figure 15 and Figure 16, were subject to further GC-MS analysis. The organic compounds in minimal medium culture were extracted using hexane and dichloromethane (DCM) for comparison. In order to assess the extraction efficiency, HPLC analysis was carried out on the aqueous phase prior and post extraction. As organic compounds were expected to be extracted into the solvent phase, disappearance of peaks in total ion chromatogram by HPLC should be seen for the more efficient solvent. The HPLC chromatograms for sample prior to extraction and the aqueous phase of sample post extraction by hexane and DCM are shown in Figure 20 and Figure 21, respectively. It can be observed that the extraction using hexane was insufficient, and DCM extraction achieved much higher efficiency, where most of the peaks in TIC disappeared (as shown in pink compared to the initial TIC in blue in Figure 21) after the extraction.

The extract from DCM extraction was then applied to GC-MS. The chromatograph is shown in Figure 22. The characteristic peaks were then matched against known molecules in the MS library for identification. Despite the limited GC-MS library data available, some of the structurally related peaks were identified as triclosan (shown in Figure 23) and 2, 4-dechloraphenol (shown in Figure 24). The compound representing a characteristic peak at 7.463 min on the GC chromatogram shows the closest match to trichlorobenzene; however, there are slight discrepancies between the GC spectrum of the characteristic peak and the reference library data for trichlorobenzene (shown in Figure 25). Therefore, the peak was

tentatively predicted to be triclorobenzene, however, it is likely that the unknown compound is not triclorobenzene but a compound having close mass spectrum to triclorobenzene.

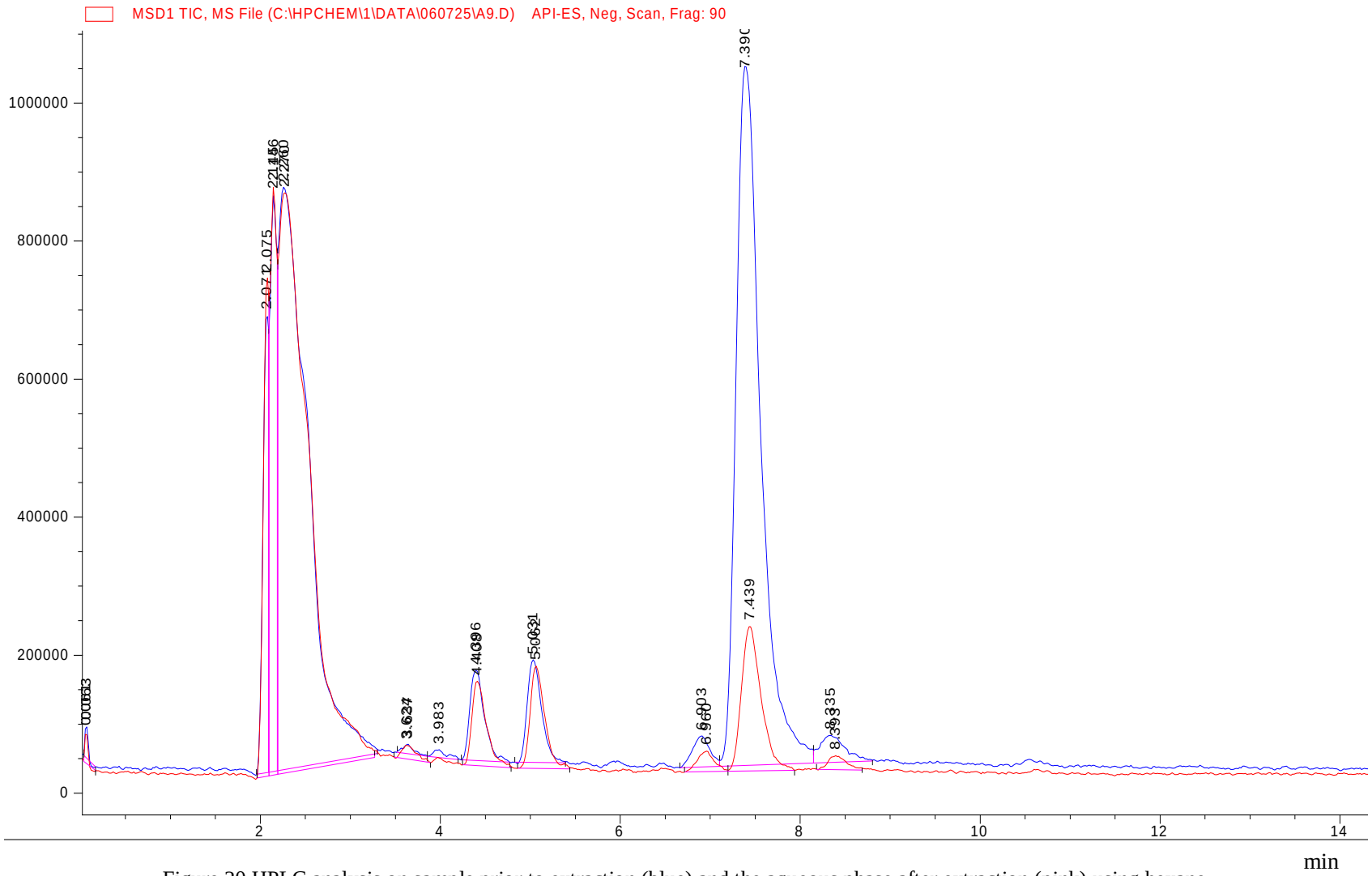


Figure 20 HPLC analysis on sample prior to extraction (blue) and the aqueous phase after extraction (pink) using hexane

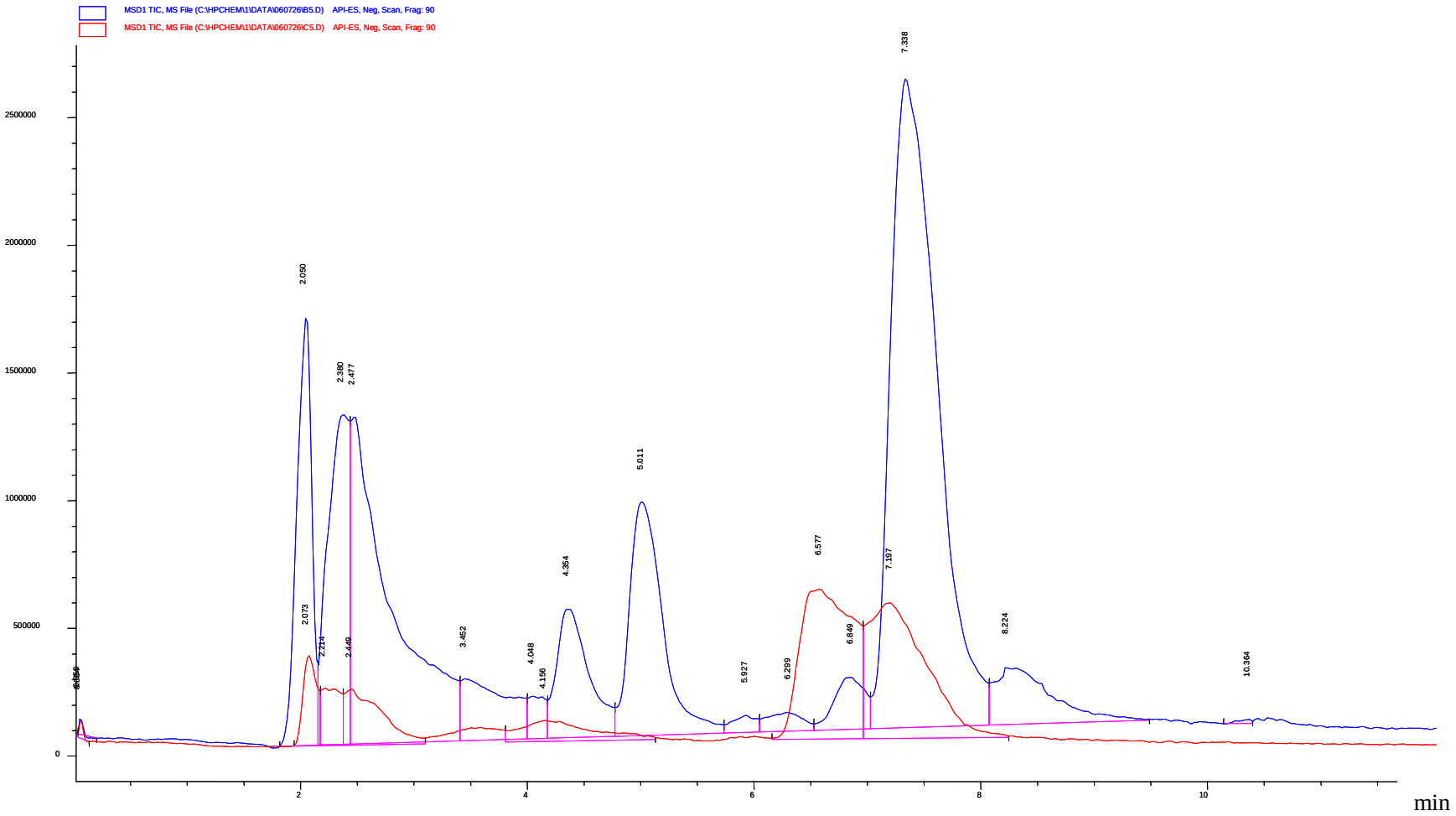


Figure 21 HPLC analysis on sample prior to extraction (blue) and the aqueous phase after extraction (pink) using DCM

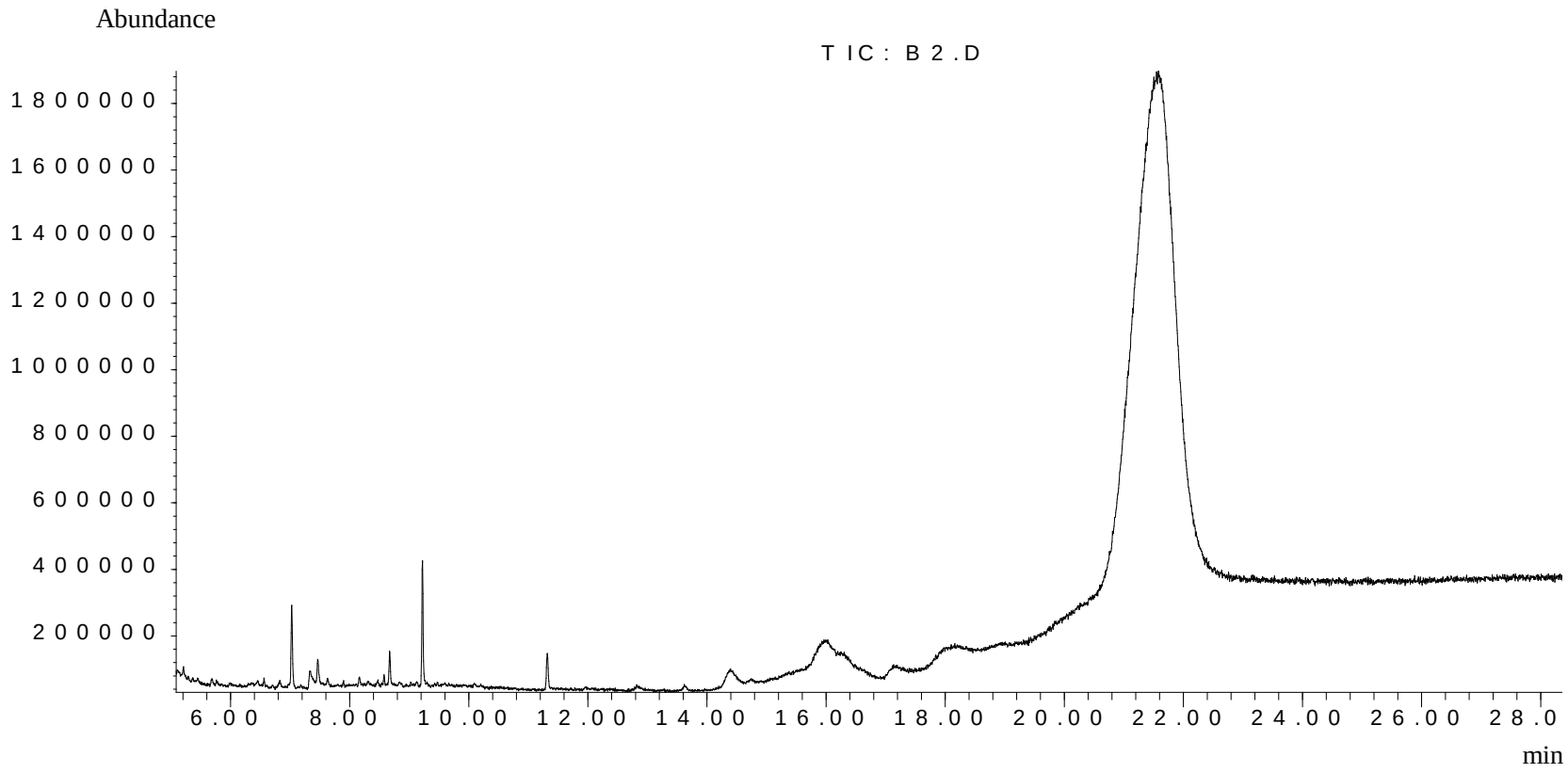
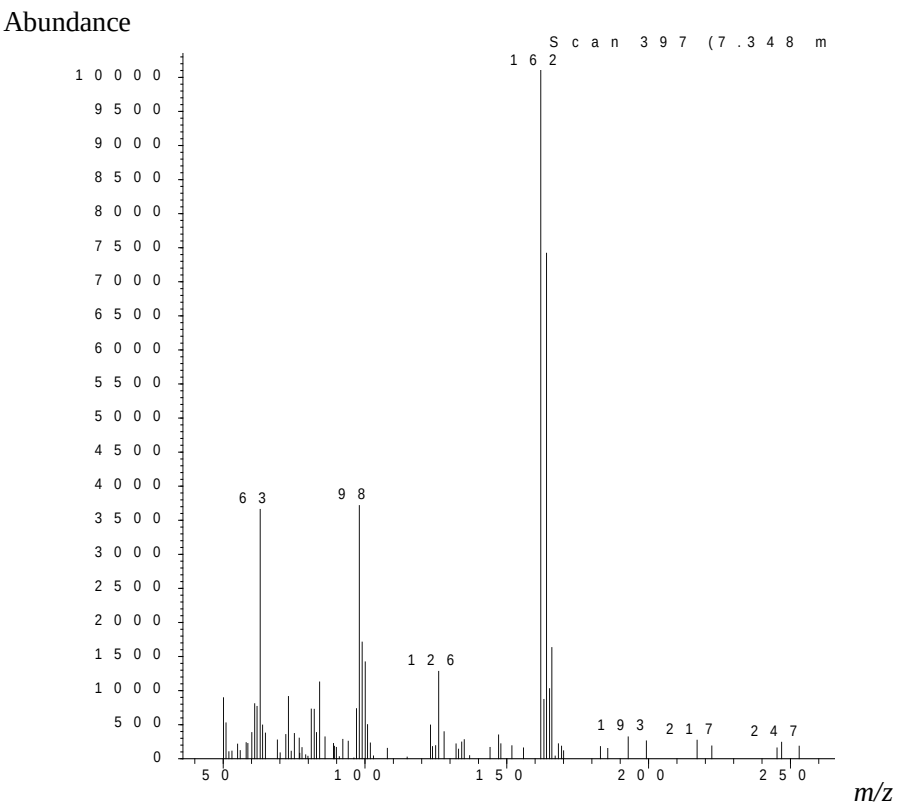
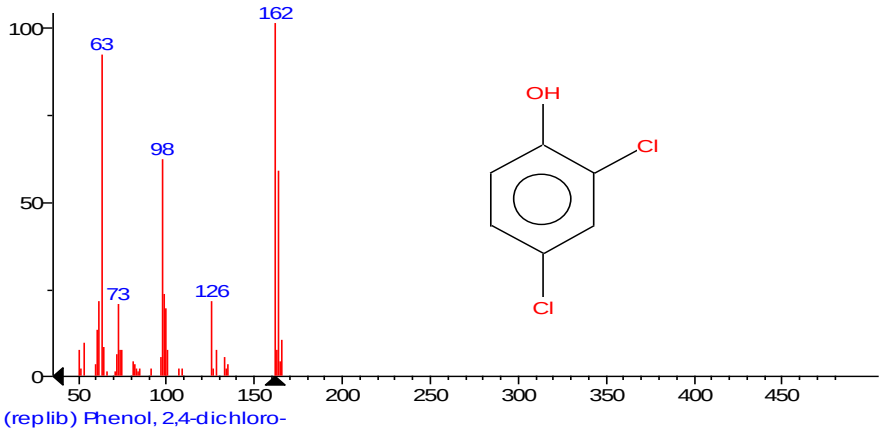


Figure 22 GC chromatogram of DCM extract from sample confirmed as containing biodegradation intermediates by HPLC

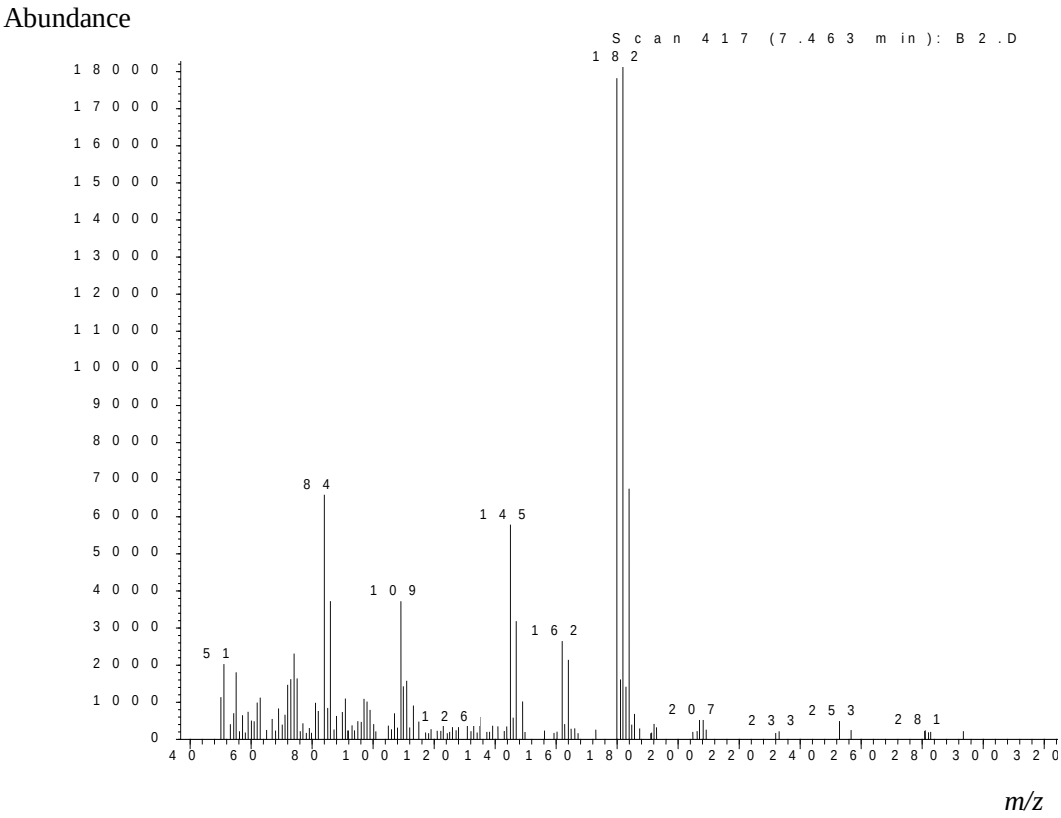


a. Mass spectrum of unknown peak for identification

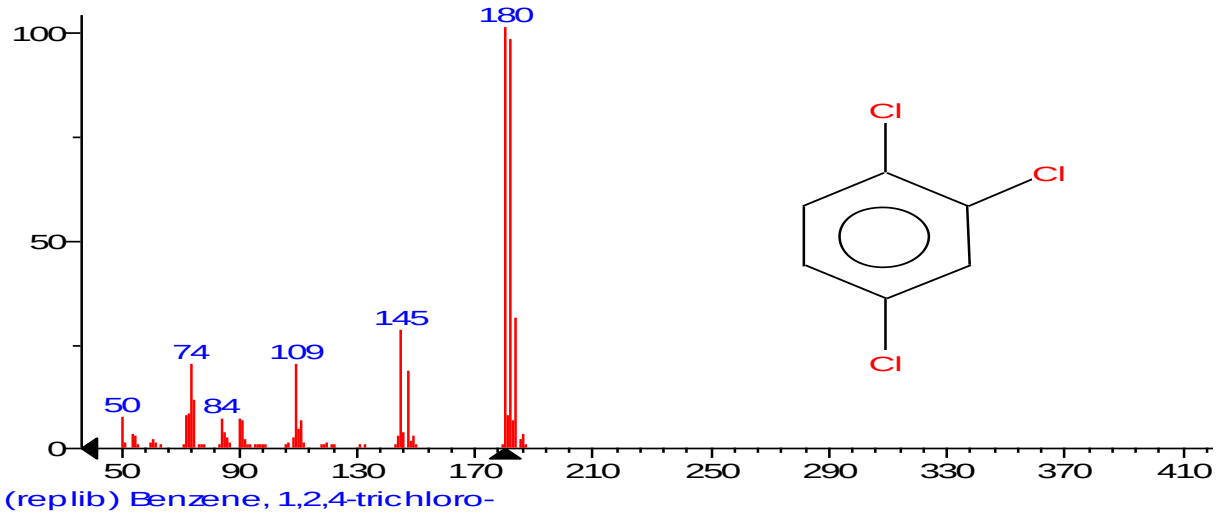


b. Mass specturm for standard molecule 2,4-dichlorophenol in the library database

Figure 23 Mass spectrum of Peak at 7.348 min on GC chromatogram

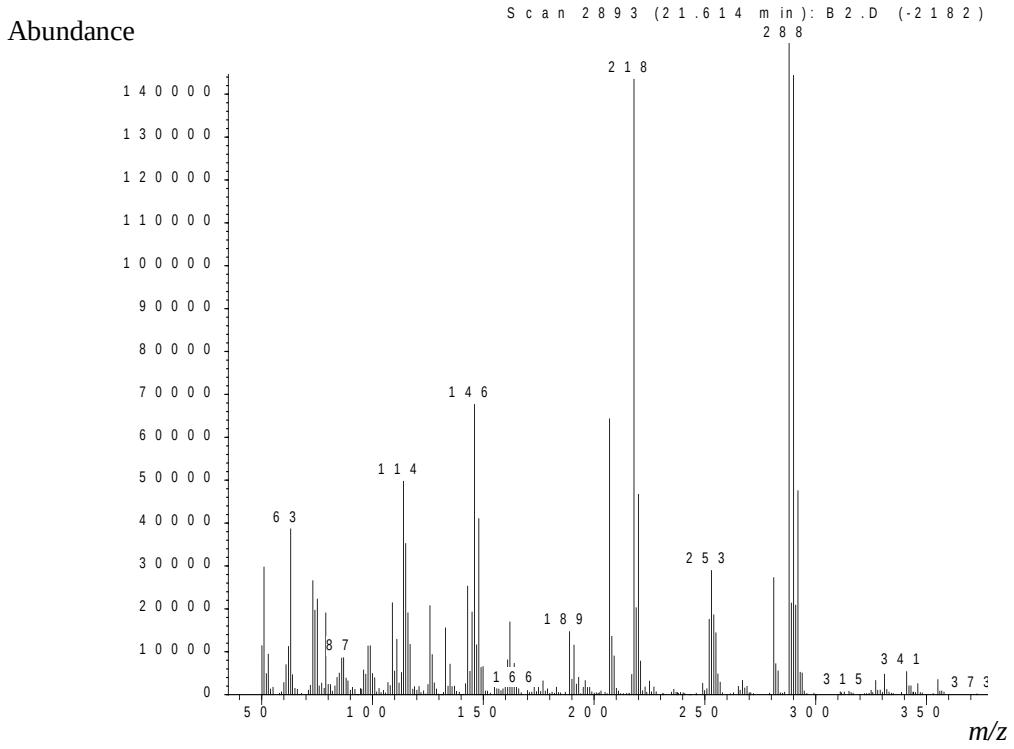


a. Mass spectrum of unknown peak for identification

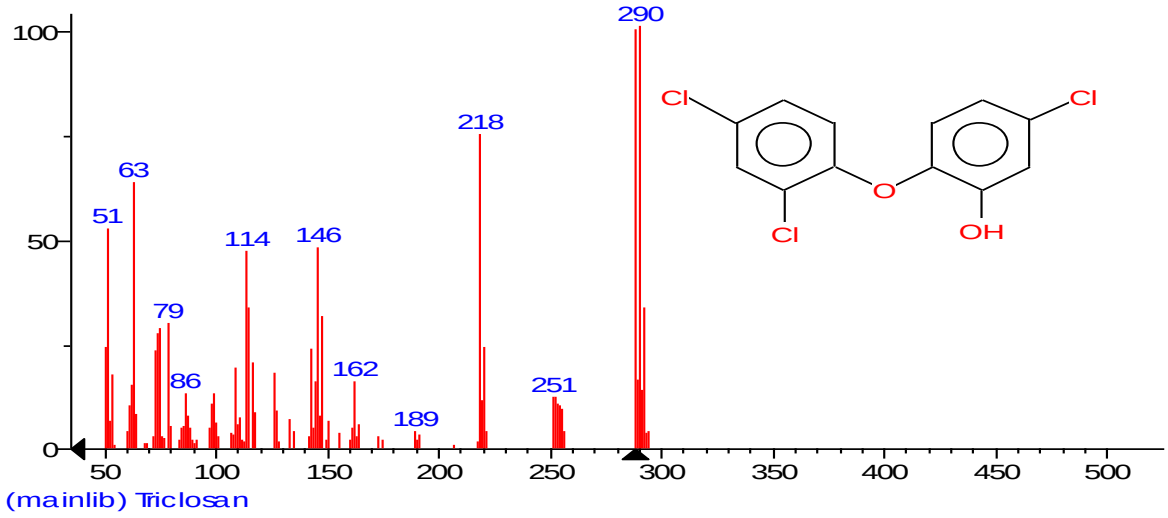


b. Mass specturm for standard molecule triclorobenzene in the library database

Figure 24 Mass spectrum of Peak at 7.463 min on GC chromatogram



a. Mass spectrum of unknown peak for identification



b. Mass specturm for standard molecule triclosan in the library database

Figure 25 Mass spectrum of Peak at 21.614 min on GC chromatogram

4.4 Discussion

The putative triclosan biodegradation intermediates or by-products were tentatively identified as $C_{12}H_7Cl_3O_3$ (2',4'-dichlorophenyl 4-chloro-6-oxo-2,4-hexadienoate), Proto-anemonin, dichloro-phenol and an unknown compound having close mass spectrum to triclorobenzene using LC-MS (shown in Figure 26) and GC-MS (shown in Figure 27 and Figure 23).

Although additional interesting peaks were revealed during LC and GC analysis, none matched reference compounds in the data base. Therefore no additional predictions on other peaks could be made conclusively in this study, which leaves this topic open for more potential research opportunities. With the improvement in the instrument analysis technology and the understanding of triclosan degradation pathway, it is anticipated that more triclosan biodegradation intermediates or metabolic by-products may be characterised in the future.

Intermediate 2',4'-dichlorophenyl 4-chloro-6-oxo-2,4-hexadienoate identified in this study is a novel compound that has not been reported in any literature. In addition, triclosan degradation by-product proto-anemonin and dichloro-phenol do not show identity to published triclosan degradation metabolites at time of thesis submission. At the time of laboratory work and data analysis of this study, there had been no triclosan biodegradation metabolites identified (Hay et al., 2001, Meade et al., 2001, Roh et al., 2009). Methyl-triclosan, which was identified in a later study (Chen et al., 2011) as one biodegradation product in activated sludge under aerobic conditions, was not identified using the method employed in this study.

In Lee's most recent study (Lee et al., 2012), 2,4-dichlorophenol was confirmed as triclosan biodegradation product, which was also confirmed to be triclosan biodegradation product by *Pseudomonas citronellolis* F12 strain in this study. There were additional four metabolites tentatively identified in Lee's most recent study based on mass spectra and the fragmentation patterns (Lee et al., 2012). These included monohydroxy-triclosan, 6-chloro-3-(2,4-dichlorophenoxy)-4-hydroxycyclohexa-3,5-diene-1,2-dione, 3-chloro-4-(5,7-dichloro-3-oxo-2,3-dihydrobenzo[1,4]dioxin-2-yl)-2-oxobut-3-enal, and 3,5-dichloro-4,6-dihydroxycyclohexa-3,5-diene-1,2-dione. Additional attack by regionselective dioxygenase at the 2,3-position, resulted in additional –OH group attached to triclosan, i.e. monohydroxy-triclosan, preceded the ring cleavage. This intermediate product then underwent meta-cleavage pathway to form 2,4-dichlorophenol. Other proposed intermediates in this recent study, such as

6-chloro-3-(2,4-dichlorophenoxy)-4-hydroxycyclohexa-3,5-diene-1,2-dione and 3-chloro-4-(5,7-dichloro-3-oxo-2,3-dihydrobenzo[1,4]dioxin-2-yl)-2-oxobut-3-enal had similar, but non-identical structures to the degradation intermediates identified in this thesis. Additional –O– and =O group was attached to the aromatic ring prior to the ring cleavage as well as to the ring cleavage product, probably as a result of the initial hydroxyl attack that was not observed in this study. Another intermediate tentatively identified in Lee's study, 3,5-dichloro-4,6-dihydroxycyclohexa-3,5-diene-1,2-dione, was not detected in this thesis. Instead, proto-anemonin was identified in this thesis as the un-chlorinated end product, whereas Lee's study didn't characterise any un-chlorinated end product. An increase of chloride level was used as an indication of dechlorination (Lee et al., 2012).

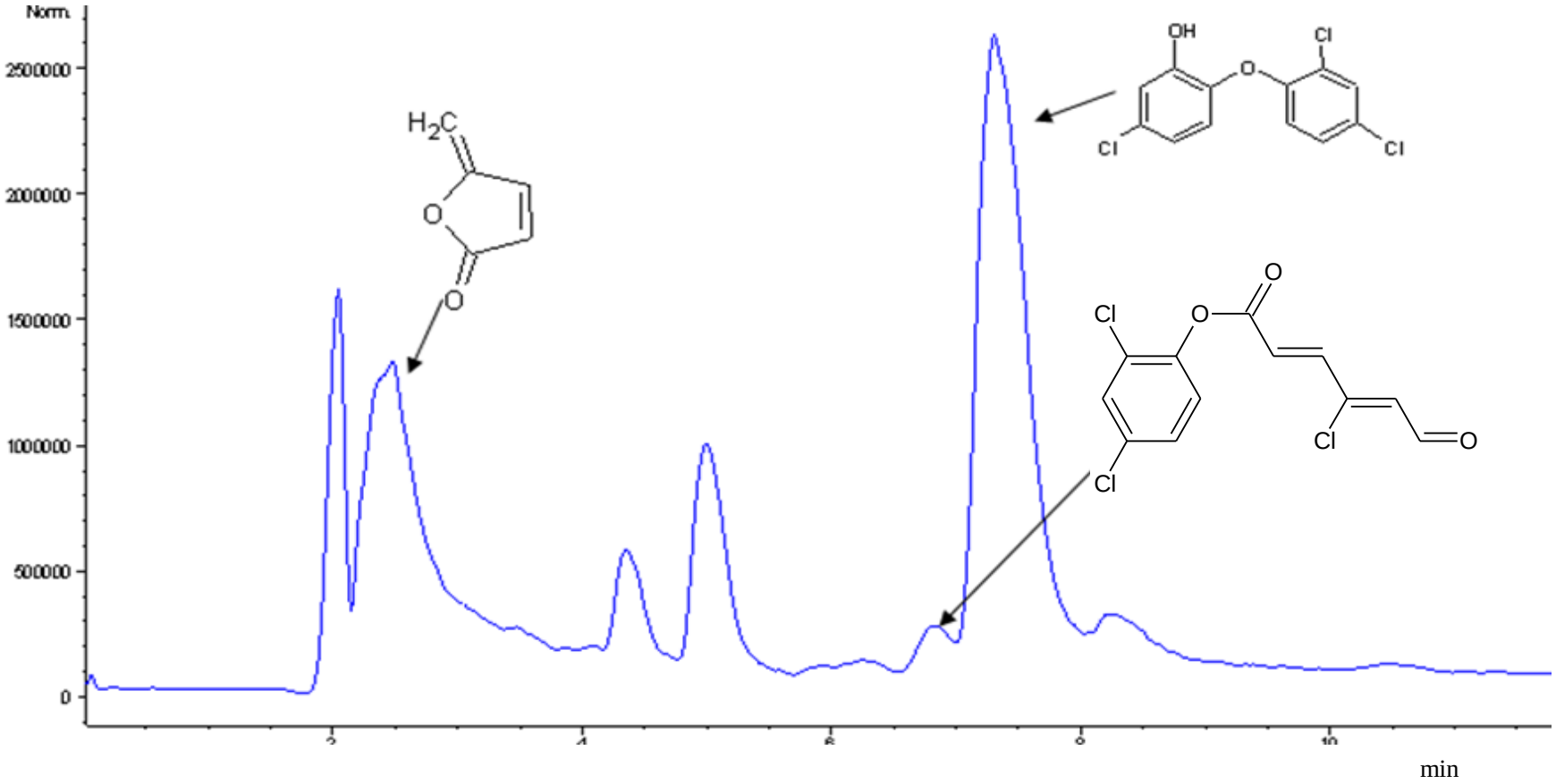


Figure 26 Putative triclosan biodegradation intermediates/by-products tentatively identified using LC-MS

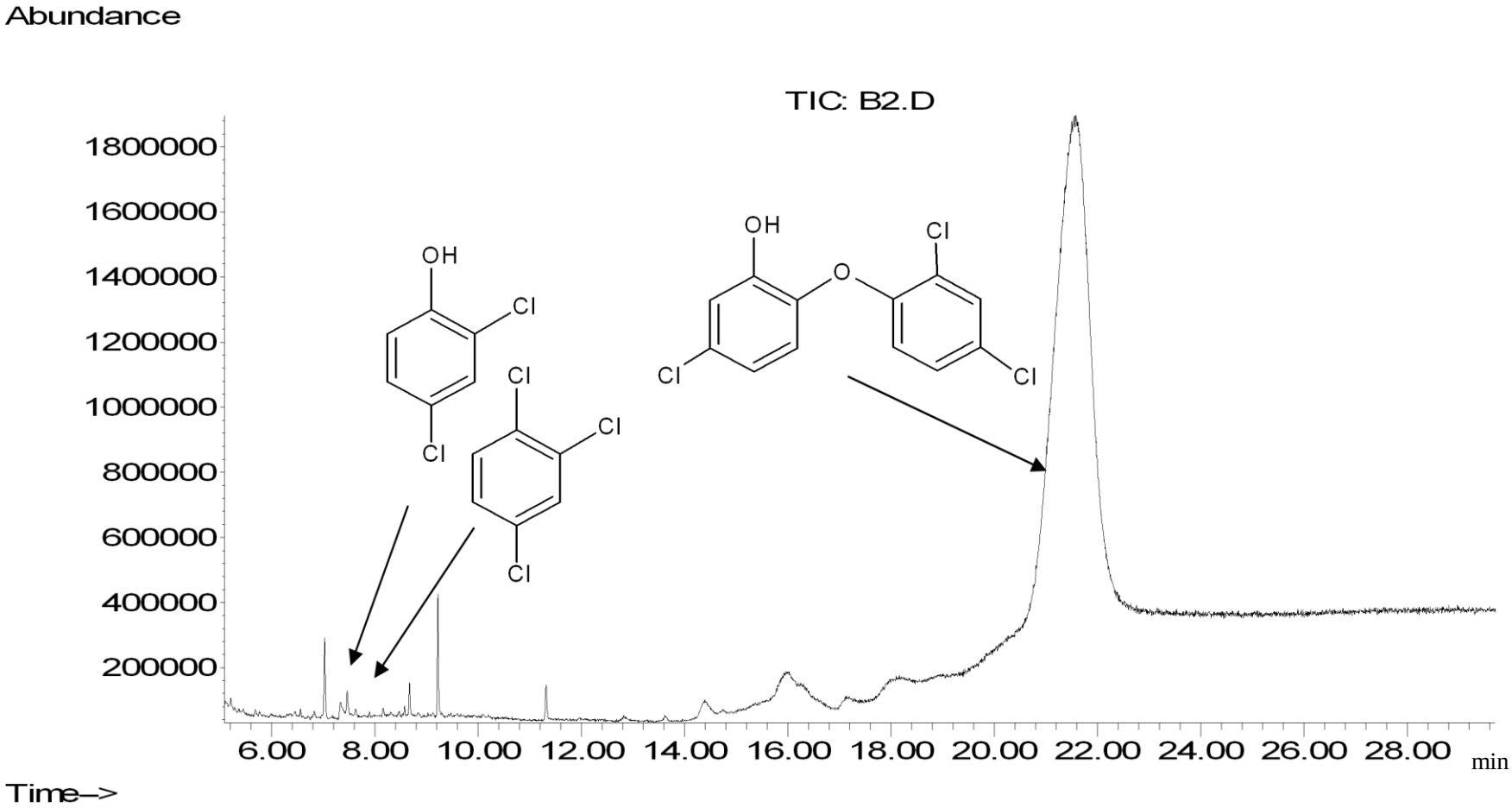


Figure 27 Putative triclosan biodegradation intermediates/by-products identified using GC-MS

The most likely triclosan degradation pathway is that triclosan undergoes a hydrolysis reaction and the ether bond breaks to form dichlorophenol and chloro catechol as by products (Figure 28). However, the intermediate analysis only confirmed the presence of dichlorophenol but no chlorocatechol was identified in the media.

Triclosan biodegradation by-products have not been characterised intensively; however, intermediates and by-products of oxidation by ferrate (Fe(VI)) were independently determined by GC-MS and rapid resolution liquid chromatography-tandem mass spectrometry in bench scale experiment, and the by-products were identified as Chlorophenol, 2-chlorobenzoquinone, 2,4-dichlorophenol and 2-chloro-5-(2,4-dichlorophenoxy)benzene-1,4-diol (Yang et al., 2011). None of these ferrate oxidation by-products were observed in the triclosan growth media in this study. Transformation products, 2,4-dichlorophenol, chlorocatechol, mono-hydroxy-triclosan and di-hydroxy-triclosan were identified as a result of ozonation of an aqueous solution of triclosan (Chen et al., 2012). These triclosan degradation products were formed through chemical oxidation processes, and they may be different to triclosan biodegradation by-products.

Chlorocatechols are toxic to most microorganisms and could inhibit subsequent metabolite reactions, therefore inhibiting the growth of bacteria or even completely killing the viable cells. However, it is possible that chlorocatechols maybe unstable in cultures and it could be further transformed to some other structure and eventually be metabolised to CO_2 and H_2O in a series of biochemical reactions. Similar reactions were observed in an earlier study on degradation of monochloro-biphenols. Isomers of chlorocatechols were produced and then *meta* cleaved. Toxicity of chlorocatechols was demonstrated by a diminished growth rate for cells exposed to 2-chlorobiphenyl, compared to a demonstrated decrease in viable cells following exposure to 3-chlorobiphenyl, which generates 3-chlorocatechol more rapidly than does 2-chlorobiphenyl (Arensdorf & Focht, 1994). Toxicity of intermediates has to be considered and well controlled when planning and interpreting biological degradation data.

The intermediate compound tentatively identified as trichloro-benzene is unlikely to be formed in the biodegradation of triclosan, as additional chlorination has to occur to lead to the production of trichloro-benzene. It is possible this mystery compound has a close MS pattern to trichloro-benzene but structurally slightly different and may be a further derivative of dichloro catechol, which is formed through the hydrolysis pathway. As shown in Figure 24, although similar, there is discrepancy of the MS spectrum of the unknown

compound in the media and the standard trichloro-benzene spectrum.

Although the hydrolysis of triclosan is possible to be the first step in its biodegradation pathway, there may also be alternative pathway used by *Pseudomonas citronellolis* strain F12 for the degradation of triclosan in minimal medium, based on results observed in the identification of other intermediates in this study.

The 2',4'-dichlorophenyl 4-chloro-6-oxo-2,4-hexadienoate was tentatively predicted to be present in the bacterial culture as a breakdown product of triclosan. This compound has a molecular weight close to triclosan, and contains a dichlorophenol structure; therefore, it is likely that the aromatic ring cleavage occurs before the hydrolysis reaction, in which 2',4'-dichlorophenyl 4-chloro-6-oxo-2,4-hexadienoate was further hydrolysed to release dichlorophenol as a biodegradation by-product. Protoanemonin was also identified to be present in the bacterial culture as a result of triclosan biodegradation. Based on these results, a potential triclosan biodegradation pathway is proposed as shown in Figure 28.

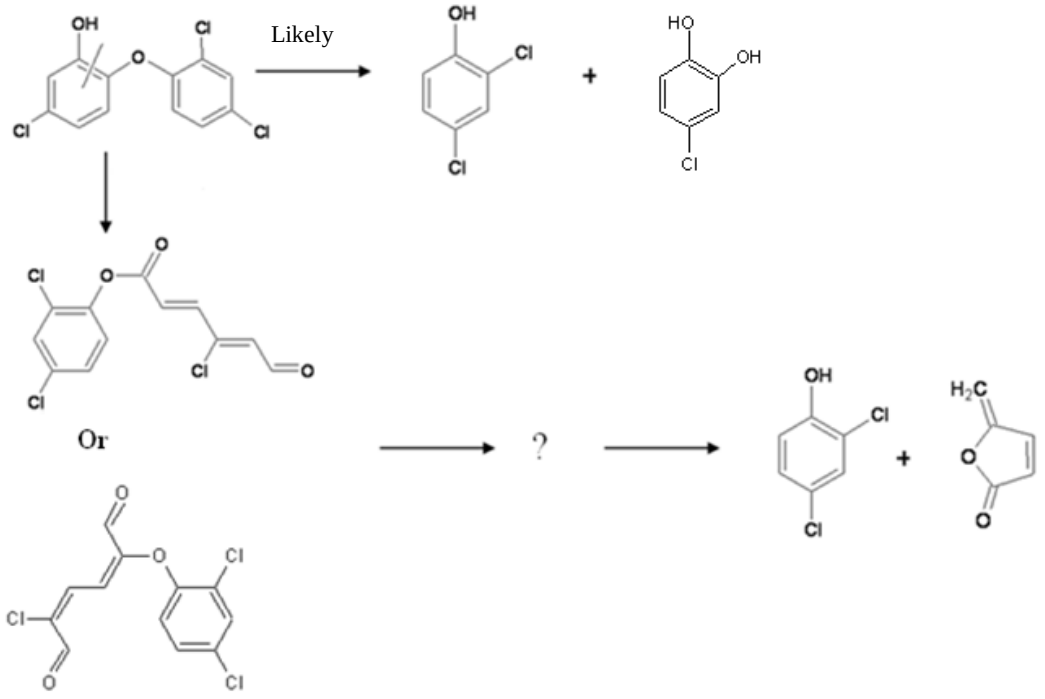


Figure 28 Potential triclosan biodegradation pathways

This alternative triclosan biodegradation pathway may involve the following three steps:

1. Aromatic ring cleavage to form 2',4'-dichlorophenyl 4-chloro-6-oxo-2,4-hexadienoate;
2. Hydrolysis to form 2',4'-dichlorophenol;

3. Conversion of hydrolysis product to 4-methylenebut-2-en-4-olide (protoanemonin).

Aromatic ring cleavage

The benzene ring is central to the structure of aromatic compounds, and the biodegradation of this structure is essential to the biodegradation of aromatic compounds. Investigations started in the 1950s, and it was found that the aromatic ring undergoes oxidation to form catechol as the initial step of the biodegradation pathway, and undergoes further ring cleavage via the catechol ortho- (or intradiol-) cleavage or the catechol meta- (or extradio-) cleavage pathway as illustrated in Figure 29 (Smith, 1990).

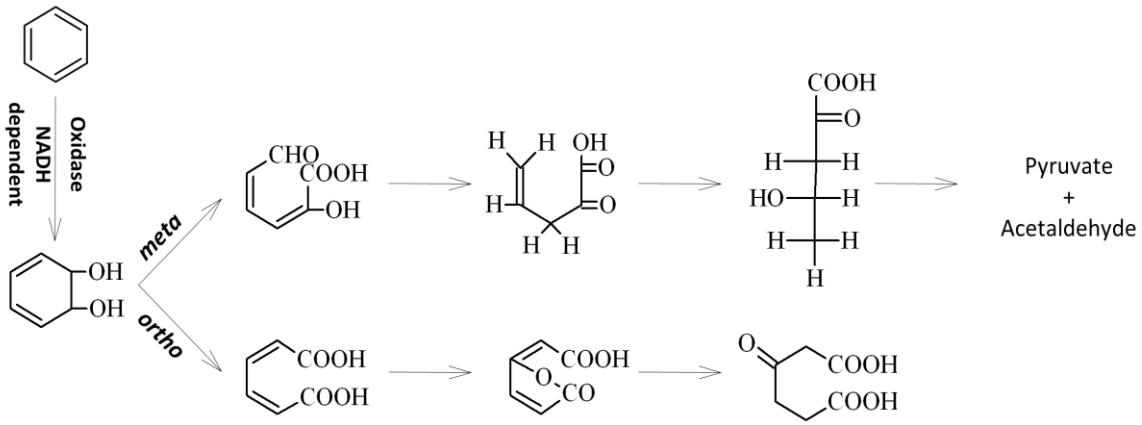
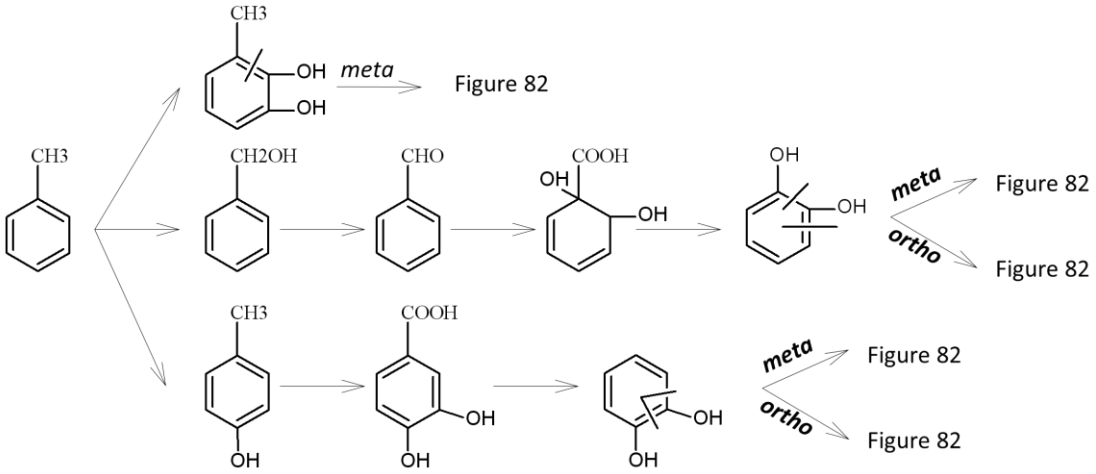
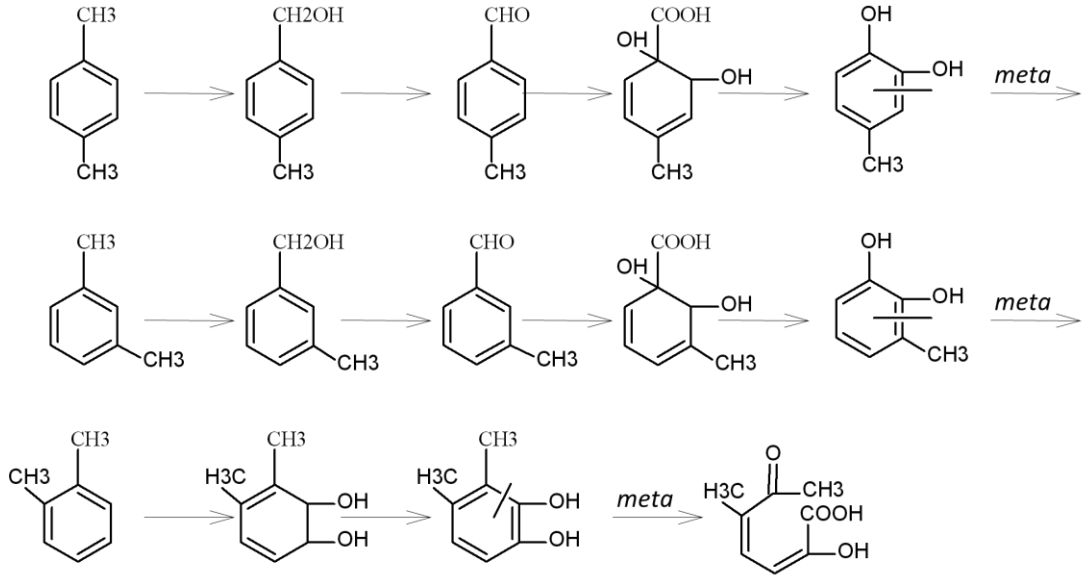


Figure 29 Catechol *meta*- and *ortho*- cleavage pathway (Smith, 1990)

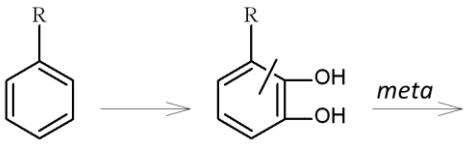
These two aromatic ring cleavage pathways form the centre of aromatic compounds biodegradation pathway. As shown in Figure 30, many aromatic compounds with substitutes undergo substitute modification, then form catechol or substituted catechol under the activity of different oxidases and dehydrogenases (Shields et al., 1991). The products of the upper degradation pathway, catechol or substituted catechol then undergo the *meta*- or *ortho*- cleavage pathway for further degradation.



a.Methylbenezene biodegradation pathway



b.Dimethyl-benzene biodegradation pathway



c.Alkyl-benzene biodegradation pathway

Figure 30 Ring cleavage of aromatic compounds (Smith, 1990)

Triclosan is a synthetic aromatic halogenated compound. Because of the difficulty in breaking a carbon-halogen bond from an aromatic ring structure, most degradation pathways of halogenated aromatic compounds start from a ring cleavage rather than a dehalogenation reaction. This is consistent with the potential pathway proposed.

As discussed, catechol formation followed by *meta*- or *ortho*- cleavage of the aromatic ring is the common biodegradation pathway for most benzene, substituted benzenes and derivatives. The first aromatic ring of triclosan contains two C-O bonds at 1, 2- position, thus having a similar structure to catechol.

Using similar rules for catechol aromatic ring cleavage, Figure 31 illustrates the possible triclosan *ortho*-cleavage pathway. A compound having molecular formula of $C_{12}H_7Cl_3O_4$ would be formed. This is inconsistent to the intermediate analysis result, in which a compound having the molecular formula of $C_{12}H_7Cl_3O_3$ was identified.

Figure 32 illustrates triclosan undergoing normal *meta*-cleavage pathway, in which a compound having molecular formula of $C_{12}H_7Cl_3O_4$ should be formed. However, this is also inconsistent to the intermediate analysis result, in which a compound having molecular formula of $C_{12}H_7Cl_3O_3$ was identified.

It is therefore proposed that triclosan undergoes a special ring cleavage pathway, either through *meta* or *ortho*-cleavage, a compound having molecular formula of $C_{12}H_7Cl_3O_3$ being formed. In normal aromatic ring cleavage pathway, one oxygen molecule is inserted to break the C-C bond and one –CHO and one –COOH group is yielded. However, in the special triclosan aromatic ring cleavage pathway, two –CHO groups are formed. This requires that, instead of two oxygen protons as of normal ring cleavage pathway, only one single oxygen proton is inserted into every cleaved bond (as shown in Figure 33). This is consistent to the intermediate analysis results in that a compound having the same molecular formula was identified as a triclosan biodegradation intermediate. This may indicate that triclosan ring cleavage mechanism is different to the normal oxidation pathway through dioxygenases as discussed later in Chapter 6.

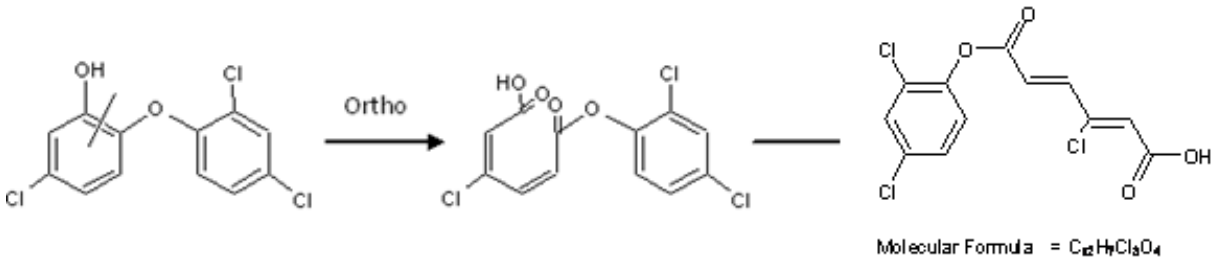


Figure 31 Triclosan undergoes normal *ortho*-cleavage

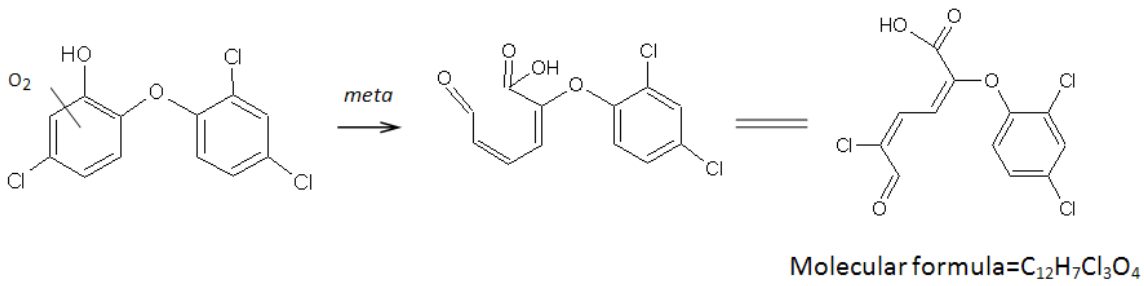


Figure 32 Triclosan undergoes normal meta-cleavage

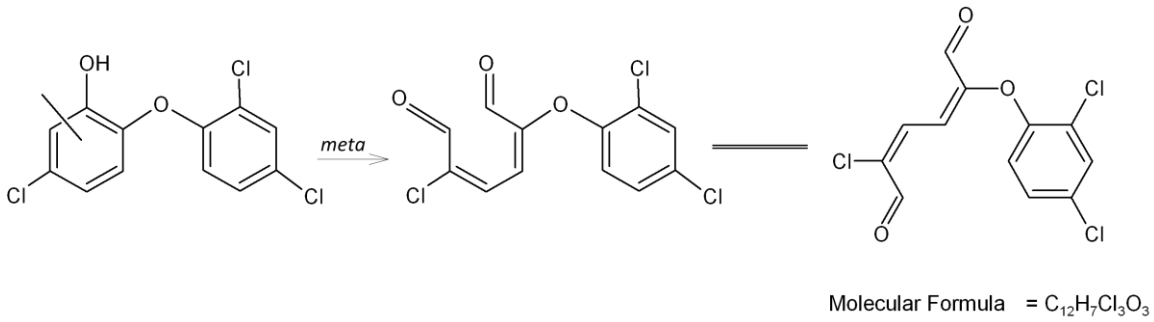


Figure 33 Triclosan undergoes special *meta*-cleavage

Hydrolysis of primary degradation intermediate

The hydrolysis of a triclosan biodegradation intermediate formed via *meta*-cleavage pathway is illustrated in Figure 35. Dichlorophenol was formed through this pathway; however, the potential second hydrolysis by-product was not identified in the intermediate analysis, and the conversion from this potential hydrolysis by-product to protoanemonin would be unlikely, as the chloro-muconate cycloisomerase recognises $–COO^-$ group (Figure 34).

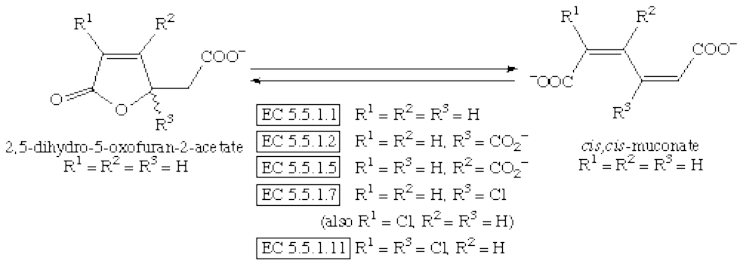


Figure 34 Function of muconate cycloisomerase (Pelzing et al., 2004)
(web page: http://www.enzyme-database.org/index.php)

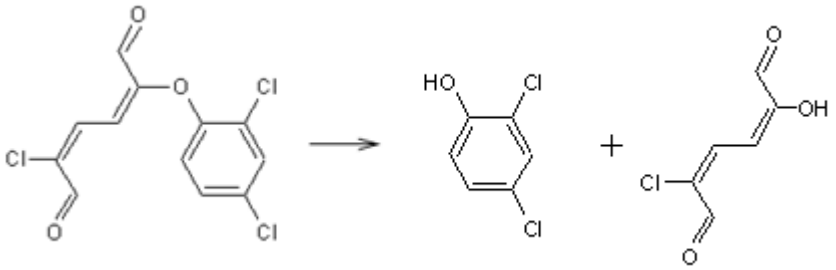


Figure 35 Hydrolysis of triclosan biodegradation *meta*-cleavage intermediate

Conversion of the second hydrolysis product to 4-methylenebut-2-en-4-olide (protoanemonin)

Protoanemonin was tentatively identified as one of the triclosan biodegradation intermediates/by-products, and this compound may be formed from 3-chloro-*cis, cis* muconate under the catalysis of chloro-muconate cycloisomerase as shown in Figure 36 (Kaulmann et al., 2001).

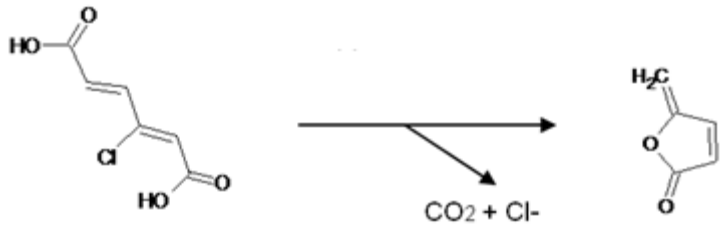


Figure 36 Conversion of chloro-muconate to protoanemonin

Proposed triclosan biodegradation pathway

Given that protoanemonin was identified as triclosan biodegradation by-product, chloro-muconate as shown in Figure 36 is likely to be the second hydrolysis product, which may be a hydrolysis product of the triclosan *ortho*-cleavage intermediate but not the *meta*-cleavage by-product. This contradicts with the identification of *meta*-cleavage intermediate of 2',4'-dichlorophenyl 4-chloro-6-oxo-2,4-hexadienoate. Therefore, it is proposed that the aromatic ring cleavage of triclosan may not have gone through the normal catechol ortho- or meta-cleavage pathway, but through a different *ortho*-cleavage

mechanism to form 2',4'-dichlorophenyl 4-chloro-6-oxo-2,4-hexadienoate. It is possible that 2',4'-dichlorophenyl 4-chloro-6-oxo-2,4-hexadienoate is the intermediate product in a two-step *ortho*-cleavage, in which the first step may involve the formation of 2',4'-dichlorophenyl 4-chloro-6-oxo-2,4-hexadienoate as illustrated in Figure 37, and the second step may involve the formation of $C_{12}H_7Cl_3O_4$ as a final product of the ortho cleavage as shown in Figure 38.

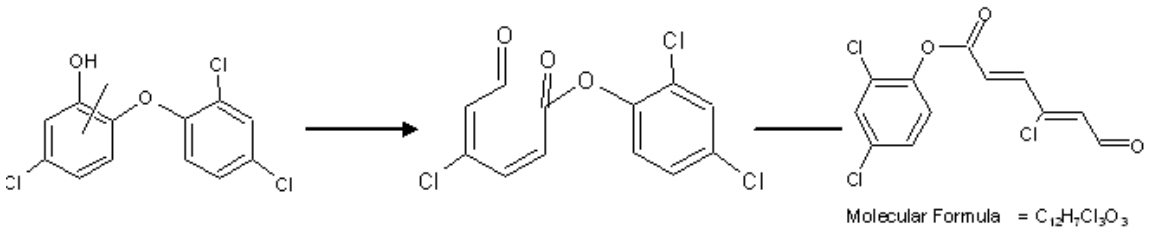


Figure 37 Triclosan undergoes special *ortho*-cleavage (Step 1) to form 2',4'-dichlorophenyl 4-chloro-6-oxo-2,4-hexadienoate

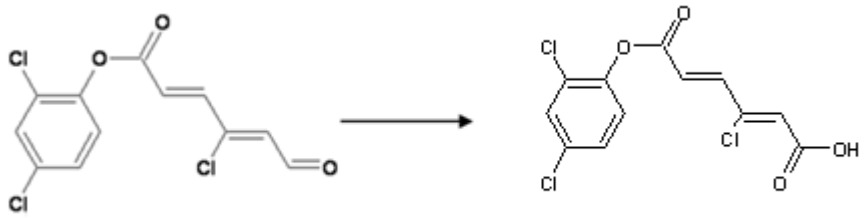


Figure 38 Step 2 following special *ortho*-cleavage of triclosan from $C_{12}H_7Cl_3O_3$ to $C_{12}H_7Cl_3O_4$

The overall triclosan biodegradation pathway was therefore proposed (as shown in Figure 39) that triclosan underwent an *ortho*-cleavage as the initial attack to form $C_{12}O_3C_{l3}H_7$, which subsequently transformed to $C_{12}H_7Cl_3O_4$ before hydrolysed to form dichlorophenol and chloro-muconate. The chloro-muconate was then converted to protoanemonin as by-product of triclosan biodegradation.

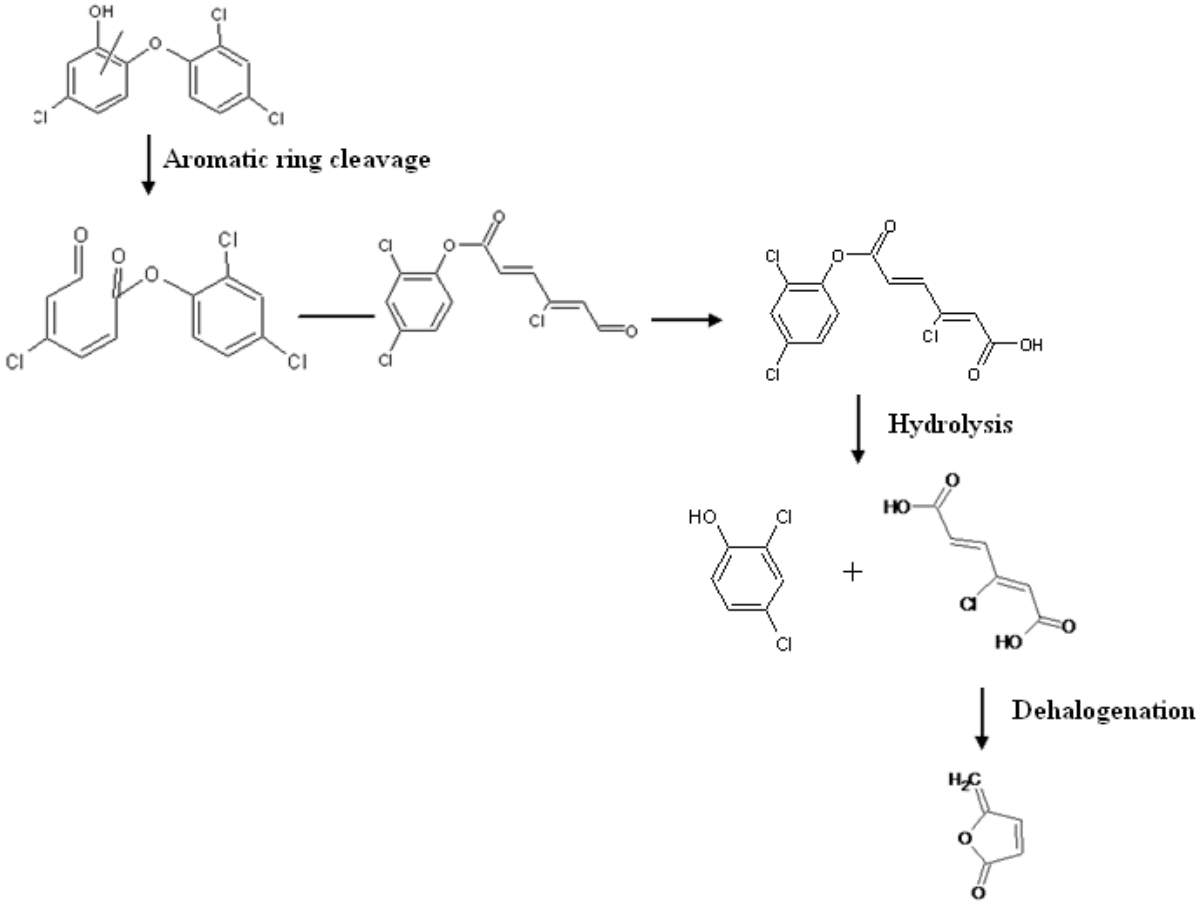


Figure 39 Proposed triclosan biodegradation pathway (chemistry)

The hydrolysis product 2, 4-dichloro phenol could be further dehalogenated and incorporated into the TCA cycle. Chloro-benzoquinone could also be formed during metabolism of chlorophenol (Roland Grey et al., 1998). Benzoquinones were found to be toxic to bacteria at a level of 20 μg/mL (Davison et al., 1999). It was not confirmed by chemical analysis in this study but it was potentially observed in the old cultures of triclosan minimal broth, which developed dark red or brown colour possibly due to the formation of benzoquinones.

Chapter 5 Genetic study on DNA determinants involved in triclosan resistance/biodegradation-Transposon mutagenesis and plasmid characterisation

5.1 Introduction

In this project, possible genes involved in regulating triclosan biodegradation were explored. By studying the genetic determinant of triclosan biodegradation, more insight can be gained into the biological mechanisms controlling the fate of triclosan in the wastewater environment. The genetic studies were carried out in two parts.

Transposon mutagenesis study was carried out initially, aimed at obtaining a bank of transposon mutants of the degradative strain for the screening of triclosan degradation deficient mutants. Triclosan degradative bacterial cells with transposon inserted into the operons that are responsible for triclosan degradation could be used to identify the responsible genes by sequencing of the genes flanking the inserted transposon DNA. The identification of triclosan degradative genes may help assess the mechanism of triclosan degradation by bacteria in the wastewater environment, and provide useful information for the potential construction of improved triclosan degradative strains.

An endogenous plasmid was observed to be present in a triclosan degrader. Temperature curing of the degradative isolate was carried out to obtain a cured strain with no plasmid. By assessing the triclosan degradation capability of this cured strain, potential plasmid mediated triclosan biodegradation was indicated. Returning of the plasmid into the cured strain as well as introducing this plasmid into some triclosan non-degraders by chemical transformation or electroporation were also attempted, aimed at characterising the functionality of this plasmid. DNA sequencing of this plasmid may provide great insight into the mechanism of triclosan biodegradation.

Transposon mutagenesis study using Tn*5*-B20

Transposon-based mutagenesis studies have provided experimental as well as bioinformatics strategies for the interpretation of the ever expanding nucleotide sequence data available in the GenBank database (DeAngelis, 1998, Gonzales et al., 1996, Hamer et

al., 2001, Kaniga & Davison, 1991, Lee et al., 2002, Llamas et al., 2000, Rajendran et al., 1994, Rossignol et al., 2001, Slater et al., 2003, Way et al., 1984, Zheng & Kathariou, 1994), despite the recently available and favourable molecular biology approaches, featuring direct, rapid and high-throughput, such as rapid nucleotide sequencing and site-directed mutagenesis (Liu & Lomonossoff, 2006, Mitchelson, 2007, *pp.* 3-44, Nunnally, 2005, Chapter 2&5, Qi & Scholthof, 2008, Seyfang & Huaqian Jin, 2004, Weiner et al., 1994).

Transposon Tn*5* is one of the well-characterised transposons that was described in 1994 as a useful tool in a mutagenesis study for the molecular analysis of *P. fluorescens (Rajendran et al., 1994)*, and has been extensively used in genetic studies (McClure et al., 1998, Poysti et al., 2007).

A transposon Tn*5*-B20 that carries the promoterless reporter gene *lacZ* in IS50L (Simon et al., 1989, van Overbeek et al., 1997) and Kan^r as selective marker was used in this study to obtain the knock out mutants of triclosan degradative strain.

One of the useful properties of transposon Tn*5* is that the relocation of the insertion elements into the target gene occurs randomly (Hayes, 2003). As transposon Tn5 randomly inserts into foreign genomic DNA, the gene where this transposon has inserted will be disrupted and the host strain will lose the particular phenotype that is conferred by this gene (Hayes, 2003). Thus a transposon mutagenesis study provides a useful tool for studying the function of a single gene associated with a particular phenotype; it is also a good tool for the location and identification of a single gene that confers a particular phenotype (Bahar et al., 1998). In the case of Tn*5*-B20, the addition of a promoterless *lacZ* gene permits expression studies of the interrupted gene to be performed under different conditions (Simon et al., 1989). Mutants with Tn*5*-B20 inserts can also be screened using the blue/white screening method.

In this study, Tn*5*-B20 was mobilized into the wild type triclosan resistant strain by a conjugative broad-host-range plasmid vector pNJ5000 (Thomas et al., 1992). Mobile plasmid pNJ5000 is a derivative of plasmid RP4, and has been used as a suicide vector, unstable in $RecA^+$ hosts (Grinter, 1983). It carries a marker gene that confers tetracycline resistance (Grinter, 1983, Grinter et al., 1989, Hepburn et al., 1985). It has been identified as a useful intermediate vector for the genetic manipulation in a $RecA^+$ host, including *E. coli, Pseudomonas,* and *Agrobacterium* (Gonzales et al., 1996, Grinter, 1983, Hepburn et al., 1985). Genetic organisation of pNJ5000::Tn*5*-B20 is shown in Figure 40.

Lack of selective pressure for pNJ5000 from the presence of tetracycline results in the loss of the plasmid from the host strain, and then the transposition of the Tn*5*-B20 carried in this plasmid could be started by the functionality of transposase encoded by genes on IS*50*R of Tn*5*-B20 (Gonzales et al., 1996, Grinter, 1983, Hepburn et al., 1985).

A diagram of methodology used in the mutagenesis study is shown in Figure 41. By screening the mutants showing a loss of resistance to triclosan, a knock-out mutant with a disrupted gene that was related to triclosan inactivation or degradation would be obtained. The disrupted gene can be further identified and characterised by sequence analysis of DNA flanking this transposon.

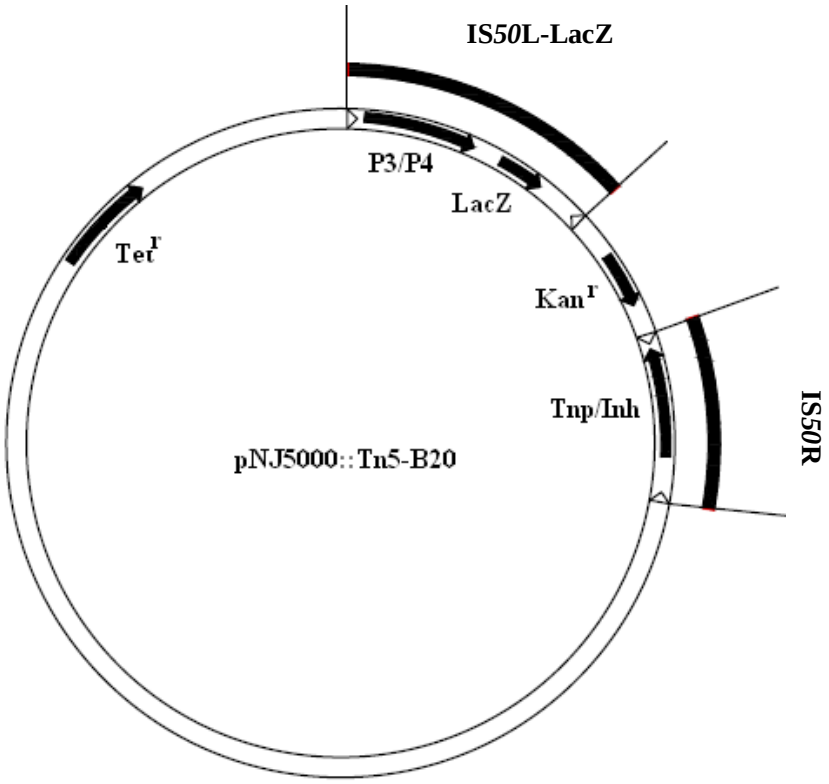


Figure 40 Illustration of genetic organisation of pNJ5000:Tn*5*-B20.
(Note: Location and size of genes are not to scale, for illustration only)

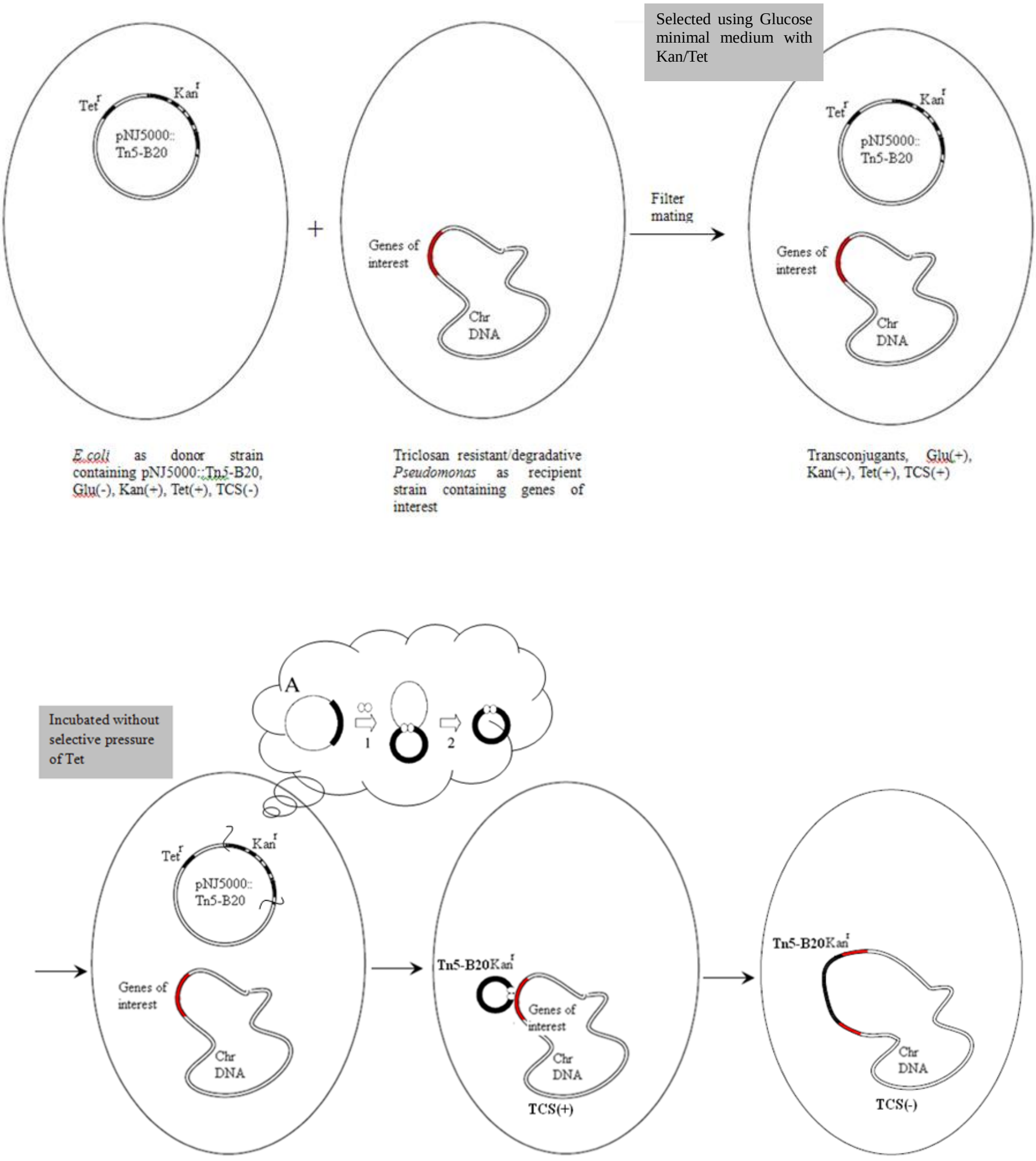


Figure 41 Illustration of methodology used for transposon mutagenesis study

Plasmid study on triclosan biodegradation

Investigation of possible plasmid-mediated biodegradation of triclosan in *Pseudomonas* strains forms the key focus of this project. The genetic study of triclosan biodegradation remains in an early stage, and no chromosomal or plasmid mediated biodegradation pathway of this compound has been characterised so far. However, plasmid-mediated biodegradation of other environmentally significant aromatic compounds has long been studied extensively (Dennis & Zylstra, 2004, Heipieper, 2007, Izmalkova et al., 2006, Jencova et al., 2004, Jencova et al., 2008, Kayser et al., 2002, Kozlova et al., 2004, Lengsfeld & Anchordoquy, 2002, Monticello et al., 1985, Shields et al., 1985, Trefault et al., 2004). *Pseudomonas* strains are notable for carrying large catabolic plasmids (Bramucci et al., 2006, Dennis & Zylstra, 2004, Don & Pemberton, 1981, Favaro et al., 1996, Nancharaiah et al., 2008), which confers biodegradative capacity to the host strains. Therefore, this study aimed to explore the possibility of plasmid-mediated biodegradation of triclosan by *Pseudomonas* strains isolated from the wastewater environment.

The functions carried on the plasmids in Gram-negative bacteria include antibiotic resistance, production of antibiotics, bacteriocins, enterotoxins, and virulence factors, and degradation of complex organic compounds (Lipps, 2008, Chapter 5).

As discussed in Section 1.4 of this thesis, a series of plasmids, evolutionarily related or not, in Gram-negative bacteria have been found to harbour the capacity to biodegrade organic compounds, such as aromatic compounds in particular (Silvia et al., 1999). Studies on the biodegradation of halogenated aromatic compounds also revealed the occurrence of plasmid determined pathways. It is important to search for novel plasmids or evolved catabolic plasmids that mediate the biotransformation of halogenated aromatic compounds (Silvia et al., 1999), so as to better understand the complicated biodegradation pathway of these compounds.

The possibility of plasmid mediated degradation of triclosan was investigated and results are presented in this chapter. Endogenous plasmid pF12 was isolated from *P. citronellolis*. Temperature curing and transfer of the plasmid by electroporation to pseudomonads and *E. coli* strains were attempted aiming to confirm the involvement of this plasmid in triclosan biodegradation.

5.2 Methods

5.2.1 Bacterial strains

Bacteria and plasmids involved in this study are listed in Table 8.

An additional 25 triclosan resistant isolates were obtained from a wastewater consortium using the method described in Chapter 3, and these were used as candidate recipients for Tn5-B20, together with the seven triclosan resistant isolates obtained as described in Chapter 3, for the filter mating experiments carried out in the transposon mutagenesis study.

5.2.2 Media and culture condition

Minimal medium with 100mg/L triclosan was used to maintain the growth of triclosan degraders unless otherwise stated, and was also used as a selective medium for electroporated strains.

LB agar and broth with the addition of 2 mg/L triclosan was used to maintain triclosan resistant isolates unless otherwise stated. Other *Pseudomonas* strains were maintained on Nutrient Agar or LB broth unless otherwise stated. *E. coli* strains were maintained on LB agar, and modified triclosan minimal medium with additional 5μg/L casamino acids and 2mg/L triclosan for selection of electroporated *E. coli* cells.

All cultures were incubated at 35°C aerobically overnight unless otherwise stated.

Table 8 List of strains and plasmids

Name	Strain	Description	Note
F12	*P. citronellolis*	Wild type triclosan degrader	Endogenous pF12
F11	*P. aeruginosa*	Wild type triclosan degrader	Endogenous plasmid, uncharacterised
C2	*P. citronellolis*	Cured strain from F12	No plasmid
C6	*P. citronellolis*	Cured strain from F12	No plasmid
10R2A06	*P. aeruginosa*	Wild type triclosan resistant isolate, non-degrader	No plasmid
RT10R2A06	*P. aeruginosa*	Derivative of 10R2A06, electroporated with pF12	Electroporated pF12
PF	*P. fluorescence*	ATCC strain	Endogenous plasmid of 5kb
DH5α	*E. coli*	Triclosan sensitive, donor	No plasmid
A118	*E. coli*	Filter mating donor, pNJ5000::Tn5-B20	Kan^r, Tet^r, Rif^r
#224	*E. coli*	Candidate donor	Amp^r, Hg^r
EpF12A	*E. coli*	Triclosan resistance at 2mg/L	Electroporated, no plasmid
EpF12B	*E. coli*	Triclosan resistance at 2mg/L	Electroporated, no plasmid
2R2A01	*P. fluorescens-25*	Wild type triclosan resistant isolate, non-degrader	
2R2A02	*P. aeruginosa*	Wild type triclosan resistant isolate, non-degrader	Filter mating recipient
2R2A03	*A.xylosoxidans ss.xylos*	Wild type triclosan resistant isolate, non-degrader	
2R2A05	*Moraxella sp.*	Wild type triclosan resistant isolate, non-degrader	Filter mating recipient
2R2A08	*P. stutzeri*	Wild type triclosan degrader, ID *P. aeruginosa* by sequencing	Filter mating recipient
10R2A01	*A. faecalis*	Wild type triclosan resistant isolate, non-degrader	Filter mating recipient
10R2A07	*P. aeruginosa*	Wild type triclosan resistant isolate, non-degrader	Filter mating recipient
R1		Wild type triclosan resistant isolate, environment	Filter mating recipient
R2		Wild type triclosan resistant isolate, environment	
R3		Wild type triclosan resistant isolate, environment	Filter mating recipient
R4		Wild type triclosan resistant isolate, environment	
R5		Wild type triclosan resistant isolate, environment	
R6		Wild type triclosan resistant isolate, environment	
R7		Wild type triclosan resistant isolate, environment	
R8		Wild type triclosan resistant isolate, environment	
R9		Wild type triclosan resistant isolate, environment	Filter mating recipient
R10		Wild type triclosan resistant isolate, environment	
R11		Wild type triclosan resistant isolate, environment	Filter mating recipient
R12		Wild type triclosan resistant isolate, environment	Filter mating recipient
R13		Wild type triclosan resistant isolate, environment	Filter mating recipient
R14		Wild type triclosan resistant isolate, environment	
R15		Wild type triclosan resistant isolate, environment	
R16		Wild type triclosan resistant isolate, environment	Filter mating recipient
R17		Wild type triclosan resistant isolate, environment	
R18		Wild type triclosan resistant isolate, environment	
R19		Wild type triclosan resistant isolate, environment	
R20		Wild type triclosan resistant isolate, environment	Filter mating recipient
R21		Wild type triclosan resistant isolate, environment	
R22		Wild type triclosan resistant isolate, environment	
R23		Wild type triclosan resistant isolate, environment	Filter mating recipient
R24		Wild type triclosan resistant isolate, environment	Filter mating recipient
R25		Wild type triclosan resistant isolate, environment	

5.2.3 *Sensitivity test*

The antibiotic sensitivity profile was tested using a replicator against candidate donor strains *E coli* A118 and E. coli #224, as well as the triclosan degrader and resistant isolates. Negative control with saline as inoculum was included. Tetracycline resistance was tested on nutrient agar and glucose minimal medium agar with and without magnesium.

For putative triclosan degraders, kanamycin, ampicillin and rifamycin resistance was tested on glucose minimal medium, whereas nutrient agar was used for *E coli* strains. Concentrations of each antibiotic used in the MIC tests, which are concentrations of antibiotic incorporated in testing plates, as described in standard method, are listed in Table 9. Other procedures followed the standard procedures (CLSI, 2003). Bacterial microbial inhibitory concentration (MIC) to an antibiotic was determined as the lowest of all concentrations of this antibiotic used in the test plates that showed no growth of the test bacterial strain at the end of the incubation period.

Table 9 Antibiotic breakpoints used in antibiotic sensitivity test

Antibiotics	**Breakpoint concentrations (mg/L)**				
Kan	0	2	5	10	20
Tet	0	1	2	5	10
Amp	0	5	10	20	50
Rif	0	10	20	50	100

5.2.4 Filter mating

Transfer of transposon Tn*5*-B20 into a triclosan degrader and a resistant isolate was carried out using bacterial biparental conjugation by means of filter mating. Wild type triclosan degraders and resistant isolates were used as recipients in the filter mating, and *E. coli* A118 carrying transposon Tn*5*-B20 on pNJ5000 was used as donor strain. Tetracycline selective medium contained an effective concentration of 10 mg/L, and kanamycin selective medium contained an effective concentration of 20 mg/L.

Filter mating was carried out according to Hepburn's method with minor modifications (Hepburn et al., 1985). Ten mL overnight culture of donor and recipient strains were harvested and washed with phosphate buffered saline (PBS) before resuspension in 2 mL

of PBS. One mL of donor culture and 0.4 mL of recipient culture were mixed and filtered using a syringe filter with a sterile cellulose nitrate (Sartorius SM11307 0.2μm) membrane filter. Two controls, one with 1mL donor only and the other with 0.4mL recipient only were included in the procedure. Membrane filters were then placed on the surface of a TSA agar plate and air dried for 5 min before incubation at 35 °C for 12 hours. Filters were then resuspended in 2 mL sterile PBS and vortexed for 2 minutes to break mating pairs. Neat and 10^{-2} dilutions of each suspension were then plated onto selective medium (GM+Kan^{20}+Tet^{10}), and incubated at 35°C until colonies appeared.

The following variables were investigated in an attempt to optimise the filter mating procedure.

Incubation period (mating): 8hr, 12hr, 24hr;

Donor: recipient ratio: 1:0.4, 1:1, 1:2, 1:5;

Selective medium: Kan^{20}+Tet^{10}, Kan^{10}+Tet^{10}, Kan^{10}+Tet^{5}

Selection of trans conjugants was based on the following antibiotic resistance/metabolism profile listed in Table 10.

Table 10 Antibiotic resistance profile of the conjugation related strains

Strain	**Growth on GM**	**Kan^r on GM**	**Tet^r on GM**	**Clearing triclosan on NA**
Donor	-	+	+	-
Recipient	+	-	-	+
Transconjugant	+	+	+	+

5.2.5 *Selection of transposition mutants*

A single colony of transconjugant obtained from the filter mating procedure was purified on GM+ Kan^{20}+Tet^{10} agar plate, and a single colony was inoculated in 10mL of LB broth without tetracycline (no selective pressure for pNJ5000). The culture was incubated aerobically at 35°C overnight. 100μl of this overnight culture was then subcultured into another 10mL fresh LB broth and incubated overnight. A control group of LB+Tc^{10} culture was included in this procedure.

The LB culture from the above step was diluted to 10^{-5} and plated onto NA+Km^{20} agar plates and incubated overnight. Colonies arising from these plates were subject to screening for Tn*5*–B20 transposition mutants.

Transposition mutants were screened by patch plating single colonies from the above NA+Km^{20} plates onto NA+Km^{20} and NA+Tc^{10}, using toothpicks. Strains that showed no growth on NA+Tc^{10} but grew on NA+Km^{20} would be the Tn*5*–B20 transposition mutants and were then subjected to the screening for a triclosan sensitive mutant.

The subcultured LB cultures with and without tetracycline selective pressure, as described above, were also diluted and plated onto GM+Tet^{10} and plain GM plates for cell counting to monitor the efficiency of transposition.

Transposition mutants obtained above needed to be further screened by patch plating onto Triclosan minimal medium and NA+Km^{20}. Strains that showed no growth on Triclosan minimal medium but grew on NA+Km^{20} would be the triclosan degradation/resistance deficient mutant having responsible gene disrupted by Tn5.

5.2.6 Plasmid extraction and analysis

Plasmids were extracted using a QIAGEN miniprep plasmid extraction kit according to manufacturer's instruction. One single colony was inoculated from the selective plate into 10 mL LB broth and incubated at 35°C with shaking for 12-16 hours. Six mL of each culture was used for one plasmid preparation and the plasmid was eluted in 40µl elution buffer.

As a comparison, plasmids were also extracted using phenol extraction, UltraClean™ 25 to 50 mL Plasmid Prep Kit and QIAGEN large construct kits. Phenol extraction was undertaken following procedures described by Dr Alex Keegan (Keegan, 2001, pp28), and the manufacturer's instruction was followed for the QIAGEN kits.

Extracted plasmid pF12 was digested using restriction enzymes *EcoRI* and *NotI* to determine the size. Ten µl of plasmid preparation was used in a total of 20µL of restriction reaction.

Extracted plasmid and digestion reaction was then evaluated with gel electrophoresis in 0.6-1% of TAE agarose. A λDNA *Eco*RI and *Hind*III digested DNA Molecular Weight Marker III (0.12-21.2kb) was used as reference to estimate the size of plasmid studied.

5.2.7 Temperature curing

Temperature curing of plasmid pF12 was carried out at 46°C with vigorous shaking. One single colony from a selective plate was inoculated into 10mL pre-warmed (46°C) LB broth and incubated overnight. One hundred µL aliquot of the liquid culture was then subcultured into another 10mL pre-warmed (48°C) fresh LB broth and incubated overnight. This was repeated three times. The liquid culture was then serially diluted and plated onto LB and selective plates in triplicate. Colonies were counted after overnight incubation. Colonies from LB plates were then patch plated onto LB and triclosan minimal plates to screen for cured strains that are no longer able to utilise triclosan as carbon source.

Plasmid extraction and agarose gel electrophoresis were performed to confirm the absence of the plasmid from the cured strains. PCR using 27f/1492r as primers were also performed on the putative cured strains, and the amplified fragments were then sequenced to check the origin to exclude contaminations.

5.2.8 Chemical transformation and Electroporation

Restoration of plasmid pF12 into the cured *Pseudomonas citronellolis* strains were attempted by chemical transformation as well as electroporation.

Purified plasmid pF12 for use in transformation and electroporation was extracted from 500mL of LB culture of F12 using QIAGEN large construct kit following manufacturer's instruction.

Chemically competent cells of *Pseudomonas* and *E. coli* were prepared and the transformation carried out according to the methods described in Section 2.9.2 of this thesis.

Electro-competent cells of *Pseudomonas* and *E. coli* were prepared according to the methods described in Section 2.9.2 of this thesis. One hundred µl ice-thawed electro-competent *E. coli* or 40 µl of ice-thawed electro-competent *Pseudomonas* cells were used for the electroporation of 10 µl of plasmid pF12 at concentration of approximately 80-100ng/µl. Diluted SOC culture was incubated at 35°C for plasmid expression with gentle shaking for one hour for *E. coli* or 3 hours for *Pseudomonas* strains. Electroporated cells were then selected on Triclosan minimal medium for *Pseudomonas* transformants, and on modified triclosan minimal medium for *E coli* transformants.

5.3 Results

5.3.1 Selection of suitable transposon and donor/recipient strains for transposon mutagenesis study

Candidate donor and recipient strains

There were two laboratory *E. coli* strains A118 and #224 containing transposons that had the potential for use in this study. Antibiotic markers involved in these two strains include kanamycin (*Kan*), tetracycline (*Tet*), rifamycin (*Rif*) and ampicillin (*Amp*).

The antibiotic resistance profiles of the triclosan degraders and resistant wild type isolates as candidate recipient strains were determined in order to choose appropriate donor/recipient strains for conjugation in transposon mutagenesis study. Bacterial ability to utilize different carbon source, such as glucose and mandelate, was also tested for all strains in order to use media containing different carbon source to select trans-conjugants.

Magnesium was found to affect the activity of tetracycline (Sompolinsky & Samra, 1972). Therefore the tetracycline sensitivity test was carried out on GM (with Mg) and GM (without Mg).

Thirty six strains, including triclosan degraders (confirmed by minimal medium growth test), triclosan resistant isolates (the rest of the wild-type resistant isolates, some may be degraders too but not confirmed by minimal medium growth test), and laboratory *E. coli* as donor candidates were tested, and MICs were determined as the lowest concentration at which no growth was observed for that strain. A list of all tested strains and MIC results are summarised in Table 11.

Table 11 Antibiotic sensitivity test of related strains (MIC mg/L)

List No on replicator	Name of strain	Growth on Glucose medium	Km MIC on GM	Rif MIC on GM	Amp MIC on GM	Tet MIC on NA	Tet MIC on GM (no Mg)	Tet MIC on GM (with Mg)
1	R25	-	no growth	no growth	no growth	>10	<1	<1
2	#244 (donor)	+	2	<10	50	2	no growth	no growth
3	A118 (donor)	-	no growth	no growth	no growth	>10	no growth	no growth
4	-ve control	-	no growth	no growth	no growth	no growth	no growth	no growth
5	R1	+	2	<10	50	>10	5	>10
6	R2	+	2	<10	50	>10	10	>10
7	R3	+	2	<10	>50	10	5	5
8	10R2A01	+	10	<10	>50	>10	5	5
9	2R2A02	+	10	<10	>50	>10	5	5
10	R4	+	>20	<10	>50	>10	>10	>10
11	R5	+	>20	<10	>50	>10	10	>10
12	2R2A01	+	>20	<10	>50	>10	>10	>10
13	R6	+	>20	<10	>50	>10	>10	>10
14	R7	+	>20	<10	>50	>10	>10	>10
15	R8	+	>20	<10	>50	>10	>10	>10
16	R9	+	5	<10	>50	10	5	2
17	R10	+	>20	<10	>50	>10	5	>10
18	2R2A03	+	>20	<10	>50	2	5	2
19	R11	+	10	<10	>50	10	5	5
20	R12	+	10	<10	>50	>10	5	5
21	R13	+	10	<10	>50	10	5	5
22	2R2A08	+	>20	<10	>50	>10	>10	>10
23	R14	+	>20	<10	>50	>10	>10	>10
24	R15	+	>20	<10	>50	>10	>10	>10
25	R16	+	10	<10	>50	>10	5	10
26	R17	+	>20	<10	>50	>10	>10	>10
27	R18	+	>20	<10	>50	>10	>10	>10
28	R19	+	>20	<10	>50	>10	>10	>10
29	R20	+	5	<10	>50	>10	5	5
30	R21	+	>20	<10	>50	>10	<1	2
31	R22	+	>20	<10	>50	>10	<1	<1
32	R23	+	2	<10	>50	10	5	5
33	10R2A07	+	5	<10	>50	10	5	5
34	2R2A05	+	2	<10	>50	10	5	5
35	2R2A08	+	5	<10	>50	10	5	5
36	R24	+	10	<10	>50	>10	5	5

It was determined that *E. coli* #224 could not be used as a donor strain, as all candidate recipient strains were intrinsically highly resistant to ampicillin, which is the selective marker carried in #224. *E. coli* A118 could be used as a donor strain, but only strains showing tetracycline MIC<5 mg/L on GM (without Mg) or Kanamycin MIC<10 mg/L on GM could be used as recipients in filter mating experiments. Triclosan resistant wild type strain 10R2A01, 10R2A07 and triclosan degrader 2R2A08 were chosen as recipient strains in the initial filter mating experiment. An extract of antibiotic resistance profile of the chosen donor and recipient strains is listed in Table 12.

Table 12 Overview of antibiotic resistance profile of chosen donor and recipient strains (mg/L)

Antibiotics	10R2A01	2R2A08	10R2A07	A118 on NA
Kan	10	5	5	>20
Tet	5	5	5	>10

The presence of pNJ5000 in *E. coli* A118 was confirmed by plasmid extraction and agarose gel electrophoresis analysis as shown in Figure 42.

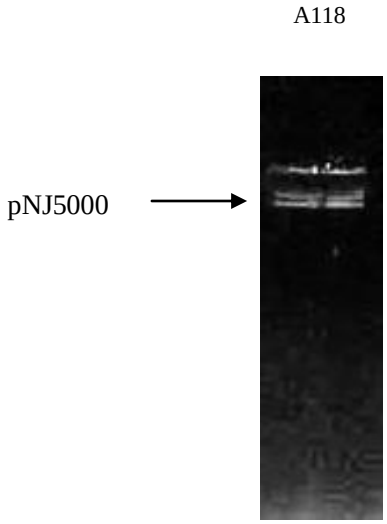


Figure 42 Presence of pNJ5000 in *E.coli* A118
(the size of plasmid was not characterised here)

5.3.2 *Construction of transposition mutant deficient in triclosan degradation or resistance*

5.3.2.1 Transfer of Tn5-B20::pNJ5000 into wild type triclosan degrader/resistant isolates

Filter mating was used for bacterial conjugation to transfer transposon Tn5-B20 into wild type putative degrader and triclosan resistant wild type isolates by "helper" pNJ5000.

Several attempts at filter mating under different conditions using different recipient strains were carried out but most of them failed, as no growth was observed on the selective plates.

Colonies grew on 10R2A01+A118 mating plates after five days of incubation on selective plates. No growth was observed on control plates.

Colonies grew on 2R2A08+A118 mating plates after five days of incubation on selective plates. Although 5 pin-point colonies were observed on a recipient-only control plate, there were more colonies on the mating plate and they were larger than those on recipient-only control plate.

Filter mating experiments were extended to other triclosan resistant wild type isolates as listed in Table 11, but no successful results were obtained.

Putative trans conjugants from 10R2A01 and 2R2A08 were purified on Glucose minimal medium containing 10 mg/L of tetracycline. Antibiotic resistance to kanamycin at 20 mg/L and tetracycline at 10 mg/L was checked. One of the putative trans conjugants from *A. faecalis* 10R2A01, namely *A. faecalis* PA11 was chosen for further study.

5.3.2.2 *Transposition of Tn5-B20*

Loss of pNJ5000 in the absence of tetracycline selective pressure

Loss of plasmid pNJ5000 in LB broth after 30-40 generations in the absence of tetracycline selective pressure was achieved by subculture of the transconjugant into LB broth in the absence of tetracycline.

The ratio of tetracycline resistant and sensitive cells in cultures with and without tetracycline is presented in Figure 43. Three consecutive overnight subcultures of putative trans conjugant PA11 in LB broth without tetracycline selective pressure resulted in 93.30% loss in tetracycline resistance, i.e. 93.30% of the trans conjugants lost the plasmid pNJ5000. In the control, where 10mg/L of tetracycline was present in the culture, the loss of tetracycline resistance was observed at a rate of only 10.30%.

	Cell count on GM	Cell count on $GM+Km^{20}+Tet^{10}$	Loss of Tet resistance
LB culture	4.9E+09	3.3E+08	93.30%
LB+Tc10 culture	2.9E+09	2.6E+09	10.30%

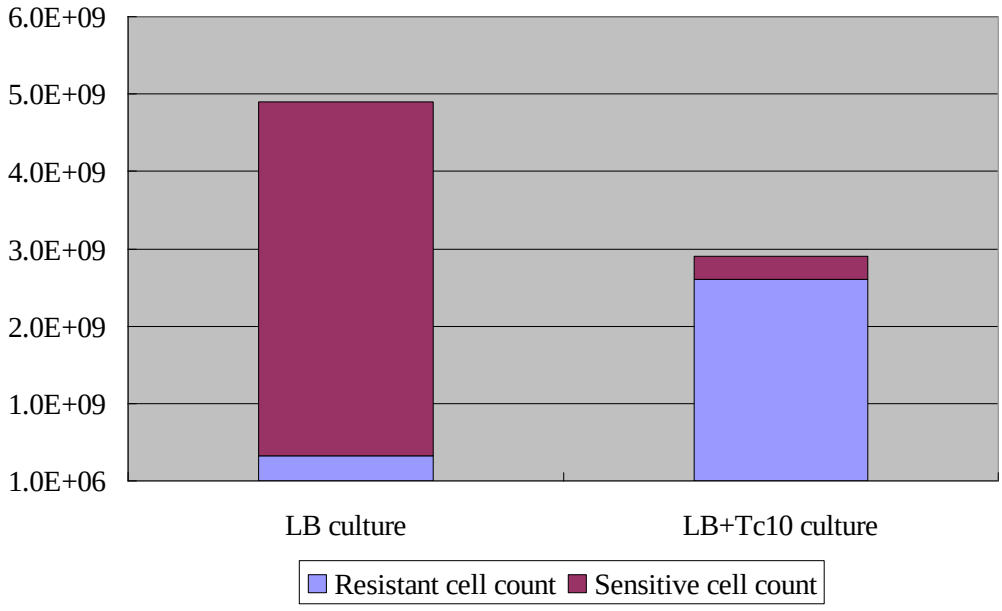
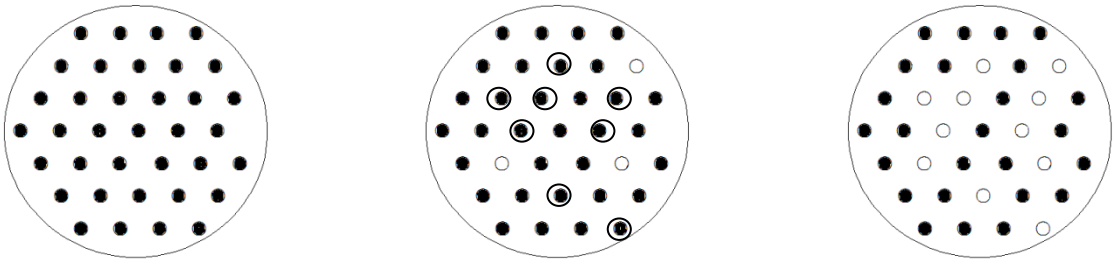


Figure 43 Loss of pNJ5000 in PA11 in the absence of selective pressure from tetracycline

Screening for Tn5-B20 transposition mutants

As shown in Figure **44**, in theory, colonies arising on the kanamycin plate but not on the tetracycline plate (as circled on kanamycin plate) would be the transposon mutants.



No antibiotics Kanamycin plate Tetracycline plate

Figure 44 Patch plating for the screening of Tn5 transposition mutant

LB cultures that lost plasmid pNJ5000 were plated on LB plates and 2000 colonies were screened by patch plating. However, no colonies were found to grow on GM+Km^{20} while showing no growth on GM+Tet^{10}. Of these colonies screened, ones that showed growth on GM+Km^{20} all showed growth on GM+Tet^{10} plate, and ones that showed no growth on GM+Km^{20} also failed to grow on GM+Tet^{10} plate. This indicates a failure of obtaining Tn*5*-B20 transposon mutant.

Screening using other putative trans conjugants and different combinations of antibiotic concentrations was also carried out, but no positive results were obtained (results not shown).

5.3.2.3 Screening of triclosan degradation deficient mutants

A triclosan degradation deficient mutant should have been detected in Tn*5* transposition mutants. However, because no mutants were detected, it was not possible to proceed to investigate any triclosan degradation deficient mutants.

5.3.3 The presence of a plasmid in triclosan degrader Pseudomonas citronellolis

The failure of the transposition study, although not conclusive, led to the observation of a plasmid present in the triclosan degrader F12 *Pseudomonas citronellolis* during plasmid

extractions carried out for the purpose of trouble shooting in the transposon mutagenesis study. Figure 45 shows the agarose gel electrophoresis picture of the plasmid, designated as pF12, extracted using the QIAGEN large construct kit. This strain was confirmed to be triclosan degrader by monitoring the increase in bacterial cell count and the corresponding decrease in triclosan concentration in the culture (as discussed in Chapter 3).

Pseudomonas strains are known to carry catabolic plasmids that confer the degradation of complex organic compounds (Bestetti & Galli, 1984, Boronin et al., 1993, Calero et al., 1989, Dennis & Zylstra, 2004, Eaton & Timmis, 1986, Kwong et al., 2000, Maeda et al., 2003). The presence of a plasmid in this degradative strain had led to the investigation on the possibility of plasmid-mediated biodegradation.



Figure 45 Presence of a plasmid in *Pseudomonas citronellolis* F12
(the size of plasmid was not characterised here)

5.3.4 Plasmid curing and screening of triclosan deficient cured strains

Temperature curing

Triclosan degrader *Pseudomonas citronellolis* F12 was tested for the ability to grow under high temperature. A range of temperatures from 38°C to 48°C was tested, and the F12 strain showed no growth at 48 °C while the growth of F12 strain at 46°C was slight. Cell counting for the colonies obtained from the third subculture under 46°C are recorded in Table 13.

It was observed that the number of cells growing on triclosan minimal medium agar was

reduced approximately 10-fold compared with those growing on nutrient agar, indicating the loss of the degradation capability of the isolate.

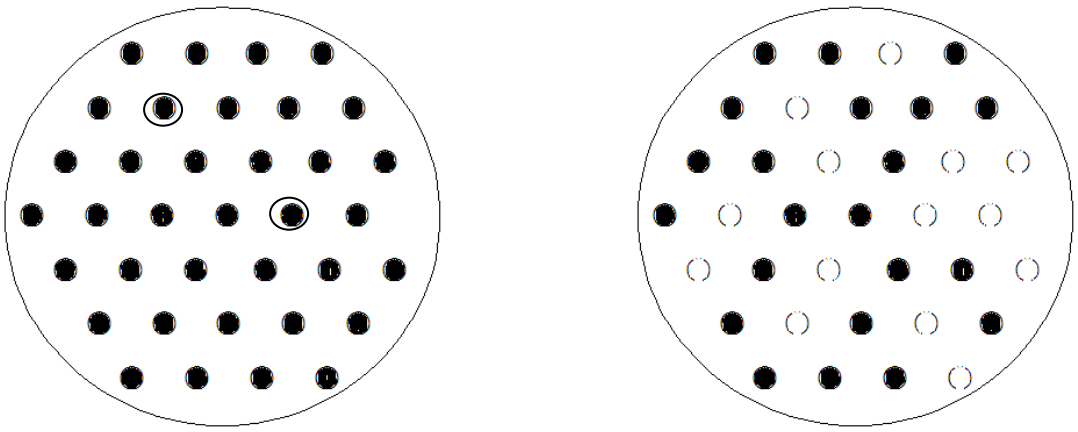
Medium	Duplicate 1	Duplicate 2	Average
Nutrient agar (# per ml)	6200	5800	6000
Modified triclosan agar (# per ml)	780	860	820

Table 13 Cell count results for plasmid curing

As seen in Table **13**, 86.33% of the cells growing at 46°C culture after 3 subcultures were cured strains showing no triclosan degradation capacity.

Screening of cured strains

Screening of cured isolates was carried out using patch plating methods as shown in Figure 46. Colonies from LB plates that showed no growth on triclosan minimal medium were considered as cured derivative of F12.



NA plate Triclosan plate

Figure 46 Screening of F12 cured strain using patch plating

Twelve strains were obtained as cured isolates out of the 38 strains tested by patch plating. Two isolates, designated as C2 and C6 were chosen for further study.

Growth deficiency on triclosan medium

Putative cured strains, designated C2 and C6, were purified on nutrient agar and the loss of

ability to grow on triclosan minimal medium and nutrient agar containing 100mg/L of triclosan was checked. Neither was found to be able to grow on triclosan minimal medium.

However, although both cured strains were found unable to grow on triclosan-nutrient agar initially, growth started after 3-5days of incubation and the capacity to grow on triclosan-containing nutrient agar was eventually restored by both strains after a few subcultures on this medium. This may indicate the mechanisms of induced triclosan resistance developed by exposure of triclosan in nutrient agar.

Identification by 16S rDNA sequencing

C2 and C6 were subjected to 16S rDNA sequencing to confirm their origin from F12 strain. The 100% identity of sequences from C2, C6 and F12 indicates that strains C2 and C6 both originated from F12 strain, excluding the possibility of contamination (Appendix 6).

Plasmid extraction by large construct kit, miniprep kit and 25mL prep kit

Due to the difficulty with plasmid extraction for low copy number large plasmids, different plasmid preparation methods (as described in Section 5.2.6) were used to confirm the loss of plasmid in cured strains as shown in Figure 47. Restriction enzymes *Not*I, *Eco*RI and *Mse*I were used to characterize and confirm the loss of plasmid from the cured strain (shown in Figure 48).

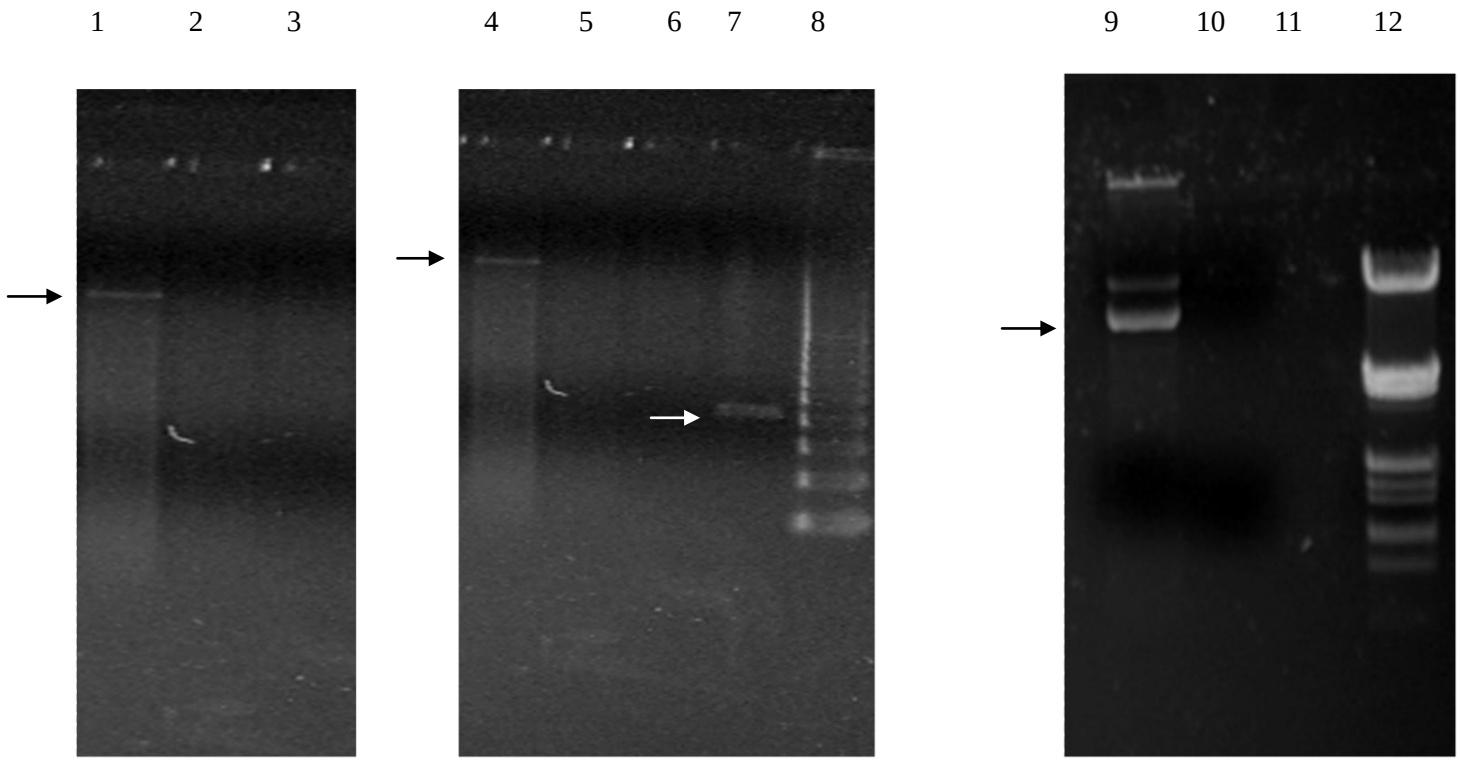


Figure 47 Loss of pF12 in cured strains observed using different plasmid extraction methods

Lane 1-3: Plasmid preparation on F12, C6, C6 (repeat) using a Mini-prep kit; Lane 4-6: Plasmid preparation on F12, C6, C6 using a QIAGEN UltraClean™ 25 to 50 mL Plasmid Prep Kit; Lane 7 Reference plasmid from a *Pseudomonas fluorescens* ATCC strain; Lane 8 500bp DNA marker; Lane 9-11: Plasmid preparation on F12, C6, C6 using a QIAGEN large construct kit; Lane 12: DNA molecular weight marker III (0.12-21.2kbp) (λDNA•EcoRI and Hind III digested).

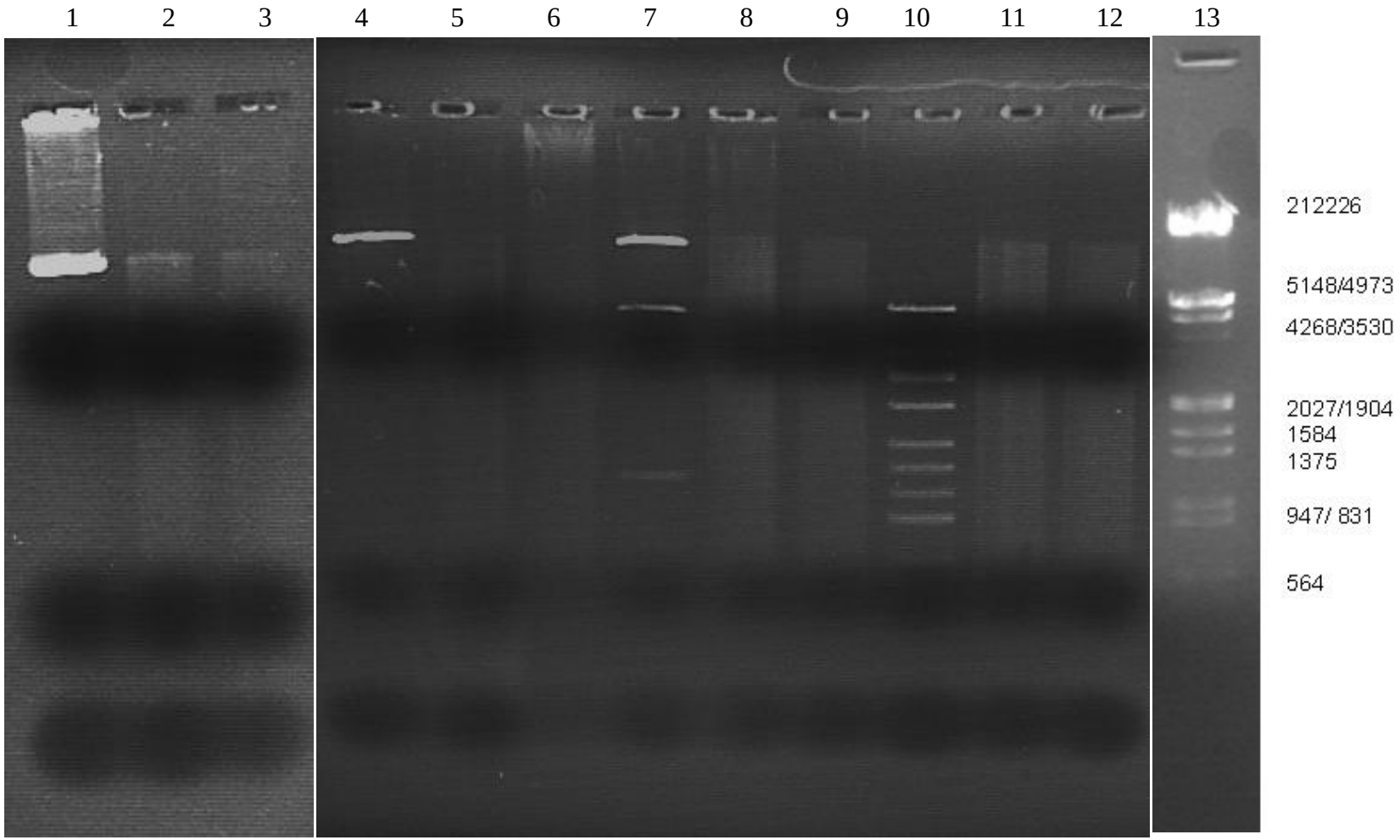


Figure 48 Loss of pF12 in cured strains (restriction digested plasmid DNA on 1% agarose gel)

Lane 1-3: Plasmid preparation of F12, C6, C6 (repeat) using large construct kit; Lane 4-6: Restriction digested (NotI) plasmid preparation of F12, C6, C6; Lane 7-9: Restriction digested (EcoRI) plasmid preparation of F12, C6, C6; Lane 10-12: Restriction digested (MseI) plasmid preparation of F12, C6, C6; Lane 13 . DNA molecular weight marker III (0.12-21.2kbp) (λDNA•*Eco*RI and *Hind* III digested);

5.3.5 Introduction of pF12 into triclosan degradation deficient strains

Introduction of pF12 into laboratory E. coli DH5α strain.

No colonies were observed when the *E. coli* DH5α strain electroporated with pF12 was cultured onto triclosan minimal medium. Selection medium was made as shown in Appendix 1.

However, a few colonies were seen on modified triclosan medium with casamino acids and a lower concentration of triclosan (2 mg/L). These colonies were identified with a Microbact™ GNB 24E test strip and gave a similar biochemical profile to the original DH5α strain (shown in Table 14), which cannot grow on this modified medium . This indicated that these colonies obtained the ability to grow on this modified medium after electroporation with pF12 (no growth was observed for the DH5α control). However, the plasmid extraction (as shown in Figure 49) failed to detect a plasmid in these electroporated *E. coli* DH5α strains.

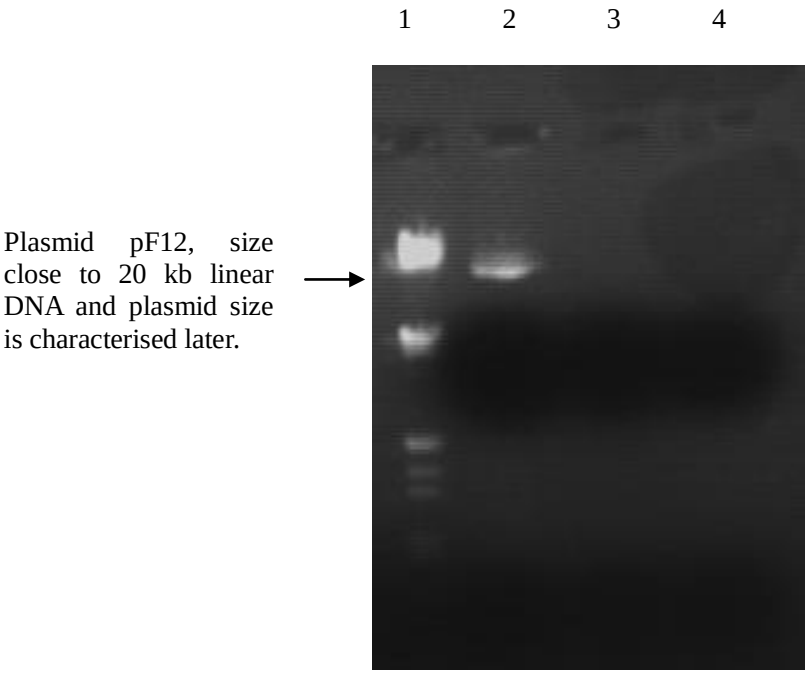


Figure 49 Introduction of pF12 into *E. coli* DH5α strain

Lane 1: DNA marker III (sizes as Figure 55)

Lane 2: Plasmid preparation of F12;

Lane 3: Plasmid preparation of EpF12A (electroporated *E. coli* DH5α);

Lane 4: Plasmid preparation of EpF12B (electroporated *E. coli* DH5α);

Table 14 Microbact™ GNB 24E test strip results on *E. coli* DH5α and electroporated *E. coli* cells

	GNB 24E																										
				GNB 12A/12E												GNB 12B											
	Oxidase	Motility	Nitrate	Lysine	Ornitrine	H_2S	Glucose	Mannitol	Xylose	ONPG	Indole	Urease	V-P	Citrate	TDA	Gelatin	Malonate	Inositol	Sorbitol	Rhamnos	Sucrose	Lactose	Arabinos	Adonitol	Raffinos	Salicin	Arginine
DH5α *E. coli* -inactive	+	+	+	–	–	–	+	+	+	–	+	–	–	–	–	–	–	–	+	+	–	–	+	–	–	–	–
	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1
	7			0			7			2			0			0			6			2			0		
EpF12A *E. coli* -inactive	+	+	+	–	–	–	+	+	+	–	+	–	–	–	–	–	–	–	+	+	–	–	+	–	–	–	–
	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1
	7			0			7			2			0			0			6			2			0		
EpF12B *E. coli* -inactive	+	+	+	–	–	–	+	+	+	–	+	–	–	–	–	–	–	–	+	+	–	–	+	–	–	–	–
	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1
	7			0			7			2			0			0			6			2			0		

Introduction of pF12 into a cured strain of P. citronellolis

Attempts were made to introduce pF12 back into cured strain *Pseudomonas citronellolis* C6; however, no colonies were observed on the triclosan minimal medium plate after electroporation, indicating a failure of this experiment, despite the attempts to optimise the plasmid extraction and electroporation method (results not shown).

Introduction of pF12 into other triclosan degradation deficient Pseudomonas strains

Two wild type triclosan resistant isolates (non-degraders), *Pseudomonas aeruginosa* 10R2A06 strain and *Pseudomonas fluorescens* 2R2A01 strain, were subjected to the electroporation of pF12 to introduce pF12 into triclosan non-degraders isolated as triclosan resistant strains from the wastewater environment as described in Chapter 3.

A few colonies were observed from the selective plate used for the electroporated *Pseudomonas aeruginosa* 10R2A06 but not for *Pseudomonas fluorescens* 2R2A01 strain. One single colony of electroporated *Pseudomonas aeruginosa* 10R2A06, designated RT10R2A06, was purified on triclosan minimal medium. A plasmid was present in this strain, and the gel picture showed a similar restriction digestion signature by *Eco*RI with the original pF12 plasmid (Figure 50).

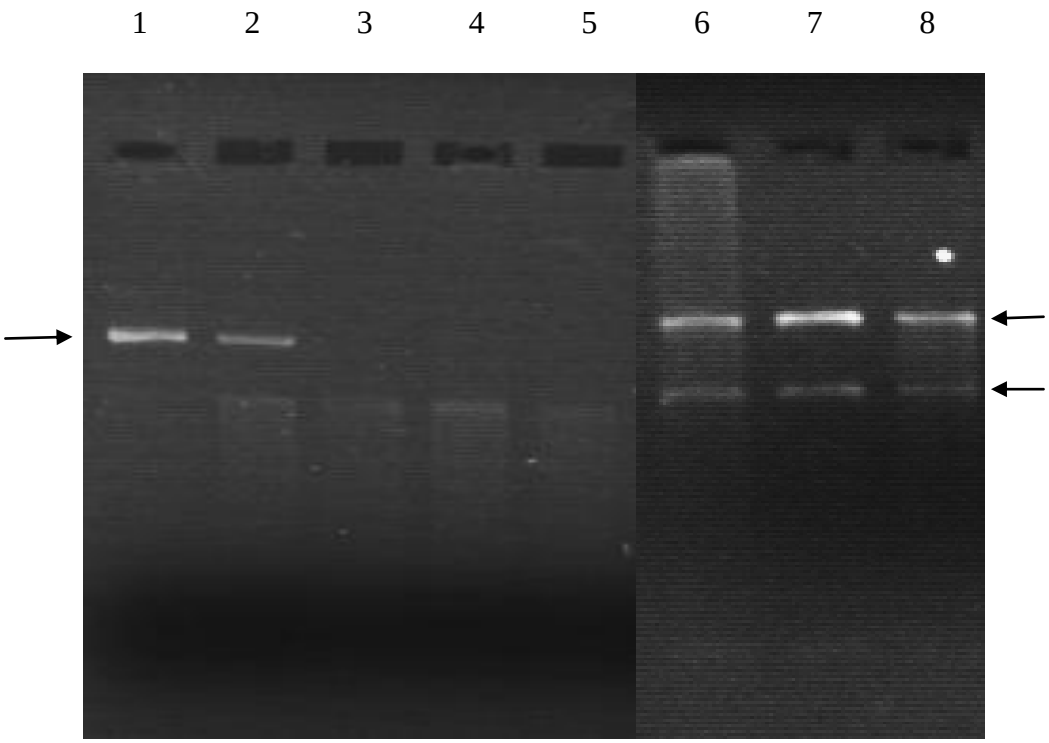


Figure 50 Introduction of pF12 into Triclosan Degradation Deficient *Pseudomonas* strains

Lane 1 Plasmid preparation from F12; lane 2 Plasmid preparation from *Pseudomonas aeruginosa* RT10R2A06 (electroporated strain) using QIAGEN large construct kit, size not characterised here; Lane 3 Plasmid preparation from *Pseudomonas aeruginosa* 10R2A06 (non-degrader); Lane 4,5 Plasmid preparation of *Pseudomonas citronellolis* C6 (cured strain); Lane 6 EcoRI digestion of plasmid preparation using QIAGEN miniprep kit from *Pseudomonas citronellolis* F12; Lane 7 Eco RI digestion of plasmid preparation using QIAGEN large construct kit from *Pseudomonas citronellolis* F12; Lane 8 *Eco*RI digestion of plasmid preparation using QIAGEN large construct kit from *Pseudomonas aeruginosa* RT10R2A06 (electroporated strain), DNA size is characterised later.

5.3.6 Plasmid characterisation

To assess its size, plasmid pF12 was digested using various enzymes, and *Eco*RI was shown to cleave this plasmid yielding three bands of various sizes as shown in Figure 51. *Not*I cut this plasmid at least once and a double digest of pF12 by *Eco*RI and *Not*I is shown in Figure 51. Other restriction enzymes that cut this plasmid resulting in discernable bands on agarose gel included *Bam*HI, *Kpn*I, *Xba*I, *Mse*I, *Nde*I, *Sac*I, *Cla*I *Sph*I, *Pst*I, *Bst*Xi, and *Sal*I (gel pictures are shown in Appendix 5).

An estimate of the plasmid size is shown in Table 15.

Table 15 An estimate of plasmid pF12 size

Restriction Enzyme	*Nde* I	*Hin*DIII	*Xba*I	*Kpn*I	*Apa*I	*Xho*I	*Bam*HI	*Eco*RI+ *Sac*I	*Eco*RI+ *Xba*I	*Eco*RI+ *Nde*I	*Sac*I	*Eco*RI	*NotI*	*EcoRI+ NotI*	*Mse*I
Fragment 1	17kb	17kb	17kb	17kb	17kb	17kb	17kb	11kb	11kb	12kb	15kb	11kb	17kb	11kb	4.5kb
Fragment 2								5kb	4kb	5kb		5kb		2.5kb	2.5kb
Fragment 3												1kb		2kb	2kb*2
Fragment 4														1kb	1.5kb
Fragment 5															1.2kb
Fragment 6															1kb
Fragment 7															0.8kb
Fragment 8															0.5kb*2
Total size	**17kb**	**17kb**	**17kb**	**17kb**	**17kb**	**17kb**	**17kb**	**16kb**	**15kb**	**17kb**	**15kb**	**17kb**	**17kb**	**16.5kb**	**16.5kb**

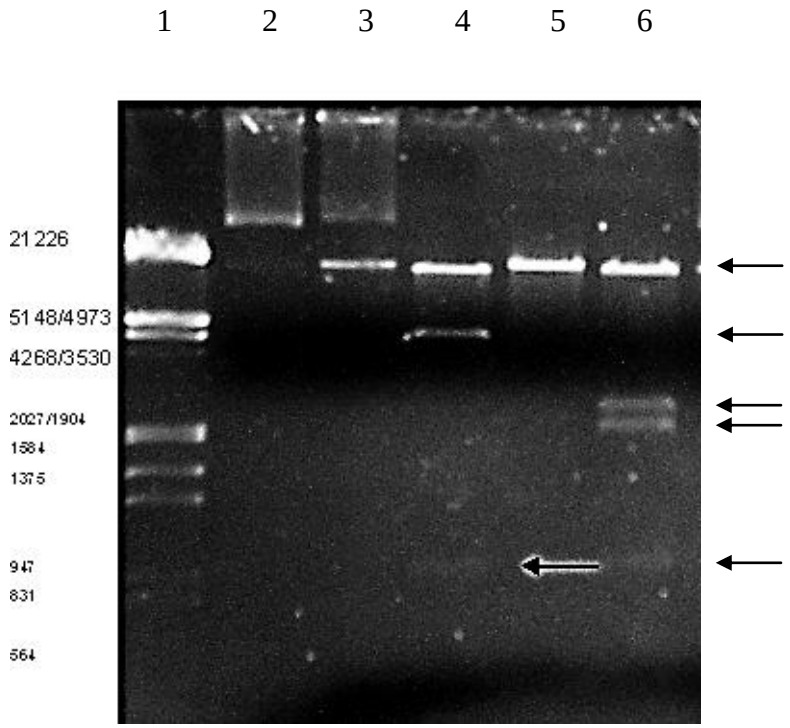


Figure 51 Restriction digest and sizing of pF12

Lane 1 DNA molecular weight marker III (0.12-21.2kbp) (λDNA•*Eco*RI and *Hind* III digested); Lane 2 Plasmid preparation from F12; Lane 3 Mock digestion of pF12 (no enzyme but nuclear free water was added); Lane 4 *Eco*RI digestion of pF12; Lane 5 NotI digestion of pF12; Lane 6 *Eco*RI & *Not*I double digestion of pF12

The size of this plasmid is approximately 15-20kb, which indicates that it is relatively small for a catabolic plasmid, and it is less likely to be a conjugative plasmid, so that natural lateral transfer of this plasmid is unlikely to occur.

5.4 Discussion

The conjugation experiment by filter mating to transfer pNJ5000::Tn5-B20 from *E. coli* A118 into the *A. faecalis* 10R2A01and *Pseudomonas aeruginosa* 2R2A08 were successful, judging from the control plates and antibiotic resistance profile of the putative transconjugant *A. faecalis* PA11 strain. However, the screening of transposon mutants from transconjugant *A. faecalis* PA11 strain failed. The failure of transposon mutagenesis study could be due to a few reasons, with potential technical defects in the method employed in this study as the major reason.

The same broad-host-range intermediate vector pNJ5000 was used to transfer narrow-host-range recombinant plasmids from *E. coli* to *Agrobacterium tumefaciens* as a preliminary technique for marker exchange (Hepburn et al., 1985). Instead of a single crossover, which inserts the whole recombinant DNA into the host plasmid, a reciprocal double crossover was screened for a replacement of the wild-type region with the modified region using a single step direct selection method (Hepburn et al., 1985).

In Hepburn's study, although the purpose of selection is different to that of this project; the direct selection of tetracycline-sensitive, kanamycin-resistant *Agrobacterium* cells was carried out using a modified method (Bochner et al., 1980, Maloy & Nunn, 1981) with additional kanamycin sulphate at 50 mg/L in the medium (Hepburn et al., 1985). The Maloy's medium (Maloy & Nunn, 1981) was made containing chlorotetracycline of 20 mg/L(inactivated by autoclaving at 121 °C for 4 hr), fusaric acid at 12 mg/L and zinc chloride at 0.1 mol/L. This medium greatly enriched for tetracycline-sensitive cells (Hepburn et al., 1985). At least 98% of colonies selected for kanamycin resistance were tetracycline sensitive (Hepburn et al., 1985), indicating a high efficiency using this selection method.

By contrast, this project used the patch plating method, which is commonly used in genetic studies in microbiology for the selection of antibiotic sensitive or resistant mutants, where a single colony was picked and inoculated onto a kanamycin-containing agar plate and a tetracycline-containing agar plate using toothpicks at a marked location on a patch plate. A small amount of inoculum of bacteria is picked and inoculated onto the antibiotic containing plates, but it would still be likely that one or more of these cells developed tetracycline resistance during incubation on patch plate, resulting in colony formation on the tetracycline-containing patch plate, which is indeed a false positive tetracycline resistance of the mutant.

Therefore, while patch plating is a well-established method for screening isolates having a particular phenotype, such as antibiotic resistance, it was found in this project that this method was not successful in screening of tetracycline sensitive *Pseudomonas* strains.

Since pNJ5000 is unstable in a RecA+ background, a 5% loss of plasmid per generation in the absence of selective pressure would be expected (Hepburn et al., 1985, Peterson et al., 1982). In Hepburn's study, no more than 10% of the *Agrobacterium* cells in a liquid culture still carried the cointegrated pNJ5000 after seven days of continuous subcultures (Hepburn et al., 1985). In this study, after three consecutive overnight subcultures of transconjugant PA11 without tetracycline present in the medium, a significant loss of pNJ5000 was observed. The plate counts for tetracycline sensitive and resistant bacteria in cultures without tetracycline selective pressure indicated that 93.3% of trans-conjugants lost pNJ5000. As there were a great number of kanamycin resistant strains seen during patch plating, this indicates that efficient transposition occurred in the culture. However, the screening of the Tn5 transposon mutant using patch plating was not successful possibly due to the induced over-expression of a multidrug efflux system in the trans-conjugants.

The mechanisms involved in tetracycline resistance in *Pseudomonas* include outer membrane permeability (Peng et al., 2005, Tseng & Bryan, 1974), *R*-factor mediated tetracycline uptake (Agersø & Sandvang, 2005, O'Hara & Kono, 1975, Tseng & Bryan, 1973) and multidrug efflux pump systems (Li et al., 1994). Active efflux of tetracycline was observed in wild-type *Pseudomonas aeruginosa* as well as single-step laboratory mutants showing a multiple-resistance phenotype (Li et al., 1994). The efflux pump systems have been identified as the major contributing factor to the general resistance of *P. aeruginosa* including tetracycline resistance (Kieboom et al., 1998, Li et al., 1994). It has also been shown that the expression of efflux pump systems may be induced by exposure to antimicrobial agents (Gupta et al., 2010, Li et al., 1994, Morita et al., 2003, Ravirala et al., 2007). In fact, the induced overexpression of the *mex* systems under exposure to tetracycline has been observed in wild-type and clinical mutants of *Pseudomonas* strains (Dumas et al., 2006, Wu et al., 2000).

Therefore, it can be concluded that, although the transposition may have been successful, under the exposure to tetracycline in the patch plate medium, the transposon mutants obtained subsequently acquired induced tetracycline resistance as a result of over-expression of a multidrug efflux pump system. This has resulted in failure to demonstrate transposition. Colonies that grew on tetracycline patch plates may well be

transposon mutants containing no tetracycline resistance marker from pNJ5000, but these colonies may be resistant to tetracycline due to the induced efflux pump system. Plasmid extraction to determine if pNJ5000 was present would help diagnose the potential problems; however, this was not further investigated due to the fact that the focus of project had been changed to the potential plasmid-mediated degradation.

Different concentrations of kanamycin (5-20 mg/L) were also investigated during trouble shooting for the selection of trans conjugants and the screening of transposon mutants in this project, but had no effect.

High level resistance to kanamycin in the trans conjugants is expected, as multiple copies of plasmid pNJ5000 containing the kanamycin resistance marker would confer a high level resistance to the host (Hepburn et al., 1985). In Hepburn's study, nutrient agar containing kanamycin at 15 mg/L with the addition of a drop of kanamycin (5 mg/ml) applied to the centre of the plate was used to select *Tn5* insertions into a recombinant plasmid (Hepburn et al., 1985). Therefore, it can be concluded that in this study it might have been better to use a different concentration of kanamycin for the selection of transconjugants and transposon mutants. While multiple copies of the kanamycin resistance marker carried on pNJ5000 in the transconjugants confers a high level of kanamycin resistance, the single copy number of a kanamycin resistance marker on the chromosome of transposon mutant would only confer low level kanamycin resistance.

Although the transposon mutagenesis study could have been technically improved, this part of the study was not pursued further in this project, due to some interesting phenomena that were observed and subsequently became the focus of further study.

While investigating growth in different media, a loss of degradation capacity by the triclosan degrader *Pseudomonas citronellolis* F12 was observed, in the absence of triclosan selective pressure. Subsequent plasmid extraction of this isolate revealed the presence of a medium sized plasmid. This directed the project to explore the possibility of a plasmid-mediated biodegradation mechanism.

Several plasmid extraction methods were attempted aiming to produce large quantities of pure plasmid from F12 strain, and the alkaline lysis method appeared to be effective. This method relies on bacterial lysis by sodium hydroxide and sodium dodecyl sulfate, followed by neutralization with a high concentration of low-pH potassium acetate buffer. This gives selective precipitation of the bacterial chromosomal DNA and other high molecular-weight cellular components. The plasmid DNA remains in suspension and is precipitated with

isopropanol in the final step. Although pure plasmid extract was obtained using this method, the procedure was complicated and required large culture and the preparation requires a few days to complete, and more importantly, the yield was low, indicated by a very weak band on an electrophoresis gel. Other plasmid DNA preparation kits used in this study were alkaline lysis method based, and the effectiveness were assessed for yield, purity of the extract and easiness of preparation. Large catabolic plasmids are always maintained in low copy numbers in the cell, perhaps owing to the metabolic burden (Sorensen et al., 2005). Although plasmid pF12 is small in size as a catabolic plasmid, it is still over 10 Kb and is much larger and probably contains less copy number than common plasmids/vectors used in genetic studies. Most of the commercially available kits for quick plasmid extractions were not efficient in the extraction of pF12. This is possibly due to a few factors including production of excessive extracellular polymeric substances (EPS) by *P. citronellolis*, plasmid size and potentially low copy number. A comparison of a number of plasmid preparation methods in this study found that the QIAGEN large construct kit was the best plasmid preparation procedure for this application.

There has been greater interest on investigations into bacterial catabolic plasmids naturally present in the soil or water body, due to the increasing amounts of toxic halogenated organic pollutants released to the environment in the last few decades (Bernhard et al., 2008, Feakin et al., 1994, Fewson, 1988, Haro & de Lorenzo, 2001, Kobayashi et al., 2009, Okpokwasili & Olisa, 1991, Sinton et al., 1986). Plasmids as well as other mobile genetic elements in environmental bacteria have been demonstrated to play an important role in the acquisition of new catabolic functions. Through the evolution of different catabolic pathways, these bacteria are important in the degradation of various environmental pollutants (Nojiri et al., 2004).

There has been no report on biodegradation of triclosan from a genetic level. A most recent report speculated that it is possible the gene involved in triclosan biodegradation might be in a plasmid based on the phenomenon of losing triclosan degradation ability after growing on rich-nutrient medium without triclosan (Lee et al., 2012). In this study, a 17kb endogenous plasmid bearing *Pseudomonas citronellolis* F12 isolated from Bolivar WWTP lagoon was found to utilise triclosan as carbon source. The loss of plasmid was observed when cultured under high temperature stress. The cured F12 strain *Pseudomonas citronellolis* C6 was shown to have lost the capability of growing on triclosan minimal medium. It is possible that this plasmid carries genetic determinants of triclosan biodegradation.

The cured strain *Pseudomonas citronellolis* C6 was also found to be unable to grow on nutrient agar containing 100 mg/L of triclosan initially. However, it developed induced capability to grow on nutrient agar containing triclosan after extended incubation. These results indicated that plasmid pF12 is triclosan related and it is likely to carry the genetic determinants of triclosan biodegradation. Biodegradation may also be a mechanism for triclosan resistance. Loss of plasmid resulted in reduced triclosan resistance, indicated by extended incubation period required by the cured strain for developing growth on nutrient agar containing triclosan. Antibiotic resistance profile was tested on wild strain F12 and the cured strain C6 against a wide spectrum of antibiotics and heavy metals. The results indicated no observable change of antibiotic resistance profile in the cured strain. This indicated that plasmid pF12 is unlikely to be directly involved in antibiotic or heavy metal resistance. The reduced resistance to triclosan is more likely to be due to the biodegradation mechanism, and the biodegradation is substrate specific, i.e. pF12 is determinant for triclosan biodegradation and triclosan does not share common biodegradation pathway with the antibiotics tested. The antibiotics and heavy metals tested may use other resistance mechanisms, as described in Chapter 1, and the resistance mechanism was not disrupted by the loss of plasmid.

The hypothesis of plasmid pF12 being a genetic determinant of triclosan biodegradation was further confirmed. Introduction of plasmid pF12 into non-pF12 bearing, triclosan non-degrader *Pseudomonas* was successful, and the recipient of pF12 obtained the ability to grow on triclosan minimal media. Although the electroporation of pF12 into the cured *Pseudomonas citronellolis* C6 strain and laboratory *E. coli* strain was unsuccessful.

The failure of *E. coli* to grow on selective triclosan minimal medium after electroporation with plasmid pF12 was not surprising, when the differences between *Pseudomonas citronellolis* and *E. coli* were considered, particularly the high intrinsic susceptibility of *E. coli* strain to triclosan (Gomez Escalada et al., 2005a). More importantly, DH5α strain is autotrophic, requiring more than one growth factor and is therefore incapable of growing on minimal medium.

Although the transfer of catabolic genes from *E. coli* to *Pseudomonas* using a transposable cassette encoding degradative genes has been reported successfully (McKay et al., 1997, Prieto et al., 1996) and the transfer of catabolic plasmid, such as TOL plasmid, has been reported; the expression of the complete pathway is often unsuccessful because of the distinct difference of promoter recognition sequences in *E coli* and *Pseudomonas* strains.

Even transfer of plasmid-encoded genes from *Pseudomonas* into *E. coli* is not always successful (Top et al., 1990).

E. coli has been widely used as host cell foreign gene expression in microbial genetics (Ahmad et al., 1990, Eck & Belter, 1993, Ju et al., 2005, Kim et al., 1994, Kitayama et al., 1996, Moon et al., 1995, Navarre et al., 2007, Ng et al., 1994, Shao et al., 1995). However, the failure in expression of foreign genes has been reported previously (Denome et al., 1993, McKay et al., 1997, Shao et al., 1995). The mechanism allowing bacteria to tolerate the acquisition of foreign DNA have been incompletely defined (Ahmad et al., 1990, Navarre et al., 2007).

The expression of foreign genes in bacteria may fail for a number of reasons, such as inefficient synthesis or efficient degradation of gene products, the synthesis of inactive proteins which may originate from improper assembly of polypeptides, or the requirement for an additional factor (or factors) not supplied by the host strain (McKay et al., 1997). The attempt to express the cluster of biphenyl dioxygenase gene products involved in chlorobiphenyl oxidation isolated from a Gram-positive *Phodococcus globerulus* P6 into *E. coli* BL21 was unsuccessful due to an unidentified post-transcriptional issue, while the same gene products were expressed successfully in *E. coli* DH5α and *Pseudomonas putida* (McKay et al., 1997). *Alcaligenes eutrophus* genes encoding for plasmid-borne resistance to cadmium, cobalt, and zinc (*czc* genes) were transferred by conjugation from *E. coli* to *A. eutrophus*, and the genes failed to express in the donor *E. coli* strain but were expressed successfully in a recipient *A. eutrophus* strain (Top et al., 1990).

Additionally, complete biodegradation of triclosan is likely to be the result of a series of catabolic reactions which may either involve a diverse bacterial community (Clarke, 2007) or a group of bacteria that carry complex catabolic capabilities as a result of a multiple cluster of related genes (Dunn & Gunsalus, 1973, Kozlova et al., 2004, Li et al., 2004, Sanseverino et al., 1993, Schluter et al., 2007, Yen & Gunsalus, 1982). Although triclosan's chemical structure is not as complicated as the structure of lignocelluloses, which requires a rich diversity of microorganisms responsible for a chain of lignocelluloses degradation-related chemical reactions (Clarke, 2007), it is possible that the plasmid pF12 may only be involved in a limited number of steps in the triclosan biodegradation pathway, while other genetic determinants in the host strain may be responsible for other steps of the biodegradation. As a consequence, when transferred to an *E. coli* strain, the biodegradation of triclosan could not occur due to the lack of other genetic determinants required to work

synergistically with pF12 in the degradation pathway.

The fact that the *E. coli* DH5α strain gained the ability to grow on modified triclosan medium containing casamino acids and a lower concentration of triclosan but no plasmid was present indicated that this *E. coli* DH5α was capable of growing on the casamino acids, but also acquired low level of triclosan resistance during the electroporation and selection processes under the selective pressure of triclosan in the selective media. This triclosan resistance is unlikely to be related to the pF12 plasmid studied.

Induced triclosan resistance has been widely observed across many bacterial genera, and the resistance varies in different species (Ledder et al., 2006). Adaptive resistance in *E. coli* has been reported previously (Braoudaki & Hilton, 2004a, Braoudaki & Hilton, 2004b, McMurry et al., 1998b, Thorrold et al., 2007). A highly significant reduction (400-fold) in triclosan susceptibility was seen in a laboratory *E. coli* strain when exposed to triclosan (Randall et al., 2004). The acquired triclosan resistance in *E. coli* has been attributed to efflux pumps (Braoudaki & Hilton, 2004a, McMurry et al., 1998b). The overexpression of an efflux pump would render the host cell resistant to the foreign compound (Chuanchuen et al., 2001, Chuanchuen et al., 2003, Chuanchuen et al., 2002, Masuda et al., 2000, Morita et al., 2003, Poole et al., 1996, Schweizer, 2003, Thorrold et al., 2007).

The successful transfer of the plasmid into non-degradative *Pseudomonas aeruginosa* by electroporation and selection using triclosan minimal medium indicated that plasmid pF12 could be involved in the degradative functionality in other species of *Pseudomonas*. The acquired ability to grow on triclosan minimal medium by this electroporated *Pseudomonas aeruginosa* confirmed that this plasmid pF12 carries genetic determinant/s of triclosan biodegradation in *Pseudomonas*.

Plasmid transfer by conjugation among different *Pseudomonas* species has been reported widely (Ehlers & Bouwer, 1999, Hallier-Soulier et al., 1999, Heinaru et al., 2009, Heuer et al., 2007, Kozdrój, 1997, Kwong et al., 2000, Latha & Lalithakumari, 2001, Mater et al., 1999). Electroporation is an effective tool used for the transfer of non-conjugative plasmids between strains of the same or different species (Baron et al., 2000, Benachour et al., 1996, Choi et al., 2006, Ravindran et al., 2006).

The horizontal transfer of genes, including mobile catabolic plasmids, was found to be a major mechanism for microorganisms to acquire new metabolic traits (Mølbak et al., 2003a, Stuart-Keil et al., 1998). A highly conserved naphthalene catabolic gene allele was

shared among phylogenetically diverse bacteria carrying naphthalene-catabolic plasmids, which indicated *in-situ* horizontal transfer of this gene cluster within the bacterial community at a coal tar-contaminated site (Stuart-Keil et al., 1998). Geologically and taxonomically diverse 2, 4-D-degrading bacteria have been found to share the *tfdABCD* genes encoding chlorocatechol and chlorophenol metabolism on mobile plasmids (Ka et al., 1994). Genes responsible for naphthalene degradation (*nahAGH)* were also found to be shared among four different *Pseudomonas stutzeri* isolates (Rossello-Mora et al., 1994). Nevertheless, the many positive results of investigations on the horizontal transfer of plasmid-mediated catabolic genes within *Pseudomonas* and among different genera of bacteria support the successful expression of the triclosan catabolic capability encoded by the pF12 in *Pseudomonas aeruginosa* after transfer from *Pseudomonas citronellolis* by electroporation in this study. Plasmids that are non-transferable can also be mobilised by other plasmids. Therefore, it is also possible that pF12 has been moved around in this way.

The failure to restore pF12 to the cured strain *Pseudomonas citronellolis* C6 may be due to technical reasons. It was found during the electroporation experiments that the preparation of *Pseudomonas citronellolis* electro-competent cells was very difficult, as this strain produced an excessive amount of the extracellular polymeric substances (EPS) that made the resuspension of cells in buffer solution extremely difficult during the preparation for competent cells. This may have resulted in the poor preparation of electro-competent cells of this cured *Pseudomonas citronellolis* C6 strain, leading to the unsuccessful electroporation. It is also possible that the EPS may protect cell membranes making electroporation difficult. Different medium was used with the aim of reducing EPS, but had little effect.

The following chapter characterises the plasmid pF12 and its potential functionality in triclosan biodegradation.

Chapter 6 Characterisation of triclosan degradative plasmid

6.1 Introduction

Biological and intermediate analysis results (shown in Chapter 4) indicated that plasmid pF12 may be associated with triclosan biodegradation in the wastewater environmental isolate *Pseudomonas citronellolis* F12. Molecular biological experiments and results are discussed in this chapter on the characterisation of the plasmid and the identification of triclosan resistance/degradation related genes.

Plasmid pF12 was extracted from the *Pseudomonas citronellolis* strain, and was digested using various endonuclease restriction enzymes. After characterisation of the plasmid by size and restriction profile, the digested plasmid fragments suitable for further study were then used for DNA cloning and sequencing.

Complete DNA sequence of Plasmid pF12 was obtained and sequence analysis was undertaken with the aim to reveal the genetic determinants of triclosan biodegradation.

6.2 Methods

6.2.1 Plasmid extraction

The plasmid pF12 was shown to be greater than 15kb in size and it was difficult to obtain a preparation of good quality concentrated plasmid DNA. A comparison of plasmid extraction methods currently available was undertaken in this study. Methods included alkaline lysis – phenol extraction, QIAGEN QIAprep Spin Miniprep kit, and QIAGEN Large-construct kit.

Cloning constructs were extracted using a QIAGEN mini prep kit according to the user's manual. Plasmids over 10kb in size or of low copy number were extracted using QIAGEN Large-construct kit following the manufacturer's instruction with minor changes. The protocol was slightly changed to offset the difficulties in extraction procedure due to the size and the low copy number of the plasmid and excessive production of EPS by *Pseudomonas citronellolis*. In order to obtain a solid bacterial pellet, Mueller Hinton III broth was used as culture medium instead of LB broth as recommended in the product instruction manual. The minimal amount of calcium and magnesium cations in Mueller Hinton III broth helped to reduce the mucoid type of growth seen in the LB broth culture, as this had prevented the successful application of this kit.

The plasmid extract was then further concentrated using an ethanol precipitation method (Summerton et al., 1983).

6.2.2 Cloning using pGEM®-5Zf(+)

pGEM®-5Zf(+) vector is a patented Promega® transcription vector having two different bacteriophage RNA polymerase promoter sequences, separated by a series of unique restriction sites into which foreign DNA can be inserted (Promega product information sheet, 2009). Vector reference points and sequence details are listed in Appendix 6.

The DNA cloning method using pGEM®-5Zf (+) vector is illustrated in **Error! Reference ource not found.**. *Nde*I digested pGEM®-5Zf (+) vector has an overhang of AT-3' which complements to the overhang of 5'-TA on the linear fragments of pF12 as a result of restriction digestion by *Mse*I. This allows the ligation to occur under the action of Ligase A

to produce a reconstructed plasmid.

E coli DH5α was used as the host strain. pGEM®-5Zf(+) containing a unique restriction site for *Nde* I digestion (having compatible end with *Mse*I digested fragment) was used as the cloning vector. This vector supports blue/white screening for recombinants. LigaFast™ Rapid DNA Ligation System from Promega Corporation was used for ligation. Ten µL of ligation reaction was assembled according to the manufacturer's instruction, and then incubated at room temperature for 5 minutes before commencing the transformation process.

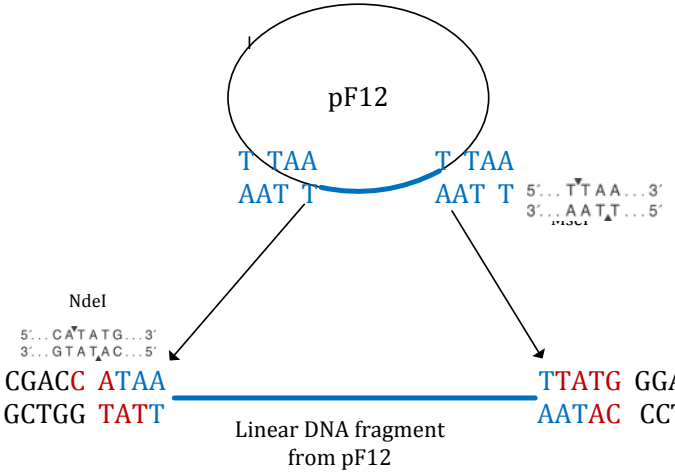
The selection of transformed cells was carried out using a combination of antibiotic marker and blue and while colony selection. Cells transformed with the ligated plasmid DNA were plated onto an X-Gal/IPTG/Amp100 plate. Plates were incubated at 37˚C overnight and white colonies were picked and purified on LB+Amp100 agar plates.

Plasmid extraction and gel electrophoresis was then performed on each single colony to screen for successful cloning and the size of inserts. Growth of transformants on triclosan minimal medium was also checked as a control method to exclude potential contamination.

pGEM®-5Zf(+) vector

5'……..TGTAA TACGA CTCAC TATAG GGCGA ATTGG GCCCG ACGTC GCATG CTCCC GGCCG CCATG GCCGC GGGAT ATCAC TAGTG CGGCC GCCTG

3'……..ACATT ATGCT GAGTG ATATC CCGCT TAACC CGGGC TGCAG CGTAC GAGGG CCGGC GGTAC CGGCG CCCTA TAGTG ATCAC GCCGG CGGAC



CAGGT CGACC ATAA TTATG GGAGA GCTCC CAACG CGTTG GATGC ATAGC TTGAG TATTC TATAG TGTCA CCTAA AT……..3'

GTCCA GCTGG TATT AATAC CCTCT CGAGG GTTGC GCAAC CTACG TATCG AACTC ATAAG ATATC ACAGT GGATT TA……..5'

Figure 52 Illustration of pF12 cloned into pGEM®-5Zf (+) vector

(Vector and primer sequences extracted from Promega® product information sheet)

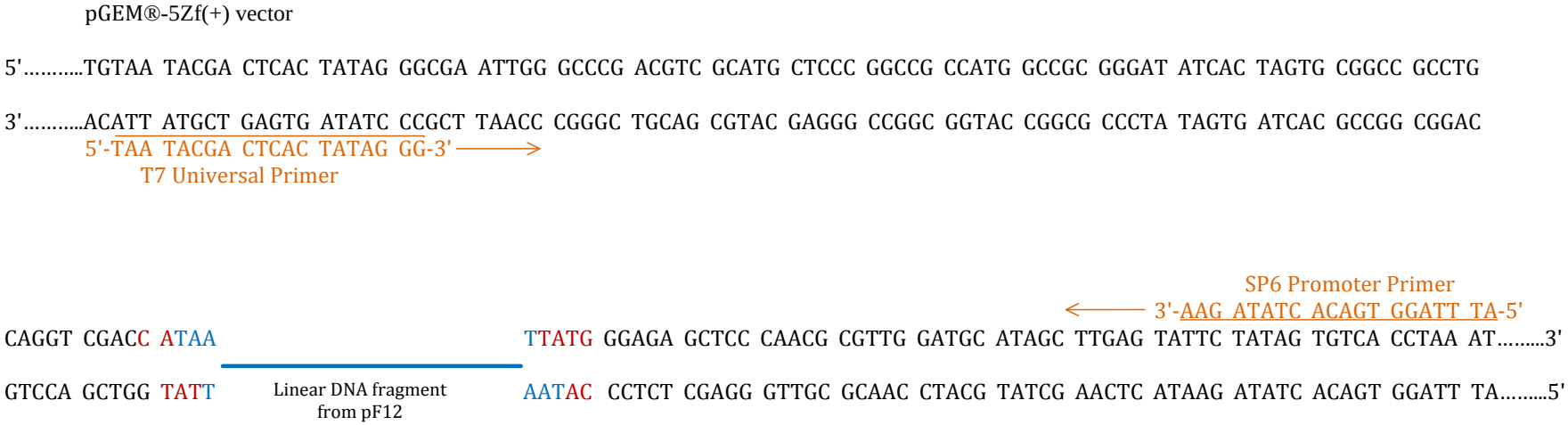


Figure 53 Illustration of DNA sequencing of cloned fragments

(Vector and primer sequences extracted from Promega® product information sheet)

6.2.3 Cloning using pWKS130 vector

E. coli DH5α strain was used as the host strain. Cloning vector pWKS130 (kindly donated by Sophia Tan, IMVS, Adelaide) containing a unique *Bam*HI recognition site was used as a vector. LigaFast™ Rapid DNA Ligation System from Promega Corporation was used for ligation.

After incomplete digestion of pF12 by *Sau*3AI, and complete digestion of the pWKS130 vector by *Bam*HI, cut plasmids were ligated using the LigaFast™ Rapid DNA Ligation System from Promega Corporation. A total of 10 µL ligation reaction was assembled according to the LigaFast™ product information. The reaction was mixed by gentle flicking and incubated at room temperature for 5 minutes before commencing transformation process.

Serial dilutions of cells transformed with ligated plasmid DNA were then plated onto X-gal/IPTG/Amp100 LB agar plates. Plates were incubated at 37˚C aerobically overnight and then 4˚C overnight to allow for colour expression. White colonies were picked and purified on LB+Amp100 agar plate.

Plasmid extraction and gel electrophoresis was then performed on each single colony to check for successful cloning and the size of inserts after restriction digestion. Growth of transformants on triclosan minimal medium was also checked.

6.2.4 Chemical transformation

Rubidium chloride – processed chemically-competent *E coli*. DH5α cells were prepared according to a method modified from User's Notes on the Biolab website (http://www.neb.com/nebecomm/tech_reference/gene_expression/RbCl_protocol.asp).
Twenty five µl of ice-thawed cells were used for the circular plasmids and 100 µl for ligation reactions. The DNA-cell suspension was incubated on ice for 1 hour, and then subjected to heat shock at 37˚C for 45 seconds. The suspension was then diluted 15-fold into SOC after incubation on ice again for 2 minutes. The diluted suspension was then incubated at 37˚C with vigorous aeration for 20 minutes for phenotypic expression. Transformants were then plated on selective agar plates containing appropriate antibiotics.

6.2.5 Restriction digest

Restriction enzymes from Promega Corporation at concentrations of 10-12 unit/µl were used in restriction digestion reactions. A total volume of 20µl restriction digest reaction was assembled in accordance with the product information. Reaction mixtures were incubated at the specified optimum temperature overnight. After digestion, 4 µL of 6× loading buffer was added and then prepared for gel electrophoresis for separation and visualization. Restriction enzymes used in cloning are listed in Table 16.

Table 16 Restriction enzymes used in cloning

	Cloning using pGEM®-5Zf(+) vector			Cloning using pWKS130 vector		
DNA	pF12	pGEM®-5Zf(+)	clones	pF12	pWKS130	clones
Enzyme	*Mse*I	*Nde*I	*Sal*I/*Sac*I	*Sau*3AI	*Bam*HI	*Xba*I/*Kpn*I

For pWKS130 clones, a double digestion with *Xba*I and *Kpn*I was performed in MultiCore® buffer. For pGEM®-5Zf(+) clones, a sequential digestion by *Sal*I and then by *Sac*I was performed. Five µL of the *Sal*I digest was added as template into *Sac*I restriction digestion reaction.

Incomplete digestion of pF12 by *Sau*3AI was performed for limited digestion durations ranging from 30 seconds to 5 minutes and then prepared for gel electrophoresis or a ligation reaction was carried out.

Uncut experimental plasmid DNA and a "mock" digestion (no enzyme) were included as controls. One µL of DNA molecular weight marker III (0.12-21.2kbp) (λDNA·*Eco*RI and Hind III digested) was run on both sides of the large agarose gels.

6.2.6 DNA Sequencing and primer walking

The general procedure for plasmid sequencing is illustrated in Figure 54.

DNA sequencing in this study was carried out using the dye-terminator sequencing method. Although new sequencing methods, such as automated high-throughput DNA sequencing

has largely replaced the dye terminator sequencing method (Mitchelson, 2007, *pp.* 3-44) in recent years, it was not available at the time of this project.

DNA sequencing of cloned pF12 fragments using appropriate primers was carried out as shown in **Error! Reference source not found.**. The pGEM®-5Zf (+) vector DNA sequence ontains T7 and SP6 RNA polymerase promoters flanking the multiple cloning region within the α-peptide coding region of β-galactosidase. Therefore, primers suitable for DNA sequencing include T7 universal primer and SP6 promoter primer. T7 Universal primer (20-mer) anneals to the T7 region of any vector containing the T7 Promoter, and SP6 promoter primer anneals to the SP6 promoter region. For pWKS130 vector, M13 universal sequencing primers (18-mer) were used.

The DNA fragment sequences can then be assembled electronically into one long, contiguous sequence by identifying 100% identical overlapping sequences between them. Gaps in the assembled sequences may be filled by "primer walking", where new primers are designed at both ends of the existing contigs and sequences get extended as a result of further DNA sequencing, i.e. "walking" into the unknown sequences. New primers can then be designed based on the new sequences obtained from previous reaction, and the whole sequence will then be obtained eventually by gradual "primer walking" through the entire sequence.

DNA Sequencing was carried out at the sequencing facility, Flinders Medical Centre, and data were analysed using Invitrogen® Vector NTI Advance 10 package (Personal Static Licence v.10 155749).

Walking primers were designed using Seqman in DNASTAR Lasergene 7 package. The primers were then subject to complementary test using online primer3 software. Walking primers were synthesised at Geneworks. Primers designed to "walk" through the whole sequence of pF12 are listed in Appendix 6.

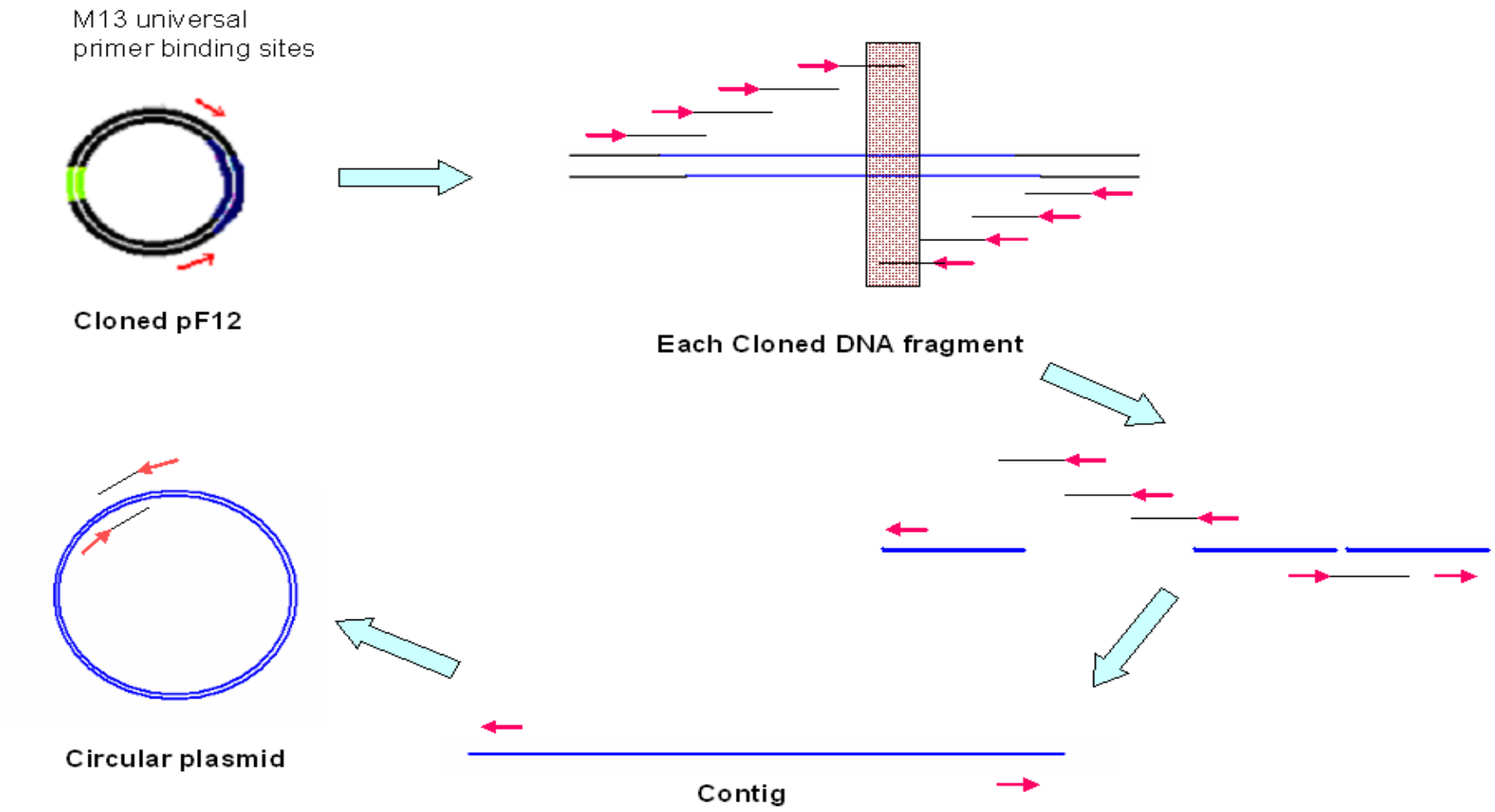


Figure 54 Illustration of plasmid sequencing procedures

6.2.7 Plasmid sequence analysis

The sequences obtained from the sequencing facility were in ABI file format and were imported into sequence analysis software. Sequences were initially analysed using DNASTAR Lasergene 7 (trial version) and then finalized and presented using Vector NTI Advance 10 (Personal Static Licence v.10 155749) from Invitrogen.

Sequences of each inserted DNA fragment were analysed firstly using ContigExpress in the Invitrogene package. Bad sequences on both ends were trimmed until 25bp contained less than 3 ambiguities. The end-trimmed sequences were then further trimmed to avoid vector contamination. As no pGEM®-5Zf (+) vector information was available in the Invitrogen package, sequences of pGEM®-5Zf (+) vector (Promega) were initially imported into the vector database of Invitrogen package using Vector NTI Explorer. After examining and adjustment of proper trimming, fragments of each cloned inserts were assembled individually to form contigs of cloned DNA inserts.

These contigs were then converted into fragments, each of which contained sequence information for each DNA insert. These fragments were then assembled together with the primer walking sequences to form a single contig containing all the sequences of the plasmid. This sequence was then exported into Vector NTI and AlignX, Blast viewer and Blast search in Invitrogen package for further DNA sequence and protein analysis.

Sequence analysis was carried out for the whole sequence of plasmid pF12, and hypothetical gene products were searched against NCBI Non-Redundant (NR) Protein Database to predict the function of each gene product. The evolution and possible function of the plasmid was then predicted based on the plasmid annotation results.

6.3 Results

6.3.1 Preparation of plasmid DNA

As shown in Figure 55, more concentrated purified plasmid DNA was extracted from *Pseudomonas citronellolis* F12 strain using QIAGEN Large-construct kit than using other methods. This kit includes an exonuclease digestion step to remove all linear DNA

including chromosomal DNA and fractioned plasmid DNA to ensure the purity of the preparation.

When run with a low percentage agarose gel, plasmid DNA was seen as bands of plasmid dimer, plasmid OC form and plasmid CCC form (super coiled) and irreversibly denatured form of plasmid as shown in Figure 56.

The initial plasmid preparation showed two distinct bands on 0.8% agarose gel. In order to determine if the two bands were multiple plasmids in this *Pseudomonas citronellolis* F12 strain or if they represent different forms of one single plasmid, the lower bright band (highlighted in Figure 55) was cut and extracted from the agarose gel using QIAGEN gel extraction kit and then run in parallel with the original plasmid preparation sample on one 0.5% gel.

As shown in Figure 56, the two samples ran a similar pattern on the gel so that we could primarily confirm that there is only one plasmid in the strain and the multiple bands seen on the low percent gel were merely different forms of the same plasmid. This was further confirmed in the restriction digestion experiment.

The same DNA samples as shown in Figure 56 were digested using restriction enzyme *Mse*I and the gel electrophoresis signature was compared. As shown in Figure 57, the size of *Mse*I digested fragments of the two DNA samples were the same, confirming that there was only single plasmid extracted from F12 and the different bands shown in individual lane of Figure 56 are merely different forms of the same plasmid.

The preparation of highly purified and concentrated intact plasmid DNA is essential for successful sequencing. It was found in this study that the recombinant plasmids derived from clones using pGEM®-5Zf (+) vector could be easily obtained using a mini prep kit due to the relatively small size (<10kb) and the high copy number of the reconstructed plasmid DNA, whereas it was more difficult to obtain reconstructed plasmids from clones using pWKS130 vector due to the lower copy number of this vector in host cells.

The concentration of pF12 prepared using the QIAGEN large construct kit was measured using BioPhotometer 6131 (V1.35). As shown in Table 17, a one in sixteen diluted DNA solution, using nuclease free water, was measured as 5.8 ng/µL. Therefore, the concentration of pF12 prepared using the QIAGEN large construct kit was 87 ng/µL.

Subsequent preparations showed an average concentration of approximately 100 ng/µL using this kit, whereas it was less than 20 ng/µL using other plasmid preparation method (data not shown).

Table 17 Concentration of pF12 plasmid preparation using the QIAGEN large construct kit

Plasmid	**Sample No.**	**O.D.**	**wavelength**	**Ratio**		**Plasmid DNA Concentration**
Blank (nfw)	Blank	0.000	A			
pF12 plasmid preparation (1/16 dilution)	#0001	0.062	A_{230}			5.8 µg/mL dsDNA
		0.115	A_{260}	1.73	260/280	
		0.067	A_{280}	1.84	260/230	
		0.011	A_{320}			
	#0002	0.062	A_{230}			5.8 µg/mL dsDNA
		0.115	A_{260}	1.73	260/280	
		0.067	A_{280}	1.84	260/230	
		0.011	A_{320}			

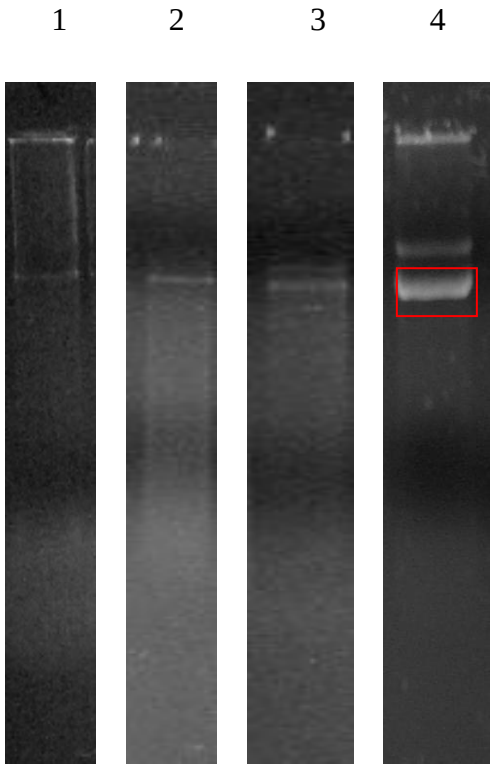


Figure 55 Comparison of different plasmid extraction method

Lane 1-4: Phenol extraction, 25mL prep kit, mini prep kit, QIAGEN large construct kit (10µL of each prepared sample) on 0.8% agarose gel with Gelstar incorporated in the gel

Note: Plasmid size is not characterised here

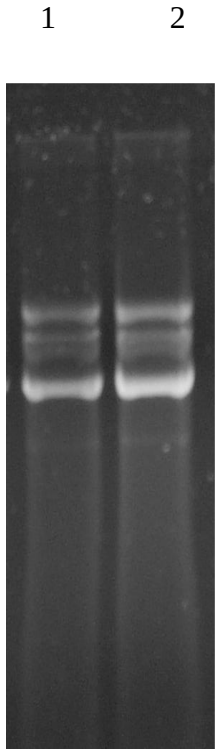


Figure 56 Comparison of initial Plasmid preparation and DNA extract of cut band from agarose gel in Figure 55 (0.5% TAE agarose gel)

Lane 1 Extract from cut gel of the bright band shown in Figure 55;

Lane 2 Initial plasmid preparation sample

Note: Plasmid size is not characterised here.

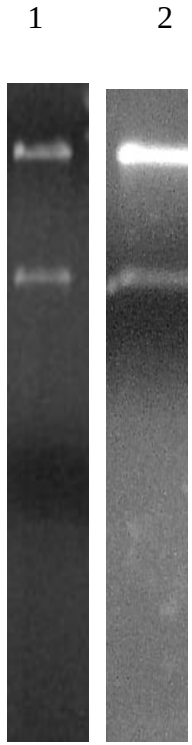


Figure 57 Comparison of restriction enzyme digested plasmid DNA and DNA extracted of cut band from agarose gel in Figure 55 (0.5% TAE agarose gel).
Lane 1 *Eco*RI digested original pF12 DNA sample

Lane2 *Eco*RI digested DNA extracted from cut gel

Note: Plasmid size is not characterised here.

6.3.2 Plasmid cloning using pGEM®-5Zf(+)

Linearised fragment DNA and vector DNA

The restriction digest pattern of pF12 with *Mse*I, and pGEM®-5Zf (+) by *Nde*I is shown in Figure 58 . The *Mse*I digest of pF12 showed 9 discernible bands sizing from 0.5kb to 4.5kb, total of approximately over 15 kb, when visualized on 0.6% agarose gel.

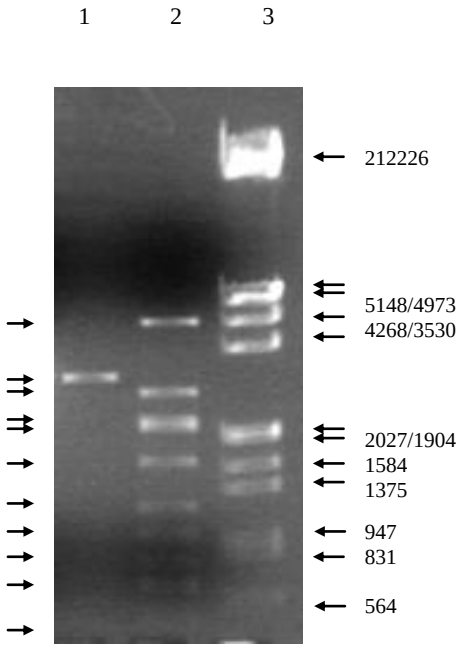


Figure 58 Restriction digestion for cloning
Lane 1: *Nde*I digest of pGEM®-5Zf (+) vector;
Lane 2: *Mse*I digest of pF12 plasmid;
Lane 3: DNA molecular weight marker III (0.12-21.2kbp)
(λDNA•*Eco*RI and *Hin*D III digested)

Ligation and transformation into E coli DH5α competent cells

Multiple colonies were observed on the selective plates for the transformants $RbCl_2$ processed DH5α competent cells transformed with pGEM®-5Zf (+). Multiple colonies were also observed on plates selective for the same competent cells transformed with the

ligation reaction. Sixteen white colonies were observed scattered among the green colonies, indicating a successful cloning.

Screening of clones

All white colonies were purified and plasmid preparation procedures were carried out using QIAGEN mini prep kit for all clones, and constructed plasmid DNA was digested using *Sal*I and *Sac*I.

Eight bands of different sizes corresponding to the restriction digest of pF12 were obtained from screening of clones as seen in Figure 59. Each recombinant plasmid was digested into two DNA fragments. The identical fragments present in Lanes 3-11 with a band size of approximately 3 kb represented the cleaved vector DNA and the other fragment in each of these samples represented the cloned fragment. Eight clones of approximately 5kb, 2.2kb, 1.8kb, 1.2kb, 1kb, 800bp, 600bp and 400bp DNA fragments could be seen from this agarose gel preparation. Some of the gel preparations obtained during screening of clones are shown in Appendix 4.

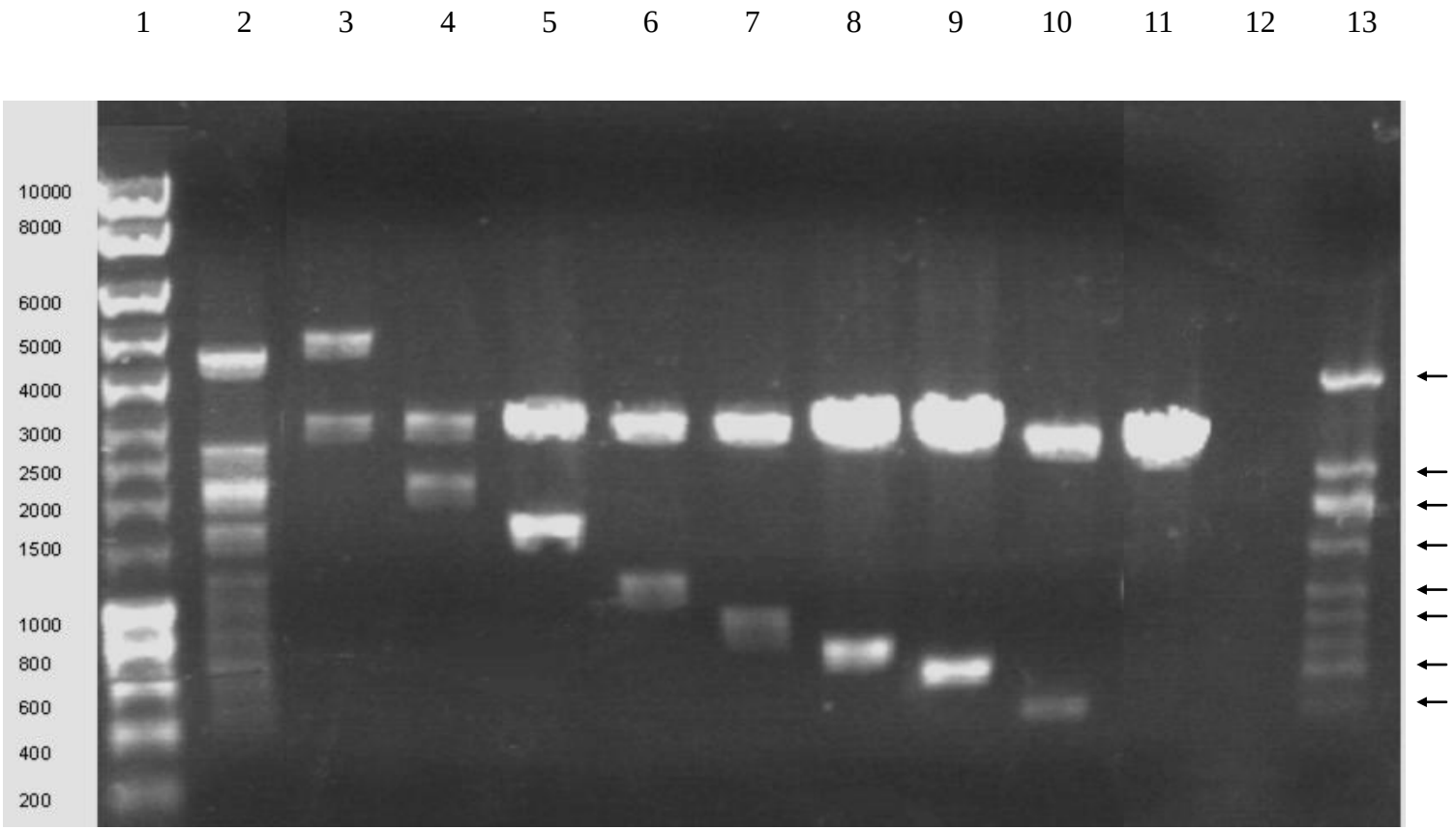


Figure 59 Plasmid cloning of pF12 using pGEM®-5Zf (+)

Lane 1 200bp DNA marker; Lane 2 MseI digested pF12; Lane 3 SalI/SacI double digested pF12 clone 1; Lane 4 SalI/SacI double digested pF12 clone 2; Lane 5 SalI/SacI double digested pF12 clone 3; Lane 6 SalI/SacI double digested pF12 clone 4; Lane 7 SalI/SacI double digested pF12 clone 5; Lane 8 SalI/SacI double digested pF12 clone 6; Lane 8 SalI/SacI double digested pF12 clone 7; Lane 10 SalI/SacI double digested pF12 clone 8; Lane 11 SalI/SacI double digested pGEM5; Lane 12 Blank; Lane 13 MseI digested pF12

6.3.3 Plasmid cloning using pWKS130

Incompletely digested pF12, at various digestion time from 30 seconds to 30 minutes, by *Sau*3AI enzyme of various concentration was used in the ligation reactions, but the resulting DNA fragments were small (data not shown). This is possibly because the recognition sequence of *Sau*3AI (GATC) consists only 4bp.

Many more white colonies were observed on the selective plates compared with the plates in the cloning using pGEM5 vector; however, only two inserted DNA fragments were obtained after screening on electrophoresis gel (Approx. 1kb and 3 kb, data not shown). No obvious cloned bands were observed in the other transformants.

6.3.4 *Sequencing and primer walking*

DNA from both the pGEM and pWKS reconstructed plasmids were extracted and sequenced. Primers for primer walking were designed based on the available sequencing results.

As a result of DNA sequencing of the cloned fragments and primer walking, the whole sequence of this plasmid was determined, with average overlapping rate of over 1.9, i.e. in average, every DNA was sequenced 1.9 times. Figure 60 is an overview of location of each sequenced fragments on this plasmid. The DNA sequencing results were verified by comparing computer generated gel diagrams with actual laboratory digested DNA electrophoresis gel preparation. The computer generated gels were based on DNA sequences obtained through DNA sequencing, and the circular sequence obtained from sequencing result was artificially digested by a computer program based on the restriction enzyme (*Eco*RI and *Not*I as shown in Figure 61 and *Mse*I as shown in Figure 62) recognition site sequence information. By comparing the computer generated gel with the actual gel electrophoresis pattern of the restriction enzyme digested plasmid DNA, any miss-assembly of the DNA sequence during the primer walking process would be identified, and a good matching of the two restriction digested gel signature indicates correct assembly of fragment sequences and therefore verifies the sequencing results.

From Figure 61 and Figure 62, it can be observed that there is a good match between the computer generated gels and the laboratory based gel patterns, indicating that the pF12 DNA sequencing result is complete and of good quality.

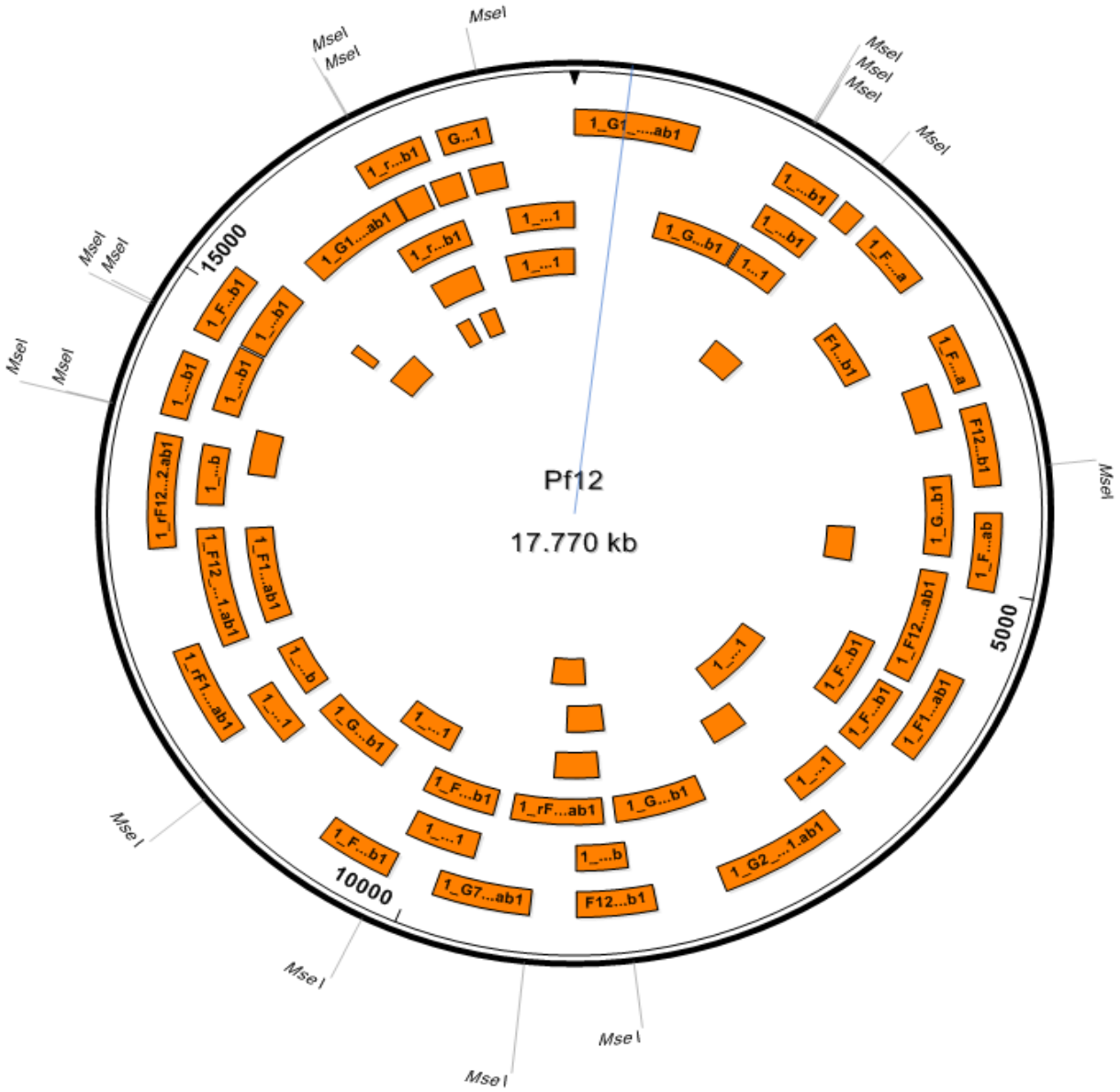
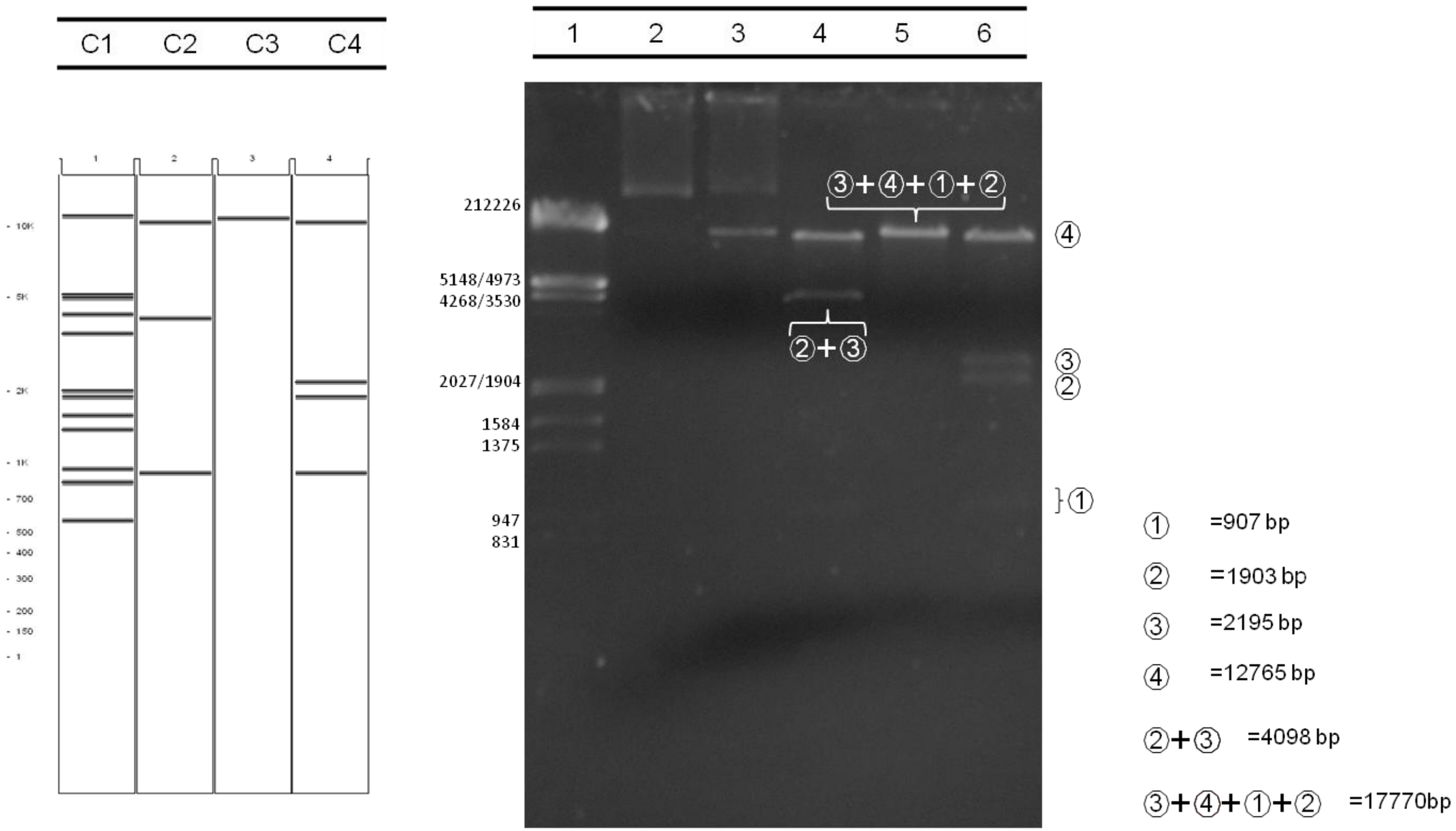
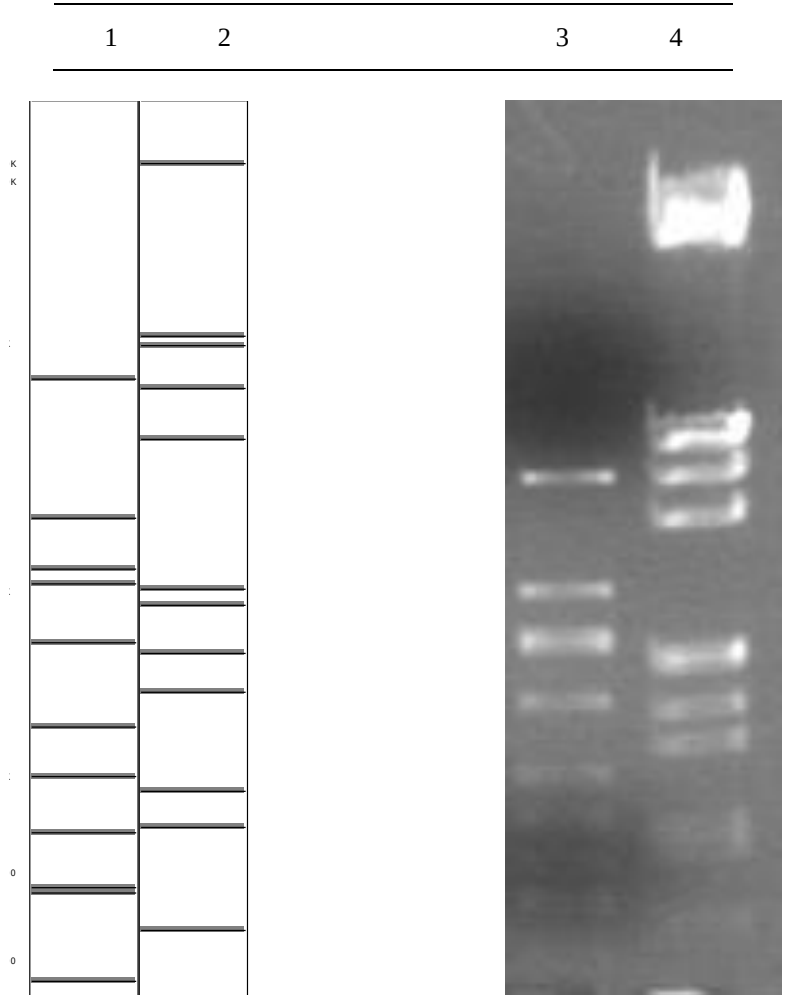


Figure 60 Overview of contigs alignment and coverage of sequences



Lane C1-C4 Computer generated DNA marker II, *Eco*RI digestion of pF12, *Not*I digestion of pF12, *Eco*RI/*Not*I double digestion of pF12, based on DNA sequencing results;
Lane 1-6 Laboratory electrophoresis gel picture: DNA marker II, pF12 uncut, Mock digest, *Eco*RI digestion of pF12, *Not* I digestion of pF12, *Eco*RI/*Not*I double digestion of pF12

Figure 61 Comparison of computer generated gel based on DNA sequences and laboratory restriction enzyme digest gel-1



Lane 1, 2: Computer generated gel: *Mse*I digestion of pF12 based on DNA sequences, DNA marker II;
Lane 3, 4: Laboratory electrophoresis gel picture of *Mse*I digested pF12, Lane 2 DNA marker II
Figure 62 Comparison of computer generated gel and laboratory restriction enzyme digest gel-2

6.3.5 Plasmid annotation

Plasmid pF12 is a circular DNA molecule of 17,770 bp. It was predicted by the sequence analysis software Vector NTI advance 10 (Invitrogen, Australia), which is based on the start and stop codons, that the plasmid pF12 could contain a total of 29 ORFs that may encode functional proteins.

The overview of the predicted proteins encoded on pF12 is shown in Figure 63. A list of predicted functions of pF12-encoded gene products with identity and E value is shown in Table 18 and summarised below.

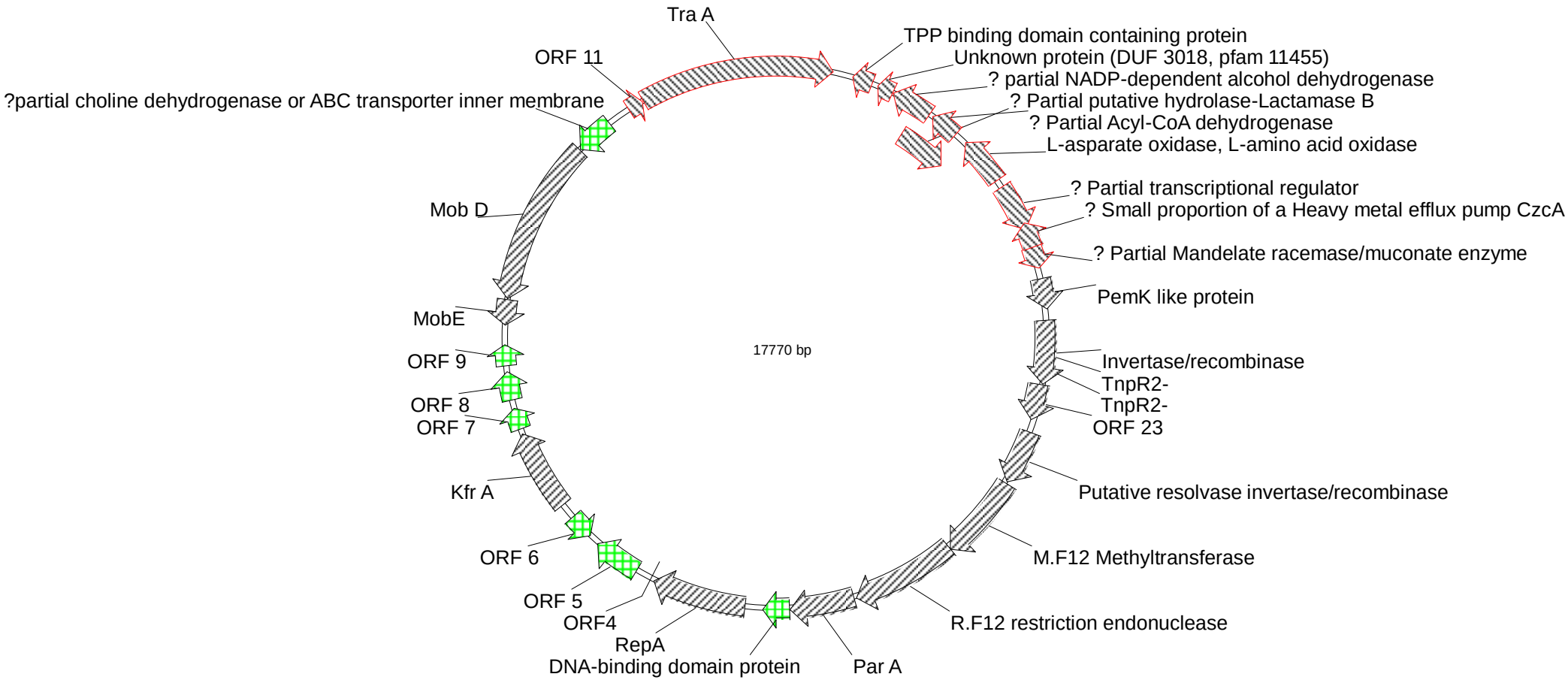


Figure 63 Overview of predicted putative proteins

Table 18 Predicted pF12-encoded gene products

Designated gene products	sequence	Closest relative from Genebank database	aa size	AC number	Identity	E-value
M.F12	14223-15122	Pac25I methyl transferase	299	YP 025343	99%	9e-176
R.F12	15122-16228	Pac25I endonuclease	333	YP 025342	98%	
Par A	16255-16933	ParA	216	ABC 70161	82%	4e-84
Putative DNA binding domain protein	16937-17218	DNA-binding domain protein	89	CAZ88706	71%	5e-10
RepA	17405-627	RepA	337	AAP22621	70%	2e-126
ORF 4	668-790	Proline-rich extension-like family protein (partial)	249	NP197482	43%	2.9
ORF5	838-1329	Unknown	694	XP001501095	29%	0.13
ORF6	1445-1711 (c)	RNA-biding S4 domain protein, unknown	674	YP002422892	37%	0.22
Kfr A	1876-2733	Kfr A-like protein	303	YP245474	high	2e-17
ORF 7	2787-3011	Unknown				
ORF 8	3099-3401	Unknown				
ORF 9	3462-3680	Unknown				
Mob E	3886-4152 (c)	Mob E	92	YP 025351	30/89	6e-07
Mob D	4166-5911 (c)	Mob D	595	YP025350	330/597	4e-176
Partial choline dehydrogenase or ABC transporter inner membrane	5921-6328 (c)	Partial choline dehydrogenase or ABC transporter inner membrane	377	EEY16583	28/82	1.4
ORF 11	6577-6738	Unknown				

Designated gene products	sequence	Closest relative from Genebank database	aa size	AC number	Identity	E-value
ORF 12	6749-8770	Unknown				
TPP binding domain containing protein	8977-9192	TPP binding domain containing protein				
ORF 14	9256-9429 (c)	Protein (pfam 11455, OUF3018)				
Partial putative hydrolase-lactamase B	9731-10336	Hydrolase-lactamase B				
Partial NADP-dependent alcohol dehydrogenase putative	9433-9873 (c)	NADP-dependent alcohol dehydrogenase putative				
Putative partial acyl-CoA dehydrogenase	9914-10219	Acyl-CoA dehydrogenase				
Putative oxidase	10352-10867 (c)	L-asparate oxidase, L-amino acid oxidase		YP_001818213 YP_002235516		
Partial transcriptional regulator	10952-11461	Transcriptional regulator				
Partial heavy metal efflux pump	11390-11659	Heavy metal efflux pump CzcA partial CD/TrbP				
Partial mandelate racemase/muconate enzyme	11654-11887	Mandelate racemase/muconate enzyme				
PemK-like protein	11999-12319	Putative PemK-like protein	106	YP361518	105/106	2e-50
TnpR2-2	12713-12871 (c)	Transposon (revertase/recombinase)	231	YP002791397	high	2e-75
TnpR2-1	12871-13023 (c)	Transposon (putative resolvases/invertases/ recombinase)	193	YP003376055	158/188	9e-73
ORF 23	13119-13499	Hypothetical protein Rpic12D_5127	127	YP002973601	119/127	1e-62

- ORF 14223-15122

The closest relative of this ORF was found to be Pac25I methyl transferase (YP 025343), which contains 299 amino acids. The identify is 99%, with E-value of 9e-176. This ORF was designated as M.F12.

- ORF 15122-16228

The closest relative of this ORF was found to be *Pac*25I endonuclease (YP 025342), which contains 333 amino acids. The identity is 98%.. This ORF was designated as R.F12.

- ORF 16255-16933

The closest relative of this ORF was found to be ParA (ABC 70161), which contains 216 amino acids. The identity is 82%, with E-value of 4e-84. This ORF was designated as ParA.

- ORF 16937-17218

The closest relative of this ORF was found to be DNA-binding domain protein (CAZ88706), which contains 89 amino acids. The identity is 71%, with E-value of 5e-10. This ORF was designated as DNA-binding domain protein.

- ORF 17405-627

The closest relative of this ORF was found to be RepA (AAP22621), which contains 337 amino acids. The identity is 70%, with E-value of 2e-126. This ORF was designated as RepA.

- ORF 668-790

The closest relative of this ORF was found to be part of Proline-rich extension-like family protein (ABC 70161), which contains 249 amino acids. The identity is 43%, with E-value of 2.9. This ORF was designated as ORF 4.

- ORF 1445-1711 (c)

The closest relative of this ORF was found to be part of a RNA-biding S4 domain protein (YP002422892), which contains 674 amino acids. The identity is 37%, with E-value of 0.22. This ORF was designated as ORF6.

- ORF 1876-2733

The closest relative of this ORF was found to be Kfr A-like protein (YP245474), which contains 303 amino acids. The E-value of 2e-17. This ORF was designated as Kfr A.

- ORF 3886-4152 (c)

The closest relative of this ORF was found to be Mob E (YP 025351), which contains 92 amino acids. The identity is 34%, with E-value of 6e-07. This ORF was designated as Mob E.

- ORF 4166-5911 (c)

The closest relative of this ORF was found to be Mob D (YP025350), which contains 595 amino acids. The identity is 56%, with E-value of 4e-176. This ORF was designated as Mob D.

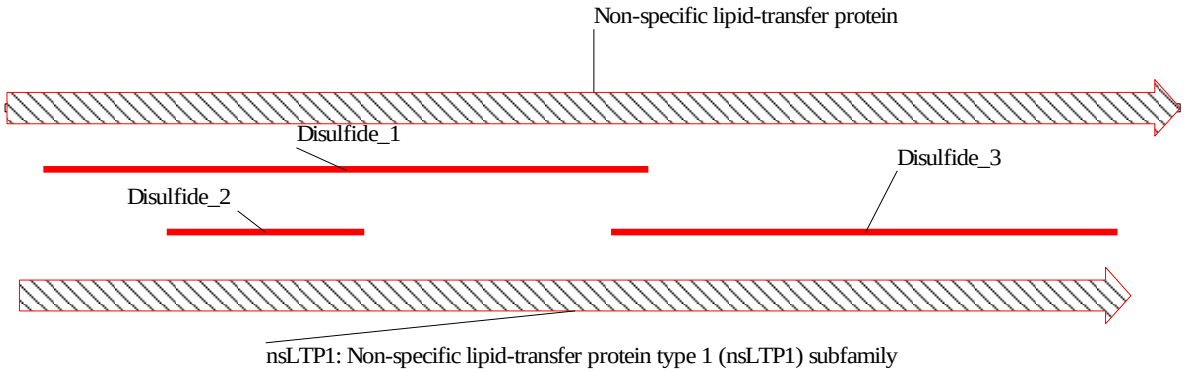
- ORF 5921-6328 (c)

The closest relative of this ORF was found to be part of a choline dehydrogenase or ABC transporter inner membrane (ZP_04994344/EEY16583), which contains 377 amino acids. The identity is 35%, with E-value of 1.4. This ORF was designated as partial choline dehydrogenase or ABC transporter inner membrane.

- ORF 8977-9192

The closest relative of this ORF was found to be a non-specific lipid transfer protein (P23802,), which contains 95 amino acids. The organization of this 95bp non-specific lipid transfer

protein and the amino acid sequence alignment with ORF 8977-9192 is shown in Figure 64.



95 bp

```
                1                                                  50
   ORF 14   (1) ---MGCQTMQQALAVASSPGARGRRPASCPSGGSGSTAAPR----QRRIC
   P23802   (1) AISCGQVSSAIGPCLAYARGAGAAPSASCQSGVRSLNAAARTTADRRAAC
Consensus   (1)    G  S   A  LA A GA A   ASC SG    AA R    R  C

               51                                          95
   ORF 14  (44) TCPRRCKAGFLGGCATWKRRTVPGRASCR----------------
   P23802  (51) NCSLKSAASRVSGLNAGKASSIPGRCGVRLPYAISASIDCSRVNN
Consensus  (51)  C  K  A  L G    K  SIPGR   R
```

Figure 64 Amino acid sequence alignment of the putative TPP binding domain containing protein and Non-specific lipid-transfer protein P23802.
Identical amino acids are highlighted in yellow, block of similar amino acids are highlighted in green, and weakly or non-similar amino acids are not highlighted. Gaps have been introduced to optimize the homology.

This ORF 8977-9192 also has significant similarity to the TPP binding module of a thiamine pyrophosphate-requiring enzyme system, BAG 45504. The amino acid sequence alignment of the ORF 8977-9192 with the thiamine pyrophosphate binding domain containing protein BAG 45504 is shown in Figure **65**.

Thiamine pyrophosphate-requiring enzymes include acetolactate synthase, pyruvate dehydrogenase (cytochrome), glyoxylate carboligase, and phosphonopyruvate decarboxylase. This domain provides functions, such as amino acid transport and metabolism / Coenzyme metabolism.

This ORF was designated as TPP binding domain containing protein.

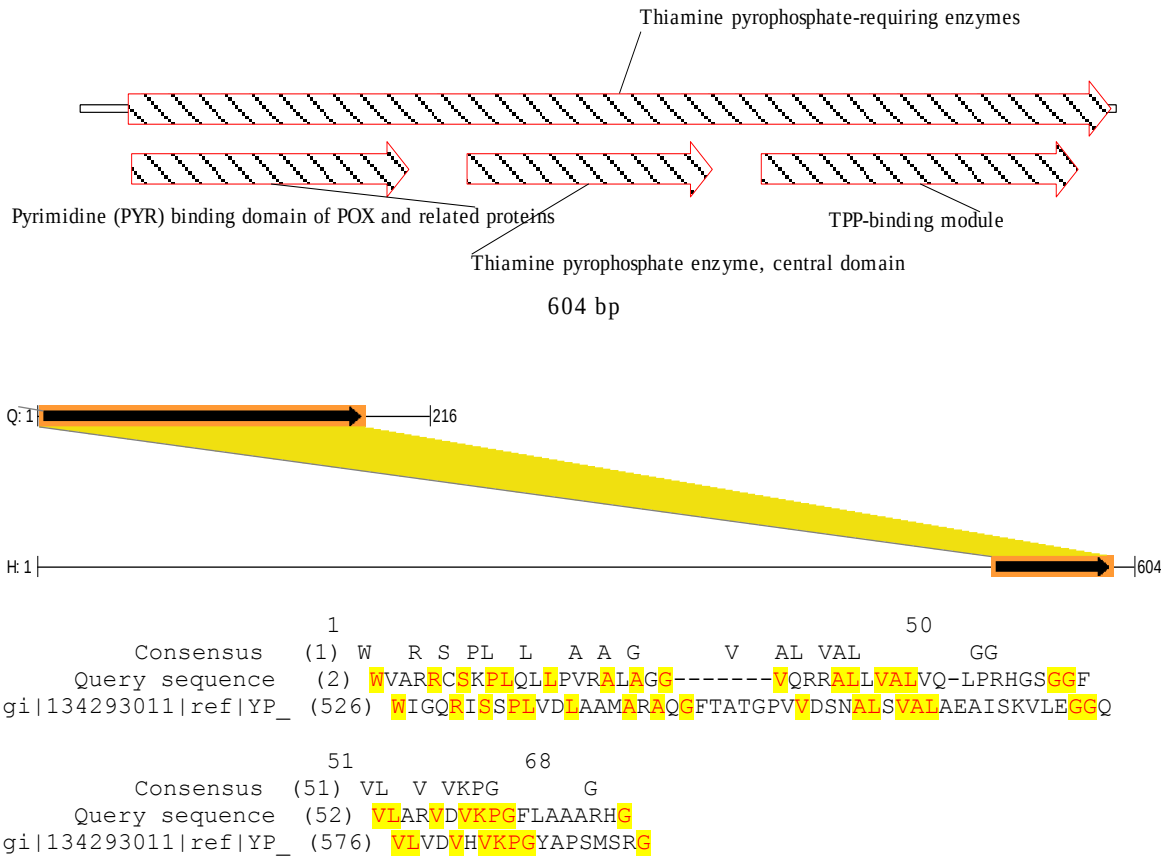


Figure 65 Amino acid sequence alignment of putative pF12 TPP binding domain containing protein and Thiamine pyrophosphate binding module BAG45604.
Identical amino acids are highlighted in yellow. Gaps have been introduced to optimize the homology.

- ORF 9256-9429 (c)

The closest relative of this ORF was found to be ParA (pfam 11455, OUF3018). This ORF was designated as ORF14.

- ORF 9731-10336

The closest relative of this ORF was found to be part of a putative hydrolase-lactamase B. This ORF was designated as partial putative hydrolase-lactamase B.

- ORF 9433-9873 (c)

The closest relative of this ORF was found to be part of a NADP-dependent alcohol dehydrogenase. This ORF was designated as partial NADP-dependent alcohol dehydrogenase.

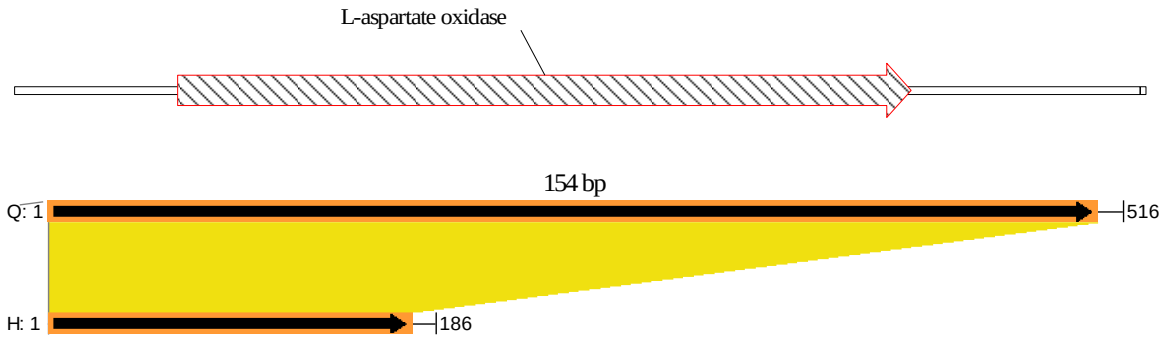
- ORF 9914-10219

The closest relative of this ORF was found to be part of an acyl-CoA dehydrogenase (ABC 70161/ZP_0470611). This ORF was designated as partial acyl-CoA dehydrogenase.

- ORF 10352-10867 (c)

The closest relative of this ORF was found to be L-asparate oxidase (gi|294624924|YP 002235516) and a L-amino acid oxidase (gi|182413147|YP_001818213).

The L-aspartate oxidase contains 154 amino acids in total and the active site contains 154 amino acids. ORF 10352-10867 (c) covers the full range of this oxidase. The alignment of this ORF with L-aspartate oxidase using NCBI BLAST program (Altschul et al., 1997) was shown in Figure 66. The alignment uses the compositional matrix adjustment method (Altschul et al., 2005). The identity between the two sequences is only 33%; however the E-value is 4e-22.




```
Score = 85.5 bits (210), Expect = 4e-22, Method: Compositional matrix adjust.
Identities = 58/176 (33%), Positives = 84/176 (48%), Gaps = 39/176 (22%)

Query  1    MQFRSRTYRDKDGNIKQGQTIQLMRYVYDAENKRGRQVMIGSFKVDDPRLPPEVEALLTP  60
            M  R +T+RDK G ++QG+T+ L R VYDA+  + R V +G+      LP E++A+LT
Sbjct  1    MHIRLQTFRDKQGRLRQGRTVALRRSVYDAKQGKSRLVQLGTVDRFARDLPRELKAVLT-  60

Query  61   QE-KAEFEEWV---------------LDTLDDVMTRSCMALDVGAGTVKDPARIWDLIDAL  105
            QE K EF +W+               L  L + +  +  AL  G    +DP R+WD I  L
Sbjct  61   QEEKGEFRQWIAARDQQISELLQRHHLGRLAEYLGLAAKALSSGV-EPEDPERLWDAIGVL  119

Query  106  ERSMTRAGH---------------ESEDDFAALD--------TLPDFIPPEKKKAP  138
            R++ +AGH               S +D   LD        +P+FIP E   AP
Sbjct  120  TRALEKAGHPKPTKERGRPVKASTPSAEDLIDLDESLVWVSENMPNFIPEELLVAP  175
```

Figure 66 Amino acid sequence alignment of ORF 10352-10867 (c) and provisional L-aspartate oxidase (gi|294624924|YP 002235516).

L-amino acid oxidase (gi|182413147|YP_001818213) is a monoamine oxidase that is involved in amino acid transport and metabolism (Geueke & Hummel, 2002).The alignment of the reference protein and ORF 10352-10867 (c) is shown in Figure 67, which is shown to cover only a small region (84/531) of the active site of this monoamine oxidase, and the identity is only 26/84.

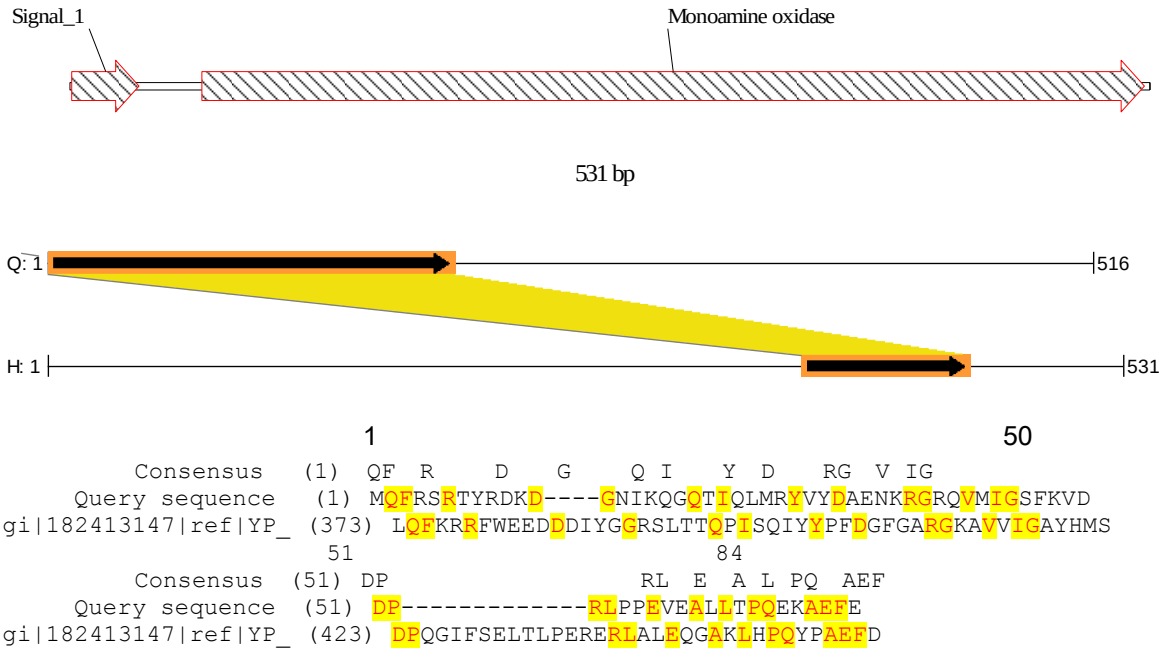


Figure 67 Amino acid sequence alignment (partial) of ORF 10352-10867 (c) and a L-amino acid oxidase (YP-001818213).
Identical amino acids are highlighted in yellow. Gaps have been introduced to optimize the homology.

ORF 10352-10867 (c) also has significant similarity to a predicted ATPase (NP_887323, data not shown), although the identical region only covers a small proportion (63aa) of the ATPase protein, which has 1219 amino acids. Despite the small coverage, the similarity is located in the active site of this protein, which indicated a potential similar function between these two molecules.

This ORF was designated as putative oxidase.

- ORF 10952-11461

The closest relative of this ORF was found to be a transcriptional regulator. This ORF was designated as partial transcriptional regulator.

- ORF 11390-11659

The closest relative of this ORF was found to be a heavy metal efflux pump CzcA. This ORF was designated as partial heavy metal effluxes pump CzcA.

- ORF 11654-11887

The closest relative of this ORF was found to be part of a mandelate racemase/muconate enzyme. This ORF was designated as partial 11654-11887.

- ORF 11999-12319

The closest relative of this ORF was found to be a PemK-like protein (YP361518), which contains 106 amino acids. The identity is 99%, with E-value of 2e-50. This ORF was designated as PemK-like protein.

- ORF 12713-12871 (c)

The closest relative of this ORF was found to be a transposon (revertase/recombinase) (YP002791397), which contains 231 amino acids. The identity is 95%, with E-value of 2e-75. This ORF was designated as TnpR2-2.

- ORF 12871-13023 (c)

The closest relative of this ORF was found to be a transposon (putative resolvases/invertases/ recombinase) (YP003376055), which contains 193 amino acids. The identity is 82%, with E-value of 9e-73. This ORF was designated as TnpR2-1.

- ORF 13119-13499

The closest relative of this ORF was found to be a hypothetical protein Rpic12D_5127 (YP002973601), which contains 127 amino acids. The identity is 95%, with E-value of 1e-62. This ORF was designated as ORF 23.

- Other unknown ORFs

No significant identities were found for ORF 838-1329, ORF 2787-3011, ORF 3099-3401, ORF 3099-3401, ORF 3462-3680, ORF 6577-6738, and ORF 6749-8770 as a result of blast search against NCBI protein library. These ORFs were designated as ORF 5, ORF7 ORF 8, ORF 9, ORF 11, and ORF12.

6.4 Discussion

Plasmid pF12 shows a modular organisation (Figure 68), with 29 ORFs (determined by computer software using start/stop codon) that are distributed between two clearly divided domains; one carries plasmid household modules and the other one encodes a catabolic functional domain.

The predicted primary functions of the plasmid pF12 household domain include plasmid replication/maintenance, mobilization, and stabilization; and the catabolic domain include putative transposon elements and a cluster of genes encoding putative triclosan related functions. The predicted proteins and functions encoded in each functional module are discussed in the following sections.

Replication/maintenance module

A significant cluster of direct repeats was found using an online program called REPFIND (http://zlab.bu.edu/repfind/). REPFIND is a program that finds clustered, exact repeats in nucleotide sequences. For each repeat cluster that it finds, it calculates a P-value, which indicates the probability of finding such a concentration of that particular repeat just by chance. Of the many possible clusters for each repeated word, REPFIND selects the one with the most significant P-value. The graphical display of the direct repeats found in pF12 DNA sequence, followed by a textual summary of each repeat cluster is attached in Appendix 5.

The bars show each position of the repeated word within the sequence; the coloured bars indicate those repeats that form the strongest cluster. The different colours serve to distinguish repeats that are close together and have no meaning beyond that. Four perfect direct repeats (AGCAGGCCGC) are found in the predicted replication and maintenance region, and may be the replication origin of this plasmid.

The presence of the putative origin of replication enables this plasmid to replicate itself in foreign (alternative) host cells (Lipps, 2008, Chapter 5), i.e. the plasmid can replicate not only in the cured strain of the original host but also when electroporated into the foreign host *Pseudomonas aeruginosa* as demonstrated in the electroporation experiment.

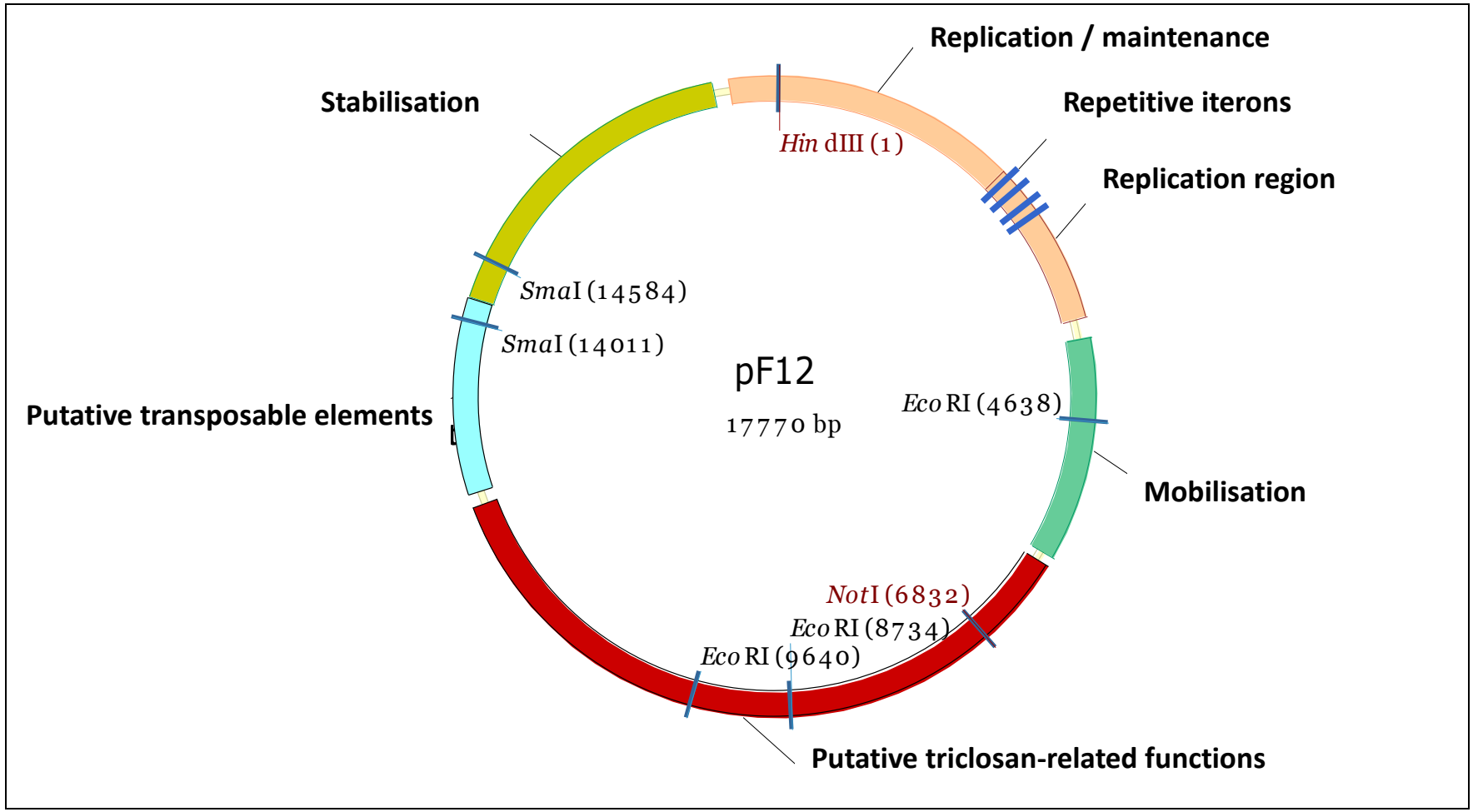


Figure 68 Modular organization of plasmid pF12

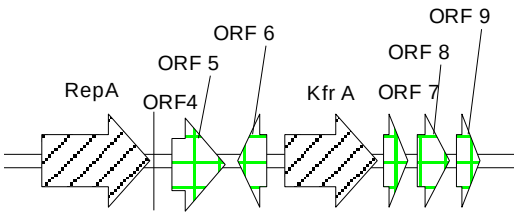


Figure 69 Genetic map of pF12 replication/maintenance region

As shown in the genetic map for this region (Figure 69), the RepA protein, which is essential for replication, is predicted to be located upstream of the hypothetical replication region and a KfrA protein encoded downstream. No other ORFs in this region were found to have high sequence homology to reference protein sequences in the Gene bank databases.

However, two gene products were predicted to be involved in plasmid replication on the basis of their location on the plasmid. The closest relatives from the Gene bank database are a proline-rich extension-like family protein (Identity 43%) and F-box and leucine-rich repeat protein (Identity 29%), respectively. KfrA-like protein belongs to pfam11740 family (a member of the super family c|13226) and functions as a plasmid replication region DNA binding N terminal protein.

The Kfr A polypeptide is a site-specific DNA-binding protein whose operator overlaps the *kfr*A promoter. It is possible one of the low identity ORF located upstream of the Kfr A protein function as a repressor for KfrA. The N-terminus encoded by *Kfr*A, containing a helix-turn-helix motif, is essential for functionality. Downstream from this family is generally an extended coiled-coil domain containing a heptad repeat segment which is probably responsible for formation of multimers, and may provide an example of a bridge to host structures required for plasmid partitioning.

DNA-binding domain protein, located upstream of the denoted replication/maintenance region, has the closest relative CAZ88706 (Identity 71%) which encodes a putative transcriptional regulator and is arbitrarily included in the region of stability, located upstream of the

replication region. However, the function of this protein maybe related to the regulation of plasmid replication and maintenance, therefore may need to be included in the replication/maintenance region. Further biochemical studies on these ORFs to determine the functions of the hypothetical proteins would help predict the replication/maintenance and regulation mechanism of this plasmid.

Mobilisation module

The predicted genetic map of the hypothetical mobilization region is shown in Figure 70. Two proteins in this region were found to have low but significant similarity to Mob E and MobD in the Gene bank databases. Part of the MobD also showed some similarity to Tra, known to be involved in a Type IV secretary pathway, which is related to plasmid conjugation.

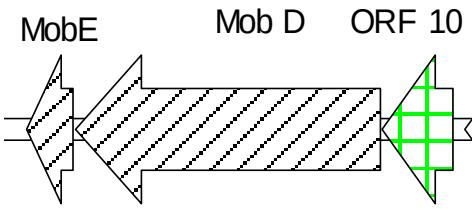


Figure 70 Genetic map of pF12 mobilization region

The lack of a complete set of *tra* genes, which is essential for bacterial conjugation and transfer, explains the failures encountered in the conjugation experiments. Although not capable of lateral transfer by conjugation, some *mob* genes (*MobD MobE*) present on this plasmid might enable this plasmid to be transferred by mobilisation. The fact that debris of the *tra* gene, although of low identity, is present in the mobilization interon of this plasmid, indicated that this plasmid may have had the conjugation capability but has lost this ability during the evolution of this plasmid.

Stabilization module

The predicted genetic map of the hypothetical stabilization region is shown in Figure 71.

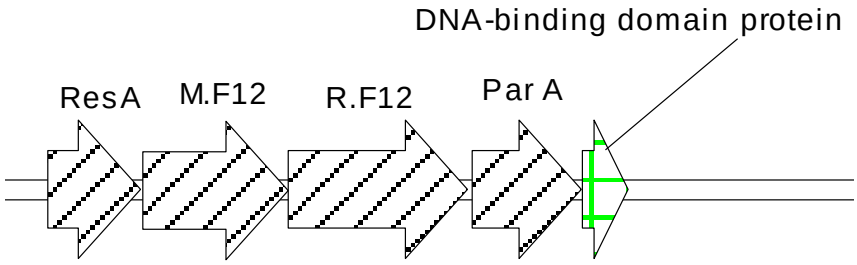


Figure 71 Genetic map of pF12 stabilization region

One key gene product of this region is predicted as partition protein, ParA, which also has similarity to cobyrinic acid a,c-diamide synthase. This enzyme has been shown to catalyse the conversion of cobyrinic acid to cobyrinic acid a, c-diamide via the intermediate formation of cobyrinic acid (Debussche et al., 1990, Fresquet et al., 2004).

The compact arrangement of the predicted gene products in the stabilization region of pF12 indicates some potential transcriptional linkage, i.e. some genes may be the precursor or regulator of another gene (Bramucci et al., 2006, Pieper et al., 2004).

Located in the plasmid stabilization module, there is also a restriction-modification system, found to be related to plasmid stability (Yeo et al., 1998a). The restriction-modification (R-M) system is a bacterial cell protection system to protect native chromosomal and plasmid DNA from foreign DNA and bacteriophages. Foreign DNA will be digested by the restriction endonuclease, whereas the chromosome and plasmid DNA originally in the host cell will be methylated by the methyl transferase and thus distinguished from the foreign DNA and avoid being digested.(Enikeeva et al., 2010) R-M systems can be encoded either by plasmids or by chromosomal DNA (Yeo et al., 1998a). The R-M system in pF12 is a type II system, which is the most common R-M system in bacteria (Yeo et al., 1998a). It consists of two enzymes, a restriction endonuclease and a methyltransferase, designated as pF12 endonuclease R.pF12 and pF12 methylase Pf12I.

Mechanisms that are involved in plasmid maintenance include post-segregational cell killing of plasmid-free cells, stable partitioning of plasmids into daughter cells and multimer resolution system (Sorensen et al., 2005). While multiple ORFs were identified to encode resolvases as well as partition protein ParA, an R-M system may contribute to the plasmid stability system. It was found recently that the R-M systems mediate plasmid segregational stability by postsegregational killing of plasmid-free cells (Kulakauskas et al., 1995). The authors proposed that a plasmid-encoded methyltransferase modifies host DNA and thus prevents its digestion by the restriction endonuclease. Plasmid loss entails degradation and/or dilution of the methylase, which will then result in double-strand breaks by the endonuclease, and will eventually cause the death of the plasmid-free cells (Kulakauskas et al., 1995).

A large number of R-M systems have been identified and some genes have been cloned since this phenomenon of R-M system was first reported in the 1950s (Yeo et al., 1998a). Two proteins encoding endonuclease and methylase in plasmid pF12 are aligned with identified R-M system proteins as shown in Figure 73 and Figure 74. High identity and similarity of pF12 restriction endonuclease and methylase with the characterised R-M system amino acid sequences can be observed in these figures.

It can be observed that the pF12 endonuclease and methylase genes were organised in a head-to-tail orientation with the methylase gene preceding and overlapping the endonuclease gene by 1 base pair (Yeo et al., 1998a). The deduced amino acid sequence of the pF12 methylase revealed significant similarity with the *Pac*25I, *Xcy*I, *Xma*I and *Cfr*9I methylases, and the pF12 endonuclease revealed significant similarity with R.Pac 25I, R.Xcy I, R.Xma I and R.Cfr 9I. All these endonucleases target similar recognition sequences and cleave between the external cytosines of the recognition sequence, i.e. 5'-C↕CCGG-3'. In addition, high identity and similarity was also found between pF12 methylase and SmaI methylase; however, the alignment of R.pF12 with R.SmaI, which recognises the same sequence but cleaves between the internal CpG , i.e., 5'-CCC↕GGG-3', revealed very low sequence similarity.

The conservation and motifs, including motif I (-GD-VLDPF-GSGT-G-V) and motif IV (VTSPY---R--) indicated that the pF12 methylase is a N4-cytosine methylase (Kwong et al., 2000, Yeo et al., 1998a).

As shown in Figure 72, the computer program identified two *Xma*I recognition sites on plasmid pF12, however, when digested with its isoschizomer *Sma*I, no separate fragments were observed on the electrophoresis gel (data not shown). This has indicated that the plasmid is resistant to cleavage by *SmaI*, probably, due to the self-protection methylation of specific sequence/s at the recognition sites of its native DNA.

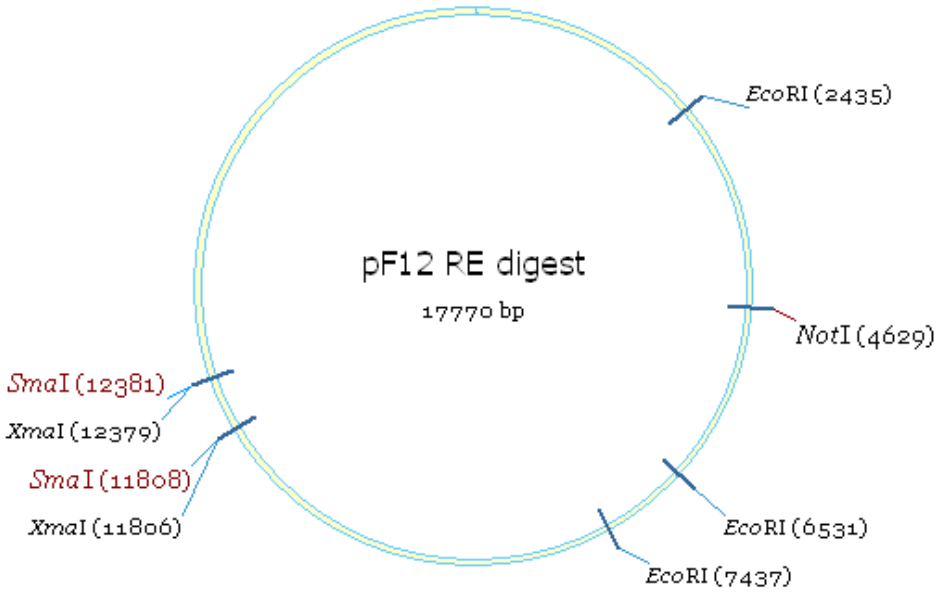


Figure 72 pF12 Restriction enzyme recognition sites by *Sma*I, *Eco*RI and *Not*I

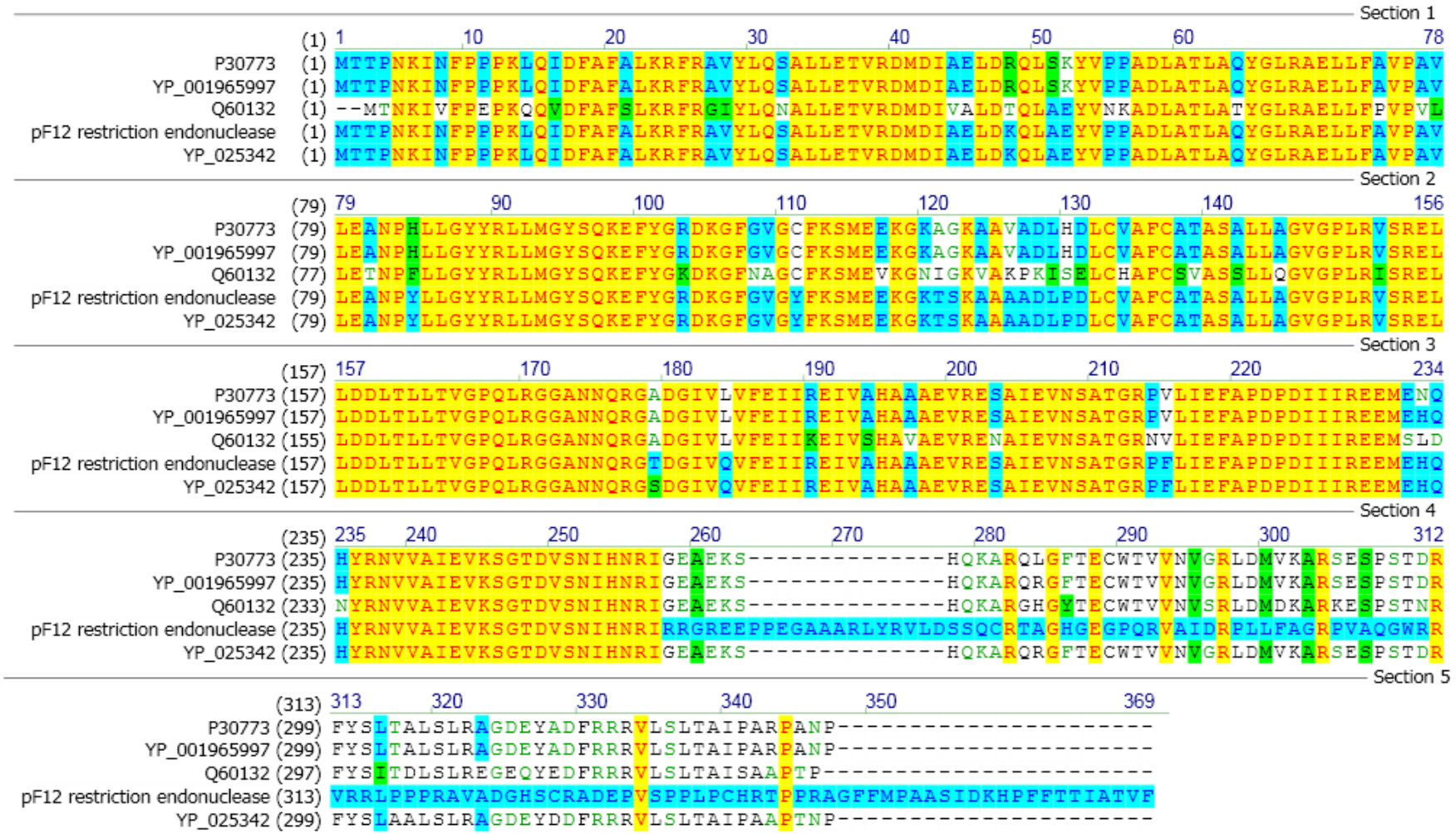


Figure 73 Amino acid sequence alignments of the pF12 restriction endonuclease, XcyI (P30773), XmaI (YP001965997), Cfr9I (Q60132), and Pac25I (YP025342) endonucleases.

Conservative amino acids are highlighted in light blue. Identical amino acids are highlighted in yellow, block of similar amino acids are highlighted in green, and weakly or non-similar amino acids are not highlighted. Gaps have been introduced to optimize the homology.

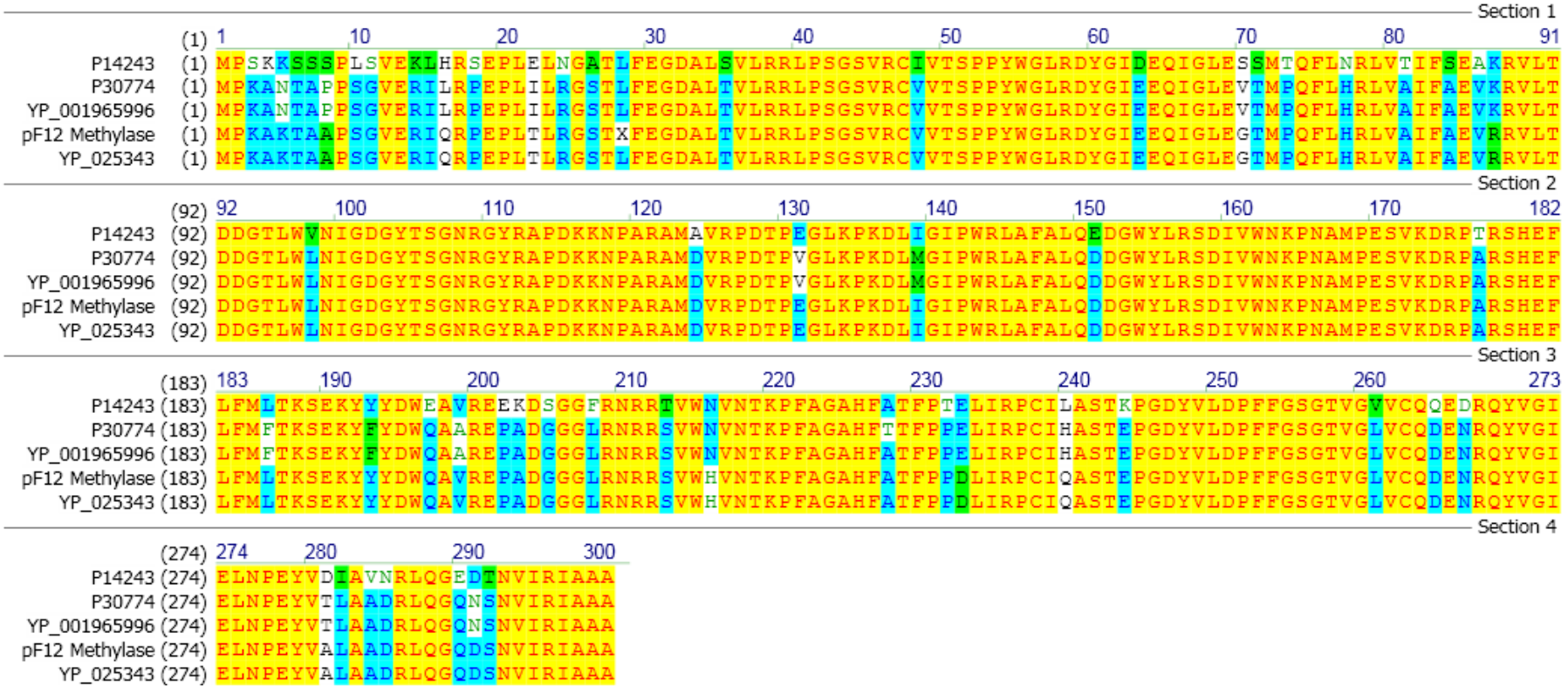


Figure 74 Amino acid sequence alignments of the pF12 methylase, XcyI (P30774), XmaI (YP001965996), Cfr9I (P14243), and Pac25I (YP025343) methylases. Conservative amino acids are highlighted in light blue. Identical amino acids are highlighted in yellow, block of similar amino acids are highlighted in green, and weakly or non-similar amino acids are not highlighted. Gaps have been introduced to optimize the homology.

Genes encoding a similar R-M system, in fact having high identity with the R-M system in pF12 are also present in the plasmid pRA2, a 33kbp native catabolic plasmid in *Pseudomonas alcaligenes*. This strain is shown to be able to degrade 2,5-xylenol, 3,5-xylenol and m-cresol via the gentisate pathway (Yeo et al., 1998a). The restriction endonuclease is a perfect isoschizomer of *Xcy*I, *Xma*I and *Cfr*9, and an imperfect isoschizomer of *Sma*I (Yeo et al., 1998a).

Furthermore, the R-M system on plasmid pF12 is located immediately downstream of a DNA resolvase *Res*A, which may be involved in DNA mobility (Yeo et al., 1998a). ORFs with motifs resembling proteins involved in DNA mobility, such as transposases, integrases, and invertases are found to be located in the vicinity of several R-M systems (Yeo et al., 1998a). These mobile elements may assist in the horizontal transfer of the R-M system between bacterial populations, as well as within the bacterial genome itself (Grinsted et al., 1990, Lett, 1988). The complete sequencing of the plasmid revealed that the R-M system is located immediately between a resolvase and a replication element (*repA*) on plasmid pF12. Although the function of the putative invertases/resolvases upstream of the F12 R-M operon is still unknown, this is still evidence suggesting that mobile elements and DNA recombination may have contributed to the distribution and subsequent acquisition of this family of R-M systems.

Mobile elements

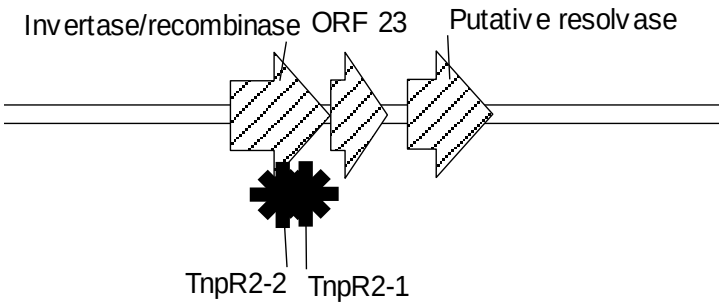


Figure 75 Genetic map of pF12 transposon-like elements

As shown in. Figure 63 and Figure 68, a region carrying transposon-like elements is located upstream of the plasmid stabilization region and the putative triclosan related functional region. Judging from the direction of the putative proteins shown in Figure **75**, it

is possible that the transposon-like element functions as the mobile element in the transfer of triclosan related gene products, which are located downstream of this region.

This region encodes three putative proteins that may function as a mobile element-transposon. It is likely that this element belongs to the Tn*3* family or derivatives (Grinsted et al., 1982, Grinsted et al., 1990, Grinsted et al., 1988, Lett, 1988, Ubben & Schmitt, 1986). Tn*3* family of transposons normally encode two gene products, both of which play a role in the formation of the final products of transposition: the *tnp*A gene encodes a transposase of about 120,000 daltons, and the *tnp*R gene encodes a resolvase, which is a site-specific recombinase (Grindley, 1985, Hayes, 2003). TnpR was tentatively predicted as the function of the first ORF in the transposon-like element module, which has high similarity to a characterised Tnp*R2* encoding a transposition protein in a fusidic acid and cadmium resistance plasmid pUB101 carried by *Staphylococcus aureus* (O'Brien et al., 2002). Although no complete Tnp*A* can be confirmed to be part of this predicted transposon-like element, an unknown ORF 23 and a putative resolvase/recombinase was predicted downstream of this putative TnpR2 protein on this plasmid. Another feature of the Tn*3* family transposons is that they have long (35-40bp) terminal inverted repeats of related sequence and duplicate a 5-bp target sequence upon transposition (Grinsted et al., 1988, Lett, 1988). No such inverted repeats were found in the predicted transposon-like element. However, transposons, namely Tn*21* and Tn*1721*, are found to be able to transpose, although at a substantially reduced frequency, when one end is completely deleted (Avila et al., 1984, Grindley, 1985, Lett, 1988, Mötsch & Schmitt, 1984). Functions of *tnpR* gene products are interchangeable among transposons, whereas functions of *tnp*A gene products may or may not be interchangeable among transposons of this family (Grindley, 1985). Therefore, it is possible to predict that one of the two proteins downstream of the Tnp*R2* like protein may function as Tnp*A* in this transposon-like element and this transposase is novel and conserved in this plasmid.

Triclosan degradation module

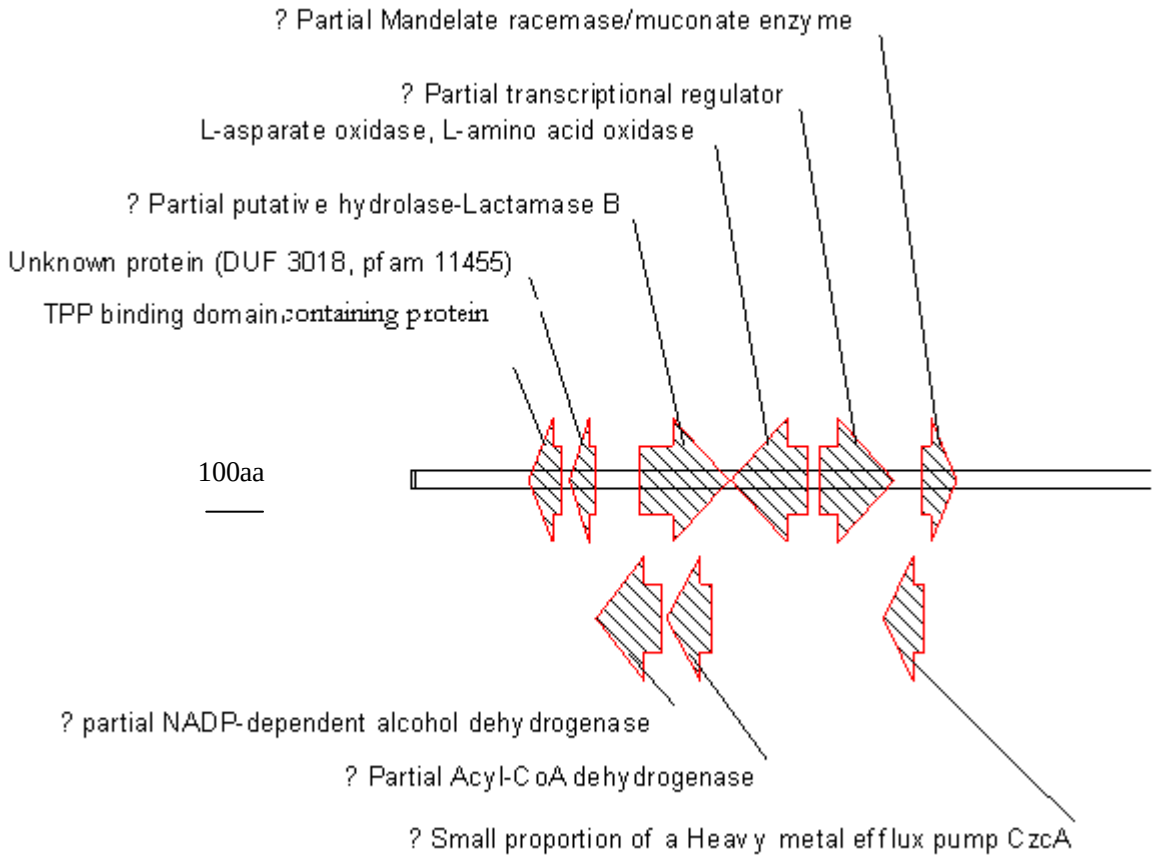


Figure 76 Genetic map of pF12 triclosan related functional region

As shown in Figure 63 and Figure 68, there is a region downstream from the putative transposon-like elements, which consists of 6-9 hypothetical proteins. The blast search results against the NCBI non-redundant protein database indicate that these genes may encode proteins with unknown functions.

The triclosan degradative related module, which is located immediately downstream of one putative transposon element, contains ORFs that have putative catabolic functions.

All gene products may carry catabolic function or may be related to drug resistance. Hypothetical protein ORF 11390-11659 has low but significant similarity to a heavy metal efflux pump (Goldberg et al., 1999, Nies, 1992, Silver, 1996), although the predicted protein only has similarity to a proportion of this characterised gene product that is involved in multidrug resistance. This indicates that the function of this plasmid may be

related to triclosan, as triclosan was found to be a substrate for multidrug resistance as discussed in Chapter 1. However, due to the small size of this putative protein, it is unlikely that this plasmid encodes the full gene cassette required for the efflux pump, which generally contains three proteins.

An L-aspartate oxidase was predicted to be located in this module, and may play a part in the triclosan degradation pathway. However, although the predicted protein covered the full length of the reference sequence (complete ORF), only 33% identity was observed, with 48% positives, 22% gaps and an E-value of 4e-22. The identity was low but significant; therefore this L-aspartate oxidase like protein may represent a major genetic determinant for triclosan degradation in this module.

Other hypothetical proteins have low but significant similarity, although may only show low coverages and identities, to some functional proteins involved in catabolic pathways, such as a NADP-dependent alcohol dehydrogenase (partial), an Acyl-CoA dehydrogenase (partial), a putative hydrolase-lactamase B, a transcriptional regulator (partial), and a Mandelate racemase/muconate enzyme (partial) as shown Figure 76. All these gene products in this region were predicted be catabolic domain protein and belong to a family of oxidoreductase enzyme, which catalyses the transfer of electrons from one molecule (electron donor) to another (electron acceptor). This group of enzymes usually utilizes NADP or NAD as cofactors. The predicted proteins are of significant interest and indicate the potential pathway of dehydrogenation, hydrolysis or oxidation of triclosan or derivatives. However, six out of nine predicted proteins in this domain have only partial coverage of the gene products in the database, and therefore the function of these hypothetical proteins remains uncertain, and needs further characterisation.

This module also contains an ORF23 having high similarity to an unknown protein, which was not fully characterised, on a plasmid pRA2 isolated from an environmental strain *Pseudomonas alcaligenes*, which was found to be capable of degrading 2,5-xylenol, 3,5-xylenol and m-cresol via the gentisate pathway (Kwong et al., 1998, Yeo et al., 1998b). Plasmid pRA2 although was not found to encode catabolic function, was demonstrated to encode a toxin-antitoxin module and contain a transposon encoding putative mercuric ion transport protein (Kwong et al., 1998, Yeo et al., 1998b).The same unpredicted protein was also found on an antibiotic resistant plasmid pRSB101 isolated in the activated sludge bacterial community, which mediates resistance to tetracycline, erythromycin,

roxythromycin, sulphonamides, cephalosporins, spectinomycin, streptomycin, trimethoprim, nalidixic acid and low concentrations of norfloxacin (Szczepanowski et al., 2004). Based on the location of this predicted unknown protein, it is possible that this ORF is also a potential triclosan-related genetic candidate that may play a role in the triclosan resistance mechanism or degradation pathway.

Similar to the pHL3 plasmid characterized in *Rhodococcus* sp. strain RHA1, which is a PCB-degrader, due to the novel genes in the catabolic gene clusters and the fact that the host genome is not yet completely known, it is difficult to assess the full catabolic role of this plasmid and how it functions in synergy with the chromosomal genes in the host (Warren et al., 2004).

However, the presence of a putative oxidase, as well as part of some efflux pump, hydrolase, dehydrogenase, mandelate racemase-coding genes, known to play central roles in the biodegradation of toxic compounds, along with the structural organization of these genes, and the putative nature of some of their regulatory genes strongly suggest that this plasmid plays an important role in the assimilation of such a compound (Warren et al., 2004). It can be speculated that the gene products in this region do not constitute a complete catabolic gene cassette encoding the entire triclosan biodegradation pathway, based on the fact that triclosan is a recalcitrant and complex compound whereas the predicted genes only shown identities to part of the reference genes and the total size of this region is only approximately 6kb.

It may be speculated that this plasmid may be evolved from an independent catabolic plasmid carrying complete catabolic genes as well as efflux pumps to fully function as a sole triclosan degradative genetic determinant; however some of these functions may have been lost and the interaction in synergy with chromosomal encoded catabolic genes was thus required. The abridged version of this plasmid as hypothesised above also implies that the selective pressure on a triclosan degradative plasmid, carrying the full functionality of a mobile plasmid, in the wastewater environment is low. However, although conjugation function has been lost, the plasmid has retained essential genes required for triclosan metabolism, which may function in synergy with some chromosomal encoded genes.

Chapter 7 General discussion, conclusion and future work

7.1 General discussion

At the time the experimental work was carried out in this project, research into triclosan biodegradation had just commenced; and little information had been published. When this study commenced, there had been attempts in previous studies to obtain pure cultures of triclosan degraders from environments such as soil, but none was successful due to the recalcitrant nature of this compound (Waller & Kookana, 2009). In this project, triclosan degradative isolates were initially obtained from a wastewater lagoon, but no degradation was observed in primary cultures. It took some time to identify potential degrader candidates (triclosan resistant strains) able to grow in triclosan medium and able to utilize triclosan as a sole carbon source. *Pseudomonas citronellolis* strain F12 was isolated and confirmed as a triclosan degradative isolate, which was able to grow, although at a slow rate, in triclosan minimal medium and caused a corresponding reduction in triclosan concentration in the culture medium. Biodegradation of triclosan by *Pseudomonas citronellolis* strain F12 was also confirmed by the degradation intermediates/by-product analysis.

Transformation of triclosan to methyl triclosan in natural water environment as well as in activated sludge under aerobic conditions was reported recently (Chau et al., 2008, Chen et al., 2011, Chen et al., 2012); however, this methylation by-product was not identified in this study, which indicated that triclosan may be transformed in diverse pathways. An identified triclosan biodegradation product, dichlorophenol, by *Pseudomonas citronellolis* strain F12 in this study has now been reported as triclosan degradation by-product in some recent studies (Kim et al., 2011, Lee et al., 2012), whereas the most recently published monohydroxy-triclosan, 6-chloro-3-(2,4-dichlorophenoxy)-4-hydroxycyclohexa-3,5-diene-1,2-dione, 3-chloro-4-(5,7-dichloro-3-oxo-2,3-dihydrobenzo[1,4]dioxin-2-yl)-2-oxobut-3-enal, and 3,5-dichloro-4,6-dihydroxycyclohexa-3,5-diene-1,2-dione were not detected in this study (Lee et al., 2012). Instead, some new intermediates, 2',4'-dichlorophenyl 4-chloro-6-oxo-2,4-hexadienoate and proto-anemonin were identified as biodegradation intermediates by *Pseudomonas citronellolis* strain F12. Triclorobenzene was also identified as the closest matching compound in the reference library for one of the intermediates; however, there is a discrepancy in the mass spectrum of the compound of interest and the reference compound. It is unlikely that triclorobenzene is present in the

bacterial culture as a breakdown product of triclosan, as the production of triclorobenzene would require chlorination to occur. It is possible that, although not chloro-catechol, this mystery compound has a close MS spectral to that of triclorobenzene, while it is actually a further metabolite of chloro-catechol that was formed in the hydrolysis of triclosan as discussed previously.

The possible triclosan degradation pathways were discussed in Chapter 4, and another alternative pathway was also tentatively proposed. This potential pathway involves a unique ring cleavage of triclosan to form 2',4'-dichlorophenyl 4-chloro-6-oxo-2,4-hexadienoate, hydrolysis of this compound subsequently to form dichlorophenol and the formation of proto-anemonin as a by-product.

Triclosan ring cleavage pathway

Because the genetic study of triclosan degradation by bacteria has not been studied extensively, there are limitations in using the NCBI Genebank protein database to identify gene products involved in the upper degradation pathway, which is likely to involve the oxidation of triclosan to some intermediates via a unique *ortho*-cleavage pathway as discussed in Chapter 4. The enzymes catalysing this reaction are likely to be novel and may be substrate specific to triclosan; therefore it would be expected to experience difficulties in identifying the enzymes involved in catalysing the upper degradation pathway, which has not been discussed and the enzymes do not exist in the current protein database. Based on the combined information resulted from the intermediate analysis and genetic studies, it is predicted that the enzymes involved in this unique *ortho*-cleavage pathway may include an L-aspartate oxidase like protein. Other oxidoreductase enzymes may also be required to complete the aromatic ring cleavage as discussed in the following sections.

Different bacterial strains; such as *Acinetobacter radioresistens*, *Pseudomonas* sp., *Arthrobacter* sp. have been found to be able to grow on benzene, and this led to the identification of the aromatic ring cleavage gene (*cat*A and homologues encoding C12O, catechol 1,2-oxygenase), which was first discovered on the bacterial chromosome (Di Gioia et al., 1998, Pessione et al., 2005, Smith, 1990). Plasmid-encoded aromatic ring cleavage genes were also identified (Smith, 1990).

The oxidation of the aromatic ring is typically the initial step of the biodegradation

pathway of this structure to form catechol or its derivatives. The subsequent ring cleavage reaction, as illustrated in Figure 29 in Chapter 4, was generally found, to date, to be catabolised by either catechol 1,2-dioxygenase (C12O) for *ortho*- or *intradiol*-cleavage or by the catechol 2,3-dioxygense (C23O) for the *meta*- or *extradio*- cleavage (Smith, 1990).The biodegradation intermediates of the *meta*-cleavage pathway include 2-hydroxymuconic semialdehyde, 2-oxopenta-4-enoate, and 4-hydroxy-2-xovalarate, and the by-products are pyruvate and acetaldehyde; whereas the intermediates of the *ortho*-cleavage pathway include muconate (*cis,cis*-muconate), muconolactone ((+)-γ-carboxymethyl-Δβ-butenolide), β-ketoadipate enol-lactone and β-ketoadipate (Smith, 1990). Alkylbenzenes biodegradation enzymes have been found to be either plasmid-borne or chromosome-encoded, although the degradation pathway was shown to be identical (Smith, 1990). While genes of alkylbenzene (and biphenyl) degradation in *Pseudomonas* sp. NCIB 10643 was found to be chromosome encoded (Smith & Ratledge, 1989), genes for isopropyl benezene biodegradation by *Pseudomonas putida* RE 204 (Eaton & Timmis, 1986) and for ethylbenezene (and 1-phynylethanol) biodegradation by *Psedomonas fluorescens* (Bestetti & Galli, 1984) were found to be sited on large plasmids (Smith, 1990).

Despite the diverse locations of the genes involved in the biodegradation of aromatic compounds, most of the catechol 1, 2-dioxygenases (C12O) were found to be chromosome encoded and may have strict substrate specificity; whereas the catechol 2, 3-dioxygenases (C23O) tend to be plasmid encoded and bears more relaxed substrate specificity. In consistence to this, it was found in this study that the triclosan aromatic ring cleavage is via a unique ortho-cleavage pathway, of which the majority of the genes may be located in the chromosome DNA, whereas only the unique gene, different to so far characterised C12O, predicted to be L-aspartate oxidase like protein, is located on the degradative plasmid. The plasmid borne aromatic ring cleavage enzyme allows the easy transfer of catabolic capability among microorganisms.

The predicted L-aspartate oxidase like protein may be of significance to this reaction. This enzyme belongs to the family of oxidoreductase specifically those acting on the CH-NH2 group of donors with oxygen as acceptor, as shown in Figure 77. This putative L-aspartate oxidase like protein on pF12 may act on the C=C group and be responsible for the incorporation of oxygen into the –O-C=C-OH group of triclosan during the ring cleavage. Due to the apparent difference in functionality required for L-aspartate oxidation and triclosan oxidation, the genes encoding the two enzymes may be different. A positive score

of 48% with an E-value of 4e-22 tentatively verified the hypothesis.

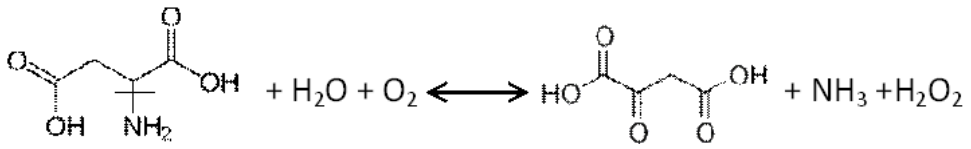


Figure 77 Function of L-aspartate oxidase

Compared to the normal *ortho*-cleavage pathway as illustrated in Figure 31, only one oxygen molecule is oxidised and attached to one molecule of triclosan instead of the dioxygenation, where two oxygen molecules are incorporated into the cleaved ring under one reaction. This single oxygen conversion would result in one –CHO and one –COOH at the ends of the cleaved bond instead of two –COOH at the ends, possibly due to the large chloroaromatic ring located close to the cleaved site, contributing a rejecting potential for the incorporation of additional oxygen molecule. Therefore, instead of catalysed by dioxygenase as is in the normal *ortho*-cleavage, the reaction may be catalysed by a potential monooxygenase.

Monooxygenase DszC was isolated from a dibenzothiophene (DBT) desulphurising bacterium *Rhodococcus* sp., and it was characterised to be responsible for the enzymatic conversion of DBT to $DBTO_2$ (Ma et al., 2006, Ohshiro et al., 2005, Ohshiro & Izumi, 2000, Ohshiro et al., 1999). In this plasmid-mediated sulphur-specific 4S biodegradation pathway (Oldfield et al., 1997), DBT is oxidized to DBT sulphone ($DBTO_2$) via DBT sulphoxide (DBTO) by DBT monooxygenase (DszC); DBT sulphone is then converted to 2-hydroxybiphenyl-2-sulfinate (HBPSi) by DBT sulphone monooxygenase (DszA), and HBPSi is desulphurised to 2-hydroxybiphenyl (2-HBP) with the release of the sulphur atom as sulphite by HBPSi desulphinase (DszB) (Bezalel et al., 1996, Gupta et al., 2007, Kayser et al., 2002, Konishi et al., 2000, Konishi & Maruhashi, 2003, Nakayama et al., 2002, Ohshiro et al., 2005, Ohshiro & Izumi, 2000, Ohshiro et al., 1999).

A recent investigation on triclosan biodegradation was carried out using ammonia-oxidizing bacteria (AOB) *Nitrosomonas europaea* and mixed AOB in nitrifying activated sludge (Roh et al., 2009). The degradation was observed only in the absence of an ammonia monooxygenase inhibitor. As a result, ammonia monooxygenase (AMO) was concluded to be responsible for triclosan degradation, although sludge samples from WWTP were able to utilize triclosan as sole carbon source regardless of the presence of

AMO inhibitor (Roh et al., 2009). It is possible that the referred AMO may bear broad substrate specificity, and is capable of catalysing the oxygenation of triclosan as proposed in the triclosan biodegradation upper pathway. Sludge samples used in this study contain a mixture of AOBs, where the more versatile bacterial population may produce other monooxygenase-like enzymes that can utilise triclosan as substrate while not being inhibited by the specific AMO inhibitor used.

AMO employed by *Nitrosomonas europaea* catalyses the oxidation of ammonia to hydroxylamine (Gilch et al., 2009). In all known AOB, the genes encoding the enzyme AMO belong to an operon with the structure of *amo*CAB (Junier et al., 2010). This family of oxygenases was also found to oxidize a wide range of aliphatic and aromatic hydrocarbons (Chang et al., 2002, Keener & Arp, 1994, Rasche et al., 1991, Roh et al., 2009, Sayavedra-Soto et al., 2010, Tran et al., 2009). It was demonstrated that, by co-metabolism, which involves the bacterial transformation of a compound while in the presence of a growth-supporting substrate, AOB can oxidize alternative substrates, including halogenated hydrocarbons that cannot support their growth (Sayavedra-Soto et al., 2010). The responses of the AOB *N. europaea* to a wide range of compounds are well studied at the physiological, biochemical and molecular levels and AMO has demonstrated a remarkably broad substrate range (Sayavedra-Soto et al., 2010).

The degradation of chlorinated hydrocarbons by AMO was proposed to be a halogen replacement reaction, producing an aldehyde or an epoxide as the by-products, which are unstable in many cases and transformed abiotically into an alcohol or an organic acid (Sayavedra-Soto et al., 2010). However, in this study, based on the intermediate analysis results, it is possible that a potential monooxygenase catalyses the oxidation of the first aromatic ring (as shown in Figure 78), breaking the C=C-O- bond to form -COO- and -CHO (aldehyde). The formation of –CHO from -COH may be catalysed by some other enzymes, possibly encoded in chromosome DNA, and this –CHO group is then oxidised to hydroxyl acid. The conversion from –COH to –CHO then to –COOH may be catalysed by enzymes involved in the normal catechol aromatic ring *ortho*-cleavage pathway. The intermediate compound in the common C12O pathway was identified in this study as 2',4'-dichlorophenyl 4-chloro-6-oxo-2,4-hexadienoate, which may then converted to 2',4'-dichlorophenyl 4-chloro-6-carboxyl-2,4-hexadienoate, becoming the substrate for the subsequent hydrolysis reaction (as shown in Figure 37 and Figure 38).

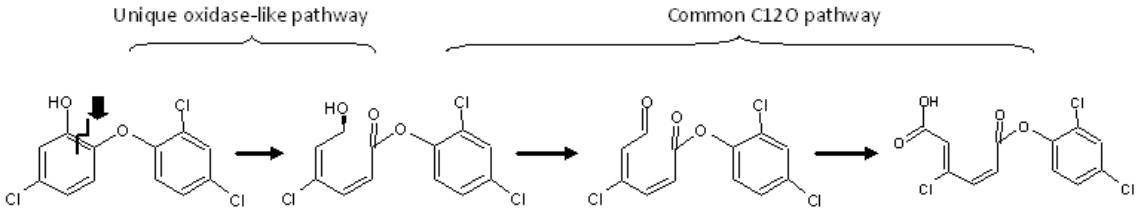


Figure 78 Proposed unique triclosan aromatic ring *ortho*-cleavage pathway

No ORF on pF12 was predicted to be a monooxygenase, but an oxidase-like protein, two potential dehydrogenases and a putative hydrolase were tentatively predicted. It is therefore possible that the oxidation of triclosan for ring cleavage to form –COO- and –CHO is catalysed by a series of enzymes, including a unique oxidase-like enzyme residing on the plasmid pF12 and some chromosome-encoded common genes involved in the normal catechol *ortho*-cleavage pathway. The plasmid-encoded unique gene is critical and functions in synergy to the common genes to give the effect of oxygen incorporation. In fact, although not yet purified with sustained activity, AMO is likely to be a multicomponent enzyme (Sayavedra-Soto et al., 2010). Dehydrogenases and hydroxylases, such as salicylate hydroxylase, salicylate 5-hydroxylase and salicylaldehyde dehydrogenase are all examples of monooxygenase in pyrene metabolism (Husain, 2008). The *alk*H gene encodes an NADH-dependent aldehyde dehydrogenase on OCT plasmid found in *P. putida* biotype A (or *P. oleovorans*, ATCC 29347). Gene *alkJ* encodes an alcohol dehydrogenase related to a number of oxidases and soluble cofactor-independent dehydrogenases that are probably linked to the electron transfer chain (van Beilen, 1996) .

In summary, catechol formation followed by *meta*- or *ortho*- cleavage of the aromatic ring is the common biodegradation pathway for most benzene, substituted benzenes and derivatives. The first aromatic ring of triclosan contains two C-O- bonds at the 1, 2-position, thus having a similar structure to catechol. However, the intermediate analysis revealed a unique oxygenation mechanism via a novel *ortho*-cleavage pathway, in which a single oxygen was incorporated onto the cleaved C=C bond to form -COO- group, and the –COH group was converted to –CHO group. The –CHO was then oxidised by –COOH to form 2',4'-dichlorophenyl 4-chloro-6-oxo-2,4-hexadienoate.

The lack of genes encoding functional oxygenases on plasmid pF12 showing significant similarity to characterised catechol 1, 2-dioxygense or monooxygenase indicated that the essential enzyme catalysing this reaction is different from the C12O or monooxygenase

characterised so far, which is consistent and verifies the unique ring cleavage pathway predicted for this reaction. The complete aromatic ring cleavage reaction also requires other gene functions that may be encoded by chromosomal DNA of the host strain. The unique oxidase-like protein functions in synergy with the common chromosome-encoded gene products involved in the normal catechol *ortho*-cleavage pathway.

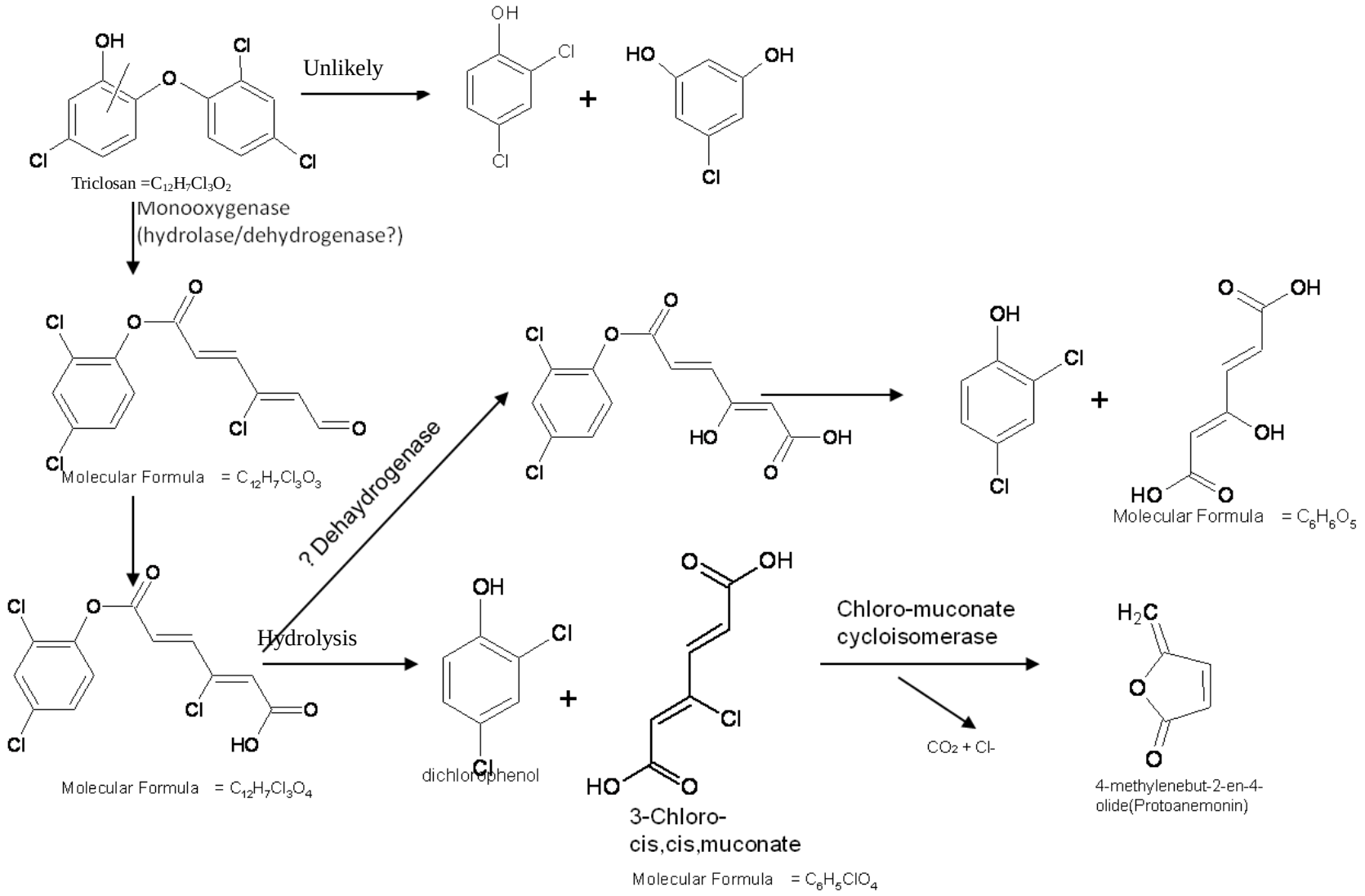


Figure 79 Possible triclosan biodegradation pathways

Hydrolysis of primary triclosan ring cleavage by-product

As discussed and shown in Figure 78, $C_{12}H_7Cl_3O_3$ was produced as an intermediate of the triclosan aromatic ring cleavage reaction and $C_{12}H_7Cl_3O_4$ was produced as a primary ring cleavage by-product. As shown in Figure 79, this primary ring cleavage by-product may experience hydrolysis prior to dehalogenation and decarboxylation as proposed in Chapter 4. It may also undergo dehalogenation prior to the separation of the second aromatic ring from the muconate structure via hydrolysis. However, the function of muconate cycloisomerase recognizes R^3 group as H, $-COO^-$ or Cl, not the –OH group as is in the alternative dehalogenation-hydrolysis pathway (as shown in Figure 34).

Based on the experimental findings and the genetic analysis of plasmid pF12, it is predicted that a hydrolase residing on plasmid pF12 may play a role in the hydrolysis of the primary ring cleavage by-product. However, it is also possible that the genes encoding hydrolysis of the primary ring cleavage by-product reside on the chromosomal DNA, similar to other degradative genes encoding the enzymes required for the triclosan degradation lower pathway. The bioinformatics analysis of the putative hydrolase revealed a low coverage, i.e. only part of the hydrolase was identified on pF12 in the triclosan catabolic module. This may indicate that the hydrolase utilized by pF12 host strain is encoded by chromosomal DNA. The ancestor of plasmid pF12 may bear hydrolases on the plasmid for catalyzing of the hydrolysis after the initial aromatic ring cleavage; however this plasmid function may have been lost, potentially due to the existence of similar chromosome-encoded hydrolases in the host strain. Hydrolysis may improve the bioavailability of the substrates (Abou-Auda et al., 2006, Aggarwal, 2009, Kurien & Scofield, 2009, Newa et al., 2008), rendering this compound and intermediates more readily degradable by microorganisms.

The putative hydrolase also showed similarity to a β-lactamase. β-lactamase over expression is a common mechanism of bacterial resistance to β-lactam antibiotics (Athanassiou et al., 2003, Danziger & Pendland, 1995, Fisher et al., 2005). The hydrolysis catalyzed by this enzyme cleaves the β-lactam ring, thus inactivating the antibiotics from β-lactam family (Kotra & Mobashery, 1998). The discovery of β-lactamases is traced back to the late 1940s (Abraham & Chain, 1940), more and more β-lactamases have emerged since then with activity against newer and more effective β-lactam antibiotics (Abraham & Chain, 1940, Fournier et al., 1994, Henriques et al., 2006, Kotra & Mobashery, 1998, Li et

al., 2002, Majiduddin et al., 2002, Muñoz et al., 1996, Quinting et al., 1997, Yang et al., 1988).

The enzymes are widely distributed in both Gram-positive and Gram-negative bacteria, and have a polyphyletic origin (Ambler, 1980). High levels of β-lactam resistance rates have been reported in clinical strains of *Pseudomonas aeruginosa* (Villegas et al., 2006), and the list of metallo-β-lactamases detected has continued to grow in various parts of the world over the last decade (Garau et al., 2004, Henrichfreise et al., 2006, Henriques et al., 2006, Hu et al., 2009, Khosravi et al., 2010, Majiduddin et al., 2002, Villegas et al., 2006, Weile et al., 2007, Yildirim et al., 2007). VIM-1, VIM-2 and VIM11 type metallo-β-lactamases were first detected in *Pseudomonas aeruginosa* isolates in Germany, US and Malysia in 2006-2007 (Henrichfreise et al., 2006, Khosravi et al., 2010, Villegas et al., 2006, Weile et al., 2007, Yildirim et al., 2007).

The β-lactamases are divided into four classes, A, B, C and D, based on the primary sequence similarity and catalytic mechanism (Ambler, 1980, Majiduddin et al., 2002). Classes A, C and D include enzymes that utilize serine in their active-site, whereas class B, also known as metallo-β- lactamases (MBLs) requires divalent metal cations (Zn^{2+}) to catalyze the hydrolysis (Ambler, 1980).

Low sequence similarities between various metallo-beta-lactamases were observed (Majiduddin et al., 2002); however, the general tertiary structure of these enzymes is very similar, which is represented as a αβ/βα sandwich structure composed of two beta-sheets at the core and five α-helices on the external faces (Hu et al., 2009, Majiduddin et al., 2002, Scott, 1973, Wang et al., 1999). Possible catalytic mechanisms involved in this group of enzymes have been discussed (Fabiane et al., 1998); however, the exact mechanism still remains unclear (Majiduddin et al., 2002).

Metallo-*beta*-lactamase have been identified in both Gram-negative and Gram-positive bacteria, and are, therefore, composed of a wide variety of enzymes in various host strains: the BcII in *Bacillus* species, CcrA in *Bacteroides fragilis*, and BlaB in *Chryseobacterium* species (Ambler et al., 1991, Galleni et al., 2001, Garau et al., 2004), to list a few.

The MBLs hydrolyse penicillins, cephalosporins and carbapenems but not aztreonam (Bush, 1998). VIM beta-lactamases have been identified in *Pseudomonas* species, and IMP enzymes were also found in *Pseudomonas aeruginosa* (Galleni et al., 2001, Majiduddin et al., 2002). Much of the genetic dissemination of β-lactamase genes has occurred via

plasmids and transposable elements (Henriques et al., 2006, Majiduddin et al., 2002, Yang et al., 1988).

Significant similarity was observed between metallo-*beta*-lactamase and the hydrolase encoded on pF12, despite the usually low similarities among metallo-beta-lactamase (Ambler, 1980, Majiduddin et al., 2002). Furthermore, the actions of triclosan and β-lactams both target bacterial cell wall biosynthesis. β-1actam antibiotics inhibit the enzymes of bacterial cell-wall biosynthesis by mimicking the three dimensional structure of the terminal portion of the peptide chain which form cross-linkages providing strength to the peptidoglycan molecule, which is critical in imparting rigidity to cell wall that is required for bacteria survival (Kotra & Mobashery, 1998). Triclosan inhibits FabI, NADH-dependent enoyl-acyl carrier protein (ACP) reductase encoding the final enzyme catalysing fatty acid biosynthesis in cell wall formation (McMurry et al., 1998b), by binding to the enoyl reductase active site at a region adjacent to the nicotinamide ring of the nucleoside co-factor (Kapoor et al., 2004a, Kapoor et al., 2004b, Roujeinikova et al., 1999a). Triclosan's phenol ring forms a face-to-face interaction with the nicotinamide ring, allowing intensive multiple points of interactions, thus preventing the enzyme from participating in the biosynthesis pathway (Stewart et al., 1999). The similar target in the cell wall biosynthesis pathway of these two groups of compounds, triclosan and β-lactams, allows the sharing of gene expression regulating system if required, and may initiate the bacterial signaling and response mechanism through a common regulatory system when under pressure. For example, when bacterial host is under the inhibitory pressure of triclosan present in culture medium, although responding to a different substrate such as triclosan other than β-lactam, the over-expression of β-lactamase may be initiated by the same signaling and response system; and the activity of this enzyme may be effective in triclosan inactivation.

However, the above is only postulation; further investigation such as structural analysis is required to confirm whether the function of this hydrolase is related to triclosan resistance conferred by the lactamase activity.

The presence of homologue to lactamase on the pF12 plasmid was also demonstrated in this project, where clearance of triclosan around bacterial colonies on cloudy agar plates containing high concentrations of triclosan (2 mg/L) was observed. Clearance of triclosan from the agar plates was also observed in previous studies, and was used as an indication of triclosan utilization/biodegradation by bacteria in these studies (Frenzel et al., 2010,

McBain et al., 2003b, Meade et al., 2001). However, the mechanism of this clearance phenomenon was not investigated. The results from this project suggest that the clearance observed on triclosan agar plate may be a result of hydrolysis catalysed by a hydrolase having low but primary sequence similarity to class B β-lactamase.

It was also demonstrated in this study that under the activity of the hydrolase, hydrolysed triclosan becomes more soluble and easier to be extracted by the organic solvent methanol (data not shown). It was observed that a much higher concentration of hydrolysed triclosan was extracted from the cleared agar than that from the uncleared agar. It is also expected that the increased solubility of hydrolysed triclosan in the culture medium will make this compound more bio-available for degrading microorganisms to utilize as a carbon source (Abou-Auda et al., 2006, Aggarwal, 2009, Kurien & Scofield, 2009, Newa et al., 2008).

Therefore, it can be concluded that this hydrolase encoded on the pF12 is an important component in the triclosan resistant/catabolic function. It could possibly catalyse the hydrolysis of the primary ring cleavage product and catalyse the subsequent hydrolysis reactions. Alternatively, it could be possible that the hydrolase confers antibiotic resistance to the host strain against triclosan as well as all β-1actam antibiotics, such as penicillins, cephalosporins cephamycins, and carbapenems, aztreonam and carbapenems (Kotra & Mobashery, 1998). Bacterial antibiotic sensitivity tests were undertaken for the original F12 strain and its cured derivative C6; however, no apparent change of antibiotic sensitivity profile was observed for the cured strain. This could be due to the incompleteness of this hydrolase in the pF12 DNA sequence, which indicating the ineffective function of this enzyme as an antibiotic resistance mechanism.

Triclosan biodegradation lower pathway

It is likely that the low pathway of triclosan biodegradation after hydrolysis is catalysed by enzymes encoded in the host chromosome. The transformation of chloro muconate is proposed and illustrated in Figure 80.

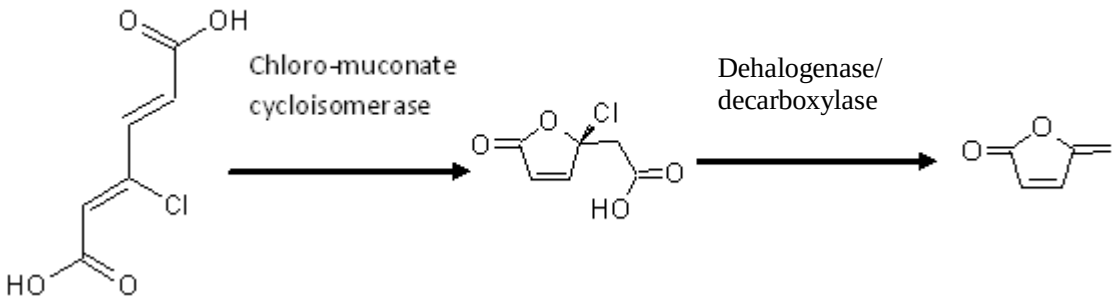


Figure 80 Proposed lower pathway of triclosan biodegradation after ring cleavage

In the triclosan degradative gene module, downstream of the transcriptional regulator, there is an ORF showing low but significant similarity to the mandelate racemase/muconate enzyme. The gene was tentatively designated as mandelate racemase/muconate enzyme. The ORF between the transcriptional regulator and the tentatively predicted mandelate racemase shows similarity to a small proportion of a heavy metal efflux pump CzcA(Goldberg et al., 1999, Poole, 2004). However, the function of those two ORFs cannot be determined based on the information obtained due to the low similarity and low coverage when compared to the reference protein from NCBI database.

Further functional investigation of these two ORFs may help determine if the degradation of triclosan is related to the mandelate pathway. The mandelate pathway, illustrated in Figure 81, involving both oxidation and decarboxylation, catalysed by the mandelate racemase, dehydrogenase and benzoylformate decarboxylase *bfdC*. In addition to the similarity to mandelate racemase/muconate enzyme, the TTP binding domain containing protein located upstream at the start of this gene cassette is closely related to a putative TPP binding domain containing protein, BAG45604, which consists of 3 proteins: TPP-binding module (aa398-582), thiamine pyrophosphate enzymes, and (Costelloe et al., 2008)s . The TPP-binding module is composed of proteins similar to *Pseudomonas putida* benzoylformate decarboxylase (*bfdC*) (Shimao et al., 1996, Tsou et al., 1990), which plays a role in the mandelate pathway (Brandt et al., 2010, Bruning et al., 2009, Henning et al., 2006), as shown in Figure 81, catalysing the conversion of benzoylformate to benzaldehyde and carbon dioxide (Johnen & Sprenger, 2009, Yep & McLeish, 2009).

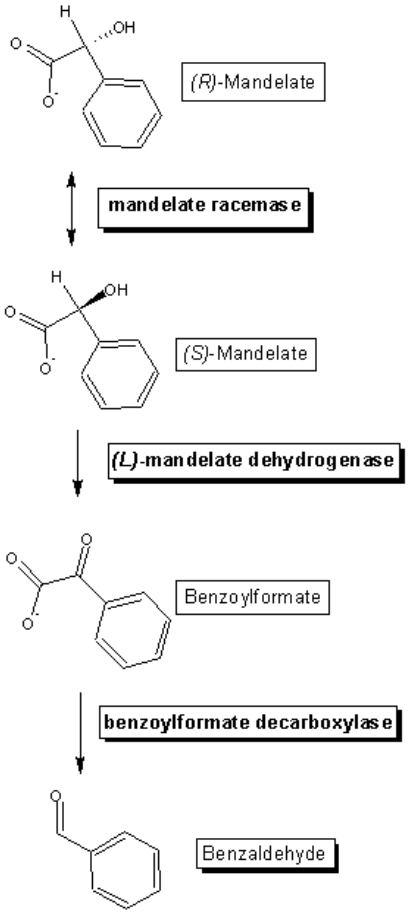


Figure 81 Illustration of mandelate pathway

(Adopted from http://umbbd.msi.umn.edu/servlets/pageservlet?ptype=r&reacID=r1049)

As discussed in Chapter 1, mandelate racemases (MR) and muconate lactonising enzymes (MLE) are the most fundamental hydrogen biocatalysis enzymes that belong to the enolase superfamily (Gunsalus et al., 1953a, Gunsalus, 1996a, Gunsalus et al., 1953b). MR and MLE are mechanistically distinct but structurally homologous enzymes (Neidhart et al., 1990). Analysis of sequence and structural similarities among proteins in this family suggests that all of their chemical reactions are mediated by a common active site architecture modified through evolution to allow the enolic intermediates to partition to different products in their respective active sites via different overall mechanisms (Babbitt et al., 1996, Gulick et al., 1998, Hasson et al., 1998).

Muconate lactonazing enzymes were found to be involved in the degradation of chlorocatechol, methylcatechol and catechol (Cámara et al., 2007, Cámara et al., 2009, Eulberg et al., 1997, Eulberg et al., 1998, Kaulmann et al., 2001, Laemmli et al., 2002, Moiseeva et al., 2002, Nikodem et al., 2003). One specific enzyme in this family,

chloro-muconate cycloisomerase was found to dechlorinate chloro-muconate and form protoanemonin as a by-product of this reaction (shown in Figure **82**) (Skiba et al., 2002, Solyanikova et al., 1995, Vollmer et al., 1999).

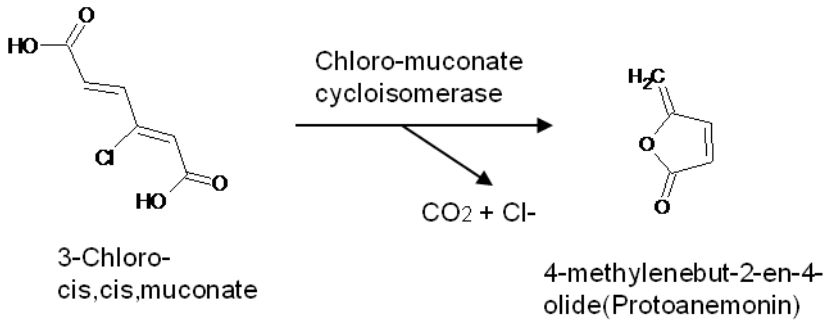


Figure 82 Function of chloro-muconate cycloisomerase

Based on the genetic analysis and biodegradation intermediate/byproduct analysis, it is predicted that biodegradation intermediate 3-chloro-cis, cis, muconate may be converted to protoanemonin under the activity of substrate specific chloro-muconate cycloisomerase (Vollmer et al., 1998, Vollmer et al., 1999), where a dehalogenation reaction occurs. This is the most commonly seen muconate cycloisomerase pathway reported (Skiba et al., 2002). Recent research has identified an alternative pathway, where the chloromuconate cycloisomerases avoid (chloro-)protoanemonin formation by increasing the rate of chloride abstraction from the enol/enolate intermediate compared to that of the addition of a proton (Kaulmann et al., 2001). Lower protoanemonin concentrations but larger amounts of maleylacetate were found to be present during the degradation of 4- and 5-chlorosalicylates in *Pseudomonas* sp. strain MT1, which suggested a novel pathway via 4-chlorocatechol, 3-chloromuconate, and maleylacetate by this strain (Nikodem et al., 2003). *Rhodococcus opacus* 1CP and *Rhodococcus rhodochrous* strain 89 were both found to undergo another dechlorination reaction, catalyzed by a muconolactone isomerase-related enzyme rather than by a specialized chloromuconate cycloisomerase (Eulberg et al., 1997, Moiseeva et al., 2002, Solyanikova et al., 2001).

Protoanemonin is toxic to bacterial cells (Blasco et al., 1997) , which may be reflected by the slow growth of degradative bacteria (Roh et al., 2009), seen in some recent triclosan degradation experiments (Aranami & Readman, 2007) and in this study.

Another possible pathway could be that hydrolysis occurs after dechlorination. The by-products of this pathway may be dichlorophenol and alkenyl acetic acid. However, alkenyl acetic acid was not detected in the intermediate/by-product analysis in this study.

Therefore, the muconate cycloisomerase pathway was proposed in this study.

Dehalogenation of polychlorinated biphenyl and ether-an area to explore

A hybrid bioinorganic catalyst Bio-Pb0 was demonstrated to cause polychlorinated biphenyls to undergo stepwise dehalogenation with preferential loss of the least sterically hindered halogen atom (Harrad et al., 2007), producing dichlorobiphenyls (33.9%), two monochlorobiphenyls (12%), and biphenyl (30.7%) as by-products of 2,4,4'-trichlorobiphenyl, and five tetrachlorinated and five trichlorinated PCB products for pentachlorinated PCB (Harrad et al., 2007). The dehalogenation of more chlorinated material (pentachlorobiphenyl) occurs much more slowly than observed for trichlorinated PCB, possibly attributable to steric hindrance (Harrad et al., 2007).

White rot fungus *Pycnoporus cinnabarinus* was found to transform biphenyl and diphenyl ether to dimers with carbon-carbon bonds in *para*-position to the hydroxyl group of the monomers (Jonas et al., 2000).

There are very limited studies on the bacterial degradation of polychlorobiphenyl ethers. Diphenyl ether was found to be utilized as diphenyl substrates for the lignin-degrading basidiomycete, *Phanerochaete chrysosporium* (Hiratsuka et al., 2005). Although the degradation was not extracellularly initiated by the lignin peroxidise (LiP), an extracellular enzyme responsible for degradation of biphenylene and dibenzo-*p*-dioxin, diphenyl ether was metabolized via an intermediate formation of hydroxylated products by intracellular enzymes (Hiratsuka et al., 2005).

Plasmid encoded biodegradation function-a novel pathway to be investigated further

Plasmids are present in all branches of the bacterial "tree of life" and have been found in all bacterial communities studied to date (Sorensen et al., 2005). Although more and more large plasmids have been sequenced in recent years, only limited amounts of plasmid-sequence data are currently available (Mølbak et al., 2003b). The comparative analyses of the available data have revealed the complexity of plasmid genetics, the capacity of these elements to replicate autonomously in permissive bacterial cells and the presence of unique sets of genes that are clearly distinct from the genes that are typically found on bacterial chromosomes (Sorensen et al., 2005). More genetic-sequence information on the components of the horizontal gene pool is required, and such

knowledge will shed light on how bacteria exchange genes and adapt to their environment (Sorensen et al., 2005).

Partial or complete sequences of a number of catabolic plasmids have been obtained and are available to provide opportunity for researchers to investigate the functions and catabolic pathways involved in the degradation of pollutant compounds (Akiyama et al., 2010, Bartilson & Shingler, 1989, Feng et al., 1997, Kwong et al., 2000, Li et al., 2004, Maeda et al., 2003, Ng et al., 1994, Shingler et al., 1992, Yano et al., 2007, Yeo et al., 1998b). Characterisation of catabolic plasmids involved in the degradation of environmental pollutants, such as toluene (pWWO), naphthalene (pND6), nicotine (pAO1), BPA (pW2), carbozole (pCAR), atrazine (pADP-1) and some non-specific catabolic plasmids including pCT14 and pJP4 have also been characterised recently (Aspray et al., 2005, Bramucci et al., 2006, Clément et al., 1995, de Lipthay et al., 2002, Dennis, 2005, Haugland et al., 1991, Johri et al., 1996, Li et al., 2004, Maeda et al., 2003, Nojiri et al., 2004, Pries et al., 1994, Rousseaux et al., 2002, Takizawa et al., 1999, Trefault et al., 2004, van der Meer, 1994).

Biodegradation of 2,4-dichlorophenoxy acetic acid and 3-chlorobenzoic acid in *Ralstonia eutroph* JMP134 are encoded by the *tfd* genes, consisting two chlorocatechol degrading gene clusters *tfd-I* and *tfd-II*, which are located in the 88 kb, *IncP-1 β*, conjugative plasmid pJP4 (Don & Pemberton, 1981, Smith & Thomas, 1987, Trefault et al., 2004). The *tfdI* gene cluster includes 2,4-dichlorophenol hydroxylase, maleylacetate reductase, dienelactone hydrolase, chloromuconate cycloisomerase, chlorocatechol-1,2-dioxygenase, LysR-type transcriptional regulator; and *tfdII* gene cluster is composed of 2,4-dichlorophenol hydroxylase, maleylacetate reductase, dienelactone hydrolase, chlorocatechol-1,2-dioxygenase, muconate cycloisomerase and *lysR*-type transcriptional regulator (Trefault et al., 2004).

While most aromatic catabolic pathways are characterised as plasmid-mediated biodegradation (Dennis & Zylstra, 2004, Fuhrmann et al., 2003, Ghosal et al., 1987, Jencova et al., Kukor & Olsen, 1991, Lee et al., 1994, Nancharaiah et al., 2008, Ravindran et al., 2006, Szczepanowski et al., 2004, Volkova et al., 2005, Yrjälä et al., 1997), chromosome-encoded catabolic pathways have been identified in different bacterial species. *E. coli* B, C and W strains were found to be able to degrade 4-hydroxyphenyl-acetic acid and homoprotocatechuate (3,4-hydroxy-phenylacetate) via an inducible, chromosomally encoded *ortho*-cleavage pathway (Burlingame & Chapman, 1983, Díaz et al., 1998, Ismail

et al., 2003).

Although the comparison with other catabolic genes reveals interesting similarities, the lack of a complete characterised gene cluster for chloro-aromatic degradative genes, such as *alk, tfd, xyl and tol* gene clusters, may suggest a novel degradative pathway and also different evolutionary origin of these plasmids (Prieto et al., 1996).

Plasmid pF12 was demonstrated to be involved in triclosan biodegradation in this study, and this coincided with a most recent study reported after this thesis was submitted for examination, which speculated possible plasmid mediated triclosan biodegradation (Lee et al., 2012). A gene cluster putatively encoding triclosan degradative function was identified to reside on this plasmid by the complete DNA sequencing of this plasmid. However, the function of some of the predicted proteins on this plasmid could not be confirmed. A schematic structure of genetic arrangement of the degradative gene cassettes is illustrated in Figure 76. The genes were annotated as *det* (degradation of triclosan) ABCDEFG, tentatively encoding TPP binding domain containing protein, unknown protein, dehydrogenase, hydrolase, oxidase, transcriptional regulator, and mandelate racemase/muconate enzyme.

Genetic organization of catabolic plasmids-origin and evolution

The genetic organization of pF12 is similar to that of a characterised catabolic plasmid isolated from a toluene degrading *Pseudomonas* strain CT14, which contains two domains: Domain I primarily contained genes for plasmid- related functions and a novel origin of replication and Domain II contained several genes from a meta-cleavage pathway for aromatic rings (Bramucci et al., 2006).

Despite the diversity of the degradation pathways, most of the aromatic catabolic plasmids sequenced so far have a modular organization (Thomas, 2000). Each module includes a cluster of genes encoding related functions (Bramucci et al., 2006, Trefault et al., 2004), such as household functions of plasmid replication, stabilization and mobilization. The genes related to catabolic functions are often located in a separate module that may be carried on some mobile elements such as inverted sequences or transposons (Bramucci et al., 2006, Maeda et al., 2003, Thomas et al., 1992, Thomas, 2000, Trefault et al., 2004,

Tsuda et al., 1999). The catabolic module may be organised into one or two operons, which encodes genes involved in a cascade of degradation pathways (Ghosal et al., 1987, Nancharaiah et al., 2008, Trefault et al., 2004). For example, the toluene degradative plasmid pWW0 contains an upper pathway operon, which encodes enzymes for oxidation of toluene to benzoate, and a lower pathway operon which encodes enzymes that cleave the aromatic ring and produce intermediates of the TCA cycle (Calero et al., 1989, Herrero et al., 1993, Nancharaiah et al., 2008, Suzuki et al., 2001).

A well characterised plasmid pJP4, which is a non-specific chloro-aromatic pollutant degradative plasmid, contains a more complicated catabolic module (Trefault et al., 2004). This functional module includes several full or truncated copies of inverted sequences and a transposon, which carries a cascade of known or unknown catabolic functions (Trefault et al., 2004). This study also proposed that the genetic recombination of mobile elements such as inverted repeats or transposons may contribute to the bacterial adaptation to chloroaromatic pollutants and the evolution of specialized chloroaromatic degradation pathways (Trefault et al., 2004). Another example is the 79kb plasmid pTDN1, which also contains catabolic genes bounded by IS sequences (Saint et al., 1990).

Plasmid pF12 investigated in this study also has a modular organisation, which includes a plasmid household function domain and a putative triclosan degradation related domain. This similar genetic organization indicates that this plasmid may be related to the biodegradative capability of the host strain. It may also suggest that this plasmid may have contributed to the bacterial adaption to chloroaromatic pollutants and originated through an evolutionary chain of specialized chloroaromatic degradation pathways. Compared to pJP4, the mobile elements on the plasmid are in a much simpler form (Trefault et al., 2004), i.e. there is only one putative transposon-like element predicted on pF12, and this element may be present in an incomplete form. This may be the result of a series of insertion, rearrangement, and deletion events that may have occurred early in the evolutionary development of this plasmid. The incomplete *mob* gene cluster and lack of complete *tra* genes in the mobilization module, which is located immediately downstream of the predicted triclosan degradative domain, indicates possible deletion events that may have occurred during the evolution of this plasmid, and these deletion events may have resulted in the loss of conjugative ability of this plasmid, as seen in the conjugation experiments carried out in this project.

The same events may have also resulted in the deletion of some catabolic genes. It may be speculated that this plasmid pF12 is an end product in the evolution of triclosan catabolic plasmids, which has already donated some of the genes encoded in the catabolic pathway to the host chromosome, and therefore has lost some of the essential genes that may have been present in the ancestors of this plasmid.

In fact, redundancy of genes involved in catabolic pathways has been either proposed or observed in previous studies on plasmid mediated metabolism of organic compounds (Bramucci et al., 2006, Harada et al., 2006, Khan et al., 2006, Pieper et al., 2004). The redundant genes may be located on the plasmid itself or in the chromosome of the host strain (Bramucci et al., 2006, Fong et al., 2000, Pieper et al., 2004, Trefault et al., 2004). It is hypothesised that when the host strain is under stress, some catabolic genes on the plasmid may be deleted through intra-molecular transposition of the transposon carrying a full catabolic interon. As a result, only part of the catabolic interon that does not have redundancy in the host chromosome will be retained and function in synergy with the chromosome encoded catabolic genes.

Two experimental findings in this study support this hypothesis:

- The screening of triclosan resistant/degradative *E coli* strain, as the recipient in the electroporation (whole plasmid or cloned fragments) experiment failed. This indicated that the plasmid alone is not able to confer triclosan resistance and degradative function to the laboratory *E coli* strain, probably due to the lack of genes responsible for triclosan resistance and the genes involved in the upper catabolic pathway, in which triclosan may be degraded to provide intermediates for TCA cycle.

- The transfer of plasmid pF12 into a non-degradative *Pseudomonas* strain by electroporation resulted in the conferring of ability to grow in triclosan medium by this recipient. This indicated that this recipient strain has all the genes required for triclosan degradation lower pathway, but lack of oxidase involved in the unique *ortho*-cleavage of the aromatic ring. *Ortho* cleavage of aromatic rings is usually chromosomally encoded, and the *meta* cleavage is often found to be plasmid encoded. This is consistent to the proposed pathway in that triclosan biodegradation underwent *ortho*-cleavage. As discussed the cleavage of triclosan aromatic ring underwent a unique pathway, only one unique gene, i.e. predicted as oxidase in this study, is required in addition to all the chromosomal genes required for the normal

Ortho-cleavage. This unique gene was donated from plasmid pF12 through electroporation, which conferred triclosan biodegradation capability to the recipient strain.

Consequently, when this plasmid was transformed by electroporation into an *E. coli* strain, the recipient did not acquire the ability to degrade triclosan probably due to the lack of other catabolic genes in the *E. coli* strain. However, when transformed into *Pseudomonas aeruginosa*, the recipient became capable of degrading triclosan and utilising it as a carbon source. This may indicate that the additional genes necessary for triclosan biodegradation exist in the *P. aeruginosa* strain, whereas they are absent from the *E. coli* strain.

Implications/significance to wastewater treatment

It is evident that bacteria in the wastewater environment may acquire the ability to degrade triclosan, should prolonged exposure to this compound favour the selection of triclosan resistant/degradative isolates from a mixed consortium. Unless through a different degradation pathway, the condition for the adaptation to occur, is that the unique gene encoding triclosan ring cleavage oxidase exists in the bacterial genome, either in chromosome or on mobile elements. The fact that the selection of triclosan degraders were difficult and this gene was found to be located on plasmid pF12, which is an abridged plasmid with most of the other catabolic genes truncated, indicate that the genes encoding enzymes required for triclosan degradation upper pathway were not widely spread among bacteria population in the wastewater consortium. There might be some unknown reasons, which require further investigation that determined the transfer of these genes into host chromosomal DNA was difficult. The uniqueness of this triclosan ring cleavage enzyme may also determine that it is difficult for the wastewater consortium bearing no triclosan degradative plasmid to obtain the degradative capability through acclimation. Therefore, inoculation/seedling with triclosan degraders are likely to be required for effective triclosan removal if no indigenous bacterium, which carries the gene encoding oxidase required for the upper pathway, is present in the wastewater. The plasmid was found to be a non-conjugative plasmid, however it could be mobilised by other elements such as transposons, or it would need to be mobilised by recombining with a conjugative plasmid for easy transfer. Mobile elements were identified to be located upstream of the triclosan catabolic region, indicating the possible transfer mechanism of this catabolic module.

This investigation provides information and background to the potential genetic engineering of efficient triclosan degradative bacteria that may be used for bio-augmentation to improve the performance of triclosan removal during wastewater treatment when/where necessary. For applications where enhanced triclosan removal is required, it is possible that the seeding of wild type or genetic-engineered triclosan degradative strains into a tertiary bio-filtration system would improve the triclosan removal efficiency through biodegradation mechanism.

Due to the slow degradation rate of triclosan and the abundance of other organic compounds, it is anticipated the biodegradation mechanism discovered in this study cannot form a major mechanism in the natural removal of triclosan in the aeration reactor during secondary wastewater treatment processes in the wastewater treatment plant; however, biodegradation may occur during the sludge treatment process, where settled sludge from primary and secondary settlers are treated to remove nutrients such as nitrogen, phosphorus and carbon before disposal. Therefore, optimisation of wastewater primary and secondary treatment process to improve the adsorption of triclosan by suspended solids and activated sludge is the key to improving triclosan reduction in wastewater treatment plant effluent. Nitrification, which normally occurs in the sludge treatment process, may enhance the biodegradation of triclosan (Roh et al., 2009). In fact, those two activities may have synergetic effect on each other, resulting in more efficient sludge treatment and triclosan degradation, if toxicity of intermediates or by-products of the reactions can be appropriately mediated (Sayavedra-Soto et al., 2010). Natural biodegradation may occur in the water and wastewater environment and a slow but efficient removal may be achieved by wastewater biological consortium.

The residence of essential gene required for triclosan biodegradation upper pathway on this plasmid explained the difficulties experienced while selecting triclosan degraders from the wastewater samples. This highlighted the importance of this plasmid and the need for further investigation.

7.2 Conclusions

Based on the microbiology experimental results, biodegradation intermediate analysis and the genetic studies, it is concluded that *Pseudomonas citronellolis* F12 stain, isolated from the wastewater environment, was capable of degrading triclosan as the sole carbon source at a slow rate, although the growth could be accelerated upon addition of casamino acids into the cultural medium. The bacterial degradation of triclosan was via a unique ortho-cleavage upper pathway, which involved the oxidation of triclosan to form 2',4'-dichlorophenyl 4-chloro-6-oxo-2,4-hexadienoate as the primary biodegradation intermediate. The activity was catalysed by a possible novel oxidase, which was encoded on a 17.7kb endogenous plasmid, namely pF12. The lower pathway included hydrolysis of the primary intermediate to dichlorophenol and chloro muconate by a putative hydrolase, possibly encoded on the plasmid. Chloro muconate was further metabolised to protoanemonin through oxidation and dehalogenation. The catabolic enzymes required for the triclosan biodegradation lower pathways are likely to be encoded by the chromosomal genes of the host strain.

Further studies are necessary to characterise the catabolic genes on the plasmid and to investigate how they interact with the host genome. In light of this information, it is then possible to choose a target strain, which carries the additional genes required for the lower pathways, and to construct an engineering bacterial strain for the fast and sensitive triclosan removal in the wastewater environment. This may involve the transfer of the degradative plasmid or only the cloning and expression of the essential genes in a recipient strain.

In this study, a hydrolase having low but significant similarity to metallo-β-lactams was tentatively identified on pF12. Cross resistance to β-lactams as well as other microbial agents sharing similar biodegradation pathways may also be expected. Care should be taken when investigating potential new pharmaceutical products of similar structure to triclosan, in case they contribute to the increased cross-resistance.

7.3 Future work

This work is an initial exploration of triclosan biodegradation. Progress in this project was made using general microbiology, genetic studies and chemical analysis. Completion of

this study suggests further research on bacterial triclosan degradation is warranted, both in genetic studies and in analytical aspects. Potential research areas of interest with the aim of identification of a new triclosan biodegradation pathway may include, but are not limited to, the following.

1. Genetic studies:

- Further transposon mutagenesis studies or equivalent mutant production.
- Cloning, expression and functional studies on triclosan-degradation-related genes as discussed previously, in particular the hydrolase, dehydrogenase, oxidase and mandelate racemase/muconate enzymes;
- Characterization and assessment of the β-lactamase B-like hydrolase and its potential involvement in antibiotic resistance, evolution and origin of this antibiotic resistance gene;
- Investigation of the possibilities of seeding the wild type triclosan degrading *Pseudomonas citronellolis* into biological filters.
- Genetic engineering of triclosan degradative genes to produce bacterial strains capable of triclosan degradation in the wastewater environment.

2. Analytical studies:

- Development of LC-ESI-MS/MS methods to identify other potential triclosan degradation intermediates that were not identified in Chapter 6 of this study;
- Further development of the isotope pattern method used in this study in combination with fragmentation analysis to study the structure of potential triclosan degradation intermediates.
- Synthesis of predicted degradation intermediates and by-products, which could be used as external standards to verify and confirm the characterization of intermediate and degradation by-products.
- Application of Nuclear Magnetic Resonance Spectroscopy (NMR) analysis to confirm the tentative triclosan degradation intermediates as well as to identify other intermediates and by-products.

Due to the difficulties in expression of triclosan degradative gene in *E. coli*, the next step in this investigation would be to clone the genes of interest into a non triclosan degradative *Pseudomonas* strain, such as *Pseudomonas aeruginosa*, or the cured F12 strain. However, the vector to be used for this cloning is critical for the success of this experiment. The vectors such as pGEM5 and PWKS130 might not be able to express stably in *Pseudomonas* strains. Use of a high copy number vector that will be stably maintained in *Pseudomonas* might be required to improve the degradative capacity. Such vectors as pKT230 and pKT231 might be considered (Bagdasarian et al., 1981, Bagdasarian & Tinmmis, 1982, Ramos et al., 1988).

Origin and derivation of this plasmid is another interesting area to explore. The complete sequence of pF12 revealed some similarities with some plasmids already characterised. Some ORFs, although not characterized fully for functionality, showed significant similarity to ORFs in other catabolic plasmids, such as pRA2 in *Pseudomonas alcaligenes*. Further investigation into the origin and evolution of this plasmid by comparing with known plasmids and exploring the possibility of horizontal transfer that might have occurred in the evolutionary development of this plasmid, may help in understanding the mechanisms of adaptation to chloroaromatic pollutants and the evolution of specialized chloroaromatic degradation pathways (Trefault et al., 2004).

The discovery of R-M system on pF12 is interesting. Often, other redundant mechanisms are involved despite the presence of the R-M system to aid in plasmid stability. Further work on R-M system to clarify the function of the endonuclease and methylase may also help to characterise the role of this R-M system in plasmid stability.

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Appendices

Appendix 1 Special media

a. Triclosan Minimal Medium (TCSM)

Solution A:

Ammonium sulphate of 0.225g was dissolved in 500ml RO water and autoclaved at 120 ˚C for 15 minutes before use.

Agar Bacterial No1 of 1.2g was added for agar medium.

Solution B (10×):

11g K_2HPO_4 and 4.25g KH_2PO_4 was dissolved in 250mL RO water; pH was adjusted to 7.0±0.2 with 10M NaOH; autoclaved at 120 ˚C for 15 minutes before use.

Solution C (100×):

Mineral salts of 9.75g $MgSO_4 \cdot 7H2O$, 2.5g $MnSO_4 \cdot H_2O$, 0.5g $FeSO_4 \cdot 7H_2O$, 0.15g $CaSO_4$ and 200µl concentrate H_2SO_4 was added in 500mL RO water; filter sterilized by 0.22µm polyethersulfone membrane (Millipore Express®) and stored under 4 ˚C for future use.

A final concentration of 10% (v/v) solution B, 1% (v/v) solution C and 89% (v/v) solution A was added for minimal medium. Triclosan (Fluke) from stock at appropriate concentrations, as described in Chapter 3-6, was added for triclosan minimal medium (TCSM).

b. Triclosan selection medium for DH5α (100 mL medium)

Vitamin B1 ×100 stock:

0.5mg/mL in MiliQ water, sterilized by 0.22 µm polyethersulfone membrane (Millipore Express®) filter.

Solution A, B and C were prepared as for TCSM.

A final concentration of 10% (v/v) solution B, 1% (v/v) solution C, 89% (v/v) solution A, 1% (v/v) VB1 stock and 0.1% (v/v) triclosan stock of 100 mg/mL was added.

c. X-gal/IPTG agar plate for blue/white screening

×1000 X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) stock solution:

This stock solution was made by dissolving X-gal (Promega) in dimethylformamide to make a 40 mg/mL solution using a glass or polypropylene tube. The tube containing the solution was wrapped in aluminium foil to prevent damage by light and was stored at -20 ℃.

×1000 IPTG (Isopropylthio-β-D-galactoside mw 238.3) stock solution

A solution of IPTG was made by dissolving IPTG (Dioxane-free, Promega) in distilled water to make a 40mg/mL solution. The solution was sterilized by filtration through a 0.22 micron disposable filter and dispensed into 0.5 mL aliquots. Stock solutions were stored at -20˚C until use.

Each stock solution of 200 µl was added into 200mL melted LB agar and mixed well before pouring the plates.

d. TFBI for preparation of competent cells

The buffer contains the following ingredients:

30 mM potassium acetate

50 mM manganese chloride

100 mM rubidium chloride

10 mM calcium chloride

15% (w/v) glycerol

Buffer was made using RO water, and pH was adjusted to 5.8 using 0.2N acetic acid. Solution was filter sterilized through a 0.22µm polyethersulfone membrane (Millipore Express®) and stored at 4 ˚C for future use.

e. TFBII for preparation of competent cells

The buffer contains the following ingredients:

10 mM mops pH 7.0

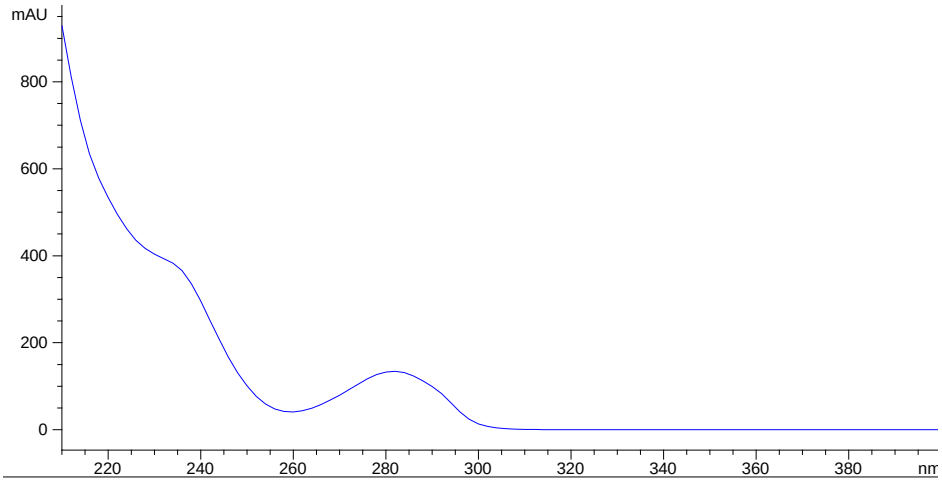
10 mM rubidium chloride

75 mM calcium chloride

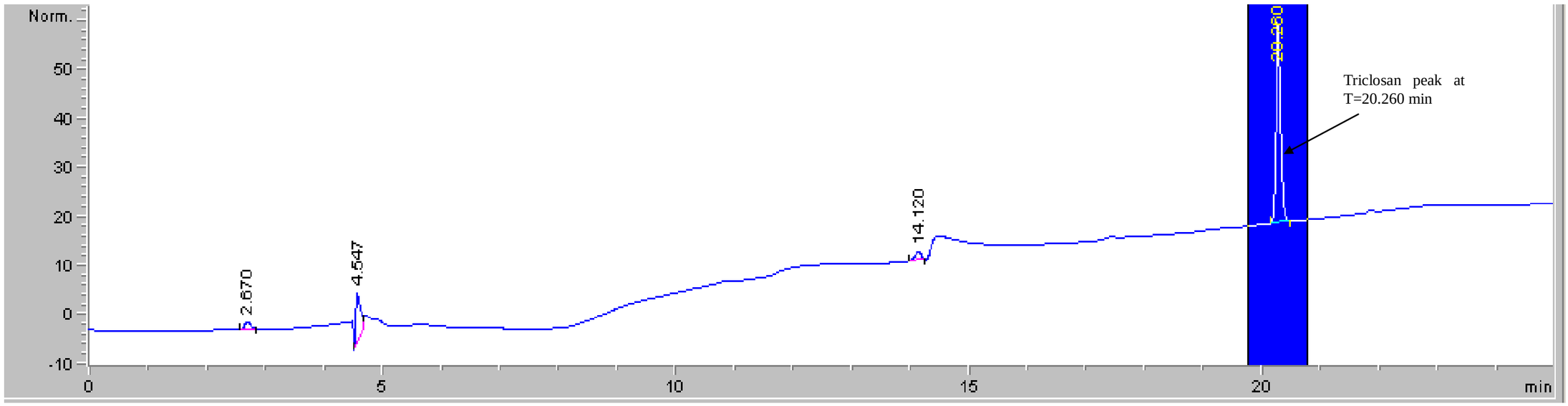
15% (w/v) glycerol

Buffer was made using RO water and was filter sterilized through a 0.22µm polyethersulfone membrane (Millipore Express®) and stored at 4˚C for future use.

Appendix 2 Characteristic UV absorbance spectrum of triclosan



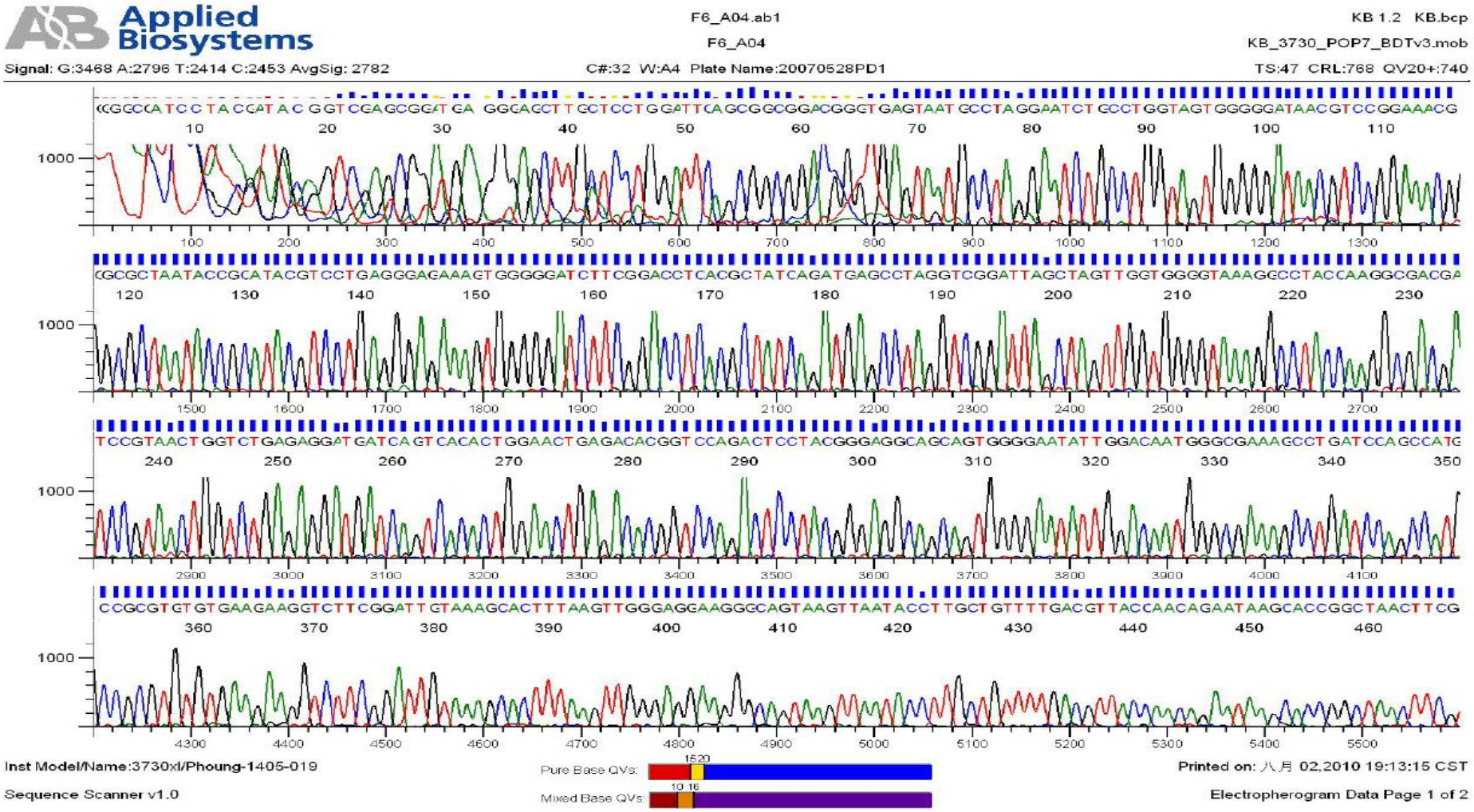
Appendix 3 Chromatogram of triclosan

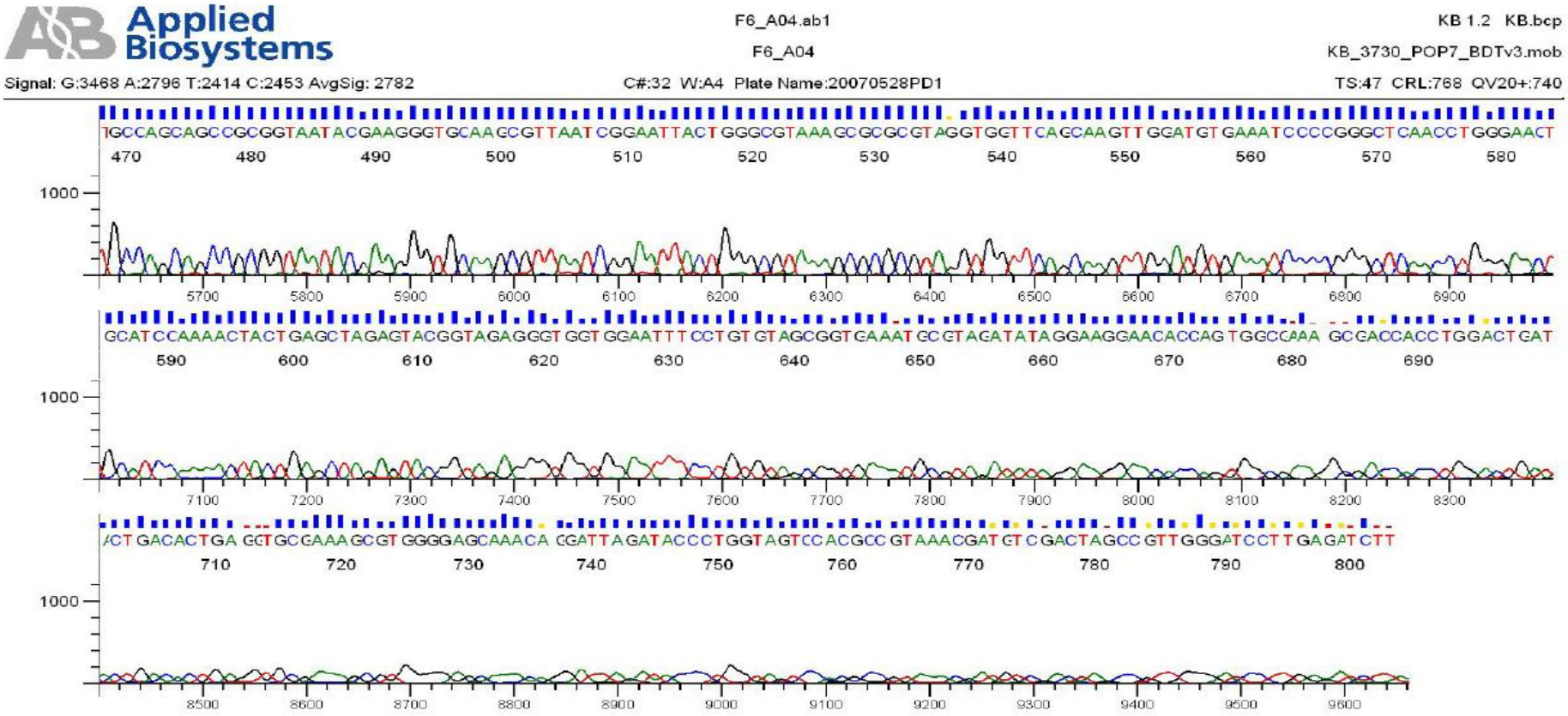


(Obtained using Alltech C8 analytical column at flow rate of 1 ml/min of a binary gradient eluant composed of 20 mM NH_4Ac and 100% CAN at a gradient of 40% ACN to 100% ACN in 30 min, where the standard containing 10 µg/mL of triclosan)

Appendix 4 Sequencing Electropherogram for 16S rDNA sequencing

a. F11 (forward)

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1000
5700 5800 5900 6000 6100 6200 6300 6400 6500 6600 6700 6800 6900
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7100 7200 7300 7400 7500 7600 7700 7800 7900 8000 8100 8200 8300
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Mixed Base QVs:
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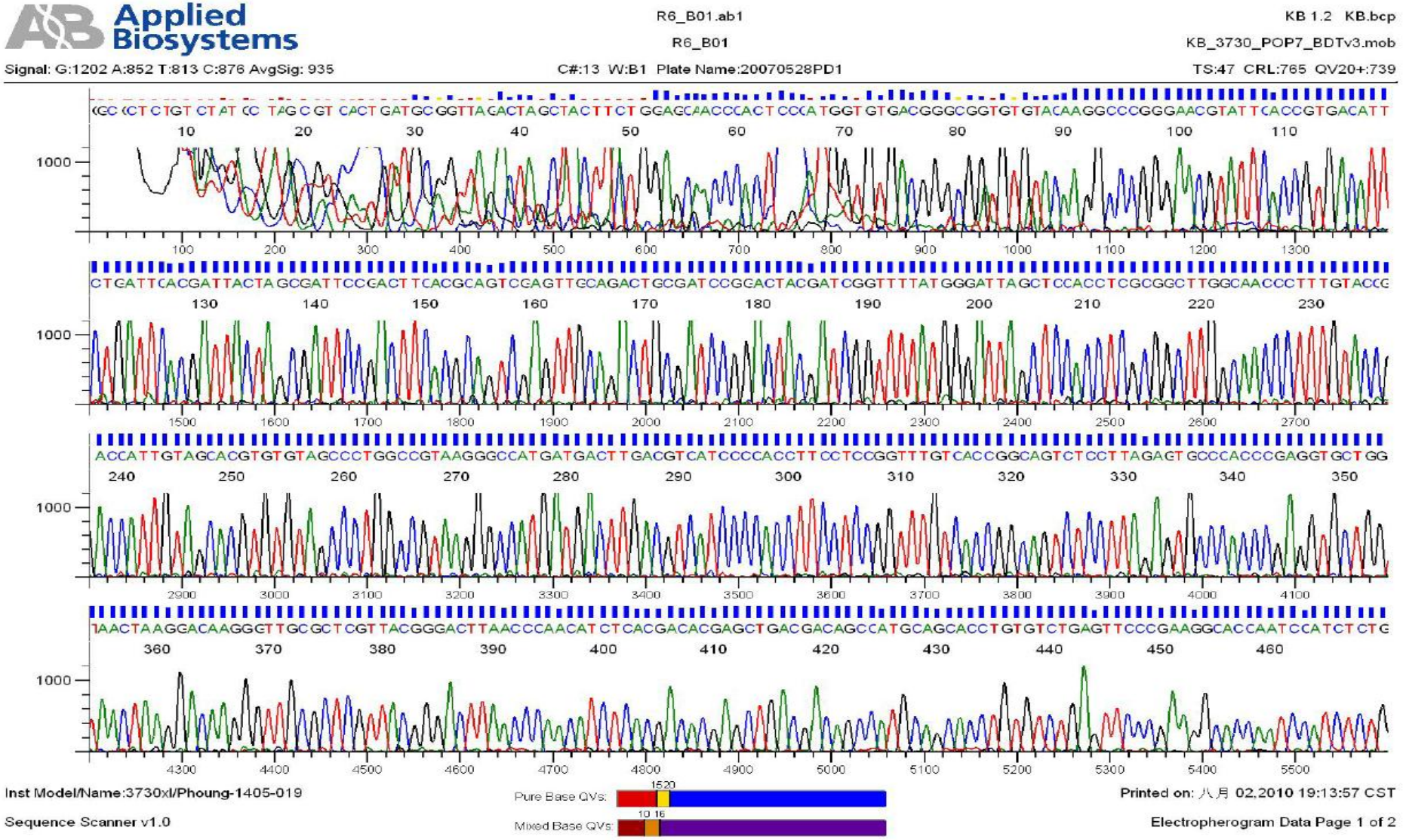
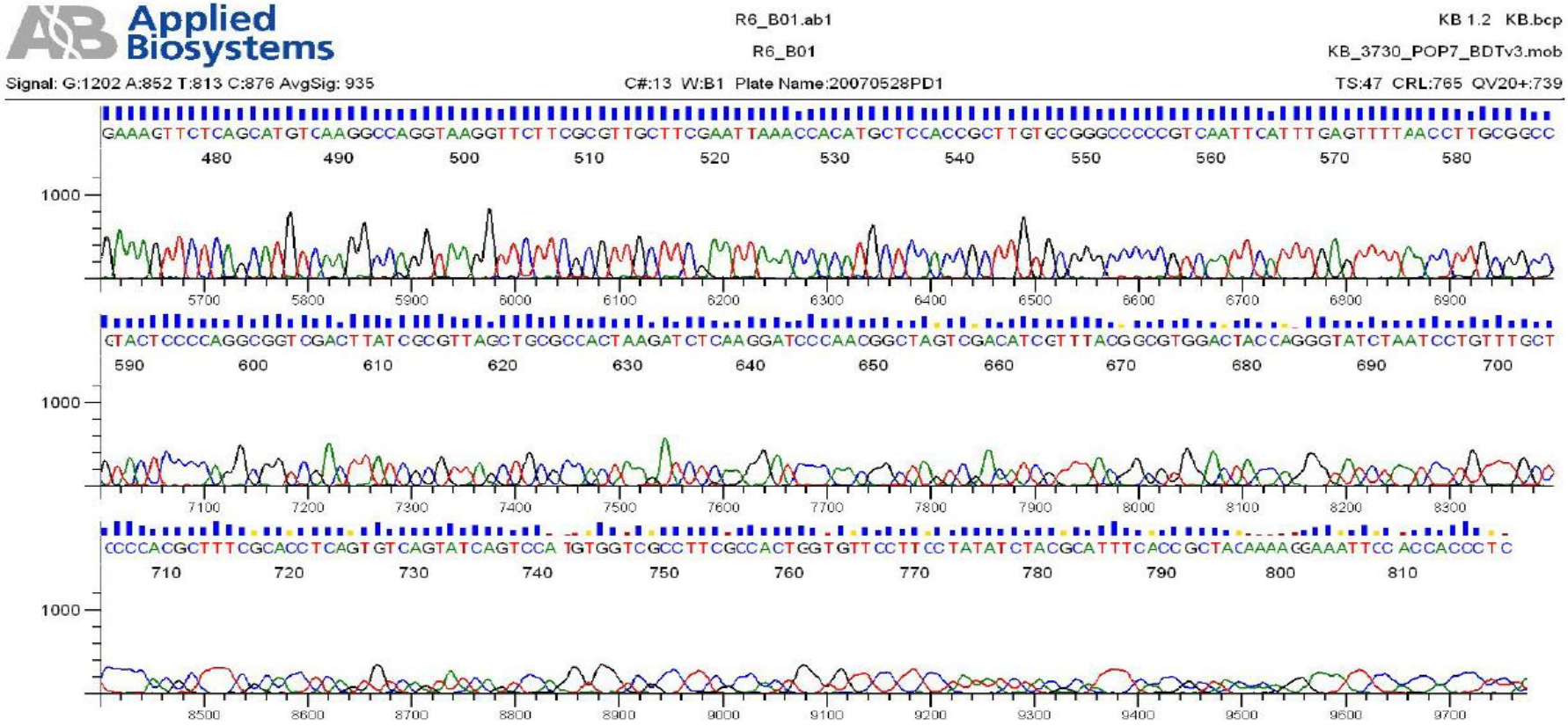
Electropherogram Data Page 2 of 2

b. F11 (reverse)





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Sequence Scanner v1.0

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Mixed Base QVs: 10 16

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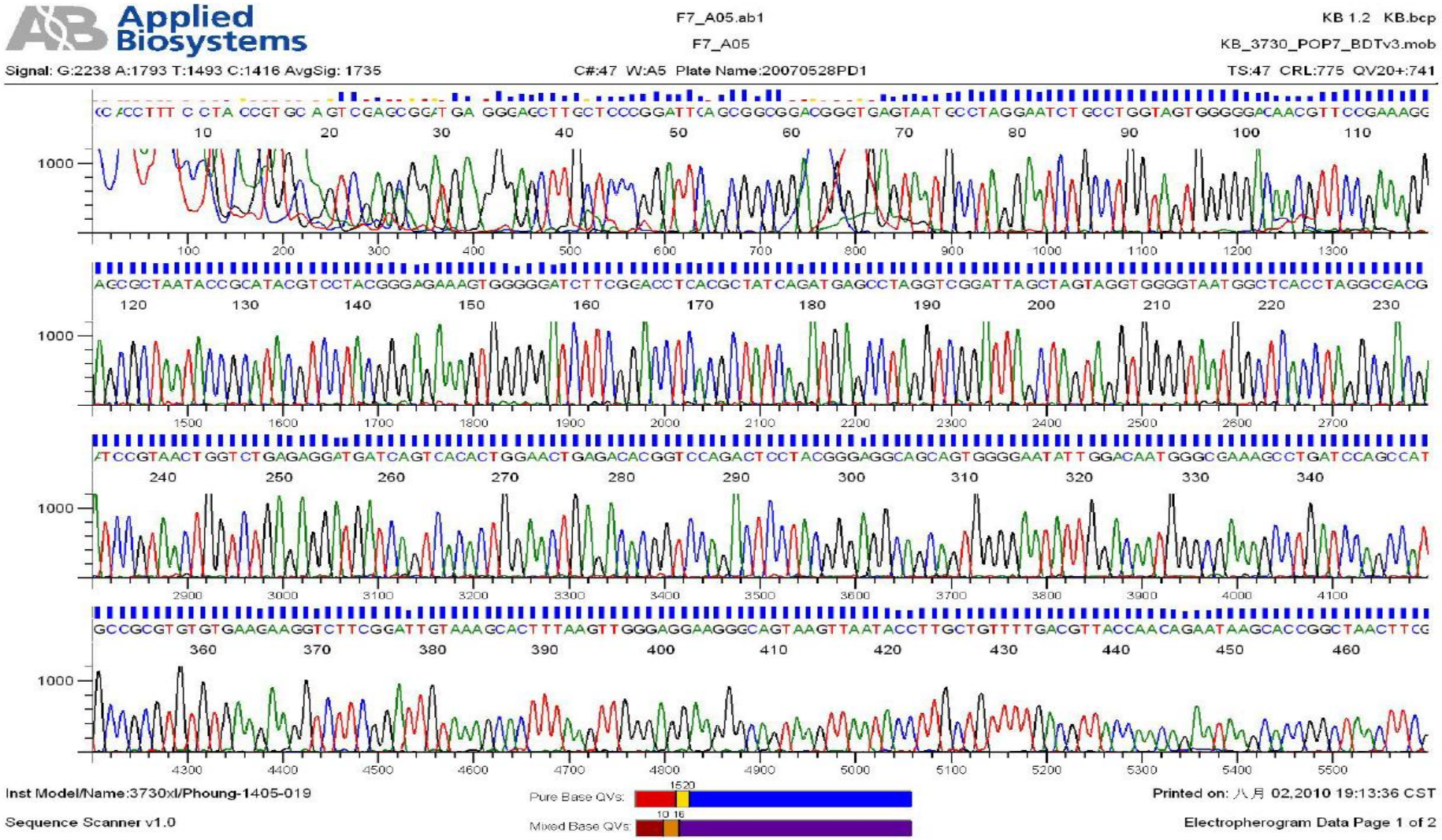
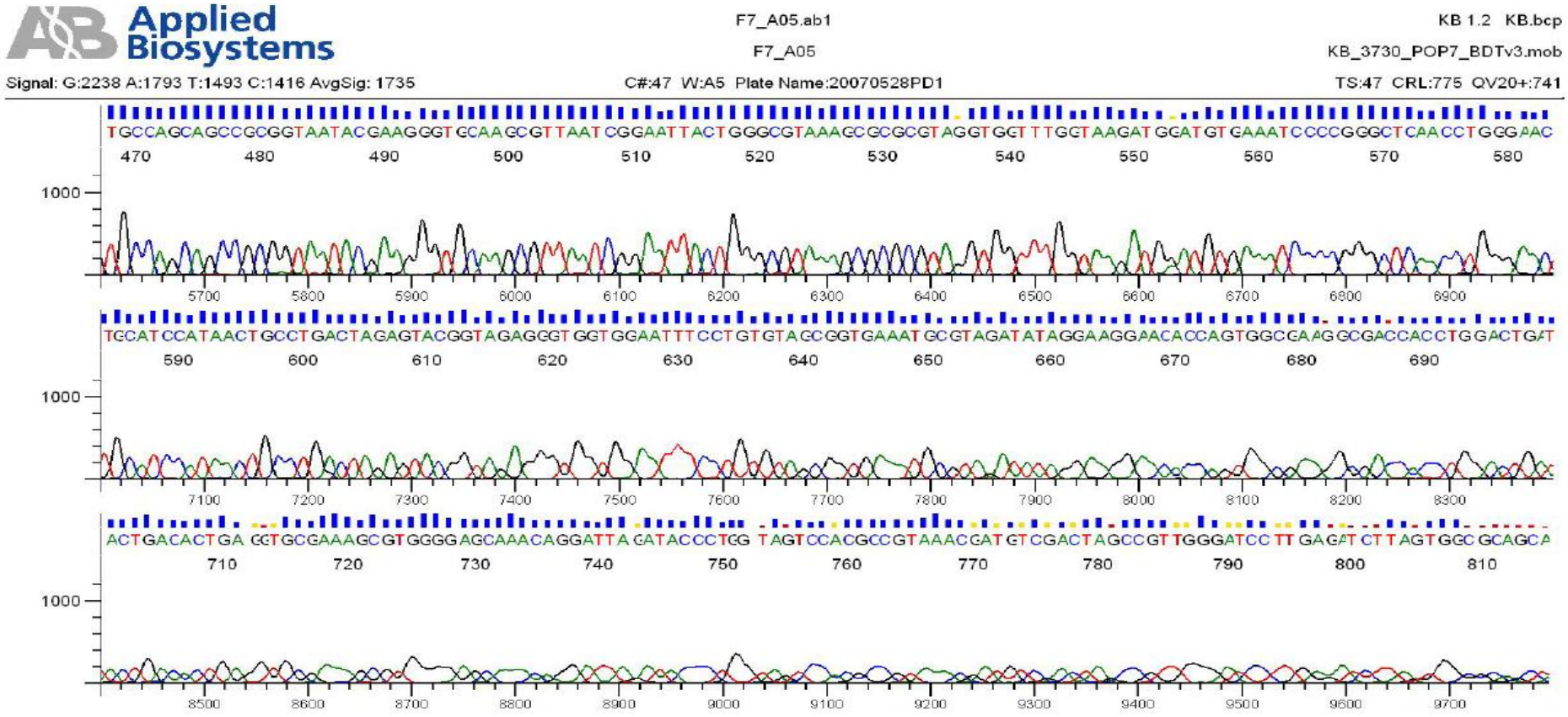
Electropherogram Data Page 2 of 2

c. F12 (forward)

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7100 7200 7300 7400 7500 7600 7700 7800 7900 8000 8100 8200 8300
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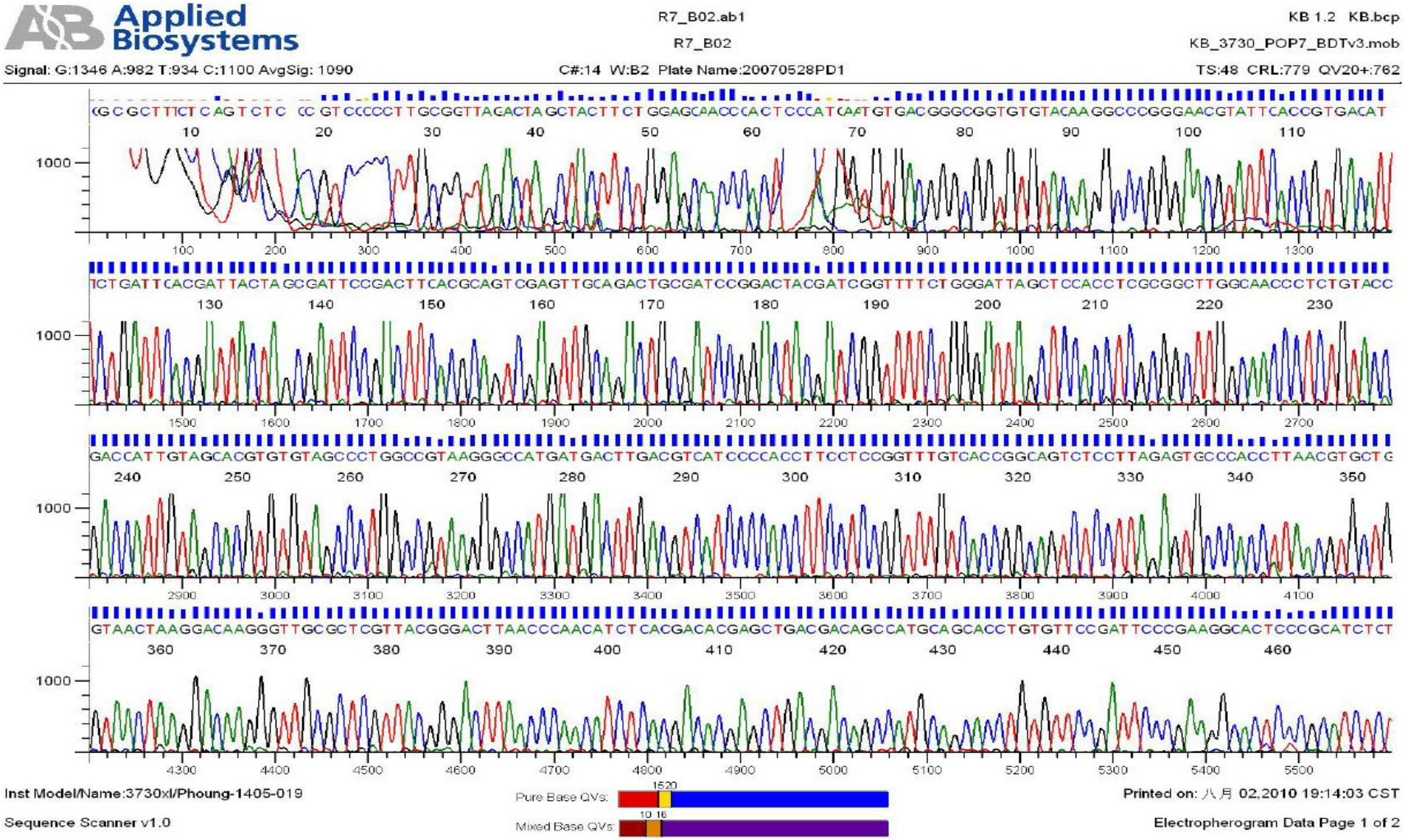
Electropherogram Data Page 2 of 2

d. F12 (reverse)



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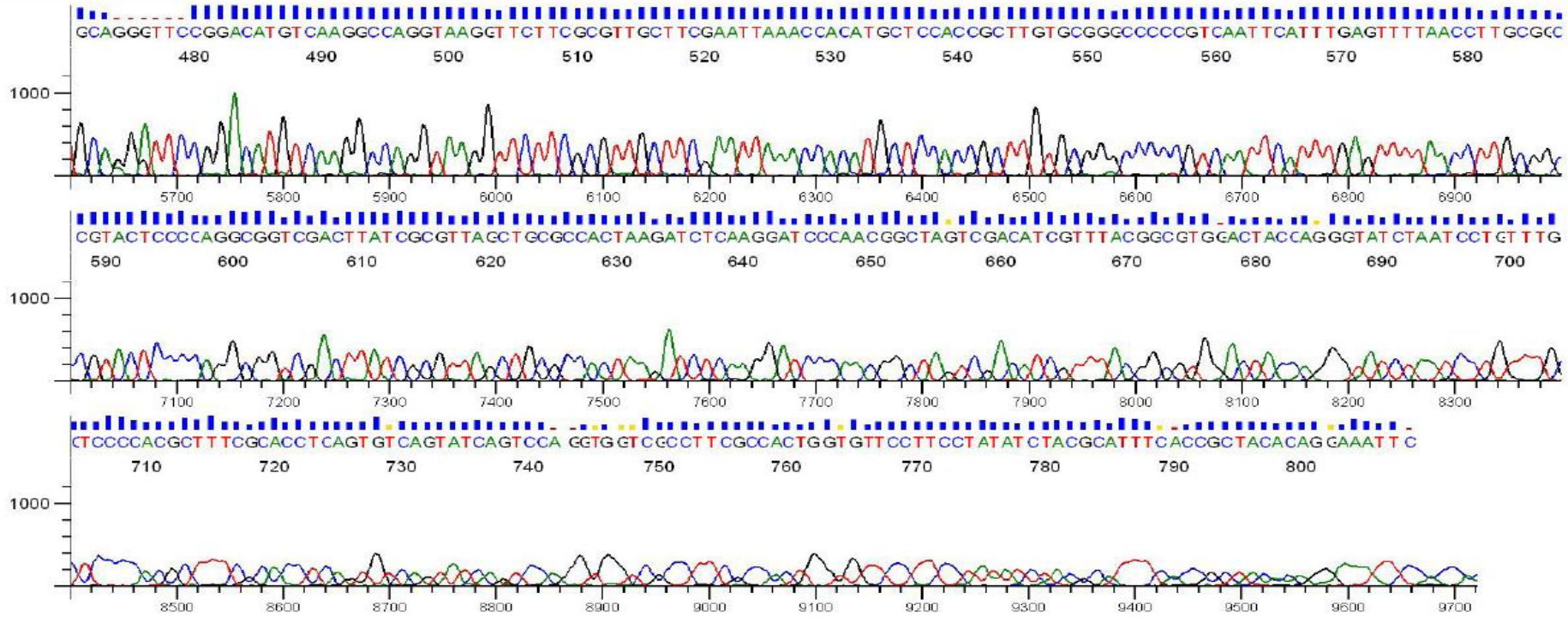

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Sequence Scanner v1.0

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Mixed Base QVs: 10 16

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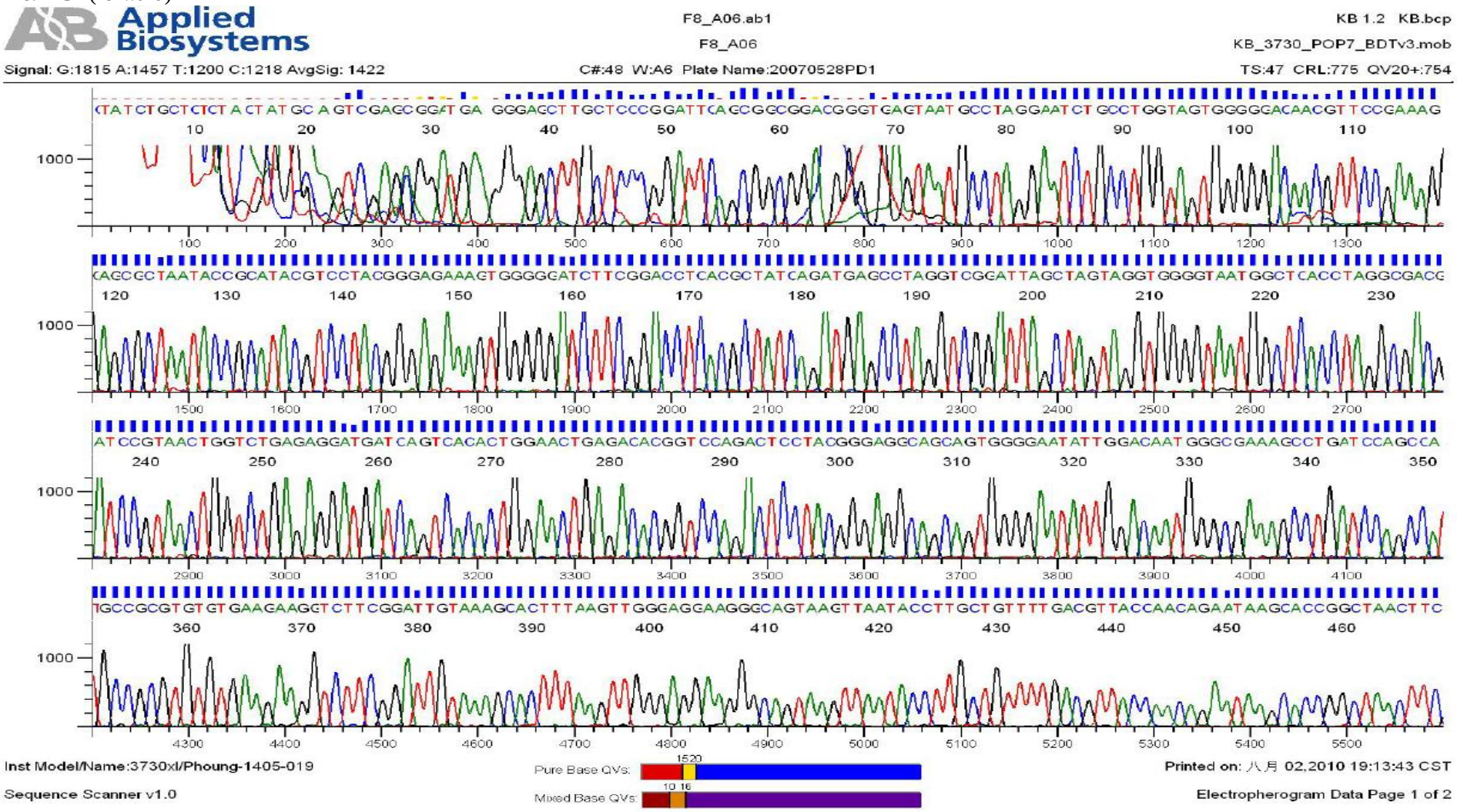
Electropherogram Data Page 2 of 2

e. C2 (forward)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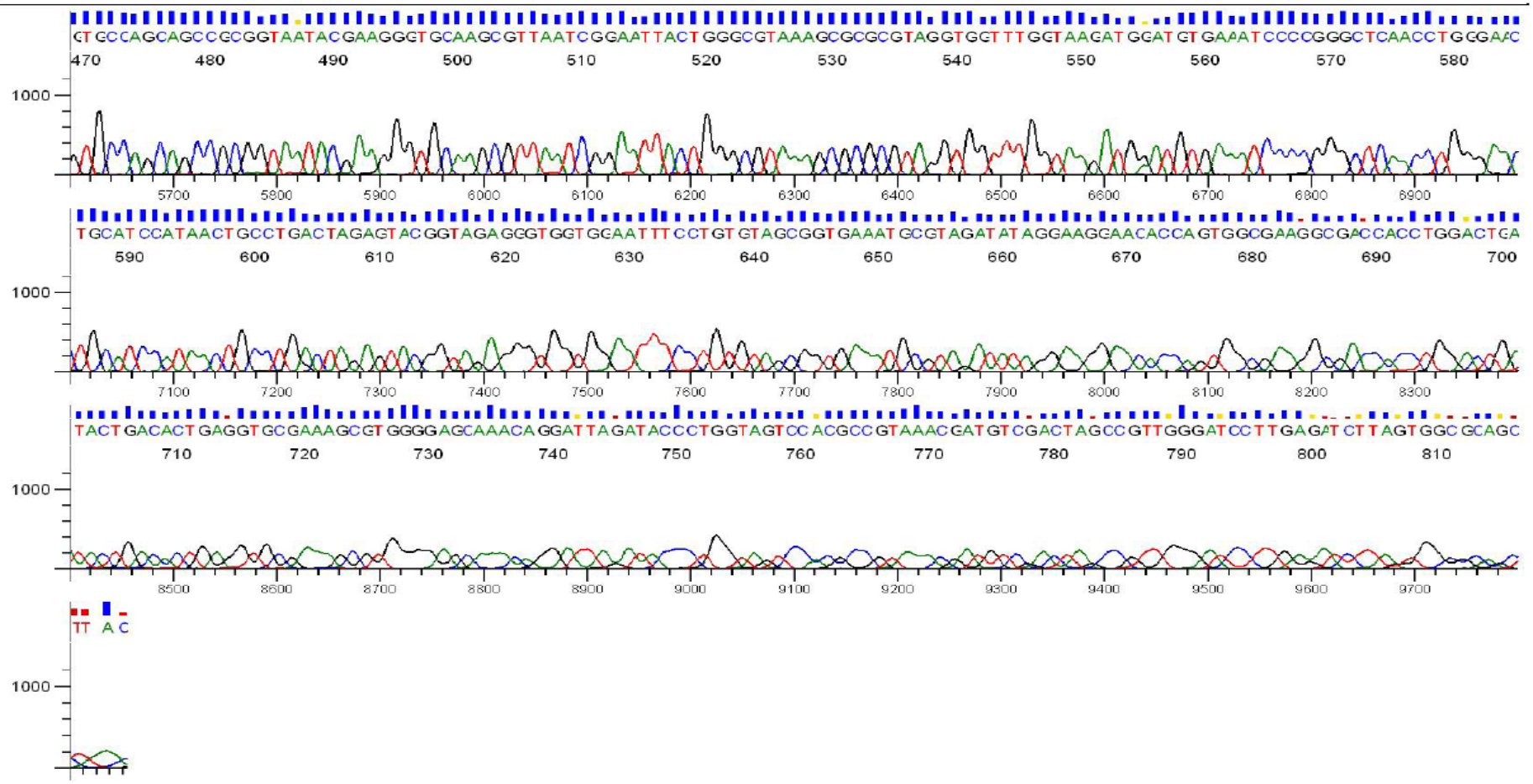

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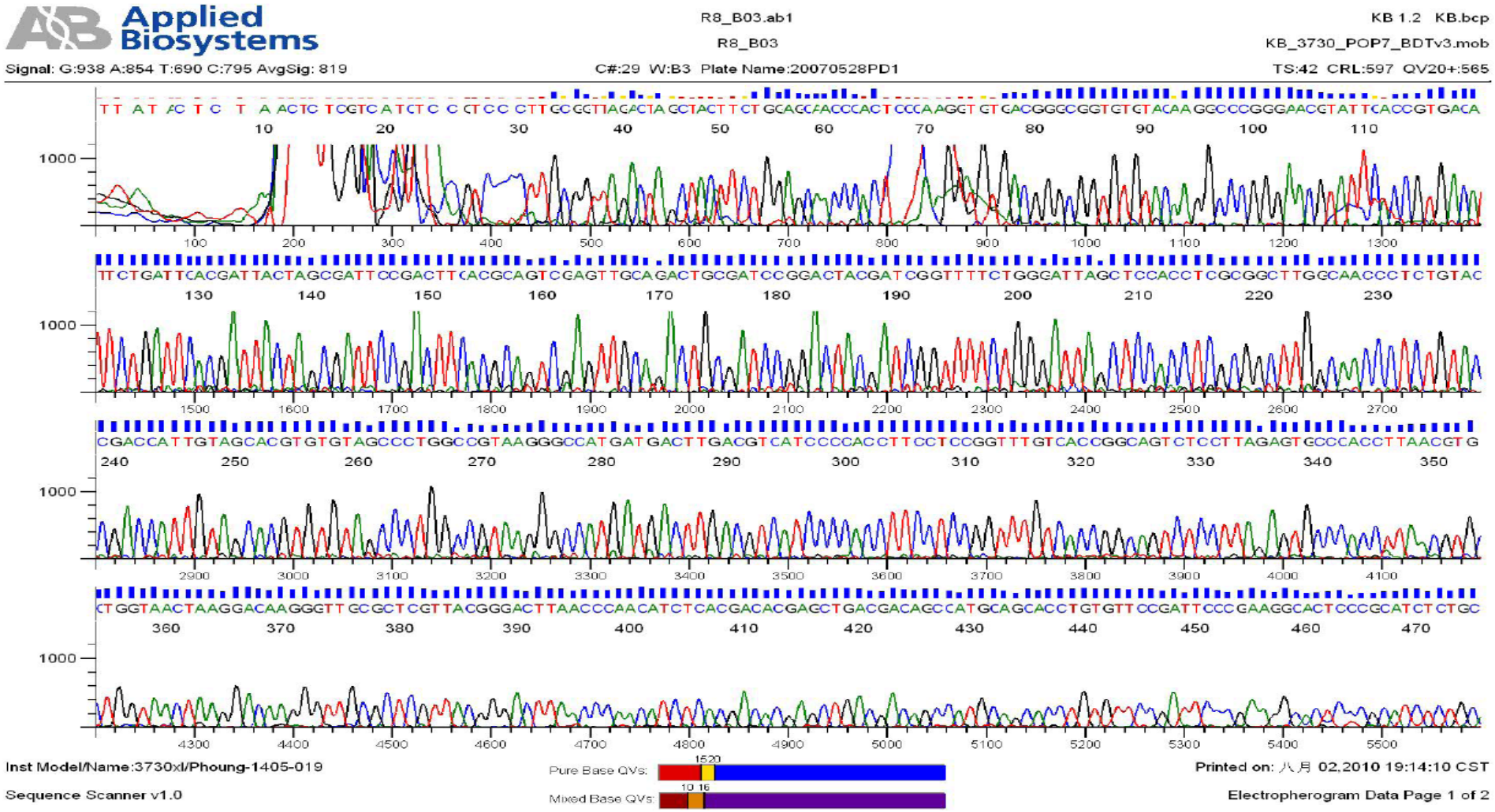
Electropherogram Data Page 2 of 2

f. C2 (reverse)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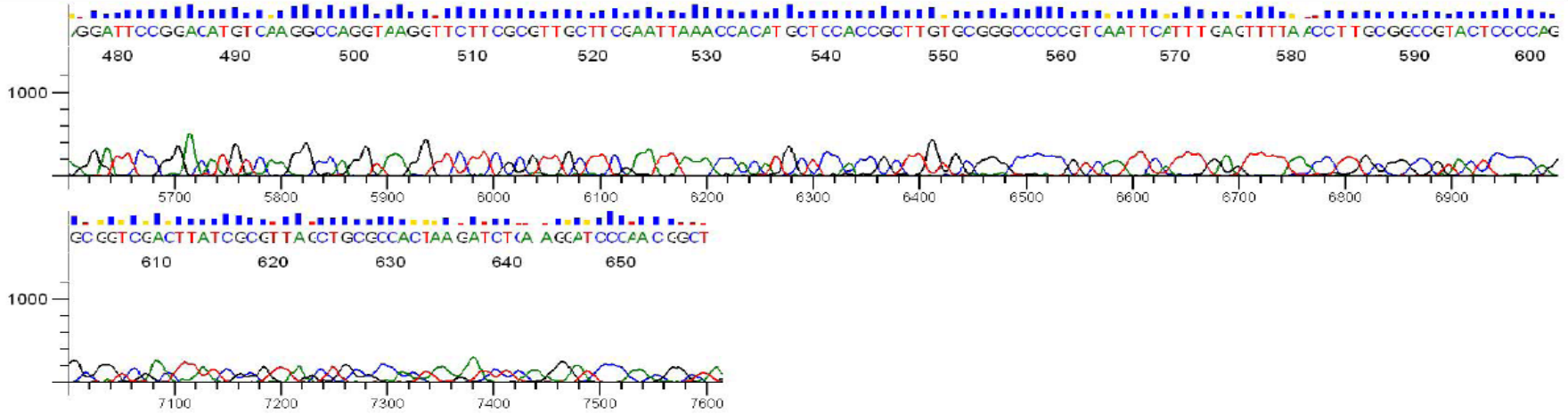

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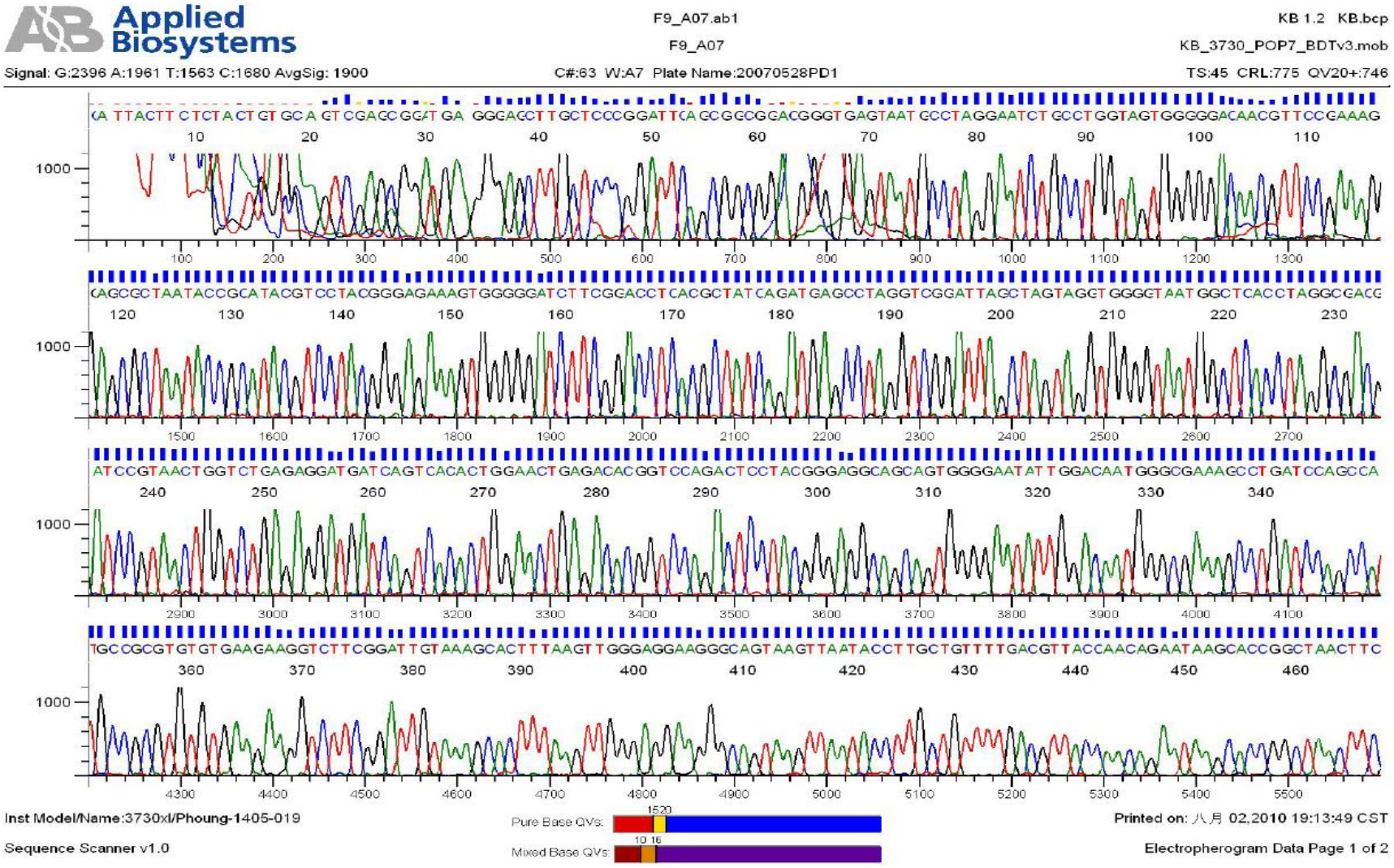
Electropherogram Data Page 2 of 2

g. C6 (forward)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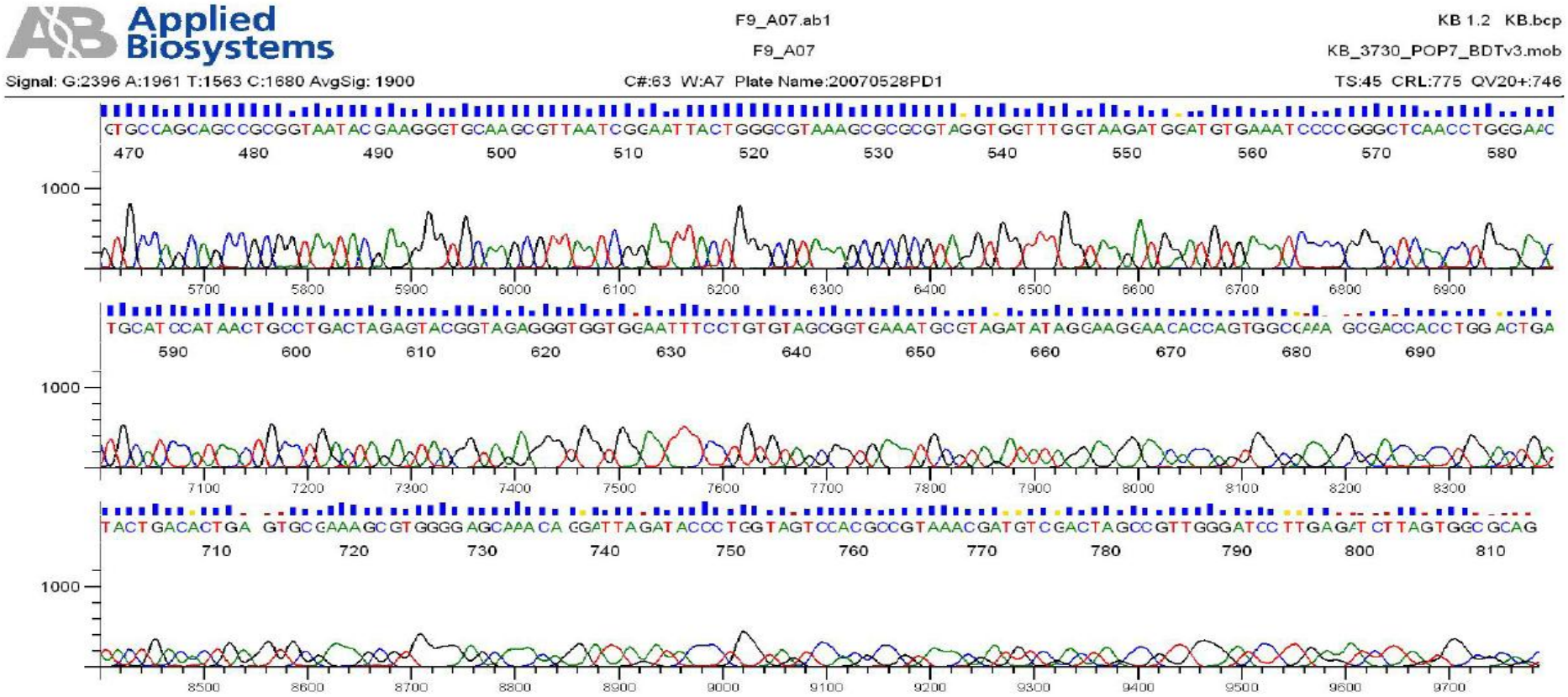
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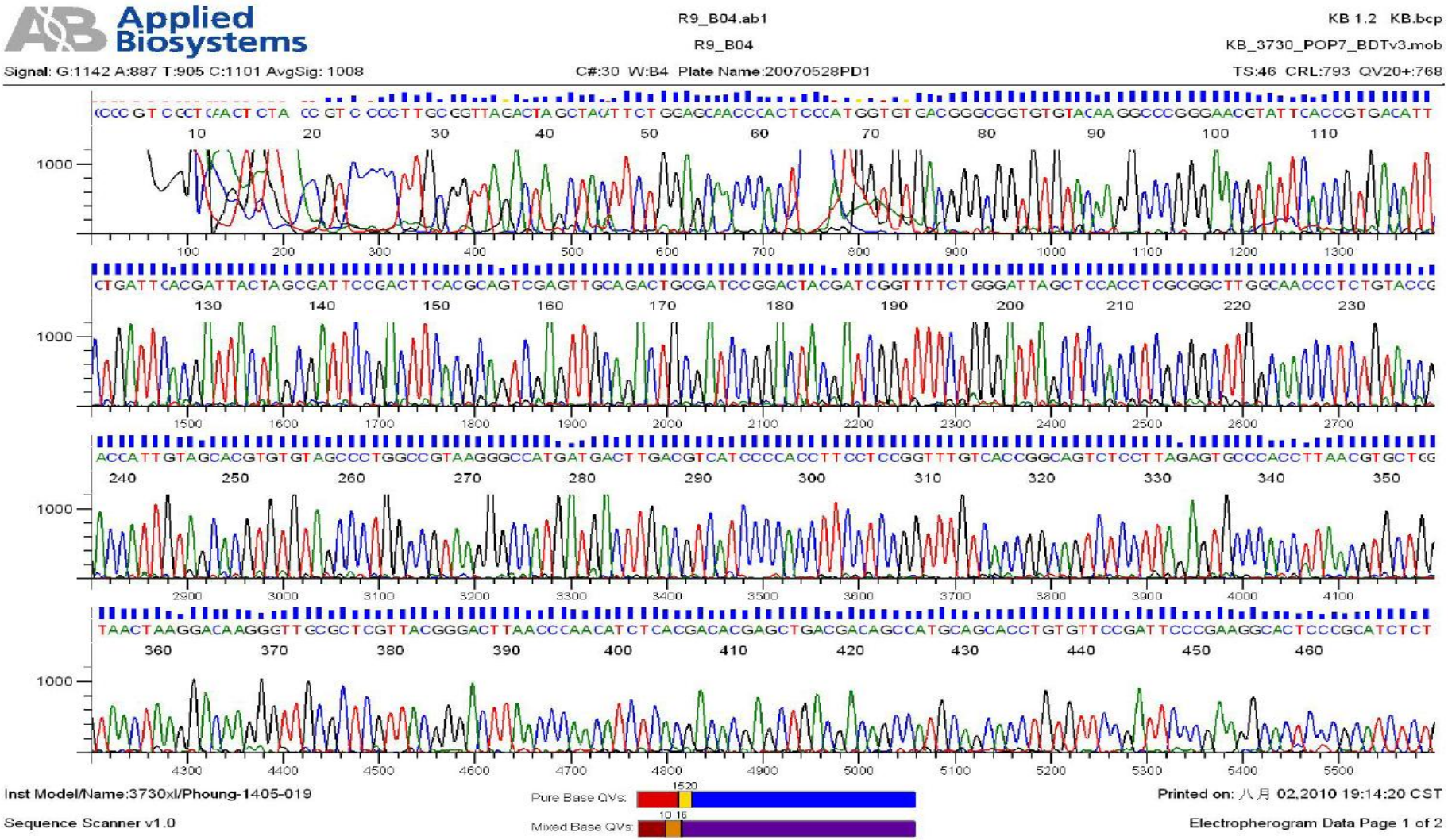
Electropherogram Data Page 2 of 2

h. C6 (reverse)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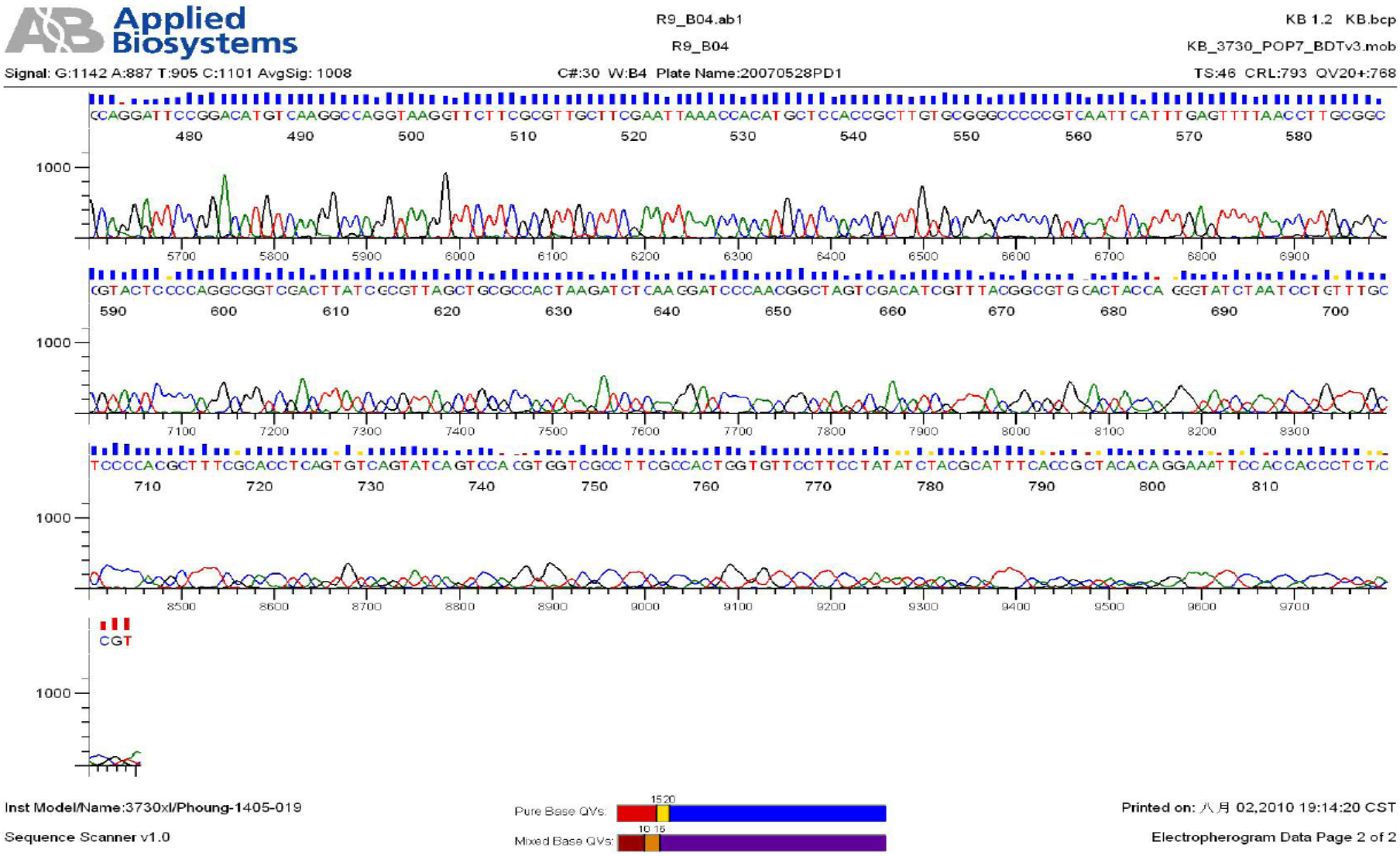
Applied Biosystems
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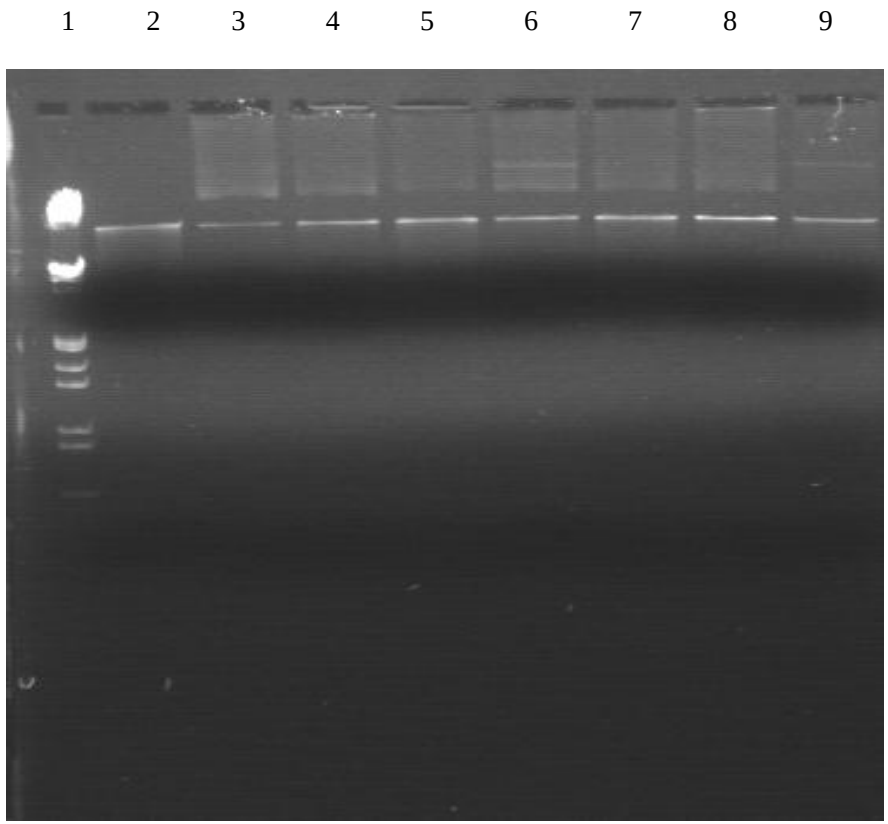
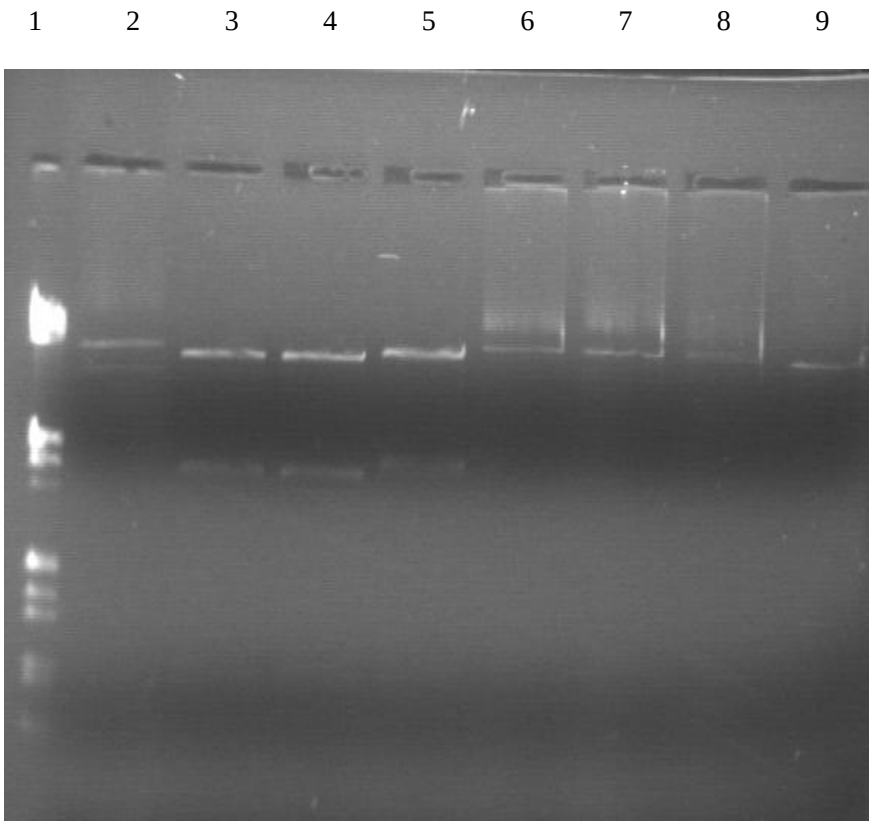
1000
5700 5800 5900 6000 6100 6200 6300 6400 6500 6600 6700 6800 6900
CGTACTCCCCAGGCGGTCGACTTATCGCGTTAGCTGCGCCACTAAGATCTCAAGGATCCCAACGGCTAGTCGACATCGTTTACGGCGTGCACTACCAGCGTATCTAATCCTGTTTGC
590 600 610 620 630 640 650 660 670 680 690 700
1000
7100 7200 7300 7400 7500 7600 7700 7800 7900 8000 8100 8200 8300
TCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCCTCTAC
710 720 730 740 750 760 770 780 790 800 810
1000
8500 8600 8700 8800 8900 9000 9100 9200 9300 9400 9500 9600 9700
CGT
1000
Inst Model/Name:3730xl/Phoung-1405-019
Sequence Scanner v1.0
Pure Base QVs:
1520
Mixed Base QVs:
10 16
Printed on: 八月 02,2010 19:14:20 CST
Electropherogram Data Page 2 of 2

Appendix 5 Restriction digestion of pF12 using various endonuclease enzymes



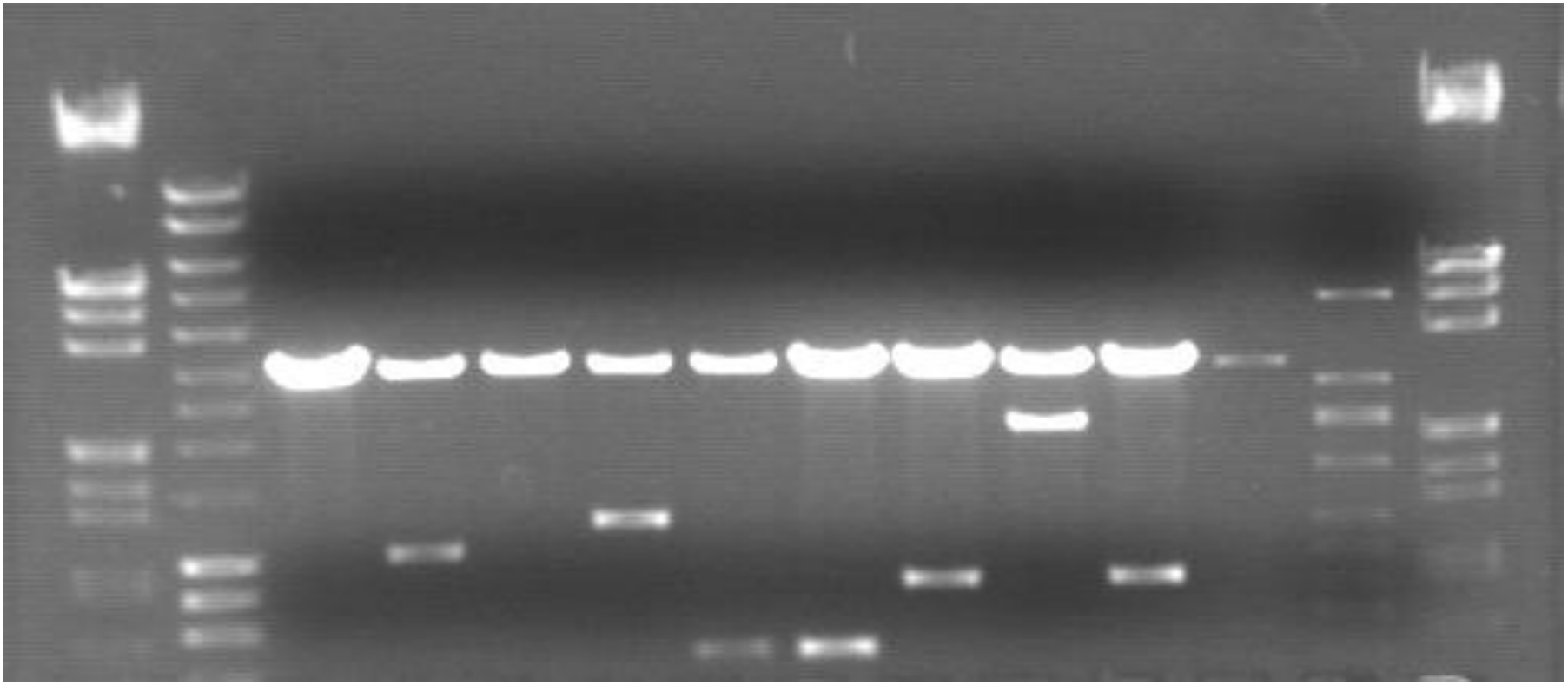
a. Lane 1: DNA Marker III;
Lane 2-8 Restriction digestion of pF12 by *Nde*I, *Hin*DIII, *Xba*I, *Kpn*I, ApaI, *Xho*I, *Bam*HI;
Lane 9 Mock digestion of pF12 (no enzyme added)



b. Lane 1: DNA marker III;
Lane 2: Mock digestion of pF12 (no enzyme added)
Lane 3-9: Restriction digestion of pF12 by *Eco*RI+*Sac*I, *Eco*RI+*Xba*I, *Eco*RI+*Nde*I, *Bam*HI, *Xba*I, *Kpn*I, *Sac*I;

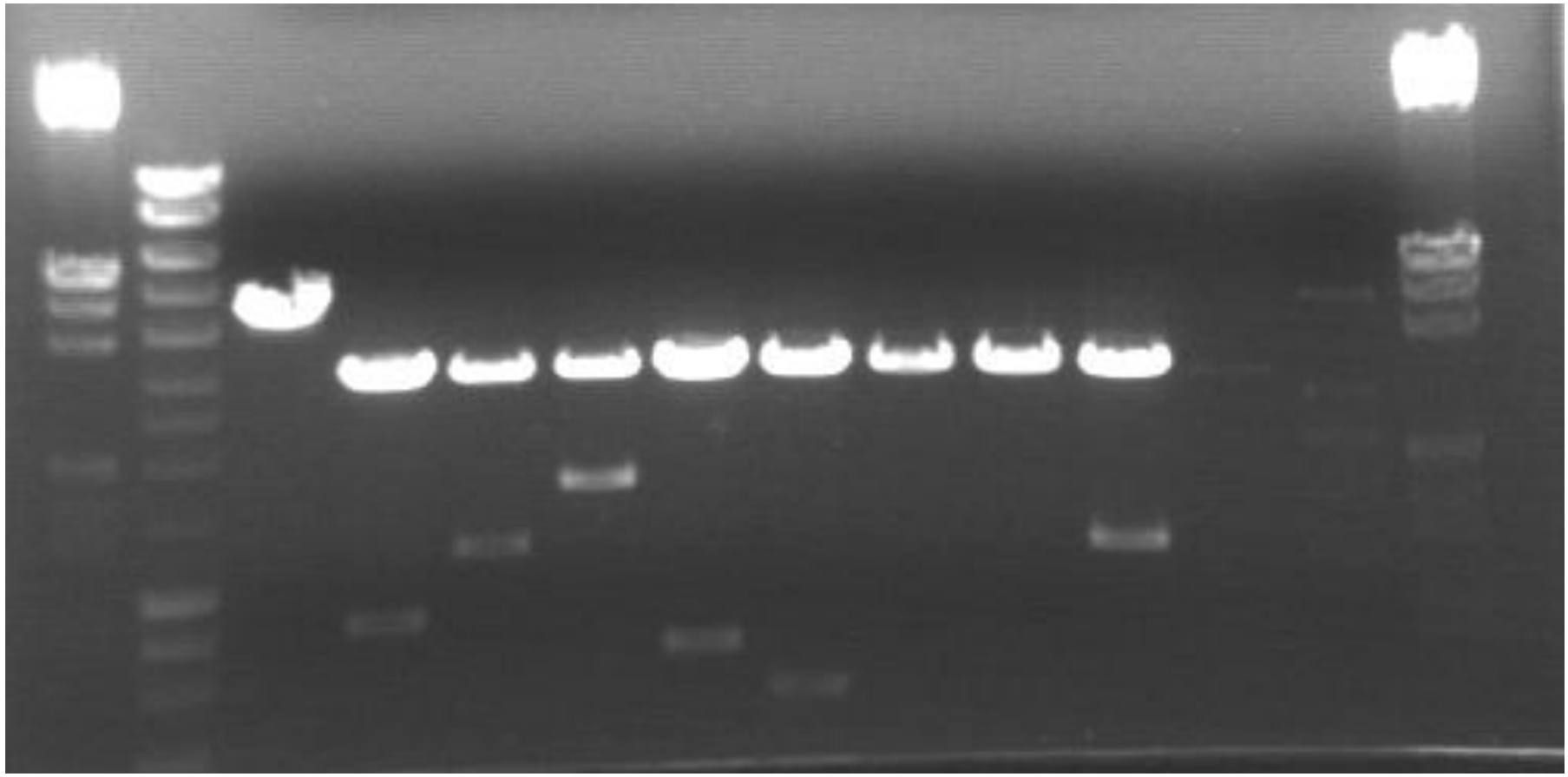
Appendix 6 Gel pictures captured during screening of cloned DNA fragments from pF12

1 2 3 4 5 6 7 8 9 10 11 12 13 14



a. Lane 1 DNA marker III; Lane 2 DNA marker; Lane 3 SalI/SacI double digested pGEM5 vector; Lane 4-11 SalI/SacI double digested pF12 clones; Lane 12 SalI/SacI double digested pGEM5 vector; Lane 13 MseI digested pF12; Lane 14 DNA marker III

1 2 3 4 5 6 7 8 9 10 11 12 13 14

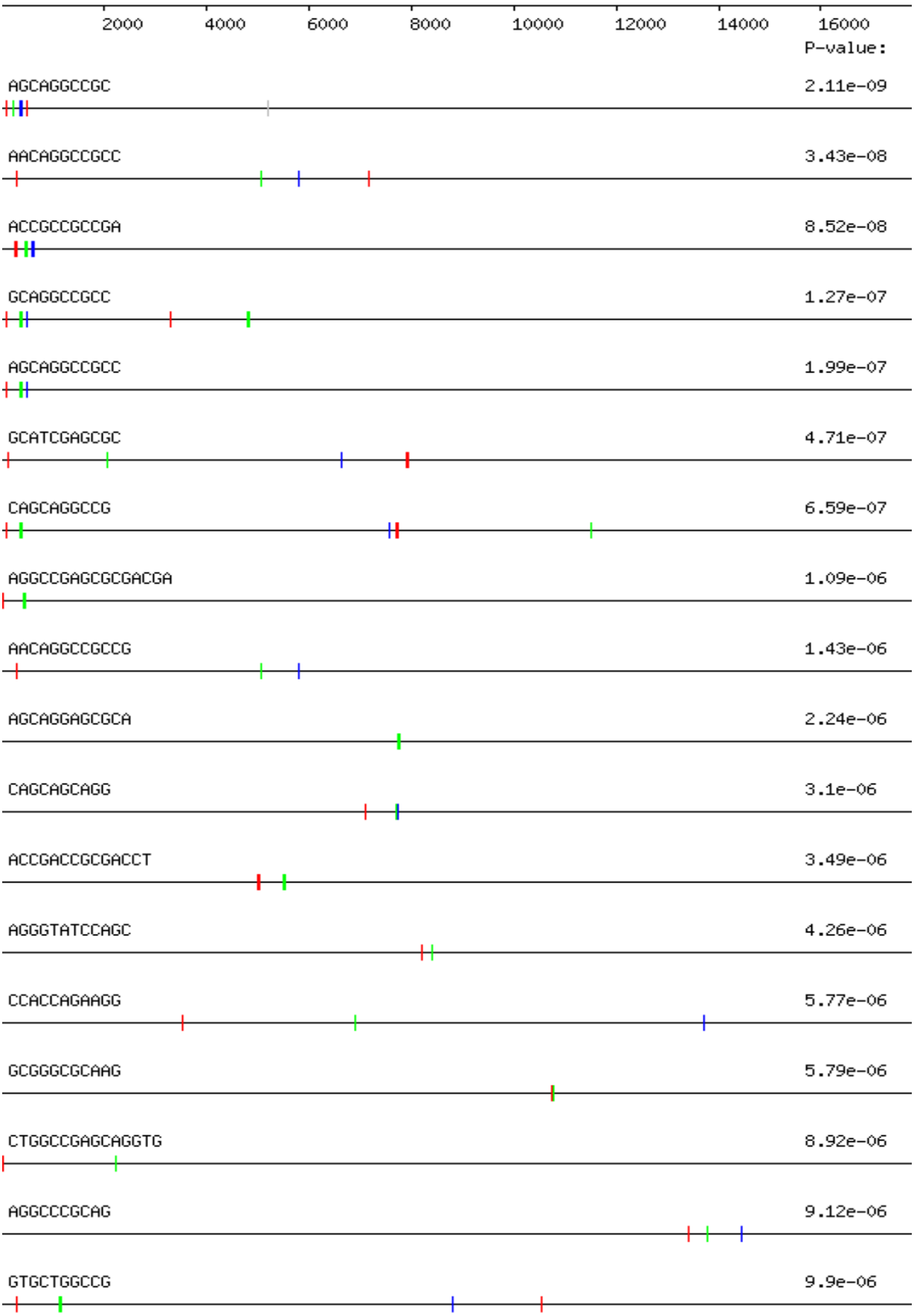


b. Lane 1 DNA marker III; Lane 2 DNA marker; Lane 3 SalI/SacI double digested pGEM5 vector; Lane 4-11 SalI/SacI double digested pF12 clones; Lane 12 SalI/SacI double digested pGEM5 vector; Lane 13 MseI digested pF12; Lane 14 DNA marker III

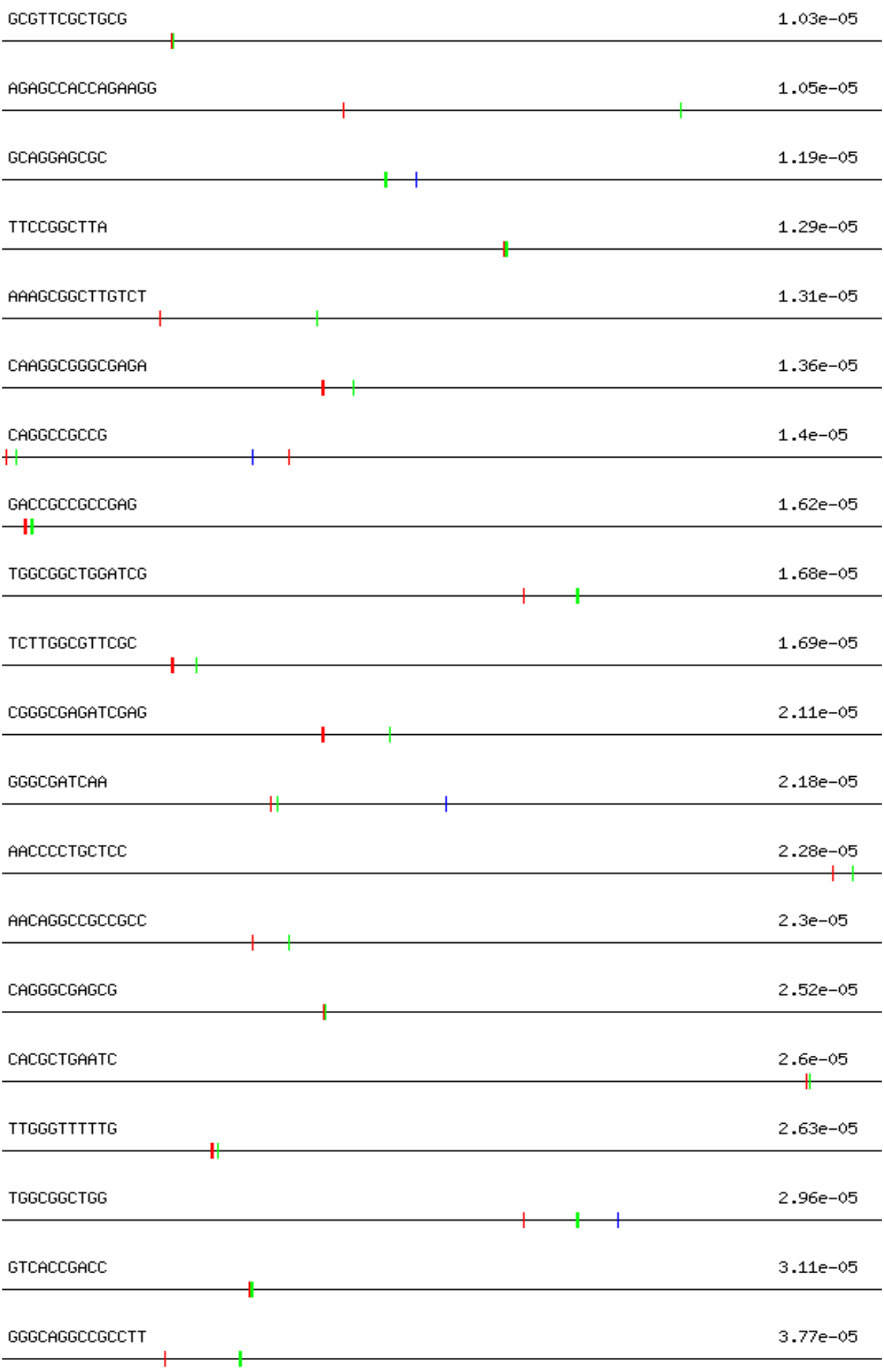
Appendix 7 A list of primers used for pF12 DNA sequencing

Primer	Sequence (5`-3`)	Primer	Sequence (5`-3`)
M13r	caggaaacagctatgacc	B3t	ggataaaccctgtggatgaagc
M13f	tgtaaaacgacggccagt	C1b	gcaagagcgcggcacgac
T7	taatacgactcactataggg	C8t	ggtttcccgagcgagagc
Sp6	atttaggtgacactatagaa	C8b	cgccaacaggatcagcagta
10t	cttgccagcgtagccatagagc	C4b	ccttgacgcccttggaacgac
13t	tggaggatgttttcagtggtggtg	C4t	gagcagcccggcaatcagc
9t	ccgctttatcaacccgctgcta	C10t	cgagcgccaaaagagttccatc
8t	accccgccagaggatgtagagg	C10b	ccgcgcagcaggttgagc
12b	tgacgcttccgagggctacttg	C6b	ctggattgtttgcccttgcttgat
12t	ccgaggccgccgaacttg	C3b	ggagggcttacgggttcg
1b	gcgcattgccggacagtga	D12t	atcgggcttgagggcaccat
1t	gccgtgcagccgactcct	D12b	gtgcttcctgctgctggctctc
3b	cgctgtaggcccgtttctgc	D16t	tggcgcttcctgatcgtttttc
4t	cgagcgattgccagattcacg	D16b	gagctttccgccgacgac
4b	gcaggggcagggcagtgtg	D17t	tcgcgcccgtaaaactccttc
5t	ggcagggctacgatgaaacacaa	D17b	tccccctccggctgaaaa
5b	ctcctgttggtcgttggcgttatt	E16t	gcgggatgacgtggttctgc
14b	gccttgtcggtggggatgg	E16b	gacccgctcgccctgctc
3t	gcagcgaagcgaggacacg	B6t	gctttccgccactcctcaa
B4b	acccagccagccgaacag	B6b	gacgcctccacgctttcac
B4t	cgttccagcgcgtctatca	B3b	cggctcggcaatggtcag
B7b	ctgcaatgggcggtcgta		

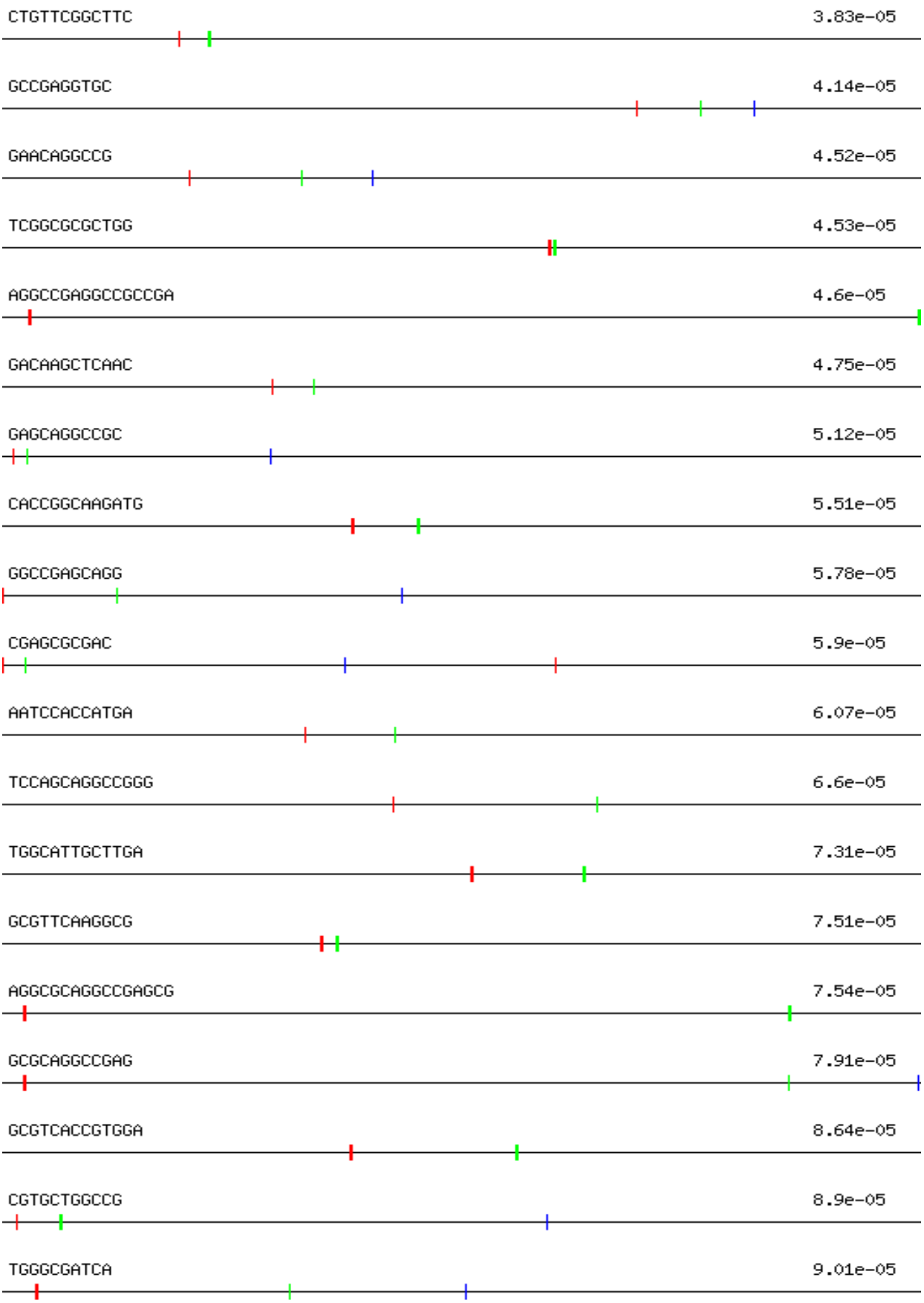
Appendix 8 Direct repeats found in plasmid pF12 DNA sequence



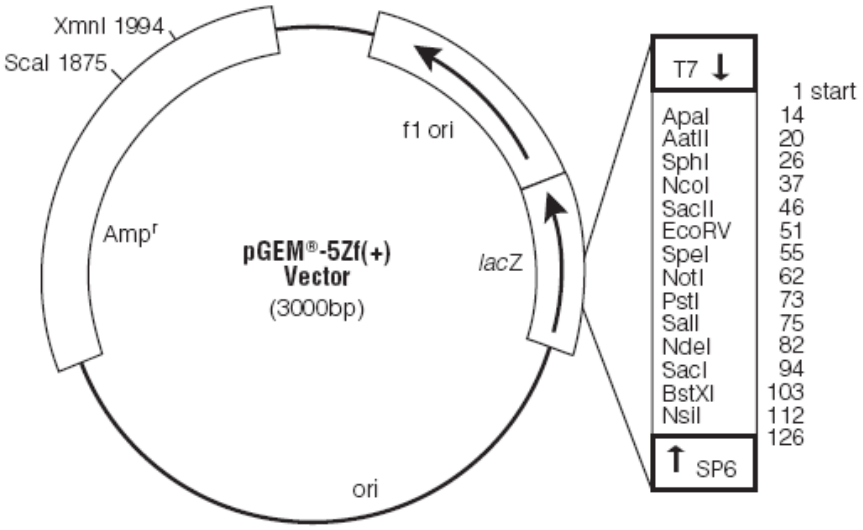
Appendix Direct repeats-Cont'd



Appendix Direct repeats-Cont’d


CTGTTCGGCTTC 3.83e-05
GCCGAGGTGC 4.14e-05
GAACAGGCCG 4.52e-05
TCGGCGCGCTGG 4.53e-05
AGGCCGAGGCCGCCGA 4.6e-05
GACAAGCTCAAC 4.75e-05
GAGCAGGCCGC 5.12e-05
CACCGGCAAGATG 5.51e-05
GGCCGAGCAGG 5.78e-05
CGAGCGCGAC 5.9e-05
AATCCACCATGA 6.07e-05
TCCAGCAGGCCGGG 6.6e-05
TGGCATTGCTTGA 7.31e-05
GCGTTCAAGGCG 7.51e-05
AGGCGCAGGCCGAGCG 7.54e-05
GCGCAGGCCGAG 7.91e-05
GCGTCACCGTGGA 8.64e-05
CGTGCTGGCCG 8.9e-05
TGGGCGATCA 9.01e-05

Appendix 9 pGEM®-5Zf(+) vector map (Promega, 2009, Product information sheet)



pGEM®-5Zf(+) vector reference points (Promega, 2009, Product information sheet)

T7 RNA Polymerase transcription initiation site	1
Multiple cloning region	10–113
SP6 RNA Polymerase promoter (–17 to –3)	124–143
SP6 RNA Polymerase transcription initiation site	126
Binding site of pUC/M13 Reverse Sequencing Primer	161–177
lacZ start codon	165
lac operon sequences	151–380; 2821–2981
lac operator	185–201
β-lactamase coding region	1322–2182
phage f1 region	2365–2820
binding site of pUC/M13 Forward Sequencing Primer	2941–2957
T7 RNA Polymerase promoter (–17 to –3)	2984–3

Appendix 10 Identification of isolates by 16S rDNA sequencing (blast search results)

a. F11

```
>dbj|AB305018.1|  Pseudomonas aeruginosa gene for 16S rRNA, partial sequence, strain:
PA1
Length=1458

 Score = 2300 bits (2550),  Expect = 0.0
 Identities = 1279/1281 (99%), Gaps = 1/1281 (0%)
 Strand=Plus/Plus

Query  1     TGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAAT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  81    TGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAAT  140

Query  61    ACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  141   ACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGC  200

Query  121   CTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201   CTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGG  260

Query  181   TCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  261   TCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA  320

Query  241   GCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  321   GCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAG  380

Query  301   AAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  381   AAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTG  440

Query  361   CTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  441   CTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAA  500

Query  421   TACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAG  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  501   TACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAG  560

Query  481   CAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  561   CAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTA  620

Query  541   GAGTACGGTAGAGGGTGGTGGAATTTCCTGTTGTAGCGGTGAAATGCGTAGATATAGGAA  600
             |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  621   GAGTACGGTAGAGGGTGGTGGAATTTCCTGT-GTAGCGGTGAAATGCGTAGATATAGGAA  679

Query  601   GGAACACCAGTGGCGAAGGCGACCACMTGGACTGATACTGACACTGAGGTGCGAAAGCGT  660
             |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  680   GGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGT  739

Query  661   GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCG  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  740   GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCG  799

Query  721   TTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTA  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  800   TTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTA  859

Query  781   CGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGT  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  860   CGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGT  919

Query  841   GGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCA  900
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Sbjct  920   GGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCA  979

Query  901   GAGATGGATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCG  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  980   GAGATGGATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCG  1039
```

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Query  961   TGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGC  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1040  TGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGC  1099

Query  1021  ACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1100  ACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC  1159

Query  1081  GTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAA  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1160  GTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAA  1219

Query  1141  GGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAG  1200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1220  GGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAG  1279

Query  1201  TCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGT  1260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1280  TCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGT  1339

Query  1261  GAATACGTTCCCGGGCCTTGT  1281
             |||||||||||||||||||||
Sbjct  1340  GAATACGTTCCCGGGCCTTGT  1360
```

b. F12

```
>emb|AM088480.1|  Pseudomonas citronellolis partial 16S rRNA gene, strain BG 6903
Length=1520

 Score = 2311 bits (2562),  Expect = 0.0
 Identities = 1286/1290 (99%), Gaps = 0/1290 (0%)
 Strand=Plus/Plus

Query  1     TAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTCCGAAAGGAGCGCTAATACCG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  98    TAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTCCGAAAGGAGCGCTAATACCG  157

Query  61    CATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  158   CATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAG  217

Query  121   GTCGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCGTAACTGGTCTG  180
             |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  218   GTCGGATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGCGACGATCCGTAACTGGTCTG  277

Query  181   AGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  278   AGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAG  337

Query  241   TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  338   TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGG  397

Query  301   TCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  398   TCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGT  457

Query  361   TTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACG  420
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Sbjct  458   TTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACG  517

Query  421   AAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGGTAAG  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  518   AAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGGTAAG  577

Query  481   ATGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCATAACTGCCTGACTAGAGT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  578   ATGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCATAACTGCCTGACTAGAGT  637

Query  541   ACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAAC  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  638   ACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAAC  697

Query  601   ACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGA  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  698   ACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGA  757

Query  661   GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGG  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  758   GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGG  817
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Query  721   ATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCC  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  818   ATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCC  877

Query  781   GCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  878   GCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT  937

Query  841   AATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCCGGAACCCTGCAGAGAT  900
             |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  938   AATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCCGGAATCCTGCAGAGAT  997

Query  901   GCGGGAGTGCCTTCGGGAATCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCG  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  998   GCGGGAGTGCCTTCGGGAATCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCG  1057

Query  961   TGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTT  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1058  TGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTT  1117

Query  1021  AAGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA  1080
             | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1118  ATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA  1177

Query  1081  AGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGT  1140
             ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1178  AGTCATCNTGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGT  1237

Query  1141  TGCCAAGCCGCGAGGTGGAGCTAATCCCAGAAAACCGATCGTAGTCCGGATCGCAGTCTG  1200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1238  TGCCAAGCCGCGAGGTGGAGCTAATCCCAGAAAACCGATCGTAGTCCGGATCGCAGTCTG  1297

Query  1201  CAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAAT  1260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1298  CAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAAT  1357

Query  1261  ACGTTCCCGGGCCTTGTACACACCGCCCGT  1290
             |||||||||||||||||||||||||||||
Sbjct  1358  ACGTTCCCGGGCCTTGTACACACCGCCCGT  1387
```

c. C2

```
>emb|AM088480.1|  Pseudomonas citronellolis partial 16S rRNA gene, strain BG 6903
Length=1520

 Score = 2241 bits (2484),  Expect = 0.0
 Identities = 1246/1250 (99%), Gaps = 0/1250 (0%)
 Strand=Plus/Minus

Query  1     CGTGACATTCTGATTCACGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1351  CGTGACATTCTGATTCACGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACT  1292

Query  61    GCGATCCGGACTACGATCGGTTTTCTGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1291  GCGATCCGGACTACGATCGGTTTTCTGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTC  1232

Query  121   TGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGT  180
             |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  1231  TGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCANGATGACTTGACGT  1172

Query  181   CATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCTTAACGTG  240
             |||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||
Sbjct  1171  CATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCATAACGTG  1112

Query  241   CTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCTCACGACAC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1111  CTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCTCACGACAC  1052

Query  301   GAGCTGACGACAGCCATGCAGCACCTGTGTTCCGATTCCCGAAGGCACTCCCGCATCTCT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1051  GAGCTGACGACAGCCATGCAGCACCTGTGTTCCGATTCCCGAAGGCACTCCCGCATCTCT  992

Query  361   GCAGGATTCCGGACATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  991   GCAGGATTCCGGACATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCA  932

Query  421   CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  931   CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGT  872
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Query  481   ACTCCCCAGGCGGTCGACTTATCGCGTWAGCTGCGCCACTAAGATCTCAAGGATCCCAAC  540
             ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  871   ACTCCCCAGGCGGTCGACTTATCGCGTTAGCTGCGCCACTAAGATCTCAAGGATCCCAAC  812

Query  541   GGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCA  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  811   GGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCA  752

Query  601   CGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCGCCACTGGTGTTCCT  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  751   CGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCGCCACTGGTGTTCCT  692

Query  661   TCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCCTCTACCGTACTCTA  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  691   TCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCCTCTACCGTACTCTA  632

Query  721   GTCAGGCAGTTATGGATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCATCTTACC  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  631   GTCAGGCAGTTATGGATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCATCTTACC  572

Query  781   AAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTTCGTATT  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  571   AAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTTCGTATT  512

Query  841   ACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTATTCTGTTGGTAACGTCAAAACAGCA  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  511   ACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTATTCTGTTGGTAACGTCAAAACAGCA  452

Query  901   AGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCT  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  451   AGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCT  392

Query  961   TCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTG  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  391   TCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTG  332

Query  1021  CCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGACTGATCATCCTCTCAGACC  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  331   CCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGACTGATCATCCTCTCAGACC  272

Query  1081  AGTTACGGATCGTCGCCTAGGTGAGCCATTACCCCACCTACTAGCTAATCCGACCTAGGC  1140
             |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  271   AGTTACGGATCGTCGCCTAGGTGAGCCTTTACCCCACCTACTAGCTAATCCGACCTAGGC  212

Query  1141  TCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCCGTAGGACGTATGCGGTAT  1200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  211   TCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCCGTAGGACGTATGCGGTAT  152

Query  1201  TAGCGCTCCTTTCGGAACGTTGTCCCCCCACTACCAGGCAGATTCCTAGGC  1250
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  151   TAGCGCTCCTTTCGGAACGTTGTCCCCCCACTACCAGGCAGATTCCTAGGC  102
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d. C6

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>dbj|AB021396.1|  Pseudomonas citronellolis DNA for 16S rRNA, strain ATCC 13674T
Length=1499

 Score = 2286 bits (2534),  Expect = 0.0
 Identities = 1276/1284 (99%), Gaps = 1/1284 (0%)
 Strand=Plus/Plus

Query  1     GCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTCCGAAAGGAGCGCTAATACCGCATA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  85    GCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTCCGAAAGGAGCGCTAATACCGCATA  144

Query  61    CGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  145   CGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCG  204

Query  121   GATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCGTAACTGGTCTGAGAG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  205   GATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCGTAACTGGTCTGAGAG  264

Query  181   GATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265   GATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG  324
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Query  241   GAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  325   GAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTT  384

Query  301   CGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  385   CGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTG  444

Query  361   ACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGG  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  445   ACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGG  504

Query  421   GTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGGTAAGATGG  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  505   GTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGGTAAGATGG  564

Query  481   ATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCATAACTGCCTGACTAGAGTACGG  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  565   ATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCATAACTGCCTGACTAGAGTACGG  624

Query  541   TAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCA  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  625   TAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCA  684

Query  601   GTGGCGAARGCGACCACCTGGACTGATACTGACACTGA-GTGCGAAAGCGTGGGGAGCAA  659
             |||||||| |||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  685   GTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAA  744

Query  660   ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCC  719
             |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  745   ACAGGATTAGATACCCTGGTNGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCC  804

Query  720   TTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAA  779
             ||||||||||||||||||   ||||||||||||||||||||||||||||||||||||||
Sbjct  805   TTGAGATCTTAGTGGCGCANNTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAA  864

Query  780   GGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT  839
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  865   GGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT  924

Query  840   CGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCCGGAATCCTGCAGAGATGCGG  899
             |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  925   CGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCCGGAATTCTGCAGAGATGCGG  984

Query  900   GAGTGCCTTCGGGAATCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAG  959
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  985   GAGTGCCTTCGGGAATCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAG  1044

Query  960   ATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTAAGG  1019
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  1045  ATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTATGG  1104

Query  1020  TGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTC  1079
             |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  1105  TGGGCACTCTAAGGAGACTGCCGGTNACAAACCGGAGGAAGGTGGGGATGACGTCAAGTC  1164

Query  1080  ATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCC  1139
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1165  ATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCC  1224

Query  1140  AAGCCGCGAGGTGGAGCTAATCCCAGAAAACCGATCGTAGTCCGGATCGCAGTCTGCAAC  1199
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1225  AAGCCGCGAGGTGGAGCTAATCCCAGAAAACCGATCGTAGTCCGGATCGCAGTCTGCAAC  1284

Query  1200  TCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGT  1259
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1285  TCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGT  1344

Query  1260  TCCCGGGCCTTGTACACACCGCCC  1283
             |||||||||||||||||||||||||
Sbjct  1345  TCCCGGGCCTTGTACACACCGCCC  1368
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Appendix 11 Alignment of cured strain DNA sequence with F12

a. Alignment of C2 sequence with F12 sequence

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 Score = 2209 bits (1196),  Expect = 0.0
 Identities = 1199/1201 (99%), Gaps = 0/1201 (0%)
 Strand=Plus/Minus

Query  1     CGTGACATTCTGATTCACGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1254  CGTGACATTCTGATTCACGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACT  1195

Query  61    GCGATCCGGACTACGATCGGTTTTCTGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1194  GCGATCCGGACTACGATCGGTTTTCTGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTC  1135

Query  121   TGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1134  TGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGT  1075

Query  181   CATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCTTAACGTG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1074  CATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCTTAACGTG  1015

Query  241   CTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCTCACGACAC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1014  CTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCTCACGACAC  955

Query  301   GAGCTGACGACAGCCATGCAGCACCTGTGTTCCGATTCCCGAAGGCACTCCCGCATCTCT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  954   GAGCTGACGACAGCCATGCAGCACCTGTGTTCCGATTCCCGAAGGCACTCCCGCATCTCT  895

Query  361   GCAGGATTCCGGACATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCA  420
             ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  894   GCAGGGTTCCGGACATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCA  835

Query  421   CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  834   CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGT  775

Query  481   ACTCCCCAGGCGGTCGACTTATCGCGTWAGCTGCGCCACTAAGATCTCAAGGATCCCAAC  540
             |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  774   ACTCCCCAGGCGGTCGACTTATCGCGTTAGCTGCGCCACTAAGATCTCAAGGATCCCAAC  715

Query  541   GGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCA  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  714   GGCTAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCA  655

Query  601   CGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCGCCACTGGTGTTCCT  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  654   CGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCGCCACTGGTGTTCCT  595

Query  661   TCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCCTCTACCGTACTCTA  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  594   TCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCCTCTACCGTACTCTA  535

Query  721   GTCAGGCAGTTATGGATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCATCTTACC  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  534   GTCAGGCAGTTATGGATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCATCTTACC  475

Query  781   AAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTTCGTATT  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  474   AAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTTCGTATT  415

Query  841   ACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTATTCTGTTGGTAACGTCAAAACAGCA  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  414   ACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTATTCTGTTGGTAACGTCAAAACAGCA  355

Query  901   AGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCT  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  354   AGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCT  295

Query  961   TCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTG  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  294   TCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTG  235
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Query  1021  CCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGACTGATCATCCTCTCAGACC  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  234   CCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGACTGATCATCCTCTCAGACC  175

Query  1081  AGTTACGGATCGTCGCCTAGGTGAGCCATTACCCCACCTACTAGCTAATCCGACCTAGGC  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  174   AGTTACGGATCGTCGCCTAGGTGAGCCATTACCCCACCTACTAGCTAATCCGACCTAGGC  115

Query  1141  TCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCCGTAGGACGTATGCGGTAT  1200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  114   TCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCCGTAGGACGTATGCGGTAT  55

Query  1201  T  1201
             |
Sbjct  54    T  54


 Score =  204 bits (110),  Expect = 5e-56
 Identities = 110/110 (100%), Gaps = 0/110 (0%)
 Strand=Plus/Minus

Query  1201  TCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCCGTAGGACGTATGCGGTAT  1260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  114   TCATCTGATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCCGTAGGACGTATGCGGTAT  55

Query  1261  TAGCGCTCCTTTCGGAACGTTGTCCCCCACTACCAGGCAGATTCCTAGGC  1310
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  54    TAGCGCTCCTTTCGGAACGTTGTCCCCCACTACCAGGCAGATTCCTAGGC  5
```

b. Alignment of C6 sequence with F12 sequence

```
>lcl|30707 F12
Length=1290

 Score = 2383 bits (1290),  Expect = 0.0
 Identities = 1290/1290 (100%), Gaps = 0/1290 (0%)
 Strand=Plus/Plus

Query  1     TAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTCCGAAAGGAGCGCTAATACCG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     TAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTCCGAAAGGAGCGCTAATACCG  60

Query  61    CATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    CATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAG  120

Query  121   GTCGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCGTAACTGGTCTG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   GTCGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCGTAACTGGTCTG  180

Query  181   AGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   AGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAG  240

Query  241   TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGG  300

Query  301   TCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   TCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGT  360

Query  361   TTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACG  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   TTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACG  420

Query  421   AAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGGTAAG  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   AAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGGTAAG  480

Query  481   ATGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCATAACTGCCTGACTAGAGT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   ATGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCATAACTGCCTGACTAGAGT  540
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Query  541   ACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAAC  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   ACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAAC  600

Query  601   ACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGA  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   ACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGA  660

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Query  841   AATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCCGGAACCCTGCAGAGAT  900
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Query  901   GCGGGAGTGCCTTCGGGAATCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCG  960
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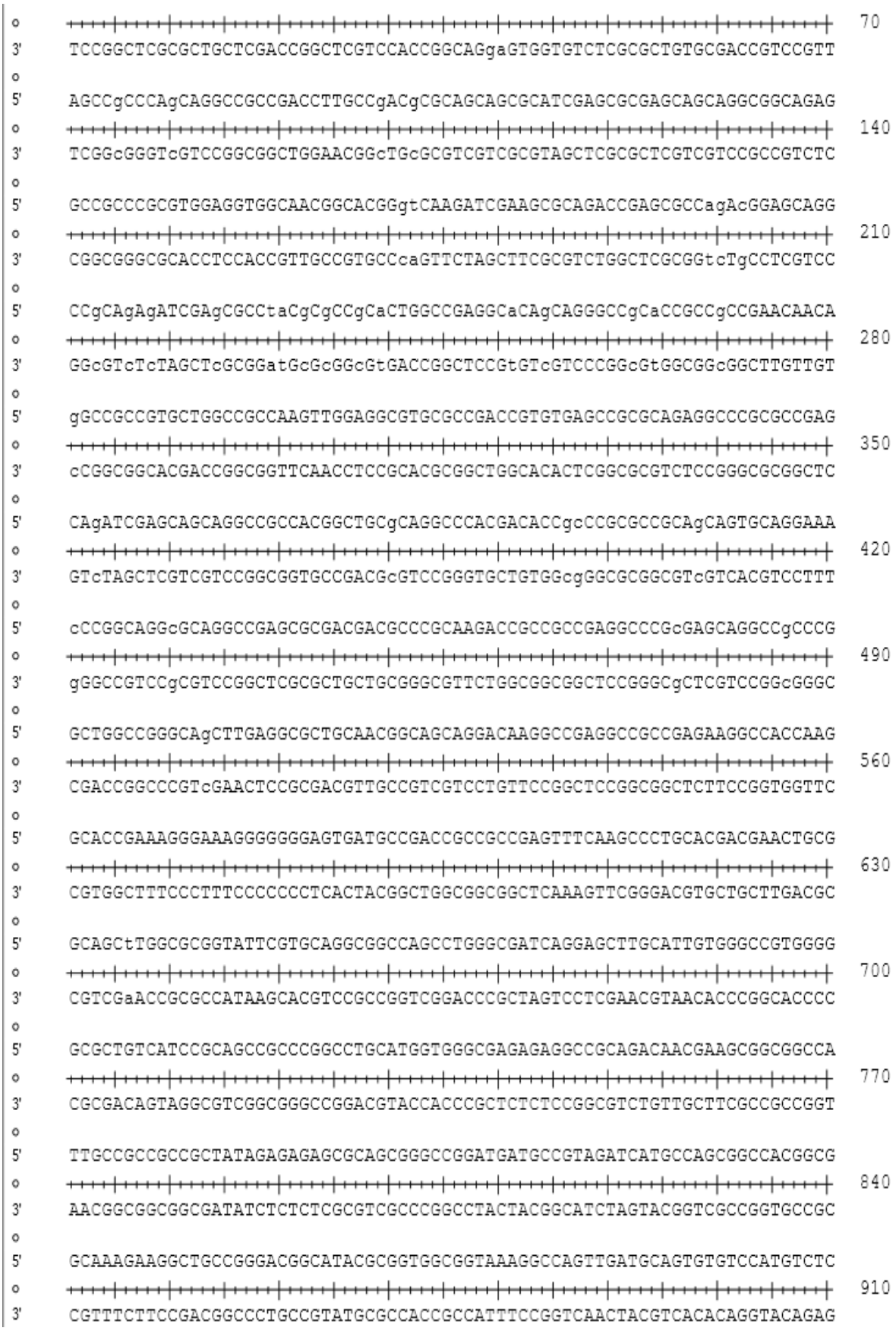
Appendix 12 Complete DNA sequence of pF12

Site summary:

"pF12" (1,17770)

Contig Length:	17770 bases
Average Length/Sequence:	542 bases
Total Sequence Length:	33634 bases
Top Strand:	36 sequences
Bottom Strand:	26 sequences
Total:	62 sequences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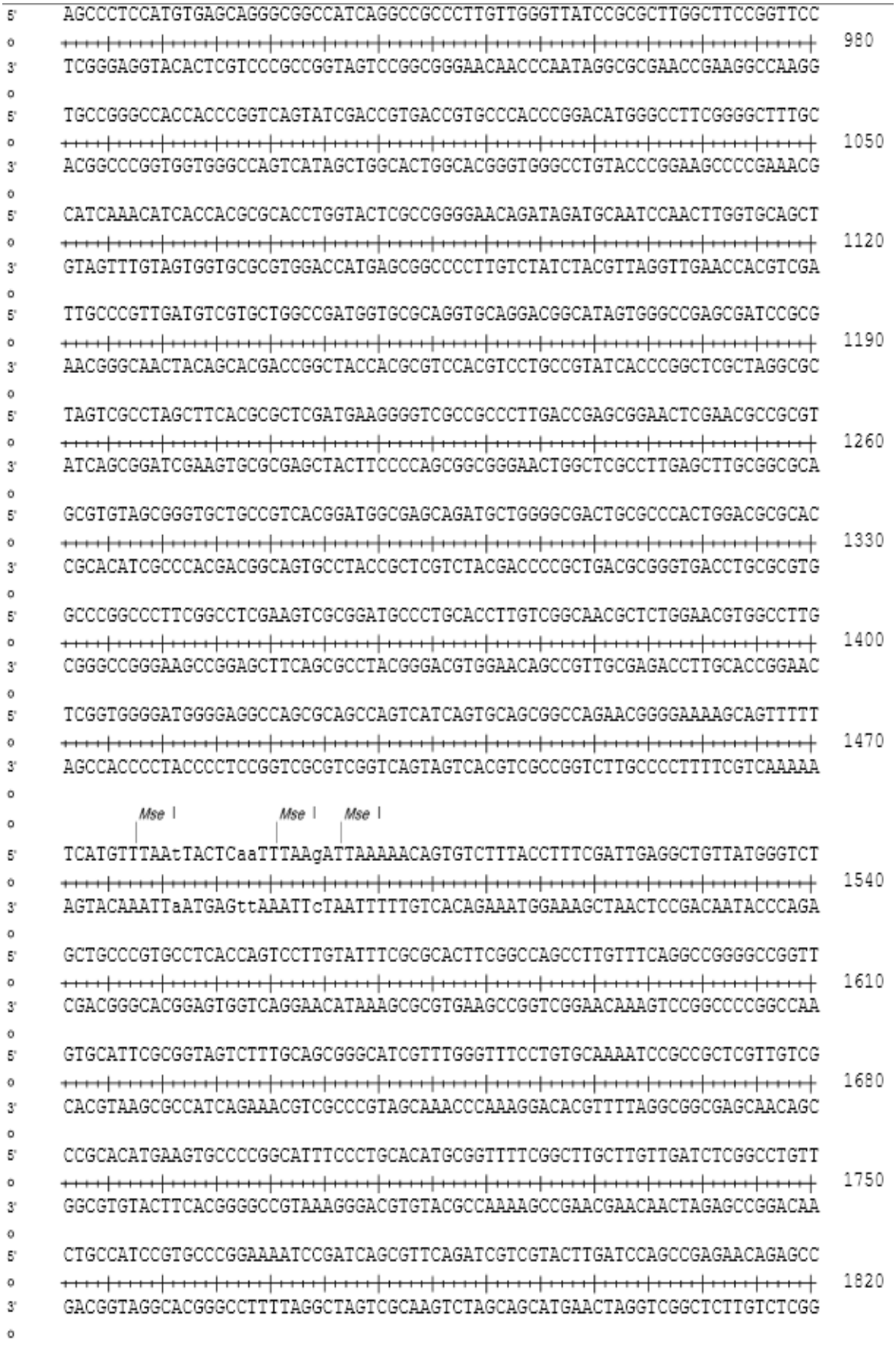
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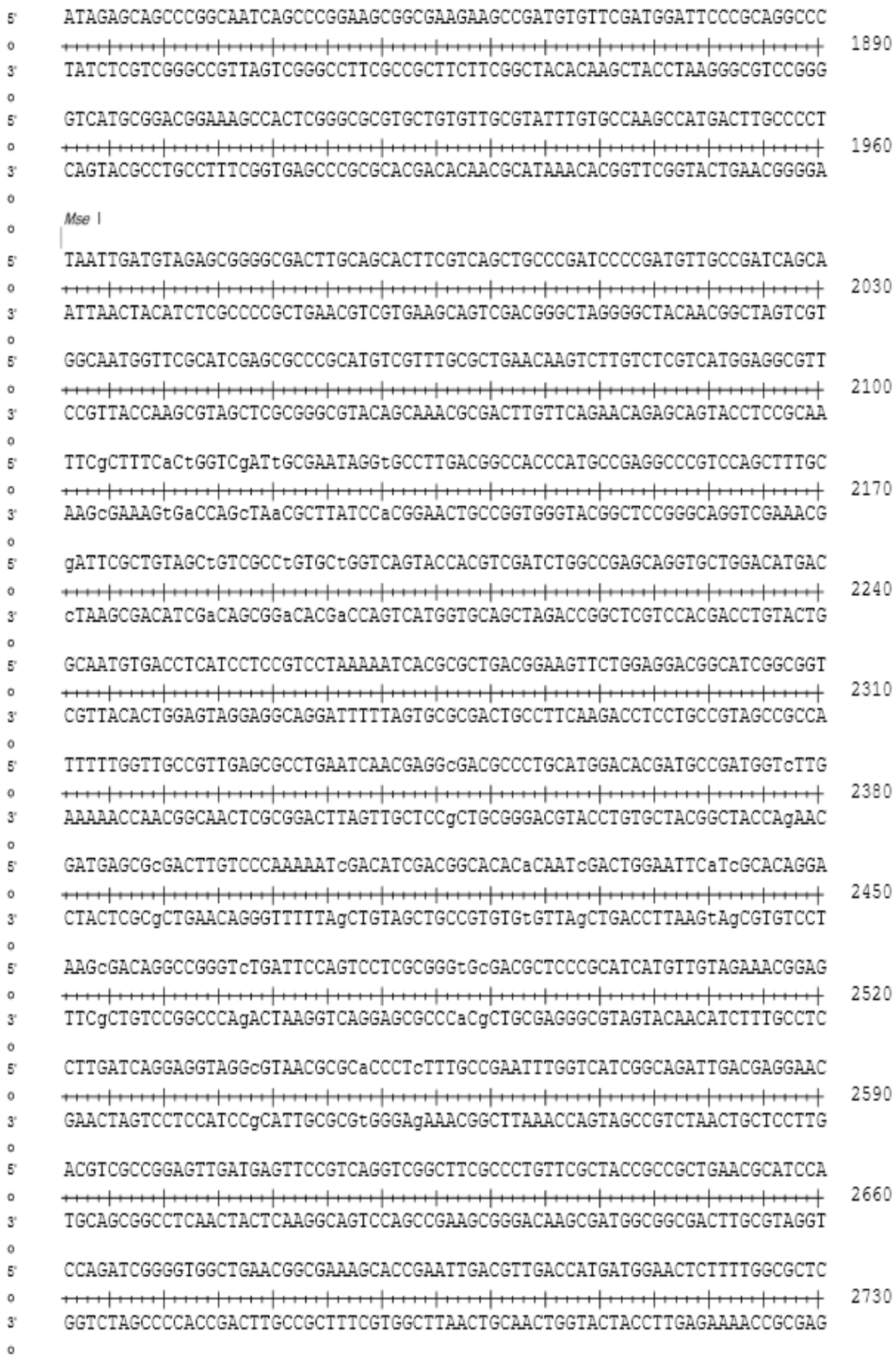
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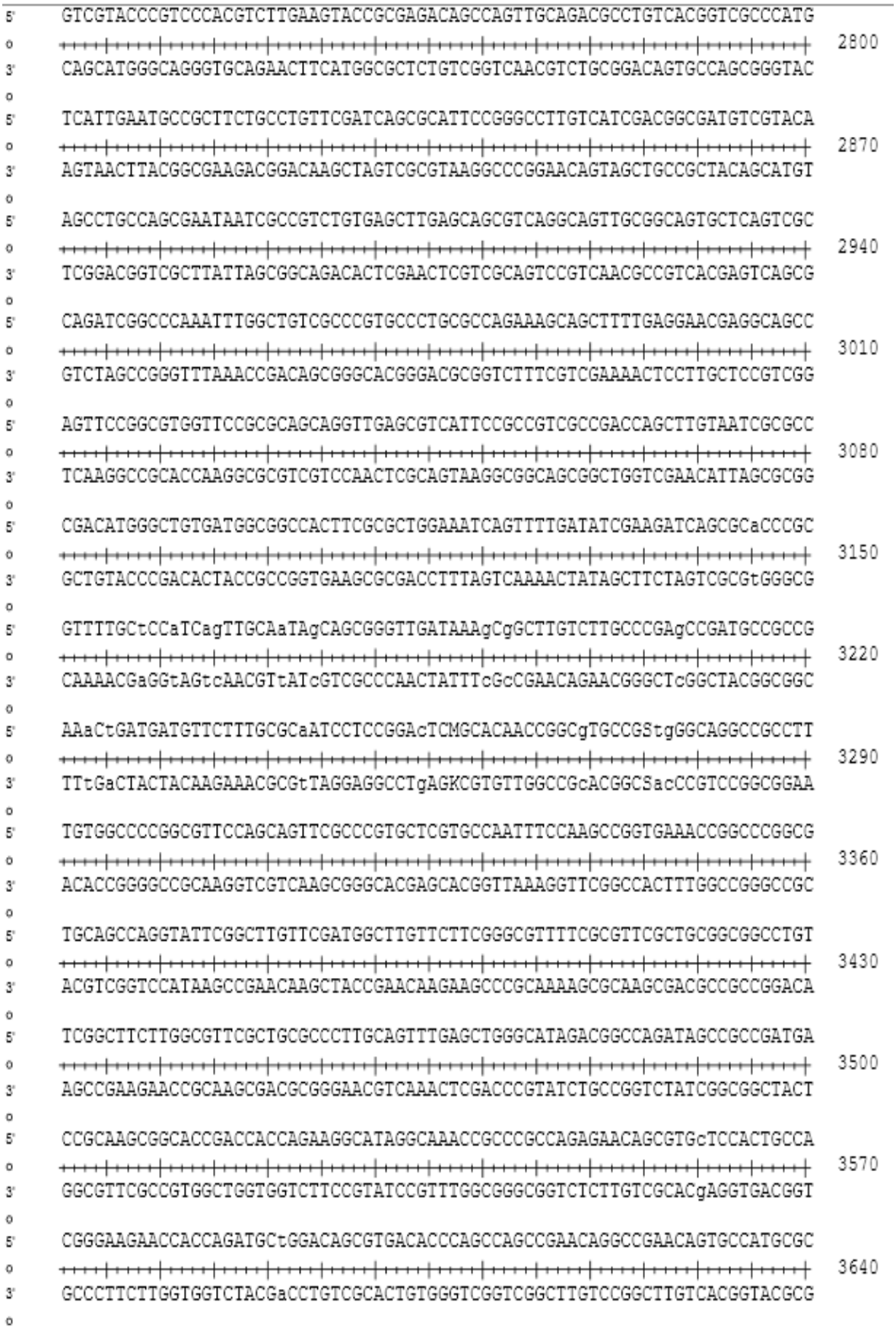
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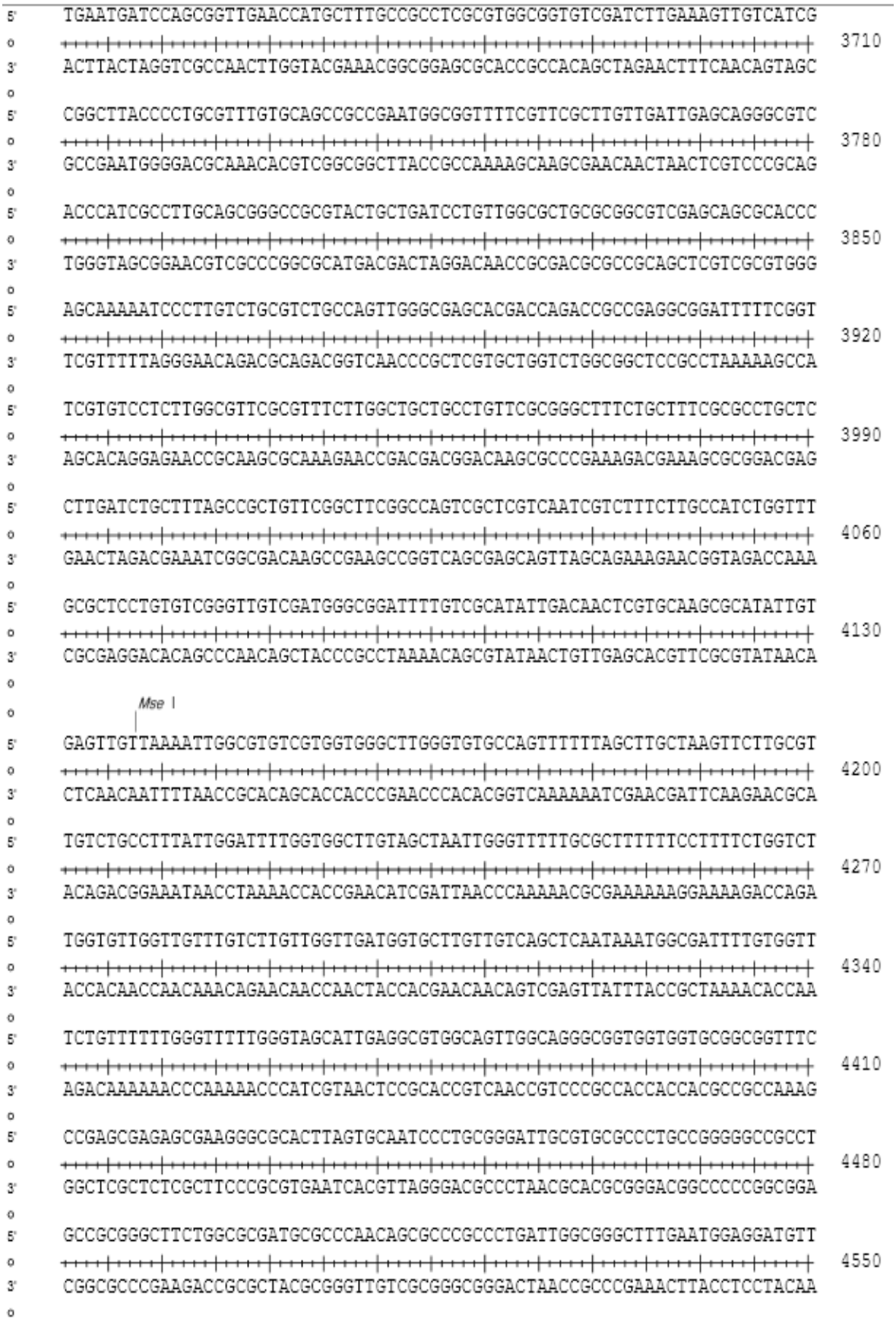
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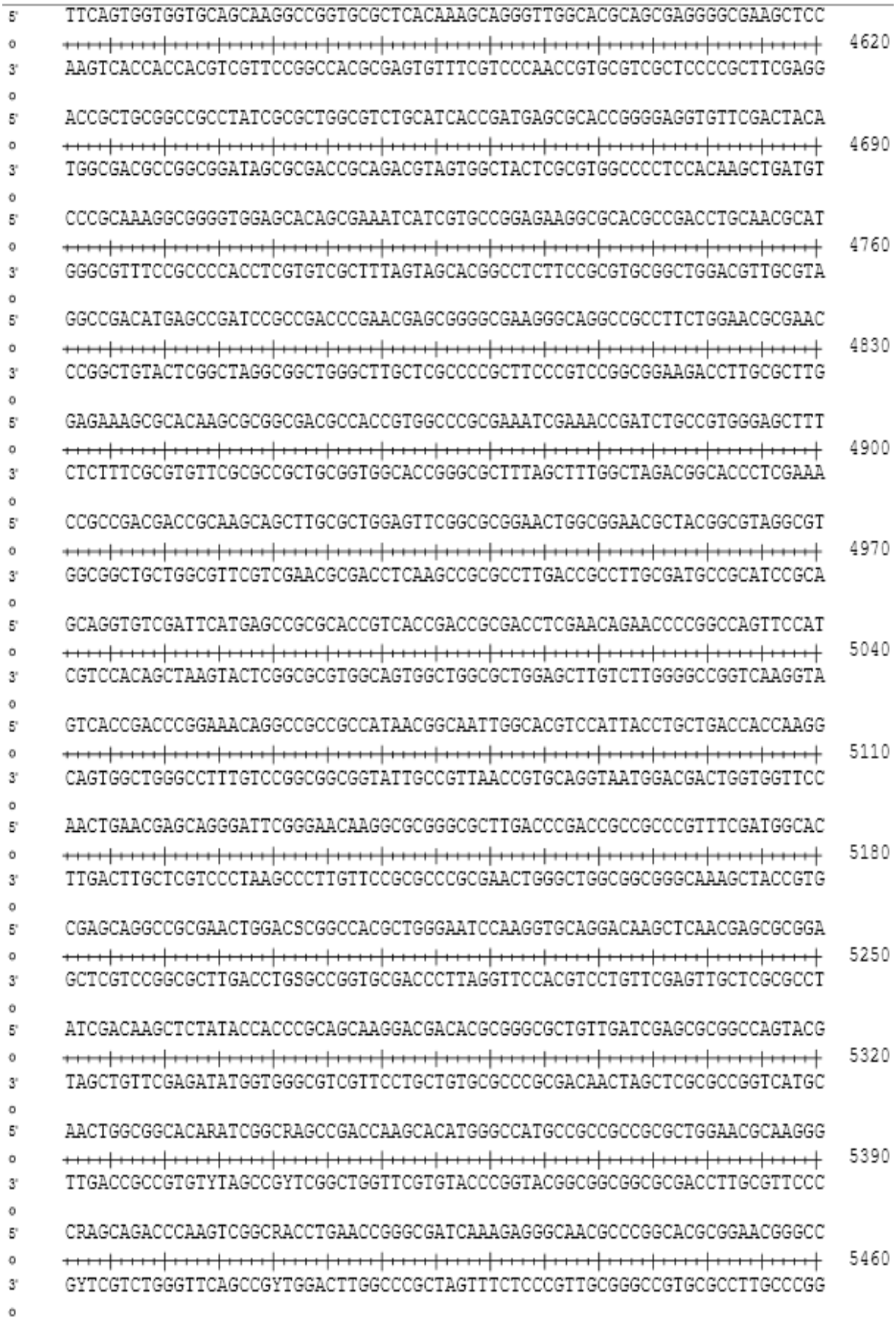
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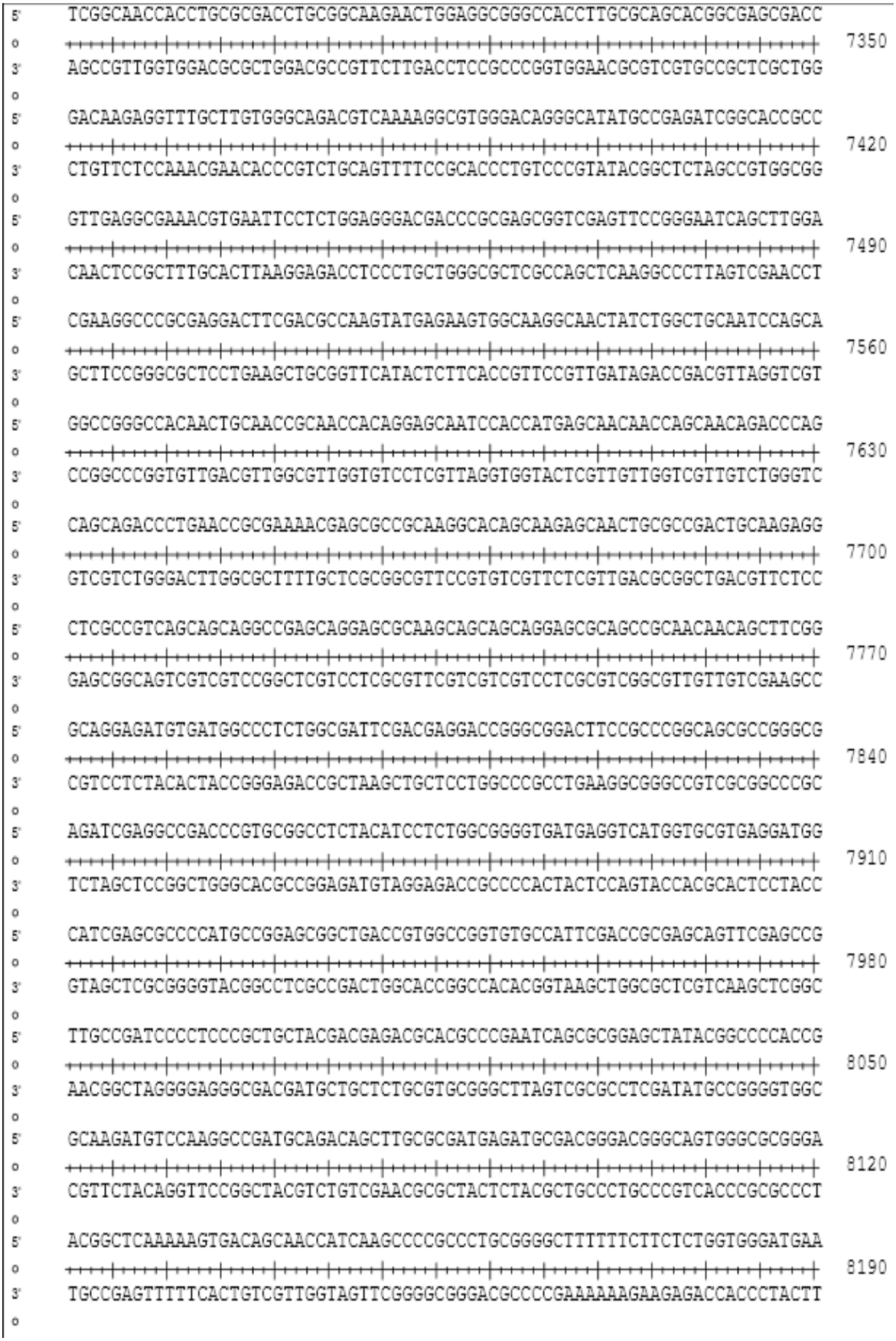
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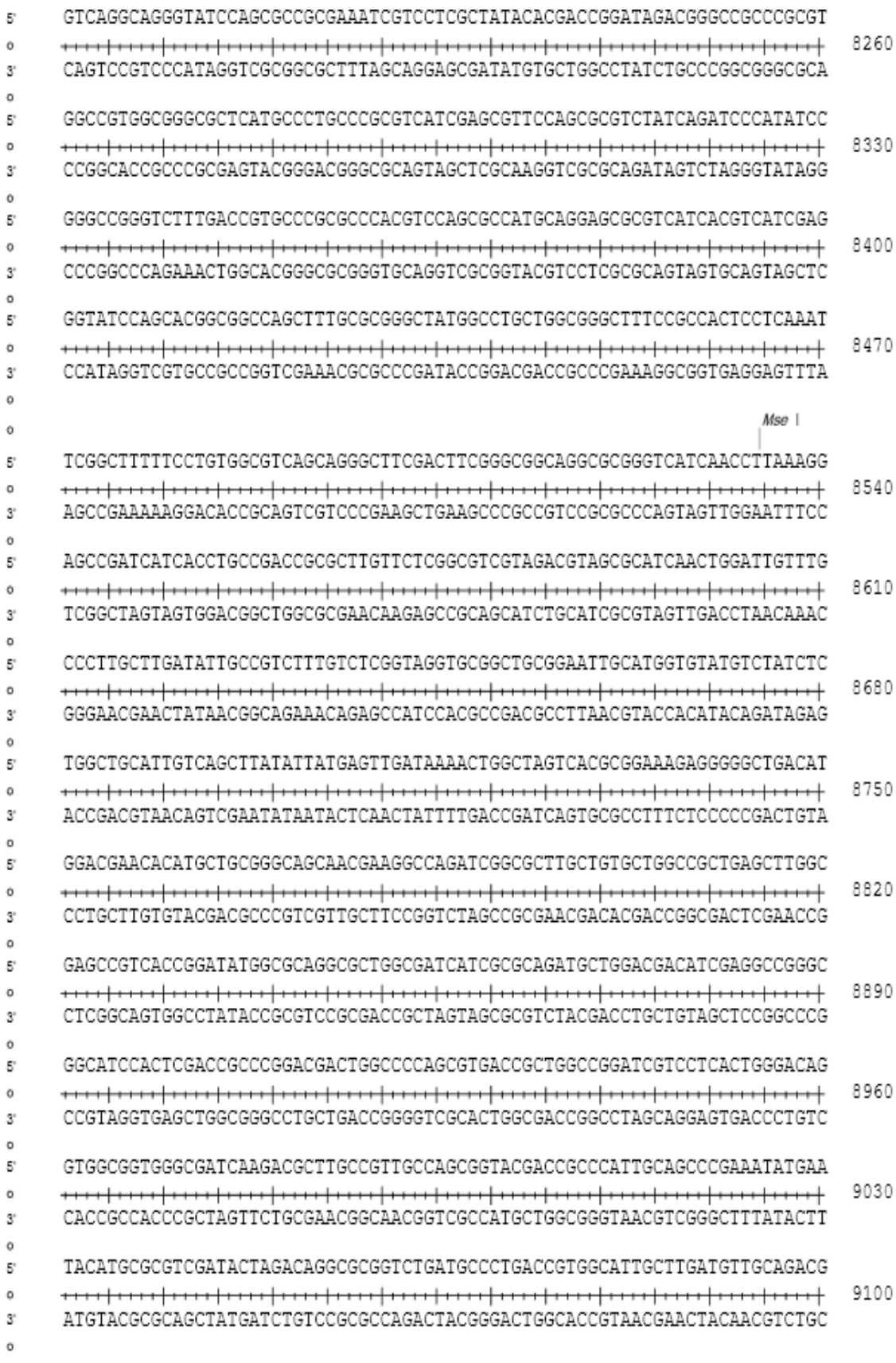
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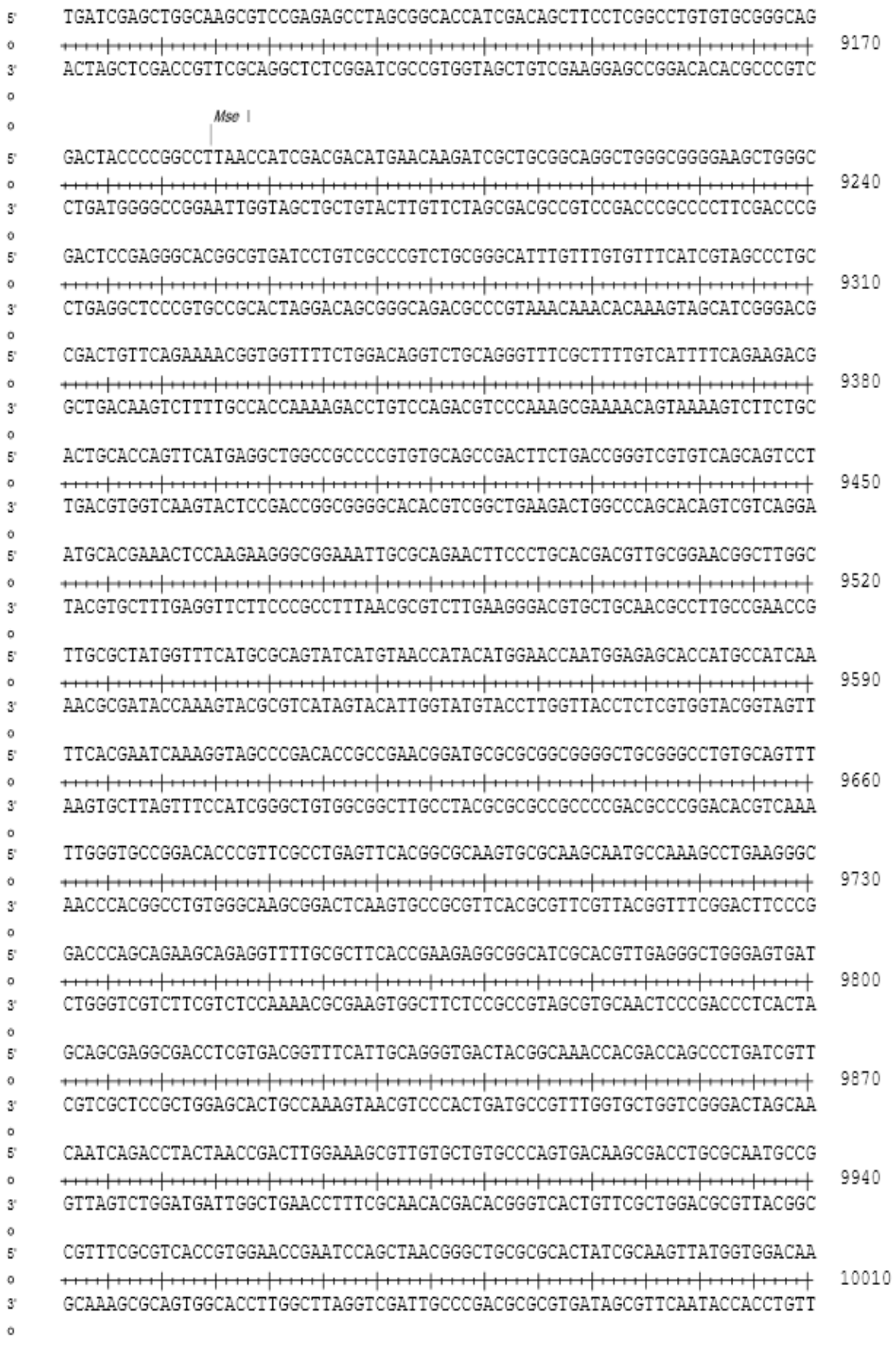
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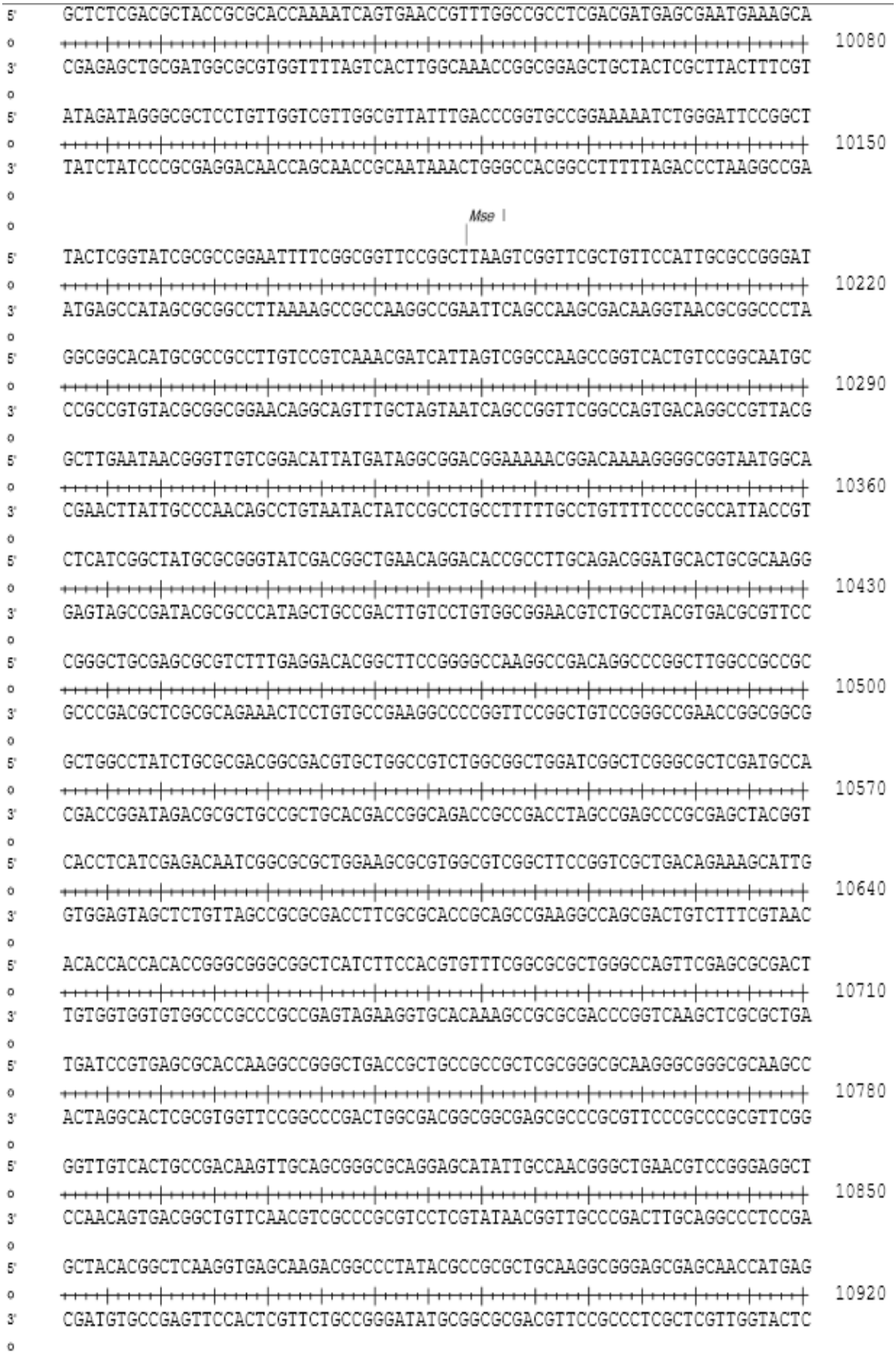
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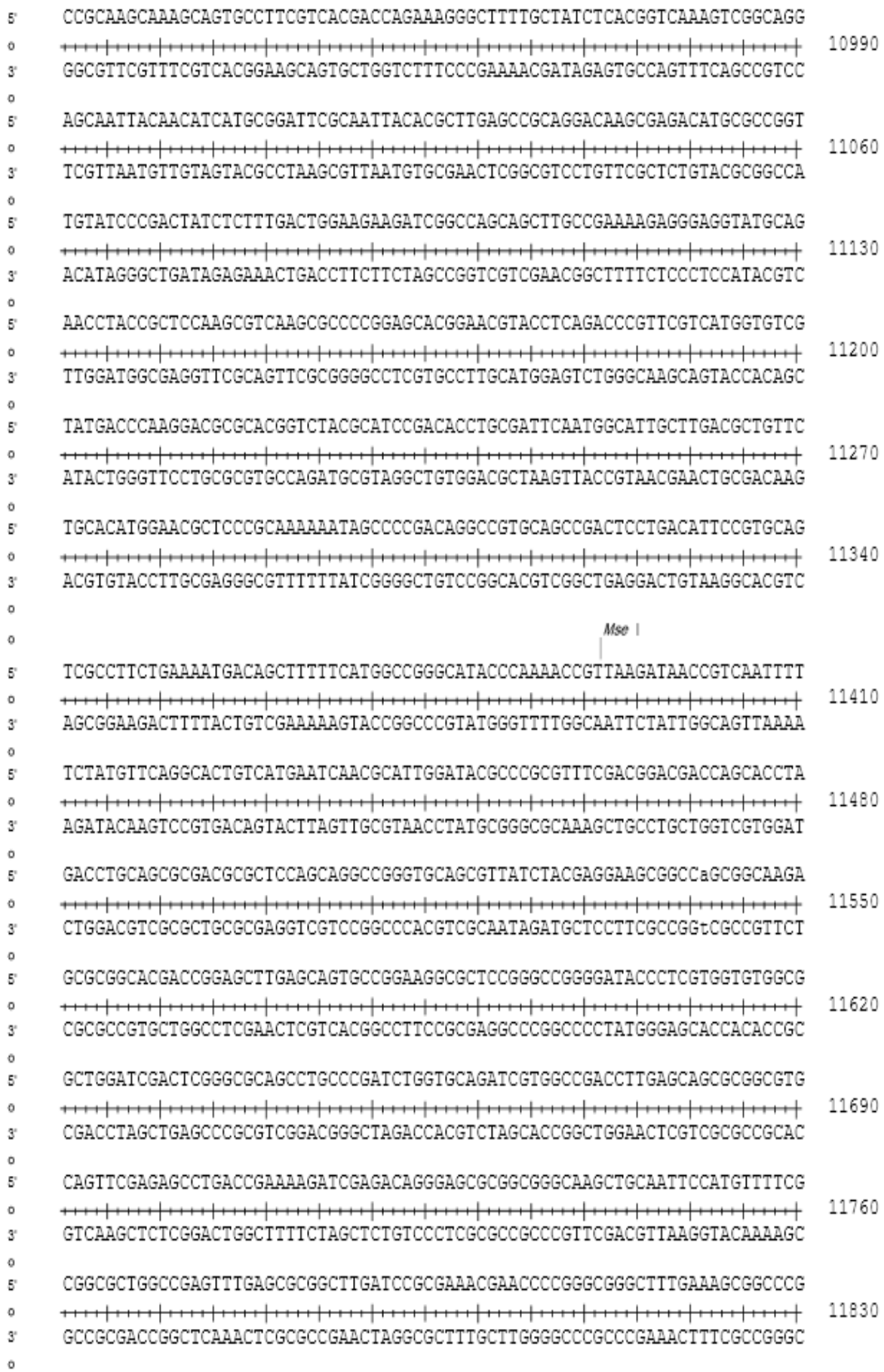
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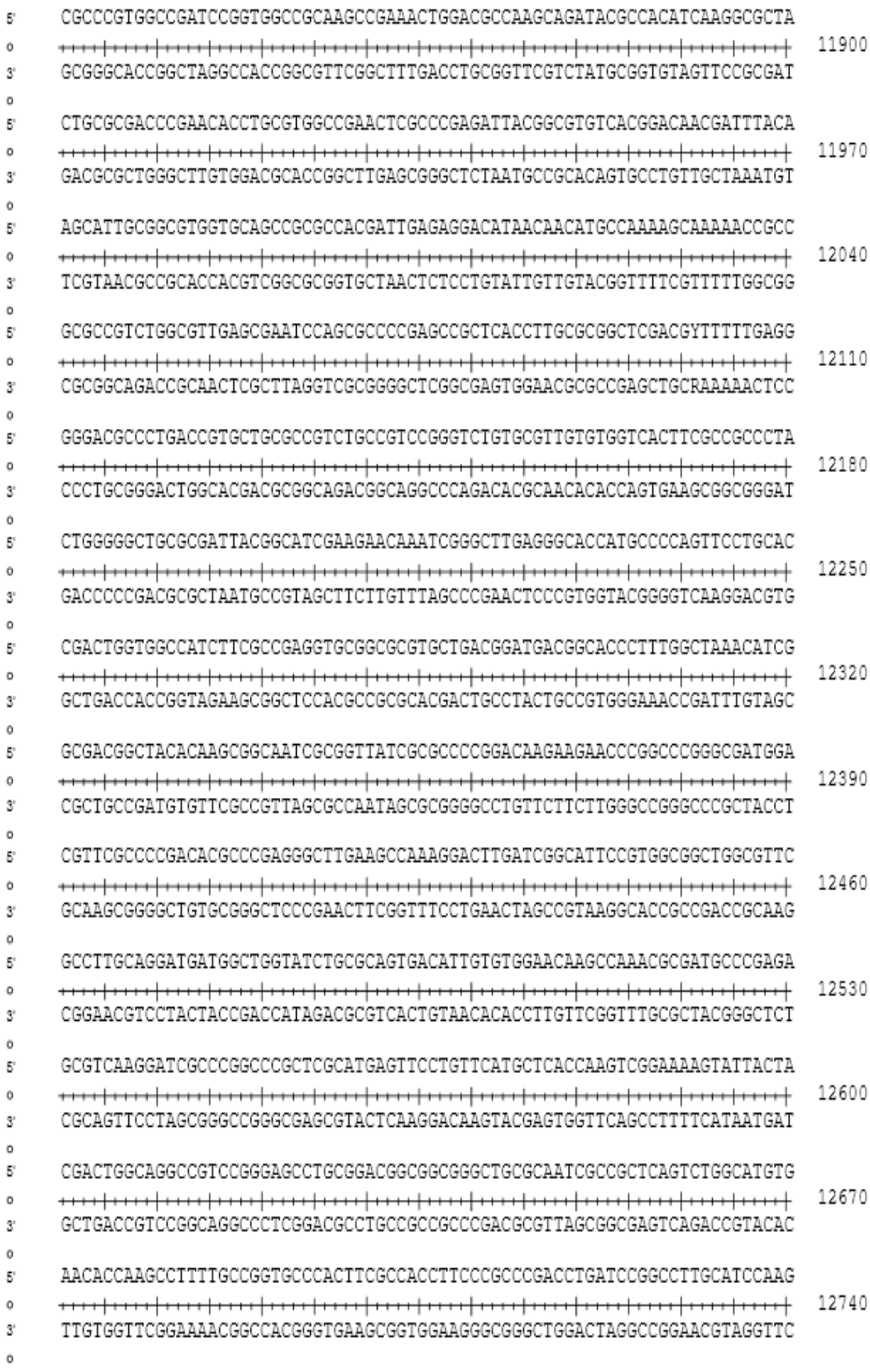
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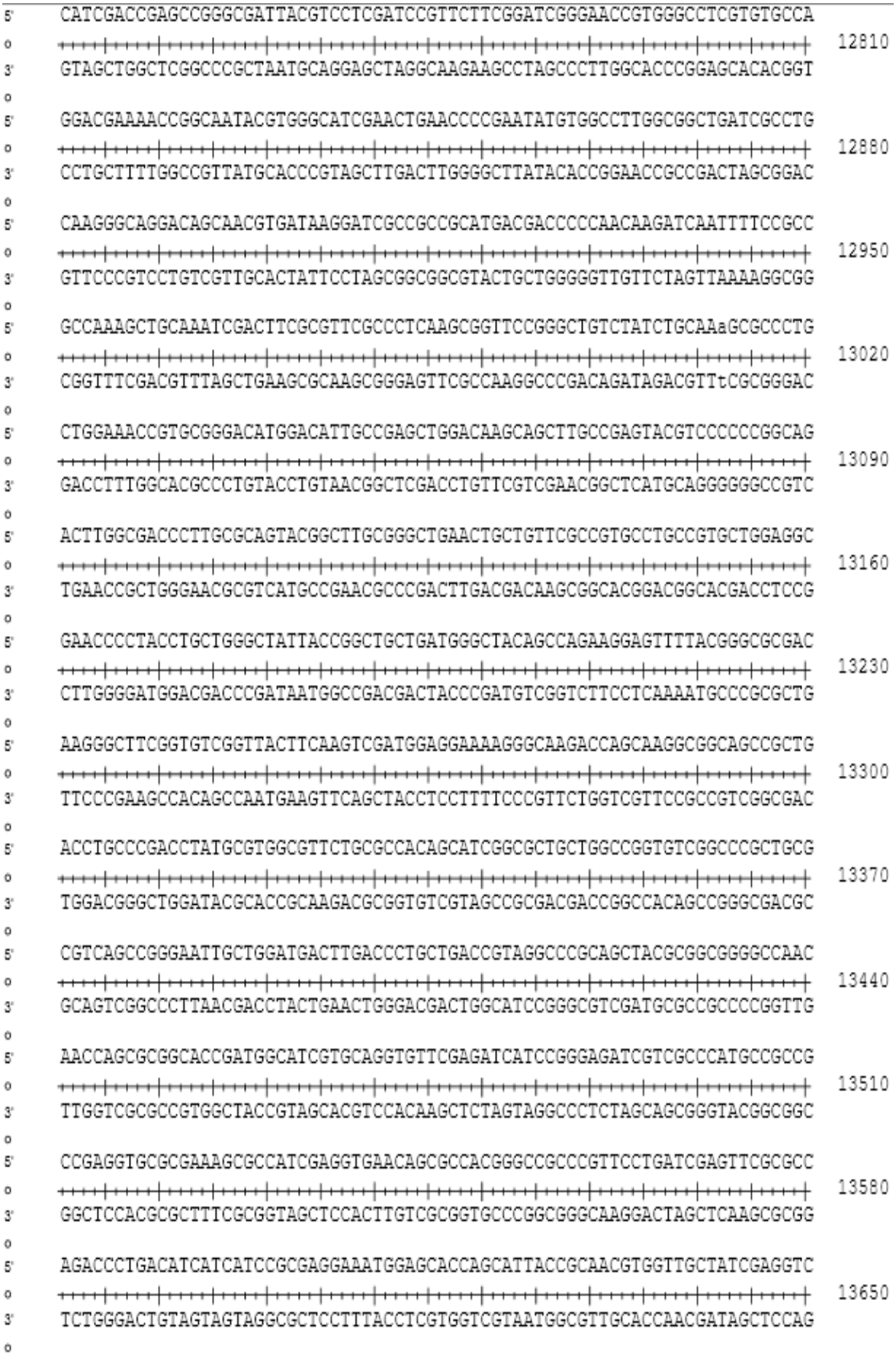
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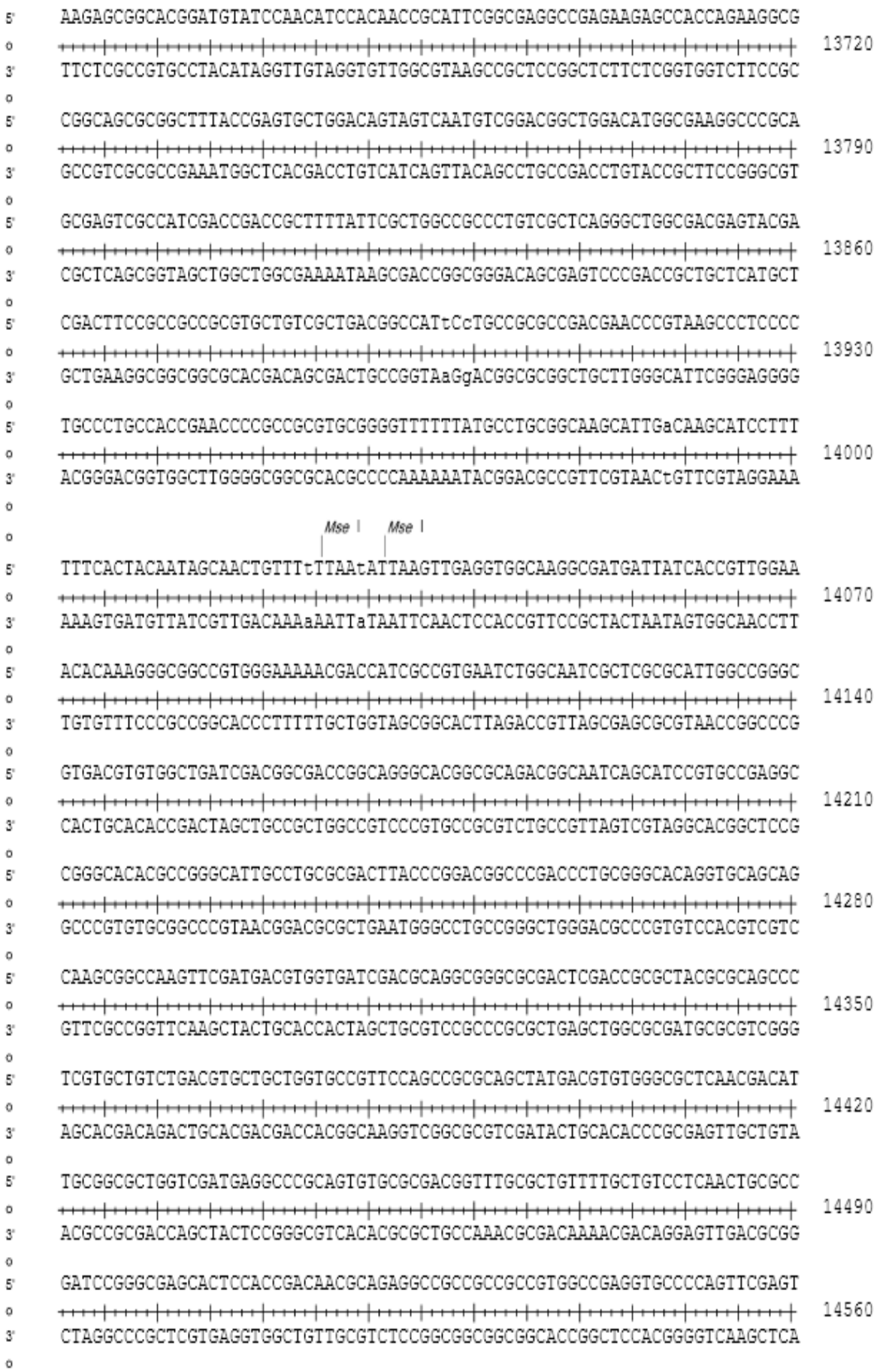
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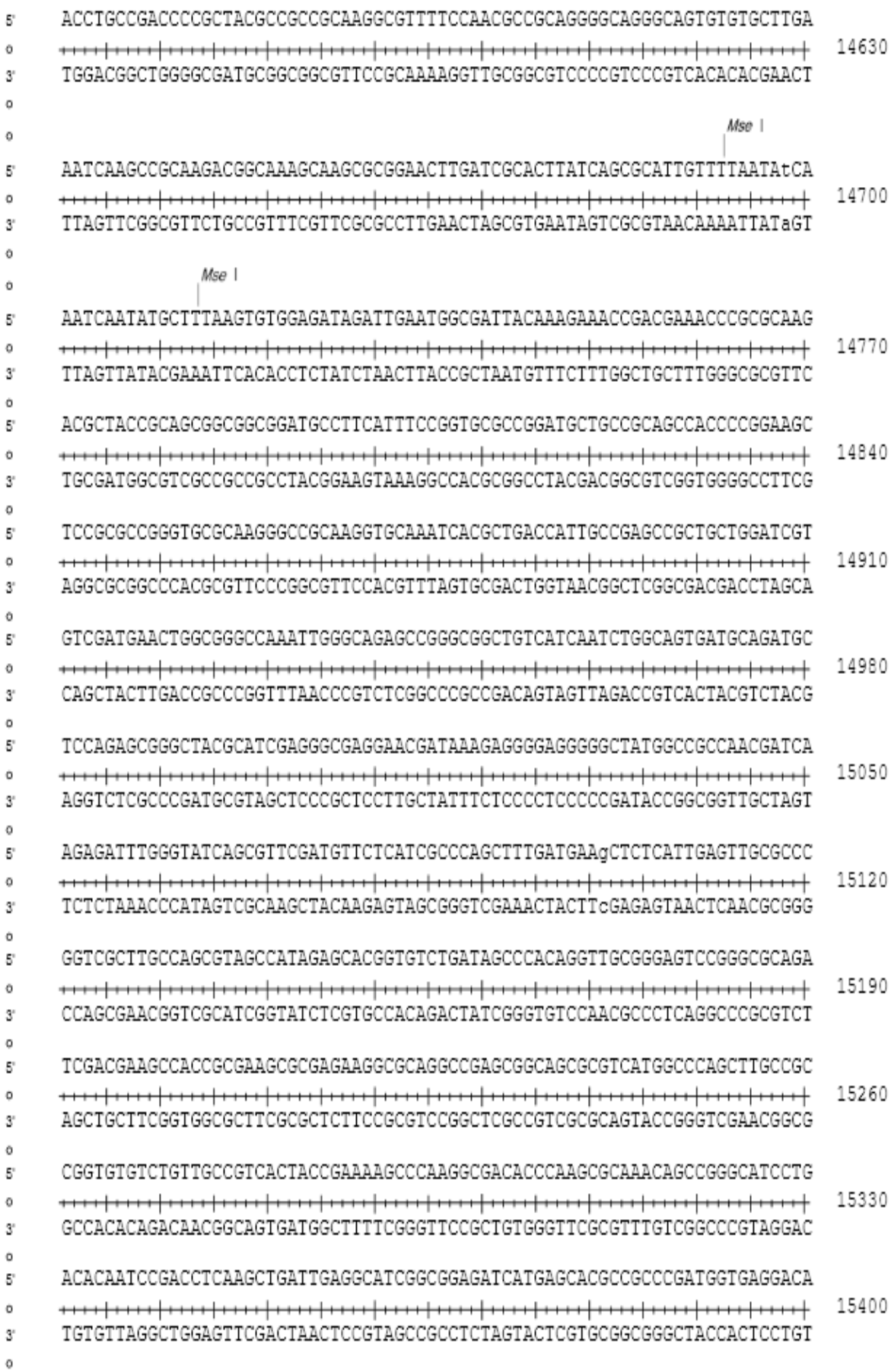
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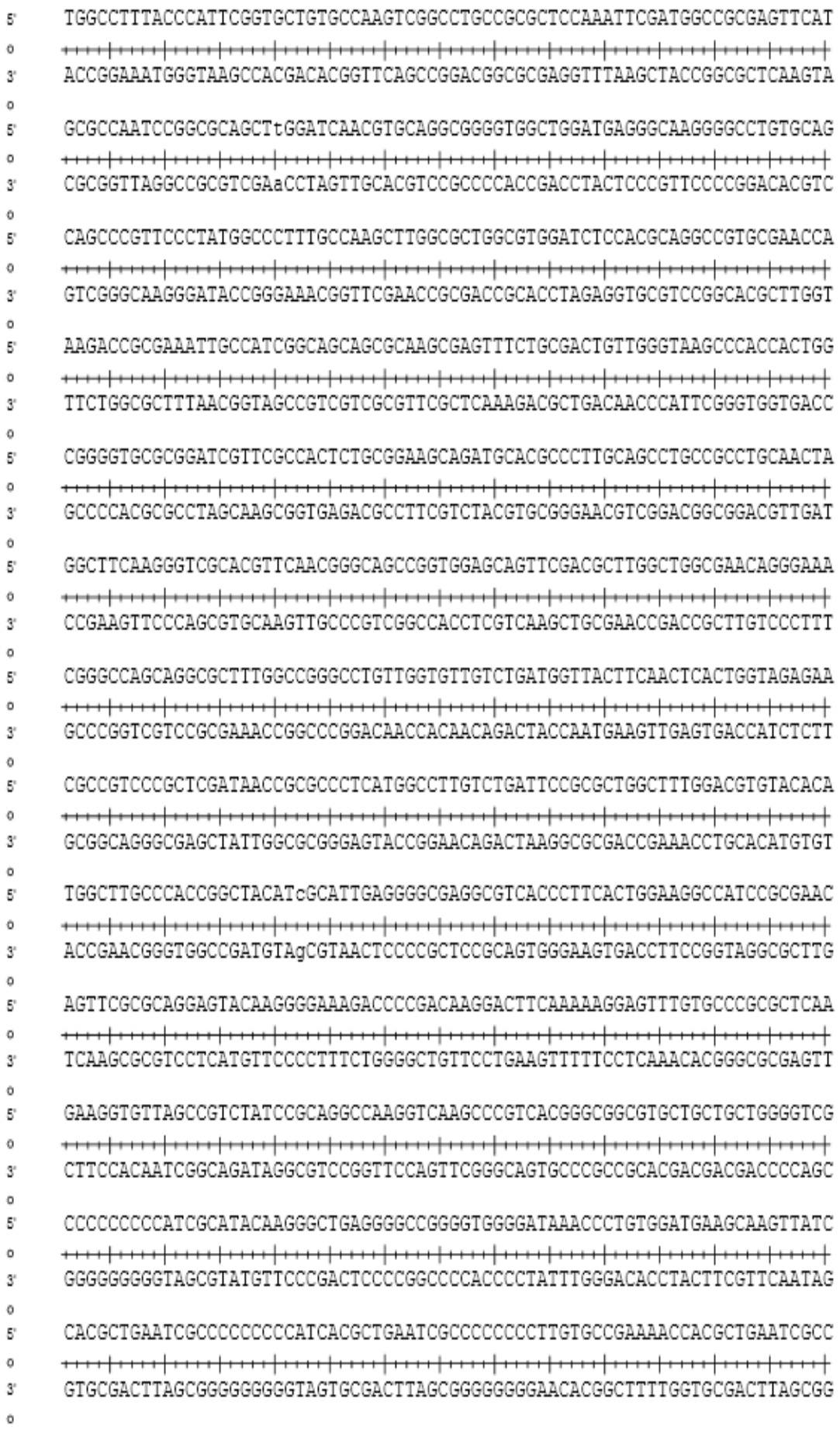
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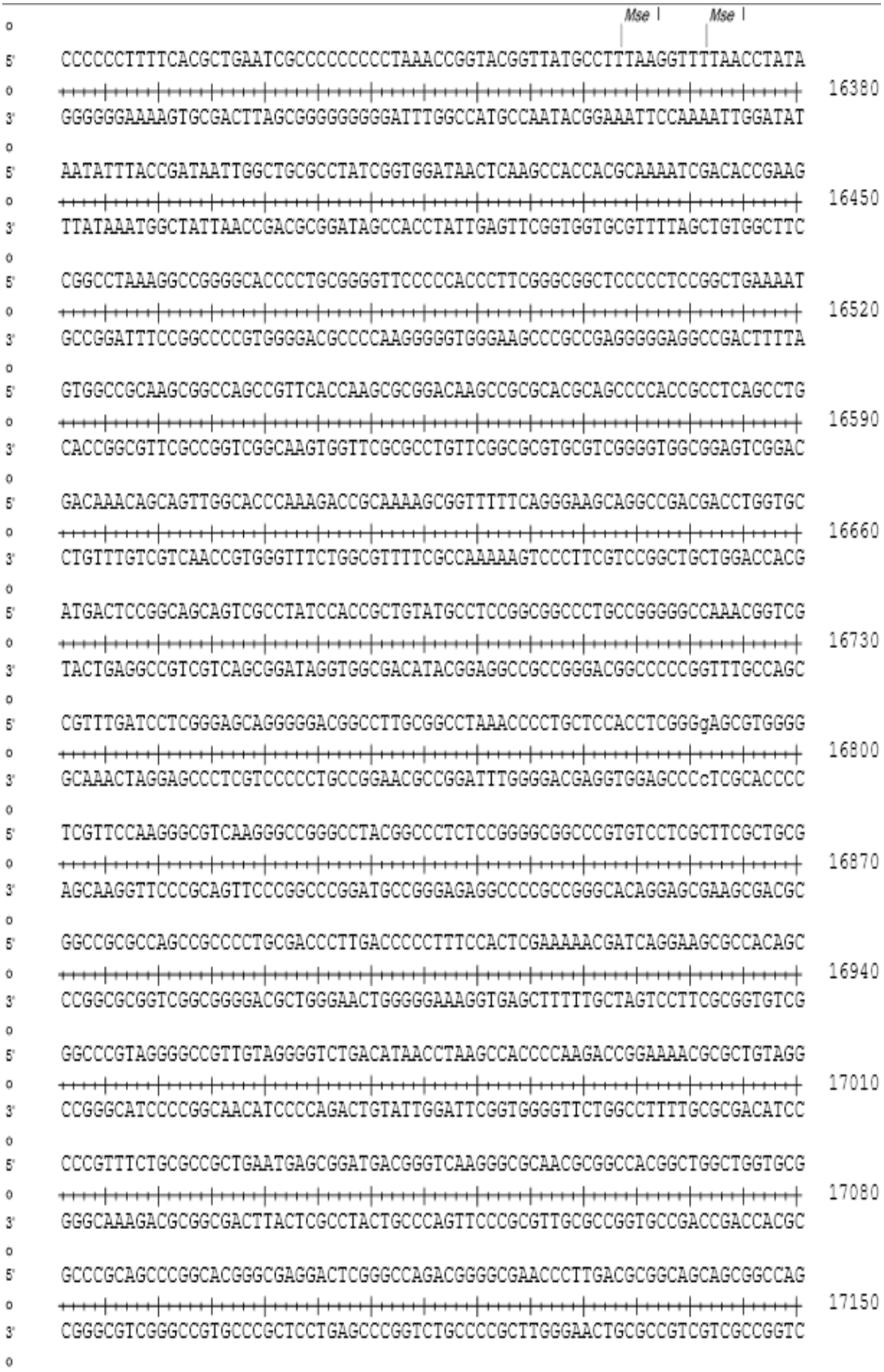
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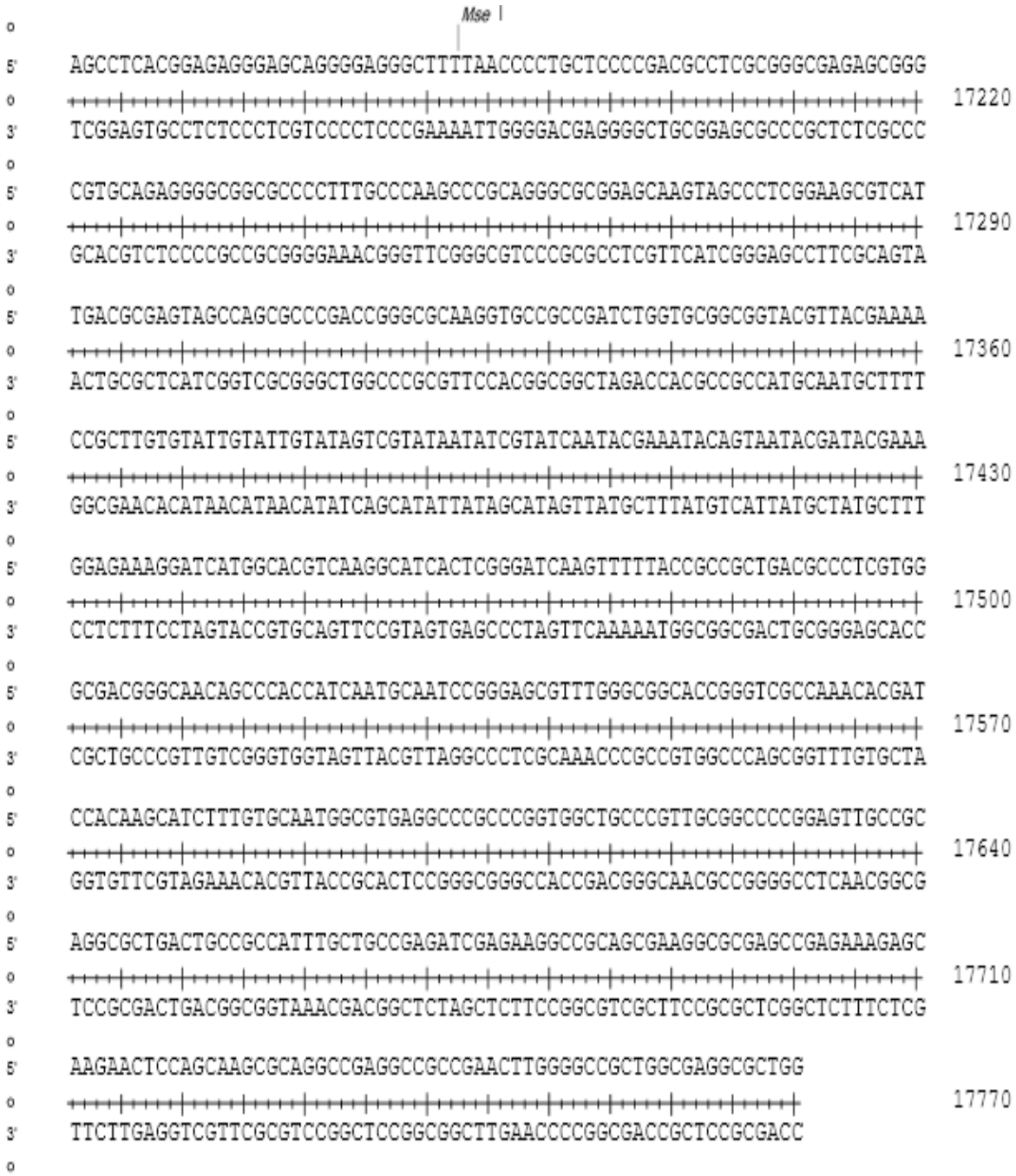
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