Role of ORF pCT0018 for Copper Homeostasis in *Listeria monocytogenes* strain DRDC8

Mei Mei Hii, B.Applied Science (Biotechnology) with Honours
University of Science Malaysia.

A thesis submitted for the Degree of Master of Science
School of Molecular and Biomedical Science
Faculty of Sciences,
The University of Adelaide
Adelaide, South Australia, Australia

(January, 2009)

Table of Contents

List of Figures

List of Tables

Abstract

Sequence analysis of part of a large plasmid carried by Australian environmental isolate of *Listeria monocytogenes* strain DRDC8 has lead to identification of an islet of genes that encode proteins similar to copper binding and transport genes found in other Gram positive bacteria. Comparative sequence analysis showed that there are at least four genes (pCT0017, pCT0018, pCT0019 and *ctpA*) on this islet predicted to be involved in copper homeostasis. One of these, *ctpA*, is predicted to encode a P-type ATPase with a function analogous to CopA, a copper transporting gene in *Enterococcus hirae*. ORF pCT0017 is likely to be a CopY-like regulatory protein which could control the expression of *ctpA*. ORF pCT0019 is predicted to be a Cu^{2+} binding protein. In addition, two genes located downstream of the *ctpA* are predicted to encode a two component regulatory system region. The predicted function of ORF pCT0018 is not clear. A related chromosomal gene (*cutR*) is predicted to also encode a copper transporting P-type ATPase.

To investigate the role of the protein encoded by pCT0018, the growth behavior of *L. monocytogenes* strain DRDC8, other strains carrying mutations within pCT0018, pCT0019, *cutR* and *ctpA*, as well as strains cured of the large plasmid, were grown under conditions of copper stress and starvation. The growth data showed that with the exception of strain DRDC8 and other strains carrying *ctpA*, most were unable to grow at higher copper concentration (> 15 mM $CuSO_4$) and suggested that the copper homeostasis genes located on the large plasmid are associated with tolerance to high levels of copper. Strain DSE955PL, which carries a *cutR* mutation and is cured of the large plasmid, was the most sensitive (<5 mM $CuSO_4$). This indicated that proteins encoded by plasmid genes work synergistically to confer tolerance to copper. Of most interest was the fact that a pCT0018 mutant was more sensitive (<15 mM $CuSO_4$) to high levels of copper than the wild type parent DRDC8 (<20 mM $CuSO_4$). This suggested that ORF pCT0018 was necessary for copper tolerance.

To investigate the effects of insertion mutations in pCT0017, pCT0018 and *ctpA* on copper uptake and export, the levels of copper accumulated by these strains was assessed using atomic absorption spectroscopy. A significant difference in copper accumulation among the bacteria strains was observed when either LEB or BHI media were used to

culture the bacteria. This data suggested that the growth medium chemicals influence the levels of copper accumulated by cells. However, the effect of these media on bacteria growth rates during copper stress was not significant. Atomic absorption analysis of intracellular copper accumulation suggested that DSE955PL and DSE955 (a chromosome mutant) were able to accumulate copper (80 - 110 $mg.g^{-1}$ dry weight of cells), whereas DRDC8 and strains carrying mutations in pCT0018, *ctpA*, and strains cured of the large plasmid, were less able to accumulate copper (30 - 70 $mg.g^{-1}$ dry weight of cells). This data suggested that *cutR* may encode a copper export system and that *ctpA* is involved in copper uptake.

To investigate the gene expression profile for pCT0018 under elevated copper, reverse transcriptase PCR was used to detect transcripts encoding pCT0017, pCT0018, pCT0019 and pCT0020 from RNA extracted from *L. monocytogenes* strain DRDC8 following culture at elevated levels of copper. Although transcripts for each of the target genes were detected, transcription was not responsive to copper, nor was the pattern of transcription consistent with that expected for a single operon.

To directly determine whether the protein encoded by the pCT0018 open reading frame was able to bind copper, this gene was cloned in pET15b in frame with an N-terminal His-tag and expressed in *E. coli*. The expressed protein was purified with a Ni-NTA column and shown to contain copper. Attempts to directly show that protein pCT0018 could bind copper by Cu-IMAC were unable to unequivocally show that the protein was immobilized on the column.

Purified protein was used to raise a polyclonal antiserum in rabbit and the antiserum was used for Western analysis to test expression of pCT0018 by wild type *L. monocytogenes* DRDC8 and specific gene mutants. Although the antiserum bound to purified protein, it was not possible to demonstrate binding to native pCT0018 in cell lysates prepared from *L. monocytogenes* DRDC8.

SDS-PAGE of cytoplasmic and cell envelope proteins isolated from *L. monocytogenes* strains was used to identify proteins expressed in response to copper stress and starvation. No significant differences in protein profiles for cytoplasmic protein were observed. However, copper-immobilized metal affinity chromatography (Cu-IMAC) showed that

expression of a number of copper binding proteins were differentially expressed by DRDC8 following growth in copper stress and starvation conditions. Three of these proteins were selected for amino sequence analysis by MALDI-TOFF MS. Two were confirmed to be *L. monocytogenes* non-heme iron-binding ferritin and a thiol peroxidase, both of which bind copper. The other protein was similar to an unknown protein from *L. monocytogenes*. Interestingly, no proteins directly implicated with the copper homeostasis islet were identified.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Mei Mei Hii and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, whendeposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Mei Mei Hii

January, 2009

Abbreviations

~	Approximately
°C	Degree Celsius
%	Percentage
µg; µl; µm	Microgram (s); Microliter (s); Micrometre (s)
aa	Amino acid
ActA	Actin A
AIDS	Acquired Immune deficiency syndrome
Amp	ampicillin
Ar	Argon
BHI	Brain Heart Infusion media
BLAST	Basic Local Alignment Search Tool
Bp	Base pairs
ca.	Circa
CAT	Catalase
CDC	Centre for Disease Control and Prevention
cm	Centimeters
CtpA	Copper Transporting P-type ATPase
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylene diamine tetra-acetic acid
EtBr	Ethidium bromide
FAE	Follicle-associated epithelium
FCA	Freund's complete adjuvant
GALT	Gut-associated lymphoid tissue
GPI-anchored protein	glycosyl-PI-anchored protein
h; min; sec	hour (s); minutes (s); seconds (s)
Hly	Haemolysin
HPKs	Histidine kinase

InlA	Internalin A
InlB	Internalin B
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kan	Kanamycin
kb	Kilobase pairs
kDa	Kilodaltons
L	Litres
LB	Luria-Bertani broth/agar
LEB	Listeria Enrichment Broth Base
LD_{50}	Median lethal dose
LLO	Listeriolysin
M; mM	Molar; Millimolar (s)
mA	Milli-amps
mg; mL; mm; μm	Milligram (s); Millilitre (s); Millimeter, Micrometre (s)
mpl	Metalloprotease
MQ	MilliQ grade pure water
mRNA	Messenger RNA
Nramp	Natural resistance-associated macrophage protein
NaAc	Sodium acetate
NEB	New England Biolabs
Ni-NTA	Nickel-nitrilotriacetic acid
nm	Nanometer
TEMED	N,N,N',N'-Di-(dimethylamino)ethane
nt	Nucleotide
OD_{600}	Optical density of 600 nm
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Phosphatidylinositol inositol
PrfA	Protein regulatory factor A
PI-PLC	Phosphatidylinositol phospholipase C
PlcB	Phosphatidylcholine-specific phospholipase C

R	Resistance
RO	Reverse osmosis
rRNA	Ribosomal ribonucleic acid
RNA	Ribonucleic acid
RNase	Ribonuclease
RR	Response regulator
RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TTBS	Tween Tris buffered saline
U	units
USA	United State Amerika
UV	Ultraviolet
v/v	volume per volume
V	Volts
w/v	weight per volume
X-Gal	5’-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Acknowledgements

I would firstly want to thank my supervisor, Dr. Connor Thomas for his patience, understanding and guidance during my studies in two years. Under his guidance, I learnt how to be a good microbiology scientist.

Secondly, I would like to thank the Sarawak Government of Malaysia for giving me the opportunity to pursue my study in Australia. I also would like to thank my company, Sarawak Biodiversity Centre (SBC) for choosing me and giving me the chance to further my study. I thank them for their understanding and support all these years.

To Mr Graham Bull, Chemistry School and Miss Lynn Waterhouse, Adelaide Microscopy Centre, for their patience and advice in helping to solve my problem in my experiment. To all my labmates, Shih-Hsun, Rethish, Samuel and Ann, thank you for your advice, experience and friendship. I appreciate all the joy and fun with you guys in the lab. To all my friends, especially Belinda, Michele, Min yan, Wendy and MeiZhi, thank you for your support and encouragement when I felt down and disappointed. I also would like to thank all the people in McColl Lab, Morona Lab and the Paton Lab, especially Dr. Tony Focareta, Dr. David Ogunniyi and Dr. Uwe Stroeher. I appreciate all the help and advice from them in my study. To other members in the school, including CSU staffs, Sergei Volgin and Garry Penney, thank you for being there to help and provide advice.

Finally, I would like to thank my parent, sisters and husband for all your love, support and couragement over the year of study. I could not have done this without you guys.