Copper Tolerance of *Listeria monocytogenes* strain DRDC8

Francesca Y Bell, B. Biotech. (Hons.) (Adelaide)

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School of Molecular and Biomedical Science
Faculty of Sciences,
The University of Adelaide
Adelaide, South Australia, Australia

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Presentation of Figures and Tables

All figures and tables referenced in this thesis are placed at the end of relevant chapters. This has been done to minimise the impact of large numbers of figures and tables on document flow and as an aid to interpretation of results and discussion sections of each chapter.

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Abstract

Listeria monocytogenes is one of the most important food-borne pathogens due to the severity of the disease it can cause. While the virulence factors required for effective colonisation and infection of mammalian hosts have been well described, other genes may modulate disease persistence. For *L. monocytogenes* strain DRDC8, the *ctpA* gene encodes a copper transporting P-type ATPase that apparently maintains intra-cellular copper ion homeostasis (Francis & Thomas, 1997a) and is also required for persistent infection of the liver and spleens of mice (Francis & Thomas, 1997b). However, the distribution of this gene is apparently limited to non-clinically derived environmental *L. monocytogenes* isolates (Bell, 2002). This may be explained by carriage of *ctpA* on plasmid DNA (Bell, 2002). Based on predictions of function and proximity to the *ctpA* gene (pCT0020), ORFs pCT0017, pCT0018, pCT0019 and *ctpA* were identified as a putative a *cop*-like operon involved in copper ion transport in *L. monocytogenes* (Bell, 2002).

Southern hybridisation analysis was used to confirm that the *ctpA* gene is carried on plasmid pCT100 in strain DRDC8. In addition, evidence to suggest that *ctpA* was encoded by bacteriophage DNA was not obtained. Furthermore, sequence analysis of DNA flanking *ctpA* identified ORFs that encode polypeptide sequences similar to proteins involved in plasmid replication and other plasmid-associated functions. Mating experiments provided evidence to show that plasmid pCT100 is not conjugative. This suggested that lateral transfer of this plasmid between cohabitating organisms may be limited.

Sequence analysis of a 37.279 kbp region of plasmid pCT100 from *L. monocytogenes* strain DRDC8 (GenBank Accession U15554) showed this plasmid had regions of gene content and organisation similar to that of other characterised *Listeria* plasmids, particularly plasmid pLI100 from *L. innocua* CLIP11262 and plasmid pLM80 from *L. monocytogenes* strain 4b H7858. Gene's common to these plasmids included those implicated in plasmid DNA replication, DNA transposition/insertion and heavy metal (cadmium) transport.

Sequence analysis of plasmid pCT100 also identified regions of DNA absent from other *Listeria* sequences. For example, a DNA region encoding a series of polypeptide

sequences similar to chromosomally-encoded proteins involved in copper transport in other Gram-positive bacteria was identified. The ORFs encoded by this region (pCT0017, pCT0018, pCT0019 and pCT0020 (*ctpA*), pCT0023, pCT0024, pCT0025, pCT0026, pCT0027) represent a novel cluster of genes implicated in copper homeostasis/tolerance that had not been previously described for other *Listeria* spp. PCR analysis was used to show that carriage of this copper gene cluster may be restricted to only some Australian *ctpA* positive *L. monocytogenes* isolates, typically of dairy and poultry origin.

In addition to these plasmid-encoded ORFs, PCR and sequence analysis identified a chromosomal ORF (*cutR*) also implicated in copper homeostasis/tolerance for strain DRDC8. *cutR* encodes a polypeptide similar to chromosomally-encoded copper-translocating P-type ATPases from other *Listeria* species.

The role of ORFs *cutR*, pCT0017, pCT0018 and pCT0019 in copper tolerance was assessed by comparison of the ability of wild type parent strain DRDC8 and variants containing independent mutations (pCT0017::*erm*, pCT0018::*erm*, pCT0019::*erm* or *cutR*::*erm*) to tolerate copper ion stress. The impact of loss of these genes (as a result of curing strain DRDC8 and *cutR*::*erm* derivatives of plasmid pCT100) on copper tolerance by DRDC8 was also examined. Minimal inhibitory concentration (MIC) and growth experiments showed that inactivation of *cutR*, pCT0018 or pCT0019, or removal of plasmid-encoded genes by curing DRDC8 of plasmid DNA, had a significant effect on copper tolerance. In addition, loss of plasmid DNA combined with disruption of *cutR* was shown to render cells completely incapable of growth in high levels of copper (14 mM $CuSO_4$). This data indicated that pCT0018, pCT0019 and *cutR* are involved in copper tolerance of *L. monocytogenes* strain DRDC8. MIC experiments also provided evidence to show that ORFs *cutR* and pCT0018 may play an additional role in tolerance to cadmium.

Interestingly, a *L. monocytogenes* mutant carrying an *erm* insertion within pCT0017 could not be constructed. However, evidence that showed that ORF pCT0017 encodes a CopY-like negative repressor protein directly implicated this ORF in copper tolerance. DNA gel shift experiments were used to show that pCT0017 protein binds to two '*cop* box-like' nucleotide sequences located upstream of the pCT0017 translation start site. Binding occurs in a copper-dependant manner that is consistent with published models of CopY-like protein function. Thus pCT0017 protein may regulate expression of

ORFs pCT0017, pCT0018, pCT0019 and *ctpA* in a copper responsive manner. This is consistent with the view that these ORFs form a *cop*-like operon involved in copper homeostasis.

In conclusion, *L. monocytogenes* strain DRDC8 displayed an exceptional tolerance to high concentrations of copper ions. The data obtained suggested that both chromosomal and plasmid-encoded genes are involved in copper homeostasis/tolerance of DRDC8. This particular strain may have acquired multiple genes involved in copper tolerance from a co-habitating Gram-positive bacterium in response to exposure to high levels of copper within the environment. Given that strain DRDC8 is an Australian dairy isolate, these genes may provide a selective advantage for survival of other *L. monocytogenes* strains in associated environments.

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 Francesca York Bell 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.

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Francesca Y Bell

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Abbreviations

°C	degrees Celsius
µg	microgram/s
µL	microlitre/s
µM	micromolar
× g	relative centrifugal force
aa	amino acid/s
AP	alkaline phoshatase
Amp	ampicillin
ATP	adenosine 5'-triphosphate
BHI	Brain Heart Infusion
bp	base pair
ca.	circa = approximately
cf.	confer = compare
CFU	colony forming units
Cm	chloramphenicol
Ctp	copper transport protein
DIG	digoxigenin
DIG-11-dUTP	digoxigenin-11-uridine 5'triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dsDNA	double stranded deoxyribonucleic triphosphate
DTT	dithiothreitol
EDTA	ethylene-diamine-tetra-acetic-acid disodium salt
Em	erythromycin
erm	erythromycin resistance gene
EtBr	ethidium bromide
g L^{-1}	grams per litre
h	hour/s
HCL	Hydrochloric acid
IPTG	isopropyl-β-D-thio-galactopyranoside

IMVS	The Institute of Medical and Veterinary Sciences
Kan	kanamycin
kbp	kilobase/s
kDA	kilodalton/s
L	litre/s
LA	Luria agar
LB	Luria Bertani broth
LIR	left inverted repeat region of Tn917
LLO	Listeriolysin O
M	molar
mg	milligram/s
min	minute/s
mL	millilitre/s
mM	millimolar
mRNA	messenger ribonucleic acid
NBT	4-Nitroblue tetrazolium chloride
ng	nanogram(s)
nm	nanometre(s)
nM	nanomolar
OD	optical density
O/N	overnight
ONPG	o-nitrophenyl-β-D-galactopyranoside
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
pM	picomolar
rbs	ribosome binding site
Rif	rifampicin
RNA	ribonucleic acid
RNase	ribonuclease

RT	room temperature
s	second/s
SDS	sodium dodecyl sulphate
SLCC	Special *Listeria* Culture Collection
Sm	streptomycin
Sp	spectinomycin
spp.	species
SSC	standard saline citrate
TAE	tris-acetate EDTA buffer
TE	tris-EDTA buffer
UTP	uridine 5’triphosphate
UV	ultraviolet light
V	volt/s/
vol	volume/s
v/v	volume per volume
w/v	weight per volume
v/v	volume/volume
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-pho	5-Bromo-4-chloro-3-indolyl-phosphate

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