

# **Copper Tolerance of** ***Listeria monocytogenes* strain DRDC8**

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## Chapter 5: Purification and Functional Analysis of the pCT0017 Protein

### 5.1 Introduction

On the basis of sequence comparisons ORF pCT0017, encoded by plasmid pCT100 in *L. monocytogenes* strain DRDC8, is predicted to encode a CopY-like copper-responsive transcriptional repressor protein that may bind specifically to DNA sequences known as *cop*-boxes. The deduced polypeptide sequence encoded by pCT0017 shares significant similarity to the CopY family of negative transcriptional regulators associated with copper transport systems. This family of proteins comprises homologues of the CopY negative transcriptional repressor of the *cop* operon (*copYZAB*) in *E. hirae* (Strausak & Solioz, 1997). The three consensus motifs characteristic of the CopY family, a C-terminal heavy-metal binding motif (CXCX<sub>4</sub>CXC) and two N-terminus conserved sequences (IX<sub>3</sub>EXEVMX<sub>2</sub>W and WX<sub>3</sub>TX<sub>2</sub>TX<sub>3</sub>RLX<sub>2</sub>K) (Vats & Lee, 2001), have been identified within the polypeptide sequence of pCT0017 (Bell, 2002).

In *E. hirae*, the homodimeric zinc protein ([Zn(II)CopY]<sub>2</sub>) CopY exhibits highly specific DNA-protein interactions that regulate expression of the *cop* operon in response to ambient copper concentrations in a biphasic fashion (Strausak & Solioz, 1997) (refer to Section 1.8.2.1 for more detail). Copper is delivered to CopY by the metallochaperone CopZ. At high cytoplasmic copper concentrations, CopZ associated Cu<sup>2+</sup> displaces the Zn<sup>2+</sup> of [Zn(II)CopY]<sub>2</sub> to convert it to [Cu(I)<sub>2</sub>CopY]<sub>2</sub>. The copper form of CopY is then unable to bind to the promoter (Portmann *et al.*, 2004). Strausak and Solioz, (1997) used *DNaseI* footprinting to show that in normal growth media, CopY dimers are bound to two separate sites in the operator between nucleotides -71 to -11, relative to its own translational start site, to repress transcription of the *cop* operon. These sites are inverted repeats called ‘*cop* boxes’, designated by the consensus sequence TACAXXTGTA (Portmann *et al.*, 2004). This motif is found in *cop*-like promoters of several different Gram-positive organisms including *Lactococcus lactis* (Magnani *et al.*, 2008; Portmann *et al.*, 2006) and *Streptococcus mutans* (Vats & Lee, 2001), and acts as the binding site for CopY-like copper-responsive repressors.

It is possible that in strain DRDC8, 'cop box'-like motifs exist upstream of the pCT0017 translational start site that act as pCT0017 protein binding sites. Extracellular copper may regulate the DNA binding activity of the pCT0017 protein and thus control expression of associated downstream genes. Given the proximity of pCT0017 to pCT0018, pCT0019, and pCT0020 (*ctpA*), these ORFs are predicted to represent a *cop*-like operon that is negatively regulated by the protein encoded by pCT0017 (Bell, 2002). An ORF encoding a CopZ-like protein has not yet been identified in strain DRDC8, thus it is unclear if the proteins encoded by pCT0018 or pCT0019 function in copper binding and delivery of ions to pCT0017.

Interestingly, attempts to relieve repression of gene expression by creating a pCT0017::*erm* mutant were not successful (described in Chapter 4 of this thesis). This suggested that mutation of ORF pCT0017 may result in loss of control of copper homeostasis, leading to lethal copper toxicity. Together, this data strongly implicates ORF pCT0017 in regulation of copper homeostasis genes. Furthermore, data presented in Chapter 4 of this thesis indicated that copper and cadmium ion homeostasis may be linked in strain DRDC8, thus pCT0017 protein could be copper and cadmium responsive.

In view of the evidence for the pCT0017 protein as a CopY-like copper-responsive transcriptional repressor protein, the aims of the work described in this chapter were to:

1. Provide experimental evidence to show that ORF pCT0017 encodes a CopY-like protein that is able to specifically bind *L. monocytogenes* DRDC8 DNA in a copper dependent manner.
2. Characterise the DNA binding sites of pCT0017 protein.

## 5.2 Experimental design

To provide evidence to show that pCT0017 protein is a CopY-like copper-responsive transcriptional repressor protein, the interaction between the pCT0017 protein and the putative promoter region relative to its own translational start site was characterised *in vitro* using DNA gel shift analysis. Gel shift analysis is a well known, widely performed technique used to characterise specific protein and DNA interactions *in vitro*. The principle behind this technique is that a protein bound to a piece of DNA will retard its

entry into and migration through a gel during electrophoresis, compared to an unbound fragment of DNA. Migration of DNA in polyacrylamide gels is dependent on conformation and size of the DNA/protein complex.

*In vitro* pCT0017 protein expression was achieved by cloning the coding region of ORF pCT0017 into the pET21-c(+) expression vector, in frame with six C-terminal histidine codons. Induction of expression of a C-terminal His-tagged pCT0017 protein in *E. coli* BL21 was followed by purification of the protein from whole cell lysates using HisLink™ Protein Purification Resin. The purified His-tagged protein was used in gel shift analysis with specific PCR amplified DIG-labelled DRDC8 DNA fragments. Gel shift analysis was used to show that His-tagged pCT0017 is capable of binding two specific DNA sites located within the putative promoter region of ORF pCT0017 in a manner that is dependent on the *in vitro* concentration of copper ions.

## 5.3 Results

### 5.3.1 Overexpression and Purification of pCT0017 Protein

#### 5.3.1.1 Construction of a pCT0017 Expression Vector

To overexpress pCT0017 protein, a 516 bp PCR product encompassing the coding region of this ORF was amplified from *L. monocytogenes* strain DRDC8 DNA using oligonucleotides pETOrfFF and pETOrfFR (refer to Table 2.8 for oligonucleotide details). pETOrfFF was designed to incorporate an *Nde*I site (CACATG) across the start codon (ATG) and pETOrfFR to incorporate a *Xho*I site (CTCGAG) across the stop codon (GAG replaces the TAG stop codon) of the ORF. The PCR product was cloned into pGEM®-T Easy, resulting in plasmid pCTCF (see Figure 5.1 for the cloning strategy used). Sequence analysis and restriction enzyme digestion was used to confirm that the plasmid carried the correct insert. Plasmid pCTCF was digested with *Nde*I and *Xho*I and the resulting 491 bp DNA fragment subcloned into similarly digested pET21-c(+) (Novagen) (refer to Table 2.3), resulting in plasmid pETCF (see Figure 5.2). Clones carrying the correct insert were identified by restriction enzyme digestion and PCR analysis using the pETOrfFF and pETOrfFR oligonucleotide pair. Sequence analysis was used to confirm that the pCT0017 insert was cloned in frame with the vector encoded histidine codons (see Appendix R, page

348 for sequence translation). The C-terminal His-tagged protein was predicted to have a molecular weight of 19.37 kDa *cf.* the native protein size of 18.4 kDa.

#### 5.3.1.2 Overexpression of pCT0017

Expression of pCT0017 protein from pETCF was induced using the Novagen T7 expression system (Studier *et al.*, 1990; Tabor & Richardson, 1985) (refer to Section 2.23). Plasmid pETCF (see Section 5.3.1.1) was used to transform the *E. coli* BL21 (DE3) host strain (refer to Table 2.2). Expression of pCT0017 was induced by incubation of strain BL21 carrying pETCF (BL21 [pETCF]) at 30°C for 6 h in the presence of 1 mM IPTG. Whole cell lysates were prepared from cultures of induced and non-induced BL21 [pETCF] (methods described in Section 2.24). SDS-PAGE (refer to Section 2.25) of whole cell lysates showed that a protein band *ca.* 19.37 kDa in size was present in samples of lysate from cultures of induced BL21 [pETCF], but not in lysates of non-induced BL21 [pETCF] (Figure 5.3). Prestained markers proteins were used as an approximate guide to molecular weight (refer Table 2.5 for details). This data indicated that a *ca.* 19.37 kDa protein was expressed from strain *E. coli* BL21 [pETCF] following induction.

#### 5.3.1.3 Purification of His-tagged pCT0017

His-tagged pCT0017 protein was purified from whole cell lysates of induced BL21 [pETCF] by gravity flow column chromatography using HisLink™ Protein Purification Resin (Promega). The cell lysate and resin column was prepared as described in Section 2.26. The concentration of imidazole required for effective washing (100 mM) and elution (500 mM) of His-tagged protein from the HisLink™ resin column was determined experimentally. Five 1 mL fractions were eluted and collected from the column and analysed by SDS-PAGE electrophoresis (Figure 5.4). His-tagged pCT0017 protein was successfully eluted in fractions 2, 3, 4 and 5. Protein mass spectrometry sequencing (refer to Section 2.27) confirmed the identity of the His-tagged pCT0017 protein (see Appendix S, page 349 for sequencing results). The protein concentration of each sample of purified protein was estimated (refer to Section 2.28), and stored at -20°C in 50 % (v/v) glycerol to preserve protein activity. Purified His-tagged pCT0017 protein is hereafter referred to as pCT0017\* protein.

### 5.3.2 Gel Shift Assay Analysis

The DNA binding capacity of purified pCT0017\* protein was characterised by gel shift assay analysis. PCR was used to amplify all DNA fragments analysed in gel shift assays. Amplification was confirmed by agarose gel electrophoresis and sequence analysis. Amplified fragments were then DIG labelled at the 3' end using terminal transferase (see Section 2.29.1). Prior to labelling, DNA concentrations were quantitated using methods described in Section 2.8.1.

For each gel shift reaction, the pCT0017\* protein was preincubated with 100 × molar excess of unlabelled competitor DNA (pre-sonicated salmon sperm DNA) to minimise non-specific protein-DNA interactions. DNA/protein binding reactions were then performed by incubating 0.5 pmol labelled DNA with pCT0017\* protein as described in Section 2.29.2. For some gel shift assays, labelled DNA was also incubated with 250 ng mL<sup>-1</sup> of non-induced BL21 [pETCF] whole cell lysate as a negative control. Binding reactions were separated on 6% non-denaturing polyacrylamide gels, followed by electroblotting of the DNA-protein complexes onto a nylon membrane as described in Section 2.29.3. Migration of pCT0017\* protein-DNA mixtures was compared to the migration 0.5 pmol of labelled DNA. Labelled DNA/protein complexes were then detected using chemiluminescence (see Section 2.29.4).

#### 5.3.2.1 Titration of pCT0017\* Protein

To ensure that the concentration of pCT0017\* protein used for detection of specific protein/DNA binding interactions by gel shift analysis did not result in non-specific DNA binding, initial binding reactions were conducted with labelled PCR amplified DNA fragments of *hly* (p234/p319 oligonucleotide pair) and *ctpA* (FB001/LM2004 oligonucleotide pair), as negative controls. Different concentrations of pCT0017\* protein were incubated with 0.5 pmol of the labelled DNA fragments and subjected to gel shift analysis. Figure 5.5 shows a Southern blot of gel shift assays performed with pCT0017\* protein concentrations ranging between 1000 – 62.5 ng mL<sup>-1</sup>. At 1000 and 500 ng mL<sup>-1</sup> protein, *ctpA* and *hly* DNA fragments were shifted by pCT0017\* protein, indicating the protein concentrations used in these binding reactions was in excess. At protein concentrations of ≤ 250 ng mL<sup>-1</sup>, *ctpA* and *hly* DNA remained unshifted by pCT0017\*

protein. Subsequently, to eliminate non-specific binding, all further gel shift assays were performed with 250 ng mL<sup>-1</sup> of pCT0017\* protein.

#### 5.3.2.2 Analysis of pCT0017\* Protein Binding to *L. monocytogenes* DNA

On the basis that pCT0017 protein is speculated to regulate expression of *ctpA* and other cotranscribed ORFs, nine DNA fragments (P1 – P9) spanning the 3' end of pCT0016 to the 5' end of *ctpA* were PCR amplified from *L. monocytogenes* DRDC8 DNA, labelled and used as the DNA targets for gel shift assays. The relative location and size of DNA fragments P1 – P9, and the oligonucleotide pairs used to amplify these fragments are shown in Figure 5.6 Panel A. The results of the gel shift assay using each of these fragments as target DNA is shown in Figure 5.6 Panel B and C. Fragments P1, P2, P3, P5, P6, P7, P8, and P9 migrated through the gel in a manner identical to control labelled DNA fragments not incubated with pCT0017\* protein, indicating that pCT0017\* protein did not interact with these fragments. However, fragment P4 (located immediately upstream of pCT0017 (refer to Figure 5.6 Panel A)), was shifted by pCT0017\* protein compared to labelled DNA control preparations (Figure 5.7). Shifting of P4 was not observed when this fragment was mixed with whole cell lysate extracted from cultures of the non-induced BL21 [pETCF] control. The data presented in Figure 5.7 also shows that the *ctpA* and *hly* negative control DNA fragments were not shifted by pCT0017\* protein. These results indicated that pCT0017\* protein was able to bind to DNA sequences within the region encompassed by fragment P4.

#### 5.3.2.3 Characterisation and Identification of pCT0017 DNA Binding Sites

To more precisely identify the DNA binding sites of pCT0017\* protein, gel shift assays were conducted with DIG-labelled PCR amplified DNA fragments P14, P24 and P34, each of which are internal to P4 (refer to Figure 5.8). Migration of fragment P24 was shifted by pCT0017\* protein compared to migration of the labelled P24 negative control. Fragments P14 and P34 were not shifted by pCT0017\* protein, and migrated relative to labelled P14 and P34 incubated without pCT0017\* protein. As expected, positive control preparations of fragment P4 were also shifted by pCT0017\* protein. These results indicated that pCT0017 protein bound to DNA sites located within the region encompassed by P24.

To further characterise the precise binding sites for pCT0017, the nucleotide sequence of fragment P24 was screened for 'cop box'-like motifs that could act as pCT0017\* protein binding sites. Two inverted repeats, TACAAATGTA (*cop* box 1) and TACAAGAGTA (*cop* box 2), consistent (with the exception of single base variation, A replacing T, in the second repeat) with the consensus TACAXXTGTA 'cop box' sequence were identified 81 bp and 47 bp, respectively, upstream of the pCT0017 start codon. Figure 5.9 Panel A shows a comparison between the DNA region carrying the putative DRDC8 *cop* boxes and the [Zn(II)CopY]<sub>2</sub> binding sites of the *E. hirae* *cop* operon promoter (Portmann *et al.*, 2004; Strausak & Solioz, 1997). Multiple alignment of the DRDC8 DNA sequence containing *cop* box 1 and *cop* box 2, the promoter *copY* sequence containing putative CopY binding sites of 14 bacterial strains (9 species) of the *Lactobacillale* order characterised by Reyes *et al.*, (2006) and the *cop* operon promoter of *E. hirae*, showed that the identified putative pCT0017 binding sites are consistent with other putative CopY binding sites (Figure 5.9 Panel B). In particular, the pCT0017 putative *cop* boxes are almost identical to those of strains of *S. agalactiae* and *S. pyogenes*. On this basis, *cop* box 1 and *cop* box 2 were identified as putative pCT0017 protein binding sites.

To show that *cop* box 1 and *cop* box 2 act as binding sites for the pCT0017 protein, 32 bp (CopBox1) and 34 bp (CopBox2) double stranded DNA fragments were constructed using complementary oligonucleotide pairs CBox1/CBox1C and CBox2/CBox2C (refer to Table 2.8 for oligonucleotide details), respectively. CopBox1 comprised the *cop* box 1 sequence flanked by 10 bp on the 5' end and 12 bp of the 3' end, and CopBox2 comprised the *cop* box 2 sequence flanked by 12 bp at the 5' and 3' ends. Figure 5.10 presents a diagrammatic representation of the location of the *cop* box 1 and *cop* box 2 motifs relative to the start codon of pCT0017, and the position and direction of oligonucleotides CBox1, CBox1C, CBox2 and CBox2C. Double stranded DNA fragments CopBox1 and CopBox2 were created by annealing equimolar amounts of oligonucleotides CBox1 with CBox1C and CBox2 with CBox2C. The mixtures were rapidly heated to 80°C followed by gradual cooling to 30°C over a period of 1 hr.

The CopBox1 (32 bp) and CopBox2 (34 bp) fragments were DIG labelled and subjected to gel shift analysis by incubation with pCT0017\* protein (Figure 5.11). Oligonucleotides GSControlF and GSControlR (complementary sequence of GSControlF)



(refer to Table 2.8 for oligonucleotide details) were designed and used to create a small double stranded negative control DNA fragment (GSControl). These control primers were designed across a 34 bp DNA region located 10 bp upstream of *cop* box 1. Gel shift analysis of the GSControl fragment confirmed that pCT0017 protein did not bind non-specifically to small DNA fragments. Gel shift assays results showed that migration of CopBox1 and CopBox2 DNA fragments were shifted by pCT0017\* protein, compared to the migration of labelled CopBox1 and CopBox2 DNA alone (Figure 5.11). Positive control preparations of fragment P4 were also shifted by pCT0017\* protein. This data suggested that *cop* box 1 and *cop* box 2 encode nucleotide sequences that act as binding sites for pCT0017\* protein.

To determine if other '*cop* box'-like sites, to which pCT0017 protein may bind, exist within the putative copper gene cluster (described in Chapter 3), nucleotide sequence spanning from the 5' end of pCT0017 to the 3' end of pCT0027 was analysed for '*cop* box'-like inverted repeats. No inverted repeats consistent with the consensus '*cop* box' motifs described in Figure 5.9 were identified.

#### 5.3.2.4 Characterisation of pCT0017\* Protein-DNA Interaction in Response to Cu<sup>2+</sup> and Cd<sup>2+</sup> Concentration

To determine if *in vitro* binding of pCT0017\* protein to *cop* box 1 and *cop* box 2 is responsive to copper or cadmium ion concentrations, gel shift assays were repeated with fragment P4 in the presence of different concentrations of CuSO<sub>4</sub> and CdSO<sub>4</sub>. The results obtained from these experiments are presented in Figure 5.12. In the presence of CuSO<sub>4</sub> at concentrations ranging from 500 μM to 14 mM (500 μM, 1 mM, 5 mM, 10 mM, 14 mM) migration of P4 was not shifted by pCT0017\* protein and was identical to that of labelled P4 DNA incubated without pCT0017\* protein (Figure 5.12 Panel A). Conversely, in the presence of CuSO<sub>4</sub> at concentrations ranging from 1 μM to 1 pM (1 μM, 500 nM, 1 nM, 500 pM and 1 pM) P4 was shifted by pCT0017\* protein, when compared to negative control labelled P4 DNA. Similarly, the positive control gel shift reaction showed that fragment P4 was shifted by pCT0017\* protein in the absence of added CuSO<sub>4</sub>. However, gel shift assays conducted in the presence of different concentrations of cadmium ions (14 mM - 1 pM) showed that fragment P4 was shifted by pCT0017\* protein at all

concentrations tested compared to labelled P4 DNA alone (Figure 5.12 Panel B). This result showed that pCT0017\* protein-DNA binding is responsive to copper ion concentrations, but not to cadmium ions.

To confirm the results obtained for gel shift analysis of fragment P4 in the presence of CuSO<sub>4</sub>, these experiments were repeated with DNA fragments CopBox1 and CopBox2 in the presence of 1 μM and 500 μM CuSO<sub>4</sub>. The results obtained are presented in Figure 5.13. In the presence or absence of 1 μM CuSO<sub>4</sub>, CopBox1 and CopBox2 are shifted by pCT0017\* protein, relative to DNA incubated without pCT0017\* protein (Figure 5.13 Panel A). Identical results were obtained for the fragment P4 positive control. Conversely, in the presence of 500 μM CuSO<sub>4</sub>, fragments CopBox1 and CopBox2 are not shifted by pCT0017\* protein and migrate through the gel at the same rate as labelled CopBox1 and CopBox2 DNA alone (Figure 5.13 Panel B). As expected, in the absence of added CuSO<sub>4</sub>, CopBox1 and CopBox2 were shifted by pCT0017\* protein. Furthermore, the positive control fragment P4 was shifted by pCT0017\* protein in the absence of CuSO<sub>4</sub>, but was not shifted in the presence of 500 μM CuSO<sub>4</sub>. The data collated from these experiments indicated that pCT0017 protein binds *cop* box 1 and *cop* box 2 in the presence CuSO<sub>4</sub> at concentrations ≤ 1 μM, however binding does not occur in the presence of CuSO<sub>4</sub> concentrations ≥ 500 μM. These results confirmed the role of pCT0017 protein as a copper responsive DNA-binding protein.

## 5.4 Discussion

Nucleotide sequence analysis of the DNA encoding ORFs pCT0017, pCT0018, pCT0019 and *ctpA* (pCT0020) strongly implicates these putative genes as a *cop*-like operon within the copper gene cluster of *L. monocytogenes* DRDC8. Furthermore, the deduced polypeptide sequence for pCT0017 has all the characteristic of a CopY regulatory protein ie. sequence similarity and three consensus motifs (C-terminal heavy-metal binding motif and two N-terminus motifs) characteristic of CopY proteins. The data presented in this chapter provides substantial support for the role of protein pCT0017 as copper responsive regulatory protein that interacts with DNA containing two ‘*cop* box’-like motifs located upstream of ORF pCT0017.

In this study, His-tagged recombinant pCT0017 protein was expressed in an *E. coli* background using the pET21-c(+) expression vector. The C-terminal His-tag enabled preparation of highly purified recombinant protein that was used for study of the interaction between this protein and DNA from *L. monocytogenes* strain DRDC8. Gel shift analysis demonstrated that the pCT0017 protein binds to a 323 bp fragment of DNA (P4) located immediately upstream of the pCT0017 start codon. Restricted electrophoretic migration of the DNA-protein complex in a polyacrylamide gel compared to that observed for the P4 DNA fragment alone was observed. The precise binding site for pCT0017 protein was further localised by gel shift experiments that showed binding to a 191 bp fragment (P24) derived from fragment P4.

Two putative 'cop box'-like motifs, *cop box 1* ((TACAAATGTA) and *cop box 2* (TACAAGAGTA) were identified within the nucleotide sequence of P24. These motifs are almost perfect matches of the consensus TACAXXTGTA inverted repeats characterised as the binding sites of CopY-like repressors other Gram-positive bacteria (Portmann *et al.*, 2004; Reyes *et al.*, 2006). The role of these motifs as binding sites for pCT0017 protein was confirmed by gel shift assays that showed that His-tagged pCT0017 protein binds specifically to two small double stranded DNA fragments encoding *cop box 1* (32 bp) and *cop box 2* (34 bp) in a copper concentration dependent fashion.

The copper responsive nature of protein pCT0017 was demonstrated by gel shift assays that showed this protein could bind to a DNA fragment (P4), which encoded the *cop box 1* and *cop box 2* motifs, at CuSO<sub>4</sub> concentrations  $\leq 1 \mu\text{M}$ , but not at CuSO<sub>4</sub> concentrations  $\geq 500 \mu\text{M}$ . This observation was confirmed by gel shift assays using the smaller (*ca.* 32 bp) DNA fragments encoding each of the 'cop box' motifs.

Evidence to suggest that the interaction of pCT0017 protein with DNA is also dependent on cadmium ion concentration could not be confirmed. The DNA-protein interaction between a fragment (P4) encoding the two *cop boxes* and the pCT0017 protein was stable at all cadmium concentrations tested. Whilst the data presented in Chapter 4 of this thesis indicated that copper and cadmium tolerance of strain DRDC8 may be linked, the gel shift data showed that pCT0017 protein-DNA interactions are not responsive to cadmium ion concentration.

The experimental findings gathered during this study overwhelmingly indicate that pCT0017 protein interacts with two 'cop box'-like motifs in a copper dependent fashion. This is similar to that described of CopY regulation of the *E. hirae* cop operon (Strausak & Solioz, 1997). On this basis, a proposed model of copper-responsive transcriptional repression by pCT0017 protein is diagrammatically represented in Figure 5.14. At low copper ions concentrations (eg.  $\leq 1 \mu\text{M}$ ), it is proposed that homodimers of the zinc form of pCT0017 protein  $[\text{Zn(II)pCT0017}]_2$  are bound to cop box 1 and cop box 2 of the pCT0017 promoter. This interaction is proposed to repress transcription of ORF pCT0017, and other cotranscribed genes. These downstream genes are likely to include pCT0018, pCT0019, and *ctpA*, however, may also include other genes associated with the DRDC8 putative copper gene cluster. At high copper concentrations (eg.  $\geq 500 \mu\text{M}$ ), an unidentified CopZ-like copper chaperone transfers copper ions to protein pCT0017. The bound zinc is displaced by  $\text{Cu}^{2+}$  ions. This form of the pCT0017 protein is unable to bind DNA and is released from the promoter, allowing transcription of associated genes to proceed. ORF pCT0018 or pCT0019 may encode a protein that functions as the chaperone protein that delivers copper to pCT0017. Alternatively, the role may be played by an uncharacterised CopZ-like protein.

Although the gel shift data described strongly support aspects of this model, further experiments are required to confirm this mechanism. Cross-linking and gel filtration experiments could be used to determine if protein pCT0017 exists as a homodimer in solution. *DNaseI* footprinting would also be useful in identifying the exact pCT0017 dimer binding sites. In addition, surface plasmon resonance analyses could be used to confirm the protein-DNA interaction and kinetics. This approach has proved useful for study of the protein-DNA interactions and has been used to characterize the interaction of CopZ with CopA P-type ATPase (Multhaup *et al.*, 2001). Displacement of zinc, by copper, from pCT0017 dimers could be monitored using 2,4-pyridylazoresorcinol. This method has been used to show zinc displacement from CopY by copper in *E. hirae* (Cobine *et al.*, 2002).

Furthermore, future studies should be directed towards establishing that pCT0017, pCT0018, pCT0019 and *ctpA* form an operon regulated by the pCT0017 protein, or alternatively, if this operon forms part of a larger cluster of genes that are all regulated by

pCT0017 protein. This could be achieved by using real-time RT-PCR analyses to identify the size of transcripts encoding pCT0017. Primer extension analysis should also be deployed to determine the transcriptional start site to define the promoter region of pCT0017. Fusion of a promoter-less reporter gene to the 5' end of pCT0017 ORF could be constructed and placed in a DRDC8 strain cured of the large plasmid. The effect of growth medium copper concentration on expression of the reporter could then be used as a means of studying expression of the *cop*-like operon. That approach has been used successfully by Vats and Lee, (2001) to study the regulation of the *copYAZ* operon from *Streptococcus mutans*.

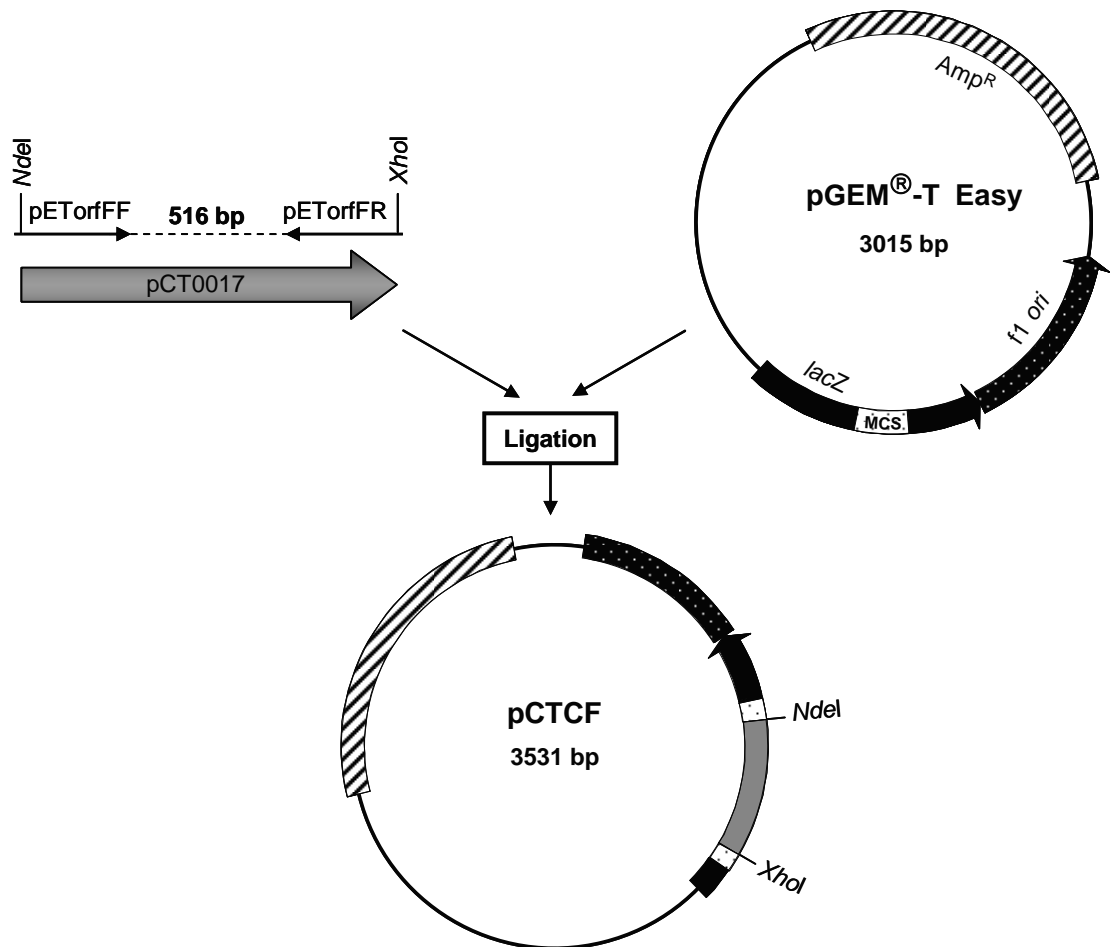
Although there is good evidence to indicate that pCT0017, pCT0018, pCT0019 and *ctpA* form a *cop*-like operon, nothing is known about the expression of these genes in response to changing environmental levels of  $\text{Cu}^{2+}$ . Nor is anything known about the shuttling of copper ions to protein pCT0017 in the cytoplasm of *L. monocytogenes* DRDC8. ORF pCT0018 or ORF pCT0019 could encode a protein that functions as a CopZ-like copper chaperone. The protein encoded by pCT0018 is a potential candidate. The polypeptide sequence has a putative C-terminal CXMXMXH metal binding motif that may be involved in binding copper ions (Dancis *et al.*, 1994; Puig & Thiele, 2002). In addition, Hii, (2009) used copper analyses and copper interaction metal affinity chromatography (Cu-IMAC) to show that recombinant pCT0018 protein can bind copper ions. However, the lack of sequence similarity between the polypeptide sequence encoded by pCT0018 and pCT0019, and known CopZ copper chaperone proteins suggests that these candidate proteins are unlikely to be CopZ-like. Examples of *cop*-like operons carried by other species of Gram-positive bacteria that contain a *copY*-like gene but not *copZ*-like genes have been described previously (Reyes *et al.*, 2006). As is the case for DRDC8, the chaperones involved in shuttling copper ions to these CopY-like proteins are yet to be identified.

The experimental data presented in this chapter that implicates protein pCT0017 as a copper responsive DNA binding protein may explain why a pCT0017 mutant could not be constructed. If pCT0017 is a CopY-like repressor protein, transcriptionally linked downstream genes (eg. *ctpA*) would be constitutively expressed in its absence. Assuming that CtpA is responsible for  $\text{Cu}^{2+}$  influx, loss of control over copper transport leading to

excess free copper ions in the cytoplasm could potentially result in lethal toxicity and cell death.

## 5.5 Conclusions

ORF pCT0017 encodes a copper responsive CopY-like protein that is able to bind specifically to DNA that encodes two 'cop box'-like sequences. These motifs are located upstream of the pCT0017 translation start site and are consistent with CopY binding sites for other Gram-positive bacteria. Interaction of the protein with the *cop* boxes only occurs at low (< 500  $\mu$ M) concentrations of copper. Thus the pCT0017 protein is likely to function as negative transcriptional repressor protein that inhibits transcription of its own gene and other cotranscribed genes in response to ambient copper concentrations. This data provides definitive evidence to support the hypothesis that ORF pCT0017 encodes a protein that is involved in copper ion homeostasis in *L. monocytogenes* strain DRDC8. The DNA binding capabilities of the pCT0017 protein does not appear to be dependent on cadmium ion concentration.



**Figure 5.1: Construction of pCTCF.**

Oligonucleotides pETorfFF and pETorfFR were used to PCR amplify the coding region of ORF pCT0017 and incorporate *NdeI* and *XhoI* sites at the 5' and 3' ends of the DNA, respectively. This 516 bp PCR product was cloned into the pGEM®-T Easy vector to create plasmid pCTCF. The insert was subsequently isolated from pCTCF using *NdeI* and *XhoI* digestion and cloned into pET-21-c(+) (see Figure 5.2).

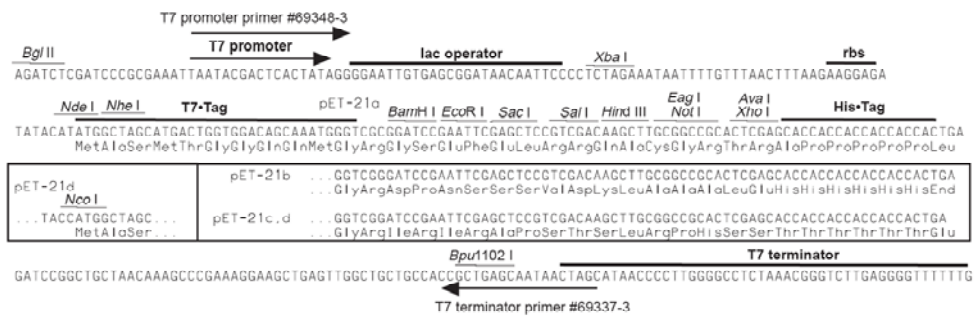
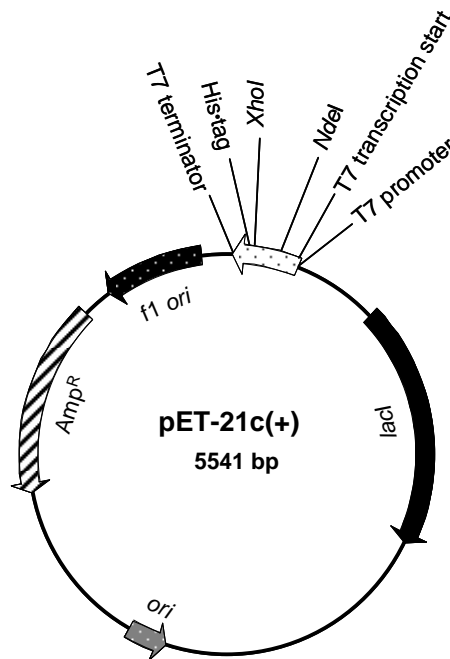
**Figure 5.2: Construction of the pCT0017 expression vector pETCF.**

Panel A. The expression vector pET-21-c(+). The cloning site is shown below the vector map.

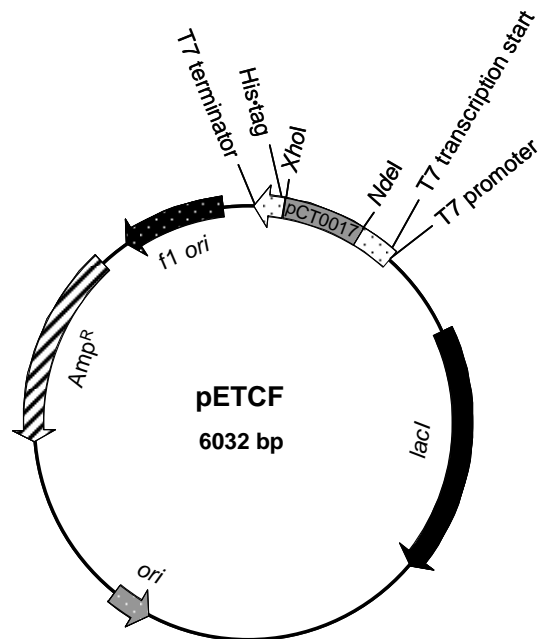
Panel B. A *NdeI/XhoI* fragment encompassing the coding region of pCT0017 was isolated from plasmid pCTCF (see Figure 5.1) using restriction enzyme digestion and cloned into *NdeI/XhoI* digested plasmid pET-21-c(+). Expression of ORF pCT0017 resulted in a polypeptide fused to a C-terminal His-tag.

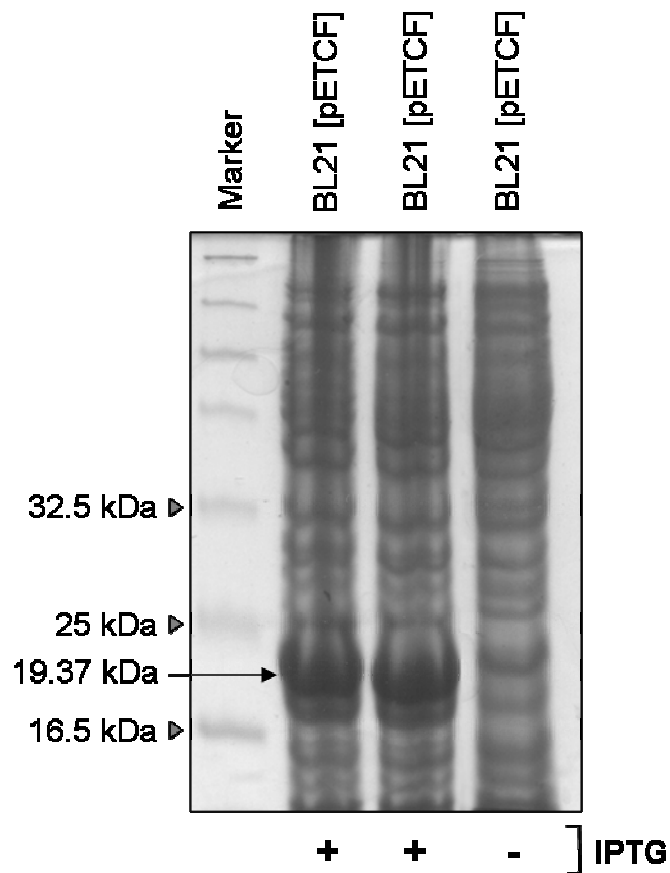


A



B

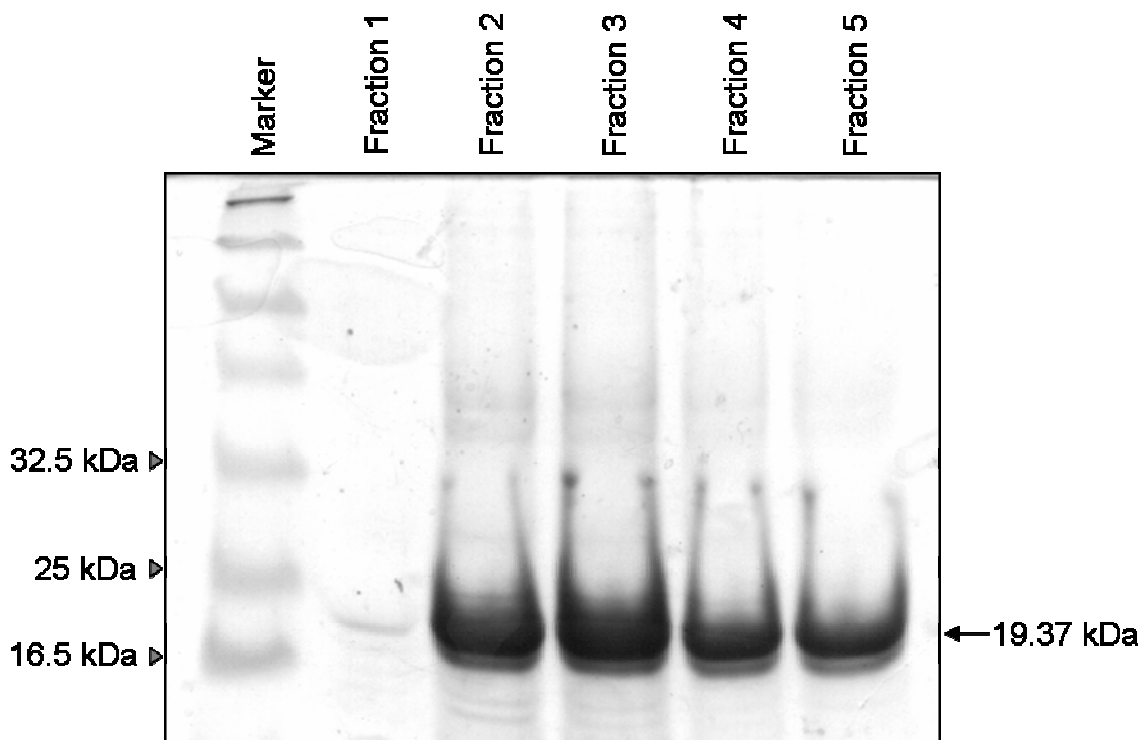




**Figure 5.3: SDS-PAGE of cell lysates of *E. coli* BL21 [pETCF].**

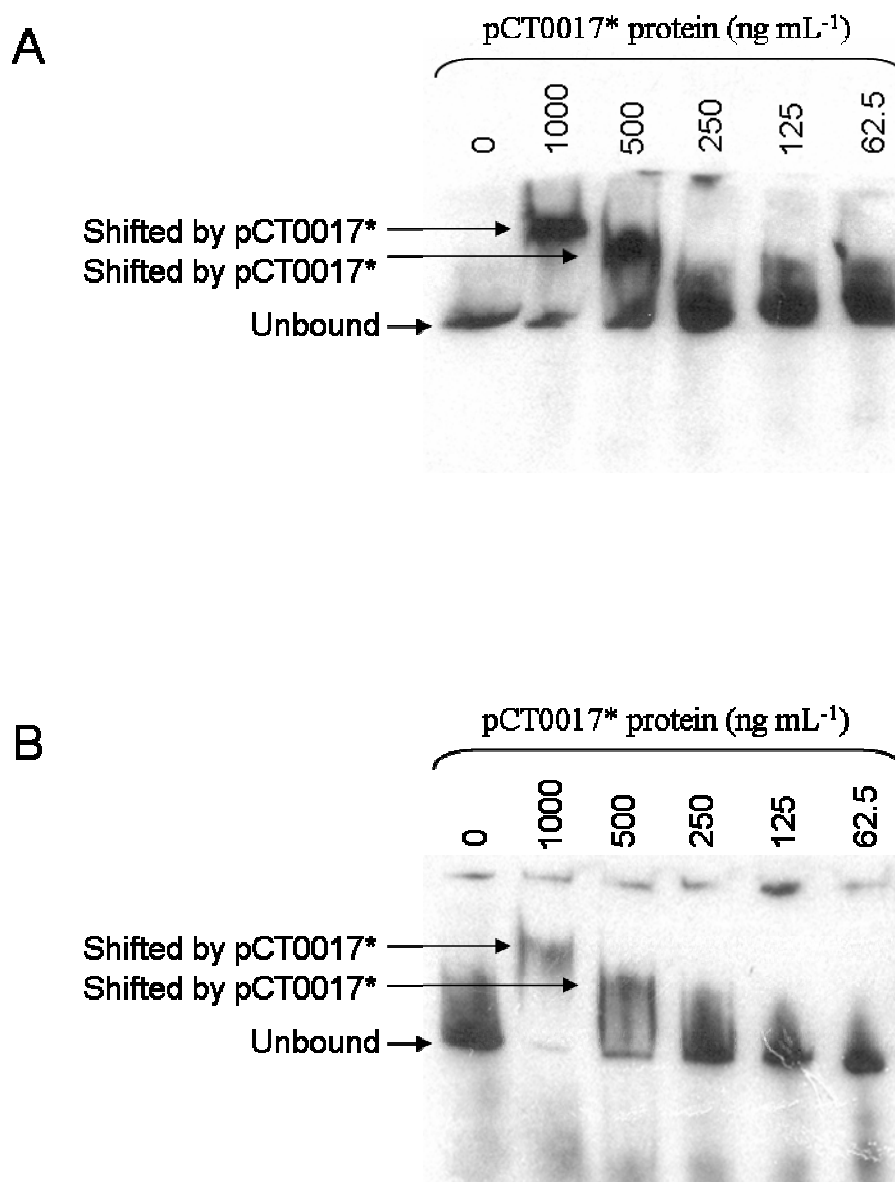
Whole cell lysates of IPTG induced and non-induced strains of BL21 carrying plasmid pETCF (BL21 [pETCF]) were electrophoresed on a 15% SDS-PAGE and stained with Coomassie blue.

The + and - signs indicate BL21 [pETCF] grown in the presence or absence, respectively, of 1 mM IPTG. The marker lane was loaded with prestained broadrange marker (see Table 2.5). The apparent molecular weight (kDa) of relevant marker bands are indicated to the left. Over-expressed pCT0017 protein (*ca.* 19.37 kDa) in whole cell lysates of induced strains of BL21 [pETCF] is indicated by the arrow. Expression of pCT0017 protein was not observed in cell lysates of the non-induced (-) BL21 [pETCF] strains.



**Figure 5.4: SDS-PAGE of affinity purified His-tagged pCT0017\* protein.**

His-tagged pCT0017 protein (pCT0017\* protein) was purified from induced BL21 [pETCF] whole cell lysate using HisLink™ resin. Samples of the 1 mL eluted fractions were electrophoresed on a 15% SDS-PAGE and stained with Coomassie blue. The marker lane was loaded with a prestained broad range marker. The apparent molecular weight (kDa) of relevant marker protein bands are indicated. The arrow indicates the band (19.37 kDa) corresponding to pCT0017\* protein in fractions 2, 3, 4 and 5.



**Figure 5.5: Titration of purified pCT0017\* protein.**

Panel A. pCT0017\* protein (0 – 1000 ng mL<sup>-1</sup>) was incubated with labelled *ctpA* fragment and used in gel shift assays. The arrows indicate DNA that is shifted by non-specific binding of excess pCT0017\* protein ( $\geq 500$  ng mL<sup>-1</sup>). Lanes without added pCT0017\* protein (0 ng mL<sup>-1</sup>) show migration of unbound DNA.

Panel B. pCT0017\* protein (0 – 1000 ng mL<sup>-1</sup>) was incubated with labelled *hly* fragment and used in gel shift assays. The arrows indicate DNA that is shifted by non-specific binding of excess pCT0017\* protein ( $\geq 500$  ng mL<sup>-1</sup>). Lanes without added pCT0017\* protein (0 ng mL<sup>-1</sup>) show migration of unbound DNA.

The gel shift assays shown were conducted without added Cu<sup>2+</sup>.

**Figure 5.6: Interaction of pCT0017\* protein with DRDC8 DNA.**

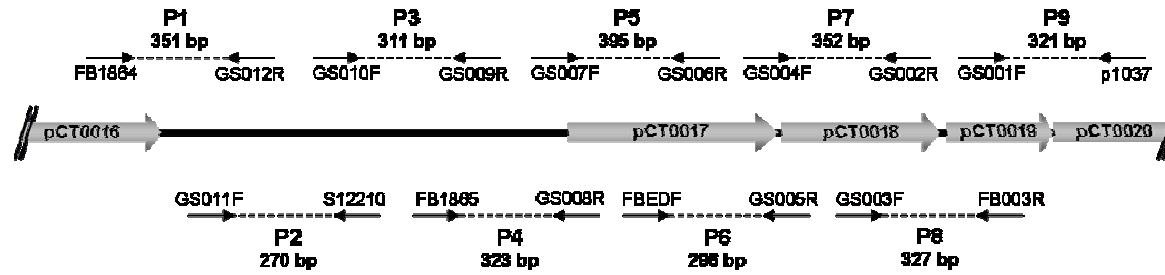
Panel A. The DNA region encoding fragments P1, P2, P3, P4, P5, P6, P7, P8, and P9 used for gel shift analysis is represented schematically (not to scale). Oligonucleotides are represented by arrows in the direction of priming and are positioned at their relative location. Fragments P1, P2, P3, P4, P5, P6, P7, P8 and P9 were amplified by PCR using the FB1864/GS012R, GS0011F/S12210R, GS010F/GS009R, FB1865/GS008R, GS007F/GS006R, FBEDF/GS005R, GS004F/GS002R, GS003F/FB003R and GS001F/p1037 oligonucleotide pairs, respectively. The respective nucleotide sequence for each oligonucleotide is listed in Table 2.7 or Table 2.8.

Panel B. Labelled fragments P1, P2, P3 and the negative control *ctpA* fragment were incubated with purified pCT0017\* protein (+). The mixtures were then used in gel shift assays. Lanes containing labelled DNA alone (-) show migration of unbound DNA. DNA in all lanes migrated to a position indicative of unbound DNA.

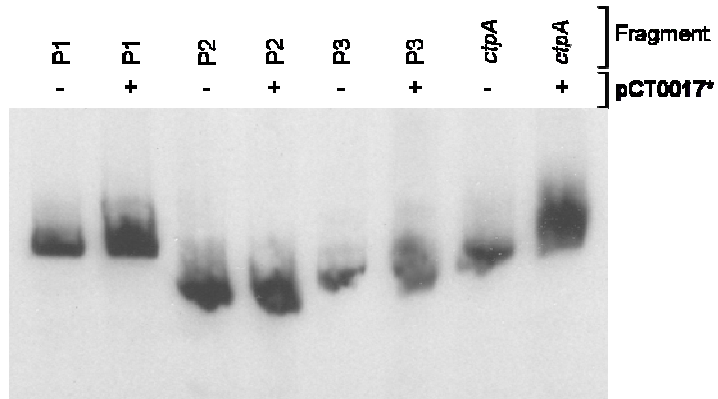
Panel C. Labelled fragments P5, P6, P7, P8, P9 and the negative control *ctpA* fragment were incubated with purified pCT0017\* protein (+). The mixtures were then used in gel shift assays. Lanes containing labelled DNA alone (-) show migration of unbound DNA for each fragment. DNA in all lanes migrated to a position indicative of unbound DNA.

The gel shift assays shown were conducted without added  $\text{Cu}^{2+}$ .

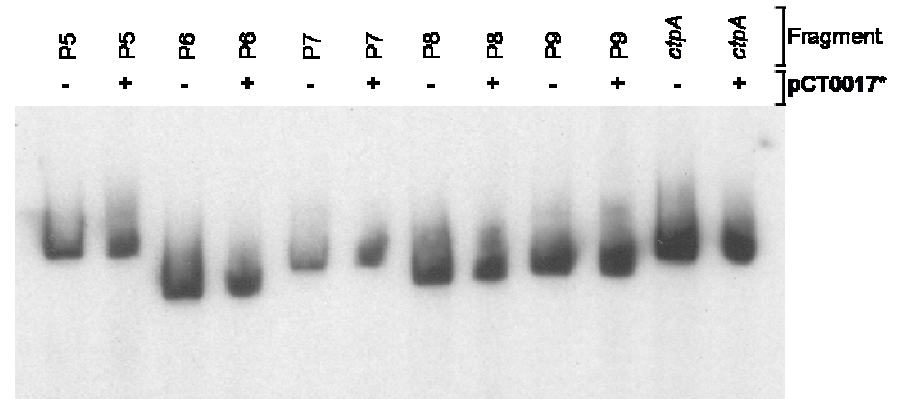
A

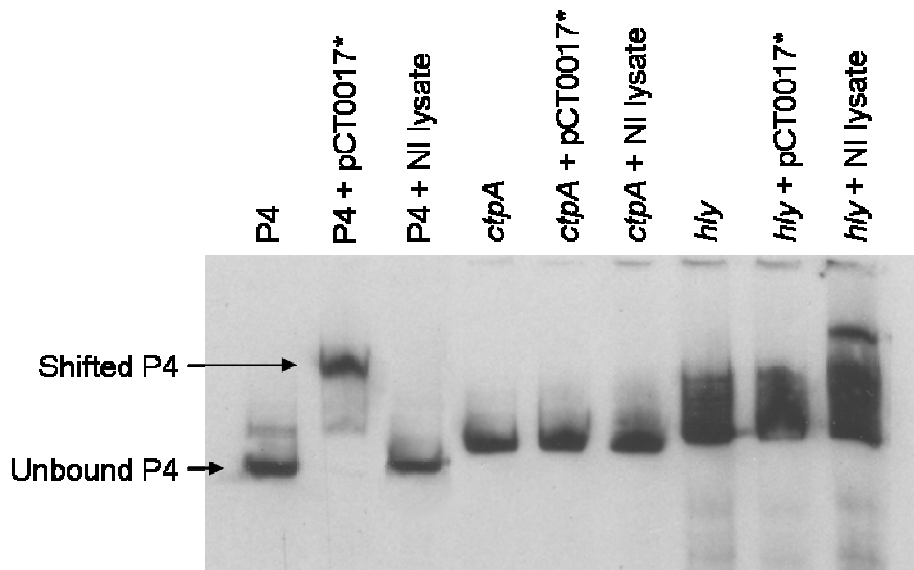


B



C





**Figure 5.7: Interaction of pCT0017\* protein with DNA fragment P4.**

Labelled fragment P4, and the negative control *ctpA* and *hly* DNA fragments were incubated with either purified pCT0017\* protein or non-induced BL21 [pETCF] whole cell lysate (NI lysate). The mixtures were then used in gel shift assays. Lanes containing labelled DNA alone (P4, *ctpA* or *hly*) show migration of unbound DNA for each fragment. Shifting of P4 by pCT0017\* protein is indicated by the top arrow and migration of unbound P4 DNA is indicated by the bottom arrow. DNA in all other lanes migrated to a position indicative of unbound DNA.

The gel shift assay shown was conducted without added  $\text{Cu}^{2+}$ .

**Figure 5.8: Interaction of pCT0017\* protein with DNA fragments P14, P24 and P34.**

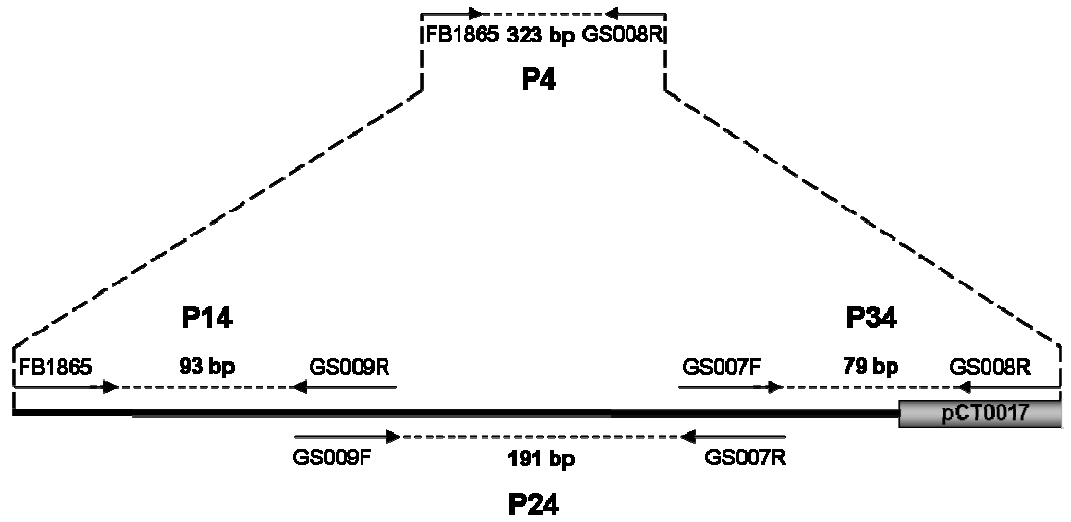
Panel A. The DNA region analysed using gel shift analysis is represented schematically (not to scale). Oligonucleotides are represented by arrows in the direction of priming and are positioned at their relative location. Products 14 (P14), 24 (P24) and 34 (P34) were amplified by PCR across the region encompassed by fragment P4 (see Figure 5.6) using the oligonucleotide pairs FB1865/GS009R, GS009F/GS007R and GS007F/GS008R respectively. The length of each DNA fragment amplified is indicated. The respective nucleotide sequence for each oligonucleotide is listed in Table 2.7 or Table 2.8.

Panel B. Labelled fragments P4, P14, P24 and P34 were incubated with purified pCT0017\* protein (+). The mixtures were then used in gel shift assays. Lanes containing labelled DNA alone (-) show migration of unbound DNA for each fragment. Shifting of the P4 positive control fragment by pCT0017\* protein is indicated by the top arrow. The migration of unbound P4 DNA is indicated by the bottom arrow in the lane containing labelled P4 DNA alone. Shifting of P24 by pCT0017\* protein is indicated (\*). Unbound P24 DNA is also indicated (#) in the lane containing DNA not incubated with protein. DNA in the lanes containing P14 and P34 incubated with pCT0017\* protein migrated to the same position as labelled P14 and P34 not incubated with protein.

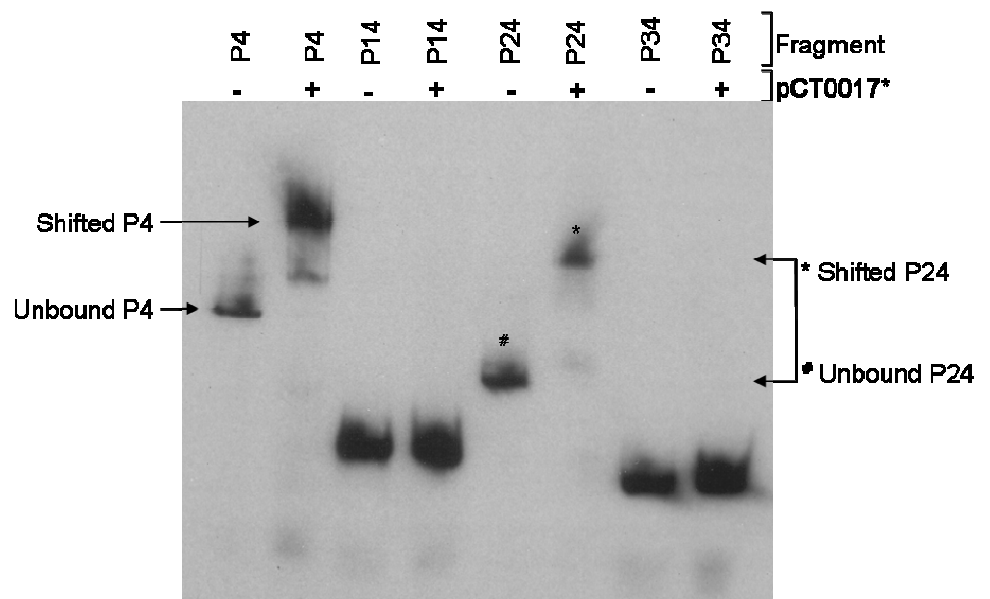
The gel shift assay shown was conducted without added  $\text{Cu}^{2+}$ .



**A**



**B**

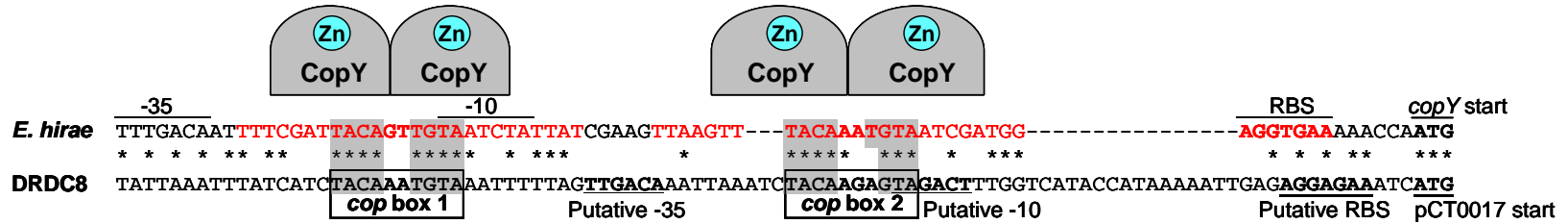


**Figure 5.9: Comparison of *cop* boxes of strain DRDC8 and other bacteria.**

Panel A. A schematic comparison between the putative promoter region of pCT0017 in DRDC8 and the *cop* operon promoter region in *E. hirae* (Strausak & Solioz, 1997). The conserved '*cop* box' inverted repeats are shaded in grey. Open boxes indicate the *cop* box 1 and *cop* box 2 motifs identified in DRDC8. A putative ribosome binding site (RBS) and putative -10 (TAGACT) and -35 (TTGACA) regions are also shown relative to the start of ORF pCT0017. The [Zn(II)CopY]<sub>2</sub>-DNA interaction of the *E. hirae* promoter is shown (Portmann *et al.*, 2004). A [Zn(II)CopY]<sub>2</sub> dimer binds to each of the two *cop* boxes and protects the regions indicated in red (Strausak & Solioz, 1997). \* Represents identical base pairs.

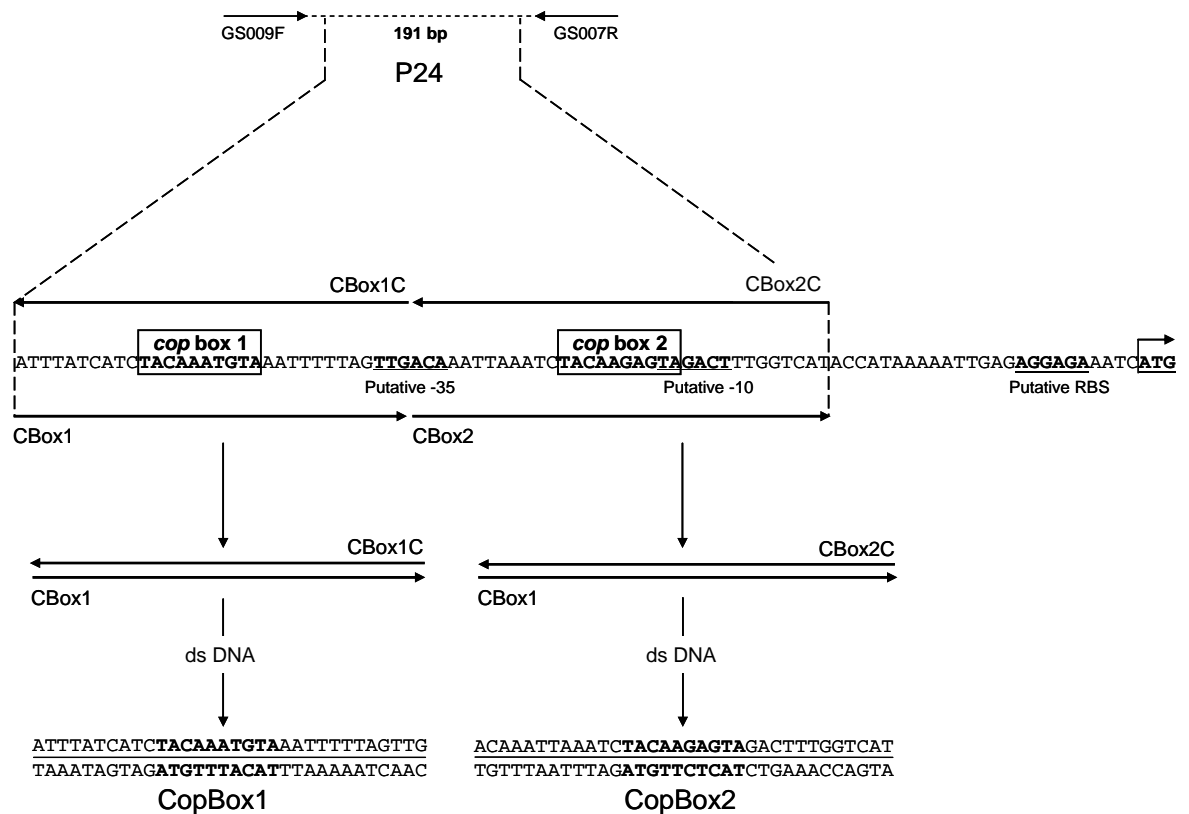
Panel B. The putative promoter region of *L. monocytogenes* strain DRDC8 containing *cop* box 1 and *cop* box 2, the promoter *copY* sequence containing putative CopY binding sites for 14 strains (9 species) (Reyes *et al.*, 2006) and the *copY* promoter of *E. hirae* (Portmann *et al.*, 2004) are shown. Open boxes indicate consensus *cop* box sequences. The numbers at both sides of the promoter *copY* sequences indicate nucleotide position with respect to the first codon of CopY. Figure modified from Reyes *et al.*, (2006).

A



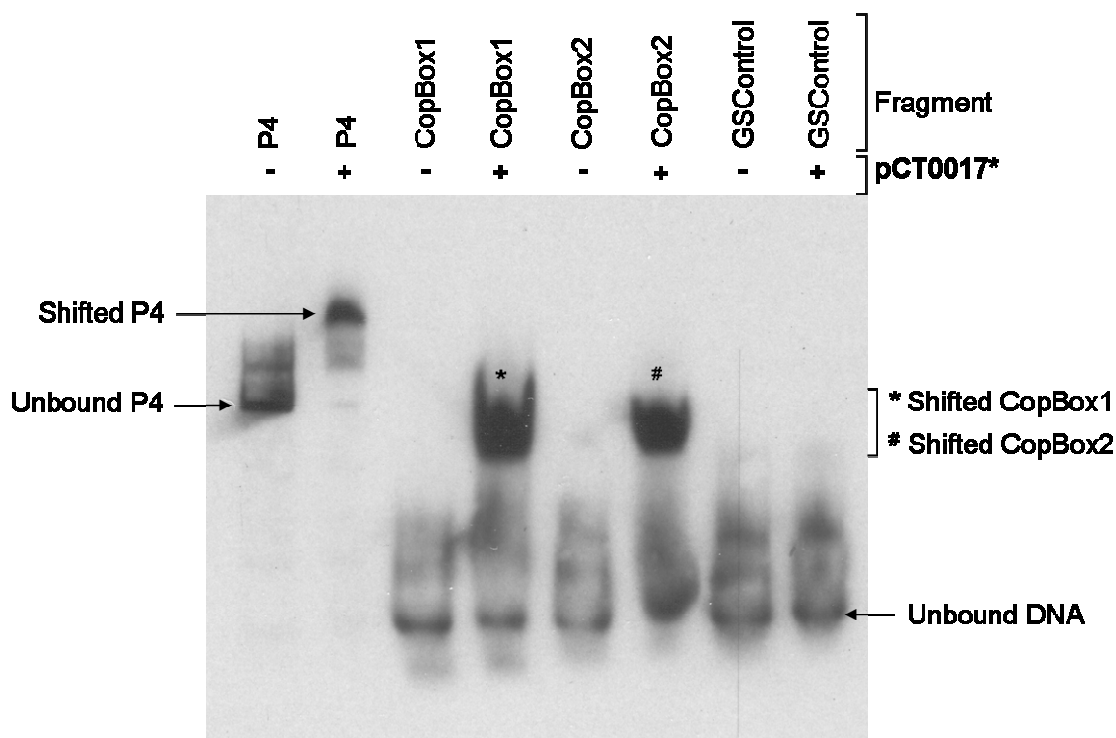
B

Strains	Promoter <i>copY</i> sequence
<i>E. hirae</i> ATCC 9770	-65 GAT TACAGTGTAACTATTATCGAAGTAAAGTTTACAAATGTAATCGATGGAGGT -10
<i>E. faecalis</i> V583	-63 GAC TACATTTGTAGTCGATAACC ----TTGAACCTTTTTTATTGATGAAGGAGGA -12
<i>L. johnsonii</i> NCC 533	-59 GTT GACAAGTGTAAC TAAAAGTTATATGAT TACATTTGTA AACGAAAGGAATATA - 4
<i>L. plantarum</i> WCFS	-50 GTT TACGGTGTAAAC TACAGTTAATTTAATTCGAAGGGTGGTC -AAACCATTGGA + 5
<i>L. lactis</i> IL1403	-51 GTT TACACGTGTAAAC TTATCTTATGAAAATGAATCGGGAAAGGAGAAAAAATGAA + 5
<i>S. mutans</i> UA159	-61 GTT GACAAATGTAGACAAAGAGGTTATAATGTATCTACAAATGTAGATGAAAGGAG - 6
<i>S. agalactiae</i> 2603V/R	-64 CTT TACAAATGTAGATTTATTT --GCTAAAATTCGTTTACAAACGTAAAT ----- -17
<i>S. agalactiae</i> 2603V/R	-67 GTT TACAATGGTAACTTATCTTGAACATA -TTCGTTTACATCTGTAAACA --ACG -15
<i>S. agalactiae</i> NEM316	-64 GTT TACAAATGTAGATTTATTT --GCTAAAATTCGTTTACAAACGTAAAT ----- -17
<i>S. pyogenes</i> MGAS315	-70 -TTTACAAATGTAGTTAAATTC --GTATAATTAATTTACAAACGTAAATATATTTG -18
<i>S. pyogenes</i> MGAS8232	-72 -TTTACAAATGTAGTTAAATTCGCGTTATAATTAATTTACAAACGTAAATATATTTG -18
<i>S. pyogenes</i> SF370	-72 -TTTACAAATGTAGTTAAATTCGCGTTATAATTAATTTACAAACGTAAATATATTTG -18
<i>S. pyogenes</i> SSI-1	-70 -TTTACAAATGTAGTTAAATTC --GTATAATTAATTTACAAACGTAAATATATTTG -18
<i>S. mitis</i> NCTC12261	-71 GTT GACAAATGTAGATTTTAAAGAGTATACTGATAAATTGTAATTGACAAATGTAGAT -16
<i>S. pneumoniae</i> R6	-65 ATT GACAAATGTAGATTTTAAAGAGTATACTGATGAGTGTAATTGACAAATGTAGAT -10
<i>S. pneumoniae</i> TIGR4	-65 ATT GACAAATGTAGATTTTAAAGAGTATACTGATGAGTGTAATTGACAAATGTAGAT -10
<i>L. monocytogenes</i> DRDC8	-84 ATCTACAAATGTAAATTTT TAGTTGACAAATTAATCTACAAGAGTAGACTTTGGT -28



**Figure 5.10: Construction of CopBox1 and CopBox2.**

Oligonucleotides CBox1, CBox1C, CBox2 and CBox2C were designed within the region encompassed by fragment P24 (refer to Figure 5.8). These oligonucleotides are represented by arrows in the direction of priming and positioned at their relative location. Oligonucleotides CBox1 and CBox2 were designed on the 5' DNA strand containing *cop box 1* and *cop box 2*, respectively. Complementary oligonucleotides CBox1C (complementary of CBox1) and CBox2C (complementary of CBox2) were designed to create a 32 bp (CopBox1) and a 34 bp (CopBox2) double stranded DNA fragment. CBox1 and CBox2 were mixed with an equimolar amount of CopBox1C and CopBox2C, respectively. The oligonucleotide CBox1/CBox1C and CBox2/CBox2C mixes were rapidly heated to 80°C and then cooled to 30°C for 1 h to create double stranded DNA fragments CopBox1 and CopBox2 respectively. The nucleotide sequences of these DNA fragments are shown.



**Figure 5.11: Interaction of pCT0017\* protein with CopBox1 and CopBox2.**

Labelled DNA fragments CopBox1 (32 bp) and CopBox2 (34 bp) (see Figure 5.10) were incubated with purified pCT0017\* protein (+). The 34 bp double stranded negative control DNA fragment GSControl and the positive control fragment P4 were also incubated with purified pCT0017\* protein (+). The mixtures were then used in gel shift assays. Lanes containing labelled DNA alone (-) show migration of unbound DNA for each fragment.

Shifting of the P4 positive control fragment by pCT0017\* protein is indicated by the top left arrow. Migration of unbound P4 DNA in the lane which contained labelled P4 DNA alone is also indicated by the bottom left arrow. Shifting of CopBox1 by pCT0017\* protein is indicated (\*). Shifting of CopBox2 by pCT0017 is also indicated (#). Migration of unbound DNA fragments in the lanes containing labelled CopBox1, CopBox2 and GSControl alone are indicated by the arrow on the right. DNA in the lanes containing GSControl incubated with pCT0017\* protein migrated to the same position as labelled GSControl alone.

The gel shift assay shown was conducted without added  $\text{Cu}^{2+}$ .

**Figure 5.12: Effect of CuSO<sub>4</sub> and CdSO<sub>4</sub> on pCT0017\* protein binding to P4.**

Labelled DNA fragment P4 was incubated with purified pCT0017\* protein (+) in the presence of different concentrations of added CuSO<sub>4</sub> or CdSO<sub>4</sub>. The mixtures were then used in gel shift assays. A lane containing labelled P4 DNA alone (-) shows migration of unbound DNA.

Panel A. Shifting of P4 by pCT0017\* protein in the absence of added CuSO<sub>4</sub> (0 mM) and in the presence of 1 μM, 500 nM, 1 nM, 500 pM and 1 pM CuSO<sub>4</sub> is indicated by the top arrow. Migration of unbound P4 DNA in the lane containing labelled P4 DNA alone is indicated by bottom arrow. Lanes containing DNA incubated with purified pCT0017\* protein in the presence of 14 mM, 10 mM, 5 mM, 1 mM and 500 μM CuSO<sub>4</sub> migrated to the same position as labelled P4 DNA alone, indicative of unbound DNA.

Panel B. Shifting of P4 by pCT0017\* protein in the absence of added CdSO<sub>4</sub> (0 mM) and in the presence of 14 mM, 10 mM, 5 mM, 1 mM and 500 μM, 1 μM, 500 nM, 1 nM, 500 pM and 1 pM CdSO<sub>4</sub> is indicated by the top arrow. Migration of unbound P4 DNA in the lane which contained labelled P4 DNA alone is indicated by the bottom arrow.



**Figure 5.13: Interaction of pCT0017\* protein with CopBox1 and CopBox2 in the presence of CuSO<sub>4</sub>.**

Panel A. Labelled fragments P4, CopBox1 and CopBox2 were incubated with purified pCT0017\* protein (+) in either the absence (0 mM) or presence of 1  $\mu$ M added CuSO<sub>4</sub>. The mixtures were then used for gel shift assays. Lanes containing labelled DNA alone (-) show migration of unbound DNA.

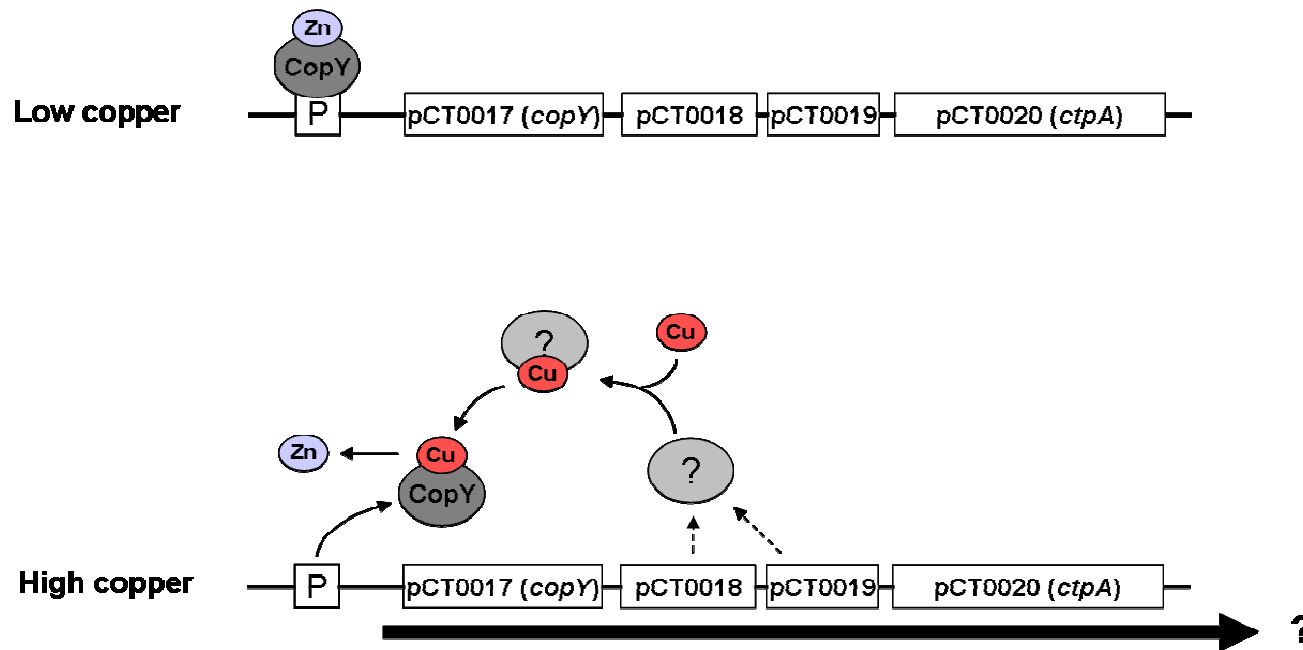
Shifting of the positive control fragment P4 by pCT0017\* protein in the absence of added CuSO<sub>4</sub> and in the presence of 1  $\mu$ M CuSO<sub>4</sub> is indicated by the top left arrow. Migration of unbound P4 DNA in the lane which contained labelled P4 DNA alone is indicated by the bottom left arrow. Shifting of CopBox1 (\*) and CopBox2 (#) by pCT0017\* protein in the absence of CuSO<sub>4</sub> and the presence of 1  $\mu$ M CuSO<sub>4</sub> is indicated. Migration of unbound CopBox1 and CopBox2 DNA in the lanes which contained labelled DNA alone is indicated by the right arrow.

Panel B. Labelled fragments P4, CopBox1 and CopBox2 were incubated with purified pCT0017\* protein (+) in either the absence (0 mM) or presence of 500  $\mu$ M added CuSO<sub>4</sub>. The mixtures were then used for gel shift assays. Lanes containing labelled DNA alone (-) show migration of unbound DNA.

Shifting of the positive control fragment P4 by pCT0017\* protein in the absence of added CuSO<sub>4</sub> is indicated by the top left arrow. Migration of unbound P4 DNA in the presence of 500  $\mu$ M CuSO<sub>4</sub> and in the lane containing labelled P4 DNA alone is indicated by bottom left arrow. Shifting of CopBox1 (\*) and CopBox2 (#) by pCT0017\* protein in the absence of CuSO<sub>4</sub> is indicated. Migration of unbound CopBox1 and CopBox2 DNA in the presence of 500  $\mu$ M CuSO<sub>4</sub> and in the lanes containing labelled CopBox1 and CopBox2 DNA alone is indicated by the right arrow.







**Figure 5.14: Proposed model of copper-responsive gene regulation by pCT0017.**

At low levels of copper ( $< 1 \mu\text{M}$ ),  $[\text{Zn(II)pCT0017}]_2$  dimers bind to *cop* box 1 and *cop* box 2 motifs of the pCT0017 promoter region (P) to repress transcription of the associated downstream genes (ie. pCT0017, pCT0018, pCT0019 and pCT0020). At high copper levels ( $> 500 \mu\text{M}$ ), an unknown copper chaperone delivers copper ions to pCT0017, which loses the bound zinc and releases the pCT0017 protein from the promoter. This relieves repression and allows transcription of associated downstream genes to proceed. The protein encoded by pCT0018 or pCT0019 may function as the copper binding protein that delivers copper to pCT0017, as indicated by the dashed arrow. Protein pCT0017 may also control expression of other downstream genes.

## Chapter 6: General Discussion

### 6.1 Introduction

*Listeria monocytogenes* is a Gram-positive, intracellular pathogen that causes listeriosis, a severe invasive illness in both humans and animals. The prevalence of listeriosis in some developed countries has reportedly increased in recent years (Garcia-Alvarez *et al.*, 2006; Koch & Stark, 2006). *L. monocytogenes* is widely distributed within the environment and capable of survival and multiplication in extreme habitats. Significantly, this organism is prevalent in food processing, distribution and storage environments (Jemmi & Stephan, 2006). Moreover, there is limited knowledge of the ecology of this deadly bacterium, particularly the mode of transmission and survival in the environment. This makes control of *L. monocytogenes* in food a significant challenge.

*Listeria* genome sequencing projects have allowed characterisation of genetic mechanisms that enable this microorganism to adapt to and withstand extreme and adverse environments. From the available sequence data, common genetic themes are evident between *Listeria* species, including genes that encode proteins involved in cation transport and homeostasis. The fact that these genes are located predominantly on plasmid DNA, suggests that plasmids may be important for survival of *L. monocytogenes* in conditions of severe cation stress. Indeed, plasmid genes encoding cadmium resistance have been suggested to confer a significant selective advantage to *L. monocytogenes* in the environment (Lebrun *et al.*, 1992). Tolerance to cation stress seems also to be important for survival and replication of pathogens such as *L. monocytogenes* where they encounter environments containing subtoxic or starvation levels of metal ions during host infection (Agranoff & Krishna, 1998). Significantly, a gene (*ctpA*) involved in copper transport has been shown to be significant for virulence of *L. monocytogenes* (Francis & Thomas, 1997b).

The *ctpA* gene encodes a copper transporting P-type ATPase (CtpA) that apparently maintains intra-cellular copper ion homeostasis in *L. monocytogenes* strain DRDC8 (Francis & Thomas, 1997a). CtpA is required for persistence of *L. monocytogenes* infection in the liver and spleens of infected mice (Francis & Thomas, 1997b).

Importantly, *ctpA* is likely to be plasmid-encoded and flanked by other genes implicated in copper ion homeostasis (This Thesis; Bell, 2002). Whilst apparently significant for virulence, the distribution of *ctpA* amongst *L. monocytogenes* isolates from clinical, environmental and food sources is limited (Bell, 2002; Francis, 1996; Webster, 2001).

One aim of this thesis was to definitively show that *ctpA* is plasmid-encoded and determine if additional accessory genes are involved in copper homeostasis in *L. monocytogenes* strain DRDC8. In addition, the work described in this thesis analysed the effects of independent mutation of ORFs implicated in copper homeostasis as well as loss of plasmid DNA on the ability of *L. monocytogenes* to tolerate cation stress. Furthermore, experimental evidence to show that ORF pCT0017 encodes a CopY-like copper-responsive transcriptional repressor protein that is able to bind specifically to DNA encoding *cop* box-like motifs was provided.

## **6.2 Plasmid-encoded Genes are Important for Environmental Survival of *L. monocytogenes* strain DRDC8**

Southern hybridisation and sequence analysis confirmed that the *ctpA* gene is encoded by plasmid pCT100 in *L. monocytogenes* strain DRDC8. While not previously characterised for *Listeria*, mechanisms for plasmid-borne copper resistance have been characterised in other Gram-positive bacteria (Hasman, 2005; Liu *et al.*, 2002; Ug & Ceylan, 2003). Sequence data gathered for part of plasmid pCT100 (as a collaborative laboratory project) also provided important information about the probable function of the proteins encoded by other genes carried on this plasmid. The part of plasmid pCT100 sequenced, indicates that this segment is similar in terms of gene content and organisation to other characterised *Listeria* plasmids, particularly plasmid pLI100 from *L. innocua* CLIP11262 and plasmid pLM80 from *L. monocytogenes* strain 4b H7858.

Conserved DNA regions were identified between plasmids pCT100, pLI100 and pLM80, particularly a plasmid DNA replication region. The existence of homologous regions between native plasmids in *Listeria* has been previously described by other authors (Flamm *et al.*, 1984; Kolstad *et al.*, 1991; Margolles & de los Reyes-Gavilan, 1998). These conserved regions are all flanked by near identical transposable DNA elements and this suggests that these plasmids may have evolved from a common ancestor plasmid. In

addition, the presence of transposase/invertase-like elements indicates that *Listeria* plasmids are mosaics created by a series of insertion and deletion events. Genetic divergence and evolution from an original source is likely to have occurred through a series of DNA acquisitions and/or deletions as a result of environmental adaptation and/or genetic exchange with other co-habiting microorganisms.

Genome sequencing has shown that the physiological and virulence potential of bacterial pathogens reflects the presence of different genes that enable different lifestyles. Indeed, the use of comparative genomics and DNA arrays has shown that *L. monocytogenes* strains are genetically diverse (Doumith *et al.*, 2004). These differences are most pronounced in genes encoding proteins that are most likely to confer traits that provide selective advantages in the environment and in infected hosts. Horizontal gene transfer of DNA encoding novel genes required to enhance opportunities for niche specific survival has clearly lead to evolutionary speciation and diversity of the *Listeria* species (Hain *et al.*, 2006a). This is a reflection of this organism's ability to adapt to and colonise a range of diverse ecological niches.

While it is evident that plasmid pCT100 of *L. monocytogenes* strain DRDC8 carries large blocks of colinear sequence that are homologous with other *Listeria* plasmids, non-conserved regions of DNA that differ substantially from other *Listeria* sequences also exist. Specifically, these regions share significant similarity to proteins encoded by the chromosomes of other Gram-positive bacteria, particularly *Enterococcus* as well as *Bacillus*, *Streptococcus* and *Lactobacillus* species. Most significantly, a DNA region encoding a series of polypeptide sequences similar to proteins involved in copper transport, regulation of copper transport, copper binding or contain copper reactive centres, has been identified. The ORFs encompassed by this region (pCT0017, pCT0018, pCT0019 and pCT0020 (*ctpA*), pCT0023, pCT0024, pCT0025, pCT0026, pCT0027) represent a cluster of genes implicated in copper homeostasis/tolerance in strain DRDC8. While *ctpA* has been shown to be involved in copper ion transport and tolerance (Francis & Thomas, 1997a), this study has shown that pCT0017, pCT0018 and pCT0019 are also involved in copper ion tolerance and are thus implicated in copper homeostasis for strain DRDC8. However, further investigation is required to substantiate that the flanking ORFs

(pCT0023, pCT0024, pCT0025, pCT0026 and pCT0027) of this gene cluster are also involved in copper ion tolerance and homeostasis.

Given that this cluster of ORFs may be restricted to a limited number of isolates including DRDC8 and only some other *ctpA* positive *L. monocytogenes* isolates, it is highly likely that this region has been acquired by horizontal gene transfer. However, the origin of this gene cluster remains a matter of speculation. Based on genetic similarity it seems likely that these genes have been acquired from chromosomal DNA of other Gram-positive bacteria, possibly from strains of *Enterococcus* and/or *Lactobacillus* species. The ubiquitous distribution of *Listeria* spp. highlights the potential for genetic transfer with other microorganisms that share common environments. Given that strain DRDC8 and other strains carrying the putative gene cluster are typically of dairy origin, this may include strains of bacteria that are prevalent within ruminant animals and other farm environments, for example, *Enterococcus* and *Lactobacillus*.

Studies of antibiotic resistance in *Listeria* have clearly shown that genetic material involved in short-term evolutionary adaptation can be successfully transferred between *Listeria* spp., and between *Listeria* and related Gram-positive bacteria including *Enterococci* (Biavasco *et al.*, 1996; Doucet-Populaire *et al.*, 1991; Poyart-Salmeron *et al.*, 1990; Roberts *et al.*, 1996). Acquisition of the cluster of genes involved in copper ion tolerance/homeostasis is likely to have occurred in response to exposure of the bacterium to conditions of severe copper ion stress. Indeed, most bacterial species that have been tested and found positive for increased tolerance to copper are environmental isolates from locations with high exposure to metals (Aarestrup & Hasman, 2004). This is discussed further in detail in Section 6.4.

The copper gene cluster of plasmid pCT100 from strain DRDC8 may have been originally acquired as a cassette/islet in a single recombination event or separate genes may have been acquired through a series of events that occurred specifically during evolution of this particular strain. Several authors have shown that mobile genetic elements such as plasmids and transposons are responsible for the acquisition of antibiotic resistance in *L. monocytogenes* and *L. innocua* (Charpentier *et al.*, 1994; Facinelli *et al.*, 1993; Flamm *et al.*, 1984; Poyart-Salmeron *et al.*, 1990; Poyart-Salmeron *et al.*, 1992; Vicente *et al.*, 1988). Acquisition of this putative gene cluster may have been mediated by horizontal gene

transfer from another bacterium followed by integration via IS transposons, integrative replicons and homologous recombination. Alternatively, bacteriophages may have played a role in acquisition. However, this study was not able to establish carriage of *ctpA* (pCT0020) by bacteriophage isolated from DRDC8.

Experiments that showed that plasmid pCT100 is not conjugative suggested lateral transfer of this plasmid between cohabitating organisms may be limited. This may explain the restricted prevalence of *ctpA* and other ORFs associated with this gene cluster to only few other isolates of environmental origins. However, it is also possible that many isolates carry *ctpA* on large plasmid, but that during infection of clinically significant hosts and/or enrichment and isolation of this bacterium from clinical or environmental specimens, the plasmid is lost. Unpublished data by Akya, (2007) has demonstrated significant loss of plasmid pCT100 by strain DRDC8 during serial culture or during passage in HeLa cell culture. This suggested that the plasmid may be unstable in the absence of specific selection and as a consequence, may be lost during growth in enrichment and selective media normally used to isolate *L. monocytogenes* from clinical and environmental samples. The fact that plasmid pCT100 encodes numerous genes implicated in copper homeostasis suggests copper levels could be a specific selective for carriage of the plasmid. Whether the copper gene cluster can be exploited to enable selection for isolates that carry this plasmid remains to be tested. It is possible that when grown in enriched laboratory media, adequate supplies of copper is present and these genes may not be required. Only specific environments may encompass sufficient copper ion stress to select for carriage of plasmid DNA. If these genes offer no advantage for survival, they could be eliminated over time.

If carriage of the plasmid pCT100 copper gene cluster is directly influenced by environmental copper levels, this may explain why it is only present in some *ctpA* positive isolates. Some *L. monocytogenes* strains may inhabit environments where *ctpA*-mediated mechanisms alone are sufficient for survival. Survival of strains that inhabit copper-rich environments may be dependant on genes such as those in the pCT100 gene cluster. These two distinct genotypes may have arisen from different insertion/deletion events in response to the environmental copper requirements.

Carriage of genes involved in copper tolerance may also confer an advantage for *L. monocytogenes* by expanding the possibility for survival and multiplication in different

environments. The fact that loss of plasmid pCT100 for strain DRDC8 is associated with a significant reduction in tolerance to both copper and cadmium stress certainly supports this proposal. These findings suggested that plasmid pCT100 could directly facilitate survival and contribute to the ecology of *L. monocytogenes* in environments containing cation stress. Moreover, given that the plasmid-encoded *ctpA* gene has been shown to be essential for persistent infection in the liver and spleens of mice (Francis & Thomas, 1997b), it is possible that proteins encoded by plasmid pCT100 assist host cell infection by *L. monocytogenes*. Whilst the fact that carriage of plasmid DNA (Kolstad *et al.*, 1992; Perez-Diaz *et al.*, 1982; Peterkin *et al.*, 1992) and plasmids encoding *ctpA* (Akya, 2007; Bell, 2002) is limited to few *L. monocytogenes* isolates of clinical origin, this observation indicates that plasmid DNA may not be critical for human infection. However, as previously discussed, it is possible that strains of *L. monocytogenes* involved in clinical infections lose plasmid DNA during parasitism of the human host and subsequent isolation (Akya, 2007).

It is interesting to speculate that genes encoded by plasmid DNA that were originally acquired to facilitate survival of this saprophytic organism in the natural environment, could assist the bacterium to establish a persistent infection in human hosts. If substantiated, this may begin to explain how this environmental organism has evolved an ability to invade and mobilise within eukaryotic cells.

### **6.3 Chromosomal and Plasmid Genes are Involved in Copper Tolerance in *L. monocytogenes* strain DRDC8**

Although copper is an essential trace element for all living cells, with roles in gene regulation, enzyme structure and catalysis, copper ions are toxic when present in excess. Cellular systems involving the acquisition, sequestration, intracellular distribution, and efflux of copper are used to respond to changes in copper ion concentrations and meet physiological requirements for copper ions, while also preventing deleterious effects caused by presence of free  $\text{Cu}^{2+}$ .

Some microbes are able to colonise environments containing concentrations of copper ions that would overwhelm chromosomally encoded copper metabolic systems like the *copYZAB* system of *E. hirae* (Strausak & Solioz, 1997). In Gram-negative bacteria, plasmid-encoded determinants that confer additional copper resistance are often found



in environments with exceptionally high levels of copper (refer to Section 6.4 for examples) (Hasman *et al.*, 2006; Munson *et al.*, 2000). In Gram-positive bacteria there are some recent reports of additional, plasmid-encoded copper resistance (Hasman, 2005; Liu *et al.*, 2002). Given that *L. monocytogenes* strain DRDC8 carries both chromosomal and plasmid-encoded genes that are implicated in copper homeostasis, this dairy isolate may possess similar mechanisms to tolerate, and colonise/survive environments containing toxic levels of copper.

*L. monocytogenes* strain DRDC8 has an exceptional tolerance (MIC) for copper. This tolerance is comparable with MIC values of other Gram-positive and Gram-negative bacteria isolated from animal environments, including strains of *E. faecalis*, *E. faecium*, *Salmonella* spp. and *E. coli* (Aarestrup & Hasman, 2004; Hasman & Aarestrup, 2002). However, these MIC values are significantly higher than that recorded for other wild type *L. monocytogenes* strains analysed, particularly strain EGD Kaufmann. Whether the gene cluster encoding proteins can alone confer this high level copper tolerance in *L. monocytogenes* has yet to be established. Nevertheless, it is of interest to note that in addition to CopA/B and CopY-like proteins, ORFs encoding a putative two-component regulatory system, a multicopper oxidase, and a prolipoprotein diacylglycerol transferase have been identified in this gene cluster. These additional proteins are also common to plasmid-encoded copper resistance systems in other Gram-positive bacteria (Hasman, 2005; Liu *et al.*, 2002; Ug & Ceylan, 2003). In *L. lactis*, these proteins apparently confer copper resistance by reducing the intracellular accumulation of copper (Liu *et al.*, 2002). It is therefore likely that in strain DRDC8, these proteins act in a similar manner to confer copper tolerance. For example, the multicopper oxidase encoded by pCT0025 may function in a similar manner to the *L. lactis* LcoC multicopper oxidase (Liu *et al.*, 2002).

The importance of the chromosomal gene (ORF *cutR*) for tolerance to copper ion stress for isolates such as EGD Kaufmann is significant. This strain does not carry *ctpA* or other ORFs associated with the DRDC8 copper gene cluster, yet is able to maintain intracellular copper homeostasis. As a *cutR*-like gene is present in other *Listeria* strains characterised to genome sequencing, it is highly probable that all *Listeria* strains carry a chromosomally-encoded *cutR* homologue. This gene is likely to be required for survival in an environment containing adequate supplies of copper. Strain DRDC8 and associated dairy and poultry

strains that carry the copper gene cluster may possess these additional plasmid-encoded copper homeostasis mechanisms for survival in copper-rich environments (refer to Section 6.4). This proposal is supported by the fact that loss of plasmid DNA has a more severe effect on the growth potential of *L. monocytogenes* DRDC8 grown under conditions of high copper concentrations (14 mM) compared to loss of *cutR* alone.

Interestingly *cutR*, together with the plasmid-encoded copper gene cluster is critical for tolerance of *L. monocytogenes* to copper stress. Loss of these genes (as a result of curing *cutR::erm* derivatives of the DRDC8 plasmid pCT100) renders *L. monocytogenes* unable to grow in media containing excess copper (14 mM). This observation indicated that these constructs cannot export the excess copper ions from the cytoplasm and suggested that a tight relationship exists between these two mechanisms to culminate copper ion tolerance. Mutation of *cutR*, or loss of pCT100 plasmid DNA alone, is not associated with such a catastrophic effect on growth of *L. monocytogenes* in conditions of copper excess. This suggested that these systems complement one another. Consequently, loss of one system allows the other to maintain a sufficient level of copper homeostasis that enables growth of *L. monocytogenes* in these conditions.

While *cutR* and the copper gene cluster may function in copper tolerance collaboratively, given that these genes are encoded by different elements of DNA they are unlikely to be transcriptional linked. A flanking chromosomal gene may be involved in regulation of expression of *cutR*. Future studies should aim to characterise the sequence data flanking *cutR* to identify if a gene encoding a protein pertaining to such function exists.

Undoubtedly ORFs pCT0018 and pCT0019 are also involved in copper tolerance of *L. monocytogenes* strain DRDC8. The fact that insertion mutation of these ORFs significantly affects the ability of *L. monocytogenes* to grow in conditions of copper excess (14 mM) strongly implicates these genes in copper ion tolerance. Furthermore, ORF pCT0017 encodes a CopY-like protein. Copper-dependant binding of pCT0017 protein to two *cop* box-like sequences located upstream of the pCT0017 translation start site is consistent with published models of CopY-like protein function (refer to Section 1.8.2.1). Although not proven experimentally, it is likely that pCT0017 protein can regulate expression of ORFs pCT0017, pCT0018, pCT0019 and *ctpA* in a copper responsive

manner by differential binding to the *cop*-box motifs. At high copper concentrations, it is likely that a chaperone protein delivers copper to pCT0017 protein that results in displacement of the bound protein from the DNA and concomitant relief of repression, thus allowing transcription to proceed. As discussed in Chapter 5, further investigation is required firstly to provide substantiating evidence for this hypothesis and secondly to identify the protein responsible for chaperoning copper ions to pCT0017.

Although there is good evidence to suggest that pCT0017, pCT0018, pCT0019 and *ctpA* form a *cop*-like operon that is regulated by the pCT0017 protein in response ambient copper concentrations, it is unknown whether pCT0017 plays a role in regulation of expression of the other ORFs associated with the *L. monocytogenes* strain DRDC8 putative copper gene cluster. The fact that other ORFs (pCT0023, pCT0024 and pCT0027) that encode polypeptides similar regulatory proteins were identified within the gene cluster suggests that these other proteins could be involved. Regulation of expression of this putative gene cluster as a whole may be the result of a collaborative effort between multiple regulatory proteins. Alternatively, this cluster of genes may not represent a single transcriptional unit; the different regulatory proteins may be responsible for regulation of expression of separate parts of the cluster in response to ambient copper concentrations.

Clearly, *L. monocytogenes* strain DRDC8 displays exceptional tolerance for copper (up to 20 mM CuSO<sub>4</sub>). However, it remains unknown what happens to the copper once it enters the cell, for example, it may be sequestered in the cell wall or precipitated inside/outside the cell. Indeed, strains of *Pseudomonas syringae* can accumulate copper in the periplasm and outer membrane as a means of preventing toxic levels of copper from entering the cytoplasm (Cha & Cooksey, 1991; Cha & Cooksey, 1993; Cooksey & Azad, 1992; Cooksey, 1993). In addition, the cell walls of strains of *Streptomyces* and *Bacillus subtilis* have large heavy metal binding capacities (Doyle *et al.*, 1980; Rho & Kim, 2002). The homogeneous distribution of adsorbed metals in cytoplasm has also been reported for copper in *Pseudomonas stutzeri* (Mattuschka *et al.*, 1994). Furthermore, heavy metal adsorption by Gram-positive *Streptomyces* has been presumed to contribute a large heavy metal binding capacity and is considered an alternative method to recover metals from waste liquid (Rho & Kim, 2002). If *L. monocytogenes* is capable of uptake and cellular sequestration of large amounts of copper into a non-toxic form, there may be potential for

these *L. monocytogenes* copper genes to be used in the development of chimeric/recombinant organisms for environmental copper waste treatment, such as harvesting copper from mining waste. Nevertheless, Hii, (2009) was unable to show that strain DRDC8 bound copper to cell walls of cultures grown in different concentrations of CuSO<sub>4</sub>. Consequently, it is probable that *L. monocytogenes* expresses proteins such as those encoded by plasmid pCT100 that export excess copper out of the cell just as fast as it is imported.

It is interestingly to speculate that cross-talk between copper and cadmium ion homeostasis in *L. monocytogenes* strain DRDC8 may occur. As previously discussed (refer to Chapter 4), this idea is not novel in Gram-positive bacteria (Solovieva & Entian, 2004). In addition to showing that plasmid-encoded genes are important for tolerance to both copper and cadmium, insertion mutation in genes implicated in copper ion homeostasis (*cutR* and ORF pCT0018), also affects tolerance of *L. monocytogenes* strain DRDC8 to high levels of cadmium ions. ORFs *cutR* and pCT0018 may have complementary roles in facilitating cadmium ion tolerance in *L. monocytogenes*. Given that *cutR* encodes a polypeptide similar to other cation transporting P-type ATPases, it is possible that CutR is involved in transport of both cadmium and copper ions. While implicated in copper homeostasis, the exact role of pCT0018 in copper tolerance is unknown. Consequently, it is difficult to hypothesise what role it might play in cadmium homeostasis. However, the finding that the pCT0017 protein DNA binding is not responsive to cadmium ions, suggested that expression of pCT0018 is unlikely to be dependant on cadmium ion concentrations. Nevertheless, the precise mechanism of the function of *cutR* and pCT0018 in cadmium homeostasis in *L. monocytogenes* strain DRDC8 remains to be investigated (see Section 6.5).

## **6.4 Exposure of *L. monocytogenes* to Copper in the Environment**

Why *L. monocytogenes* strain DRDC8 has acquired multiple genes that encode exceptional tolerance to copper is not clear. Copper is an essential trace metal but in addition to its role in normal growth of the animal, it also seems to have an “antibiotic” effect when used in high doses. Copper sulphate has been widely used since 1955 as a growth promoter in pig diets (Edmonds *et al.*, 1985). Copper apparently elicits an antibacterial effect on the

bacteria of the pig gut that alleviates sub-clinical infections and as a result leads to an increase in pig growth (Shurson *et al.*, 1990; Stansbury *et al.*, 1990). High levels of copper also have a growth promoting effect in broiler chickens (Ewing *et al.*, 1998; Pesti & Bakalli, 1996; Skrivan *et al.*, 2000). Copper has also been used extensively as a key component of bactericides and fungicides used in agriculture for control of bacterial and fungal diseases of crop plants, fruits and vegetables. Furthermore, copper has been introduced into soils via sewage sludge, mine effluents and industrial waste. Subsequently, since the mid-1980s copper resistant strains of bacterial pathogens have been isolated following exposure to high levels of  $\text{Cu}^{2+}$  ions in agricultural environments (Brown *et al.*, 1992; Trevors, 1987).

A well described example of a bacterial pathogen isolated from a high copper environment carrying plasmid-encoded copper resistance was strains of *E. coli* isolated from the effluent of an Australian piggery where pigs had been fed a diet containing copper sulphate as a growth promoter (Tetaz & Luke, 1983). In these strains, copper resistance was conferred on a 78-megadalton conjugative plasmid (pRJ1004) by the *pco* operon (Brown *et al.*, 1992; Tetaz & Luke, 1983). Copper-resistant strains of *Pseudomonas syringae* have also been isolated from tomato fields in California where solutions containing  $\text{CuSO}_4$  were applied as an antifungal agent. In these strains copper resistance was encoded by a plasmid-borne *cop* operon (Bender & Cooksey, 1987). Studies have shown that the *pco* and *cop* operons are closely related (Brown *et al.*, 1995; Bryson *et al.*, 1993). Copper-resistant strains of *Xanthomonas campestris* pv. *vesictoria* have also been isolated from pepper and tomato plants to which copper compounds were frequently applied (Voloudakis *et al.*, 1993). The copper resistance determinant of those strains was encoded on a large conjugative plasmid. Similar examples of copper-resistant enteric bacteria have been isolated from the United Kingdom (Williams *et al.*, 1993). In addition, copper resistant strains of the Gram-positive bacteria *Enterococcus faecium* and *Enterococcus faecalis* have been isolated from pigs, where  $\text{CuSO}_4$  was being used in large amounts as feed additive. In these strains, the *tcrB* gene, which is located on a conjugative plasmid, conferred copper resistance (Aarestrup *et al.*, 2002; Hasman & Aarestrup, 2002).

In view of the fact that copper resistance is relatively common, it seems likely that copper tolerant strains of *L. monocytogenes* will be common to environments characterised

by elevated copper concentrations. Thus, strain DRDC8 may have acquired multiple genes involved in copper tolerance in response to exposure to high levels of copper within the dairy environment. Moreover, in the absence of appropriate selection, it is understandable why clinical strains do not carry plasmid-encoded copper homeostasis genes. The fact that strain DRDC8 is an Australian dairy isolate is significant in understanding how this bacterium, and other related *L. monocytogenes* dairy strains, may have been exposed to high concentrations of copper. Copper-deficiency, associated with low blood copper levels, low levels of ceruloplasmin (a copper dependent metabolite) and low liver copper, has been described in cattle in New South Wales (Lloyd Davies *et al.*, 2003). In the South East of South Australia, copper deficiencies in cattle are recommended to be treated by administration of Cu directly to animals (McFarlane *et al.*, 1990). Certainly, this method is used to treat copper deficiency, termed ‘swayback’, in Australian sheep (Saylor & Leach, 1980). It has also been reported that pasture development in the South East of South Australia is reliant on copper enriched fertilisers (McFarlane *et al.*, 1990). It is thus likely that some Australian dairy environments contain high levels of copper, possibly from direct supplementation of the animal host with copper or grazing on pastures fertilised with copper enriched compounds. This would undoubtedly create a copper rich environment which resident *L. monocytogenes* isolates would require multiple genes involved in tolerance to copper to survive.

Given that farm animals, particularly cattle, and their environments may represent a reservoir for human *L. monocytogenes* infection (Nightingale *et al.*, 2004) (refer to Section 1.5.1), the identification of *L. monocytogenes* strains that carry genes which confer a selective advantage for survival in certain dairy environments may have important implications for their prevalence within Australian food processing systems and thus human listeriosis. Ruminant farm ecosystems have been shown to maintain a high prevalence of *L. monocytogenes* isolates that have been linked to human infection (Nightingale *et al.*, 2004). In addition, survival of *L. monocytogenes* in dairy industry environments is important where outbreaks of disease in humans have been traced to the consumption of unpasteurised milk and have also been traced back to pasteurized milk (Oliver *et al.*, 2005). Moreover, isolates from food and food processing environments that are linked to human infection may be derived from animal environments where copper

supplementation has selected for strains carrying copper resistance genes. Genes acquired for survival and persistence in copper rich dairy environments could potentially enhance *L. monocytogenes* ability to mount a successful infection in a mammalian host.

*L. monocytogenes* is likely to encounter different cation stresses during host infection. For example, infection can dramatically alter the concentration of trace elements within humans and laboratory animals in response to systemic inflammation (Beisel, 1977). *L. monocytogenes* strains adapted to cation stress may be better equipped to infect a host cell. In fact, mutation in *ctpA* leads to a reduction in persistence in the liver of infected mice, where  $\text{Cu}^{2+}$  availability is assumingly limited (Francis & Thomas, 1997b). Thus exposure of strain DRDC8 and other related isolates to copper rich dairy environments could potentially enable *L. monocytogenes* to establish a more persistent infection by overcoming cation stress encountered during host invasion.

## 6.5 Future Directions

Understanding how *L. monocytogenes* is able to adapt its cellular physiology to overcome various forms of environmental stress is an important step in developing better methods of controlling *L. monocytogenes* infection. Genome sequencing projects represent a means to gain a better understanding of the involvement of plasmids in enhancing the virulence and environmental adaptation potential of *L. monocytogenes* strains. These projects also provide insight into the survival strategies particular *L. monocytogenes* strains use to persist in the different ecological niches. Therefore, future work should aim to fully characterise plasmid pCT100 from *L. monocytogenes* strain DRDC8. This will allow identification of niche specific genes and proteins used by this particular strain for survival and proliferation within the dairy environment.

Future studies should certainly focus on deciphering the mechanisms that underpin the ability of this pathogen to survive and proliferate under such extreme copper stress. In particular, the mechanisms by which *L. monocytogenes* strain DRDC8 maintains copper homeostasis should be further investigated. For example, while it is known that *ctpA* encodes a copper transporting P-type ATPase and *cutR* is likely to encode a similar protein, it is unknown if they function in copper influx or efflux. Copper uptake experiments would be useful to establish the functionality of proteins encoded by the

putative copper homeostasis genes by analysing the effect of their mutation on copper transport. Establishing their function will assist in determining which gene/s are involved in maintaining copper ion homeostasis in different environmental conditions.

It is also important for future studies to confirm that all of the ORFs associated with the putative copper gene cluster are in fact involved in copper ion homeostasis. Analysing the effects of individual mutation of each ORF on the ability of *L. monocytogenes* to maintain copper ion homeostasis is important to confirm this hypothesis. To avoid complication of data analysis, methods of deletion or conditional mutation should be used to eliminate interference with cotranscribed genes and other pleiotropic effects that can be caused by insertion mutations. Functional analysis of these ORFs would be assisted by cloning the entire gene cluster into a vector capable of replication in *L. monocytogenes* and transforming this into a plasmid-less variant of DRDC8. Alternatively, as previously mentioned in Chapter 4, this could be done in a non DRDC8 background such as *L. monocytogenes* strain EGD Kaufmann, which is naturally sensitive to low concentrations of copper. MIC and growth experiments conducted using media containing different concentrations of copper could be used to determine if these constructs acquire increased copper tolerance. Selective mutation of each ORF carried on this construct could then be used to identify loss of function.

In addition, more research is required to determine if this gene cluster comprises a large transcriptional unit or if they represent smaller, separately transcribed units. Real-time RT-PCR analyses would provide fundamental information as to which genes are expressed and under what conditions. Primer extension analysis should also be deployed to determine the transcriptional start site and define the promoter region. Western analysis using antibodies against the specific proteins could also be used to look at protein expression under different conditions. This information is absolutely critical to deciding whether or not there is any worth in studying this system any further. If these genes are not expressed, they may be non-functional. While unlikely, since mutation/loss of these genes clearly affects *L. monocytogenes* tolerance to copper stress, it is difficult to make meaningful conclusions without knowing if these genes are expressed.

On the basis that this study has provided limited evidence to suggest that *cutR* and pCT0018 play an auxiliary role in cadmium tolerance, further investigation is required to



substantiate this hypothesis. Firstly, the effect of cadmium on growth of *cutR* and pCT0018 mutants could be analysed using growth experiments in the presence of cadmium ions. Secondly, Northern hybridisation analysis of RNA isolated from wild type and pCT0018 and *cutR* mutant strains following induction with  $\text{Cd}^{2+}$  would be useful to determine if cadmium effects of transcription of these genes. Thirdly, the use of  $\text{Ni}^{2+}$  affinity chromatography, using methods described by Solovieva & Entian, (2004), could determine their ability to bind cadmium ions. Moreover, affinity chromatography analysis would also be useful in confirming that pCT0017 protein-DNA interactions are not responsive to cadmium by showing that the pCT0017 protein does not bind to cadmium.

Genes encoded by plasmid DNA originally acquired to facilitate environmental survival of this saprophytic organism, have the potential to play auxiliary roles in enabling the bacterium to establish a more persistent infection in human hosts. Thus more investigation is required to establish if the presence of the putative copper gene cluster impacts on the ability of *L. monocytogenes* to persist during infection. This could be achieved by through a comparison of *in vivo* persistence of specific mutants (individual mutation of the different copper cluster genes) and plasmid-less strains versus wild type DRDC8 and other strains carrying the gene cluster through competition experiments used by (Francis & Thomas, 1997b).

Given that strain DRDC8 is an Australian dairy isolate, characterising the virulence and environmental adaptation potential of this strain may have significance for the Australian dairy industry. Future studies could ultimately lead to novel ways to interrupt transmission within the dairy industry and thus help to prevent foodborne disease.

## 6.6 Conclusions

In conclusion, this study has described some important genetic features of the large plasmid pCT100 isolated from *L. monocytogenes* strain DRDC8. The mechanisms by which *L. monocytogenes* strain DRDC8 tolerates copper ion stress were also investigated by this work.

The principal conclusions are as follows:

3. The *ctpA* gene, which has significance for virulence of *Listeria monocytogenes*, is encoded by plasmid pCT100 in strain DRDC8.
4. Sequence analysis of plasmid pCT100 from *L. monocytogenes* strain DRDC8 has shown that its genetic content and organisation is similar to plasmids from other *Listeria* spp.
5. The identification of unique regions of DNA on plasmid pCT100 which encode amino acid sequences that share significant similarity to proteins from other species of Gram-positive bacteria indicates that this plasmid has been subject to insertion/deletion events.
6. Plasmid pCT100 encodes a cluster of genes implicated in copper ion homeostasis for strain DRDC8.
7. Plasmid-encoded genes are necessary for tolerance of *L. monocytogenes* to cation stress.
8. In addition to CtpA, *L. monocytogenes* strain DRDC8 carries a chromosomal gene (*cutR*) that encodes a putative copper-translocating P-type ATPase. *cutR* and *ctpA* may function collaboratively to maintain copper ion homeostasis.
9. ORFs pCT0017, pCT0018 and pCT0019 are also involved in copper ion tolerance in strain DRDC8.
10. *cutR* and pCT0018 insertion mutants display altered tolerance to cadmium. This indicated that these genes may play associative roles in cadmium homeostasis.
11. ORF pCT0017 encodes a copper responsive CopY-like transcriptional repressor protein that is able to bind specifically to DNA that encodes two *cop* box-like sequences located upstream of the pCT0017 translation start site.
12. Binding of the pCT0017 protein to *L. monocytogenes* strain DRDC8 DNA is inhibited at high concentrations of copper ions.
13. ORFs pCT0017, pCT0018, pCT0019 and *ctpA* may form a *cop*-like operon. Expression of this operon is likely to be regulated by pCT0017 in response to ambient copper concentrations. This operon may form part of a larger cluster of genes that function to maintain copper ion homeostasis in strain DRDC8.

14. *ctpA* and other ORFs associated with the copper gene cluster are also harboured by other Australian strains of *L. monocytogenes* isolated from dairy and poultry environments. These genes may provide a selective advantage for *L. monocytogenes* persistence in Australian food processing systems and thus human infection by conferring resistance to conditions of extreme copper stress.

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