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Iron-Dependent Regulation of Gene Expression in
Corynebacterium pseudotuberculosis

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BSc (hons) MSc

A thesis submitted in fulfilment of the requirements for the degree of Doctor of
Philosophy at the Faculty of Veterinary Medicine, University of Glasgow

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***GOLD** is for the mistress—silver for the maid—
Copper for the craftsman cunning at his trade.”*

“Good!” said the Baron, sitting in his hall,
“But Iron—Cold Iron—is master of them all.”

Rudyard Kipling
1865-1936

Abstract

This study set out to analyse *C. pseudotuberculosis* within an environment relevant to that which would be encountered within its natural host. The impact of the availability of iron within the growth environment of numerous bacteria has been widely reported, and an equivalent investigation was conducted to determine whether the same was true of *C. pseudotuberculosis*. To this end, a novel chemically-defined medium was designed, which supported the growth of *C. pseudotuberculosis*, but in which the concentration of specific growth factors could be manipulated. Subsequently, iron was shown to be essential for *C. pseudotuberculosis* growth, and analysis of secreted protein profiles revealed differential expression between low- and high-iron growth conditions. Furthermore, growth experiments conducted in the defined medium revealed that *C. pseudotuberculosis* is capable of obtaining iron from the host iron-binding proteins, transferrin and lactoferrin. Subsequently, *C. pseudotuberculosis* was discovered to produce a siderophore, which is likely to contribute to the organism's ability to acquire iron *in vivo*.

Continuing with the investigation of iron, it was observed that *C. pseudotuberculosis*, *C. ulcerans* and *C. diphtheriae*, when infected with a corynebacteriophage carrying the diphtheria toxin-encoding gene (*tox*), have all been shown to produce the toxin in an iron-dependent manner (Maximescu, *et al.* 1974). Significant work has been conducted in *C. diphtheriae*, which has shown the involvement of an iron-dependent regulatory protein, the diphtheria toxin repressor or DtxR, is essential for the iron-dependent regulation of *tox* expression. Therefore, it was considered likely that an equivalent regulator also existed within *C. pseudotuberculosis* (and *C. ulcerans*). A previous study conducted in this laboratory had allowed the PCR-amplification of a small fragment of a gene sharing similarity to *C. diphtheriae* DtxR (Malloy, 2004). In the current study, a 6.5 kb chromosomal locus, which contained the sequence previously amplified by PCR, was cloned and sequenced. Subsequently, this chromosomal locus was found to be equivalent to that present in other sequenced corynebacteria, in that the genes encoding (from 5' - to 3' -) an RNA-polymerase sigma factor (*sigB*), the DtxR protein (*dtxR*), a UDP-galactose 4-epimerase (*galE*), and 2 further proteins were all present. The transcription of *sigB*, *dtxR* or *galE* was analysed and found to be linked. Furthermore, although the expression of these genes themselves was not found to be controlled in an iron-

dependent manner, *C. pseudotuberculosis* was shown to control the expression of known *C. diphtheriae* DtxR-regulated genes in direct response to environmental iron concentration.

In order to confirm the involvement of *C. pseudotuberculosis* DtxR in the regulation of expression of target genes, a highly-efficient allele-replacement mutagenesis procedure was adapted and enhanced for use in this organism, which included the employment of a novel enrichment procedure to aid in the isolation of the desired mutants. Subsequently, a DtxR-deficient mutant derivative was created, allowing comparisons of expression of target genes to be conducted in the isogenic mutant and wild-type parent strains, under low- and high-iron growth conditions. The results of these studies confirmed *C. pseudotuberculosis* DtxR in the regulation of the *C. pseudotuberculosis* ferrous acquisition gene (*fagA*); however, the results proved that the serine protease (*cp40*) was not regulated by DtxR.

Towards the end of the study, the partial *C. pseudotuberculosis* genome sequence became available, which allowed the identification of a gene, designated ORF02949, which potentially-encoded the siderophore. Although preliminary attempts to confirm the gene as that of the siderophore were unsuccessful, a putative DtxR-binding motif was found immediately upstream of the gene, and the involvement of DtxR in its regulation, in an iron-dependent manner was subsequently demonstrated. Taken together, the results presented in this thesis confirm the importance of iron to *C. pseudotuberculosis*, and reveal the existence of an iron-dependent regulator which is involved in regulating the expression of multiple target genes.

Declaration

The work reported in this thesis was carried out under the supervision of Dr Michael C. Fontaine and Professor Willie Donachie at the Moredun Research Institute and Professor David G. E. Smith at the Moredun Research Institute and the Faculty of Veterinary Medicine, University of Glasgow. All results presented, unless otherwise stated, are the sole work of this author, as is the composition of this thesis.

Signed:

Date:

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Abbreviations

°C	Degrees centigrade
Acc #	Accession number
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
<i>bfrA</i>	Bacterioferritin
<i>bfrB</i>	Ferritin
BHI	Brain heart Infusion
BHIA	Brain Heart Infusion agar
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
<i>C. pstb</i>	<i>Corynebacterium pseudotuberculosis</i>
CCDM	<i>Corynebacterium</i> Chemically Defined Medium
CDM	Chemically Defined Medium
cDNA	Complementary Deoxyribonucleic acid
CLA	Caseous Lymphadenitis
cm	Centimetre
Cp40	Corynebacterial Protease 40
DAB	3,3'-Diaminobenzidine
DCO	Double crossover
ddH ₂ O	Double distilled water
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP's	Deoxynucleotide triphosphates
Dps	DNA-binding proteins of starved cells
DSO	Double stranded origin
DT	Diphtheria Toxin
DtxR	Diphtheria toxin repressor
EDDA	ethylenediaminediacetic acid
EDTA	ethylenediaminetetraacetic acid
EF 2	Elongation factor 2
ETOH	Ethanol
FA	Formaldehyde
Fe ²⁺	Ferrous Iron (reduced)
FeCl ₃	Ferric Chloride
Fe ³⁺	Ferric Iron (oxidised)
g	Grams
G+C	Guanine and Cytosine
h	Hours
H ₂ O ₂	Hydrogen Peroxide
HIB	Heart Infusion Broth
ICP-MS	Inductively coupled plasma mass spectrometry
IdeR	Iron-dependent regulator
iPCR	Inverse Polymerase Chain reaction
IROMP	Iron regulated outer membrane protein

IRP	Iron regulated protein
kb	Kilobase
kDa	Kilodalton
kV	Kilovolts
L	Litres
LC-ESI MS/MS	Liquid Chromatography electrospray ionisation tandem mass spectrometry
M	Moles
mF	Millifarad
mg	Milligrams
min	Minutes
ml	Millilitres
MOPS	3-[N-morpholino] propanesulfonic
M _r	Relative molecular weight
mRNA	Messenger Ribonucleic acid
MSG	Monosodium Glutamate
NADPH	Nicotinamide adenine dinucleotide phosphate
NCTC	National Collection of Type cultures
ng	Nanograms
NIS	Nonribosomal Peptidase Synthetase-Independent Siderophore
NRPS	Nonribosomal Peptidase Synthetase
ONPG	<i>o</i> -nitrophenyl- β -D-galactoside
ORF	Open Reading frame
PAI	Pathogenicity Island
PBS	Phosphate buffered Saline
pCARV	<i>Corynebacterium</i> allele replacement vector
PCR	Polymerase Chain reaction
PFGE	Pulsed Field Gel Electrophoresis
PLD	Phospholipase D
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain reaction
RC	Rolling circle
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
rPLD	Recombinant Phospholipase D
RT-PCR	Reverse Transcription Polymerase chain reaction
SAP	Shrimp alkaline phosphatase
SCO	Single crossover
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sec	Seconds
SOB	Super optimal Broth
SSC	Saline-sodium citrate
SSE	Sodium salicylate extract
SSO	Single stranded origin
TAE	Tris-acetate EDTA
Tbps	Transferrin binding proteins

TBS	Tris Buffered saline
T _m	Melting temperature
Ts plasmid	Temperature sensitive plasmid
TW	Tween ⁸⁰
Tween ⁸⁰	Polyoxyethylenesorbitan monooleate
U	Units
v/v	volume for volume
w/v	weight for volume
Zur	Zinc uptake Regulator
Δ	Delta
μg	Micrograms
μl	Microlitres
Ω	Ohms

Chapter One
General Introduction

1.0 The corynebacteria

The Corynebacteria form a diverse group of organisms occupying a variety of ecological niches including skin, soil and sewage. Corynebacteria are Gram-positive bacteria with a high content of guanine and cytosine (G+C) nucleotides in their DNA (Connell, 2001). The genus forms part of the order of bacteria known as “Actinomycetales”, within which the corynebacteria belong to a suprageneric taxon (or sub-order) referred to as the Mycolata or Corynebacterianeae (Stackebrandt, 1997) (Zhi, *et al.* 2009). This taxon includes mycobacteria, nocardiae and rhodococci, where grouping is based on similarities between the complex cell wall structures of the three genera (Barksdale, *et al.* 1981; Dover, *et al.* 2004). The chemical structure of the cell wall of *Corynebacterium*, *Mycobacterium* and other related genera has been extensively studied. It has been shown that lipids make up a large portion of the cell wall; the most significant of these lipids being mycolic acid. When compared to mycobacteria and nocardia, corynebacteria have the shortest chain mycolic acids which are known as corynemycolic acids.

The genus *Corynebacterium* was originally created in 1896 by Lehmann and Neumann, to accommodate the diphtheria bacillus (*Corynebacterium diphtheriae*) and a few other species pathogenic to animals (Jones, 1986). Over the years, however, many other non-spore-forming, irregularly-stained, Gram-positive species, both aerobic and anaerobic, were assigned to the genus. Essentially, this resulted in a collection of heterogeneous, unrelated organisms (Jones, 1986). The heterogeneity of the genus became evident upon analyses of cell wall composition (Jones, 1986). Subsequently, as more biochemical information (such as cell wall chemistry and lipid composition) became available the genus became more precisely-defined based on the cell wall characteristics. The genus *Corynebacterium* is now confined to species that are characterised by the presence of arabinogalactan and *meso*-diaminopimelic acid (*meso*-DAP) in their cell wall, contain corynemycolic acid, and have a G + C content of 51-63% (Barksdale, 1970; Jones, 1986; Schleifer & Kandler, 1972).

Often referred to as “diphtheroids”, corynebacteria are non-motile, non-spore forming, straight to slightly-curved Gram-positive rods. On occasion, corynebacteria appear as cocci or coccobacilli with club-shaped ends, hence the term coryne (the Greek word for club). Interestingly, staining can sometimes be irregular, and when viewed under a microscope the

bacteria show a palisade arrangement which has been described as reminiscent of the appearance of Chinese letters (Brown & Olander, 1987). These characteristics are probably due to the cell surface composition and the resulting hydrophobic interactions of the external lipid layer, together with the irregular nature of the dividing septa, since corynebacteria divide by snapping division (Hard, 1969b).

1.1 Other notable corynebacteria

1.1.1 Non-pathogenic corynebacteria

Corynebacterium glutamicum is the work-horse of the genus *Corynebacterium*, and is one of the most biotechnologically-significant bacterial species in use today. The importance of this bacterium lies in its ability to overproduce L-glutamate commonly known as Mono-sodium-glutamate (MSG), and annual production of this amino acid lies in excess of two million tons (Burkovski, 2008; Kimura, 2003). The mechanism of L-glutamate overproduction by *C. glutamicum* exploits the organism's auxotrophy for biotin. This phenomenon was first observed by Shiio *et al.* (1962) who demonstrated that excess biotin in the culture medium lowers the cellular permeability of amino acids hence, there is less L-glutamate released in excess biotin and, in contrast, low biotin levels in the growth medium results in an overproduction of L-glutamate (Shiio *et al.* 1962). Interestingly, even in the presence of excess biotin, *C. glutamicum* can be induced to overproduce L-glutamate. This phenomenon is achieved by the addition of a detergent, such as polyoxyethylene sorbitane monopalmitate (Tween 40), or polyoxyethylene sorbitane monostearate (Tween 60) (Duperray *et al.* 1992). Significantly, polyoxyethylene sorbitane monolaurate (Tween 20) and polyoxyethylene sorbitane monooleate (Tween 80) are not able to induce the overproduction of L-glutamate; however, the reasons behind this are not known (Kimura, 2003). The constant drive to improve the yield of glutamate has led to the initiation of molecular biological analyses, including novel genetic manipulations and proteomic analyses. Significantly, the techniques being developed to improve the productivity of *C. glutamicum* in an industrial context may well aid in expanding our understanding of other corynebacterial species, including those pathogenic for humans and animals. In addition to *C. glutamicum* the closely-related non-pathogenic bacterium *Corynebacterium efficiens* is also used in the food industry for the large-scale production of glutamate. Unlike *C. glutamicum*, *C. efficiens* is capable of growing and

producing glutamate at 40°C. For the glutamate industry the thermostability of the organism is a valuable feature since it reduces the considerable cost of the cooling of *C. glutamicum* cultures, in order to dissipate the heat which builds up during the glutamate fermentation process (Nishio, *et al.* 2003).

1.1.2 The pathogens

1.1.2.1 *Corynebacterium diphtheriae*

Corynebacterium diphtheriae is an important human pathogen, being the aetiological agent of the disease, diphtheria. The first stage of *C. diphtheriae* infection in humans starts with colonisation of the throat. The organism generally gains entry following inhalation of aerosols from an infected person or an asymptomatic carrier. The first symptoms of the resulting highly-infectious, potentially-fatal, disease include malaise, low-grade fever, sore throat and loss of appetite. As the infection develops, a greyish membrane forms in the throat which can extend to the lungs. The membrane, which consists of a complex of fibrin, bacteria and inflammatory cells, adheres to underlying tissue which, when attempts are made to physically remove the membrane, can bleed. In more serious cases, once *C. diphtheriae* infection has become systemic, a protein toxin, known as diphtheria toxin (DT), is produced which can cause damage to internal organs (Salyers & Whitt 1994). Experimentally, when large doses of DT were administered parenterally into susceptible animals, typical diphtheria-like systemic lesions were observed, including, myocarditis, polyneuritis, and focal necrosis in organs such as the liver, kidneys and adrenal glands (Holmes, 2000). Fortunately, as a result of a successful vaccination programme, diphtheria has been under control (with no significant outbreaks) for many years in developed countries; however, the disease has recently been making a remarkable return, specifically within Eastern Europe, where vaccine uptake has not been sufficient to maintain herd immunity (Golaz, *et al.* 2000). Vaccination is achieved by administering toxoided DT, a formalin-inactivated derivative which results in a protective antitoxic immune response in vaccinated individuals.

1.1.2.2 *Corynebacterium ulcerans*

Another corynebacterial species, *Corynebacterium ulcerans*, has been identified, which is normally associated with disease in cows and domestic animals such as dogs and cats; however, in recent times, in addition to *C. diphtheriae*, *C. ulcerans* has also been implicated as a cause of diphtheria-like disease in humans (Gubler, *et al.* 1990; Lartigue, *et al.* 2005; Tiwari, *et al.* 2008).

1.1.2.3 *Corynebacterium jeikeium*

Another important pathogenic corynebacterial species is *Corynebacterium jeikeium*. This opportunistic pathogen causes septicaemia and endocarditis in immunocompromised humans, and attempts to combat infection are often without success due to the organism's resistance to a number of antibiotics (Balci, *et al.* 2002).

1.2 *Corynebacterium pseudotuberculosis*

Corynebacterium pseudotuberculosis is the aetiological agent of a variety of chronic and suppurative conditions in a number of mammalian species, most notably, caseous lymphadenitis (CLA) of small ruminants and ulcerative lymphangitis of horses (Hughes & Biberstein, 1959; Knight, 1969; Lopez, *et al.* 1966; Miers & Ley, 1980). In 1888 the French veterinarian, Edmond Isidore Etienne Nocard, first described an organism from a case of lymphangitis in a cow. Three years later, Hugo von Preisz isolated a similar bacterium from renal abscesses in a ewe (Preisz & Giunard 1891). The organism subsequently became known as the Preisz-Nocard bacillus. In 1894, Preisz described the organism in detail and branded it *Bacillus pseudotuberculosis ovis*, the name pseudotuberculosis, meaning “false tuberculosis” derived as a result of the similarities between the lesions and the caseous nodules commonly observed during infections with *Mycobacterium tuberculosis* (Preisz, 1894). However, in 1911 Buchanan suggested abandoning the subspecies designation, resulting in the name *B. pseudotuberculosis*. Subsequently, several investigators observed the noticeably diphtheroid morphology of the organism (*i.e.* sharing similarities with *Corynebacterium diphtheriae*). Later, in the 1923 edition of the Bergey's manual, the organism was presented as *Corynebacterium ovis*, due to its frequent association with disease in sheep; however, by the sixth edition of “the manual”, the name had been officially changed back to *Corynebacterium*

pseudotuberculosis, due to the recognition that the organism caused disease in a range of animal species (Rogosa, *et al.* 1974).

C. pseudotuberculosis is a facultative anaerobe and facultative intracellular pathogen. Dimensions of the organism vary from 0.55 by 0.40µm to 1.2 by 0.50µm (Hard, 1969a). When grown on blood agar, *C. pseudotuberculosis* produces pale grey colonies that are matt in appearance. Due to the waxy (high-lipid) nature of the cell wall, *C. pseudotuberculosis* colonies can be pushed along the agar surface, and tend to spatter when introduced to a flame (Quinn, *et al.* 1994). In liquid media, the bacterium has a tendency to form clumps; again, this is due to the high lipid content of its outer layer. Significantly, cell wall lipids account for as much as 11.3% of the dry weight of the bacterial cell (Ionedo & Silva, 1979).

1.2.1 Phenotypic characterisation

Many early attempts were made to characterise strains of *C. pseudotuberculosis* by assessment of biochemical characteristics (Barakat, 1984; Biberstein, *et al.* 1971; Muckle & Gyles, 1982). *Corynebacterium pseudotuberculosis* is strongly catalase- and urease-positive and is able to ferment glucose, galactose, maltose and mannose (Brown & Olander, 1987). Interestingly, these biochemical traits are identical in isolates of *C. pseudotuberculosis* from different mammalian species, with the exception of the ability to reduce nitrate to nitrite. It was originally reported that *C. pseudotuberculosis* isolates from horses and cattle were uniformly able to reduce nitrate, whereas isolates from sheep and goats were not. These developments led to the creation of the term “biotype” whereby strains were separated into two distinct biotypes in an overly-simplistic attempt to label isolates from diseases in different host species. Non-nitrate reducing (or nitrate-negative) strains responsible for causing CLA in sheep and goats were assigned to biotype I, whereas nitrate-reducing (or nitrate-positive) strains responsible for causing infections in cattle and horses were assigned to biotype II (Biberstein, *et al.* 1971). Later studies involving the comparative biochemical characterisation and restriction endonuclease analysis of chromosomal DNA of *C. pseudotuberculosis* isolates demonstrated that the variation in the ability to reduce nitrate coincided with different restriction patterns, whereby nitrate-positive strains produced one pattern, while nitrate-negative strains produced another. As a consequence, the terms ‘biovar equi’ for nitrate-positive strains and ‘biovar ovis’ for nitrate-negative strains were coined (Songer, *et al.* 1988).

However, it has subsequently become apparent that the relationship between biotype/biovar and specific host type is not as clear-cut as was once thought. In recent years, nitrate negative strains have been isolated from horses (Biberstein, *et al.* 1971; Connor, *et al.* 2000). Therefore, different approaches to characterising the relationships between isolates have been taken. Other methods of characterising strains have included serotyping (Barakat, 1984; Sutherland, *et al.* 1989) immunoblotting (Muckle, *et al.* 1992) and molecular characterisation (Connor, *et al.* 2000; Costa *et al.* 1998; Sutherland *et al.* 1996). Interestingly, the molecular studies, although they were carried out using different methods of characterisation, are in agreement with regards to the grouping of nitrate-negative ovine and caprine isolates and the designation “biovar *ovis*” (Biberstein, *et al.* 1971; Connor, *et al.*, 2000; Muckle, *et al.*, 1992). Using pulsed-field gel electrophoresis (PFGE), Connor *et al.* (2000) genotyped 50 UK isolates of *C. pseudotuberculosis* which included both sheep and horse isolates (Connor, *et al.* 2000). Pulsed-field gel electrophoresis using *Sfi*I and *Sma*I restriction endonucleases identified six different pulsotypes within the clonally-related group of ovine and caprine isolates. The results of that study led the authors to suggest that *C. pseudotuberculosis* isolates of ovine and caprine origin within the UK are clonally-related (Connor, *et al.* 2000). Furthermore, a later study addressed the molecular genotyping of a panel of multinational *C. pseudotuberculosis* isolates (Connor, *et al.* 2007). Unexpectedly, Connor *et al.* (2002) observed that, irrespective of their country of origin, ovine and caprine *C. pseudotuberculosis* isolates are clonally related, leading them to suggest that the differences in the manifestation of CLA in sheep and goats in different countries is more likely to be attributed to fundamental differences in the host species themselves, in addition to other factors such as animal age or environmental pressures (al-Rawashdeh & al-Qudah, 2000; Connor, *et al.* 2007). Significantly, these findings may have important implications with regards future vaccine development and vaccination policies.

Significantly, transmission of *C. pseudotuberculosis* biovar equi strains from horses to sheep and goats grazing on the same pasture has not been shown to occur. In an experiment carried out by Brown *et al.* (1985) kid goats were experimentally infected intradermally with *C. pseudotuberculosis* strains originally isolated from goats or horses (Brown, *et al.* 1985). Five kids were administered a caprine-derived strain, whilst a further five were administered an equine-derived strain. At *post mortem*, those goats infected with the caprine strain were observed to have multiple lesions, both peripherally and in visceral locations. The goats that

received the equine strain had an initially more prominent reaction at the site of inoculation, but at the end of 4 months 1 animal had no evidence of diseases and the other 3 had minimal abscesses found only in the draining lymph node (Brown, *et al.* 1985). Therefore, it would appear that, as originally postulated by Songer (1988), the two biovars have diverged to the extent that biovar equi strains cannot infect sheep and goats. Superficially, it would appear the same is not true of infection of horses and cattle with nitrate-negative strains. However, the analysis of the nitrate-negative horse isolates conducted by Connor *et al.* (2000) revealed that it was genetically-distinct to the ovine and caprine isolates included in the same study, in addition to being distinct from other nitrate-positive horse isolates (unpublished data). Therefore, it would appear that there is a further “type” of *C. pseudotuberculosis* in circulation, which does not correspond to those previously described (Connor, *et al.* 2000).

1.2.2 Virulence factors

Significantly, there has never been a report of an avirulent *C. pseudotuberculosis* strain. However, there is relatively little information available in the published literature regarding specific *C. pseudotuberculosis* factors that contribute to the organism’s ability to colonise and persist within susceptible host species. The reasons for this are several-fold but stem, at least in part, from the sporadic research attention that *C. pseudotuberculosis* has received over the years. In addition, much of the existing literature supports several commonly-held hypotheses regarding *C. pseudotuberculosis* pathogenicity; one example being the involvement of mycolic acid in the ability of *C. pseudotuberculosis* to survive within macrophages. Significantly, this hypothesis was a result of early electron microscopy studies carried out at a time when our understanding of host and pathogen biology, and the host/pathogen interaction, was completely different to what it is nowadays. These early experiments were conducted within the confines of the available methodologies and knowledge of the times, and thus far have not been substantiated by modern molecular methods. However, due to the many times these hypotheses have been cited throughout the subsequent years of research on *C. pseudotuberculosis*, they have become convincing fact. Nevertheless, it is potentially unsafe to assume that all resulting hypotheses concerning factors contributing to *C. pseudotuberculosis* virulence are correct. Despite this fact, the majority of modern *C. pseudotuberculosis* research has focused upon factors identified decades previously. In some cases these hypotheses have already been substantiated with modern molecular methods. The hypothesis which implicates

the Phospholipase D-exotoxin (PLD) as being an important virulence factor (Carne & Onon, 1978) has since been substantiated with molecular methods (Hodgson, *et al.* 1992; McNamara, *et al.* 1994), however, there are still other putative virulence factors which are yet to be proven. The absence of a genome sequence and suitable tools for the genetic manipulation of *C. pseudotuberculosis* has made it virtually impossible to identify other potential research targets until very recently. It is therefore understandable why there has not been a vast amount of research effort focused on the identification of novel virulence determinants, furthermore therein lays a reason why there is a lack of evidence to establish claims of previous ones.

1.2.2.1 Mycolic acid

As previously stated, the corynebacteria have a particularly complex cell wall structure, and like other members of the mycolata the cell wall has been implicated in the virulence of *C. pseudotuberculosis* (Carne, *et al.* 1956; Hard, 1972; Muckle & Gyles, 1983; Tashjian & Campbell, 1983). A lipid (subsequently thought to be mycolic acid) of *C. pseudotuberculosis* was shown to elicit cytotoxic properties (Carne, *et al.* 1956). Using petrol ether, Carne *et al.* (1956) extracted the lipid from *C. pseudotuberculosis* without impairing the viability of the organism. When injected intradermally in guinea pigs or rabbits, this toxic lipid caused clinical signs including local oedema, congestion and haemorrhagic necrosis (Carne, *et al.* 1956). It is important to note that in this study there was no mention of the amount of lipid administered, which may have been far in excess of what would be encountered by the host during a natural infection. As a consequence these results must be interpreted with caution. In a further experiment, Carne *et al.* (1956) noted that leukocytes which had engulfed *C. pseudotuberculosis* prior to being maintained under tissue culture conditions, rapidly degenerated and died. Carne *et al.* (1956) subsequently concluded, that the capacity of *C. pseudotuberculosis* to resist digestion by phagocytes, contributed to the eventual formation of abscesses. Later, studies by Muckle and Gyles (1983) postulated that the virulence of any particular strain of *C. pseudotuberculosis* may be related to the cell wall lipid component of that strain. By extracting the lipid component of 25 different isolates of *C. pseudotuberculosis* and inoculating each into groups of mice, the researchers demonstrated that the strain with the greatest lipid content produced most abscesses (Muckle & Gyles, 1983). While this work supported that of Carne *et al.* (1956), it is also possible that other cell-surface anchored/associated components were being extracted along with the lipid, and that some

other, as yet undetermined, factor may be responsible for the apparent differences in virulence observed between strains.

According to a later study, it is the high lipid content of the cell wall which allows *C. pseudotuberculosis* to evade digestion by cellular enzymes, and subsequently to persist as a facultative intracellular parasite (Hard, 1972); however, it is important to note that this hypothesis was based exclusively upon analyses of electron micrographs. It is entirely possible that other factors are associated with the organism's ability to survive within macrophages. For example, in the related Actinomycete *M. tuberculosis*, the mechanism by which this organism is able to survive killing within macrophages has been well-described at a molecular level. Most bacteria, once internalised by a macrophage, are destroyed by lysosomes; however, mycobacteria are capable of evading delivery to lysosomes, and hence are able to survive within macrophages. Interestingly, the ability of mycobacteria to avoid lysosomal degradation is only a trait observed with viable cells (Armstrong & Hart, 1971; Hasan, *et al.* 1997); a phagosome which contains internalised dead bacteria rapidly becomes a phagolysosome, suggesting compounds synthesised by living mycobacteria impede the mechanisms by which phagosomes and lysosomes fuse (Pieters & Gatfield, 2002). Although it has yet to be the focus of research attention, it is entirely possible that a similar mechanism exists in *C. pseudotuberculosis*, and it should be recognised that this would not be identified by scrutiny of electron micrographs alone.

A further study by Jolly (1965) demonstrated that, when brought into contact with *C. pseudotuberculosis in vitro*, murine macrophages usually only phagocytose a single organism (Jolly, 1965a); however, it was observed that bacterial multiplication was initiated immediately, and several generations were seen within 5 hours. Subsequently, it was reported that degeneration of infected macrophages occurred when the number of organisms reached 30 or 40 (Jolly, 1965a). Later, using electron microscopy to look at the viability of phagocytosed *C. pseudotuberculosis* in caprine mammary macrophages, Tashjian and Campbell (1983) showed the bacteria not only survived in macrophages but that they remained viable (Tashjian & Campbell, 1983). While the results of these studies clearly show that *C. pseudotuberculosis* is able to survive and replicate within macrophages, they do not prove the involvement of mycolic acid, and therefore further work is required to prove or refute the original hypothesis.

1.2.2.2 Phospholipase D

Phospholipase D is a powerful exotoxin, secreted into the extracellular milieu by *C. pseudotuberculosis*. It was first characterised by Carne in 1940, and has since been detected in all strains of *C. pseudotuberculosis* that have been recovered from infected mammalian species (Carne, 1940; Songer, *et al.* 1988). The closely-related *Corynebacterium ulcerans* is also known to produce PLD, and the proteins from the two species share 98 % similarity and 80 % identity with each-other (McNamara, *et al.* 1995).

Phospholipids form an important part of the surface membranes of all mammalian cells; hence, a bacterial enzyme with specific activity against them is likely to exert a significant effect upon the infected host (Batey, 1986). Bacterial phospholipases comprise a diverse group of enzymes produced by a variety of Gram-positive and Gram-negative bacteria. The enzymes manifest a range of effects *in vivo* and *in vitro*, from minor alterations in cell membrane composition and function, to lethality at low concentrations (Songer, 1997; Soucek, *et al.* 1971; Titball, 1997). Phospholipases are able to hydrolyze one or more ester linkages in glycerophospholipids; the letters A-D are used to differentiate between phospholipases and to denote the specific phospholipid ester bond that is cleaved (Ansell, 1964). Specifically, PLD cleaves sphingomyelin into choline and ceramide-1-phosphate (Bernheimer, *et al.* 1980). Interestingly, an enzyme similar to PLD, showing comparable bioactivity, is found in the venom of the *Loxosceles* genus of spider. The causative toxin is known as sphingomyelinase D, and this observation demonstrates the ubiquitous nature of the phospholipase enzymes (Bernheimer, *et al.* 1985).

In 1965, Jolly demonstrated that PLD was involved in the pathogenesis of *C. pseudotuberculosis*. The investigator showed that an antitoxin against PLD, which was in the form of serum from a horse which had received multiple injections of sub-lethal doses of toxin, was capable of eliminating toxin-induced vascular permeability in the skin of sheep which subsequently reduced the transport of bacteria to the local lymph nodes (Jolly, 1965c). Later, the potency of PLD was demonstrated *in vivo*, when high doses of the enzyme administered intravenously, intraperitoneally and subcutaneously, were observed to cause rapid death in laboratory rodents and gnotobiotic small ruminants (Hsu, *et al.* 1985; Muckle & Gyles, 1983). Significantly, these experiments were conducted using much higher concentrations of PLD than would normally be encountered during a natural infection, and for

this reason toxæmia is not typical of CLA *in vivo* (Yozwiak & Songer, 1993). Interestingly, *in vitro*, PLD causes lysis of sheep erythrocytes when grown in synergy with *Rhodococcus equi* cholesterol oxidase (Bernheimer, *et al.*, 1980; Muckle & Gyles 1986; Prescott, *et al.* 1982). Moreover, an inhibitory affect on the haemolytic activity of staphylococcal β -toxin can be observed when erythrocytes are pre-treated with PLD (Souckova & Soucek, 1972). The toxin has been shown to cause dermonecrosis and increase vascular permeability by hydrolysis of vascular and lymphatic endothelial cell walls (Egen, *et al.* 1989; Songer, 1997). As a result of these observations, PLD has been implicated in aiding the dissemination of *C. pseudotuberculosis* within the host (Batey, 1986). The increase in vascular permeability, (resulting from the hydrolysis of sphingomyelin), leads to leakage of plasma from small blood vessels at the site of infection into the surrounding tissues which then floods lymphatic spaces and allows the bacteria to be transferred to regional lymph nodes (Carne, *et al.* 1956; Jolly, 1965c; Onon, 1979).

More recently, the *C. pseudotuberculosis* PLD gene was cloned, and subsequent sequence analysis revealed that it encoded a protein of 31.4 kDa (Hodgson, *et al.* 1990). When expressed in *E. coli* (under the control of its own promoter), the resulting protein had sphingomyelinase activity (Hodgson, *et al.* 1990). Hodgson *et al.* (1992) disrupted the chromosomally-located PLD-encoding gene from *C. pseudotuberculosis*, thus obtaining a PLD-deficient (Toxminus) strain (Hodgson, *et al.* 1992). Interestingly, when put into an experimental sheep model, virulence was reduced by at least two log doses, and these results clearly demonstrated that PLD contributes to the virulence of *C. pseudotuberculosis* (Hodgson, *et al.* 1992). The investigators also noted that the wild-type bacteria persisted in the host for at least 18 weeks (the duration of the experiment), whereas the PLD⁻ mutant strain was resolved at the site of inoculation within a few weeks. In addition, the mutant bacteria could not be cultured from any of tissue samples obtained from challenged animals at necropsy. These results add to the seminal work on PLD with respect to virulence by suggesting that PLD contributes to the *in vivo* survival of *C. pseudotuberculosis*. Later, McNamara *et al.* (1994) also made a PLD-deficient mutant using allelic exchange mutagenesis to introduce a nonsense mutation in the wild-type chromosomal gene (McNamara, *et al.* 1994). When the resulting PLD-deficient strain was used in goat challenge studies, similar results to those previously observed by Hodgson *et al.* (1992) were seen, in that wild-type bacteria could be recovered from primary lesions and popliteal lymph nodes of infected goats,

whereas the PLD-deficient mutant could only be recovered from the inoculation site of one goat which had received a large (5×10^6 cfu) inoculum of the mutant. Although both investigators demonstrated that PLD affects the formation of abscesses and the subsequent survival and spread of the bacteria within the host, McNamara *et al.* (1994) suggested that further attenuation would be required before a PLD-deficient mutant strain could be used as a vaccine (McNamara, *et al.* 1994). This is because, at the site of inoculation, the abscessation of the goat which received the 5×10^6 cfu inoculum was indistinguishable from that of the wild-type strain.

1.2.2.3 Serine protease

Walker *et al.* (1994) described the identification of a 40 kDa protein antigen from *C. pseudotuberculosis* (Walker, *et al.* 1994). The majority of native protein was found to reside in the culture supernatants, suggesting the 40 kDa protein was secreted, although there was also a suggestion that the protein may be non-covalently attached to the cell (Walker, *et al.* 1994). Significantly, sheep vaccinated with the 40 kDa antigen preparation in an aluminium hydroxide adjuvant, were protected against infection with *C. pseudotuberculosis*, exhibiting, an 82 % reduction in the proportion of infected sheep and a 98 % reduction in number of lung lesions. When compared to the levels of protection observed with the commercially available vaccines at the time of the publication, the researchers stated that their 40 kDa protein antigen vaccine compared favourably (Eggleton, *et al.* 1991a; Eggleton, *et al.* 1991b).

A year later, researchers in the same laboratory cloned the 40 kDa protein structural gene and subsequent DNA sequence analysis revealed an ORF of 1,137 bp, encoding a protein of 379 amino acids (Wilson, *et al.* 1995). Wilson *et al.* (1995) overexpressed the recombinant 40 kDa protein and subsequent biochemical analyses of both the recombinant and native protein revealed associated proteolytic activity. Furthermore, preliminary characterisation of the protein revealed that it had serine protease-type activity; however, there was no significant homology with previously published DNA or protein sequence data found in the databases at that time, leading the authors to suggest that it is a novel protein, possibly belonging to an uncharacterised subclass of serine proteases. Interestingly, protease activity could not be detected in culture supernatants; in fact, protease activity could only be detected after purification of the native protein. Since other serine proteases require conformational change

and cleavage modifications for maximal activity the authors suggest conversion of the inactive form to the active form of the protein may require conformational change and/or post-translational modification (by an autocatalytic self cleavage process). The 40 kDa protein, characterised as having serine protease activity, was subsequently designated Corynebacterial protease 40 (*cp40*; acc. # U10424). The function of the Cp40 is not known, and Wilson *et al.* (1995) alluded to the function by comparison with other characterised serine proteases, which have been shown to have roles in aiding intracellular survival, tissue destruction and helping bacteria to evade the host immune system (Maeda & Molla, 1989; Wilson, *et al.* 1995). The authors also suggested further characterisation of the serine protease is essential to allow an understanding of its contribution to pathogenesis (Wilson, *et al.* 1995).

1.3 Caseous lymphadenitis

Caseous lymphadenitis (CLA or CL) is a chronic, suppurative disease affecting farmed small ruminants, including sheep and goats, worldwide. CLA is caused by infection with *C. pseudotuberculosis*, and although rarely fatal, infections can last for most, if not all, of an animal's life. If this infection is not managed, it can disseminate rapidly through a flock or herd, significantly affecting welfare and productivity as a result. Rather than being an acute disease, CLA is a subtle, often-recurring complaint, classically associated with the formation of pyogranulomas which appear sporadically in various lymph nodes, in particular the superficial lymph nodes (*i.e.* those nodes which can be palpated externally, including the mandibular, parotid, inguinal, prescapular, retropharyngeal, popliteal and mammary lymph nodes) of infected animals (Valli and Parry, 1993). On occasion, internal lymph nodes (*e.g.* mediastinal, bronchial and lumbar lymph nodes) and visceral organs such as the liver, kidney, spleen, brain and most frequently the lungs are also affected (Batey, 1986; Hard, 1969a, 1972; Jensen, 1974; Jolly, 1965b; Jubb & Kennedy, 1970; Marsh, 1965). Lesions within superficial lymph nodes can be externally visible as distinct swellings, often accompanied by the loss of overlying hair, and these observations can be used in diagnosing the presence of CLA within a flock/herd. In contrast, because lesions are hidden within affected animals, the visceral form of CLA is impossible to diagnose, unless at *post mortem*; however, signs of weight loss and ill-thrift are commonly observed in animals with CLA. In this respect, the visceral manifestation is thought to be an important factor contributing to the debilitating condition referred to as “thin ewe syndrome” (Gates, *et al.* 1977).

1.3.1 Prevalence of CLA

CLA is of major economic consequence to sheep producers in many parts of the world, including North and South America, Australia, New Zealand, Europe and South Africa, this global prevalence reflecting the major small ruminant farming areas (M. Smith & Sherman, 1994; Williamson, 2001). The establishment of CLA infection within livestock, in particular sheep, has been an issue for decades in many countries. As early as the 1930s, CLA was recognised as “extensively affecting the flocks of most mutton exporting countries” (Cesari, 1930). For some time it has been suggested that the origins of infection were in Europe, and that the global spread of *C. pseudotuberculosis* followed the exportation of sheep by the 18th century colonial powers. In particular, the Merino breed, which originated in Spain and was widely valued for its characteristics as a dual meat and wool animal, was first exported to South Africa and subsequently Australia and the Americas. This early exportation is thought to have assisted in the spread of *C. pseudotuberculosis* (Paton, 2000). It was therefore surprising, that the first case of CLA in the UK was not identified until 1990, when the disease was thought to have entered via an infected goat from Europe (Lloyd, *et al.* 1990). Unfortunately, since it was first identified CLA has become increasingly prevalent in the UK, with the last published prevalence report revealing that as many as 18 % of terminal sire breeds were affected by the disease (Baird, *et al.* 2001). In the years following the initial assessment of prevalence, incidences of disease have been reported the length and breadth of the British Isles, Northern Ireland and the Republic of Ireland. As a result, the prevalence report of 2001 is likely to be a significant under-representation of the current rate of infection, since rather than being a disease restricted to breeding animals alone, CLA has become well-established within the national flock (Baird, *et al.* 2004).

1.3.2 Economic consequences of CLA

The Economic consequences of CLA are several. Primarily, CLA is responsible for decreased post-slaughter meat yield from production animals, through the necessity to trim off infected lesions, or through outright carcase condemnation in severe cases. In addition, significantly decreased wool yields have been reported in CLA-affected animals in Australia, where it was shown over a 12 month period that sheep challenged with, but not vaccinated against, *C. pseudotuberculosis* produced 0.20 kg less clean wool than unchallenged controls (the annual

wool yield from a healthy sheep is *ca.* 4 kg (Paton, *et al.* 1988); it is currently not known whether the same is also true of sheep in the UK. A reduction in the reproductive efficiency of breeding stock, increased rates of culling and increased mortality of breeding stock are also important economic factors associated with *C. pseudotuberculosis* infection (Williamson, 2001), not to mention reduced live weight gain due to ill thrift in infected animals.

1.3.3 Pathogenicity and disease in sheep

1.3.3.1 Primary infection

Infection with *C. pseudotuberculosis* typically occurs via skin or mucus membrane wounds, such as those introduced by shearing. The bacterium has also been reported to be able to penetrate through intact skin (Smith & Sherman, 1994); however, this observation is open to some criticism and further work would be required to prove or refute such a statement. Following entry into the host, *C. pseudotuberculosis* rapidly spreads to the local draining lymph nodes; it is here that multiple micro-abscesses or micro-pyogranulomas develop, and merge to form larger abscesses (Baird & Fontaine, 2007). The caseation observed within CLA lesions is thought to result from a constant cycle of phagocytosis, bacterial multiplication and cellular degeneration. Pepin *et al.* (1991) studied the early phase of infection in lambs and reported that early pyogranulomas contained clumps of bacteria and cellular debris, in addition to a reasonably high proportion of eosinophils which gave the purulent core a slightly green tinge (Pepin, *et al.* 1991a). Subsequently, the predominant cell type within the lesion became monocytes/macrophages; this stage was closely followed by encapsulation of the lesion (Pepin, *et al.* 1994). As the encapsulated pyogranulomas grow in size, there is a constant degradation and re-synthesis of the fibrous capsule around the expanding lesion. On dissection of a mature abscesses, an 'onion ring' appearance is exhibited, which is typical of CLA (Batey, 1986; Hard, 1969b). As lymph node abscesses mature they can rupture through a fistula, draining infective purulent discharge into the environment (Williamson, 2001). The rupture of superficial lymph node abscesses can result in the release of many millions of bacteria (it has been estimated that each gram of pus contains between 1×10^6 and 5×10^7 cfu (Brown & Olander, 1987)) into the environment, which has been shown to contaminate pens, water buckets, shearing clippers, *etc.*, thereby facilitating transmission of *C. pseudotuberculosis* to other susceptible animals. Furthermore, although unable to multiply, *C.*

pseudotuberculosis has been shown to be able to survive for long periods outside of the host, and the extent of this survival time is enhanced in the presence of particulate fomites such as wood, straw and faeces (Augustine & Renshaw, 1986; Williamson, 2001). Significantly, viable *C. pseudotuberculosis* has been isolated from straw, hay and wood up to 8 weeks after inoculation with pus from a CLA lesion. Viable organisms have also been isolated up to 8 months from soil, where it is thought that survival is enhanced by shaded, moist conditions and low temperatures (Augustine & Renshaw, 1986; Brown & Olander, 1987; Rizvi, *et al.* 1997), such as could be argued to be prevalent within the British Isles. *C. pseudotuberculosis* has also been shown to survive in prepared commercial sheep dips for at least 24 hours (Nairn & Robertson, 1974), although this is no longer an issue in the UK since it is no longer a legal requirement to dip sheep. Once a ruptured abscess has healed over, it is not uncommon for further abscessation to occur within the same animal at the same or other sites, months or even years later. This reoccurrence of abscesses is likely to be a result of a previous infection in which the animal was unable to completely eliminate the infecting organism (Williamson, 2001).

As stated above, CLA occurs in both sheep and goats, and generally there are many similarities in the way that the disease manifests in both species. It is primarily the superficial lymph nodes that are affected, and, in both sheep and goats the visceral form of the disease is thought to occur less frequently but is more severe. Interestingly, the distribution and form of the CLA lesions in sheep and goats around the world have been described as ‘varying’ by some authors (Batey, 1986; Brown & Olander, 1987; Rizvi, *et al.* 1997; Williamson, 2001). In Australia, the superficial lymph nodes of the shoulder and flank of sheep are the sites most commonly associated with CLA lesions; however, in the UK a different pattern is seen, whereby abscesses develop predominantly in the superficial lymph nodes of the head and neck (Baird, *et al.* 2001). Significantly, in other countries, this “UK sheep” lesion pattern is more commonly associated with manifestation of CLA in goats, whereby the parotid lymph node is often affected, followed by the prescapular, and then by other nodes usually confined to the head and neck area (Brown & Olander, 1987).

In Australia the visceral form of CLA is relatively rare, although, it is thought that the associated lung abscesses could be involved in transmission of *C. pseudotuberculosis* via the airways (Ellis, *et al.* 1987; Paton, *et al.* 1995; Stoops, *et al.* 1984). In the USA, the visceral form of CLA is more widespread and is regarded as the most common cause of chronic

wasting disorder or thin ewe syndrome (Paton, 1997). Being a relatively new disease in the UK, there have been few reports of the extent of visceral infection; however, anecdotal evidence would suggest the prevalence of lung lesions in approximately 25 % of naturally-infected animals, and this observation is borne out through the results of experimental challenges of sheep with a UK *C. pseudotuberculosis* strain (Fontaine, *et al.* 2006).

1.3.3.2 Dissemination

Once *C. pseudotuberculosis* has gained entry into a host animal, macrophages migrate to the invasion site and engulf the organism (Pepin, *et al.* 1991b). As discussed previously, conclusive proof is still required to confirm that the high lipid content of the *C. pseudotuberculosis* cell wall enables the bacterium to evade the action of lysosomal enzymes and subsequently survive within the macrophage phagosome (Carne, *et al.* 1956; Hard, 1972). However, whatever the actual method of intracellular survival, once engulfed, surviving intracellular organisms can rapidly multiply within macrophages, resulting in the degeneration and subsequent death of the infected cells, thereby releasing *C. pseudotuberculosis* to be engulfed by secondary macrophages (Batey, 1986; Hard, 1969a, 1972; Jolly, 1965b). The organism is then able to spread through the lymphatic system to secondary sites such as regional lymph nodes and internal organs including, the lungs, spleen and kidney, where new infectious foci are established (Batey, 1986; Hard, 1969a, 1972; Jolly, 1965b). The dissemination process is thought to be aided by the inflammation that occurs as part of the host innate immune response, since in sheep, inflammation results in increased vascular permeability, which increases lymph flow, prostaglandin levels and polymorphonuclear cell numbers in efferent lymph fluid (Johnston, *et al.* 1979). As discussed above, it has also been suggested that vascular permeability, and hence dissemination of the infecting organism, is facilitated by the action of PLD (Carne, *et al.* 1956; Jolly, 1965c; Onon, 1979).

Following dissemination of *C. pseudotuberculosis* within leukocytes, these immune cells are thought to be temporarily removed from the circulation by localisation in the lungs, liver and spleen. It is thought this occurs prior to redistribution to sites of predilection (Hay, 1982). Thakur *et al.* (1977) used whole body scans to follow isotype-labelled leukocytes. The researchers revealed a concentration of cells in the lung tissue, suggestive of pooling of blood or temporary entrapment of leukocytes (Thakur, *et al.* 1977). Batey (1986) suggested that the

work by Thakur *et al.* (1977) supports the observed incidence of *C. pseudotuberculosis* infection within different tissues. In particular, Batey (1986) postulated that retention of degenerating infected immune cells within the lungs of sheep is responsible for the subsequent development of micropyo-granulomas, eventually, leading to fully-developed CLA abscesses within the lungs (Batey, 1986).

1.4 *C. pseudotuberculosis* infection in other species

1.4.1 *C. pseudotuberculosis* infection in horses

Infection with *C. pseudotuberculosis* in horses causes ulcerative lymphangitis, an excoriating, suppurative inflammation of the lymphatic system. The condition, which is usually confined to the distal portion of the limbs of affected animals, is generally thought to be associated with poor husbandry practices, and is rarely encountered nowadays, except for in the USA (Brown & Olander, 1987). More recently in the USA, *C. pseudotuberculosis* has been reported as the causative-agent of a syndrome in horses known as Pigeon fever (synonyms; dry land distemper, Wyoming strangles, false strangles). Pigeon fever is recognised as a problem, particularly in the Western United States, and clinical signs of the disease include lameness, fever, weight loss and lethargy, in addition to the development of very deep abscesses and multiple sores along the midline and groin areas. In some cases the abscesses can spread to the back, and also develop internally. The disease was coined “pigeon fever” due to the nature of the abscesses, which cause swellings on the pectoral muscles, which subsequently resemble a pigeon’s chest (Brown & Olander, 1987; Hughes & Biberstein, 1959). In rare cases, *C. pseudotuberculosis* has been associated with cases of equine abortion (Miers & Ley, 1980; Rumbaugh, *et al.* 1978) and equine mastitis (Addo, *et al.* 1974).

The observation that the majority of *C. pseudotuberculosis* infections in horses occur predominantly in the Western United States of America, has led to the suggestion that there may be a specific reason for this geographical clustering within the USA. Some authors have suggested that arthropod vectors in this area may have facilitated spread of the organism between horses (Knight, 1969; Miers & Ley, 1980). Spier *et al.* (2004) performed real-time PCR analyses of flies that were collected from an area which surrounded that inhabited by infected horses. The researchers demonstrated the presence of the *C. pseudotuberculosis* (as

determined by successful PCR amplification of the PLD-encoding gene) in a number of the samples, thereby implicating the flies in the carriage of this organism. The most prevalent arthropod vector found to be carrying *C. pseudotuberculosis* was the housefly (*Musca domestica*), where around 20 % were shown to be carriers (Spier, *et al.* 2004).

1.4.2 *C. pseudotuberculosis* infection in cattle

Infections of cattle with *C. pseudotuberculosis* were previously thought to be exceptionally rare, with the only clinical case having been described in 1980; mastitis, arising through a laceration of the teat resulted in clinical signs including a swollen, hard and tender udder quarter, watery milk containing clots, fever, and severe weight loss (Adekeye, *et al.* 1980). Subsequently however, reports of infections of cattle have increased, although it is unknown whether this is as a result of increased prevalence or increased attention. *Corynebacterium pseudotuberculosis* has been isolated from cattle with ulcerative granulomatous lesions (Yeruham, *et al.* 2004), otitis (Duarte & Hamdan, 2004) and a visceral form of ulcerative lymphangitis (Shpigel, *et al.* 1993).

Significantly, *C. pseudotuberculosis* lesions in the bovine host are distributed quite differently to those in the equine host, since only rarely do lesions occur on the limbs of cattle. In Egypt, *C. pseudotuberculosis* is the causative agent of an endemic disease of buffalo called oedematous skin disease (OSD), which is characterised by swellings and thickening of the skin in the hind or fore limbs, the belly and dewlap (Selim, 2001); lesions are usually associated with the drainage lymph nodes. Oedematous skin disease primarily manifests during the summer months, although sporadic cases can develop throughout the year, which would implicate the involvement of a seasonally-occurring vector in the promotion of disease transmission.

1.4.3 Zoonotic Infections

Reports of *C. pseudotuberculosis* infections in humans are somewhat rare, but are usually associated with patients' occupations. In particular, workers in regular contact with sheep (*e.g.* shepherds, shearers, abattoir-workers and butchers) are more likely to be affected (Baird

& Fontaine, 2007), and clinical signs include suppurative granulomatous lymphadenitis (Mills, *et al.* 1997). Significantly, there have been no fatal cases of *C. pseudotuberculosis* infection of humans reported. The first published case was in 1966 (Lopez, *et al.* 1966), and reports of subsequent cases have been sporadic (Goldberger, *et al.* 1981; Keslin, *et al.* 1979). Recently, the description of a case of necrotizing lymphadenitis in a 12 year old child was described (Join-Lambert, *et al.* 2006). The child contracted the infection after having had contact with sheep whilst on holiday in rural France. The organism that was isolated from the infection was nitrate-negative *C. pseudotuberculosis* isolate which was susceptible to a range of antibiotics *in vitro*. However, two separate treatments with amoxicillin/clavulanate were unsuccessful. The infection increased in severity which prompted the use of intravenous antimicrobial therapy and the subsequent surgical resection of affected tissue. Intravenous antimicrobial therapy was continued for 4 months after the surgery followed by 6 months of oral therapy and after two years no relapse had occurred. In addition, a second report occurred in a 63 year old woman who was living in rural France. The first treatment introduced was antibiotherapy, which was followed by a second course of antibiotics. Unfortunately neither was able to clear the infection which left surgery as the only option for a cure (Hemond, *et al.* 2009). It has been suggested that human *C. pseudotuberculosis* infections are under reported in countries such as Australia where ovine CLA is particularly prevalent, since often affected human lymph node abscesses are not cultured after excision, and hence the causative agent of the infection is not determined (Baird & Fontaine, 2007).

1.5 Antimicrobial therapy

Although *C. pseudotuberculosis* isolates are routinely sensitive to a wide variety of antimicrobial compounds, the generally-held hypothesis is that it is extremely difficult, if not impossible, to successfully cure *C. pseudotuberculosis*-infected animals with antibiotics. There are two reasons for this; firstly, it is thought that the organism is protected from commonly employed antibiotics during stages of the infection when the bacteria are intracellular (Williamson, 2001). Secondly, antibiotics are thought to be unable to penetrate the thick encapsulations around the typical pyogranulomas and the thick caseous pus within the lesion (Williamson, 2001). Recently however, there have been reports of the successful use of antibiotics to cure sheep of infection with *C. pseudotuberculosis* (Senturk & Temizel, 2006; Ural, 2008). Senturk and Temizel (2006) used a combination of rifamycin and

oxytetracycline antibiotics to treat CLA in a naturally-infected sheep (2-3 years of age, weighing 54-61 kg). Rifamycin is often used to combat infections involving *M. tuberculosis* and *Mycobacterium leprae* since the antibiotic is known to kill susceptible intracellular bacteria. Senturk and Temizel (2006) took 10 CLA-affected animals, and over a period of 10 days treated twice-daily with rifamycin, interspersed with injections of oxytetracycline at intervals of 3 days. The researchers observed a decrease in the size of CLA-lesions in the external lymph nodes and described the result as a clinical resolution of the lesions (Senturk & Temizel, 2006). However, the report did not describe how long the animals were apparently “disease free”, and did not involve detailed *post mortem* analyses. Furthermore, the study used antibiotics not licensed for veterinary use, at least in the UK. In a published, critical response to this study, it has been suggested that further and thorough studies are essential before antibiotic treatment of CLA cases becomes a viable alternative to culling (Baird, 2006).

In a more recent study Ural *et al.* (2008) assessed the efficacy of kanamycin for controlling *C. pseudotuberculosis* infection in goats. The researchers demonstrated a statistically significant difference ($p < 0.05$) in the clinical recovery of kanamycin-treated goats compared to a placebo group (Ural, 2008). These results suggested that the antibiotic was effective at reducing clinical signs of CLA in the goat model; however, as with the previous study by Senturk and Temizel (2006) the animals were not followed up for a significant period of time after they had apparently been cured. In this study, it was entirely possible, and likely, that a recrudescence of infection would occur in kanamycin-treated animals, since the antibiotic cannot penetrate macrophages, and hence would have no access to *C. pseudotuberculosis* cells resident within macrophages at the time of treatment.

1.6 Vaccines against *C. pseudotuberculosis*

Interestingly, although there is very little known about the virulence mechanisms employed by *C. pseudotuberculosis*, there has been a substantial volume of work carried out with respect to the development of vaccines against infection with this organism. Historically, the use of cellular components, bacterin vaccines and the *C. pseudotuberculosis* PLD exotoxin have demonstrated some protection against experimental infection (Brodgen, 1995; Cameron, *et al.* 1972; Jolly, 1965c). Jolly (1965) demonstrated that PLD was able to confer some protection against *C. pseudotuberculosis* infection in sheep (Jolly, 1965c). Later, Cameron *et al.* (1972)

determined the ovine immune response to vaccination, following administration of an experimental vaccine composed of formalin-killed, whole cells of *C. pseudotuberculosis* (Cameron, *et al.* 1972). The researchers concluded that vaccination with the bacterin vaccine could protect sheep against the lethal effects of sub-acute infection but not against the formation of the lesions associated with chronic infections (Cameron, *et al.* 1972). Subsequently, attempts to develop an effective CLA vaccine concentrated on the use of whole cell vaccines or bacterins as it was generally considered that a toxoid vaccine would not be sufficient to confer protection against *C. pseudotuberculosis* due to the chronic nature of the infection in sheep.

In Australia, since management strategies alone were found to be unable to prevent the spread of CLA, efforts to develop an effective vaccine were increased in the 1970s. As a result, further evidence demonstrating the ability of the *C. pseudotuberculosis* PLD exotoxin to protect against CLA was reported (Burrell, 1978; Nairn, *et al.* 1977); this influential work led to the development of the first commercial CLA vaccine, Glanvac™ (Eggleton, *et al.* 1991a). The Glanvac™ vaccine consists of a combined clostridial and corynebacterial components, including formalin-inactivated PLD obtained from filtered and concentrated *C. pseudotuberculosis* culture supernatants (Burrell, 1983). The vaccine has been reported to confer statistically-significant protection against infection with *C. pseudotuberculosis* (Burrell, 1983; Eggleton, *et al.* 1991a; Eggleton, *et al.* 1991b; Eggleton, *et al.* 1991c); however, Burrell (1983) stated that the culture supernatants used to prepare Glanvac™ contain other *C. pseudotuberculosis* antigens which vary from batch to batch (Burrell, 1983), and hence affect the efficacy of different vaccine batches. Subsequent to the release of Glanvac™, there has been a steady flow of publications in which research groups have attempted to improve upon the level of protection obtained by focusing upon the development of alternative CLA vaccines. Another reason for needing to improve upon the already significant protection achieved with Glanvac™, was as a result of the general requirement for a single dose vaccine (at present Glanvac™ requires lambs to be revaccinated 4 weeks after an initial vaccination). One way in which this has been attempted was through the use of live attenuated vaccines. Since PLD has been shown to be an important virulence factor of *C. pseudotuberculosis* (Carne & Onon, 1978) a group of researchers in Australia attempted to create a mutant derivative of the PLD-encoding gene in *C. pseudotuberculosis*, and test its ability to act as a live attenuated vaccine (Hodgson, *et al.* 1992). Hodgson *et al.* (1992) demonstrated that single

subcutaneous inoculation with 10^5 cfu of the mutant strain (designated Toxminus), resulted in an absence of the clinical disease usually associated with wild-type *C. pseudotuberculosis* infection; however, at higher doses (10^7 cfu) transient abscesses were observed at the site of inoculation. Later, in order to overcome these problems the same group evaluated the use of Toxminus as a live oral vaccine (Hodgson, *et al.* 1994). During this work Hodgson *et al.* (1994) postulated that by creating a *pld* strain, its usefulness as live oral vaccine was reduced since it no longer contained one of the major immunodominant antigens (Hodgson, *et al.* 1994). In order to overcome this, the researchers subsequently used the Toxminus strain to introduce a plasmid borne copy of PLD which had been mutated in such a way that it secreted inactive PLD (Hodgson, *et al.* 1994). The PLD-encoding gene was toxoided by site specifically mutating PLD His-20 to Tyr, a substitution which had been shown previously to remove enzymatic activity (Haynes, *et al.* 1992). However, in contrast to subcutaneous vaccination of sheep with Toxminus, oral vaccination did not provide significant protection against challenge with wild-type *C. pseudotuberculosis* (Hodgson, *et al.* 1994). Using a goat infection model McNamara *et al.* (1994) also assessed the utility of a *C. pseudotuberculosis* *pld* variant as a live attenuated vaccine. The researchers mutated the PLD-encoding gene of a Whetten 1 goat strain of *C. pseudotuberculosis* by replacing it with an allele containing a nonsense mutation. Subsequently, virulence of the mutant strain (designated W1.31r1) was compared to wild-type by inoculating goats (McNamara, *et al.* 1994). The researchers demonstrated that the mutant bacteria had a greatly reduced ability to induce a clinical response in goats (McNamara, *et al.* 1994). However, neither of these *C. pseudotuberculosis* mutants have been suitable for use as live attenuated vaccines due to the infection at the sites of inoculation being indistinguishable to that of wild-type.

Subsequent to the release of Glanvac™, other commercial CLA vaccines have been developed, including Case-Bac and Caseous D-T (Colorado Serum Laboratories; Denver, CO, USA) which are either based on killed whole-cell preparations or toxoided PLD. However, these latter vaccines are of unproven efficacy and are unlicensed for use outside of their country of origin (USA).

To date there is no routinely-licensed vaccine available in the UK for use against CLA. However, the production of a so-called “autogenous” CLA vaccine is possible under an emergency license issued by the Veterinary Medicines Directorate (VMD). Autogenous

vaccines are adjuvanted bacterins derived from *C. pseudotuberculosis* isolates from flock outbreaks. These vaccines are only intended for use in animals on the holding in which the strain used to prepare the vaccine has derived (Fontaine, *et al.* 2006). Unfortunately, however, in addition to the relatively high cost of preparation, there is very little data available to support the efficacy of these vaccines. Fontaine *et al.* (2006) assessed an experimental autogenous vaccine (formalin-killed *C. pseudotuberculosis*) and showed that it was sufficient to prevent the spread of the *C. pseudotuberculosis* challenge strain beyond the site of inoculation in an ovine experimental model of CLA. Although these results confirm that autogenous vaccination does confer significant protection, it is not known what the exact formulation of the commercial autogenous vaccine is, and whether an equivalent level of protection can be achieved with this vaccine, especially under field conditions. In addition, it is unknown whether an autogenous vaccine will confer protection against infection with heterologous *C. pseudotuberculosis* strains. In this respect, it has been shown by PFGE analysis that more than a single strain of *C. pseudotuberculosis* may be in circulation within a single flock at any given time, presumably due to multiple importations of the disease through purchase of new animals, *etc.* (Fontaine, *et al.* 2006).

The ability of Glanvac™ to protect against experimental challenge with a heterologous UK *C. pseudotuberculosis* strain (heterologous because the PLD in Glanvac™ was derived from a different strain to that used for the challenge) was recently assessed, and this work described the only scientific investigation into the capacity of Glanvac™ to protect against the UK form of CLA (Fontaine, *et al.* 2006). It was demonstrated that, overall, the prevalence of *C. pseudotuberculosis*-infected loci was reduced within Glanvac™ immunised animals as compared to unvaccinated, challenged control animals; however, only one of six vaccinated animals appeared completely free from infection, while the others had infections at multiple internal loci. It was therefore concluded that Glanvac™ was not able to prevent the dissemination of the challenge strain beyond the primary site of infection; however, it was interesting to note that none of the vaccinated animals were found to have lung lesions (Fontaine, *et al.* 2006). Glanvac™ was never licensed for use within the European Union; however, this has not prevented sheep producers from (legally) purchasing the vaccine from abroad and (illegally) administering it to their animals. This introduced a potential complication with respect to a newly-introduced CLA diagnostic test (so-called “Elitest CLA”; M. C. Fontaine, unpublished data), which is currently provided on a UK-wide

flock/group-testing basis by the Scottish Agricultural College (Perth Veterinary Centre, Bertha Park View, Perth, Perthshire, Scotland). The test, described in a preliminary form by Fontaine *et al.* (2006), utilises a recombinant PLD protein to detect anti-PLD antibodies in sheep with CLA, using an ELISA-based procedure (Fontaine, *et al.* 2006). Given that Glanvac™ is also PLD-based, there was the possibility that illegally-vaccinated animals would cause false-positive reactions in the blood test, which would be unlikely to be explained due to lack of information regarding vaccine usage. However, the purchase of the vaccine in the UK has recently become possible under an emergency license which may be issued by the VMDAs such, by removing the illegality of the use of Glanvac™, it is hoped that producers will be more likely to report the use of the vaccine when they sell-on their animals, so that prospective buyers can bear this in mind when deciding whether to undertake CLA blood-testing.

As part of the same experiment involving the assessment of Glanvac™ in the UK, Fontaine *et al.* (2006) also assessed the efficacy of a recombinant PLD protein (rPLD) and a killed whole-cell vaccine supplemented with rPLD to protect against infection with the homologous *C. pseudotuberculosis* strain. Significantly, the rPLD vaccine (which had been completely inactivated by treatment with SDS) induced a greater level of protection than had been observed with Glanvac™. Although it was acknowledged that the experimental rPLD vaccine was derived from the challenge strain (whereas Glanvac™ was not), the overall similarity between the PLD sequences in different *C. pseudotuberculosis* strains is so great that there was essentially nothing to discriminate between homologous and heterologous challenge in this case. It would therefore seem that the recombinant derivative of PLD conferred greater protection than Glanvac™, which is in contrast to a previous study in Australia, whereby it was found that a recombinant, inactivated derivative of PLD did not induce the same level of protection in vaccinated animals as Glanvac™ did (Burrell, 1983). In addition, the results of the Fontaine *et al.* (2006) study would tend to disprove the hypothesis that residual PLD activity in PLD-based vaccines is required for protection. In their study, Fontaine *et al.* (2006) also used a combined rPLD and formalin-killed whole cell vaccine, and demonstrated that this combination conferred 100% protection against experimental infection of sheep with *C. pseudotuberculosis* (Fontaine, *et al.* 2006).

1.7 Iron-regulated proteins as vaccines

Iron is essential for life, and bacteria have developed numerous mechanisms by which they obtain it from their immediate environment. As a result of the absolute requirement for it, interference with iron-acquisition mechanisms can lead to the death of the bacterial cell. As a result, the proteins involved in iron-acquisition have been targeted in the development of novel vaccines, and to date there have been several publications documenting the successful application of such vaccines (Banerjee-Bhatnagar & Frasch, 1990; Chhibber & Bajaj, 1995; Chibber & Bhardwaj, 2004; Gilmour, *et al.* 1991). Early studies, such as that conducted by Banerjee-Bhatnagar *et al.* (1990) described the *potential* use of iron-regulated proteins (IRPs), including a 70 kDa transferrin receptor, as components in a vaccine against group B meningococcal disease. The researchers noted that their vaccine, which was an outer membrane protein preparation from bacteria grown under low-iron conditions in order to induce expression of IRPs, induced significant levels of anti-IRP antibodies. However, despite postulating the use of IRPs as a vaccine, and noting that IRPs are major outer membrane proteins expressed *in vivo* and are required for bacterial survival, the researchers did not assess vaccine efficacy in their study (Banerjee-Bhatnagar & Frasch, 1990). In 1991, Gilmour *et al.* assessed the efficacy of a vaccine containing *Pasteurella haemolytica* sodium salicylate extract (SSE) and IRPs (the latter having been induced by growth under low-iron conditions) in the protection against an experimental model of lamb pasteurellosis (Gilmour, *et al.* 1991). During the trial the group also tested a vaccine containing SSE only (previously shown to confer protection against a different strain of *P. haemolytica*). Interestingly, the researchers demonstrated significant protection ($p < 0.005$) against *P. haemolytica* using the vaccine which incorporated both SSE and IRPs whereas the SSE only vaccine did not confer protection against the strain under investigation. The researchers proposed that the enhanced protection observed with the SSE-IRP vaccine was as a direct result of the IRPs. Furthermore, they also suggested that the IRPs may have enhanced the protective efficacy of their vaccine by stimulating the production of antibodies to antigens produced *in vivo* in response to naturally provoked iron depletion (Gilmour, *et al.* 1991).

Subsequent to these initial reports, other groups have studied the use of IRPs as vaccine candidates. Using a rat infection model, Chibber and Bajaj (1995) investigated the immunogenicity of a polysaccharide-iron-regulated cell surface protein conjugate vaccine

against *Klebsiella pneumoniae*-induced lobar pneumonia (Chhibber & Bajaj, 1995). The vaccine was found to be highly protective in a rat infection model, influencing a decrease in bacterial counts by 3.17 log cycles (Chhibber & Bajaj, 1995). A much later publication described protection against infection in a mouse experimental model of peritonitis, elicited by iron-regulated outer membrane proteins (IROMPs) of *Salmonella typhi* (Chibber & Bhardwaj, 2004). In that study the researchers used a conjugative vaccine consisting of *S. typhi* polysaccharide and IROMPs which had been prepared from the same strain. The vaccine preparation was tested in male BALB/c mice, and following two vaccinations the animals were challenged intraperitoneally with 10^9 organisms per ml of *S. typhi*. Quantitative bacterial counts determined from the spleens, Peyer's patches and livers of mice immunised with the conjugated vaccine demonstrated a 4-fold decrease in bacterial counts compared to unvaccinated mice (Chibber & Bhardwaj, 2004). The researchers noted that the conjugate vaccine elicited both a humoral and cell-mediated immune response. It was postulated that the higher antibody levels seen with the conjugate compared to the controls (where animals were separately immunised with each subunit component of the conjugate vaccine) may have contributed towards the protection against challenge, since antibodies against IROMPs have been shown to interfere with iron uptake, leading to protection of mice against infections (Banerjee-Bhatnagar & Frasch, 1990; Lucier, *et al.* 1996).

A recent investigation by Aranda *et al.* (2009) employed a slightly different approach to study the protective capacity of iron-regulated proteins (Aranda, *et al.* 2009). The researchers created a double knockout of a *Streptococcus suis* strain to create a mutant which was deficient in both the zinc/manganese regulator (*adcR*) and the iron-uptake regulator (*fur*). The mutant strain, designated UA5002 *AdcR*⁻/*Fur*⁻, overexpressed a set of immunogenic, cell-surface proteins, which were able to confer significant protection against *S. suis* challenge in a mouse model of infection (Aranda, *et al.* 2009).

1.8 Iron-homoeostasis

Although iron is an essential requirement for most living organisms, unless its concentration is tightly-regulated it is highly toxic. Under physiological conditions, iron exists mainly in the extremely insoluble ferric form (Fe^{3+}); however, in the presence of molecular oxygen, it reacts via the Fenton reaction to produce highly-reactive oxygen species (ROS), hydroxyl radicals

and ferryl iron (Touati, 2000). Excess ROS (Greenberg & Demple, 1989) are detrimental to living organisms, resulting in DNA damage (Touati, 2000). Due to excess iron being highly toxic to the host as well as the pathogen, availability of iron within extracellular fluid and on the mucosal surfaces is highly restricted; in mammals this is achieved by sequestration of free-iron by iron-binding glycoproteins. The predominant mammalian iron-binding protein in serum and extracellular fluid is transferrin, whereas lactoferrin dominates in mucosal secretions and milk (Andrews, *et al.* 2003; Wooldridge & Williams, 1993). Other important sources of iron within the host are haem-containing proteins, such as haemoglobin. It is estimated that serum contains as much as 1.56 mg/ml of iron (Burriel & Heys, 1997); however, much of this is not readily accessible to bacterial pathogens (Litwin & Calderwood, 1993).

For bacterial pathogens, the availability (or otherwise) of iron contributes to successful colonisation or clearance from the host. As a result, one arm of the mammalian non-specific immune defence has evolved to exploit this iron requirement. During infection, iron availability is limited by the host in a process known as hypoferremia, which has been recognised as a component of the acute-phase response (Litwin & Calderwood, 1993; Otto, *et al.* 1992). The mechanisms of hypoferremia are poorly understood, however, it can be induced by intact microbes and certain microbial products such as endotoxin and mycolic acid, and is mediated through the increased release of various cytokines (interleukin-1 and tumour necrosis factor alpha in particular (Beutler & Cerami, 1987; Dinarello, 1984)). As a result, iron saturation in serum decreases concurrently with an increase in the level of transferrin, which binds free-iron, rendering it inaccessible (Litwin & Calderwood, 1993; Muller, *et al.* 1983; Weinberg, 1984). In addition, during the inflammatory response, the level of lactoferrin within the blood is also increased. The release of apo-lactoferrin by leukocytes occurs during degranulation and phagocytosis, which, in turn, increases the local concentration of lactoferrin at sites of infection (Leffell & Spitznagel, 1975; Wright & Gallin, 1979). Interestingly, several studies have shown that animals whose serum levels of iron have been reduced by an iron-deficient diet, show increased resistance to bacterial infection. Moreover, resistance to infection is reversed if the iron deficient host is injected with sufficient iron to restore levels to that of normal serum (Hart, *et al.* 1982; Puschmann & Ganzoni, 1977).

1.9 Genes Involved In Iron Sequestration

Bacteria have evolved a number of diverse mechanisms which enable them to scavenge iron from their environment, whether it be in or out of a mammalian host. Furthermore, because iron-limitation is such an important factor, the expression of many of these genes is regulated in direct response to the levels of environmental iron (Hantke, 2001). A common method of iron acquisition is through the production and secretion of low molecular weight, high-affinity, iron-scavenging molecules known as siderophores (which are discussed in detail in Chapter 6).

Significantly, bacteria which have been genetically-manipulated to be deficient in the biosynthetic or transport apparatus required for siderophore production and utilisation, are not always restricted in their ability to colonise and persist within the host. This is because, in addition to siderophores, bacteria have also evolved other mechanisms of obtaining iron from their environment, which is somewhat unsurprising given the importance of iron to the cell. Certain bacteria possess “siderophore-independent” mechanisms of iron acquisition, the best-characterised of which involve the direct binding of the host iron-binding protein (*e.g.* transferrin, lactoferrin and haemoglobin) to substrate-specific receptors located on the outer membrane, prior to transport of the bound protein into the cell. The receptors, Transferrin-binding proteins (Tbps), involved in the binding of transferrin and lactoferrin have been well studied in Gram-negative pathogenic *Neisseria* and *Haemophilus* species (Mietzner & Morse, 1994). The process of iron delivery is via a periplasmic-binding protein-mediated active transport mechanism involving the Ferric iron-binding protein (Fbp) (Mietzner & Morse, 1994). In contrast, siderophore-independent mechanisms of iron acquisition are less well understood in Gram-positive bacteria. In *Mycobacterium avium* subsp. *paratuberculosis*, an extracellular ferric reductase has been identified, which is able to remove iron from ferric ammonium citrate, ferritin and transferrin by reduction of the metal (Rodriguez & Smith 2004). In *C. diphtheriae*, a study by Schmitt *et al.* (1997) demonstrated that the organism was able to utilise iron from haem and haemoglobin when in an iron-limited growth environment (Schmitt, 1997b). Later, several genes involved in haem utilisation were identified in *C. diphtheriae* (Schmitt, 1997b; Wilks & Schmitt, 1998), including *hmuO*, encoding the first bacterial haem oxygenase. Subsequent work to assess the function of HmuO, by sub-cloning and expression of the protein in *E. coli*, revealed that the enzyme had haem-cleaving activity

and enabled *C. diphtheriae* to obtain iron from haem-containing compounds such as haemoglobin (Wilks & Schmitt, 1998).

1.10 Microbial “sensing” of the environment

In order to adapt to diverse niches, bacteria are required to sense and respond to their environment. Such environmental conditions include temperature, pH and ion concentration, including iron. The “sensing” mechanism is achieved by bacterial regulatory proteins which adapt accordingly to changes in environmental conditions. Regulation occurs by both activation and repression of gene expression by so-called stand-alone transcriptional regulatory proteins (*e.g.* Zur (Smith, *et al.* 2008)) and by two-component regulatory systems (*e.g.* PhoR/S (Kocan, *et al.* 2006)). Stand-alone transcriptional regulatory systems involve a receptor protein forming a complex with a specific ligand (in the case of Zur, the ligand is zinc (Smith, *et al.* 2008)); subsequently, the receptor/ligand complex is able to function directly as a transcriptional activator or repressor by binding to specific promoters, which leads to the modulation of gene expression (Miller, *et al.* 2007). In contrast to the relatively simple stand-alone regulators, two-component regulatory systems are more complex since they involve a membrane-bound sensor histidine kinase (the first component) which is required to phosphorylate a transcriptional activator (the second component) which is then able to regulate gene expression (Miller, *et al.* 2007). Briefly, on stimulation by an extracellular signal the histidine kinase domain of the sensor protein is autophosphorylated. Signal transduction takes place when the phosphoryl group is transferred to the equivalent response regulator protein. Phosphorylation of the regulator protein subsequently adjusts its binding affinity for target promoter regions, and, as a result this affects the frequency of transcription of specific genes or operons (Graham, *et al.* 2002).

Often, an interrelationship between groups of genes which are controlled by different regulatory proteins exists. In such situations, one regulator is able to exert an affect on the expression of another regulator, thus allowing the “global” co-ordination of expression of multiple groups of genes required for multiple different processes and produced in response to varying environmental stimuli. Several examples of such “global regulatory networks” have been described, one example being the Control of virulence Regulator” (CovR) of *Streptococcus pyogenes*. CovR is part of a two-component regulatory system (CovR/S) which negatively controls expression of both proven and putative virulence factors, including capsule

and cysteine protease (Graham, *et al.* 2002). The expression of these virulence-associated genes is regulated in response to growth phase and the external environment of the bacteria (Churchward, 2007). The CovR/S system regulates, either directly or indirectly (by influencing the expression of genes encoding other transcriptional regulators), 15% of the genes within the *S. pyogenes* genome (Graham, *et al.* 2002).

1.11 Iron-regulation in corynebacteria

Very little is known about the differential expression of *C. pseudotuberculosis* genes in response to environmental iron concentrations (or any other condition for that matter); however, there has been a significant amount of research conducted on this subject in other corynebacteria, principally *C. diphtheriae* and *C. glutamicum*. In *C. diphtheriae*, the principle virulence determinant is diphtheria toxin (DT), which the organism must produce in order for it to cause the severe respiratory disease, diphtheria. Diphtheria toxin is an A-B toxin that catalyses the ADP-ribosylation of a host cell protein essential for protein synthesis (Salyers, 1994). So called A-B toxins are composed of two parts, these being an enzymatically-active portion, and a receptor-binding portion (Salyers & Whitt 1994). In the case of DT, susceptible cells are killed by the toxin catalyzing ADP ribosylation of elongation factor 2 (EF 2), resulting in the inhibition of protein synthesis (Collier, 1967; Honjo, *et al.* 1971; Iglewski & Kabat, 1975). Significantly, DT is extremely toxic with a lethal dose of *ca.* 0.1 µg/kg body weight for humans and susceptible animals (Holmes, 2000).

In 1936, Pappenheimer and Johnson demonstrated that the addition of low concentrations of iron to the growth medium resulted in the inhibition of DT production by *C. diphtheriae* (Pappenheimer, & Johnson 1936). It was later found that DT was expressed at maximal levels only during the “decline phase” of the bacterial growth cycle, when iron becomes a growth rate-limiting substrate (Pappenheimer, 1955). It was noted some years ago that DT, despite being essential for the development of diphtheria, is not produced by all *C. diphtheriae* strains. Those strains without DT were generally regarded as nontoxigenic, and infections with these strains tended to manifest as superficial, self-limiting sore throat and cold-like symptoms. However, more recently there have been documented cases in which systemic disease has resulted from infection with a nontoxigenic *C. diphtheriae* isolate (Zasada, *et al.* 2005), and it

is currently thought that these nontoxigenic isolates could act as a reservoir for the re-emergence of toxigenic *C. diphtheriae* strains in the UK (De Zoysa, *et al.* 2005). Uchida *et al.* (1971) demonstrated that rather than being a normal component of the *C. diphtheriae* genome, the DT structural gene, *tox*, was in fact carried by several corynebacteriophages, including the well characterised phage β (Uchida, *et al.* 1971). Lysogeny of susceptible strains with *tox*-carrying (*tox*⁺) corynebacteriophages was essential to allow production of DT; however, the expression of the *tox* gene itself was shown to be dependent on the physiological state of the host bacterial cell (Murphy, *et al.* 1974). Using an *E. coli* system to express DT from β -phage DNA, Murphy *et al.* (1974) proved that the synthesis of DT was not dependent on iron alone. The addition of iron to the medium had no effect on the synthesis of DT and other phage-encoded proteins, whereas the addition of *C. diphtheriae* extracts resulted in differential expression of DT with and without iron supplementation (Murphy, *et al.* 1974). Therefore, the expression of the *tox* gene was clearly shown to be dependent on the physiological state of the *natural* bacterial host.

The observation that the physiology of toxigenic *C. diphtheriae* strains influenced *tox* expression led to the hypothesis that the organism carries a gene encoding an apo repressor, which, in the presence of iron, forms an active complex that specifically binds to the *tox* operator thereby blocking transcription of the gene (Murphy & Bacha 1979). This hypothesis was confirmed some time later when Boyd *et al.* (1990) and Schmitt and Holmes (1991) described the molecular cloning of an iron-dependent regulatory element; the chromosomally-encoded Diphtheria toxin Repressor (DtxR) protein (Boyd, *et al.* 1990; Schmitt & Holmes, 1991). The *dtxR* gene encodes a 226 aa polypeptide with a molecular weight of 25,316 kDa, which shares some homology to the *E. coli* Fur protein. In the presence of iron, DtxR was shown to bind to a specific DNA motif within the *tox* operator, preventing the binding of RNA polymerase to the *tox* promoter, thereby preventing transcription of *tox*. However, in the absence of iron, DtxR was found to be unable to bind the *tox* operator; thus the iron-dependent transcription of *tox* was proven (Tao, *et al.* 1994). In addition to DT of *C. diphtheriae*, the expression of other bacterial toxins has also been shown to be repressed in the presence of iron. Examples include the Shiga toxin from *Shigella dysenteriae* type I (Van Heyningen & Gladstone, 1953), Shiga-like toxin I from *E. coli* (Calderwood & Mekalanos, 1987), and exotoxin A from *Pseudomonas aeruginosa* (Bjorn, *et al.* 1978).

1.12 DtxR-like proteins in other bacteria

Since its discovery, DtxR has become the founding member of a rapidly expanding group of bacterial, metal-ion-dependent, regulatory proteins that have been identified in numerous Gram-positive and acid fast genera (Feese, 2001), including: *Streptococcus* (Jakubovics, *et al.* 2000; Kitten, *et al.* 2000), *Staphylococcus* (Hill, *et al.* 1998), *Bacillus* (Que & Helmann, 2000), *Escherichia* (Patzner & Hantke, 2001), *Treponema* (Hardham, *et al.* 1997), *Streptomyces* (Gunter-Seeboth & Schupp, 1995), *Mycobacterium* (Doukhan, *et al.* 1995) and *Rhodococcus* (Boland & Meijer, 2000). The DtxR-like transcriptional regulator has been characterised for some of these bacteria, and it has become apparent that they can be neatly divided into two distinct groups, defined by the ability of the regulator to exert an effect through the binding of either iron or manganese.

Oram *et al.* (2004) assayed chromosomal DNA from 42 different corynebacterial strains, representing 33 different species, for the presence of a highly conserved region of the *dtxR* gene (Oram, *et al.* 2004). Interestingly, the researchers demonstrated that all strains tested incorporated this conserved region, including a strain of *C. pseudotuberculosis*, ATCC 19410 (Oram, *et al.* 2004). Concurrently, a strain of *C. pseudotuberculosis*, isolated from a sheep in an outbreak of CLA in the Scottish Borders (Connor, *et al.* 2000), was assessed for the presence of a *dtxR*-like gene (Malloy, 2004); a *ca.* 0.3 kb PCR product was amplified from this strain using oligonucleotide primers based upon conserved regions within the *dtxR* genes of other sequenced corynebacteria, and subsequent analysis of sequence of the PCR product revealed that it encoded a fragment of a *C. pseudotuberculosis* gene sharing homology with the *C. diphtheria dtxR* gene. However, despite these findings which suggest the presence of a *dtxR*-homologue in *C. pseudotuberculosis*, there has yet to be any report of the involvement of a DtxR homologue in the regulation of gene expression in this species.

Following the sequencing of the *C. glutamicum* genome (Kalinowski, *et al.* 2003), and the subsequent discovery of a DtxR-like protein in this species, several studies ensued. DtxR was shown to be vital for the iron-dependent regulation of gene expression in *C. glutamicum* (Brune, *et al.* 2006; Wennerhold & Bott, 2006). By comparing the global gene expression of a *dtxR* deletion mutant with that of the wild-type using DNA microarrays Wennerhold and Bott (2006) determined the DtxR regulon of *C. glutamicum* (Wennerhold & Bott, 2006). Furthermore, a computer-based approach was used to identify putative DtxR binding sites in

the *C. glutamicum* genome. The results demonstrated that around 60 genes are likely to be controlled by DtxR in *C. glutamicum*, thus forming the DtxR regulon. In addition, since several of these genes themselves encode transcriptional regulators, such as *ripA*, it was postulated that a multitude of further genes are controlled indirectly by DtxR; hence, *C. glutamicum* DtxR was referred to as a “master regulator” of gene expression (Wennerhold & Bott, 2006).

The iron-dependent regulation of gene expression in other actinomycetes, such as *M. tuberculosis*, *R. equi* and *R. erythropolis*, occurs through the action of the global regulator, IdeR (Boland & Meijer, 2000; Rodriguez, *et al.* 2002). IdeR is a homologue of the *C. diphtheriae* DtxR protein (Boland & Meijer, 2000; Schmitt, *et al.* 1995; Tao, *et al.* 1994), and acts in the same way as DtxR, by binding to target DNA sequences in the presence of iron, resulting in the repression of gene expression. In *M. tuberculosis*, IdeR is an essential gene (Rodriguez, *et al.* 2002), however, the same is not true of its non-pathogenic relative *M. smegmatis*, where IdeR has been shown to be non-essential (Dussurget, *et al.* 1996).

1.13 Overall objectives and aims of the study

Currently there is limited scientific knowledge regarding *C. pseudotuberculosis* factors associated with virulence, and there is essentially nothing known about how these (and other) genes are regulated. The availability of genome sequences has facilitated the expansion of knowledge in these areas for other pathogenic bacteria, including the corynebacteria; however, at the time the work described in this thesis was initiated, no genome sequence was available for *C. pseudotuberculosis*.

Due to the importance of iron for bacterial viability, and the fact that mammalian host species restrict the bio-availability of iron, it was considered likely that low environmental iron concentrations would be a trigger for the switching on of the expression of *C. pseudotuberculosis* genes involved in host colonisation and persistence, including those associated with iron acquisition. This hypothesis was further strengthened by reports of iron-dependent regulation of virulence gene expression (through DtxR) in the closely-related pathogen, *C. diphtheriae*, and also by the characterisation of the partial sequence of a gene

encoding a DtxR-like regulator in *C. pseudotuberculosis* (Malloy, 2004; Oram, *et al.* 2004). Therefore, with a view to identifying factors associated with *C. pseudotuberculosis* pathogenicity, the overall objective of this study was to determine the effects of iron on gene expression, both by analysis of the general response to varying environmental iron concentrations, but also by conducting a more in-depth investigation into the possible involvement of a DtxR-like transcriptional regulator. In addition to expanding our understanding of *C. pseudotuberculosis* phenotypes associated with growth in a low-iron environment (these phenotypes being of relevance to growth *in vivo*), and of a novel, potential *C. pseudotuberculosis* regulatory mechanism, it was also hoped that these studies would lead to the identification of novel virulence-associated determinants that could, in future, be exploited for the development of improved diagnostic and preventative reagents for CLA.

Chapter Two

Materials and Methods

2.1 Chemicals

Unless otherwise stated all chemicals were from purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd., Dorset, UK), and restriction endonucleases and modifying enzymes were purchased from Promega (Promega UK Ltd.; Hampshire, UK).

Sterilisation of media and reagents was achieved by autoclaving (121°C for 15 min) or by filtering. In the latter case, small volumes were sterilised by passage through 0.2 µm pore diameter syringe filters (Millipore; Watford, UK), while larger volumes were drawn through 0.2 µm Stericup[®] filter units (Millipore) under vacuum.

Oligonucleotide primers were synthesised to-order by Sigma-Genosys Ltd. (Haverhill, Suffolk, UK) or by Integrated DNA Technologies (Coralville, IA, USA), and are presented in **Tables 2.3, 2.4, 2.5 and 2.6**. Stock solutions were prepared by reconstituting lyophilised primers to 100 µM in ddH₂O. Oligonucleotides were subsequently stored at -20°C until required.

2.2 Bacterial strains, plasmids and media

2.2.1 *Escherichia coli*

E. coli strains used for general cloning purposes (**Table 2.1**) were routinely cultured using Luria-Bertani (LB) medium or nutrient agar (**Appendix one**). For maintenance of plasmids (**Table 2.2**), media were supplemented with the appropriate antibiotics at the following concentrations: erythromycin (300 µg/ml), kanamycin (50 µg/ml), spectinomycin (100 µg/ml) or ampicillin (100 µg/ml).

2.2.2 *Corynebacterium pseudotuberculosis*

The *C. pseudotuberculosis* isolate 3/99-5 used throughout this study was a virulent field isolate which originated from an outbreak of CLA in the Scottish Borders (Connor, *et al.*, 2000). The isolate is nitrate negative, which is typical of ovine isolates of *C. pseudotuberculosis*. This strain has been extensively studied in-house and has been used in the

establishment of a standardised ovine experimental model of CLA. As standard, *C. pseudotuberculosis* isolates were maintained on Brain Heart Infusion (BHI) agar (Oxoid Ltd.; Basingstoke, Hampshire, UK), or cultured in BHI broth (Oxoid) supplemented with 0.05% (v/v) polyoxyethylene sorbitane monooleate (Tween 80[®]) from a filter-sterilised 20% (v/v) stock solution (Dubos & Middlebrook, 1948; McGinley, *et al.* 1985a; McGinley, *et al.* 1985b; Riley, *et al.* 1979). Both BHI broth and agar media were reconstituted as recommended by the manufacturer; however, the BHI medium was filter-sterilised prior to use rather than autoclaved as previous experience in our lab had shown improved growth of *C. pseudotuberculosis* in filtered medium. Prior to use, 0.05 % (v/v) filter-sterilised Tween 80[®] was added and the medium was subsequently referred to as BHIT.

In addition to BHI media, a *Corynebacterium* chemically-defined medium (CCDM) was developed as part of this study (**Chapter 3**). For ease of reference, the final, optimised recipe is presented here: Each litre of CCDM consisted of 10.38 g/l RPMI-1640 (Sigma, R 8755), 86 mM L-glutamic acid and 10 % (w/v) glucose. Media components were dissolved in 900 ml ddH₂O prior to adjusting the pH to 4.0 and supplementing with NaHCO₃ to 23 µM final concentration. The pH was then adjusted to 7.1 prior to the medium being made up to a final volume of 1 l. The medium was subsequently stored at 4°C protected from light. Prior to use, the CCDM was supplemented with 0.05 % (v/v) filter-sterilised Tween 80[®]. Where required, BHI and CCDM were supplemented with the appropriate antibiotics, at the following concentrations: erythromycin (0.05 µg/ml), kanamycin (50 µg/ml), spectinomycin (100 µg/ml) and ampicillin (100 µg/ml).

Table 2.1 Bacterial strains

Bacterial strain	Genotype	Source/Reference
<i>E. coli</i>		
electroMax DH5 α -E	(F- ϕ 80/ <i>lacZ</i> Δ M15 Δ (<i>lacZYA</i> -argF) U169 <i>recA1 endA1 hsdR17</i> (rk-, mk+) <i>gal- phoA supE44 λ- thi-1 gyrA96 relA1</i>)	Invitrogen, UK
One shot $^{\circ}$ TOP 10 chemically competent cells	(F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80/ <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG</i>)	Invitrogen, UK
<i>C. pseudotuberculosis</i>		
3/99-5	Nitrate-negative virulent field isolate	(Connor, <i>et al.</i> 2000)
<i>C. pstb</i> Δ <i>cp40</i>	Serine protease (<i>cp40</i>) deletion derivative of <i>C. pstb</i> 3/99-5	This study
Cp- Δ <i>dtxR</i>	Diphtheria toxin repressor (<i>dtxR</i>) deletion derivative of <i>C. pstb</i> 3/99-5	This study

Table 2.2 Plasmid vectors

Plasmid	Description	Selective antibiotic	Source or reference	Chapter
Zero [®] Blunt TOPO [®]	Routine <i>E. coli</i> cloning vector	Kanamycin	Invitrogen, UK.	5
pCR [®] II-TOPO [®]	Routine <i>E. coli</i> cloning vector	Kanamycin	Invitrogen, UK.	5
pCAW001	Zero [®] Blunt TOPO [®] :: inverse PCR product from <i>KpnI</i> digested gDNA	Kanamycin	This study	5
pCAW002	Zero [®] Blunt TOPO [®] :: inverse PCR product from <i>HindIII</i> digested gDNA	Kanamycin	This study	5
pCAW003	Zero [®] Blunt TOPO [®] :: inverse PCR product from <i>XbaI</i> digested gDNA	Kanamycin	This study	5
pSFKT2	Temperature sensitive shuttle plasmid	Kanamycin	(Nakamura, <i>et al.</i> 2006)	4
pG ⁺ host 9	Source of <i>ermAM</i> Em ^r gene	N/A	(Maguin, <i>et al.</i> 1996)	4
pSPZ	<i>lacZ</i> reporter gene containing plasmid	Spectinomycin	(Oram, <i>et al.</i> 2006)	5/6
toxP	pSPZ :: <i>tox</i> promoter region	Spectinomycin	(Oram, <i>et al.</i> 2006)	5
Irp3P	pSPZ :: <i>irp3</i> promoter region	Spectinomycin	R. K. Holmes personal communication	5
pCAW004	pSPZ :: <i>sigB</i> promoter region	Spectinomycin	This study	5
pCAW005	pSPZ :: <i>dtxR</i> promoter region	Spectinomycin	This study	5
pCAW006	pSPZ :: <i>galE</i> promoter region	Spectinomycin	This study	5
pCAW007	pSPZ :: <i>fagA</i> promoter region	Spectinomycin	This study	5/6
pCAW008	pSPZ :: <i>cp40</i> promoter region	Spectinomycin	This study	5
pCAW009	pSPZ :: <i>ORF 02949</i> promoter region	Spectinomycin	This study	6
pCARV001	pCARV ::224bp <i>cp40</i> fragment	Erythromycin	This study	4
pCARV002	pCARV ::432bp <i>cp40</i> fragment	Erythromycin	This study	4
pCARV003	pCARV ::750bp <i>cp40</i> fragment	Erythromycin	This study	4
pCARV004	pCARV ::1020bp <i>cp40</i> fragment	Erythromycin	This study	4
pCARV005	pCARV :: $\Delta cp40$	Erythromycin	This study	4
pCARV006	pCARV :: $\Delta dtxR$	Erythromycin	This study	5
pCARV007	pCARV :: $\Delta 02949$	Erythromycin	This study	6
pWSK29	<i>E. coli</i> cloning/expression plasmid	Ampicillin	(Wang & Kushner, 1991)	5
pWSK29/DtxR	pWSK29 :: DtxR	Ampicillin	This study	5
pEP2	Shuttle vector <i>E. coli</i> and <i>Corynebacterium</i>	Kanamycin	(Messerotti, <i>et al.</i> 1990)	5
pCAW010	pEP2 :: DtxR	Kanamycin	This study	5

2.3 Measurement of Bacterial Growth

The Bioscreen C system (Oy Growth Curves Ab Ltd., Helsinki, Finland) was used to allow the automated measurement of bacterial growth characteristics based upon optical density measurements at user-defined wavelengths. Essentially, the apparatus is a computer-controlled incubator/reader/shaker which uses a unique micro-plate format (10 × 10 wells); with space for 2 plates it is possible to run 200 samples simultaneously. The optical density of bacterial cultures is recorded over a user-defined period of time and at user-defined time intervals.

The Bioscreen C was used to assess *C. pseudotuberculosis* growth in a variety of media; however, a standard experiment was carried out as follows: Five ml of the desired medium was inoculated with a single colony of *C. pseudotuberculosis* and incubated overnight at 37°C with shaking at 225 rpm. The following day 250 µl was taken from a 6 ml aliquot of fresh medium and placed into micro-plate, in triplicate (media blank controls). The remaining medium was inoculated 1/100 with the overnight culture and a 250 µl aliquot was placed into the Bioscreen micro-plate, in triplicate. The machine was then programmed to measure and record the growth of the cultures in each well every 15 min, at 37° × 225 rpm.

2.4 Nucleic Acid Extraction and Purification

2.4.1 Small scale extraction and purification of plasmid DNA

For routine analysis of recombinant clones, rapid extraction and purification of plasmid DNA from *E. coli* was performed using the QIAprep Spin Miniprep Kit (Qiagen; Qiagen Ltd., West Sussex UK.). The procedure was carried out according to the manufacturer's instructions and employs a modified alkaline lysis method (Birnboim & Doly, 1979) combined with the use of silica membrane-containing microcentrifuge spin columns to purify plasmid DNA. Briefly, a 5 ml overnight culture of *E. coli*, propagated in LB broth containing an appropriate selective antibiotic, was centrifuged at 3, 893 × *g* for 15 min at 4°C. The resulting bacterial pellet was re-suspended in 250 µl of buffer P1, containing RNase, and subsequently transferred to a fresh microcentrifuge tube. Two hundred and fifty µl of buffer P2 was added to the solution and the tube was mixed thoroughly. Following this step, 350 µl of buffer N3 was added and mixed well by inverting of the tube. The tube was then centrifuged at 17, 970 × *g* for 10 min at room temperature in a microcentrifuge, and then the cleared supernatant was transferred to a

QIAprep spin column which was subsequently centrifuged, as before, for 1 min. The column was washed with 750 µl of buffer PE and centrifuged twice for 1 min, as before, to ensure the complete removal of residual ethanol. The spin column was then placed into to a clean microcentrifuge tube and 30 µl of elution buffer (10 mM Tris-HCl, pH 8.0) was added directly to the surface of the silica membrane. The tube was then incubated for 1 min at room temperature before eluted plasmid DNA was collected in the microcentrifuge tube by centrifugation as before for 1 min.

2.4.2 Large scale extraction and purification of plasmid DNA

To provide large quantities of highly-pure plasmid DNA for procedures such as cloning, DNA sequencing, *etc.*, the QIAprep Maxiprep Kit (Qiagen) was employed. The principle of this procedure was identical to that of the miniprep procedure above (**Section 2.4.1**), whereby cells were lysed using a slightly modified version of the alkaline lysis method described by Birnboim and Doly (1979). In addition, the use of spin columns was replaced by gravity flow columns, to ensure reduced contamination of plasmid preparations with *E. coli* chromosomal DNA. So as not to overload the binding-capacity of the silica resin in the columns, a maximum of 100 ml of cultures of *E. coli* containing high-copy-number plasmids or 500 ml of cultures of *E. coli* containing low-copy-number plasmids was used. Briefly, a single colony of *E. coli* transformed with the desired plasmid was used to inoculate 5 ml of LB medium containing the appropriate selective antibiotic. After incubation overnight at 37°C × 225 rpm, the culture was diluted 1/500 into LB broth containing the appropriate antibiotic. This culture was incubated overnight, as before, prior to harvesting bacteria by centrifugation at 3, 893 × *g* for 15 min at 4°C. The bacterial pellet was thoroughly re-suspended in 10 ml of buffer P1, prior to the addition of 10 ml of buffer P2. Subsequently, following thorough mixing of the tube contents by gentle inversion of the tube 4-6 times, cells were lysed during a 5 min incubation at room temperature. Finally, the lysate was neutralised by addition of 10 ml of chilled buffer P3; the tube contents were immediately mixed by gentle inversion 4-6 times, followed by incubation on ice for 20 min. The lysate was subsequently cleared by filtration through a 1 µM syringe filter (Millipore). A Qiagen-tip 500 (column) was equilibrated by the addition of 10 ml buffer QBT. The filtered lysate was added to the equilibrated Qiagen-tip 500 and allowed to empty by gravity flow. Subsequently, the column was washed twice with 30 ml buffer QC prior to elution of plasmid DNA with 15 ml of buffer QF. To precipitate

DNA, 10.5 ml of isopropanol was added to the eluate, and immediate centrifugation was conducted at $9,500 \times g$ for 15 min at room temperature. The resulting DNA pellet was washed with 5 ml of 70 % (v/v) ethanol and centrifuged as above for 10 min. Finally, the supernatant was carefully decanted and the pellet was allowed to air dry prior to re-suspension in a suitable volume of ddH₂O.

2.4.3 Extraction and purification of *C. pseudotuberculosis* genomic DNA

Genomic DNA was harvested from cells from 5 ml overnight cultures of *C. pseudotuberculosis*, propagated in BHIT. Cells were harvested by centrifugation at $3,893 \times g$ for 15 min at 4°C. Immediately before use, 5 mg of lysozyme was dissolved in 500 µl of buffer P1 (**Appendix one**). In addition 2 µl/ml RNase A (10 mg/ml) was added to the buffer P1/lysozyme solution which was subsequently used to re-suspend the cell pellet. Following incubation at 37°C for at least 1 hr, the cell suspension was transferred to a 1.5 ml tube containing *ca.* 500 µl of 10 µm diameter zirconium beads. Homogenisation was then performed for 3×20 sec at a speed rating of 5.5 on a FastPrep® instrument (Q.Biogene; Middlesex, UK). The tubes were then centrifuged briefly in at $17,970 \times g$ in a microcentrifuge and the supernatant was transferred to a fresh 1.5 ml microcentrifuge tube. Then, 5 mg proteinase K and 50 µl of a 10 % (v/v) solution of N-lauryl-sarcosine were added to the supernatant prior to a further overnight incubation at 55°C. Following this incubation, the DNA was extracted with 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1); the solution was mixed by gently inverting the tube numerous times, and then centrifugation was performed at $17,970 \times g$ for 5 min at room temperature. The top layer of the supernatant was transferred into a clean 1.5 ml microcentrifuge tube and a further round of extraction was carried out with chloroform:isoamyl alcohol (24:1). Subsequently, the DNA was precipitated by addition of 2 volumes of absolute ethanol and 0.1 volumes of 3 M sodium acetate (pH 5.2). Precipitated DNA was harvested by centrifugation at $17,970 \times g$ for 20 min at 4°C, and the resulting pellet was washed by addition of 500 µl of 70 % (v/v) ethanol and centrifuging as before for 10 min. Finally, the ethanol was carefully decanted, and the DNA pellet was air-dried prior to re-suspension in 50-100 µl of ddH₂O.

2.4.4 Isolation of genomic DNA for Colony PCR

Two-hundred μl of well shaken Insta-Gene™ Matrix (Bio-Rad; Bio-Rad laboratories Ltd., Hertfordshire, UK.) was placed into a 1.5 ml microcentrifuge tube. Using a toothpick, a single colony of *C. pseudotuberculosis* was transferred into the Insta-Gene™ Matrix solution. The mixture was then vortexed briefly before being placed in a hot-block at 100°C for 8 min. The solution was then vortexed again prior to being centrifuged at 17, 970 $\times g$ for 3 min in a microcentrifuge. The DNA-containing supernatant was then transferred to clean 1.5 ml microcentrifuge tube, and between 3-20 μl was used as template in subsequent polymerase chain reactions.

2.4.5 Extraction and purification *C. pseudotuberculosis* RNA

C. pseudotuberculosis total RNA was extracted and purified based upon a method described by Huser *et al.* (2003) which employs the RNeasy mini kit (Qiagen) with some modifications (Huser, *et al.*, 2003). Briefly, a single colony of *C. pseudotuberculosis* was used to inoculate 5 ml of medium which was subsequently incubated at 37°C \times 225 rpm overnight. The culture was diluted 1/100 into 5 ml of fresh medium and the bacteria were grown to mid log phase before being harvested at 3,893 $\times g$ at 4°C. The entire supernatant was removed and the pellet was immediately snap-frozen in a dry ice/ethanol bath. The pellet was then re-suspended in 1 ml of RLT buffer (containing 10 μl of β -mercaptoethanol). To achieve 5×10^9 bacterial cells (previously shown to result in an optimal yield of RNA) a 500 μl aliquot of the suspension was taken and made up to 800 μl with RLT. The suspension was transferred to a screw cap tube containing *ca.* 500 μl of 10 μm zirconium glass beads. The beads/cells suspension was homogenised twice in the Fast prep® instrument (Q.Biogen) at a speed rating of 6.5 for 30 sec with a 5 min interval on ice. Following homogenisation the tube was centrifuged briefly in a microcentrifuge at 17, 970 $\times g$ to pellet the beads and cell debris. The supernatant, containing the RNA was transferred to a clean 1.5 ml tube and 250 μl of absolute ethanol was added and mixed by pipetting. The sample was then transferred to an RNeasy mini column and centrifuged for 15 sec at 10,000 $\times g$. At this stage, on-column digestion of genomic DNA with DNase I was carried out using the RNase-free DNase set (Qiagen) according to the manufacturer's instructions. Briefly, 350 μl of buffer RW1 was pipetted into the RNeasy mini column and centrifuged for 15 sec at at least 10,000 $\times g$. Following this wash, 10 μl of DNase

I stock solution was added to 70 μl of buffer RDD and the solution was mixed gently by inverting the tube; this was then pipetted directly onto the membrane of the RNeasy column and incubated at room temperature for 15 min. The column was washed with 350 μl of buffer RW1 followed by centrifugation for 15 sec at $10,000 \times g$. Following the DNase I digestion the column was washed twice with 500 μl of buffer RPE, the first time by centrifuging for 15 sec at $10,000 \times g$ and the second time for 2 min. Subsequently, following removal of the RPE flow-through, the RNeasy mini column was further centrifuged, as before, to eliminate any carry-over of RPE buffer which may interfere with the amplification of cDNA from the RNA. The RNeasy mini column was transferred to a fresh 1.5 ml microcentrifuge tube, and total bacterial RNA was eluted from the column by adding 50 μl of RNase free water directly to the silica resin in the column and centrifuging for 1 min at $10,000 \times g$.

Prior to using the purified mRNA in RT-PCR experiments, a further round of DNase I digestion was performed to ensure complete removal of contaminating DNA. This was achieved using amplification grade DNase I (Invitrogen), according to the manufacturer's instructions. Briefly, 1 μg of RNA was transferred to a 0.2 ml RNase-free tube, to which was added $1\times$ DNase I reaction buffer and 1 U DNase I. The reaction volume was made up to 10 μl with RNase free water, and incubation was performed at room temperature for 15 min. The DNase I was then deactivated by heating to 65°C for 10 min in the presence of 25 mM EDTA. Subsequently, RNA was stored at -20°C for subsequent use.

2.5 General DNA manipulations

2.5.1 Restriction endonuclease digests

Restriction endonuclease digestions were conducted, essentially as detailed in the manufacturer's supplied instructions. Digests were performed in 1.5 ml microcentrifuge tubes, containing the appropriate restriction endonuclease reaction buffer to $1\times$ final concentration, a final concentration of acetylated BSA of 0.1 $\mu\text{g}/\mu\text{l}$, an appropriate volume of ddH₂O and *ca.* 5-10 U of restriction endonuclease per μg of DNA. To minimise star activity, restriction digests were performed in sufficient volumes to ensure that the final concentration of glycerol (from the restriction enzyme storage buffer) was $\leq 10\%$ (v/v). Subsequently,

restriction digests were incubated at the recommended temperature for the enzyme for between 1-4 hrs.

2.5.2 Dephosphorylation of DNA

Shrimp alkaline phosphatase (SAP) was used to remove the phosphate groups of digested vector DNA to prevent re-circularisation of vector during ligation reactions. Dephosphorylation was performed in 30-50 µl reaction volumes, containing 1× SAP buffer and 1 U SAP per µg DNA. Reactions were incubated at 37°C for 15 min, prior to heat inactivation at 65°C.

2.5.3 End-filling of DNA fragments

The large (Klenow) fragment of DNA polymerase I (Invitrogen) was used to fill-in sticky-ended restriction endonuclease termini or to remove the 3'-adenine residue frequently incorporated into amplified DNA fragments during PCR with *Taq* polymerase. Reactions were conducted in 30 µl volumes, containing 1× React[®] 2 buffer (Invitrogen), 66 µM final concentration of each of dATP, dTTP, dCTP and dGTP, between 0.5-1.0 µg of DNA and 0.5 U of Klenow polymerase (from a 0.5 U/µl stock solution prepared by diluting the enzyme in Klenow Dilution Buffer (Invitrogen)). Reactions were mixed briefly by vortexing, centrifuged briefly at 17, 970 × *g* in a microcentrifuge, and then incubated for 20 min on ice. Subsequently, reactions were terminated by the addition of 0.6 µl of 0.5 M EDTA followed by heat-inactivation of the enzyme at 75°C for 20 min. The resulting blunt-ended DNA was ready for use in blunt-cloning reactions.

2.5.4 Ligation of DNA fragments

Ligations were performed according to the traditional method employing T4 DNA ligase, or by using a Quick Ligation Kit purchased from NEB (New England Biolabs UK Ltd., Hertfordshire, UK). For the former method, ligation reactions were conducted in 20 µl volumes, containing 1× Ligase Buffer (containing ATP), a 1:9 ratio of vector:insert DNA and 2 Weiss Units of T4 DNA ligase. Reactions were incubated overnight at 4°C in a chilled water bath, prior to heat inactivation of the ligase at 65°C for 10 min.

Quick ligations were conducted according to the manufacturer's supplied instructions. Briefly, for each ligation, 50 ng of vector DNA was combined with a 3-fold molar excess of insert in a clean 1.5 ml microcentrifuge tube and the mixture was made up to a final volume of 10 μ l. Ten μ l of 1 \times Quick ligation reaction buffer was added to the tube. The tube was then vortexed briefly and 1 μ l of Quick T4 DNA ligase was added and mixed again by vortexing. The reaction was centrifuged briefly at 17, 970 $\times g$ in a microcentrifuge at room temperature, and subsequently incubated at room temperature (25°C) for 5 min. Following incubation the reaction was chilled on ice.

For both methods, where ligated DNA was to be used for subsequent bacterial transformations, the heat-inactivated ligation mixtures were de-salted by dialysing on Millipore VS membranes (**Section 2.7.3**).

2.5.5 Polymerase Chain Reaction

Polymerase Chain Reactions were conducted using different DNA polymerase enzymes according to the downstream applications for which amplified DNA was to be used, and PCR was routinely performed in 50 μ l reaction volumes using 0.25 ml flat-topped PCR tubes (Elkay Lab Products (UK) Ltd; Hampshire, UK). In addition, template DNA varied in concentration (and hence volume) according to source.

2.5.5.1 KOD DNA Polymerase

For amplification of DNA fragments for subsequent cloning, KOD Hot Start Polymerase (Novagen; Merck Chemicals, Nottingham, UK) was used, which generates PCR products without 3'-overhangs (Mizuguchi, *et al.* 1999). PCRs routinely contained 1 \times Polymerase Buffer, 1.5 mM MgSO₄, 0.2 mM each of dATP, dTTP, dCTG and dGTP, 0.3 μ M of each oligonucleotide primer and 0.02 U/ μ l of KOD Hot Start Polymerase. Thermal cycling was typically conducted for 95°C for 2 min as an initial denaturation step, followed by 30-40 cycles of a 95°C for 20 sec denaturation step, a 10 sec annealing step (temperature dependent on primers) and a 70°C extension step (extension time dependent on length of target being amplified e.g 1min/kb).

2.5.5.2 Platinum *Taq* Polymerase

For amplification of complementary DNA (cDNA) fragments, Platinum® *Taq* Polymerase (Invitrogen) was used according to the manufacturer's supplied instructions. Reactions routinely contained 1× PCR Buffer, 0.2 mM each of dATP, dTTP, dCTP and dGTP, 1.5 mM MgCl₂, 0.2 μM of each primer and 1 U Platinum® *Taq* DNA polymerase. Thermal cycling regularly involved a preliminary denaturation step at 94°C for 2 min, followed by 25-35 cycles of a 94°C for 30 sec denaturation step, a 30 sec annealing step (temperature dependent on primers) and a 72°C extension step for 1 min per kb of target sequence). A final extension step at 72°C for 10 min was then performed.

2.5.5.3 Inverse PCR

An “inverse PCR” technique was used to amplify unknown regions of DNA flanking a known portion of the *C. pseudotuberculosis dtxR* gene (Ochman, Gerber, & Hartl, 1988). The protocol was essentially identical to standard PCR (**Section 2.5.5.1**), with the exceptions being the way that the template DNA was prepared and the fact that inverse PCR primers orientate in the opposite direction to conventional PCR primers (since they are based upon known DNA sequences and are used to amplify unknown upstream and/or downstream flanking sequences). Genomic DNA was digested to completion with a suitable restriction endonuclease (as determined by Southern hybridisation). Subsequently, the digested DNA fragments were gel purified (**Section 2.7.1**), and circularised by self-ligation with T4 DNA ligase in a 20 μl reaction containing 3 μg DNA (**Section 2.5.4**). Finally, following heat-inactivation of the ligase, the DNA was de-salted by dialysis against ddH₂O (**Section 2.7.3**).

Inverse PCR was conducted as described in **Section 2.5.5.3**. A total of 1 μl of circularised genomic DNA was used as template in 50 μl reactions. Thermal cycling was conducted for 30 cycles, as described, using a primer annealing temperature of 56°C for 1 min and an extension temperature of 72°C for 2.5 min.

For inverse PCR amplification of larger DNA fragments, following circularisation of genomic template DNA, long-range PCR was carried out using the Expand Long Template PCR system (Roche Diagnostics Ltd., West Sussex, UK.), which is designed to allow amplification of DNA fragments up to 20 kb in length. For the work described in this thesis, the supplied “Buffer 1” was used, which is optimised for amplification of DNA fragments of ≤9 kb. Long-

range inverse PCR was performed in 50 µl reactions, containing 20 µl of circularised DNA as template, 0.2 mM each of dATP, dTTP, dCTP and dGTP, 0.3 µM *dtxR_inv*(fwd) primer, 0.3 µM *dtxR_inv* (rev) (**Table 2.3**), 1× Buffer 1 and 0.75 µl polymerase mix (3.75 U). Following a preliminary denaturation step at 94°C for 2 min, thermal cycling was conducted for 25 cycles, comprising a 94°C denaturation step for 10 sec, a 30 sec annealing step at 55.5°C and a 68°C extension step for 8 min. Subsequently, amplified DNA fragments were purified as above (**Section 2.7.1**).

2.5.5.4 Reverse Transcription-PCR (RT-PCR)

Ribonucleic acid was reverse transcribed using SuperScript™ III First Strand Synthesis System (Invitrogen) following the manufacturer's instructions. Briefly, 19.2 ng of random hexamers, 769 µM of dNTP's cumulatively and 10 µl of DNase I-digested RNA solution, containing 1 µg of total RNA, were added to a nuclease-free 0.2 ml microcentrifuge tube. The solution was made up to a total volume of 13 µl with nuclease-free water and the mixture was heated to 65°C for 5 min, followed by incubation on ice for at least 1 min. Tubes were pulse centrifuged, following which First Strand buffer was added to 1× final concentration in addition to 40 U RNaseOUT™ recombinant RNase inhibitor and 1 µl SuperScript™ III RT (or 1 µl nuclease free water for negative controls) for subsequent cDNA synthesis. The solution was mixed by pipetting up and down and the tubes were incubated at 25°C for 5 min. Tubes were subjected to a further incubation at 50°C for 1 hr, followed by inactivation of the enzyme by heating to 70°C for 15 min.

2.4.5.5 Quantitative real time reverse transcription PCR (qRT-PCR)

Quantitative real time reverse transcription PCR was carried out using OMNiMix HS (TaKaRa Bio Inc., CA, USA), a pre-dispensed blend of reagents with hot start *Taq* polymerase, which are supplied as lyophilised beads and are specifically designed for use with the Cepheid SmartCycler® system (Cepheid, CA, USA). Oligonucleotide primers were specific for the target gene(s) of interest (**Table 2.6**) and the probes (**Table 2.7**) included 5' 6-carboxyfluorescein and 3' black hole quencher 1 modifications. Working stocks of primers and probes were made up to 10 µM and 2 µM, respectively. The qRT-PCR procedure was performed according to the manufacturer's supplied instructions. Briefly, a single OMNiMix HS lyophilised bead (sufficient for two 25 µl reactions) was reconstituted in 4 µl of each primer stock, 3 µl of probe stock and 48 µl of ddH₂O. Subsequently, 1 µl of cDNA (**Section 2.5.5.2**) was added to each 24 µl aliquot of reconstituted OMNiMix, in smart-cycler tubes

(Cepheid, CA, USA) in a cold rack. The resulting 25 µl reaction mixes contained 1.5 U of TaKaRa hot start polymerase, 200 µM dNTP mix, 4 mM MgCl₂ and 25 mM HEPES pH 8.0, and care was taken to not touch or scratch the windows of the smart-cycler tubes (which can affect the fluorescence). Immediately prior to thermal cycling, reactions were centrifuged briefly in a microcentrifuge at 17, 970 × *g* to remove air bubbles which can affect fluorescence; thermal cycling was conducted at 75°C for 75 sec followed by 50 cycles of 95°C for 5 sec and 59°C for 40 sec. During cycling, fluorescence was monitored and logged. The standard curve for each primer and probe set was determined prior to the actual cDNA reactions. The standard curve was achieved using 1 µg DNA diluted 10-fold in nuclease-free water to give a range of dilutions down to 1×10⁻⁸ µg DNA.

2.6 Agarose gel electrophoresis

2.6.1 Analysis of DNA samples

PCR products, restriction endonuclease digests and other DNA samples were analysed by electrophoresis through agarose gels, using the Mini-Sub Cell GT Cell electrophoresis apparatus (BioRad). Briefly, agarose gels were prepared, containing a range of molecular biology-grade agarose concentrations from 0.8-2.0 % (w/v) (depending on the size of DNA fragments to be analysed) in 0.5 × TAE buffer (**Appendix one**). The TAE was heated in a microwave oven until the agarose had completely melted, following which the molten agar solution was cooled to *ca.* 56°C prior to addition of GelRed (Cambridge Biosciences, Cambridge, UK) to 1:10,000. The solution was subsequently poured into a mini-gel casting tray, with a comb. The gel was allowed to set prior to submersion in an electrophoresis tank containing 0.5× TAE buffer. Subsequently, DNA samples were prepared in a 1× final concentration of Blue/Orange loading dye (Promega), and loaded into wells in the gel. Each mini-gel was run at 100 V/cm until the dye-front had reached the end of the gel. Then, DNA fragments were visualised over UV light using an AlphaImager (GRI AlphaInnotech; UK) gel documentation system, and linear DNA sizes were determined in comparison to a DNA ladder molecular weight marker (Promega).

Table 2.3 **Miscellaneous oligonucleotide primers**

Primer	Sequence (5'-3') [*]	Target	Reference
<i>dtxR</i> _inv (fwd)	TCGTAGATGGTCCGCA	bp 2091-2106 <i>C. pstb dtxR</i> locus	This study
<i>dtxR</i> _inv (rev)	TGGCTTAGATATCCACAAGG	bp 2326-2345 <i>C. pstb dtxR</i> locus	This study
pCARV MCS F	ACACAGGAAACAGCTATGACC	bp 4594-4614 pCARV vector	This study
pCARV MCS R	AAACGACGGCCAGTGAAT	bp 4679-4696 pCARV vector	This study
<i>dtxR</i> 1 F	AGCCCAAACCATTGAGGTAG	bp 962-981 <i>C. pstb dtxR</i> locus	This study
<i>dtxR</i> 1 R	TGTATGCGGCTGTACAGGAG	bp 3838-3857 <i>C. pstb dtxR</i> locus	This study
$\Delta dtxR$ AE 1	gtcgac AGGACTCAGAAGAACCACTA ^a	bp 1006-1025 <i>C. pstb dtxR</i> locus	This study
$\Delta dtxR$ AE 2	ctcgag ATCTTTCATTTCAACCTCTT ^b	bp 2045-2065 <i>C. pstb dtxR</i> locus	This study
$\Delta dtxR$ AE 3	ctcgag TAAAGGGTACCCACATGAAACT ^b	bp 2735-2756 <i>C. pstb dtxR</i> locus	This study
$\Delta dtxR$ AE 4	gtcgac GAGAAAACGAATGCAGCAG ^a	bp 3777-3795 <i>C. pstb dtxR</i> locus	This study
$\Delta dtxR$ DCO F	ATCTAATTTACCACCATAAAA	bp 1788-1809 <i>C. pstb dtxR</i> locus	This study
$\Delta dtxR$ DCO R	GAAGAAAAGACCAAGTTTGTTA	bp 3068-3089 <i>C. pstb dtxR</i> locus	This study
cp40 F	cccggg GCACAGTGACCCAAGGA ^c	bp 4-20	Acc. # U10424
cp40 0 224bp	cccggg AGAGCGGAAGTTATGCCTA ^c	bp 245-263	Acc. # U10424
cp40 1 432bp	cccggg CTGGAGCTTGTCTTTCCAA ^c	bp 450-468	Acc. # U10424
cp40 2 750bp	cccggg CGGAGTTCCATGTCTACG ^c	bp 759-776	Acc. # U10424
Cp40 4 1020bp	cccggg CTGGCAGGAATTGATCTTG ^c	bp 1041-1059	Acc. # U10424
cp AE 1	gtcgac GCGGACTTTGTAAAGTTTGC ^a		
cp AE 2	ctcgag GCGTGAGACTGATCGAGG ^b	bp 217-234	Acc. # U10424

cp AE 3	ctcgag TTCACCACACTCAAACCGAC ^b	bp 1240-1259	Acc. # U10424
cp AE 4	gtcgac GACCTTTTTGTTACCGTGC ^a		
Δcp40 DCO F	CTTGCCCAGGATTAAATGC		
Δcp40 DCO R	CGCCCGTGAGATTATTTTT		
<i>dtxR</i> pWSK29 A	AGACAAACTCCGCGCCTAC	bp 1766-1784 C. <i>pstb dtxR</i> locus	This study
<i>dtxR</i> pWSK29 B	AACCTCGTGGCCTTGTTTCG	bp 2811-2829 C. <i>pstb dtxR</i> locus	This study
AE 249 1	gtcgac TATTTATCTGGTTCTCAACG ^a	bp 4025-4044 02949 locus	This study
AE 249 2	ctcgag GGAAGATCGATAAAACAAAG ^b	bp 4956-4975 02949 locus	This study
AE 249 3	ctcgag GGAAAGGATATATACATTGA ^b	bp 8246-8265 02949 locus	This study
AE 249 4	gtcgac GCACTATAAAACGCTATCAT ^a	bp 9214-9233 02949 locus	This study
Δ0249 DCO F	CCCAACACTGATTAAGAAAGC	bp 4789-4809 02949 locus	This study
Δ0249 DCO R	GCAACCAGAGTAGATGATCCT	bp 8415-8435 02949 locus	This study
<i>dtxR</i> _01	GTGAAGGATCTGGTCGATACC	Degenerate primers	(Malloy, 2004)
<i>dtxR</i> _03	CCAGCGGCAGGCTTCGTCTGTG	Degenerate primers	(Malloy, 2004)
pCARV_MCS F	ACACAGGAAACAGCTATGACC	pCARV	This study
<i>dtxR</i> _SX R	TGTATGCGGCTGTACAGGAG	bp 3838-3857 C. <i>pstb dtxR</i> locus	This study
Erm_probe F	TGGAAATAAGACTTAGAAGC	bp 3303-3322 pCARV	This study
Erm_probe R	CGACTCATAGAATTATTTCC	bp 4156- 4175pCARV	This study
<i>dtxR</i> _comp F	AGACAAACTCCGCGCCTAC	bp 1766-1784 C. <i>pstb dtxR</i> locus	This study
<i>dtxR</i> _comp R	AACCTCGTGGCCTTGTTTCG	bp 2811-2829 C. <i>pstb dtxR</i> locus	This study

oligonucleotide-incorporated restriction endonuclease recognition sites are indicated by lower-case letters and the suffixes **a**, **b** and **c** refer to *SalI*, *XhoI* and *XmaI* respectively.

Table 2.4 Sequencing primers for the *C. pseudotuberculosis* *dtxR* locus

Primer	Sequence (5'-3')	Target	Reference
M13 F	CGCCAGGGTTTTCCCAGTCACGAC	bp 452-475 inverse PCR product	TOPO Blunt (Invitrogen)
M13 R	GAGCGGATAACAATTTACACAGG	bp 185-208 inverse PCR product	TOPO Blunt (Invitrogen)
pCAW004 1.0	AGCATAACACCGAATTG		This study
pCAW004 2.0	GGTACCCACATGAACTCCT	bp 2740-2759 <i>C. pstb</i> <i>dtxR</i> locus	This study
pCAW004 1.1	TTCCATGGCTCGGATAAG	bp 1188-1205 <i>C. pstb</i> <i>dtxR</i> locus	This study
pCAW004 2.1	GCAGGAGCATATGGAAG	bp 3256-3272 <i>C. pstb</i> <i>dtxR</i> locus	This study
pCAW004 1.2	TCACCGATCTCTTGACAA	bp 7193-7211 <i>C. pstb</i> <i>dtxR</i> locus	This study
pCAW004 2.2	TCGTTTTCTCATCATGACA	3786-3804 bp <i>C. pstb</i> <i>dtxR</i> locus	This study
pCAW004 1.3	GAATTTTTCGCTAACAAGC	bp 6660-6678 <i>C. pstb</i> <i>dtxR</i> locus	This study
pCAW004 2.3	AAAGCTTCCCCACGGCT	bp 4337-4353 <i>C. pstb</i> <i>dtxR</i> locus	This study
pCAW004 1.4	AGCAAGAAATTTCTCGAACT	bp 6114-6133 <i>C. pstb</i> <i>dtxR</i> locus	This study
pCAW004 2.4	TGGATAGCGCGTCATGC	bp 4870-4886 <i>C. pstb</i> <i>dtxR</i> locus	This study
pCAW004 1.5	ACCAAAGCGTTCTACGAGGT	bp 5598-5617 <i>C. pstb</i> <i>dtxR</i> locus	This study
pCAW004 2.5	CTTGAGCACAGGCCAGT	bp 5381-5397 <i>C. pstb</i> <i>dtxR</i> locus	This study

Table 2.5 Primers for PCR amplification of promoter regions

Primer	Sequence (5'-3')	Target	Reference
<i>sigB</i> P F	agatct TTGGGGCAACGATGGAGGT ^a	bp 601-619 <i>C. pstb dtxR</i> locus	This study
<i>sigB</i> P R	gtcgac GCGACTTCCACGGTCAATCA ^b	bp 841-860 <i>C. pstb dtxR</i> locus	This study
<i>dtxR</i> P F	agatct CGGAATTGACGAGTCTAA ^a	bp 1415-1432 <i>C. pstb dtxR</i> locus	This study
<i>dtxR</i> P R	gtcgac TAGATGGTCCGCAGATAC ^b	bp 2086- 2103 <i>C. pstb dtxR</i> locus	This study
<i>galE</i> P F	agatct AGAGCGGCGGCTTGTTGATG ^a	bp 2392-2411 <i>C. pstb dtxR</i> locus	This study
<i>galE</i> P R	gtcgac CGGCTTCCTTATTGCCAGTAG ^b	bp 2849-2869 <i>C. pstb dtxR</i> locus	This study
<i>fagA</i> P F	agatct TGGGATCTGGATGGAATAGAG ^a	bp 2774-2794 <i>fagA</i> gene	Acc. # AF401634
<i>fagA</i> P R	gtcgac ACCCAAAGCGTGCTTAAC ^b	bp 3268-3285 <i>fagA</i> gene	Acc. # AF401634
<i>cp40</i> P F	agatct GGATACGCCAAAGGAAAC ^a	<i>cp40</i> gene	
<i>cp40</i> P R	gtcgac TTGCTGATCGCTCTTCTG ^b	bp 532-549 <i>cp40</i> gene	Acc. # U10424
<i>sid</i> P F	agatct TTGGAATTAGTCAAGTCCCT ^a	bp 4640- 4659 ORF02949	This study
<i>sid</i> P R	gtcgac ATCATGGTAGCCAAGGCTA ^b	bp 4896-4914 ORF02949	This study

oligonucleotide-incorporated restriction endonuclease recognition sites are indicated by lower-case letters and the suffixes **a** and **b** refer to *Bgl*II and *Sal*I respectively

Table 2.6 qRT-PCR primers

Primer	Sequence (5'-3')	Target	Reference
<i>recA</i> F	TTGTGATGTTCGGCGTATCC	bp 921-940 <i>recA</i> gene	Acc. # U30387
<i>recA</i> R	ACTACCTTTAACCGCGTCCTGTT	bp 973-995 <i>recA</i> gene	Acc. # U30387
<i>sigB</i> B F	CTTCCTTGCGACACTCTGACAT	bp 1564-1585 <i>C. pstb</i> <i>dtxR</i> locus	This study
<i>sigB</i> d R	CGAATTGTGCGCCAATTTG	bp 1677-1695 <i>C. pstb</i> <i>dtxR</i> locus	This study
<i>dtxR</i> C F	AACGTGACGGGCTCGTTGTA	bp 2202-2221 <i>C. pstb</i> <i>dtxR</i> locus	This study
<i>dtxR</i> c R	GCGAGGCGATGTTTACGCAT	bp 2282-2301 <i>C. pstb</i> <i>dtxR</i> locus	This study
<i>galE</i> G F	ACAAGTCTGCGCTACTTCAA	bp 3232-3251 <i>C. pstb</i> <i>dtxR</i> locus	This study
<i>galE</i> a R	GGCCTAAAGCAACTTGGAGAATT	bp 3315-3337 <i>C. pstb</i> <i>dtxR</i> locus	This study
<i>dtxR-galE</i> F F	ACGGTCGCGTGATTATTACG	bp 2652-2671 <i>C. pstb</i> <i>dtxR</i> locus	This study
<i>dtxR-galE</i> b R	GCTCCGCCGGTGACAA	bp 2759-2774 <i>C. pstb</i> <i>dtxR</i> locus	This study
<i>fagA</i> F	AGCGGTTGCACTGGCCGCAA	bp 2157-2176 <i>fagA</i> gene	Acc. # AF401634
<i>fagA</i> R	GTGCTCACAATCGCTTGCGCT	bp 2277-2297 <i>fagA</i> gene	Acc. # AF401634

Table 2.7 qRT-PCR probes

Probe	Sequence (5'-3') ^a	Target	Reference
<i>recA</i>	AAGGACGGGCAGGATGCCATCG	bp 949-970 <i>recA</i> gene	Acc. # U30387
<i>sigB</i> SB	TGAACAAGACGTGATCAGAATGCGTTA	bp 1616-1642 <i>C. pstb</i> <i>dtxR</i> locus	This study
<i>dtxR</i> DR	TTCAAATGACGCCCCACTGGGC	bp 2241-2261 <i>C. pstb</i> <i>dtxR</i> locus	This study
<i>galE</i> GE	ATCGGCGAAAATCGCGAAGTTGA	bp 3274-3296 <i>C. pstb</i> <i>dtxR</i> locus	This study
<i>dtxR-galE</i> DG	ACCTAGCTCACGCAGTACGAATCGAAGAAAT	bp 2703-2733 <i>C. pstb</i> <i>dtxR</i> locus	This study
<i>fagA</i>	ATAGATGTAGCCATGGCATCA	bp 2230-2250 <i>fagA</i> gene	Acc. # AF401634

^a 5'-6-carboxyfluorescein and 3'-black hole quencher 1 modifications

2.6.2 Analysis of RNA samples

To visualise total RNA, formaldehyde (FA) gels were prepared. Briefly, for each gel, 1.2 g of molecular biology grade agarose was added to 10 ml of 10× FA gel buffer (**Appendix one**) and the solution was made up to 100 ml with RNase-free water. The mixture was heated in a microwave to melt the agarose, following which the molten agarose was cooled to *ca.* 56°C prior to the addition of 1.8 ml of 37 % (12.3 M) formaldehyde and GelRed to 1: 10,000. Gels were cast as above (**Section 2.6.1**), then submerged in an electrophoresis tank containing 1× FA gel running buffer. Each gel was equilibrated in FA for at least 30 min prior to the addition of RNA samples; RNA samples were prepared in RNA loading buffer (**Appendix one**) to a final concentration of 1×, heated to 65°C for 5 min, then chilled on ice prior to gel loading. Subsequently, each mini-gel was run at 36 V/cm until the dye-front had reached the end of the gel.

2.7 DNA Clean up

2.7.1 DNA extraction from agarose gels

Following electrophoresis through agarose gels, DNA bands were visualised over UV light (as described in **section 2.6.1**), prior to being excised using a clean scalpel blade, and placed into

a clean 1.5 ml microcentrifuge tube. The weight of gel slices was determined (by subtracting the weight of an empty tube from the weight of a tube + gel slice) and 100 μ l of GENECLAN[®] *Turbo* salt solution was added per 0.1 g of gel slice. Each gel slice/salt solution was incubated at 55°C for approximately 5 min (or until the gel had melted), aided by frequent vortexing. No more than 600 μ l of DNA/salt solution was added to a GENECLAN[®] *Turbo* cartridge and catch tube. The cartridge was centrifuged at $17,970 \times g$ for *ca.* 15 sec in a microcentrifuge, and then the flow-through in the catch tube was discarded. Cartridge-bound DNA was washed by addition of 500 μ l of GENECLAN[®] *Turbo* wash buffer, followed by further centrifugation at $17,970 \times g$ for 15 sec. Finally, the catch tube was emptied, and the cartridge was centrifuged for a further 4 min at $17,970 \times g$ to ensure there were no traces of ethanol contaminating the DNA. The GENECLAN[®] *Turbo* cartridge containing bound DNA was placed into a clean 1.5 ml microcentrifuge tube and 30 μ l of ddH₂O was added directly to the GLASSMILK[®]-embedded membrane. The tube was then incubated at room temperature for 5 min prior to a final centrifugation at $17,970 \times g$ for 1 min to elute the DNA.

2.7.2 Isolation of DNA from PCR reactions and other enzymatic solutions using GENECLAN[®] Turbo Kit (Q. Biogene)

In order to purify DNA from PCR and other enzymatic reactions, the GENECLAN[®] *Turbo* kit (Q. Biogene) was employed, according to the manufacturer's instructions. Briefly, DNA-containing solutions were supplemented with 5 volumes of GENECLAN[®] *Turbo* salt solution. Subsequently, GENECLAN[®] *Turbo* cartridges and catch tubes were used to purify DNA, as described in **Section 2.7.1**, ensuring that no more than 600 μ l of DNA/salt solution was added to each cartridge.

2.7.3 De-salting of ligations prior to transformation

To reduce the risk of arcing during bacterial transformations involving electroporation, rapid dialysis of ligation reactions was performed to remove excess salts, using Millipore Type VS 0.025 μ M filter discs (Millipore). VS filter discs were placed (shiny-side-up) in Petri dishes containing ddH₂O. After a brief wetting period, ligation reactions were pipetted carefully onto the centre of discs and allowed to sit for approximately 10 min at room temperature. Subsequently, ligation reactions were transferred to clean microcentrifuge tubes and either used immediately for bacterial transformation or stored at -20°C for subsequent use.

2.8 Bacterial transformation

2.8.1 Preparation of *E. coli* chemically-competent cells

A 10 ml aliquot of LB broth containing the appropriate antibiotic was inoculated with a single colony of the *E. coli* strain to be transformed. The culture was incubated overnight at 37°C \times 225 rpm, then diluted 1/100 into 100 ml of SOB medium (Appendix one). The culture was incubated at 37°C \times 225 rpm for ca. 2 hrs, until an OD_{600nm} of 0.3 was reached. The culture was then chilled on ice for 20 min prior to harvesting cells by centrifugation at 3,893 \times g for 10 min at 4°C. Cells were washed by re-suspension of the pellet in 20 ml of ice-cold, sterile Transformation Buffer (Appendix one), then harvested by centrifugation as previously stated. Cells were then re-suspended in 2.5 ml of ice-cold Transformation Buffer and incubated on ice for 30 min. Subsequently, 90 μ l of DMSO was then added. Finally, cells were incubated on ice for a further 10 min prior to the transfer of 100 μ l aliquots into pre-chilled 0.2 ml microcentrifuge tubes. Cell aliquots were snap frozen in a dry ice/ ethanol bath prior to being aliquoted and stored at -80°C.

2.8.2 Transformation of *E. coli*

2.8.2.1 Chemical transformation

A 50-100 μ l aliquot of chemically competent *E. coli* cells from **section 2.8.1** or One shot[®] TOP 10 cells (Invitrogen) were taken from storage at -80°C and thawed on ice. The cells were

then mixed with 0.5-1 µg plasmid DNA and incubated on ice for 30 min. Subsequently, cells were heat shocked at 42°C for 45 sec prior to incubating on ice for a further 5 min. A 500-900 µl aliquot of SOB (**Appendix one**) was added to the cell suspension, which was then incubated at 37°C × 225 rpm for 1 hr. Ten-fold serial dilutions of the transformed cells were then prepared in LB broth, and 100 µl aliquots were plated on agar media containing the appropriate antibiotics. Finally, plates were incubated at 37°C until single colonies had developed.

2.8.2.2 Electroporation

An aliquot of electrocompetent *E. coli* ElectroMAX™ DH5α-E™ cells (Invitrogen, Paisley, UK) was removed from -80°C storage and thawed on ice. Subsequently, 1 µg of plasmid DNA was added to the defrosted cells and mixed by gently stirring with a pipette tip. The mixture was then incubated on ice for 10 min before being transferred into a pre-chilled, 0.1 cm electrode gap electroporation cuvette (Bio-Rad). The cells were electroporated in a Gene Pulser Xcell™ Electroporator (Bio-Rad) at 200 Ω, 25 µF and 2.0 kV, and immediately transferred to 500 µl of SOC medium in a sterile 1.5 ml microcentrifuge tube. Subsequently, the cell suspension was incubated at 37°C × 225 rpm for 1 hr. Following incubation the cell suspension was plated onto antibiotic containing LB nutrient agar prior to overnight incubation at 37°C.

2.8.3 Preparation of electrocompetent *C. pseudotuberculosis*

Electrocompetent *C. pseudotuberculosis* cells were prepared to facilitate transformation by electroporation. Twenty ml of BHIT broth was inoculated with a colony of *C. pseudotuberculosis*, and the culture was incubated overnight at 37°C × 225 rpm. Subsequently, the culture was diluted 1/10 into 200 ml of BHI broth containing 2.5 % (w/v) glycine. The culture was incubated, as above, for *ca.* 4 hrs to an OD_{600nm} of between 0.6-0.8, and then cells were harvested by centrifugation at 3,893 × *g* for 15 min at 4°C. The cell pellet was gently re-suspended in 100 ml of ice cold, sterile electroporation buffer (15 % (v/v) glycerol, 0.01 % (v/v) Tween 20), and centrifugation was conducted as before; this washing procedure was carried out a total of 3 times. Cells were then re-suspended in 1 ml of ice cold electroporation buffer, and 65 µl aliquots of the cell suspension were transferred into pre-chilled 0.2 ml microcentrifuge tubes. Cells required for immediate transformation were stored

on ice, while those that were not required immediately were snap frozen in a dry ice/ethanol bath and stored at -80°C for future use.

2.8.4 Transformation of *C. pseudotuberculosis*

Plasmid DNA was introduced into *C. pseudotuberculosis* by electroporation. If competent cells were stored at -80°C, an aliquot was allowed to thaw gently on ice prior to use. To an aliquot of cells was added 1 µg of highly-pure plasmid DNA, and the cells and DNA were mixed gently by stirring with a pipette tip. Following a *ca.* 15 min incubation on ice, the cells were transferred to a pre-chilled, 0.1 cm electrode gap electroporation cuvette (Bio-Rad), and electroporation was conducted in a Gene Pulser Xcell™ Electroporator (Bio-Rad) at 200 Ω, 25 µF and 1.8 kV. Immediately, cells were transferred to a 14 ml polypropylene Leighton tube containing 10 ml of room temperature BHIT, and incubated without agitation for 2 hrs at 37°C (or the permissive replication temperature for the plasmid). Cells were harvested by centrifugation at $3,893 \times g$ for 15 min at 4°C, and then re-suspended in 200 µl of BHIT. Ten-fold serial dilutions of the transformed cells were prepared in PBS, prior to the plating of 100 µl aliquots onto the appropriate media containing the necessary selective antibiotics. Finally, plates were incubated at a temperature permissive for plasmid replication until single colonies had developed.

2.9 Southern hybridisation

Southern hybridisation (blotting) was carried out using a standard capillary-transfer method. Briefly, restriction endonuclease digested DNA samples were separated by electrophoresis through a 20 cm × 15 cm, 2 % (w/v) agarose gel for 12 hrs at 50 V/cm. Subsequently, DNA was depurinated by soaking the gel in 0.25 M HCl for no longer than 10-15 min. Four sheets of Whatman™ 3 MM paper (Whatman Ltd., UK) were cut to the size of the gel tray, ensuring a 5 cm overhang at each end. A sheet of Zeta-Probe Genomic Tested (GT) blotting membrane (Bio-Rad) was cut to the size of the gel tray, and pre-wet in ultra pure ddH₂O for 5 min. The Whatman™ 3MM paper sheets were placed on an inverted gel casting tray which was then placed into the bottom of a deep dish and saturated with 0.4 M NaOH. To ensure good capillary transfer of DNA, bubbles were removed using a plastic pipette in a rolling motion

over the filter paper. The gel was then carefully placed onto the filter paper; again bubbles were removed, and a small amount of 0.4 M NaOH was used to cover the gel to prevent it from drying out. Plastic laboratory wrap was used to cover the whole dish and a clean scalpel blade was used to cut a small gel sized window into the plastic wrap (thereby allowing the gel to be exposed but preventing the 0.4 M NaOH from evaporating elsewhere). The pre-wet Zeta-Probe GT membrane was then placed over the gel surface and a further 2 pieces of filter paper were cut to the exact size of the gel, pre-wet, and placed onto the Zeta-Probe GT membrane/gel stack. A pile of pre-cut paper towels, a glass plate and a lead weight were placed on top of the membrane/gel stack and transfer was allowed to continue for 24 hrs. Following transfer, the membrane was separated from the gel, rinsed briefly in 2× SSC (**Appendix one**) and allowed to air dry. The DNA was cross-linked to the membrane by microwaving for 2 min on high in a 950 W microwave oven, ensuring constant turning to prevent scorching.

2.9.1 Digoxigenin-dUTP (DIG) Labelling of DNA

DNA probes used for Southern hybridisation experiments were labelled using the DIG high prime labelling and detection starter kit (Roche), according to the manufacturer's instructions. Briefly, approximately 1 µg linear or supercoiled template DNA was added to ddH₂O to a final volume of 16 µl. The DNA was denatured by heating in a boiling water bath for 10 min followed by chilling in an ice water bath. The DIG High prime solution was mixed thoroughly, 4 µl was added to the denatured DNA, and the resulting mixture was vortexed and centrifuged briefly at 17, 970 × *g*. To achieve efficient labelling of DNA the mixture was incubated overnight at 37°C. The reaction was stopped by the addition of 2 µl of 0.2 M EDTA (pH 8.0) and by heating to 65°C for 10 min.

2.9.2 Hybridisation

Prior to use in hybridisations, DIG-labelled probes were denatured by boiling for 5 min in a boiling water bath before being chilled in an ice water bath. The Zeta-Probe GT membrane onto which DNA had been transferred was first pre-hybridised by placing it in a sealable tray and submerging it in hybridisation fluid. The fluid contained 0.25 M sodium phosphate (pH

7.2) and 7 % SDS. The membrane was incubated at 65°C for 30 min ensuring complete and even coating with the solution. Following pre-hybridisation the fluid was removed and replaced with fresh hybridisation fluid. The denatured DIG labelled probe (**Section 2.9.1**) was added to the hybridisation fluid and mixed by swirling. All bubbles were removed to avoid background noise on the blot. The tray was placed in a Hybaid hybridisation oven (Thermo-Scientific, UK) overnight at 65°C with agitation. Following the incubation the hybridisation fluid and probe were removed from the membrane which was washed twice in 20 mM sodium phosphate (pH 7.2), 5 % (w/v) SDS at 65°C for 30 min. The membrane was then subjected to two further washes in 20 mM sodium phosphate (pH 7.2), 1 % (w/v) SDS at 65°C for 30 min.

2.9.3 Immunological detection of DIG labelled probes

Immunological detection of hybridised, DIG-labelled probes was carried out according to the manufacturer's instructions. Briefly, the hybridised membrane was rinsed in DIG washing buffer (**Appendix one**) and then incubated in 100 ml of 1× blocking solution (**Appendix one**) for 30 min at room temperature. The blocking solution was removed and replaced with 20 ml of antibody solution (Anti-Digoxigenin-AP, diluted 1:10,000 in 1× blocking solution) at room temperature. The membrane was incubated for a further 30 min in antibody solution at room temperature. This solution was removed and the membrane was subjected to two 15 min washes in washing buffer. Following these washes, the membrane was equilibrated for 2-5 min in 20 ml detection buffer. The developing solution was removed and the membrane was placed on laboratory plastic wrap, DNA-side-up. One ml of chemiluminescent substrate for alkaline phosphatase (CSPD) was added to the membrane which was immediately covered by a second piece of laboratory plastic wrap and all air bubbles removed. Excess liquid was squeezed out of the membrane which was then incubated at 37°C for 10 min to enhance the luminescent reaction. The membrane was then used to expose a piece of X-ray film (Kodak® X-Omat AR Film, Kodak, UK) for 15-20 min at room temperature. The X-ray film was then developed using an X-ray developer (Optimax 2010 X-Ray film processor; Protec GmbH & Co., Germany)

2.10 General Proteomic Techniques

2.10.1 Isolation of exported proteins from *C. pseudotuberculosis*

In order to observe differences in the exported protein profiles of *C. pseudotuberculosis* grown under high- and low-iron conditions (**Chapter 3**), 10 ml of CCDM was inoculated with single colony of *C. pseudotuberculosis* and grown overnight at 37°C × 225 rpm.. The overnight culture was diluted to an OD_{600nm} of 0.1 in a total volume of 200 ml of iron-restricted CCDM and non-restricted CCDM in plastic disposable flasks (Nalgene, Rochester, NY, USA), and incubation was continued as before for *ca.* 7 hrs until log phase growth was reached. Discrepancies between the OD_{600nm} of the cultures were adjusted by addition of CCDM to obtain equivalent cell numbers between the 2 cultures. The cultures were transferred to 50 ml disposable Falcon polypropylene tubes (BD-Biosciences, Oxford, UK) and centrifuged for 15 min at 3,893 × *g* and 4°C. The resultant supernatants were filter-sterilised to remove any cell debris, and supplemented with Complete protease inhibitor cocktail (Roche), to 1× final concentration, and stored on ice to prevent proteolytic degradation of the exported proteins. Subsequently, proteins were concentrated using Amicon Ultra-15-5K M_r cut-off centrifugal filters (Millipore), at 3,893 × *g* at 4°C. The concentrated samples were then buffer-exchanged 3 times in PBS (**Appendix one**). Subsequently, the concentrated protein solutions were transferred to 1.5 ml microcentrifuge tubes and stored at -20°C until required.

2.10.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

C. pseudotuberculosis exported proteins were visualised by SDS-PAGE using the Bio-Rad mini protean II apparatus (Bio-Rad). Glass plates were cleaned with isopropanol to remove any debris. The plates were then clipped into the gel casting stand and 10 % resolving gel mixture (**Appendix one**) was poured in between the two glass plates to about 2 cm from the top. The resolving gel was covered with isopropanol whilst it was setting to provide a uniform interface between the resolving and stacking gels. Once the resolving gel had polymerised the isopropanol was poured off and the gel was rinsed with ddH₂O. A 5 % stacking gel mixture (**Appendix one**) was poured onto the solid resolving gel and a Teflon comb was placed in between the plates. Once the stacking gel had polymerised, the comb was removed and the

apparatus was placed into the mini protean II electrophoresis tank. If only one gel was being run in the tank at a time, a blank cartridge was used to seal the inner chamber of the tank. Both the inner and outer chambers were filled with $1\times$ running buffer (**Appendix one**). Five-10 μ l of the exported protein samples were transferred to clean microcentrifuge tubes, and 1-2 μ l of $5\times$ SDS-PAGE sample loading buffer (**Appendix one**) was added to each. Samples were then boiled in a hot block for 5 min, centrifuged in a microcentrifuge at $17,970\times g$ for 3 min, and loaded into the wells of the gel. Protein M_r markers were chosen according to the type of post-staining technique to be used; SeeBlue[®] Plus2 pre-stained standards (Invitrogen) were used for Coomassie staining or Mark 12TM standards (Invitrogen) were used for silver staining.

2.10.3 Visualisation of proteins in SDS-PA gels

2.10.3.1 Colloidal Coomassie blue staining

Electrophoresed proteins for LC-ESI-MS/MS analysis were stained with colloidal Coomassie brilliant blue (Genomic Solutions, MI, USA). The gel was fixed in a solution of 40 % (v/v) methanol, 10 % (v/v) acetic acid for 30 min in a plastic tub. The gel was then stained overnight in 80 ml of colloidal Coomassie and 20 ml of methanol. Prior to downstream protein analysis, gels were de-stained in 20 % methanol for at least 1 hr.

2.10.3.2 Silver staining

To allow greater staining sensitivity, silver staining of protein gels was carried out using the SilverQuestTM Silver staining kit (Invitrogen) following the manufacturer's instructions. Each gel was rinsed in ddH₂O and placed in a plastic tub containing 100 ml of fixing solution (40 % (v/v) ethanol, 10 % (v/v) acetic acid). The gel was microwaved on full power in a 950 W microwave oven for 30 sec, and immediately agitated at room temperature for 5 min on a rotary shaker. The fixative solution was removed, and the gel was placed in 30 % (v/v) ethanol and microwaved on high power for a further 30 sec and agitated again for 5 min at room temperature. The ethanol was decanted and 100 ml of sensitizing solution (30 ml ethanol, 10 ml sensitizer and 60 ml ddH₂O) was added to the tub containing the washed gel. The gel/sensitising solution was subjected to further microwaving on high power for 30 sec prior to agitation for 2 min. The sensitizing solution was decanted and the gel was washed twice in 100 ml ddH₂O; each wash step included 30 sec of microwaving and 2 min agitation at room temperature. Following these washes the gel was subsequently placed into 100 ml of staining

solution (1 ml stainer and 99 ml ddH₂O), microwaved on high power for 30 sec and agitated at room temperature for 5 min. The staining solution was decanted and the gel was washed with 100 ml of ddH₂O for less than 1 min. The gel was then placed into developing solution (10 ml developer, 1 ml of enhancer and 89 ml ddH₂O) and incubated for 5 min at room temperature with agitation. Once the desired band intensity was achieved, 10 ml of stopper solution was added to the developer solution/gel and the gel was washed in ddH₂O.

2.10.4 Drying of SDS-PAGE Mini-Gel's

Mini-gels were dried using the DryEase® mini-gel drying system (Invitrogen) following the manufacturer's instructions. Briefly, 35 ml of Gel-Dry™ drying solution was added to the mini-gel which was incubated at room temperature for 15 min. Two sheets of cellophane were immersed in Gel-Dry™ and one sheet was then placed over the gel drying base. The Mini-gel was then placed on top of the pre-wet cellophane followed by the mini-gel and a further piece of pre-wet cellophane. Bubbles and wrinkles were removed from the gel and the top of the DryEase™ frame was clamped around the mini-gel. The apparatus was allowed to air dry for approximately 12 hrs.

2.10.5 Western blotting

Following separation by SDS-PAGE, proteins were immediately transferred to nitrocellulose membranes (BioRad). Briefly, the top left corner of the each membrane was removed to aid orientation post-transfer. The nitrocellulose membrane and two pieces of Whatman™ 3MM filter paper were pre-soaked in Tris-glycine transfer buffer (**Appendix one**), then the membrane and gel were placed in-between the two pieces of filter paper and two sponges. Using the Bio-Rad Mini Trans-Blot™ apparatus, the gel was clamped into the assembly with the gel closest to the cathode core. To keep the apparatus cool at high voltages an ice block and stir bar were placed in the tank, which was then placed on a magnetic stirrer. The chamber was filled with 1× Tris-glycine transfer buffer and run at 400 mA for 30 min.

Following transfer, the nitrocellulose membrane to which proteins were bound was blocked in 1 % (w/v) Top-Block™ (Fluka Chemical Corp. Buchs, Switzerland) for 1 hr. The Top-

BlockTM was then poured off and replaced with 15 ml of PBS containing 0.05 % (v/v) Tween 20 (PBST) containing CLA-positive sheep sera diluted 1:500. The membrane was incubated with the sheep sera for 1 hr at room temperature on a rotary shaker, and then washed three times for 5 min in 15 ml of PBST on a rotary shaker. The membrane was then incubated in 15 ml of PBST containing a 1:5,000 dilution of a horseradish peroxidase-conjugated mouse monoclonal anti-goat/sheep IgG antibody (clone GT-34; Sigma) for 1 hr, with shaking. The membrane was washed twice with PBST, as before, then incubated in 3,3'-diaminobenzidine (DAB) substrate (prepared from a DAB tablet (Sigma) according to the manufacturer's instructions), at room temperature until bands were visible. The membrane was then rinsed with ddH₂O and dried in filter paper.

2.11 Allele replacement mutagenesis

2.11.1 Targeted plasmid integration into the *C. pseudotuberculosis* chromosome

An individual colony of *C. pseudotuberculosis* which had been transformed with a pCARV-based plasmid construct was grown in BHIT broth supplemented with 0.05 µg/ml erythromycin (Chapter 4). The culture was incubated at 25°C (the permissive replication temperature for the plasmid) × 225 rpm until, an OD_{600nm} of 0.8 was reached. The temperature of incubation of the culture was subsequently shifted to 37°C (the non-permissive replication temperature for the plasmid) for 6-9 generations (*ca.* 9 hrs). Ten-fold serial dilutions were then prepared in PBS containing 0.05 % (v/v) Tween 80[®] and 100 µl aliquots of each dilution were plated onto BHI agar containing 0.05 µg/ml of erythromycin. The plates were incubated at 37°C until bacterial colonies had developed. At this stage, bacteria resistant to erythromycin at 37°C were considered to have undergone a single homologous recombination event between plasmid and chromosomal sequences, resulting in the integration of the plasmid into the chromosome; although the temperature was not-permissive for plasmid replication, the integrated plasmid replicated along with the bacterial chromosome, allowing expression of the plasmid-encoded erythromycin resistance determinant.

2.11.2 Secondary cross-over mutagenesis

A single co-integrate colony was inoculated into 5 ml BHIT supplemented with erythromycin, and the culture was incubated overnight at 37°C × 225 rpm. Subsequently, the culture was serially-diluted down to 1.0×10^{-5} into a 5 ml volume of fresh BHIT (without erythromycin) and incubation was continued at 25°C (the permissive replication temperature for the plasmid). Once the culture had reached stationary phase, 10-fold serial dilutions were prepared in PBS containing 0.05 % (v/v) Tween 80[®]. Appropriate dilutions were plated onto BHI agar and incubated at 37°C until single colonies had developed. Subsequently, individual colonies were replica plated onto erythromycin-containing and erythromycin-free BHI agar plates to identify those which had undergone a secondary recombination event that caused excision of the plasmid from the chromosome, resulting in the loss of plasmid-encoded antibiotic resistance.

2.11.3 Ampicillin enrichment of secondary crossover mutations

To facilitate the identification of *C. pseudotuberculosis* secondary cross-over mutants which had undergone excision of pCARV-based constructs from the chromosome, a novel antibiotic enrichment procedure was devised. Allele-replacement mutagenesis was performed exactly as detailed in **Sections 2.11.1** and **2.11.2** above. However, following dilution to 1.0×10^{-5} and shifting of the incubation temperature to 25°C (**Section 2.11.2**) until the culture had reached stationary-phase, serial dilutions were not plated immediately. Rather, the stationary-phase culture (containing secondary cross-over mutants) was further diluted 1:100 in 5 ml of BHIT, and incubated at 37°C until early log-phase growth (*ca.* OD_{600nm} 0.4) was reached. At this point erythromycin was added to the inhibitory concentration (0.05 µg/ml) and the culture was incubated for a further 2 hrs to allow expression of the plasmid-encoded erythromycin resistance gene. Finally, the culture was supplemented with ampicillin to 100 µg/ml, and incubation was continued at 37°C. The OD_{600nm} of the culture was measured regularly until growth was observed to be static. At this point it was assumed that erythromycin-resistant cells (which still contained chromosomally-integrated plasmid) would have been killed by the bactericidal affect of the ampicillin, while the bacteriostatic affect of erythromycin upon non-erythromycin-resistant cells (which had undergone plasmid excision from the chromosome)

would have protected these cells from the harmful effects of the ampicillin. Subsequently, non-lysed cells were harvested by centrifugation at $3,893 \times g$ for 15 min), and washed twice with PBS containing 0.05 % (v/v) Tween 80 to remove residual ampicillin. Ten-fold serial dilutions of the culture, which had been enriched in the proportion of plasmid excision mutants to non-excised plasmid integrants, were then prepared in PBS containing 0.05 % (v/v) Tween 80, and 100 μ l aliquots were plated onto BHI agar. Plates were incubated at 37°C to allow bacterial growth, and then single colonies were replica plated onto erythromycin-containing and erythromycin-free BHI agar plates to allow identification of cells having undergone secondary cross-over events, resulting in the loss of plasmid (and hence erythromycin-resistance).

2.12 β -galactosidase promoter-fusion assays

A single colony of *C. pseudotuberculosis*, transformed with a pSPZ-derived construct (**Chapter 5**), was used to inoculate 5 ml of CCDM containing spectinomycin (100 μ g/ml), and the culture was incubated overnight at 37°C \times 225 rpm; cultures were prepared in triplicate. Subsequently, cultures were diluted to an OD_{600nm} of 0.1, and incubated, as before, for *ca.* 6 hrs until mid log-phase had been reached. The OD of each culture was recorded, and a 100 μ l aliquot of each was then transferred to glass test tubes. For each culture aliquot, bacteria were lysed by the addition of 900 μ l of Z buffer (**Appendix one**), followed by 40 μ l of 0.1 % (w/v) SDS and 150 μ l of chloroform. The tubes were then sealed with Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA) and vortexed for 30 sec. To assess expression of the *lacZ* reporter gene, 200 μ l of a 4 mg/ml solution of *o*-nitrophenyl- β -D-galactoside (ONPG) was added. Subsequently, tubes were incubated at room temperature to allow colour development for up to 1 hr depending on the speed of the colour change. Enzymatic reactions were stopped by addition of 500 μ l of 1 M Na₂CO₃ to a final concentration of 0.5 M, and the length of incubation was recorded. The whole mixture was subsequently transferred to 2 ml tubes and centrifuged at $17,970 \times g$ for 5 min at room temperature. Following centrifugation, the upper, aqueous phase was transferred to a cuvette and the OD_{420nm} was measured and recorded. Subsequently, the level of expression was determined, expressed in “Miller units”, according to the following equation: $1000 \times (A_{420nm} - 1.75) \div (\text{length of incubation}) \times \text{cell volume (0.1 ml)} \times A_{600nm}$.

2.13 Siderophore Assay

To allow quantification of siderophore in culture supernatants, a colourimetric microtitre plate assay was conducted. Five ml cultures of *C. pseudotuberculosis* were propagated (in triplicate) in high- and low-iron-containing growth media for *ca.* 12 hrs at 37°C × 225 rpm until stationary growth-phase was reached. Subsequently, cultures were diluted 1:10 in CCDM, in order to record an accurate OD_{600nm} of each culture. Then, each neat culture was centrifuged at 3,893 × *g* for 20 min at room temperature, and the supernatants (containing siderophore) were decanted into clean tubes. Following this, 100 µl of each culture supernatant was transferred to individual wells of a 96-well, flat-bottomed microtitre plate (Nunc™, Rochester, NY, USA). To allow quantification of siderophore, a standard curve was prepared using an 80 µM stock of EDDA. One hundred µl of the stock EDDA solution was transferred into 100 µl Tris-buffered saline (TBS), pH 7 (**Appendix one**) in wells B1 and C1 of the microtitre plate. Then, 100 µl of TBS was added to wells B1 and C1, and serial 2-fold dilutions of the EDDA were prepared in TBS, across the plate from left to right. The final 100 µl was discarded, resulting in duplicate rows, with each well containing 100 µl of EDDA solution at the following concentrations: 40 µM, 20 µM, 10 µM, 5 µM, 2.5 µM and 1.25 µM and again 40 µM, 20 µM, 10 µM, 5 µM, 2.5 µM and 1.25 µM in the remaining 6 columns resulting in a duplicated plate. Subsequently 100 µl of CAS assay buffer (**Appendix Two**) was added to each well and the plate was incubated at room temperature, in the dark, for 2 hrs. Following incubation, the OD_{630nm} of each microtitre plate well was determined in a ELX 808 ultra microplate-reader (Bio-Tek Instruments Inc. VT, USA) A result indicative of chelator presence turned the normally blue CAS assay buffer to pink, due to stripping the iron from the dye. Subsequently, siderophore units were divided by OD_{600nm} to correct differences in culture density.

2.14 DNA Sequencing

Automated sequencing of DNA was carried out by the Moredun Proteomics Facility (Moredun Research Institute) on a Mega BACE sequencer (Amersham Biosciences) using sequence specific primers (**Table 2.4**).

2.15 Computational analyses

Basic DNA sequence analyses, including mapping of restriction endonuclease recognition sites, open reading frame (ORF) predictions, translation and multiple DNA and protein sequence alignments were carried out using the Clone Manager Professional Suite Version 8 (Sci-Ed Scientific Ltd., Cary, NC, USA). In addition, translated *C. pseudotuberculosis* ORFs were identified by comparison with sequences resident in the National Center for Biotechnology Information (NCBI) non-redundant database, using the Basic Local Alignment Search Tool (BLAST), accessed via the NCBI WWW portal (<http://www.ncbi.nlm.nih.gov>).

2.16 Statistical analyses

Data was analysed and graphs were drawn using Microsoft® Office Excel 2003 (Microsoft®; CA, USA). Significant differences were assessed by the Student's *t*-test and ANOVA, which were performed using the Minitab® 15.1 software (Minitab™, UK). All errors bars that are shown throughout this are a result of the standard deviation of the mean. Statistical advice was provided by Dr Mintu Nath and Jill Sales at Biomathematics and Statistics Scotland (BioSS; University of Edinburgh, Kings Buildings, Edinburgh, Scotland).

2.17 Proteomic data interrogation

Proteins were identified following a match of at least two peptides, those peptides with lower scores were inspected manually and only included if a series of 4 or more continuous *y* or *b* ions were observed. Obtaining matches to a number of peptides from a single protein provides a very high level of confidence that the results are correct. Results obtained from proteomic studies were analyzed using Mascot™ software²¹ (Matrixscience, London, U.K.), SwissProt (Swiss Institute of Bioinformatics), NCBI nr databases (National library of Medicine and National Institutes of Health) and an in-house Mascot database derived from *C. diphtheriae*, *C. efficiens* and *C. glutamicum* genomes.

Chapter Three

Investigation of the effects of environmental iron on *Corynebacterium pseudotuberculosis*

3.0 Introduction

Iron is an essential element which is required by the majority of living organisms, an exception being some lactobacilli (Archibald, 1983). It contributes to numerous vital biological processes, including respiration, the trichloroacetic acid cycle, oxygen transport, gene regulation and DNA biosynthesis (Andrews, *et al.* 2003). Iron is the fourth most abundant metal in the Earth's crust, implying that there is a plentiful supply; however, under physiological conditions iron exists in an extremely insoluble ferric form. Furthermore, as part of the host's innate defence against invading microorganisms, free-iron is bound by host iron-binding proteins such as transferrin and lactoferrin. In order to cause infection, bacteria need to multiply; however, in order to multiply they need to acquire essential nutrients, including iron. Bacteria generally require 10^{-7} to 10^{-5} M of iron to achieve optimal growth (Andrews, *et al.* 2003), however, in the mammalian host, the concentration of free-iron is normally around 10^{-18} M which is far below the required level (Bullen, *et al.* 1978). Therefore, due to its general unavailability, microorganisms have evolved a number of mechanisms to obtain iron from their environment, and these mechanisms are often closely-linked to virulence (Litwin & Calderwood, 1993).

Host iron-binding proteins are varied; the majority of iron being bound by hemoproteins such as haemoglobin. Ferritin, another host-iron binding protein, is involved in iron storage and detoxification. Interestingly, ferritin analogs are found in bacteria, where they are known as bacterioferritin, and are also involved in the storage of intracellular iron (Mietzner & Morse, 1994; Theil, 1987). Haemoglobin and ferritin are intracellular iron sources, being found within host cells, while transferrin and lactoferrin are extracellular. Transferrin is responsible for transporting extracellular iron between cells, and circulates in the plasma and lymphatic fluids (Welch, 1992), while lactoferrin tends to be associated with mucosal secretions and phagocytic granules (Welch, 1992) (Mietzner & Morse, 1994). Lactoferrin and transferrin are also involved in the first line of defence against invading microorganisms by binding extracellular free-iron. At decreased pH, lactoferrin retains its affinity for iron, whereas iron is dissociated from transferrin at pH <4.5 (Welch, 1992) (Mietzner & Morse, 1994). The primary function of lactoferrin is to act as an inhibitor of microbial growth, but unlike transferrin it is not thought to be involved in eukaryotic iron transport (Mietzner & Morse, 1994).

Bacteria use several different mechanisms to sequester iron. The type of iron sequestration employed largely depends on the bacterium in question. The iron/haem acquisition systems in bacteria can generally be separated into two groups; the first involves direct contact between the source of iron and the bacterial surface, while the second mechanism involves compounds (known as siderophores), that are secreted by the bacteria into the extracellular medium to scavenge iron (Wandersman & Delepelaire, 2004).

In order to become a successful pathogen, an organism is required to “sense” its environment and respond quickly to environmental changes by modulating the expression of relevant genes. Environmental factors including pH, osmolarity, temperature and amino acid concentration, all exert an effect on bacterial gene expression including those genes associated with virulence (Miller, *et al.* 1989). It is not surprising then that being an essential requirement for growth, the concentration of environmental iron is also “sensed” by bacteria. In *C. pseudotuberculosis*, the sensing of environmental iron concentrations and the concomitant effect on pathogenesis is not yet fully understood. Billington *et al.* (1992) identified the so-called ferric iron acquisition genes (*fag*) operon, comprising three genes (*fagABC*) shown to be involved in iron acquisition. These genes were subsequently associated with *C. pseudotuberculosis* virulence (Billington, *et al.* 2002). However, to-date there has been no other published research on the requirement of iron or its potential link to gene expression (and therefore virulence). Interestingly, iron has been shown to play a very important role in the regulation of expression of virulence genes in the closely related human pathogen *C. diphtheriae*, and there has been a plethora of work carried out in this area.

Upon infection with a microorganism, and as part of the innate defence mechanism, the host specifically limits free-iron. This shift from a high to low-iron environment is an important environmental signal for bacteria to coordinate the regulation of gene expression (Litwin & Calderwood, 1993), and it is a signal which is interpreted in each of two ways; primarily, the bacterium recognises that it must acquire iron, but secondly it recognises that it has entered a hostile environment (*i.e.* the host) and must initiate attempts to colonise and persist. Subsequently, in addition to iron-acquisition genes, the expression of many genes involved in virulence are also regulated in response to environmental iron concentration. With this in mind, it was thought likely that by assessing the influence that iron had on *C. pseudotuberculosis* gene expression, it might eventually be possible to identify novel

phenotypes, which could be the focus of further study in order to discover specific factors contributing to the pathogenicity of the organism. There is no doubt that the natural host is the best system in which to study an organism; however, this environment is very complex, and hence it is difficult to dissect the multiple, often subtle influences that are exerted upon the pathogen by multiple host factors. Therefore, for the purposes of this study, a preliminary aim was to develop a defined growth medium, which would allow significant *in vivo* conditions to be mimicked. Using such a medium, it was anticipated that environmental iron concentrations could be altered so as to assess the outcome on growth and gene expression (determined by observation of phenotypes and assessment of changes in protein profiles) of *C. pseudotuberculosis*.

3.1 Results

3.1.1 Development of a chemically defined medium to investigate iron-regulation of gene expression *C. pseudotuberculosis*

A major objective of the work described in this chapter was to develop a chemically-defined medium (CDM) which would support the rapid growth of *C. pseudotuberculosis*, and, in which the concentrations of specific growth factors, including iron, could be easily and accurately manipulated. Furthermore, to facilitate downstream proteomic analyses, it was important that the CDM contained as few contaminating proteins as possible. Significantly, an effective CDM was an essential resource, required for all subsequent experiments on the iron-regulation of gene expression, described throughout this thesis.

3.1.2 Growth Characteristics of *C. pseudotuberculosis* in rich media

In order to define optimal growth conditions that could subsequently be exploited in the development of a CDM, it was important to determine the definitive growth of *C. pseudotuberculosis* in a rich, undefined medium. Routinely, corynebacteria are grown in Brain Heart Infusion broth (BHI). This undefined medium is very rich and provides all the necessary nutrients for the bacterium to flourish; however, the presence of contaminating proteins in the BHI could potentially result in background noise in proteomic analyses. In addition, and more importantly, the exact composition of the medium is unknown with respect to the abundance of specific growth factors and nutrients. The growth of *C. pseudotuberculosis* in BHI (typical results are shown in **Figure 3.1**) reached an OD_{600nm} of 1.8 after 15 hr, and this was used as the gold standard level of growth to compare the success of the subsequently developed CDM.

Due to the waxy nature of the *Corynebacterium* cell wall, polyoxyethylene sorbitane monooleate (Tween 80[®]), a non-ionic surfactant and emulsifier, is typically added to liquid cultures to prevent the bacteria clumping (Dubos & Middlebrook, 1948; McGinley, *et al.* 1985a; McGinley, *et al.* 1985b; Riley, *et al.* 1979). Furthermore, Tween 80[®] also serves as a metabolite, subsequently enhancing growth (Dubos & Middlebrook, 1948). The addition of 0.05 % (v/v) Tween 80[®] to liquid cultures became a standard procedure throughout this thesis.

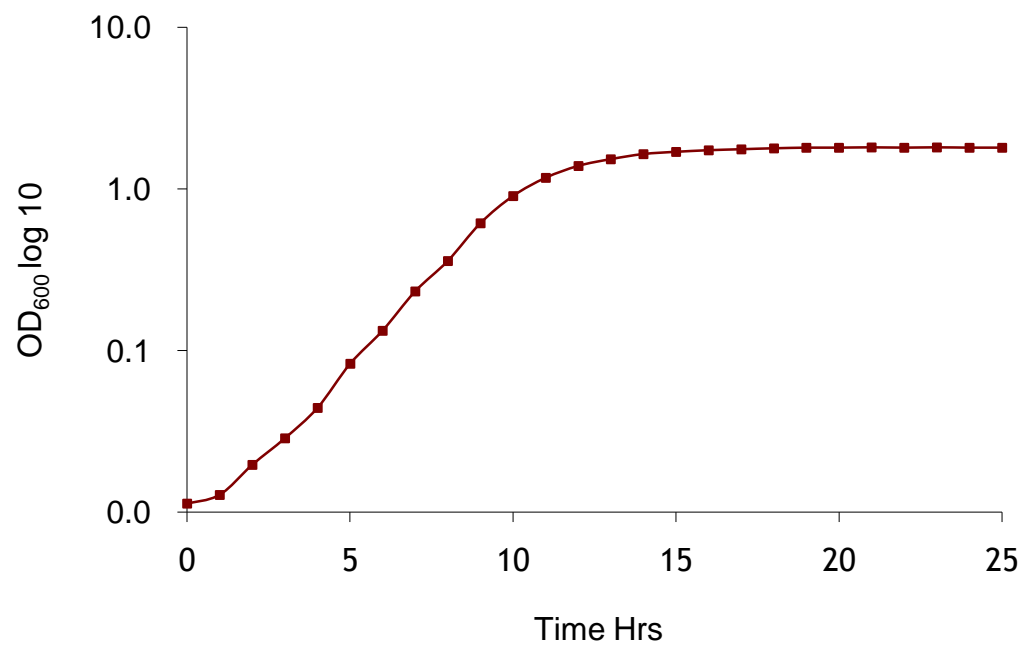


Figure 3.1 Typical growth curve of *C. pseudotuberculosis* 3/99-5 in BHIT broth at 37°C with constant shaking.

3.1.3 *C. pseudotuberculosis* chemically defined medium

A CDM which has been successfully used in the culture of the closely-related organism *Corynebacterium renale* (VanEseltine, *et al.* 1978), was used as a guide to help determine the composition of a *C. pseudotuberculosis* CDM. Significantly, many previously described CDM, including the one mentioned above, are composed of a remarkably complex blend of inorganic salts, amino acids and vitamins. Such media are time-consuming to prepare and frequently suffer from inconsistencies in performance due to their complex nature and the increased likelihood of errors during preparation. Hence, in order to ensure consistency in the quality of the CDM, in addition to facilitating ease of preparation, the utility of commercially available cell-culture media as a base for a novel *C. pseudotuberculosis* CDM was determined. The evaluation of two commercially available media, Hanks Balanced Salt Solution (HBSS; Sigma-Aldrich, UK, catalogue no. H 8264), and Roswell Park Memorial Institute medium 1640 (RPMI-1640; Sigma-Aldrich, UK, catalogue no. R 8755) were carried out to assess their ability to support the growth of *C. pseudotuberculosis*. **Table 3.1** compares the ingredients of the CDM (VanEseltine, *et al.*, 1978), RPMI-1640 Sigma-Aldrich, UK, catalogue no. R 8755 and HBSS Sigma-Aldrich, UK, catalogue no. H 8264).

Initially, growth of *C. pseudotuberculosis* in HBSS was assessed. Unfortunately, this medium was found not to be able to support the growth of the organism, and after 24 hr incubation the OD_{600nm} of the culture was <0.1. The second cell culture medium to be evaluated was RPMI-1640. The RPMI-1640 medium, although it contains some amino acids, is typically used in conjunction with a further preparation which provides supplementary amino acids. The addition of the supplement increased the concentration of amino acids in the RPMI-1640 which had the affect of substantially improving growth over RPMI-1640 alone (data not shown). Also, the addition of a carbon source (glucose) was found to be essential (data not shown), as RPMI-1640 contains only 2 g/L of glucose whereas the published CDM contained 10 g/L (VanEseltine, *et al.*, 1978). Another addition to the RPMI-1640 was 12 g/L of L-glutamic acid. Again, this was to increase the concentration to mimic that of the published CDM (VanEseltine, *et al.*, 1978). Significantly, the addition of 10 µM manganese and zinc chloride respectively, made no difference to the growth of *C. pseudotuberculosis* in the newly developed CDM (data not shown).

The modified/supplemented RPMI-1640 medium was subsequently designated “*Corynebacterium* Chemically-Defined Medium (CCDM). In order to confirm that CCDM could reproducibly support the growth of *C. pseudotuberculosis*, single colonies were picked from BHI agar plates and used to inoculate CCDM. Following incubation for 24 hr, the results clearly demonstrated that the novel CCDM was capable of sustaining growth of *C. pseudotuberculosis* (data not shown). In addition, growth experiments were carried out using overnight *C. pseudotuberculosis* cultures, propagated in CCDM to inoculate fresh CCDM. Following incubation for 24 hr, sub-cultured *C. pseudotuberculosis* had reached an OD_{600nm} of 1.6; a typical growth curve is presented in **Figure 3.2**. This showed that the CCDM contained sufficient nutrients/growth factors such that *C. pseudotuberculosis* was not relying on intracellular stores during primary culture. In addition, the final OD_{600nm} was almost equivalent to the gold standard BHI.

Table 3.1 Comparison of growth media ingredients

Component	RPMI-1640 (cat no. R 8755)* g/L	CDM (VanEselatine) g/L	HANKS (cat no. H 8264) g/L
Inorganic salts	0.1	0.065	0.185
Ca(NO ₃) ₂ •4H ₂ O			
MgSO ₄ (anhydrous)	0.04884	0.021	0.097
KCl	0.4	0.4	0.4
KH ₂ PO ₄	-	0.68	0.06
NaHCO ₃	-	0.5	0.35
NaCl	6.0	6.2	8.0
Na ₂ HPO ₄ (anhydrous)	0.8	2.8	0.045
Amino acids			
L-Arginine	0.2 (10)	-	-
L-Asparagine (anhydrous)	0.05 (2.8)	-	-
L-Aspartic Acid	0.02 (1.0)	-	-
L-Cystine•2HCl	0.065 (2.5)	0.05	-
L-Glutamic Acid	0.02 (1.0)	12.7	-
L-Glutamine	0.3	-	-
Glycine	0.01 (0.5)	-	-
L-Histidine	0.015 (0.75)	-	-
Hydroxy-L-Proline	0.02 (1.0)	0.5	-
L-Isoleucine	0.05 (2.5)	-	-
L-Leucine	0.05 (2.5)	-	-
L-Lysine•HCl	0.04 (2.0)	-	-
L-Methionine	0.015 (0.75)	0.03	-
L-Phenylalanine	0.015 (0.75)	-	-
L-Proline	0.02 (1.0)	-	-
L-Serine	0.03 (1.5)	-	-
L-Threonine	0.02 (1.0)	-	-
L-Tryptophan	0.005 (0.25)	-	-
L-Tyrosine	- (1.16)	-	-
L-Tyrosine•2Na•2H ₂ O	0.02883	-	-
L-Valine	0.02 (1.0)	-	-
Vitamins	0.0002	0.0004	-
D-Biotin			
Choline Chloride	0.003	-	-
Choline Bitartrate	-	-	-
Folic Acid	0.001	-	-
myo-Inositol	0.035	0.5	-
Niacinamide	0.001	-	-
p-Amino Benzoic Acid	0.001	-	-
D-Pantothenic Acid•½Ca	0.00025	-	-
Pyridoxine•HCl	0.001	-	-
Riboflavin	0.0002	-	-
Thiamine•HCl	0.001	0.01	-
Vitamin B-12	0.000005	-	-
Other			
D-Glucose	2.0	10	-
Glutathione (reduced)	0.001	-	-
HEPES	-	-	-
Phenol Red•Na	-	-	-

*Values in parentheses relate to RPMI amino acid solution

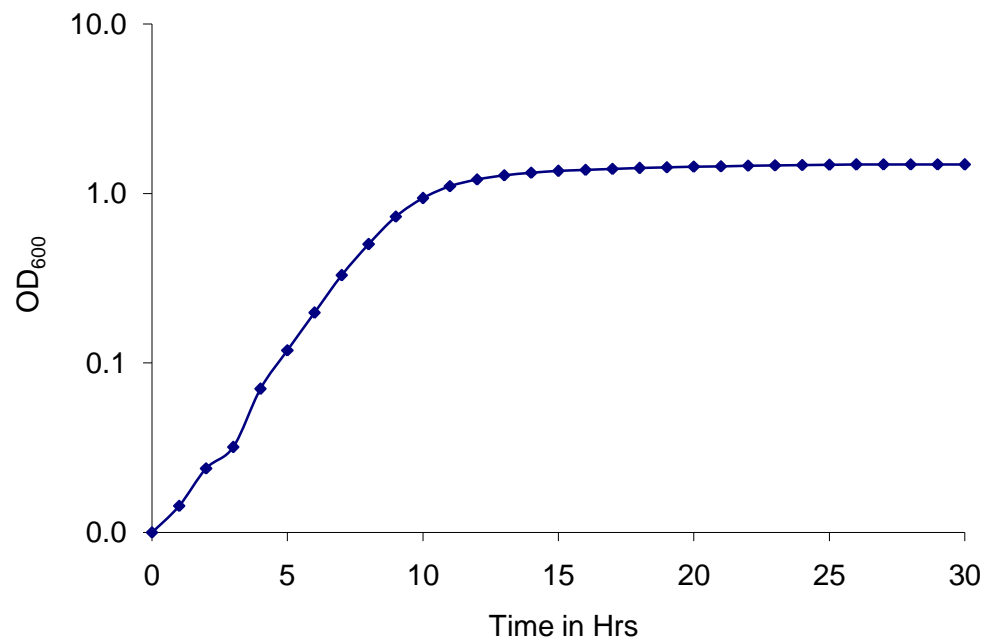


Figure 3.2 Typical growth curve of *C. pseudotuberculosis* 3/99-5 in CCDM

3.1.4 Modulating the iron content of CCDM

With the intention of using the CCDM as a tool to investigate iron-regulation of gene expression in *C. pseudotuberculosis*, the next and most critical step was to determine suitable high- and low-iron growth conditions. Importantly, it was necessary to obtain a high-low iron differential which had an effect on gene expression, while maintaining growth at a level sufficient to provide adequate cell density for subsequent analyses. The literature accompanying the RPMI-1640 (which was purchased in powdered form) and the other chemicals added to create CCDM did not state that iron was present. However, the observation that *C. pseudotuberculosis* was able to grow in CCDM meant that there were either traces of iron present, or that the organism did not require iron for growth. In order to prevent the leaching of iron from contaminated glassware, plastic containers were used during preparation of the CCDM. Therefore, the most likely scenario was that minor contamination with iron was a result of the water and/or commercial chemicals used to prepare the CCDM.

The growth of *C. pseudotuberculosis* in CCDM was comparable to that in BHI. However, because only trace (contaminant) levels of iron were present, it was thought possible that the addition of further iron to the CCDM potentially could improve the growth of *C. pseudotuberculosis*. To determine the effect of iron on the growth of the organism, CCDM was supplemented with ferric chloride (FeCl_3) to 1 μM , 10 μM and 100 μM final concentration, and the outcome on the growth of *C. pseudotuberculosis* was assessed (**Figure 3.3**). Interestingly, the addition of FeCl_3 did not improve the growth of *C. pseudotuberculosis*. Interpreting this result led to the conclusions that either sufficient contaminating iron was present in CCDM to promote growth, or that *C. pseudotuberculosis* did not require iron for growth.

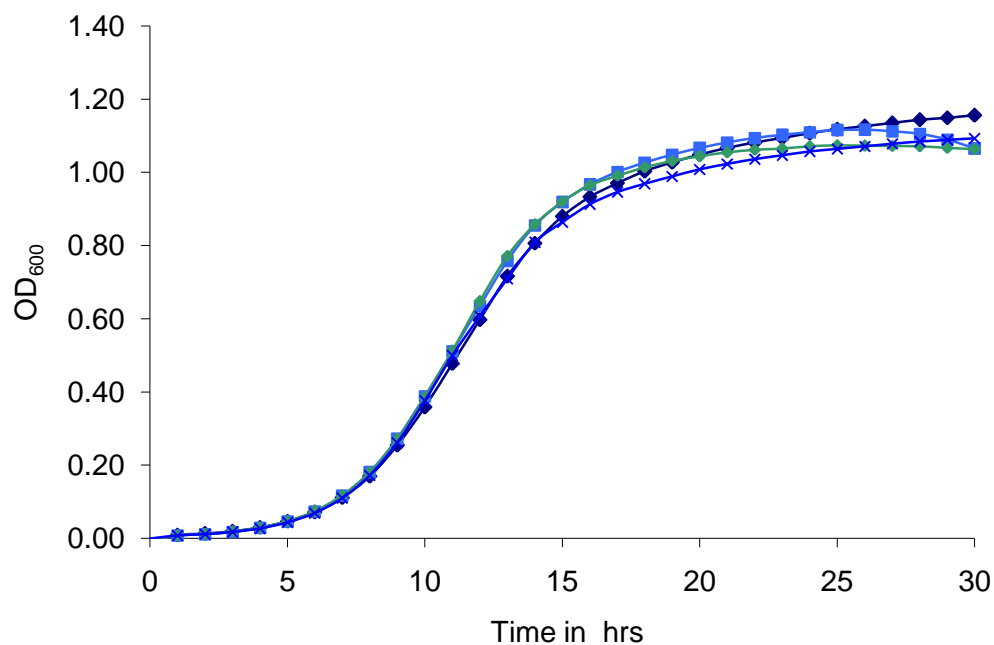


Figure 3.3 Growth of *C. pseudotuberculosis* in CCDM supplemented with varying concentrations of FeCl₃. CCDM (—◆—), CCDM + 1 μM FeCl₃ (—■—), CCDM + 10 μM FeCl₃ (—◆—), CCDM + 100 μM FeCl₃ (—×—). For ease of interpretation, data is plotted on a linear scale.

To determine whether *C. pseudotuberculosis* requires iron for growth, the iron-load in CCDM was reduced by addition of the powerful iron-chelator, 2', 2' dipyridyl (subsequently referred to as dipyridyl). *Corynebacterium* chemically defined medium was supplemented with a range of concentrations of dipyridyl from 200-450 μM (final concentration). The results of these experiments are presented in **Figure 3.4** where it is apparent that with increasing dipyridyl concentration, there is a concomitant decrease in growth of *C. pseudotuberculosis*. Therefore, it was assumed that *C. pseudotuberculosis* did require iron for growth, and that contamination of the CCDM with trace amounts of iron was sufficient to allow growth of the organism. In the course of assessing the outcome of supplementing CCDM with dipyridyl, a suitable low-iron growth medium was determined, based upon the observation that a 300 μM final concentration of dipyridyl resulted in a reduction of growth to a maximum $\text{OD}_{600\text{nm}}$ of *ca.* 1.0. This level of growth was deemed sufficient to have reduced the growth satisfactorily to exert an effect on iron-dependent gene expression, while allowing sufficient bacterial growth to provide material for downstream analyses.

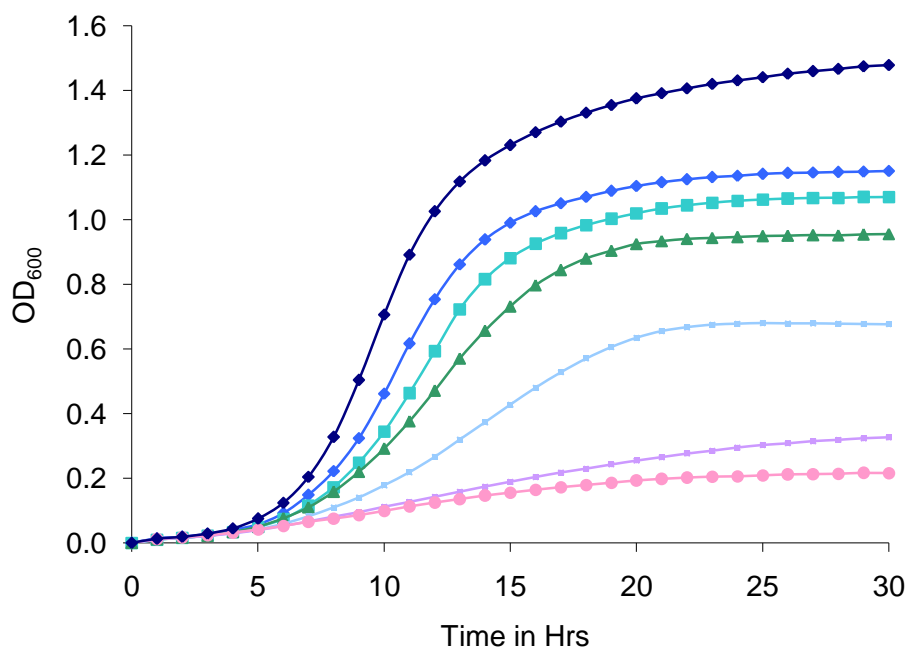


Figure 3.4 Growth of *C. pseudotuberculosis* in CCDM supplemented with varying concentrations of the iron chelator dipyriddy. CCDM (◆), CCDM + 200 μM (◆), 250 μM (■), 300 μM (▲), 350 μM (■), 400 μM (■), 450 μM (●), dipyriddy. For ease of interpretation, data is plotted on a linear scale.

3.1.5 Concentration of metal ions in *C. pseudotuberculosis* growth media

In order to determine the levels of metal ions present in the CCDM, it was analysed in comparison to BHI, both with and without the addition of Tween 80[®]. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analyses were performed by staff at the Analytical Services Department of the Scottish Agricultural College (Bush Loan, Penicuik, Midlothian, Scotland) on a Perkin Elmer Optima 4300DV mass spectrometer. The ICP-MS procedure is highly sensitive and capable of determining the concentrations of a range of metals, and is based on coupling together inductively coupled plasma as a method of producing ions which are separated and detected using a mass spectrometer. However, it is unable to measure iron concentration accurately below 230 µg/ml. The results of ICP-MS analyses are presented in **Table 3.2**, and clearly show that all assessed metal ions were reduced in quantity in CCDM-based media as compared to BHI. Unfortunately however, the concentration of iron in CCDM was too low to be quantified, being below the limit of sensitivity of 230 µg/ml, so it was not possible to determine the relative concentrations of iron in CCDM as compared to dipyrityl-treated CCDM. As expected, the addition of Tween⁸⁰ did not have a significant effect on the composition of the media.

Table 3.2. ICP-MS analysis of *C. pseudotuberculosis* growth media

	BHI	BHI + (Tween 80)	CCDM	CCDM + (Tween 80)	CCDM + dipyridyl
Sulphur	127	123	36.3	35.5	33.8
Magnesium	18.9	18.6	7.95	8.10	7.71
Calcium	20.7	20.6	13.9	13.9	13.2
Aluminium	356	340	<80.0	<80.0	<80.0
Iron	866	843	<230	<230	<230
Manganese	275	270	<4.0	<4.0	<4.0
Copper	<11.0	<11.0	<11.0	<11.0	<11.0
Zinc	827	822	<9.0	<9.0	<9.0
Phosphorus	597000	598000	167000	170000	162000
Cadmium	<14.0	<14.0	<14.0	<14.0	<14.0
Chromium	20.0	19.9	<13.0	<13.0	<13.0
Nickel	<30.0	<30.0	<30.0	<30.0	<30.0
Lead	<110	<110	<110	<110	<110
Boron	224	220	<45	<45	<45

Units of concentration are µg/ml except sulphur, magnesium and calcium which are mg/ml. Analyses were carried out by the Analytical Services Department of the Scottish Agricultural College

3.1.6 *C. pseudotuberculosis* acquisition of iron from host iron-binding proteins

3.1.6.1 Transferrin

In order to determine whether *C. pseudotuberculosis* was able to acquire iron from the host iron-binding protein, transferrin, 1 mg/ml of holo (iron-saturated)-transferrin was added to aliquots of CCDM, with and without a final concentration of 300 μ M dipyriddy (Figure 3.5). No difference in the growth profile was observed following the addition of transferrin to CCDM without dipyriddy, suggesting that the CCDM alone contains sufficient iron to support the growth of the organism. In contrast, the addition of holo-transferrin to dipyriddy-supplemented CCDM resulted in the restoration of growth, after a *ca.* 5 hr lag-phase, from the previously observed OD_{600nm} of *ca.* 1.0 to the CCDM-equivalent OD_{600nm} of *ca.* 1.6. This data suggests that *C. pseudotuberculosis* is able to sequester iron from transferrin.

To further demonstrate the capacity of *C. pseudotuberculosis* to acquire iron from transferrin, CCDM was supplemented with apo (iron-free)-transferrin, which has a high-affinity for iron. As can be seen in Figure 3.6, *C. pseudotuberculosis* was still able to grow in CCDM supplemented with 1mg/ml of apo-transferrin, although the final OD_{600nm} was slightly lower than observed in CCDM supplemented with dipyriddy and holo-transferrin (or CCDM alone). These results are consistent with the preliminary sequestration of free-iron by transferrin, followed by the acquisition of transferrin-bound iron by *C. pseudotuberculosis*. In addition, these results further demonstrate that *C. pseudotuberculosis* is able to acquire iron from transferrin.

3.1.6.2 Lactoferrin

An equivalent experiment to that described for transferrin was conducted, whereby aliquots of CCDM (with and without dipyriddy) were supplemented with lactoferrin to 1 mg/ml. It should be noted that the lactoferrin used in this experiment, which was purchased from Sigma-Aldrich, was obtained from bovine colostrum, and it was not possible to ascertain from the manufacturer's accompanying data whether it was the apo- or holo-form.

Following the supplementation of cultures with lactoferrin a preliminary lag in growth of *ca.* 5 hr was observed (Figure 3.7), following which growth proceeded at a rate slower than observed in CCDM, but faster than in CCDM supplemented with dipyriddy. The final OD_{600nm} of lactoferrin supplemented cultures was *ca.* 1.6, equivalent to growth in CCDM. These data,

which were equivalent to the growth of *C. pseudotuberculosis* in CCDM supplemented with dipyridyl and holo-transferrin, revealed that the organism was able to acquire iron from lactoferrin.

3.1.6.3 Haemoglobin

To further investigate the ability of *C. pseudotuberculosis* to utilise host iron binding proteins as sources of iron, CCDM was supplemented with haemoglobin to a final concentration of 1 mg/ml. Unfortunately the addition of haemoglobin to the CCDM resulted in solubility issues, and these experiments were not pursued further.

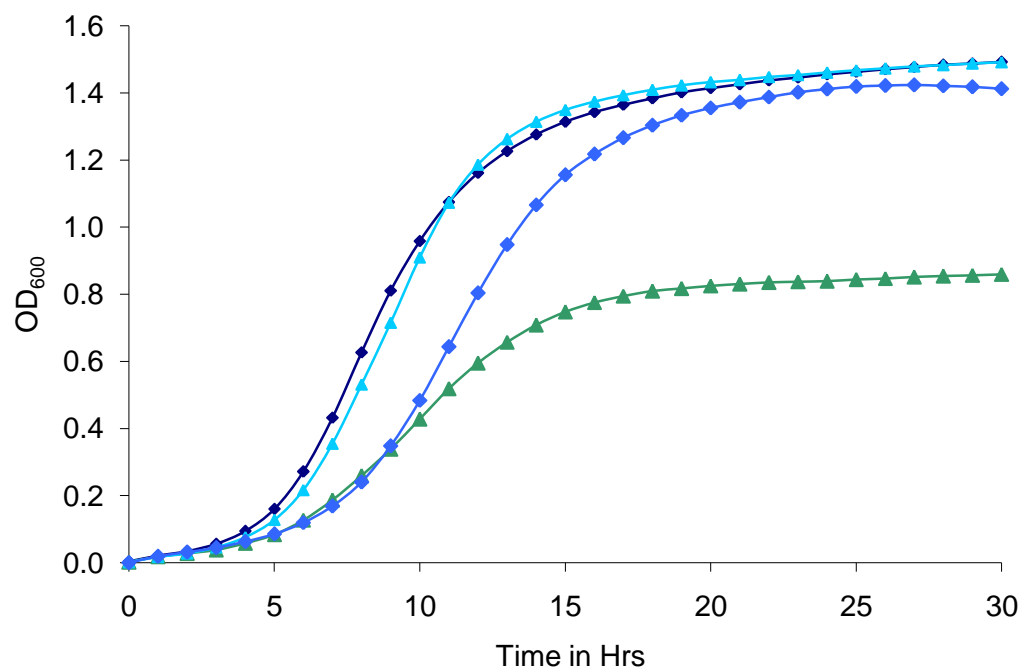


Figure 3.5 Growth of *C. pseudotuberculosis* 3/99-5 in CCDM supplemented with dipyrindyl and transferrin. CCDM (—◆—), CCDM + 300 µM dipyrindyl (—▲—), CCDM + 1 mg/ml holo-transferrin (—▲—), CCDM + 1 mg/ml holo-transferrin + 300 µM dipyrindyl (—◆—). For ease of interpretation, data is plotted on a linear scale.

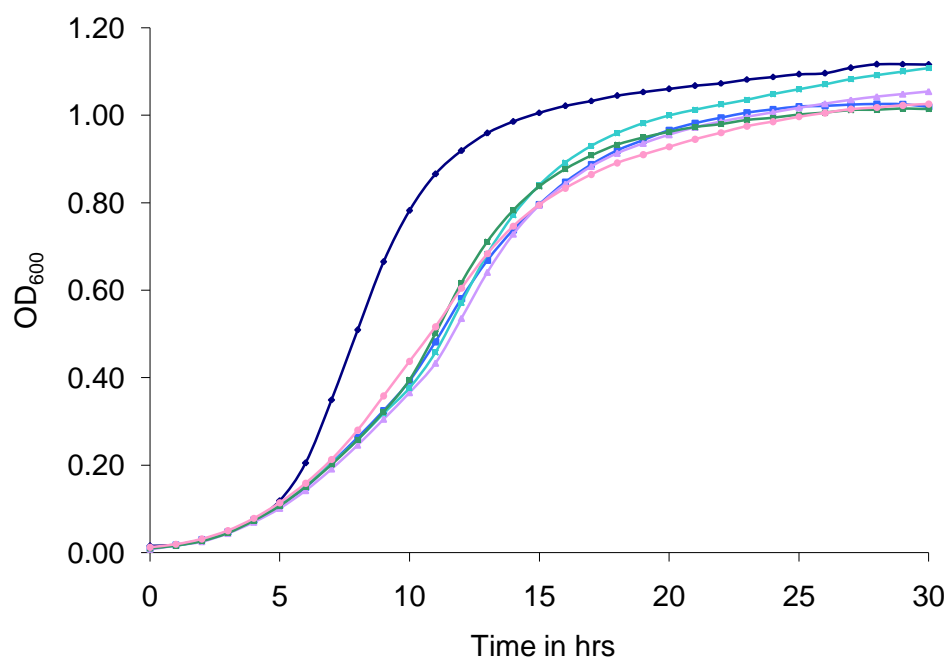


Figure 3.6 Growth of *C. pseudotuberculosis* 3/99-5 in CCDM supplemented with varying concentrations of apo-transferrin. CCDM (—◆—), CCDM + 5 μM (—◆—), 10 μM (—▲—), 15 μM (—■—), 20 μM (—■—), 25 μM (—●—) apo-transferrin. For ease of interpretation, data is plotted on a linear scale.

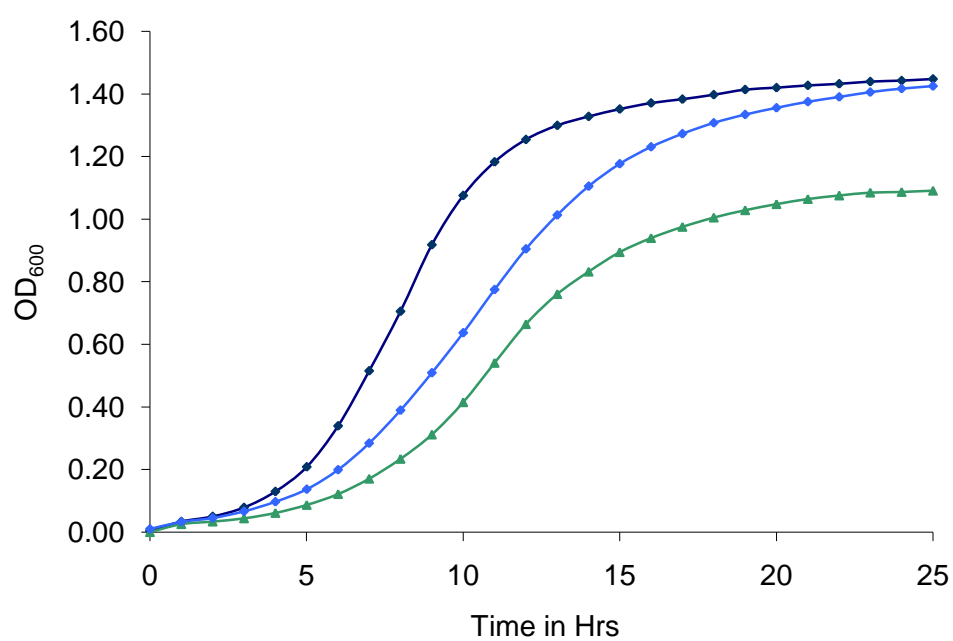


Figure 3.7 Growth of *C. pseudotuberculosis* 3/99-5 in CCDM supplemented with lactoferrin.

CCDM (—◆—), CCDM + 300 μM dipyrldyl (—▲—), CCDM + 1 mg/ml lactoferrin (—◆—). For ease of interpretation the graph is presented on a linear scale.

3.1.7 Environmental iron-dependent production of exported proteins

The successful development of CCDM, and the determination of high- and low-iron growth conditions, provided the opportunity to investigate the effect of variation in environmental iron concentration on gene expression, as determined by the observation of differences in protein profiles from *C. pseudotuberculosis* grown under different conditions. As a preliminary investigation, *C. pseudotuberculosis* was cultured to mid exponential growth-phase in high- and low-iron-containing CCDM. Culture volumes were scaled up in order to obtain enough material for subsequent analyses. However, it was observed that when using 500 ml flasks containing 100 ml of culture volume, the observed OD_{600nm} of the high-iron culture reached approximately 3.0, while that of the low-iron culture reached approximately 1.5. This difference in growth between the Bioscreen (high-iron 1.6 and low-iron 1.0) and culture flask final OD_{600nm} was consistently reproducible and most likely derived from different levels of aeration between the two culture methods. However, despite differences in the final OD_{600nm} values, the growth profiles were identical between methods; only the final OD varied.

The culture supernatants containing *C. pseudotuberculosis* exported proteins were normalised by diluting the high-iron culture to an equivalent OD_{600nm} to the low-iron culture; hence equivalent cell numbers/ml were obtained. An equivalent volume of each culture supernatant was supplemented with protease inhibitors to prevent protein degradation prior to being concentrated and buffer-exchanged in PBS using 5 kD M_r cut-off centrifugal filters. Subsequently, equal volumes of the concentrated protein preparations were separated by SDS-PAGE, and visualised by silver staining.

To allow optimal separation of both large and small proteins, the protein preparations were analysed on both 6 % and 10 % gels (**Figure 3.8**). As can be seen from **Figure 3. 8**, it would appear that there was more protein present in the low-iron derived sample compared to that of the high-iron derived sample. Given that the culture volume had been normalised by cell number, and the CCDM contained no contaminating protein, one could hypothesise that the apparent increase in protein production is a result of iron stress.

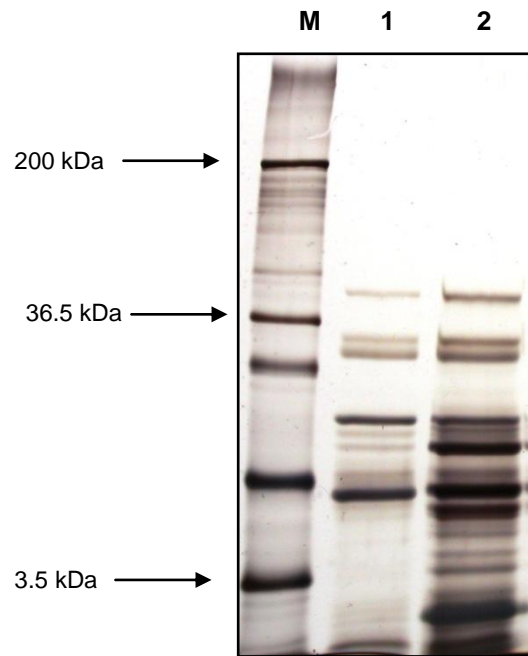
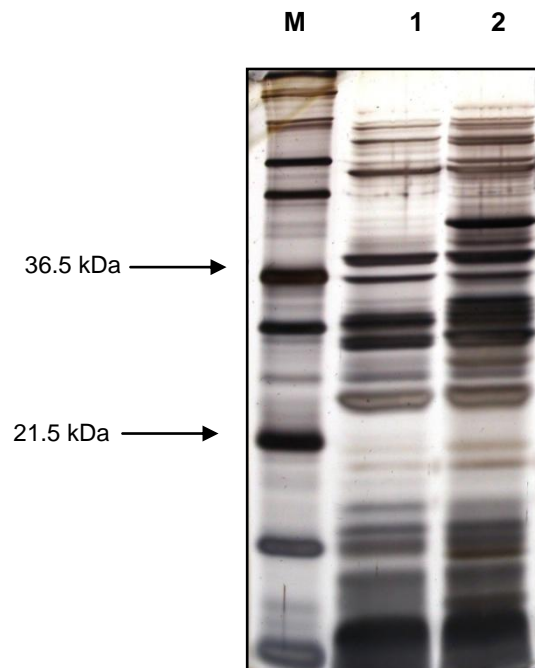
Panel A)**Panel B)**

Figure 3.8 Supernatant proteins derived from *C. pseudotuberculosis* 3/99-5 cultures. Shown are examples of a 6 % SDS- PA gel (panel A) and a 10% SDS-PA gel (panel B). In both panels samples correspond to proteins derived from high- (lane 1) and low- (lane 2) iron growth conditions. A Mark 12™ standards (Invitrogen) molecular weight marker (M) was used.

3.1.8 Western blot analysis of *C. pseudotuberculosis* exported proteins

Following growth of *C. pseudotuberculosis* under high and low-iron conditions *in-vitro*, an experiment was carried out to ascertain whether any of the exported proteins were produced *in vivo*. Pooled sera from sheep known to have CLA (hence, infected with *C. pseudotuberculosis*), were used to probe the *C. pseudotuberculosis* exported protein preparations by Western blot analysis (**Figure 3.9**). Proteins elaborated from *C. pseudotuberculosis* grown under high- and low-iron conditions were separated through a 10 % SDS-PA gel and transferred to a nitrocellulose membrane prior to conducting Western blots. As can be seen in **Figure 3.9**, no proteins were recognised in a negative control Western blot conducted using sera from CLA-free sheep. In contrast, several proteins were recognised in the blot probed with CLA-positive sera, in sample lanes containing proteins isolated from bacteria grown under both high- and low-iron conditions. Significantly, several of the bands appeared to be the same between each preparation; however, in addition there were other bands which were either less abundant or completely absent from the high-iron culture. Furthermore, there was evidence of one band being more abundant in the high-iron culture.

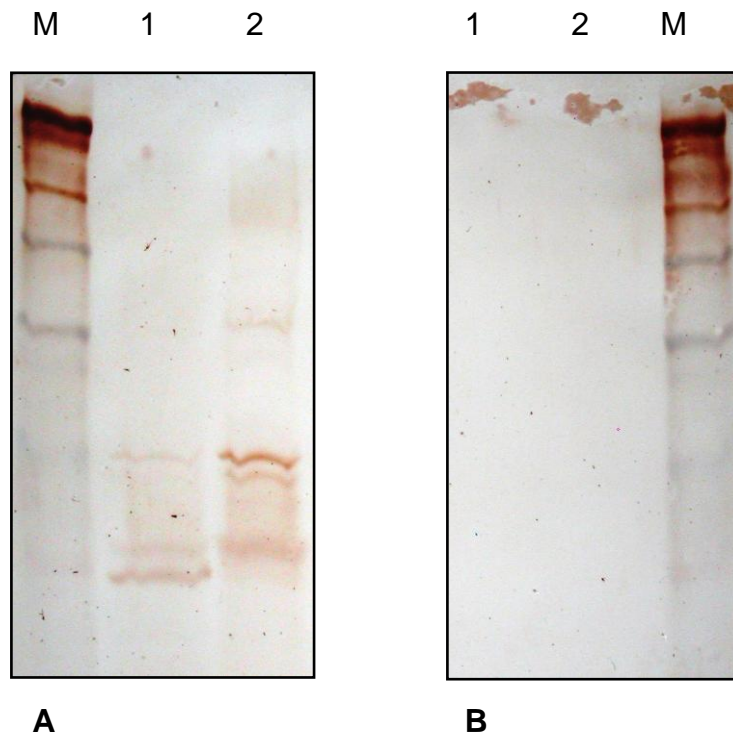


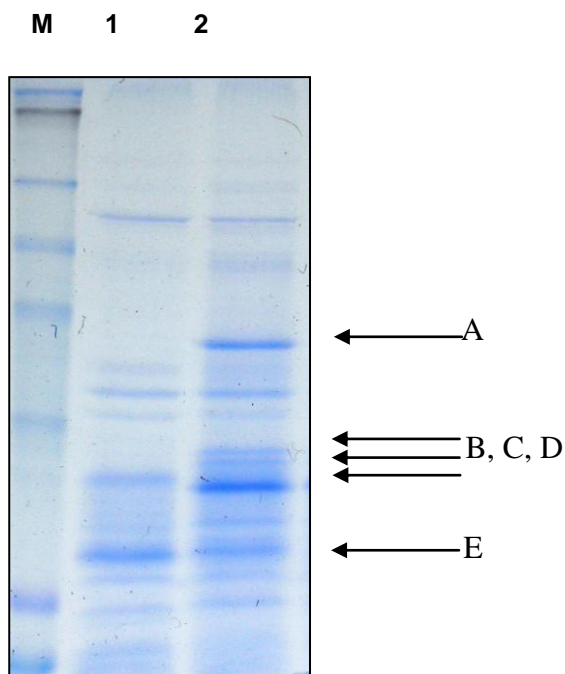
Figure 3.9 Western blot analysis of *C. pseudotuberculosis* exported proteins. Primary antibodies to probe membrane-bound proteins were either pooled serum samples from sheep with CLA (**panel A**) or from sheep naïve to *C. pseudotuberculosis* (**panel B**). Sample lanes correspond to exported proteins obtained from cultures propagated in CCDM (**Lane 1**) or CCDM containing 300 μ M dipyrityl (**Lane 2**). Molecular weight markers (**M**) were SeeBlue® Plus2 (Invitrogen).

3.1.9 Preliminary proteomic analyses

In an attempt to identify *C. pseudotuberculosis* exported proteins, a preliminary proteomic investigation was conducted. *Corynebacterium pseudotuberculosis* was cultured under low-iron conditions. Subsequently, exported proteins were separated through a 10 % SDS-PA gel and visualised by staining with colloidal Coomassie blue (**Figure 3.10**). Five unique bands (*i.e.* bands that weren't apparent in the exported proteins isolated from high-iron growth conditions) were excised and analysed by liquid chromatography, electrospray ionisation, tandem mass spectrometry (LC-ESI-MS/MS)(Batycka, *et al.* 2006).

At the time this work was conducted, there was no *C. pseudotuberculosis* genome available; hence, successful identification of proteins from the MS-analysed peptides was dependent on similarity with proteins in the publicly-available databases. Interrogation of Mascot databases compiled from publicly-available protein sequences revealed a significant Molecular weight search (Mowse) score with two published *C. pseudotuberculosis* protein sequences (**Figure 3.10**). The first of these proteins, with a Mowse score of 301, and matching peptides across 37% of the published sequence was identified as phospholipase D (Accession no. L16587). The second of the proteins, with a Mowse score of 104, and matching peptides across 10 % of the published sequence, was the *C. pseudotuberculosis* serine proteinase, Cp40 (Accession no. U10424).

Panel A



Panel B

<i>Corynebacterium</i> Hits	Identifier (GI)	Origin	Mowse Score	No. of peptides matched	% coverage	Band
Serine proteinase	531383	<i>C. pseudotuberculosis</i>	104	4	10	D
Phospholipase D precursor	289914	<i>C. pseudotuberculosis</i>	301	7	37	B, C

N.B. Bands A and E resulted in no significant hits within the publicly available databases

Figure 3.10 Colloidal Coomassie stained (10 %) SDS-PAGE gel, showing *C. pseudotuberculosis* 3/99-5 exported proteins purified from cultures cultivated under high- (lane 1) and low-iron (lane 2) growth conditions. The letters A-E identify the bands that were subsequently excised and analysed by LC-ESI-MS/MS. (Panel A). Significant hits with *C. pseudotuberculosis* through NCBI databases (Panel B)

3.2 Discussion

In most labs, *C. pseudotuberculosis* is routinely cultured in/on BHI, which is a rich medium; however, BHI originates from animal extracts, and as such, it contains proteins which could complicate downstream proteomic analyses. Therefore, utilising BHI for the purposes of this project was not an option, which led the requirement to develop a chemically-defined medium (a CDM being defined as a medium consisting of purified ingredients, the exact composition of which is known). Although a major focus of the work described in this chapter was to develop such a CDM, its purpose was simply to facilitate downstream experiments in which the iron-dependent regulation of gene expression in *C. pseudotuberculosis* could be studied. Therefore, the presentation of extensive growth data acquired during the development of the CDM has been deliberately restricted to the minimum required to highlight the performance of *C. pseudotuberculosis* in the subsequently developed medium, CCDM. In order to develop a CDM it is desirable to have prior knowledge of the growth requirements of the organism for which the CDM is required. Fortuitously, there was a published recipe for CDM for another actinomycete, *C. renale* (VanEseltine, *et al.* 1978). Significantly, the ingredients previously recommended for the successful growth of *Corynebacterium* (VanEseltine, *et al.* 1978) revealed similarities to commercial cell culture media. It was therefore the intention to examine the growth of *C. pseudotuberculosis* in two cell culture media, HBSS and RPMI-1640. The use of a commercial cell culture medium was appealing for two reasons. Firstly, the constituents would be consistent, and therefore batch to batch variation would be negligible. Secondly, cell culture media are purchased in the form of pre-prepared powders, which are reconstituted in water. Thus, utilising a commercially-available cell culture medium as a base for a CDM required less time and effort to prepare compared to the laborious task encountered when generating a CDM from individual components.

The CDM was evaluated by comparing the maximum growth of *C. pseudotuberculosis* in the novel medium, to the maximum growth in BHI, the gold standard (allowing an OD_{600nm} of 1.8 to be reached in Bioscreen C experiments). The first cell culture medium to be assessed was HBSS. Unfortunately, this medium was not able to support the growth of *C. pseudotuberculosis*. In contrast, the constituents of RPMI-1640 were similar in many ways to the components of previously reported corynebacteria CDM. As a result, an RPMI-1640-based medium was chosen for further development. Typically, for eukaryotic cell culture, RPMI-

1640 is supplemented with a further complement of amino acids. Again, this mixture comes pre-prepared, hence is very easy to use, simply being added to the re-suspended RPMI-1640 medium. The addition of the amino acid supplement resulted in a final amino acid concentration similar to that of the previously reported CDM. However, further optimisation was required in order to improve the RPMI-1640-based medium such that it was similar in composition to the CDM developed by VanEselstine *et al* (1978); in particular, the levels of glucose and L-glutamic acid were increased. The resulting CDM designated *Corynebacterium* CDM (CCDM) was successfully shown to support the growth of *C. pseudotuberculosis*. Significantly, the maximum OD_{600nm} of *C. pseudotuberculosis* grown in CCDM in Bioscreen C experiments was 1.6; a favourable result, given that the maximum OD_{600nm} in BHI was OD₆₀₀ 1.8. Interestingly, when cultures were scaled-up to allow the provision of proteins for mass spectrometric analyses, it became apparent that a larger culture vessel resulted in cultures reaching a much higher cell density (as determined by OD_{600nm} values). *Corynebacterium pseudotuberculosis* is a facultative anaerobe, able to grow under aerobic and anaerobic conditions; however, in hindsight this result is perhaps not surprising as the oxygen tension of cultures is known to be important for other actinomycetes, even those that are facultative anaerobes. For example, Moore and James (1982) recognized that *Mycobacterium bovis* BCG requires a sufficient oxygen tension to support logarithmic phase growth *in vitro*; consequently, growth was dependent on the depth of the culture (Moore & James, 1982).

The main objective of the work presented in this chapter was to recreate *in vitro*, the low-iron conditions faced by *C. pseudotuberculosis* *in vivo*. At the time this study began, very little was known about the *C. pseudotuberculosis* requirement for iron. Preliminary experiments using dipyriddy were conducted to determine whether removal of medium-containing iron resulted in a decrease in growth rate, and eventual cessation of growth at higher concentrations of dipyriddy. The results of these experiments showed that removal of increasingly more iron resulted in a decreasing ability of *C. pseudotuberculosis* to grow. However, although dipyriddy is a strong chelator of iron, it is acknowledged that it also binds other transition metals. In this respect, although it was considered most likely that iron-chelation was affecting growth, there was a slight possibility that removal of other transition metals, such as manganese and zinc, were affecting the ability of *C. pseudotuberculosis* to grow in chelated CCDM. In order to assess the importance of iron over other transition metals experiments examining the interaction between *C. pseudotuberculosis* and transferrin or lactoferrin were

performed. Clearly, the results presented in **Figures 3.5, 3.6 and 3.7** reveal that *C. pseudotuberculosis* is able to acquire iron from transferrin and lactoferrin, allowing a restoration of growth to an equivalent OD_{600nm} to that observed in un-chelated CCDM. Since lactoferrin and transferrin only bind host iron, these experiments provided the necessary evidence to show that the reduction in growth induced by supplementing CCDM with dipyrindyl was a direct result of the reduction in availability of iron.

In addition to proving that *C. pseudotuberculosis* requires iron for growth, these experiments also provided evidence of one of the means by which *C. pseudotuberculosis* is able to acquire iron from the host, which is of relevance since transferrin is present within lymphatic fluid and blood, and may therefore serve as a major source of iron for the organism. Following the addition of transferrin to dipyrindyl-treated CCDM, the growth of *C. pseudotuberculosis* was restored to a level equivalent to that in CCDM alone; however, there was a slower transition to logarithmic-phase of growth. It was considered likely that the slow transition was a result of the bacterium sensing the low-iron environment and adjusting accordingly, in order to acquire iron from transferrin. However, it is also possible that it was due to a requirement for the pH of the culture to drop (observed during bacterial growth), allowing the release of iron from transferrin, as has been described elsewhere (Welch, 1992) (Mietzner & Morse, 1994). However, *in vivo* the pH would not be expected to drop in a similar manner to that observed in *in vitro* culture, and it is possible, therefore, that localised reduction in pH, such as may be observed as a result of H⁺ transport across the cell membrane, may be responsible for the iron release. In order to test this hypothesis, however, further work would be required to determine the physical interaction of *C. pseudotuberculosis* with transferrin.

Corynebacterium pseudotuberculosis has also been associated with cases of mastitis in sheep, goats and cows, most likely through introduction of the organism to the mammary gland following rupture of supra-mammary lymph node abscesses. Lactoferrin is the principle iron-binding protein in milk, and is similar to transferrin except that it has a 300-fold greater affinity for iron. Furthermore, lactoferrin is usually less than 10 % iron saturated (Bezwooda & Mansoor, 1989; Mietzner & Morse, 1994). Interestingly, the addition of lactoferrin to CCDM resulted in a similar *C. pseudotuberculosis* growth pattern to that seen previously when dipyrindyl-treated CCDM was supplemented with apo-transferrin (**Figures 3.6 and 3.7**), in that the transition from lag to exponential growth phase was slow. There could be several reasons

for this, one of them being that the lactoferrin, being less than 10 % saturated, acts in the same way as apo-transferrin by binding the iron in the CCDM prior to *C. pseudotuberculosis* scavenging the iron back. This would support the hypothesis that the slow transition between growth phases is due to the bacteria sensing their low-iron environment and modulating gene expression of iron acquisition genes accordingly. Significantly, the release of iron from lactoferrin has been shown not to be affected by pH (Welch, 1992; Mietzner & Morse, 1994), and, as such it is likely that *C. pseudotuberculosis* uses some other, as yet undefined method for obtaining iron from this molecule. In addition, it may well be that an equivalent mechanism is also employed by the organism to obtain iron from transferrin. Significantly, both lactoferrin and transferrin are known to be bacteriostatic (Weinberg, 1993), and restrict bacterial growth by limiting iron availability; however, some bacterial pathogens have evolved methods to retrieve iron from host iron binding proteins, thus permitting growth once more (Ratledge & Dover, 2000).

In the course of the current study, a preliminary attempt was made to address the question of whether differences in the ability of *C. pseudotuberculosis* isolates from different host species were better or less able to acquire iron from transferrin or lactoferrin. A preliminary experiment was conducted to determine the differences in the ability of an ovine-derived (3/99-5) and bovine-derived strain of *C. pseudotuberculosis* to utilise transferrin and lactoferrin as a source of iron. Unfortunately, the clumping of the bovine isolate during growth, even in high concentrations of Tween 80, interfered with the ability to accurately measure growth, either by OD-based measurements or plate counts, and also made it difficult to compare results between the two strains, and for this reason the results are not presented in this thesis. However, the results did tend to suggest that the bovine strain preferred lactoferrin as a source of iron (data not shown); however, clearly more work is required to prove or refute this preliminary observation.

Whilst studying the effect of iron-chelation on the growth of *C. pseudotuberculosis*, it became apparent that the CCDM contained a sufficient amount of iron to support growth, and that addition of further iron made no difference to growth (until higher concentrations were reached when toxicity issues arose). Significantly, this work has highlighted that *C. pseudotuberculosis* is not a particularly fastidious organism, and is able to grow well in the presence of very little iron. It is likely the presence of trace amounts of iron in CCDM was

either a consequence of contamination of the RPMI-1640 components themselves, or of the water used to re-suspend the medium. The use of Chelex 100 (Sigma) may have provided a more consistent means of controlling iron content within CCDM; however, preliminary attempts to do this were time-consuming and problematic, and for this reason dipyriddy was used instead.

This thesis contains the first report of the acquisition of iron from transferrin and lactoferrin by *C. pseudotuberculosis*. As mentioned above, the mechanism(s) by which the organism acquires iron from these molecules has not yet been determined, and no attempt has been made to address this issue in the work presented in this thesis. However, in *C. diphtheriae* the acquisition of iron from transferrin has been shown to be siderophore-dependent (Schmitt, 1997b). A mutant of *C. diphtheriae* (designated HC1) which lacks a siderophore uptake mechanism was unable to utilize transferrin as an iron source. Furthermore, other *C. diphtheriae* siderophore transport mutants were also unable to obtain iron from holo-transferrin (Schmitt, 1997b). It may be that, like *C. diphtheriae*, *C. pseudotuberculosis* also requires siderophore to utilize transferrin-bound iron. However, to date there has been no published evidence of a siderophore produced by *C. pseudotuberculosis*. In this respect, the assessment of siderophore by *C. pseudotuberculosis* was conducted as part of the work carried out towards this thesis, and these results are presented in their entirety in Chapter 6, alongside a more thorough discussion of siderophore-dependent iron acquisition.

In general, little is known about siderophore-independent iron-uptake systems; however, more information exists for some bacteria than others. Besides siderophore, other mechanisms by which bacteria utilize transferrin and lactoferrin-bound iron have been described, and include receptor-mediated transport systems specific for host-iron complexes (Andrews, *et al.* 2003). However, the ability to acquire iron from transferrin and lactoferrin in a siderophore-independent manner has been mainly studied in the Gram-negative species, *Neisseria* (Cornelissen & Sparling, 1994). Species in this genus have independent transferrin and lactoferrin receptors, which facilitate binding of these molecules at the bacterial cell-surface and allow iron to be stripped, resulting in the extracellular release of the (intact) iron-free transferrin or lactoferrin molecule (Andrews, *et al.* 2003). Another method that some bacteria employ to exploit iron sources *in-vivo*, is to convert extracellular ferric iron into the more usable ferrous iron (Andrews, *et al.* 2003). The enzyme involved in this is ferric reductase, and

ferric reductase activity has been identified in several bacteria including *E. coli* (Coward, 2002). At low pH (~4.5) iron can be easily mobilised from transferrin (Mietzner & Morse, 1994). Subsequently, bacteria have evolved mechanisms to reduce the environmental pH and retrieve iron from transferrin. As yet, it is not possible to confirm which method *C. pseudotuberculosis* employs in order to utilise transferrin and lactoferrin-bound iron, however, this is an interesting field of investigation that could result in the identification of potential novel virulence determinants in *C. pseudotuberculosis*.

Haem is the most abundant source of iron in the mammalian host. Interestingly, the closely related human pathogen *C. diphtheriae* is known to scavenge iron directly from haem by employing haem oxygenase, HmuO (Schmitt, 1997b). It may be that *C. pseudotuberculosis* shares a similar mechanism in order to exploit haem-containing proteins as an iron source. However, being a facultative intracellular parasite able to survive in macrophages, (where the principle iron sources utilised are ferritin and the transferrins) it may be that *C. pseudotuberculosis* does not require the use of haem (an extracellular source of iron). The ability of *C. pseudotuberculosis* to utilise iron from haem sources was investigated, unfortunately, without success. When CCDM was supplemented with haem there were solubility issues, which subsequently resulted in a lack of bacterial growth. Given more time, this would be an interesting area to explore. In order to do this it would be necessary to solubilise the haem in a suitable solvent, prior to supplementation of CCDM.

As part of the work presented in this chapter, the affect of environmental iron availability on *C. pseudotuberculosis* gene expression and hence protein production, was investigated. During this preliminary work, only exported proteins (those found in culture supernatant) were analysed and no attempt was made to assess differences in cell wall-anchored proteins; primarily this was due to the fact that the failure to identify *C. pseudotuberculosis* proteins by MS-based approaches rendered obsolete the requirement to assess cell wall material following disappointing results with secreted proteins. However, it was known at the start of this project that the addition of Tween 80[®] to the *C. pseudotuberculosis* cultures would likely have an affect on *C. pseudotuberculosis*, as has been described previously in relation to cell morphology (Chevalier, *et al.* 1988). In addition, it is highly likely that surface-associated proteins would also have been removed from the cell surface by the action of the detergent;

hence, although referred to as “exported proteins”, they included a range of proteins both associated and un-associated with the cell surfaces.

Interestingly, the exported protein profile of *C. pseudotuberculosis* cultured in low-iron CCDM differed significantly to that of cells cultured in high-iron medium. Furthermore, the concentration of proteins exported was much higher under low-iron conditions compared to high-iron. While it remains to be proven, it may be that the increased concentration of proteins demonstrated under low-iron conditions, was due to bacterial sensing of the extracellular environment, resulting in attempts to acquire more iron through the up-regulation of genes involved in iron acquisition, and the down-regulation of genes involved in iron storage, such as ferritin and bacterioferritin (both ferritin and bacterioferritin have been shown to be up-regulated under high-iron conditions in *M. tuberculosis* (Rodriguez, *et al.* 2002)).

The exported proteins from bacteria grown in high- and low-iron media were analysed by Western blot. Pooled sera from CLA-positive sheep detected a number of bands in both protein preparations, however a clear difference was observed between the actual bands that were recognised. These results indicated that at least some of the proteins in the “exported” fraction were produced *in vivo*. Furthermore, infected sheep obviously produced antibodies against these proteins, some of which were differentially produced between high- and low-iron growth conditions. Those proteins which were produced in greater amounts in high-iron medium may well have been associated with iron storage (such as bacterioferritin, although the size of the bands in relation to the reported size of iron storage proteins was not compared), while those over-produced in low-iron medium may well have been associated with iron-acquisition. A significant fact that can be concluded from these results is that iron-limitation, *in vivo*, is clearly an environmental signal to which *C. pseudotuberculosis* responds, resulting in changes in gene expression leading to the production or up-/down-regulation of specific proteins. Significantly, while “immunogenicity” does not necessarily correlate with “protectivity”, it would be interesting to determine what these differentially-produced, immunogenic proteins are, since they could form the basis of targets for the development of novel therapeutics.

While sufficient to demonstrate that differences in environmental iron concentrations were able to induce differential protein production (and hence gene expression) in *C.*

pseudotuberculosis, another objective of the work conducted towards this thesis was to identify novel *C. pseudotuberculosis* “factors” that may contribute to virulence. Therefore, it was decided to conduct more in-depth analyses of differentially-produced proteins using a previously described mass spectrometric technique. As described in the results, the ability to identify *C. pseudotuberculosis* proteins from MS data was hampered by the absence of a relevant database to interrogate with the MS data. In addition, a significant flaw in the experimental design was noticed after-the-fact, in that the only bands excised from SDS-PA gels for subsequent analysis were from proteins obtained from cultures grown under low-iron conditions. In order to confirm that iron was implicated in inducing differential protein production, equivalent bands from cultures grown under low- and high-iron conditions should have been excised and analysed, to allow direct comparison of results. In addition, it is generally recognised that each apparently single band in a one-dimensional gel can actually contain many different proteins. Therefore, a positive identification of a protein from a 1D gel does not prove that the differentially-produced protein was identified, only that a protein within that particular band had been identified. In this respect, it is of relevance that one of the two *C. pseudotuberculosis* proteins identified by MS analysis in this study was PLD significantly, the *pld* gene has previously been shown not to be iron-regulated (Billington, *et al.* 2002), suggesting that the MS-derived observation made in this study was incorrect. Published information pertaining to the second identified protein, Cp40, is scarce, and up-regulation of expression of the *cp40* gene has not been described under low-iron conditions. Therefore, it was unknown at this stage in the study whether the MS prediction of Cp40 as the product of an iron-regulated gene was correct (although work presented in Chapter 5 eventually proved that it was not). Irrespective of whether or not the finding was correct, further work would be required to provide conclusive proof in favour of the observation. Alternatively, a more appropriate analytical method (such as 2D gel electrophoresis to identify differentially-produced proteins, or a quantitative MS approach) could be employed to more accurately determine differences in the protein profiles from bacteria grown under high- and low-iron conditions. However, advances in other areas of this project, presented in the following chapters, led to the decision not to pursue this line of investigation any further, partly due to lack of time, but particularly due to the lack of a genome sequence with which to build a meaningful database for interrogation with MS data.

Chapter Four

Site-Directed Mutagenesis of *Corynebacterium pseudotuberculosis*

4.0 Introduction

To date, genetic manipulation of *C. pseudotuberculosis* has been limited in comparison to other more frequently studied corynebacterial species. This has, in part, been due to the lack of an available genome sequence, limiting the number of potential mutagenesis targets, in addition to a lack of mutagenesis tools. Mutagenesis of putative virulence genes from pathogenic bacteria is a very powerful tool in molecular bacteriology. Mutant derivatives that are deficient in the expression of specific genes can be successfully created, and subsequently analysed *in vitro* or preferably *in vivo*, thereby providing definitive answers to the contribution of specific genes to virulence (*i.e.* fulfilling “molecular Koch’s postulates”). To-date, mutants of *C. pseudotuberculosis* have been constructed, using transposon (Tn) mutagenesis (Dorella, *et al.* 2006) and allele exchange mutagenesis (Billington, *et al.* 2002; Hodgson, *et al.* 1992; McNamara, *et al.* 1994; Simmons, *et al.* 1997). Targeted (site-directed) allele-exchange is by far the most useful of the two approaches. However, the technique as it has been applied to *C. pseudotuberculosis*, is imprecise, complicated and difficult to reproduce.

Site-directed, allele-exchange mutagenesis is a technique which may be used to introduce specific changes into known chromosomal gene(s); such changes can range from a single nucleotide substitution to deletion of most, if not all, of a gene coding sequence. The process involves homologous recombination (also known as Campbell-type recombination), which is a natural phenomenon that allows species to adapt more quickly to their environment by speeding up the process of evolution; this occurs by recombination between two DNA molecules which share identical (or highly-similar) nucleotide sequences within the region to be recombined. Subsequently, both molecules break at an equivalent site within the same region, then complementary base-pairing results in the ligation of one molecule to the other, resulting in a “crossover” or “strand-exchange”. In nature, homologous recombination has been described as a means of adaptive evolution in several bacterial species. For example, some *Streptococcus suis* strains have been shown to possess a type-II restriction-modification system (designated *SsuDATII*) which other strains lack (Sekizaki, *et al.* 2001b). Comparison of the nucleotide sequence between strains possessing and lacking *SsuDATII* system revealed that the *SsuDATII* system had originally inserted into the *S. suis* chromosome via illegitimate recombination and was subsequently transferred among *S. suis* strains by homologous recombination (Sekizaki, *et al.* 2001a; Sekizaki, *et al.* 2001b). Takamatsu *et al.* (2002) suggest

that a series of genetic exchanges, illustrated by the *SsuDAT1I* system, may also be occurring in other genes and, that this process has been an important factor in the evolution of *S. suis*, a bacterium in which different strains exhibit various combinations of virulence markers (Takamatsu, *et al.* 2002); the authors provide evidence to suggest that the *sly* gene (encoding suilysin, a haemolysin) was acquired by a series of gene transfers and subsequently spread among strains, and that this type of transfer is a common occurrence in *S. suis* (Takamatsu, *et al.* 2002).

In experimental practice, a wild-type chromosomal gene may be swapped with an *in vitro*-constructed, mutated derivative by recombination between homologous DNA sequences flanking the gene/region of interest in both the wild-type chromosomal gene and a mutant derivative of that gene. The DNA containing the mutagenised gene may be single- or double-stranded in form; however, the process is facilitated if the mutated gene is cloned into a suitable plasmid. In this way the plasmid may be easily manipulated prior to delivery into the target bacterial population by transformation of competent cells (either naturally-competent or induced, *e.g.* by electroporation), or alternatively by conjugation. In addition, carriage of mutated genes on a plasmid facilitates a two-step allele-replacement process, as discussed below.

Plasmids used for allele-replacement must ultimately be removed from the bacterial host after the desired mutation has been constructed, and hence these plasmids are usually (but not always) designed in such a way as to be removed in a straight forward manner. Some are simply plasmids which do not possess an origin of replication compatible with the target bacterial species, and hence they are not able to replicate. However, non-replicative vectors are problematic, since, in general, the frequency of homologous recombination is very low, and they are therefore only suitable for use in bacteria that can be transformed to a high frequency (Biswas, *et al.* 1993). Other more sophisticated plasmids overcome the problem of low transformation efficiencies by being able to replicate in the target host, but only under certain specific conditions (*e.g.* normally only under a particular permissive temperature, (Hashimoto & Sekiguchi, 1976)). Introduction of a conditionally-replicative suicide vector into a single bacterial cell can allow the propagation of a large population of daughter cells, all of which will contain the plasmid if the cells are grown under permissive conditions. With such large starting populations of transformed cells, the likelihood of rare homologous

recombination events occurring is increased, especially since it has been reported that replication of the plasmid itself stimulates homologous recombination (Noirot, *et al.* 1987). Alternatively, some plasmids are designed to be able to induce the death of host cells in a controllable manner. For example, some carry the *sacB* gene encoding a sucrose transporter which is lethal in some Gram-positive bacteria (Jager, *et al.* 1992). Often plasmids used for allele replacement are referred to as “suicide vectors”, alluding to the fact that they can be removed from the bacterial population essentially, at will.

Under permissive conditions, conditionally-replicating plasmids are able to replicate autonomously in the cytoplasm of the host cell, where they are stably maintained by antibiotic selection, permitted by a plasmid-encoded antibiotic-resistance gene. The majority of conditionally-replicating plasmids are temperature-sensitive (Ts), in that they are only able to replicate at a specific incubation temperature, normally below a critical cut-off temperature. In Ts vectors, the gene encoding the protein responsible for plasmid replication is mutated (either naturally or by artificial means such as by hydroxylamine mutagenesis) such that the resulting protein becomes unstable (and non-functional) at elevated temperatures (Humphreys, *et al.* 1976). As such, by shifting the incubation temperature of cultures containing Ts plasmids from a permissive to non-permissive temperature, the ability of the plasmid to replicate is lost. At this point, the plasmid is no longer segregated into daughter cells at cell division, and is rapidly lost from the bacterial population. As a result, the net effect is the loss of the antibiotic-resistance phenotype from the cell population.

Traditionally, a large proportion of targeted allele-replacement experiments have attempted gene replacement with a concurrent, double-crossover process, whereby strand exchange between plasmid- and chromosome-borne sequences occurs simultaneously at the 5'- and 3'-regions flanking the target gene; the result is a direct swapping of the target gene with the mutated derivative. Significantly, because of the extremely low frequency of recombination events, a concurrent double-crossover event is highly unlikely, so identification of the desired mutants is normally facilitated by the introduction of a mutation in the form of a gene disruption with an antibiotic-resistance cassette, which allows direct selection for cells carrying the mutation. Experimentally, while a double-crossover is the most direct approach to mutagenesis, the low chance of occurrence combined with the nature of the induced mutation is such that other approaches are more favourable.

An alternative means of using homologous recombination to achieve gene replacement is by using a two-step process. This involves selecting for cells having undergone an initial recombination event (so-called “co-integrants”), in which the plasmid carrying the mutated gene has been integrated into the chromosome (**Figure 4.1**). At this stage, the plasmid replicates along with the chromosome, and expression of the plasmid-borne antibiotic-resistance marker takes place, even under conditions not-permissive for replication of the plasmid in cytoplasmic form. Once a Ts plasmid has integrated into the chromosome, a second recombination event can occur, which results in the excision of the plasmid (**Figure 4.2**). This process is facilitated by incubation of co-integrants at the permissive temperature for plasmid replication, which causes the integrated plasmid to become unstable, increasing the likelihood of excision (Biswas, *et al.* 1993); this is in contrast to non-replicating plasmids, which can be difficult to excise (Nakamura, *et al.* 2006; Schwarzer & Puhler, 1991). Subsequently, plasmid-integrand cells having undergone a second recombination event are selected for, in which the plasmid is excised from the chromosome. As presented in Figures 4.1 and 4.2, whether this two-step recombination procedure results in the wild-type or mutant gene being left in the chromosome depends on whether successive recombination events occur at opposite ends of the target gene (to generate a mutant) or at equivalent ends (to retain the wild-type gene).

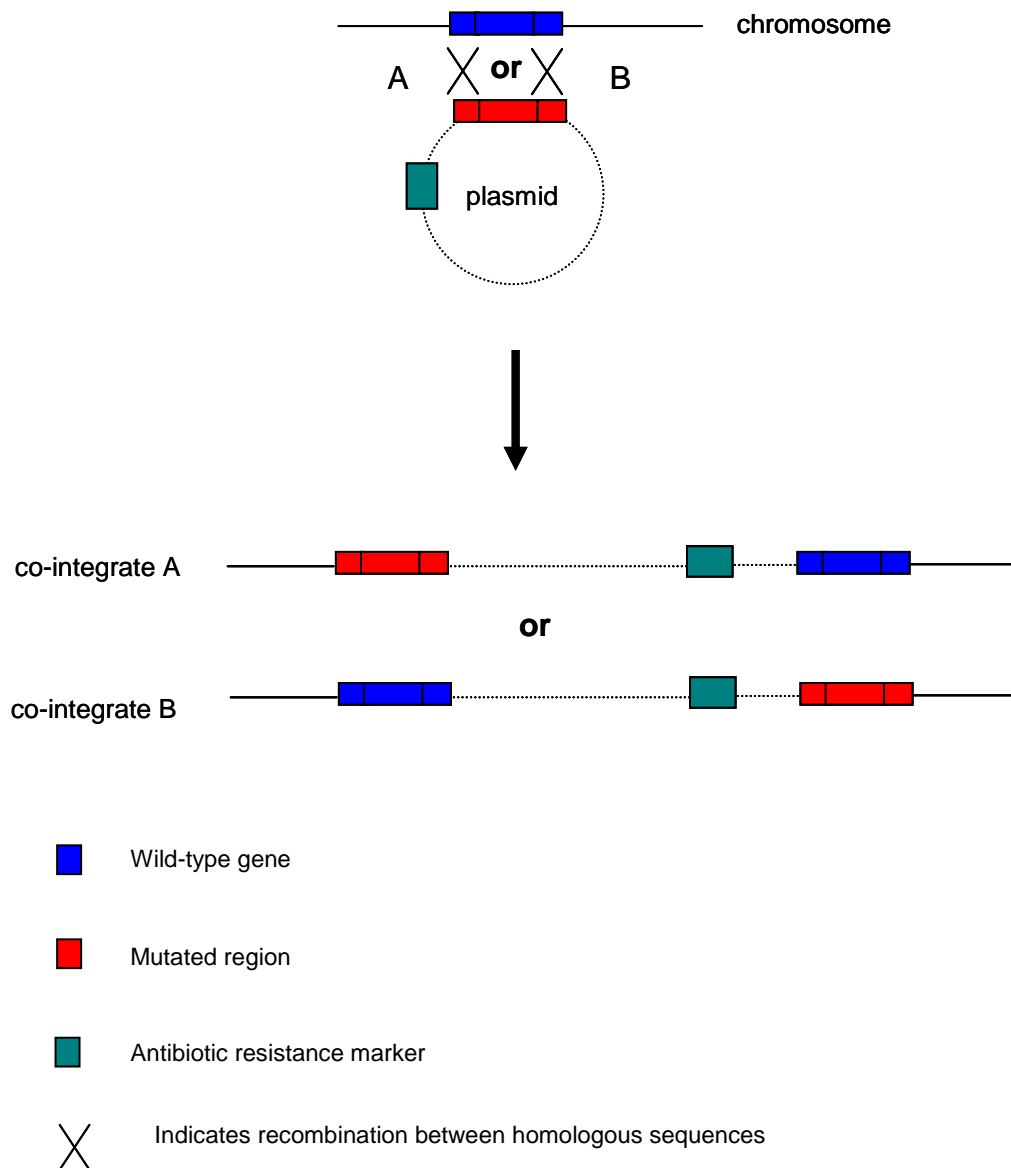


Figure 4.1 Schematic representation of single crossover integration (SCO)

An SCO results in the integration of the plasmid into the chromosome, resulting in a co-integrant. The crossover event can happen between homologous regions flanking either side (A or B) of the mutated region. The end at which the crossover event takes place determines whether the integrated plasmid lies upstream (co-integrate A) or downstream (co-integrate B) of the wild-type target gene.

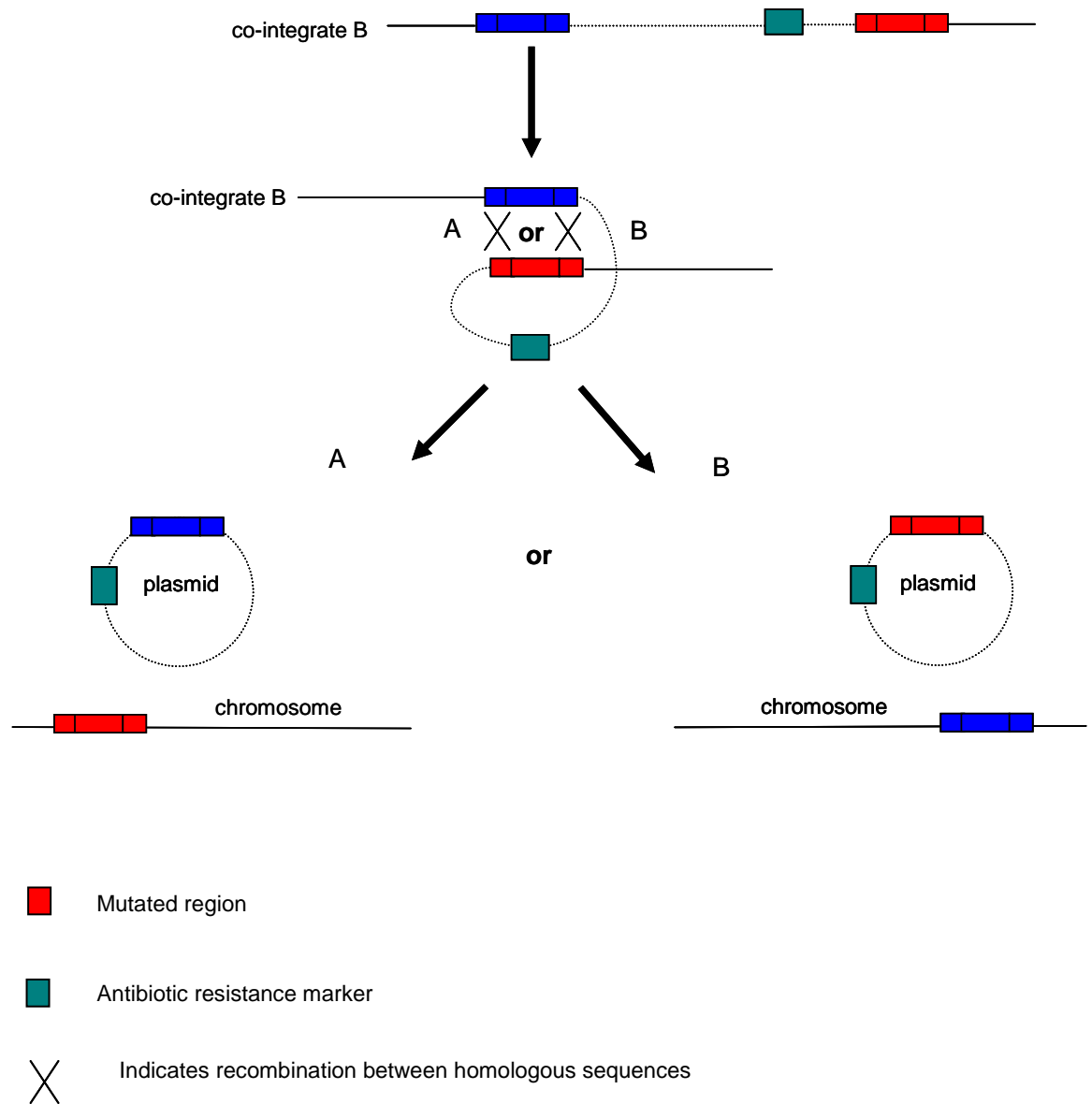


Figure 4.2 Schematic representation of secondary or double crossover (DCO)

DCO is the second step in the two step recombination procedure. Using co-integrate B as an example (**Figure 4.1**) it is possible to observe that there are two likely outcomes. Recombination at the opposite end from that that led to initial plasmid integration results in the plasmid excising, carrying with it the wild type gene and leaving the mutant derivative in the chromosome.

In recent years, a number of *C. pseudotuberculosis* genes have been cloned and sequenced including *pld* (Hodgson, *et al.* 1990; Songer, *et al.* 1990), *aroQ* (Simmons, *et al.* 1997), *cp40* (Wilson, *et al.* 1995) and *fagABC* (Billington, *et al.* 2002). Significantly, in order to fulfil molecular Koch's postulates, several investigators have attempted to construct *C. pseudotuberculosis* mutants, deficient in the production of specific (putative) virulence-associated proteins. Unfortunately however, allele-replacement mutagenesis of *C. pseudotuberculosis* has proven to be extremely problematic, as a result of which reports of success have been limited. To-date, the only successful reports of site-directed mutagenesis in *C. pseudotuberculosis* have involved non-replicative suicide plasmids (Billington, *et al.* 2002; Hodgson, *et al.* 1992; McNamara, *et al.* 1994; Simmons, *et al.* 1997). Hodgson *et al.* (1992) constructed a *pld*-deficient mutant of *C. pseudotuberculosis* by targeted mutagenesis of the *pld* gene. A recombination plasmid was constructed by cloning a fragment of *pld* (which had part of the gene deleted and replaced with an erythromycin resistance gene) into the plasmid pEP2 (Zhang, *et al.* 1994). The resulting plasmid (pBTB58) was used to transform wild-type *C. pseudotuberculosis* by electroporation. Subsequently, *C. pseudotuberculosis* isolates containing the recombination plasmid, were transformed with pEP3, which contains the same origin of replication as pEP2 but harbours a different antibiotic resistance gene. To maintain the plasmid pEP3 and promote the loss of pBTB58 by exploiting plasmid incompatibility, the investigators sub-cultured cells containing both plasmids. Finally, a mutant was identified in which a double-crossover recombination event had incorporated the erythromycin gene into the chromosome resulting in a disruption of *pld* and the subsequent loss of pBTB58 from the cell. While undoubtedly successful, this indirect and inefficient approach necessitated the insertion of a selectable marker into the chromosome, and also left the mutant containing a plasmid (pEP3) with an antibiotic-resistance gene which it previously did not have.

McNamara *et al.* (1994) constructed a mutant derivative of *pld in vitro*, into which a frame-shift deletion had been introduced; subsequently, the mutant construct was used to replace the wild-type chromosomal gene with the mutant derivative. To create their mutant strain, the authors used an *E. coli* plasmid vector, unable to replicate in *C. pseudotuberculosis*. The resulting recombinant plasmid, designated pJM11ar, contained the erythromycin-resistance gene from the plasmid pNG2 (a *C. diphtheriae* plasmid that is functional in both *E. coli* and *C. pseudotuberculosis* (Serwold-Davis & Groman, 1986)), and the mutated *pld*. The plasmid was integrated into the chromosome of *C. pseudotuberculosis* by homologous recombination, and

in the absence of antibiotic selective pressure the integrated plasmid resolved from the chromosome (and was subsequently lost from the cell), creating the *pld*-deficient mutant. Using a similar approach, Simmons *et al.* (1997) created a mutant *C. pseudotuberculosis* strain in which the *aroQ* gene, encoding the 3-dehydroquinase enzyme involved in aromatic amino acid biosynthesis, was insertionally inactivated by introduction of a mutant derivative of the gene interrupted with an erythromycin resistance cassette (Simmons, *et al.* 1997). More recently, Billington *et al.* (2002) constructed a *fagB*-deficient mutant of *C. pseudotuberculosis*, the gene being thought to be involved in the acquisition of iron. In order to create the mutation, a chloramphenicol resistance gene was used to disrupt *fagB in vitro*, the resulting mutant gene was cloned into the non-replicative plasmid pJGS102, and allele-replacement was performed to replace the wild-type *fagB* gene with the insertionally inactivated derivative (Billington, *et al.* 2002).

As summarised above, the majority of the limited allele-replacement studies conducted in *C. pseudotuberculosis* thus far have involved the use of a non-replicative plasmid to achieve the desired mutations. As such, these studies were subject to the inherent limitations of the mutagenesis approach employed; primarily low frequencies of recombination resulting in poor efficiency of mutant creation. Significantly, it is perhaps for this reason that all but one study, namely that of McNamara *et al.* (1994), involved creating mutations by disrupting the coding sequence of target genes by insertion of a selectable marker to facilitate downstream identification of the desired mutants. The use of such a strategy is flawed for two reasons; primarily this type of mutation can induce polar effects on the transcription or translation of downstream genes (especially those in an operon), such that the resulting mutant phenotype may not actually be due to a mutation in the target gene, but due to altered expression of some other, undetermined gene. The inactivation of non target genes can occur by polar termination (Berg, 1996), and the promoters of the selectable marker genes can also alter the expression of adjacent genes in the chromosome (Wang & Roth, 1988). In addition, inclusion of antibiotic resistance markers into bacterial pathogens increases the risks associated with biosecurity, and if the bacterium being mutagenised is intended for use in food manufacturing or as a live, attenuated vaccine, it is imperative that no antibiotic resistance marker(s) remain in the chromosome at the end of the mutagenesis procedure.

Over the years, some of the most extensive work into factors affecting homologous recombination in Gram-positive bacteria has been conducted at l'Institut National de Recherche Agronomique (INRA), in France. A broad Gram-positive-host-range, Ts derivative of a lactococcal plasmid was used by Biswas *et al.* (1993) to investigate the integration of different sized homologous fragments into the *L. lactis* chromosome. The Ts vector, pG⁺host 5, used in the study was shown to have potential use as a delivery vector in many Gram-positive bacterial species (Maguin, *et al.* 1992); however, it has been reported that the use of the pG⁺host plasmids may be extended to other Gram-positive bacteria but plasmid replication, thermosensitivity, background integration and level of drug resistance must be determined in each new host (Biswas, *et al.* 1993). Significantly, pG⁺host 5, which at one time was commercially-available, was found to be inherently unstable, and a new derivative, pG⁺host 9 was created in its place (Maguin, *et al.* 1996). This vector has been used with great success in the targeted mutagenesis of numerous genes in numerous Gram-positive species (*e.g.* (Fontaine, *et al.* 2003)); unfortunately however, the vector was shown to be unable to replicate in *C. pseudotuberculosis* (M. C. Fontaine, personal communication).

Compared to *C. pseudotuberculosis*, methods of allele-replacement mutagenesis in other corynebacterial species are more advanced. Of the various approaches taken to-date, the most versatile system was reported by Nakamura *et al.* (2006), who exploited the *C. glutamicum* cryptic plasmid, pBL1 (Santamaria, *et al.* 1985) and the *E. coli* cloning vector, pK1, to construct the *C. glutamicum*/*E. coli* shuttle vector, pSFK6. Subsequently, pSFK6 was subjected to *in vitro* mutagenesis using hydroxylamine, prior to being introduced into *C. glutamicum*. The investigators then isolated a mutant plasmid which was stably maintained at 26°C but not at 34°C; following sequence analysis, the mutation thought to be responsible for the temperature-sensitivity of the plasmid was identified within the gene encoding the protein responsible for plasmid replication. Using site-directed mutagenesis the investigators were able to construct a plasmid carrying only this mutation (rather than numerous, random, hydroxylamine-induced mutations) which was then cloned into pSFK6, and the resulting Ts plasmid was designated pSFKT2. Subsequently, Nakamura *et al.* (2006) successfully used pSFKT2 to create a deletion in the glutamate dehydrogenase gene of *C. glutamicum*.

For the purposes of facilitating downstream experiments in this study, it was necessary to obtain a means of creating unmarked, in-frame deletion mutants of *C. pseudotuberculosis*,

rather than mutants containing insertionally-inactivated genes. The primary reason for this was to ensure that any changes in the phenotype, pathogenicity, *etc.* of the mutants was due to the imposed mutations rather than downstream polar effects. Furthermore, because it was anticipated that the method would be employed both in this project, but subsequently as a general molecular biological tool in the laboratory in which this project was conducted, an efficient and reproducible method was desirable, to ensure rapid progress and to avoid the problems associated with the use of non-replicative plasmid vectors. Following a review of the available literature pertaining to allele-replacement mutagenesis systems in *C. pseudotuberculosis* and other actinomycetes, it was felt that the pSFKT2 Ts vector previously used in *C. glutamicum* (Nakamura, *et al.* 2006) would be most suitable. For this reason, efforts were made to adapt this system of mutagenesis for use in *C. pseudotuberculosis*.

4.1 Results

4.1.1 Construction and utilisation of pCARV in *C. pseudotuberculosis*

A Ts plasmid vector, pSFKT2, (a kind gift from J. Nakamura, Dept. of Bioengineering, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama, Japan)(Nakamura, *et al.* 2006) previously designed for use in *C. glutamicum* was successfully used to transform electrocompetent *C. pseudotuberculosis*. The pSFKT2 plasmid, which carries a kanamycin resistance determinant, was shown to be able to replicate in *C. pseudotuberculosis* at the permissive temperature of 26°C, as determined by recovering transformed cells at 26°C on BHI agar plates (with kanamycin). Subsequently, colonies deriving from transformed cells were picked and sub-cultured onto fresh BHI agar plates (with kanamycin) and incubated at 37°C for 4 days. The absence of any bacterial growth from these plates indicated that the plasmid was temperature-sensitive at the elevated temperature of 37°C, and also indicated that non-specific integration of pSFKT2 into the *C. pseudotuberculosis* chromosome was not occurring. Interestingly, when cells transformed with pSFKT2 were cultured at 26°C on BHI agar containing kanamycin an irregular colony morphology was observed, in that they were slightly raised, and had a more crenated and less waxy appearance than normally encountered with wild-type *C. pseudotuberculosis* (**Figure 4.3**).

The reason for kanamycin inducing an irregular *C. pseudotuberculosis* colony morphology was unknown. However, in order to alleviate this problem, which could not be ruled out as being detrimental to the bacterium, it was decided to replace the kanamycin resistance gene of pSFKT2 with another antibiotic resistance determinant. Importantly, in order to facilitate a novel enrichment process (discussed in **Section 4.1.4**), a resistance determinant against a bacteriostatic antibiotic was desirable; hence the kanamycin gene was swapped with an erythromycin resistance gene. This was achieved by excising a *ca.* 2.7 kb *HpaI/StuI* fragment from pSFKT2, which contained the kanamycin resistance gene, but also contained two open reading frames (ORF 2 and 3; (Nakamura, *et al.* 2006)). Subsequently, the *ermAM* erythromycin resistance gene from pG⁺host 9 (Maguin, *et al.* 1996), a kind gift of E. Maguin, Laboratoire de Génétique Microbienne, INRA, Jouy-en-Josas, France), was obtained by digesting the vector with *Bam*HI; the resulting *ca.* 1.1 kb DNA fragment was blunt-ended, and cloned into *HpaI/StuI* digested pSFKT2. The resulting *ca.* 5,666 bp recombinant plasmid,

designated pCARV (for *Corynebacterium* Allele Replacement Vector; **Figure 4.4**), was propagated in *E. coli* for preparation of stocks for downstream experiments. The pCARV vector was shown to be capable of replication in *C. pseudotuberculosis* in the presence of erythromycin at 26°C, and, despite the deletion of ORFs 1 and 2, pCARV retained its temperature sensitivity at 37°C. Furthermore, non-specific chromosomal integration of pCARV was not apparent in *C. pseudotuberculosis*.

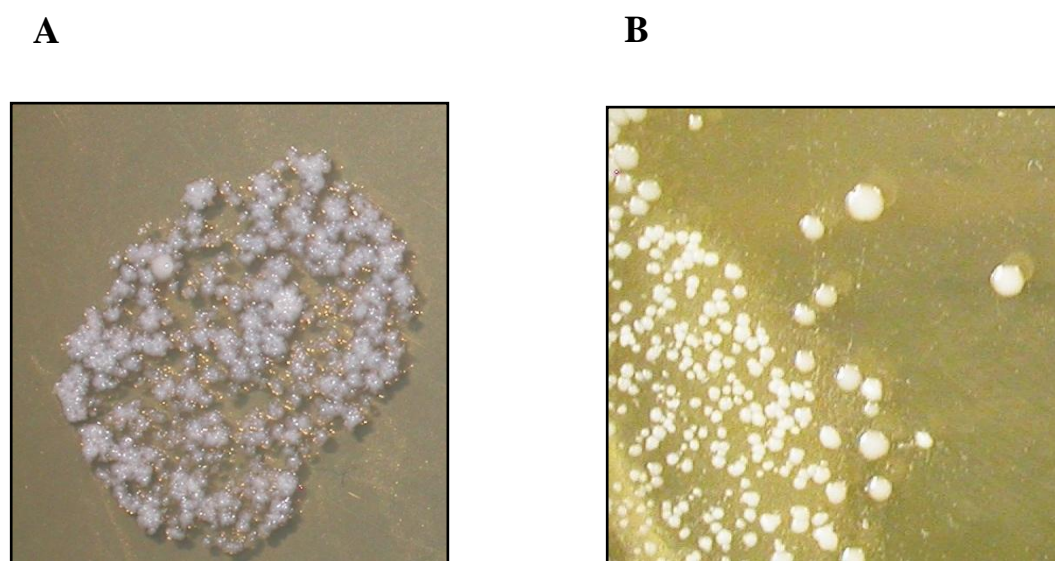


Figure 4.3 *Corynebacterium pseudotuberculosis* colony morphology.

Plasmid-containing *C. pseudotuberculosis* was cultured on BHI agar containing either kanamycin (panel A) or erythromycin (panel B). Plasmid-free *C. pseudotuberculosis* cultured on BHI agar without antibiotics exhibited identical colony morphology to that in panel B (data not shown).

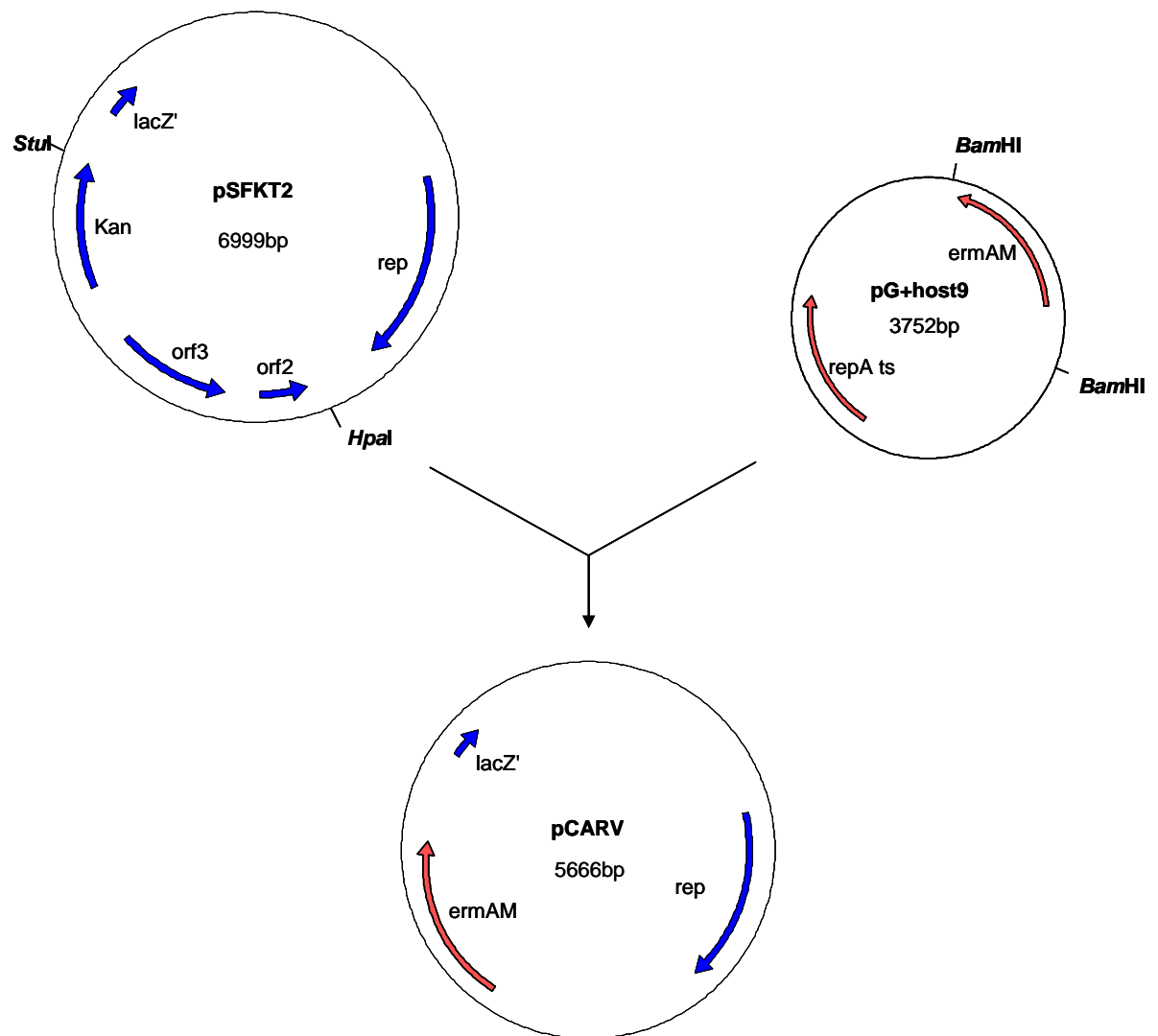


Figure 4.4 Cloning strategy for the construction of pCARV

A *ca.* 2.7 kb *HpaI/StuI* fragment from pSFKT2, which contained the kanamycin resistance gene, but also contained two open reading frames (ORF 2 and 3) was excised. Subsequently, the *ermAM* erythromycin resistance gene from pG⁺host 9 was obtained by digesting the vector with *BamHI*; the resulting *ca.* 1.1 kb DNA fragment was blunt-ended and cloned into the *HpaI/StuI* digested pSFKT2. The resulting 5,666 bp recombinant plasmid was designated pCARV (*Corynebacterium* Allele Replacement Vector).

4.1.2 Chromosomal integration of pCARV-based constructs

The suitability of pCARV as an allele-replacement mutagenesis vector in *C. pseudotuberculosis* was assessed, initially by determining the relationship between the length of homologous sequences and the resulting frequency of homologous recombination. This was facilitated by creating a series of recombinant pCARV-based constructs containing varying-sized fragments of the *C. pseudotuberculosis* *cp40* gene ((Wilson, *et al.* 1995); acc. # U10424). The *cp40* gene fragments were amplified by PCR using oligonucleotide primers that incorporated *Xma*I restriction endonuclease recognition sites to facilitate downstream cloning of PCR products into *Xma*I-digested pCARV (**Table 2.3**). PCR was conducted using *C. pseudotuberculosis* 3/99-5 genomic DNA as template, using the primers *cp40* F and *cp40_0_224* to amplify a 224 bp fragment, the primers *cp40* F and *cp40_1_432* to amplify a 432 bp fragment, the primers *cp40* F and *cp40_2_750* to amplify a 750 bp fragment, and the primers *cp40* F and *cp40_4_1020* to amplify a 1,020 bp fragment of the *cp40* gene. The resulting recombinant plasmids were designated pCARV001, pCARV002, pCARV003 and pCARV004, respectively. To facilitate downstream experiments in *C. pseudotuberculosis*, large-scale, highly-pure preparations of the plasmids were obtained from cultures of *E. coli* transformed with each plasmid.

The approach to mutagenesis adopted in this study was a two-step process involving the chromosomal integration of plasmid constructs, followed by a secondary plasmid excision step (as discussed above). However, for the experimental assessment of the pCARV mutagenesis system, by creating recombinant plasmids containing fragments of the wild-type *cp40* gene, no attempt was being made in the first instance to create mutant *C. pseudotuberculosis* strains; rather the intention was only to measure the rate of homologous recombination driving the integration and subsequent excision of the recombinant plasmids from the chromosome. As a first step, the rate of integration of the pCARV-based vectors into the *C. pseudotuberculosis* chromosome by homologous recombination (giving rise to so-called bacterial “co-integrates”) was determined. Plasmid integration was conducted as described in **Section 2.11.1**, and the integration frequency of each plasmid construct was calculated according to the following equation: $(100 \div \text{total cell number}) \times \text{erythromycin-resistant cells}$. The results of triplicate experiments revealed an average integration frequency of 1.81×10^{-1} , 2.23×10^{-1} , 2.80×10^{-1} and

4.75×10^{-1} for pCARV001, pCARV002, pCARV003 and pCARV004 respectively. Significantly, as presented in **Figure 4.5**, the rate of plasmid integration increased linearly with increasing length of homologous sequences.

4.1.3 Secondary crossover recombination

Single *C. pseudotuberculosis* colonies, deriving from cells with integrated pCARV-based constructs, were used as the starting point for the next experimental phase. A second recombination event between homologous vector-borne and chromosomal sequences drives the excision of the vector from the chromosome. The experimental procedure detailed in **Section 2.11.2** was followed, and the resulting excision frequency of each of the plasmid constructs from the *C. pseudotuberculosis* chromosome was determined, according to the equation: $(100 \div \text{total number of colonies}) \times \text{number of erythromycin resistant colonies}$. The data from triplicate experiments is presented in **Table 4.1**, and it is readily apparent that, although the general trend was undoubtedly an increase in excision rate with increasing length of homologous sequences, the variability within the triplicate experiments for each particular construct was extensive.

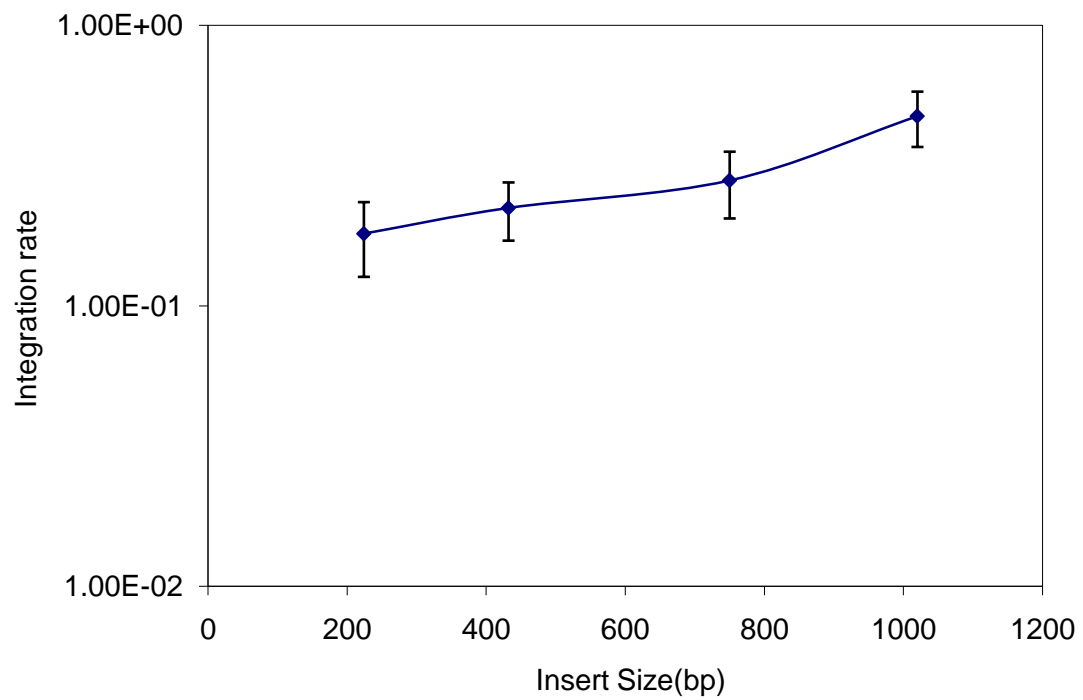


Figure 4.5 Integration rates of pCARV-based constructs containing cp40 fragments into the *C. pseudotuberculosis* chromosome by homologous recombination

Table 4.1 pCARV001-004 plasmid excision rates

Size of insert (bp)	Percentage Excision Rate
224	0-13 %
432	0-50 %
750	2-54 %
1,020	23-69 %

4.1.4 Application of a novel enrichment method to isolate secondary crossover mutants

Although the assessment of the plasmid excision rate revealed a frequency of recombination much higher than observed for initial plasmid integration (the reasons for this are discussed in **section 4.2**), it was felt that a means of enriching cultures to increase the chance of identifying mutants having undergone a secondary recombination event would be a useful tool. Such an enrichment process would be particularly useful in cases where very short lengths of homologous sequence were available to drive homologous recombination, where the resulting rate of plasmid excision would be low. Therefore, a novel enrichment process was developed which exploited the erythromycin resistance determinant in pCARV, which was deliberately used to replace the kanamycin resistance determinant of pSFKT2 (**Section 4.1.1**).

To test the hypothesis, the series of pCARV constructs pCARV001-004, were screened both with and without enrichment (**Tables 4.1 and 4.2**). The enrichment method was made possible by the observation that different antibiotics are either bactericidal or bacteriostatic. Erythromycin, which is a macrolide antibiotic, acts by inhibiting translocation of peptidyl tRNA from the A to the P site on the ribosome by binding to the 50S ribosomal subunit of 70S rRNA. This has the affect of interfering with the production of functionally useful proteins, resulting in bacteriostasis until such time as the erythromycin is removed from the bacterium's environment. In contrast, ampicillin is a bactericidal beta-lactam antibiotic which acts as a competitive-inhibitor of the transpeptidase enzyme, which is required for bacterial cell wall synthesis (AHFS Drug information® 2006, 2006 ed, American Society of Health-System

Pharmacists). The outcome of this competition is the inhibition of the formation of cross-links in the peptidoglycan layer (the third and final stage of bacterial cell wall synthesis), which ultimately leads to cell lysis; significantly however, due to its mode of action, the antibiotic is only effective against actively-growing cells.

For ease of reference, the preliminary enrichment method developed as part of this thesis is presented in **Chapter 2, Section 2.11.3**. During the culture steps required to allow downstream identification of mutants having undergone plasmid excision from the chromosome, a large proportion of the bacterial co-integrate population will not have undergone plasmid excision. Significantly, these cells retain pCARV-encoded erythromycin resistance, while those that have lost the plasmid become sensitive to the antibiotic. Therefore, following the normal culturing process to allow plasmid excision, cultures were diluted into fresh medium containing erythromycin, and incubated at 37°C (the non-permissive temperature for plasmid replication) for a period of time sufficient to allow expression of the resistance gene and the subsequent growth of resistant cells (with a concurrent inhibition of the growth of non-resistant cells). Subsequently, cultures were supplemented with ampicillin, which only affected actively-growing cells, *i.e.* the undesired co-integrates. Cultures were monitored by measuring optical density, until the OD_{600nm} had dropped to its lowest point and had become stable. Subsequently, cultures were washed to remove antibiotics, and 10-fold serial dilutions were replica-plated onto either antibiotic-free BHI agar plates, or BHI agar containing erythromycin. Subsequently, the numbers of colonies deriving from cells having undergone excision events was determined as a percentage of the total bacterial population. The results of these calculations are presented in **Table 4.2**, where it is evident that the novel ampicillin-enrichment procedure significantly increased the likelihood of identifying cells having undergone secondary crossover events. This was most evident for the pCARV-based constructs containing the shortest *cp40* fragments; for example, cells having undergone excision of the 224 bp-containing construct, pCARV001, had increased from a maximum of 13 % of the population without enrichment to 73 % following enrichment.

Table 4.2 pCARV001-004 plasmid excision rates following ampicillin enrichment

Size of insert (bp)	Proportion of population having undergone plasmid excision
224	25-73 %
432	43-97 %
750	45-89 %
1,020	48-81 %

4.1.5 Site-directed mutagenesis of the *C. pseudotuberculosis* *cp40* gene

Having determined the suitability of the pCARV plasmid as a system of allele-replacement mutagenesis of *C. pseudotuberculosis*, based upon the ability to integrate and excise the plasmid from the bacterial chromosome, the next logical step was to obtain conclusive proof that the vector could be used to deliver *in vitro*-constructed mutations into target chromosomal genes, to generate clean, well-defined, isogenic mutant strains, free from foreign plasmid sequences within the chromosome. To this end, a deletion derivative of the *cp40* gene was constructed by first amplifying the 5'- and 3'-chromosomal regions flanking *cp40* from *C. pseudotuberculosis* genomic DNA using the primers cpAE1 and cpAE2 ("Fragment 1", upstream 5'-region) and cpAE3 and cpAE4 ("Fragment 2", downstream 3'-region). The Fragment 1 amplicon comprised 1,032 bp of upstream flanking sequence including the first 10 bp of the *C. pseudotuberculosis* *cp40* gene, while Fragment 2 comprised 919 bp of downstream flanking sequence, containing the last 122 bp of the *cp40* gene. Significantly, the length of both fragments was equivalent (at 1,042 bp and 1,041 bp for Fragment 1 and Fragment 2, respectively), so as not to bias a higher rate of homologous recombination at one end of the *cp40* gene. The two PCR amplicons were digested with *Xho*I (enabled by the enzyme recognition site having been engineered into the primers cpAE2 and cpAE3) and ligated to each other (3'-Fragment 1 to 5'-Fragment 2), prior to reamplification of the *ca.* 2.1 kb fragment by PCR with the primers cpAE1 and cpAE4. The result was the creation of a derivative of *cp40* (designated $\Delta cp40$) which contained an in-frame deletion that had effectively removed *ca.* 1,000 bp of the intact, wild-type gene. Subsequently, the $\Delta cp40$ gene

was cloned into pCARV, facilitated by the *SalI* restriction endonuclease recognition site engineered into primers cpAE1 and cpAE4. The resulting recombinant plasmid, designated pCARV005, was subsequently propagated in *E. coli* DH5 α , prior to being introduced into *C. pseudotuberculosis* by electroporation. Then, allele-replacement, followed by ampicillin-enrichment, was conducted as described in **Chapter 2, Section 2.11**, prior to the identification of erythromycin-sensitive isolates, having lost pCARV005 from the chromosome. A total of 10 erythromycin-sensitive isolates were screened to determine whether recombination had resulted in either the wild-type *cp40* or the mutant $\Delta cp40$ derivative gene being left in the chromosome. Genomic DNA was extracted from each of the isolates, and PCR was conducted using the primers $\Delta cp40$ DCO F and $\Delta cp40$ DCO R; it was anticipated that a 1,299 bp fragment would be amplified from isolates containing the wild-type gene, while a 292 bp fragment would be amplified from $\Delta cp40$ -containing isolates. Amplified PCR products were analysed by agarose gel electrophoresis (**Figure 4.6**). Of the 10 isolates, 9 were confirmed as having excised pCARV005 from the chromosome, as determined by the amplification of only a single DNA fragment corresponding to the wild-type or mutant phenotype. In contrast, a single isolate was identified as still containing chromosomally-integrated pCARV005, as determined by the presence of both the 1,299 bp and 292 bp bands. Of the 9 plasmid excision isolates, a 1,299 bp fragment was amplified from 4, confirming that primary and secondary recombination events had occurred at equivalent loci, resulting in plasmid excision and the wild-type gene being left in the chromosome. In contrast, a 292 bp fragment was amplified from 5 of the 9 isolates, confirming that primary and secondary recombination events had occurred at opposite loci, resulting in plasmid excision and the mutant gene being left in the chromosome.

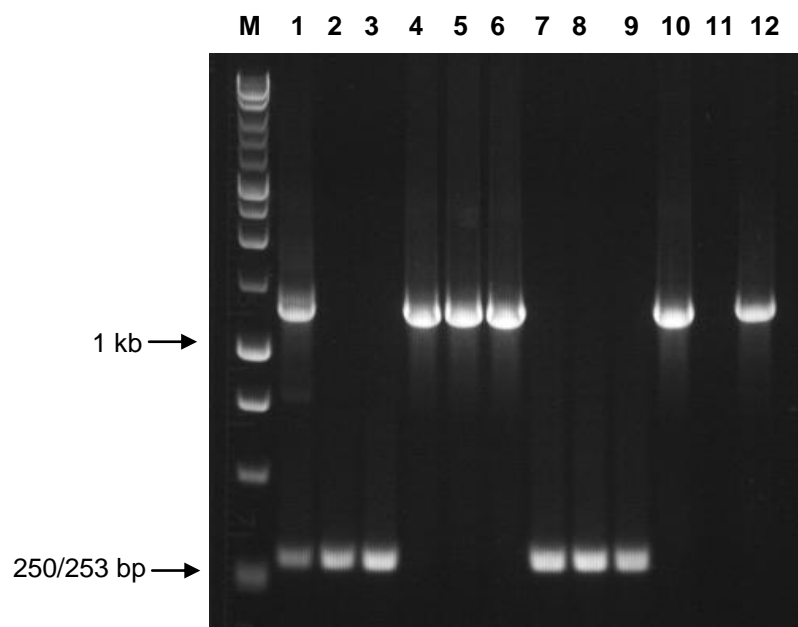


Figure 4.6 PCR analysis of *C. pseudotuberculosis* secondary-crossover mutants

PCR products visualised following electrophoresis through a 1 % (w/v) agarose gel. Lanes correspond to 1kb marker (M), co-integrate (Lane 1), $\Delta cp40$ (Lanes 2, 3, 7, 8 and 9), wild-type *cp40* (Lanes 4, 5, 6 and 10)., and *C. pseudotuberculosis* wild-type control PCR (Lane 12).

4.2 Discussion

In order to fulfil molecular Koch's postulates, *i.e.* confirming the contribution of a specific gene product to the pathogenicity of an organism, it is essential that efficient and straight-forward method(s) are available to allow manipulation of target gene(s) within the organism of interest, without the need for excessive and extensive experimentation. Until the work described in this chapter was conducted, no such system of genetic manipulation existed for *C. pseudotuberculosis*. As discussed in **Section 4.0**, above, limited reports of targeted genetic manipulation of this organism have been forthcoming (*e.g.* (Billington, *et al.* 2002; Hodgson, *et al.* 1992; McNamara, *et al.* 1994; Simmons, *et al.* 1997)); however, these have all been conducted using low-efficiency, non-replicative plasmid vectors, or have exploited plasmid incompatibility, and hence these systems are not ideal when a high-throughput process of mutant generation is required. Furthermore, the majority of these mutagenesis studies have focused upon creating gene disruptions through insertion of antibiotic resistance cassettes, an approach that facilitates the identification of allele-replacement mutants through antibiotic selection. However, it is generally accepted that such an approach is unfavourable due to the possibility of downstream polar effects which may affect the expression of other gene(s) in addition to the target gene.

In general, the need to create *C. pseudotuberculosis* mutants has been limited, primarily because of the lack of information pertaining to valid targets for mutagenesis. However, as part of the work presented in the following chapters of this thesis, the availability of a means of creating well-defined mutations within target genes was absolutely essential to allow experimental progress. In addition, while not the focus of the current study, the genome of the virulent UK *C. pseudotuberculosis* strain, 3/99-5, was being sequenced as part of a parallel project within the same laboratory. Therefore, there was a clear requirement for a highly-efficient mutagenesis system downstream of the analysis of the genome sequence, since it was anticipated that a range of suitable mutagenesis targets would be identified, allowing a thorough analysis of the role of these targets in the virulence of the organism. A pre-requisite for such a mutagenesis system was that it was based upon a conditionally-replicating plasmid to circumvent problems associated with low-transformation frequencies. In addition, it was essential that the system be relatively rapid and reproducible. It was the intention that the creation of clean, in-frame deletion mutants would be the preferred approach in this study, and

hence no extraneous DNA sequences (including the mutagenesis plasmid itself) could remain within the bacterium (either within the chromosome or the cytoplasm) following mutagenesis. Given this approach, it was considered likely that the downstream identification of the desired mutants could be a time-consuming process, and hence the need for a suitable means of enriching for the required mutants was desirable.

Taking all of these factors into account, it was apparent that none of the pre-existing *C. pseudotuberculosis* mutagenesis systems were applicable. Previous work in the same laboratory had assessed the suitability of the pG⁺host 9 broad-host-range, allele-replacement vector for use in *C. pseudotuberculosis*; however, the plasmid had been shown not to replicate in this actinomycete host (M. Fontaine, personal communication). Significantly, while the work described in this thesis was being conducted, a manuscript detailing the development of a temperature-sensitive plasmid, pSFKT2, for allele-replacement mutagenesis of *C. glutamicum* was described (Nakamura, *et al.* 2006). This plasmid was derived from hydroxylamine mutagenesis of a parent plasmid. The plasmid pSFKT2 was generously donated by J. Nakamura. Preliminary analyses revealed that the plasmid was able to be used to transform *C. pseudotuberculosis*, conferred resistance to kanamycin at 25°C (the permissive temperature for plasmid replication), was unable to replicate at 37°C, and did not integrate non-specifically into the *C. pseudotuberculosis* chromosome.

Despite preliminary indications that pSFKT2 would be suitable for use with *C. pseudotuberculosis*, it was noticed that colonies of *C. pseudotuberculosis* grown on BHI agar containing kanamycin exhibited an unusual morphology. A similar observation had been made previously within the same laboratory, using a different plasmid which also carried a kanamycin-resistance marker (M. C. Fontaine, personal communication), and it was therefore decided that the kanamycin marker in pSFKT2 would be changed. Significantly, this decision enabled two further distinct issues to be addressed, which led to the creation of pCARV. Primarily, the choice of replacement antibiotic resistance marker was made so as to facilitate a downstream enrichment process for mutant recovery. In addition, the overall size of pSFKT2 was reduced by removal of sequence encoding 2 ORFs (designated ORF 2 and ORF 3), which had previously been shown to be non-essential for plasmid replication (Goyal, *et al.* 1996). The deletion was anticipated to have the effect of increasing transformation efficiencies with the derivative plasmid, in addition to increasing plasmid stability, and as expected, deletion of

ORF 2 and ORF 3 appeared to make no difference to the ability of the plasmid to replicate in *C. pseudotuberculosis*. Furthermore, deletion of unnecessary sequences was conducted in order to reduce the metabolic load that the plasmid imposed upon the host cell. Metabolic load can be defined as the proportion of the host cell's resources, either in the form of energy or raw materials such as amino acids, that are required to maintain and express foreign DNA, as either RNA or protein, in the cell (Glick, 1995). A certain amount of cellular energy is needed to maintain a plasmid in the host cell, and increasing plasmid size requires increasing amounts of cellular energy (Cheah, *et al.* 1987; Glick, 1995). Significantly, the observed colony morphology of *C. pseudotuberculosis* containing pCARV was equivalent to wild-type *C. pseudotuberculosis*, suggesting that erythromycin was exerting a less-harsh effect on cells.

It has been previously reported that a log-linear relationship between length of homologous sequence and the frequency of chromosomal integration, exists in *Lactococcus lactis* (Biswas, *et al.* 1993) and *Lactobacillus sake* (Leloup, *et al.* 1997); log-linear models postulate a linear relationship between independent variables, *e.g.* the length of homologous sequence, and the logarithm of the dependent variable (such as frequency of chromosomal integration). The integration frequencies observed in *Lactococcus* and *Lactobacillus* were similar, but were observed over a specific range of homologous sequence lengths, where short sequence lengths were (unsurprisingly) found to be a rate-limiting factor for recombination. In *L. lactis*, Biswas *et al.* (1993) observed a log-linear relationship between frequency of integrations per cell and length of homology over sequence lengths between 356 bp and 2,552 bp, whereby integration frequencies of between 10^{-5} and 10^{-2} were observed. The authors noted a minimum threshold length of 330 bp for efficient homologous recombination, and further reported that, with fragments larger than 2,552 bp, recombination frequencies appeared to plateau. Similarly in *L. sake*, Leloup *et al.* (1997) observed a log-linear relationship between homologous sequence length and integration frequency for DNA fragments in the range of 0.3 kb to 1.2 kb, whereby integration frequencies of between 10^{-5} to 10^{-3} were recorded. In addition, these investigators reported an almost constant recombination rate for larger inserts. Interestingly, the integration rates in *C. pseudotuberculosis* observed in the present study were somewhat higher than previously reported, being in the range of 2×10^{-1} to 5×10^{-1} ; however, a log-linear relationship between homologous sequence length and integration frequency was also observed (**Figure 4.5**). Although the results from the studies performed with different bacteria appear similar, it is not possible to make direct comparisons due to the different systems used in each study. In

addition to the different organisms, a different plasmid was used in each case, and the target genes were also not equivalent; all of these factors could have affected the observed integration rates (Biswas, *et al.* 1993). Other studies investigating the integration of different sized homologous fragments have been carried out in *Bacillus subtilis* (Michel & Ehrlich, 1984) and *E. coli* (Michel & Ehrlich, 1984), however, these studies reported linear or exponential integration frequencies, rather than log-linear.

Interestingly, previous studies in *B. subtilis* have shown that recombination between homologous chromosomal DNA sequences are stimulated when an active plasmid replicon is integrated in their proximity (Noirot, *et al.* 1987). There are two main replication mechanisms observed in plasmids, these being theta type and rolling circle (RC) (del Solar, *et al.* 1998). Noirot *et al.* (1987) reported that an active rolling circle replicon that has integrated into the bacterial chromosome can stimulate homologous recombination between flanking repeated sequences between 20 to 450 times more than a non-replicating plasmid (Noirot, *et al.* 1987). Biswas *et al.* (1993) showed that, in *L. lactis*, in the absence of replication of the integrated plasmid, secondary homologous recombination was less than 0.25 %, which is consistent with the excision frequency seen in *L. lactis* when a non-replicating plasmid was used (Leenhouts, *et al.* 1991). Furthermore, as already alluded to above, it has been postulated that the type of replication mechanism employed by the plasmid plays a significant role in the rate of recombination (Biswas, *et al.* 1993; Noirot, *et al.* 1987). Noirot *et al.* (1987) postulated that the single-stranded DNA generated during rolling circle-type plasmid replication plays a role in the initiation of the recombination process. Later, Biswas *et al.* (1993) published findings which agreed with the previous hypothesis. In addition, Morel-Deville and Ehrlich (1996) demonstrated that theta-replication had only a moderate effect on influencing recombination rate as compared to rolling circle replication (Morel-Deville & Ehrlich, 1996), presumably due to the observation that with theta-type replication there are no single-stranded intermediates, resulting in a greater stability of integrated plasmids (Tsuchida, *et al.* 2008). In their studies in *Lactococcus*, Biswas *et al.* (1993) observed between 1-40 % gene replacement when direct selection was not available (*i.e.* a selectable marker was not inserted into the target gene), whereas between 50-98 % was observed when selection for the replaced gene was available; these variable but high recombination rates were attributed to rolling circle replication of the integrated plasmid. Significantly, the plasmid, pCARV, used during this investigation derived from pSFKT2, contains an origin of replication from the *C. glutamicum* cryptic plasmid, pBL1

(Santamaria, *et al.* 1985), which is known to replicate via a rolling circle mechanism (Fernandez-Gonzalez, *et al.* 1994). The rates of secondary recombination driving plasmid excision in *C. pseudotuberculosis* in this study were between 0-69 %, dependent upon the length of homologous sequence. Therefore, it is tempting to hypothesise that the replication mechanism of pCARV influences the apparently high, but variable rates of excision, given that plasmid co-integrants were cultured in the absence of antibiotic selection and at a temperature permissive for plasmid replication, in order to enhance the recombination rate; this fortuitous factor enhances the suitability of this vector for use in allele-replacement experiments.

Mutations within genes that are important to the survival of the bacteria can be extremely difficult to isolate; however, methods of enriching for the required mutant have been described. One of the most popular methods in Gram-positive bacteria is the use of the *sacB* gene as a counter-selectable marker on a plasmid (Oram, *et al.* 2006; Pelicic, *et al.* 1997; Pelicic, *et al.* 1996a, 1996b, 1996c; Schafer, *et al.* 1994). This enrichment process employs the *B. subtilis sacB* gene, encoding levansucrase, the expression of which is lethal to Gram-positive bacteria in the presence of 10 % (w/v) sucrose. Therefore, growth on sucrose can be used as a means to eliminate co-integrates (or those that have retained plasmid DNA). This enrichment process has also been successfully applied to enrich for *C. glutamicum* and *C. diphtheriae* allele-replacement mutants (Oram, *et al.* 2006; Schafer, *et al.* 1994). As an alternative approach during this investigation, a novel method of enriching secondary-crossover cultures was developed, in order to increase the likelihood of identifying the desired mutants. This method, which relies upon the allele-replacement plasmid encoding a bacteriostatic antibiotic-resistance marker, is equally as applicable to the pCARV mutagenesis system described here as it is to any other allele-replacement vector, as long as that vector carries a bacteriostatic antibiotic-resistance marker. Significantly, given that the rate of secondary crossover using pCARV is already naturally high, due to the rolling circle replication of the plasmid, it could be argued that the ampicillin-enrichment method would be even more suitable for use with low-efficiency mutagenesis systems, such as non-replicative allele-replacement plasmids. Given the extremely low rate of recombination with these vectors, any means of enriching cultures to facilitate mutant identification would be a distinct advantage. In addition, the inclusion of such an enrichment method would arguably negate the need to use antibiotic-resistance markers to create disruptions in target genes. Irrespective of

its applicability to other systems, when combined with the pCARV mutagenesis system the ampicillin-enrichment procedure offers an extremely powerful tool to render the identification of secondary crossover mutants a routine procedure, rather than necessitating tedious replica-plating procedures to screen many colonies for an erythromycin-sensitive phenotype. In this study, homologous recombination between sequences as short as 224 bp was assessed. The ampicillin-enrichment process is particularly well-suited for inclusion when short sequence lengths are available for recombination, although the minimum number of homologous bases required to drive homologous recombination in *C. pseudotuberculosis* was not assessed in this study.

Although successful, the ampicillin-enrichment procedure presented here is only in a preliminary form, and could still benefit from some improvement; however, there was not sufficient time available in the current study to pursue this further. The most likely way of fine-tuning the procedure would be with respect to the length of time the secondary-crossover cultures are incubated in the presence of ampicillin. Despite a drop and subsequent levelling-off of the OD_{600nm}, it is apparent that a number of erythromycin-resistant co-integrants still remained at the end of the process (as evidenced by a <100 % mutant recovery rate). Therefore, further rounds of culture dilution and incubation in ampicillin-containing medium may well improve the process further. Irrespective, there can be no doubt that the enrichment process, even in its current form, is a powerful addition to the normal allele-replacement process. The process is generally applicable to the enrichment of cultures to allow subsequent identification of almost any gene mutation; however, there are potential side-effects resulting from the use of the procedure that could prove deleterious in some rare instances (as discussed in Chapter 5).

During this study, a mutant derivative of *C. pseudotuberculosis* was constructed, which was deficient in the production of Cp40, through the introduction of an in-frame deletion within the *cp40* gene. While it would be interesting to study this mutant further, since Cp40 has been implicated in *C. pseudotuberculosis* virulence (Walker, *et al.* 1994; Wilson, *et al.* 1995), there was not sufficient time available to pursue this further. Rather, the generation of the mutant was used to prove that the pCARV mutagenesis system, in combination with the ampicillin-enrichment procedure were capable of achieving the desired result in a timely and uncomplicated manner. It can be concluded therefore, that the system described here provides a high-efficiency means of generating clean mutations within specific target genes (or

genomic regions). The use of a Ts vector negates the requirement for a high transformation frequency, since incubation of a single transformed cell under conditions permissive for plasmid replication can provide a large population of daughter cells, all containing the same plasmid.

Chapter Five

The Iron Dependent Regulatory Protein DtxR of *Corynebacterium pseudotuberculosis*

5.0 Introduction

Knowledge available regarding virulence mechanisms in *C. pseudotuberculosis* is limited. However, by comparison the information available regarding its close relatives, including *C. diphtheriae* and *C. glutamicum*, is immense. This is due, in part, to the availability of relevant genome sequences. The influence of the environment on bacterial gene expression has been well-studied for many bacteria. This is particularly true of the affect of environmental iron concentration on the expression of *C. diphtheriae* genes, including virulence genes (discussed in Chapter 1). In *C. diphtheriae* the principal virulence determinant is diphtheria toxin (DT), encoded by the *tox* gene which is carried by *tox*⁺ corynebacteriophages; however, the regulatory protein responsible for controlling the expression of *tox* in response to environmental iron concentration is the product of a chromosomally-located gene, *dtxR*, which encodes the so-called Diphtheria Toxin Repressor (DtxR).

In the presence of iron, DtxR combines with Fe²⁺ resulting in a conformational change that allows it to bind to specific sequences within the promoter-encoding regions of target genes. Binding of the DtxR/Fe²⁺ complex to the promoter region subsequently impedes RNA polymerase from binding to the promoter; thus, transcription is prevented. In contrast, under conditions of low environmental iron, DtxR is unable to bind Fe²⁺, and subsequently is restricted to a conformation that is incapable of binding to target DNA sequences. As a result, transcription of target genes is permitted. A similar regulatory protein is known to exist in *E. coli*. The protein, known as Fur, is similarly capable of modulating the expression of target genes in response to environmental iron concentrations. However, despite an equivalent role, it is interesting to note that DtxR is not able to control the expression of Fur-regulated genes, and *vice versa*. This was demonstrated through the observation that Fur was unable to regulate β -galactosidase expression from a *tox* promoter-*lacZ* transcriptional fusion, and DtxR was incapable of regulating the expression of the outer-membrane proteins of a Fur-deficient strain of *E. coli* (Boyd, *et al.* 1990). Despite their lack of interchangeability, the two proteins share 25 % amino acid homology (Tao, *et al.* 1994), and DtxR and Fur have therefore been designated the prototypes of two different types of bacterial iron-dependent regulatory proteins (Holmes, 2000).

The crystallographic structure of the *C. diphtheriae* DtxR protein has been determined (Qiu, *et al.* 1995). The elucidation of this structure has increased understanding of the basis of iron induced DtxR activation and DNA binding. The DtxR protein in *C. diphtheriae* consists of three domains. Domain 1 contains a classic helix-turn-helix DNA-binding motif which spans amino acids 1 to 73 (Qiu, *et al.* 1995) (Schiering, *et al.* 1995). Amino acids 74 to 140 make up the second domain which is required for dimerization and metal binding. The third domain, consisting of amino acids 141 to 226 has a topology resembling SH₃ domains consistent with signal transduction proteins (Qiu, *et al.* 1996). SH₃ domains are β -sheet-rich structures, originally identified in eukaryotic Src proteins, which modulate metal-binding and regulation (Pohl, *et al.* 1999). Once it has bound iron, DtxR undergoes a conformational change which assists the binding of a second iron molecule to the complex. The dimeric complex is thus stabilised and is subsequently able to bind to the operator sequence of specific DtxR regulated genes, thereby preventing RNA polymerase binding to the promoter region of the gene (Tao, *et al.* 1994). DtxR binds to 19 bp operators with the consensus sequence 5'-TWAGGTTAGSCTAACCTWA-3', where W = A or T and S = C or G (the consensus sequence is in essence a perfect 9 bp palindrome interrupted by a single C or G nucleotide (Schmitt & Holmes, 1994; Tao & Murphy, 1994)).

Both *C. pseudotuberculosis* and *C. ulcerans* are known to produce DT when lysogenised with a *tox*-containing corynebacteriophage. Furthermore, these corynebacteria, like *C. diphtheriae*, produce DT in an iron-dependent manner (Maximescu, *et al.* 1974; Wong & Groman, 1984). There have been several cases of *C. ulcerans* causing diphtheria-like disease in humans (Gubler, *et al.* 1990; Lartigue, *et al.* 2005; Tiwari, *et al.* 2008). In the UK, isolation of any toxigenic *Corynebacterium*, including *C. pseudotuberculosis* and *C. ulcerans*, requires notification of local and national communicable disease control agencies (Efstratiou & George, 1999). By analogy with *C. diphtheriae*, control of expression of DT in *C. ulcerans* and *C. pseudotuberculosis* is likely to be controlled by a DtxR-like protein (Oram, *et al.* 2004).

In 1995, Oguiza *et al.* identified a *dtxR* homologue in the non-pathogenic *Brevibacterium lactofermentum* (*B. lactofermentum* has since been reclassified as *C. glutamicum* ATCC 13869 (Liebl, *et al.* 1991) (Usuda, *et al.* 1996), and this is how it will subsequently be referred to throughout this thesis). The *dtxR* gene (accession no. L35906) shared homology with *dtxR* of

C. diphtheriae. The authors subsequently hypothesised that a family of DtxR-like regulatory elements may be present in other corynebacteria (Oguiza, *et al.* 1995). In keeping with this hypothesis, a fragment of a *dtxR*-homologue was subsequently identified in *C. pseudotuberculosis* (Malloy, 2004; Oram, *et al.* 2004). Oram *et al.* (2004) used PCR to determine the presence of *dtxR* homologues within the genomes of 42 different corynebacterial isolates, representing 33 different species including both pathogenic and saprophytic organisms. The investigators identified *dtxR*-homologues in all 42 species of the genus *Corynebacterium*, including *C. pseudotuberculosis* (Oram, *et al.* 2004).

In *C. diphtheriae*, iron-activated DtxR is not only involved in the regulation of DT but it is also involved in the regulation of other genes implicated in the acquisition and storage of iron. Following the identification of a functional siderophore in *C. diphtheriae* (Russell, *et al.* 1984), expression of the genes involved in biosynthesis and transport of the siderophore were shown to be regulated by DtxR (Kunkle & Schmitt, 2005). Another DtxR-regulated gene, *hmuO*, encoding a haem oxygenase enzyme, was identified in *C. diphtheriae* by Schmitt (1997). Haem oxygenase enzymes catalyse the release of iron from haem, resulting in the oxidation of haem to biliverdin and CO (Schmitt, 1997a, 1997b). The HmuO enzyme of *C. diphtheriae* shares a high degree of homology with eukaryotic haem oxygenase enzymes (Schmitt, 1997b). Significantly, HmuO has been shown to be at least partially responsible for the utilisation of iron from haem in *C. diphtheriae* (Kunkle & Schmitt, 2007). Expression of *hmuO* is activated by the presence of haem and haemoglobin in the environment, but DtxR is responsible for its repression. In addition to controlling the expression of siderophore-related genes and *hmuO*, DtxR has recently also been shown to regulate genes involved in the oxidative stress response of *C. diphtheriae* (Oram, *et al.* 2002).

A significant body of work has led to the recognition of DtxR as a transcriptional regulator of iron metabolism in *C. diphtheriae* (Schmitt & Holmes, 1994; Tai, *et al.* 1990) and *C. glutamicum* (Brune, *et al.* 2006; Wennerhold & Bott, 2006). However, in *C. glutamicum*, DtxR has also been shown to regulate the expression of other regulatory components (Wennerhold, *et al.* 2005), and thus is involved in a global regulatory network. The Regulator of iron proteins, RipA, represses the expression of metabolism proteins, such as aconitase (an iron-containing protein) in *C. glutamicum*, however the RipA-encoding gene is itself regulated by DtxR in an iron-dependent manner. As a consequence, in *C. glutamicum*, DtxR has been

shown to be a “master regulator” of iron-dependent gene expression (Wennerhold, *et al.* 2005), although the same scenario has yet to be proven for other corynebacteria.

In addition to its more well-recognised role as a repressor, it is interesting to note that in *C. glutamicum*, DtxR has also been shown to be able to activate the expression of some genes; this observation is analogous to the IdeR regulatory protein in *M. tuberculosis* (Gold, *et al.* 2001; Wennerhold & Bott, 2006; Brune, *et al.* 2006). Wennerhold and Bott (2006) and Brune *et al.* (2006) identified a number of genes that, in a *C. glutamicum dtxR*-deficient mutant strain, showed decreased levels of mRNA for several genes, therefore suggesting that expression of these genes was up-regulated by DtxR (Brune, *et al.* 2006; Wennerhold & Bott, 2006). The genes identified included those encoding proteins which are involved in the assembly and repair of iron-sulphur clusters as well as genes involved in iron storage and oxidative stress.

As discussed previously in Chapters 1 and 3, as part of the innate defence against invading microorganisms the mammalian host specifically limits the concentration of circulating iron in an attempt to combat microbial infection. Therefore, the “sensing” of environmental iron concentrations by microbes is an indicator of whether or not they have gained entry into a host animal. As a result, the expression of virulence genes (*i.e.* genes that are essential for a pathogenic microbe to colonise and persist within a host) is often linked to environmental iron concentrations. Significantly, the introduction of a synthetic construct into *M. tuberculosis*, resulting in constitutive expression of *C. diphtheriae dtxR*, caused reduced virulence in a mouse model of tuberculosis (Manabe, *et al.* 1999). The most likely explanation for this observation was that DtxR was interfering with the normal *M. tuberculosis* iron-dependent response *in vivo*. Interestingly, the DtxR-like regulator shown to exist in *M. tuberculosis* (Doukhan, *et al.* 1995) is actually the so-called iron dependent regulator, IdeR (Schmitt, *et al.* 1995), discussed above, and it is significant that both DtxR and IdeR appear to be functionally interchangeable, and are therefore members of the same family of proteins.

In *C. pseudotuberculosis*, it is interesting to note that the most well-documented virulence factor, PLD, was shown by one research group not to be expressed in an iron-dependent manner (Billington, *et al.* 2002). The same group also identified the *C. pseudotuberculosis fagABC* operon, encoding ferric iron acquisition genes (Billington, *et al.* 2002). Significantly,

Billington *et al.* (2002) showed that the expression of the *fag* genes occurred in an iron-dependent manner *in-vitro*. Furthermore, a putative DtxR-like binding locus was identified just upstream of the first gene in the *fag* operon, although at that time a functional DtxR homologue had yet to be confirmed in *C. pseudotuberculosis*. The authors constructed a *fagB(C)* mutant, which showed reduced virulence when compared to its isogenic, wild-type parent in an experimental model of CLA in goats. Although tantalisingly close to proving the involvement of a DtxR-like regulator in the expression of the *fag* genes, Billington *et al.* (2002) did not pursue this research further.

Brune *et al.* (2006) carried out a series of experiments in order to understand the regulatory network of DtxR in *C. glutamicum*. In order to achieve this, the investigators used a series of methods including transcriptomics, bioinformatics, *in vitro* assays and comparative genomics. Subsequently, the DtxR protein of *C. glutamicum* was recognised as having a major role in controlling genes involved in iron metabolism. Generally, DtxR in *C. glutamicum* was shown to act as a repressor of genes involved in iron uptake and utilisation, and an activator of genes involved in iron storage and DNA protection (Brune, *et al.* 2006). Interestingly, the DtxR binding loci of some repressed genes were situated within the deduced promoter region, whereas the DtxR binding loci of some activated genes were located upstream of the promoter region. Due to the observation that decreased expression of these activated genes occurred in a DtxR-deficient *C. glutamicum* mutant (as measured by DNA microarray hybridisation and real-time RT-PCR), Brune *et al.* (2006) hypothesised that the location of the DtxR binding loci of the activated genes was more consistent with an activating function of the DtxR protein (Brune, *et al.* 2006; Madan Babu & Teichmann, 2003).

The complete genome sequence of *C. diphtheriae* NCTC 13129 has become publicly available relatively recently (Cerdeno-Tarraga, *et al.* 2003). Analysis of the sequence surrounding the *dtxR* gene revealed a gene encoding an RNA sigma (σ) factor, *sigB*, located 224 bp upstream and a gene encoding UDP-galactose-4-epimerase, *galE*, 22 bp downstream of *dtxR* (Cerdeno-Tarraga, *et al.* 2003). The σ -factor, SigB, is thought to have homology with σ^{70} -type factors, which are known to be involved in bacterial stress responses (Oram, *et al.* 2006). It was previously noted that the *galE* gene of *C. glutamicum* shared homology with *C. diphtheriae* *galE* (Boyd, *et al.* 1990; Oguiza, *et al.* 1996a), the deduced amino acid sequences of both sharing significant homology with UDP-galactose 4-epimerases from other Gram-positive

genera (Oguiza, *et al.* 1996a). The actual function of the protein encoded by *galE* in *C. diphtheriae* has not yet been determined; however, UDP-galactose and UDP-glucose of other bacteria are substrates for the production of exopolysaccharides on the surface of the cell.

The lack of understanding in relation to *C. pseudotuberculosis* virulence mechanisms has hindered the understanding of disease pathogenesis and hence the development of new CLA vaccines. Using the wealth of information available regarding the close relatives of *C. pseudotuberculosis* it was thought possible that a comparative study could be undertaken; however, it was considered likely, due to species divergence (and the fact that *C. glutamicum* is non-pathogenic) that an informed selection exercise (essentially a fishing-exercise) to determine the presence of other corynebacterial virulence genes in *C. pseudotuberculosis* would be problematic with a low chance of success. In contrast, the involvement of DtxR in the regulation of gene expression in *C. diphtheriae* and *C. glutamicum*, combined with the suggestion of a DtxR-like regulator in *C. pseudotuberculosis* (Malloy, 2004) (Oram, *et al.* 2004) and a putative DtxR-binding motif identified upstream of the *C. pseudotuberculosis* *fag* operon (Billington, *et al.* 2002), suggested that focusing research on the equivalent regulator in *C. pseudotuberculosis* would be a means of identifying information regarding virulence mechanisms in this organism. Therefore, the work in this chapter aimed to characterise the *dtxR* locus of *C. pseudotuberculosis* and to determine whether it functioned as a regulator of gene expression.

5.1 Results

The work described in this chapter was conducted simultaneously with that presented in the next chapter. Interestingly, during the laboratory work described in Chapter 6 it became apparent that, although the CCDM “high-iron” growth medium described in Chapter 3 was sufficient to support maximal growth of *C. pseudotuberculosis* in culture, the level of iron in the medium was insufficient to permit full repression of iron-responsive genes. Therefore, it became necessary to increase the concentration of available iron in the CCDM by supplementing with FeCl₃ to 10 µM final concentration (**Figure 5.1**). This new “high-iron” medium, which supported an equivalent level of bacterial growth in culture but ensured minimal expression of iron-responsive genes, became the *de facto* high-iron growth medium used to generate the data presented in this and the following chapter.

5.1.1 Characterization of a *C. pseudotuberculosis* chromosomal locus containing *dtxR*

Previously in our laboratory, a 312 bp fragment of a *dtxR*-homologue was identified in *C. pseudotuberculosis* (Malloy, 2004). To determine the rest of the sequence of the *dtxR*-homologue, and to characterise the chromosomal locus in which the gene lay, inverse PCR (iPCR) was employed (**Figure 5.2**), which is a technique designed by Ochman *et al.* (1988) to amplify unknown DNA sequences that flank a region of known sequence (Ochman, *et al.* 1988).

In the first instance, to determine which restriction endonucleases would generate chromosomal DNA fragments containing the *dtxR*-like gene, of an optimal size for iPCR, a Southern blot was performed. Several restriction endonucleases were chosen based on their ability to cut relatively frequently within GC-rich DNA sequences. *C. pseudotuberculosis* 3/99-5 genomic DNA was digested to completion with either *Kpn*I, *Spe*I, *Nde*I, *Hind*III, *Bam*HI, *Xba*I, *Bgl*II, *Pst*I, *Eco*RI or *Nco*I, and separated by agarose gel electrophoresis prior to Southern blot analysis. The blot was hybridised with a DIG-labelled probe consisting of the original 312 bp *dtxR* PCR product identified by Malloy (2004), and 3 restriction endonucleases producing fragments of a size suitable for subsequent amplification by PCR were identified (**Figure 5.3**).

As presented in **Figure 5.3**, the restriction endonucleases *KpnI*, *HindIII* and *XbaI* generated genomic DNA fragments of *ca.* 0.9 kb, 3 kb, 6.5 kb respectively, which contained the *dtxR*-like gene; these were considered to be of a suitable size for PCR amplification. Significantly, Southern blot analysis also confirmed the presence of a single copy of the *dtxR* homologue on the *C. pseudotuberculosis* chromosome (**Figure 5.3**). Having identified appropriate restriction endonucleases to generate optimally-sized DNA fragments, fresh genomic DNA was digested to completion with *KpnI*, *HindIII*, and *XbaI*. Subsequently, linearised genomic DNA fragments were circularised by ligation to create templates for inverse PCR.

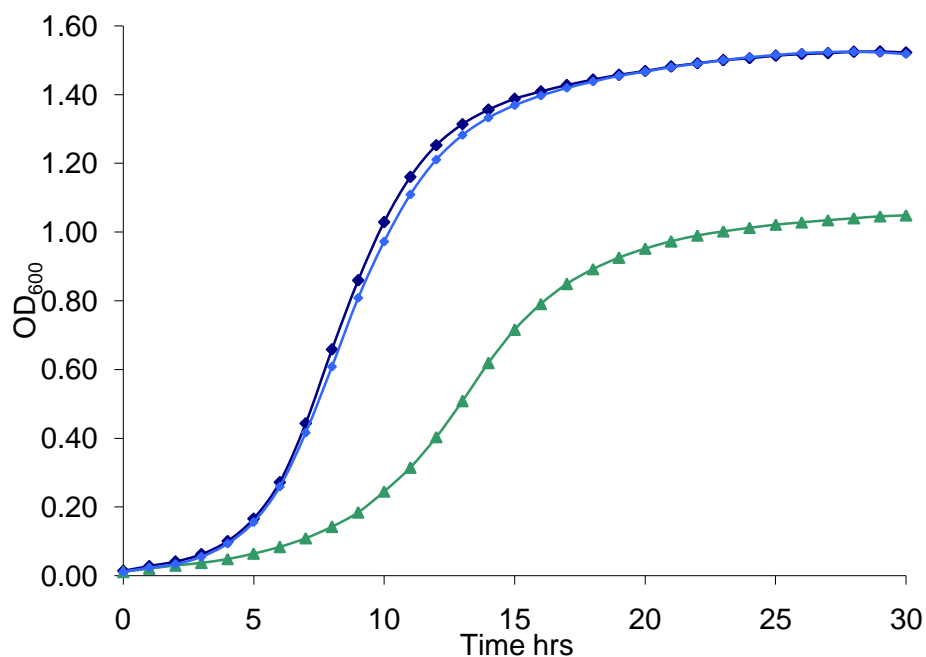


Figure 5.1 Analysis of the affect of iron on *C. pseudotuberculosis* growth. *C. pseudotuberculosis* 3/99-5 was cultured in CCDM (—◆—), CCDM supplemented with 2',2'-dipyridyl to 300 μ M final concentration (—▲—), or CCDM supplemented with dipyridyl and FeCl₃ to 10 μ M final concentration (—◆—). Despite supplementing chelated CCDM with a 10-fold greater amount of FeCl₃ than had been used previously, growth of *C. pseudotuberculosis* in the iron-restored medium was still equivalent to that in CCDM alone.

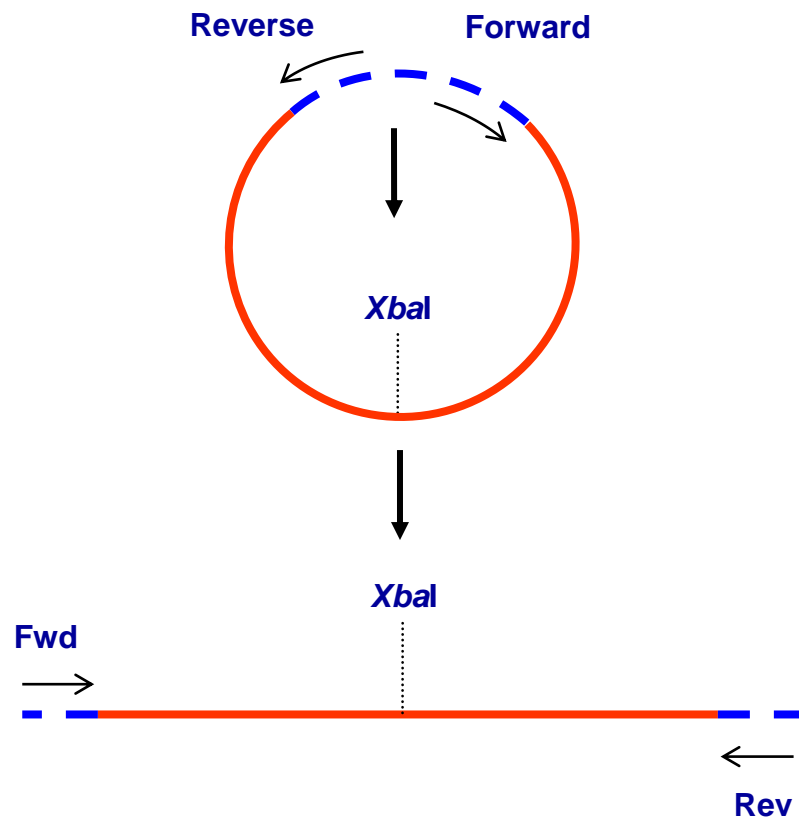


Figure 5.2 Schematic representation of inverse PCR

The inverse PCR method (Ochman, *et al.* 1988) uses straightforward PCR; however, oligonucleotide primers are orientated in the reverse direction to that used normally. The template is a restriction fragment (in this example the restriction endonuclease is *XbaI*) that has been self-ligated to form a circular piece of DNA. Amplicons are subsequently cloned and sequenced thereby allowing identification of unknown sequence surrounding a known DNA sequence

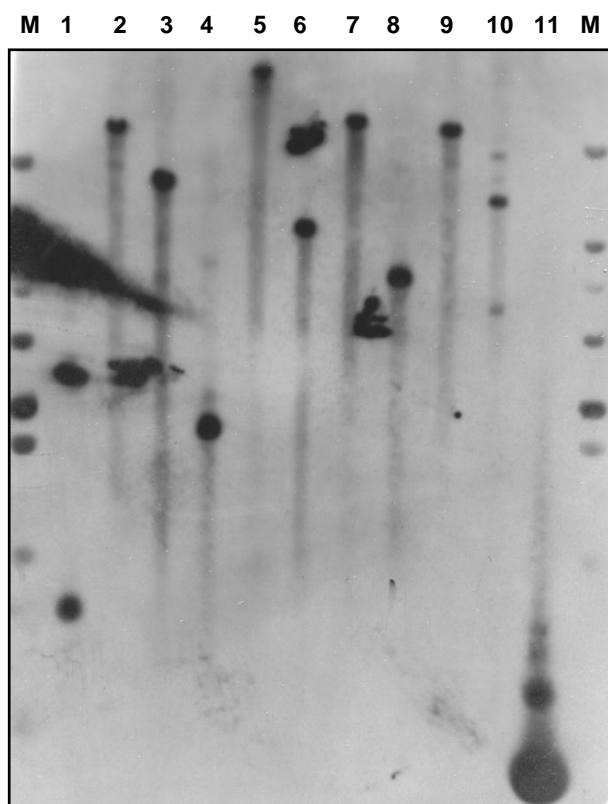


Figure 5.3 Southern blot analysis of *C. pseudotuberculosis*

Samples in lanes 1-10 correspond to *C. pseudotuberculosis* genomic DNA digested with *KpnI*, *SpeI*, *NdeI*, *HindIII*, *BamHI*, *XbaI*, *BglII*, *PstI*, *EcoRI* and *NcoI*, respectively. M is indicative of 1 kb DNA ladder (Invitrogen), and the 312 bp PCR product containing a fragment of the *dtxR*-homologue was included as a positive control (lane 11). The probe corresponded to a DIG-labelled, 312 bp fragment of the *C. pseudotuberculosis* putative *dtxR*-like gene.

Inverse PCR was conducted using the primers *dtxR*_inv (fwd) and *dtxR*_ inv (rev) (**Table 2.3**). Amplified blunt-ended DNA products were separated through a 1 % (w/v) agarose gel, stained with gel red and visualised over UV light (**Figure 5.4**). DNA bands were excised and cloned into pCR[®]Blunt II TOPO[®]. The resulting recombinant plasmids (designated pCAW001, pCAW002, and pCAW003, respectively) were analysed by restriction endonuclease digestion and sequenced, in the first instance, using the M13 forward and reverse primers (**Table 2.4**) which annealed either side of the pCR[®]Blunt II TOPO[®] multiple cloning site. The new DNA sequences were used to design further sequencing primers, and so on until the inserts in each of the 3 recombinant plasmids had been sequenced.

From the sequencing data obtained, the region of the *C. pseudotuberculosis* chromosome surrounding the original 312bp *dtxR*-homologous sequence was assembled (**Figure 5.5**). The DNA sequences amplified by iPCR from *Kpn*I and *Hind*III digested DNA were all present within the larger 6.5kb *Xba*I iPCR product. By sequencing all 3 plasmid constructs it was possible to confirm the integrity of the assembled chromosomal region. Open reading frames (ORFs) were identified within the sequences and subsequent BLAST analysis of the 6.5 kb *C. pseudotuberculosis* contig. revealed homology with *C. diphtheriae* (acc. # NC_002935) *dtxR* (locus tag DIP1414), *galE* (locus tag DIP1415), and 2 conserved hypothetical proteins of *C. diphtheriae*. In addition, a partial ORF sharing homology with the *sigB* (locus tag DIP1413) of *C. diphtheriae* was present upstream of the ORF encoding the DtxR homologue. Additionally, a partial ORF, which shared homology with a putative helicase of *C. diphtheriae* (locus tag DIP1418) was identified at the 3'-end of the 6.5 kb contig.

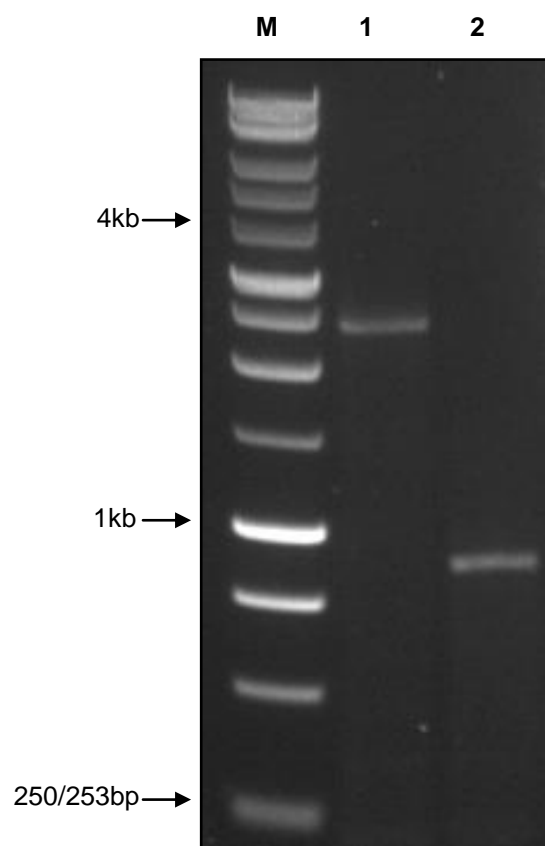


Figure 5.4 Inverse PCR products

Sample lanes correspond to 1 kb marker (M), the *Hind*III iPCR product (*ca.* 3kb) (lane 1) and the *Kpn*I iPCR product (*ca.* 900bp) (lane 2).

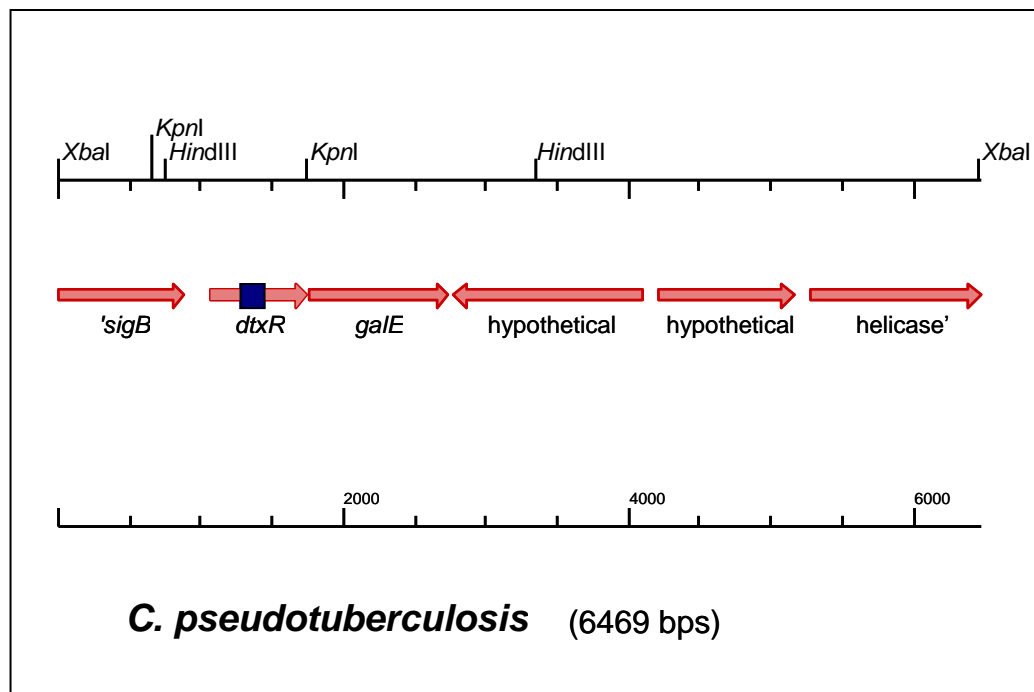


Figure 5.5 Schematic representation of the *C. pseudotuberculosis* locus containing the *dtxR*-homologue

The *ca.* 6.5 kb fragment generated by inverse PCR using *XbaI*-digested *C. pseudotuberculosis* genomic DNA.

The blue square indicates the previously-determined 312 bp region of the *dtxR*-homologous gene upon which the inverse PCR primers were designed.

Interestingly, an identical gene order with a high overall level of sequence homology was observed between the locus of *C. pseudotuberculosis* containing the *dtxR*-homologous gene and other sequenced corynebacteria. The translated products of the *C. pseudotuberculosis* ORFs were compared with the equivalent proteins from *C. diphtheriae* and *C. glutamicum*. Significantly, subsequent to the inverse PCR work, and as part of a separate project in the same laboratory, the *C. pseudotuberculosis* 3/99-5 genome was partially sequenced. Fortuitously, although the genome had not been analysed in any great depth, one of the several available contigs was found (by BLAST analysis) to contain the sequence corresponding to the *dtxR*-like region amplified by iPCR. Subsequently, it was possible to obtain the 5'-region of the *sigB* homologue of *C. pseudotuberculosis* which was lacking in the 6.5 kb iPCR fragment. The translated product of the *sigB* homologue was subsequently found to share a high level of homology with *C. diphtheriae* and *C. glutamicum*, with 92% identity and 97% similarity with SigB of *C. diphtheriae*, and 89% identity and 95% similarity with that of *C. glutamicum*. The sequence of the DtxR-like protein of *C. pseudotuberculosis* shared 79% identity and 87% similarity with *C. diphtheriae* and 74% identity and 84% similarity with *C. glutamicum*. The GalE homologue of *C. pseudotuberculosis* shared 81% identity and 90% similarity with that of *C. diphtheriae* and 78% identity and 87% similarity with *C. glutamicum*. For the sake of this study, only the *sigB*, *dtxR* and *galE* genes were a focus of comparisons, since previous work carried out by Oram *et al.* (2006) on *C. diphtheriae* had identified these genes as being linked and therefore worthy of further analysis in *C. pseudotuberculosis* (Oram, *et al.*, 2006).

The DtxR-like protein of *C. pseudotuberculosis* was aligned with the DtxR proteins of *C. diphtheriae* and *C. glutamicum* (**Figure 5.6**). The sequence encoding the predicted DNA-binding domain (domain 1) lay within a region of generally-high sequence conservation, particularly between *C. pseudotuberculosis* and *C. diphtheriae*. Similarly, domain 2, required for dimerization of DtxR, was also highly conserved between all three corynebacteria. Interestingly, however, domain 3 was highly variable between all three species.

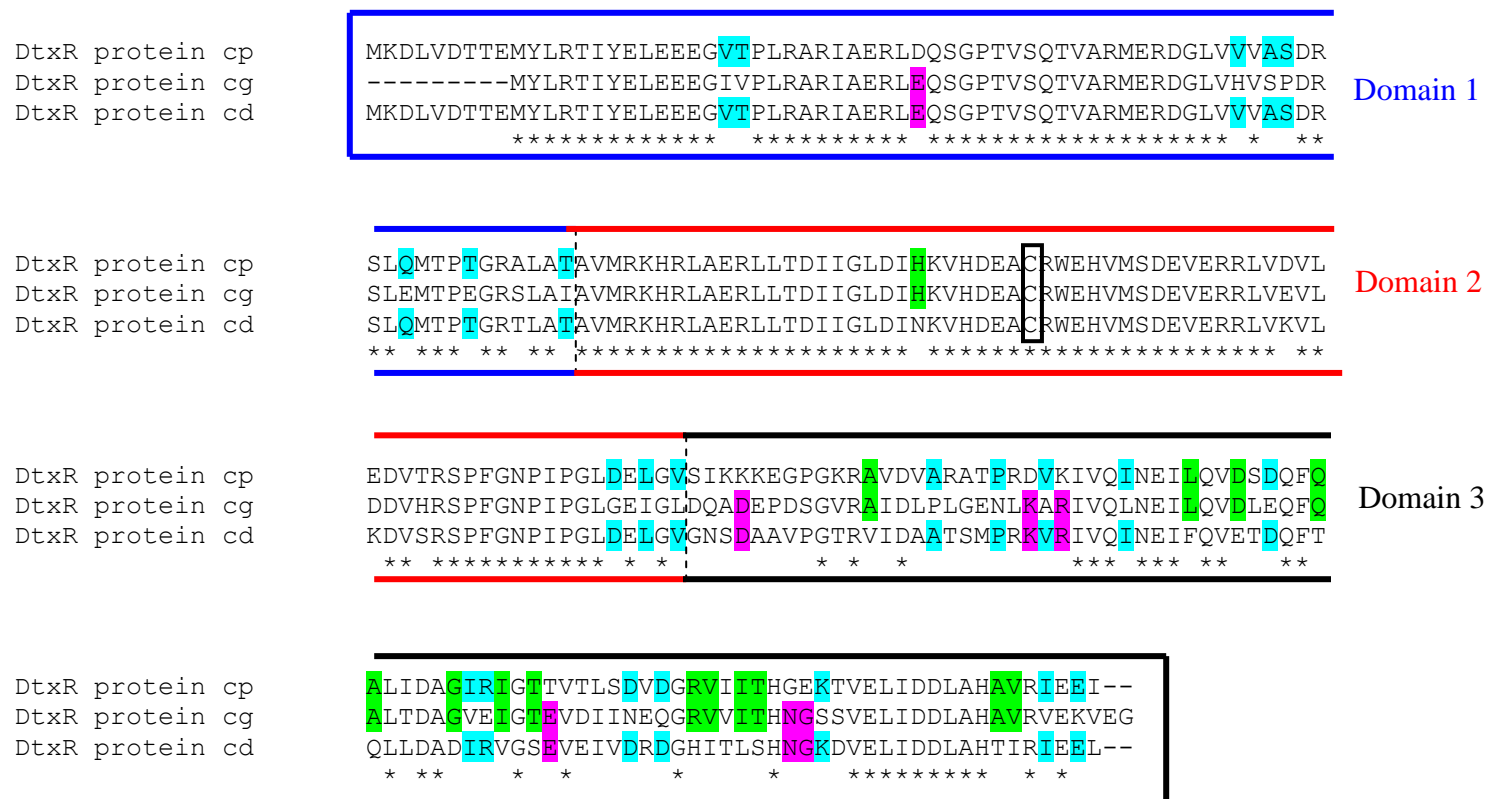


Figure 5.6

Clustal Wallace multiple alignment of *C. pseudotuberculosis* (cp), *C. glutamicum* (cg) and *C. diphtheriae* (cd) DtxR protein.

The predicted domains are labeled 1-3. Regions of homology are indicated by a star. Blue highlighter indicates homology between *C. pseudotuberculosis* and *C. diphtheriae* only, green indicates homology between *C. pseudotuberculosis* and *C. glutamicum* only and pink indicates homology between *C. glutamicum* and *C. diphtheriae* only. Domain 1 - classic helix-turn-helix DNA binding-motif (amino acids 1-73 in blue lines). Domain 2 - Required for dimerisation of DtxR and contains binding sites for two Fe^{2+} ions per monomer (amino acids 74-140 in red lines), (Also shown is the Cys-102). Domain 3 - shares homology with SH3 domains found in some eukaryotic signal transduction proteins (amino acids 141-266 in black lines).

5.1.2 Analysis of expression of genes within the *C. pseudotuberculosis* locus containing the *dtxR*-homologue

Having identified the locus containing a *dtxR*-homologue, it was essential to determine whether the *dtxR*-like gene was actually expressed in *C. pseudotuberculosis*. In the first instance this was achieved by PCR amplification of the original 312 bp fragment of the gene with the primers *dtxR*_01 and *dtxR*_03 (Table 2.3), using reverse-transcribed mRNA extracted from a log-phase culture of *C. pseudotuberculosis*. As expected, a fragment of the correct size was successfully amplified from the reverse transcribed mRNA, while absence of genomic DNA contamination was confirmed by the failure to amplify a product from non-reverse-transcribed mRNA (Figure 5.7); therefore, expression of the *dtxR*-like gene was confirmed.

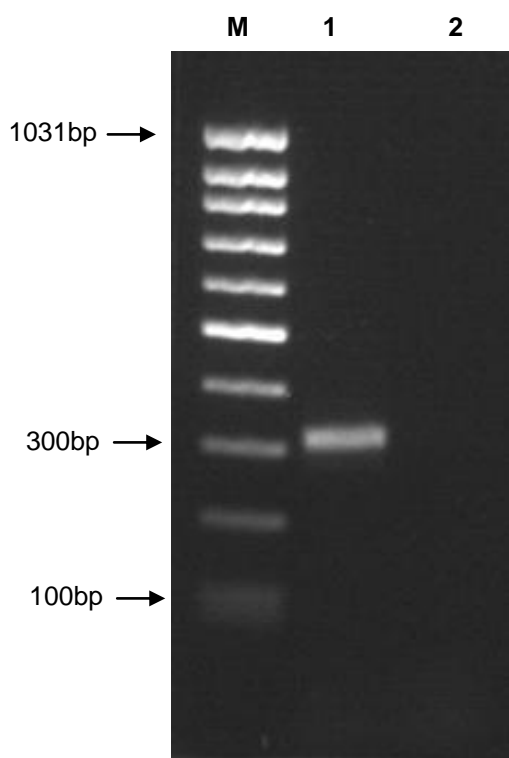


Figure 5.7 The 312bp transcript of the *C. pseudotuberculosis* *dtxR* gene amplified by RT-PCR

Gel lanes correspond to 100 bp DNA ladder (M), 312 bp fragment of the *dtxR*-like gene amplified from reverse-transcribed mRNA (Lane 1) and control reaction using non-reverse-transcribed mRNA (Lane 2).

Having confirmed the expression of the *dtxR*-homologue by RT-PCR, an experiment was conducted to determine whether *C. pseudotuberculosis* contained a functional DtxR regulator. This was achieved using the plasmid pSPZ (Oram, *et al.* 2006), containing a promoterless *lacZ* gene, a spectinomycin resistance gene and a stable pNG2 replication origin for *C. diphtheriae*, to study the outcome of environmental iron concentrations on the expression of known *C. diphtheriae* DtxR-regulated genes by measurement of β -galactosidase production. Previously created pSPZ constructs were obtained from Prof. R. K. Holmes (University of Colorado at Denver and Health Sciences Centre, School of Medicine, Dept. of Microbiology, Aurora, Colorado, USA), which comprised the promoter-containing regions of the *tox* (Oram, *et al.*, 2006) and *irp3* (R. K. Holmes, personal communication) genes cloned upstream of the promoterless *lacZ* gene in pSPZ. These constructs were introduced into wild-type *C. pseudotuberculosis* by electroporation, and promoter activity was assessed by measuring β -galactosidase activity under high- and low-iron culture conditions. Significantly, as shown in **Figure 5.8** *lacZ* expression was found to be up-regulated under conditions of low-environmental iron for both the *tox* and *irp3* promoters in *C. pseudotuberculosis* (the data shown is representative of 3 distinct experiments). The data was statistically analysed using a paired t-test, the outcome of which confirmed that environmental iron concentration was exerting a highly significant effect upon the level of expression of *irp3* ($p=0.011$). Furthermore, the expression of *tox* also appeared to be affected by iron concentration (albeit to a lesser extent than *irp3*), although variability in the data over subsequent experiments resulted in fairly large error-bars, with the result that statistical analysis only suggested a trend towards significance ($p= 0.068$). While these results confirmed the presence of a DtxR-like regulator in *C. pseudotuberculosis*, they only circumstantially implicated the product of the *dtxR*-like gene in this process. Therefore, further experiments were required to confirm (or refute) whether the product of this gene was a functional regulator of gene expression.

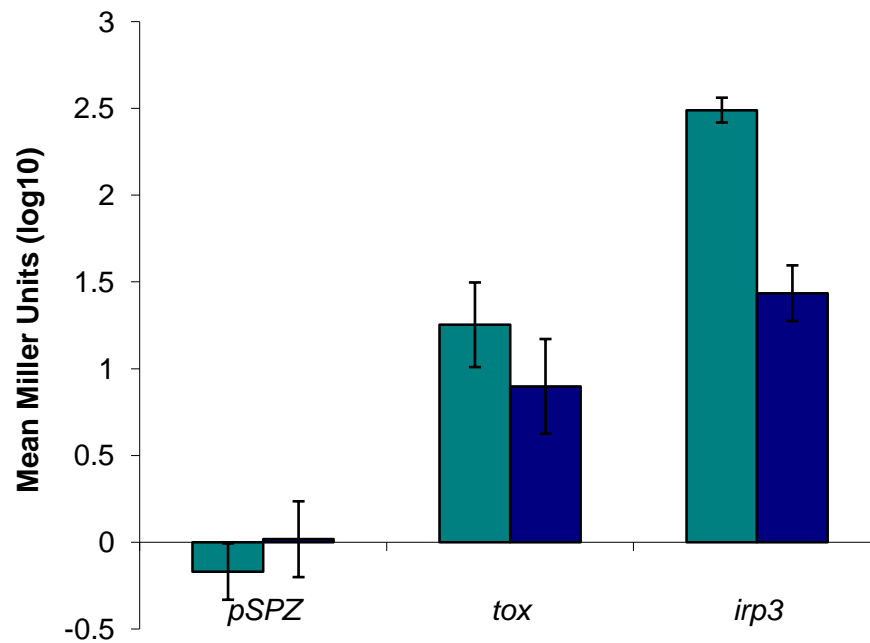


Figure 5.8 Assessment of the affect of environmental iron concentration on the expression of *tox*- and *irp3*-promoter/lacZ fusions in *C. pseudotuberculosis*. Promoter fusion assays were undertaken to determine whether the expression of genes encoding known *C. diphtheriae* iron-regulated genes were regulated in response to environmental iron concentrations when in *C. pseudotuberculosis*. Statistical analyses using a paired t-test confirmed that environmental iron concentration was exerting a highly significant effect upon the level of expression of *irp3* ($p= 0.011$); *tox* also appeared to be affected by iron concentration albeit to a lesser extent than *irp3* ($p= 0.068$). Bars correspond to low-iron (■), high-iron (■).

5.1.3 Assessment of the involvement of *C. pseudotuberculosis* DtxR in the iron-dependent regulation of *tox*

To confirm the involvement of the product of the *C. pseudotuberculosis* *dtxR*-homologue in the iron-dependent regulation of known DtxR-regulated *C. diphtheriae* genes, a further promoter fusion assay was carried out in *E. coli* DH5 α , using the pSPZ construct containing the *tox* promoter. *E. coli* DH5 α was transformed with either the pSPZ construct alone or with that construct in addition to pWSK29/DtxR, which contained the *C. pseudotuberculosis* *dtxR*-like gene cloned into pWSK29 (Wang & Kushner, 1991). Using the PCR primers *dtxR*_comp F and *dtxR*_comp R (Table 2.3) a 1063 bp fragment which started 291 bp upstream of the *dtxR*-like gene (so as to include the promoter region) and finished 96 bp downstream of *dtxR*, was amplified. The amplicon was sub-cloned into pCR[®]II-TOPO[®] vector (Table 2.2) to create pCR[®]II-TOPO[®]/*dtxR*. Subsequently, a *ca.* 1063 bp fragment was excised from the pCR[®]II-TOPO[®]/*dtxR* construct using the restriction endonuclease *Eco*RI. The 1063 bp fragment, which included the full *dtxR* gene and promoter region, was then blunt-end ligated into a similarly digested pWSK29 vector to create pWSK29/DtxR (Table 2.2). Promoter fusion assays were carried out under high- and low-iron growth conditions as previously. This experiment was only conducted on a single occasion (with triplicate samples); therefore, rather than subjecting the data to statistical analysis equivalent to that described above (which corresponded to data gathered in triplicate on 3 different occasions), the level of repression of the *tox* promoter under high- and low-iron growth conditions was determined by calculating the repression ratio (low-iron/high-iron). The repression ratio was calculated in order to highlight the differential expression of *tox* when DtxR is present. In the presence of DtxR the repression ratio was 2.12; however, when DtxR was absent the repression ratio was markedly lower at 0.89. These results confirm that the *C. pseudotuberculosis* DtxR-homologue was capable of regulating a known *C. diphtheriae* DtxR regulated gene in an iron-dependent manner (Figure 5.9). Given its equivalent function, for ease of nomenclature the *C. pseudotuberculosis* *dtxR*-homologue will henceforth be referred to as *dtxR*.

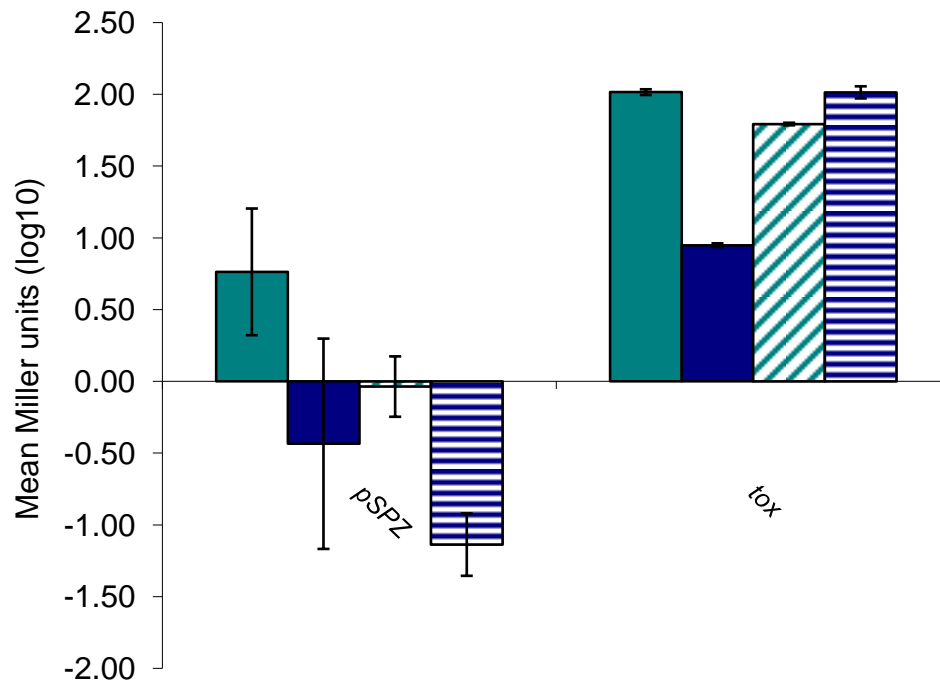


Figure 5.9 Assessment of *C. pseudotuberculosis* DtxR regulation of *tox*.

A further promoter fusion assay was carried out in *E. coli* DH5 α , using the pSPZ construct containing the *tox* promoter (*toxP*). *E. coli* DH5 α were transformed with the pSPZ construct, while a further transformant was obtained by introduction of the pSPZ construct in addition to pWSK29/DtxR, which contained the *C. pseudotuberculosis dtxR*-like gene cloned into pWSK29. Bars correspond low-iron + DtxR (■), high-iron + DtxR (■), Low-iron - DtxR (green dashes), high-iron - DtxR (blue dashes). In the presence of DtxR the *tox* promoter was differentially expressed (repression ratio of 2.12) whereas when DtxR was absent the repression ratio was markedly reduced (0.89).

5.1.4 Analysis of expression of the genes within the *C. pseudotuberculosis* *dtxR* chromosomal locus

Having proven the involvement of *C. pseudotuberculosis* DtxR in the control of expression of a *C. diphtheriae* DtxR-regulated gene, experiments were conducted to investigate the expression of the genes within the *C. pseudotuberculosis* *dtxR*-containing region. This was done with a view to determining whether DtxR was involved in regulation of its own structural and surrounding genes, as has been described for the Fur regulator in *Helicobacter pylori* and *Neisseria meningitidis* (Delany, *et al.* 2003; Delany, *et al.* 2002). In order to assess the transcription of the *C. pseudotuberculosis* *sigB*, *dtxR* and *galE* genes, promoter fusions were constructed. In **Figure 5.10** is presented a schematic representation of the *dtxR* locus of *C. pseudotuberculosis*, indicating the upstream regions which were used in promoter fusion assays. To determine the promoter activity of *sigB*, a 261bp fragment (derived from the ongoing genome sequence project) was amplified using the primers *sigB*_P_F and *sigB*_P_R (**Table 2.5**), and cloned upstream of the *lacZ* reporter gene in pSPZ. Similarly, fragments of 690bp and 479bp were cloned into pSPZ from sequences upstream of *dtxR* and *galE* using primers *dtxR*_P_F and *dtxR*_P_R, and *galE*_P_F and *galE*_P_R respectively. The resulting plasmids were designated pCAW004, pCAW005 and pCAW006 respectively. The fragment upstream of *dtxR* incorporated approximately 690bp so as to include the intergenic region between *sigB* and *dtxR*. The constructs were subsequently introduced into wild-type *C. pseudotuberculosis* and the promoter activity was determined by measuring β -galactosidase activity (**Figure 5.11**). The results indicated that a functional promoter lay upstream of *sigB*, and also upstream of *dtxR*; however, in contrast, there was no detectable promoter activity upstream of *galE*. Furthermore, despite the evidence of functional *sigB* and *dtxR* promoters, there was no evidence of environmental iron concentration exerting an effect upon the level of expression of these genes, as determined by there being no difference in the level of β -galactosidase production under either condition. A paired t-test was employed to statistically analyse the data, which confirmed that there was no evidence to implicate iron concentration in the regulation of gene expression of *sigB* ($p=0.507$), *dtxR* ($p=0.293$) or *galE* ($p=0.448$).

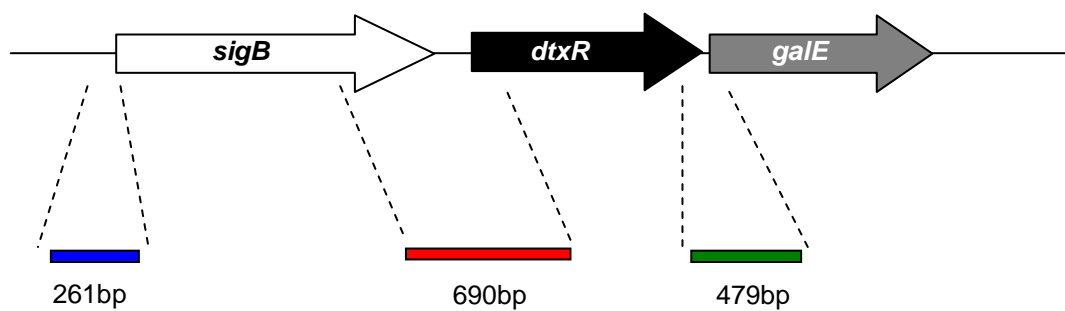


Figure 5.10 Schematic representation of the *C. pseudotuberculosis* *dtxR* locus.

Intergenic sequences which were assessed for the presence of functional promoters are highlighted.

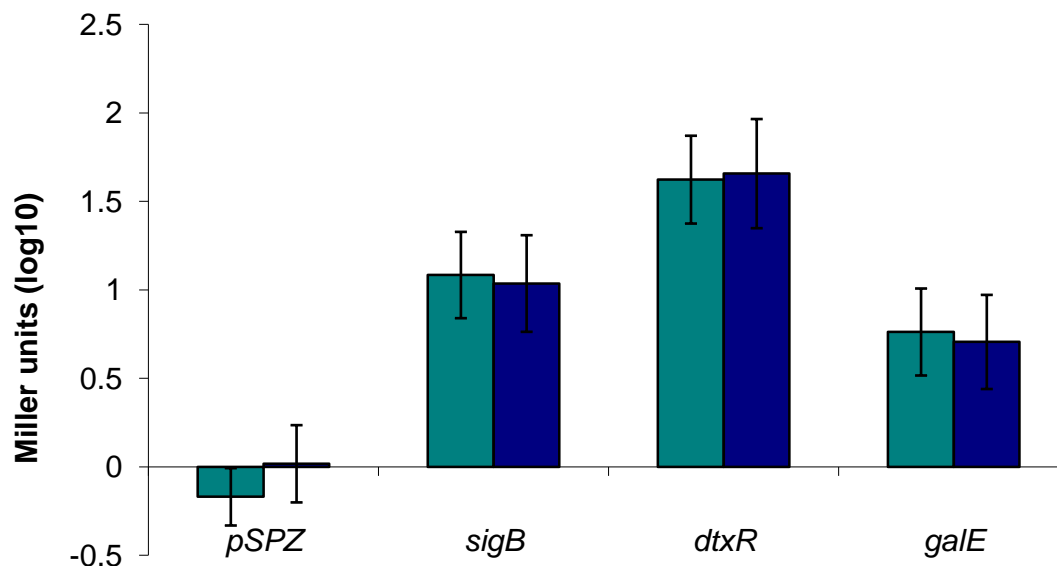


Figure 5.11 Determination of functional promoters within the *dtxR* chromosomal locus, using *pSPZ/ lacZ* fusions. Promoter fusion assays were undertaken to determine whether the expression of the genes within the *C. pseudotuberculosis dtxR* locus were regulated in response to environmental iron concentrations. A paired t-test was employed to statistically analyse the data, which confirmed that there was no evidence to implicate iron concentration in the regulation of gene expression of *sigB* ($p=0.507$), *dtxR* ($p=0.293$) or *galE* ($p=0.448$). Bars correspond to Low-iron (■) and high-iron (■).

To further assess the expression of the *sigB*, *dtxR* and *galE* genes in *C. pseudotuberculosis*, quantitative reverse transcription PCR (qRT-PCR) was used to determine the relative transcript abundance of these genes. For qRT-PCR, RNA was extracted from bacterial cells harvested from exponentially-growing cultures of *C. pseudotuberculosis* 3/99-5, grown under either high- or low-iron conditions; each experiment was conducted in triplicate. Oligonucleotide primers were designed to amplify transcripts from *sigB*, *dtxR*, the *dtxR-galE* intergenic region and *galE* (**Figure 5.12**). Primers for the *sigB* transcript annealed 226 bp upstream of the 3' end of *sigB* (**B, d with blue probe in Figure 5.12**). To amplify the *dtxR* transcript, primers were designed to anneal 145 bp downstream of the 5' end of *dtxR* (**C, c with red probe in Figure 5.11**). A transcript spanning the intergenic region of *dtxR-galE* was also measured, whereby primers annealed 82 bp upstream of the 3' end of *dtxR* and incorporated 200 bp of *galE* (**F, b with yellow probe in Figure 5.12**). Finally, the *galE* gene transcript was measured using primers which annealed 483 bp downstream of the 5' end of *galE* (**G, a with green probe in Figure 5.12**). Transcript abundance was measured relative to the housekeeping gene *recA* (accession no. U30387). In addition, *fagA* (accession no. AF401634), the expression of which had previously been shown to be down-regulated under high-iron growth conditions (Billington, *et al.*, 2002), was used as positive control.

A graphical representation of the qRT-PCR results is presented in **Figure 5.13**. A statistical paired t-test analysis of the results of qRT-PCR experiments demonstrated no statistically-significant difference between the expression of either *sigB* ($p=0.414$), *dtxR* ($p=0.452$) or *galE* (0.356) under high- or low-iron growth conditions. In general, *sigB* was only expressed at low levels under both growth conditions. Interestingly, *galE* was observed to be co-transcribed with *dtxR*, consistent with the failure to identify a functional *galE* promoter in promoter fusion assays. In contrast, qRT-PCR data revealed a significant difference ($p=0.003$) between the expression of *fagA* under high- and low-iron growth conditions; transcription of *fagA* had increased 16-fold under low-iron compared to high-iron conditions.

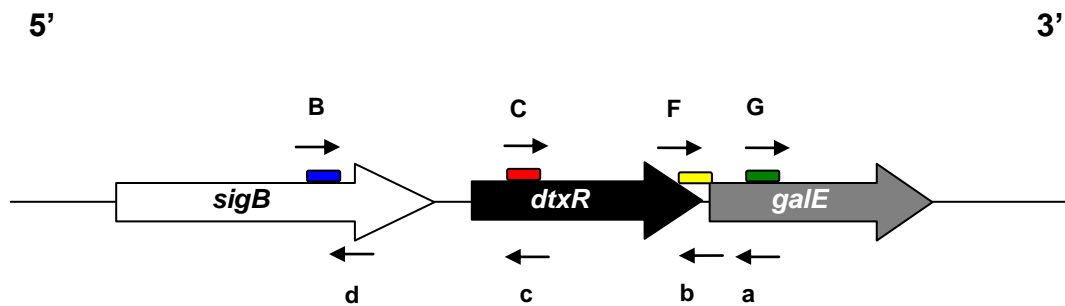


Figure 5.12 Schematic representation of the *C. pseudotuberculosis* *dtxR* chromosomal locus highlighting the locations of qRT-PCR primer and probe binding sites.

Primers that were designed to amplify *sigB* transcripts annealed 226 bp upstream of the 3' end of *sigB* (**B, d with blue probe**). To amplify *dtxR* transcripts, primers were designed to anneal 145 bp downstream of the 5' end of *dtxR* (**C, c with red probe**). Transcripts spanning the intergenic region of *dtxR-galE* were also measured, whereby primers annealed 82 bp upstream of the 3' end of *dtxR* and incorporated 200 bp of *galE* (**F, b with yellow probe**). The *galE* gene transcripts were measured using primers which annealed 483 bp downstream of the 5' end of *galE* (**G, a with green probe**).

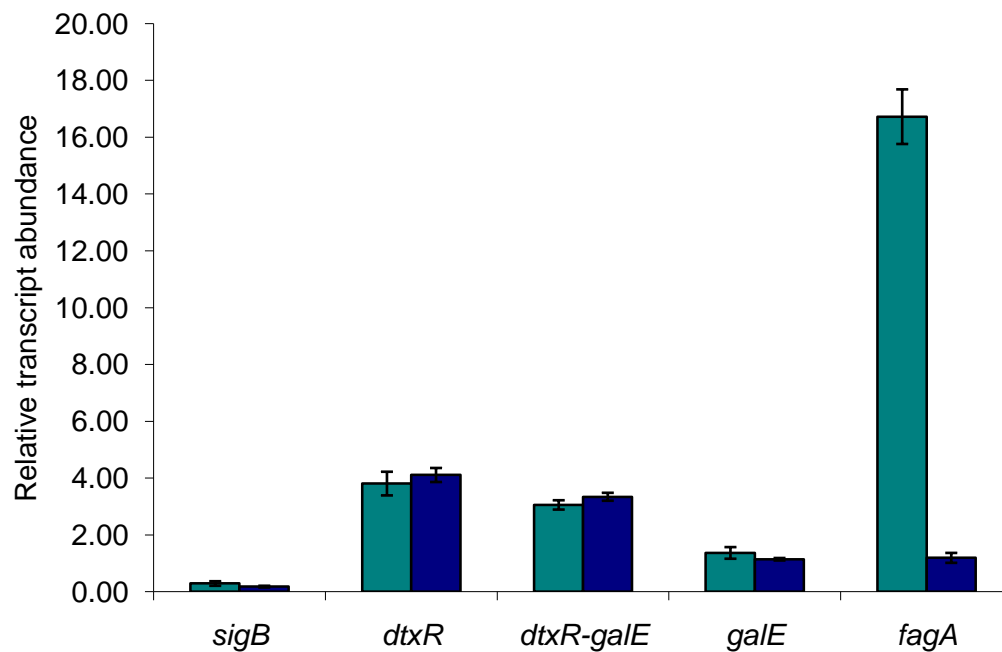


Figure 5.13 Relative transcript abundance of genes within the *C. pseudotuberculosis* *dtxR* chromosomal locus. The expression of each gene within the *C. pseudotuberculosis* *dtxR* locus was analysed following extraction of mRNA from cells cultured in high- and low-iron media. Statistical analysis of the data provided evidence to suggest that there was no significant difference between expression of *sigB* ($p=0.414$), *dtxR* ($p=0.452$) or *galE* ($p=0.356$) when grown under low- or high iron conditions. However, there was a significant difference between the expression of *fagA* under low-iron compared to high-iron conditions ($p=0.003$). Bars correspond to low-iron (teal) and high-iron (dark blue).

Previously, Oram *et al.* (2006) demonstrated that in *C. diphtheriae*, *sigB*, *dtxR* and *galE* are transcribed as a polycistronic message (*i.e.* exist in an operon structure) (Oram, *et al.* 2006). Taking into account the similarities already seen between the *dtxR* loci of *C. diphtheriae* and *C. pseudotuberculosis*, it was thought likely the same operon structure also existed within the *dtxR* locus of *C. pseudotuberculosis*. Therefore, to determine whether *sigB* was expressed as part of a polycistronic mRNA, *sigB-dtxR-galE* transcription was analysed by conventional RT-PCR. Using the primers *sigB*_B_F and *galE*_a_R (**Table 2.6**), it was possible to amplify an expected sized fragment of *ca.* 2 kb, from mRNA isolated from *C. pseudotuberculosis* cells harvested in exponential growth-phase (**Figure 5.14**). In contrast, an equivalent band was not amplified in a control reaction containing non-reverse-transcribed mRNA. Therefore, this result confirmed the existence of a transcript spanning *sigB-dtxR-galE*, and hence proved the co-expression of all 3 genes.

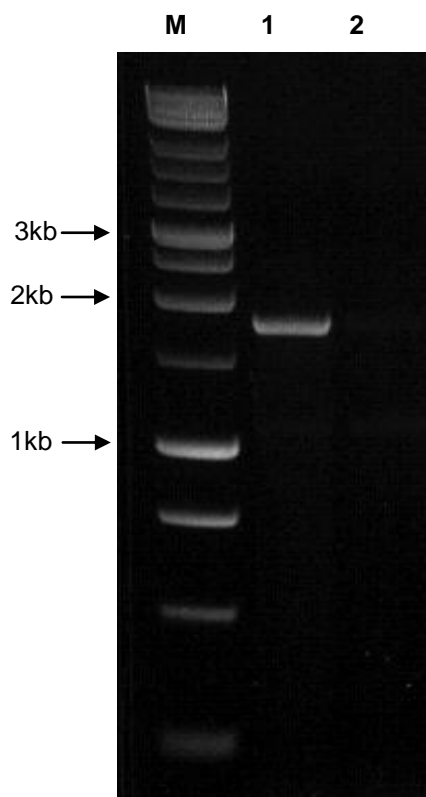


Figure 5.14 Detection of *sigB-dtxR-galE* transcripts using RT-PCR

Samples correspond to 1kb DNA ladder (M), the *sigB-dtxR-galE* transcript (Lane 1) and an equivalent negative control reaction using non-reverse-transcribed mRNA as template (Lane 2)

5.1.5 Iron-dependent expression of other *C. pseudotuberculosis* genes

Using promoter fusion assays, experiments were undertaken to determine whether the expression of genes encoding published *C. pseudotuberculosis* virulence factors were regulated in response to environmental iron concentrations. With the exception of *pld* (the phospholipase D-encoding gene shown previously not to be expressed in an iron-dependent manner (Billington, *et al.* 2002)), the only virulence-related genes available in the NCBI sequence repository were *fagA* and the serine protease-encoding gene, *cp40* (accession number U10424). As stated above, the *fagA* gene had already been shown by Billington *et al.* (2002) to be regulated in response to environmental iron concentrations, and this result had been corroborated by the data presented in **Section 5.1.4**. Computational analysis of the *fagA* and *cp40* nucleotide sequences was performed, in order to determine whether either of the genes contained a putative DtxR-binding motif. Significantly, a putative DtxR-binding motif (TTAGTTTAGGCTAAACTGG) was identified 122 bp upstream of the first nucleotide of *fagA*, corroborating a previous report (Billington, *et al.* 2002). Furthermore, *cp40* was also observed to contain a putative DtxR-binding motif (GAAGTTATGCCTACCGTAA), although it was located 34 bp within the start of the gene, which was not consistent with the location of other DtxR-binding motifs in other genes.

Promoter fusions of *fagA* and *cp40* were constructed in pSPZ and the resulting recombinant plasmids were designated pCAW007 and pCAW008 respectively. This was achieved by cloning a 511 bp fragment corresponding to 211 bp within the 5' end of the gene to 300 bp upstream of *fagA* and a 488 bp fragment corresponding to 161 bp upstream of *cp40*. Even though *fagA* had already been shown to be expressed in an iron-dependent manner through qRT-PCR analysis (**Section 5.1.4**), it was included here as a positive control. The two plasmids were used to transform wild-type *C. pseudotuberculosis*, and β -galactosidase production was measured under high- and low-iron growth conditions (**Figure 5.15**). A paired t-test was applied to statistically analyse the data and, as expected, *fagA* was found to be expressed in an iron-dependent manner ($p=0.013$), corroborating previous qRT-PCR results. However, no significant difference in β -galactosidase production was detected under high- and low-iron growth conditions, in bacteria containing the *cp40* promoter fusion vector ($p=0.103$).

Previously, it had been possible to prove the involvement of *C. pseudotuberculosis* DtxR in the iron-dependent control of expression of *C. diphtheriae tox*, using *E. coli* as an experimental host (**Section 5.1.3**). By calculating the repression ratio it was possible to see that in the presence of DtxR the *tox* promoter was being repressed under high iron with a repression ratio of 2.12, whereas when DtxR was absent the repression ratio was less markedly less at 0.89. These experiments were conducted during a working visit to the laboratory of Prof. Randall Holmes at the University of Colorado at Denver and Health Sciences Centre, School of Medicine, Dept. of Microbiology, Aurora, Colorado, USA. With respect to *fagA*, although the results obtained thus-far had proven the differential expression of the gene in response to iron concentration, and a putative DtxR-binding motif had been identified upstream of the gene, the actual involvement of *C. pseudotuberculosis* DtxR in regulating *fagA* expression had yet to be proven. To achieve this objective, it was decided to undertake an equivalent experiment to that conducted previously in America, using *E. coli* as a “DtxR-deficient” host in which promoter fusion assays could be conducted in the presence/absence of a plasmid-encoded copy of the *C. pseudotuberculosis dtxR*-like gene. However, attempts to replicate the conditions of the assay back in the UK were, unfortunately, unsuccessful.

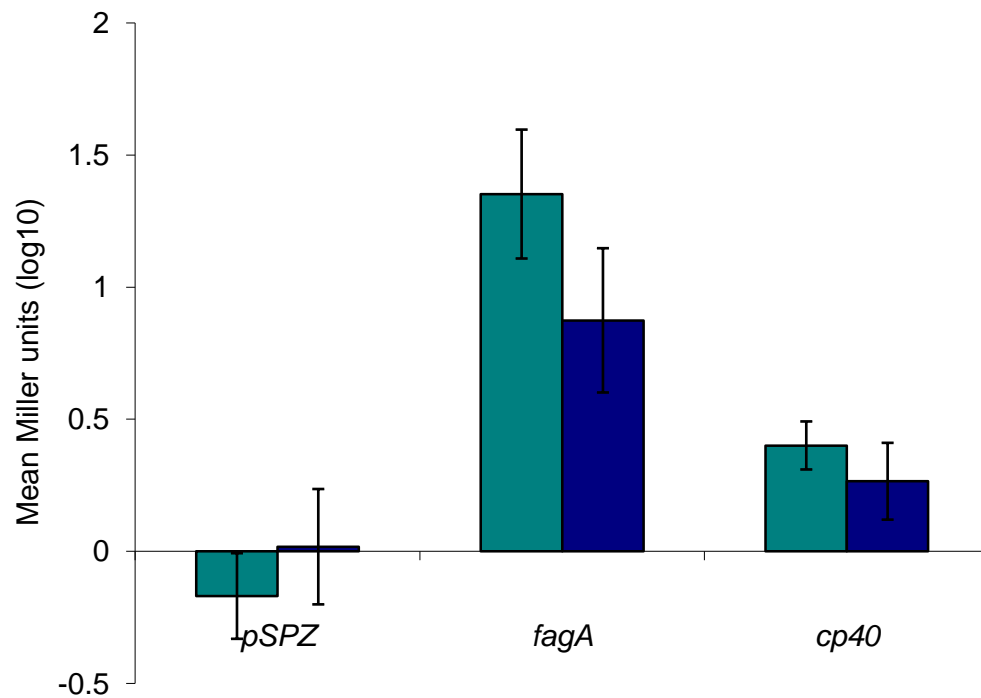


Figure 5.15 Iron-dependent regulation of gene expression of genes encoding published *C. pseudotuberculosis* virulence factors

Promoter fusion assays were undertaken to determine whether the expression of genes encoding published *C. pseudotuberculosis* virulence factors were regulated in response to environmental iron concentrations. Statistical t-tests confirm *fagA* is differentially expressed with respect to iron ($p=0.013$), whereas *cp40* is not ($p=1.03$). Bars correspond to low-iron (■), and high-iron (■).

5.1.6 Construction of a *dtxR*-deficient mutant strain of *C. pseudotuberculosis*

Due to the inability to assess the role of *C. pseudotuberculosis* DtxR using a plasmid-based system in *E. coli*, it was decided to generate a *dtxR*-deficient mutant of *C. pseudotuberculosis* 3/99-5 so that the expression of putative DtxR-regulated genes could be compared in the mutant and isogenic, wild-type parent strains. In order to create the mutant strain, the targeted allele-replacement mutagenesis system described in Chapter 4 was used. In the first instance, a deletion derivative of the *C. pseudotuberculosis* *dtxR* gene was constructed *in vitro*. PCR with the primers $\Delta dtxR_AE_1$ and $\Delta dtxR_AE_2$ (**Table 2.3**) was used to amplify a 1,022 bp fragment from immediately upstream of *dtxR*, which included the first 9 bp of the 5' end of the *dtxR* gene. Likewise, PCR using the primers $\Delta dtxR_AE_3$ and $\Delta dtxR_AE_4$ was used to amplify a 1,020 bp fragment from immediately downstream of *dtxR*, which included the last 3 bp of the 3' end of the *dtxR* gene. The two fragments were then ligated together (using the incorporated *XhoI* site), and the *ca.* 2 kb ligation product was amplified by PCR using primers $\Delta dtxR_AE_1$ and $\Delta dtxR_AE_4$; the resulting mutant gene, comprising a deletion of the majority of the *dtxR* coding sequence, was designated $\Delta dtxR$. The $\Delta dtxR$ amplicon was then cloned into *SalI* digested pCARV (facilitated by primer-encoded restriction endonuclease recognition sites), and the resulting vector, designated pCARV006, was used to transform *C. pseudotuberculosis*. Subsequently, the plasmid-borne $\Delta dtxR$ was used to replace the wild-type chromosomal gene by homologous recombination. In the first instance, bacterial cells having undergone integration of pCARV006 into the chromosome were identified by virtue of their resistance to erythromycin at 37°C (the non-permissive temperature for plasmid replication). These intermediary mutants (containing both chromosomal wild-type and plasmid-borne mutant *dtxR* genes) were analysed by PCR using primers pCARV_MCS F and *dtxR*_SX R (**Table 2.5**), specific for the vector and the insert (**Figure 5.16**). It was expected that if homologous recombination had occurred upstream of the $\Delta dtxR$ construct that a *ca.* 2,500 bp fragment would be amplified, corresponding to the region spanning the mutant gene; however, if recombination had occurred downstream of $\Delta dtxR$ then a *ca.* 3,000 bp fragment would be amplified, corresponding to the region spanning the wild-type gene. Significantly, due to the inclusion of the vector-specific pCARV_MCS F primer, a product could only be amplified from genomic DNA containing integrated plasmid. The data presented in **Figure 5.16** revealed

that homologous recombination was occurring successfully between homologous chromosomal and plasmid-borne sequences present on either side of $\Delta dtxR$.

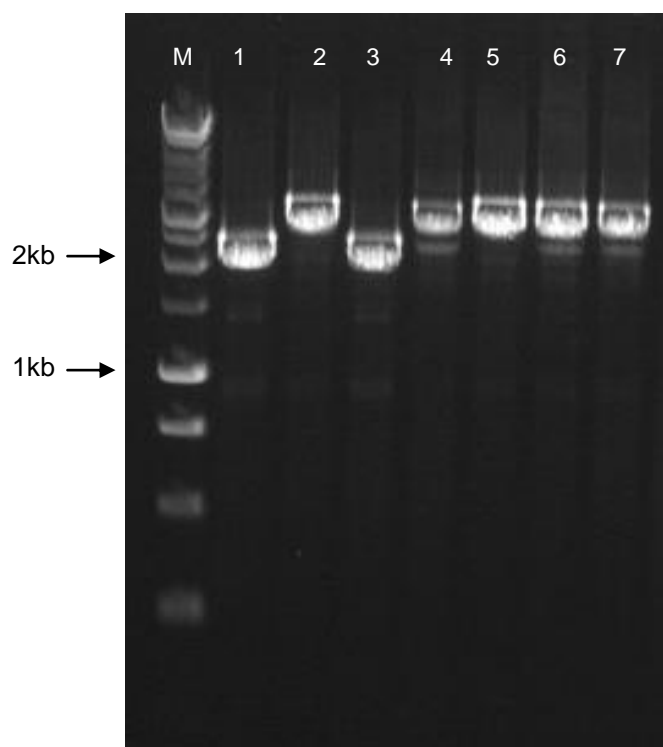


Figure 5.16 Analysis of single-crossover intermediate mutants of *C. pseudotuberculosis*. Cells having undergone chromosomal integration of pCARV006 were analysed by PCR to determine at which side of $\Delta dtxR$ recombination was occurring. Sample lanes correspond to 1 kb DNA ladder (M), co-integrants arising through recombination at the 5'-end of $\Delta dtxR$ (Lanes 1 and 3) or the 3'-end of $\Delta dtxR$ (Lanes 2, 4, 5, 6 and 7).

A single colony deriving from a cell in which pCARV006 had integrated into the chromosome was used to inoculate a quantity of BHI containing erythromycin. The culture was subsequently grown to stationary phase at 37°C × 225 rpm. The culture was then diluted (to 1×10^{-5}) in BHI and incubated at the permissive temperature for the plasmid (26°C), without any antibiotic selection. During this incubation, the ability of the integrated plasmid to replicate at the lowered temperature caused it to become unstable in the chromosome, driving its excision; subsequently, the absence of antibiotic selection resulted in the excised plasmid being lost from cells. In keeping with the allele replacement protocol developed in chapter 4, ampicillin enrichment of cells which had undergone secondary cross-over events was carried out in order to reduce the number required to be replica plated. Following ampicillin enrichment, 10-fold serial dilutions of the resulting culture were then plated on BHI agar and, following incubation, single colonies were replica plated onto BHI agar with and without antibiotic selection (allowing identification of cells from which the plasmid had excised). Subsequently, a number of colonies were identified which derived from cells in which a secondary cross-over event had occurred. Single colonies were screened by PCR with the primers $\Delta dtxR_DCO_F$ and $\Delta dtxR_DCO_R$, which spanned the region of the chromosome containing *dtxR*; it was anticipated that a wild-type chromosomal *dtxR* gene would give rise to a 1,259 bp fragment, while the $\Delta dtxR$ gene would give rise to a fragment of 559 bp. Unfortunately, in all cases, PCR screening led only to the identification of cells having excised the plasmid to leave the wild-type gene in the chromosome (data not shown). Despite repeated attempts to obtain a *dtxR*-deficient mutant, only the wild-type strain could ever be recovered, despite using low-iron growth media to prevent iron-toxicity. However, it was subsequently decided to omit the ampicillin-enrichment step, and at that point it became possible to isolate a mutant derivative containing the $\Delta dtxR$ gene in the chromosome.

The mutant *C. pseudotuberculosis* strain, designated Cp- $\Delta dtxR$, was checked to ensure that the *dtxR* deletion was as expected. Genomic DNA extracted from the wild-type or the Cp- $\Delta dtxR$ mutant was used as template in PCR reactions using the primers $\Delta dtxR_DCO_F$ and $\Delta dtxR_DCO_R$ (Table 2.3). As expected, these primers resulted in the amplification of a 1,259 bp fragment from the wild-type strain; however, the equivalent PCR product for the $\Delta dtxR$ strain was *ca.* 0.7 kb shorter, confirming the deletion within the chromosomal *dtxR* gene (Figure 5.17). In addition to PCR analysis, Southern blots were also performed, using probes specific for either *galE* or the erythromycin-resistance gene of pCARV (Figure 5.18).

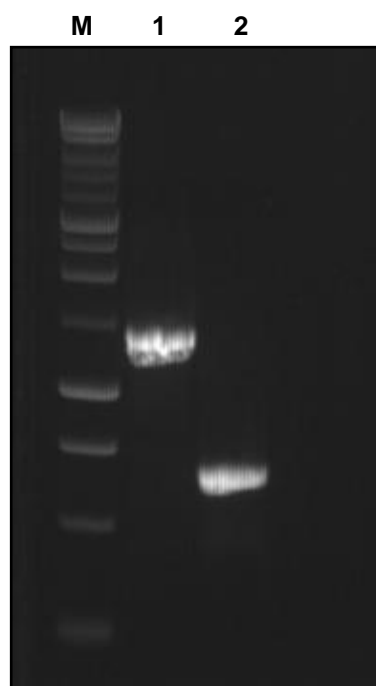


Figure 5.17 PCR analysis of the region across *dtxR* in *C. pseudotuberculosis* wild-type and Cp- $\Delta dtxR$ strains

Sample lanes correspond to 1 kb DNA ladder (M), *dtxR* from the wild-type strain *ca.* 1.2 kb (Lane 1) and $\Delta dtxR$ from the Cp- $\Delta dtxR$ strain *ca.* 0.6 kb (Lane 2)

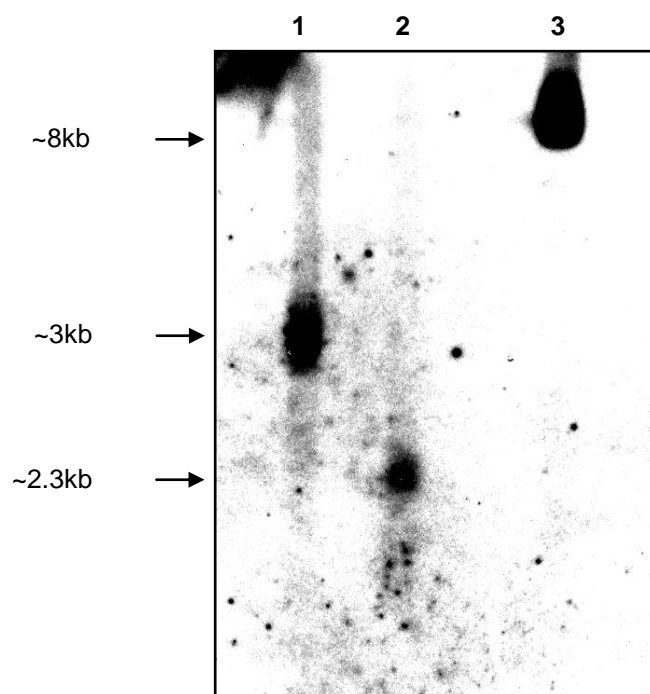


Figure 5.18 Southern Blot analysis of *C. pseudotuberculosis* wild-type and Cp- Δ dtxR genomic DNA

Sample lanes correspond to *dtxR* from the wild-type strain (Lane 1), Δ *dtxR* from the Cp- Δ *dtxR* strain (Lane 2) and pCAW002 (positive control) (Lane 3).

Genomic DNA from the Cp- Δ *dtxR* mutant and wild-type parent strains was digested to completion with *Hind*III (a previous Southern blot had revealed that *Hind*III produced a chromosomal fragment which contained both the *dtxR* and *galE* genes, and this observation was corroborated by *in silico* restriction analysis of the *ca.* 6.5 kb *dtxR* contig). As a result of the deletion within the *dtxR* gene of the mutant strain, probing with *galE* revealed a difference in size between the wild-type and mutant of *ca.* 0.7 kb; the mutant being smaller. In order to confirm that pCARV had been completely excised from the chromosome of the Cp- Δ *dtxR* mutant, and had subsequently been lost from the cell due to incubation at a temperature non-permissive for plasmid replication, the same *Hind*III-digested genomic DNA was probed with the DIG-labelled erythromycin-resistance gene from pCARV. As expected, no hybridisation was observed, confirming that the plasmid was absent (data not shown).

In order to ensure that no polar effects had occurred on the transcription of other genes in the *sigB-dtxR-galE* operon, RT-PCR analysis was carried out as it had been previously for the analysis of the transcription of these genes in the wild-type strain. Messenger RNA was extracted from exponential-phase cultures of the wild-type and Cp- Δ *dtxR* strains, and RT-PCR was conducted using the primers *sigB_B_F* and *galE_a_R* to detect the presence of a *sigB-dtxR-galE* transcripts. As expected, transcripts were detected in mRNA from both strains; however, the Cp- Δ *dtxR* transcript was *ca.* 0.7 kb smaller than that of the wild-type, corresponding to the size of the deletion within *dtxR* (**Figure 5.19**). No transcripts were detected in Cp- Δ *dtxR* mutant or wild-type parent mRNA that had not been reverse transcribed, confirming the absence of genomic DNA contamination.

In addition to the genomic and transcriptional analyses of the Cp- Δ *dtxR* mutant strain, phenotypic differences between it and the isogenic wild-type parent were also observed. When grown on high-iron-containing medium, the colony size of the Cp- Δ *dtxR* mutant appeared to be small in comparison with those of the wild-type parent grown on the same medium. Furthermore, preliminary experiments were conducted to assess the tolerance of the mutant versus wild-type to H₂O₂ exposure. Briefly, stationary phase *C. pseudotuberculosis* cultures were plated as a lawn onto BHI agar plates containing 100 μ M of 2', 2'-dipyridyl. A 5 mm WhatmanTM filter disc was placed directly onto the centre of each plate, and 10 μ l of 30 %, 3 % or 0.3 % hydrogen peroxide (diluted in ddH₂O) was pipetted directly onto each filter disc. Subsequently, plates were incubated at 37°C for 48 hrs to allow bacterial growth and the

zone of growth inhibition surrounding the filter discs was measured. Although sufficient time was not available to undertake a thorough analysis, preliminary results suggested that the Cp- $\Delta dtxR$ mutant survived less-well than the wild-type in a hydrogen peroxide inhibition assay (data not shown).

5.1.7 Analysis of *C. pseudotuberculosis* DtxR regulation of target genes

As stated above, in order to unequivocally determine the involvement of DtxR in the iron-dependent regulation of *C. pseudotuberculosis* genes, *cp40* and *fagA*, promoter fusion assays were carried out using the wild-type and Cp- $\Delta dtxR$ mutant strains as hosts for plasmid constructs. This experiment meant it was possible to directly compare the extent of repression of each gene when DtxR was present (wild-type) and absent (Cp- $\Delta dtxR$). The *C. pseudotuberculosis* strains were transformed with the promoter fusion plasmids pCAW007 and pCAW008 and subsequent differences in the level of β -galactosidase production from each construct, under high- and low-iron growth conditions, was recorded for each strain (**Figure 5.20**). A paired t-test was used to compare the expression of *fagA* and *cp40* under high- and low-iron growth conditions in the Cp- $\Delta dtxR$ mutant strain. The results suggest there was not a significant difference between the expression of *fagA* ($p=0.086$) or *cp40* ($p=0.329$) under high or low-iron growth conditions. These results indicated that the absence of DtxR affected the influence of iron on repression of the iron-regulated *fagA* gene expression. In addition ANOVA was employed to statistically analyse the difference between the two strains (wild-type and Cp- $\Delta dtxR$). The analysis provided statistical evidence to suggest that there was a significant difference in gene expression of *fagA* in wild-type compared to Cp- $\Delta dtxR$ ($p=0.005$). This suggested that, as predicted, the absence of DtxR had an effect on iron-dependent regulation of *fagA* gene expression under differential iron growth conditions. The difference between the level of expression of *fagA* under high-iron conditions was greater than the difference under low-iron conditions (wild-type mean at high-iron was 0.87 ± 0.06 and Cp- $\Delta dtxR$ high-iron was 1.42 ± 0.06 , compared to wild-type at low-iron which was 1.35 ± 0.06 and Cp- $\Delta dtxR$ at low-iron was 1.54 ± 0.06) which indicated an interaction effect between strain and iron. In contrast, the statistical evidence suggested that there was no difference ($p=0.290$) between *cp40* gene expression in wild-type or Cp- $\Delta dtxR$, and that the

difference in expression of *cp40* in wild-type compared to Cp- Δ *dtxR* was similar at both high and low levels of iron.

5.1.8 Complementation of the *C. pseudotuberculosis* *dtxR* mutant

The pEP2 vector (Radford & Hodgson, 1991) was used to provide a plasmid-borne copy of the wild-type *C. pseudotuberculosis* *dtxR* gene. This was achieved by performing an *EcoRI* digest on the previously constructed pWSK29/*dtxR* (Table 2.2), thereby excising the full *C. pseudotuberculosis* *dtxR* gene and its promoter region, which was subsequently cloned into *EcoRI*-digested pEP2; the resulting construct was designated pCAW010 (Table 2.2). This construct was used to transform Cp- Δ *dtxR* and thereby provide an *in trans* copy of a fully-functional *C. pseudotuberculosis* DtxR protein. Under high- and low-iron conditions the growth of the complemented mutant was compared to that of the wild-type parent strain and the uncomplemented Cp- Δ *dtxR* mutant (Figure 5.21). Significantly, the growth of the complemented mutant under high-iron conditions was restored to that of wild-type, confirming that deletion of the *dtxR* gene had given rise to the observed phenotype. Preliminary analyses demonstrated that sensitivity to hydrogen peroxide was also restored to wild-type levels (data not shown).

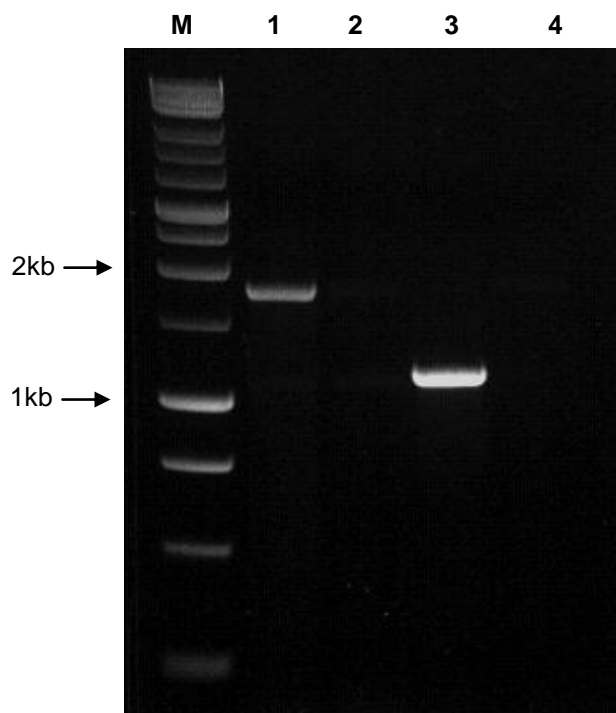


Figure 5.19 Determination of the presence of *sigB-dtxR-galE* polycistronic mRNA in *C. pseudotuberculosis* wild-type and *Cp-ΔdtxR* strains

Sample lanes correspond to 1 kb DNA ladder (M), *sigB-dtxR-galE* transcript from wild-type cDNA with and without reverse transcriptase (Lanes 1 & 2) and *sigB-dtxR-galE* transcript from *Cp-ΔdtxR* cDNA with and without reverse transcriptase (Lanes 3 & 4).

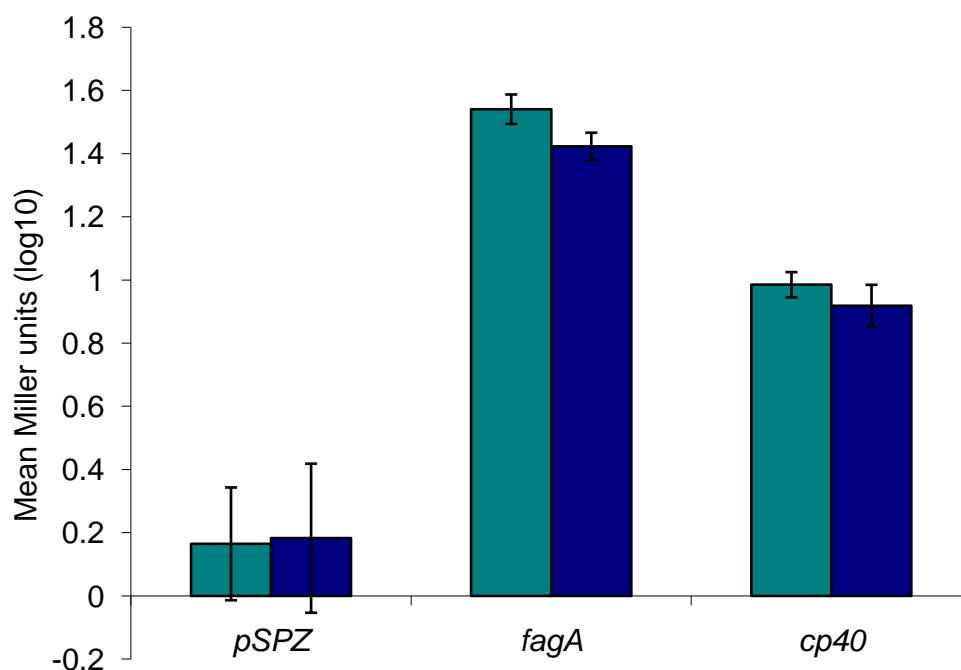


Figure 5.20 Assessment of DtxR-regulation of *C. pseudotuberculosis* genes

The Cp- Δ *dtxR* strain was used as a host for pCAW007 and pCAW008 in a promoter fusion assay. Beta-galactosidase activity was measured and Miller units were calculated to determine the expression of *fagA* and *cp40* under high- and low-iron conditions under the control of the Cp- Δ *dtxR* mutant host. A paired t-test indicated that expression of *fagA* in Cp- Δ *dtxR* was not differentially expressed ($p=0.086$) and nor was *cp40* ($p=0.329$) implicating DtxR in the regulation of *fagA*. Bars correspond to low-iron (■) and high-iron (■) growth conditions.

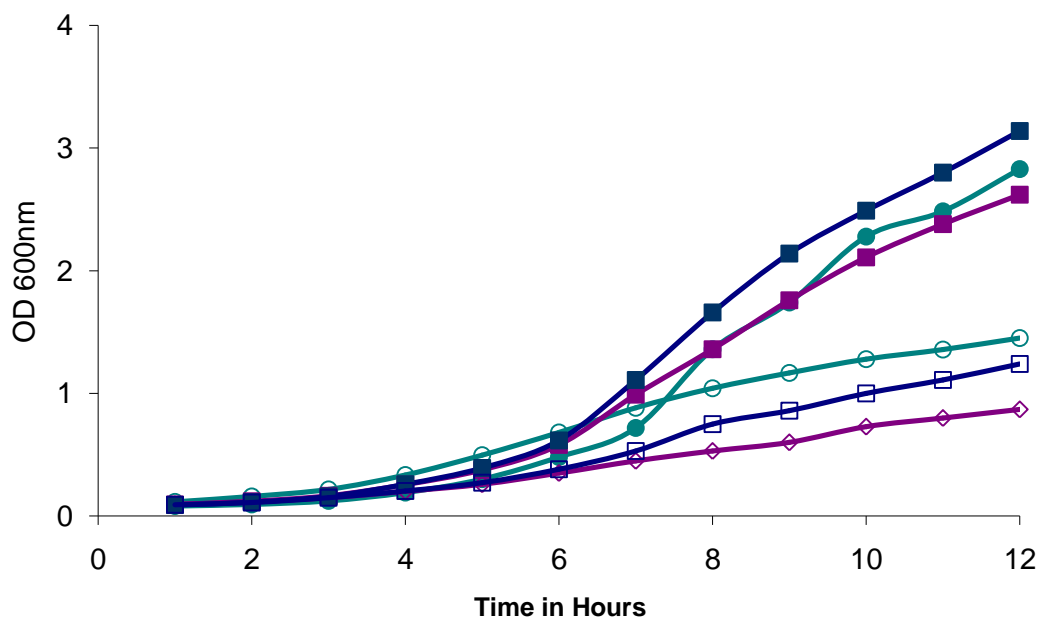


Figure 5.21 Growth of *C. pseudotuberculosis* wild-type, Cp-ΔdtxR and Cp-ΔdtxR + DtxR strains under high- and-low iron conditions

The graph corresponds to wild-type grown under high-iron conditions (●), wild-type grown under low-iron conditions (○), Cp- ΔdtxR grown under high-iron conditions (■), Cp- ΔdtxR grown under low-iron conditions (□), Cp- ΔdtxR + DtxR grown under high-iron conditions (■), Cp- ΔdtxR + DtxR grown under low-iron conditions (□)

5.1.9 Transition metal activation of *C. pseudotuberculosis* DtxR

In vitro, DtxR of both *C. diphtheriae* and *C. glutamicum* have been shown to bind transition metals other than iron (namely zinc and manganese), resulting in an effect on the ability of DtxR to regulate gene expression (Qiu, *et al.* 1995; Tao, *et al.* 1995). To determine whether *C. pseudotuberculosis* DtxR was also activated by other transition metal ions, the *fagA* promoter fusion vector, pCAW007, was introduced into wild-type *C. pseudotuberculosis* 3/99-5 and the transformed strain was cultured with and without zinc and manganese (for the purpose of this study, in addition to iron, only the transition metals zinc and manganese were assessed). Following transformation with pCAW007, wild-type *C. pseudotuberculosis* was cultured in CCDM containing 10 μM (final concentration) of either zinc, manganese or iron (as a positive control) in addition to CCDM alone to determine background levels of DtxR repression through binding of metal ions already in the medium. The low-ion medium was prepared in the same way as it had been throughout this thesis by adding 300 μM of dipyrldyl to CCDM (**Figure 5.22**). In order to compare the level of repression that resulted from the different metal ions, the “repression ratio” was calculated by dividing the mean Miller units observed under CCDM with 300 μM dipyrldyl (low-ion) conditions by the mean Miller units observed under high-ion for each metal-ion and CCDM only as a control. The results of these experiments revealed that, in this *in vitro* assay, manganese was able to activate DtxR (repression ratio of 2.6), although iron was clearly the more potent metal ion (repression ratio of 3.3). In contrast, there appeared to be little repression of DtxR in the presence of zinc, with a repression ratio of 1.8 which was less than that observed in CCDM only (repression ratio of 2.0).

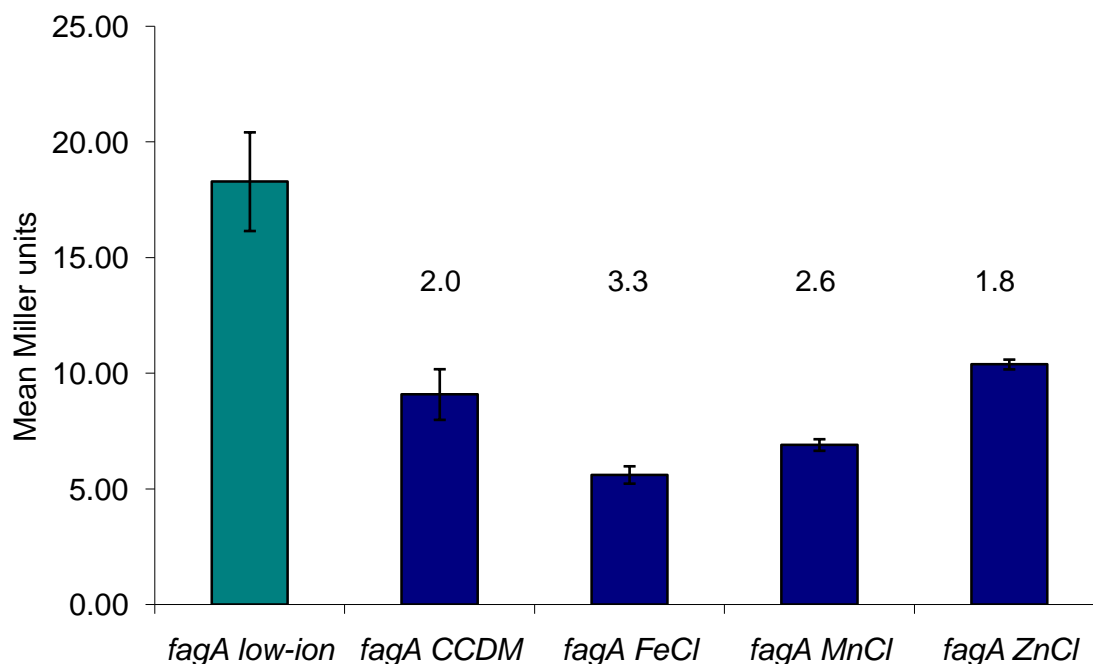


Figure 5.22 Promoter fusion assay to determine the efficiency of zinc and manganese in the activation of DtxR and subsequent repression of the *fagA* promoter

The *fagA* promoter fusion vector, pCAW007, was introduced into wild-type *C. pseudotuberculosis* 3/99-5. The transformed strain was grown under low-ion conditions (CCDM with the addition of 300 μ M dipyrityl ■), and high-ion (10 μ M final concentration of manganese, zinc and iron ■); CCDM was included as a control to determine the level of background repression. The repression ratio (indicated above the bars) was calculated by dividing the mean Miller units under low-ion conditions by the mean Miller units under high-ion for each metal-ion and CCDM only as a control. The repression ratio highlights the extent of the repression on the *fagA* promoter in the presence of different metal ions. The background level of repression seen with CCDM alone was 2.0, zinc showed a repression ratio of 1.8, manganese was 2.6 and iron, which was the most potent repressor had a repression ratio of 3.3.

5.2 Discussion

Previously in our laboratory a 312 bp fragment, of what has now been confirmed in this study to correspond to the *dtxR* gene of *C. pseudotuberculosis*, was amplified by PCR cloned, and sequenced. Using that novel sequence, a strategy for the inverse PCR amplification of the remainder of the gene and the surrounding chromosomal region was devised, resulting in the recovery of a *ca.* 6.5 kb fragment containing homologues of the *sigB*, *dtxR*, *galE* and helicase genes described previously in other sequenced corynebacteria, including *C. diphtheriae* (Cerdeno-Tarraga, *et al.* 2003) and *C. glutamicum* (Oguiza, *et al.* 1996b) (Kalinowski, *et al.* 2003). Interestingly, a similar gene arrangement has been observed for two of the genes in the locus in the other actinomycetes, *M. tuberculosis*, *M. smegmatis*, and *Rhodococcus equi*, whereby a *sigB* gene has been shown to lie immediately upstream of *ideR*, the equivalent of *dtxR* (Boland & Meijer, 2000; Doukhan, *et al.* 1995). Interestingly, in *R. equi*, unlike *M. tuberculosis* and *M. smegmatis*, a *galE* gene also lies immediately downstream of *dtxR*. The similarities within this locus indicate that the linkage, of *sigB* and *dtxR* (*ideR*) at least, is highly-conserved among members of the order Actinomycetales. The reason for the linkage of *sigB* and *dtxR* (*ideR*) is currently unknown; however, Doukhan *et al.* (1995) suggest a functional significance (Doukhan, *et al.* 1995).

The predicted *C. pseudotuberculosis* DtxR protein sequence has been shown to share *ca.* 70 % homology with *C. glutamicum* DtxR, and 90 % homology with that of *C. diphtheriae*. In addition to this high level of sequence homology, alignment of the sequences of the 3 proteins has revealed that domains 1 and 2, which are involved in DNA- and metal ion-binding respectively (Qiu, *et al.* 1995) (Schiering, *et al.* 1995), are highly conserved between all three species, particularly *C. pseudotuberculosis* and *C. diphtheriae* (**Figure 5.6**). Domain 3 of DtxR is known to contribute ligands to the metal binding site in domain 2. Furthermore, domain 3 is predicted to control the activity of the protein at specific promoters (Love, *et al.* 2004; Oram, *et al.* 2005). Interestingly, domain 3 was highly variable between *C. pseudotuberculosis*, *C. diphtheriae* and *C. glutamicum*, an observation which has been reported previously for comparisons between sequenced corynebacteria (Oram, *et al.* 2005). Oram *et al.* (2005) discovered DtxR domain 3 in *C. diphtheriae* contains immunodominant epitopes, and is required for full repression of *tox* in an *E. coli* reporter system. However, it is not required for DNA binding *in vitro* (Oram, *et al.* 2005). Oram *et al.* (2005) also suggested

that, in some members of the DtxR family, the variability within the third domain potentially affects the intra-molecular interactions that this domain has with domains 1 and 2. Furthermore, Oram *et al.* (2005) reported that the biological activity, which is a result of domain 3 interactions, is also variable and subsequently species-specific roles for the third domain of these transcriptional regulators is, as yet, undefined (Oram, *et al.* 2005).

In vitro, DtxR, including that of *C. pseudotuberculosis* during this work, has been shown to be capable of binding several different metal ions, resulting in the conformational change that permits the protein to act as a repressor of gene expression. However, *in vivo* DtxR would appear to be almost entirely iron-specific (Groman & Judge, 1979). In *in vitro* studies, DT production in *C. diphtheriae* did appear to be repressed in the presence of manganese; however, a 1,000-fold higher concentration of manganese than iron was required to achieve the same level of repression (Groman & Judge, 1979). It had previously been discovered that the Cysteine residue at amino acid 102, (Cys-102) is involved in heavy metal ion activation of DtxR (Tao & Murphy, 1993). Later, using site-directed mutagenesis, Geudon *et al.* (2003) investigated the effect of substituting specific amino acids on the metal ion specificity of DtxR and MntR (a manganese dependent DtxR-homologue). The investigators made changes to the two variable residues in the metal-sensing site, and subsequently converted DtxR to a manganese responsive regulator (Guedon & Helmann, 2003). Furthermore, the researchers were also able to make the manganese responsive MntR protein responsive to iron in a similar way. These results suggest that the Cys-102 residue is responsible for the specificity of *C. diphtheriae* DtxR for iron, *in vivo* (Guedon & Helmann, 2003). Oram *et al.* (2004) reported that the highly conserved sequence of the *Corynebacterium* DtxR-like proteins that they had investigated, exhibited a much higher overall homology to iron-responsive members of the DtxR family, than to those that are responsive to manganese (Oram, *et al.* 2004). One can postulate then, that due to the high degree of homology between *C. pseudotuberculosis* DtxR and *C. diphtheriae* DtxR and, given that the same Cys residue resides at amino acid position 102 in *C. pseudotuberculosis*, it is likely that DtxR of *C. pseudotuberculosis* is iron-specific *in vivo*. Certainly, the results of the analyses of the affect of different transition metal ions on *C. pseudotuberculosis* DtxR function in the laboratory environment proved that iron was the preferred transition metal. However, even though manganese also resulted in the repression of target gene expression, it is worth noting that the levels of accessible manganese *within the*

natural host (i.e. sheep/goats) is likely to be significantly less than the equivalent levels of iron.

The conservation of the *dtxR* locus in the genus *Corynebacterium* is rather interesting; one could hypothesise that this region has been highly conserved due to its importance to the survival and fitness of the bacteria in question. However, a recent publication by Nakamura *et al.* (2003) suggests that the integrity of the genomes of sequenced corynebacteria is due to the absence of the *recBCD* genes. The authors believe the absence of the RecBCD pathway in corynebacteria could have prevented frequent genome inversions, giving rise to greater genomic stability and hence the likelihood that gene order will be conserved over stretches of the genome (Nakamura, *et al.* 2003).

Having identified the conserved nature of the *dtxR* locus of *C. pseudotuberculosis* and that of other sequenced corynebacteria, the next logical step in this study was the investigation of the functionality of the protein. In order to confirm the ability of *C. pseudotuberculosis* to regulate known *C. diphtheriae* DtxR regulated genes, the promoter regions of two known DtxR regulated genes which had previously been fused to a *lacZ* reporter gene in pSPZ (*toxP* and *irp3P*) (Oram, *et al.* 2006)(Holmes R.K. personal communication). Interestingly, when *toxP* and *irp3P* were introduced into wild-type *C. pseudotuberculosis* and grown under high- and low-iron conditions, both the *tox* and *irp3* promoters were found to be regulated in response to environmental iron concentrations (**Figure 5.8**). Although the expression of *tox* was only found to show a trend towards significance ($p=0.068$) between low- and high-iron growth conditions in *C. pseudotuberculosis*, it is likely this was due to the relatively low levels of expression observed with this promoter, which subsequently led to high variability within the data. Given a larger data set, it is possible that the differential expression of *tox* would have been more evident. Significantly however, the expression of *tox* in the natural host, *C. diphtheriae*, has also been shown to be relatively low (Oram, *et al.* 2006).

Despite proving iron-dependent expression of DtxR-regulated *C. diphtheriae* genes in *C. pseudotuberculosis*, the above experiment did not confirm the involvement of *C. pseudotuberculosis* DtxR in this regulation. For this reason, a further experiment was conducted, using *E. coli* as a DtxR-deficient host to assess the capacity of the product of the recombinant *C. pseudotuberculosis dtxR* gene to repress expression from the *tox* promoter.

Significantly, *C. pseudotuberculosis* DtxR was able to repress the *tox* promoter in an iron-dependent manner, proving that *C. pseudotuberculosis* and *C. diphtheriae* DtxR proteins are functionally compatible. It is also interesting to note that the DtxR homologues of *C. glutamicum*, *M. tuberculosis* (IdeR) and *R. equi* (IdeR) are also able to repress the *C. diphtheriae tox* promoter in an iron-dependent manner in an *E. coli* host (Boland & Meijer, 2000; Oguiza, *et al.* 1995; Schmitt, *et al.* 1995). These prior observations, combined with the results of this study, demonstrate the conservation between the functions of these important proteins.

Oram *et al.* (2004) previously assayed chromosomal DNA of 42 different isolates of *Corynebacterium*, including a *C. pseudotuberculosis* strain, for the presence of a *dtxR* gene. The portion of the genes assessed contained the sequence encoding domains 1 and 2 (the helix-turn-helix DNA-binding motif and the metal ion binding site). Interestingly, the investigators noticed an extremely high level of conservation in the region of the *dtxR* genes that they tested, leading them to postulate that these iron-dependent gene regulators are under strong selective constraints (Oram, *et al.* 2004). However, whether this statement is true, considering the fact that corynebacteria appear to be unable to undergo significant genetic rearrangements, due to the lack of recombination enzymes (Nakamura, *et al.* 2003), remains to be determined, since the strong selective constraints postulated may simply be an *inability* to change.

To further characterise the *C. pseudotuberculosis dtxR* locus, investigation of the transcriptional regulation of *dtxR* and surrounding genes was explored. This was achieved using two methods, firstly, by the use of promoter fusion assays to identify regions possessing promoter activity within the locus, and secondly by using qRT-PCR, to assess the relative transcript abundance of the genes within the *dtxR* locus. The promoter fusions, constructed by cloning fragments from immediately upstream of *sigB*, *dtxR* and *galE* respectively, into the pSPZ *lacZ* fusion vector and introducing the constructs into wild-type *C. pseudotuberculosis*, confirmed the presence of promoters for both the *sigB* and *dtxR* genes; however no promoter was identified upstream of *galE*, suggesting that no promoter is present for this gene. It is not surprising that the *galE* gene does not possess its own promoter in *C. pseudotuberculosis* since there is only a 15 bp gap between the 3' end of *dtxR* and the 5' end of *galE*. The results observed in this study are in agreement with those reported previously for *C. diphtheriae*

(Oram, *et al.* 2006), whereby only two promoter regions were identified within the *dtxR* locus of that organism. In the course of their study, Oram *et al.* (2006) used promoter fusion assays and RNase protection assays to determine the position of the promoters and equivalent to the situation in *C. pseudotuberculosis*, the *C. diphtheriae* promoters were only found upstream of *sigB* and *dtxR*.

In *C. diphtheriae*, *galE* is co-transcribed with *dtxR* (Oram, *et al.* 2002; Oram, *et al.* 2006). The resulting mRNA is said to be bicistronic, implying the promoter region of *dtxR* produces a single mRNA molecule that encodes two separate gene transcripts, *dtxR* and *galE*. The co-transcription of *dtxR* and *galE* has also been recognized in other actinomycetes. For example, Oguiza *et al.* (1996) investigated the transcriptional analysis of the *dtxR* and *galE* genes of *C. glutamicum* ATCC 13869. In nutrient rich broth, the researchers demonstrated that these genes formed part of an operon that is actively transcribed as a bicistronic mRNA. They also reported that *dtxR*-homologue could be expressed as a monocistronic transcript under certain conditions (Oguiza, *et al.* 1996a). In *R. equi*, IdeR (the DtxR homologue) shares greater homology with *M. tuberculosis* (75%) than with *C. diphtheriae* (57%); however, the organisation of the genes in the locus in which the IdeR structural gene lies was observed to be more similar to that of *C. diphtheriae*, in that there was a *galE* gene just downstream of *ideR* (Boland & Meijer, 2000). Furthermore, Boland *et al.* (2000) postulated that, due to the small sequence gap between *ideR* and *galE* in *R. equi*, these genes too are likely to be co-transcribed (Boland & Meijer, 2000). In *C. glutamicum* ATCC 13032, the order of genes in the *dtxR* locus is the same as that of *C. diphtheriae* and *C. pseudotuberculosis*; it is therefore likely that *dtxR* and *galE* are also co-transcribed in *C. glutamicum*.

Reasons for the co-transcription of *dtxR* and *galE* are unclear; however, galactose is an important component in corynebacterial cell wall biosynthesis, since the galactan in arabinogalactan, which is an integral cell wall component, is a polymer of galactose (Dover, *et al.* 2004). Furthermore, in *C. diphtheriae*, transcription of *dtxR* occurs constitutively at a low level (Schmitt & Holmes, 1991). Being required for cell wall biosynthesis, one could argue that galactose is constantly required by the cell; in short, *galE* is required to manufacture galactose, galactose is essential in the construction of the cell wall, and therein lays the requirement for *galE* to be constitutively expressed. If two genes are constitutively expressed

it makes sense for them to be co-transcribed as this results in less of a burden on the bacterial cell's resources.

To verify the results seen with the promoter fusion assays, qRT-PCR was subsequently employed to assess the expression of the genes in the *C. pseudotuberculosis dtxR* locus. Interestingly, the *galE* transcript was apparently less abundant than that of the co-transcribed *dtxR-galE*. In a similar experiment carried out in *C. diphtheriae*, Oram *et al.* (2006) previously suggested that if *galE* can be transcribed both from a promoter in the *dtxR-galE* intergenic region and also by extension of *dtxR*-specific transcripts that initiate further upstream, then the transcripts detected with the *galE* probe should be more abundant than the transcripts detected by the *dtxR-galE* intergenic probe (Oram, *et al.* 2006). Therefore, by analogy it is possible to propose that *C. pseudotuberculosis galE* is co-transcribed with *dtxR*, similarly to the scenario in *C. diphtheriae*.

In *C. diphtheriae* and *C. glutamicum*, co-transcription of both *sigB-dtxR* and *dtxR-galE* has been described (Oguiza, *et al.* 1996a, 1996b; Oram, *et al.* 2006). However, in *C. diphtheriae*, co-transcription of *sigB-dtxR-galE* has also been described (Oram, *et al.* 2006). In the current study, an equivalent *C. pseudotuberculosis* polycistronic message was detected, proving that the transcription of genes within the *C. pseudotuberculosis dtxR* locus is closely similar to those of the equivalent locus in *C. diphtheriae*. In contrast however, it is interesting to note that, although the arrangement of the *sigB-ideR* genes in *Mycobacterium* sp. is similar to that of *Corynebacterium*, the genes are not co-transcribed in this species (Smith, *et al.* 1998) (Lee, *et al.* 2008). Therefore, there are clearly differences in the expression of these genes among members of the order Actinomycetales.

Larisch *et al.* (2007) demonstrated that SigB of *C. glutamicum* is implicated in the regulation of gene transcription during the transition from exponential- to stationary-growth phase (Larisch, *et al.* 2007). Furthermore, Oram *et al.* (2006) discovered that, in *C. diphtheriae*, *sigB* transcripts were more abundant following changes in several environmental conditions, including exposure to acid, cold, heat, ethanol and SDS; this suggested a possible role for SigB in the *C. diphtheriae* response to environmental stress conditions (Oram, *et al.* 2006). Similar results were seen in the closely related *Brevibacterium flavum*, where expression of *sigB* was significantly enhanced after exposure to several different stress conditions

(Halgasova, *et al.* 2001). It is possible SigB of *C. pseudotuberculosis* is also involved in the regulation of gene expression during the transition to stationary-growth phase, or perhaps more appropriately in protection of the cell under conditions of environmental stress (such as the nutrient limitation associated with the shift towards stationary-growth phase). The co-transcription of *sigB* and *dtxR* could be a means of facilitating the cell's preparation for the environmental changes that ensue at stationary phase of growth. Certainly, with respect to nutrient limitation, it is highly likely that environmental iron concentration will be low; therefore, DtxR may need to up regulate iron sequestration genes and down regulate iron storage genes.

The *E. coli* Fur protein is, like DtxR, an iron-dependent transcriptional regulator. Interestingly, in some bacteria, such as *Helicobacter pylori* and *Neisseria meningitidis*, the expression of the Fur-encoding gene has itself been shown to be autoregulated in response to environmental iron concentrations (Delany, *et al.* 2003; Delany, *et al.* 2002). Delany *et al.* (2002) suggested that this type of negative feedback loop is critical in the homeostasis of iron. Since it is such an important element, essential, yet toxic to bacteria in large quantities, intracellular concentrations of iron are required to be tightly regulated; fine tuning via a negative feedback loop dampens the extremes of responses (Delany, *et al.* 2002). It is well documented that DtxR and Fur, although undertaking the same activity, are actually very different proteins. It was therefore decided to determine whether *C. pseudotuberculosis* *dtxR*, like *fur*, is autoregulated in response to environmental iron concentration. In order to determine whether DtxR of *C. pseudotuberculosis* was able to regulate expression of its own structural gene in an iron-dependent manner, both the promoter fusion assays and qRT-PCR experiments were carried out using bacteria cultured under high- and low-iron conditions. Interestingly, and in agreement with the results of studies carried out with *C. diphtheriae* (Oram, *et al.* 2006), the two putative promoters upstream of *sigB* and *dtxR* did not appear to be regulated in response to environmental iron concentration in *C. pseudotuberculosis*. It therefore seems that, unlike Fur in some bacteria, DtxR is not autoregulated in response to iron, at the very least not in either *C. diphtheriae* or *C. pseudotuberculosis*.

Having assessed the transcription of genes within the *C. pseudotuberculosis* *dtxR* locus, the involvement of DtxR in the regulation of other target genes was studied. The promoter regions of two previously published *C. pseudotuberculosis* genes (*fagA* and *cp40*) were

investigated in promoter fusion assays, in order to determine whether they were expressed differentially under high- and low-iron growth conditions. Previously, Billington *et al.* (2002) identified a DtxR-like binding motif upstream of *fagA*, although the authors made no attempt to study the potential involvement of DtxR (or a DtxR-homologue) in the regulation of this gene (Billington, *et al.* 2002). Billington *et al.* (2002) using promoter fusion assays did however show that *fagA* was expressed in response to environmental iron concentrations. The results of the promoter fusion assay carried out during this investigation are in agreement with the results of Billington *et al.* (2002). Moreover, the results of the qRT-PCR experiments conducted during this study, whereby *fagA* transcript was observed to be 16-fold higher under low-iron growth conditions as compared to high-iron conditions, also support these results.

In addition to *fagA*, another *C. pseudotuberculosis* gene, *cp40*, was also assessed to determine whether it was expressed in response to environmental iron conditions. The *cp40* gene encodes a 40 kDa protein that shares homology with serine proteases (Wilson, *et al.* 1995). Wilson *et al.* (1995) undertook a study whereby immunodominant proteins produced by *C. pseudotuberculosis* during infection of sheep were identified. Cp40 was shown to be one of the first *C. pseudotuberculosis* proteins to which sheep responded immunologically. Subsequently, vaccination of sheep with recombinant Cp40 was found to partially-protect against subsequent challenge with *C. pseudotuberculosis*. On the basis of these observations, Cp40 has been implicated as contributing to disease pathogenesis, although no role for this protein has yet been defined. Since DtxR is known to regulate the expression of virulence genes in other bacteria, it was thought possible that the same may be true of *cp40* in *C. pseudotuberculosis*; this hypothesis was strengthened by the identification of a putative DtxR-binding motif within the 5'-region of the *cp40* gene. Despite this hypothesis, however, the results of this study confirm that the expression of *cp40* is not regulated by DtxR, nor is it affected by variations in iron-concentration through an alternative regulatory mechanism. Of significance, it is worth noting that DtxR-binding motifs are normally found within the promoter-containing region upstream of DtxR-regulated genes, and not within the genes themselves; however, the presence of a predicted DtxR-binding domain so close to the 5'-end of *cp40* made a convincing case for further study.

To unequivocally prove the involvement of DtxR in the iron-dependent regulation of *C. pseudotuberculosis* genes, a mutant derivative of *C. pseudotuberculosis* 3/99-5 was

constructed, which was deficient in production of DtxR. The mutant, designated Cp- Δ dtxR, was derived from the deletion of a ca. 699 bp portion of *dtxR*. The generation of a *C. pseudotuberculosis* *dtxR* mutant proved to be significantly more difficult than was initially anticipated; however, in retrospect the difficulties experienced are perhaps not so surprising. Previous efforts to construct a *cp40* deletion mutant of *C. pseudotuberculosis* 3/99-5, using the allele-replacement mutagenesis procedure described in Chapter 4, were successful, with the desired mutant strain being constructed quickly and without problems. It was therefore rather perplexing as to why, using the same method, the isolation of a *dtxR* mutant became such an ordeal. Several hypotheses were considered in order to explain why a *dtxR* deletion derivative of *C. pseudotuberculosis* could not be obtained. Firstly, it was thought possible that the *dtxR* gene was essential, and if this were the case then all attempts to construct a deficient mutant would be pointless. Confusingly, evidence was found to be available in the published literature to both corroborate and refute this hypothesis. On the one-hand, investigators were reportedly unable to construct an *ideR*-deficient derivative of *M. tuberculosis*, unless a fully-functional copy of the gene was expressed *in trans* (Rodriguez, *et al.* 2002). Substantiating this train of thought, further evidence hinting at the fact that a *dtxR* mutation in *C. pseudotuberculosis* may be lethal was derived from a report by Oram *et al.* (2004), whereby it was suggested that a 275 bp fragment of the *dtxR*-homologue of *C. pseudotuberculosis* shared more homology with *Mycobacterium ideR* than that of other corynebacterial species (Oram, *et al.*, 2004). Previously, *dtxR* of *C. diphtheriae* was thought to be an essential gene; however, Oram *et al.* (2002) conclusively demonstrated that the gene was not essential by isolating a viable transposon insertion mutant that was unable to produce DtxR (Oram, *et al.* 2002). The researchers subsequently created an in-frame *dtxR* deletion derivative of *C. diphtheriae* (Oram, *et al.* 2006). Furthermore, a *dtxR* deletion mutant was also isolated from *C. glutamicum* (Wennerhold & Bott, 2006) (Brune, *et al.* 2006), and an *ideR* deletion mutant was isolated from the non-pathogenic *Mycobacterium smegmatis* (Dussurget, *et al.* 1996). Given the similarity between the *C. pseudotuberculosis* and *C. diphtheriae* *dtxR* loci determined throughout the course of this study, it was considered likely that, as with *C. diphtheriae*, the *dtxR* gene of *C. pseudotuberculosis* was also not essential.

A second hypothesis for why a *C. pseudotuberculosis* *dtxR* mutant could not initially be obtained was that a *dtxR* deletion mutant of *C. pseudotuberculosis* would be more sensitive to iron toxicity than its wild-type parent, and therefore a mutant strain would be less likely to

survive under high-iron growth conditions. Both *C. diphtheriae* and *C. glutamicum dtxR* mutants were observed to grow more slowly under high-iron conditions, suggesting that iron toxicity was a problem (Oram, *et al.* 2006; Wennerhold & Bott, 2006). Similarly, Brune *et al.* (2006), in the construction of their *dtxR*-deletion derivative of *C. glutamicum*, suggested that a *dtxR* mutation resulted in a conditionally lethal phenotype depending on the amount of iron that is supplied in the medium (Brune, *et al.* 2006). Similar results have also been seen in other Gram-positive species. For example, in *Streptococcus equi* subspecies *equi*, a deletion in *eqbA* (a *dtxR*-homologue) resulted in a small colony phenotype when grown on high-iron medium (Heather, *et al.* 2008); the researchers hypothesised that the small colony phenotype was due to iron toxicity which was, in turn, due to a lack of regulation of genes involved in iron sequestration. In order to overcome the potential issue of iron toxicity, the chelator, 2', 2' dipyridyl, was added to the BHI medium used to culture *C. pseudotuberculosis* during attempts to mutagenise *dtxR*. Chelation of excess iron within the growth medium has been an approach that has been successfully used to prevent the toxic affects of iron on iron-sensitive *dtxR*-homologue deletion derivatives of other bacteria, and has been achieved through the use of (for example) chelex-100 (Dussurget, *et al.* 1996), nitrilotriacetic acid (NTA) (Heather, *et al.* 2008) and ethylenediamine-*N*, *N*-diacetic acid (EDDA) (Oram, *et al.* 2006). However, despite these reports in the literature, it was extremely frustrating when, even after the addition of an iron chelator, the isolation of a *dtxR*-deficient *C. pseudotuberculosis* mutant was still not possible.

Although it had previously been successfully used to facilitate the recovery of a *C. pseudotuberculosis* mutant in which the *cp40* gene had been deleted, the constant difficulties in isolating a *dtxR* mutant led to the scrutiny of the allele exchange method itself. During the isolation of a *dtxR* mutant and in order to potentially improve the likelihood of isolating a mutant, ampicillin enrichment of secondary cross-over cultures was employed (**Chapter 4**). Significantly, ampicillin enrichment of secondary crossovers had previously been used, with great success, to enhance the recovery of mutants. Since this novel ampicillin enrichment protocol had not caused problems with the recovery of a *cp40* mutant strain, it was not considered likely to cause problems with the recovery of mutants of any other gene. However, it subsequently became apparent that the enrichment method may inadvertently have been the source of the problem. The ampicillin-enrichment process, described in detail in Chapter 4, utilises ampicillin to kill erythromycin-resistant cells still harbouring the pCARV mutagenesis

plasmid within the chromosome. Recently Kohanski *et al.* (2007) investigated the mode-of-action of three major classes of bactericidal antibiotics including β -lactams, of which ampicillin is a member. β -lactams are cell-wall synthesis inhibitors, and interfere with normal cell wall biosynthesis, inducing cell death through lysis. However, Kohanski *et al.* (2007) proposed that a side-effect of the normal route of killing resulted in an alternative phenomenon which is fundamental to all classes of bactericidal antibiotics. This mechanism involves the release of harmful hydroxyl radicals from lysed cells; these radicals are normally formed intracellularly as a function of metabolism-related NADH depletion, leaching of iron from iron-sulphur clusters, and subsequent stimulation of the Fenton reaction ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO}^\cdot + \text{HO}$) (Kohanski, *et al.* 2007). This leaching of iron results in increased intracellular free-iron which, subsequently, via the Fenton reaction, results in the generation of oxygen radicals and oxidative stress (Imlay, *et al.* 1988) (Kohanski, *et al.* 2007). It is well documented that bacteria which are unable to produce iron-regulatory proteins are in fact more susceptible to oxidative stress than their wild-type counterparts (Touati, 2000). Deregulation of the control of iron acquisition/uptake in mutants of iron regulatory proteins produces iron overload which, via the Fenton reaction in an aerobic environment, generates ferryl and hydroxyl radicals leading to increased oxidative stress (Dussurget, *et al.* 1996; Touati, 2000). Such oxidative stress susceptibility has been documented in iron-dependent regulatory mutants of several Gram-positive bacteria, including *C. diphtheriae* (Oram, *et al.* 2002), *M. smegmatis* (Dussurget, *et al.* 1996) and *M. tuberculosis* (Rodriguez, *et al.* 2002). In addition, the same phenomenon has been observed in some Gram-negative species, including *E. coli*, whereby deletion of the *fur* gene resulted in hypersensitivity to oxidative stress (Touati, *et al.* 1995).

Both DtxR and Fur play a role in protecting the cell from oxidative stress (Touati, *et al.* 1995) (Oram, *et al.* 2002). The primary role of the Fur regulated DNA-binding proteins of starved cells (Dps) from *E. coli* is to protect DNA from the combined affects of ferrous iron and H_2O_2 which result in the production of hydroxyl free radicals via the Fenton reaction (Martinez & Kolter, 1997; Zhao, *et al.* 1991). The Dps protein catalyses oxidation of ferrous iron to ferric iron by hydrogen peroxide, and thereby prevents hydroxyl radical formation via the Fenton reaction. In *E. coli*, Dps is induced in response to oxidative or nutritional stress. Furthermore, in *M. tuberculosis* iron-rich environments prompt the up-regulation of iron storage molecules to increase mechanisms of protection against iron-mediated oxidative damage (Rodriguez & Smith, 2003). When grown under high-iron conditions, *M. tuberculosis* up-regulates

bacterioferritin (*bfrA*) and ferritin (*bfrB*), both of which are involved in iron storage, and catalase peroxidase (*katG*) which catalyses the dismutation of hydrogen peroxide into water and molecular oxygen (Rodriguez & Smith, 2003). In *C. diphtheriae*, it has been predicted that the starvation-inducible Dps is regulated via DtxR (Yellaboina, *et al.* 2004). DtxR-regulated Dps in *C. diphtheriae* (Yellaboina, *et al.* 2004), *C. glutamicum* (Wennerhold & Bott, 2006), and presumably also *C. pseudotuberculosis*, plays a role in protection from oxidative stress. It is therefore likely that a deletion in the *dtxR* gene of these species would result in the deregulation of Dps, resulting in a mutant that is more susceptible to oxidative stress. Therefore, it is possible that the free radicals released into the culture medium following ampicillin-induced lysis of *C. pseudotuberculosis* cells resulted in the unexpected (and unwanted) killing of the very cells that the ampicillin treatment was supposed to enrich for; hence, the only excision mutants ever identified corresponded to the wild-type *dtxR* genotype. Certainly, by omitting the ampicillin-enrichment step, and by utilising a low-iron growth medium during allele-replacement experiments, the desired *dtxR*-deficient mutant was finally isolated very quickly thereafter.

During preliminary hydrogen peroxide inhibition assays, the *dtxR*-deficient strain was found to be hypersensitive to H₂O₂, as compared to its wild-type parent strain (data not shown). This data would tend to support the free-radical killing hypothesis with respect to the previous inability to isolate a *dtxR*-deficient mutant. Interestingly, up-regulation of *ideR* was detected in *M. tuberculosis*-infected macrophages (Hobson, *et al.* 2002). Rodriguez and Smith (2003) postulated that the induction of IdeR could contribute to the bacterial response to macrophage oxidative defence mechanisms (Rodriguez & Smith, 2003). Oxidative defence mechanisms or 'the oxidative burst' occurs following ingestion of bacteria by a macrophage. Following ingestion, bacteria are compartmentalised into a phagosome. The macrophage undergoes a burst of oxygen consumption which is a result of an NADPH oxidase complex (Iyer, 1961; Sbarra and Karnovsky, 1959); NADPH oxidase catalyses the reduction of oxygen at the expense of NADPH, resulting in the production of superoxide and other highly reactive oxidising agents. The resulting reactive oxygen species are essential for killing a number of microorganisms (Steinbeck, *et al.* 1992) (Chanock, *et al.* 1994) (Hampton, *et al.* 1998). *C. diphtheriae* DtxR (Oram, *et al.* 2002) and *M. smegmatis* IdeR (Dussurget, *et al.* 1996) mutants have been shown to be more susceptible to oxidative stress. Since *C. pseudotuberculosis* causes infection by surviving within macrophages and disseminating throughout the host's

lymphatic system, it follows that a *C. pseudotuberculosis* *dtxR* mutant would potentially be unable to survive the macrophage oxidative burst; however, further experiments are required to support this hypothesis.

Analysis of *C. pseudotuberculosis* DtxR regulation of *fagA* and *cp40* genes was conducted in order to unequivocally implicate the DtxR regulator in the iron-dependent expression of putative *C. pseudotuberculosis* virulence genes. Statistical analyses (ANOVA) provided strong evidence to suggest that, under differential iron concentrations, *fagA* gene expression was affected by the presence/absence of a functional DtxR protein. In contrast, statistical evidence suggested that *cp40* gene expression was not significantly different in wild-type or Cp- Δ *dtxR* under differential iron concentrations. These data corroborate the previous results seen in wild-type experiments whereby the *fagA* gene was expressed differentially under high- and low-iron growth conditions; however, *cp40* was not. Therefore, it was apparent that DtxR was intimately involved in the iron-dependent regulation of *fagA*, but was not with *cp40*. Interestingly, although a t-test performed to assess the levels of *fagA* expression in the mutant strain under high- and low- iron conditions did not reveal a statistically-significant difference, it was noted that the level of expression under high-iron conditions was consistently slightly lower than the equivalent in low-iron conditions, suggesting that some DtxR-independent regulatory mechanism may also affect the expression of this gene. (**Figure 5.20**). In this respect, it should be noted that similar reports of DtxR(or DtxR-like)-independent regulation of expression of DtxR-regulated genes has been reported for close relatives of *C. pseudotuberculosis*. In *M. smegmatis*, inactivation of IdeR resulted in only partial derepression of siderophore biosynthesis (Dussurget, *et al.* 1996). Dussurget *et al.* (1996) suggested the existence of a second repressor, going on to hypothesise that, due to the importance of iron, it is very likely that a second repressor exists. In *C. diphtheriae*, Oram *et al.* (2006) have suggested that iron-dependent regulation in *C. diphtheriae* is due to both DtxR-dependent and DtxR-independent mechanisms, however to-date it is unclear what the DtxR-independent mechanism(s) might be (Oram, *et al.* 2006). Given the similarities encountered throughout the course of this study, it is possible that the same DtxR-independent regulation reported in *C. diphtheriae* is also occurring in *C. pseudotuberculosis*; however, the statistical analysis carried out during this project suggests the residual repression is not significant (at the 95 % confidence limit). Therefore, in *C. pseudotuberculosis* at least, it may be that the suggestion of iron-dependent differential expression in Cp- Δ *dtxR* maybe just an

artefact. Previous reports in other bacteria based their conclusions as to the involvement of another (non-DtxR) iron-responsive regulatory mechanism on non-statistical methods; therefore, it is currently unclear whether an alternative regulator (which would presumably form part of a global regulatory network) actually exists or not.

In order to attempt to supplement the results obtained from reporter gene assays conducted in the *dtxR*-deficient mutant strain, equivalent assays were conducted following complementation of the mutant with a functional *dtxR* gene. The full *C. pseudotuberculosis* *dtxR* gene and its promoter were cloned into pEP2 (Radford & Hodgson, 1991) (a small plasmid able to replicate in *C. pseudotuberculosis*) and the resulting construct (designated pCAW010) was used to transform the *dtxR*-deficient mutant. Unfortunately, following transformation of the complemented mutant with the promoter fusion constructs, it became apparent that there was a problem when antibiotic selection was imposed to maintain the two plasmids within the bacterial population. Addition of either kanamycin (for the pEP2-based construct) *or* spectinomycin (for the pSPZ-based construct) permitted growth of cultures, however, when both antibiotics were added at the same time, bacteria were unable to grow. Subsequently, it became apparent that pEP2 (Radford & Hodgson, 1991) and pSPZ (Oram, *et al.* 2006) contain the same stable replication origin from the plasmid pNG2 (Messerotti, *et al.* 1990). Plasmids containing the same origin of replication are generally considered incompatible (Austin & Nordstrom, 1990; Nordstrom & Austin, 1989; Novick, 1987), resulting in an inability of such plasmids to stably co-exist within a single bacterial cell. Partly because of competition for growth factors, cells containing smaller plasmids tend to have a growth advantage over cells containing their larger counterparts. Of relevance to the experiments described here, pSPZ is approximately 10 kb in size, compared to pEP2 which is around 3kb. No efforts were made in the current study to determine which plasmid was being lost most rapidly; however, it seems logical that the pEP2-based construct would be preferentially-retained because of its smaller size. Although it would have been possible to utilise a different, compatible plasmid, sufficient time was not available to pursue this work. Despite the inability to use the complemented *dtxR*-deficient mutant to perform promoter fusion assays, it was possible to observe the affect of complementation on the ability of the mutant to grow under high- and low-iron conditions. Significantly, following complementation, the growth of the mutant was restored to that of its wild-type parent under both high- and low-iron conditions (**Figure 5.21**).

In vivo, *C. diphtheriae* DtxR regulates gene transcription in response to changes in iron concentrations; however, *in vitro* DtxR is able to bind to specific DNA sequences in the presence of other transition metal ions including, cadmium, cobalt, manganese, nickel and zinc (Schmitt & Holmes, 1993, 1994; Tao, *et al.* 1992). During this study, the ability of DtxR to utilise manganese and zinc in place of iron was tested. In order to achieve this, the ability of DtxR to bind to the *fagA* promoter region (and hence regulate transcription) was assessed in the presence of these different transition metal ions. Interestingly, in the presence of manganese, DtxR was able to repress *fagA* expression; however, zinc did not have an equivalent effect. Despite the ability to utilise manganese, the presence of iron resulted in a more potent repression of *fagA* expression. This observation is in keeping with a previous report for *C. diphtheriae*, in which it was demonstrated that DtxR in complex with Fe^{2+} was the most potent inhibitor of DT production and therefore bound more strongly to DtxR loci (Groman & Judge, 1979) (**Figure 5.22**). Moreover, in *C. diphtheriae*, cobalt, nickel and manganese were all found to inhibit DT toxin production at a concentration that did not inhibit bacterial growth. However, Tao *et al.* (1992) demonstrated that in order for zinc to repress toxin production it required a 10-fold increase in concentration compared to iron; significantly, this 10-fold increase in concentration resulted in bacterial growth inhibition (Tao & Murphy, 1992). The repression ratio calculated for zinc (1.8) was below that of the CCDM (2.0) (**Figure 5.22**), which was employed to calculate background repression; one can postulate that the low level of repression observed with zinc could be due to the zinc inhibiting growth or exerting a toxic effect upon the cell, or may simply be because zinc is not bound by *C. pseudotuberculosis* DtxR.

In summary, the work described in this chapter has highlighted the usefulness of a comparative approach when trying to understand the pathogenesis of a bacterium that has not yet been sequenced (although as a result of an ongoing sequencing project, during the later stages of this PhD study a partial genome sequence became available). Using the vast amount of knowledge available regarding the involvement of iron in the regulation of virulence genes in the important human pathogen *C. diphtheriae* as a starting block, it has been possible to begin unravelling the iron-regulatory mechanisms of *C. pseudotuberculosis*. This work has shown that there are many similarities with regards to the iron-dependent regulation of gene expression by DtxR in *C. pseudotuberculosis* and *C. diphtheriae*. The confirmation of *C. pseudotuberculosis* DtxR as a regulator suggests that the expression of numerous other *C.*

pseudotuberculosis genes will be under DtxR control. Since many of the genes under DtxR control are associated with virulence in other bacteria, the same will likely be true of *C. pseudotuberculosis*. As such, the identification of these genes could have a dual outcome by increasing our understanding of how *C. pseudotuberculosis* causes disease, but also by providing potential targets for the development of diagnostic assays and/or vaccines.

Chapter Six

Identification of a *Corynebacterium pseudotuberculosis* Siderophore

6.0 Introduction

In oxygen-containing environments, the availability of iron is limited. For the invading microorganism, this problem is confounded by the mammalian host's ability to further deplete available iron as part of its innate defence mechanism against infection. To counteract this defence, in order to successfully colonise and persist within their selected host, pathogenic microorganisms have developed mechanisms that enable them to sequester iron from host-iron binding proteins; one of the most common mechanisms being through the production of siderophores. Siderophores are low molecular weight (<1 kDa) chelating agents, which are synthesised by many bacteria and fungi in response to low-iron growth conditions (Neilands, 1995); their high affinity for ferric iron makes these molecules very efficient at fulfilling the micro-organism's iron requirements.

The biosynthesis of siderophores can occur via two different mechanisms; and the mechanism employed depends on the type of siderophore being synthesised. Siderophores can be divided into three main classes, these being catecholates (which include phenolates (Miethke & Marahiel, 2007)), hydroxamates and carboxylates, although more recently mixed types have been identified, such as Mycobactin T of *M. tuberculosis*, which is a phenolate-hydroxamate (Miethke & Marahiel, 2007) siderophore. In general siderophores are synthesised either dependently or independently of a nonribosomal peptide synthetase (NRPS) (Miethke & Marahiel, 2007). NRPSs are multi-modular enzymes that produce peptide products of a particular sequence without an RNA template, rather, the order of NRPS domains specifies the order of monomeric amino acids activated and incorporated (Crosa & Walsh, 2002). NRPSs are responsible for the assembly of thousands of natural peptides yielded by a wide range of microorganisms (Marshall, *et al.* 2002). NRPS-dependent siderophore biosynthesis has been studied in detail in several human pathogens, examples of which include enterobactin (catecholate) synthesis in *E. coli*, and yersiniabactin (phenolate) synthesis in *Yersinia* (Crosa & Walsh, 2002). NRPSs have been shown to be partially involved in the synthesis of hydroxamate and carboxylate siderophores; however, generally these types of siderophores are assembled by NRPS-independent siderophore (NIS) mechanisms (Miethke & Marahiel, 2007). An example of a well studied hydroxamate NIS is aerobactin. The aerobactin gene cluster consists of five genes, four of which (*iucABCD*) direct aerobactin synthesis, with the fifth (*iutA*) encoding the siderophore receptor protein (de Lorenzo, *et al.* 1986), involved in uptake

of siderophore from the extracellular environment. Significantly, some siderophores which are synthesised via NRPS-independent mechanisms have been implicated in virulence (Miethke & Marahiel, 2007), an example being aerobactin, which is thought to be a key virulence factor in many pathogenic strains of *E. coli* (Warner, *et al.* 1981; Gibson & Magrath, 1969).

On arrival at the bacterial cell surface there are several mechanisms by which a siderophore's iron load can be transferred into the cell. One such mechanism is through reduction, whereby siderophore-bound Fe^{3+} undergoes a reaction catalysed by free-extracellular or membrane-bound ferric reductases (Miethke & Marahiel, 2007), resulting in its conversion into the ferrous state, a form which is readily usable by the bacterial cell, and one which results in the release and subsequent recycling of the siderophore. The second and most common mechanism for siderophore-bound iron uptake is by the import of the iron-siderophore complex into the cytosol. Due to the differences in cellular structure, uptake of siderophores occurs by different mechanisms in Gram-negative and Gram-positive bacteria. In Gram-negative bacteria, because of the presence of an outer membrane (OM) siderophore uptake occurs via a two-step process. Firstly, prior to transport across the OM into the periplasmic space, the iron-siderophore complex is bound by an OM receptor molecule. Then with the help of the TonB complex (The TonB complex "transduces" proton-motive energy to the receptor to allow substrate translocation (Miethke & Marahiel, 2007)), the receptor-bound siderophore is released into the periplasm. Subsequently, the complex binds to a specific periplasmic receptor of an inner membrane ABC transporter (a transmembrane protein that functions in the transport of a wide variety of substrates across extra- and intracellular membranes) prior to transportation across the cytoplasmic membrane into the cytoplasm (Schneider & Hantke, 1993) (Brown & Holden, 2002). Ironically, although siderophore uptake by Gram-positive bacteria could be considered simpler due to the absence of an OM, the process is actually less-well understood. In Gram-positive bacteria, the cell wall-located siderophore-binding receptor is generally considered to be a lipoprotein which is anchored into the cytoplasmic membrane through an N-terminal lipid moiety (Schneider & Hantke, 1993; Kunkle & Schmitt, 2005). Subsequent transport of the siderophore through the cytoplasmic membrane requires the involvement of an ABC-type transporter (Schneider & Hantke, 1993; Kunkle & Schmitt, 2005).

Once siderophore-bound iron has been delivered into the cell it must be removed from the siderophore into the cytosol, and it is generally thought that there are two mechanisms of iron-release. Firstly, similarly to the release of iron extracytoplasmically, the reduction of siderophore-bound Fe^{3+} to Fe^{2+} , by intracellular ferric reductases, followed by the spontaneous release or competitive sequestration of the reduced species can occur (Miethke & Marahiel, 2007). A second mechanism is a result of ferrisiderophore (iron-laden siderophore) hydrolysis. During this process specialised enzymes are employed to hydrolyse the ferrisiderophore thereby releasing its iron load. However, hydrolytic iron release results in the destruction of the siderophore scaffold; hence, it is more costly to the cell compared to ferrisiderophore reduction, whereby the siderophore can be re-secreted (Cooper, *et al.* 1978; Miethke & Marahiel, 2007).

More than 25 years ago, the presence of an extracellular factor that promoted the uptake of iron in *C. diphtheriae* was discovered (Russell & Holmes, 1983). Following this finding, further work provided evidence to show that, under low-iron growth conditions *C. diphtheriae* produced a siderophore (Russell, *et al.* 1984). A year later, Russell *et al.* (1984) identified a siderophore-deficient mutant of *C. diphtheriae* which did not produce siderophore; however, although the mutant, referred to as HC6, did not produce siderophore, it was found to be able to utilise exogenous aerobactin (Russell, *et al.* 1984). A siderophore which is synthesised by one bacterium but opportunistically utilised by another is referred to as a xenosiderophore. In order to utilise xenosiderophores, a bacterium must possess ferric-chelate reductases and/or uptake systems for siderophores not synthesised by themselves (Miethke & Marahiel, 2007). Russell *et al.* (1984) postulated that the *C. diphtheriae* siderophore and aerobactin may be transported by a similar mechanism (Russell, *et al.* 1984), although the mechanism of transport of the *C. diphtheriae* siderophore or the xenosiderophore had not been identified. Significantly, much later Wennerhold and Bott (2006) alluded to the fact that *C. glutamicum*, strain ATCC 13032, likely employs siderophore produced by other microorganisms to acquire iron (Wennerhold & Bott, 2006); hence, the utilization of xenosiderophores may be a common feature among the corynebacteria.

The discovery of the *C. diphtheriae* siderophore initiated a significant amount of downstream work. In 1997, Schmitt *et al.* characterised a lipoprotein, for which the structural gene, *irp1*, shared homology with genes involved in iron acquisition and was shown to be regulated by

DtxR (Schmitt, *et al.* 1997). Although potentially associated with iron-acquisition, the researchers demonstrated that Irp1 was unable to complement either a HC6 siderophore-deficient mutant, or other *C. diphtheriae* mutants defective in iron uptake, designated HC1, HC3, HC4 and HC5 (Cryz, *et al.* 1983). Irp1 was also shown not to affect siderophore production or regulation in the strains tested (Schmitt, *et al.* 1997). Qian *et al.* (2002) later sequenced the *irp1* operon and demonstrated that it encoded a *C. diphtheriae* ABC transport system, the function of which has yet to be elucidated. Irp1 was subsequently designated Irp1A and shown to share homology with FhuD from *B. subtilis*; however, the Irp1 operon could not complement the defects in siderophore-dependent iron transport in the HC1, HC3, HC4 and HC5 mutants (Qian, *et al.* 2002). Significantly, Qian *et al.* (2002) noted that the *irp1* operon was not present in the genome of *C. diphtheriae* NCTC 13129; thereby demonstrating that the ABC transport system is not present in all strains of *C. diphtheriae* (Qian, *et al.* 2002). Concurrently, Qian *et al.* (2002) identified a further iron regulated operon *irp6ABC*. Both *irp1* and *irp6* operons demonstrate gene organisation typical of ABC transport systems for Gram-positive bacteria; however, there is no significant homology between the lipoprotein receptors Irp6A and Irp1A, even in the N-terminal signal sequences (Qian, *et al.*, 2002). Irp1B and Irp1C proteins each share 30 % homology with Irp6B whereas Irp6C and Irp1D share 34 % homology (Qian, *et al.* 2002). Interestingly, clones containing the *irp6* genes were shown to complement the deficiency in growth and siderophore regulation in the HC1, HC4 and HC5 iron uptake mutant strains (Qian, *et al.* 2002). As a consequence, the investigators hypothesised that the *irp6* operon encodes an ABC transporter which is involved in the high affinity siderophore-dependent uptake in *C. diphtheriae* (Qian, *et al.* 2002).

To-date, the structure of the *C. diphtheriae* siderophore has yet to be determined, and it is not actually known whether *C. diphtheriae* produces more than one siderophore (Kunkle & Schmitt, 2005; Allen & Schmitt, 2009). However, Kunkle and Schmitt (2005) recently identified a genetic locus associated with siderophore biosynthesis and transport in *C. diphtheriae* (Kunkle & Schmitt, 2005). The investigators conducted a BLAST analysis of predicted open reading frames within the sequenced genome of *C. diphtheriae* strain, NCTC 13129 (accession # NC_002935), and subsequently identified a gene (locus tag DIP0586), the predicted product of which shared homology with the aerobactin biosynthetic enzymes IucA and IucC. This gene, referred to as *ciuE*, also lay downstream of four other genes, designated *ciuA*, *ciuB*, *ciuC* and *ciuD* (DIP 0582-DIP 0585), the predicted products of which shared

significant homology to ABC-type iron transport systems (Kunkle & Schmitt, 2005). Kunkle *et al.* (2005) noticed that the *ciuE* gene appears to be a fusion of two *iucC/iucA*-like genes. Somewhat confusingly, the authors reported that the predicted product of *ciuE* shows similar relatedness to both IucA and IucC in both halves of the protein; furthermore, there is not a significantly higher amino acid similarity to either IucA or IucC in either the N-terminal region or C-terminal section of CiuE (Kunkle & Schmitt, 2005). With regards to CiuE of *C. diphtheriae*, which appears to be a fusion protein, it is not known whether the protein has two distinct enzymatic activities within the single peptide (Kunkle & Schmitt, 2005). Since CiuE shared significant homology with the aerobactin biosynthesis proteins, IucA and IucC, the investigators wanted to determine whether CiuE was involved in the synthesis of a *C. diphtheriae* siderophore; subsequently, an in-frame *ciuE* mutant was constructed in two *C. diphtheriae* strains and siderophore production was assessed. A significant decrease in the production of siderophore was observed in both CiuE-deficient strains of *C. diphtheriae*, although siderophore production equivalent to the wild-type parent strains could be restored by complementation with a fully-functional copy of *ciuE*. It was therefore proven conclusively that *ciuE* is a siderophore biosynthesis gene (Kunkle & Schmitt, 2005). Interestingly, Kunkle and Schmitt (2005) provided evidence to suggest, that the *irp6* operon, previously thought to be involved in high-affinity siderophore-dependent uptake in *C. diphtheriae* (Qian, *et al.* 2002), was not necessary for the uptake of the *ciuE* product, through the observation that an *irp6A* mutant was still able to utilise *C. diphtheriae* siderophore. However, a *ciuA* mutant was not able to uptake the siderophore, providing strong evidence for the involvement of *ciuA* in siderophore transport in *C. diphtheriae*. In contrast to the previous observation that an *irp6A* mutant was not able to utilise aerobactin, supplementation of the CiuA-deficient mutant strain with aerobactin restored growth. These results suggested to the researchers that Irp6A is in fact essential for the uptake of aerobactin but not *C. diphtheriae* siderophore, and CiuA is essential for *C. diphtheriae* siderophore uptake but not that of aerobactin (Kunkle & Schmitt, 2005).

Schmitt *et al.* (1997b) demonstrated that the *C. diphtheriae* siderophore uptake mutant HC1 (Cryz, *et al.* 1983), unlike the wild-type parent strain, was unable to obtain iron from transferrin, although it was able to obtain iron from haem and haemoglobin (Schmitt, 1997b). The ability of *C. diphtheriae* to utilise haem has been shown to be via a siderophore-independent mechanism (Schmitt, 1997b; Bibb, *et al.* 2005), and it was subsequently

suggested that the acquisition of iron from transferrin in *C. diphtheriae* is dependent on a functional siderophore uptake system (Schmitt, 1997b). As part of the work described in this thesis, the ability of *C. pseudotuberculosis* to utilise transferrin-bound iron was established (**Chapter 3**). By analogy with the work by Schmitt *et al.* (1997b), it was considered entirely possible that a functional siderophore may also be associated with the ability of *C. pseudotuberculosis* to acquire iron from transferrin; however, to date there has been no siderophore detected in this organism. Subsequently, the aim of the work described in this chapter was to determine the potential involvement of siderophore in *C. pseudotuberculosis* iron acquisition.

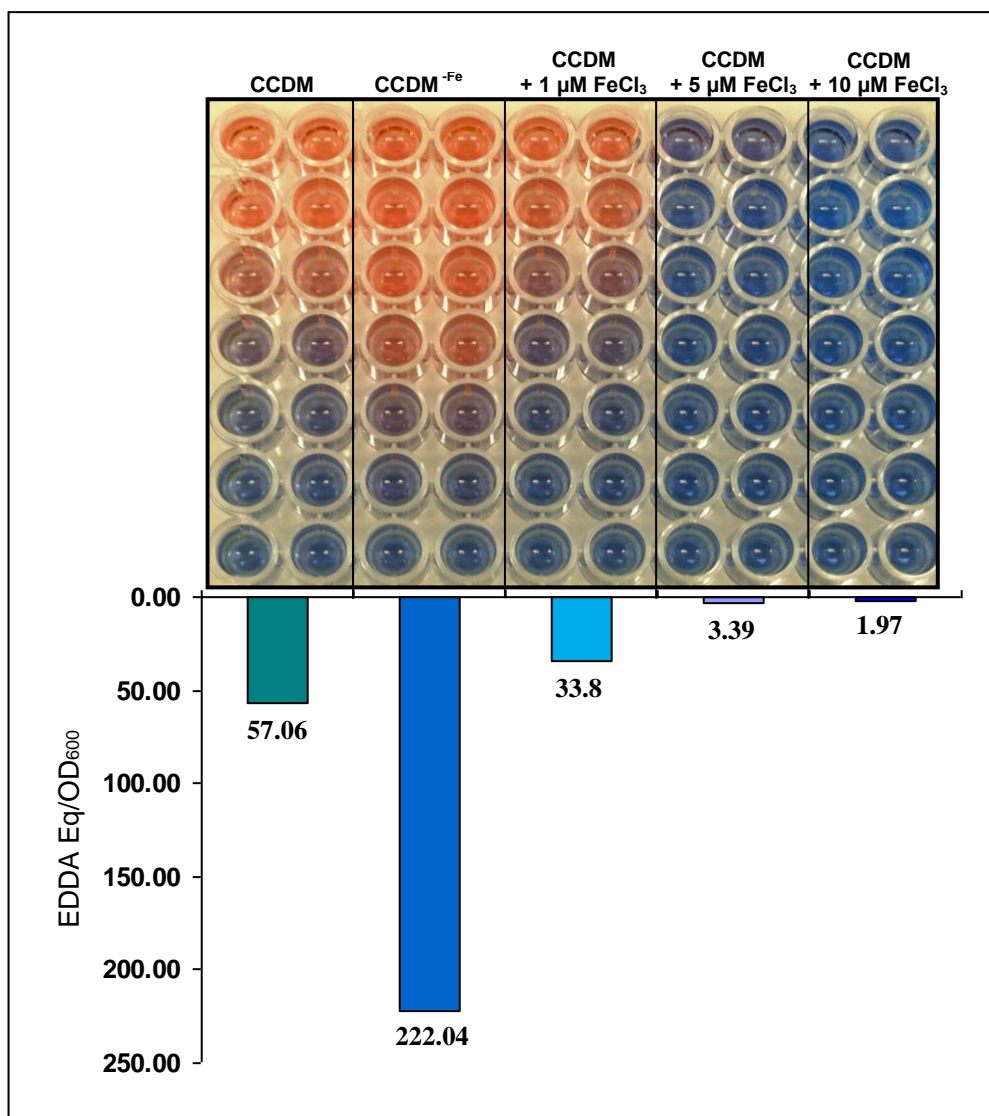
6.1 Results

6.1.1 Production of siderophore by *C. pseudotuberculosis*

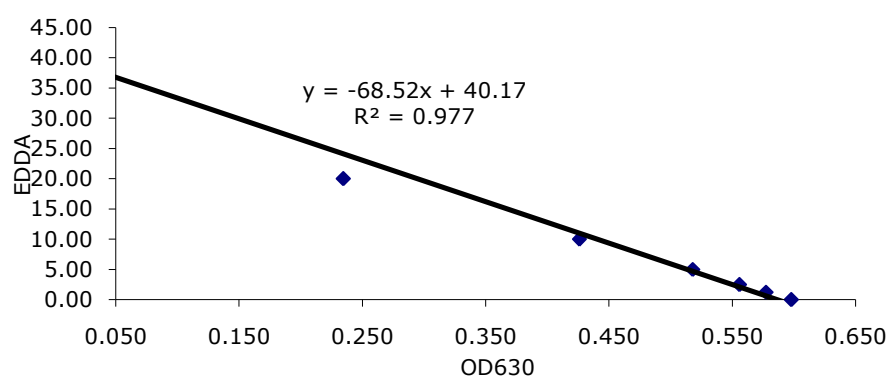
A CAS assay was performed (Schwyn & Neilands, 1987) in order to ascertain whether *C. pseudotuberculosis* produces a siderophore when subjected to low-iron growth conditions. Production of siderophore was assessed by calculating “siderophore units” using a standard curve of known EDDA concentrations (ranging from 1-20 μM), per absorbance of the culture (EDDA Eq/OD_{600nm}) (**Figure 6.1**). Significantly, results of preliminary experiments, presented in **Figure 6.1**, revealed that the low-iron conditions previously determined in Chapter 3 resulted in a considerable amount of siderophore being produced by *C. pseudotuberculosis*. In CCDM (characterised as the high-iron growth medium in chapter 3) the production of 57.06 EDDA Eq/OD_{600nm} was observed as compared to 222.04 EDDA Eq/OD_{600nm} of siderophore produced in CCDM containing dipyrindyl (low-iron growth conditions).

The affect of supplementing CCDM with different concentrations of iron was investigated. Increasing concentrations of FeCl₃ were added to the CCDM, and with increasing concentration came a concurrent decrease in siderophore production. Addition of FeCl₃ to final concentrations of 1 μM , 5 μM and 10 μM , resulted in the production of 33.8, 3.39 and 1.97 EDDA Eq/OD_{600nm} of siderophore, respectively. This data revealed that, although the addition of further iron had no affect on the level of *C. pseudotuberculosis* growth (as compared to CCDM alone; **Chapter 3, Figure 3.3**), the near complete termination of siderophore production was not observed until the medium contained 10 μM FeCl₃. The inverse relationship between iron concentration and siderophore production suggested that siderophore production is regulated by extracellular iron concentration. At this point it was considered possible that, if it was necessary to supplement CCDM to a final concentration of 10 μM FeCl₃ in order to terminate siderophore production, the same may well be true in order to regulate other genes which are expressed in an iron-dependent manner.

Panel A



Panel B

**Figure 6.1 CAS assay for the detection of *C. pseudotuberculosis* siderophore production**

Panel A) Production of siderophore by *C. pseudotuberculosis* was assessed following growth in either CCDM, CCDM deferrated by addition of 2',2'-dipyridyl to 300 μM (CCDM^{-Fe}), or CCDM supplemented with varying amounts of FeCl₃. Samples were analysed in duplicate in a microtitre plate assay, and production of siderophore was evidenced by the formation of an orange-pink colour. Sample lanes correspond to CCDM (■), CCDM^{-Fe} (■), CCDM + 1 μM FeCl₃ (■), CCDM + 5 μM FeCl₃ (■) and CCDM + 10 μM FeCl₃ (■). Panel B) Standard curve.

6.1.2 Optimal growth conditions for the study of iron-dependent regulation of gene expression

Following the identification of optimal low- and high-iron conditions, the CAS assay was performed a further 3 times to ensure that meaningful results were obtained (**Figure 6.2**). Each time, consistent levels of siderophore production were observed in either high- or low-iron medium, confirming the observation of siderophore production, and also confirming the iron-dependent nature of production. A paired t-test was employed to statistically analyse the data. The analyses provided evidence to suggest there was a statistically significant difference between the amount of siderophore produced under low-iron conditions compared to the amount of siderophore produced under high-iron conditions ($p=0.011$).

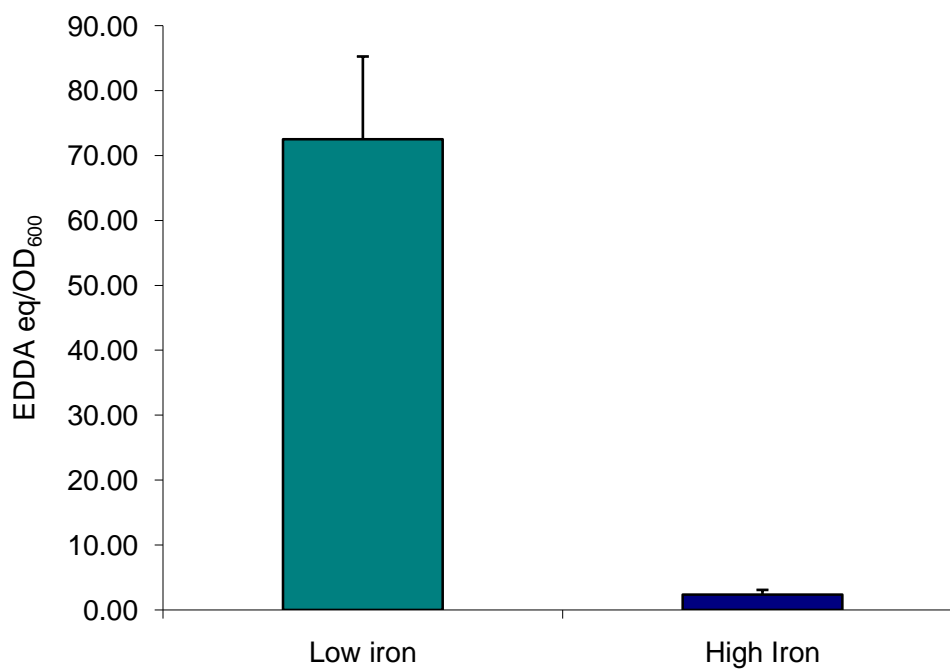


Figure 6.2 Siderophore production by *C. pseudotuberculosis* under low- and high-iron growth conditions

This figure illustrates an average of three CAS assay experiments in which *C. pseudotuberculosis* was grown under low iron (CCDM with 300 μ M 2', 2' dipyridyl) and high iron (CCDM with 10 μ M FeCl₃) conditions. Bars correspond to low-iron (■), high-iron (■), and the difference in the level of expression was statistically significant ($p=0.011$).

6.1.3 *C. pseudotuberculosis* chromosomal siderophore-encoding locus

As mentioned in Chapter 5, towards the end of this PhD study, the near complete sequence of the *C. pseudotuberculosis* 3/99-5 genome became available. Next Generation sequencing was conducted using a Roche 454 sequencing apparatus (GS-FLX Instrument) by 454 Life Sciences (Branford, Connecticut, USA). Subsequently, the sequence provider assembled sequence reads into 8 contiguous sections, comprising an estimated *ca.* 98 % of the complete *C. pseudotuberculosis* genome. The subsequent (and ongoing) analyses of this genome sequence, including gap-closure, are being conducted as part of a separate project, and will not be discussed here. However, ORF predictions and a preliminary genome annotation was conducted by staff of the Bioinformatics Unit within the Moredun Research Institute, and the annotated sequence was made available as a means of attempting to link a specific chromosomal locus (or loci) to the observed siderophore-producing phenotype.

Analysis of the annotated gene list led to the identification of a 3,390 bp open reading frame, designated here as ORF02949 (**Figure 6.3**). The translated product of this ORF shared 52 % identity and 68 % similarity over its entire length with the previously published siderophore biosynthesis-encoding gene, *ciuE* of *C. diphtheriae* strain NCTC 13129 (locus tag DIP0586). A protein of 1,129 amino acids was predicted using the Clone Manager 9 software package. Analysis of the region immediately upstream of ORF02949 revealed a further 4 predicted ORFs, designated ORF02945 (905 bp), ORF02946 (563 bp), ORF02947 (1,031 bp) and ORF02948 (821 bp) (**Figure 6.3**). Significantly, the translated product of each of these 4 open reading frames shared homology with ABC-type iron transport systems, the translated product of each ORF corresponding to a periplasmic binding protein (a possible lipoprotein (Kunkle & Schmitt, 2005)), 2 permease components and an ATPase component, respectively. Comparison of the putative *C. pseudotuberculosis* siderophore transporter proteins with the previously described *C. diphtheriae* Ciu ABC-type siderophore transporter (Kunkle & Schmitt, 2005) revealed significant homology. The translated product of ORF02945 shared 76 % identity and 84 % similarity across its entire length with *C. diphtheriae* CiuA (locus tag DIP0582). The translated product of ORF02946 shared 66 % identity and 82 % similarity with *C. diphtheriae* CiuB (locus tag DIP0583), the translated product of ORF02947 shared 75 % identity and 87 % similarity with *C. diphtheriae* CiuC (locus tag DIP0584), and the translated

product of ORF02948 shared 69 % identity and 84 % similarity with *C. diphtheriae* CiuD (locus tag DIP0585).

Despite a significant level of sequence conservation between the equivalent siderophore-encoding loci of *C. pseudotuberculosis* and *C. diphtheriae*, it was apparent that there had been some divergence of sequence between the two species. During their analysis of the *C. diphtheriae* NCTC 13129 genome sequence, Cerdeno-Tarraga *et al* (2003) revealed 13 regions which were absent in the non-pathogenic *C. glutamicum* and *C. efficiens* (Cerdeno-Tarraga, *et al.* 2003). Analysis of these regions led to the identification of anomalies in nucleotide composition, such as G+C content, and led to the speculation that they encoded pathogenicity islands (Cerdeno-Tarraga, *et al.* 2003). Significantly, it was observed that some of these islands encoded iron-uptake systems and a potential siderophore biosynthesis system. However, it is unfortunate that the Ciu-encoding locus of *C. diphtheriae* was not characterised until a few years later, and although likely, it is not stated that the siderophore biosynthesis system identified by Cerdeno-Tarraga *et al.* (2003) is that encoding the Ciu locus. What is interesting is that analysis of the chromosomal region immediately flanking the *C. pseudotuberculosis* siderophore-encoding locus revealed a putative open reading frame, which shared homology with transposase IS3510b for *C. jeikeium*. Analysis further upstream and downstream revealed that the *C. pseudotuberculosis* siderophore-encoding locus lay in an entirely different region of the chromosome than the (possible) equivalent locus in *C. diphtheriae*, and it would therefore appear, at least from preliminary analysis, that the acquisition of siderophore by both of these species has occurred as a result of independent bacteriophage lysogeny events, at different locations within the chromosome.

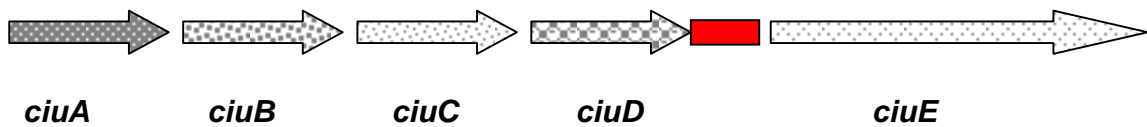
Panel A**Panel B**

Figure 6.3 Schematic representation of the *C. pseudotuberculosis* and *C. diphtheriae* siderophore loci

The *C. pseudotuberculosis* locus (**panel A**) incorporates the putative siderophore biosynthesis gene homologue (ORF02949), and demonstrates the predicted DtxR binding locus (shown in red). Also within the locus are ORFs 02945-02948 which share significant homology to an ABC-type transport system. The 309 bp region upstream of ORF02949 that was used in subsequent promoter fusion assays is indicated by the black bar. The *C. diphtheriae* locus (**panel B**) incorporates the siderophore biosynthesis gene (*ciuE*), and the predicted DtxR binding locus (shown in red). The genes *ciuA*-*ciuD* are thought to encode an ABC-type transport system (Kunkle & Schmitt, 2005).

6.1.4 Complementation of a *C. diphtheriae* siderophore-deficient mutant with *C. pseudotuberculosis* siderophore

Given the predicted divergence in sequence, it was thought interesting to establish whether the *C. pseudotuberculosis* siderophore was functionally homologous to that encoded by *ciuE* of *C. diphtheriae*. Supernatant from cultures of *C. pseudotuberculosis* grown in high-iron growth medium (containing no siderophore) and low-iron growth medium (containing un-purified siderophore) were used to supplement a *C. diphtheriae* *ciuE*-knockout strain (S. L. Zajdowicz personal communication) to determine whether the growth of the mutant (which was unable to grow under low-iron growth conditions) could be restored. The siderophore bioassay was carried out in collaboration with Dr S. L. Zajdowicz in the Department of Microbiology, University of Colorado at Denver and Health Sciences Centre, whilst on a working visit to Prof. Randall Holmes' lab. *Corynebacterium diphtheriae* strain C7(-) Δ *ciuE* was grown in an 80:20 mixture of PGT medium (Oram, *et al.*, 2002) (with 10 g/L casamino acids) and CHI broth (chelex resin-treated Heart Infusion broth) for 18 h. Heart Infusion broth with Tween⁸⁰, containing 60 μ M EDDA to chelate iron, was inoculated with *C. diphtheriae* strain C7(-) Δ *ciuE* to a final concentration of 10^6 bacteria/ml. An overlay of 1% (w/v) agarose containing 0.01% triphenyltetrazolium chloride was added, and wells were cut into the agar plate. Sample containing 25 μ l of each siderophore-containing sample was added to the wells and growth stimulation was measured after incubation for 18 h at 37°C. An example of a bioassay is shown in **Figure 6.4** where supernatant containing siderophore from cultures of *C. pseudotuberculosis* propagated in either CCDM, CCDM containing 300 μ M dipyridyl, or CCDM containing 1 μ M FeCl₃ or 10 μ M FeCl₃ is shown. A sample of *C. diphtheriae* siderophore, which was in the form of supernatant taken from a culture of *C. diphtheriae* grown under low-iron conditions, was used as a positive control. As shown, supplementing the *C. diphtheriae* *CiuE* mutant with supernatant from wild-type *C. pseudotuberculosis* grown in low-iron medium resulted in a restoration of the ability of the *C. diphtheriae* mutant to grow under iron-restricted conditions; however, in contrast, supplementing the mutant with the high-iron medium did not result in the restoration of growth. These results suggest that the siderophore produced by *C. pseudotuberculosis* under low-iron growth conditions is similar enough in terms of function to be useable by *C. diphtheriae*.

Figure 6.4 Complementation of a *C. diphtheriae* C7 (-) Δ *ciuE* with *C. pseudotuberculosis* siderophore containing supernatant

Sample wells correspond to supernatant from cultures of *C. pseudotuberculosis* propagated in CCDM (1), CCDM containing 300 μ M dipyridyl (2), CCDM containing 1 μ M FeCl₃ (5) and CCDM containing 10 μ M FeCl₃ (3). A positive control was included corresponding to siderophore-containing supernatant obtained from a culture of *C. diphtheriae* propagated under low-iron growth conditions (4). Growth of the mutant strain is apparent by the presence of a brown colony surrounding each respective well.

6.1.5 Analysis of expression of the *C. pseudotuberculosis* putative siderophore-encoding gene

A 309 bp DNA fragment containing 271 bp of the chromosomal region upstream of the putative *C. pseudotuberculosis* siderophore biosynthesis gene (ORF02949), and 4 bp into the 5'-end of the gene itself (**Figure 6.3**), was amplified by PCR using the primers Sid_P_F and Sid_P_R (**Table 2.5**), and cloned into the promoterless *lacZ* reporter gene plasmid, pSPZ (Oram, *et al.*, 2006). The resulting construct, designated pCAW009, which was considered likely to have incorporated the promoter-containing region of the *C. pseudotuberculosis* gene, was introduced into wild-type *C. pseudotuberculosis*, and β -galactosidase activity resulting from expression of the promoterless *lacZ* gene under the control of an ORF02949 promoter was subsequently measured under high- and low-iron growth conditions. The use of pCAW007, the *fagA* containing construct, which was previously proven to be regulated by DtxR in Chapter 5, was employed as a positive control in these experiments. Assays were performed in triplicate on three separate occasions, and results confirmed that a functional promoter-encoding sequence was present within the cloned 309 bp fragment (**Figure 6.5**). Moreover, the promoter was observed to drive expression of the *lacZ* reporter gene in an iron-dependent manner, since under high-iron growth conditions β -galactosidase activity was significantly less than that observed under low-iron conditions. Statistical analyses (by way of a paired t-test) corroborated this with evidence to suggest that a statistically significant difference ($p=0.018$) existed between expression of ORF02949 under low-iron conditions compared to expression under high-iron conditions. While these results confirmed the iron-dependent expression of the putative siderophore-encoding gene, it was not clear whether DtxR was involved in the iron-dependent regulation of this gene in *C. pseudotuberculosis*, equivalent to DtxR control of *ciuE* expression in *C. diphtheriae* (Kunkle & Schmitt, 2005).

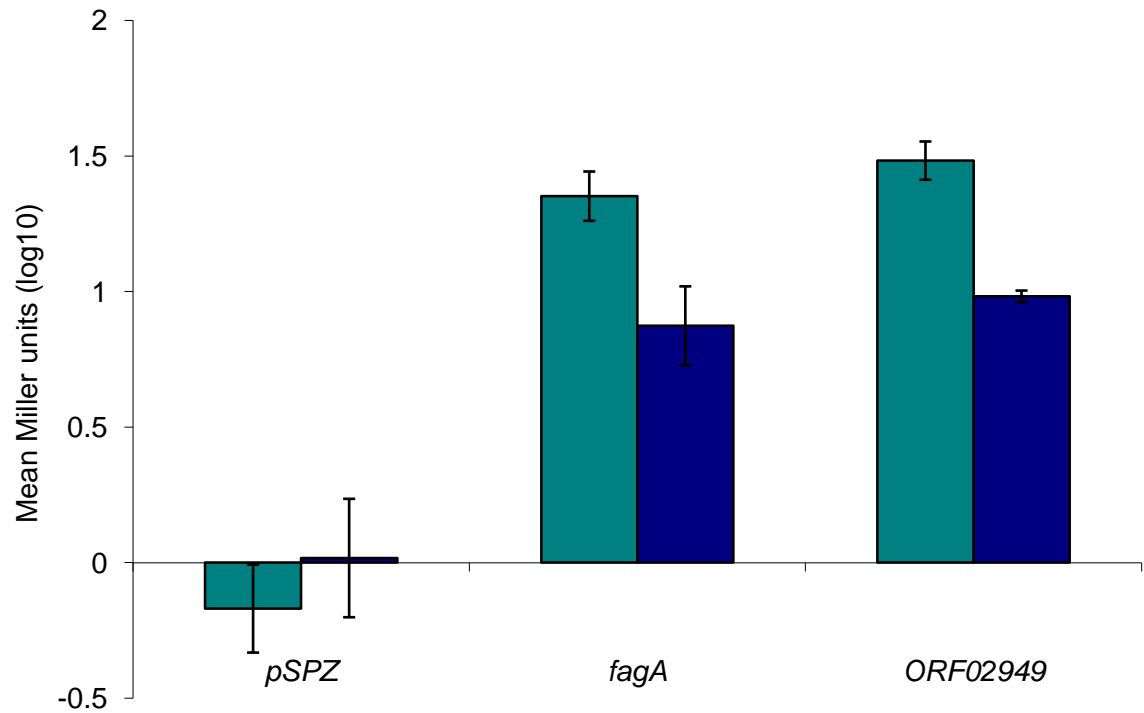


Figure 6.5 Iron-dependent regulation of *C. pseudotuberculosis* putative siderophore biosynthetic gene expression

The putative promoter containing region of ORF02949 was fused to promoterless *lacZ*, and β -galactosidase production was measured under high- and low-iron conditions in wild-type *C. pseudotuberculosis*. The *fagA* gene, previously been shown to be regulated in an iron-dependent manner was included here as a positive control. Bars correspond to low-iron (■) and high-iron (■), and ORF02949 was found to be differentially expressed in response to iron in a statistically significant manner ($p=0.018$).

6.1.6 Involvement of DtxR in the regulation of the *C. pseudotuberculosis* putative siderophore-encoding gene

Further analyses were conducted in order to determine whether DtxR was involved in the regulation of expression of the *C. pseudotuberculosis* putative siderophore-encoding gene. In order to identify putative DtxR-binding loci in *C. pseudotuberculosis* genes, the software package “fuzznuc”, available through the European Molecular Biology Online Software Suite (EMBOSS; <http://bioweb2.pasteur.fr/docs/EMBOSS/fuzznuc.html>) was used to search for specific nucleic acid motifs within the *C. pseudotuberculosis* 3/99-5 chromosomal region corresponding to ORFs 02945-02949. The search was conducted using the *C. diphtheriae* consensus DtxR-binding motif, 5'-TWAGGTWAGSCTWACCTWA-3', where W = A or T and S = C or G. To allow for divergence between species, up to five mismatches were allowed in the search, which was conducted by staff of the Moredun Bioinformatics Unit. Significantly, a DtxR-like binding motif (sequence 5'-TAAAGGTAGCCTTGGCTAC-3') was identified immediately (20 bp) upstream of the putative siderophore biosynthesis gene, ORF02949 (**Figure 6.3**). Furthermore, a DtxR-like binding motif (sequence 5'-TTAGGTTTGCTTTGCATAC-3') was identified immediately (29 bp) upstream of the putative ABC-transporter gene, ORF02945 however due to lack of time the promoter region of this gene was not analysed for DtxR-dependent regulation.

In order to confirm whether the promoter upstream of ORF02949 was regulated by DtxR in an iron-dependent manner, a promoter fusion assay was conducted using the DtxR-deficient *C. pseudotuberculosis* strain (Cp- Δ dtxR) as a host for pCAW009 (**Figure 6.6**). As a positive control, the fusion construct containing the *C. pseudotuberculosis* *fagA* promoter, pCAW007, was also used to transform the Cp- Δ dtxR strain. If expression of the putative siderophore-encoding gene was under the control of DtxR in *C. pseudotuberculosis*, then it would be expected that there would be no difference between the levels of expression of the *lacZ* gene in pCAW009 in the DtxR-deficient host strain under high- and low-iron conditions. The average results obtained from 3 promoter fusion experiments are presented in **Figure 6.6**. A paired t-test was employed to analyse the difference between expression of ORF02949 under high- and low-iron growth conditions in the DtxR-deficient host strain Cp- Δ dtxR. The results provided evidence to suggest that there was no significant difference in expression ($p=0.240$), implicating DtxR in the regulation of this gene. In addition, an ANOVA was employed to

compare the expression of ORF02949 between wild-type and Cp- $\Delta dtxR$. The ANOVA provided statistical evidence to suggest there was a significant difference in gene expression of ORF02949 in wild-type as compared to Cp- $\Delta dtxR$ ($p=0.009$). Furthermore, the difference in expression of ORF02949 between wild-type and Cp- $\Delta dtxR$ was greater under high-iron growth conditions, compared to the expression in the two strains under low-iron growth conditions. Expression of ORF02949 in wild-type under high-iron conditions was 0.982 ± 0.08 compared to expression in the Cp- $\Delta dtxR$ under high-iron conditions which was 1.316 ± 0.08 ; these results indicated an interaction effect between the strain and iron.

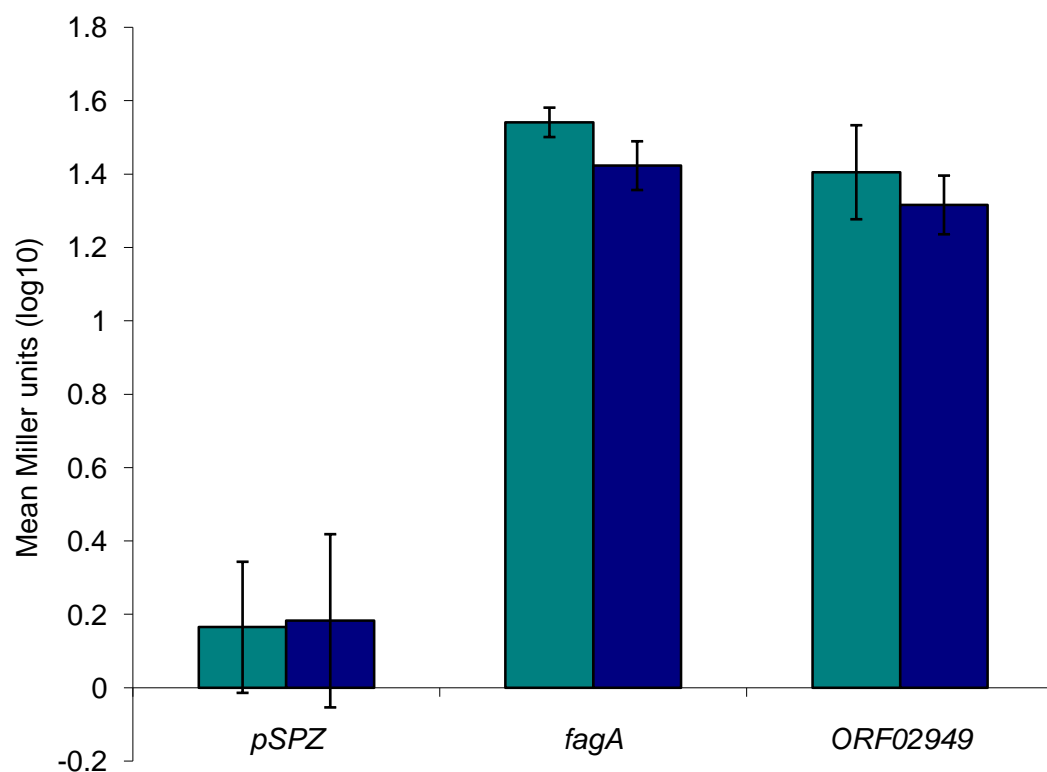


Figure 6.6 Assessment of DtxR-regulation of *C. pseudotuberculosis* putative siderophore biosynthetic gene expression.

The pCAW009 (containing *ORF02949*) construct was used to transform *Cp-ΔdtxR* and β -galactosidase expression was measured under high- and low-iron conditions. Again, pCAW007 (*fagA*) was included as a positive control since it had previously been shown to be regulated in an iron-dependent manner (Chapter 5). Bars correspond to low-iron (■) and high-iron (■). A paired t-test indicated that expression of *ORF02949* was not differentially expressed ($p = 0.240$) in the mutant *Cp-ΔdtxR* strain implicating DtxR in the regulation of the putative siderophore biosynthesis gene.

6.1.7 Construction of a putative siderophore-deficient mutant of *C. pseudotuberculosis*

Using the allele-replacement method previously developed during this work (Chapter 4), attempts were made to construct a mutant of *C. pseudotuberculosis* 3/99-5, deficient in ORF0249; in this way it was hoped that the involvement of this gene in *C. pseudotuberculosis* siderophore production would be confirmed. Using the PCR primers AE_249_1 and AE_249_2 (Table 2.3) a 951 bp fragment was amplified from immediately upstream of ORF0249 which included the first 65 bp of the 5' end of ORF0249. Similarly PCR primers AE_249_3 and AE_249_4 (Table 2.3) were used to amplify a 988 bp fragment from immediately downstream of ORF0249 which included the last 53 bp of the 3' end of ORF0249. The two fragments were then ligated together (using the incorporated *Xho*I site); the ca. 2 kb product was amplified by PCR using primers AE_249_1 and AE_249_4 and the resulting mutant gene product was designated Δ ORF0249. The Δ ORF0249 deletion derivative was cloned into *Sal*I digested pCARV (facilitated by primer-encoded restriction sites) and the resulting recombinant plasmid, designated pCARV007, was used to transform *C. pseudotuberculosis*. Subsequently, an attempt was made to replace the wild-type, chromosomal ORF0249 gene with the plasmid-borne Δ ORF0249 by homologous recombination, as described in Chapter 2. Bacterial cells which were assumed to have undergone chromosomal integration of pCARV007 were identified by virtue of their ability to grow in the presence of erythromycin at 37°C (the non-permissive temperature for plasmid replication). A single colony deriving from a cell in which pCARV007 had integrated into the chromosome was used to inoculate a quantity of BHI medium containing erythromycin. Subsequently, the second stage in the mutagenesis protocol was conducted in order to obtain a derivative of *C. pseudotuberculosis* in which the wild-type gene had been replaced with Δ ORF0249. Using primers Δ 0249_DCO_F and Δ 0249_DCO_R (Table 2.3), a number of colonies which had derived from cells in which the pCARV007 plasmid had excised were screened by PCR. Unfortunately PCR carried out on the cells in which the plasmid had excised did not result in an amplicon of the correct size, confirming that a mutant had not been obtained. Unfortunately however, due to lack of time in this final stage of the project, it was not possible to pursue the construction of the mutant any further.

6.2 Discussion

In Chapter 3 of this thesis, results pertaining to the ability of *C. pseudotuberculosis* to utilise transferrin-bound iron were presented. In addition, a previously published study revealed that the related human pathogen, *C. diphtheriae*, requires a functional siderophore to obtain iron from transferrin (Schmitt, 1997b). Taken together, it was thought possible that these observations might be linked, and that *C. pseudotuberculosis* may therefore also produce a siderophore; however, despite decades of research attention, no siderophore had previously been reported for this organism. The aim of the work presented in this chapter was therefore to determine whether *C. pseudotuberculosis* produces a siderophore; if, having successfully demonstrated siderophore production, the next objective was then to use a comparative approach in order to identify the genes responsible for the siderophore biosynthesis.

In order to detect a functional siderophore, CAS assays were carried out. The CAS assay (Schwyn & Neilands, 1987) provides a useful, straight-forward and relatively rapid method of detection of all types siderophore; hence, it is a powerful tool to allow screening for this phenotypic attribute, either on solid media or in liquid assays, and no prior knowledge of the type of siderophore an organism produces is required. The assay is based on the colour change that occurs during the transfer of ferric ion from its intense blue complex to the siderophore, resulting in an orange-pink colour. In order to ensure the best chance of detecting a siderophore, *C. pseudotuberculosis* was initially grown in CCDM supplemented with 300 μ M (final concentration) of dipyrindyl, the so-called “low-iron growth medium” utilised throughout the work described in this thesis. In this way, a liquid CAS assay, assessing serial-dilutions of culture supernatant, allowed the detection of a *C. pseudotuberculosis* siderophore (**Figure 6.1**). Significantly, the work described here is the first documentation of a siderophore produced by this organism.

The siderophore, aerobactin, into which much research has been conducted, has been shown to be synthesised by a NRPS-independent (NIS) pathway, and utilises two siderophore synthetase enzymes, IucA and IucC (de Lorenzo, *et al.* 1986) (Challis, 2005). Other siderophores produced via NIS biosynthesis pathways include vibrioferrin of *Vibrio parahaemolyticus*, staphylobactin of *Staphylococcus aureus* and anthrachelin of *Bacillus anthracis* (Challis, 2005), and it is interesting to note that each of these pathways utilises at least one enzyme with significant sequence similarity to the aerobactin siderophore

synthetases (IucA and IucE) (Challis, 2005). It is perhaps of relevance that often, pathogens that biosynthesise siderophore via a NRPS-independent pathway require a functional siderophore to achieve full virulence (Miethke & Marahiel, 2007; Challis, 2005). An example of this has been reported for the porcine pathogen, *Bordetella bronchiseptica*, whereby strains that are unable to produce the NIS siderophore, alcaligin, are apparently reduced in virulence in swine (Register, *et al.* 2001).

Recently, a gene (designated *ciuE*) sharing significant homology to the genes encoding aerobactin biosynthesis enzymes IucA and IucC, was identified in *C. diphtheriae* (Kunkle & Schmitt, 2005). IucA and IucC are the prototypes of NRPS-independent siderophore synthetases that catalyse aerobactin synthesis; significantly, all known NRPS-independent siderophore synthesis pathways utilise at least one enzyme with high sequence similarity to the aerobactin synthetases (Challis, 2005; Miethke & Marahiel, 2007). Immediately upstream of *ciuE* was reported the presence of four genes, designated *ciuA*, *ciuB*, *ciuC* and *ciuD* (thought to be in an operon structure), the predicted products of which have significant similarities to ABC-type iron transport systems. The *ciuA* gene encoded a protein which was similar to other iron uptake proteins of Gram-positive bacteria; in particular, lipoproteins; the *ciuB* and *ciuC* genes were predicted to encode the permease components of an ABC transport system while *ciuD* shared similarities to the ATPase component (Kunkle & Schmitt, 2005). Subsequently, Kunkle and Schmitt (2005) created an in-frame deletion derivative of the *ciuE* and *ciuA* genes in *C. diphtheriae* (Kunkle & Schmitt, 2005). The researchers demonstrated that deletion of 95 % of the *ciuE* gene resulted in a significant decrease in siderophore activity relative to wild-type strains under low-iron growth conditions, indicating that the *ciuE* mutant has a defect in siderophore production. However, the presence of a plasmid carrying a functional copy of the *ciuE* gene resulted in wild-type levels of siderophore being produced, providing strong evidence for the role of CiuE in siderophore biosynthesis. Additionally, a deletion in the *ciuA* gene resulted in a severe defect in iron uptake and the ability to obtain iron from *C. diphtheriae* siderophore, implicating this protein in the transport of siderophore.

The utilisation of the hydroxamate xenosiderophore, aerobactin, by *C. diphtheriae* has been reported (Russell, *et al.* 1984). Interestingly, the *C. diphtheriae* siderophore, CiuE, tested negative in Arnow and Csaky tests for phenolate and hydroxamate siderophore (Russell, *et al.* 1984), and it was subsequently postulated that the properties of the corynebacterial

siderophore were not identical to aerobactin. Interestingly, partially-purified *C. diphtheriae* siderophore manifested a positive reaction to the phenolate spray test (which detects all phenolate compounds) in a study conducted by Russell *et al.* (1984), however, the Arnow test is considered to be more stringent (Russell, *et al.* 1984), and the non-phenolate classification of the *C. diphtheriae* siderophore would appear to stand at this time. Since the structure of the *C. diphtheriae* siderophore has yet to be elucidated, conclusive evidence as to what type of siderophore it is does not exist. Furthermore, it is not known whether the bacterium produces more than one siderophore, although a defined CiuE-deficient mutant of *C. diphtheriae* did not appear to produce any other siderophore that would facilitate growth of the mutant under reduced-iron growth conditions (Kunkle & Schmitt, 2005).

The use of xenosiderophores by corynebacteria has been well documented (Russell, *et al.* 1984) (Wennerhold & Bott, 2006; Billington, *et al.* 2002). For example, Wennerhold and Bott (2006) inspected the *C. glutamicum* ATCC 13032 genome and were unable to identify obvious siderophore biosynthesis genes; the authors subsequently suggested that this *C. glutamicum* strain copes with iron starvation by using a variety of siderophores produced by other microbes (Wennerhold & Bott, 2006). Interestingly however, it has been suggested that another *C. glutamicum* strain (ATCC 14067) does in fact produce a cyclic catecholate-type siderophore (Budzikiewicz *et al.* 1997 in Wennerhold and Bott (2006)) (Wennerhold & Bott, 2006). On the one-hand it is possible that strain-specific differences exist among different *C. glutamicum* strains, with respect to the ability to produce siderophore, however, there has been significant debate over the accuracy of the data pertaining to the ability of *C. glutamicum* ATCC 14067 to produce a cyclic catecholate siderophore (Dertz, *et al.* 2006).

Like *C. diphtheriae*, the structure of the *C. pseudotuberculosis* siderophore described here has yet to be elucidated. Therefore, it is not currently possible to conclusively determine whether the type of siderophore produced is the same in both organisms. However, an interesting experiment to study the functionality of the *C. pseudotuberculosis* siderophore was conducted, whereby the *C. pseudotuberculosis* siderophore was used to complement a *C. diphtheriae* CiuE-deficient mutant (**Figure 6.4**). During a working visit to the laboratory of Prof. Randall Holmes at the University of Colorado at Denver and Health Sciences Centre, and in collaboration with Dr Sheryl Zajdowicz of Prof. Holmes' lab, a bioassay was conducted. The results of the bioassay revealed that the *C. pseudotuberculosis* siderophore (produced only

under low-iron growth conditions and secreted into the culture supernatant) was effectively utilised by the *C. diphtheriae* mutant, allowing acquisition of iron and subsequent growth, in a manner identical to that observed upon supplementing the mutant strain with culture supernatant containing wild-type *C. diphtheriae* supernatant. These results indicate that *C. diphtheriae* is able to utilise *C. pseudotuberculosis* siderophore; however, it should be noted that the results shown here do not prove that the siderophore types are the same, and there is also a possibility that the *C. pseudotuberculosis* siderophore was transported into the *C. diphtheriae* cell via another transporter. In this respect, the alternative putative ABC-transporter, Irp6 has been suggested to be involved in the uptake of xenosiderophores in *C. diphtheriae* (Kunkle & Schmitt, 2005). Therefore, unless equivalent experiments were conducted using siderophore-deficient mutant strains, also deficient in either the Ciu or the Irp6 ABC-transporter, it is not possible to prove that the *C. pseudotuberculosis* and *C. diphtheriae* siderophores are functionally similar; however, it would seem highly likely that they are, given the 52 % identity and 68 % similarity between the two proteins.

CCDM growth medium supplemented with a range of concentrations of FeCl_3 was assessed to determine whether the *C. pseudotuberculosis* siderophore was produced in response to declining environmental iron concentrations. Results showed conclusively that the lower the concentration of iron in the growth medium was, the greater was the level of siderophore production. However, a further interesting observation was also made at this point. Previously during this investigation, high-iron growth conditions had been determined, and utilised in preliminary experiments, and the amount of iron (in the form of FeCl_3) used to supplement CCDM for high-iron growth was based upon the observation that an FeCl_3 concentration beyond 1 μM did not improve the growth of *C. pseudotuberculosis* in CCDM any further. However, despite the fact that this concentration was sufficient to allow optimal growth, during the analyses which were conducted to investigate whether siderophore was produced in response to environmental iron concentration, it was observed that 10 μM (final concentration) of FeCl_3 was required for maximum repression of siderophore production. Obviously, this concentration was significantly higher than that of the medium previously designated optimal for high-iron growth. It was considered entirely possible that if siderophore production was repressed more-fully by this higher concentration of iron, then the same may also be true of the repression of other genes which were regulated in a similar manner; as a

result, an FeCl₃ concentration of 10 µM was used for all subsequent experiments as well as experiments in Chapter 5 which were carried out concurrently with the work presented here.

Following the discovery of a *C. pseudotuberculosis* siderophore it was desirable to identify the responsible genes. Efficient generation of *C. pseudotuberculosis* transposon random-insertion libraries has been achieved in our laboratory (unpublished data) and by others (Dorella, *et al.* 2006). Initially, it was intended that such a library would be screened in order to identify mutants deficient in siderophore production, through which it would be possible to identify the genes responsible. However, towards the end of this investigation it was fortuitous that a partial *C. pseudotuberculosis* genome sequence became available as part of a separate study in the same laboratory. Therefore, rather than constructing a transposon-insertion library and subjecting it to time-consuming screening, it was considered a quicker approach to scan the preliminary annotation of the genome sequence with a view to identifying anything relating to a siderophore-encoding sequence. As discussed above, a locus which at this time is simply designated ORFs 02945-02949, which shared significant (between 54-84%) amino acid homology to the *C. diphtheriae* *ciu* locus (NCTC 13129 locus tag DIP 0582-DIP 0586) was identified. However, were more time available within the current study, it would have been interesting to complement the bioinformatic approach with the molecular approach of screening a transposon-insertion library for siderophore-deficient mutants. In that way it would have been possible to corroborate the involvement of certain genes in the production/transport of siderophore, and relate these findings back to the bioinformatic predictions. In addition, it would have been possible to identify potential regulatory components involved in siderophore production.

It is common for genes involved in siderophore biosynthesis and transport to be regulated in an iron-dependent manner (Hantke, 2001). To determine whether the genes in the *C. diphtheriae* *ciu* locus were regulated in response to iron concentration, Kunkle and Schmitt (2005) analysed the transcription of these genes under varying environmental iron conditions. The researchers identified two putative promoter regions, one upstream of the *ciuA* gene and the other upstream of the *ciuE* gene (Kunkle & Schmitt, 2005), both of which were regulated in an iron-dependent manner. Similarly in *C. pseudotuberculosis*, a DtxR binding locus was identified upstream of the *C. diphtheriae* *ciuA* and *ciuE* equivalent genes, ORF02945 and ORF02949. As part of the comparative approach of this investigation, and, in order to further

characterise the putative siderophore locus (ORFs 02945-02949) in *C. pseudotuberculosis*, a promoter fusion assay was employed to study the promoter activity upstream of the ORF 02949 in *C. pseudotuberculosis*; however, due to lack of time it was not possible to study the promoter upstream of ORF02945. The 309 bp chromosomal fragment, taken from immediately upstream of the putative siderophore-encoding gene (ORF02949) was cloned into pSPZ (Oram, *et al.* 2006), and expression of the *lacZ* reporter gene from the resulting construct (designated pCAW009) was assessed in the natural host, *C. pseudotuberculosis*. Following growth under high- and low-iron conditions, and using the *fagA* gene promoter as a positive control (**Chapter 5**), β -galactosidase activity was measured. In agreement with what had been seen previously for *ciuE* in *C. diphtheriae*, the predicted promoter region upstream of *C. pseudotuberculosis* ORF02949 was shown to be functional, as determined by its ability to drive expression of the promoterless *lacZ* gene. In addition, mirroring the observations of the CAS assays, ORF02949 was shown to be expressed in an iron-dependent manner, with greater expression occurring in low-iron growth medium (**Figure 6.5**).

The identification of a functional DtxR protein in *C. pseudotuberculosis* (**Chapter 5**), allowed the investigation of any involvement of DtxR in the regulation of iron-dependent expression of the putative siderophore-encoding gene. The observation that ORF02949 contained a potential DtxR-binding motif immediately upstream lent weight to the potential involvement of DtxR in the regulation of this gene. In order to confirm the involvement of DtxR in the regulation of expression of ORF02949, the pCAW009 promoter fusion vector was introduced into the DtxR-deficient *C. pseudotuberculosis* mutant (Cp- Δ dtxR) constructed during the work presented in Chapter 5. Interestingly, the results demonstrated that ORF02949 was indeed regulated by DtxR in an iron-dependent manner. As expected, the lack of a functional DtxR protein resulted in a non-significant difference between the expression of ORF02949 under high- and low-iron growth conditions (as demonstrated by a paired t-test). This result was as expected since ORF02949 was shown to be expressed in an iron-dependent manner in wild-type *C. pseudotuberculosis* and it was highly likely this iron-dependent regulation was a result of the DtxR protein. As was noted with the expression of *fagA* (previously shown to be regulated by DtxR in Chapter 5) there was a significant difference between the expression of ORF02949 in wild-type and Cp- Δ dtxR strains ($p=0.009$). Significantly, this difference was more apparent at the higher level of iron suggesting an interaction effect between strain and iron.

Previously, Billington *et al.* (2002) identified a genetic locus in a goat-derived *C. pseudotuberculosis* isolate that contained open reading frames which shared significant homology to genes encoding iron transport systems. The researchers identified an operon designated *fagABC*, which encoded proteins that collectively resemble cytoplasmic membrane transport systems for iron-siderophore uptake (Billington, *et al.* 2002). The researchers reported that, in particular, the genes shared a high degree of similarity to proteins involved in uptake of the catecholate siderophore enterochelin (enterobactin) (Billington, *et al.* 2002), although no *C. pseudotuberculosis* siderophore was detected in that study. Subsequently, the researchers constructed a *fagB*-deficient knock out strain, and examined the ability of the mutant to acquire iron from FeCl_3 , FeSO_4 , hemin, haemoglobin and transferrin. Interestingly, the mutation within the *fag* operon had no effect on the ability of *C. pseudotuberculosis* to obtain iron from any of the sources tested. The researchers subsequently hypothesised that the effect of the mutation in the Fag uptake system was masked by another uptake system with similar specificity; they also suggested that the products of the *fag* genes are responsible for the uptake of a xenosiderophore, which they hypothesised was supported by the fact that there was no siderophore biosynthetic gene(s) clustered with the *fag* genes (Billington, *et al.* 2002). It is interesting to postulate that the *C. pseudotuberculosis* putative siderophore biosynthesis gene (ORF02949) and upstream putative siderophore transporter-encoding genes (ORFs 02945, 02946, 02947, 02948), which collectively share more homology with the *ciuE* locus of *C. diphtheriae* than with any other gene so far reported in any other bacterium, are in fact the true *C. pseudotuberculosis* siderophore biosynthesis and transport genes. Furthermore, it could be possible that the *fagABC* operon is the *C. pseudotuberculosis* equivalent of the *C. diphtheriae* *irp6ABC* operon, involved in the uptake of xenosiderophores; however, although an attractive hypothesis, further work is required to confirm this, and it is significant to note that a BLAST search of the *C. diphtheriae* *irp6ABC* genes against the (ca. 98 % complete) *C. pseudotuberculosis* genome did not identify any significant homologues. Therefore, while it is entirely possible that the *fag* and *irp6* loci encode genes with a similar function, they have either been acquired independently, or have diverged significantly over time.

Unfortunately, because of a lack of time remaining at the end of this project, it was not possible to conclusively prove that *C. pseudotuberculosis* ORF 02949 encodes the siderophore biosynthesis gene, although the available evidence would tend to support this hypothesis. Preliminary attempts to construct a defined mutant deficient in the expression of the

ORF02949 failed. It subsequently appeared likely that this was due to experimental error and that the initial mutagenesis construct was incorrect; however, a second attempt was not possible due to time constraints. At some point it will be imperative to revisit this work to allow confirmation of the involvement of this gene in siderophore production by *C. pseudotuberculosis*. Subsequently, it would be possible to conduct a variety of assays, including assessment of the mutant's ability to utilise transferrin and bacterial xenosiderophores as sources of iron. Further interesting work could involve the construction of mutant strains in which the ORF02945 and *fagA* genes had been serially-deleted; it would then be possible to determine whether such a mutant was still able to uptake both *C. pseudotuberculosis* siderophore and xenosiderophores. This would provide evidence as to which transport system is being controlled by which locus, since if a deletion in ORF02945 was found to prevent the uptake of *C. pseudotuberculosis* siderophore but a deletion in *fagA* did not then it could be postulated that ORF02945 is involved in transport of *C. pseudotuberculosis* siderophore while *fagA* is not.

At this time, no attempt has been made to give the *C. pseudotuberculosis* siderophore biosynthetic and transport genes any more meaningful designation, this being primarily due to the fact that further analysis of this region is required to conclusively confirm the role that these genes play in siderophore production and uptake. However, the apparent absence of any other siderophore-encoding gene from the *C. pseudotuberculosis* genome sequence so-far available would tend to implicate ORF02949 as the only candidate for the siderophore biosynthesis gene. Through their proximity to ORF02949 and their homology with the *ciuABCD* ABC transporter genes of *C. diphtheriae*, ORF02945, ORF02946, ORF02947 and ORF02948 are likely candidates for the *C. pseudotuberculosis* siderophore-uptake machinery. Although further work is required to conclusively prove (or disprove) this hypothesis, the previous observation by Billington *et al.* (2002) that the Fag-deficient *C. pseudotuberculosis* mutant was still able to acquire iron from transferrin would suggest that, if siderophore is required for such a process in *C. pseudotuberculosis*, then the only other possible siderophore transporter identified thus-far is that encoded by ORFs 2945-2948.

In conclusion, the work described in this chapter has lead to the discovery of a novel *C. pseudotuberculosis* siderophore, which is produced in response to declining environmental iron conditions. The translated products of the ORFs encoded within the genetic locus which

has been putatively associated with biosynthesis and transport of the siderophore have been shown to share between 54-84 % homology (dependent on the ORF) to the proteins encoded by the *ciu* locus of *C. diphtheriae*, which is responsible for the production of siderophore in that organism. However, it should be noted that a significant degree of divergence of sequence has occurred between the two loci, and more importantly that the two loci exist in completely different regions of the *C. pseudotuberculosis* and *C. diphtheriae* chromosomes. There is evidence that the locus in *C. pseudotuberculosis* has been acquired by horizontal gene transfer and although the siderophore-encoding locus in either *C. pseudotuberculosis* or *C. diphtheriae* may have resulted from infection with the equivalent (*e.g.*) bacteriophage, it would appear that these acquisitions occurred independently of each other. Irrespective of the origin of the siderophore-encoding locus, it is clear that further work into this aspect of *C. pseudotuberculosis* virulence would be worthwhile with respect to beginning to elucidate the organism's ability to survive and persist within its chosen host.

Chapter Seven
General Discussion

In recent years, CLA has become endemic in the UK and, in addition to issues of animal welfare, is becoming of increasing concern as a cause of financial loss to the sheep-producing industry. Vaccination options against CLA are limited, especially in the UK, and it is therefore imperative that novel strategies for combating this disease are developed. At the simplest level, such strategies could be focused upon the development of improved diagnostic reagents, to allow diseased animals to be identified more efficiently. However, the increasing incidence of CLA within the national flock is such that it is becoming increasingly expensive (and therefore unlikely) that the disease will be combated by a “test and cull” strategy alone. Rather, the majority of sheep-producing communities, both in the UK and elsewhere, all look to the development of an effective vaccine as a solution to the problem. Traditionally, the vast majority of sub-unit vaccines against bacterial diseases are based upon derivatives of bacterial virulence proteins (a relevant example being the Diphtheria vaccine, which contains toxoided *C. diphtheriae* toxin). This highlights the link between vaccine development and the fundamental science upon which it is based. In order to identify an effective vaccine candidate, it is first necessary to understand the involvement of that candidate in the natural infection process. As such, it is often through studies into microbial pathogenicity that suitable vaccine candidates are identified. Taking all of this into account, the basic lack of understanding in relation to *C. pseudotuberculosis* has clearly been a sticking point, not only in the development of new CLA vaccines but also of diagnostic reagents and other methods to limit disease spread.

Taking the above into account, the impact of the work described in this thesis is significant, first and foremost with respect to building upon the weak foundations that formed the level of understanding of *C. pseudotuberculosis* and its interaction with the ovine/caprine host that existed when the work described here was begun. As a result of this work, genetic manipulation, which was previously a difficult and relatively imprecise methodology, is now a routine procedure. In this respect, the work described here has paved the way for several other studies already underway in this laboratory. Through this work, it is anticipated that our understanding not only of *C. pseudotuberculosis* itself, but of the comparative similarities between it and the other members of the “diphtheria” group of organisms will increase, allowing us to gain further insight into the evolution of these organisms from their common host, and begin to unravel the factors which contribute to host-specificity. This type of approach will be particularly interesting with respect to *C. pseudotuberculosis*, since of all the

diphtheria group it appears to be the only species which has undergone further evolutionary changes that have resulted in the emergence of distinct *C. pseudotuberculosis* biotypes, which are specific for either ovine/caprine or equine hosts. Undoubtedly the *C. pseudotuberculosis* genome project, ongoing in this laboratory, will expand our understanding of this divergence; however, without tools with which to study novel determinants, the availability of genome sequence(s) is of limited benefit.

Significantly, the allele-replacement system altered and optimised during the course of this study was one previously reported for use in *C. glutamicum*. Based upon the applicability of the system within both *C. glutamicum* and *C. pseudotuberculosis*, it is extremely likely that the same system is equally as applicable to use in other corynebacterial species, including *C. diphtheriae*. Currently, in this latter species, the most frequently used system of mutagenesis is by the plasmid pK19mobsacB (Schafer, Kalinowski, & Puhler, 1994). This system works by RP4-mediated conjugation (rather than transformation of electrocompetent cells) and a modified *sacB* gene to aid the isolation of organisms which have undergone secondary recombination. However, pK19mobsacB is a non-replicative vector and, as discussed in detail in chapter 4, non-replicative vectors are less efficient than replicative vectors such as pCARV. Furthermore, corynebacterial plasmids are frequently able to replicate in mycobacterial hosts (*e.g.* pEP2, based upon the pNG2 plasmid (Messerotti, *et al.* 1990)), and it is therefore entirely possible that the pCARV mutagenesis vector is equally applicable to the genetic manipulation of this related actinomycete genus.

While the mutagenesis vector utilised in this study derived from a plasmid previously used in *C. glutamicum*, it is important to note that it was changed significantly during the course of the work described here. In the first instance, the deletion of ORFs 1 and 2 served to reduce the overall size of the plasmid, thus increasing the ease by which the plasmid could be introduced into host cells by electroporation, but also decreasing the metabolic burden to the host cell. In addition, the replacement of the kanamycin resistance marker with an erythromycin resistance marker facilitated the application of a novel approach to the selection of secondary cross-over mutants through the ability to inhibit the growth of the “desired” cells through the action of the bacteriostatic antibiotic erythromycin prior to killing unwanted cells through the action of ampicillin. The author is not aware of such an approach having been reported previously, and it has the significant advantage of enriching secondary cross-over cultures for the desired cells,

greatly facilitating downstream screening for the identification of the desired mutant. This is particularly important with respect to allele-replacement by homologous recombination between extremely short sequences. In the current study, the smallest fragment size assessed for the ability to drive homologous recombination in the chromosomal region surrounding the *cp40* gene was 250 bp. It was thought in advance of this work being conducted that using a fragment of this size would render it extremely difficult to isolate cells having undergone subsequent homologous recombination events. However, in reality, although the frequency of recombination using this length of fragment was clearly lower than with larger fragments, the rate of recombination was still determined to be up to 13 % (although ranging from 0-13 %). Therefore, in combination with the novel antibiotic enrichment procedure, there is clearly the capacity to undertake targeted homologous recombination experiments using even shorter sequence lengths. This is particularly significant in cases where very little DNA sequence information exists for the target region. While to some extent the existence of a genome sequence negates the importance of being able to conduct targeted gene replacement with very short sequences, it must be noted that genome sequences do not exist for all strains/species/genera which could potentially be mutagenised using this plasmid system. The other important point to make is that the mutagenesis plasmid pCARV could be manipulated further to allow its use in human-pathogenic organisms where it is unethical to use erythromycin antibiotic resistance markers due to the routine clinical use of the antibiotic to treat human infections; an important case in point is *C. diphtheriae*. In such cases the novel enrichment procedure could still be used if the erythromycin antibiotic resistance marker was replaced with another bacteriostatic antibiotic; however, it would seem that the improvement in recombination resulting from the build up of single-stranded intermediates associated with the replication of pCARV, might be sufficient in many cases to allow efficient mutagenesis without the need to employ the enrichment process.

During this project the newly developed allele mutagenesis procedure was applied to *C. pseudotuberculosis* in order to create a mutant strain, deficient in the production of serine protease (Cp40). The key reason for using this gene to trial the novel mutagenesis protocol was due to the accessibility of the published gene sequence prior to the availability of the partial *C. pseudotuberculosis* genome sequence; however, of added interest was the fact that Cp40 had also previously been shown to exhibit immunogenic properties (Wilson, *et al.*, 1995). Interestingly, an *M. tuberculosis* serine protease was implicated in the intracellular

survival of the pathogen within macrophages (Vandal, *et al.* 2008). *M. tuberculosis* survives within macrophages by preventing fusion of phagosomes with lysosomes; however, it also persists within acidic phagolysosomes. Using ratiometric fluorescence measurements, Vandal *et al.* (2008) demonstrated that *M. tuberculosis* is acid resistant and is able to control its intrabacterial pH in acid *in vitro* as well as in both activated and non-activated macrophages (Vandal, *et al.* 2008). The researchers also demonstrated that a mutant strain disrupted in ORF Rv3671c (encoding a predicted serine protease) was unable to control its intrabacterial pH and survive within activated macrophages; furthermore, the Rv3671c mutant derivative was severely attenuated in mice (Vandal, *et al.* 2008). With this in mind, it is tempting to speculate a similar role may exist for the Cp40 serine protease. Subsequently, it would be of great interest to characterise the properties of the $\Delta cp40$ strain further. In particular, exposing the mutant strain to varying concentrations of acid, and comparing its growth to that of the wild-type strain grown under the same conditions could be achieved easily; thereby making it possible to observe what effect the deletion of the *cp40* gene has with regards to the acid tolerance of *C. pseudotuberculosis*. These initial experiments could be followed up by comparing the intracellular survival of the mutant against that of wild-type *C. pseudotuberculosis*, which could be achieved using flow cytometry or gentamycin killing assays in the first instance. Further experiments, as the tools become available, could include microarray analysis of gene expression in macrophages; these experiments would allow the comparative gene expression between mutant and wild-type. This work highlights the use of an ‘informed selection’ exercise whereby genes that are involved in virulence in closely related organisms (such as the well studied pathogens *C. diphtheriae* and *M. tuberculosis*) can be assessed for a similar role in *C. pseudotuberculosis*.

It is significant that, through the course of the work described here, greater similarities have been shown to exist between *C. pseudotuberculosis* and *C. diphtheriae* than might have been thought. Until a full comparative analysis of the ongoing *C. pseudotuberculosis* genome has been conducted, it is not possible to speculate as to the extent of these similarities; however, at least with respect to the conservation of and expression of genes within the *dtxR* chromosomal locus, and the action of *C. pseudotuberculosis* DtxR as a regulator of target gene expression under low-iron growth conditions, the similarities between the two species are striking. Since DtxR in *C. diphtheriae* is associated with the regulation of genes associated with iron storage and homeostasis, it seems highly likely that the same will also be true of *C.*

pseudotuberculosis. However, it seems that at least some virulence genes which have evolved to be expressed under the control of DtxR, are likely to vary between the two species, since these factors are likely to have been subjected to the greatest environmental pressure to change, being intimately associated with the bacterium and its association with its chosen host. Significantly however, on infection with a *tox* positive bacteriophage, both *C. pseudotuberculosis* and *C. ulcerans* are capable of producing the potent diphtheria toxin in an iron-dependent manner, and, as such in the UK, isolation of any toxigenic *Corynebacterium*, including *C. pseudotuberculosis* and *C. ulcerans*, requires notification of local and national communicable disease control agencies (Efstratiou & George, 1999). The presence of these toxigenic strains of corynebacteria, other than *C. diphtheriae*, highlight the importance of employing a comparative approach to determine DtxR-regulated genes of *C. pseudotuberculosis*, since there may be other important DtxR-regulated genes which are involved in virulence of *C. pseudotuberculosis* and/or *C. ulcerans* that have, as yet, not been identified.

During the early stages of this work, a chemically defined medium was developed. At the time the medium was developed, several factors were kept in mind. Firstly, it was necessary to create a medium which was relatively simple to make; furthermore, the medium was required to be easily manipulated in order to study different parameters of growth; for example, the effect of different metal ions, the concentration of amino acids and the effect of different carbon sources. The resulting medium, so-called CCDM, was designed in such a way that these parameters, and indeed other such parameters, could be easily studied. The CCDM was proven to be useful in determining the effect of environmental iron concentrations on growth of *C. pseudotuberculosis* and preliminary experiments demonstrated that the CCDM was capable of supporting *C. diphtheriae* growth, however these experiments highlighted the requirement for optimisation of the CCDM when growth of organisms other than *C. pseudotuberculosis* was required since the medium has been developed specifically with *C. pseudotuberculosis* in mind.

During the initial stages of this investigation the ability of *C. pseudotuberculosis* to utilise iron from various sources was determined (**Chapter 3**). Interestingly, *C. pseudotuberculosis* was able to utilise iron from host iron binding proteins transferrin and lactoferrin. Previously, Schmitt *et al.* (1997) had suggested that in order for *C. diphtheriae* to utilise iron from

transferrin, it was necessary for there to be a functional siderophore uptake system (Schmitt, 1997b). Taking into account the many similarities observed between the two species with regards to iron mechanisms it appeared likely that the same be true for *C. pseudotuberculosis*. Prior to this study however, there had been no previously documented evidence of a *C. pseudotuberculosis* siderophore. Continuing with the comparative theme, and making use of the partial *C. pseudotuberculosis* genome that became available during the later stages of this project, BLAST analysis identified a locus which shared significant homology to the Ciu locus of *C. diphtheriae*, which encodes siderophore biosynthesis and transport genes. Using this information, a more thorough examination of the iron-dependent expression of ORF02949 (a putative siderophore biosynthesis gene) was carried out. In addition, functional assays were employed to prove the existence of a siderophore in *C. pseudotuberculosis*. Functional assays identified a *C. pseudotuberculosis* siderophore, however, until such a time that an isogenic mutant of ORF02949 is created it is not possible to confirm or refute whether the ORF02949 gene is responsible for the expression of *C. pseudotuberculosis* siderophore. Further work could include testing the mutant strain for its ability to produce siderophore and obtain iron from transferrin. Also since there is no information regarding the *C. pseudotuberculosis* siderophore it would be interesting to elucidate the structure of the siderophore and to compare it to that of *C. diphtheriae* siderophore as and when this information becomes available. In order to identify genes encoding the siderophore transport machinery, it would be interesting to screen a transposon library using the CAS assay.

The work from this project has added significantly to the available information regarding the mechanisms by which iron-dependent genes of *C. pseudotuberculosis* are regulated. Furthermore, the identification of the DtxR-dependent regulation of *fagA*, together with previous research by Billington *et al.* (2002), has demonstrated that DtxR plays a role in the regulation of virulence genes in *C. pseudotuberculosis*. Further work is required to determine whether any other DtxR regulated genes are involved in virulence. Since the genome has become available, using bioinformatics, it is now possible to identify DtxR binding loci. Importantly, genes that are DtxR-regulated could be involved in pathogenesis. Using the newly developed allele replacement mutagenesis system, it would be interesting to construct mutations in these genes and perform *in vitro* and *in vivo* assays in order to determine their involvement in pathogenesis of *C. pseudotuberculosis*. In addition, it would be interesting to revisit the application of proteomic approaches in an attempt to identify exported proteins

from *C. pseudotuberculosis* grown under high- and low-iron conditions; particularly, with the intention of complementing previous work which identified DtxR-regulated genes. By comparing the exported proteins profiles under high- and low-iron resulting from the Cp- Δ dtxR strain, wild-type and complemented Cp- Δ dtxR it would be possible to identify those proteins that are regulated by iron, via DtxR in *C. pseudotuberculosis*. To complement this work it may also be valuable to perform a further Western blot with CLA positive sera in an attempt to identify the antigenic proteins regulated by DtxR/iron. Once these proteins are identified, it would be possible to create recombinant proteins, and subsequently assess the vaccine potential of the recombinant proteins in more detail using, for example, *in vivo* and *in vitro* virulence studies. Such experiments could be carried out in a mouse model of infection or preferably using the well-established sheep infection model that has been created at Moredun Research Institute.

In conclusion, this project has added significantly to the understanding of *C. pseudotuberculosis* and possible mechanisms by which it causes disease. Furthermore, the conducted work has provided important tools for the growth/manipulation of the organism which previously did not exist. Using these newly available tools, it has been possible to demonstrate the existence of a functional DtxR protein, which has not only been shown to be a functional homologue of the *C. diphtheriae* DtxR, but has also been shown to be involved in the regulation of several *C. pseudotuberculosis* genes, at least one of which (*fag*) has currently been shown to be associated with virulence. In addition, the first evidence of a *C. pseudotuberculosis* siderophore has been presented. A putative siderophore biosynthesis gene has been identified and shown to be regulated by iron, via DtxR. The ‘user friendly’ chemically defined medium described here has allowed the investigation of iron-dependent gene and protein expression. This tool will allow subsequent studies into the DtxR-regulation of, and more generally, iron or metal ion regulated genes. In addition, the newly developed effective allele replacement mutagenesis system for *C. pseudotuberculosis*, along with the genome sequence, will promote the construction of *C. pseudotuberculosis* isogenic mutants that will ultimately provide evidence for the mechanisms of pathogenesis of *C. pseudotuberculosis*. Armed with the understanding that these tools will facilitate, the development of novel control strategies against this organism could eventually see effective control of, and perhaps even eradication of, CLA from the UK.

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Appendices

Appendix One: Common Buffers and reagents

Reagent	Recipe
Buffer P1	50 mM Tris-HCl, 10 mM EDTA (pH 8.0)
FA gel buffer (10 x)	200 mM 3-[N-morpholino]propanesulfonic (MOPS), 50 mM sodium acetate and 10 mM EDTA, adjusted to pH 7 with NaOH
FA gel running buffer (1 x)	100 ml 10 x FA gel buffer, 20ml of 37 % (12.3 M) formaldehyde 880 ml of RNase free water
Luria- Bertani (LB) Agar	1 % w/v Bacto-tryptone; 0.5 % w/v Bacto yeast extract; 1 % w/v NaCl; 1.5 % agar
Luria- Bertani (LB) Broth	1 % w/v Bacto-tryptone; 0.5 % w/v Bacto yeast extract; 1 % w/v NaCl
Phosphate buffer pH range 7-9	0.1 M K_2HPO_4 adjusted to required pH with 0.1 M KH_2PO_4
Phosphate-buffered saline	0.1 M phosphate buffer pH 7.5 with 0.9% w/v NaCl in ddH ₂ O
RNA loading buffer (5 x)	16 µl saturated aqueous bromophenol blue solution, 80 µl 500 mM EDTA, pH 8, 720 µl 37 % (12.3 M) formaldehyde, 2 ml 100 % glycerol, 3084 µl formamide, 4 ml 10 x FA gel buffer, RNase free water up to 10 ml
SDS-PAGE Resolving gel mixture (10 %)	10% w/v N,N'-methylenebisacrylamide 375 mM Tris-HCl (pH 8.8), 0.1% SDS, 0.025% ammonium persulphate (APS), 0.005% N, N, N', - tetramethylethyldiamine (TEMED)
SDS-PAGE Running buffer (10 x)	25 mM Tris, 192mM glycine and 0.1 %

	SDS in 1 L ddH ₂ O
SDS-PAGE sample loading buffer (5 x)	0.125 M Tris-HCl pH 6.8; 20% v/v glycerol; 0.01% w/v SDS; 0.02% v/v bromophenol blue; 0.4 ml β-mercaptoethanol
SDS-PAGE Stacking gel buffer	0.5 M Tris-HCl (pH 6.8)
SDS-PAGE Stacking gel mixture (5 %)	1.15 ml ddH ₂ O, 0.33 ml N,N'-methylenebisacrylamide [30 % (w/v)], 0.5 ml stacking gel buffer 0.02 ml 10 % SDS, 0.02 ml 10 % APS 0.002 TEMED
SOB medium	2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl ₂ , 10 mM MgSO ₄
SOC medium	Identical to SOB except with addition of 20 mM glucose
SSC buffer (20 x)	3 M NaCl and 0.3 M sodium citrate (pH 7)
TAE buffer	40 mM Tris-acetate, pH 8.0; 1 mM EDTA in ddH ₂ O
Transformation buffer	14 ml rubidium chloride (2.6 g into 111 ml of ddH ₂ O), 1.14 ml 1 M manganese chloride, 1.5 ml 1 M Calcium chloride, 1.0 ml 1 M potassium acetate, 7.5 ml of 50 % sucrose. Filter sterilised
Tris-buffered saline (10x) pH 7	0.5 M Tris-HCl pH 8.8; 1.5 M NaCl adjusted to pH 7.0 with HCl in ddH ₂ O
Tris-glycine buffer (transfer buffer)	25 mM Tris, 192 mM glycine, 20 % methanol
Z buffer	0.06 M Na ₂ HPO ₄ , 0.04 M NaH ₂ PO ₄ , 0.01 M KCl and 0.001M MgSO ₄ and 2.7 µl/ml B-mercaptoethanol
10 × blocking buffer	

1 × blocking solution	1: 10 dilution of 10 × blocking solution with maleic acid buffer
DIG washing buffer	0.1 M maleic acid, 0.15 M NaCl, 0.3 % (v/v) Tween 20

Appendix Two: Siderophore assay solutions

Reagent	Recipe
1 M HCl	4 ml conc. HCl (12 M) diluted in 44 ml ddH ₂ O (48 ml total)
10 mM FeCl₃ in 100 mM HCl	Dissolve 0.135 g FeCl ₃ .6H ₂ O + 5 ml 1 M HCl in 50 ml ddH ₂ O
2 mM CAS	Dissolve 0.061 g CAS dye in 50 ml ddH ₂ O; cover conical in foil to protect from light
10 mM HDTMA	Dissolve 0.182 g hexadecyl –trimethyl-ammonium bromide in 50 ml ddH ₂ O with stirring
Pipes Buffer	Dissolve 4.307 g pipes in 75 ml ddH ₂ O, add ~ 8 ml 12 M HCL to pH 5.6
CAS Assay buffer	Stir in 6 ml 10 mM HDTMA, 0.15 ml 10 mM FeCl ₃ in 100 mM HCl, and 7.5 ml CAS into the 75 ml pipers buffer (pH 5.6) solution. Add ddH ₂ O to 100 ml. Store at RT in plastic bottle covered in foil. Solution should not be colloidal, and will gradually become dark blue as dye binds to iron