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Investigation of the molecular biology and contribution to virulence of Bordetella bronchiseptica urease

David McMillan

University of Wollongong

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Investigation of the molecular biology and contribution to virulence of *Bordetella bronchiseptica* urease

A thesis submitted in fulfilment of the requirements for the award of the degree

**Doctor of Philosophy**

from

University of Wollongong

by

David McMillan, BSc (Hons)

Department of Biological Sciences

1999
**Declaration**

This thesis is submitted in accordance with the regulations of the University of Wollongong in fulfilment of the degree of Doctor of Philosophy. It does not include any material previously published by another person except where due reference is made in the text. The experimental work described in this thesis is original work and has not been submitted for a degree in any University or Institution.
Acknowledgments

This thesis could not have been completed with the support of many friends and family. Firstly I would like to thank my supervisor, Dr Mark Walker, for his invaluable advice on the research and for also giving me the inspiration to continue when progress was slow.

I also extend my gratitude to the people of Lab111. Thanks then to Peter, Adam, Tania, Jason, Nick, Darren, Yan, Jody and Tamantha. To my friends outside of the lab, who provided me with recreation and relaxation when it was most needed, I also thank you. Some of you may have moved on, but I thank you no less.

Thanks also go to Dr C. A. Guzmán and Dr G. S. Chhatwal for hosting me at the GBF in Germany. Dr Guzmán also undertook the mouse colonisation and intracellular invasion assays.

Finally, I thank my parents. They have provided me with love, a home and unswerving support over the years taken to complete this thesis.
Abstract

*Bordetella bronchiseptica* is a common pathogen of the mammalian respiratory tract. Infection by this organism results in a number of respiratory conditions which may be fatal to the host. Urease is a potential virulence determinant of *B. bronchiseptica* that has been implicated in the pathogenesis of several other microorganisms. The urease operon of *B. bronchiseptica* is encoded within an 8.9 kb DNA fragment which contains the structural genes (*ureA, ureB* and *ureC*) and accessory genes (*ureD* and *ureG*) homologous to urease genes from other bacteria. Uniquely, the *ureE* and *ureF* genes are fused to form a hybrid protein, UreEF, which may result in tighter coordination of the putative functions of the individual accessory genes. The operon contains an additional open reading frame, UreJ, found only also in the *Alcaligenes eutrophus* urease operon. UreJ is also 37% homologous with HupE from *Rhizobium leguminosarum* bv. viciae, and may be involved in nickel transport across the bacterial membrane. A transcriptional activator, designated *Bordetella bronchiseptica* urease regulator (*bbuR*), is located directly upstream and in the opposite orientation to the urease operon. *BbuR* shares homology with members of the LysR regulatory protein family. Other members of this family have been shown to regulate the expression of urease in *Klebsiella aerogenes* (NAC), and to induce the expression of a set of genes in *Escherichia coli* (OxyR) which protects the bacteria from phagolysosomal attack after intracellular invasion. A putative *BbuR* binding site (5'-ATA-N9-TAT-3'), identical to the NAC-binding consensus sequence, was also found 27 bp upstream of the urease promoter in *B. bronchiseptica*. The results of regulation studies show that urease is repressed by the *Bordetella virulence gene* locus (*bvg*). Urease was not inducible by 10 mM urea nor up-regulated in nitrogen limiting conditions. To investigate the role of *BbuR* in the regulation of urease, *BbuR* mutants of the BB7865 and the avirulent strain BB7866 were constructed by homologous recombination. The *BbuR* deficient BB7865 B1 lost the ability to regulate the expression of urease, suggesting that *BbuR* may be an intermediary in the *bvg* regulation of urease. As *BbuR* is
homologous to OxyR, and urease has been suggested to protect bacteria intracellularly, an evaluation of the intracellular survival of urease-negative mutants of BB7865 and BB7866 in comparison to their urease-positive parental strains was also undertaken. This experiment demonstrated that increasing the concentration of urea in the assay increased survival of the wild-type but not urease-negative strains after 24 h, suggesting that urease may have a role in intracellular survival. To further address the role of urease in respiratory infection, we compared the ability of BB7865, the bbuR mutant strain BB7865 B1 which constitutively expresses urease, and the urease negative mutant BB7865 U5 to colonise and persist on the murine respiratory tract. The results showed that the constitutive expression, or abolition of urease activity, had no significant effect on respiratory infection in this model. A DNA probe containing the gene encoding UreA of *B. bronchiseptica* hybridised to chromosomal DNA from *Bordetella pertussis* Tohama I indicating the presence of cryptic urease genes in this urease negative species. PCR primers designed to amplify part of ureD and the urease promoters from *B. bronchiseptica* were also able to amplify identically sized DNA fragments from *B. bronchiseptica*, *B. pertussis* and *B. parapertussis* ATCC15311. Nucleotide sequence analysis of these regions revealed no differences in the ureD open reading frame between each species. A cluster of mutations in both *B. pertussis* and *B. parapertussis* was found upstream of the urease promoter, in a region proximal to the bbuR promoter. The inability of *B. pertussis* to produce urease may therefore reflect mutations in regulatory elements, and not mutations in the urease locus itself. The biochemical data together with the results from the intracellular invasion experiments suggest that urease is somehow involved in post-infection processes. The possible presence of compensatory mechanisms may explain why this hypothesis is not supported by the murine respiratory colonisation assay data.
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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>adenylate cyclase</td>
</tr>
<tr>
<td>Ap</td>
<td>ampicillin</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCG</td>
<td><em>Mycobacterium bovis</em> strain bacillus Calmette-Guerin</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>bvg</td>
<td><em>Bordetella virulence gene</em> locus</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>Cm</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>CMI</td>
<td>cell mediated immunity</td>
</tr>
<tr>
<td>dH₂O</td>
<td>distilled water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNT</td>
<td>dermonecrotic toxin</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FHA</td>
<td>filamentous haemagglutinin</td>
</tr>
<tr>
<td>g</td>
<td>9.8 ms⁻²</td>
</tr>
<tr>
<td>GTP</td>
<td>guanine triphosphate</td>
</tr>
<tr>
<td>GLN</td>
<td>glutamine synthesis</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>Hly</td>
<td>haemolysin</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-d-galactopyranoside</td>
</tr>
<tr>
<td>Km</td>
<td>kanamycin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>NAC</td>
<td>Nitrogen assimilation control protein</td>
</tr>
<tr>
<td>nm</td>
<td>nanometres</td>
</tr>
<tr>
<td>NTR</td>
<td>Nitrogen regulatory system</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>rif</td>
<td>rifampicin</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Tc</td>
<td>tetracycline</td>
</tr>
<tr>
<td>TrisBase</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
</tbody>
</table>
TrisHCl
UV
V
vag
vrg
Xgal

Tris(hydroxymethyl)aminomethane hydrochloride
ultraviolet
volt
virulence activated gene
virulence regulated gene
5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside
INTRODUCTION

1.1. Overview

*Bordetella bronchiseptica* is a Gram-negative respiratory pathogen of wild and domestic animals. Infection by *B. bronchiseptica* is associated with various respiratory conditions, including acute tracheobronchitis in dogs (Bemis *et al.*, 1977; Wright *et al.*, 1973), feline bordetellosis (Jacobs *et al.*, 1993) and bronchopneumonia in guinea pigs (Ganaway *et al.*, 1965). *B. bronchiseptica* has also been implicated as a factor in the development of both mild and severe forms of swine infectious atrophic rhinitis (Roop *et al.*, 1987). Although *B. bronchiseptica* infections of primates have been reported (Good and May, 1971; Graves, 1970), the bacterium rarely infects humans. Adhesins, involved in the attachment to the ciliated epithelial cells of the respiratory tract, and toxins that cause cellular damage mediate the pathogenicity of *B. bronchiseptica*. Many of the same virulence determinants are also found in *Bordetella pertussis* and *Bordetella parapertussis*, the two respiratory pathogens most closely related to *B. bronchiseptica*. Urease is a potential virulence factor of *B. bronchiseptica* and *B. parapertussis* that is not shared by *B. pertussis*. In other microorganisms, urease has been implicated as having a major role in virulence. The major aims of this thesis was therefore to characterise urease at the molecular level and to investigate the potential contribution of this enzyme to the virulence of *B. bronchiseptica*.

1.2. The Genus *Bordetella*

The Genus *Bordetella* consists of seven species. Four of these species, *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. avium* have been extensively characterised (Pittman and Wardlaw, 1981). *B. pertussis* is the causative agent of potentially fatal infant whooping cough. As such it has been comprehensively researched and much of the information gained on the *Bordetella* Genus has been provided through the study of this organism. *B. parapertussis* produces a milder
form of whooping cough in children and has also been isolated from sheep (Porter et al., 1994). *B. bronchiseptica* is a pathogen of a broad range of mammalian species, particularly those reared in close confinement (Goodnow, 1980). *B. avium* is an avian respiratory pathogen, and the cause of diseases such as turkey coryza (Kersters et al., 1984).

Three new species have recently been proposed for the *Bordetella* Genus. *B. holmesii* was isolated from the blood of immuno-compromised patients and is associated with septicaemia, endocarditis and respiratory failure (Tang et al., 1998; Weyant et al., 1995). *B. hinzii* was also isolated from immunocompromised humans, but is primarily known as a bird pathogen (Cookson et al., 1994; Vandamme et al., 1995). *Bordetella tremantum* is also a human pathogen, isolated from wounds and ear infections (Vandamme et al., 1996). Currently little of the basic biology or pathogenicity of these organisms is known.

1.2.1. Phylogenetic relationship of *Bordetella* species.

Several different methods have been used to demonstrate the similarity of *B. bronchiseptica, B. pertussis* and *B. parapertussis*. These include antigenic relationships (Eldering and Kendrick, 1938; Ferry and Klix, 1918), nutritional requirements (Fukumi et al., 1953; Proom, 1955), bacteriophage typing (Rauch and Pickett, 1961), DNA base composition (De Ley, 1968), DNA hybridisation (Kloos et al., 1981), multilocus enzyme electrophoresis (Musser et al., 1986) and pulsed field gel electrophoresis of DNA macro-restriction digests (Khattak and Mattewa, 1993). There is greater than 99% identity between the 23S rRNA genes of *B. bronchiseptica, B. pertussis*, and *B. parapertussis* (Muller and Hildebrandt, 1993). Analysis of the 16S rRNA loci of these species, *B. avium* and *B. holmesii* shows a less than 2% difference in their nucleotide sequences (Figure 1.1). When compared to species representing 25 genera, only *Alcaligenes faecalis, Alcaligenes xylosoxidans* and *Oxalobacter formigenes* possessed greater than 90% identity to
the 16S rRNA genes of these *Bordetella* species (Weyant et al., 1995). The limited genetic diversity within the *Bordetellae* has led some to question the validity of classifying the members of this Genus as individual species (Kloos et al., 1981; Musser et al., 1986).

**Figure 1.1.** Evolutionary relationships in the Genus *Bordetella*. The 16S rRNA sequences of each species are more than 98% identical (Weyant et al., 1995). The 16S rRNA sequence for *B. hinzii* and *B. tremantum* are unknown at present.

Most recently, a combination of multilocus enzyme electrophoresis and typing of three insertion sequences was used to infer evolutionary relationships between the *Bordetella* species, and between isolates within species (van der Zee et al., 1997). This study, like others before it, found that *B. parapertussis* was more closely related to *B. bronchiseptica* than *B. pertussis*. The study also found that genetic diversity within *B. bronchiseptica* isolates generally correlated with the host organism. Similarly, ovine *B. parapertussis* isolates clustered together, separate from the human *B. parapertussis* isolates.

### 1.2.2. *Bordetella* bronchiseptica

*B. bronchiseptica* is a pathogen of wild, farm and laboratory animals, first isolated from the respiratory tracts of dogs suffering canine distemper (Ferry, 1910; Ferry, 1911). The bacteria was placed in several genera including *Haemophilus, Brucella*, and *Alcaligenes* until the creation of the *Bordetella* Genus in 1952 (Moreno-Lopez, 1952). The ease of transmission of this bacterium makes animals reared in close...
Introduction

Confinement, such as guinea pig colonies, dogs in shelters, or swine in piggeries particularly susceptible to infection. Rabbits (Webster, 1924), seals (Heje et al., 1991; Munro et al., 1992), monkeys (Good and May, 1971), skunks (Switzer et al., 1966) and koalas (McKenzie et al., 1979) provide good examples of the broad host range of *B. bronchiseptica*.

Infection by *B. bronchiseptica* is characterised in most mammals by the development of bronchopneumonia which may be accompanied by coughing and nasal discharges. In some instances, notably rabbits, infection may be asymptomatic (Cotter and Miller, 1994). *B. bronchiseptica* adheres to the ciliated epithelial cells in the upper respiratory tract but does not invade underlying tissues. The cilia normally remain intact, except in cases where the smaller bronchi become blocked by purulent discharges (Bemis et al., 1977; Thompson et al., 1976). An inflammatory response against the infection is characterised by the infiltration of polymorphonuclear leucocytes into the mucosa of the respiratory tract. In non-lethal cases of canine infection, the clinical signs of disease first appear 2-3 days after infection, and lasts from several days to 1-2 weeks (Thompson et al., 1976). In pigs, infection by *B. bronchiseptica* is also associated with atrophic rhinitis, an economically significant disease in the swine industry (Roop et al., 1987). Infection by *B. bronchiseptica* alone induces a mild form of the disease whereas dual infection with *Pasteurella multocida* results in the development of a particularly severe form of atrophic rhinitis (Chanter et al., 1989; Sakano et al., 1992).

1.2.3. Phenotypic Modulation

A defining characteristic of *B. bronchiseptica, B. pertussis* and *B. pertussis* is their ability to undergo a reversible change in phenotype in response to environmental stimuli. This behaviour was first comprehensively studied by Lacey (1960), who described three distinct phenotypic phases; an X-phase, a C-phase and an intermediate I-phase. The X and C phases are now commonly described as virulent...
and avirulent phases, or \textit{bvg}-positive and \textit{bvg}-negative phases. In the virulent phase, \textit{virulence activated genes} (\textit{vag’s}) including filamentous haemagglutinin (FHA), fimbriae, adenylate cyclase/haemolysin and pertactin are expressed (Table 1.1). Colonies grow more slowly in the virulent phase, and appear rounded and shiny. In the avirulent phase, these \textit{vag’s} are no longer expressed and a second class of genes are activated. The \textit{virulence repressed genes} (\textit{vrg’s}) include those involved in flagella biosynthesis (Akerley \textit{et al.}, 1992; West \textit{et al.}, 1997) and siderophore production in \textit{B. bronchiseptica} (Giardina \textit{et al.}, 1995). Avirulent \textit{Bordetellae} grow faster and colonies appear flatter with a matt finish. In the laboratory, the addition of magnesium sulphate (greater than 20 mM) or nicotinic acid (greater than 5 mM) to growth media, or reduction of the incubation temperature to less than 30°C are commonly used to modulate the expression of \textit{bvg}-regulated genes. While several other modulating agent have also been identified (Melton and Weiss, 1993), the true environmental signals important to phenotypic modulation \textit{in vivo} are still to be determined.

1.2.3.1. \textit{The bvg locus}

The coordinated regulation of \textit{vag’s} and \textit{vrg’s} is controlled by the \textit{Bordetella virulence gene} (\textit{bvg}) locus (Weiss and Falkow, 1984). This locus encodes two proteins, homologous to other two-component signal transduction systems (Arico \textit{et al.}, 1989; Uhl and Miller, 1996b). This family is characterised by a His-Asp-His-Asp phosphorelay mechanism, and the presence of additional receiver and C-terminal output domains in the sensor molecule. BvgS is a transmembrane environmental sensory protein and BvgA is a transcriptional activator. Although most research of the \textit{bvg} locus has been undertaken in \textit{B. pertussis}, the genetic similarities between \textit{bvg} of \textit{B. pertussis} (\textit{bvg}\textsubscript{BP}) and \textit{bvg} of \textit{B. bronchiseptica} (\textit{bvg}\textsubscript{BB}) allow insights into the mechanism of \textit{bvg}\textsubscript{BP} to be applied to \textit{bvg}\textsubscript{BB}. The two loci differ at 198 nucleotides, resulting in 61 amino acid changes within BvgS and no changes in BvgA. Most differences (40 amino acid changes) are located
Table 1.1. Bvg-regulated genes of \textit{Bordetella}.

<table>
<thead>
<tr>
<th>Virulence factor</th>
<th>Reference</th>
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<tr>
<td><strong>virulence activated genes</strong></td>
<td></td>
</tr>
<tr>
<td>adenylate cyclase (cya)</td>
<td>Betsou \textit{et al}., 1995</td>
</tr>
<tr>
<td>bordetella virulence gene locus (bvg)</td>
<td>Scarlato \textit{et al}., 1990</td>
</tr>
<tr>
<td>dermonecrotic toxin (dnt)</td>
<td>Pullinger \textit{et al}., 1996</td>
</tr>
<tr>
<td>fimбриae (fim)</td>
<td>Boschwitz \textit{et al}., 1997; Savelkoul \textit{et al}., 1996</td>
</tr>
<tr>
<td>lipopolysaccharide (wlb)</td>
<td>Allen \textit{et al}., 1998a</td>
</tr>
<tr>
<td>pertactin (prn)</td>
<td>Li \textit{et al}., 1992</td>
</tr>
<tr>
<td>pertussis toxin (ptx)</td>
<td>Locht and Keith, 1986; Nicosia \textit{et al}., 1986</td>
</tr>
<tr>
<td>Porin like protein (ompQ)</td>
<td>Finn \textit{et al}., 1995a</td>
</tr>
<tr>
<td>serum resistance locus (brk)</td>
<td>Fernandez and Weiss, 1994; Rambow \textit{et al}., 1998</td>
</tr>
<tr>
<td>tracheal colonisation factor (tcf)</td>
<td>Finn and Stevens, 1995b</td>
</tr>
<tr>
<td>type III secretion (bsc)</td>
<td>Yuk \textit{et al}., 1998</td>
</tr>
<tr>
<td><strong>virulence repressed genes</strong></td>
<td></td>
</tr>
<tr>
<td>acid phosphatase</td>
<td>Chhatwal \textit{et al}., 1997</td>
</tr>
<tr>
<td>motility</td>
<td>Akerley \textit{et al}., 1992; West \textit{et al}., 1997</td>
</tr>
<tr>
<td>siderophore (alc)</td>
<td>Giardina \textit{et al}., 1995</td>
</tr>
<tr>
<td>vrg-6</td>
<td>Beattie \textit{et al}., 1990</td>
</tr>
<tr>
<td>vrg-18</td>
<td>Beattie \textit{et al}., 1990</td>
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<tr>
<td>vrg-73</td>
<td>Beattie \textit{et al}., 1990</td>
</tr>
</tbody>
</table>

within the periplasmic sensor region of BvgS (Arico \textit{et al}., 1991). The introduction of a single copy of \textit{bvgB}_{\text{Bp}} into the chromosome carrying a defective \textit{bvgB}_{\text{Bb}} locus restores the virulent phenotype in \textit{B. bronchiseptica} (Martinez de Tejada \textit{et al}., 1996; McGillivray \textit{et al}., 1989; Monack \textit{et al}., 1989). However, these strains are not as sensitive to modulating signals as the wild type \textit{B. bronchiseptica} strain. Replacement of the \textit{B. pertussis} sensor domain of BvgS with the \textit{B. bronchiseptica} sensor domain restored the sensitivity of \textit{B. bronchiseptica} carrying \textit{bvgB}_{\text{Bp}}, indicating that this region alone was involved in conferring modulating sensitivity. \textit{B. bronchiseptica} expressing \textit{bvgB}_{\text{Bb}} or \textit{bvgB}_{\text{Bp}} are indistinguishable in their ability to colonise the rat respiratory tract. The importance of the differences in modulating
sensitivities of the bvg loci to the pathogenesis of B. bronchiseptica and B. pertussis remains undetermined (Martinez de Tejada et al., 1996).

1.2.3.2. BvgS

BvgS is a 135 kDa protein that senses and responds to environmental stimuli. The protein consists of a transmembrane sensor domain connected to the transmitter, receiver, and C-terminal output domains via a linker region. Mutations in any of the domains may eliminate or alter the pattern of expression of bvg-regulated proteins (Beier et al., 1995; Miller et al., 1992). Signal transduction in BvgS is mediated by a phosphorelay mechanism (Figure 1.2). In response to an extracellular stimulus encountered by the sensor domain, a signal is transduced across the bacterial membrane, through the linker to the transmitter. Autophosphorylation of His-729 of the transmitter then occurs, receiving the γ-phosphate of ATP. The phosphate group is then transferred to Asp-1023 of the receiver domain. The receiver may then transfer the phosphate group to His-1172 in the C-terminus output domain, or pass it on to surrounding water molecules, mediating dephosphorylation of BvgS (Uhl and Miller, 1996a). From the C-terminus the phosphate group can be passed on to Asp-54 in BvgA, or donated back to the receiver (Uhl and Miller, 1996a).

1.2.3.3. BvgA

BvgA is a 23 kDa transcriptional activator that contains a receiver domain at the N-terminus (residues 1-115) (Arico et al., 1989) and DNA binding domain with a likely helix-turn-helix motif at its C-terminus (Boucher et al., 1994). Mutations in either of these domains ablates BvgA activity. Binding of BvgA to target promoters is dependent on its phosphorylation by BvgS. In contrast to unphosphorylated protein, phosphorylated BvgA has an increased affinity for the fha promoter and also has affinity for the ptx and cya promoters (Boucher et al., 1994; Boucher and Stibitz, 1995; Gross et al., 1992; Karimova et al., 1996; Roy and Falkow, 1991).
In response to an external signal, a phosphorelay mechanism transfers a phosphate group from the transmitter domain of BvgS to BvgA (A through D). Phosphorylated BvgA is then able to interact with Bvg-regulated promoters.

*Figure 1.2. The bvg locus.* In response to an external signal, a phosphorelay mechanism transfers a phosphate group from the transmitter domain of BvgS to BvgA (A through D). Phosphorylated BvgA is then able to interact with Bvg-regulated promoters.

*In vivo* there are differences in expression of *bvg*-regulated mRNA species in response to both temporal and chemical modulation. At 25°C, the promoters of *fha*, *ptx* and *cya* are inactive. When transferred from 25°C to 37°C, *fha* transcription occurs in less than 10 min, whereas *ptx* and *cya* mRNA transcription is still minimal after 2 hours (Scarlato et al., 1991). When MgSO₄ is added to *B. pertussis* cells grown in non-modulating conditions, transcription of *fha*, *ptx* and *cya* mRNA is repressed within 6 min, whereas transcription of *bvg* mRNA persists for 2 hours (Scarlato et al., 1990). In *E. coli*, the products of the *bvg* locus alone are sufficient for expression of the *fha* and *bvg* genes, but not *ptx* or *cya* (Miller et al., 1989). Single amino acid point mutations in the C-terminal DNA binding domain of BvgA abolish expression of *cya* and *ptx-phoA* fusions but not *fha*...
expression (Stibitz, 1994). The different transcriptional pattern of *fha* and *bvg* on the one hand, and *ptx* and *cyA* on the other may reflect different sensitivities of their respective promoters to phosphorylated BvgA. In support of this hypothesis, the time taken to induce transcription of *cyA* after shifting *B. pertussis* from 22°C to 37°C can be shortened if the cytoplasmic BvgA concentration is artificially elevated (Prugnola *et al.*, 1995; Scarlato *et al.*, 1991).

In light of the differential affinity of BvgA for target promoter sequences, and the differential expression of *bvg*-regulated mRNA species in response to environmental conditions, a model is evolving that invokes the cytoplasmic concentration of phosphorylated BvgA as a temporal regulator of individual *bvg*-regulated genes (Scarlato *et al.*, 1991). When a *Bordetella* spp. first colonises the respiratory tract, 'early' *bvg*-activated genes requiring low phosphorylated BvgA concentrations are switched on. These are likely to encode proteins essential for initial colonisation events such as FHA and the products of the *bvg* locus. After colonisation has been established, the cytoplasmic concentration of phosphorylated BvgA increases to a level sufficient for expression of 'late' genes that include the toxins *ptx*, *cyA* and other factors relevant to the elaboration of disease.

1.2.3.4. *BvgR*

It is not clear at present how expression of the *vrg*'s is controlled by the *bvg* locus. BvgA may either bind directly to *vrg* regulatory elements to repress transcription or alternatively, BvgA may stimulate the synthesis of an intermediary regulatory protein. In support for the second model, mutations in a single open reading frame (ORF) directly downstream of the *bvg* locus have been shown to result in the constitutive *bvg*-independent expression of *vrg-6* and *vrg-73* in *B. pertussis* (Merkel and Stibitz, 1995). The ORF, designated *bvgR*, encodes a 32 kDA protein which is expressed maximally in non-modulating conditions (Merkel *et al.*, 1998a). A similar sized protein, possibly BvgR, has been shown to bind to the regulatory
elements of vrg-6 (Beattie et al., 1993). The vrg-6 regulatory element is found in another three vrg's and is located within the coding region of these genes (Beattie et al., 1990; Beattie et al., 1993). Mutation of this conserved region in vrg-6 results in the constitutive expression of this locus. Replacement of the vrg-6 promoter with a non bvg-regulated promoter has no effect on modulation of vrg-6 expression, indicating that all elements responsible for regulation of vrg-6 are located within the regulatory element.

1.2.3.5. Bvg-activated genes

The vag's of B. bronchiseptica and B. pertussis have been extensively characterised and the majority are common to both species. A notable exception is pertussis toxin which is only expressed in B. pertussis (Arico and Rappuoli, 1987). Most virulence determinants have been characterised in B. pertussis but some direct studies of virulence factors in B. bronchiseptica have been undertaken. Sequence analysis of virulence determinants of B. bronchiseptica and B. pertussis also shows that there is a very limited difference in their respective amino acid sequences.

Adenylate cyclase/haemolysin

Adenylate cyclase is a secreted multifunctional RTX toxin that possesses adenylate cyclase (AC) and haemolytic (Hly) activities. The calmodulin dependent AC catalytic domain is located in the N-terminus and the haemolytic domain found in the remaining portion of the molecule. The haemolytic domain interacts with the eukaryotic cell membrane, forming a pore through which the calmodulin dependent AC domain is translocated (Gueirard and Guiso, 1993; Rogel and Hanski, 1992). Once internalised, AC stimulates the synthesis of large amounts of cyclic cAMP which disrupts physiological cellular functions. This in turn reduces the ability of phagocytic cells to respond to B. bronchiseptica infection. AC activity has also been shown to be sufficient for the killing of macrophages in vivo (Khelef et al., 1993).
Colonisation of the murine respiratory tract by \textit{B. pertussis} requires both the AC and Hly activities (Khelef \textit{et al}., 1992). Balb/c mice vaccinated with AC/Hly and challenged with \textit{B. bronchiseptica} are protected against infection, and bacteria are cleared from the lung within 10 days, a rate similar to mice vaccinated with a whole cell vaccine. Antibodies to AC/Hly were detected two weeks after intranasal inoculation, earlier than either FHA or pertactin antibodies. In bronchialveolar lavage fluid, AC/Hly antibodies were detected 5 to 13 weeks after infection, and pertactin antibodies never detected (Gueirard and Guiso, 1993), implying that AC/Hly is a critical virulence determinant in early infection processes. Another study has shown however, that naturally occurring AC/Hly mutants can be isolated from pigs which do not present with symptoms of atrophic rhinitis (Novotny \textit{et al}., 1985a). Thus in pigs, AC/Hly may be required for the induction of disease, but is not required to maintain infection. The importance of AC/Hly to virulence may therefore be host dependent.

\textit{Dermonecrotic toxin}

Dermonecrotic toxin (DNT) is an 160 kDa protein that when injected intradermally, induces necrosis in mice, rabbits and other animals. \textit{In vitro}, DNT changes cell morphology, inhibits expression of cell differentiation markers, blocks cytokinesis and stimulates DNA and protein synthesis (Horiguchi \textit{et al}., 1991; Horiguchi \textit{et al}., 1993). DNT is believed to cause these alterations by binding to rho, a GTP binding protein involved in regulating many cellular functions (Horiguchi \textit{et al}., 1995). Cell extracts with high DNT activity cause severe nasal turbinate atrophy in pigs similar to \textit{B. bronchiseptica} infection and is therefore considered a major virulence determinant in the infection of swine (Hanada \textit{et al}., 1979). However, the role of DNT is unclear in the infection of other mammals as Gueirard and Guiso (1993), reported no difference in the ability of a \textit{B. bronchiseptica} DNT mutant to infect mice. A DNT-deficient \textit{B. pertussis} mutant is also fully virulent in both mouse and rat models of whooping cough infection (Weiss and Goodwin, 1989).
Filamentous haemagglutinin (FHA) is one of several adhesins of *B. bronchiseptica*. FHA possesses distinct attachment sites that mediates binding to monocytes and macrophages, ciliated epithelial cells, and non-ciliated epithelial cells respectively (Cotter *et al.*, 1998). *B. bronchiseptica* deficient in expression of FHA are incapable of binding to the porcine nasal epithelium or to the rat L2 epithelial cell line (Cotter *et al.*, 1998; Ishikawa and Isayama, 1988). *B. bronchiseptica* engineered to ectopically express FHA in the *bv*-*g*-negative phase adhere to L2 cells as efficiently as wild type *B. bronchiseptica*, indicating that FHA alone is sufficient for attachment to this cell line in vitro. *B. bronchiseptica* carrying a deletion in *fhaB* have a reduced ability to colonise the murine nasal septum, and were incapable of colonising the trachea (Cotter *et al.*, 1998), indicating that FHA is a requirement for successful colonisation.

Fimbriae

Fimbriae is another major adhesin of *Bordetella* species. The fimbrial operon, downstream of the *fhaB* gene encodes the accessory structural gene *fimA*, the accessory genes *fimB* and *fimC* and the fimbrial minor subunit *fimD* (Boschwitz *et al.*, 1997). The genes encoding the major fimbrial subunits Fim2 and Fim3 are found in other parts of the chromosome. Fimbriae are only expressed in the *bv*-*g*-positive phase of *B. bronchiseptica*. As both *bv*-*g*-positive and *bv*-*g*-negative strains of *B. bronchiseptica* are able to attach to HeLa cells (Savelkoul *et al.*, 1996), *B. bronchiseptica* also expresses *bv*-*g*-independent, or *bv*-*g*-repressed adhesins.

Reactivity to a monoclonal antibody against an epitope of Fim2 showed that fimbrial variation correlates with host species (Burns Jr *et al.*, 1993). Strains from guinea pigs and pigs showed the least variation in their fimbrial profiles, and dogs the greatest. Fimbrial variation may therefore play a key role in the determination of host specificity for individual *B. bronchiseptica* strains.
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Lipopolysaccharide

The importance of lipopolysaccharide (LPS) to host specificity and the pathogenicity of *Bordetella* spp. has until recently been overlooked in favour of research into other *bvg*-regulated virulence determinants. The LPS band B molecules of *B. bronchiseptica* and *B. pertussis* share a common core consisting of Lipid A linked to a branched oligosaccharide consisting of heptose, glucose, glucuronic acid, glucosamine and galactosaminuronic acid. LPS band A consists of LPS band B plus a trisaccharide consisting of *N*-acetyl-*N*-methyl-fucosamine, 2,3-dideoxy-di-*N*-acytymannosaminuronic acid and *N*-acytymannosamine (Allen *et al.*, 1998b). In addition, *B. bronchiseptica* LPS also possesses an O-side chain consisting of repeat units of 2,3-dideoxy-di-acetyl-galactosaminuronic acid.

*B. bronchiseptica* isolates grown in the presence of magnesium sulphate or at 25°C have different LPS profiles to strains grown in non-modulating conditions, indicating that LPS production is a *bvg*-regulated phenotype in this species (van den Akker, 1998). Although *B. pertussis* also expresses LPS in a temperature dependent fashion, there are contradictory reports regarding the *bvg* locus' involvement (Ray *et al.*, 1991; van den Akker, 1998). Isolates of *B. bronchiseptica* from pigs, dogs and humans also demonstrated conserved LPS profiles that correlated with the host organism (van den Akker, 1998). As is the case with fimbrial variation, the greatest diversity was seen in LPS profiles of dog isolates (Burns Jr *et al.*, 1993). Comparison of *B. parapertussis* from sheep and humans showed isolates from each host also had distinct LPS profiles. Extending this observation, the LPS profiles of *B. parapertussis* from humans resembled the LPS profiles of *B. bronchiseptica* strains isolated from humans. These results may indicate that, like fimbriae, the LPS profile is essential in governing host specificity of *B. bronchiseptica* strains.
In comparison to bvг-negative *B. bronchiseptica*, *B. pertussis* and other bacteria, bvг-positive *B. bronchiseptica* has a pronounced resistance to antimicrobial cationic peptides. Analysis of two *B. bronchiseptica* transposon mutants with decreased resistance to these peptides demonstrated that in each case, the transposon had integrated into genes involved in LPS biosynthesis (Banemann *et al.*, 1998). The composition of LPS may therefore be important in defining which peptides are able to access and translocate across the bacterial membrane, and therefore determine which peptides have antimicrobial activity against *B. bronchiseptica*.

**Pertactin**

Pertactin is the third major adhesin of *Bordetella* species. This 68 kDa protein is located on the bacterial outer membrane and has been shown to mediate attachment to a number of eukaryotic cell lines (Everest *et al.*, 1996). Monoclonal antibodies against pertactin inhibit both the attachment and the internalisation of *B. pertussis* into HeLa cells (Leninger *et al.*, 1992). In *B. bronchiseptica* the presence of antibodies against pertactin correlates with protection against atrophic rhinitis in pigs. A pertactin deficient strain of *B. bronchiseptica* was also shown to be unable to induce atrophy, or protect piglets when used as a vaccine (Novotny *et al.*, 1985b).

**Serum Resistance**

The *brk* locus protects *B. pertussis* from killing by human serum by providing resistance to attack from the classical complement pathway (Fernandez and Weiss, 1994). *brk* mutants have a reduced ability to adhere to a human lung fibroblast cell line, and consequently also show a reduced invasive capacity. bvг-negative *B. pertussis* is approximately 100 times more susceptible to human serum than wild type *B. pertussis* indicating that *brk* is another bvг-activated phenotype. The *brk* locus encodes two proteins. BrkA is partially homologous to pertactin. BrkB is homologous to two proteins of unknown function from *Escherichia coli* and *Mycobacterium leprae* and is predicted to be a cytoplasmic membrane protein.
Work by Rambow et al., (1998) showed that three of four *B. bronchiseptica* strains containing the *brk* locus express BrkA, and that these strains are resistant to killing by different animal sera. One strain, *B. bronchiseptica* RB50, which did not express BrkA, was susceptible to killing by a commercially available sera from guinea pigs, but not other sera. Analysis of the killing of RB50 showed that in contrast to *B. pertussis*, both complement and antibodies are required for the bactericidal activity of sera. To assess the contribution of BrkA to the serum resistance in *B. bronchiseptica*, intact copies of *brkAB* were introduced into RB50. This did not confer serum resistance to this strain however. Similarly, in *B. bronchiseptica* expressing *brk*, the mutation of *brkA* did not significantly reduce resistance to sera, indicating that *brk* is either not involved in serum resistance, or that *B. bronchiseptica* has other serum resistance factors in addition to the *brk* locus.

**Type III secretion**

Type III secretion systems are found in several pathogenic and invasive bacteria and are commonly used to translocate proteins directly into host cells. Using differential display PCR, a 420 bp fragment, encoding a partial open reading frame, BscN, homologous to the YscN protein involved in type III secretion in *Yersinia* spp., was identified in *B. bronchiseptica* (Yuk et al., 1998). Cloning and sequencing of the DNA surrounding this fragment in *B. bronchiseptica* identified a further four ORF’s that were homologous to genes involved in type III secretion in other species. *B. bronchiseptica* carrying deletions in bscN were unable to secrete a set of proteins of unknown function. Secretion of previously characterised proteins such as FHA and AC/Hly remained unaffected by mutation of BscN however. *bscN* mutants have a reduced ability to survive for long periods in rat tracheas, although survival in the nasal septum remained unaffected, providing further evidence that different *bvg*-regulated factors are required for initial colonisation and long-term infection. The *bscN* mutants also show an impaired ability to induce
cytotoxicity to the rat epithelial L2 cell line. Wild type but not BscN mutant strains of *B. bronchiseptica* were found to mediate the tyrosine dephosphorylation of L2 proteins (Yuk *et al.*, 1998). Type III secretion of the tyrosine phosphatase YopH by *Yersinia* is associated with the inhibition of the phagocytic activity of macrophages in this species (Andersson *et al.*, 1996).

1.2.3.6. *Bvg*-repressed genes

*Bvg*-repressed genes are expressed in the absence of *BvgA* and *BvgS*. In comparison to *vag*’s, little characterisation of the *vrg*’s has been undertaken. Consequently, the function most *vrg*’s found is unknown. The genes involved in flagella biosynthesis and motility are known to be negatively regulated by the *bvg* locus in *B. bronchiseptica* (Akerley *et al.*, 1992) as are the siderophore genes in some strains (Giardina *et al.*, 1995). Five *vrg*’s have been identified using Tn*PhoA* fusions in *B. pertussis* (Beattie *et al.*, 1990), and another 22 using 2D gel electrophoresis of outer membrane proteins (Stenson and Peppler, 1995). The functions of these proteins are unknown at present.

Motility

The regulation of motility and flagella biosynthesis involves a complex transcriptional cascade (Akerley *et al.*, 1992). The *frl* gene has been proposed as the apex of this cascade in *B. bronchiseptica* (Akerley and Miller, 1993). Synthesis of *frl* mRNA is correlated with the production of flagella, and only occurs in *B. bronchiseptica* grown in modulating conditions. Cloning of the *fhaB* promoter in front of *frl* results in production of flagella in non-modulating conditions, in conjunction with *virulence-activated genes* (Akerley *et al.*, 1995). *frl* also complements mutations in *flhDC* in *E. coli*, a locus required for expression of all other known flagella genes in this species.
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The function of flagella in relation to *B. bronchiseptica* pathogenesis remains unresolved. Expression of flagella outside of the host would enable *B. bronchiseptica* the movement to seek out nutrients. Flagella may also have a role in vivo however. Flagella has been implicated as a factor involved in attachment to eukaryotic cells in other bacteria, and may have a similar role in *B. bronchiseptica*, particularly as *bvg*-negative *B. bronchiseptica* but not *B. pertussis* are able to attach to HeLa cells (Savelkoul et al., 1996). Non-motile *frl* mutants colonise rat tracheas equally as well as wild type *B. bronchiseptica*. Recombinant *B. bronchiseptica* that produce flagella as a *bvg*-activated phenotype have reduced ability to colonise rat tracheas when compared to wild type, suggesting that inappropriate flagella production may actually be detrimental to infection, and indicate one reason why flagella production is *bvg*-regulated. West et al., (1997) were able to show that a non-motile avirulent mutant of *B. bronchiseptica* had a reduced ability to invade and persist in HeLa cells. Further analysis of flagella expression on the virulence of *B. bronchiseptica* is therefore needed to explain these apparently contradictory results.

Siderophore

Iron is a nutritional requirement for almost all bacteria, and the ability to acquire this metal is an important virulence factor in many pathogenic species (Kang et al., 1996). In mammals iron is sequestered intracellularly, and extracellular iron is bound to lactoferrin or transferrin. Infectious bacteria have evolved two strategies to scavenge the ions from these molecules. Many secrete small molecules called siderophores which are highly affinitive for iron, and can appropriate them from lactoferrin or transferrin. The iron-loaded siderophore then binds to specific receptors on the bacterial surface, and the iron is then transported across the membrane. Other bacteria bind transferrin and lactoferrin directly to the cell surface, allowing direct iron removal. *B. bronchiseptica* and *B. pertussis* use both strategies to acquire iron (Menozzi et al., 1991).
In *Bordetella* species the major siderophore is the macrocyclic dihydroxamate alcaligin (Agiato and Dyer, 1992; Moore *et al*., 1995). Like other siderophores, alcaligin is regulated by iron availability through the Fur (ferric iron uptake regulation) protein (Brickman and Armstrong, 1995). In some *B. bronchiseptica* strains alcaligin synthesis is also regulated by the *bv* locus (Giardina *et al*., 1995). Of 114 *B. bronchiseptica* isolates examined, 64 showed *bv*-repression of alcaligin synthesis. The *bv*-regulation of alcaligin also correlated with phylogenetic lineage, and mammalian host species. Predominantly, isolates from swine show *bv* independent synthesis of siderophore, whereas in other mammals, the majority of isolates had *bv*-regulated alcaligin biosynthesis operons. Alcaligin synthesis was therefore suggested to be important in the infection of some animals, but not others.

*Bvg*-repressed phenotypes in *B. pertussis*

A large number of unidentified *bv*-repressed genes have been identified in *B. pertussis*. The proteins encoded by *vrg*-6 and *vrg*-18 identified by Beattie *et al*., (1990) have strong N-terminal hydrophobic regions, indicating they are likely to be membrane-bound or secreted proteins. Mutation of *vrg*-6 was originally shown to result in reduced infection of rat lungs and tracheas when compared to wild type strains (Beattie *et al*., 1992), but was contradicted in a later report (Martinez de Tejada *et al*., 1998). While *B. bronchiseptica* and *B. parapertussis* contain DNA sequences that hybridise to a *vrg*-6 gene probe, they do not express the gene product, indicating that *vrg*-6 is not involved in virulence in these species (Beattie *et al*., 1992). Strains that constitutively express *vrg*s in *B. pertussis* or strains that are deficient in *vrg* expression are able to colonise the lungs and tracheas of infected mice as efficiently as wild type strains. A strain constitutively expressing *vrg*s had a reduced ability to cause disease however, and survived *in vivo* for a longer period than other strains (Merkel *et al*., 1998b). Thus while *vrg*s may not be involved in establishing infection or causing disease, they may be important in the maintenance of chronic infection.
1.2.3.7. Bvg-intermediate genes

After the discovery of the bvg locus little or no research on the intermediate phenotype as described by Lacey (1960) was carried out. It was assumed that this phenotype, produced in semi-modulating conditions, was the result of the over-lap in expression of some vag’s and vrg’s. Recently Cotter and Miller (1997) have shown that a new class of antigens unique to this intermediate phase are produced. They demonstrated that when grown in semi-modulating conditions a subset of bvg-activated genes including fha and bvg are expressed, but others such as prn and cya showed little or no expression. Analysis of outer membrane profiles of B. bronchiseptica also showed that unique proteins, not expressed in fully modulating or non-modulating conditions, were also expressed when B. bronchiseptica was grown in semi-modulating conditions. A bvg-intermediate phase-locked mutant displayed an increased resistance to nutrient limitation, but has a reduced ability to colonise the rat respiratory tract. The reduced virulence of the phase-locked mutant may either be due to the absence of some vag’s required for colonisation, or the presence of bvg-intermediate proteins which may be exceptionally immunogenic.

1.2.3.8. Phase variation

In addition to phenotypic modulation, B. bronchiseptica and B. pertussis may undergo an irreversible change to the avirulent phenotype at a frequency of approximately $10^{-3}$-$10^{-6}$. Genetic analysis phase variants has shown that frame-shift or deletions mutations within the bvg locus are responsible for the production of this isogenic phenotype (Monack et al., 1989; Stibitz et al., 1989). For example, deletion of a 50 base pair fragment from within the bvg locus of BB7866 renders this strain isogenically avirulent (Monack et al., 1989). The virulent phase phenotype can be restored to phase-variant strains of B. bronchiseptica by introducing single copies of the bvg locus into the chromosome. The relevance of the phase variants to the pathogenicity of B. bronchiseptica remains unclear.
1.2.3.9. **What is the biological role of the bvg locus**

Although the bvg locus regulates a large class of molecules that may or may not be involved in virulence, little definitive research investigating the importance of modulation in *B. bronchiseptica* has been undertaken. Cotter and Miller (1994) have demonstrated that virulent phase is sufficient and a requirement for the initial colonisation of guinea pigs and that the bvg-negative phase is required for survival in nutrient depleted conditions. Experiment such as these have led to a model where the bvg-negative phase is only expressed by *B. bronchiseptica* when residing outside of the host. The increased temperature encountered by *B. bronchiseptica* in the initial infective stage would stimulate expression of vag's through the bvg locus. While this model is attractive it does not completely answer the question of the biological relevance of bvg. *B. pertussis* does not appear to have an environmental reservoir outside of the human host. Following the above model, it is difficult to envisage when *B. pertussis* would switch to the avirulent phase. Some authors have suggested that the bvg locus is retained by *B. pertussis* because BvgA is required for expression of vag's. Others have suggested that *B. pertussis* may encounter two intracellular environments, one requiring vag's and the other vrg's (Merkel et al., 1998b). One potential environment where the expression of vrg's and the repression of vag's might be advantageous is intracellularly. By inactivating virulence determinants, which may cause damage to the host cell, the bacteria enhances its ability to evade host immune responses. As *B. bronchiseptica* appears to be more successful than *B. pertussis* at intracellular survival (see below), *B. bronchiseptica* may also modulate gene expression intracellularly.

1.2.4. **Intracellular survival of Bordetella species**

Some pathogenic microorganisms have the capacity to invade and survive within eukaryotic cells. The intracellular environment is rich in nutrients and may also be used by bacteria to evade host immune responses. Bacteria that survive intracellularly have developed specialised abilities that protect them from the anti-
microbial activity of these cells. These abilities include inhibition of phagosome-lysosome fusion, escape from the phagocytic vacuole, inhibition of acidification of the phagolysosomal compartment, resistance to lysosomal enzymes and adaptation to low pH (Alpuche-Aranda et al., 1992; Falkow et al., 1992; Finlay and Falkow, 1988; Finlay and Falkow, 1989). Although *B. bronchiseptica*, *B. pertussis* and *B. parapertussis* have traditionally been considered non-invasive, various studies have now established that these species can invade and survive within different cultured eukaryotic cell lines, including macrophages (Friedman et al., 1992; Masure, 1992; Saukkonen et al., 1991), HeLa cells (Ewanowich et al., 1989a; Lee et al., 1990; Savelkoul et al., 1993), epithelial cells (Schipper et al., 1994), CHO cells (Mouallem et al., 1990), polymorphonuclear leukocytes (Steed et al., 1991) and dendritic cells (Guzmán et al., 1994a; Guzmán et al., 1994b).

The treatment of eukaryotic cells with cytochalasin D, a microfilament inhibitor, results in a dramatic reduction in the uptake of *B. pertussis* or *B. bronchiseptica*, indicating that both these species are internalised via receptor mediated endocytosis (Ewanowich et al., 1989a; Guzmán et al., 1994b). *bvg*-positive and *bvg*-negative *B. bronchiseptica* have similar invasive capabilities when using epithelial, dendritic or macrophage cell lines (Banemann and Gross, 1997; Guzmán et al., 1994a; Schipper et al., 1994). After internalisation the number of viable *B. bronchiseptica* is maintained for several days, followed by a gradual decrease in bacterial cell numbers. In epithelial carcinoma cells, *B. bronchiseptica* are recovered 7 days post-infection while in dendritic cells, significant numbers of bacteria are recovered 3 days after infection (Guzmán et al., 1994a; Schipper et al., 1994). Of further interest is the fact that two studies have shown that avirulent strains of *B. bronchiseptica* have a significant survival advantage over virulent strains in long term survival studies (Banemann and Gross, 1997; Schipper et al., 1994). As many *vag’s* are toxic to eukaryotic cells, it is possible that low-level expression of *vag’s* by wild type *B. bronchiseptica* may account for the reduced persistence of
Introduction

virulent *B. bronchiseptica*. After internalisation, phagosomes containing *B. bronchiseptica* fuse with lysosomes (Banemann and Gross, 1997; Guzmán et al., 1994a). *B. bronchiseptica* may remain in the phagolysosome, or escape to the cytoplasm (Schipper et al., 1994) or a separate compartment within the cell (Guzmán et al., 1994a). Bacterial cells that escape to the new regions of the cell retain normal morphology, whereas cells that remain in the phagolysosome display some changes in morphology, presumably due to the hostile nature of this environment. Escape from the phagosome may represent one strategy *B. bronchiseptica* uses to maintain an intracellular presence.

In contrast to *B. bronchiseptica*, only bvg-positive strains of *B. pertussis* are internalised (Ewanowich et al., 1989a). After uptake, the numbers of viable *B. pertussis* cells immediately begins to decrease. In comparison to the results for *B. bronchiseptica* quoted above, only minimal numbers of *B. pertussis* are obtained 5 days after infection of epithelial cells and no cells recovered 24 h after infection of dendritic cells. *B. pertussis* is unable to escape from the phagolysosomal compartment, but is capable of inhibiting phagosome-lysosome fusion (Steed et al., 1991).

While *B. pertussis* and *B. bronchiseptica* are invasive, their strategy and ability to survive intracellularly as witnessed using eukaryotic cell lines are clearly distinct. The relevance of these observations to the in vivo infective processes of Bordetellae is not well understood. The specific factors required for invasion and persistence of both *B. bronchiseptica* and *B. pertussis* have also yet to be elucidated. *B. bronchiseptica* and *B. pertussis* produce several oxidoreductases, including superoxide dismutase (SOD) (Graeff-Wohlleben et al., 1997; Khelef et al., 1996) and catalase (DeShazer et al., 1994) which are presumed to be involved in intracellular survival. These enzymes detoxify reactive oxygen metabolites in the phagolysosomal compartment which are toxic to bacterial cells. The abolition of
sodB activity, or the deletion of the catalase gene katA, does not alter the ability of B. pertussis to survive within polymorphonuclear leucocytes however (DeShazer et al., 1994; Khelef et al., 1996). sodA and sodB mutants of B. pertussis and B. bronchiseptica also survive equally well as the parental strains in a mouse respiratory infection model (Graeff-Wohlleben et al., 1997). As inactivation of sodA, sodB or katA does not impair intracellular survival, redundant oxidoreductase enzymes may exist which compensate for mutations in any of these genes. B. bronchiseptica mutants deficient in the bvg-repressed phenotypes flagella or acid phosphatase also have a reduced invasive ability (Chhatwal et al., 1997; West et al., 1997). Acid phosphatase is a factor involved in inhibiting the intracellular respiratory burst (Baca et al., 1993) and has also recently been shown to also be under the control of the ris locus in B. bronchiseptica. ris encodes for another two component sensory system whose gene products are upregulated after intracellular invasion. ris mutants express a different class of proteins in comparison to wild type B. bronchiseptica, have an increased sensitivity to oxidative stress, and are cleared more quickly from murine lungs (Jungnitz et al., 1998).

The pathological importance of intracellular survival is not clear for any of the Bordetellae. Several studies have shown that infection of mice with B. bronchiseptica or B. pertussis induces CD4+ Th1 type T-cells associated with cell-mediated immune (CMI) responses which are normally against intracellular pathogens (Gueirard et al., 1996; Mills et al., 1993; Redhead et al., 1993). This CMI response has been shown to be sufficient in clearing B. pertussis from the lungs of infected mice (Mills et al., 1993), indicating its importance in protective immunity. Further, infection by B. pertussis results in secretion of Interleukin-12 by macrophages, a response which has also been reported following infection by other intracellular bacteria including Toxoplasma gondii, Listeria moncytogenes and Mycobacterium tuberculosis (Mahon et al., 1996).
The intracellular stage of infection may represent a dormant stage where *Bordetella* can efficiently evade the host immune response, and may provide a mechanism for the long-term persistence of chronic *Bordetella* infection (Mills et al., 1993). If true, the invasive bacteria may try to limit damage the host cell by down-regulating toxin expression which in turn, would enhance the host cells chances of being noticed by the host immune responses. A simple way for *Bordetella* to do this would be to inactivate the *bvg* locus intracellularly. One study by Masure (1992) demonstrating that adenylate cyclase is inactivated after intracellular invasion by *B. pertussis* has been published, although a second paper by the same author contradicted these results (Masure, 1993). Banemann and Gross (1997) have shown that *B. bronchiseptica* is also able to down-regulate adenylate cyclase production after intracellular invasion.

### 1.3. Urease

#### 1.3.1. Introduction

Urea is a simple organic compound, produced as a mammalian waste product, and through purine catabolism. Urea is found in urine, blood serum and other body fluids. Significant amounts of urea are also found in marine and terrestrial environments due to the excretion of urea and through the degradation of uric acid, the waste product of birds and reptiles (Mobley and Hausinger, 1989). As a consequence of its abundance, many plants, fungi and bacteria have developed enzymes to utilise this compound.

Urease (urea amidohydrolase E.C. 3.5.1.5) is a metalloenzyme found in Gram-negative and Gram-positive bacteria, cyanobacteria, plants and fungi. This enzyme catalyses the decomposition of urea to ammonia and carbamate (Figure 1.3). The spontaneous degradation of the carbamate yields another molecule of ammonia and
carbon dioxide (Mobley et al., 1995b). In plants, fungi and some bacteria, urease is involved in maintaining nitrogen balance by providing an ammonium source. In other bacteria urease contributes to virulence. Urease has been implicated in the production of stomach ulcers, gastric carcinoma, the formation of urinary stones and other gastro and urinary tract infections (Mobley et al., 1995b).

UREASE

\[
\begin{align*}
\text{NH}_2\text{CONH}_2 + \text{H}_2\text{O} & \rightarrow \text{NH}_3 + \text{NH}_2\text{COOH} \\
\text{NH}_2\text{COOH} & \rightarrow \text{NH}_3 + \text{CO}_2
\end{align*}
\]

Figure 1.3. Urea is hydrolysed by urease to produce ammonia and carbon dioxide (Mobley et al., 1995b).

Urease is an historically important enzyme. The urease of the plant C. ensiformis was the first protein to be crystallised (Sumner, 1926) and also the first to demonstrate a requirement for nickel (Dixon et al., 1975). While this urease was the first extensively characterised, the majority of research is now focussed on the ureases found in micro-organisms. This is due to a developing role for this enzyme in the pathogenesis of many bacteria, and also the ease of study of microbial enzyme systems in comparison to eukaryotic systems.

One other enzyme, urea amidolyase (EC 3.5.1.45), found in yeasts and green algae is able to catabolise urea (Nishiya and Imanaka, 1993). This bifunctional enzyme possesses both urea carboxylase and allophante hydrolase activity. The first step, the carboxylation of urea, is an ATP and biotin dependent reaction. The resulting product, allophante, is then hydrolysed to form two bicarbonate ions and two ammonium ions. Urea amidolyase shows no homology to urease and will not be discussed further in this thesis.
1.3.2. Genetics of Urease

The DNA sequence of *P. mirabilis* urease subunit genes was first reported in 1989 (Jones and Mobley, 1989). Since then more than 30 complete sequences have been provided (Table 1.2). These include sequences from the micro-organisms *K. aerogenes* (Lee et al., 1992; Mulrooney and Hausinger, 1990), *B. subtilis* (Cruz-Ramos et al., 1997), *H. pylori* (Clayton et al., 1990; Cussac et al., 1992; Labigne et al., 1991) and *M. tuberculosis* (Clemens et al., 1995) as well as sequences from the plant and yeast species. In each case the structural genes are found in a single locus and are oriented in the same direction. An exception is the urease from the cyanobacteria *Synechocystis*. Sequencing of the *Synechocystis sp. PCC6803* genome has shown the urease genes to be scattered throughout the chromosome of this species (Kaneko et al., 1996). Urease loci are generally found in the chromosome, but are also located on plasmids in some *Enterobacteriaceae* (Collins and Falkow, 1990; D’Orazio and Collins, 1993a). In nearly all cases the three structural genes are found as a contiguous uninterrupted unit. The two exceptions are the loci of *R. meliloti* (Miksch et al., 1994b) and *Alcaligenes eutrophus* (Piettre and Toussaint, 1998).

The subunit genes are surrounded by a number of accessory genes, whose products are required for a catalytically active urease. The four most common accessory genes, *ureD, ureE, ureF* and *ureG* are found in almost every locus. An exception is the urease gene cluster of *B. subtilis* which is the only one thus far sequenced that does not contain any accessory genes (Cruz-Ramos et al., 1997). Whether this urease does not require the products of accessory genes to function, or the accessory are located on some other part of the chromosome is yet to be determined. In addition to the major accessory genes, some loci possess unique accessory genes. *H. pylori* and *Streptococcus salivarius* possesses an additional gene (Chen et al., 1996; Cussac et al., 1992) and *Bacillus* sp. Strain TB-90
Table 1.2. Cloned and sequenced urease genes.

<table>
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<th>Species</th>
<th>Genes</th>
<th>Accession No.</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>Actinobacillus pleuropneumoniae</td>
<td>ABCXEFGD</td>
<td>U89957</td>
<td>Bosse and MacInnes, 1997</td>
</tr>
<tr>
<td>Alcaligines eutrophus</td>
<td>D1D2A(ORF1)BCEFG</td>
<td>M31834</td>
<td>Piettre and Toussaint, 1998</td>
</tr>
<tr>
<td>Bacillus pastuerii</td>
<td>ABCEFGD</td>
<td>U29368</td>
<td>You et al., 1995</td>
</tr>
<tr>
<td>Bacillus sp. strain TB-90</td>
<td>ABCEFGDHI</td>
<td>D14439</td>
<td>Maeda et al., 1994</td>
</tr>
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<td>Bacillus subtilis</td>
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<td>Y08559</td>
<td>Cruz-Ramos et al., 1997</td>
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<td>Canavalia ensiformis b</td>
<td>ure</td>
<td>M65260</td>
<td>Riddles et al., 1995</td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>ABC</td>
<td>Y10356</td>
<td>Dupuy et al., 1997</td>
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<td>ure</td>
<td>U81509</td>
<td>Yu et al., 1997</td>
</tr>
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<td>Escherichia coli (inducible)</td>
<td>RDAB.FG</td>
<td>L03307</td>
<td>D'Orazio and Collins, 1993a</td>
</tr>
<tr>
<td>Filobasidiella neoformans var. neoformans b</td>
<td>ure</td>
<td>AF006062</td>
<td>Cox et al., 1997</td>
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<td>ABC</td>
<td>M16772</td>
<td>Krueger et al., 1987</td>
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<td>U32736, L42023</td>
<td>Fleischmann et al., 1995</td>
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<td>Helicobacter felis c</td>
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<td>L25079</td>
<td>Solnick et al., 1994</td>
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<td>AB</td>
<td>AF066836</td>
<td>Shen et al., 1998</td>
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<td>AB</td>
<td>L33462</td>
<td>Solnick et al., 1995</td>
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<tr>
<td>Helicobacter pylori c</td>
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<td>M60398, M84338</td>
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<td>X51816</td>
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<td>Rhizobium meliloti</td>
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<td>Mirosch et al., 1994</td>
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<td>Schizosaccharomyces pombe b</td>
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<td>AB002590</td>
<td>Tange, 1997</td>
</tr>
<tr>
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<td>ABC..FG</td>
<td>Z25136</td>
<td>Jose et al., 1994</td>
</tr>
<tr>
<td>Streptococcus salivarius</td>
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<td>U35248</td>
<td>Chen et al., 1996</td>
</tr>
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<td>Streptomyces coelicolor</td>
<td>(AB)C</td>
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<td>Redenbach et al., 1996</td>
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<td>AF035751</td>
<td>Sakamoto et al., 1998</td>
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<td>AB001339</td>
<td>Kaneko et al., 1996</td>
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<td>Neyrolles et al., 1996; Willoughby et al., 1991; de Koning Ward et al., 1994</td>
</tr>
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<td>L24101</td>
<td>Sebba et al., 1998</td>
</tr>
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<td>Yersinia pestis</td>
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<td>AF095636</td>
<td>Riot et al., 1997</td>
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<td>Yersinia pseudotuberculosis</td>
<td>ABCEFGD</td>
<td>U40842</td>
<td>Riot et al., 1997</td>
</tr>
</tbody>
</table>

a Only cloned and sequenced genes are depicted. In many cases the accessory genes have not yet been sequenced.

b In plant species, urease consists of a single subunit.

c In this species ureA corresponds to a fusion between the genes for the β and γ subunits. ureB is equivalent to the γ subunit.

d In this species ureH corresponds to the normal accessory gene ureD.
another two (Maeda et al., 1994). Both Alcaligenes eutrophus (Piettre and Toussaint, 1998) and R. meliloti (Miksch et al., 1994b) contain genes of unknown function that separate the urease structural genes.

Transcriptional analysis of the urease gene cluster has only been undertaken in the urea inducible locus of E. coli. The introduction of polar mutations, and their subsequent complementation identified three transcriptional units, ureDABC, ureEF and ureG. Analysis of mRNA showed an increased level of expression of all seven genes in the urease loci in response to urea, suggesting the promoter of each transcript was inducible. Subsequently the ureD and ureG promoters were shown to be inducible by urea. However, only a low level constitutive promoter was found upstream of UreE. The increase in ureE and ureF transcription levels was attributed to readthrough from ureDABC (D'Orazio and Collins, 1993b). As the expression of inducible loci requires the regulatory protein UreR, transcriptional control of non-inducible loci may differ to the scenario described here.

1.3.3. Biochemistry of Urease

1.3.3.1. Protein Structure

Microbial ureases contain three distinct α, β and γ subunits, also known as UreC, UreB and UreA. The β and γ subunits are small, 10-14 kDa and 8-10 kDa in size respectively. The α is much larger, ranging from 60 to 72 kDa (Collins and D'Orazio, 1993a). The exceptions to the tri-subunit conformation of bacterial ureases are those from the Helicobacter Genus. In these ureases the two smaller β and γ subunits have fused into a single protein (Dunn et al., 1990; Hu and Mobley, 1990). The urease of C. ensiformis is comprised of a single 91 kDa subunit (Takishima et al., 1988). Each of the microbial α, β and γ subunits corresponds to adjacent regions of the monomeric plant urease, indicating a shared evolutionary ancestor for both prokaryotic and eukaryotic enzymes. Based on the relative intensity of subunit bands in Coomassie stained polyacrylamide gels, the trimeric
ureases were believed to have an \((\alpha_2\beta\gamma)_2\) conformation. X-ray crystallography of *Klebsiella aerogenes* urease has now revealed this enzyme to have an \((\alpha\beta\gamma)_3\) conformation (Jabri *et al*., 1995). The similarity between the trimeric urease would suggest that they all have 1:1:1 subunit ratios, a stoichiometry that agrees with that of *C. ensiformis* and *Helicobacter* ureases (Dunn *et al*., 1990; Takishima *et al*., 1988).

The urease subunits of different organisms show a high degree of similarity, with not less than 50% homology between any two. As expected, the highest degree of homology is between ureases of the same Genus. Analysis of the complete amino acid subunit sequences of UreA, UreB and UreC together (Mobley *et al*., 1995b) using the PAUP algorithm identified three potential phylogenetic trees, making the assignation of evolutionary relationships between species difficult. Of interest was the fact that within these trees there is no clear separation of Gram-negative or Gram-positive bacteria, or between bacteria and plant, indicating that horizontal transfer of the urease genes appears to have occurred several times.

Based on amino acid identity, the three urease subunits are not similar to any other proteins in the Genbank database. However, analysis of the quaternary structure of *K. aerogenes* urease has identified a set of proteins with similar structural conformation. A single urease \((\alpha\beta\gamma)\) unit consists of four structural domains. Two of the domains, an \((\alpha\beta)\) barrel with an elliptical axis and a \(\beta\) domain are found on the \(\alpha\) subunit. The elliptical \((\alpha\beta)\) barrel, and position of the metal binding site in relation to this barrel is remarkably similar to the two zinc containing enzymes adenosine deaminase and phosphodiesterase (Jabri *et al*., 1995). Each of these proteins has a common reaction mechanism, in which the metal ion deprotonates a solvent molecule for nucleophilic attack on the substrate (Holm and Sander, 1997). Using the common motifs of these proteins as a starting point, a large enzyme superfamily, unrelated by amino acid similarity, was identified. This superfamily
Introduction

includes deaminases, dihydroorotases, allantoinases, hydantoinases, and several other proteins, previously unrelated to others in protein databases. The majority of proteins in this superfamily (including urease) are amidohydrolases, that catalyse the hydrolysis of an amide from the substrate (Holm and Sander, 1997). Each member of the superfamily requires one or two metal ions, and retains four histidine and an aspartic acid residue that serve as conserved ligands to these ion(s). As two of the member families of the superfamily have representatives in Archea, Eubacteria and Eukaryota, this superfamily is believed to have very ancient evolutionary origins.

1.3.3.2. The urease active site

When expressed in the absence of nickel, an inactive form of urease is produced. In vitro, the addition of both nickel and bicarbonate containing buffers to the medium are required for partial activation of the apoprotein (Park and Hausinger, 1995b). The two nickel ions in the K. aerogenes active site are separated by 3.5 Angstrons, and bridged by a carbamylated lysine. Carbamylation of Lys217 is achieved through a reaction with carbon dioxide, highlighting the requirement of this molecule. The first nickel ion is coordinated by the carbamylated lysine and two histidine ligands (His246 and His272). The second nickel ion is coordinated also by the carbamylated lysine, two histidine (His134 and His136), an aspartate (Asp360) and a solvent molecule. Other important residues in the active site include His320, the general base believed to be involved in urea hydrolysis and His219, which is likely to be involved in stabilising the binding of the urea substrate in the active site.

Based on the X-ray crystallographic data provided by Jabri et al., the model for the catalysis of urea first proposed by Dixon et al., (1980) has been extended. In this model, urea is proposed to bind the nickel ion coordinated by only three ligands. The binding is stabilised by His219, which also may aid in polarising the carbonyl
group of the urea molecule. A nitrogen ion from His219 then deprotonates from
the water molecule coordinated to the second nickel ion. The nucleophile generated
then attacks the carbonyl group of urea resulting in its decomposition to ammonia
and a nickel bound carbamate. The carbamate is then free to dissociate from urease
and spontaneously degrades to ammonia and carbon dioxide (Jabri et al., 1995).

1.3.3.3. Accessory proteins

In vivo, the assembly of urease into an active enzyme is a complex process that
requires several accessory proteins. The four common accessory proteins, UreD,
UreE, UreF and UreG are found in almost all urease loci. Deletions in ureD, ureF
and ureG result in the loss of activity in K. aerogenes (Lee et al., 1992) that
correlates with a loss of nickel from the catalytic site of the apoprotein. UreE
mutants also have reduced urease activities that also correspond to reduced nickel
content (Lee et al., 1992). Thus each of the accessory proteins is believed to have a
role in the assimilation of nickel into the active site of urease. Based on amino acid
identity, UreE is potential nickel donor to urease, and UreG contains a possible
nucleotide binding domain (see below). The sequences of UreD and UreF do not
resemble any other in protein databases and can not be used to infer any functional
role in urease activation.

UreD

Overexpression of ureD in conjunction with the urease subunit genes lead to the
formation of complexes containing 1-3 UreD molecule per apoprotein (Park et al.,
1994). The addition of nickel results in the partial activation of these complexes
and is accompanied by the dissociation of UreD from the now active urease.
Further examination of the activation of UreD-urease apoprotein complexes showed
that at low bicarbonate concentrations, UreD increased the rate of urease activation
(Park and Hausinger, 1995a). The presence of UreD also reduced the proportion of
inactive nickel-containing forms of urease produced. Thus UreD is believed to
be a chaperone that increases the efficiency of enzyme activation at low bicarbonate concentrations by holding the apoprotein in a conformation competent for nickel uptake. Moreover, UreD may also inhibit the non-productive binding of nickel to the active site (e.g., binding of nickel to apoprotein that lacks bound carbon dioxide), thereby reducing the formation of non-functioning forms of the enzyme (Park and Hausinger, 1995a).

UreE

Deletions in ureE reduce, but do not eliminate urease activity (Lee et al., 1992). While UreE is not homologous to proteins in the Genbank database, several UreE proteins possess histidine rich carboxy termini (Jones and Mobley, 1989; Lee et al., 1992) which have previously been shown to be involved in nickel binding. Experiments using equilibrium dialysis revealed that purified UreE from K. aerogenes is able to reversibly bind six nickel ions per dimer (Lee et al., 1993). UreE molecules truncated by the removal of the histidine rich carboxy terminal are still able to bind two nickel ions per dimer and cells containing the urease gene clusters with the truncated UreE retain 75% activity compared to cells with wild type UreE. Thus while the carboxy termini is involved in the binding of nickel, it is not essential in the donation of nickel to the active site. This portion of UreE may therefore function in the sequestering of nickel (Brayman and Hausinger, 1996) so that in times of nickel shortage the metal ions may be donated to the urease apoprotein.

UreF

The role of UreF remains unclear. UreF shows no homology to other proteins, and is only present in low concentrations in cell extracts in comparison to the urease subunits and other accessory proteins (Lee et al., 1992). Protein complexes containing UreD-UreF-urease and UreD-UreF-UreG-urease apoprotein have been identified that exhibit different activation properties to apoprotein and UreD-apo complexes (Park and Hausinger, 1995a). Moncrief and Hausinger (1996)
speculated that the UreD-UreF-apoprotein complex is an intermediary prior to the formation of UreD-UreF-UreG-apoprotein. One hypothesis suggests that UreF (and other accessory proteins) are able to inhibit the assimilation of nickel into the active site prior to the carbamylation of the lysine residue. Another hypothesis is that UreF assists in the transfer of carbon dioxide into the active site. Neither hypothesis has been supported by any evidence thus far.

UreG

UreG is highly conserved across bacterial species, and is also homologous to the HypB protein of *E. coli*. HypB is involved in nickel processing for the three hydrogenases of *E. coli* and is capable of binding and hydrolysing ATP. UreG contains a P-loop motif that in other bacteria has been shown to participate in the binding of ATP or GTP (Lee *et al.*, 1990). Attempts to show that the P-loop binds or hydrolyses either ATP or GTP in UreG have met with failure however (Moncrief and Hausinger, 1997). Site directed mutagenesis of the P-loop resulted in the formation of inactive urease, and the inability to produce UreD-UreF-UreG-apoprotein complexes. Thus while nucleotide binding to the P-loop has not been demonstrated, this domain is still obviously important in the *in vivo* activation of urease. When *ureD, ureF* and *ureG* were expressed in a gene cluster containing mutations in structural genes and *ureE* a complex containing UreD-UreF-UreG was produced that was able to bind an ATP-linked agarose resin. Similar complexes were produced when the UreG P-loop variant was used but these were unable to bind the ATP-linked agarose resin (Moncrief and Hausinger, 1997). The formation of the UreD-UreF-UreG complex may therefore be required before nucleotide binding by the P-loop is possible.

Other accessory proteins

Apart from the major accessory genes described above, several urease gene clusters possess unique accessory genes. *Bacillus* sp. strain TB-90 contains two additional accessory genes, *ureH* and *ureI* (Maeda *et al.*, 1994). *E. coli* strains harbouring
ureH or ureI negative constructs still possess urease activity, but the level of activity is directly proportional to the concentration of nickel in the growth medium. UreH is homologous to HoxN, a transmembrane nickel transporter (Eberz et al., 1989), and also has strong hydrophobic regions. Together UreH and UreI are likely to comprise a transmembrane nickel-transporter. UreI from H. pylori and S. salivarius are similar, but share little homology to UreI of Bacillus sp. strain TB-90 (Chen et al., 1996; Cussac et al., 1992). Although the function of UreI in these species remains undetermined, the presence of 5 strong hydrophobic regions suggests it is also a transmembrane protein, probably involved in transmembrane nickel transport.

1.3.4. Regulation of urease expression

Although ureases are evolutionary related, and have similar genetic conformations, they are regulated in several different manners. Examples of nitrogen regulation (Bender, 1991), urea inducibility (Jones and Mobley, 1988), pH control (Sissons et al., 1990), constitutive expression as well as more specialised forms of regulation have all been provided. Some of these methods of regulation are expanded on below.

1.3.4.1. Nitrogen regulation

The best example of nitrogen regulation of urease is provided by K. aerogenes. The global nitrogen regulatory system has been extensively studied in this bacterium and consists of three main features; the GLN system, the NTR system, and NAC regulated operons. The GLN system controls the assimilation of ammonia into glutamate and also regulates the NTR system, which in turn regulates the nitrogen assimilatory control protein, NAC (Bender, 1991). When ammonia, the preferred nitrogen source of K. aerogenes is abundant, operons involved in the catabolism of alternative nitrogen sources such as histidine utilisation (hut), tryptophan utilisation, asparagine utilisation (Bender, 1991), and urease (Friedrich
and Magasanik, 1977) are repressed. When ammonia is limiting, expression of operons involved in catabolism of alternative nitrogen sources increase (Schwacha and Bender, 1993).

Each of the alternative nitrogen regulated pathways is controlled by the NTR system, which consists of three proteins. NTRA is an alternative sigma factor ($\sigma^{54}$) that in conjunction with the RNA polymerase core enzyme is required for transcription from specific nitrogen regulated promoters (Collins et al., 1993b). NTRB is a phosphotransferase that mediates both the phosphorylation and dephosphorylation of NTRC, a regulatory protein that when phosphorylated (NTRC-P), binds upstream of, and enables transcription from, $\sigma^{54}$ dependent promoters (Bender, 1991). The GLN system is able to alter the ratio of NTRC/NTRC-P by increasing the dephosphorylating activity of NTRB in conditions of nitrogen-excess. Thus in nitrogen abundance, phosphorylation of NTRC is reduced, resulting in lowered expression from NTRC-P/$\sigma^{54}$ dependent promoters. In nitrogen limiting conditions, the GLN system does not contribute to the dephosphorylating activity of NTRB, and a greater proportion of NTRC becomes phosphorylated. Consequently, expression from NTRC-P/$\sigma^{54}$ dependent promoters also increases.

The NTR system is a requirement, but not sufficient for the expression of all NTR-controlled operons. Some operons, namely, histidine utilisation (hut), proline utilisation (put) and the urease operon also require the expression of NAC (Bender, 1991). NAC is a 32 kDa protein homologous to the LysR family of transcriptional regulators, and is itself regulated by the NTR system (Best and Bender, 1990). While NAC mutants are unable to express hut, put and ure, the expression of other NTR-regulated operons remain unaffected (Macaluso et al., 1990). When nac expression is placed under the control of the IPTG inducible tac promoter, expression of NAC regulated operons becomes IPTG dependent. Nitrogen
limitation and the NTR system had no effect on expression on the NAC-regulated operons in this system, indicating that NAC alone is sufficient for expression of these genes (Schwacha and Bender, 1993). Further, using this system, it was demonstrated that the hut, put and ure promoters have different sensitivities to NAC. Urease expression could be detected in the absence of IPTG induction of NAC, whereas hut expression required 1 mM IPTG for expression.

Gel shift analysis demonstrates that NAC binds directly to the promoters of hut, put and ure (Goss and Bender, 1995). Analysis of regions of these promoters protected from DNase I digestion by NAC identified a possible 5'-ATA-N9-TAT-3' consensus sequence that contains the 5'-T-Nn-A-3' sequence proposed to be the generalised LysR binding motif (Goethals et al., 1992). The hut and put promoters have been identified as σ70 dependent. A DNA sequence resembling a σ70 dependent promoter exists upstream of the ure promoter but no experimental evidence for this or any other candidate promoter has been supplied. As the operons directly controlled by the NTR system utilise σ54 promoters, NAC links the σ54 dependent NTR system to operons utilising σ70 dependent promoters.

Regulation of urease by nitrogen availability has been reported in other bacteria. In M. tuberculosis, urease activity is increased 10-fold when the asparagine content of the growth media is depleted (Clemens et al., 1995). In the presence of ammonia urease activity is repressed seven fold in Rhizobium meliloti (Miksch and Eberhardt, 1994c). An open reading frame found upstream and required for expression of urease in R. meliloti also possesses a NTRA binding site (Miksch, 1994a) that is the signature of many NTR regulated enzymes. Although urease activity is also nitrogen regulated in B. subtilis, it does not have an identifiable NTR regulatory system (Atkinson and Fisher, 1991).
1.3.4.2. Inducible ureases

The ureases of some Enterobacteriaceae including *P. mirabilis*, *P. vulgaris* and the plasmid encoded urease loci of some strains of *E. coli*, *Providencia stuartii* and *Salmonella* are inducible by urea (D'Orazio and Collins, 1993a; Jones and Mobley, 1988). A transcriptional regulator, UreR, has been identified in *P. mirabilis* and *E. coli* that controls induction of urease in these species (D'Orazio and Collins, 1993b; Nicholson et al., 1993). The *ureR* gene is located approximately 400 bp upstream of *ureD* and is transcribed divergently to the urease locus. The open reading frame encoded by *ureR* contains a Helix-Turn-Helix DNA binding motif and a domain associated with the AraC family of transcriptional regulators. Plasmid constructs of the *P. mirabilis* or *E. coli* urease locus lacking *ureR* do not respond to urea induction. Transcriptional fusions of a promoterless LacZYA to *ureD* result in 20-30 fold increase in β-galactosidase expression in *E. coli* (D'Orazio and Collins, 1993b) and a 40-fold increase in expression in *P. mirabilis* (Island and Mobley, 1995) when both *ureR* and urea is present. The presence of UreR in other bacteria with inducible ureases remains unconfirmed. Colony blots of *Proteus vulgaris* and *Providencia* species did not hybridise to a *ureR* probe under stringent conditions. Given the high degree of similarity between *P. mirabilis* and *P. vulgaris* ureases, and their close taxonomic relationship, it would seem unlikely for these two species to have different regulatory mechanisms however (Nicholson et al., 1993).

1.3.4.3. Regulation during swarm cell differentiation

*P. mirabilis* undergoes a form of differentiation called swarming migration. This behaviour is characterised by cells on the edge of colonies undergoing a change in morphology from short rods to long multinucleate swarm cells, and a dramatic increase in production of flagella. The cells migrate away from the colony until they revert to the short vegetative form. In liquid culture, urease activity of differentiated swarm cells is increased two-fold above the induced level of non-swarming cells (Jin and Murray, 1987), and on solid agar five times (Allison et al.,
1992). The increase in expression of urease activity was accompanied by an increase in expression of two other virulence determinants, haemolysin and metalloprotease. Analysis of mRNA transcripts demonstrated expression of urease and haemolysin correlated with changes in expression of flagellin, a marker of swarming, suggesting the coordinate regulation of these three genes by an unknown factor (Allison et al., 1992).

1.3.4.4. Regulation of urease in Bacillus subtilis

Regulation of urease is complex in B. subtilis (Wray, et al., 1997). Three promoters have been identified upstream of the urease locus which control expression. The predominant promoter, ureP3 is controlled by three proteins, CodY (a DNA binding protein), GlnR and TnrA. Each of these genes has previously been shown to be involved in the regulation of other genes involved in nitrogen catabolism and assimilation. When grown in minimal medium containing glucose and amino acids, CodY represses expression from the ureP3 promoter 60-fold and from the ureP2 promoter 30-fold. GlnR also represses expression from the ureP3 promoter 55-fold. Urease expression from ureP3 is increased 10-fold by TnrA in nitrogen depleted media. In addition, it was shown that urease activity also increases at the end of exponential growth, when the bacteria were ready to initiate sporulation. This increase resulted from an increase in transcription from the ureP2 promoter which utilises the σH RNA polymerase subunit, instead of σ1, the B. subtilis homologue of σ54. This increase in activity was attributed to the fact that σH levels also increase at the onset of sporulation. The complex nature of the regulation of urease may be attributed to the fact that during the life-cycle of B. subtilis urea is obtained from different sources.
1.3.5. Contribution to Pathogenesis

1.3.5.1. Helicobacter pylori

*Helicobacter pylori* is a spiral bacterium associated with duodenal ulcers, gastritis and gastric carcinoma. The bacterium is found in the gastric mucosa and attached to the epithelial cells (Evans *et al.*, 1992). It is postulated that urease protects *H. pylori in vivo* by hydrolysing urea to produce a cloud of ammonia that surrounds the bacteria and neutralises gastric acid. *H. pylori* is sensitive to acid in the absence of physiological urea concentrations (Marshall *et al.*, 1990) and urease negative *H. pylori* mutants are unable to colonise the gastric mucosa of gnotobiotic piglets (Eaton *et al.*, 1991) or the nude mouse (Tsuda *et al.*, 1994). Urease negative mutants of closely related *H. mustelae* are similarly diminished in their ability to colonise the ferret stomach (Andrutis *et al.*, 1995).

Ammonia, released by the decomposition of urea, has also been shown to be directly toxic to gastric cells. Neither urea alone, nor urease positive or urease negative mutants of *H. pylori* cultured in the absence of urea are cytotoxic to HEp-2 or KatoIII cells. The addition of urea to the urease positive strain induces a cytotoxic effect on the eukaryotic cells which was most pronounced in the gastric derived KatoIII cell line (Segal *et al.*, 1992). In addition gastric CRL 1739 cells cultured in the presence of *H. pylori* and urea present a much greater impairment of viability than cells in which the urease inhibitor acetohydroxamic acid was also present (Smoot *et al.*, 1990).

Ammonia can also exert direct toxic effects on intracellular junctions, resulting in alterations of gastric mucosal permeability. *H. pylori* has been observed to localise in close proximity to and within the junctions of gastric epithelial cells (Segal *et al.*, 1992). Comparisons of the effect of *H. pylori* on cultured Caco-2 cells showed that the greatest disruption to cell junctions was produced by a urease positive strain.
in the presence of urea. *H. pylori* alone, or urease negative *H. pylori* in the presence of urea had a negligible effect on junction integrity.

1.3.5.2. *Proteus mirabilis*

Urease has been implicated a major virulence factor of bacteria in urinary tract infections, including the formation of infection stones, pyelonephritis, and the encrustation of urinary catheters (Mobley and Hausinger, 1989). *P. mirabilis* has been implicated as one of several ureolytic organisms associated with these conditions. While the number of cases of urinary tract infection attributed solely to *P. mirabilis* is diminishing (Mobley *et al.*, 1995b) it remains the focus of most investigations. To assess the importance of urease to the virulence of *P. mirabilis*, a recombinant urease negative strain was constructed by allelic exchange. When compared using a mouse model of ascending urinary tract infection, the urease negative mutant required an infectious dose 1000 times greater than the wild type to initiate 50% infection in female CBA/J mice. The urease negative strains were also unable to induce the formation of urinary stones, and only caused mild pyelitis. When compared to the wild type, the urease negative strain was also cleared more quickly from the urine, bladders and kidneys. As kidneys are the favoured niche of *P. mirabilis*, the inability to colonise this site demonstrates the critical importance of urease to the virulence of this organism (Jones *et al.*, 1990).

Infection stones form as a result of the precipitation of struvite ([MgNH₄PO₄·6H₂O]) and carbon apatite ([Ca₁₀(PO₄)₅CO₃]) in the urinary tract. Experiments clearly show that both urease and urea were required for the precipitation and encrustation of mineral salts onto glass rods in human or synthetic urine (Griffith *et al.*, 1976). Increased alkalinity caused by the decomposition of urea results in the supersaturation and reduced solubility of magnesium and calcium, and their eventual precipitation. Pyelonephritis is an acute or chronic inflammation of the kidney, caused by presence of excessive amounts of ammonia. While non-
ureolytic bacterial species can induce acute pyelonephritis, the presence of urea-splitting bacteria contributes to significant increases in inflammation and tissue damage (Mobley and Hausinger, 1989). Comparisons of tissue damage caused by urease positive and urease negative *P. mirabilis* also demonstrated the significance of urease in determining the severity of the disease (Braude and Siemienski, 1960; Jones et al., 1990).

1.3.5.3. *Staphylococcus saprophyticus*

*Staphylococcus saprophyticus* is a bacterium associated with female urinary tract infections. Rats infected transurethrally with a urease negative strain of *S. saprophyticus* produced lowered levels of inflammation in bladders than urease positive strains, and were recovered at a significantly reduced rate. Infection with urease positive *S. saprophyticus* also resulted in more severe lesions on the bladder wall, and the formation of bladder stones (Gatermann et al., 1989a; Gatermann and Marre, 1989b).

1.3.5.4. *Yersinia spp.*

*Yersinia enterocolitica* and *Yersinia pseudotuberculosis* are ureolytic bacteria that exist as free living organisms and intracellular pathogens. After being ingested with contaminated food or water the bacteria pass through the stomach and colonise the intestinal mucosa, before invading M-cells of the lamina propria. *Yersinia* infection is associated with mesenteric lymphadenitis, gastroenteritis, reactive arthritis and septicaemia (Riot et al., 1997; Young et al., 1996).

The urease of *Y. enterocolitica* is activated 780 fold at pH 2 in comparison to pH 7. At pH of less than three *Y. enterocolitica* is also acid resistant in the presence of 0.3 mM urea. Urease has been postulated to protect *Yersinia* during passage through the acidic conditions found in the stomach. Fewer numbers of urease negative *Y. enterocolitica* compared to the wild type are recovered from the ileum of mice 90
min after gastric inoculation, reflecting a reduced ability of mutants to survive transit through the stomach (de Koning Ward and Robins-Browne, 1995). In contrast to Y. enterocolitica, Y. pseudotuberculosis is acid resistant only when urea concentration exceed 20 mM. As this concentration is 15-fold higher than found in the stomach, urease does not appear to protect Y. pseudotuberculosis from the acid conditions found in the stomach (Riot et al., 1997).

1.3.5.5. *Mycobacterium tuberculosis*

Urease has been postulated to be involved in maintaining the persistence of *M. tuberculosis* intracellularly. Urease may contribute to the intracellular survival of this species by liberating ammonium, which has previously been shown to inhibit phagosome-lysosome fusion (Gordon et al., 1980), and may also inhibit phagolysosomal acidification. The attenuated *Mycobacterium bovis* strain bacillus Calmette-Guerin (BCG) is the current vaccine against *M. tuberculosis*. A urease deficient mutant of BCG created by allelic exchange (Reyrat et al., 1995) shows no difference when compared to the urease positive BCG strain in their ability to invade and multiply within cultured human macrophages. In vivo, there was a slight decrease in both the multiplication and persistence of the urease negative BCG in comparison to the wild type strain recovered from the lungs of mice (Reyrat et al., 1996). Thus while urease may contribute to survival it does not appear essential. It must be remembered however that the BCG strain is avirulent in humans, and urease may have a more pronounced effect on the survival in virulent *M. tuberculosis*.

1.3.5.6. *Actinobacillus pleuropneumoniae*

The highly contagious *Actinobacillus pleuropneumoniae* is the causative agent of porcine pleuropneumonia. The dose required to achieve a 50% lethal dose of *A. pleuropneumoniae* in mice is the same for urease positive and urease deficient strains. Pigs infected with urease negative *A. pleuropneumoniae* present with
symptoms of pleuropneumonia that are indistinguishable from urease positive strains suggesting that urease is not a significant virulence determinant in this species (Cabrero et al., 1997).

1.3.5.7. *Bordetella bronchiseptica*

To investigate the contribution of urease to the virulence of *B. bronchiseptica*, a mixed infection model was used to compare the abilities of a urease positive and an isogenic urease negative strain to colonise the respiratory and gastrointestinal tracts of guinea pigs (Monack and Falkow, 1993). The recovery of the urease negative mutants from both tracts actually exceeded recovery of the parental strain. One explanation for this observation is that urease may be highly immunogenic, and strains expressing urease may induce a greater inflammatory response. The urease activity of the parental strain may also be sufficient to mask the lack activity in the urease deficient strain, negating any deleterious effects mutation of this locus may have during infection.

1.4. **Project Aims**

Urease is a highly conserved protein that serves as a provider of a nitrogen source, and in the pathogenesis of other prokaryotes. As a virulence factor, urease protects bacteria form acidic environments, induces cytotoxic damage, and may inhibit phagosome lysosome fusion. Apart from the study of Monack and Falkow, (1993), no work to characterise urease, investigate its regulation, or to define a role in the life-cycle of *B. bronchiseptica* has been undertaken. In this thesis we have sought to address these issues by investigating the molecular biology of urease, and assess its contribution to pathogenesis in *in vitro* and *in vivo* settings.
METHODS

2.1. Bacterial strains, plasmids and media

Bacterial strains and plasmids used in this study are listed in Tables 2.1. Unless otherwise specified all *Bordetella* strains were grown on Bordet Gengou agar (Bordet and Gengou, 1906) supplemented with 15% defibrinated horse blood and 1% glycerol. *Bordetellae* were also grown in SS-X or SS-C broth, both of which are modified versions of Stainer and Scholte medium (Stainer and Scholte, 1971). The use of SS-X as growth medium resulted in the growth of *Bordetellae* in the *bv*-positive phase. *Bordetellae* grown in SS-C (containing 5 g.l\(^{-1}\) magnesium sulphate) express the *bv*-negative phenotype. All *E. coli* strains were grown on Z agar (Walker *et al.*, 1988) or in Luria Bertani (LB) broth (Sambrook *et al.*, 1989). Recipes for growth media and all solutions used are given in Appendix I. Growth media was supplemented with antibiotics when appropriate to the following concentrations: 50 \(\mu\)g.ml\(^{-1}\) kanamycin; 100 \(\mu\)g.ml\(^{-1}\) ampicillin; 50 \(\mu\)g.ml\(^{-1}\) cephalexin; 50 \(\mu\)g.ml\(^{-1}\) chloramphenicol and 50 \(\mu\)g.ml\(^{-1}\) tetracycline. Isopropyl-\(\beta\)-d-galactopyranoside (IPTG, 0.04 mM) and 5-bromo-4-chloro-3-indolyl-\(\beta\)-D-galactopyranoside (Xgal, 0.004%) were also added where appropriate (Sigma Chemical Co., Australia). Unless otherwise specified all incubations were at 37°C. Liquid media incubations were agitated in an orbital shaker incubator at 200 rpm (Edwards Instrument Co., Australia).

2.2. Genetic techniques

2.2.1. Basic manipulations of DNA

Restriction endonucleases were purchased from Boehringer-Mannheim (Germany). Purified DNA was electrophoresed in 0.7-1.0% agarose (ICN Biochemicals, USA)/ 1xTAE gels in a BIORAD mini-sub electrophoresis chamber (BIORAD,
### Table 2.1. Bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Source or reference</th>
</tr>
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<tr>
<td><strong>Bordetella bronchiseptica</strong></td>
<td></td>
</tr>
<tr>
<td>BB7865</td>
<td>ure⁺ bvɡ⁺</td>
</tr>
<tr>
<td>BB7865 U4</td>
<td>ure⁻ bvɡ⁺ Km₁</td>
</tr>
<tr>
<td>BB7865 U5</td>
<td>ure⁻ bvɡ⁺ Km₁</td>
</tr>
<tr>
<td>BB7865 B1</td>
<td>bbur⁻ Km₁</td>
</tr>
<tr>
<td>BB7866</td>
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</tr>
<tr>
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<td>ure⁻ ΔbvɡS Km₁</td>
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</tr>
<tr>
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</tr>
<tr>
<td>294 Rif²</td>
<td>rif²</td>
</tr>
<tr>
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<td>λpir</td>
</tr>
<tr>
<td>InvαF²</td>
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<td>XL1-Blue MRF' Kan</td>
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<tr>
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</tr>
<tr>
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<td>Tc² Ap²</td>
</tr>
<tr>
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<tr>
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<td>Ap²</td>
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</tr>
<tr>
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</tr>
<tr>
<td>pHSG398Norl</td>
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</tr>
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</tr>
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</tr>
<tr>
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<td>Ap², Kmᵣ</td>
</tr>
<tr>
<td>pMC48</td>
<td>Kmᵣ</td>
</tr>
</tbody>
</table>
USA). Gels were stained by immersion in 0.5 µg.ml⁻¹ ethidium bromide for 30 min and visualised under ultra-violet (UV) radiation.

2.2.2. Ligation of DNA

Ligation of DNA was achieved by incubating 0.5 µg of restricted DNA at 65°C for 1 min in a 36 µl final volume. The sample was subsequently incubated for 10 min each at 37°C, room temperature and 10°C after which 4 µl of 10x ligation buffer and 1 µl (1 unit) of T4 DNA ligase (Boehringer-Mannheim) was added. The mixture was then incubated at 10°C overnight.

2.2.3. Preparation and transformation of competent E. coli

Competent cells were prepared using the method of Cohen et al., (1972). Bacteria were inoculated into 200 ml of LB broth and grown to an OD560 of 0.4. After harvesting by centrifugation at 4,000g, the bacterial pellet was placed on ice for 10 min. The cells were resuspended in 100 ml of chilled 0.1 M MgCl₂, recentrifuged, and suspended in 10 ml of chilled 0.1 M CaCl₂. Competent cells were kept on ice if used during the next seven days, or mixed with an equal volume of 50% glycerol and placed at -70°C for long term storage.

DNA to be transformed (5-20 µl) was added to 200 µl of competent cells and left on ice for 1 h. The transformation mixture was then incubated at 37°C for 5 min, the bacteria collected by spot centrifugation and resuspended in 500 µl of LB broth. The cultures were then incubated at 37°C for another hour and plated onto selective agar.

2.2.4. DNA extraction from agarose gels

DNA was extracted from agarose gels using BRESA-CLEAN (Bresatec, Australia). DNA was first electrophoresed in a 0.7-1.0% agarose gel, the band of interest
excised, and placed in a 1.5 ml microfuge tube. Three volumes of BRESA-SALT was added and incubated at 55°C for 5 min, or until the agarose had liquefied. Approximately 10 μl of BRESA-BIND was then added, and the mixture incubated at room temperature for 5 min to allow the matrix to bind the DNA. The BRESA-BIND/DNA complex was pelleted by spot centrifugation and the supernatant discarded. The pellet was washed once in BRESA-WASH in a volume equivalent to the volume of BRESA-SALT used earlier in the procedure. After centrifugation to remove the wash buffer, the pellet was resuspended in dH2O to a volume twice that of the BRESA-BIND used. The mixture was incubated at 55°C for 5 min, centrifuged, and supernatant containing purified DNA transferred to a new microfuge tube.

2.3. Cosmid Cloning of DNA

2.3.1. Preparation of DNA

Partial digests of chromosomal DNA was achieved by serial dilution of the restriction endonuclease Sau3A. In a microfuge tube, chromosomal DNA, dH2O and Sau3A restriction buffer were added to a final 120 μl volume. In another 5 microfuge tubes, DNA, dH2O and restriction endonuclease buffer was made up to 60 μl. A 1 μl aliquot of Sau3A was pipetted into the first tube. After mixing, 60 μl was transferred to the second tube. Another 60 μl was then taken from the second tube and transferred to the third tube. The process was repeated for all tubes. Each digestion mixture was incubated for 30 min and then electrophoresed on a 0.7% agarose gel. The restriction digests with partially digested DNA with a minimum fragment sizes of 35-40 kDa were pooled together, the DNA ethanol precipitated and resuspended in 100 μl of dH2O.

Cosmid DNA was prepared by first digesting pHc79 with BamHI. After denaturation of the enzyme at 65°C for 10 min, the DNA was ethanol precipitated
and resuspended in 100 μl of dH₂O. The partially digested chromosomal DNA and fully digested cosmid DNA were then mixed and ligated.

2.3.2. Packaging into λ phage

Packaging into λ phage was achieved using the Boehringer-Mannheim packaging kit. A 3 μl aliquot of the ligation mixture was added to the λ packaging components on ice, followed by addition of the λ tail components. The sample was incubated at room temperature for 60 min without shaking. Treated E. coli 294 rif™ cells were added and incubated at 37°C for 1 h. The cell suspension was then added to 5 ml of LB broth and allowed to express for 75 min at 37°C before being plated onto selective agar.

2.3.3. Treatment of cells for λ phage transfection

E. coli 294 rif™ were inoculated into 200 ml of LB Mg Mal medium and grown overnight at 37°C. The cells were then centrifuged at 8,000g for 15 min, the pellet resuspended in 2 ml of 10 mM MgSO₄ and shaken rapidly for 60 min at 37°C. The cells were left on ice till used.

2.4. Chromosomal DNA Extraction

Chromosomal DNA was extracted using the method described by Priefer et al. (1984). After growth overnight on solid agar, a loopful of bacteria was collected, resuspended in 25 ml of 1 M NaCl and shaken vigorously on ice for 60 min. The suspension was centrifuged at 12,000g for 10 min and the pellet resuspended in 25 ml of chilled TES buffer. The centrifugation was repeated and the pellet subsequently resuspended in 10 ml of chilled TE buffer containing 0.1 mg.ml⁻¹ lysozyme (Sigma Chemical Co.). After incubation at 37°C for 15 min, a 0.6 ml aliquot of 10% SDS, 0.5% protease dissolved in TE buffer was added and the solution incubated overnight at 37°C to solubolise membranes and digest proteins.
A 5 ml aliquot of TE saturated phenol was added, the solution vigorously shaken and centrifuged at 12,000g for 10 min. The upper aqueous layer was transferred to a new centrifuge tube and the phenol extraction repeated. A 1 ml aliquot of diethyl ether was then added to the aqueous layer, the tube shaken and centrifuged at 16,000g for 5 min. The upper organic layer containing any residual phenol was discarded and the ether extraction repeated. DNA was precipitated by the addition of 1.5 ml of 3 M sodium acetate and 25 ml of chilled 100% ethanol. After centrifugation at 16,000g for 30 min the ethanol was decanted, the pellet air-dried and dissolved in 300-500 μl of sterile dH2O containing 20 μg.ml⁻¹ RNase A (Sigma Chemical Co.).

2.5. Plasmid extraction

2.5.1. Alkaline Lysis

Small-scale extraction of plasmid DNA form E. coli was accomplished via alkaline lysis (Birnboim and Doly, 1979). Bacteria was grown overnight in 1.5 ml liquid cultures with selective antibiotics. The cultures were then transferred to 1.5 ml microfuge tubes and the bacteria collected by centrifugation at 16,000g for 1 min. The pellet was washed in 100 μl of a 10 mM EDTA, 25 mM TrisHCl (pH 8.0) solution, centrifuged as before and resuspended in another 100 μl of the same solution. A 200 μl sample of 0.2 M NaOH, 1% SDS solution was then added, and the mixture placed on ice for five minutes. A 150 μl aliquot of 3 M sodium acetate (pH 4.8) was added, the suspension mixed quickly and thoroughly by inversion and placed on ice for another 20 min. The cells were then centrifuged at 10,000g for 3 min and the supernatant collected. After the addition of 0.8 ml of ethanol the sample was placed on ice for 15 min before centrifugation for 5 min at 16,000g to precipitate DNA. The supernatant was discarded and the pellet suspended in 0.4 ml of 0.2 M sodium acetate, 25 mM TrisHCl (pH 8.0). The precipitation was repeated.
using 0.88 ml of ethanol and the pellet suspended in 50 µl of dH2O containing 20 µg.ml\(^{-1}\) RNase A.

2.5.2. **QIAGEN plasmid extraction protocol**

The QIAGEN midi-prep kit (QIAGEN, USA) was used to extract large amounts of plasmid DNA. Bacteria were inoculated into 150 ml of LB broth supplemented with appropriate antibiotics and grown overnight. The cells were harvested by centrifugation at 5,000g for 10 min and resuspended in 4 ml of buffer P1 containing 100 µg.ml\(^{-1}\) RNase A. After the addition of 4 ml of buffer P2 to lyse the cells, the suspension was incubated for 5 min at room temperature. The solution was neutralised by the addition of 4 ml of Buffer P3, and incubated for a further 15 min on ice. The cells were then centrifuged at 16,000g for 30 min, and the supernatant applied to a QIAGEN midi-column previously equilibrated with 4 ml of buffer QBT. The supernatant was allowed to pass through the column via gravity flow. The column was washed twice with 10 ml of Buffer QC and bound plasmid DNA then eluted with 5 ml of Buffer QF. The DNA was precipitated by the addition of 3.5 ml of isopropanol and subsequently pelleted by centrifugation at 16,000g for 30 min. The supernatant was removed, the pellet washed with 2 ml of 70% ethanol and resuspended in 300 µl of dH2O.

The QIAprep Spin Mini-prep kit (QIAGEN) was used to isolate plasmid DNA used in automated DNA sequencing as DNA extracted by this method was of greater purity than when other extraction protocols were employed. The extraction protocol, as described by the manufacturer, is essentially the same as the QIAGEN midi-prep kit extraction protocol. The QIAprep kit utilises centrifugable microfuge spin-columns in place of the larger column of the Midi-kit, and also uses smaller culture and buffer volumes.
2.6. Polymerase chain reaction

2.6.1. The PCR protocol

The polymerase chain reaction (PCR) was used to amplify segments of DNA from both chromosomal and plasmid DNA. The general reaction mix used to amplify PCR products is outlined in Table 2.2. The reaction mix was optimised, when appropriate, to generate increased yields or purer PCR fragments. Optimisation included altering the concentrations of DNA or primer, or variation of the MgCl₂ concentration.

<table>
<thead>
<tr>
<th>Table 2.2. The PCR reaction mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR buffer 1x</td>
</tr>
<tr>
<td>Primer 1 0.4 μM</td>
</tr>
<tr>
<td>Primer 2 0.4 μM</td>
</tr>
<tr>
<td>dNTP's 0.4 μM each</td>
</tr>
<tr>
<td>DNA 80 pg.μl⁻¹</td>
</tr>
<tr>
<td>Polymerase 1 μl (1 unit)</td>
</tr>
</tbody>
</table>

*Pfu* polymerase (Stratagene, USA) was chosen to amplify the chromosomal DNA fragments as it is known to have a lower error rate than other DNA polymerases. *Taq* polymerase was used to amplify DNA from plasmids. Primers were synthesised by Auspep (Australia) or Gibco-Life Technologies (USA) and normally contained flanking restriction sites (Appendix II). The PCR reactions were carried out in a 100 μl final volume when using the Hybaid thermocycler (Hybaid, United Kingdom) or in 50 μl volume when using the GeneAmp 9600 System (Perkin-Elmer, USA). When the Hybaid thermocycler was used, a 100 μl mineral oil overlay was placed over the reaction mix. The GeneAmp machine did not require this overlay. The cycling parameters for PCR are described in Table 2.3. The optimal annealing temperature for each PCR was determined empirically. The extension temperature was altered from 72°C when using *Taq* polymerase to 74°C.
when using Pfu polymerase. Each PCR was performed in duplicate, and the products generated cloned using one of the procedures described below.

<table>
<thead>
<tr>
<th>Denaturing temp.</th>
<th>Annealing temp.</th>
<th>Extension temp.</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>variable</td>
<td>72°C</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5 min</td>
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<table>
<thead>
<tr>
<th>Denaturing temp.</th>
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<th>Extension temp.</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>variable</td>
<td>72°C</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5 min</td>
<td></td>
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</tbody>
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<table>
<thead>
<tr>
<th>Denaturing temp.</th>
<th>Annealing temp.</th>
<th>Extension temp.</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>variable</td>
<td>72°C</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 min</td>
<td></td>
</tr>
</tbody>
</table>

2.6.2. Cloning of PCR products produced by Taq polymerase

Taq polymerase generates an extra deoxyadenosine base on the 3' end of PCR products. The TA Cloning kit (Invitrogen, USA) utilises these single nucleotide overhangs in the direct cloning of PCR products into the vector pCR2.1. pCR2.1 is supplied as a linearised vector containing single deoxythymidine overhangs on its 3' ends, enabling high efficiency sticky ended ligation of the PCR products. The ligation reaction mix consisting of 1-4 µl of fresh PCR product, 1 µl of 10x ligation buffer, 2 µl of pCR2.1 (50 ng) and 1 µl of T4 DNA ligase in a 10 µl total volume was incubated at 14°C overnight and then transformed into E. coli INVαF'.

Transformation involved the addition of 2 µl of 0.5 M β-mercaptoethanol and 2 µl of the ligation mix to a vial containing 50 µl of competent E. coli INVαF'. The transformation mix was placed on ice for 30 min and then subjected to heat shock for 30 sec in a 42°C water bath. The vial was returned to the ice for another 2 min.
A 250 μl aliquot of SOC medium was added and the vial incubated for 1 h at 37°C. The transformation mix was then plated out on LB agar supplemented with Xgal, IPTG and either ampicillin and kanamycin.

2.6.3. Cloning of blunt ended PCR products

The Stratagene pCR-Script Cloning kit was used to clone blunt-ended PCR fragments produced by Pfu polymerase. The ligation mix consisted of 1 μl of pCR-Script (10 ng), 1 μl of 10x ligation buffer, 0.5 μl or 10 mM rATP, 2-4 μl of PCR product, 1 μl of Srf restriction enzyme and 1 μl of T4 DNA ligase (4 Units) in a final 10 μl volume. The ligation mix was incubated for 1 h at room temperature and then at 65°C for 10 min.

The strategy for transformation into E. coli XL1-Blue MRF' Kan is similar to that described for the TA Cloning Kit. Briefly, 0.7 μl of β mercaptoethanol was added to 40 μl of competent cells and agitated for 15 min. A 2 μl sample of the ligation mix was added to the tube, and the samples incubated on ice for a further 30 min. The transformation mix was then heat shocked at 42°C for 45 sec and placed on ice for 2 min. A 450 μl sample of SOC media pre-heated to 42°C was added and the sample incubated for 1 h at 37°C before plating onto LB agar supplemented with ampicillin, Xgal and IPTG.

2.7. Southern hybridisation

2.7.1. Southern transfer and hybridisation

Transfer of DNA to positively charged nylon membranes (Boehringer-Mannheim) was performed by the method described by Southern (1975). DNA was restricted with the appropriate endonuclease and electrophoresed on a 0.7% agarose gel at low voltage (10-20 V) overnight. The gel was subjected to 2 x 10 min washes in 0.25 M HCl to acid cleave the DNA, followed by a 2 min wash in dH2O. The
DNA was denatured by 2x10 min washes of the gel in 1.5 M NaCl, 0.5 M NaOH, and neutralised by four 10 min washes in 1 M TrisHCl, 1.5 M NaCl (pH 8.0).

The gel was then placed on Whatman filter paper pre-soaked in 2xSSC that overlayed a glass plate. Both ends of the filter paper draped over the edge of the plate into 10xSSC, forming a wick. Plastic wrap was then placed around the edges of the gel to stop any ‘short-circuiting’ of the gel. A positively-charged nylon membrane, and three pieces of Whatman filter paper, cut to the size of the gel were pre-soaked in 2xSSC and placed on the gel. Tissue paper, another glass plate and a small weight (approx 500 g) was then placed on top of the membrane. The transfer of DNA to the membrane typically proceeded for 12-18 h. The DNA was then fixed to the membrane by cross-linking at 254 nm for 2 min using the UV Stratalinker (Stratagene).

After transfer, the membrane was transferred to a hybridisation bottle (Hybaid) containing 10 ml of prehybridisation buffer and incubated for 6 h at 65°C in a Hybaid Mini-oven MkII. The radio-labelled DNA probe was then denatured by incubation at 95°C for 10 min and added to the prehybridisation solution. Hybridisation of the probe to the membrane occurred overnight at 65°C. The membrane was washed successively in 10 ml of 5xSSC, 2xSSC and 1xSSC at 42°C, each containing 0.2% SDS for 10 min and exposed to X-ray film (Fuji, Japan). Alternatively, the membrane was exposed to a phosphor screen which was scanned using a Storm 840 Phosphor-imager (Molecular Dynamics, USA).

2.7.2. Radio-labelling and purification of DNA probes

\[^{32}\text{P}\]-dATP radio-labelling of DNA probes was achieved using the Gibco nick translation system (Gibco-Life technologies). A 5 µl portion of solution A1 (containing all dNTP’s except dATP) was added to 1 µg of probe DNA, 2-5 µl of \[^{32}\text{P}\]-dATP (Bresatec), and dH2O to give a final volume of 45 µl. Solution C,
containing DNA polymerase and DNase I, was added and the mixture incubated for 1 h at 15°C. The reaction was terminated by the addition of 5 µl of Solution D.

Unincorporated nucleotides were removed from labelling mix by centrifugation. To construct the centrifuge column manually a small amount of glass wool was placed in the bottom of a 1 ml syringe. The syringe was filled with Sephadex G50 in STE buffer previously equilibrated at 65°C. The column was centrifuged for 4 min at 2,000g to pack the Sephadex G50 and the process repeated till the beads filled 0.9 ml of the column. The radio-labelled probe was then applied to the column which was centrifuged as before. The radio-labelled probe passed through the column and was collected in a microfuge tube, while the unincorporated nucleotides remained trapped in the column.

Alternatively, the radio-labelled probed was separated from unincorporated nucleotides using BIORAD BioSpin columns. These columns fit in the top of microfuge tubes, and contain the Bio-Gel P-6 matrix in 1xSSC buffer. After draining excess liquid, the column was centrifuged at 1,000g for 2 min and the labelling mix pipetted onto the column. The column was then centrifuged for 4 min at 1,000g, and the labelled probe collected in a microfuge tube.

2.8. Nucleotide sequence determination and analysis

Plasmid DNA for nucleotide sequencing was extracted using the QIAprep Spin Miniprep Kit (QIAGEN) and sequenced using the Sanger method (Sanger et al., 1977). Reaction mixes were prepared using Perkin-Elmer Dye Terminator or dRhodamine Terminator ready reaction mixes. Prior to addition to the reaction mix, the DNA was first dialysed against milli-Q dH2O for 30 min using Millipore VS 0.025 µM filter membranes (Millipore, USA). Universal M13 forward and reverse primers were purchased from Bresatec, whilst internal primers were synthesised and purchased from Auspep or Gibco-Life Technologies. A typical sequencing
Methods

The reaction mix contained 8 μl of ready reaction mix, 100-250 ng of DNA, 3-10 pmol of primer and 1 μl of DMSO in a 20 μl volume. Only Milli-Q dH₂O was used in the preparation of reaction samples. The amplification reaction was carried out under the conditions listed in Table 2.4.

| Table 2.4. Amplification parameters for DNA sequencing. |
|--------------------------|----------------|----------------|
| Denaturing temp. | 95°C | 10 sec |
| Annealing temp. | 50°C | 5 sec |
| Extension temp. | 60°C | 4 min |
| cycles | 25 |

At completion of the amplification, the extension products were precipitated by the addition of 2 μl of 3 M sodium acetate (pH 4.6) and 50 μl of 95% ethanol. The reactions were placed on ice for 10 min, before centrifugation at 16,000g for 30 min. The liquid was decanted, and the pellet washed in 200 μl of 70% ethanol. The pellet was subsequently dried using a vacuum centrifuge.

The DNA sequence samples were electrophoresed on a 4% acrylamide gel using a Perkin-Elmer ABI Prism 377 DNA sequencer. The gel was cast according to directions in the ABI Prism 377 user manual. Prior to loading onto the gel the samples were dissolved in 4 μl of DNA sequence loading dye and heated for 3 min at 95°C.

Individual sequences were assembled into a contiguous DNA fragment using AutoAssembler software (Perkin-Elmer). Potential ORFs were then identified by comparing the sequence to the Non Redundant Genbank database (NR database) located at ANGIS (Australian National Genomic Information Service; http://www.angis.su.oz.au) using the blastx algorithm (Altschul, et al., 1990). The potential ORFs were then individually compared to the NR database to identify the most homologous proteins. The optimal alignment of the deduced *Bordetella*
*bronchiseptica* ORFs and most homologous protein sequences was then determined using the ClustalW program (Thompson *et al.*, 1994).

**2.9. Conjugation of *E. coli* and *B. bronchiseptica***

Due to the difficulty of transformation of *Bordetellae*, conjugation was used to introduce recombinant plasmids into *B. bronchiseptica*. The description of the construction and relevant features of each recombinant plasmid that was transferred to *B. bronchiseptica* is described in the Results section. Each plasmid was first introduced into an appropriate donor strain (*E. coli* JM109Δpir or S17Δpir). For all conjugations the donor strain, and recipient strain were mixed in a 1:1 ratio in 0.7% sterile saline and incubated overnight on Bordet Gengou agar. Colonies were then collected, suspended in 0.7% saline and serially diluted onto Bordet-Gengou or SS-X agar supplemented with the appropriate antibiotics. Any resultant colonies that grew were then screened for the presence of absence of the relevant phenotypes and also subjected to Southern analysis.

**2.11. Urease assays**

A coupled enzyme assay was used as the basis to measure urease activity in *Bordetella* spp. (Kaltwasser and Schlegel, 1966). This method measures the rate of reduction of NADH that accompanies the conversion of ammonia (a urease end-product) and α-ketoglutarate to glutamate. *Bordetella* strains grown on Bordet Gengou agar were inoculated into 200 ml of SS-X broth and grown to an OD$_{560}$ of 0.8. Aliquots of 900 µl were collected, centrifuged for 1 min and the supernatant discarded. The pellet was washed and resuspended in 90 µl of PBS. The resuspension was then transferred to a 5 ml tube containing 2,400 µl of urease reaction mix, 50 µl of a 10 mg.ml$^{-1}$ glutamic dehydrogenase and 150 µl of dH$_2$O. Aliquots of 900 µl were removed and added to three 1 ml cuvettes. The cuvettes were placed in the spectrophotometer, and left for approx 1 min for extinction
readings at 365 nm to settle and remain constant. After the settling period, 100 μl of 100 mM urea was added to two of the cuvettes, and 100 μl of dH2O added to the third. The optical density of the solutions at 365 nm was measured 3 and 6 min after the addition of these solutions. The addition of water to the third cuvette enabled the measurement of background levels of NADH reduction. All samples were tested independently at least in duplicate. Results are expressed as a mean ± standard error of the mean. One unit of urease activity was defined the amount of activity releasing 2 μM NH4+ per minute.

2.12. Tissue culture methods and invasion assays

HeLa cells (American Type Culture Collection CCL2, Rockville, MD) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and 5 mM glutamine (Gibco Laboratories, Germany) in an atmosphere containing 5% CO2 at 37°C. Cells were seeded at a concentration of approximately 5 x 10⁴ per well in 24-well Nunclon DeltaR tissue culture plates (Inter Med NUNC, Denmark), incubated for 18 h, and then washed twice with complete medium. B. bronchiseptica was grown for invasion assays on Bordet Gengou agar for 24 h, recovered with sterile swabs, suspended in complete DMEM and the suspensions adjusted spectrophotometrically to an optical density at 540 nm corresponding approximately to 4 x 10⁷ colony forming units (CFU) ml⁻¹. A 0.5 ml sample of the suspension was then added to each well of the HeLa-containing tissue culture plates and the plates incubated statically for 2 h. Supernatant fluids were subsequently discarded and the cells washed twice with PBS to remove nonadherent bacteria. The medium was replaced with 0.5 ml of complete DMEM supplemented with 100 μg.ml⁻¹ of gentamicin (Sigma Chemical Co., Germany) and incubated at 37°C for 2 or 24 h to kill remaining extracellular bacteria. The supernatant was then discarded and the cells washed twice with PBS to remove residual gentamicin. Cells were lysed by the addition of 0.5 ml of water to each well. The number of CFU recovered from each well was determined by plating 10-
fold dilutions on Bordet Gengou or brain heart infusion agar using a Spiral Plater model C (Spiral Biotech, Inc., USA). The results reported are mean values of three independent assays with standard deviations. Incubation of B. bronchiseptica suspended to a density equal to that used in the invasion assays in DMEM supplemented with gentamicin at 100 mg.ml\(^{-1}\) for 2 h resulted in greater than 6 orders of magnitude reduction in CFU. The results obtained were analysed by analysis of variance and Student's t-test. Differences were considered significant at \(P \leq 0.05\).

2.13. Construction of urease mutants of B. bronchiseptica

Mutations in the urease genes of B. bronchiseptica were generated via transposon mutagenesis (Shojaei, 1992) using the suicide vector pUT::miniTn5/Km2 (Herrero et al., 1990). This construct was designed with the cognate transposase located on the transposon donor replicon, but outside of the inverted repeats of the mini-transposon, such that it is not carried with the transposon during transposition. As a consequence, the integrated transposon is not subject to transposon mediated gene rearrangements and deletions and cannot further transfer to other replicons. The donor strain, E. coli SM10 \(\lambda\)pir containing pUT::miniTn5/Km2 and recipient strain (B. bronchiseptica BB7865 or B. bronchiseptica BB7866) were mixed in a 1:1 ratio in 0.7% sterile saline and incubated overnight on Z agar or Bordet Gengou agar. Colonies were then collected, suspended in 0.7% saline and appropriate dilutions plated onto SS-X agar supplemented with kanamycin (selection for mini-transposon) and cephalexin (selection for B. bronchiseptica; negative selection for E. coli). The urease activity of mutant strains was determined by growth on urea agar (Oxoid, United Kingdom). Urease-negative strains were identified by their inability to turn such media pink after incubation at 37°C for 48 h. Urease-negative strains of B. bronchiseptica BB7865 were further tested for the retention of the virulent phenotype (ie. bvg-positive) as evidenced by haemolytic activity on Bordet Gengou agar.
RESULTS

3.1. Molecular analysis of the urease locus of *B. bronchiseptica*

Many bacterial urease operons have been identified through classical genetic techniques, and others operons are being discovered through the rapidly advancing field of whole genome sequencing. Without exception, the subunit proteins that make up an active urease are highly conserved. Analysis of the promoter region of urease sequences often also identifies signature domains involved in the regulation of urease operons that may include the NAC and NTRC binding sites in nitrogen regulated operons. Sequencing of the inducible loci of *P. mirabilis* and *E. coli* has also assisted to identify the transcriptional activators involved in the regulation of urease in these species.

3.1.1. Characterisation of urease deficient mutants of BB7865 and BB7866

Transposon mutagenesis utilising miniTn5/Km2 had been previously used by Shojaei (1992) to generate five urease-negative mutants of *B. bronchiseptica*; three from BB7866 and two from BB7865. Each mutant was originally identified by its ability to grow on Bordet Gengou agar supplemented with kanamycin, and by it's inability to change the colour of urease agar plates from yellow to pink within 24 h at 37°C. Quantification of the urease activity of these mutants showed that each possessed an activity of less than 1 U.l⁻¹, representing less than 3% of the activity of the parental strain (Figure 3.1a). Southern analysis of *EcoRI* restricted chromosomal DNA of each of the strains using pUT::miniTn5/Km2 as the probe confirmed the successful integration of the mini-transposon into each of the mutants (Figure 3.1b). The differences in sizes of the reactive bands of the mutants on the Southern blot also demonstrated the random integration of the mini-transposon into the *B. bronchiseptica* chromosome.
Figure 3.1 (a) Comparison of the urease activity of BB7865, BB7866 and urease-deficient mutants derived from these strains. Each strain was grown in SS-C at 37°C to an OD560 of 0.8. Results are expressed as a percentage of the urease activity of the respective parental strain. (b) Southern blot analysis of chromosomal DNA from BB7866 U1, BB7866 U2, BB7866 U3, BB7865 U4, BB7865 U5, BB7865 and BB7866 (lanes 1-7) restricted with EcoRI and probed against pUT::miniTn5/Km2. DNA Molecular weight markers (in kb) are shown on the right. The size of the reactive band of each strain is indicated on the left.
3.1.2. Cloning of the urease gene locus of \textit{B. bronchiseptica} BB7866 U1

To analyse the region of chromosomal DNA disrupted by transposon mutagenesis, mini\textit{Tn5}/\textit{Km2} and DNA flanking either end was cloned from the mutant BB7866 U1 into the cosmid vector pHC79. This was easily achievable due to the presence of the selectable kanamycin resistance marker within the transposon. From the recombinant cosmid, pMC1, two smaller fragments were subcloned. A 5.9 kb \textit{EcoRI} fragment including mini\textit{Tn5}/\textit{Km2} and putative urease genes flanking one end of the mini-transposon was cloned into pHSG399, creating pMC2. A second 6.8 kb \textit{NotI} fragment, that included mini\textit{Tn5}/\textit{Km2} and DNA flanking the other end of the mini-transposon was cloned into pHSG398\textit{NotI}, generating pMC4. pHSG398\textit{NotI} is a 2.2 kb plasmid derived from pHSG398 in which the polylinker cloning site has been modified with the introduction of \textit{NotI}, \textit{EcoRI}, \textit{SalI}, \textit{HindIII} and \textit{NotI} restriction sites. The restriction maps of the two plasmids, pMC2 and pMC4, were determined and the position and orientation of the mini-transposon in the \textit{B. bronchiseptica} BB7866 U1 genome ascertained (Figure 3.2).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.2.png}
\caption{Restriction map of mutated urease genes of \textit{B. bronchiseptica} BB7866. The site of integration of the mini-transposon in the \textit{B. bronchiseptica} BB7866 U1 genome is indicated by the open bar.}
\end{figure}
A 2.2 kb PstI restriction fragment immediately adjacent to the transposon was excised from pMC2 and cloned into pUC18 to construct pMC3. Preliminary sequence analysis indicated that pMC3 contained a DNA fragment that encoded a partial ORF homologous to the bacterial urease subunit protein UreA. Southern analysis of EcoRI restricted chromosomal DNA of the five urease-mutants probed with pMC3 revealed that in each case, the same 11.0 kb EcoRI fragment, present in the parental strains, had been disrupted (Figure 3.3). The size of the reactive band in for each urease mutant was the same as when the blot was probed with miniTn5/Km2. In the case of BB7866 U3 and BB7865 U4, two fragments hybridised with the pMC3 probe. It is possible, that in these two cases, the suicide vector that was used to induce the urease mutation, had integrated into a site contained within the DNA used as the probe. As miniTn5/Km2 carries an EcoRI site, two separate EcoRI fragments from BB7866 U3 and BB7865 U4 would then hybridise with the probe used. The relative intensity of each band would also indicate which fragment carried the larger amount of DNA homologous to the probe.

Figure 3.3. Southern analysis of BB7866 U1, BB7866 U2, BB7866 U3, BB7865 U4, BB7865 U5, BB7865 and BB7866 (lanes 1-7 respectively) restricted with EcoRI and probed with pMC3.
3.1.3. Sequence analysis of the urease operon

Each of the plasmids pMC2, pMC3 and pMC4 was used in the subsequent DNA sequence analysis of the urease locus in *B. bronchiseptica*. Two other plasmid constructs were used to aid in the sequencing of this locus. pMC5 is a pUC18 derivative with a 1.9 kb *BamHI/EcoRI* fragment cloned from adjacent the mini-transposon in pMC4. pMC6 contained a 1.8 kb PCR fragment from BB7866 that overlaps the site of integration of the mini-transposon in BB7866 U1. This fragment was amplified using the primers BB7 and BB11 and cloned into pCR2.1. This step ensured that any mutations introduced into the urease cluster by the integration of the mini-transposon were identified and corrected. The full strategy used to produce all sequencing constructs is outlined in Figure 3.4. Analysis of the plasmids pMC2, pMC3, pMC4, pMC5 and pMC6 enabled 8.9 kb of contiguous sequence to be generated. The full DNA sequence of the urease gene cluster is depicted in Figure 3.5 and is deposited in the Genbank database (Accession Number AF000579).
Figure 3.4 Subcloning the mutated urease gene from pMC1. A 5.9 kb EcoRI fragment and 6.8 kb NotI fragment was subcloned from pMC1 into pHSG399 and pHSG398NotI respectively to construct pMC2 and pMC4. A 2.2 PstI fragment was further subcloned from pMC2 to construct pMC3. A 1.9 kb BamHI/EcoRI region from pMC4 was also cloned into pUC18 to construct pMC5. Plasmids are not drawn to scale and only relevant features are shown. The genes depicted are: ampicillin resistance gene (Ap, open arrow); chloramphenicol resistance gene (Cm, open arrow); tetracycline resistance gene (Tc, open arrow); miniTn5/Km2 (open box); β-galactosidase gene (LacZ, black arrow); mutated urease sequences (shaded boxes).
Figure 3.5. Nucleotide and deduced amino acid sequence of the urease gene cluster of *B. bronchiseptica*. Ten potential genes, *orfl*, *bbuR*, *ureD*, *ureA*, *UreI*, *ureB*, *ureC*, *ureEF*, *ureG* and *orfl0* are identified. *orfl* and *bbuR* are transcribed in the reverse orientation to the other genes and the deduced amino acid sequence for these genes are italicised. Putative promoters for the urease operon and *bbuR* are indicated by dots above the sequence. Putative Shine-Dalgarno sequences are underlined. A possible transcription termination site for the urease locus is indicated by arrows. The putative BbuR binding sequence is boxed. Two direct repeats proximal to the *bbuR* promoter are also indicated by arrows.
Results

E I L L P R C I L D P H P P D F L V A

68

CACGCTAGCGGCAGCCTGGG

1560

CGCCGGTGCAGACGCACCG

1620

GTGGCCGGATGGGGTGGGG

1680

CGCCGGTGCAGACGCACCG

1740

GTGGCCGGATGGGGTGGGG

1800

CGCCGGTGCAGACGCACCG

1860

GTGGCCGGATGGGGTGGGG

1920

CGCCGGTGCAGACGCACCG

1980

GTGGCCGGATGGGGTGGGG

2040

CGCCGGTGCAGACGCACCG

2100

GTGGCCGGATGGGGTGGGG

2160

CGCCGGTGCAGACGCACCG

2220

GTGGCCGGATGGGGTGGGG

2280

CGCCGGTGCAGACGCACCG

2340

GTGGCCGGATGGGGTGGGG

2400

CGCCGGTGCAGACGCACCG

2460

GTGGCCGGATGGGGTGGGG

2520

CGCCGGTGCAGACGCACCG

2580

GTGGCCGGATGGGGTGGGG

2640

CGCCGGTGCAGACGCACCG

2700

GTGGCCGGATGGGGTGGGG

2760

CGCCGGTGCAGACGCACCG

2820

GTGGCCGGATGGGGTGGGG

2880

CGCCGGTGCAGACGCACCG

2940

GTGGCCGGATGGGGTGGGG

3000

CGCCGGTGCAGACGCACCG

3060

GTGGCCGGATGGGGTGGGG
Results

72

ALRPAT LPAVHAC ACAMWAL

7500

GGCGCCGCGGGCGGACGC

7560

CCCGCTGGATGAGCAGCGAC

7620

AGCCGGGCTGGTGGCGC

7680

RGFRS UreG MHDISS

7800

GCAGAAAGGCCAAGACGAC

7860

ATTTGACCCAGCTACGACGAC

7920

GCGCCAGGACGCATCCATCA

8040

TGCACTACCGAGATTGTCGAC

8100

GGGGACCTGGCGGCGGACGG

8160

AGCCTCTTCGCTAGTACACC

8220

GGGCGGGCCTGGCATCACCA

8280

CTTCTTCGCTAGTACACC

8340

TGCACTACCGAGATTGTCGAC

8400

GGGGACCTGGCGGCGGACGG

8460

AGCCTCTTCGCTAGTACACC
A search of the Non-Redundant protein Database located in ANGIS using the blastx algorithm, revealed ten putative ORF’s within the derived sequence. The relative position of each open reading frame, and it’s identity is shown in Figure 3.6. Five of the deduced ORFs correspond to the conserved structural proteins, UreA, UreB, UreC, and accessory proteins UreD and UreG. The genes encoding these proteins were all transcribed in the same direction and contained their own Shine-Dalgarno sequences. An ORF unique to the *B. bronchiseptica* urease operon corresponds to a fusion between what is normally UreE and UreF. UreE has no identifiable stop codon and is transcribed in the same frame as UreF. UreF itself however has its own ATG start site and a potential Shine-Dalgarno sequence [5′-GGCGA-3′] upstream of the ATG start site. Gene fusions are not uncommon in urease loci. The three structural subunits of bacterial ureases are found as a single protein in plant ureases (Takishima *et al.*, 1988). The two smaller urease structural subunit proteins of *H. pylori* are also fused together (Dunn *et al.*, 1990; Hu and Mobley, 1990).

![Figure 3.6. Physical map of the cloned DNA fragments used to sequence the urease gene cluster. Subclones containing the urease genes (pMC2-pMC6) are indicated by thick lines. The position of open reading frames within the DNA sequence are indicated by open arrows.](image)

A potential promoter region for the urease operon with -35 region (5′-TTGGAT-3′) and -10 region (5′-ATTAAT-3′) was detected upstream from the UreD ATG start codon (nucleotides 2413 to 2445). A possible transcription termination site
was also found downstream of UreG (nucleotides 8562 to 8588). Following the termination signal, a truncated ORF, extending to the 3' end of the sequence was discovered. This ORF showed no homology to others in the Genbank database. The DNA sequence was also analysed for the presence of signature DNA binding motifs. No BvgA or BvgR binding sites were discovered. Motifs representing NTRA and NTRC binding sites were also absent.

3.1.4. Alignment of the \textit{B. bronchiseptica} urease subunit proteins

The predicted molecular weights of UreA, UreB, UreC, UreD, UreEF and UreG proteins was 11 kDa, 11 kDa, 61 kDa, 31 kDa, 46 kDa and 28 kDa respectively. The predicted amino acid sequences of the \textit{B. bronchiseptica} urease proteins were compared with other proteins in the non-redundant protein database using the Blastp algorithm. The optimum alignment of the five most homologous sequences to the ORF's, UreA, UreB, UreC, UreD, UreEF and UreG was determined using the ClustalW alignment program. The results are depicted in Figure 3.7. It was apparent that the individual subunits are generally most homologous to the urease subunits of \textit{A. eutrophus} (Genbank Accession No. Y13732), \textit{K. aerogenes} (Genbank Accession No. M36068) and \textit{P. mirabilis} (Genbank Accession No. M31834). The UreA, UreB and UreC ORFs from \textit{B. bronchiseptica} were 84%, 63% and 70% identical to the corresponding subunits from \textit{A. eutrophus}, 79%, 69% and 69% identical to the corresponding subunits from \textit{K. aerogenes}, and 73% 67% and 68% identical to those from \textit{P. mirabilis}. The accessory proteins UreD and UreG were 43% and 75% homologous to those from \textit{A. eutrophus}, 33% and 66% homologous to those from \textit{K. aerogenes} and 28% and 59% identical to those from \textit{P. mirabilis} respectively. The region corresponding to UreE from UreEF in \textit{B. bronchiseptica} was 39% homologous to UreE from \textit{A. eutrophus} and 38% homologous to UreE from both \textit{K. aerogenes} and \textit{P. mirabilis}. The sequence spanning what is normally UreF was 54%, 31% and 31% identical to UreF from the above mentioned species.
**Figure 3.7.** Alignment of the deduced amino acid sequences from *B. bronchiseptica* with urease subunits from other bacteria. Amino acids identical to that of the *B. bronchiseptica* sequence are indicated by dots; differences are shown by upper case letters. Dashes represent gaps introduced into the amino acid sequence to optimise alignment. Abbreviations used: *B.b.*, *Bordetella bronchiseptica*; *A.e.*, *A. eutrophus* (Piettre and Toussaint, 1998); *K.a.*, *Klebsiella aerogenes* (Mulrooney and Hausinger, 1990); *K.p.*, *Klebsiella pneumoniae* (Collins et al., 1993); *E.c.*, *E. coli* (D'Orazio and Collins, 1993); *P.v.*, *Proteus vulgaris* (Morsdorf and Kaltwasser, 1990); *P.m.*, *P. mirabilis* (Jones and Mobley, 1989); *S.s.*, *Synechocystis* sp. strain PCC6803 (Kaneko et al., 1996); *R.m.*, *Rhizobium meliloti* (Miksch et al., 1994b); *A.e.*, *A. pleuropneumonia* (Bosse and MacInnes, 1997); *B.p.*, *Bacillus pasteurii* YYBE (You et al., 1995); *H.i.*, *Haemophilus influenzae* (Fleischmann et al., 1995).
UreA

B.b. MELTRIEKLLITAILLA ERRARGKLINPETVALIT AAMEGRODGVTAEMLESS
A.e. ---...A....R.......R.......
K.s. ---...L.......S.......
K.p. ---...L.......
P.v. ---...L.......
P.v. ---...L.......

B.b. TRILGRDEVMEGVPEMISNI QVEVFTFODKLTHVTPPDPV
A.e. TV..ED..DA..PE.A....V..H.I.
K.s. RHV.T.QX.PD.A.....S.V...II
K.p. RHV.T.QX.PD.A.....S.V...II
E.c. RTL.TAQK.KD...C.....VSI.D.I.
P.v. RAIV.TAQK.KD...C.....VSI.D.I.

UreB

B.b. MIPGEILTEPGQIELNVGRPTLTIAWNEGDRPIQVGSHYHFAEANNALVFRED
A.e. LMPAD....E....A.A..VSVT.A.T...F...Y.T.A......T
E.c. KVNHAL.D....A.E.Q.Q.A.H...Y.V.D.K.E.N
K.a. YHVK.A..T.A.CRW.E.HV.P.K.QQ
P.m. RVNAAL.D....A.E.K..Q.A.HVY.V.E.R.AKE
S.S. MAT.I.P..E...D.S.C.N.A.T...V.A...Q...D.

UreC

B.b. MTRISRSAYAEIYGPTWGVGGDRLVRADTLLLAEVEKDHTIFGEVKFGGGKVIRDGM
A.e. M.S..V.HWG-L.G.R.AAI.W.
P.v. WSAE..V..L..1...HWG-R.TV.N.D.V.
P.m. WSAE..V..L..1...HWG-R.VV.N.D.V.
P.m. WSAE..V..L..1...HWG-R.VV.N.D.V.
P.a. ..G..M..A.L.LV.HWG-VR....F..V.
P.m. PG...I....K.GM.S
R.m. ..I..AI..K.G...
B.b. ASTEWAGEGLIVTAGAIDTHIFICPQQIEEALATMTMGGGTGPATGSLATTSTSG
A.e. ..YISR..L...NV.QPD..R..AV.A....A.C.
P.m. PG..I....K...G.TI.M.F.LC.LHCTC.P.
K.a. ..YISR..L...NV.QPD..R..AV.A....A.C.
P.m. PG..I....K...G.TI.M.F.LC.LHCTC.P.
R.m. ..I..AI..K.G...

Results
All ureases described thus far have a number of conserved amino acids. These include four histidines and an aspartate residue which act as ligands to the two nickel ions in the active site of urease. In *B. bronchiseptica* these residues are located at His-139, His-141, His-251, His-277 and Asp-365 in UreC. Other conserved amino acids include UreC Lys-222 which is carbamylated in the active protein, and His-325, the general base believed to be involved in catalysis. The carboxyl terminus of UreE in some bacteria is histidine rich and may be involved in the binding of nickel. In *B. bronchiseptica* UreEF, the region matching the carboxyl terminus of UreE contains only 3 histidines. A conserved GTP-binding motif, GGPVGSGTK is also located from amino acids 22 to 30 in UreG.
3.1.5. Phylogenetic analysis of UreC

ClustalW alignments demonstrate the similarity between proteins, but can not be used to infer phylogenetic lineage. The GCG program 'protpars' was therefore used to investigate the evolutionary relationships between 18 different UreC proteins. The results of this analysis is shown in tree form in Figure 3.8. As *A. eutrophus* is more closely taxonomically related to *B. bronchiseptica* than the other bacteria examined, it is not unexpected UreC from this species showed the closest evolutionary similarity to *B. bronchiseptica*. Other closely related proteins included those from *K. aerogenes* and *P. mirabilis*. These results are in agreement with the alignments generated by the ClustalW analysis. Another feature of the tree is the placement of the four sequences from *C. ensiformis*, *G. max*, *M. morganni* and *S. pombe*. *C. ensiformis* and *G. max* are plants, *M. morganni* a bacterium and *S. pombe* a fungus. Their placement on the tree on a separate branch may provide evidence for the horizontal gene transfer across different phyla.

![Figure 3.8. Phylogenetic analysis of UreC. Sequences were aligned using the 'protpars' algorithm from the GCG package.](image-url)
3.1.6. Analysis of UreJ

The *B. bronchiseptica* urease cluster contains an ORF, designated UreJ, found in only one other urease operon. The *ureJ* gene, situated between *ureA* and *ureB*, exhibited 50% identity to ORF1 from the urease locus of *Alcaligenes eutrophus* (Genbank Accession No. Y13732). The only other significant amino acid sequence homology (37% identity) was with HupE, a protein of unknown function encoded by the hydrogenase operon of *Rhizobium leguminosarum* bv. *viciae* (Hildago, et al., 1992). Figure 3.9a shows the alignment between *ureJ*, *orf1* and *hupE*. The *ureJ* gene is transcribed in the same orientation to that of other genes in the urease gene cluster. A hydrophilicity plot showed that like HupE, UreJ has several strong hydrophobic regions, suggesting it is also a transmembrane protein (Figure. 3.9b).

3.1.7. Analysis of BbuR

An ORF, designated *bbuR*, (*B. bronchiseptica* urease regulator), located upstream of *ureD* and transcribed in the opposite direction to the urease operon, was identified as sharing homology with the LysR family of transcriptional factors (Figure 3.10). A helix-turn-helix domain common in the N-terminal region of LysR-type proteins was also identified in BbuR (amino acids 31 to 52) using the method of Dodd and Egan (1990). This family contains many transcriptional activators from both prokaryotes and eukaryotes, which are required for the expression of a variety of target genes. Within the LysR family, BbuR demonstrated significant homology to NAC, a protein required for urease expression in *K. aerogenes* and the OxyR proteins of *Erwinia carotovora* (Calcutt et al., 1996), *Xanthomonas campestris* (Loprasert et al., 1997) and *E. coli* (Christman et al., 1989). The BbuR open reading frame is preceded by the sequence 5’-AAAGGG-3’ which may act as a Shine-Dalgarno sequence. A potential promoter region with -35 region (5’-TTAATT-3’) and -10 region (5’-
Figure 3.9. (a) Alignment of UreJ from *B. bronchiseptica* (B.b.) with Orf1 of *A. eutrophus* (A.e.) (Piettre and Toussaint, 1998) and HupE of *Rhizobium leguminosarum* bv. viciae (R.l.) (Hildago *et al.*, 1992). (b) Hydrophilicity plot of UreJ. Hydropathy was calculated using the method of Kyte and Doolittle (1982), with a window of 11 amino acids. Hydrophobic regions are indicated by negative values.
Figure 3.10. Homology of BbuR with other LysR proteins. Amino acids identical to that of the B. bronchiseptica sequence are indicated by dots; differences are shown by upper case letters. Dashes represent gaps introduced into the amino acid sequence to optimise alignment. The helix-turn-helix motif is indicated above the sequence. Abbreviations used: B.b., Bordetella bronchiseptica; E.c, Erwinia carotovora OxyR (Calcutt et al., 1996); X.c, Xanthomonas campestris OxyR (Loprasert et al., 1997); E.cl, E. coli Mor (Warne et al., 1990); E.c2, E. coli OxyR (Christman et al., 1989); K.a, K. aerogenes NAC (Bender, 1991).

ATAAT-3') was found upstream from the \textit{bbuR} ATG start codon (nucleotides 2128 to 2147). Two direct repeat sequences 5'-ATTATTTNCAATA-3' separated by three base pairs was located upstream of the putative \textit{bbuR} promoter (base pairs 2167-2195), which may be involved in binding another transcriptional activator regulating \textit{bbuR} expression. Tandem direct repeats have been shown to be involved in pertussis toxin operon regulation in \textit{B. pertussis} (Gross and Rappuoli, 1988). A putative BbuR binding region, 5'-ATA-N9-TAT-3', homologous to NAC-binding consensus sequences (Goss and Bender, 1995), and LysR-binding
consensus sequences, 5'-T-N_{11}-A-3', was found 42 base pairs upstream from the urease promoter region. Downstream of $bbuR$, another open reading frame, designated ORF1, was identified that shared homology at both the DNA and amino acid level to ORF7 (Valentin, et al., 1995; Genbank Accession No. L36817) from the operon necessary for biosynthesis of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in *Alcaligenes eutrophus* (results not shown).

3.2. Characterisation of urease activity of *B. bronchiseptica*

Ureases are regulated by several different mechanisms, and often, the mode of regulation may give an indication of the role urease plays in a microorganism's life cycle. To date no work characterising the regulation of urease has been undertaken in *B. bronchiseptica*. The investigation of parameters, known to influence expression of urease in other species, was therefore carried out in *B. bronchiseptica*. Sequence analysis also identified an open reading frame, $bbuR$, upstream of the urease locus that encodes a LysR-like regulatory protein. The location and orientation on $bbuR$, and the presence of a sequence that resembles the putative LysR binding site proximal to the urease promoter make BbuR an attractive candidate regulator of urease in *B. bronchiseptica*.

3.2.1. Urease activity of *B. bronchiseptica* BB7865 and BB7866

The initial characterisation of urease in *B. bronchiseptica* involved the assessment of its expression BB7865 and BB7866. Freshly grown cultures of each strain were inoculated into SS-X to an OD$_{560}$ of approximately 0.1 and the growth and urease activity monitored hourly (Figure 3.11a). Both strains grew at a similar rate and reached stationary phase at a comparable optical density. The urease activity of BB7866 increased in parallel with the optical density of the culture. While BB7865 showed a similar trend to BB7866, the urease activity was markedly lower at the
equivalent growth level. Urease activity was not detected in culture supernatants (data not shown), indicating that urease was cell-associated.

The differences in urease activity in BB7866 and BB7865 when grown in SS-X suggested that the \textit{bvg} locus may be involved in the regulation of this enzyme in \textit{B. bronchiseptica}. This hypothesis was tested by growth of BB7866 and BB7865 in SS-X and SS-C media at both 37°C and 30°C as these factors are known to modulate expression of the \textit{bvg} locus (Figure 3.11b). In SS-X at 37°C BB7865 showed a low urease activity (3.2±0.35 U.l⁻¹) in comparison to BB7866 (26.1±1.6 U.l⁻¹). In SS-C however, the urease activity of BB7865 increased such that there was no significant difference between the activities of the two strains. At 30°C, BB7865 demonstrated low urease activity levels in both SS-X and SS-C whereas BB7866 maintained the higher level of activity. Growth in the presence of an intact \textit{bvg} locus therefore appears to repress urease activity in \textit{B. bronchiseptica}. The absence of \textit{bvg} gene products is a requirement, but not sufficient, for the derepression of urease activity in BB7865 however. To analyse the effect of urea inducibility and nitrogen limitation, growth media was either supplemented with 10 mM urea, or 18.6 g.l⁻¹ sodium citrate (a carbon source) used in place of L-glutamate (a nitrogen and carbon source). Growth in these conditions had no discernible effect on the urease activity of either strain (Figure 3.11b).
**Figure 3.11.** (a) Growth and expression of urease by BB7866 and BB7865. Expression of urease in both strains is correlated with growth rate. The results shown are representative of one of several repeat experiments. (b) Urease activity of *B. bronchiseptica* in varying growth conditions. In BB7865 urease activity is repressed when grown in non-modulating conditions or at 30°C. Urease is constitutively produced by BB7866. No significant variance in the urease activity of either strain was evident in the presence of 10 mM urea or in nitrogen limiting conditions.
3.2.2. Mutation of bbuR

To determine whether BbuR was involved in the regulation of urease in *B. bronchiseptica*, homologous recombination was used to disrupt *bbuR* in BB7865 and BB7866. Two primers, BB18 and BB19, designed with flanking EcoRI recognition sequences, were used to amplify an internal 0.5 kb segment of *bbuR* from pMC3. The resulting PCR product was cloned into pCR2.1, transformed into *E. coli* INVαF' and colonies screened for the presence of the PCR product. One plasmid containing the PCR product, designated pDT1 was purified, digested with EcoRI and electrophoresed on a 0.7% agarose gel. The 0.5 kb band was excised from the gel, purified using BRESA-CLEAN and ligated into the EcoRI site of the kanamycin resistant suicide vector pJP5603 (Figure 3.12a). After transformation into *E. coli* JM109λpir, colonies were screened for the presence of pJP5603 containing the insert. One such plasmid, pMC48, was introduced into *E. coli* S17λpir. Matings between *E. coli* S17λpir harbouring pMC48 and BB7866 or BB7865 yielded several kanamycin resistant *B. bronchiseptica* mutants. Southern hybridisation of XhoI restricted chromosomal DNA of two mutants, BB7865 B1 and BB7866 B8 demonstrated the presence of pMC48 homologously recombined into the *bbuR* gene of each of these mutants (Figure 3.12b).

When assayed for urease activity, BB7866 B8 showed no difference in activity in comparison to BB7866, regardless of the incubation temperature or growth media. In contrast there was a difference in the expression of urease in BB7865 B1 when compared to BB7865. Urease activity was constitutively expressed in all conditions tested for BB7865 B1, indicating that the ability to regulate urease activity has been lost by this strain (Figure 3.13).
Figure 3.12. (a) Construction of the recombinant vector for homologous recombination with the *bbuR* gene of BB7865 and BB7866. A 0.5 kb internal fragment of the *bbuR* genes was PCR amplified from pMC3 (shaded box) and cloned into pCR2.1. The fragment was then subcloned into the mobilisable suicide plasmid pJP5603, allowing transfer to a recipient chromosome. (b) Southern blot analysis of *XhoI* restricted chromosomal DNA from BB7865, BB7866, BB7865 B1 and BB7866 B8 (lanes 1-4) probed with pMC48.
Figure 3.13. Comparison of the urease activities of (a) BB7865 and (b) BB7866 with their \textit{bbuR} deficient derivatives BB7865 B1 and BB7865 B8. Bacteria were grown in SS-X or SS-C at both 30°C and 37°C and urease activity assayed. Data represent the means of at least three independent experiments ± standard error.
3.3. Analysis of urease activity in other *Bordetella* spp.

3.3.1. Urease activity of *B. pertussis* and *B. parapertussis*

Examination of the urease activity of other *Bordetella* species revealed only *B. parapertussis* to be ureolytic. *B. parapertussis* ATCC15311 expressed urease in both SS-X and SS-C, albeit at lower levels than *B. bronchiseptica*. Like *B. bronchiseptica* BB7865 however, the urease activity of *B. parapertussis* was greatest when grown in SS-C at 37°C. *B. pertussis* Tohama I did not express urease when grown at 37°C in SS-X or SS-C. *B. avium* ATCC35086 also did not possess urease activity in SS-X. This strain of *B. avium* was unable to grow at 37°C in SS-C (Figure 3.14).

![Figure 3.14. Urease activity of *B. pertussis*, *B. parapertussis* and *B. avium* grown at 37°C.](image)

Expression of urease in *B. parapertussis* appears to be *bvg* regulated. *B. avium* was not ureolytic, and the urease activity in *B. pertussis* was not significantly greater than zero.

3.3.2. Southern blot and PCR analysis of the urease genes of *B. pertussis* and *B. parapertussis*

It has previously been shown that the genes expressing particular phenotypes (e.g. pertussis toxin, flagella) in one species of the *Bordetella* Genus are often present but cryptic in the other species. In the case of the cryptic pertussis toxin genes in *B. bronchiseptica* and *B. parapertussis*, mutations located in the promoter region are responsible for lack of expression from this locus (Arico and Rappuoli, 1987).
Southern hybridisation of EcoRI restricted chromosomal DNA of *B. pertussis* Tohama I, *B. parapertussis* ATCC15311 and *B. avium* ATCC35086 using pMC3 as a probe revealed these species may contain cryptic urease genes. The size of the reactive band from the two *B. bronchiseptica* strains (11 kb) differed from that of *B. pertussis* (12.5 kb) and *B. parapertussis* (11.5 kb) (Figure. 3.15a). Overexposure of the blot also revealed a 13.5 kb *B. avium* DNA fragment also shared some homology with the probe (data not shown).

Two primers BB1 and BB2 were designed to amplify 587 bp fragments from *B. bronchiseptica* that encompassed the beginning of *ureD* and promoter regions of the urease operon in these species. Using these primers, fragments of identical size were amplified from the chromosomes of *B. bronchiseptica* BB7866, *B. bronchiseptica* BB7865, *B. pertussis* and *B. parapertussis* (Figure. 3.15b). The bands from *B. bronchiseptica* BB7865 and *B. bronchiseptica* BB7866 were generated in more stringent conditions (60°C annealing temperature, 4 mM MgCl₂) than for *B. pertussis* and *B. parapertussis* (45°C annealing temperature, 8 mM MgCl₂). Attempts to amplify similar bands in *B. avium*, even in low stringency conditions, proved unsuccessful. Each of the PCR products was cloned into pCR-Script to enable DNA sequence analysis to be undertaken.

When the DNA sequences of the PCR product from each species were compared (Figure 3.16), no differences in the 277 bp *UreD* coding region in *B. bronchiseptica* and the corresponding regions in *B. parapertussis* and *B. pertussis* were found. The 150 base pairs immediately preceding *UreD*, which encompass the potential promoter and BbuR binding regions contained a single point mutation in *B. pertussis*, and no mutations in *B. parapertussis*. Significantly more mutations in the DNA sequences of *B. pertussis* (29 changes) and *B. parapertussis* (28 changes) when compared to *B. bronchiseptica* were found further upstream (150 to 312 base pairs upstream), in a region proximal to the
Figure 3.15 (a) Southern blot analysis of EcoRI restricted chromosomal DNA from *B. bronchiseptica* BB7866, *B. bronchiseptica* BB7865, *B. pertussis Tohama I*, *B. parapertussis* ATCC15311 and *B. avium* ATCC35086 (Lanes 1-5 respectively) probed with pMC3. With the exception of *B. avium*, all species hybridised strongly to the pMC3 probe. Overexposure of the blot revealed *B. avium* to hybridise weakly to the probe. The size of individual bands are described in kilobases and indicated by arrows. (b) PCR of *B. bronchiseptica* BB7866, *B. bronchiseptica* BB7865, *B. pertussis Tohama I*, *B. parapertussis* ATCC15311 and *B. avium* ATCC35086 using primers designed after partial DNA sequencing of the urease genes from *B. bronchiseptica*.
putative regulatory elements for $bbuR$. Only a single difference was found when comparing the sequences of $B. pertussis$ and $B. parapertussis$ in this region.

![Figure 3.16. Comparison of the nucleotide sequences of the $B. bronchiseptica$, $B. pertussis$ Tohama I and $B. parapertussis$ ATCC15311 DNA fragment spanning the urease promoter region and first open reading frame (UreD). Nucleotides identical to that of the $B. bronchiseptica$ sequence are indicated by dots. The putative urease promoter is indicated by dots above the sequence. The putative BbuR binding sequence is boxed. The $ureD$ ATG start codon is shown in bold. Abbreviations: Bb, $B. bronchiseptica$; Bpp, $B. parapertussis$; Bp, $B. pertussis$.](image-url)
3.4. *In vitro and in vivo* analysis of urease mutants of *B. bronchiseptica*

Urease has been recognised as a virulence factor in many bacteria. In *M. tuberculosis* and some other pathogens, urease is suggested to contribute to intracellular survival by inhibition phagolysosomal fusion, or by increasing the phagolysosomal pH. To address the role of urease in the intracellular invasion and survival of *B. bronchiseptica*, the recovery of urease mutant and wild-type strains were compared in a HeLa invasion assay. The abolition of urease activity has also been shown to result in the impairment of colonisation and persistence in some pathogenic bacteria (de Koning Ward and Robins-Browne, 1995; Eaton *et al.*, 1991; Jones *et al.*, 1990; Tsuda *et al.*, 1994). Urease has also been postulated to be involved in the virulence of some respiratory pathogens although evidence to support such claims is weak (Cabrero *et al.*, 1997; Reyrat *et al.*, 1996). In a study using a mixed guinea-pig infection model, urease deficient-mutants of *B. bronchiseptica* were found to survive more readily than the parental wild-type strains (Monack and Falkow, 1993). In the mixed model, urease deficiency may be masked by the activity of the wild-type strain. Therefore a more appropriate study comparing the recovery of urease mutants and wild type *B. bronchiseptica* from separate murine hosts was undertaken. Both the intracellular survival assay and murine colonisation assay were performed by Dr C. A. Guzmán at the Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany using the strains developed in this thesis.

3.4.1. *Intracellular survival of urease-mutants of B. bronchiseptica*

It has been demonstrated that *bvg*-positive *B. pertussis* and *B. parapertussis* are able to invade HeLa cells (Ewanowich *et al.*, 1989a; Ewanowich *et al.*, 1989b), whereas both *bvg*-positive and *bvg*-negative *B. bronchiseptica* can be taken up and survive intracellularly (Ewanowich and Peppler, 1990; Guzmán *et al.*, 1994a;
Guzmán et al., 1994b; Savelkoul et al., 1993; Schipper et al., 1994). In order to analyse the potential role played by urease in the uptake and intracellular survival of B. bronchiseptica, bvg-positive and bvg-negative urease-mutants were compared in an in vitro assay using HeLa cells (Figure. 3.17). An initial reduction in the number of viable bacteria recovered per well 4 h after infection with the urease-mutants was observed, which was more evident for the bvg-positive derivatives. However, when the intracellular survival was assessed 24 h after infection, a significantly increased number of bacteria were recovered from cells infected from four of the five urease-deficient mutants. The increase in survival of B. bronchiseptica BB7865 U4 was above but not significantly different from the parental strain.

**Figure 3.17.** Invasion of HeLa cells by B. bronchiseptica. Results are expressed as the mean percentage of the colony forming units (CFU) recovered per well from the parental strains for the three experiments. The error bars represent standard deviations. Differences between the parental and mutant strains were considered significant at $P < 0.05$ (*).

The infection assay was carried out in the presence of 0.5 mM urea (present in the foetal calf serum). However, the physiological concentration of urea in plasma is considerably higher (3-8 mM, Altman and Dittmer, 1974). Therefore the
invasiveness and survival of BB7865, BB7866 and the corresponding urease-deficient strains, BB7865 U5 and BB7866 U2 was evaluated in the presence of different urea concentrations (0.5-33.5 mM). Our results showed that after 4 h infection (Figure 3.18a) all bacterial strains exhibited an increase in intracellular survival at higher urea concentrations. After 24 h (Figure 3.18b), increasing the concentration of urea increased the recovery of urease-positive parental strains, but not the urease-mutants. The difference in survival after 24 h between parental and urease-mutant strains was therefore dependent on the urea concentration. At low urea concentrations significantly more cells of the urease-mutants were recovered. As the urea concentration increased the difference in survival decreased between urease-positive and urease-negative strains so that at 16 and 33.5 mM urea, recovery of the urease-mutants was not significantly greater than the parental strains. All colonies isolated 24 h after infection maintained the antibiotic resistance marker of the mini-transposon mutant. The bvg-positive haemolytic phenotype was retained by the BB7865 derivatives (data not shown).
Figure 3.18. Invasion of HeLa cells by *B. bronchiseptica* at varying urea concentrations. Recovery of *B. bronchiseptica* BB7865, *B. bronchiseptica* BB7865 U5, *B. bronchiseptica* BB7866 and *B. bronchiseptica* BB7866 U2 after 4 (a) and 24 h (b). The results for panels (a) and (b) are expressed as a mean percentage of the control (infection assay of each individual strain performed in the presence of 0.5 mM urea). Numbers over the control indicate absolute values for recovery of the control strain. The error bars represent standard deviations.
3.4.1. In vivo analysis of urease mutants of B. bronchiseptica

In this study, a mouse model was used to determine the abilities of BB7865 B1 and BB7865 U5 mutants to colonise and persist in the respiratory tract in comparison with the wild-type strain. Approximately $10^5$ bacteria were resuspended in 1% Casamino acids in PBS and administered intranasally to 4 week old female BALB/c mice. Groups of animals (n=4) were sacrificed at 2 h and at 5, 12, 20 and 40 days after challenge. The lungs were homogenised using a Polytron PT 1200 homogeniser (Kinematica, Switzerland) and the number of viable microorganisms determined by plating appropriate dilutions onto Bordet Gengou agar supplemented with cephalexin. The results in Figure 3.19 show that similar progressive bacterial growth was observed during the first 5 days post-infection in all the strains tested. After this time, bacterial numbers declined gradually, but were still recoverable 40 days post-infection in all bacterial strains. No significant differences in the survival rate of mice infected with any of the strains was detected at any of the time points.

![Graph showing colonisation and persistence of BB7865, BB7865 B1 and BB7865 U5 after intranasal inoculation of mice.](image)

**Figure 3.19.** Colonisation and persistence of BB7865, BB7865 B1 and BB7865 U5 after intranasal inoculation of mice. The number of viable *B. bronchiseptica* at 2 h and at 5, 12, 20 and 40 days post-inoculation was determined by serial dilution of homogenised murine lung tissue onto Bordet-Gengou agar. Data points represent the geometric mean of four individual mice.
DISCUSSION

*B. bronchiseptica* is a pathogen of veterinary significance that infects most mammals, but rarely humans. Most work to date on this organism has concentrated on a number of coordinately regulated virulence determinants common to other members of the Genus. The long term aim of such studies is in the development of vaccines and antimicrobial drugs that may limit the prevalence and duration of infection. Studies that focus on the differences between individual *Bordetella* species have been less widespread, but are starting to emerge. Such research may enlighten us as to what factors are important in defining host specificity and in the understanding of which factors are vital in virulence for each species. Urease is a potential virulence determinant of *B. bronchiseptica*, and several other pathogenic bacterial species, that is not shared by *B. pertussis*. Defining a role for this enzyme in the life-cycle of *B. bronchiseptica* may help in understanding the pathogenesis of this species.

The complete urease gene cluster in *B. bronchiseptica* is described for the first time in this thesis. While similar to other urease operons, these genes are unique in containing fused *ureE* and *ureF* gene products, and in the presence of the open reading frame *ureJ*, situated between *ureA* and *ureB*. The only other urease locus to contain a similar open reading frame is that from *A. eutrophus*. In this species the role of the UreJ homologue has also not been described. UreJ was identified as also being homologous to HupE from *R. leguminosarum* bv. viciae (Hildago et al., 1992). HupE is involved in the H₂ uptake (*hup*) system that recycles hydrogen produced in the nitrogen reduction process of this bacterium, increasing the efficiency of symbiotic nitrogen fixation. This is accomplished by shuttling electrons from H₂ oxidation into the electron transport chain at the level of ubiquinone. The *hup* locus includes two genes which encode the structural proteins for hydrogenase (HupS and HupL), and accessory genes responsible for the production of an active hydrogenase (eg. hypE, hypD, hypC and hypD). Like
urease, hydrogenases are nickel containing enzymes, and requires the accessory genes to form a catalytically active enzyme. Interestingly, the \textit{hup} locus of \textit{R. leguminosarum} bv. 

viciae is the only hydrogenase cluster to contain a \textit{hupE} analogue (Vignais and Toussaint, 1994). The strong hydrophobic regions in UreJ indicate this protein is probably embedded in the membrane. It has been suggested that HupE along with HupC and HupD in \textit{R. leguminosarum} bv. 

viciae forms a membrane bound complex that reduces ubiquinone to ubiquinol with H$_2$. Whilst the role of UreJ in the \textit{B. bronchiseptica} urease operon is unclear, we speculate that UreJ might be involved in nickel transport. The construction of an active urease requires the three structural subunit proteins, UreA, UreB and UreC, and the four accessory proteins UreD, UreE, UreF and UreG, involved in nickel assimilation. As the genes expression of these seven genes is sufficient for the activation of urease in other bacteria, other accessory genes are likely to encode proteins that have regulatory functions or are involved in nickel transport.

The requirement for nickel transport across the membrane has been recognised for some time, and initially but incorrectly, it was thought one or more of the more common accessory genes may be involved in this process. The only accessory gene located in the urease operons identified thus far as being involved in nickel transport is UreH from \textit{B. subtilis}. Mutations in UreH that reduce urease activity can be overcome by increasing the nickel content in the growth medium. The NixA protein has a similar function in \textit{H. pylori} (Mobley \textit{et al.}, 1995a), although it is not located in the urease operon. Both UreH and NixA are homologous to \textit{Alcaligenes eutrophus} HoxN (Eberz \textit{et al.}, 1989; Eitinger and Friedrich, 1991) and \textit{Bradyrhizobium japonicum} HupN (Fu \textit{et al.}, 1994); transmembrane nickel transporters encoded by hydrogenase gene clusters. Other proteins from the urease loci of \textit{H. pylori} and \textit{S. salivarius} have also been predicted to be membrane proteins involved in nickel transport, but to date no direct evidence for this role has been supplied (Chen \textit{et al.}, 1996; Cussac \textit{et al.}, 1992). It is tempting to suggest that
UreJ may be part of a similar nickel transport system, although no homology to any known nickel transporter was apparent. The positioning of ureJ between ureA and ureB is also rare. Apart from A. eutrophus, the urease gene cluster from Rhizobium meliloti is the only other operon to have extra genes inserted between the structural genes. This cluster contains ureA, ureB, ureC and another four ORFs, three of which are situated between the structural genes. There was no homology detected between these unknown ORFs and any other known protein. Mutations in any of the three unknown ORFs that did not disrupt the ureA, ureB and ureC structural genes did not alter urease levels. However mutations in ureB and one unknown ORF did abolish hydrogenase activity (Miksch et al., 1994b).

Another link between ureases and hydrogenases are the two homologous proteins UreG and HypB. When determining the proteins most similar to UreG of B. bronchiseptica, the HypB proteins of Methanococcus jannaschii (Borodovsky et al., 1996), Rhodobacter capsulatus (Xu and Wall, 1991) and E. coli (Lutz et al., 1991) demonstrated a limited but significant similarity to UreG from B. bronchiseptica. UreG itself is more highly conserved than other accessory proteins highlighting its importance in urease activation. The fact that ureases and hydrogenases both require additional genes for the successful incorporation of nickel suggests a possible shared evolutionary precursor for these accessory genes.

Uniquely, the UreE and UreF genes are fused into a single open reading frame in the B. bronchiseptica urease operon. This fusion may not adversely effect the function of either protein however. From research on other accessory proteins, it was demonstrated that UreE was able to reversibly bind nickel, suggesting it is the nickel donor for urease (Brayman and Hausinger, 1996). The role of UreF is presently unclear. One suggestion is that UreF in conjunction with the accessory proteins UreD and UreG binds to the urease apoprotein and prevents nickel incorporation until the correct formation of the catalytic site (Moncrief and
Hausinger, 1996). UreF may alternatively be required for the diffusion of carbon dioxide into the active site. If either model is correct then a hybrid UreEF may still carry out both these functions, with the nickel ions being stored on UreEF until the correct formation of the active site.

In this study a transcriptional activator *bbuR*, was identified upstream of the urease operon. BbuR shares homology with members of the LysR regulatory protein and several factors suggested that BbuR may be involved in the regulation of urease activity. Firstly, *bbuR* is adjacent and in the opposite orientation to the urease gene operon. Secondly, the NAC protein of *K. aerogenes* which directly controls urease expression is a LysR protein, and also homologous to BbuR. A putative BbuR binding site is also located upstream of the urease promoter. The proposed consensus sequence for LysR binding proteins is 5'-T-Nn-A-3'. In *K. aerogenes* the three NAC controlled promoters, *hutUp*, *putP* and *ureDp* all contain the consensus sequence 5'-ATA-NQ-TAT-3' (Goss and Bender, 1995), and an identical site is found upstream of the urease promoter in *B. bronchiseptica*. Binding of BbuR to this sequence may be important in the transcription of urease mRNA in *B. bronchiseptica*. Mutation of *bbuR* by homologous recombination did result in the constitutive expression of urease in BB7865, suggesting that BbuR is involved in the regulation of urease. The mutations, introduced into *bbuR* via homologous recombination resulted in the incorporation of the whole plasmid into the *bbuR* gene. The possibility that the integration of this large amount of DNA may result in downstream polar effects which result in its constitutive expression of the urease locus can not be ruled out. Complementation of the *bbuR* mutation with an intact copy of *bbuR* is one strategy that could be used to confirm the role of this gene in regulation of urease. Alternatively, mutations that do not cause polar effects, such as in frame-deletions or site directed mutagenesis of specific nucleotides, could be introduced into *bbuR*. 
A direct repeat region was also found proximal to the putative bbuR promoters. The repeats had the consensus sequence 5'-ATTATTTNCAATT-3'. The promoter region of the B. pertussis pertussis toxin operon contains two direct repeat sequences. Removal of part of either repeat region results in reduced or total loss of pertussis toxin expression (Gross and Rappuoli, 1988). The direct repeats proximal to the bbuR promoter regions may be similarly important for the expression of BbuR, raising the possibility that urease is controlled via a complex regulatory cascade.

The greatest homology to BbuR was not with NAC, however, but with a sub-class of LysR transcriptional activators that are inducible by hydrogen peroxide. In some bacteria (eg. E. coli and E. carotovora) the addition of small amounts of hydrogen peroxide induces the expression of a set of genes that protect the bacterium from levels of hydrogen peroxide that are normally lethal. This is accomplished through the increased expression of OxyR, a LysR protein, which in turn increases expression of a number of other loci, including catalase and glutathione reductase (Christman et al., 1989). Hydrogen peroxide is one constituent of the eukaryote phagolysosome which has anti-microbial activity. Stimulation of a class of enzymes that protect bacteria from oxidative attack and other phagolysosomal enzymes is one method by which bacteria may survive intra-phagosomally. B. bronchiseptica is able to survive for long periods intracellularly and therefore has defence mechanisms against eukaryote host cell anti-microbial activity (Guzmán et al., 1994a). Induction of urease in the phagolysosome may result in increased pH and inactivation of lysosomal enzymes, enabling the bacterium to survive until it can escape to another intracellular compartment. Ammonia production has also been reported to inhibit phagosome-lysosome fusion (Gordon et al., 1980). Urease activity may help to disrupt the fusion of these two vesicles, giving the bacterium greater time to mount a defence against lysosomal enzymes.
This study has shown urease to be a virulence repressed phenotype of *B. bronchiseptica*. Other virulence repressed genes in *B. bronchiseptica* include those involved in flagella biosynthesis and motility in *B. bronchiseptica* (Akerley and Miller, 1993; Akerley et al., 1992), and also the genes involved in alcaligin biosynthesis (Giardina et al., 1995). A large number of vrg's whose function remains undetermined are also found in *B. pertussis*. Virulent *B. bronchiseptica* BB7865 expresses urease in the presence but not absence of magnesium sulphate at 37°C. A similar result was observed in virulent *B. bronchiseptica* 5376 (data not shown). The isogenically avirulent strain *B. bronchiseptica* BB7866 expressed urease constitutively at levels similar to up-regulated *B. bronchiseptica* BB7865, providing further evidence for *bvg* regulation of urease. At 30°C however, urease activity was not up-regulated in virulent strains, even in the presence of magnesium sulphate. In contrast, the *bvg*-repressed flagella biosynthesis phenotype of *B. bronchiseptica* is expressed at 30°C in the presence of magnesium sulphate (Akerley et al., 1992). A second temperature dependent mechanism of urease regulation may therefore be operating in *B. bronchiseptica*. As high levels of urease activity were observed in the avirulent strain at 30°C, *bvg* may also regulate the postulated temperature sensitive regulator.

A model which may explain these observations would include a repressor of urease, possibly BbuR, which is activated by the transcriptional activator BvgA in response to non-modulating conditions detected by the environmental sensory protein BvgS (eg. absence of magnesium sulphate). Additionally, in response to modulating conditions detected by BvgS (eg. presence of magnesium sulphate) a temperature sensitive regulator would repress urease activity at 30°C but not at 37°C. Melton and Weiss (1989) have provided evidence that in modulating conditions brought about by low temperature (28°C) the *bvg* mRNA transcript encoding BvgS and BvgA is still produced. On the other hand, in the presence of magnesium sulphate the *bvg* mRNA transcript is not transcribed (Melton and
Discussion

Weiss, 1989). The differential expression of the bvg locus in response to different modulating signals may therefore be relevant to regulation of urease at 30°C. Mutation of BvgS (as is the case in B. bronchiseptica BB7866) would remove both repression of urease by low growth temperature and the absence of magnesium sulphate.

The results of this thesis suggest that both the products of the bvg locus and BbuR contribute to the regulation of urease in B. bronchiseptica. Several different experiments could be used to confirm these findings. Firstly, gel shift mobility assays could be used to investigate the interaction between the promoter regions of ure and bbuR and purified BvgA or BbuR. No putative BvgA binding site was identified in the ure and bbuR regulatory elements, so it is unlikely that evidence of BvgA binding to this region would be gained. How the bvg locus does regulate expression of the virulence repressed genes remains open to speculation. In B. pertussis, BvgR has shown to be involved in regulation of some loci, and conserved elements have been found within some vrg's (Beattie et al., 1993). However, anecdotal evidence by Martinez de Tejada et al., (1996) suggests that BvgR is not expressed in B. bronchiseptica. A putative BbuR binding site near the urease promoter gives credence to BbuR's involvement in urease regulation. This argument could be strengthened however, if an interaction between the BbuR binding site and the protein itself could be directly demonstrated. Another aspect that could be investigated is the expression of BbuR in response to changes in modulating conditions. At present, there is no assay (apart from its effect on urease regulation) to detect the expression of BbuR. Linking the bbuR promoter elements to a reporter gene, such as a promoterless LacZ gene, and reintroducing the construct back into B. bronchiseptica is one strategy that would allow the analysis of expression of BbuR. Alternatively, to remove any background effects, the bbuR/LacZ fusion and the bvg locus could be transferred to E. coli and then analysed.
Similar levels of recovery of \textit{bvgs}-positive and \textit{bvgs}-negative strains of \textit{B. bronchiseptica} have been observed after invasion of epithelial cells (Schipper \textit{et al.}, 1994) or dendritic cells (Guzmán \textit{et al.}, 1994a). After invasion of HeLa cells however, significantly more bacteria are recovered from \textit{bvgs}-positive strains (Savelkoul \textit{et al.}, 1993). The results observed in this study suggest that two parameters seemed to be affecting bacterial survival after 4 h of infection. Firstly, the absence of a functional urease resulted in decreased survival of the mutants, suggesting that urease indeed plays a role in invasion and initial intracellular survival, possibly by increasing the pH in the phagosome due to the production of ammonia. Secondly, increasing the urea concentration in the assay resulted in increased recovery of all bacteria regardless of their urease phenotype. The presence of urea may therefore increase the permissiveness of HeLa cells to bacterial invasion. Alternatively in the presence of high substrate concentrations, the ammonia released by the wild-type strains will be increased, and any residual activity of the urease-mutants may be potentiated. After 24 h at low urea concentrations, the recovery of urease-mutants was greater than the parental strains. Experiments carried out by Monack and Falkow (1993) suggest that urease-negative strains of \textit{B. bronchiseptica} are able to colonise the lungs of guinea pigs better than urease positive strains. It may be that at low urea concentrations, there is no benefit to be gained by possessing urease, and that there is a significant metabolic cost in producing the enzyme. As the urea concentration was increased, urease activity had a positive impact on the cell numbers of the parental strains, so that at high urea concentrations recovery of the urease-mutants was not significantly greater than parental strains. \textit{B. bronchiseptica} BB7866 was actually recovered at a significantly higher rate than \textit{B. bronchiseptica} BB7866 U2 at the highest urea concentration used in the invasion assays. Therefore there is a benefit to be gained by urease producing strains when urea is present in sufficient quantities.
B. bronchiseptica is internalised via a phagocytic process (Guzmán et al., 1994b). The different strategies that are employed by bacteria to survive intraphagosomes include inhibition of phago-lysosome fusion (Buchmeier and Heffron, 1991; Zeigler, 1983), inactivation of lysosomal enzymes (Fields et al., 1989) and escape from the phagosome to another cellular compartment or cytoplasm (McDonough et al., 1993; Rikisha and Ito, 1980). Inhibition of phagosome-lysosome fusion has been suggested as one method by which B. pertussis can survive intracellularly (Steed et al., 1991). However, phagosome-lysosome fusion has been demonstrated to occur in B. bronchiseptica cell-invasion (Guzmán et al., 1994a).

B. bronchiseptica may survive intracellularly at least in part by inhibiting phagolysosomal enzyme activity. Upon internalisation the bvg-locus may be switched off, resulting in the expression of virulence repressed determinants such as urease. Adenylate cyclase, a bvg-activated toxin, is down-modulated after entry into macrophages, presumably in a bvg-regulated manner (Banemann and Gross, 1997; Masure, 1992). After phago-lysosome fusion the degradation of urea by urease may protect B. bronchiseptica by increasing the phagolysosomal pH to levels that impair lysosomal enzyme activity. The bacterium may then survive in a modified cellular compartment (Guzmán et al., 1994a) or escape to the cytoplasm (Schipper et al., 1994) during persistent chronic infection.

Together, the biochemical data showing that urease is expressed at 37°C, and the intracellular survival data suggest that urease is an important factor after the infection of the host organism. However, the abolition of urease activity had no effect on the ability of B. bronchiseptica to colonise and persist in the lungs of mice after intranasal inoculation. It is possible that in the case of B. bronchiseptica the effect of the loss of urease activity may be overcome by compensatory mechanisms. Both B. pertussis and B. bronchiseptica produce a superoxide dismutase (Graeff-Wohlleben et al., 1997; Khelef et al., 1996), and B. pertussis also expresses a catalase (DeShazer et al, 1994). Both these enzymes are believed to protect the
bacterium from oxidative attack after intracellular invasion of phagocytes. A direct link between SOD and catalase activity and virulence in *Bordetellae* could not be established however, as *sodA*, *sodB* and *katA* mutants are as virulent as wild-type strains in murine infection models (DeShazer et al., 1994; Khelef et al., 1996; Graeff-Wohlleben et al., 1997). It was suggested that the loss of SOD or catalase activity may be compensated by the activity of other redundant oxidoreductases, as is the case in *E. coli* and *Salmonella* (Farr and Kogoma, 1991). The existence of redundant mechanisms for intracellular survival in *B. bronchiseptica* may be relevant in explaining the discrepancy between the intracellular survival data and *in vivo* colonisation results.

Comparison of the DNA sequences from *B. bronchiseptica*, *B. parapertussis* and *B. pertussis* revealed there were no differences in DNA sequence in the coding regions of *ureD* of these three species, and only a single point mutation in *B. pertussis* in the 150 base pairs preceding *ureD* that encompass the promoter region for the urease locus. A large number of mutations were found upstream of the urease locus in a region proximal to the putative *bbuR* promoter. These results suggest that the lack of urease expression in *B. pertussis* may be due to mutations in regulatory loci. Similarly, Arico and Rappuoli (1987), have shown that mutations in the promoter regions of the silent pertussis toxin genes of *B. parapertussis* and *B. bronchiseptica* are responsible for abrogation of pertussis toxin expression in these two species. Finally, although urease may be important in the pathogenesis of *B. bronchiseptica*, and to some extent *B. parapertussis*, the loss of urease activity by *B. pertussis* is not detrimental to the virulence of this organism.
CONCLUSION

Although urease has been implicated in the pathogenesis of many bacterial species, little research into the molecular biology and function of this enzyme has been undertaken in *B. bronchiseptica*. This study has provided the complete urease gene cluster DNA sequence and has also identified a potential regulatory protein, BbuR, the gene for which is found upstream of the urease locus. Mutation of BbuR results in the constitutive expression of urease, which was shown to be under the control of the *bvg* locus in other experiments. Thus the *bvg* locus may exert its influence on urease through BbuR. BbuR was shown to be homologous to the LysR family of transcriptional regulators. Two other members of this family, NAC and OxyR, which are homologous to bbuR are relevant in attempting to assign a role for BbuR.

Intracellular survival assay showed that for urease-positive strains, the presence of urea improved their ability to be recovered from Hela cells. Thus urease may be important in the intracellular survival of this organism. Further experiments, such as linking the urease promoter to a reporter gene whose expression can be monitored after intracellular invasion (such as Green Fluorescent Protein) may be used to confirm the intracellular expression of urease by *B. bronchiseptica*. Although the murine colonisation assays did not shown that urease deficient *B. bronchiseptica* had an impaired colonisation ability, it is possible that redundant enzyme systems may compensate for the loss of urease activity *in vivo*. 
REFERENCES


References


References


References


APPENDIX 1

Growth media and solutions

Media

BG agar
Bordet Gengou agar 3%
Glycerol 1%

Z agar
Glucose 1 g.l⁻¹
CaCl₂ 0.37 g.l⁻¹
NaCl 1 g.l⁻¹
Bactotryptone 1 g.l⁻¹
Yeast extract 5 g.l⁻¹
agar 15 g.l⁻¹

LB broth
Bactotryptone 10 g.l⁻¹
Yeast extract 5 g.l⁻¹
NaCl 10 g.l⁻¹

LB Mg Mal medium
Bactotryptone 10 g.l⁻¹
Yeast extract 5 g.l⁻¹
NaCl 5 g.l⁻¹
Glucose 1 g.l⁻¹
Maltose 4 g.l⁻¹
MgCl₂.H₂O 1 g.l⁻¹

Soc medium
Bactotryptone 2%
Yeast Extract 0.5%
NaCl 10 mM
KCl 2.5 mM
pH 7.0

MgCl₂ 10 mM (autoclaved separately)
glucose 20 mM (filter sterilised)
**Modified Stainer and Scholte medium**

**SS base**
- L-glutamate: 10.72 g.l\(^{-1}\)
- L-proline: 0.24 g.l\(^{-1}\)
- KH\(_2\)PO\(_4\): 0.5 g.l\(^{-1}\)
- KCl: 0.2 g.l\(^{-1}\)
- MgCl\(_2\).6H\(_2\)O: 0.1 g.l\(^{-1}\)
- CaCl\(_2\): 0.02 g.l\(^{-1}\)
- TrisBase: 6.075 g.l\(^{-1}\)
- NaCl (for SS-X): 2.5 g.l\(^{-1}\)
- MgSO\(_4\) (for SS-C): 5.0 g.l\(^{-1}\) (autoclaved separately)
- x100 Salts

**100x Salts**
- L-cysteine: 0.4 g.100ml\(^{-1}\)
- FeSO\(_4\).7H\(_2\)O: 0.1 g.100ml\(^{-1}\)
- Ascorbic acid: 0.2 g.100ml\(^{-1}\)
- Nicotinic acid: 0.04 g.100ml\(^{-1}\)
- Glutathione: 1.0 g.100ml\(^{-1}\)

**Solutions**

**STE buffer**
- TrisHCl: 10 mM
- EDTA: 1 mM
- NaCl: 0.1 M
  - pH 8.0

**10x TAE Buffer**
- TrisBase: 48.44 g.l\(^{-1}\)
- Sodium Acetate: 27.22 g.l\(^{-1}\)
- EDTA: 7.44 g.l\(^{-1}\)
  - pH 8.0

**TE Buffer**
- TrisHCl: 100 mM
- EDTA: 25 mM
  - pH 8.0

**TES Buffer**
- TrisHCl: 100 mM
- EDTA: 25 mM
- NaCl: 150 mM
  - pH 8.0
Media and Solutions

Agarose gels
Agarose 0.7-1.0%
TAE Buffer 1x

DNA loading dye
Bromophenol blue 0.05%
Glycerol 75% v/v
TE Buffer 25% v/v

PBS
NaCl 8 g.l⁻¹
KCl 0.2 g.l⁻¹
Na₂HPO₄ 1.44 g.l⁻¹
KH₂PO₄ 0.24 g.l⁻¹

Urease assay reagent mix
TrisHCl (pH 8.0) 48 mM
ketoglutaric acid 1 mM
NADH 0.375 mM

QIAGEN Buffers

Buffer P1
RNase A 100 μg.ml⁻¹
TrisHCl 50 mM
EDTA 10 mM
pH 8.0

Buffer P2
NaOH 200 mM
SDS 1%

Buffer P3
Potassium Acetate 2.55 M
pH 4.8

Buffer QBT
NaCl 750 mM
MOPS 50 mM
ethanol 15%
pH 7.0

0.15% Triton X-100
**Buffer QC**

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**Southern Solutions**

**20 x SSC**

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**Prehybridisation solution**

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**Radio-Labelling Solutions**

**Solution A1**

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<td>MgCl₂</td>
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**Solution C**

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<tr>
<td>PMSF</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>glycerol</td>
<td>50% (v/v)</td>
</tr>
<tr>
<td>nuclease free BSA</td>
<td>100 µg.ml⁻¹</td>
</tr>
</tbody>
</table>
### Solution D

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$EDTA</td>
<td>300 mM</td>
</tr>
<tr>
<td>pH 8.0</td>
<td></td>
</tr>
</tbody>
</table>

### DNA sequence loading Dye

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formamide</td>
<td>5 parts</td>
</tr>
<tr>
<td>EDTA (pH 8.0)</td>
<td>1 part</td>
</tr>
</tbody>
</table>
### APPENDIX II

**Primers for PCR and DNA Sequencing**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal Forward</td>
<td>5'-GTAAAACGACGGCCAGT-3'</td>
</tr>
<tr>
<td>Universal Reverse</td>
<td>5'-AATGATGCTGCTTCTTCTGACTAGTGG-3'</td>
</tr>
<tr>
<td>BB1</td>
<td>5'-GGATCCCTTGACTCTCTACGAGCAGCGG-3'</td>
</tr>
<tr>
<td>BB2</td>
<td>5'-GAATTCGCAATAATGGCGCATTGCG-3'</td>
</tr>
<tr>
<td>BB4</td>
<td>5'-GAAGGGGAGTAAGAAGATG-3'</td>
</tr>
<tr>
<td>BB7</td>
<td>5'-GCTCGTCCATAGCTACCTTC-3'</td>
</tr>
<tr>
<td>BB8</td>
<td>5'-GAACGCTACGCGACTAGTGC-3'</td>
</tr>
<tr>
<td>BB9</td>
<td>5'-CACCACGAGGCCTCCAGCG-3'</td>
</tr>
<tr>
<td>BB11</td>
<td>5'-CATGATGCGAAGATCCCGGAC-3'</td>
</tr>
<tr>
<td>BB12</td>
<td>5'-GAAGATGTTGATGGTACCC-3'</td>
</tr>
<tr>
<td>BB15</td>
<td>5'-CTCGGCGAAGGTAGTGGTAC-3'</td>
</tr>
<tr>
<td>BB16</td>
<td>5'-CTCTTCTTCGTTCCAGGGAC-3'</td>
</tr>
<tr>
<td>BB17</td>
<td>5'-ACCTCCCAAACCGTGCTTAG-3'</td>
</tr>
<tr>
<td>BB18</td>
<td>5'-GGGAAATTCCAAACAGTTATCGAGGGAGCCTG-3'</td>
</tr>
<tr>
<td>BB19</td>
<td>5'-GGGAATTCCTATGCCCGCTACTTCCTGAC-3'</td>
</tr>
<tr>
<td>BB20</td>
<td>5'-TTCTCCCATCATGACGCTCCGGA-3'</td>
</tr>
<tr>
<td>BB21</td>
<td>5'-CTTTGCCACGAGCACAGGT-3'</td>
</tr>
<tr>
<td>BB22</td>
<td>5'-ATCCGCGGAGCCAGGCAGCAGTTTAT-3'</td>
</tr>
<tr>
<td>BB23</td>
<td>5'-AACAATGCTGCTCCGCGCAAT-3'</td>
</tr>
<tr>
<td>BB24</td>
<td>5'-TGAGCAAGGCGATGACCGGAT-3'</td>
</tr>
<tr>
<td>BB25</td>
<td>5'-AACCTGCTGGAAGACCCCTTC-3'</td>
</tr>
<tr>
<td>BB26</td>
<td>5'-AGAGTTCGCGACGACATC-3'</td>
</tr>
<tr>
<td>BB27</td>
<td>5'-TGCTTCTGTGTGCCTGGGATA-3'</td>
</tr>
<tr>
<td>BB29</td>
<td>5'-GAGACCCTTGGGTATGCGG-3'</td>
</tr>
<tr>
<td>BB30</td>
<td>5'-CCTGACTCTCCCAGCT-3'</td>
</tr>
<tr>
<td>BB31</td>
<td>5'-TATGCTTTTCGTGGCCGGA-3'</td>
</tr>
<tr>
<td>BB32</td>
<td>5'-TCCAGCCACGAAAGACATA-3'</td>
</tr>
<tr>
<td>BB33</td>
<td>5'-TTGGTGAAACAAGAATTC-3'</td>
</tr>
<tr>
<td>BB34</td>
<td>5'-AGCACTGGGACCAAAGGCGAA-3'</td>
</tr>
<tr>
<td>BB35</td>
<td>5'-CGGAACTTACCAAAACACATCT-3'</td>
</tr>
<tr>
<td>BB37</td>
<td>5'-ACCAGGATGCTCCCTTCTG-3'</td>
</tr>
<tr>
<td>BB38</td>
<td>5'-TCAAGACCTGAGTTGAACCTAC-3'</td>
</tr>
<tr>
<td>BB39</td>
<td>5'-AGCAAGGACCTGCCCTTA-3'</td>
</tr>
<tr>
<td>BB40</td>
<td>5'-AGCTTCTGATGTTTTGGG-3'</td>
</tr>
</tbody>
</table>
APPENDIX III

Publications arising from this thesis

Manuscripts


McMillan, D. J., E. Medina, C. A. Guzman and M. J. Walker. Expression of urease does not effect the ability of *Bordetella bronchiseptica* to colonise and persist in the murine respiratory tract. *In press*.

Oral presentations and posters

