Rational attenuation of *Bordetella bronchiseptica* for vaccine purposes

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Rational Attenuation of *Bordetella bronchiseptica* for Vaccine purposes.

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

**Doctor of Philosophy**

from

The University of Wollongong

by

Jason McArthur

Department of Biological Sciences

2002
Declaration

This thesis is submitted in accordance with 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. The experimental work described in this thesis is original work and has not been submitted for a degree at any other university or institution.

/ Jason McArthur
Acknowledgments

There are a large number of people I wish to thank for their contributions and support during the production of this thesis.

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Finally, I would like to thank mum and dad who through their never ending support have given me the confidence and opportunity to complete this part of my education.
**ABSTRACT**

*Bordetella bronchiseptica* is a Gram-negative respiratory pathogen of a variety of animals. This bacterium causes kennel cough in dogs, bronchopneumonia in rabbits and guinea pigs, and has been associated with the disease atrophic rhinitis in swine. To investigate whether *B. bronchiseptica* could be attenuated through disruption of aromatic compound biosynthesis, the *aroA* gene of *B. bronchiseptica* was PCR amplified and cloned. DNA sequence analysis of the area surrounding the *aroA* gene revealed it to be part of a mixed-function superoperon in the order of *gyrA*, *serC*, *pheA*, *tyrA*, *aroA*, *cmk*, *rpsA* and *ihfB* that has been shown to be well conserved through wide phylogenetic distances. The *aroA* ORF was disrupted by inserting a kanamycin resistance cassette within it. This construct was used to produce an insertion mutation in *aroA* of *B. bronchiseptica* via homologous recombination. This mutation resulted in strain CMJ25 that displayed a slower growth rate in minimal medium when compared to the parental strain but was not completely auxotrophic for aromatic compounds. Growth rate comparable to that of the parental strain was exhibited when aromatic supplements were added to the medium or when CMJ25 was subjected to growth conditions resulting in the strain displaying a *bvg*-repressed phase. CMJ25 was not attenuated in a murine model of respiratory infection. To obtain an aromatic amino acid auxotrophic mutant of *B. bronchiseptica* that was attenuated *in vivo*, CMJ25 was subjected to rounds of mutagenesis using the gentamycin resistant mini-transposon, mini-Tn5/Gm. The resulting strain CMJ60, was unable to grow in minimal SS-X medium in the absence of aromatic amino acid (aamix) supplementation, specifically when tryptophan was not present. The region of the chromosome containing the transposon was cloned and DNA sequence analysis revealed that the mini-transposon had inserted into an ORF encoding *trpE*. Analysis of the sequence surrounding *B. bronchiseptica trpE* was performed using the genome sequence currently being assembled at the Sanger Center (http://www.sanger.ac.uk/projects/B_bronchiseptica). This revealed *trpE* to be organised into an operon with genes that code for other tryptophan biosynthetic enzymes, *trpG*, *trpD* and *trpC*. The presence of a putative *trpL* leader sequence upstream of *trpE* may indicate that *B. bronchiseptica* uses an attenuation mechanism to regulate
expression of this operon. Compared to the parental wild-type strain, the mutant displayed significantly reduced abilities to invade and survive within the mouse macrophage-like cell line J774A.1 \textit{in vitro} and in the murine respiratory tract following experimental intranasal infection. Mice vaccinated with CMJ60 displayed significant dose-dependent increases in \textit{B. bronchiseptica}-specific antibody responses, and exhibited increases in the number of \textit{B. bronchiseptica}-reactive spleen cells in lymphoproliferation assays. Immunised animals were protected against lung colonisation after challenge with the wild-type parental strain and the humoral immune response in vaccinated and protected mice was directed primarily against \textit{B. bronchiseptica} lipopolysaccharide. These results demonstrate that \textit{B. bronchiseptica}, when made auxotrophic for aromatic compounds, is attenuated in mice and can be used as a live intranasal vaccine that elicits strong and protective humoral and cell-mediated immune responses. Such a strain may not only be utilised for the prevention of \textit{B. bronchiseptica} associated disease, but could also be used to deliver heterologous antigens to a variety of mammalian species.
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<tr>
<td>A</td>
<td>absorbance</td>
</tr>
<tr>
<td>aamix</td>
<td>aromatic supplements</td>
</tr>
<tr>
<td>Ap</td>
<td>ampicillin</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BG</td>
<td>Bordet Gengou</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>bv g</td>
<td><em>Bordetella</em> virulence gene</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>Cp</td>
<td>cepahlexin</td>
</tr>
<tr>
<td>dH2O</td>
<td>distilled water</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>dinucleotide triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine-tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>g</td>
<td>relative centrifugal force 9.8ms$^{-2}$</td>
</tr>
<tr>
<td>G</td>
<td>gauge</td>
</tr>
<tr>
<td>Gm</td>
<td>gentamycin</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-galactosidase</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases</td>
</tr>
<tr>
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</table>
min  minute
ng  nanogram
nm  nanometer
OD  optical density
ORF  open reading frame
PBS  phosphate buffered saline
pmol  picomoles
 PAGE  polyacrylamide gel electrophoresis
r  resistant
Rif  rifampicin
RNase A  ribonuclease A
rpm  revolutions per minute
sec  seconds
SDS  sodium dodecyl sulphate
SS-X  Stainer Scholte medium
Tc  tetracycline
TEMED  N,N,N',N'-Tetra-methylethlenediamine
t-Octylphenoxypolyethoxyethanol
Triton X-100  units
U  microgram
μm  micron
UV  ultra violet
V  volts
X-Gal  5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
1. Introduction

1.1 The genus *Bordetella*

The *Bordetella* genus consists of eight species that are responsible for a variety of respiratory diseases in humans and animals. The genus consists of *Bordetella pertussis*, *B. bronchiseptica*, *B. parapertussis* (human), *B. parapertussis* (ovine), *B. avium*, *B. hinzii*, *B. holmsei* and *B. trematum*. The species best characterised is *B. pertussis* which is the causative agent of the contagious childhood disease whooping cough. During a *B. pertussis* infection the pathogen colonises the ciliated epithelia of the trachea and bronchi. Symptoms of the disease include bronchopneumonia and severe paroxysmal coughing episodes which if left untreated can lead to neurologic damage or death. *B. parapertussis* (human) produces a milder form of whooping cough in humans (Heininger et al., 1994). *B. parapertussis* (ovine) are isolated from the respiratory tract of sheep where the infection can cause pneumonia and predispose animals to secondary invaders (Porter et al., 1994). *B. bronchiseptica* has a broad host range with the infection of confinement-reared animals being a considerable economic problem (Goodnow, 1980). *B. avium* is a respiratory pathogen of birds and has been found responsible for the diseases avian bordetellosis and turkey coryza (Kersters et al., 1984). Very little is known about the pathogenesis of the remaining three members of the *Bordetella* genus. *B. hinzii* is also an avian pathogen but has been isolated from immuno-compromised humans (Cookson et al., 1994; Vandamme et al., 1995). *B. holmsei* was isolated from the blood of immunocompromised humans and is thought to have played a role in endocarditis, respiratory failure and septicemia (Weyant et al., 1995). *B. trematum* was isolated from wounds and ear infections of humans (Vandamme et al., 1996).

Various methods have been used to determine the genetic similarities displayed between members of the *Bordetella* genus. These include DNA sequence relationships (Kloos et al., 1981), comparative multilocus enzyme electrophoresis (Musser et al., 1986), pulse field gel electrophoresis of DNA digests (Khattach and Matthews, 1993), ribosomal DNA sequence similarities (Muller and Hildebrandt, 1993; Weyant et al., 1995) and distribution
of insertion sequences (van der Zee et al., 1997). The high similarity of both 16S (>98%) and 23S rRNA (99%) gene sequences confirms the close relationship between members of the *Bordetella* genus and suggests a recent evolutionary origin. This lack of genetic divergence has also lead to the suggestion that members be classified into subspecies rather than species. van der Zee et al. (1997) using multilocus enzyme electrophoresis and typing of three insertion sequences have shown the population structure of the *Bordetella* genus to be predominantly clonal with very little horizontal gene transfer and also suggests that both *B. pertussis* and *B. parapertussis* may have evolved from distinct clones of *B. bronchiseptica*.

### 1.1.1 *B. bronchiseptica*

*B. bronchiseptica* is an aerobic, Gram-negative, non-spore forming, motile coccobacilli. Motility is achieved by a peritrichous flagella. In biochemical assays, *B. bronchiseptica* tests positive for urease, catalase, cytochrome oxidase, citrate utilisation, nitrate reduction and tetrazolium reduction. It is negative for tyrosine hydrolysis, indole and acid production during oxidation-fermentation of glucose and maltose media (Goodnow, 1980).

*B. bronchiseptica* was first isolated from dogs suffering canine distemper in 1911 (Ferry, 1911). It has since been isolated from a variety of animal species that includes monkeys, cats, guinea pigs, rabbits, mice, pigs, horses, foxes, skunks, koalas and seals (Goodnow, 1980). Humans are not considered as a natural host for *B. bronchiseptica* however, human infections have been documented in immuno-compromised patients (Woolfrey and Moody, 1991; Dworkin et al., 1999). *B. bronchiseptica* exhibits a diverse and broad host range but is more prevalent in confinement reared animals, such as swine in piggeries, dogs in shelters and laboratory animals. Respiratory diseases caused by *B. bronchiseptica* include infectious tracheobronchitis (kennel cough) in dogs, snuffles in rabbits and has been associated with atrophic rhinitis in pigs (Giles, 1986).

Infections associated with *B. bronchiseptica* are typically chronic, often asymptomatic and generally difficult to clear. *B. bronchiseptica* adheres to and colonises the ciliated
respiratory epithelia of the upper respiratory tract (Matsuyama and Takino, 1980; Nakai et al., 1988). The bacteria induce an inflammatory response in lungs which is characterised by the infiltration of polymorphonuclear leucocytes, such as neutrophils, into the mucosa of the respiratory tract (Gueirard et al., 1996). In dogs, the disease is characterised by a hacking cough that is followed by gagging and retching. In most of the cases the infections are self-limiting and last for 1-2 weeks however, complicated cases can lead to bronchopneumonia and death (Keil and Fenwick, 1998). In pigs, the disease atrophic rhinitis has a significant global economic impact on the swine industry. The disease is characterised by atrophy of nasal turbinate bones, snout deformation, pneumonia and poor growth. Although infection with *B. bronchiseptica* alone is enough to induce a mild form of the disease, it is now believed that *Pasteurella multocida* plays the major pathogenic role in atrophic rhinitis. Infection by *B. bronchiseptica* is thought to condition the nasal mucosa allowing *P. multocida* to colonise more readily as a secondary invader (Giles, 1986).

1.2 Phenotypic modulation

During its life cycle, *B. bronchiseptica* can encounter a diverse range of environments. For it to survive and successfully mediate infection of appropriate hosts *B. bronchiseptica* must be able to sense and respond accordingly to the conditions being presented. To do this *B. bronchiseptica* has the ability to undergo a freely reversible change in phenotype in response to environmental stimuli. This process is referred to as phenotypic or antigenic modulation.

Lacy (1960) showed that *Bordetella* spp. could alternate between three distinct phenotypic phases in response to changes in the growth medium, i.e. X-phase, C-phase and I-phase, an intermediate between the two. The X and C phases are commonly referred to as virulent and avirulent phases, or $bv$g-activated and $bv$g-repressed phases, respectively. In the $bv$g-activated phase numerous virulence activated genes ($vags's$) are expressed (Table 1.1) and result in the production of various adhesins and toxins used by the bacterium to initiate an infection. Colonies in the $bv$g-activated phase are rounded with a glistening appearance
and produce a clear zone of haemolysis when grown on Bordet-Gengou (BG) agar. In the
*bvg*-repressed phase, *vag’s* are no longer expressed and a different class of genes known as
virulence repressed genes (*vrg’s*) are activated (Table 1.1). Avirulent *Bordetellae* grow
faster with colonies having a flat appearance, matt finish and are non-haemolytic. The
environmental signals sensed for phenotypic modulation are not known however in the
laboratory, growth at low temperatures (less than 30°C) or in the presence of nicotinic acid
(greater than 5 mM) or in the presence of the sulphate anion (greater than 20 mM) are
commonly used as modulators (Melton and Weiss 1993).

1.3 *Bordetella* virulence gene locus

The *Bordetella* virulence gene (*bvg*) locus is responsible for a two component, signal
transduction system that coordinately regulates the expression of *vag’s* and *vrg’s* (Weiss
and Falkow, 1984). This locus encodes two proteins, BvgS a transmembrane
environmental sensory protein and BvgA, a DNA binding transcriptional activator (Uhl and
Miller, 1996). BvgS and BvgA belong to a subfamily of signal transducing proteins with a
characteristic four step His-Asp-His-Asp phosphorelay mechanism (Parkinson and Kofoid,
1992). This type of system has been found in a variety of prokayotes and eukaryotes and
are used for regulating a diverse range of cellular processes in response to a large array of
environmental stimuli (Parkinson and Kofoid, 1992; Chang *et al.*, 1993; Gross, 1993; Ota
and Varshavsky, 1993)
Table 1.1. Bvg-regulated genes of *Bordetella.*

<table>
<thead>
<tr>
<th>Virulence Factor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Virulence activated genes</strong></td>
<td></td>
</tr>
<tr>
<td>Adenylate cyclase-haemolysin (cya)</td>
<td>(Betsou <em>et al.</em>, 1995)</td>
</tr>
<tr>
<td>Dermonecrotic toxin (dnt)</td>
<td>(Pullinger <em>et al.</em>, 1996)</td>
</tr>
<tr>
<td>Filamentous haemagglutinin (fha)</td>
<td>(Cotter <em>et al.</em>, 1998), (Relman <em>et al.</em>, 1989)</td>
</tr>
<tr>
<td>Fimbriae (fim)</td>
<td>(Savelkoul <em>et al.</em>, 1996), (Boschwitz <em>et al.</em>, 1997)</td>
</tr>
<tr>
<td>Lipopolysaccharide (wlb)</td>
<td>(Allen <em>et al.</em>, 1998)</td>
</tr>
<tr>
<td>Pertactin (prn)</td>
<td>(Li <em>et al.</em>, 1992)</td>
</tr>
<tr>
<td>Pertussis toxin (ptx)</td>
<td>(Locht and Keith, 1986)</td>
</tr>
<tr>
<td>Serum resistance locus (brk)</td>
<td>(Fernandez and Weiss, 1994; Rambow <em>et al.</em>, 1998)</td>
</tr>
<tr>
<td>Tracheal colonisation factor (tcf)</td>
<td>(Finn and Stevens, 1995)</td>
</tr>
<tr>
<td>Type III secretion (bsc)</td>
<td>(Yuk <em>et al.</em>, 1998)</td>
</tr>
<tr>
<td><strong>Virulence repressed genes</strong></td>
<td></td>
</tr>
<tr>
<td>Motility</td>
<td>(Akerley <em>et al.</em>, 1992)</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>(Chhatwal <em>et al.</em>, 1997)</td>
</tr>
<tr>
<td>Siderophore (ale)</td>
<td>(Giardina <em>et al.</em>, 1995)</td>
</tr>
<tr>
<td>vrg-6</td>
<td>(Beattie <em>et al.</em>, 1990)</td>
</tr>
<tr>
<td>vrg-18</td>
<td>(Beattie <em>et al.</em>, 1990)</td>
</tr>
<tr>
<td>vrg-73</td>
<td>(Beattie <em>et al.</em>, 1990)</td>
</tr>
</tbody>
</table>

A diagrammatic representation of the *bvg* locus is shown in Figure 1.1. The genes encoding *bvgA* and *bvgS* are transcriptionally linked while *bvgR* (a gene encoding a Bvg activated repressor protein) located downstream from *bvgS*, is transcribed in the opposite direction (Arico *et al.*, 1989; Merkel *et al.*, 1998a). The expression of the *bvg* locus is regulated by four promoters, P1.4 (Roy *et al.*, 1990; Scarlato *et al.*, 1990). The promoters P1, P3 and P4 are autoregulated by BvgA while transcription from P2 is constitutive. The P4 promoter produces an RNA transcript that is complementary to the 5′ untranslated region of the *bvg* mRNA’s and may be involved in the stabilisation of these transcripts by preventing secondary structure formation (Scarlato *et al.*, 1990). Comparison of the *bvgAS*
loci of *B. bronchiseptica* and *B. pertussis* has found them to be highly conserved. The predicted amino acid sequences of BvgA are identical while there are 61 amino acid changes within BvgS. The majority (40) of these changes were found to be located within the periplasmic sensor region of BvgS (Arico *et al.*, 1991). Martinez de Tejada *et al.* (1996) have found that these two sensory molecules respond to the same signals albeit at differing sensitivities. This has lead to the hypothesis that the BvgAS regulatory system may serve different roles in the two species.

![Diagram of Bvg operon](image)

**Figure 1.1** Diagrammatic representation of the *Bordetella* virulence gene (*bvg*) operon. Genes encoding BvgA, BvgS and BvgR are shown as arrows. The four *bvg* promoters and their relative position are indicated with bent arrows.

### 1.3.1 BvgS

BvgS is a 135 kDa transmembrane sensor kinase capable of sensing and responding to environmental stimuli. This complex protein consists of four domains. An N-terminal periplasmic sensor domain connected via a 159 amino acid linker region to the cytoplasmically located transmitter, receiver and output domains (Figure 1.2). Signal transduction involves a phosphorylation cascade that results in the conversion of an environmental stimulus to a cellular signal (Beier *et al.*, 1995).

Unlike many other sensor kinases, BvgS is active under most conditions and is only down regulated when specific modulating signals are encountered. When BvgS is active the phosphorylation cascade begins with the kinase activity of the transmitter domain autophosphorylating itself at a conserved histidine(729) residue. The phosphate group is
transferred to an aspartic acid(1023) residue of the receiver domain. This phosphate can now be donated to histidine(1172) of C-terminus output domain or to surrounding water molecules forming inorganic phosphate and thereby dephosphorylating the system. From the output domain the phosphate can be either transferred back to the receiver or onto the aspartic acid(54) residue of BvgA and thereby activating it (Uhl and Miller, 1996).

Figure 1.2 The BvgAS phosphorelay cascade. BvgS is a transmembrane protein containing a periplasmic sensor domain, a linker region, a transmitter, a receiver and an output domain. BvgA is a transcriptional activator and consists of a receiver domain and a DNA binding helix-turn-helix (HTH) motif. Signal inputs detected by the sensor domain are relayed through the membrane causing autophosphorylation of transmitter at His729. The phosphoryl group is then transferred to an aspartic acid residue (Asp1023) of the receiver domain. Asp1023 can then donate the phosphoryl group to His5172 of the output domain which then donates it to BvgA (Asp54). BvgA is now capable of binding to promoter regions of bvg activated genes and initiating transcription.


1.3.2 BvgA

BvgA is a 23 kDa transcriptional activator that is composed of two domains, a receiver domain at the N-terminus and a DNA binding domain at the C-terminus with a helix-turn-helix (HTH) motif that is typical of response regulators (Arico et al., 1989; Boucher et al., 1994). Once phosphorylated it is believed that BvgA~P forms dimers (Perraud et al., 2000) and display an increased affinity for specific sequences within bvg-activated promoters (Boucher et al., 1994; Beier et al., 1995; Steffen et al., 1996).

As mentioned earlier Bordetella are capable of regulation between distinct phenotypic phases. Recently it has become clear that the expression of various virulence operons have also been shown to be regulated, in a differential manner. Earlier studies using B. pertussis have shown that when the bvg regulon is induced with a temperature shift, fha is expressed within minutes while expression of ptx does not occur for hours (Scarlato et al., 1991a). Similarly when MgSO₄ is added to B. pertussis grown in non-modulating conditions, ptx expression is shut down at lower concentrations than those required to shut down the expression of fha (Scarlato and Rappuoli, 1991c; Stibitz, 1998). These data suggest that differential regulation is due to the differences in the sensitivity of the two promoters to the concentration of BvgA~P. The basic architecture of these promoters consists of an upstream primary BvgA binding site to which it binds with high affinity and a secondary binding region, located further downstream that has a much lower affinity for BvgA (Boucher and Stibitz, 1995; Boucher et al., 1997). The high affinity binding of BvgA at the primary site is associated with a heptanucleotide repeat consensus sequence, TTT(C/G)NTA (Roy and Falkow, 1991; Zu et al., 1996; Karimova and Ullmann, 1997). Secondary binding regions appear to lack any apparent recognition sequences (Boucher et al., 2001). At the ptx promoter there is cooperative binding of BvgA~P dimers along the sequence between the initial binding site and the promoter allowing BvgA~P to interact with RNA polymerase and activate transcription (Boucher and Stibitz, 1995; Marques and Carbonetti, 1997). For fha, a single BvgA~P dimer binds to the secondary site and this is cooperative with binding at the primary site. Boucher et al. (2001), have proposed that the binding of BvgA~P dimer to the secondary site is stabilised through protein-protein interactions with the upstream BvgA~P dimer bound at the primary site and the
downstream RNA polymerase. This type cooperative interaction between regulatory proteins bound at high and low affinity sites within promoter regions has been described for the ComA regulator and the ResD activator of *Bacillus subtilis* (Roggiani and Dubnau, 1993; Paul *et al.*, 2001), the FixJ activator of *Rhizobium meliloti* (Galinier *et al.*, 1994), and the CsrR regulator of *Streptococcus pyogenes* (Miller *et al.*, 2001).

It is this difference in affinities displayed by the various promoter regions that allow the concentration of BvgA~P to dictate the differential activation of virulence genes. This coordinated timing of expression for specific virulence factors may play a critical role during the infection process however, the exact role differential regulation of virulence factors has in *Bordetella* pathogenesis is currently unknown (Kinnear *et al.*, 2001). When this type of regulation is combined with the phosphorelay of the sensitive BvgAS system responding to changes in environmental signals, it allows *Bordetella* to respond, both appropriately and efficiently using the one regulatory system to a variety of changes encountered throughout its life cycle.

### 1.3.3 BvgR

While the *bv*g locus is responsible for the activation of a variety of virulence factors, such as *fha*, *cya* and *ptx*, a second set of genes which is repressed by the *bv*g locus has also been elucidated (Knapp and Mekalanos, 1988). Five virulence repressed genes (*vrg's*) have been identified in *B. pertussis*, *vrg6, vrg18, vrg24, vrg53,* and *vrg73*. In four of these genes a 32 bp conserved sequence element was revealed, unusually located within the coding region for that particular gene (Beattie *et al.*, 1990). In studies where the sequence element of *vrg6* was mutated, this resulted in constitutive expression and loss of modulation responsiveness (Beattie *et al.*, 1993). In nonmodulated cells this conserved sequence element of *vrg6* was also shown to be bound by a 34 kDa protein that was activated by *bv*g (Beattie *et al.*, 1993). Merkel *et al.* (1998a), have shown the locus required for expression of repressor activity is located immediately downstream from *bv*g*S* and has been designated *bv*g*R* (Figure 1.1). It is believed that *BvgR* is the actual repressor protein that binds to the conserved sequence elements of *vrg's*. The *bv*g*R* regulation of gene expression
has been shown to play a role in respiratory infection of mice with *B. pertussis* as constitutive expression of *bvgr*-repressed genes interfered with pathogenesis (Merkel *et al.*, 1998b). Expression of *bvgr* in *B. bronchiseptica* has not yet been demonstrated.

### 1.3.4 Bvg-intermediate phase

It has recently been discovered that when *Bordetella* is grown in the presence of semi-modulating conditions, i.e. intermediate concentrations of modulating signals, it expresses a phenotypic phase distinct from the well characterised *bvgr*-activated or *bvgr*-repressed phases (Cotter and Miller, 1997). This *bvgr*-intermediate (*Bvgi*) phase is characterised by the lack of expression of *Bvg*-repressed phenotypes, expression of a subset of *Bvg*-activated virulence factors and expression of newly discovered phenotypes that are unique to this phase. Studies using a mutant strain locked in a *Bvg*-intermediate phase showed it to display an increase resistance to nutrient limitation, a tendency for autoagglutination and a reduced ability to colonise the rat respiratory tract. This has led to the proposal that the *Bvgi* phenotype may play a critical role in aerosol transmission between hosts (Cotter and Miller, 1997; Stockbauer *et al.*, 2001). Recent identification and characterisation of BipA, a *Bvg*-intermediate phase protein (Stockbauer *et al.*, 2001), and analysis of its promoter region (Deora *et al.*, 2001) has revealed *BvgA–P* as the regulator of *bipA* transcription. Depending on its intracellular concentration, *BvgA–P* can function as either an activator or repressor of *bipA* transcription and reinforces the complex nature of the *BvgA–S* regulatory system and the subtle responses it is capable of.

### 1.3.5 Phase variation

*B. bronchiseptica* and *B. pertussis* may also undergo an irreversible change to an *bvgr*-repressed phenotype via a process known as phase variation (Peppler and Schrumpf, 1984). Studies of phase variants has revealed that the phase variation process involves frameshift mutations (Stibitz *et al.*, 1989) or deletions of variable length within the *bvgr* locus (Monack *et al.*, 1989). Phase variants of *B. bronchiseptica* occur at a frequency of $10^3$ to $10^6$ with these cells being permanently locked into a *bvgr*-repressed phenotype and are not capable of
switching to a bvg-activated phenotype, even when grown in the presence of modulators. The exact mechanisms involved in phase variation remains to be determined and the relevance to pathogenicity of \textit{B. bronchiseptica} remains unclear.

1.4 Biological role of the bvg locus

The molecular basis for phenotypic modulation has been discussed, however the selective advantage provided by this ability in pathogenesis still remains to be resolved. As more evidence is gathered it has become clear that the coordinated regulation for the production of virulence factors is beneficial for the infection process. In \textit{B. bronchiseptica} it has been demonstrated that the bvg-activated phase is sufficient and a requirement for the respiratory colonisation of rabbits (Cotter and Miller, 1994). However negative regulation exerted by bvg over vrg's has been found to be equally important as expression of virulence determinants during the infection process. Akerley et al. (1995), have shown that ectopic expression of flagella, a bvg-repressed phase specific phenotype, in the bvg-activated phase resulted in a reduced ability for tracheal colonisation of rats. Combined with the knowledge that antibodies against Bvg-repressed phase factors could not be detected, suggests that \textit{B. bronchiseptica} do not switch in the Bvg-repressed phase during infection.

\textit{B. bronchiseptica} is also capable of survival and multiplying under conditions of severe nutrient limitation (Porter et al., 1991; Porter and Wardlaw, 1993). The ability to grow in such harsh environments appears to be enhanced by the expression of the bvg-repressed phenotype (Cotter and Miller, 1994), although what features of the bvg-repressed phase contribute to this are currently unknown. This has lead to the proposal that the Bvg-repressed phase of \textit{B. bronchiseptica} may contribute to transmission by allowing it to survive outside the host in an environmental reservoir. BvgAS regulation may therefore be responsible for mediating the transition from infection of the host to survival outside it.

A role for BvgAS regulation in \textit{B. pertussis} is not as clearly defined. The current knowledge surrounding \textit{B. pertussis} transmission and pathogenesis suggests it is incapable of survival outside the host. This has made it difficult to envisage a role for BvgAS
regulation of virulence factors in this bacterium. The Bvg-repressed phenotypes of *B. pertussis* and *B. bronchiseptica* are very different. *bvgs*-repressed genes of *B. bronchiseptica* appear to be involved in survival outside the host while the functions of *bvgs*-repressed genes of *B. pertussis* are currently unknown. Like *B. bronchiseptica*, the *bvgs*-activated phase of *B. pertussis* is necessary for respiratory infection and ectopic expression of *bvgs*-repressed phenotypes in the *bvgs*-activated phase has detrimental effects on the infection process (Martinez de Tejada et al., 1998). In addition, repression of *bvgs*-repressed genes (which is controlled by the *bvgs*AS locus) is also critical for the infection process. Mutants harbouring a defective *bvgs*R gene were found to be significantly attenuated in their ability to cause disease in a mouse model of infection relative to the wild-type strain (Merkel et al., 1998b). The role of BvgAS regulation may therefore be more subtle in *B. pertussis*. Evidence for modulation to the Bvg1 phase in vivo is seen in sera, collected from children recovering from pertussis infections, that contains antibodies which recognise specific Bvg1 antigens (Martinez de Tejada et al., 1998). Another possible role for *bvgs* regulation is during intracellular invasion. *B. bronchiseptica* strains in a *bvgs*-repressed phase have a significant long term survival advantage over *bvgs*-activated strains when internalised by a variety of eukaryotic cell types (Schipper et al., 1994; Banemann and Gross, 1997). By repression of Bvg-activated factors it is believed that this reduces the damage caused to host cells and may enhance the bacterium’s ability to evade immune responses promoting longevity of infection. This is supported by the discovery that adenylate cyclase expression, after intracellular invasion, is down regulated by both *B. pertussis* (Masure, 1992) and *B. bronchiseptica* (Banemann and Gross, 1997).

### 1.5 Intracellular survival of *Bordetella* species

*Bordetella* species colonise the ciliated epithelium of the respiratory tract and have traditionally been considered non-invasive pathogens. Numerous studies now indicate that these bacteria can invade and survive in a variety eukaryotic cells. These include respiratory epithelial cells (Roberts et al., 1991; Schipper et al., 1994; Bassinet et al., 2000), non-respiratory epithelial cells (Ewanowich et al., 1989b; Lee et al., 1990; Mouallem et al., 1990; Savelkoul et al., 1993; Everest et al., 1996), macrophages
(Saukkonen et al., 1991; Friedman et al., 1992; Masure, 1992; Brockmeier and Register, 2000), polymorphic leukocytes (Steed et al., 1991) and dendritic cells (Guzman et al., 1994a; Guzman et al., 1994b). Internalisation of *Bordetella* spp. is via an endocytic process as a result of bacterial attachment mediated through a specific receptor binding mechanism (Ewanowich et al., 1989a; Guzman et al., 1994b). Intracellular invasion and survival is dependent on the Bvg-activated phase for *B. pertussis* (Ewanowich et al., 1989b) while *B. bronchiseptica* can invade and survive in both the Bvg-activated and Bvg-repressed phase (Schipper et al., 1994; Guzman et al., 1994a; Banemann and Gross, 1997). After internalisation, *B. bronchiseptica* numbers are maintained for several days at which point they start to decline. In contrast *B. pertussis* cell numbers immediately start to decline upon internalisation. *B. bronchiseptica* has been shown to be cytotoxic for HeLa cells (van den Akker, 1997) and mouse macrophages (Banemann and Gross, 1997). This effect is restricted to when the bacteria are located externally and are in a Bvg-activated phase. Intracellular bacteria did not appear to be cytotoxic for eukaryotic cells (Banemann and Gross, 1997; Forde et al., 1998; Brockmeier and Register, 2000). *B. pertussis* is cytotoxic for macrophages by inducing apoptosis and this is believed to be mediated through the actions of adenylate-cyclase haemolysin toxin (Khelef and Guiso, 1995). But as for the case with *B. bronchiseptica*, intracellular bacteria are not cytotoxic for eukaryotic cells (Banemann and Gross, 1997).

Bacteria that are capable of invading and surviving within eukaryotic cells have developed numerous strategies to defend against the antimicrobial activity of these cells. These strategies include escape from the phagocytic vacuole, inhibition of respiratory burst, inhibition of phagosome-lysosome fusion, inhibition of acidification of the phagolysosomal compartment or resistance to low pH and resistance to lysosomal enzymes (Finlay and Falkow, 1997). After internalisation *B. bronchiseptica* is contained within phagosomes, usually singly, that are first surrounded by then fused with lysosomes (Guzman et al., 1994a; Banemann and Gross, 1997; Forde et al., 1998). It appears that *B. bronchiseptica* can either remain in the phagolysosome, escape to the cytoplasm (Schipper et al., 1994), or escape to another unfused compartment within the cell (Guzman et al., 1994a). However, escaping as a mechanism of evading killing seems unlikely as such escape occurs at much
lower levels than that seen for other intracellular bacteria such as *Shigella* and *Listeria*,
which are known to use this mechanism (Goldberg and Sansonetti, 1993; Karuanasagar et al., 1993). *B. pertussis* has demonstrated no ability to escape from the phagolysosomal compartment, but is capable of inhibiting phagosome-lysosome fusion (Steed et al., 1991; Steed et al., 1992). Acidification of phagosomes is one of the factors that contributes to bacterial clearance. It has been demonstrated that *B. bronchiseptica* has a higher acid tolerance than *B. pertussis* and it is believed that this may account for the different survival rates seen for the two species when they are located within phagosomes after internalisation (Schneider et al., 2000; Zimna et al., 2001).

Phagocytosis of *Bordetella* induces a process known as respiratory burst. This involves the production of various toxic oxygen products including superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals within the phagosome (Steed et al., 1992). A number of bacteria are capable of inhibiting respiratory burst as a mechanism of intracellular survival (Kossack et al., 1981; Horwitz, 1983; Riley and Robertson, 1984; Charnetzy and Shuford, 1985; Akporiaye et al., 1990), but *B. pertussis* does not possess this ability (Steed et al., 1992). To detoxify these reactive oxygen metabolites *B. pertussis* and *B. bronchiseptica* produce several oxidoreductases, including an iron-containing superoxide dismutase (SodB) (DeShazer et al., 1994a), a manganese-containing superoxide dismutase (SodA) (Graeff-Wohlleben et al., 1997) and catalase (*katA*) (DeShazer et al., 1994b). Studies with strains containing mutations in these genes have failed to show a decrease in the ability of such mutants to survive within eukaryotic cells or colonise the murine respiratory tract (DeShazer et al., 1994b; Khelef et al., 1996). However, as protection from respiratory burst is most likely achieved through a combination of enzymatic activities, deletions of only one enzyme may not be sufficient to alter the behaviour of the bacterium. Another factor that is involved in inhibiting respiratory burst is acid phosphatase (Baca et al., 1993). *B. bronchiseptica* produces a 40 kDa acid phosphatase that is not expressed by other *Bordetella* species. Mutants that cannot produce acid phosphatase display a reduced invasive ability in eukaryotic cells (Chhatwal et al., 1997). Expression of acid phosphatase in *B. bronchiseptica* is under the control of the *ris* locus. This locus encodes for another two component sensory system whose gene products are upregulated after intracellular
invasion (Jungnitz et al., 1998). Mutants defective in ris expression are more susceptible to phagosomal acidification, more susceptible to oxidative stress and are unable to persist in the lungs of mice (Jungnitz et al., 1998; Zimna et al., 2001).

Another factor which may play a role in intracellular survival and is uniquely expressed by *B. bronchiseptica* is urease. Urease is a multi-subunit enzyme responsible for catalysing the conversion of urea to ammonia and CO$_2$ (Mobley et al., 1995). In *B. bronchiseptica* there are 8 genes associated with urease biosynthesis and these are organised into an operon (McMillan et al., 1998). Urease appears to be a bvg-repressed phenotype, however it fails to be expressed at 30°C, even in the presence of the modulating sulphate anion. This indicates a more complex mechanism of regulation may exist for urease expression in *B. bronchiseptica* (McMillan et al., 1996). Ureases from a variety of bacteria have been implicated in the pathogenesis of a number of diseases including the formation of urinary stones, encephalopathy and gastric ulcers (Mobley and Hausinger, 1989). McMillan et al. (1996) showed that urease producing strains had an advantage over non-producing strains for survival within HeLa cells in the presence of urea. It has been suggested that this benefit may be due to urease breaking down urea to produce ammonia which can then be used as an additional nitrogen source. A defence strategy involving urease may be operating in the phagolysosome. The degradation of urea by urease may increase the pH of the phagolysosome to levels that impair lysosomal enzyme activity, thereby allowing the bacterium to survive in a modified cellular compartment. This is supported by the fact that urease is only expressed at 37°C, indicating a possible *in vivo* role for this enzyme.

1.6 *Bvg*-activated genes (*vags*)

*Bordetella* species produce an arsenal of *vag* products that play a specific role in the pathogenesis of these organisms. These include adhesins, which the bacterium uses for adherence to a variety of hosts cells and tissues, and toxins that are responsible for the pathological effects of the disease and modulation of the immune response. Much of the research characterising these factors has focused on *B. bronchiseptica* and *B. pertussis* which share the majority of known *vags*. The most notable exception being pertussis toxin
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which is only expressed by *B. pertussis*. Both *B. parapertussis* and *B. bronchiseptica* possess the pertussis toxin operon but in these organisms it remains transcriptionally silent (Arico and Rappuoli, 1987).

1.6.1 Filamentous hemagglutinin

Filamentous hemagglutinin (FHA) is one of the major adhesins of *Bordetella* expressed in the *bvg*-activated phase (Stibitz et al., 1988). FHA is a large, hairpin shaped molecule that is highly immunogenic and it is this fact that has seen this antigen included as a primary component of acellular pertussis vaccines. This protein is initially produced as a 367 kDa precursor which is modified at its N-terminus (Jacob-Dubuisson et al., 1996) and cleaved at the C-terminus (Renauld-Mongenie et al., 1996) to produce the mature 220 kDa FHA protein. FHA can be associated with the cell surface or be secreted via an accessory protein within the outer membrane, FhaC (Jacob-Dubuisson et al., 1999).

FHA possesses at least 3 different binding domains responsible for adherence to a variety of cell types. The Arg-Gly-Asp (RGD) triplet, situated in the middle of FHA, promotes attachment to monocytes and macrophages (Relman et al., 1989). FHA also mediates attachment to ciliated respiratory epithelial cells and macrophages via a carbohydrate recognition domain (Saukkonen et al., 1991; Prasad et al., 1993), and to non-ciliated epithelial cells via a heparin binding domain situated at the amino terminal end of FHA (Hannah et al., 1994) which is also responsible for the FHA-mediated hemagglutination of *B. pertussis* (Menozzi et al., 1994).

An *in vivo* role for FHA has been difficult to determine. Much of the work done with *B. pertussis* in various animal models have been conflicting and appears to be hindered by lack of a natural animal host in which to conduct these types of experiments. Using FHA mutants of *B. bronchiseptica*, one with a deleted *fhaB* gene and the other expressing FHA ectopically in the *Bvg*-repressed phase, it has been demonstrated that FHA is both necessary and sufficient for adherence to rat lung epithelial cells *in vitro* (Cotter et al., 1998). Using a rat model of respiratory infection, it was also determined that FHA is a
requirement for tracheal colonisation, but is not sufficient alone when it is expressed without other virulence factors in the Bvg-repressed phase (Cotter et al., 1998). FHA was not required to establish tracheal colonisation in anaesthetised animals, suggesting a possible role for FHA may be to overcome the clearance activity of the mucociliary escalator. Mutant strains unable to express FHA failed to be recovered from the trachea of experimentally infected rats. This result suggests FHA may contribute to long-term tracheal persistence (Cotter et al., 1998). FHA may also contribute to long term persistance through a number of immunomodulatory functions that have been put forward for this protein. Mechanisms that could be responsible for these functions may result from the adherence of Bordetella to macrophages and other leucocytes which is mediated by FHA binding to specific receptor sites and could directly disrupt signal transduction systems of these cells or may allow for more efficient delivery of toxins that may kill these phagocytic cells (Saukkonen et al., 1991; Steed et al., 1991). FHA has been shown to promote apoptosis in macrophages and human bronchial epithelial cells and could therefore interfere with immune responses by inducing cell death in specific immune defence cells (Abramson et al., 2001). Further, the interaction of purified FHA with receptors on J774 murine macrophages results in suppression of the proinflammatory cytokine IL-12 which could delay development of Th1 immune responses (McGuirk and Mills, 2000).

1.6.2 Fimbriae

Fimbriae are long, filamentous membrane-associated structures that extend from the bacterial surface and are believed to have a possible role in mediating binding of the bacterium to the upper respiratory surface. There are two predominant fimbrial phenotypes of Bordetella, Fim2 and Fim3 which are encoded by two unlinked loci, fim2 and fim3 (Livey et al., 1987; Mooi et al., 1987). The major subunits encoded by these genes are stacked to form the long filamentous structure with a common minor subunit, encoded by fimD, located at the tip (Willems et al., 1993). The gene fimD is organised into the fimbrial biogenesis gene cluster with fimB and fimC (Willems et al., 1992). The high homology displayed by FimB and FimC with those proteins essential for fimbrial biogenesis in E. coli (Pap), suggests these proteins may serve to transport and anchor fimbriae in the cell
membrane (Locht et al., 1992). Located at the 5' end of the gene cluster is fimA. In B. pertussis this gene is truncated and silent however, in B. bronchiseptica fimA is intact and capable of expressing a new fimbrial subtype, FimA (Willems et al., 1992; Boschwitz et al., 1997). Two other loci capable of encoding major fimbrial subunits have also been identified. These include fimX, which is expressed at very low levels, and fimN which appears to be B. bronchiseptica specific (Riboli et al., 1991; Kania et al., 2000).

The expression of fimbrial genes in Bordetella is positively regulated by the bvg locus. Different strains are capable of expressing different fimbrial serotypes and this is dependent on the level of transcription of the major fimbrial subunit genes. Serotypes may be altered by a process know as fimbrial phase variation and involves transcriptional regulation of individual fimbrial major subunit genes. Promoter regions of these genes contain a long stretch of cytosine residues located between the putative -10 box and the BvgA binding site. Alteration in the number of cytosine residues can occur via slip-strand mispairing which in turn changes the distance between the -10 box and the BvgA binding site thereby affecting promoter activity. Genes with longer C-stretches have been shown to have enhanced gene expression (Willems et al., 1990; Kania et al., 2000). Bacteria can therefore express any combination of the major fimbrial subunit genes and may use this mechanism to escape immune recognition.

Fimbriae are believed to mediate binding of Bordetella to the respiratory epithelium via the major fimbrial subunits. These subunits have been shown to contain heparin binding domains and have an affinity for sulphated sugars (Geuijen et al., 1996). Fimbriae also mediate the binding of Bordetella to monocytes through FimD binding to the very late antigen-5 present on their surface (Hazenbos et al., 1995). Mutant strains of B. pertussis defective in fimbriae production display a reduced ability to multiply in the respiratory system of mice (Mooi et al., 1992; Geuijen et al., 1997). Using B. bronchiseptica mutants, Mattoo et al. (2000) have shown that fimbriae enhance the efficiency of establishment of tracheal colonisation and are essential to tracheal persistence in the rat respiratory system. In addition to this observation, fimbriae were shown to specifically enhance the induction of Th2 immunity (Mattoo et al., 2000).
1.6.3 Pertactin

Mature pertactin is a 68 kDa outer membrane protein and has been shown to mediate adherence to a variety of eukaryotic cells *in vitro* (Everest *et al.*, 1996). The pertactin protein contains an Arg-Gly-Asp (RGD) motif as well as a number of proline-rich and leucine-rich repeats which are believed to play a role in eukaryotic cell binding (Leininger *et al.*, 1991; Emsley *et al.*, 1994). Pertactin is transcribed from the *prn* gene as a 93.5 kDa precursor (Charles *et al.*, 1988). Processing of this precursor includes removal of the 34 amino acid N-terminal signal peptide (Makoff *et al.*, 1990) and cleavage of a 30 kDa polypeptide (p.30) from the C-terminal (Capiau *et al.*, 1990). Sequences of *prn* from *B. bronchiseptica*, *B. pertussis* and *B. parapertussis* show the C-terminus region to be highly conserved, suggesting a functional relevance and as the C-terminus is detected in outer membrane preparations it is believed to be involved in export of pertactin to the outer membrane (Charles *et al.*, 1994).

Mutant strains of *B. pertussis* deficient in pertactin production displayed no difference from the wild-type in its ability to invade HEp2 cells or colonise the respiratory tracts of mice (Roberts *et al.*, 1991). Although its role in pathogenesis remains unclear, it is a strong immunoprotective antigen that has been used in a variety of subunit and live vaccines (Shahin *et al.*, 1990; Strugnell *et al.*, 1992; Roberts *et al.*, 1993; Greco *et al.*, 1996).

1.6.4 Serum resistance locus

The *brk* locus of *B. pertussis* has been shown to confer resistance to killing by complement and may also play a role in adherence to eukaryotic cells (Fernandez and Weiss, 1994). BrkA, the product of the *brk* locus, is homologous to pertactin (Li *et al.*, 1992) and tracheal colonisation factor, a virulence determinant that is uniquely expressed by *B. pertussis* (Finn and Stevens, 1995). Like pertactin and tracheal colonisation factor, BrkA is produced as a 103 kDa precursor that is processed to a 73 kDa N-terminal domain and a 30 kDa C-terminal domain that remains in the outer membrane and may play a role in the export of
the N-terminal domain to the cell surface (Fernandez and Weiss, 1994). Unlike *B. pertussis*, BrkA does not confer resistance to complement in *B. bronchiseptica* (Rambow et al., 1998). *B. bronchiseptica* has previously been shown to be naturally more resistant to complement than *B. pertussis* (Byrd et al., 1991), and it is believed that differences in the two species LPS structures may contribute to this observation.

### 1.6.5 Lipopolysaccharide (LPS)

LPS structures expressed by Gram-negative bacteria are highly toxic and immunogenic molecules that constitute a major component of the cell membrane. LPS is believed to contribute to pathogenicity in a number of ways including mediation of adherence to host cells, antigenic variation and molecular mimicry (Preston et al., 1996). The general structure of LPS from Gram-negative bacteria consists of 3 domains. Lipid A, the membrane component; the core, a non-repeating oligosaccharide; and the O-antigen consisting of a distinct repeating oligosaccharide. *Bordetella* LPS vary among species. *B. pertussis* LPS lacks a repetitive O-antigen and resolves as two distinct bands when separated by sodium dodecyl sulphate-polyacrylamide electrophoresis (Peppler, 1984). The faster migrating band B, consists of lipid A and a core oligosaccharide, while addition of a trisaccharide to band B creates the larger LPS molecule that is referred to as band A (Caroff et al., 1990). *B. bronchiseptica* produces LPS that is composed of band A and band B as well as a form containing an O-antigen structure. *B. parapertussis* contains LPS that lacks band A, has a modified band B and contains the same O-antigen structure as *B. bronchiseptica* (Di Fabio et al., 1992).

Temperature has been found to be a modulator of LPS expression in *Bordetella*. In *B. pertussis* this form of regulation is independent of the *bv* locus however, in some strains of *B. bronchiseptica* *bv* does appear to regulate LPS biosynthesis (van den Akker, 1998). LPS expression among *Bordetella* spp. is variable and appears to be host species specific. This has been demonstrated well in *B. bronchiseptica* which is capable of infecting a wide variety of mammalian hosts. Isolates of *B. bronchiseptica* from dogs, pigs rabbits and humans have all been shown to display different LPS profiles (Le Blay et al., 1997;
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Gueirard et al., 1998; van den Akker, 1998). Biosynthesis of full-length LPS is essential for the expression of full virulence in mice. Mutants of B. pertussis, B. parapertussis and B. bronchiseptica which are only capable of producing band B LPS were all shown to be defective when compared to the parental strain, in colonisation of the respiratory tract of BALB/c mice (Harvill et al., 2000). It was also hypothesised that the different distal LPS structures expressed by the three species appear to play different roles during respiratory infection. For B. bronchiseptica the distal LPS structures are important for resisting clearance by the adaptive immune response indicating that they contribute to the persistent infections that are a characteristic of the chronic diseases caused by this organism (Harvill et al., 2000). The LPS of B. bronchiseptica has also been shown to be important for resistance to antimicrobial peptides. Many mucosal surfaces possess this type of innate immunity which can also be encountered within professional phagocytes. It is believed that the highly charged O-antigen of B. bronchiseptica LPS shields the negative charges present on the membrane and prevents attack by the peptides (Banemann et al., 1998). Resistance to antimicrobial peptides was also shown to be affected by the bvg locus, with a Bvg-repressed phase locked strain of B. bronchiseptica showing a significant increase in susceptibility (Banemann et al., 1998).

1.6.6 Dermonecrotic toxin

Dermonecrotic toxin (DNT) is a 140 kDa, heat-labile, cytoplasmic protein which is produced by all Bordetella species and is regulated by the bvg locus (Cowell et al., 1979; Nakai et al., 1985; Horiguchi et al., 1989; Zhang and Sekura, 1991). DNT produces characteristic skin lesions when injected intradermally into test animals such as mice, rabbits and guinea pigs and is lethal at low doses for mice when injected intravenously (Bordet and Gengou, 1909; Iida and Okonogi, 1971; Livey and Wardlaw, 1984; Horiguchi et al., 1992).

Using an osteoblastic cell line, MC3T3-E1, purified DNT from B. bronchiseptica was shown to induce morphological changes, stimulate DNA replication and inhibit differentiation into osteoblasts (Horiguchi et al., 1991; Horiguchi et al., 1993). These
effects are achieved by DNT mediated activation of the GTP binding protein RhoA via deamination of Gln-63 which is converted into Glu (Horiguchi et al., 1995; Horiguchi et al., 1997). This results in tyrosine phosphorylation of focal adhesion kinase and paxillin with activation of these two molecules leading to alterations in the actin cytoskeleton and assembly of focal adhesins (Lacerda et al., 1997).

The effects of DNT on Bordetella pathogenesis is not clear. Studies have suggested that DNT production may not be required for virulence in the murine model of infection (Weiss and Goodwin, 1989; Gueirard and Guiso, 1993). However, upon intravenous injection of DNT, mice displayed atrophy of the spleen and suppressed in vivo antibody responses against T-cell dependent and T-cell independent antigens (Horiguchi et al., 1992). A more recent study has shown that a strain genetically modified to abolish DNT production was similar in virulence for mice when compared to the parental strain. However, this strain was shown to produce far less turbinate atrophy and appeared to alter the immune function of infected animals (Magyar et al., 2000). Pigs experimentally infected with strains of B. bronchiseptica that were unable to produce DNT have also been shown to exhibit decreased turbinate atrophy and bronchopneumonia when compared to animals infected with the parental strains (Roop et al., 1987; Magyar et al., 1988; Brockmeier et al., 2002). This suggests DNT does play some role during the infection process.

1.6.7 Tracheal Cytotoxin

Tracheal Cytotoxin (TCT) is a 921 Da disaccharide tetrapeptide which corresponds to a monomer of peptidoglycan that all Gram-negative bacteria produce in their polymeric cell wall (Cookson et al., 1989b). Only Bordetella spp. and Neisseria gonorrhoeae have been shown to secrete these fragments into the environment during exponential growth (Sinha and Rosenthal, 1980; Rosenthal et al., 1987). Other Gram-negative bacteria recycle this peptidoglycan fragment and transport it back into the cytoplasm (Park, 1995).

TCT belongs to the class of molecules known as muramyl peptides. These peptides have a wide variety of biological activities including cytotoxicity, adjuvanticity and enhancement
of cell mediated immunity (Luker et al., 1995). Purified TCT from *B. pertussis* culture supernatant produces the same specific damage to ciliated cells that is seen during a *B. pertussis* infection (Cookson et al., 1989a). *In vitro* studies using cultured hamster tracheal epithelial (HTE) cells and human nasal epithelial biopsies have shown the damage caused by TCT to be specific to the ciliated cell population and includes mitochondrial blebbing, disruption of tight junctions and extrusion of ciliated cells (Goldman and Herwaldt, 1985; Wilson et al., 1991). The cytopathic effects observed are believed to be due to a TCT-dependent increase in nitric oxide (NO) (Heiss et al., 1993a; Heiss et al., 1994). TCT has also been shown to stimulate interlukin-1 (IL-1) production in HTE cells which can activate expression of inducible nitric oxide synthase and in turn leads to the production of high levels of NO (Heiss et al., 1993b). NO can inhibit mitochondrial function and DNA synthesis within the epithelial cell itself as well as nearby host cells (Heiss et al., 1994). Recent evidence suggests that TCT acts synergistically with endotoxin to induce the production of NO and that NO is not produced in the ciliated cells of the respiratory tract but in adjacent secretory cells (Flak and Goldman, 1999). TCT has also been shown to be cytotoxic for neutrophils which are an important defence mechanism within the respiratory tract through the phagocytosis and killing of bacteria (Cundell et al., 1994).

### 1.6.8 Adenylate cyclase toxin / haemolysin (CyaA)

Adenylate cyclase toxin / haemolysin (CyaA) is a secreted, bifunctional protein of 1706 amino acids (Glaser et al., 1988). The adenylate cyclase activity is located within the N-terminal catalytic domain of the first 400 amino acids (Ladant et al., 1989). The remaining 1306 amino acid C-terminal domain is required for binding of the toxin and delivery of the catalytic domain into the cytosol of eukaryotic cells (Sakamoto et al., 1992). This C-terminal domain also possess a weak haemolytic activity and is homologous to the *E. coli* haemolysin (HlyA) as well as other members of the RTX (repeat in toxin) family of calcium dependent, pore-forming cytotoxins. These toxins contain a series of nine amino acid repeats, rich in glycine and aspartic acid residues that are thought to play an integral role in calcium binding (Coote, 1992; Rose et al., 1995).
CyaA toxin is encoded by the cyaA gene which is part of an operon that contains cyaB, cyaD and cyaE which are involved in toxin secretion (Glaser et al., 1988). Located upstream from cyaA is cyaC, which is transcribed in the opposite direction and is involved in the activation of CyaA (Barry et al., 1991). CyaA is synthesized as a protoxin that is activated through the actions of CyaC catalysing the palmitoylation of an internal lysine residue (Lys-983) (Hackett et al., 1994; Basar et al., 2001). Upstream of cyaA there is a site for binding by phosphorylated BvgA (Karimova et al., 1996).

CyaA binds to target cells via the αMβ2 integrin (CD11b/CD18) which is mostly found on neutrophils, macrophages, dendritic and natural killer cells (Guermonprez et al., 2001). The catalytic domain is then internalised into the cell cytosol via a calcium and temperature dependent mechanism that also involves the plasma membrane potential (Rogel and Hanski, 1992; Karimova et al., 1998). Inside the cell CyaA is activated by calmodulin and catalyses the production of cAMP from ATP which in neutrophils and macrophages leads to inhibition of chemotaxis, phagocytic function and superoxide generation (Confer and Eaton, 1982; Hanski and Farfel, 1985). CyaA has also been shown to induce macrophage apoptosis in vitro (Khelef et al., 1993; Khelef and Guiso, 1995; Bachelet et al., 2002) and inhibit phagocytosis of B. pertussis by human neutrophils (Weingart and Weiss, 2000). In vivo mutants of B. pertussis deficient in CyaA production exhibit a decreased ability to cause lethal infections (Goodwin and Weiss, 1990), fail to promote an influx of inflammatory cells (Khelef et al., 1994) and induce less apoptosis in neutrophils and macrophages in the lungs of infected mice when compared to infections by the parental strain (Gueirard et al., 1998). Using a CyaA deficient mutant of B. bronchiseptica, Harvill et al. (1999) demonstrated using a variety of mouse strains that were either immunocompetent, T and B cell deficient or neutropenic, that neutrophils are essential to the early defence against B. bronchiseptica infection. Taken together these data suggest that CyaA targets phagocytes such as macrophages and neutrophils thereby disrupting the early innate antibacterial immune response and favouring the development of adaptive immunity that controls later stages of infection.
1.6.9 Type III secretion

A type III secretion system has recently been identified in *B. bronchiseptica* based on homology to the type III secretion system of *Yersina* spp. (*yscN*) (Woestyn *et al.*, 1994). Type III secretion systems allow Gram-negative bacteria to translocate effector molecules into the plasma membrane or cytoplasm of eukaryotic cells through a conserved needle-like injection apparatus. These molecules alter a variety of eukaryotic signal transduction pathways which effects bacterial-host interaction (Lee, 1997).

Expression of the type III secretion system in *B. bronchiseptica* is regulated by *bvg* (Yuk *et al.*, 1998). Mutants harbouring a deletion in *bscN* (encoding an ATPase that is believed to provide energy for the secretion of effector proteins) displayed a decrease in the secretion of several proteins, were unable to dephosphorylate specific host proteins and were less cytotoxic for L2 epithelial cells (Yuk *et al.*, 1998) and J744A1 mouse macrophage-like cells (Yuk *et al.*, 2000). A functional type III secretion system was also seen to inhibit NF-κB translocation to the nucleus. NF-κB is a family of transcription factors involved in the regulation of host immunostimulatory responses which includes pro-inflammatory genes and therefore may provide a mechanism for down regulating recruitment of inflammatory cells (Yuk *et al.*, 2000).

In vivo, the type III secretion system of *B. bronchiseptica* has been shown to be required for persistent tracheal colonisation in mice and rats (Yuk *et al.*, 1998; Yuk *et al.*, 2000). Mice infected with a mutant strain deficient in type III secretion produced higher anti-*Bordetella* antibodies than animals infected with the wild-type strain and were hypervirulent for mice lacking functional T and B cells (SCID phenotype). *B. bronchiseptica* was also shown to induce apoptosis of infiltrating inflammatory cells in the lungs which was dependent on a functional type III secretion system (Yuk *et al.*, 2000). These data suggest the type III secretion system of *B. bronchiseptica* may facilitate the evasion and modulation of inducible host immune responses that function in the lower respiratory tract and may therefore facilitate the chronic infections cause by *B. bronchiseptica*.
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1.7 Bvg-repressed genes (vrgs)

Bvg-repressed genes (vrgs) are expressed at times when BvgA is dephosphorylated. In *B. pertussis* 27 vrgs have been identified but the function of these proteins is currently unknown (Beattie et al., 1990; Stenson and Peppier, 1995). The genes involved in flagella biosynthesis and siderophore production are the most well studied vrgs of *B. bronchiseptica*. As mentioned earlier the bvg-repressed phase of *B. bronchiseptica* has been implicated in survival in the external environment (see Biological role of the bvg locus) and intracellularly within host cells (see Intracellular survival of *Bordetella* species). Further investigation will help to identify and elucidate the role vrgs play in *B. bronchiseptica* pathogenesis.

1.7.1 Siderophore

Iron is an essential nutrient for the majority of living organisms as it is a co-factor for many biological oxidation-reduction reactions (Neilands, 1981). In mammals, free Fe concentrations are too low to support microbial growth as the majority is sequestered intracellularly and extracellular iron is bound to lactoferin and transferrin (Weinberg, 1978). Pathogenic bacteria have evolved two ways to scavenge iron. Most secret small molecules called siderophores which due to their high affinity for Fe, can bind it directly from lactoferin and transferrin. The siderophore can then bind to specific siderophore receptors on the bacterial surface where the Fe is transported across the bacterial membrane. Alternatively bacteria can express exogenous siderophore receptors that can bind other Fe-protein complexes such as enterobactin, heme, hemoglobin, ferichrome and desferal and use these as Fe sources (Beall and Hoenes, 1997). Both strategies are employed by *B. bronchiseptica* and *B. pertussis* to acquire iron (Menozzi et al., 1991).

*B. bronchiseptica* produces and secretes the siderophore alcaligin, a macrocyclic dihydroxamate Fe-chelating compound (Moore et al., 1995). Alcaligin is encoded by the *alcABCDER* operon (Kang et al., 1996). The *alcR* locus encodes an AraC type activator that is essential for expression of the *alc* operon and the alcaligin receptor gene *fauA* (Beaumont et al., 1998; Brickman and Armstrong, 1999). AlcR-mediated transcriptional
activation is extremely sensitive to alcaligin concentrations which acts as an inducer (Brickman et al., 2001). The alc operon has also been shown to be regulated by the Fur (ferric iron uptake regulation) protein which responds to free Fe concentrations (Brickman and Armstrong, 1995). Fur represses transcription of the alc operon by binding to promoter sequences upstream of alcA and upstream of alcR (Giardina et al., 1997; Kang and Armstrong, 1998). Alcaligin expression has also been shown to be repressed by bvg however this mode of repression appears to be influenced by the host species. Giardina et al. (1995), found that 94% of pig isolates were bvg-independent whereas 78% of isolates from other mammals displayed bvg-repression of alcaligin biosynthesis. This suggests that alcaligin expression may be important for colonisation of the porcine respiratory tract but not critical for other hosts. As human Bordetella strains (i.e. B. pertussis) also produce alcaligin independently of bvg it was suggested that it may also be essential for colonisation of human hosts (Giardina et al., 1995). Strains of B. bronchiseptica and B. pertussis containing mutations in alcR were shown to be defective in alcaligin biosynthesis but proved to be unhindered in their ability to colonise the mouse respiratory tract (Pradel et al., 1998). Register et al. (2001), using a alcaligin mutant containing a mutated alcA showed that siderophore production is required for maximum virulence in swine, although this gene product is not essential for colonisation.

1.7.2 Motility

When grown under modulating conditions, B. bronchiseptica expresses a peritrichous flagella and exhibits a motile phenotype (Akerley et al., 1992). Biosynthesis of flagella in B. bronchiseptica is controlled by frl (flagella regulatory locus) consisting of the frlA and frlB genes. The frlAB locus encodes an activator of flagella genes and expression is directly controlled by BvgAS at the transcriptional level (Akerley and Miller, 1993). Mutating the frl locus results in a non-motile phenotype and these mutants were found to colonise the rat trachea equally as the wild-type B. bronchiseptica. Additionally, a recombinant strain of B. bronchiseptica that expresses flagella in the Bvg-activated growth phase displayed a reduced ability to colonise rat trachea when compared to the wild-type strain (Akerley et al., 1995). Taken together, as well as the fact that reactive antibodies
towards flagella cannot be detected in the sera of rats infected with wild-type *B. bronchiseptica*, these data suggest that flagella expression is not required during infection and inappropriate expression may actually be detrimental (Akerley *et al*., 1995).

### 1.8 Vaccines against *B. bronchiseptica*

*B. bronchiseptica* is capable of infecting a wide range of mammalian species. Of particular economic importance is the infection of dogs and pigs. *B. bronchiseptica* is the causative agent of infectious tracheobronchitis (i.e. kennel cough) in dogs and bordetellosis in pigs which in the presence of other factors can lead to atrophic rhinitis. With disease often endemic in confinement-reared swine and high density kennels, treatment involves a combination environmental management practices, chemotherapeutic agents and vaccination programs. There are numerous vaccines available for the prevention of *B. bronchiseptica* associated diseases in both dogs and pigs. Vaccine preparations can be categorised into three main classes which consist of killed whole-cell bacterins, subunit vaccines containing purified antigenic extracts and avirulent live cultures. However, data from the United States where vaccination rates are up to 42%, suggests only 10.5% of swine herds are free from atrophic rhinitis (Register *et al*., 2001). Although swine producers in both the United States and Europe continue to use *B. bronchiseptica* vaccines, their effectiveness remains questionable (Backstrom, 1999). New vaccines for the prevention of *B. bronchiseptica* infections with more efficacious properties are the focus of much research around the world.

#### 1.8.1 Whole-cell bacterins of *B. bronchiseptica*

Bacterin vaccine preparations consist of chemically or heat-inactivated whole cells of *B. bronchiseptica* and are administered either intramuscularly or subcutaneously. Adjuvanted bacterin vaccines have been commercially available since the 1970’s and have proven to reduce the prevalence of clinical atrophic rhinitis in swine although do not eliminate the condition (Giles, 1986). Matherne *et al*. (1987), has also shown that a commercially available porcine bacterin reduced the severity of the disease in guinea pigs but failed to
prevent tracheal colonisation. This lack of protection against colonisation is believed to be due to the vaccines inability to induce a secretory IgA response which play an important protective role at mucosal surfaces. Bacterins consisting of killed whole cells of P. multocida and B. bronchiseptica have also been proven to reduce the prevalence of turbinate atrophy, and progeny from vaccinated sows grow significantly faster (Kabay et al., 1992). Vaccination of sows induces passive colostral protection to B. bronchiseptica infections in the serum of sucking piglets, which represents an effective means of controlling the disease (Giles, 1986).

In dogs, bacterin vaccines have proven to be less effective. These vaccines generate a high degree of systemic immunity which is important for the prevention of the disease but may not prevent infection (Keil and Fenwick, 1998). In some cases the use of bacterin vaccines has resulted in swelling and abscess formation at the site of injection and has even resulted in lameness in a significant number of animals (Goodnow, 1980). Colostral antibodies passed on to unvaccinated puppies from bacterin vaccinated animals has been shown to inhibit development of systemic immunity, necessitating multiple vaccinations (Keil and Fenwick, 1998).

1.8.2 Live avirulent vaccines
Shimizu (1978), isolated a live, avirulent, temperature sensitive mutant of B. bronchiseptica which when delivered intranasally to guinea pigs provided protection from B. bronchiseptica induced pneumonia. Intranasal immunisation of dogs with a live, avirulent strain resulted in a 95% reduction in the occurrence of clinical tracheobronchitis after challenge with wild-type B. bronchiseptica. Vaccinated animals also showed a significant reduction in the number of B. bronchiseptica cells isolated from nasal secretions when compared to non-vaccinated animals (Shade and Goodnow, 1979). Bey et al. (1981), demonstrated using the same attenuated strain of B. bronchiseptica that a secretory IgA response was observed upon intranasal immunisation of dogs. A correlation between the occurrence of IgA in the nasal secretions of vaccinated dogs and protection against disease was observed. A number of live, avirulent vaccines are now commercially available for
intranasal administration in dogs. The majority of these preparations not only contain an attenuated strain of *B. bronchiseptica* but also contain attenuated strains of canine parainfluenza virus and canine adenovirus which are the viral factors associated with the early stages of infectious tracheobronchitis in dogs. These vaccines are not affected by maternal antibodies and have been shown to induce the production of systemic as well as secretory IgA antibodies and are therefore considered to be the most appropriate vaccine to use in young dogs (Keil and Fenwick, 1998). Information concerned with defining the nature of the attenuation for these strains is not available and in some cases has not been determined. Due to the undefined nature of these vaccine strains it is not known if or how likely reversion to full virulence will occur in these strains.

### 1.8.3 Subunit vaccines

Subunit vaccines consist of antigenic extracts or purified protein components of *B. bronchiseptica*. Vaccines prepared in this manner minimise contamination with LPS that may be contained in bacterin formulations and therefore produce less side effects. Side effects associated with whole-cell *B. pertussis* immunisations saw a decline in public confidence and a decrease in the use of these vaccines. This lead to the development of several new acellular vaccines for the control of whooping cough. A subcellular *B. bronchiseptica* vaccine containing a cell wall extract has been shown to induce significantly higher levels of protection from turbinate damage than a whole-cell bacterin (Goodnow, 1980). In dogs, there are a number of subunit vaccines commercially available for use. Vaccinated dogs will clear a *B. bronchiseptica* challenge from the nasal passages more rapidly than control animals and show a decreased rate of *B. bronchiseptica* shedding (Keil and Fenwick, 1998). In young dogs colostral antibodies can inhibit the immunologic response and therefore multiple doses must be given.

Immunisation of numerous host species with purified proteins from *B. bronchiseptica* have been shown to induce high levels of protection when subsequently challenged with the wild-type organism. Purified FHA from *B. bronchiseptica* has been used to immunise pigs. Colonisation by wild-type *B. bronchiseptica* in vaccinated animals was shown to be
significantly lower than that seen in unvaccinated controls and pigs vaccinated with a whole-cell vaccine (Hansen et al., 1983). Immunisation of pregnant sows with purified pertactin has also been shown to induce protection in the resulting piglets when subsequently challenged with virulent *B. bronchiseptica* (Kobisch and Novotny, 1990). Gueirard and Guiso (1993), have demonstrated that immunisation of mice with purified CyaA from *B. bronchiseptica* protected mice from colonisation with the wild-type organism as well as a whole-cell vaccine preparation. Using a fimbrial antigen based subunit vaccine, Jacobs et al. (1993) found that vaccinated kittens were protected and showed no signs of respiratory disease after aerosol challenge with *B. bronchiseptica* when compared to non-vaccinated controls. Vaccinated animals were also shown to clear the challenge bacteria more quickly than controls. Although these results suggest that purified component vaccines can provide effective protective immunity, the costs involved in the purification and manufacturing procedure may ultimately prove to costly for widespread use of subunit vaccines in veterinary situations.

1.9 Live attenuated vaccines

1.9.1 Advantages of live attenuated vaccines

With research in pathogenicity of microorganisms and vaccine development, there has been a significant reduction in the prevalence of many infectious diseases of man and animals. Yet, infectious diseases still represent a considerable economic burden to both the health care system and animal producers world wide. Vaccination has proved to be the most cost effective measure to combat infectious agents. However, there are many infectious diseases for which effective vaccines are not yet available and numerous vaccines currently in use which could benefit from the application of new vaccine strategies aimed at improving efficacy, safety, stability, ease of administration and cost effectiveness.

Live attenuated bacteria have been used successfully for active immunisation and have stimulated much interest as a novel strategy for the generation of new vaccines and the improvement of vaccines currently in use that are not completely satisfactory. The
majority of infectious diseases are initiated by pathogens entering a host via mucosal surfaces and it is therefore beneficial to induce local mucosal immunity through vaccination to block infection and prevent disease. Most vaccines currently available for use are administered parentally which results in strong systemic responses but generally induce poor mucosal immunity. Attenuated bacteria can act as live mucosal immunogens by establishing a limited infection that mimics the natural infection process without causing disease. This leads to activation of the humoral, cellular and mucosal arms of the immune system (Drabner and Guzman, 2001; Galen and Levine, 2001). All mucosal surfaces are linked through an integrated lymphoid system termed mucosa-associated lymphoid tissue (MALT) (McDermott and Bienenstock, 1979). The MALT is composed of lymphocytes grouped into follicles covered by an epithelial cell layer containing specialised M-cells. These cells take up foreign molecules allowing them to be presented to T-cells and macrophages and thereby recognised as antigens (Locht, 2000). Lymphocytes activated in the MALT can enter the lymphatic and then the systemic circulation which is used to transport these cells to distal mucosal sites, seeding them for specific antigenic reactivity (Springer, 1994). B-lymphocytes at these sites also produce secretory IgA which plays a critical role in the control of microbial colonisation at these mucosal surfaces (Mestecky, 1987).

With the development of recombinant DNA technology, it is now possible to create safe, genetically stable mutants specifically for vaccine purposes. Attenuated bacteria with genetically defined mutations confers an increased safety profile that enables a greater acceptance over live avirulent strains in which the nature of the attenuation is not known and therefore is perceived to have a greater potential to revert to full virulence. The most characterised pathogen to be genetically attenuated is Salmonella, which when delivered orally can invade the intestinal mucosa through M-cells and infect professional antigen presenting cells. This leads to the stimulation of bacteria-specific cell-mediated and humoral immunity. A large number of attenuated Salmonella strains have been produced and tested in animal models to assess the level of attenuation and immunogenicity displayed by the particular strain (see later section 1.9.3 Strategies for rational attenuation).
1.9.2 Intranasal administration of attenuated bacteria

More recently the administration of immunogens to the respiratory tract have been used and found to produce stronger immune responses than those obtained after oral administration of the same vaccine. The respiratory route has a number of advantages over oral delivery: 1. Antigens avoid the acidic environment of the stomach; 2. Immune responses elicited are higher after intranasal vaccination; 3. In addition to local mucosal immunity, systemic immune responses are more easily achieved by intranasal delivery; 4. The respiratory tract contains less commensal microorganisms than the gut and therefore presents an environment with less ecological competition (Locht, 2000). A number of attenuated pathogens have been used as intranasal vaccinations. These include Bacille Calmette-Guérin (BCG), B. pertussis, B. bronchiseptica, P. multocida and Actinobacillus pleuropneumoniae (see Table 1.2). Attenuated Salmonella vectors have also been used for the delivery of a variety of heterologous antigen via intranasal administration. Intranasal vaccinations were found to produce higher antigen-specific antibody titres when compared to other delivery via other mucosal routes (Hopkins et al., 1995), induce immunity at distal mucosal sites (Nardelli-Haefliger et al., 1997), and produce increased serum responses upon repeated intranasal immunisations (Barry et al., 1996).

1.9.3 Strategies for rational attenuation

Studies performed by Bacon et al., (1950) using Salmonella demonstrated that certain auxotrophies (i.e. dependence on purines and other aromatic compounds) could attenuate pathogens. Early attempts at attenuating Salmonella involved chemical mutagenesis produced attenuated strains that were genetically undefined. However, this work did lead to the production of the first licensed attenuated bacterial vaccine for human use, Ty21a (Germanier and Furer, 1975). As individual genes were identified as targets for attenuating mutations, this approach has since been replaced by more defined genetic techniques. A number of strategies have been employed to rationally attenuate bacterial pathogens. These can be broadly categorised into two main groups; those that involve mutations within genes encoding virulence determinants and those that involve mutations within genes that are involved in the biosynthesis of essential metabolic requirements.
Table 1.2 Attenuated pathogens used as intranasal vaccines.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Attenuation</th>
<th>Protection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCG</td>
<td>Unknown</td>
<td>In humans</td>
<td>(Rosenthal et al., 1968)</td>
</tr>
<tr>
<td><em>B. pertussis</em></td>
<td><em>aroA</em></td>
<td>In mice</td>
<td>(Roberts et al., 1990)</td>
</tr>
<tr>
<td></td>
<td>Pertussis toxin</td>
<td>Strong in mice</td>
<td>(Mielcarek et al., 1998)</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>Unknown</td>
<td>Strong in pigs</td>
<td>(de Jong, 1987)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>Unknown</td>
<td>Strong in dogs</td>
<td>(Bey et al., 1981)</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td><em>apxIIC</em></td>
<td>Strong in pigs</td>
<td>(DiGiacomo et al., 1987)</td>
</tr>
<tr>
<td></td>
<td>Toxin-activating protein</td>
<td>Poor in field trials</td>
<td>(Deeb et al., 1989)</td>
</tr>
</tbody>
</table>

Modified from Locht, (2000).

1.9.3.1 Disruption of virulence determinants

A virulence determinant can be defined as a component of a pathogen that damages the host and can include components essential for viability (Casadevall and Pirofski, 1999). Disruption of certain genes encoding these virulence determinants or the genes responsible for their regulation can produce strains displaying an attenuated phenotype. Table 1.3 lists a number of virulence associated genes that have been specifically disrupted in a variety of bacterial species to render them both avirulent and immunogenic for mice and other animal models.

1.9.3.2 Disruption of biosynthetic house keeping genes

The majority of work done on genetically defined attenuated pathogens have examined the effects of auxotrophic mutations. Disruption of biosynthetic pathways can render microorganisms auxotrophic for specific metabolic requirements that may not be available at different stages of the infection process. Unless the required compounds are supplied exogenously the bacterium cannot survive. This has lead to the creation of strains that produce only a limited infection therefore being attenuated *in vivo*, but retaining their immunogenicity. House-keeping genes that have been disrupted to attenuate bacterial pathogens have been listed in Table 1.4.
### Table 1.3

Virulence associated genes that have been disrupted to rationally attenuate bacterial pathogenesis.

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>Enzyme</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td><em>phoP phoQ</em></td>
<td>Regulation of intracellular survival</td>
<td>(Miller et al., 1989)</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td><em>cya crp</em></td>
<td>Adenylate cyclase and cyclic AMP receptor protein</td>
<td>(Curtiss and Kelly, 1987)</td>
</tr>
<tr>
<td><em>Salmonella choleraesuis</em></td>
<td><em>cya crp</em></td>
<td>Adenylate cyclase and cyclic AMP receptor protein</td>
<td>(Kennedy et al., 1999)</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td><em>ompR</em></td>
<td>Transcriptional activation of porins</td>
<td>(Dorman et al., 1989)</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td><em>ompC ompF</em></td>
<td>Porins</td>
<td>(Chatfield et al., 1991)</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td><em>htrA</em></td>
<td>Stress response</td>
<td>(Chatfield et al., 1992)</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td><em>sseC</em></td>
<td>Type III secretion</td>
<td>(Medina et al., 1999)</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td><em>sseD</em></td>
<td>Type III secretion</td>
<td>(Medina et al., 1999)</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td><em>flgM</em></td>
<td>Flagella synthesis regulation</td>
<td>(Schmitt et al., 1994)</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td><em>crp cdt</em></td>
<td>Cyclic AMP receptor protein and colonisation of deep tissue</td>
<td>(Zhang et al., 1997)</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td><em>ctx</em></td>
<td>Cholera toxin</td>
<td>(Mekalanos et al., 1983)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td><em>sarA agr</em></td>
<td>Global regulatory loci</td>
<td>(Kielian et al., 2001)</td>
</tr>
<tr>
<td><em>Salmonella enterica</em></td>
<td><em>guaBA</em></td>
<td>GMP synthetase and IMO dehydrogenase</td>
<td>(Wang et al., 2001)</td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em></td>
<td><em>yadA sodA</em></td>
<td><em>Yersina</em> adhesion A and superoxide dismutase</td>
<td>(Igwe et al., 1999)</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td><em>sap</em></td>
<td>Secreted aspartyl proteinases</td>
<td>(Hube et al., 1997)</td>
</tr>
<tr>
<td><em>Brucella abortus</em></td>
<td><em>omp2S</em></td>
<td>outer membrane protein</td>
<td>(Edmonds et al., 2001)</td>
</tr>
<tr>
<td><em>Pasteurella haemolytica</em></td>
<td><em>lktC</em></td>
<td>Transacylase gene of leukotoxin operon</td>
<td>(Highlander et al., 2000)</td>
</tr>
<tr>
<td><em>Brucella abortus</em></td>
<td><em>lon</em></td>
<td>Stress response protease</td>
<td>(Robertson et al., 2000)</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td><em>slyA</em></td>
<td>Virulence transcriptional regulator</td>
<td>(Watson et al., 1999)</td>
</tr>
<tr>
<td><em>Salmonella enterica</em></td>
<td><em>poxA</em></td>
<td>Peroxidase</td>
<td>(Kaniga et al., 1998)</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td><em>erp</em></td>
<td>exported repeated protein</td>
<td>(Berthet et al., 1998)</td>
</tr>
</tbody>
</table>
Table 1.4  House keeping genes that have been disrupted to rationally attenuate pathogens.

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>Enzyme</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aeromonas salmonicida</em></td>
<td>$aroA$</td>
<td>5-Enolpyruvylshikimate-3-phosphate synthase</td>
<td>(Vaughan et al., 1993)</td>
</tr>
<tr>
<td><em>Aeromonas hydrophila</em></td>
<td>$aroA$</td>
<td>5-Enolpyruvylshikimate-3-phosphate synthase</td>
<td>(Moral et al., 1998)</td>
</tr>
<tr>
<td><em>Bordetella pertussis</em></td>
<td>$aroA$</td>
<td>5-Enolpyruvylshikimate-3-phosphate synthase</td>
<td>(Roberts et al., 1990)</td>
</tr>
<tr>
<td><em>Pasteurella haemolytica</em></td>
<td>$aroA$</td>
<td>5-Enolpyruvylshikimate-3-phosphate synthase</td>
<td>(Homchampa et al., 1994)</td>
</tr>
<tr>
<td><em>Pasteurella multocida</em></td>
<td>$aroA$</td>
<td>5-Enolpyruvylshikimate-3-phosphate synthase</td>
<td>(Homchampa et al., 1992)</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>$aroA$</td>
<td>5-Enolpyruvylshikimate-3-phosphate synthase</td>
<td>(Hiøseth and Stocker, 1981)</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>$aroA$</td>
<td>5-Enolpyruvylshikimate-3-phosphate synthase</td>
<td>(Dougan et al., 1987)</td>
</tr>
<tr>
<td><em>Yersinia pestis</em></td>
<td>$aroA$</td>
<td>5-Enolpyruvylshikimate-3-phosphate synthase</td>
<td>(Oyston et al., 1996)</td>
</tr>
<tr>
<td><em>Neisseria gonorrhoeae</em></td>
<td>$aroA$</td>
<td>5-Enolpyruvylshikimate-3-phosphate synthase</td>
<td>(Chamberlain et al., 1993)</td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td>$aroC$</td>
<td>Chorismate synthase</td>
<td>(Bernardini et al., 2001)</td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td>$aroC$ $purE$</td>
<td>Chorismate synthase and 5-Phosphoribosyl-5-aminomidazole</td>
<td>(Bernardini et al., 2001)</td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td>$aroA$ $aroD$</td>
<td>5-Enolpyruvylshikimate-3-phosphate synthase and 3-dehydroquinate dehydratase</td>
<td>(Verma and Lindberg, 1991)</td>
</tr>
<tr>
<td><em>Corynebacterium pseudotuberculosis</em></td>
<td>$aroQ$</td>
<td>3-Dehydroquinate dehydratase</td>
<td></td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>$aroA$ $aroC$</td>
<td>5-Enolpyruvylshikimate-3-phosphate synthase and chorismate synthase</td>
<td>(Dougan et al., 1988)</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>$aroA$ $purA$</td>
<td>5-Enolpyruvylshikimate-3-phosphate synthase and adenylsuccinate synthetase</td>
<td>(Stocker et al., 1983)</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>$aroC$ $aroD$</td>
<td>Chorismate synthase and 3-dehydroquinate dehydratase</td>
<td>(Tacket et al., 1992)</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>$dac1$ $nag1$ $hsk1$</td>
<td>N-acetylglucosamine catabolic pathway</td>
<td></td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>$leuD$</td>
<td>alpha-isopropylmalate isomerase small subunit</td>
<td>(Hondalus et al., 2000)</td>
</tr>
<tr>
<td><em>Edwardsiella ictaluri</em></td>
<td>$purA$</td>
<td>Adenylosuccinate synthetase</td>
<td>(Lawrence et al., 1997)</td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td>$dsbA$</td>
<td>Disulphide oxidoreductase</td>
<td>(Yu, 1998)</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>$purC$</td>
<td>5-Phosphoribosyl-5-aminomidazole 4-N-scocinocarboxamide synthetase</td>
<td>(Jackson et al., 1999)</td>
</tr>
</tbody>
</table>
A number of studies have shown that strains harboring different single or multiple mutations can differ markedly in their immunogenicity and the type of immune responses generated. As increasing numbers of strains harboring different attenuating mutations become characterised, it is now becoming possible to create customised vaccines which have been optimised to specifically elicit the type of response associated with protective immunity (Drabner and Guzman, 2001).

1.9.4 Attenuation through disruption of aromatic amino acid biosynthesis

The most common strategy employed for the attenuation of pathogens has been the disruption of genes involved in the biosynthesis of aromatic amino acids (Table 1.4). In bacteria this process consists of a linear biochemical pathway called the shikimate or common pathway which converts phosphoenol pyruvate and erythrose-4-phosphate into chorismate, a branch point compound that is subsequently converted into aromatic amino acids, para-aminobenzoic acid (PABA), 2,3-dihydroxybenzoic acid (DHBA), para-hydroxybenzoate and ortho-succinylbenzoic acid (Pittard, 1987) (Figure 1.3). The aroA gene has been most frequently targeted in mutational studies. This gene encodes the enzyme 3-enolpyruvylshikimate-5-phosphate synthase and converts shikimate to 5-enolpyruvyl-3-phosphate. A mutation in the linear upper pathway, before the production of chorismate renders bacteria auxotrophic for the aromatic compounds produced in the lower pathways. The aromatic amino acids, phenylalanine, tyrosine and tryptophan are essential metabolic requirements but are readily available within mammalian tissue while the compounds para-hydroxybenzoate and ortho-succinylbenzoic acid are not requirements for bacterial survival. It is the inability to produce PABA and DHBA, which are essential bacterial requirements that are not available within mammalian tissue, that leads to attenuation during an infection (Hoiseth and Stocker, 1981). A number of bacterial pathogens, including *B. pertussis*, have been attenuated through rational attenuation of the aroA gene (see Table 1.4). *B. pertussis* is the causative agent of the childhood disease whooping cough and is closely related to *B. bronchiseptica* but is confined to human hosts. Roberts *et al.* (1990), constructed an aroA mutant of *B. pertussis* CN2992F, a derivative of
the Wellcome whole-cell vaccine strain CN2992, which is only modulated by temperature. *B. pertussis* <i>aroA</i> was shown to be attenuated in its ability to colonise the lungs of mice when compared to the wild-type parental strain after aerosol exposure. Mice given three doses of *B. pertussis* <i>aroA</i> were protected against lung colonisation and infection when subsequently challenged with the wild-type strain. Vaccinated mice produced a more rapid anti-*B. pertussis* humoral antibody response after challenge when compared to unvaccinated controls which remained high for several weeks.

**Figure 1.3** The shikimate or common pathway for aromatic amino acid biosynthesis in bacteria. Phosphoenol pyruvate and erythrose-4-phosphate are converted into the branch point compound chorismate. This compound is subsequently converted into the aromatic amino acids, tryptophan, tyrosine, phenylalanine and other aromatic compounds. The names of the enzymes catalysing the various reactions are given and the genes encoding these proteins are shown in italics. Adapted from Pittard, (1987) and Cardenas and Clements, (1992).
1.10 Specific Aims

The effectiveness of current vaccines available for the prevention of *B. bronchiseptica* infection is questionable (Backstom, 1999). Live attenuated strains of *B. bronchiseptica* have been shown to induce protective immunity and numerous live, avirulent vaccines are now commercially available for intranasal administration in dogs. However, the nature of the attenuation is often undefined making it difficult to predict the stability of these vaccine strains. A common strategy used to construct attenuated strains has been the disruption of genes involved in the biosynthesis of aromatic amino acids. Strains produced using this strategy are easily characterised and have been successfully used as live vaccines for a number of other bacterial pathogens. It is currently not known if *B. bronchiseptica* can be attenuated through disruption of biosynthesis of aromatic compounds. The aims of this thesis were to construct an aromatic auxotrophic strain of *B. bronchiseptica* which was attenuated and immunogenic. This was to be achieved by firstly cloning and characterising the *aroA* gene of *B. bronchiseptica*. The *aroA* gene fragment was then to be used to mutate the *aroA* gene of *B. bronchiseptica* via homologous recombination. Mutants were to be characterised genetically, phenotypically and *in vivo* using a mouse model of respiratory infection. The immunogenicity of the mutant strains in mice were assessed and the ability of the strain to act as a live vaccine was determined by examining the rates of clearance of a wild-type challenge infection in vaccinated and non-vaccinated animals.
2. Materials and Methods

2.1 Bacterial strains, plasmids and growth conditions

Bacterial strains used in this study are described in Table 2.1 while plasmid vectors are listed in Table 2.2. Recipes for all media and buffers are listed in Appendix I. *B. bronchiseptica* strains were grown at 37°C on Bordet-Gengou (BG) agar (Bordet and Gengou, 1906) (Difco Laboratories) containing 1% glycerol w/v and 10% v/v defibrinated horse blood for the determination of haemolytic activity and colony morphology. To test the Aro phenotype, *B. bronchiseptica* strains were grown in minimal Stainer Scholte (SS-X) (Stainer and Scholte, 1971) broth or on agar containing 1.5% w/v Noble agar (Sigma) with or without aromatic supplements (aamix). When added to media the aamix consisted of tyrosine, tryptophan and phenylalanine at a final concentration of 40 μg ml⁻¹, and 2,3-dihydroxybenzoate and p-aminobenzoic acid at 10 μg ml⁻¹. To repress bvg activity, SS-X medium containing 40 mM MgSO₄ instead of NaCl, (SS-C) was used. Motility assays were performed in SS-X medium containing 0.35% w/v Noble agar and were incubated for 24 h at 37°C. *E. coli* strains were routinely grown on Z agar (Walker et al., 1988), in Luria Bertani (LB) broth (Sambrook et al., 1989) or on M9 minimal medium. For transfection with λ phage, *E. coli* 29A Rif was grown in LB broth containing 0.4% maltose and 10 mM MgCl₂. When required, antibiotics were added to the medium at the following concentrations: ampicillin (Ap) 100 μg ml⁻¹; kanamycin (Km) 50 μg ml⁻¹; tetracycline (Tc) 20 μg ml⁻¹; rifampicin (Rif) 100 μg ml⁻¹; gentamycin (Gm) 25 μg ml⁻¹ and cephalaxin (Cp) 50 μg ml⁻¹. Isopropyl-β-D-galactopyranoside (IPTG) 0.04 mM and 5-bromo-4-chloro-3-indoly1-β-D-galactopyranoside (X-Gal) 40 μg ml⁻¹ were added to medium when required. All incubations were performed at 37°C unless otherwise stated. Liquid cultures were agitated during incubation in an orbital-shaking incubator (Paton Industries) at 180 rpm when required.
Table 2.1  Bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype / relevant phenotype</th>
<th>Source / reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. bronchiseptica</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K8744/lb</td>
<td>Pig isolate; Bvg-positive, Cp'</td>
<td>P. Blackall</td>
</tr>
<tr>
<td>CMJ25</td>
<td>aroA mutant of K8744/lb, Cpr, Km', Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>CMJ60</td>
<td>aroA trpE mutant of K8744/lb, Cp', Km', Ap', Gm'</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB2829</td>
<td>glnV42 LAM aroA354 rpoS396 rph-1 F'</td>
<td>Pittard and Wallace, (1966)</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>supE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lac' F' [proAB' lac6 lacZΔM15 Tn10(ter')]</td>
<td>Stratagene</td>
</tr>
<tr>
<td>S17-lapir</td>
<td>recA hsdR thi pro ara RP-4-2-Tc::Mu-Km::Tn7 lapir (Tp' Sm')</td>
<td>Simon et al., (1983)</td>
</tr>
<tr>
<td>294 Rif</td>
<td>hsdR hsdM+ thi endA recA</td>
<td>Talmadge and Gilbert, (1980)</td>
</tr>
</tbody>
</table>

Abbreviations:  Cp, cephalixin; Km, kanamycin; Ap, ampicillin; Rif, rifampicin; Gm, gentamycin; Tp, trimethoprim; Sm, streptomycin.

2.2 DNA Manipulations

2.2.1 Isolation and purification of DNA

2.2.1.1 Chromosomal DNA extraction

Chromosomal DNA of *Bordetella* strains was extracted using a genomic extraction kit supplied by QIAGEN following the manufacturer’s instructions. Briefly, bacterial cells were grown overnight in liquid broth, harvested by centrifugation and resuspended in 3.5 ml of Buffer B1 (Appendix I). Cells were lysed by adding lysozyme (2.2 mg/ml) and proteinase K (0.6 mg/ml) and incubating at 37°C for 60 min. Proteins were then denatured through the addition of 1.2 ml of Buffer B2 (Appendix I) and incubation at 50°C for 30 min. The lysate was then vortexed for 10 sec and applied to a QIAGEN genomic column (tip-100). The column was washed twice with 7.5 ml of Buffer QC (Appendix I) and bound genomic DNA was then eluted with 5 ml of Buffer QF (Appendix I). The DNA
was precipitated by the addition of 3.5 ml of isopropanol and collected by centrifugation at 10,000 \( g \) for 30 min. The DNA pellet was then washed with 2 ml of cold 70% ethanol and allowed to air dry for 10 min. To dissolve the purified genomic DNA, 500 \( \mu l \) of TE buffer was added to the pellet and incubated on a shaker overnight at room temperature.

Table 2.2  Plasmid vectors used in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Phenotype</th>
<th>Source / Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR-Script</td>
<td>Ap(^{+}), lac(Z\alpha) cloning vector for PCR fragments</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBluescript</td>
<td>Ap(^{+}), lac(Z\alpha) cloning vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pHSG398</td>
<td>Cm(^{+}), lac(Z\alpha)</td>
<td>Takeshita \textit{et al.},\ (1987)</td>
</tr>
<tr>
<td>pHC79</td>
<td>Ap(^{+}), Tc(^{-}) cosmid cloning vector</td>
<td>Hohn and Collins,\ (1980)</td>
</tr>
<tr>
<td>pGP704Sal</td>
<td>Ap(^{+}), pGP704 (Miller and Mekalanos, \ 1988) with SaI site removed</td>
<td>This study</td>
</tr>
<tr>
<td>pH45(\Omega)Km</td>
<td>Ap(^{+}), Km(^{-}), Source of the 2.2 kb Km(^{-}) gene</td>
<td>Fellay \textit{et al.},\ (1987)</td>
</tr>
<tr>
<td>pJMC6</td>
<td>Ap(^{+}), 1.3 kb (aroA) gene fragment in pCR-Script</td>
<td>This study</td>
</tr>
<tr>
<td>pJMC9</td>
<td>Ap(^{+}), 1.3 kb (aroA) gene fragment in pGP704Sal</td>
<td>This study</td>
</tr>
<tr>
<td>pJMC10</td>
<td>Ap(^{+}), pJMC9 with a 90 bp SaI deletion in (aroA)</td>
<td>This study</td>
</tr>
<tr>
<td>pJMC13</td>
<td>Ap(^{+}), pJMC10 with 2.2 kb Km(^{-}) gene at SaI</td>
<td>This study</td>
</tr>
<tr>
<td>pCOS24</td>
<td>Ap(^{+}), cosmid containing (aroA)</td>
<td>This study</td>
</tr>
<tr>
<td>pJMC25</td>
<td>Ap(^{+}), 4.5 kb fragment of pCOS24 in pBluescript</td>
<td>This study</td>
</tr>
<tr>
<td>pJCOS60</td>
<td>Ap(^{+}), cosmid containing chromosomal region of CMJ60 harbouring the mini-Tn5/Gm</td>
<td>This study</td>
</tr>
<tr>
<td>pJMC61</td>
<td>Ap(^{+}), 7.2 kb (BamH1) fragment of pJMC60 in pBluescript</td>
<td>This study</td>
</tr>
<tr>
<td>pJMC65</td>
<td>Ap(^{+}), 6.1 kb (SphI) fragment of pJMC60 in pBluescript</td>
<td>This study</td>
</tr>
</tbody>
</table>

\textit{Mini-transposons}  

| pBSL202::mini-Tn5/Gm | Gm\(^{+}\)                                                               | Alexeyev \textit{et al.},\ (1995) |

Abbreviations:  
Cp, cephalxin; Km, kanamycin; Ap, ampicillin; Rif, rifampicin; Gm, gentamycin; Tc, tetracycline; lac\(Z\alpha\), \(\beta\)-galactosidase

\textit{2.2.1.2 Plasmid DNA extraction}  
For small scale extraction of plasmid DNA, an alkaline lysis system in the form of the FlexiPrep kit from Pharmacia Biotec was used. Bacterial cells were grown overnight in liquid LB broth and 1 ml aliquots were then centrifuged at 16,000 \( g \) for 5 min to produce
Methods

Cell pellets. These pellets were resuspended in 200 μl of Solution 1 (Appendix I) and cells were lysed by the addition of 200 μl of Solution 2 (Appendix I). Cellular debris was precipitated through the addition of 200 μl of Solution 3 (Appendix I) and this material was removed via centrifugation at 16,000 g for 5 min. DNA present in the supernatant was precipitated using isopropanol and collected by centrifugation at 16,000 g for 5 min. This crude DNA pellet was then resuspended in 150 μl of Sephaglas slurry which binds to the DNA. Any contaminating material is removed with wash steps using 200 μl of Wash buffer (Appendix I), then 350 μl of 70% ethanol. DNA was eluted from the matrix with 50 μl of dH₂O and stored at -20°C until required.

To extract large amounts of plasmid DNA, the QIAGEN midi-prep kit was used. Bacteria were grown overnight in 150 ml of LB broth supplemented with appropriate antibiotics. The cells were harvested by centrifugation at 5,000 g for 10 min and resuspended in 4 ml of Buffer P1 (Appendix I) containing 100 μg.ml⁻¹ RNase A. After the addition of 4 ml of Buffer P2 (Appendix I) 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 (Appendix I), and incubated for 15 min on ice. Precipitated cellular debris was then removed by centrifugation at 16,000 g for 30 min, and the supernatant applied to a QIAGEN midi-column (Tip-100) previously equilibrated with 4 ml of buffer QBT. The supernatant was allowed to pass through the column via gravity flow. The column was washed 20 ml of Buffer QC (Appendix I) and the bound plasmid DNA was then eluted with 5 ml of Buffer QF (Appendix I). The DNA was precipitated by the addition of 3.5 ml of isopropanol and subsequently pelleted by centrifugation at 16,000 g for 30 min. The supernatant was removed and the pellet washed with 2 ml of 70% ethanol. After air drying, the DNA pellet was resuspended in 300 μl of dH₂O.

High quality plasmid DNA suitable for use in DNA sequencing reactions was obtained via the QIAprep spin kit. The extraction protocol, as described by the manufacturer, is essentially the same as the QIAGEN midi-prep kit extraction protocol. Briefly, bacterial cells were grown overnight and 1 ml aliquots were then centrifuged at 16,000 g for 5 min to
produce cell pellets. Pellets were resuspended in 250 µl of Buffer P1 (Appendix I) and cells were lysed by the addition of 250 µl of Buffer P2 (Appendix I). Cellular debris was precipitated through the addition of 350 µl of Buffer N3 (Appendix I) and this material was removed via centrifugation at 16,000 g for 5 min. The resulting lysate was then loaded onto a QIAprep column and spun for 60 sec. Plasmid DNA present in the lysate bound to the silica matrix of the column which was then washed with 750 µl of Buffer PE (Appendix I). DNA was eluted with 50 µl of dH₂O and stored at -20°C.

2.2.2 Polymerase Chain Reaction

2.2.2.1 PCR amplification

The polymerase chain reaction (PCR) was used to amplify specific chromosomal DNA segments from *B. bronchiseptica*. The reaction mix used to amplify PCR products is outlined in Table 2.3. When required, the reaction mix was optimised by altering the concentrations of template DNA or primer, or varying the concentration MgCl₂.

Amplifications of chromosomal DNA fragments were performed using *Pfu* polymerase (Stratagene, USA). Primers were synthesised by Auspep (Australia) or GibcoBRL-Life Technologies (USA) and individual sequences have been listed in Appendix II. PCR reactions were carried out in a 100 µl final volume when using the Hybaid thermocycler.
Table 2.3  Components of a typical PCR reaction and their concentration.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td>8 ng</td>
</tr>
<tr>
<td>5’ primer</td>
<td>0.25 μm</td>
</tr>
<tr>
<td>3’ primer</td>
<td>0.25 μm</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>0.4 mM each</td>
</tr>
<tr>
<td>10x Buffer</td>
<td>1x</td>
</tr>
<tr>
<td>DNA polymerase</td>
<td>2.5 units</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>8 mM</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to final volume</td>
</tr>
</tbody>
</table>

(Hybaid, United Kingdom) or in 50 μl volume when using the GeneAmp 9600 System (Applied Biosystems, USA). The cycling parameters used are described in Table 2.4 with the optimal annealing temperature for each PCR being determined empirically.

Table 2.4  The PCR cycling parameters used in this study.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Duration</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Annealing</td>
<td>Variable</td>
<td>3.5 min</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1.5 min</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>40 sec</td>
<td>35</td>
</tr>
<tr>
<td>Annealing</td>
<td>Variable</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1.5 min</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>Annealing</td>
<td>Variable</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>5 min</td>
<td></td>
</tr>
</tbody>
</table>
2.2.2.2 Cloning of PCR products

To clone blunt-ended PCR fragments produced by *Pfu* polymerase, the pCR-Script Cloning kit (Stratagene, USA) was used. Essentially, PCR products are cloned into the *SrfI* restriction site of pCR-Script and transformed into supercompetent *E. coli* which in turn is plated onto medium to allow for blue/white selection of possible clones. The ligation mix consisted of 10 ng of pCR-Script, 1 μl of 10x ligation buffer, 10 mM rATP, 2-4 μl of PCR product, 5 units of *SrfI* restriction enzyme and 4 units of T4 DNA ligase in a final volume of 10 μl. Ligation was allowed to proceed for 1 h at room temperature and then the enzyme was inactivated by heating at 65°C for 10 min.

Supercompetent *E. coli* XL1-Blue MRF' Kan cells were supplied by the manufacturer as 40 μl aliquots and were thawed on ice prior to use. β-mercaptoethanol (0.7 μl) was added to the cells which were agitated for 15 min on ice. 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 a further 2 min. SOC media (Appendix I), pre-heated to 37°C, was then added to the cells which were incubated for 1 h at 37°C before plating onto LB agar supplemented with ampicillin, X-gal and IPTG.

2.2.3 Restriction endonuclease analysis of DNA

The various restriction endonucleases used in this study were purchased from Roche (Roche Diagnostics, Australia). Digestions were conducted in 1.5 ml microfuge tubes and consisted of 5 to 10 μl of DNA, 2 μl of the appropriate 10x restriction buffer, dH₂O to 19 μl and 1 μl of the required restriction enzyme. The tube was then incubated at 37°C for 1 h to allow digestion, then heated at 65°C for 10 min to denature the enzyme.

2.2.4 Conversion of cohesive ends to blunt ends

When required, the cohesive ends of DNA fragments produced by restriction enzymes were converted to blunt ends using the Klenow fragment of *E. coli* DNA polymerase I (Roche
Diagnostics, Australia). The reaction mix containing the digested DNA fragment to be blunt ended consisted of 0.5 mM of each dNTP, 100 mM MgCl₂, 100 mM Tris-HCl (pH 8) and 2.5 units of the Klenow enzyme. The mixture was incubated at 30°C for 30 min, then stopped by inactivating the enzyme through heating for 20 min at 70°C.

2.2.5 Ligation of DNA

Ligation reactions were set up in a final volume of 40 μl in a ratio of 3:1 of insert to vector DNA. The appropriate amounts of DNA, and dH₂O to a final volume of 35 μl was added to a 1.5 ml microfuge tube which was heated at 65°C for 1 min, 37°C for 10 min, room temperature for 10 min and on ice for 10 min. 4 μl of 10x ligation buffer and 2 units of T₄ DNA ligase (Roche Diagnostics, Australia) was added and the tube was incubated at 10°C overnight. The ligase enzyme was then deactivated at 65°C for 10 min.

2.2.6 Competent cell preparation and transformation of DNA

E. coli were routinely made chemically competent to facilitate the uptake of foreign DNA. Bacteria were inoculated into 200 ml of LB broth and grown to an A₅₆₀ of 0.5. Bacterial cells were harvested by centrifugation at 4,000 g for 10 min and the pellet was then placed on ice for a further 10 min. 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 and remained viable for several days.

For transformation of plasmid DNA, 5 to 15 μl of the DNA sample was mixed with a 200 μl aliquot of competent cells and incubated on ice for 1 h. The transformation mixture was then incubated at 37°C for 5 min and the bacteria collected by spot centrifugation. The pellet was then resuspended in 500 μl of LB broth and incubated at 37°C for 1 h before being plated onto selective agar.
2.2.7 Agarose gel electrophoresis

Electrophoresis of DNA was performed in 0.85% agarose/TAE gels using BioRad electrophoresis equipment. Typically, electrophoresis was for 2 h at 60-80 volts. However, for Southern hybridisation which requires a high level of resolution, gels were run at 12 volts overnight. DNA was stained by immersion in a 1 µg/ml solution of ethidium bromide for 30 min and visualised with ultra-violet light. To determine the size of DNA fragments, λ bacteriophage DNA (Roche Diagnostics, Australia) digested with HindIII restriction enzyme or 1 kb ladder (GibcoBRL-Life Technologies) were electrophoresed as molecular weight markers.

2.2.8 Elution of DNA from agarose gels

When required DNA was extracted from agarose gels using the Bresa-Clean procedure (Bresatec, Australia). DNA was first electrophoresed in a 0.85% agarose gel and stained with ethidium bromide. The band of interest was then excised and placed in a 1.5 ml microfuge tube containing three volumes of Bresa-Salt. The tube was then incubated at 55°C and mixed regularly until all the agarose had melted. Approximately 5-10 µl of Bresa-Bind was then added, and the mixture incubated at room temperature for 5 min to allow the silica matrix to bind the DNA. This complex was pelleted by spot centrifugation and washed once in Bresa-Wash using a volume equivalent to the initial volume of Bresa-Salt. After centrifugation to remove the wash buffer, the pellet was air dried and resuspended in dH2O to a volume twice that of the Bresa-Bind used. To assist dissociation of the DNA from the Bresa-Bind the mixture was incubated at 55°C for 5 min, centrifuged, and supernatant containing purified DNA transferred to a new tube.

2.2.9 DNA sequence analysis

Automated DNA sequencing was performed using a Perkin Elmer ABI prism 377 DNA sequencer. Reaction mixes, utilising the Sanger method (Sanger et al., 1977), were conducted with Perkin Elmer BigDye terminator cycle sequencing ready reaction mixes. Plasmid DNA to be sequenced was extracted using the QIAprep Spin Miniprep Kit
Methods

(QIAGEN) and dialysed against Milli-Q water for 30 min using Millipore 0.025 μm filter membranes (Millipore, USA). Universal M13 forward and reverse primers were purchased from Perkin Elmer or Bresatec, whilst internal primers were synthesised and purchased from GibcoBRL-Life Technologies. A typical sequencing reaction mix contained 8 μl of ready reaction mix, 100-250 ng of double stranded template DNA, 3.2 pmol of primer and 1 μl of DMSO and dH₂O to 20 μl. Amplification was performed in a Perkin Elmer GeneAmp 9600 thermocycler using the conditions listed in Table 2.5 for a total of 25 cycles.

Table 2.5 Amplification parameters for DNA sequencing.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>50°C</td>
<td>15 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>60°C</td>
<td>4 min</td>
</tr>
</tbody>
</table>

After amplification, the extension products were precipitated by the addition of 2 μl of 3 M sodium acetate (pH 4.6) and 50 μl of 100% ethanol. The mixture was then placed on ice for 10 min, before centrifugation at 16,000 g for 30 min. The supernatant was removed and the pellet washed with 200 μl of 70% ethanol and air dried.

Electrophoresis of the DNA samples was through a 48 cm, 0.2 mm thick, 4% acrylamide gel containing 6M urea and buffered with TBE. Gel preparation and casting was performed according to the ABI prism 377 user manual. Electrophoresis was performed for 10 h at a constant 37 W.

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 National Centre for Biotechnology Information (NCBI) non redundant protein database located at ANGIS (Australian National Genomic Information Service; http://www.angis.su.oz.au) using the blastX algorithm. The optimal alignment of
the deduced *B. bronchiseptica* ORFs and most homologous protein sequences was then determined using the ClustalW program (Thompson *et al.*, 1994). DNA sequence of the operon regions surrounding the DNA sequences determined in this study was obtained from the *B. bronchiseptica* genome sequencing project currently in progress at the Sanger Centre (http://www.sanger.ac.uk/projects/B_bronchiseptica).

### 2.2.10 Southern hybridisation analysis

The transfer of DNA to positively charged nylon membranes (Roche Diagnostics, Australia) was performed by the method of Southern, (1975). DNA was restricted with endonucleases and electrophoresed on a 0.85% agarose gel at low voltage (12 V) overnight and visualised with ethidium bromide staining. DNA in the gel was depurinated by submersion in 0.25 M HCl for 5 min, then rinsed by a 2 min wash in dH2O.

#### 2.2.10.1 Alkaline transfer

The Southern transfer apparatus can be seen in Figure 2.1 and was set up as follows. A wick consisting of 2 sheets of Whatman blotting paper was positioned over a glass plate support that was spanning a large glass dish. The wick was then moistened with 0.4 M NaOH transfer buffer and air bubbles were removed. The dish was then filled with transfer buffer to a level that saw the ends of the wick fully immersed. The gel, with wells removed, was inverted and carefully lowered onto the wick without the introduction of air bubbles. To ensure that the transfer solution moves only through the gel and not around it, the gel was surrounded with plastic wrap. The nylon membrane, precut to cover the entire surface of the gel, was pre-wet in dH2O and lowered onto gel. This was followed by 4 pieces of pre-wet Whatman paper of identical size. A stack of absorbent paper approximately 10 cm high was placed onto the filter paper, followed by a glass plate and a 500 g weight. The DNA was allowed to transfer overnight. The membrane was then rinsed in 2x SSC for 10 min and allowed to air dry. DNA was then fixed to the membrane by UV-crosslinking in a Stratagene UV Stratalinker 1800 for 3 min at 254 nm.
2.2.10.2 DNA probe preparation

DNA that was to be used as a hybridisation probe was radio-labelled with $[^{32}\text{P}]-\text{dATP}$ using the GibcoBRL nick translation system (GibcoBRL-Life Technologies). The labelling reaction was set up using 1 μg of probe DNA, 5 μl of solution A1 (containing all dNTP's except dATP), 5 μl of $[^{32}\text{P}]-\text{dATP}$ (Bresatec) and dH$_2$O to 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. To stop the reaction 5 μl of Solution D containing 300 mM Na$_2$EDTA (pH 8) was added.

Unincorporated nucleotides were removed from labelling mix by passing it through a 1 ml Sephadex spin column. The spin column was constructed in a 1 ml syringe that had been plugged at the bottom with sterile glass wool. The syringe was filled with Sephadex G50 (Amersham Pharmacia Biotec) in STE buffer previously equilibrated at 65°C. To pack the Sephadex G50, the column was centrifuged for 4 min at 2,000 g and the process repeated until a bed volume of 0.9 ml was reached. The labelling reaction mix was diluted to 100 μl with STE buffer then applied to the column. The column was centrifuged as before and the
eluant collected in a 1.5 ml microfuge tube. The column was washed with 100 μl of STE buffer that was collected in the same tube.

2.2.10.3 Hybridisation and detection
Membranes were pre-wet with 0.5 M NaHPO₄ and inserted into a Hybaid® bottle. The NaHPO₄ solution was poured off and 20 ml of hybridisation buffer (Appendix I) was added. The membranes were then incubated at 65°C for 10 min in a Hybaid Mini Oven Mk II. The hybridisation was replaced with fresh buffer then incubated for a further 60 min. The radio-labelled probe was denatured by heating to 100°C for 5 min, then allowed to cool on ice. After cooling, the probe was diluted with the addition of 800 μl of hybridisation buffer at 65°C and added to the Hybaid bottle containing the membranes. The probe was allowed to hybridise to the membranes overnight at 65°C. To remove any non-specific binding membranes were then washed twice for 30 min at 65°C using Southern Wash 1 (Appendix I) and twice for 30 min at 65°C using Southern Wash 2 (Appendix I).

After washing, the membranes were blotted onto filter paper and sealed into plastic bags before exposure to X-ray film (Fuji). Exposure times varied according to the level of radioactivity being displayed by the blot. The film was developed by immersion for 2 min in X-ray developer (Kodak), followed by rinsing in water and then fixed for 2 min in X-ray fixer (Kodak). The film was then rinsed thoroughly in water before being air dried.

2.2.11 Colony blotting
To screen large numbers of colonies when attempting to identify required clones, colony blotting was used. Circular, positively charged nylon membranes (Hybond N+, Amersham Pharmacia Biotec) were overlaid directly onto the surface of a nutrient agar plates. Using sterile toothpicks, colonies were transferred onto the membrane as well as a master plate and the plates were incubated at 37°C overnight to allow bacterial growth. The membranes were then removed and treated in the following manner to lyse the bacteria and fix the DNA released to the membrane. Membranes were placed onto blotting paper saturated
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with Solution 1 and incubated for 3 min. Excess solution was removed from the membrane by placing it onto dry blotting paper before it was then placed on Solution 2 for 5 min. This process was repeated for Solutions 3 and 4 in which the membranes were incubated for 5 min each. The membranes were then allowed to air dry for 30 min before the DNA from the colonies was fixed to the membrane by UV-crosslinking in a Stratagene UV Stratalinker 1800 for 3 min at 254 nm. Hybridisation and detection using specific radiolabelled probes was performed as described in the section 2.2.10.3 above.

2.2.12 Cosmid cloning

A genomic library of *B. bronchiseptica* chromosomal DNA was prepared using cosmids. Cosmids are vectors that are capable of cloning large DNA fragments (35-40 kb) which greatly reduces the amount of screening required when attempting to identify specific genomic regions. Chromosomal DNA to be cosmid cloned was partially digested to the required length using a serial dilution of the restriction endonuclease *Sau3A*. To a 1.5 ml microfuge tube, chromosomal DNA, dH₂O and *Sau3A* restriction buffer were added to a final volume of 120 µl. In another 5 microfuge tubes, DNA, dH₂O and restriction endonuclease buffer was made up to 60 µl. A 1 µl aliquot containing 2 U of *Sau3A* was pipetted into the first tube, mixed, then 60 µl was transferred to the second tube. This 2 fold dilution process was repeated for all tubes. The reactions were incubated for 30 min at 37°C and then heated to 65°C for 10 min to inactivate the enzyme. The digested DNA was electrophoresed on a 0.85% agarose gel and tubes with fragment sizes in the range of 35-40 kb were identified.

Cosmid vector DNA (pHC79) was prepared by digesting with *BamH1* which produced compatible ends to those produced by *Sau3A*. After inactivation of the enzyme at 65°C for 10 min, the DNA was mixed with the partially digested chromosomal DNA and ligated. An aliquot of 2-5 µl of the ligation mix was packaged into λ phage using the Max Plax packaging kit (Epicentre Technologies, USA). The packaging reaction was allowed to proceed for 2 h at room temperature. *E. coli* 294 Rif cells were prepared for transfection by centrifuging an overnight culture at 8,000 g for 15 min. The resulting cell pellet was
resuspended in 2 ml of 10 mM MgSO₄ and shaken rapidly for 60 min at 37°C. Cells were kept on ice until required. Transduction was accomplished by mixing the packaging reaction with the treated cells and incubating at 37°C for 60 min. 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 Protein manipulations

2.3.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

2.3.1.1 Preparation of whole cell lysates and LPS extraction

Whole cell lysates were prepared by pelleting an appropriate number of cells in a 1.5 ml microfuge tube and then resuspending in 100 μl of PBS. Added to this was an equal volume of PAGE sample buffer and the tube boiled for 10 min.

Extraction of *B. bronchiseptica* lipopolysaccharide (LPS) was performed by Nicholas West (University of Wollongong) according to the following method. Cells were grown to late log phase (A₅₆₀ of 1) in SS-C at 25°C or SS-X at 37°C. Cells were concentrated to an A₅₆₀ of 1.5 in PBS and a 500 μL aliquot was pelleted and resuspended in 100 μl of dH₂O. To this tube, an equal volume of LPS sample buffer (6% SDS; 6% β-mercaptoethanol; 10 mM DTT; 46% glycerol; 60 mM Tris, pH 8; 0.1% bromophenol blue) was added and the sample boiled for 10 min. To digest protein in the samples, proteinase K was added to a final concentration of 50 μg/ml and the sample incubated at 37°C overnight. Samples were then boiled for 10 min and a second volume of proteinase K, equal to the first, was added before incubation at 55 °C for 3 h. LPS samples were stored at -20°C until required.

2.3.1.2 Electrophoresis and staining

Proteins were resolved using SDS-PAGE through a 4% polyacrylamide stacking gel and 12% running gel (35:0.8 acrylamide : bis-acrylamide, Bio-Rad). Electrophoresis took place in a Bio-Rad Mini Protean II gel apparatus for 1-2 h at 100 V in a Tris/glycine buffer
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(Appendix I). Protein bands within the gel were stained with Coomassie Brilliant Blue G250 (Sigma). Low range molecular mass markers (Bio-Rad) were used for size determination on Coomassie stained gels where as pre-stained low range molecular mass markers (Bio-Rad) were electrophoresed on gels intended for western blotting.

LPS was visualised by oxidative silver staining (Tsai and Frasch, 1982). After electrophoresis, gels were fixed overnight in a 40% ethanol, 5% acetic acid solution. The fixing solution was then replaced with 0.7% periodic acid in 40% ethanol, 5% acetic acid and allowed to oxidase for 7-10 min. The gels were washed 3 times for 15 min with Milli-Q water. After washing, the gels were stained with fresh silver staining reagent containing 2 ml of concentrated ammonium hydroxide, 28 ml of 0.1 M NaOH, 5 ml of 20% (w/v) silver nitrate and Milli-Q water to 150 ml. Gels were allowed to stain for 10 min, then were washed 3 times in Milli-Q water for 10 min. Once washed, gels were developed in a 200 ml solution containing 10 mg citric acid and 100 μl of 37% formaldehyde. When the bands reached the required intensity, development was terminated by replacing the development solution with Milli-Q water.

2.3.2 Western blot analysis

2.3.2.1 Transfer to polyvinylidene (PVDF) membrane

Proteins that had been separated by SDS-PAGE were transferred to PDVF membrane (Immobilon-P, Millipore) for western blot analysis. Following electrophoresis, gels with the stacking gel removed, were equilibrated for 15 min in western transfer buffer (Appendix I). PVDF membrane cut to the same size as the gel was pre-wet in methanol and allowed to equilibrate in transfer buffer for a similar time period. Protein transfer was accomplished using a Bio-Rad Mini Trans Blot Cell at 30 V and run overnight.

2.3.2.2 Detection of proteins

After proteins had been transferred to PVDF membrane, non-specific binding sites were blocked by incubating the membrane in blocking solution (5% skim milk powder in PBS)
for 60 min with gentle rocking at room temperature. The blocking solution was then discarded and the membrane washed 3 times for 10 min each with PBS. Sera collected from mice that had been infected with *B. bronchiseptica* was diluted appropriately in blocking buffer then applied to the membrane as primary antibody. After a 90 min incubation, the antibody solution was discarded and the membrane washed 3 times with PBS. To detect where the primary antibody had bound to the membrane, a goat anti-mouse horseradish peroxidase conjugated antibody (Kirkegaard and Perry Laboratories, Gaithersberg Maryland) was added as the second antibody at a dilution of 1:1000. After a 60 min incubation, the antibody solution was again discarded and the membrane washed 3 times with PBS. The blot was then developed by the addition of western development solution (Appendix I) to the membrane. Upon reaching the required colour intensity the development solution was discarded and the membrane washed with dH₂O, then air dried.

2.3.3 *Enzyme linked immuno-sorbent assay (ELISA)*

2.3.3.1 *Coating ELISA plates with whole cell antigen*

*B. bronchiseptica* was grown on BG agar, scraped off and resuspended in PBS/10% methanol. This cell suspension was then adjusted to an A₅₆₀ of 5 which was then aliquoted and used as the whole cell antigen stock for all ELISA’s. The optimal concentration for coating immunoassay plates (96 flat bottom well, medium binding capacity; Griener) with whole cell *B. bronchiseptica* as antigen was determined by titration and found to be at an A₅₆₀ of 0.05. With the stock adjusted to the correct concentration in coating buffer (Appendix I), 50 µl was added to each well and the immunoassay plate spun at 420 g for 10 min to coat the bottom of the wells with cells. Immediately after centrifugation unbound antigen was removed and the plates were allowed to air dry for 30 min.

2.3.3.2 *Immuno-sorbent assay*

Non-specific binding was blocked by the addition of 80 µl per well of blocking solution (Appendix I) and incubation at 37°C for 60 min. The plates were then washed 3 times with wash buffer (Appendix I) before the addition of mouse serum or lung wash, serially diluted
3-fold down the plate, and incubation at 37°C for 90 min. The plates were then washed 4 times with wash buffer, then 50 μl of horseradish peroxidase-labelled goat anti-mouse IgG, IgM or IgA (Kirkegaard and Perry Laboratories, Gaithersberg Maryland) diluted 1:2000 in blocking buffer was added to each well. After incubation for 60 min at 37°C, the plates were washed 5 times and bound labelled second antibody was detected by the addition of 50 μl of orthophenylenediamine (Sigma) substrate reagent (Appendix I) to each well. The reaction was allowed to proceed at room temperature until the required colour intensity was reached at which point the reaction was stopped by the addition of 25 μl of 2M H2SO4. Plates were read at 490 nm with a SpectraMax 250 microplate spectrophotometer (Molecular Devices). Titres for anti-*B. bronchiseptica* antibodies were taken as the dilution required to reach an A490 of 0.1 and was determined using the SoftMax program (Molecular Devices).

### 2.4 Microbiological techniques

#### 2.4.1 Replica plating
Sterile wooden toothpicks were used to remove (pick) individual bacterial colonies from plates and patch them onto replica plates selecting for the desired phenotype. This was performed in sets of 50 colonies per plate.

#### 2.4.2 Bacterial conjugation
Conjugation was used to introduce recombinant plasmids into *B. bronchiseptica*. Bacterial strains to be mated were grown on appropriate solid medium with one loopful of growth taken for each strain and mixed in 500 μl of sterile 0.7% saline solution. After thorough mixing, the conjugation mixture was plated onto non-selective BG agar and incubated overnight at 37°C. A loopful of the resultant growth was then resuspended in 1 ml of sterile saline, tenfold serially diluted onto selective agar and incubated at 37°C until single colonies were produced.
2.4.3 Transposon mutagenesis

Random transposon mutagenesis was used to mutate genes of *B. bronchiseptica* involved in the synthesis of aromatic compounds. The mini-Tn5 derivative transposon was contained on the suicide plasmid pBSL202::miniTn5/Gm (Figure 2.2) (Alexeyev *et al*., 1995). This construct was designed with the transposase gene located on the vector outside the inverted repeats of the mini-transposon and is therefore not transposed into the chromosome during transposition. This results in more stable insertions as secondary transpositions, deletions or inversions cannot occur (de Lorenzo *et al*., 1990). Conjugations between *B. bronchiseptica* CMJ25 and the donor *E. coli* S17-1λpir containing pBSL202::miniTn5/Gm were performed as described above. Transconjugates were selected for on SS-X medium supplemented with gentamycin (selection for mini-transposon), cephalexin (selection for *B. bronchiseptica*; negative selection for *E. coli*) and containing aamix to maintain any aromatic amino acid auxotrophic mutants generated. Mutants were screened for the inability to grow on minimal SS-X medium via replica plating.

2.4.4 Motility assays

Motility assays of selected *B. bronchiseptica* strains were performed in SS-C or SS-X medium containing 0.4% agar. Single colonies were stabbed into the semi-solid agar and incubated for 48 h at 37°C. Motility was seen as a halo like appearance as the bacterial growth spread out from the point of inoculation.
Methods

Figure 2.2 Diagrammatic representation of pBSL202::miniTn5/Gm. This transposon was used to mutate genes of *B. bronchiseptica* involved in the synthesis of aromatic compounds. The transposable element contains the polylinker of pBluescriptII and the gene for gentamycin resistance. The plasmid backbone consists of the Tn5 transposase gene, the gene for ampicillin resistance, the RP4 oriT region which enables vectors to be conjugally transferred and the R6K origin of replication that restricts the maintenance of these vectors to hosts that can provide *in trans* the initiator protein encoded by R6K gene *pir*, such as in *E. coli* S17-1/λpir. Abbreviations; Gm', gentamycin resistance; tnp, transposase; Ap', ampicillin resistance; RP4 oriT, origin of transfer; R6K ori, origin of replication; MCS, multiple cloning site or poly linker; (I) inner and (O) outer insertion sequences (19 bp, IS50).

2.4.4 Invasion assays

*B. bronchiseptica* strains were tested for their ability to survive in the macrophage-like cell line J774A.1 (ATCC TIB 67). Cells were maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 1% (v/v) foetal calf serum, 2 mM glutamine and 1.5 g/l NaHCO₃ in an atmosphere containing 5% (v/v) CO₂ at 37°C. J774A.1 cells were seeded at a concentration of approximately 2 x 10⁶ per well in 24-well tissue culture plates (Inter Med NUNC, Roskilde, Denmark), and then washed twice with complete DMEM. *B. bronchiseptica* was grown for invasion assays on BG 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 $4 \times 10^7$ colony forming units (CFU) ml$^{-1}$. A 0.5 ml sample of the suspension was then added to each well tissue culture plates and incubated for 2 h. Non-adherent bacteria remaining in the culture supernatant were removed by aspiration and the cells were then washed twice with PBS. The medium was replaced with 0.5 ml of complete DMEM supplemented with 10 μg.ml$^{-1}$ of polymyxin B (Sigma) and incubated at 37°C for 2 or 24 h to kill any remaining extracellular bacteria. The supernatant was then discarded and the cells washed twice with PBS to remove residual polymyxin B. 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 BG agar using a Spiral Plater model C (Spiral Biotech, Inc., USA). The results reported are mean values of three independent assays with standard deviations. Results were analysed by analysis of variance and Student's t-test with differences considered significant at $P < 0.05$.

2.4.6 Murine respiratory infection model

To examine the in vivo respiratory infection by *B. bronchiseptica*, female BALB/c mice (Animal Resource Centre, Perth, Australia) at age 6 weeks were used. To confirm that animals from each lot were *Bordetella* free, four individual mice were sacrificed and an aliquot of homogenised lung tissue was plated onto BG agar. For infection and challenge studies, bacteria were grown to mid-log phase in supplemented or unsupplemented SS-X broth, washed and resuspended in the appropriate volume of PBS. Mice were anaesthetised using an intraperitoneal injection containing xylazine (0.25 mg) and ketamine (1.25 mg) and a 25 μl aliquot of the bacterial suspension containing $10^5$ or $10^6$ cells was administered intranasally (12.5 μl per nare) using an air-displacement pipette. The mice were then allowed to recover.

2.4.6.1 Surgical procedures

To monitor the bacterial load in the lungs, at various times after infection four mice per time point were sacrificed. Under aseptic conditions, an anaesthetised mouse was dissected
to expose the thorax and abdomen, then exsanguinated by cardiac puncture. Lungs were removed and homogenised in PBS with tissue grinders (Kontes, Vineland New Jersey, USA). Appropriate dilution's were then plated onto aamix supplemented or unsupplemented SS-X agar and CFU determined after incubation for 3 days.

Four mice per treatment were also used to investigate the immune response following vaccination and challenge studies. Mice were again anaesthetised, dissected and blood was collected by cardiac puncture. When required for lymphocyte proliferation studies spleens were collected aseptically. Lung washes were collected by pertracheal cannulation and lavaging 0.7 ml of PBS containing 2 mM phenylmethylsulfonyl fluoride. Lung washes were immediately placed on ice after collection and subsequently stored at -20°C until required.

2.4.7 Lymphocyte proliferation assays

Splenocytes were isolated and released by teasing spleens into 5 ml 1 x RPMI 1640 (Life Technologies). Cells were collected by centrifugation at 800 g for 10 min and red blood cells were lysed with 5 ml AMCO (0.83% NH₄Cl₂ 0.1% KHCO₃, 0.01M EDTA). Cells were washed twice in 5 ml PBS containing 1% glucose and recovered by centrifugation (800 g for 5 min). Cells were then resuspended in 1 ml Complete Medium, RPMI containing 100 unit Penicillin/100 μg Streptomycin, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 0.06 mM β-Mercaptoethanol, 10% Fetal Calf Serum. An aliquot of the suspension was diluted tenfold in Trypan Blue and counted using a haemocytometer. The cell concentration was then adjusted to 5 x 10⁶ cells/ml in Complete Medium. 50 μl of cell suspension (2.5 x 10⁵ cells) in triplicates was added to a 96 well flat bottom cell culture plate (Interpath) containing 200 μl of complete media alone, mitogen or antigen (heat-killed K8744/1b cells at 64 ng/well). The plate was incubated for 72 h at 37°C in 5% CO₂ in air. At the end of the incubation period, 50 μl (0.5 μCi) of ³H thymidine (Amersham, TRK120) was added to each well and the plate was incubated for a further 18 h. Cells were harvested with a PHD cell harvester (Cambridge Technology) onto glass fibre filter strips. The filters were punched out into insert vials (Pony vials, Packard, 6000286) containing 2
ml scintillant (Ultima Gold scintillation fluid, Packard, 60133). Tubes were then mixed vigorously and radioactivity were counted for 1 min with a Packard 4000 series liquid scintillation counter (model 4530). Results were expressed as stimulation index which were determined by cpm (stimulated)/cpm (media only).
Results I

3. Construction of *B. bronchiseptica aroA*

3.1 Cloning of *B. bronchiseptica aroA*

When constructing attenuated bacterial strains for vaccine purposes it is essential that defined, non-reverting mutations are introduced into the genome. The construction of auxotrophic mutants has been a popular method for the generation of rationally attenuated strains and disruption of the pre-chorismate pathway through mutation of *aroA* has been a common strategy in a number of studies. It currently has not been determined if *B. bronchiseptica* will be attenuated with a mutation in *aroA*. In this study the *aroA* gene of *B. bronchiseptica* was specifically targeted for mutation via homologous recombination.

To do this the *aroA* gene of *B. bronchiseptica* was PCR amplified, cloned and mutated via insertion of a Km\(^r\) gene within the coding region of *aroA*. This construct was then used to produce an aromatic amino acid auxotrophic mutant of *B. bronchiseptica* K8744/1b via homologous recombination at *aroA*.

3.1.1 PCR amplification of *B. bronchiseptica aroA*

PCR amplification of the *aroA* gene from *B. bronchiseptica* K8744/1b chromosomal DNA was performed with oligonucleotide primers, one containing a flanking EcoRI restriction site (underlined), 5'GAATTTCATGAGCGGATTGGCATAT3' and 5'CGCGTACACGTCGAAATAAT3' which were designed using the *B. pertussis aroA* gene sequence (Roberts *et al.*, 1990). The amplification procedure resulted in a 1.3 kb product (Figure 3.1) that was confirmed to be the *aroA* gene of *B. bronchiseptica* by partial DNA sequence analysis (data not shown). This fragment was subsequently cloned into pCR-Script (stratagene), forming plasmid pJMC6 (Figure 3.2).
Results

Figure 3.1 Agarose gel electrophoresis of a 1.3 kb DNA fragment containing the PCR amplified aroA gene fragment of *B. bronchiseptica* K8744/lb. Lane 1 contains the PCR product and lane 2 contains DNA standards (1 kb ladder, Gibco) with sizes indicated in bp.

To clone the surrounding area of the genome, a cosmid library was generated by partially digesting *B. bronchiseptica* K8744/lb chromosomal DNA with Sau3A to give DNA in the size range of 40-50 kb, followed by subsequent ligation into the *BamH*1 site of the cosmid vector pHC79. The resulting recombinant cosmid DNA was packaged into λ phage and used to infect *E. coli* 294 Rif. The recombinant clones were screened using the [α-\(^{32}\)P]dATP labelled, PCR-amplified aroA gene fragment of *B. bronchiseptica* as a probe. Out of 500 recombinant colonies screened, a single positive clone containing cosmid pJCOS24 was identified (Figure 3.3).

In order to identify a smaller sized DNA fragment to subclone and sequence, pJCOS24 was digested with the restriction enzyme ClaI and fragments containing the aroA gene were identified via Southern blotting using the PCR amplified aroA gene fragment of *B. bronchiseptica* K8744/lb (Figure 3.4). A 4.5 kb ClaI fragment within cosmid pJCOS24 was found to hybridise with the aroA gene probe and was subsequently cloned into pBluescript SK+, forming pJMC25.
Results

Figure 3.2  Cloning of the \textit{B. bronchiseptica} \textit{aroA} gene. Specific oligonucleotide primers (arrows) were constructed based on the \textit{aroA} gene sequence of \textit{B. pertussis} (Roberts \textit{et al}., 1990) and used to amplify the \textit{aroA} gene of \textit{B. bronchiseptica} K8744/1b using PCR. The PCR product was ligated into the \textit{SrfI} site of pCR-Script producing recombinant plasmid pJMC6. Abbreviations; Ap, ampicillin resistance; \textit{f1} (+) origin, \textit{f1} filamentous phage origin of replication; \textit{lacZ}, \textit{\beta}-galactosidase gene; ColE1, plasmid origin of replication.

Both pJMC6 and pJMC25 were transformed into \textit{E. coli} AB2829 \textit{aroA} to determine whether either of these plasmids were able to complement the \textit{aroA} mutation of this strain. AB2829 containing either plasmid failed to grow on minimal M9 medium. However, full growth was seen once aamix was added to the medium (data not shown). This indicates that the 1.3 kb fragment of pJMC6 or the 4.5 kb fragment of pJMC25 were unable to complement the \textit{aroA} mutation in \textit{E. coli} AB2829.
Figure 3.3  Autoradiogram of a colony blot generated during screening of the cosmid library generated to clone the area of genome surrounding the aroA gene in B. bronchiseptica K8744/1b. The blot was probed with the 1.3 kb PCR amplified aroA gene fragment of B. bronchiseptica K8744/1b. A single positive clone was detected and is indicated by a circle. The plasmid pJMC6 was dotted onto the membrane as a positive control in these experiments and is indicated by an arrow.

3.2 Molecular analysis of the aroA locus of B. bronchiseptica.

3.2.1 DNA sequence analysis of the aroA operon

The 4.5 kb ClaI insert of pJMC25 was sequenced in both directions. DNA sequence analysis of this fragment revealed the presence of 5 open reading frames with significant homology to the predicted amino acid sequences of pheA (chorismate mutase/prephenate dehydratase), tyrA (prephenylate dehydrogenase), aroA (5-enolpyruvylshikimate-3-phosphate synthase), cmk (monophosphate kinase) and rpsA (ribosomal protein S1). Promoter or terminator sequences were not identified between these genes, which were transcribed in the same direction, suggesting that they are organised into a putative operon (Figure 3.5 A, shaded arrows). The translational stop signal (bold) of tyrA overlaps with the translational start signal (underlined) of aroA ATGA, indicating that these genes may be
translationally coupled. These sequence data are shown in Figure 3.5 B and have been submitted to the GenBank database under the accession number AF182427.

![ agarose gel electrophoresis of Clal digested pJCOS24 DNA with the corresponding Southern blot been shown in lane 2. ](image)

This gene arrangement is the same as that seen in one of the contigs of the genome sequence of *B. bronchiseptica* (strain RB50) currently being assembled at the Sanger Centre (http://www.sanger.ac.uk/projects/B_bronchiseptica). From this data the ORF's surrounding this region were determined and their relative positions can be seen in Figure 3.5 A. The predicted amino acid sequences from these ORF's were compared to the NCBI non-redundant protein database using the BlastX algorithm and identity was assigned based on homology. Putative promoter sequences consisting of -35 and -10 regions were found 169 bp and 144 bp upstream of the *gyrA* start codon respectively and inverted repeat terminator sequences were found 41 bp downstream of the *ihfA* stop codon indicating that the full operon containing *aroA* may include *gyrA, serC, pheA, tyrA, aroA, cmk, rpsA* and *ihfB*. 
Results

Figure 3.5  The *B. bronchiseptica* operon containing *aroA*. (A) Physical map of the chromosomal region containing the *aroA* gene of *B. bronchiseptica*. Shaded regions correspond to the DNA sequences determined during this study. Putative genes identified based on amino acid sequence homology with other known genes include: *gyrA* (DNA gyrase), *serC* (3-phosphoserine aminotransferase), *pheA* (chorismate mutase/prephenate dehydratase), *tyrA* (prephenylate dehydrogenase), *aroA* (5-enolpyruvylshikimate-3-phosphate synthase), *cmk* (monophosphate kinase), *rpsA* (ribosomal protein S1), *ihfB* (integration host factor B). Scale is given in kilobases. (B) Diagramatic representation of the sequencing strategy employed for pJMC25. The position of primers used and the subsequent sequence generated has been represented by arrows. Individual primer sequences are listed in Appendix II. (C) Nucleotide and deduced amino acid sequence of the *gyrA*-serC-pheA-tyrA-aroA-cmk-rpsA-ihfB* operon of *B. bronchiseptica*. Putative Shine-Dalgarno sequences are in bold, terminator repeats are underlined and -35 and -10 promoter regions are shown in red. DNA sequence data obtained from the 4.5 kb *ClaI* fragment contained in pJMC25 is shown as non-italicised text. The sequences surrounding this region was obtained from the *B. bronchiseptica* genome sequencing project at the Sanger Centre (http://www.sanger.ac.uk/Projects/B._bronchiseptica) and is shown in italics.
Results

1 kb

pJMC25

For Rev

GyrA

1000 2000 3000 4000

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ATTCCGTTAAA CCCACGGGAA CCGCCGGGAT CGCCATCTTG TAGATTGAC

GCAAGCCTGGC CCAGGGCGCCC CCAAAAGGAG CAAAGGATTAG CAAATTGGTAG AATTGGCGTG

CTTGCGGCGG GCCCGCGCGG GTTTCCTCC GTTTTTCCTA GCTTACTATC TTAAAGGACA

TTACCAGGTCT CGGAGCTATCG CTTAGACTAC ATTCTGATGC CACTGCCGCG GCAGATCTAT

AACCGGCGGG CCGCCGCGGG CTTTTCTTCC CTTTTCTTCC CCTTCTCTAC

K S A R I V G D V I G K Y H P H G D Q S

GTATACTGCC CCAATCGTCG CATCTCTGGC GACCAGATCG CCGGAGAC TTATCTGATC

VA Y D T I V R M A Q D F S M R Y M L V D
Results

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G K R T P I T E Y T R H G R T K G M I

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serC

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DSSGLTI
EQT
VQAVLDF

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WRA*

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TGACGACGCC
CGTTAGGCAA

rpsA
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CAACCTAATG
TCTTCCGTTT

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NLMSVSTSASIVGEN

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GTTCGCAGAA
AGCCTCAAGA

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3.2.2 Alignment of the deduced amino acid sequences of B. bronchiseptica genes

The predicted amino acid sequences of the B. bronchiseptica genes in the aroA operon were compared with other proteins in the NCBI non-redundant protein database using the BlastP algorithm. The optimal alignment of the most homologous sequences to the GyrA, SerC, PheA, TyrA, AroA, Cmk, RpsA and IhfB ORF’s was determined using the Clustal W alignment program. Results are depicted in Figure 3.6. The identity and similarity percentages of the homologous sequences from other bacteria used in the alignments is given in Table 3.1.
Figure 3.6  Clustal W alignment of the deduced amino acid sequences from *B. bronchiseptica* with homologous sequences from other bacteria. Amino acids identical to that of the *B. bronchiseptica* sequence are indicated by dots (.), whereas differences are shown. Dashes (−) represent gaps introduced into the amino acid sequence to optimise alignment. (A) Alignment of the deduced amino acid sequence encoded by the *gyrA* genes from *B. bronchiseptica* (BbgyrA), *Methylovorans* sp. (Methyl; Ac. aac34892), *Neisseria meningitidis* (Nmenin; Ac. a81089), *Serratia marcescens* (Smarce; Ac. aab95117), *Neisseria gonorrhoeae* (Ngonor; Ac. p48371), *Vibrio cholerae* (Vchole; Ac. e82221) and *E. coli* (Ecoli; Ac. p09097). (B) Alignment of the deduced amino acid sequence encoded by the *serC* genes from *B. bronchiseptica* (BbserC), *Pseudomonas aeruginosa* (Paerug; Ac. h83250), *E. coli* (Ecoli; Ac. p23721), *Salmonella typhimurium* (Styphm; Ac. p55900), *V. cholerae* (Vchole; Ac. g82233) and *Yersinia enterocolitica* (Yenter; Ac. p19689). (C) Alignment of the deduced amino acid sequence encoded by the *pheA* genes from *B. bronchiseptica* (BbpheA), *N. meningitidis* (Nmenin; Ac. h81196), *Pseudomonas stutzeri* (Pstutz; Ac. p27603), *Xanthomonas campestris* (Xcampi; Ac. g3153199), *Xylella fastidiosa* (Xfasti; Ac. b82572), *Campylobacter jejuni* (Cjejun; Ac. h81450) and *E. coli* (Ecoli; Ac. p07022). (D) Alignment of the deduced amino acid sequence encoded by the *tyrA* genes from *B. bronchiseptica* (BbtyrA), *N. meningitidis* (Nmenin; Ac. a81199), *Caulobacter crescentus* (Ccresc; Ac. aak24195), *Deinococcus radiodurans* (Dradio; Ac. a75434), *P. stutzeri* (Pstutz; Ac. aa047362), *Aquifex aeolicus* (Aqaeol; Ac. b70451) and *Streptococcus pneumoniae* (Spneum; Ac. aal0035). (E) Alignment of the deduced amino acid sequence encoded by the *aroA* genes from *B. bronchiseptica* (BbaroA), *B. pertussis* (Bpertu; Ac. p12421), *Klebsiella pneumoniae* (Kpneum; Ac. p24497), *Y. pestis* (Ypesti; Ac. q60112), *Y. enterocolitica* (Yenter; Ac. p19688) and *Salmonella galerium* (Sgaler; Ac. p22299). (F) Alignment of the deduced amino acid sequence encoded by the *cmk* genes from *B. bronchiseptica* (Bbcmk), *Haemophilus influenzae* (Hinflu; Ac. p43893), *E. coli* (Ecoli; Ac. p23863), *Bacillus subtilis* (Bsubti; Ac. p38493), *Mycobacterium leprae* (Mlepra; Ac. s72954) and *A. aeolicus* (Aqaeol; Ac. o67907). (G) Alignment of the deduced amino acid sequence encoded by the *rpsA* genes from *B. bronchiseptica* (BbrpsA), *N. meningitidis* (Nmenin; Ac. g81099), *P. aeruginosa* (Paerug; Ac. c83250), *E. coli* (Ecoli; Ac. p02349), *X. fastidiosa* (Xfasti; Ac. b82559), *V. cholerae* (Vchole; Ac. b82141) and *H. influenzae* (Hinflu; Ac. q48082). (H) Alignment of the deduced amino acid sequence encoded by the *ihfB* genes from *B. bronchiseptica* (BbihfB), *P. aeruginosa* (Paerug; Ac. q51473), *Pasteurella multocida* (Pmulti; Ac. q9cm18), *X. fastidiosa* (Xfasti; Ac. q9paq8), *N. meningitidis* (Nmenin; Ac. q9tjh3) and *V. cholerae* (Vchole; Ac. q9kqt4).
Results

Bbrcmk  VPRMRHERPIG  DLVDLARQFG  AGIEYLGQAG  YPPRLIGGGS  IRVDPAVPVE  GSJVSSQFHTA  177

BbaroA  LLMAPVLAR  RSQDITTEV  VGELISKFPI  EITLNLARFT  GSVSRDQGR  AFTIARDAY  237
Bpeptu  S.LAP---TV.AI  K.V.R  D.H.KT  E.ENQAYQ  R.IVGNQO  231
Kpnem  S.LAP---TV.AI  K.V.R  D.H.KT  E.ENQAYQ  R.IVGNQO  231
Ypesti  S.LAP---TV.AI  K.V.R  D.H.KT  E.ENQAYQ  R.IVGNQO  231
Yenter  S.LAP---TV.AI  K.V.R  D.H.KT  E.ENQAYQ  R.IVGNQO  231

MTAI  SG A....LCKAM  MTD  I.VA....AG  T..SSVSRGL  357
MI..V...SG  A....LCYAL  357
KLFKEIINNK  DL..QMAEGR  345
DEPAVAAVAQ  AKQLGWDVLD  SGLYRLTAL  AALRGLPAT  60

BbaroA  GWATRGRGVRV  AEGRLKAFD  ADFNLFDPA  MTAAELALVA  DFCPRRNLNG  SNRIVKEDRI  417

F. Cmk

Bbrcmk  MAISASAGAA  PVITIDGPTA  SOKGTIAHRV  AKQGLWVDVL  SGALGRLTAL  AALRGLPAT  60
Hinflu  MI.V...SG  A.LCYAL  .EK.YAL  .I.V...Q.KTL  50
Ecoli  MI.V...SG  A.LCYAL  .EK.YAL  .I.V...Q.KTL  50
Bsubti  MEKK  LS.A.A  A.S.V.EI  EKKSYYMJ  T.M.AITY  QENVVL  54
Mplea  MTKD  I.VA...AG  T.SSVSRG  SRE.ARY  T.M.MMT  .V.A.IDPA  53
Aqael  MK.A...S  S.V.KII  SQK.IPYE  T.LV.TY  VS.KFQK  50

Bbrcmk  DEPAVAAAVQ  ALDVFDPGD-  ---HVLYEGR  --DAG-HEIRQE  EVGNYASRIA  AYPVRQL  115
Hinflu  N.TDL.E.L.R  H.IQ.IPQN  GEVINIF.AM  .VS-RL  .TQ  ADA.KV  VPQK.S  109
Ecoli  S.D.LVLS  H.IQ.IPQN  GEVINIF.AM  .VS-RL  .TQ  ADA.KV  VPQK.S  114
Bsubti  EKL ELLK  R.IELITTK  DQGK  .FNT  IS.VQ  IA  KHR.S  113
Mplea  AA.IGESWM  KQVQLS.HD  ----RFG.GE  VS.S...T  TQAV.AVS  .I.A.VR  107
Aqael  I.DKLFSLFV  KVFVKPQGTK-  ----E.I.K  PVNEEDL  .S.KEL  SI.EF.ER  110

Bbrcmk  ERQRFAQPP  G-LVADGRDM  GTFVFFPSAL  KJLFVADVEA  RAQRRCKCRI  EKCGSNL  174
Hinflu  QL.QD.AKND  .......  N.VQ  L.D.SA.E  K.Y...Q  N.NQ.FAQ  168
Ecoli  R.E.L  .......  N.VQ  L.D.SA.E  K.Y...Q  N.NQ.FAQ  168
Bsubti  K.QLGEGK  VM...I  H.L.N.EV  L.S.E  K.YEENV  K.FVD.YT  172
Mplea  DL.QMAEGR  SV.VE...I  L.P  T.SP.T  R.ND.NV  AS.SADDY  167
Aqael  KLFKEINNK  Q.M...VE  A.HI.E.PV  K.VT.SPE  K.YE.K  L.YEVSFEE  165
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| **BbrpsA** | GELVTGTITG | KVGGVLTVTN | NIGAFPLGPG | LDLRPVKT | TPGQKLRE | KVIKDRKKN | 179 |
| **Paerug** | ———S ——— | ———T ——— | ———S ——— | ———I ——— | ———N ——— | ——— | 104 |
| **Ecoli** | ———S ——— | ———T ——— | ———K ——— | ———L ——— | ———E ——— | ——— | 104 |
| **Vchole** | ———A ——— | ———F ——— | ———Q ——— | ———Q ——— | ——— | ——— | 104 |
| **Hinflu** | ———A ——— | ———V ——— | ———Q ——— | ———Q ——— | ——— | ——— | 104 |

| **BbrpsA** | GEEVEVMVLE | IDEDRRRISL | GMKQCRQFPW | EEFATNFKR | DKVRGAIKSI | TDFGTVFVLP | 399 |
| **Nmenin** | ———S ——— | ———T ——— | ———S ——— | ———Q ——— | ——— | ——— | 325 |
| **Paerug** | ———S ——— | ———T ——— | ———S ——— | ———Q ——— | ——— | ——— | 324 |
| **Ecoli** | ———K ——— | ———T ——— | ———A ——— | ———I ——— | ———L ——— | ——— | 324 |
| **Vchole** | ———K ——— | ———T ——— | ———Q ——— | ———Q ——— | ——— | ——— | 324 |
| **Hinflu** | ———A ——— | ———N ——— | ——— | ——— | ——— | ——— | 323 |

| **BbrpsA** | DPWVLGAEV | PQGTRLFKG | TNLTDYGAFF | EVEAGIEGLV | HVSEMDWNTK | NVDPKRKVL | 359 |
| **Nmenin** | ———S ——— | ———T ——— | ———S ——— | ———Q ——— | ——— | ——— | 325 |
| **Paerug** | ———S ——— | ———T ——— | ———S ——— | ———Q ——— | ——— | ——— | 324 |
| **Ecoli** | ———K ——— | ———T ——— | ———A ——— | ———I ——— | ———L ——— | ——— | 324 |
| **Vchole** | ———K ——— | ———T ——— | ———Q ——— | ———Q ——— | ——— | ——— | 324 |
| **Hinflu** | ———A ——— | ———N ——— | ——— | ——— | ——— | ——— | 323 |

<p>| <strong>BbrpsA</strong> | GEEVEVMVLE | IDEDRRRSL | GMKQCRQFPW | EEFATNFKR | DKVRGAIKSI | TDFGTVFVLP | 419 |
| <strong>Nmenin</strong> | ———S ——— | ———T ——— | ———S ——— | ———Q ——— | ——— | ——— | 325 |
| <strong>Paerug</strong> | ———S ——— | ———T ——— | ———S ——— | ———Q ——— | ——— | ——— | 324 |
| <strong>Ecoli</strong> | ———K ——— | ———T ——— | ———A ——— | ———I ——— | ———L ——— | ——— | 324 |
| <strong>Vchole</strong> | ———K ——— | ———T ——— | ———Q ——— | ———Q ——— | ——— | ——— | 324 |
| <strong>Hinflu</strong> | ———A ——— | ———N ——— | ——— | ——— | ——— | ——— | 323 |</p>
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Table 3.1  
Relatedness of the genes used in the Clustal W alignments of the deduced amino acid sequences from *B. bronchiseptica* with homologous sequences from other bacteria.

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<th>Gene</th>
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<th>Identity (%)</th>
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#### 3.3 Construction of aromatic amino acid auxotrophic mutants of *B. bronchiseptica*

Ideally for the construction aromatic amino acid auxotrophic mutants of *B. bronchiseptica*, double cross-over deletion or insertion mutations were hoped to be produced. As *B. bronchiseptica* is relatively insensitive to a large variety of antibiotics, a number of researchers have produced specialised vectors specifically for the production of double cross-over mutations in *B. bronchiseptica*. These vectors allow for selection of the first recombination event then can have positive selection applied for the second recombination event that results in the removal of the plasmid vector leaving the mutated gene of interest stably inserted within the chromosome. One such vector is pSS1129 (a Gm<sup>r</sup> derivative of pRTP1 (Stibitz *et al.*, 1986)) and was used in the construction of a urease mutant of *B. bronchiseptica* (Monack and Falkow, 1993). This vector was requested from D. M. Monack but it was not provided. The alternate strategy we followed involved cloning the 1.3 kb *aroA* gene into the Ap<sup>r</sup> suicide vector pGP704Sal, then mutating it by inserting a Km<sup>r</sup> gene within the coding region of *aroA*. This construct was then mated into *B. bronchiseptica* and the initial homologous recombination event was selected with Km. As this system fails to provide positive selection for the second recombination event, it was envisioned that a second cross-over event would occur if the single cross-over transconjugate was continuously subcultured without Ap selection.
The 1.3 kb aroA fragment of pJMC6 was cloned as an EcoRI fragment into pGP704Sal forming pJMC9. pGP704Sal is a derivative of pGP704 that was digested with BglII and XbaI, Klenow treated, then re-ligated resulting in a plasmid lacking the following restriction sites from its polylinker; BglII, PstI, SalI, BamH1, HinDIII and XbaI. This plasmid can be mobilised from E. coli strain S17-1λpir into B. bronchiseptica via the RP4 oriT but cannot replicate in B. bronchiseptica because of the absence of the pir gene product supplied in trans by the host E. coli strain. When pJMC9 was subsequently digested with SalI and re-ligated, it resulted in a 90 bp deletion within the aroA gene fragment producing pJMC10. A 2.2 kb kanamycin resistance cassette was excised from pH45ΩKm with EcoRI, Klenow treated and ligated into the now unique SalI site of pJMC10, which had also been Klenow treated, to create pJMC13 (Figure 3.7). After conjugation between S17-1λpir containing pJMC13 and B. bronchiseptica K8744/lb, co-integrates were selected for on SS-X agar plates containing aamix, Km and Cp. Aro' mutants were identified by their reduced ability to grow on SS-X medium lacking aamix.

One such Aro' transconjugate was designated CMJ25. Analysis of CMJ25 chromosomal DNA demonstrated that the aroA gene had been disrupted through integration of pJMC13 into the chromosome (Figure 3.8). The labelled 1.3 kb PCR amplified aroA gene fragment reacted with a 5.2 kb fragment in Clal digested chromosomal DNA of K8744/lb. In the mutant strain CMJ25, the probe reacted with a band of 11.2 kb which corresponds to the Clal fragment containing integrated pJMC13 (Figure 3.9). Attempts to yield pJMC13 from CMJ25 (ie. detection of a double crossover event) by continuous subculturing in the presence of Km but without Ap selection were unsuccessful. Although using the construct in pJMC13 for mutation of aroA results in an intact aroA gene being formed, it was believed that this gene would not be expressed. As the translational stop codon of tyrA overlaps with the translational start codon of aroA it is thought these genes are translationally coupled. Therefore the newly formed aroA gene that resulted from integration of pJMC13 into the chromosome would not contain a functional Shine Dalgarno sequence.
Figure 3.7 Construction of the recombinant plasmid pJMC13 for homologous recombination with the aroA gene of K8744/1b. The 1.3 kb aroA gene of pJMC6 was cloned into the mobilisable suicide plasmid pGP704Sal forming pJMC9. A SalI deletion within the aroA gene produced pJMC10 and into this now unique SalI site a 2.2 kb kanamycin resistance cassette from pHP45ΩKm was cloned forming pJMC13.

Abbreviations: Ap, ampicillin resistance; f1 (+) origin, f1 filamentous phage origin of replication; lacZ, β-galactosidase gene; ColEl, plasmid origin of replication; mob RP4, origin of transfer; ori R6K, origin of replication; MCS, multiple cloning site or polylinker; Km, kanamycin resistance; (E), EcoRI; (S), SalI; (B), BamHI.
Figure 3.7  Construction of aro\textit{A} mutant CMJ25 via homologous recombination. Recombinant plasmid pJMC13 was introduced into K8744/1b via bacterial conjugation. Transconjugates which had undergone homologous recombination at the aro\textit{A} gene were selected for using Km (selecting for pJMC13) and Cp (selecting for \textit{B. bronchiseptica}). Aro\textsuperscript{+} mutants were identified by their reduced ability to grow on minimal SS-X medium. Only single cross-over mutants were identified.

3.3 Phenotypic characterisation of \textit{B. bronchiseptica} aro\textit{A}

The growth rate of CMJ25 in SS-X broth supplemented with aamix was similar to that of the wild-type strain K8744/1b. However, it was found that CMJ25 was also capable of growth in SS-X broth without supplementation, but had a significantly slower growth rate than that of K8744/1b or CMJ25 grown with aamix supplementation (Figure 3.10). Given that CMJ25 was able to grow in the absence of aamix, the ability of CMJ25 to revert to an Aro\textsuperscript{+} wild type phenotype was investigated. These tests involved culturing CMJ25 in aamix supplemented SS-X broth with and without Km selection. After overnight growth a dilution series of the culture was then plated onto SS-X medium with and without aamix
supplementation and with and without Km selection. Colonies that were seen to grow on unsupplemented medium were considered to be revertants. These tests showed that CMJ25 was reverting to a Aro+ phenotype at a rate of $10^{-4}$. No differences were seen between cultures grown with Km selection or without. Further investigations via Southern blot analysis found the aroA gene was disrupted in the revertant strains and hybridisation patterns were identical to the original mutant strain, CMJ25 (Figure 3.11). All revertant colonies displayed a characteristic colony morphology that was different to that of K8744/1b or CMJ25. A representative colony was chosen and designated CMJ25R for further characterisation.

Figure 3.8 Southern blot analysis of B. bronchiseptica K8744/1b and CMJ25. Chromosomal DNA was restricted with Clal and probed with the 1.3 kb PCR amplified aroA gene from K8744/1b. The Clal fragment containing aroA in K8744/1b was 5.2 kb (lane 1) and in CMJ25 was 11.2 kb (lane 2) which is consistent with disruption of aroA through single crossover integration of pJMC13 into the chromosome.
On unsupplemented SS-X agar at 37°C, wild-type strain K8744/1b grew to single colonies. CMJ25 on this medium displayed only slight growth on the primary streak and failed to produce single colonies. Full growth was restored upon the addition of aaMix to the medium. The revertant strain CMJ25R was able to grow fully on SS-X agar without supplementation indicating it no longer required aromatic supplementation to grow on minimal medium (Figure 3.12 A). The revertant strain CMJ25R exhibited a colony morphology that was typical of *B. bronchiseptica* in the Bvg-repressed growth phase. The wild-type strain K8744/1b and the mutant strain CMJ25, when grown on BG agar in the absence of modulators at 37°C, form small, domed, glistening haemolytic colonies. When CMJ25R was grown on BG agar under the same conditions it forms large, flat, nonhaemolytic colonies (Figure 3.12 B). Motility in *B. bronchiseptica* is specific to the Bvg-negative phase. Motility assays with K8744/1b and CMJ25 showed a non motile phenotype when grown in unsupplemented SS-X at 37°C, as expected. CMJ25R was found
to be motile under these conditions, again indicating that it is in a Bvg-negative phase (Figure 3.12 C). When grown under modulating conditions on SS-C minimal medium at 20°C, CMJ25 was able to grow to single colonies in a manner similar to that seen for K8744/1b and CMJ25R (Figure 3.12 D). These data suggest that when CMJ25 is modulated into a Bvg-repressed growth phase it no longer requires aromatic supplementation to grow. Furthermore, revertants such as CMJ25R most likely represent phase variants of CMJ25 that have undergone a mutation in bvg, permanently locking such strains into a Bvg-repressed phase. When in a Bvg-repressed phase, *B. bronchiseptica* does not express any *vags* and may therefore have less metabolic pressure being placed upon it. This may allow the growth rate to increase to that of the wild-type strain. Or alternatively, specific features of the Bvg-repressed phase of *B. bronchiseptica* may be allowing the bacterium to bypass actual dependence on the upper shikimate pathway for the production of aromatic compounds. Since growth of CMJ25 was never completely abolished in the absence of aromatic supplementation, expression of *aroA* or activity of its gene product cannot be ruled out by this study as an explanation for the phenotype displayed by CMJ25.

![Image of Southern blot analysis](image)

**Figure 3.10** Southern blot analysis of *B. bronchiseptica* K8744/1b, CMJ25 and CMJ25R. Chromosomal DNA was restricted with *ClaI* and probed with the 1.3 kb PCR amplified *aroA* gene from K8744/1b. The *ClaI* fragment containing *aroA* in K8744/1b was 5.2 kb (lane 1) and in CMJ25 was 11.2 kb (lane 2) which is consistent with disruption of *aroA* through single crossover integration of pJMC13 into the chromosome.
Results

3.4 Colonisation of murine lungs by *B. bronchiseptica* strains

3.4.1 Dose optimisation for *B. bronchiseptica* K8744/1b

It has previously been shown that different isolates of *B. bronchiseptica* can vary substantially in LD$_{50}$ for mice (Gueirard and Guiso, 1993). Therefore to establish a non-lethal dose of *B. bronchiseptica* K8744/1b that is still capable of inducing a persistent infection for BALB/c mice, animals were infected with three different dosages of the *B.
Results

*brachiseptica* K8744/1b wild-type strain. Symptoms of the infection and the lung colonisation profile were monitored over time. Mice that were given a dose of $10^5$ CFU’s showed no ill-effects. The colonisation profile for this dose showed bacterial numbers to increase 24 fold for the first 2 days post infection at which point numbers started to decline slowly. Bacteria were shown to persist for at least 35 days in the lungs (Figure 3.13). Mice that were given a dose of $10^6$ CFU’s displayed no symptoms for the first 8 h post infection. However, by day 1 mice had taken on a ruffled appearance and were less active. Over day 2 and 3 mice were still maintaining a ruffled appearance and had lost weight but had been observed eating. On day 4, one of the 35 mice infected was found dead but all others were seen to be improving. By day 6 all individuals looked to be in good health having a normal appearance and displaying regular activity and preening levels. From the colonisation profile (Figure 3.13) after the initial dose of $10^6$ cells, bacterial numbers in the lung increased 9 fold for the first 2 days. At day 4, bacterial numbers were at a similar level to those seen on day 2. After day 4 bacterial numbers were seen to decline at a similar rate as that observed for the $10^5$ dose. Bacteria were shown to persist at very low levels for at least 40 days. Mice that were given a dose of $10^7$ CFU’s displayed no symptoms for the first 8 h post infection. At 18 h post infection mice had begun to show severe symptoms. Individuals had taken on a ruffled appearance and had watery discharges coming from their eyes. Their breathing had become laboured and was readily observable. Activity levels had decreased and individuals remained huddled in their nest. Symptoms did not improve over the next day and early on day 3 mice were deemed moribund and were euthanased. The colonisation profile for this infection saw a rapid increase in bacterial numbers. Over the 3 days bacteria had increased over 40,000 fold at which point the trial was terminated (Figure 3.13). Based on these experiments a dosage of $10^5$ CFU’s was chosen to be used in future trials. This dosage produced a persistent infection lasting over 30 days, but did not exhibit noticeable symptoms or result in death. The colonisation profile generated using this dosage is similar to that seen by others using different isolates of *B. bronchiseptica* (Gueirard and Guiso, 1993; Gueirard et al., 1996; Jungnitz et al., 1998; Harvill et al., 1999).
Figure 3.13  Determining a non-lethal respiratory infection of *B. bronchiseptica* K8744/1b in BALB/c mice. Mice were infected intranasally with either $10^5$ (filled squares), $10^6$ (unfilled diamonds) or $10^7$ (filled circles) CFU’s on day 0. At each time point mice were euthanased, lungs removed, homogenised and the number of viable bacterial cells per lung was determined. The plot shows averages, plus and minus standard error for 3 to 4 mice per time point.

3.5.2 *Lung colonisation by B. bronchiseptica aroA*

It is currently not known what role, if any, the Bvg-negative growth phase and *bvg* repressed genes play *in vivo*. As the ability of CMJ25 to bypass the *aroA* mutation seems to be repressed by Bvg, this mutant was investigated to determine if it was able to express the required metabolic machinery during the course of an infection which would enable it to survive and successfully colonise the murine host. Mice were infected by the intranasal route with a sub-lethal dose of either K8744/1b or CMJ25 and the number of viable bacteria present in the lungs was determined at different time intervals (Figure 3.14). The
strains demonstrated an equal ability to establish a limited infection in the murine lung. During the first 2 days after infection, both strains rapidly multiplied then, over the next 30 days were slowly cleared from the lungs. To examine the in vivo stability of the aroA mutation in CMJ25, bacteria isolated from CMJ25 infected mice on day 4 and day 10 were examined and shown to be phenotypically identical to the inocula (i.e. haemolytic, Km\(^{+}\) and Cp\(^{+}\); data not shown).

**Figure 3.14**  Clearance from the murine lung of the parental *B. bronchiseptica* strain K8744/1b (filled squares) and the aroA mutant CMJ25 (unfilled triangles) following intranasal infection of BALB/c mice. Results represent the mean colony forming units per lung of 4 mice per time point for the respective strains. Error bars represent standard errors.

### 3.5.3 The serum antibody response of mice infected with *B. bronchiseptica*

The serum obtained during the course of *B. bronchiseptica* K8744/1b and CMJ25 infection was assayed for specific antibodies by ELISA against whole cell *B. bronchiseptica* as antigen. Specific IgG and IgM responses were observed soon after infection. For both
strains the specific IgG response steadily increased during the course of infection whereas the IgM response increased up to day 14 and day 18 then levels were seen to slowly decrease. Specific IgA antibodies were only detected at low levels, late in the course of infection (Figure 3.15 A and B).

As CMJ25 was able to colonise and persist in the lungs of mice in a manner similar to that of the wild-type organism, sera from animals infected with CMJ25 was examined to determine if it reacted uniquely with antigenic polypeptides that failed to be recognised by sera from animals infected with K8744/1b. Western blots were used to ascertain if there was a difference in the antigenic profile being recognised by sera from mice infected with either CMJ25 or K8744/1b. Whole-cell extracts from K8744/1b and CMJ25 as well as purified LPS from K8744/1b grown in SS-X and SS-C were separated by SDS-PAGE and probed with serum from mice 33 days after inoculation with CMJ25. Pre-immune sera from mice was unreactive (data not shown). Serum from mice infected with CMJ25 detected a number of large proteins that were either specific for a Bvg-positive growth phase or common to both Bvg-positive and Bvg-repressed phases of growth (Figure 3.14 C). Purified LPS from Bvg-positive and Bvg-repressed growth phases (Figure 3.15 C, lanes 5 and 6) was also reactive in western blots. A similar pattern of recognition was seen in western blots probed with sera from mice infected with the wild-type strain B. bronchiseptica K8744/1b (data not shown).
Figure 3.15  Comparison of the serum antibody responses of mice infected with *B. bronchiseptica* (K8744/1b) or *B. bronchiseptica aroA* (CMJ25). *B. bronchiseptica* specific antibody responses in the serum of mice infected with CMJ25 (A) or K8744/1b (B). Graphs represent specific IgG, IgM and IgA anti-*B. bronchiseptica* titres determined by ELISA. (C) Western blot of whole-cell lysates of strains K8744/1b (lanes 1 and 2) and CMJ25 (lanes 3 and 4) grown in SS-X (x) or SS-C (c) broth at 37°C and purified LPS from strain K8744/1b (lanes 5 and 6) grown under the same conditions. Lysates and LPS were separated on a 10% SDS-PAGE gel, transferred to polyvinylidene difluoride membrane and probed with pooled sera from four mice infected with CMJ25. Size of molecular weight markers in kDa are shown on the right.
Results II

4. Construction of *B. bronchiseptica aro*A *trp*E

4.1 Transposon mutagenesis of *B. bronchiseptica* aroA

When CMJ25 is modulated into a Bvg-repressed growth phase it no longer requires aromatic supplementation to grow. One possibility is that specific features of the Bvg-repressed phase of *B. bronchiseptica* may be allowing the bacterium to bypass actual dependence on the upper shikimate pathway for the production of aromatic compounds. In an attempt to identify gene(s) that may be responsible for this ability and to obtain an aromatic amino acid auxotrophic mutant of *B. bronchiseptica* unable to grow in minimal medium, CMJ25 was subjected to rounds of mutagenesis using the gentamycin resistant mini-transposon, mini-Tn5/Gm (Alexeyev et al., 1995). Out of 3,000 gentamycin resistant colonies screened, one was identified as being unable to grow in minimal SS-X medium and was designated CMJ60. When the growth of this strain was examined over time it was found to be unable to replicate in SS-X broth containing Km and Gm, in the absence of aamix supplementation. However in the presence of aamix in the growth medium, the growth rate of CMJ60 was similar to that of the wild-type strain (Figure 4.1).

To further characterise the specific growth requirements of CMJ60, the effect of removing single aromatic amino acid supplements was examined for this strain and compared to both wild type *B. bronchiseptica* and CMJ25 (Table 4.1). K8744/1b grew well under all conditions, CMJ25 recorded growth under all conditions but was considerably slower than the wild-type strain. Strain CMJ60 failed to grow when tryptophan was removed from the medium indicating that a gene involved in tryptophan biosynthesis had been mutated by the transposon.
Results

Figure 4.1 Growth of the parental *B. bronchiseptica* strain K8744/lb compared to mutant CMJ60. K8744/lb (triangles) and mutant CMJ60 (squares) were cultured in SS-X broth at 37°C (unfilled triangles or squares) or in SS-X broth supplemented with aromatic amino acids at 37°C (filled triangles or squares). CMJ60 was cultured in the presence of Km and Gm.

To identify the gene disrupted by the insertion of the mini-transposon, a cosmid library of *Sau*3A partially digested chromosomal DNA of CMJ60 was cloned into the *Bam*H1 site of pHC79 and transfected into *E. coli* 294 Rif. A recombinant cosmid containing the transposon was selected via gentamycin resistance and designated pJCOS60. After digesting pJCOS60 with either *Bam*H1 or *Sph*I, suitably sized fragments containing the gentamycin transposon were identified via Southern blotting using the radio-labelled gentamycin gene from pBSL202::miniTn5/Gm as a probe (Figure 4.2A). The 7.2 kb *Bam*H1 and the 7.4 kb *Sph*I fragments of pJCOS60 were excised from agarose gels and purified before being cloned into pBluescript forming pJMC61 and pJMC65 which were used for DNA sequence analysis (Figure 4.2B).
Table 4.1 Characterisation of the growth of \textit{B. bronchiseptica} strains on minimal SS-X medium lacking one of the five aromatic supplements being used to sustain growth of the mutant strains of \textit{B. bronchiseptica}.

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<tr>
<td>K8744/1b</td>
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</tr>
<tr>
<td>CMJ25</td>
<td>+/-</td>
</tr>
<tr>
<td>CMJ60</td>
<td>+/-</td>
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</table>

+  normal growth
+/- slow growth
-  no growth

4.2 Molecular analysis of the \textit{trp} operon of \textit{B. bronchiseptica}

4.2.1 DNA sequence analysis of the \textit{trp} operon

DNA sequence analysis revealed that the mini-transposon had disrupted the \textit{trpE} gene by inserting at base 901 (GenBank accession number AF266751). The gene \textit{trpE} encodes the enzyme anthranilate synthase, component I and catalyses the conversion of chorismate to anthranilate (Figure 4.3). Analysis of the region surrounding this gene was undertaken by examining the genome sequence of \textit{B. bronchiseptica} (RB50) currently being assembled at the Sanger Centre (http://www.sanger.ac.uk/projects/B_bronchiseptica). The putative identity of the ORF’s was determined through deduced amino acid sequence homology (Figure 4.4A). Upstream of the \textit{trpE} start codon, putative promoter sequences consisting of -35 (235 bp upstream) and -10 regions (211 bp upstream) were identified as well as a potential \textit{trpL} leader peptide that begins 190 bp upstream of the \textit{trpE} start codon and encodes 15 amino acids which includes a methionine start codon and four tryptophan codons which may act as a possible attenuation mechanism for the \textit{trp} operon. Included in the operon with \textit{trpE} are genes that code for other tryptophan biosynthetic enzymes, \textit{trpG}, \textit{trpD} and \textit{trpC} (Figure 4.3). Potential inverted repeat terminator sequences are located 41 bp down stream of the \textit{trpC} stop codon.
Figure 4.2  Subcloning the Gm' transposon from pJCOS60. (A) Lanes 1 and 2 shows agarose gel electrophoresis of BamH1 and Clal digested pJCOS60 DNA with the corresponding Southern blot being shown in lanes 3 and 4. λHinDIII DNA standards are indicated on the left in kb. The 7.2 kb BamH1 and the 7.4 kb Sphi fragments of pJCOS60 identified as containing the Gm' transposon has been indicated on the agarose gel with a black circle. (B) Cloning of the BamH1 and the Sphi fragments of pJCOS60 into pBluescript to form pJMC61 and pJMC65.
**Figure 4.3** The pathway of tryptophan biosynthesis. The branch point compound from the shikimate pathway for aromatic amino acid biosynthesis chorismate, is converted into tryptophan via a number of intermediate compounds. The names of the enzymes catalysing the various reactions are given and the genes encoding these proteins are shown in italics. Adapted from Pittard (1987).
Results

Figure 4.4  The *B. bronchiseptica* operon containing *trpE*.  (A) Physical map of the chromosomal region containing the *trpE* gene of *B. bronchiseptica*.  Shaded regions correspond to the DNA sequences determined during this study.  Putative genes identified based on amino acid sequence homology with other known genes include: *trpL* (leader peptide), *trpE* (3-phosphoserine aminotransferase), *trpG* (chorismate mutase/prephenate dehydratase) and *trpC* (prephenylate dehydrogenase).  Scale is given in kilobases.  (B) Diagrammatic representation of the sequencing strategy employed for pJMC65 and pJMC61.  The position of primers used and the subsequent sequence generated has been represented by arrows.  Individual primer sequences are listed in Appendix II.  Scale is given in bp.  (C) Nucleotide and deduced amino acid sequence of the *trp* operon of *B. bronchiseptica*.  Putative Shine-Dalgarno sequences are in bold, terminator repeats are underlined and -35 and -10 promoter regions are shown in red.  DNA sequence data obtained from the 7.2 kb *BamH*I and the 7.4 kb *Sph*I fragments contained in pJMC61 and pJMC65 is shown as non-italicised text.  The sequences surrounding this region was obtained from the *B. bronchiseptica* genome sequencing project at the Sanger Centre (http://www.sanger.ac.uk/Projects/B_bronchiseptica) and is shown in italics.
Results

A

trpL  trpE  trpG  trpD  trpC

B

\[
\begin{array}{c}
250 \quad 500 \quad 750 \quad 1000 \quad 1250 \quad 1500 \quad 1750 \\
\end{array}
\]

\[
\begin{array}{c}
\text{trpE} \\
\text{TrpER2} \quad \text{TrpER1} \quad \text{TrpE5} \\
\text{TrpE3} \quad \text{TrpE1} \quad \text{TrpER6} \\
\text{For pJM65} \quad \text{For pJM61} \quad \text{TrpER5} \\
\text{250 bp}
\end{array}
\]

C

\[
\begin{array}{c}
\text{CACTGGATTT} \quad \text{CCACGGCGAA} \quad \text{CGCAAGCCG} \quad \text{CAGCAGACCA} \quad \text{CCGGCGCGTG} \quad \text{AAAGCCCGCC} \\
\text{GTGACCTAAA} \quad \text{GGGTGGCGTT} \quad \text{GGGGCTGGGC} \quad \text{GGCCTGTTGT} \quad \text{GGCCGGCGAC} \quad \text{TTTCGGCGGC} \\
\text{GGTACGCCCG} \quad \text{GTGTCGACCC} \quad \text{AACACCGACG} \quad \text{TGGCACGTAC} \quad \text{ATGAACGCTC} \quad \text{GGCCCTCGAA} \\
\text{GCCATGCGGG} \quad \text{CAGCAGTGGG} \quad \text{TTGGTCTGTG} \quad \text{AGCGGTCGATG} \quad \text{TACTTGCGAG} \quad \text{CAAGGAGGTT} \\
\text{60} \\
\text{GTGACCTAAA} \quad \text{GGTGACCGTT} \quad \text{GGGGCTGGGC} \quad \text{GGCCTGTTGT} \quad \text{GGCCGGCGAC} \quad \text{TTTCGGCGGC} \\
\text{GGTACGCCCG} \quad \text{GTGTCGACCC} \quad \text{AACACCGACG} \quad \text{TGGCACGTAC} \quad \text{ATGAACGCTC} \quad \text{GGCCCTCGAA} \\
\text{GCCATGCGGG} \quad \text{CAGCAGTGGG} \quad \text{TTGGTCTGTG} \quad \text{AGCGGTCGATG} \quad \text{TACTTGCGAG} \quad \text{CAAGGAGGTT} \\
\text{120} \\
\text{CGGTACGCCCG} \quad \text{CCGGCGCGGC} \quad \text{AAAGCCCCCG} \quad \text{CCGGCGCGGC} \quad \text{AAAGCCCCCG} \quad \text{CCGGCGCGGC} \\
\text{CCAAATGCAC} \quad \text{GGTTTACGCG} \quad \text{M R} \quad \text{AACGCCTCGT} \quad \text{TTGCGGAGCA} \quad \text{N A S} \\
\text{GGGTTACGCG} \quad \text{CCGCAACCAC} \quad \text{TTGGTCTGTG} \quad \text{AGCGGTCGATG} \quad \text{TACTTGCGAG} \quad \text{CAAGGAGGTT} \\
\text{240} \\
\text{TTCCCGGGCCC} \quad \text{TGGACGTATC} \quad \text{TTCCCGGGCCC} \quad \text{TGGACGTATC} \quad \text{TGGACGTATC} \quad \text{TGGACGTATC} \\
\text{CGGTACGCCCG} \quad \text{CCGGCGCGGC} \quad \text{AAAGCCCCCG} \quad \text{CCGGCGCGGC} \quad \text{AAAGCCCCCG} \quad \text{CCGGCGCGGC} \\
\text{GCCATGCGGG} \quad \text{CAGCAGTGGG} \quad \text{TTGGTCTGTG} \quad \text{AGCGGTCGATG} \quad \text{TACTTGCGAG} \quad \text{CAAGGAGGTT} \\
\text{300} \\
\end{array}
\]

trpL

\[
\begin{array}{c}
\text{CCAAATGCAC} \quad \text{AACGCCACGC} \quad \text{TGGGCGGCGT} \quad \text{CCGCTGTTGT} \quad \text{GGGCGCCGGC} \quad \text{AGATGTCGAC} \\
\text{GGGTGTCGTC} \quad \text{GGGGCTGGGC} \quad \text{GGCCTGTTGT} \quad \text{GGCCGGCGAC} \quad \text{TTTCGGCGGC} \\
\text{GGGTCAAGCCG} \quad \text{CCGCAACCAC} \quad \text{TTGGTCTGTG} \quad \text{AGCGGTCGATG} \quad \text{TACTTGCGAG} \quad \text{CAAGGAGGTT} \\
\text{180} \\
\end{array}
\]

trpE

\[
\begin{array}{c}
\text{GTACGAGACC} \quad \text{CGACCATGCT} \quad \text{GAACGTGAAT} \quad \text{TCAAGCGGCT} \quad \text{GGCGCGCCAA} \quad \text{GGCTACGAA} \\
\text{CATGTCCTG} \quad \text{CTCTGTCGTC} \quad \text{ATTTTGCCGA} \quad \text{TACCGGATAC} \quad \text{CGGACGCGTT} \quad \text{CCGATGTTG} \\
\text{GCATTCCCTT} \quad \text{GGTCGCCGAA} \quad \text{ACCTACCCTG} \quad \text{ACCTACGAC} \quad \text{GGCCGCTGGG} \quad \text{ATCTACGCT} \\
\text{CTGTAAGGGGA} \quad \text{CAGGCACCCT} \quad \text{TGGATCGGCG} \quad \text{TGGAGCTGGT} \quad \text{GGGCGACCG} \quad \text{TACAGTGGAG} \\
\text{R I P L V A E T Y A D L D T P L A Y I L} \\
\text{AGCTGCGCCCA} \quad \text{CAGCAGCGCC} \quad \text{CGATGACGC} \quad \text{CTGATCGGAG} \quad \text{TGGATCGTGG} \\
\text{TCGGCTGCTG} \quad \text{GGTACCTGCG} \quad \text{CCGACTACGC} \quad \text{GGCCACGC} \quad \text{AGGCACGC} \\
\text{K L A H S G P Q G G R M S C L M E S V V} \\
\end{array}
\]
Results

TCCGTGCTGA GCGACACCGG CCATGGCTGTG ATGCGCAACT TCCTGACCCG CTGAACCCCT 2460
AGGCCGACGT CGCTGTCGTCC GGTACCACGC TACGGTGTGGT GAGGCTGACT GCCATGACCC 2520
AGGACATGCG TGCCGGGCTGG GCTGACGACG ATCCGCTTGTG ATGCGCCAGC GCTGACGACG 2580
GAGATCTTCC AGGACAGAAAAT GCTGACGACG ATCCGCTTGTG ATGCGCCAGC GCTGACGACG 2640
AGGCCGTAGC GCAGAGCCGT CTGCTGCTGG GACGCTGGGGG CTGTCGTGACT GGGCGACGTA 2700
ATCACGCCGCG CCGCGGCTCC CTACGCTTGGT ATGCGCCTGC TGATGCGCGG CGAGCTGTCG 2760
TAGTTGCCGCG CGGCGACGCC CTACGCTGCTG ACGGCTGGGG GCTCGACAGC ELS 2820
ACCACCGCCCA TGGTTCGTGCC GCGCGGCTGG GACGCTGGGGG CTGTCGTGACT GGGCGACGTA 2880
GTCTGTCGAC GCGTGGCCGG GCGCGGCTGC ACCGCTACGT GCTCCGTGCTG CACGACCTTC 2940
GCCACCGCCCA TGGTTCGTGCC GCGCGGCTGG GACGCTGGGGG CTGTCGTGACT GGGCGACGTA 3000
GCCACCGCCCA TGGTTCGTGCC GCGCGGCTGG GACGCTGGGGG CTGTCGTGACT GGGCGACGTA 3060
GAGTGCGGGC AGGATTTGGCC GCTGACGAGC GGGCGCGCAG CTTGGAGCGC GCAGCAGCCC 3120
GGCCGGGTGG TCACCGGCTGA CTTCTTGCAA CGGCGCCACG CGTTCCTTAA CCCCCACGCG 3180
GCCACCGGTGC CTGATTTGGCC GCGCGGCTGG GACGCTGGGGG CTGTCGTGACT GGGCGACGTA 3240
GCCACCGGTGC CTGATTTGGCC GCGCGGCTGG GACGCTGGGGG CTGTCGTGACT GGGCGACGTA 3300

S V L S E H G  HAL  M R N F L T R *
trpD
S V L S E H G  HAL  M R N F L T R *

I T A A A Q V M R E  FA T  P V V T P N P

T A M F V A  AAA  G V P  TAKE  G N R

S A S S S S G  SAD  V L E  ALGA  N L Q

N A I L N Q L M

G V F V H P D L G V I Q V R V L R L G S

G V F V H P D L G V I Q V R V L R L G S

R H V L V H G K D G M D E A S L G A A

T M V G E L K D G V V R E Y E I H P E D

Y G L S M M S N R G I K V S N R E E S R
4.2.2 **Alignment of the deduced amino acid sequences from** *B. bronchiseptica* **trp genes**

The predicted amino acid sequences of the *B. bronchiseptica* genes in the *trp* operon were compared with other proteins in the NCBI non-redundant protein database using the BlastP algorithm. The optimal alignment of the most homologous sequences to the TrpE, TrpG, TrpD and TrpC ORF's was determined using the Clustal W alignment program. Results are depicted in Figure 4.5. The identity and similarity percentages of the homologous sequences from other bacteria used in the alignments is given in Table 4.2.
Figure 4.5  Clustal W alignment of the deduced amino acid sequence encoded by the trp operon of *B. bronchiseptica* with homologous sequences from other bacteria. Amino acids identical to that of the *B. bronchiseptica* sequence are indicated by dots (.), whereas differences are shown. Dashes (-) represent gaps introduced into the amino acid sequence to optimise alignment. (A) Alignment of the deduced amino acid sequence encoded by the *trpE* genes from *B. bronchiseptica* (*BbtrpE*), *Pseudomonas syringae* (*Psyri*; Ac. p21689), *Acinetobacter calcoaceticus* (*Acalco*; Ac. p23315), *Aquifex aeolicus* (*Aqscol*; Ac. o66849), *Rhodobacter sphaeroides* (*Rspiar*; Ac. p95646), *Clostridium thermocellum* (*Ctherm*; Ac. p14953), *Mycobacterium tuberculosis* (*Mtuber*; Ac. o06127) and *Arthrobacter globiformis* (*Aglobi*; Ac. p96556). (B) Alignment of the deduced amino acid sequence encoded by the *trpG* genes from *B. bronchiseptica* (*BbtrpG*), *Nitrosomonas europaea* (*Neurop*; Ac. t44524), *Pseudomonas putida* (*Pputid*; Ac. nnps2p), *N. meningitidis* (*Nmenin*; Ac. h81135), *Salmonella typhimurium* (*Styphm*; Ac. p06193), *Acinetobacter calcoaceticus* (*Acalco*; Ac. p00902) and *Klebsiella aerogenes* (*Kaerog*; Ac. p06194). (C) Alignment of the deduced amino acid sequence encoded by the *trpD* genes from *B. bronchiseptica* (*BbtrpD*), *N. meningitidis* (*Nmenin*; Ac. a81136), *X. fastidiosa* (*Xfasti*; Ac. c82835), *N. europaea* (*Neurop*; Ac. t44525), *P. aeruginosa* (*Paerug*; Ac. p20574), *A. calcoaceticus* (*Acalco*; Ac. p00500) and *Methanococcus jannachii* (*Mjanna*; Ac. q57686). (D) Alignment of the deduced amino acid sequence encoded by the *trpC* genes from *B. bronchiseptica* (*BbtrpC*), *P. aeruginosa* (*Paerug*; Ac. p20577), *N. europaea* (*Neurop*; Ac. t44526), *X. fastidiosa* (*Xfasti*; Ac. a82833), *A. calcoaceticus* (*Acalco*; Ac. p00911), *Bradyrhizobium japonicum* (*Bjapon*; Ac. p94327) and *N. meningitidis* (*Nmenin*; Ac. h81216).
A. TrpE

BbtrpE --- --- MTE LEPKALAAGQ YNIRPLVAQT YADOLTPLAI YLKLHASSQF GRRMCLAMES 53
Psynin ---- NR E...IR...E... AR... L.F...S... L.D...Q...N... Y.L... 48
Acalco ---- MTSL TQ.EQ.KTA... T...VYQR... L.T...SV...Far.KDY... --TOAYF... 49
Aqueol --MLNLNS EVRELSN... V...VTL... LV.TE...S... F...KEK... --QPNI... 52
Rpspher ---- MTSF ES.ERG.KWA... Q...QIV.R... RL...T.VSL... M...EAR... --TTDFML... 49
Ctherm ---- MFYT DEVKIM.KD... I...VTM.V... ME.ISL... FKRPEE... --SSCFL... 50
Muterb MHADDLAAT.S R.DFR.L.I.AE HRV.VTVRKEN... L...SE...S...AR...AN... --RPFTF... 56
Aglobi MQLUGIISGP... EREFEL... AHR...SRLV...L...R.AE...L...AGL... --R...QG... --QFTFP... 56

BbtrpE VUGVRFFGNY SFGLPRTAV IRASGSTTEV LQDGHVIAETH E----GDLPA FIESQYARFK 109
Psynin .Q...KW... .I...C... M.VH.HVVS... TH...VEI.SL... DV----E... D.VT.FK... 105
Acalco .E.N...WA... .I...GES... FSCNGAQT... KNAQGNIT.Q SC----A.FQ Y.RD...SQ... 106
Rpspher .I...KW... .I...GNS... LTRGKQIV...Q... NERAFFRDKQA PQFFE...V.E...TLRALI...Esr... 109
Ctherm .E...KWA... .I...KPNFL... VESQION.II... REINGSGRSVP... ----...N.VZ I.KGIMG... 106
Muterb AEN.RSWS.W AG.P.A LTVRESQAGAV GAVPKDAPI G----R... ALQVTELLA... 112
Aglobi AAV.GAWS... --SRS.AT LTTKQDQAGAW... GEPGAPVPGV... D--N...VD A.RDITEALR... 112

BbtrpE VA----LRPGM PRFPGCLAYG FGFYSTVVRHE QGRPLPAVFK PFMDGGEEPTD IMLHVDUEL... 166
Psynin VP...XMH .I...ALV... .I...Q...V...X...C.V... K...ANCNP.D... --L.V... L.MVS.AV... 158
Acalco .F...KDTLF .I...HNTTD... .E...Q... M...SNVPEAD... -----V...L... WMMILSKTV... 162
Rpspher IE----MPADL PIAA.F... L...MI.LV... HLP.IN...D... --L.L... AV.MRPSVSA... 160
Ctherm GN--.N.L... .N...AV... .LI...Y... NLNPVNP----ED D--.M...L ECFHMPF...VL... 157
Muterb T.DRQE..E... EL.S..MV.F... A...M.RL... RLERF...--E...D... --E...LCL... LATDVA... 166
Aglobi TDR----.PO.L DTS.P.V.F... L...WE...L... KTR.P...ED----EQE...L... NA.LN.TM... 163

BbtrpE -----ERRD FOKADYLAAV ABAKYEAIAG DNVQTVQGQVQ IAKFFPRDAPL SYLRA...LRSN... 273
Psynin ADP--VF.SS .TQG.E... DITQ.L...Q... L...QPS.R... MSID.KA...I... D...CF... 270
Acalco ---- HFSCL TG.KEF.ES... ETV...R... .V...HR... MVSD.DGD... QV...H... 268
Aqueol FK.--.W.SN T.EFEFDI... Q...I...Q... L...VLS.R... FR.RKGNM... NI...V...F... 271
Rpspher --EM.SN .TSEG.K... EK...D.R... IF...VPS.R... W.QD...LP.F A...S...KT... 273
Ctherm KYNELAVPSN IS.E.FCRN... LK...Q.RD... IF...VLS.R... LCVTENENF... NI...V...I... 273
Muterb ----RPEPRH AQ TRVRE...G.I... EYLVQD... EAF...VPS.R... FEMOTDVOD... IV...I...V... 272
Aglobi ----DFAASQOQER WNEPE...L L.D...AV.E.D... FIE.EVSR... FEMECGAS... D.V...N...VT... 280

BbtrpE BPSYMYFW---- ----FDGFQ VVGASPEILV RQEQQIDDGQ... TRSQTISIRPL ATGKRTKGTPG... 327
Psynin T.F...T.F... T...N...H... S...S...V... .V...DN... --V.D... I..P...A...E... 316
Acalco ---- LPVQQQ HTLDWTMFH... I...S...S... L.DG...----TA...V... TA.Q...Y... 320
Aqueol ---- YLD... ----DIQL... L...LM... ----L...Q... E...R...E... R...ET...I... 317
Rpspher ...F.F...F... ...F...G... ...LDR... ----EV...V...I... A...A... 319
Ctherm ---- ...VLK ......G.YR II...S.M... V...NG... ----IVETC.I... R...I... 319
Muterb ---- LLQV PNSDGAS... I...S...A... TVHEG... ----WA...TH...I... W.R...R... 332
Aglobi ----IFSIL EDADAG-QQYS I...S...A... TVQGE... ----DVITH... I...S...K... P...V... 332

BbtrpE EEDLALAEAL QDKEFRAEN VMLIDLARND VGRAGEISVG RVSĐMVATI... YSHVMHLVSN... 387
Psynin A.....EED... LS...H.I... L...G... --S...T... K...L...TEK... N...I... 376
Acalco ---- EQ... L.E.I... L...G... ----SK.K... Q.Т.R...I... N...I... 380
Aqueol ---KR.EE... LS.E... L...V... S...I...K...T... E...K...ENF...I... I...D... 377
Rpspher ---- R.K...L... L...L.G... ----K...T... PTEKFI... I...I... K...I... 379
Ctherm ---- E.EK... LS.E... L...V.G... S...IF...K... I.A...K...N.L...H... V...T... 379
Muterb D.VL.EK... D...L...V.G... S...L...CFF.T... T...E.YSH... ----T... 392
Aglobi D.K.A.E... L.Q... L...V.S... LSK.CVA... D...TQ.F.EV... F...I...T... 392
### Results

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### B. TrpG

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B. TrpG
C. TrpD

**BbtrpD**
MTIAAEAELT RCIEHREIFH DEMLHMLRL MRGELSPQAIA SALLMGLRVK KETVGEITNA 60
Xfasti ---.S.T.P.K... .C...LQ .V.Q... .NA... GTHV .I.A... .AG 60
Neurop ------------- .C...A... .IE...A... .S.N... VMT .V.T... .R.S... 38
Acalco --.N.Q...N .H.TKINHI.TQ .AQ...EDV... .Q...ATEA .G...M... G.SD... 58

**BbtrpD**
AQVNRERATP VVTPNFQDLL DMCGTGGDS HTFNTSSTAM FVAAAGVPI AHNHRASS 120
Nmnnin ---A....P..E--- .PLE.AEG... .K....S... .S...S 119
Xfasti .T...SR... .N.QORT... .W...C... .T...T 120
Neurop ---.L.VR... .IE...DAS... .T...C... .S...Q... .G...G 98
Paerug VA...L.DG .QL.TL.K.V... .V...A...I...V.S.A...S...V...G... .A...G 118
Mjanna .KI...SL... .INP...V.PK... .T...NL...APA...VS.Y...P...KA... 115

**BbtrpD**
SSGSADVLEA LGANLQPITPE EVACVAAATG IGFMFPAHHH GAKMNVAAR KelGVRTIPN 180
Xfasti ---.STE...Q..L... .V.GCI... .V...I... .P...QV.S... .M...M... 180
Neurop ---.V...Q...Q... .I.I...Q.D... .I.TS.RV... .F...H... .V...V... 158
Paerug ---.A...Y.S.E--- .R..DVT... .V...QV... .K...Y...G.P... .L...L 178
Acalco SL...Q .A.I...D.MQ... .TER...REM... V.L...L...K...Y...VGP... .I...S... 178
Mjanna .V...NVPIT... .R.K..EK.I...L..HFP...P...F.T...I...V...I... 175

**BbtrpD**
ILGPLNPGAC AANQLMIGVFH PDLVQIGQVRV LERLGSRLVL VVHHQGDMHE ASLAGAATMVG 240
Acalco L...VR... .VR...V... .K...SQT...M...E...D...POAQ...E...K...GRKKE... AEQK...AELIV 297
Mjanna V...N...N... .NY...YD... .EK..TEK... .L..N...LGK...G...I.F.E...EVEG ---AK...X...V... 290

**BbtrpD**
ELKGVDREVY EIHPEDYGLS MMS-NGRGIKV SNEESRALV IEALDNVDG-- ---VARDVA 295
Xfasti ---.R.G...R... .R...V...A... .VS...MA... .VSP...ISN.Q... .E...PA...EML...LVD...A...Q...P... ---P...L.V... 295
Neurop ---.NA... .SVQ...E... .RAA...A-TS...Q... .N.T.DAQ.ML...LSV...H...P... ---P...L... 273
Paerug ---.VR...V...K... .V.R...K... .SQT...M...E...D...POAQ...E...K...GRKKE... AEQK...AELIV 297
Acalco N...T.W VLN...VN...P SQT...-S...I... EDSN...K... .K.D...K...GKSDS... TGEK...ANM...273
Mjanna ---.R...N.IK.S--- .Y...E.Y...K... KAK...ED...RG...GDA...NAXI...G...IFE...EVEG ---AK...X...V... 290

**BbtrpD**
LNAGLAIYAG NKADSIPAEAL ALAFETISNG SARAALKFFC AYTRKFQK-- ---343
Xfasti ---.V...V... .V.QD.GH.V... .VLFD...R...DI...DRY... .RP...VY... ---344
Neurop ---.V... .V... .VAR...W... .ET...DMLAS...A.K.Q.MQA...E...SNOQVA... ---321
Paerug ---.M...P... .D.L.T.LH...I...Q...HDLHT...L...E.MD... .AVV...EEN...AQ... ---349
Acalco ---.G...VS... .L.T.YQKV...V... .HDI.YG... .Q...L.E.MI.S...E...KALKYNA... NN...349
Mjanna ---.AF...I... .EE.KDVE...I... .K..EKS.DE... .K.L...K...DI...E.Y.BG... ---336

D. TrpC

**BbtrpC**
MNDPABA KILAVKAEV ATARQMSEAR ELLREARDAQR DVRQFAQAIE DKSQGRGAV 56
Paerug ---.MSVPTV.Q... .R...R... .E...AR...E...L.RS.D...AP...N.L...EARRKKE... 58
Xfasti ---.SN...T... .I.WV.I...I... .EL.LH...Q... .LVARCADLP TP...G.LQ...AT.AH.D... 56
Byjapn ---.S...T... .ETY.R...I... .A.KRAQPI.S...V.A...K.TQ.KG...AP...L..R...K...AHTN.DF...L... 56
Nmnnin ---.T...N... .T...Q... .AQKA...AE... .HIRTLEAB.E...A...P...S...IDS.R...G.HRLNL... 56

**BbtrpC**
IAEIKKASPS KGVLRNFDPP AEAAASYAHM GAACLSSVLTD QVFQGSHDN LRRARAACS 116
Acalco ---.V... .LI...A... .POQ.KA...A... .T...P...S...H.LH...MVA...T... 116
Byjapn ---.V... .LI...R... .POQ.KA...A... .T...P...S...H.LH...MVA...T... 116
Nmnnin ---.V... .LI...R... .POQ.KA...A... .T...P...S...H.LH...MVA...T... 116

119
### Table 4.2
Relatedness of the genes used in the Clustal W alignments of the deduced amino acid sequences from *B. bronchiseptica* with homologous sequences from other bacteria.

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<th>Gene</th>
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<th>Identity (%)</th>
<th>Similarity (%)</th>
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<td>64.4</td>
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<td><em>A. calcoaceticus</em></td>
<td>47.7</td>
<td>57.1</td>
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<td><em>A. aeolicus</em></td>
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<td>56</td>
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<td></td>
<td><em>R. sphaeroides</em></td>
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<td>56.6</td>
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<td><em>C. thermocellum</em></td>
<td>39.8</td>
<td>52.9</td>
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<td><em>M. tuberculosis</em></td>
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<td><em>A. globiformis</em></td>
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4.3 Level of attenuation of *B. bronchiseptica aroA trpE*.

4.3.1 *In vitro intracellular survival of B. bronchiseptica aroA trpE.*

The ability of the parental wild-type strain K8744/1b and the *aroA trpE* strain CMJ60 to survive within the macrophage-like cell line J774A.1 was investigated. Compared to the parental strain, CMJ60 displayed significantly reduced abilities to invade and survive J774A.1 cells after both 2h (P<0.05) and 24h (P<0.01) post-infection respectively (Figure 4.6).

![Figure 4.6](image)

**Figure 4.6** Intracellular survival of the parental *B. bronchiseptica* strain K8744/1b (filled) and the *aroA trpE* mutant CMJ60 (unfilled) at 2 and 24 h in the macrophage-like cell line J774A.1. Results represent the mean colony forming units per well of triplicate wells for the respective strains. Error bars represent standard deviations. Variation between K8744/1b and CMJ60 was considered significant at P≤ 0.05 (*).

4.3.2 *Survival of B. bronchiseptica aroA trpE within the murine respiratory tract.*

The level of attenuation of CMJ60 following experimental intranasal infection of mice was examined. Mice were infected with a sub-lethal dose of either K8744/1b or CMJ60 and the number of viable bacteria present in the lungs was determined at different time intervals (Figure 4.7A). Compared to K8744/1b, the mutant strain CMJ60 was significantly
Results

attenuated in its ability to survive in the murine respiratory tract. CMJ60 failed to replicate in the murine respiratory tract and was cleared more rapidly than the wild-type strain K8744/1b. Reduction of cell numbers to undetectable levels from the lung was achieved by day 35 for CMJ60 and day 45 for K8744/1b. To examine the in vivo stability of the mutations in CMJ60, all bacterial colonies isolated from CMJ60 infected mice on day 16 were examined and shown to be phenotypically identical to the inocula (ie. haemolytic, auxotrophic, Km\(^t\), Gm\(^t\) and Cp\(^t\); data not shown) suggesting 100% in vivo stability of the vaccine strain for at least 16 days post-immunisation. The serum obtained during the course of the murine *B. bronchiseptica* K8744/1b and CMJ60 infection was assayed for specific antibodies by ELISA against whole cell *B. bronchiseptica* K8744/1b as antigen. For the wild-type infection, specific IgM was found to steadily increase from day 2 until day 18, after which titres started to decline. Specific IgG was not seen until day 9 with titres increasing up to day 26 where they leveled out. Specific IgA antibodies were only detected at low levels, late in the course of infection (Figure 4.7B). However, for the CMJ60 infection specific IgM only increased slightly up until day 10 at which point titres started to decline. Only slight increases in specific IgG titres were seen from day 16 to day 35 at which point levels started to decline. A small increase in specific IgA was seen on day 35 but were again undetectable by day 45 (Figure 4.7C).
Figure 4.7  Clearance from the murine lung of the parental *B. bronchiseptica* strain K8744/1b (filled squares) and the *aroA trpE* mutant CMJ60 (unfilled triangles) following intranasal infection of BALB/c mice (A). Results represent the mean colony forming units per lung of 4 mice per time point for the respective strains. Error bars represent standard deviations. Variation between K8744/1b and CMJ60 was considered significant at P< 0.05 on days 2, 4 and 9. *B. bronchiseptica* specific antibody responses in the serum of mice infected with K8744/1b (B) or CMJ60 (C). Graphs represent specific IgG (solid bars), IgM (dotted bars) and IgA (cross-hatched bars) anti-*B. bronchiseptica* titres determined by ELISA. Results represent the titres of pooled sera for 4 mice per time point.
Results III

5. *B. bronchiseptica aroA trpE* as a live vaccine

5.1 Immune responses following vaccination and boosting with *B. bronchiseptica aroA trpE*

As demonstrated in the previous chapter, mice infected with CMJ60 failed to elicit a strong humoral response against *B. bronchiseptica* whole cell antigens. Here we examine the effects of booster immunisations on the magnitude of the immune responses generated. The anti-*B. bronchiseptica* immunological response of mice were examined in non-immunised mice and in mice intranasally immunised with $1 \times 10^5$ CMJ60 viable cells according to Table 5.1. Immune responses were determined 10 days after the last dose was administered.

**Table 5.1** Outline of the vaccination and boosting protocol followed to examine the effects of multiple doses of CMJ60 upon the elicitation of immune responses in BALB/c mice.

<table>
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<th>Group</th>
<th>Day 0</th>
<th>Day 14</th>
<th>Day 28</th>
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<tbody>
<tr>
<td>0 Dose</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>1 Dose</td>
<td>$10^5$ CFU's</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2 Dose</td>
<td>$10^5$ CFU's</td>
<td>$10^5$ CFU's</td>
<td>NA</td>
</tr>
<tr>
<td>3 Dose</td>
<td>$10^5$ CFU's</td>
<td>$10^5$ CFU's</td>
<td>$10^5$ CFU's</td>
</tr>
</tbody>
</table>

Abbreviations: NA, not applicable; CFU's, colony forming units.

Non-immunised mice did not exhibit significant anti-*B. bronchiseptica* immune responses. In contrast the serum anti-*B. bronchiseptica* IgG titre increased by over 10-fold following a single boost at day 14 ($P<0.05$) and over 100-fold following booster immunisations at day 14 and 28 ($P<0.05$). A moderate increase in serum IgA levels was also observed (Figure 5.1A). The anti-*B. bronchiseptica* IgG titre was significantly increased in lung lavages following a single immunisation ($P<0.05$). Anti-*B. bronchiseptica* IgG and IgA titres were significantly increased in lung lavages following two ($P<0.05$) or three doses ($P<0.05$)
Results

Compared with non-immunised mice, vaccinated animals exhibited a significant increase (P<0.05) in the number of *B. bronchiseptica*-reactive spleen cells in lymphoproliferation assays only after the second boost (Figure 5.2).

![Figure 5.1](image)

**Figure 5.1** Levels of *B. bronchiseptica*-specific IgG (filled bars) and IgA (unfilled bars) in mouse serum (A) and lung lavage (B) samples in non-immunised mice (0 doses), mice intranasally immunised with CMJ60 (1 dose), mice intranasally immunised then boosted with CMJ60 on day 0 and day 14 (2 doses), and mice immunised then boosted with CMJ60 on day 0, day 14 and day 28 (3 doses). Results represent the titres of 4 mice per treatment. Error bars represent standard deviations. Variation between non-immunised and vaccinated mice was considered significant at P≤ 0.05 (*).

5.2 Protection afforded to mice following vaccination with *B. bronchiseptica aroA trpE*

5.2.1 Clearance of *B. bronchiseptica* after vaccination and challenge

The level of protection afforded following intranasal immunisation and boosting of mice with 1 x 10⁵ CMJ60 viable cells on day 0, day 14 and day 28 was examined. Non-immunised and vaccinated mice were challenged with 1 x 10⁶ wild-type K8744/1b viable cells on day 38. Clearance of the challenge strain from the lungs of mice was monitored by determining the number of viable bacteria present in the lungs at various time intervals. Only prototrophic *B. bronchiseptica* colonies were isolated post-challenge, screened for the
ability to grow without aromatic amino acid supplementation on minimal SS-X medium. This thereby enabled isolation and quantification of challenge bacteria (K8744/1b) only, even in the presence of the vaccine strain.

Figure 5.2 Levels of *B. bronchiseptica*-reactive spleen cells in non-immunised mice (unfilled bars), mice intranasally immunised with CMJ60 (filled bar, 1 dose) mice intranasally immunised then boosted with CMJ60 on day 0 and day 14 (filled bar, 2 doses), and mice immunised then boosted with CMJ60 on day 0, day 14 and day 28 (filled bar, 3 doses). Error bars represent standard deviations. Variation between non-immunised and vaccinated mice was considered significant at P<0.05 (*).

Compared with non-vaccinated mice, a significant reduction in the ability of the wild-type K8744/1b strain to colonise the lungs of CMJ60 vaccinated mice was observed. In non-vaccinated mice, the wild-type infection showed a typical profile with bacterial numbers increasing for 4 days after initial inoculation, then being slowly cleared from the lung over the next 23 days (Figure 5.3). The wild-type infection of mice vaccinated with CMJ60 failed to replicate within the lung and was rapidly cleared to low levels 8 days after initial inoculation (Figure 5.3).
Results

Figure 5.3  Clearance from the murine lung of the parental *B. bronchiseptica* strain K8744/1b in non-immunised mice (unfilled diamonds) and mice immunised then boosted with CMJ60 on day 0, day 14 and day 28 (filled squares). Results represent the mean colony forming units per lung of 4 mice per time point. Error bars represent standard deviations. Variation between non-immunised and vaccinated mice was considered significant at P < 0.05 4h post-challenge and on days 2 and 4.

5.2.2 Immune responses following vaccination and challenge with *B. bronchiseptica*

In order to investigate the immunological responses involved in protection of vaccinated mice, we have examined groups of mice for humoral responses and the presence of *B. bronchiseptica*-reactive spleen cells on day 34 (4 days prior to challenge) and day 51 (13 days post-challenge). Compared with non-vaccinated controls, vaccinated and challenged mice exhibited elevated *B. bronchiseptica*-specific serum IgG and IgA titres and lung lavage IgG and IgA at 13 days post-challenge with only the increase in serum IgA found to be significant (Figure 5.4). Compared with non-vaccinated mice, an increase in the number of *B. bronchiseptica*-reactive spleen cells in vaccinated animals was observed 13 days post-challenge, however this increase was not statistically significant (Figure 5.5).
Figure 5.4  Levels of *B. bronchiseptica*-specific IgG (A and B) and IgA (C and D) in mouse serum (A and C) and lung lavage (B and D) samples in non-immunised mice (unfilled bars) and mice immunised then boosted with CMJ60 on day 0, day 14 and day 28 (filled bars). Samples were analysed 4 days prior to challenge (-4) and 13 days post-challenge (13). Results represent the titres of 4 mice per treatment. Error bars represent standard deviations. Variation between non-immunised and vaccinated mice was considered significant at $P \leq 0.05$ (*).
An investigation was undertaken to determine the target antigens recognised by the humoral immune response in both vaccinated mice and mice that had been both vaccinated and challenged. The antibody response against both *B. bronchiseptica* K8744/1b whole cell extracts and purified lipopolysaccharide was examined. Clearly, a significant proportion of the serum antibody response was directed against *B. bronchiseptica* K8744/1b lipopolysaccharide, and specifically against O-antigen (Figure 5.6). Serum obtained post challenge, also recognised 2 unidentified polypeptides with apparent molecular weight of 63 and 89 kDa (Figure 5.6E).
Results

**Figure 5.3** Characterisation of the antigens recognised by the humoral response of both vaccinated and vaccinated and challenged mice. (A) Silver-stained purified *B. bronchiseptica* K8744/1b LPS (lane 1) and Coomassie-stained *B. bronchiseptica* K8744/1b whole cell extract (lane 2). (B-E) Western blot analysis of K8744/1b LPS (lane 1) and whole cell extract (lane 2) using 4 pooled sera obtained from: non-immunised mice (B); mice intranasally immunised then boosted with CMJ60 on day 0 and day 14 (C); mice immunised then boosted with CMJ60 on day 0, day 14 and day 28 (D); and mice immunised and boosted with CMJ60 on day 0, day 14 and day 28 then challenged on day 38 (E). Sera were collected 10 days after immunisations and 13 days after challenge. Arrows in panel E indicate the 89 kDa and the 63 kDa bands being recognised by serum obtained post challenge.
6. Discussion

*B. bronchiseptica* is a Gram-negative respiratory pathogen of a variety of animal species. This bacterium is the causative agent of infectious tracheobronchitis in dogs, bronchopneumonia in rabbits and guinea pigs, and has been associated with the disease atrophic rhinitis in swine (Goodnow, 1980). With disease often endemic in confinement-reared swine and high density kennels, *B. bronchiseptica* infections represent a considerable economic burden to the agricultural and veterinary industries. Currently there are numerous vaccines commercially available for the prevention of *B. bronchiseptica* associated diseases in both dogs and pigs. These are available in the form of killed whole-cell bacterins, subunit vaccines containing purified antigenic extracts or live avirulent cultures. The undefined nature of many vaccines and the lack of efficacy displayed by others has resulted in research focusing on the application of new vaccine strategies aimed at improving efficacy, safety, stability, ease of administration and cost effectiveness of vaccines for the prevention of *B. bronchiseptica* infections. Attenuated strains harbouring genetically defined mutations may represent one such strategy.

Using primers based on the *aroA* sequence of *B. pertussis*, it was possible to amplify the *aroA* gene of the closely related species *B. bronchiseptica*. To clone the surrounding area of the genome a cosmid library was generated and the *aroA* gene fragment was used as a DNA probe to identify a recombinant cosmid containing the *aroA* gene. Both the 1.3 kb *aroA* gene fragment in pJMC13 and the 4.5 kb fragment in pJMC25, subcloned from pCOS24, failed to complement the *aroA* mutation in *E. coli* AB2829. This may indicate that the transcriptional or translational signals contained within these fragments were not recognised in *E. coli* or the *B. bronchiseptica* aroA gene product failed to complement the *E. coli* aroA defect.

The *aroA* gene of *B. bronchiseptica* is situated in close proximity and in the same orientation as a number of other genes which, due to the lack of obvious transcription start and stop sites, most likely form an operon in the order of *gyrA*, *serC*, *pheA*, *tyrA*,
aroA, cmk, rpsA and ihfB. A very similar gene organisation is seen in *Pseudomonas stutzeri* (Xie et al., 1999). This mixed-function supraoperon contains the genes SerC-(pdxF)-aroQ-pheA-HisHb-tyrAc-aroF-cmk-rpsA and this linkage has been shown to be well conserved through wide phylogenetic distances (Xie et al., 1999). Upstream of this operon in *P. stutzeri* is an ORF encoding the gyrA gene which encodes DNA gyrase, a DNA topoisomerase. This is similar to the gene arrangement seen in *B. bronchiseptica*. However in *P. stutzeri*, gyrA does not form part of the mixed-function supraoperon as 268 bp downstream is a stem loop structure that is likely to be a transcription termination signal (Xie et al., 1999). It is believed that this high degree of conservation of genetic organisation may represent a global relationship based on regulatory requirements (Xie et al., 1999). Within the *B. bronchiseptica* DNA sequence, putative promoter sequences consisting of -35 and -10 regions were found 169 bp and 144 bp upstream of the gyrA start codon respectively and inverted repeat terminator sequences were found 41 bp downstream of the *ihfA* stop codon.

The gene serC encodes the enzyme 3-phosphoserine aminotransferase which is involved in the biosynthesis of serine. The genes pheA and tyrA encode proteins with a role in aromatic amino acid biosynthesis and are involved in the conversion of chorismate to phenylalanine and tyrosine respectively. The pheA and tyrA genes in *Lactococcus lactis* are also organised into an operon with aroA (Griffin and Gasson, 1995). As the translational stop signal of tyrA overlaps the translational start signal of aroA, this may indicate that these genes are translationally coupled. In other organisms, genes involved in aromatic amino acid biosynthesis that are translationally coupled include trpB with trpA (Aksoy et al., 1984) and trpE with trpD (Nichols et al., 1980). In such cases it was shown that efficient translation of the gene directly downstream is dependent on the translation of the end of the gene upstream (Platt and Yanofsky, 1975; Oppenheim and Yanofsky, 1980). This may have implications for the regulation of aroA in *B. bronchiseptica* as it would therefore be directly affected by the mechanisms controlling the transcription and translation of tyrA. Regions where cistrons overlap, or are only separated by a small number of base pairs, may represent an efficient means of translating a polycistronic messenger and are areas where gene fusion events could give
rise to new multi-function proteins. This may have been the case in *Neurospora crassa* where the *trpB* and *trpA* functions are contained in the one protein (Matchett and DeMoss, 1975) and may also have given rise to the pentafunctional AroM proteins of fungi and yeast (Hawkins *et al.*, 1993). Downstream from *aroA*, the gene *cmk* encodes cytidine monophosphate kinase that is involved in the conversion of CMP and dCMP to CTP and dCTP respectively, *rpsA* encodes the ribosomal protein S1 and *ihfA* encodes integration host factor.

Previously a vector (pSS1129; a Gm\(^{\prime}\) derivative of pRTPl) (Stibitz *et al.*, 1986) had been developed and used to construct unmarked deletions in the *B. bronchiseptica* chromosome (Monack and Falkow, 1993). However this vector was not provided by these authors upon repeated request. Therefore, an alternative strategy was employed to develop *aroA* mutations in the *B. bronchiseptica* chromosome. The PCR amplified, 1.3 kb *aroA* gene fragment was cloned into the Ap\(^{\prime}\) suicide vector pGP704Sal and mutated by inserting a Km\(^{\prime}\) gene cassette within the coding region of *aroA*. This construct was then conjugally transferred into *B. bronchiseptica* and the initial homologous recombination event was selected with Km. Transconjugates displaying Km resistance and a reduced ability to grow on unsupplemented minimal medium were continuously subcultured without Ap selection in an attempt to produce a second cross-over event that would yield vector sequences from the chromosome of *B. bronchiseptica*. This process was hampered by the *B. bronchiseptica* cultures undergoing phase variation and becoming permanently locked into a Bvg-negative phenotype after only two passages. An avirulent Bvg-negative phenotype would be an undesirable feature of a live vaccine strain as it would be unable to express any of the *bvg*-activated virulence factors and would therefore be limited in its ability to initiate an infection. This mutant would also be likely to display decreased immunogenicity as a result of the poor colonisation potential and due to many of the *vags* expressed by *B. bronchiseptica* being highly immunoreactive.

The construct used in pJMC13 for mutation of *aroA* results in an intact *aroA* gene being formed, however it was believed that this gene would not be expressed. As the
translational stop codon of \( tyrA \) overlaps with the translational start codon of \( aroA \) it is thought these genes are translationally coupled. Therefore the newly formed \( aroA \) gene that resulted from integration of pJMC13 integration into the chromosome would not contain a functional Shine Dalgarno sequence. This \( aroA \) gene has also been separated from it natural promoter sequence but transcription initiated from vector sequences cannot be ruled out. The phenotype displayed by CMJ25 suggest that the biosynthesis of aromatic compounds had been affected (Figure 3.10). Since growth was not completely abolished, expression of \( aroA \) or activity of its gene product cannot be ruled out by this study. Although the newly formed \( aroA \) gene that resulted from integration of pJMC13 into the chromosome would not contain a function Shine Dalgarno sequence, translation initiated from vector encoded sequences may be possible. However, the sequence of the plasmid vector pGP704 is currently incomplete thereby making this analysis impossible (see http://genome-www2.stanford.edu/vectordb/vector_descrp/PGP704.html). Vectors containing a counterselectable marker would enable selection pressure for a second recombination event, leaving only the inactivated copy of \( aroA \) in the chromosome. Alternatively an internal \( aroA \) fragment could be used to produce single cross-over mutants without forming a duplicated intact \( aroA \).

As CMJ25 was able to grow in the absence of aamix, the stability of the mutation contained in CMJ25 was investigated. Reversion tests where CMJ25 was grown in aamix supplemented SS-X broth, with and without Km selection showed that this strain was reverting to a Aro\( ^+ \) phenotype (i.e. a wild-type like growth rate) at a rate of \( 10^{-4} \). No differences were seen between cultures grown with or without Km selection and Southern blot analysis found that the \( aroA \) gene remained disrupted in these revertant strains indicating the single cross-over mutation was stable. The reversion rate seen for CMJ25 is comparable to that for phase variation in \( B. bronchiseptica \) (Monack et al., 1989) and upon further analysis it was determined that the reversion occurring was in fact the strain undergoing phase variation. One reason why CMJ25 is able to grow at a rate similar to that of the wild-type strain when in a Bvg-negative phase may be due to a decrease in the metabolic load on the cell. As \( B. bronchiseptica \) does not express \( vags \) when in a Bvg-negative phase the bacterium may have more resources available to allow
it to grow faster. *B. bronchiseptica* is also capable of survival and multiplying under conditions of severe nutrient limitation (Porter *et al*., 1991; Porter and Wardlaw, 1993), and this ability to grow in such harsh environments appears to be enhanced by the expression of the Bvg-negative phenotype (Cotter and Miller, 1994). Therefore, specific features of the Bvg-negative phase of *B. bronchiseptica* may allow this bacterium to bypass actual dependence on the upper shikimate pathway for the production of aromatic compounds.

Both CMJ25 and the wild-type parental strain colonised and persisted in the mouse respiratory tract with equal ability (Figure 3.13). As CMJ25 is able to grow unrestricted when in a Bvg-negative phase, an examination of sera from infected animals was done to ascertain whether CMJ25 was calling upon any Bvg-repressed gene products to survive *in vivo*. Sera from mice infected with either the wild-type or CMJ25 strain was reacted with whole-cell extracts and compared to determine if any unique antigenic polypeptides were recognised. Serum from mice infected with CMJ25 detected a number of large proteins that were either specific for a Bvg-activated growth phase or common to both Bvg-activated and Bvg-repressed phases of growth. However this recognition profile was the same as that seen with sera from mice infected with the wild-type strain. This observation suggests that either no unique Bvg-repressed factors are expressed *in vivo* or that an immune response to such Bvg-repressed factors is not being elicited or not being detected by the methods used. Anti-*B. bronchiseptica* titres determined during the course of infection show CMJ25 to be as immunogenic as the wild-type parental strain.

To obtain an aromatic amino acid auxotrophic mutant of *B. bronchiseptica* that was attenuated *in vivo*, CMJ25 was subjected to rounds of mutagenesis using the gentamycin resistant mini-transposon, mini-Tn5/Gm. The resulting strain CMJ60, was unable to grow in minimal SS-X medium in the absence of aamix supplementation. Upon further examination it was found that CMJ60 failed to grow when tryptophan was removed from the medium indicating that a gene involved in tryptophan biosynthesis had been mutated by the transposon. DNA sequence analysis revealed that the mini-transposon had inserted into an ORF encoding *trpE*. It was not determined if the auxotrophy for
tryptophan observed for this strain was due to the inactivation of \textit{trpE} or due to pleiotrophic effects on downstream genes. Analysis of the region surrounding \textit{B. bronchiseptica} \textit{trpE} revealed this gene to be organised into an operon with genes that code for other tryptophan biosynthetic enzymes, \textit{trpG}, \textit{trpD} and \textit{trpC}. However, unlike in \textit{E. coli} it appears that all of the genes involved in tryptophan biosynthesis are not contained within this one operon. In \textit{E. coli} the \textit{trpA} and \textit{trpB} genes encoding the \(\alpha\) and \(\beta\) subunits of tryptophan synthase are located downstream of \textit{trpC} within the \textit{trp} operon (Yanofsky, 1971). The location of these genes within the genome of \textit{B. bronchiseptica} will only be able to be determined when the finishing/gap closure of the shotgun sequence reads is completed for the \textit{B. bronchiseptica} genome project currently being undertaken at the Sanger Centre (http://www.sanger.ac.uk/Projects/B_bronchiseptica). The presence of a putative \textit{trpL} leader sequence upstream of \textit{trpE} may indicate that \textit{B. bronchiseptica} uses an attenuation mechanism to regulate expression of this operon.

When compared to the wild-type parental strain, reduced numbers of intracellular CMJ60 were recovered after co-cultivation with the macrophage-like cell line J774A.1. Intracellular invasion and survival has been proposed as a mechanism which allows \textit{Bordetella} infections to persist in the host, promoting either an asymptomatic carrier state for \textit{B. pertussis} infections (Steed \textit{et al.}, 1991) or the chronic course of a \textit{B. bronchiseptica} infection (Goodnow, 1980). It appears that CMJ60 is attenuated in its ability to survive the initial internalisation process which may therefore effect disease progression by this strain. The intracellular environment has been put forward as an environment where mutants defective in aromatic compound synthesis cannot obtain all of the requirements for survival (Hoiseth and Stocker, 1981). However, bacterial numbers within the J774A.1 cells increased between the 2 and 24 h period for the mutant strain indicating that CMJ60 is capable of surviving and replicating within eukaryotic cells. Invasion into host cells has been utilised by numerous pathogens to avoid host immune responses and to multiply in a nutrient rich environment. However, a number of these pathogens have been successfully attenuated through the introduction of defined mutations affecting the upper shikimate pathway of aromatic compound biosynthesis. These include \textit{Salmonella}

Upon experimental intranasal infection of mice, CMJ60 was significantly attenuated in its ability to survive in the murine respiratory tract when compared to the wild-type parental strain. The mutant strain failed to replicate in the lungs and was cleared at a faster rate than the wild-type strain. The insertion mutations of CMJ60 were found to be genetically stable for at least 16 days *in vivo*. Mice infected with one dose of CMJ60 produced only low levels of anti-*B. bronchiseptica* titres in the serum. Antibodies were only produced late in the course of the infection when numbers of CMJ60 cells were at low levels in the lung. The rapid reduction in cell numbers after experimental infection with CMJ60 may not have allowed enough time for optimal exposure of *B. bronchiseptica* to the immune system thereby preventing elicitation of a strong humoral response.

Booster immunisations using the intranasal route have been shown to be more efficient than booster immunisations delivered via the oral route when using attenuated *S. typhi* (Barry et al., 1996). The effect of booster immunisations on the immune response of mice experimentally infected with CMJ60 was examined. Anti-*B. bronchiseptica* IgG titres were shown to be significantly higher in the serum after two and three doses of the mutant strain and this corresponded with a significant increase in IgA titres present in the lungs. Mice given three doses of CMJ60 also displayed a significantly higher number of *B. bronchiseptica*-reactive spleen cells when compared to non-vaccinated control mice. Vaccination of mice using a three dose immunisation regime promoted the elicitation of strong anti-*B. bronchiseptica* cellular and humoral immune responses, which were also found to be protective for mice when challenged with the virulent parental strain. The humoral immune response in vaccinated and protected mice was directed primarily against *B. bronchiseptica* lipopolysaccharide post challenge and also recognised 2 unidentified polypeptides with apparent molecular masses of 63 and 89 kDa. The exact immune mechanism by which protection is afforded to vaccinated animals has not been delineated in this study.
Natural *B. bronchiseptica* infections are characterised by efficient establishment, long term persistence and the absence of chronic disease. It has recently been suggested that this is achieved via modulation of host immunity. Mechanisms employed by *B. bronchiseptica* to modulate host immunity include CyaA decreasing normal innate immune functions and type III secreted proteins down-regulating the humoral response in the host (Harvill *et al.*, 1999; Yuk *et al.*, 2000). These mechanisms and others yet to be characterised, facilitate persistent infection in the respiratory tract by *B. bronchiseptica* during the infection process. Administering booster immunisations of the vaccine constructed in this study lead to the induction of a strong serum IgG response and local immunity in the respiratory tract in the form of a secretory IgA response. Induction of anti-*Bordetella* antibodies is critical to neutralise the effects of toxins produced by *Bordetella* species and has been shown to confer protection against disease (Mills *et al.*, 1998; Harvill *et al.*, 1999; Hellwig *et al.*, 2001). Mice vaccinated with CMJ60 were also shown to produce a strong cell mediated response. Cell-mediated immunity is believed to also play a role in controlling *Bordetella* infections by mediating the physical elimination of bacteria from the respiratory tract (Mills *et al.*, 1993; Redhead *et al.*, 1993).

Anti-*B. bronchiseptica* titres generated after vaccination in the challenge trial (Figure 5.4) were lower than those obtained from mice given the same vaccination protocol in the previous trial (Figure 5.1). The reason for this is unknown and variability in the immune responses generated by this vaccine strain will have to be thoroughly investigated. Mice displayed no symptoms during the course of immunisations used to vaccinate animals. However, a more thorough examination investigating undesirable side effects associated with the use of this strain will have to be undertaken in animal species that are most likely to benefit from *B. bronchiseptica* vaccination. This is of particular importance to the swine industry where weight gain and feed conversion are important parameters that could be influenced by vaccine side effects and are of considerable economic significance to producers. The efficacy of this vaccine should also be compared to the vaccines commercially available for vaccination against *B. bronchiseptica* infections.
The attenuated vaccine presented in this study contains antibiotic resistance markers used for selecting the insertion mutations introduced into this strain. Clearly this would not be a candidate for use in commercial situations but has allowed a prototype vaccine strain to be generated and characterised. This has lead to the identification of genes involved in aromatic compound biosynthesis as targets for attenuation in *B. bronchiseptica*. As *B. bronchiseptica* is capable of infecting a wide range of mammalian hosts, the resulting strain may not only be utilised for the prevention of *B. bronchiseptica* associated disease, but could also be used to deliver heterologous antigens to a variety of mammalian species.
Conclusions

*B. bronchiseptica* is the causative agent of infectious tracheobronchitis in dogs and bordetellosis in pigs which in the presence of other factors can develop into the disease atrophic rhinitis. Despite the widespread use of commercially available *B. bronchiseptica* vaccines, respiratory disease associated with this organism remains a significant problem for the industries involved in the breeding of these animals. New vaccines for the prevention of *B. bronchiseptica* infections with more efficacious properties remain the focus of much research around the world. This study indicates the genes *aroA* and *trpE* are potential mutagenic targets for the construction of attenuated strains, auxotrophic for aromatic compounds.

The *aroA* gene of *B. bronchiseptica* was found to be organised into a mixed-function superoperon that has been shown to be well conserved through wide phylogenetic distances. The insertion mutation introduced into *aroA* via homologous recombination failed to produce an auxotrophic strain of *B. bronchiseptica* completely reliant upon exogenous aromatic compounds. This strain also failed to be attenuated in a murine model of respiratory infection. An attenuated vaccine strain was produced using random transposon mutagenesis that resulted in a strain auxotrophic for tryptophan. Genetic analysis revealed the transposon had inserted within the *trpE* gene, the first ORF of a *trp* operon containing genes that code for other tryptophan biosynthetic enzymes, *trpG*, *trpD* and *trpC*. The mutant was attenuated both in the ability to survive in the macrophage-like cell line J774A.1 and in the murine lung. The strain was genetically stable for at least 16 days *in vivo* but failed to elicit strong humoral responses after initial exposure. However, upon administration of boosting doses this attenuated strain promoted the elicitation of efficient anti-*B. bronchiseptica* cellular and humoral immune responses, which were able to protect mice against challenge with a virulent strain. The humoral immune response in vaccinated and protected mice was directed primarily against *B. bronchiseptica* lipopolysaccharide. This prototype strain demonstrates that *B. bronchiseptica*, rendered auxotrophic for aromatic compounds, is attenuated in mice and
can be used as a live intranasal vaccine that elicits strong and protective humoral and cell-mediated immune responses.
References


References


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APPENDIX I

Growth media and solutions

Growth Media

**BG agar**

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**Z agar**

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**LB broth**

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**LB Mg Mal medium**

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**M9 minimal medium**

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**M9 salts (5x)**

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Appendix

KH₂PO₄  15 g.l⁻¹
NaCl  2.5 g.l⁻¹
NH₄Cl  5 g.l⁻¹
Thiamine  10 mg.l⁻¹

**Soc medium**

Bactotryptone  2%
Yeast Extract  0.5%
NaCl  10 mM
KCl  2.5 mM
MgCl₂  10 mM (autoclaved separately)
glucose  20 mM (filter sterilised)
pH 7.0

**Modified Stainer and Scholte medium**

**SS base**

L-glutamate  10.72 g.l⁻¹
L-proline  0.24 g.l⁻¹
KH₂PO₄  0.5 g.l⁻¹
KCl  0.2 g.l⁻¹
MgCl₂.₆H₂O  0.1 g.l⁻¹
CaCl₂  0.02 g.l⁻¹
TrisBase  6.075 g.l⁻¹
NaCl (for SS-X)  2.5 g.l⁻¹
MgSO₄ (for SS-C)  5.0 g.l⁻¹ (autoclaved separately)
x100 Salts  10 ml.l⁻¹
pH 7.6

**100x Salts**

L-cysteine  4 mg.ml⁻¹
FeSO₄.₇H₂O  1 mg.ml⁻¹
Ascorbic acid  2 mg.ml⁻¹
Nicotinic acid  0.4 mg.ml⁻¹
Glutathione  10 mg.ml⁻¹
### aamix (100x)

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### Solutions

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<td>1.44 g.L(^{-1})</td>
</tr>
<tr>
<td>KH(_2)PO(_4)</td>
<td>0.24 g.L(^{-1})</td>
</tr>
<tr>
<td>pH</td>
<td>7.6</td>
</tr>
</tbody>
</table>

#### QIAGEN Buffers

#### Buffer P1

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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</thead>
<tbody>
<tr>
<td>RNase A</td>
<td>100 µg.ml(^{-1})</td>
</tr>
<tr>
<td>TrisHCl</td>
<td>50 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
</tr>
<tr>
<td>pH</td>
<td>8.0</td>
</tr>
</tbody>
</table>
**Buffer P2**
NaOH  
SDS  

**Buffer P3**
Pottassium Acetate  
pH 4.8  

**Buffer B1**
EDTA  
TrisHCl  
Tween-20  
TritonX-100  
RNaseA  
pH 8  

**Buffer B2**
GuHCl  
Tween-20  
pH 5.5  

**Buffer QBT**
NaCl  
MOPS  
ethanol  
Triton X-100  
pH 7.0  

**Buffer QC**
NaCl  
MOPS  
ethanol  
pH 7.0
**Buffer QF**

NaCl 1.25 M
MOPS 50 mM
ethanol 15%
pH 8.2

**Southern Solutions**

**20 x SSC**
Sodium citrate 88.2 g.l⁻¹
Sodium Chloride 175.3 g.l⁻¹
pH 7.0

**Hybridisation buffer**
EDTA 1 mM
NaHPO₄ 40 mM
SDS 7%

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

**Washing solution 1 and 2**
EDTA 1 mM
NaHPO₄ 40 mM
SDS 5%

**Washing solution 3 and 4**
EDTA 1 mM
NaHPO₄ 40 mM
SDS 1%
Radio-Labelling Solutions

Solution A1

dCTP 0.2 mM
dGTP 0.2 mM
dTTP 0.2 mM
TrisHCl (pH 7.8) 500 mM
MgCl₂ 50 mM
β-mercaptoethanol 100 mM

Solution C

DNA polymerase I 0.4 U.µl⁻¹
DNase I 40 µg.µl⁻¹
TrisHCl (pH 7.5) 10 mM
Mg-Acetate 5 mM
β-mercaptoethanol 1 mM
PMSF 0.1 mM
glycerol 50% (v/v)
nuclease free BSA 100 µg.ml⁻¹

Solution D

Na₂EDTA 300 mM
pH 8.0

Gel electrophoresis solutions

8x PAGE running buffer

Tris base 15 g.l⁻¹
Glycine 72 g.l⁻¹
SDS 5 g.l⁻¹
pH 8.3

PAGE sample buffer

TrisHCl (pH6.8) 60 mM
SDS 1%
β-Mercaptoethanol 1%
Glycerol 10%
BPB 0.01%
Appendix

4% acrylamide gels (separating)
Acrylamide 35%/bis-acrylamide 0.8% stock 250 μl
TEMED 25 μl
Ammonium persulfate (10% stock) 8 μl
SDS (10% stock) 25 μl
dH2O 1.59 ml
0.5 M TrisHCl (pH 6.8) 630 μl

12% acrylamide gels (resolving)
Acrylamide 35%/bis-acrylamide 0.8% stock 1.5 ml
TEMED 50 μl
Ammonium persulfate (10% stock) 15 μl
SDS (10% stock) 50 μl
dH2O 2.2 ml
1.5 M TrisHCl (pH 8.8) 1.75 ml

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

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

10x TBE buffer
Tris base 108 g.l⁻¹
Boric acid 55 g.l⁻¹
Na₂EDTA.2H₂O 8.3 g.l⁻¹
pH 8.3
Appendix

**Sequencing gels (48 cm)**

- Urea: 28.8 g
- Acrylamide (40%): 8.5 ml
- Milli Q H₂O: 35 ml
- TBE filtered (10x stock): 8 ml
- TEMED: 55 µl
- Ammonium persulfate (10%): 400 µl

**DNA sequence loading Dye**

- Formamide (50 mg.ml⁻¹): 5 parts
- EDTA (25 mM, pH8.0): 1 part

**Western hybridisation solutions**

**Blocking solution**

- Low fat instant milk powder: 5%
- PBS: 1x

**Western transfer buffer**

- Tris base: 3.1 g.l⁻¹
- Glycine: 14.4 g.l⁻¹
- Methanol: 20%
- pH 8.3

**Development Solution**

**PartA**

- H₂O₂ (30%): 20 µl
- dH₂O: 30 ml

**PartB**

- 4-chloro-1-napthol: 30 mg
- Methanol: 10 ml
ELISA solutions

Coating buffer
Solution 1: Na$_2$CO$_3$ 1.3 g in 250 ml
Solution 2: NaHCO$_3$ 1.7 g in 400 ml
Add solution 1 to solution 2 until the pH is 9.6

Wash buffer
PBS 1x
Tween 20 0.05%
pH 7.4

Blocking buffer
PBS 1x
Tween 20 0.05%
BSA 1%
pH 7.4

Peroxidase substrate buffer
Na$_2$HPO$_4$ 0.28 g in 100 ml
Adjust the pH to 5 with solid citric acid

Substrate preparation
Add 10 mg of o-phenylenediamine dihydrochloride and 10 µl of 30% H$_2$O$_2$ to 25 ml of peroxidase substrate buffer.
## APPENDIX II

**Primers For DNA Sequencing and PCR**

For the *aroA* locus

<table>
<thead>
<tr>
<th>Primer Identification</th>
<th>Sequence (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal Forward (For)</td>
<td>GTAAACGACGAGCCAGT</td>
</tr>
<tr>
<td>Universal Reverse (Rev)</td>
<td>AATGCTCTTTGATCAGT</td>
</tr>
<tr>
<td>DNAK2</td>
<td>ATGGCCGACAACGCCGTTCC</td>
</tr>
<tr>
<td>DNAK3</td>
<td>GTCCCCGTGTGGCTTG</td>
</tr>
<tr>
<td>DNAK4</td>
<td>GTACGGCAGCGTTCAGT</td>
</tr>
<tr>
<td>RPSA1</td>
<td>GTGAATCGTGCAGT</td>
</tr>
<tr>
<td>RPSA2</td>
<td>ACACGCAGCTG</td>
</tr>
<tr>
<td>RPSA3</td>
<td>TAGCCGGCGATGCAAGTAG</td>
</tr>
<tr>
<td>CMK</td>
<td>CGCATGCTACTGG</td>
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<tr>
<td>CMK2</td>
<td>GAACGCGAGCTGTCACC</td>
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<tr>
<td>CMK3</td>
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<tr>
<td>TYRA</td>
<td>AGATCCGGGCTTGTTG</td>
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<tr>
<td>TYRA2</td>
<td>ACGAGGGAGAGGCATCAAGGCC</td>
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<tr>
<td>TYRA3</td>
<td>GGTCAAGCCTGTCATCC</td>
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<td>BB812</td>
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<td>P1</td>
<td>GAATTCATGAGCGGATATTGCC</td>
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<td>3</td>
<td>TGAGGCTAGTGCAGG</td>
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<td>4</td>
<td>GATCCAGGCCGGCATAGC</td>
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<td>6</td>
<td>CGCGTACACGTCGAAAATAAT</td>
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For the *trpE* locus

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<th>Primer Identification</th>
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<tbody>
<tr>
<td>Universal Forward (For)</td>
<td>GTAAAACGACGGCCAGT</td>
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<tr>
<td>Universal Reverse (Rev)</td>
<td>AATGTGTCCTTTGTGATCTGG</td>
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<tr>
<td>TRPE1</td>
<td>TTCTGGGAAGTGC CGCCGCTC</td>
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<tr>
<td>TRPE2</td>
<td>AACGCCAGCGGGTCGCTTCGTG</td>
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<td>TRPE3</td>
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<td>67REV1</td>
<td>ATGAGCAGCATGGATGTGC</td>
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<td>67REV2</td>
<td>CCTTCACCTACAAACCTGGTG</td>
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<tr>
<td>67FOR1</td>
<td>GCACGTACAGGCTGCCGTTC</td>
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<tr>
<td>TRPER1</td>
<td>GCACCTACCTGATGGTATGTG</td>
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<tr>
<td>TRPER2</td>
<td>CCCGACATGACTGAACCTTG</td>
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<td>TRPER5</td>
<td>AGCGCATCGGCACTCCAAGTCC</td>
</tr>
<tr>
<td>TRPER6</td>
<td>AAGTCGCTTGAGCAATATACC</td>
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APPENDIX III

Publications


Patents


Conference Oral Presentations
