CHARACTERISATION, RECOMBINANT EXPRESSION AND IMMUNOGENICITY OF BHLP29.7, AN OUTER MEMBRANE LIPOPROTEIN OF BRACHYSPIRA HYODYSENTERIAE

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DECLARATION

I hereby declare that the work presented in this thesis has been performed by myself, except where otherwise clearly stated in the text, and that it has not been previously submitted for application for a degree at any University.

Signed ………………………………………
Dedicated to my wife, Pei Ling,
and my daughter, Kaitlin
Swine dysentery (SD) is an important endemic infection in many piggeries, and control can be problematic. In this study, the gene encoding a 29.7 kDa outer membrane lipoprotein of the causative intestinal spirochaete *Brachyspira hyodysenteriae*, was identified and sequenced. An 816 bp hypothetical open reading frame (ORF) was identified, with a potential ribosome binding site, and putative –10 and –35 promoter regions upstream from the start of the ORF. The 29.7 kDa outer membrane lipoprotein was designated Bhlp29.7 and the encoding gene named *bhlp29.7*.

The amino acid sequence of Bhlp29.7 included a 19 residue hydrophobic signal peptide, incorporating a potential signal peptidase cleavage site and membrane lipoprotein lipid attachment site. *In silico* analysis of this protein together with lipidation studies further supported its probable outer membrane localisation. Comparison of the Bhlp29.7 sequence with public sequence databases showed that it had up to 40% similarity with the D-methionine substrate-binding outer membrane lipoprotein (MetQ) of a number of bacterial pathogens. The Bhlp29.7 gene was detected in all 48 strains of *B. hyodysenteriae* examined, and in *Brachyspira innocens* strain B256ᵀ, but not in 10 other strains of *B. innocens* or in 42 strains of other *Brachyspira* spp. The gene was sequenced from *B. innocens* strain B256ᵀ and from 11 strains of *B. hyodysenteriae*. The *B. hyodysenteriae* genes shared 97.9-100% nucleotide sequence identity and had 97.5-99.5% identity with the gene of *B. innocens* strain B256ᵀ. The Bhlp29.7 gene was subsequently cloned and expressed as a histidine fusion-protein in an *Escherichia coli* expression system.

An ELISA test using recombinant his-tagged Bhlp29.7 (His<sub>6</sub>-Bhlp29.7) as the detecting antigen was developed and evaluated. The threshold value of the test was chosen to provide a highly stringent assessment of the disease status of a herd. The sensitivity and specificity of the test was 100%. When the test was applied to sera from eight herds with suspected SD, four
gave ELISA values indicating that the herds were diseased. The remaining four herds gave ELISA values below the threshold value. These results indicated that the Bhlp29.7-ELISA was useful as an indirect test for exposure of a herd to *B. hyodysenteriae* and may be a helpful complement to current methods of SD diagnosis.

Recombinant His<sub>6</sub>-Bhlp29.7 was evaluated as a vaccine subunit for prevention of SD. The His<sub>6</sub>-Bhlp29.7 was shown to be immunogenic in mice following two intramuscular injections. Vaccination of mice with His<sub>6</sub>-Bhlp29.7 provided full protection after oral challenge with *B. hyodysenteriae*. In two experiments, intramuscular and oral vaccination of pigs with the His<sub>6</sub>-Bhlp29.7 resulted in a 50% reduction in incidence of SD compared to unvaccinated control pigs (*P*=0.047). This is the first subunit vaccine shown to provide pigs with protection from SD. Further work is needed to optimise delivery routes and adjuvants for commercial development of the vaccine.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CBB</td>
<td>Coomassie Brilliant Blue</td>
</tr>
<tr>
<td>CSPD</td>
<td>Chemiluminescent substrate for alkaline phosphatase development</td>
</tr>
<tr>
<td>DAB</td>
<td>3-3’ Diaminobenzidinetetrahydrochloride</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FIA</td>
<td>Freund's Incomplete Adjuvant</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Mab</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MBP</td>
<td>Maltose-binding protein</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
</tr>
</tbody>
</table>
### ABBREVIATIONS (CONTINUED)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni-NTA</td>
<td>Nickel-nitrilotriacetic acid</td>
</tr>
<tr>
<td>OMP</td>
<td>Outer membrane protein</td>
</tr>
<tr>
<td>ORF</td>
<td>Open-reading frame</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline containing 0.05% (v/v) Tween 20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethysulphonyl fluoride</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline containing 0.05% (v/v) Tween 20</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethyl-ethylenediamine</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TSA</td>
<td>Trypticase soy agar</td>
</tr>
<tr>
<td>TSB</td>
<td>Trypticase soy broth</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>

xxvi
CHAPTER 1: LITERATURE REVIEW

1.1. GENERAL INTRODUCTION

Swine dysentery (SD) is a contagious severe mucohaemorrhagic diarrhoeal disease of pigs which primarily occurs in pigs from weaning up to 20 weeks of age, although the disease may also occur in breeding herds where it affects sows (Alexander and Taylor, 1969; Meyer, 1978; Achacha and Messier, 1991; Harris et al., 1999). Pigs affected by SD show signs of dehydration, emaciation and rapid loss of weight. In severe cases, the disease can cause death (Alexander and Taylor, 1969; Olson, 1974). SD is potentially one of the most economically significant diseases of pigs and occurs in most pig-producing countries, including Australia (Hampson and Trott, 1995; Hampson et al., 1997). Economic losses due to SD result mainly from growth retardation, costs of drug medication, and mortality (Hampson et al., 1997; Harris et al., 1999). Internationally SD remains a major problem, and the worldwide market for an effective solution is substantial.

This chapter provides a review of the current literature relating to aspects of serological detection and prevention of SD, with the key emphasis on antigens which have been identified as putative immunogens for the development of serological assays and vaccine subunits. The chapter concludes with an outline of the main aims and objectives of the project.
1.2. AETIOLOGY OF SD

The causative agent of SD was first identified and characterized in 1971 (Taylor and Alexander, 1971; Taylor and Blakemore, 1971), and named *Treponema hyodysenteriae* (Glock and Harris, 1972; Harris *et al.*, 1972). This anaerobic spirochaete was later transferred to the new genus *Serpula* (Stanton *et al.*, 1991), then to *Serulina* (Stanton, 1992), and now has been reassigned to the genus *Brachyspira* as *Brachyspira hyodysenteriae* based on results of 16S rRNA gene sequence analysis (Ochiai *et al.*, 1997). *B. hyodysenteriae* is a Gram-negative, anaerobic, loosely coiled, β-haemolytic spirochaete. It is six to 11 μm long and 0.32 to 0.38 μm in diameter. The organism consists of a helical protoplasmic cylinder enveloped by a loose outer membrane (Fig. 1.1). It possesses seven to 14 periplasmic flagella which are inserted sub-terminally in two rows at the ends and overlap at the centre of the protoplasmic cylinder (Harris *et al.*, 1972; Meyer, 1978; Blaha and Gunther, 1985; Lee *et al.*, 1993).

![Figure 1.1](image_url)

Figure 1.1. Transmission electron micrograph of B78, the type strain of *B. hyodysenteriae*, showing eight periplasmic flagella at each cell end, and the blunt ends of the cell.
**B. hyodysenteriae** is an actively motile bacterium which displays the ability to move through semi-solid and liquid media. The most commonly used liquid medium for propagation is comprised of Trypticase Soy Broth (TSB) supplemented with 10% (v/v) foetal bovine serum, whilst the most commonly used solid medium is Trypticase Soy Agar (TSA) supplemented with 5% (v/v) sheep blood. **B. hyodysenteriae** also requires both cholesterol and a phospholipid for growth. Cells grow more rapidly at 42°C than at 37°C, although they do not grow at temperatures between 25°C and 30°C. The presence of strong β-haemolysis around the growth on agar plates containing blood is characteristic of **B. hyodysenteriae** (Kinyon and Harris, 1974; Lemcke *et al.*, 1979; Kunkle *et al.*, 1986; Stanton, 1987). The other four *Brachyspira* species that colonise the pig are all weakly haemolytic (Table 1.1).

### 1.3. CLINICAL SIGNS

The most consistent indication of SD is diarrhoea, although the severity of the clinical signs may vary considerably. The first evidence of disease in most animals is the passage of soft, yellow to grey faeces (Meyer, 1978). Reduced appetite and increased rectal temperature may also be evident in some animals. More severely affected animals are anorexic and depressed. In the subsequent stages of the disease, large amounts of mucus and flecks of blood can be seen in the faeces. As the diarrhoea progresses, the faeces becomes dark and watery containing mucus, blood and white microfibrinous exudate. The hindquarters of affected animals are often wet and stained with faecal discharge. Affected pigs generally appear gaunt with arched backs, protruding ribs and have a rough hairy coat. Prolonged diarrhoea usually leads to dehydration, weight loss, weakness, incoordination, emaciation and eventually death. The cause of death in most pigs is usually associated with dehydration, acidosis, hyperkalaemia and toxaemia (Harris *et al.*, 1999).
**Table 1.1.** Biochemical properties used to differentiate between the *Brachyspira* species which colonise pigs.

<table>
<thead>
<tr>
<th>Species</th>
<th>Haemolysis</th>
<th>Indole</th>
<th>Hippurate</th>
<th>Flagella per cell</th>
<th>α-glucosidase</th>
<th>α-galactosidase</th>
<th>β-glucosidase</th>
<th>D-Ribose</th>
<th>L-Fructose</th>
<th>D-cellulose</th>
<th>MLEE grouping</th>
<th>Mol% G + C content</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. hyodysenteriae</em></td>
<td>S</td>
<td>+*†</td>
<td>-</td>
<td>22 - 28</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>I</td>
<td>25.8</td>
</tr>
<tr>
<td><em>B. intermedia</em></td>
<td>W</td>
<td>+</td>
<td>-</td>
<td>24 - 28</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>II</td>
<td>25</td>
</tr>
<tr>
<td><em>B. innocens</em></td>
<td>W</td>
<td>-</td>
<td>-</td>
<td>20 - 26</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>III</td>
<td>25.6</td>
</tr>
<tr>
<td><em>B. mardochii</em></td>
<td>W</td>
<td>-</td>
<td>(v)</td>
<td>22 - 26</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>27</td>
</tr>
<tr>
<td><em>B. pilosicoli</em></td>
<td>W</td>
<td>- (v)</td>
<td>+</td>
<td>8 - 12</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>VI</td>
<td>24.9</td>
</tr>
</tbody>
</table>


**Key:** S – strong, W – weak, v – variable, + – positive, - – negative, +/- – strains can be positive or negative.

*†* indole negative strains have been described (Hommez *et al.*, 1998; Fellström *et al.*, 1999).

MLEE, multilocus enzyme electrophoresis

ND, not determined
1.4. PATHOGENICITY

SD is associated with the proliferation of *B. hyodysenteriae* in the lumen of the large intestine and invasion of the colonic crypts (Kinyon and Harris, 1979). SD is initiated after susceptible pigs ingest dysenteric faeces containing *B. hyodysenteriae*. An inoculum of $10^5$ colony forming units (cfu) is usually sufficient to cause SD (Kinyon and Harris, 1979). The spirochaetes survive passage through the acidic environment of the stomach, presumably protected in faeces, and eventually arrive in the large intestine. The ability of *B. hyodysenteriae* to colonise is influenced by factors in the local environment, particularly those associated with the diet and the resident microflora (Hampson *et al.*, 2006a). *B. hyodysenteriae* may have a selective advantage in colonising the colon since it can utilise substrate amounts of oxygen, and its ability to dispose of NADH in several ways also contributes to its increased metabolic efficiency (Stanton, 1989). *B. hyodysenteriae* is motile due to the action of the periplasmic flagella, which allows the spirochaete to move efficiently through viscous material such as mucus to gain access to the epithelial cells in the colon. The motility of the spirochaete is essential for its virulence (Rosey *et al.*, 1995; Rosey *et al.*, 1996). *In vitro* studies have shown that *B. hyodysenteriae* elicits a chemotactic response to mucus (Lysons *et al.*, 1984; Kennedy *et al.*, 1988), and it is proposed that the chemotactic response together with strong motility, enables the spirochaetes to penetrate the mucus layer and invade the colonic crypts. Strains apparently lacking this strong chemotactic response are thought to be less virulent (Milner and Sellwood, 1994). At the colonic crypts, the presence of the spirochaete stimulates an outpouring of mucus into the lumen. Clinical signs and lesions begin to develop as *B. hyodysenteriae* numbers reach $10^6$ cfu per cm$^2$ of mucosa (Hughes *et al.*, 1977; Whipp *et al.*, 1979). At this time, there is a shift in the microflora composition in the colon from predominantly Gram positive in healthy animals to predominantly Gram negative in pigs with dysentery (Pohlenz *et al.*, 1984).
At the lumen, motile *B. hyodysenteriae* cells enter goblet cells in the colonic crypts and penetrate intracellular gaps in the epithelium (Sueyoshi and Adachi, 1990). There is an associated loss of cohesion between colonic enterocytes subsequently resulting in necrosis and shedding of the epithelium. The spirochaetes attach to the luminal surface and enter the disrupted epithelial cells. Some spirochaetes may also be seen in the lamina propria, particularly around blood vessels. Bleeding occurs from small blood vessels located under eroded areas of epithelium. Although typical lesions have been associated with the presence of *B. hyodysenteriae* in the cytoplasm of epithelial cells and within the lamina propria of the colonic tissue, invasion may not be essential for lesion production (Glock *et al.*, 1974; Albassam *et al.*, 1985). *In vitro* attachment of *B. hyodysenteriae* to intestinal cell lines also indicated that attachment of the spirochaete did not necessarily cause cytopathological changes or invasion of the infected cells. The frequency of attachment in these systems was also dependent on the viability of the organism (Knoop *et al.*, 1979; Wilcock and Olander, 1979; Bowden *et al.*, 1989). It has not been clearly demonstrated whether attachment is a significant feature in the disease.

The mechanisms responsible for the enterocyte disruption and subsequent necrosis, which are central characteristics of SD, have not been fully elucidated. Two possible toxic components which may contribute to lesion production have been described and characterised. These include: 1) the endotoxic effect of the spirochaete’s lipopolysaccharide (LPS) (Baum and Joens, 1979a; Nuessen *et al.*, 1982; Nuessen *et al.*, 1983), and 2) the cytotoxic effects of the haemolysin(s) secreted by *B. hyodysenteriae* (Kinyon and Harris, 1979; Knoop, 1981; Kent *et al.*, 1988; ter Huurne *et al.*, 1993).

*B. hyodysenteriae* does not invade beyond the lamina propria of the large intestine and the lack of significant lesions and/or the presence of the spirochaete in other organs suggest that the enteric lesions directly characterise the entire pathogenesis of SD (Glock *et al.*, 1974). Diarrhoea and the

6
presence of mucus and blood in the faeces is a consequence of colonic malabsorption and erosive colitis. The colonic malabsorption, and subsequently fluid loss, is caused by the failure of the epithelial transport system to actively transport sodium and chloride from the lumen to the blood (Argenzio et al., 1980; Schmall et al., 1983). As much as 30-50% of the daily extracellular fluid volume of a pig is presented to the colon for absorption in the form of exogenous secretions, and failure to reabsorb these exogenous secretions exclusively results in fluid losses by the animal. Therefore, colonic malabsorption alone is sufficient to account for the progressive dehydration and death associated with the disease.

1.5. PATHOLOGICAL CHANGES

1.5.1. Macroscopic lesions

A consistent characteristic of SD is the presence of lesions in the large intestine but not the small intestine. Colitis and typhlitis are the main gross findings. There is a characteristic catarrhal inflammation and superficial necrosis of the mucosa of the colon and caecum. In the acute stages, hyperaemia and oedema of the walls and mesentery of the large intestine are typical changes. The inflammation may also induce congestion of the mesenteric lymph nodes and the formation of small amounts of clear ascites as well as a rugose appearance of the colon covered with mucus, blood and fibrin. The contents of the colon are soft and watery. As the disease advances, the mucosal surface becomes reddened with a granular appearance, and fibronecrotic diphtheritic membranes develop (Fig. 1.2). As the lesions become more chronic, strands of fibrin become visible on the surface of the colonic mucosa giving the appearance of superficial necrosis. Large numbers of \textit{B. hyodysenteriae} can be detected on the mucosal surface of these lesions (Hughes et al., 1977; Griffin and Hutchings, 1980; Harris et al., 1999).
1.5.2. Microscopic lesions

In the acute stage of SD, vascular congestion, together with accumulation of fluids and leucocytes results in the obvious thickening of the colonic mucosa. Hyperplasia and swelling of the goblet cells lead to the production of a thick column of mucus. Epithelial cells at the base of the crypts may become elongated and hyperchromic. An accumulation of neutrophils is commonly observed in and surrounding capillaries near the lumen, and this increases as necrosis occurs. Focal areas of haemorrhage occur in the superficial lamina propria resulting in the exposure of capillaries and a typical blood-flecked appearance of the colonic contents. Later changes consist of the accumulation of large amounts of fibrin, mucus and cellular debris in the mucosal crypts and on the luminal surface of the large intestine. Increased numbers of neutrophils may also be observed in the lamina propria. Chronic changes include milder hyperaemia and oedema, although these changes are not specific. Superficial necrosis may be extensive but deep ulceration is not typical, unless there are secondary infections with *Salmonella* spp.. Advance superficial necrosis of the

Figure 1.2. Macroscopic lesions of the large intestine of a pig suffering SD.
mucosa often occurs, and usually is covered by a thick and fibrinous pseudomembrane. In all stages of the disease, *B. hyodysenteriae* cells are found in the lumen and within the crypts, although large numbers are only found in the acute stages (Hughes et al., 1977; Harris et al., 1999).

1.6. IMMUNITY

1.6.1. Humoral immunity

Studies of immune mechanisms in SD have concentrated on antibody mediated immunity, and these have shown that there is no direct relationship between serum antibody titres and protection against SD. A proportion of pigs that have recovered from SD, naturally or after medication, will be protected from subsequent challenge for up to 17 weeks after the initial exposure (Joens and Glock, 1979). Joens et al. (1979) used a microtitration agglutination test (MAT) to investigate the serological status of 29 pigs that had been challenged with *B. hyodysenteriae* and then allowed to recover. Circulating antibodies against *B. hyodysenteriae* from recovered pigs were observed for only approximately eight weeks after inoculation. Thirteen of the 29 pigs developed diarrhoea when re-exposed to the organisms after one to 28 days, however only two suffered from clinical dysentery. Elevated antibody titres also have been demonstrated in infected and convalescent pigs by other workers, but again there was no clear relationship between the magnitude of titres, degree of protection, and frequency or duration of faecal shedding of *B. hyodysenteriae* (Fisher and Olander, 1981; Fernie et al., 1983).

Experiments in which mouse peritoneal cells were used to phagocytose *B. hyodysenteriae* showed that opsonisation with antibody was an important event in the process. The opsonising antibody worked best if it had been raised against the same serotype of *B. hyodysenteriae*, although some effects were seen with heterologous serotype antisera (Nuessen and Joens, 1982). These serotype-specific antigens are thought to be largely lipopolysaccharide (LPS) in nature (Baum and Joens,
1979b). Pigs vaccinated with formalinised bacterins also have shown serotype specificity in their host defence mechanisms, although some cross-protection between serotypes does occur (Parizek et al., 1985).

Joens et al. (1983) further demonstrated the serotype-specific nature of protective immunity against SD in the colon using colonic loops prepared in pigs. Pigs were infected with one or other strains of different serotypes of *B. hyodysenteriae* and allowed to recover. A series of surgically-prepared ligated colonic loops were then inoculated either with homologous serotype strains or with other heterologous serotype strains. Generally, lesions developed in the loops inoculated with heterologous serotypes, but not in the loops inoculated with homologous serotypes. A limited amount of cross-protection between serotypes was observed in some loops, but overall immunity appeared to be largely serotype specific, and therefore probably directed against LPS antigens. It is uncommon for herds to be infected with more than one strain of *B. hyodysenteriae*, so that serotype-specific immunity is unlikely to be important in preventing a given strain proliferating whilst allowing another of a different serotype to establish itself (Combs et al., 1992).

Ligated colonic loops prepared in pigs have also been used to demonstrate passive protection against SD (Joens et al., 1985). When immune serum, containing complement components, and the homologous strains of *B. hyodysenteriae* were inoculated into colonic loops, 83% (ten of 12 loops) were protected from developing lesions of SD. Of these 12 loops, protection was obtained in 72% (eight of 11 loops) inoculated with the homologous organism and antiserum that had been heat-inactivated to remove complement components, whilst only 23% (three of 13 loops) inoculated with heat-inactivated antiserum and heterologous isolates were protected. This suggests that complement components and serum IgG secreted into the colon may contribute to the protection seen in convalescent pigs.
Antibodies directed against LPS have been found in sera and colonic secretions of convalescent pigs using enzyme-linked immunosorbent assays and immunoblotting (Egan et al., 1983; Wannemuehler et al., 1988; Wannemuehler and Galvin, 1994). Following infection, an increase in B. hyodysenteriae-specific circulating and local antibodies can be detected. High levels of circulating IgG in the serum tend to be correlated with the duration of clinical signs rather than with the degree of protection against SD, whilst secretory IgA levels in the colons, faeces and bile indicate recent exposure to B. hyodysenteriae (Rees et al., 1989). In one study, a production peak of coproantibody (predominantly IgA, with IgG and IgM) was detected in the colon prior to the production of circulating antibody (Jenkins and Roberts, 1980). This suggested that methods which stimulate mucosal immunity may be a good means to induce protection against SD.

1.6.2. Cell-mediated immunity

Cell mediated immunity also may be important in SD since there is evidence for inhibition of peripheral blood leukocyte migration, a delayed hypersensitivity response, and a T-cell proliferative response in the peripheral blood of pigs convalescent from SD (Jenkins et al., 1982; Kennedy et al., 1992; Waters et al., 1999a; Waters et al., 1999b). In mouse models of the disease, however, there are no significant changes in T-cell subsets in the lamina propria, and changes observed in local mast cell population numbers were not correlated with development of lesions following infection (Nibbelink and Wannemuehler, 1992).

It has been well established that CD4+ T-cells are involved in the induction of inflammatory bowel diseases (Cong et al., 2000; Strober et al., 2002). Inflammatory bowel diseases may result from deficient regulation of CD4+ T-cell responses to antigens from normal large intestinal microflora resulting in a consequent cytokine imbalance (Cong et al., 2000; Annacker and Powrie, 2002; Kullberg et al., 2003). During SD, CD4+ and γδ T-cells in the peripheral blood and colonic lymph nodes of intramuscularly immunised pigs have been shown to proliferate to B. hyodysenteriae
antigens *ex vivo*. These circulating CD4+ T-cells secrete interferon-γ (INF-γ) upon stimulation with *B. hyodysenteriae* antigens during the infection, and this response was down-regulated by exogenous interleukin-10 (IL-10) post-infection. In addition, INF-γ expression in the colonic lymph nodes of SD pigs was up-regulated (Waters *et al.*, 1999a; Waters *et al.*, 1999b; Hontecillas *et al.*, 2002; Hontecillas and Bassaganya-Riera, 2003). These results suggested that a predominant T helper type 1 response was established during *B. hyodysenteriae*-induced colitis, contributing to the overall inflammation and necrosis of the colon.

Recently, Jonasson *et al.* (2004) investigated levels of circulating leukocytes and lymphocyte subpopulations in pigs before and after experimentally induced SD. By comparing results from pigs that did or did not succumb to disease, the authors deduced that γδ T-cells and CD8+ cells may be associated with susceptibility to infection, whilst monocytes and CD4+ CD8+ T-cells appeared to be the major responding lymphocytes to infection. More recently, a report showed that *ex vivo* γδ T-cell responses to *B. hyodysenteriae* antigens were not different between control pigs and pigs experimentally challenged with *B. hyodysenteriae* (Hontecillas *et al.*, 2005). In addition, γδ T-cells of infected pigs were significantly depleted from the epithelial layer, although their numbers in the lamina propria maintained unaltered. No differences were found in the numbers of circulating and colonic lymph node γδ T-cells between control and dysenteric pigs. Most recently, Jonasson *et al.* (2006) showed that following experimental infection of pigs with *B. hyodysenteriae*, increased levels of circulating monocytes, neutrophils and CD8α+ cells correlated with the development of clinical SD. During the recovery period, γδ T cells were found to increase and high levels of circulating neutrophils were also observed. Pigs with SD showed decreased levels of CD21+ cells in the blood during disease and recovery, but developed *B. hyodysenteriae*-specific antibodies from the first day of recovery. Taken together, these results indicate that
infection with *B. hyodysenteriae* induces a mucosal T-cell response and points to these responses being important contributors to the immunopathogenesis of SD.

Although it has been suggested that both the peripheral and mucosal T-cell response of the host contributes to the development of lesions associated with *B. hyodysenteriae*-induced colitis, the implication of these responses to the development of more effective vaccines still remains unclear. Further dissection of the nature of the mucosal T-cell response during SD would be beneficial.

1.7. DIAGNOSIS

SD is a relatively common disease which occurs in all major pig-producing countries (Roncalli and Leaning, 1976; Hampson and Trott, 1995; Hampson *et al.*, 1997; Harris *et al.*, 1999). When SD first enters a herd, exposure of the pigs to low infectious doses initially results in disease in only one or two pigs. As infectious faeces accumulate, the disease continues to spread to the remaining members of the herd. The rate of spread depends on the type of contact with the infected material. Once the infection becomes endemic within a piggery, the disease spectrum can vary from being mild, transient or unapparent, to severe and even fatal. Medication strategies on individual piggeries may mask clinical signs. Whether or not obvious disease is present, *B. hyodysenteriae* may persist in infected pigs, or in other reservoir hosts such as mice, or the environment. All these sources pose potential for transmission of the disease between herds (Hampson and Trott, 1995; Hampson *et al.*, 1997; Harris *et al.*, 1999).

1.7.1. Conventional methods

Various methods for the diagnosis of SD have been used over the years. In the first instance, observation of the clinical signs of the disease, including the presence of bloody and/or mucus-containing diarrhoea, are commonly suggestive indications (Harris *et al.*, 1999). Similar clinical signs however may also be seen in salmonellosis, swine fever and intestinal spirochaetosis.
A definitive diagnosis of SD still involves the isolation and identification of *B. hyodysenteriae* from the faeces or mucosa of the diseased pigs (Jensen, 1997; Harris et al., 1999). Major problems involved in achieving this include the slow growth and fastidious nutritional requirements of the bacteria, and confusion due to the presence of morphologically similar spirochaetes in the normal flora of the pig intestine (Hudson et al., 1976a; Joens and Harris, 1980). Fortunately, these other species are weakly β-haemolytic (Fig. 1.3). Biochemical reactions of *B. hyodysenteriae*, and comparison with those of other porcine *Brachyspira* species are shown in Table 1.1.

![Figure 1.3](image)

**Figure 1.3.** Demonstration of strong β-haemolysis produced by *B. hyodysenteriae* type strain B78^T^ (A), and weak β-haemolysis produced by *B. innocens* type strain B256^T^ (B) on a blood agar plate. Taken from Hampson *et al.*, 2006a.
1.7.2. Molecular biological methods

Significant improvements in diagnosis of SD in individual pigs were achieved with the development of specific and sensitive nucleic acid-based assays, such as DNA probes (Jensen et al., 1990; Sotiropoulos et al., 1993; Harel and Forget, 1995) and polymerase chain reaction (PCR) assays (Elder et al., 1994; Harel and Forget, 1995; Leser et al., 1997; Atyeo et al., 1998; Atyeo et al., 1999; La et al., 2003; La et al., 2005) for the detection of spirochaetes in faeces. It has been suggested that PCR can detect as few as 10 spirochaete cells per gram of faeces, however in practical applications the limit of detection of PCR consistently has been reported to be in the range of $10^3$-$10^4$ cells per gram of faeces. Although PCR can be a sensitive and rapid method for detection of *B. hyodysenteriae*, it requires the availability of a diagnostic laboratory with specialised equipment. The inability of PCR to detect low numbers of cells present in the faeces also limits its application for the detection of carrier animals with subclinical infections.

1.7.3. Serological methods

Generally there is little difficulty in isolating and identifying *B. hyodysenteriae* in pigs with clinical SD. The main difficulty comes in detecting herds with subclinical infections, and individual healthy carrier animals which may be introduced into uninfected herds. As a consequence, there is a clear need to develop an effective diagnostic tool capable of detecting *B. hyodysenteriae* infection at the herd and individual pig level. Colonisation by *B. hyodysenteriae* elicits a strong immunological response against the spirochaete, hence indirect evidence of exposure to *B. hyodysenteriae* can be obtained by measuring circulating antibody titres in the blood of infected animals. These antibody titres have been reported to be maintained at low levels, even in animals that have recovered from SD (Joens et al., 1979; Fisher and Olander, 1981; Joens et al., 1982). Serological tests therefore have considerable potential for detecting recovered carrier pigs that may otherwise have undetectable numbers of spirochaetes in their large intestines.
A number of techniques have been developed to demonstrate the presence of circulating antibodies directed against *B. hyodysenteriae*. These have included indirect fluorescent antibody tests (IFAT), haemagglutination tests (HAT), microtitration agglutination tests (MAT), complement fixation tests (CFT) and enzyme linked immunosorbent assays (ELISA) using either lipopolysaccharide (LPS) or whole sonicated spirochaetes as antigen. All these tests can be useful for detecting infected herds, but they are less useful at identifying individual infected pigs. To date, no completely sensitive and specific assays are available for the detection of antibodies against *B. hyodysenteriae* in infected pigs.

1.7.3.1. Indirect fluorescent antibody test (IFAT)

Lee and Olson (1976) examined sera from 119 pigs that were exposed to *B. hyodysenteriae*, and then medicated with various drugs. They used an indirect fluorescent antibody test (IFAT) to detect serum antibodies generated against *B. hyodysenteriae* in these treated pigs and compared the results with those for 20 non-medicated pigs that had recovered naturally. Fluorescence was observed when the sera from 18 of the 20 non-medicated pigs were used, but none of the sera from the medicated pigs reacted at high titre. The more efficacious drugs apparently reduced the severity of the disease and resulted in the production of lower titres, which were still detectable in this simple assay. The specificity of the reaction for *B. hyodysenteriae* was not investigated.

1.7.3.2. Haemagglutination test (HAT)

Using sera from pigs experimentally infected with *B. hyodysenteriae*, Jenkins *et al.* (1976) were able to detect antibody against *B. hyodysenteriae* in a haemagglutination (passive haemolysis) test as early as one week after infection. Peak titres occurred six weeks after infection. In this test red blood cells were coated with guinea pig complement. Absorption of immune sera with 5% (v/v) sheep red blood cells reduced the haemolytic titres, indicating that some of the haemolysis of unabsorbed sera was probably due to heterogeneous antibodies. When hyperimmune sera were
absorbed with organisms such as *Escherichia coli* and *Salmonella* spp. there was no effect on the resultant titre. It was therefore suggested that the haemagglutination test could be a useful diagnostic tool as it was rapid to perform, required minute quantities of reagents and was highly sensitive. However, the macroscopic scale of the reactions (two millilitre final volume) would limit the number of samples which could be processed at one time. A small-scale variation of the assay in microtitre plates may overcome this problem. The specificity of this assay requires testing using sera from healthy pigs from a range of herds.

1.7.3.3. Complement fixation test (CFT)

Adachi *et al.* (1979) compared the specificity of agglutinating and complement fixing antigens for the detection of antibody to *B. hyodysenteriae*. They found that agglutination reactions differed between strains. In contrast the complement fixation test showed reactions with all strains, although these were slightly weaker with heterogeneous strains than with the homologous strain. Furthermore, agglutination tests had the advantage of being about two to eight fold more sensitive than the CFT at detecting antibody against *B. hyodysenteriae*. The CFT for SD has not been developed further.

1.7.3.4. Microtitration agglutination test (MAT)

Joens *et al.* (1978; 1980) developed a microtitration agglutination test to detect serum antibody to surface antigens of *B. hyodysenteriae*. This test was used to detect antibodies against *B. hyodysenteriae* in experimentally infected pigs, and was shown to have a high sensitivity and repeatability. Peak agglutinating titres occurred three to four weeks after the pigs were experimentally challenged with the organism. Only low cross-reacting antibody titres were produced against isolates of *Brachyspira innocens*, a non-pathogenic commensal spirochaete that commonly colonises pigs, thus suggesting that the test was specific. The inactivated whole bacterial antigen used in this test remained stable for up to ten days.
Diarra et al. (1994; 1995) evaluated the usefulness of MAT, HAT and haemolysis for the detection of antibody against *B. hyodysenteriae* in pig sera. They concluded that a boiled cell suspension used as antigen in MAT was reliable for the diagnosis of SD on a herd basis. They also found that upon boiling the antigen, cross-reactions between *B. hyodysenteriae* and *B. innocens* were significantly reduced. Although this test appeared to be reasonably specific and sensitive, it lacked standardization to allow its use for routine diagnosis. The antigen used consisted of a complex mixture of inactivated whole bacteria, which would differ in composition depending on the culture conditions used. An incubation time of 18 to 36 hours was required, making this test rather slow for diagnostic testing. In general, pigs usually develop circulating antibody titres to *B. hyodysenteriae* from one to two weeks after inoculation (Joens et al., 1979; Joens et al., 1982). MAT detected agglutinating antibody titres three to four weeks post-inoculation, suggesting that this test did not have sufficient sensitivity for detecting pigs with low titres of antibody. An overlying disadvantage of the MAT is that it can only be used to measure total antibody produced against the spirochaete, thus subjecting it to variations depending on the strain of *B. hyodysenteriae* the pig was infected with.

**1.7.3.5. Enzyme-linked immunosorbent assay (ELISA) with LPS as antigen**

An enzyme-linked immunosorbent assay (ELISA) using LPS extracted by the hot phenol-water method as a plate coating antigen has been used for the detection of antibody against *B. hyodysenteriae* infection in swine (Joens et al., 1982). Infected pigs were detected within one to two weeks post-inoculation, and circulating antibody could be demonstrated in convalescent pigs for as long as 19 weeks post-inoculation. Egan et al. (1983) compared the accuracy and sensitivity of MAT and ELISA and concluded that the ELISA was more useful, but it still was not suitable for detection of individual infected pigs because false-positive and false-negative results occurred. *B. hyodysenteriae* isolates have been divided into nine or more serogroups based on their LPS
components and cross-reactivities with absorbed antisera (Lau and Hampson, 1992; Hampson et al., 1997). Knowing this, it is expected that no one extract of LPS can be used as a general antigen in the ELISA. Each serum must be tested with a range of different LPS antigen standards because of the serotype specificity of the LPS (Egan et al., 1983). Therefore, knowledge of the serogroups of the *B. hyodysenteriae* present in an area is necessary so that appropriate LPS extracts can be used (Mhoma et al., 1992). Even then, false-negative reactions may arise from infections with members of new or undetermined serogroups. It is known that common LPS reactivities exist within serogroups (Wannemuehler et al., 1988; Lau and Hampson, 1992), however, there is no evidence showing common LPS reactivities across serogroups. For field conditions, the LPS-ELISA appears to be a robust tool for analysis of multiple samples and can be completed with reasonably short incubation times. However, without a suitable general antigen specific for *B. hyodysenteriae*, this form of ELISA remains problematic for general use.

### 1.7.3.6. Enzyme linked immunosorbent assay (ELISA) with sonicated bacteria as antigen

SD has been detected in herds by the use of an ELISA in which serum antibodies are measured using whole sonicated cells of *B. hyodysenteriae* that are used to coat the wells of the ELISA plate (Wright et al., 1989; Smith et al., 1991). This form of ELISA appears to be sensitive enough to detect the majority of infected herds. When tested at a herd level, it correctly identified 90% of individually infected pigs (Wright et al., 1989). Occasionally, however, it can give false negative results, but its most serious limitation is that it can give non-specific cross-reactivity (Burrows et al., 1984; Wright et al., 1989). However, the specificity and sensitivity of this form of ELISA for detection of antibody to *B. hyodysenteriae* in pigs can be improved when coupled with immunoblotting against outer envelope extracts of *B. hyodysenteriae* (Smith et al., 1991). The use of sonicated spirochaetes in ELISA may be a useful tool for screening herds, however due to the many cross-reactivities between *B. hyodysenteriae* and other related spirochaetes, it still cannot be used as a definitive test for SD. Even when coupled with immunoblotting, the possibility of cross-
reactivity still exists. The increased complexity of the overall technique also makes this less suitable for applications in the field, and it does not appear to have been developed further.

1.8. CONTROL AND PREVENTION

A number of methods are employed to control SD, varying from the prophylactic/therapeutic use of antimicrobial agents, to complete de-stocking of infected herds. The on-going use of antimicrobial agents in commercial piggeries is coming under increasing public pressure, and resistance to the agents is developing. On the other hand, de-stocking is an extremely expensive option, and is not always successful in eradicating the disease. As a result, there is a growing worldwide demand for the development of an effective vaccine for the preventative control of SD. The following section reviews the common methods currently employed for control of SD in piggeries, with greater emphasis placed on vaccination.

1.8.1. Antimicrobial drugs

Control of SD in endemically diseased herds generally involves the use of prophylactic antimicrobials, usually provided in the feed (Harris et al., 1999). Those that have been successfully employed to control the disease include tiamulin, ronidazole, carbadox, lincomycin, dimetridazole, tylosin and valnemulin (Rainier et al., 1980; Burch, 1982; Olson, 1986; Wood and Lysons, 1988; Smith et al., 1990; Buller and Hampson, 1994; Dalziel, 1996; Fellstrom et al., 1996; Molnar, 1996; Hayashi et al., 1997; Karlsson et al., 2001; Karlsson et al., 2002; Karlsson et al., 2003; Rohde et al., 2004). With the use of prophylactic drugs the incidence and severity of SD may be reduced. A disadvantage of this approach is that immunity may not develop and although the animals look healthy, subclinical infection may occur in the herd resulting in outbreaks after the withdrawal of the chemotherapeutics (Harris et al., 1999). Furthermore, antibiotic resistance in medicated herds appears to be becoming more common (Blaha et al., 1987; Buller and Hampson, 1994; Molnar, 1996; Gresham et al., 1998; Karlsson et al., 1999; Karlsson et al., 2001; Karlsson et
al., 2002; Karlsson et al., 2004a; Karlsson et al., 2004b; Lobova et al., 2004; Pringle et al., 2004; Rohde et al., 2004) and concerns are growing about the use of certain of these drugs in food animals, particularly in relation to the possible presence of drug residues in meat. Strict hygiene also needs to be maintained in medicated herds to reduce the level of challenge, and to prevent the entry of other strains of \textit{B. hyodysenteriae} into the piggery, as these may have different patterns of drug resistance or degree of virulence (Robertson et al., 1992).

1.8.2. Eradication

Ideally the best long-term approach to the control of SD is to eliminate \textit{B. hyodysenteriae} from infected herds. This can be achieved by partial destocking followed by the use of prophylactic drugs at therapeutic levels of medication on all animals in the herd. Unfortunately this may not be successful since there are likely to be reservoirs of infection, such as waste lagoons or infected rodents on the property serving as a source of later outbreaks (Songer et al., 1978; Olson, 1992). Eradication of SD from herds is most successfully achieved by complete or partial depopulation of stock in combination with strict hygiene and medication procedures (Janc et al., 1982). This is difficult to carry out in large herds, but has frequently been used to eliminate chronic SD (Muirhead, 1987; Harris et al., 1999). In the full depopulation method, all pigs are removed from the premises for periods of up to eight weeks and the environment is thoroughly cleaned, after which repopulation with new stock from a high health status herd may be carried out. This removes potential sources of infection and reduces the likelihood of re-entry of the disease.

1.8.3. Vaccination

A proportion of pigs which have recovered from acute SD are protected from disease when subsequently re-exposed to \textit{B. hyodysenteriae} (Joens et al., 1979), thus indicating that the infection can induce a protective immune response. Despite this, numerous attempts to develop vaccines to control SD have met with limited success, either because they have provided inadequate protection
on a herd basis, or they have been too costly and difficult to produce to make them commercially viable (Galvin et al., 1997). Bacterin vaccines provide some level of protection (Fernie et al., 1983; Parizek et al., 1985; Hampson et al., 1993; Diego et al., 1995; Waters et al., 1999a; Waters et al., 1999b), but they tend to be LPS serogroup-specific, which then requires the use of multivalent bacterins. Furthermore, they are difficult and costly to produce on a large scale because of the fastidious anaerobic growth requirements of the spirochaete. One study even reported that immunisation with a *B. hyodysenteriae* bacterin exacerbated dysentery (Olson et al., 1994). Several attempts have been made to develop attenuated live vaccines for SD, including laboratory passaged strains (Hudson et al., 1974; Lysons et al., 1987), and specific inactivation of genes encoding haemolysin (Hyatt et al., 1994), flagella components (Rosey et al., 1996; Kennedy et al., 1997) or NADH oxidase (Stanton et al., 1999). This approach has the disadvantage that attenuated strains show reduced colonisation, and hence cause reduced immune stimulation. There also is reluctance on the part of producers and veterinarians to use live vaccines for SD because of the possibility of reversion to virulence - especially as very little is known about genetic regulation and organization in *B. hyodysenteriae*.

Until recently, one vaccine for SD was commercially available in the USA. This vaccine was based on a proteinase digest of the *B. hyodysenteriae* whole cells and was formulated for serotypes 1 and 2 (the main types present in the USA) (Waters et al., 1999b). The vaccine provided only partial protection, although pigs showing clinical signs usually had mild diarrhoea and continued to gain weight. The vaccination was unable to provide sterilising immunity since protected pigs still continued to shed the spirochaete. The immunity did not appear to be mediated by the LPS content of the vaccine as pigs vaccinated with purified LPS were not protected despite their sero-conversion. Although the immunological role of LPS remains unclear, it must still be important since challenge of vaccinated pigs with heterologous strains does not result in the same level of protection (Hampson et al., 2006a).
The use of recombinant subunit vaccines is an attractive alternative to other methods, since the products would be well-defined (highly advantageous for registration purposes), and relatively easy to produce on a large scale. A number of attempts have been made to identify surface exposed proteins from *B. hyodysenteriae* that could be used as recombinant vaccine components, but again no successful vaccine has yet been made. A much more global approach is needed for the identification of potentially useful immunogenic recombinant proteins from *B. hyodysenteriae*. Likely targets would reside in the spirochaete outer membrane (i.e. surface exposed lipoproteins and transmembrane proteins/porins), as well as secreted proteins involved in the pathogenesis of the disease. These proteins are likely to induce an immune response during natural infection, although it is possible that other surface proteins that are not expressed during active infection could also be used to induce a protective immune response. Several proteins have been investigated for their potential efficacy for providing protection in pigs against SD. These proteins will be reviewed in more detail in the following sections.

1.8.3.1. Haemolysin

*B. hyodysenteriae* produces strong β-haemolysis after four to six days when grown on sheep blood agar, whilst non-pathogenic *B. innocens* produces only weak β-haemolysis (Hudson *et al.*, 1976a; Kinyon *et al.*, 1977; Kinyon and Harris, 1979). Many workers have considered that the presence of the strong β-haemolytic activity is associated with the pathogenicity of SD (Hughes *et al.*, 1975; Lysons *et al.*, 1991; ter Huurne *et al.*, 1993). The haemolysins of many bacterial species, including *E. coli* (van den Bosch *et al.*, 1982), *Actinobacillus pleuropneumoniae* (Frey and Nicolet, 1988), *Listeria monocytogenes* (Geoffroy *et al.*, 1987) and *Leptospira interrogans* (del Real *et al.*, 1989) have been associated with virulence.
A *B. hyodysenteriae* haemolysin has been purified and injected into ligated ileal and colonic loops in germ-free pigs (Lysons *et al.*, 1991). The epithelium of the colon was severely damaged, and the changes were similar to those seen in natural cases of SD. The haemolysin therefore was considered to be a potent cytotoxin for pig enterocytes and a virulence factor in SD. However, after at least 80 subcultures *in vitro*, a strain of *B. hyodysenteriae* lost its pathogenicity to pigs but retained its haemolytic activity (Hudson *et al.*, 1974). Other factors therefore also are liable to be involved in virulence.

Saheb *et al.* (1980) characterised a purified *B. hyodysenteriae* haemolysin as being oxygen-resistant, proteinase K-sensitive, heat-labile and inactivated over a wide pH range between 2 to 10. The haemolysin, with a molecular mass of 74 kDa, was probably a polypeptide or a protein associated with lipids and nucleotides. The size of purified haemolysin of *B. hyodysenteriae* has been estimated to be 68 kDa by SDS-PAGE (Knoop, 1981) and 19 kDa (Kent *et al.*, 1988) or 74 kDa (Saheb *et al.*, 1980; Hyatt *et al.*, 1994) by gel filtration. The conflict over the molecular mass may be due to the different methods of extraction and the use of different elution buffers. Equally, it was later recognised that *B. hyodysenteriae* possessed at least three haemolysins (ter Huurne *et al.*, 1993; 1994), so there is the possibility that different molecules were being examined.

The gene *tly*, encoding a *B. hyodysenteriae* haemolysin, was cloned and sequenced by Muir *et al.* (1992). This gene was present in single copy in all pathogenic serotypes of *B. hyodysenteriae* tested, and was not present in the weakly β-haemolytic non-pathogenic organism *B. innocens*. The recombinant 26.9 kDa protein encoded by the *tly* gene was EDTA insensitive, thermolabile, haemolytic for erythrocytes, and cytotoxic for several eukaryotic cell lines. To elucidate the molecular basis of virulence, a *tly*Δ (previously named *tly*) mutant strain of *B. hyodysenteriae* was constructed by transformation of a disrupted *tly*Δ gene (ter Huurne *et al.*, 1992). The recombinant mutant was able to colonise the caecum of mice, but produced fewer caecal lesions than the wild-
type *B. hyodysenteriae*. This virulence test indicated that the *tlyA*-encoded haemolysin may be an important contributor of virulence, in combination with numerous other factors. Hyatt *et al.* (1994) tested the virulence of *tlyA* mutants in pigs and demonstrated that pigs previously inoculated intragastrically with *tlyA* mutants were partially protected against subsequent challenge with a virulent *B. hyodysenteriae* strain.

Two other genes associated with β-haemolysis, designated *tlyB* and *tlyC*, were later identified and characterised based on their ability to induce a haemolytic phenotype in *E. coli* (ter Huurne *et al.*, 1994). When expressed in *E. coli*, the *tlyB* and *tlyC* genes encoded heat-labile, protease-sensitive proteins which exhibit *in vitro* haemolytic and cytotoxic activity. The calculated molecular weights of the *tlyB* and *tlyC* gene products were 93.3 kDa and 30.8 kDa, respectively. *TlyB* was similar to other bacterial Clp protease proteins, whereas *TlyC* showed no homology with other protein sequences. Southern hybridization showed that these genes are present as a single copy on the chromosome of *B. hyodysenteriae*.

More recently, a distinct gene (*hlyA*) has been characterised that encodes an 8.93 kDa polypeptide of *B. hyodysenteriae* which has haemolytic activity (Hsu *et al*. 2001). Haemolysis-negative *E. coli* strains expressing the *HlyA* gene was β-haemolytic on blood agar. The recombinant haemolysin had identical protease and lipase sensitivities and electrophoretic mobility to those of native *B. hyodysenteriae* β-haemolysin. It now appears that the *tly* genes may be regulatory elements required for haemolysin production, rather than encoding the haemolysins themselves (Hampson *et al.*, 2006a). Nevertheless, the outcomes of the experiments on both the *tlyA* and *hlyA* genes emphasise the likely role of haemolysin(s) in the virulence of *B. hyodysenteriae*. 
1.8.3.2. Flagellar proteins

The periplasmic flagella of spirochaetal cells are structurally and chemically similar to the external flagella of other bacteria (Johnson, 1977; Li et al., 2000). However, unlike other bacterial flagella, they are permanently wound around the cell body and are entirely enclosed by the outer sheath (Canale-Parola, 1984). The periplasmic flagella consist of three structural components: the filament, the hook, and the basal body (Nauman et al., 1969; Holt, 1978; Dettori et al., 1987; Charon et al., 1992).

Intestinal spirochaetes possess two wound bundles of periplasmic flagella which are inserted subterminally at each cell end, and extend toward the opposite pole of the cell. The flagella are located between the outer membrane and the cytoplasmic cylinder and thus are completely contained within the outer envelope (Canale-Parola, 1978; Dettori et al., 1987). The periplasmic flagella are essential for the typical motility of spirochaetes. The flagella of Treponema, Spirochaeta, Borrelia and Brachyspira overlap in the central portion of cells, unlike leptospiral flagella (Johnson, 1977; Li et al., 2000). The diameter of periplasmic flagella is approximately 12-25 nm (Blaha and Gunther, 1985). Most bacterial flagella have only one major flagella protein, but the flagella of spirochaetes are complex and contained multiple major proteins (Holt, 1978; Cockayne et al., 1987). Miller et al. (1988) purified B. hyodysenteriae periplasmic flagella and observed six major bands by SDS-PAGE electrophoresis, with approximate molecular mass of 39, 29, 27, 22, 21 and 18.5 kDa respectively. All these proteins cross-reacted in immunoblots with antiserum raised against B. innocens.

Kent et al. (1989) demonstrated that purified periplasmic flagella from eight serotypes of B. hyodysenteriae and two non-pathogenic intestinal spirochaetes had similar SDS-PAGE profiles, with molecular masses of 43.8, 38, 34.8, 32.8 and 29.4 kDa. Five major periplasmic flagella polypeptides were identified and these proteins were present in all the other serotypes as well as in
non-pathogens. Convalescent serum also showed a strong antibody response to the periplasmic flagella polypeptides in an immunoblot assay. This investigation indicated that the periplasmic flagella of \textit{B. hyodysenteriae} are major immunodominant antigens.

Koopman \textit{et al.} (1992a) purified and characterised the major components of the periplasmic flagella of \textit{B. hyodysenteriae} strain C5. The periplasmic flagella were composed of two sheath proteins (molecular masses 44 and 35 kDa) and three core proteins (37, 34, and 32 kDa). The 44 kDa sheath protein and the 34 kDa core protein were further characterised by cloning the corresponding genes and DNA sequence analysis of these. The 44 kDa sheath protein consisted of 320 amino acids, and was homologous to the flagella sheath proteins of the spirochaetes \textit{T. pallidum} and \textit{S. aurantia}, but not to any other flagella proteins (Koopman \textit{et al.}, 1992b). The 34 kDa core protein consisted of 285 amino acids and had significant similarity with the core flagellins of other spirochaetes (Koopman \textit{et al.}, 1993).

Li \textit{et al.} (1993) reported that a periplasmic flagella sheath protein of molecular mass 44 kDa was present in all \textit{B. hyodysenteriae} reference strains, but absent in \textit{B. innocens}. In contrast, the flagella core proteins of molecular masses 39, 35, 32, 30 and 29 kDa antigenically cross-reacted with the periplasmic flagella proteins of \textit{B. innocens}. Therefore, they suggested that the 44 kDa flagella protein was a species-specific antigen of \textit{B. hyodysenteriae}. The difference in number and molecular mass of periplasmic flagella proteins reported by different researchers may be related to the different methods used to extract the flagella, and to subsequent electrophoretic analysis.

Boyden \textit{et al.} (1989) cloned the gene that codes for an \textit{B. hyodysenteriae} periplasmic flagella antigen of molecular mass 25 kDa, and this cloned antigen was used to immunise CF-1 mice. Partially purified preparations of the recombinant product were injected intraperitoneally into CF-1 mice model, and these were protected against oral challenge with both homologous and
heterologous serotypes of *B. hyodysenteriae*. Gabe *et al.* (1995) cloned a 38 kDa periplasmic flagella protein of *B. hyodysenteriae* into *E. coli*, and vaccinated pigs with this encoded protein. This recombinant protein did not protect the animals from experimental challenge with an inoculum of the homologous strain. The incubation period however was shorter in control pigs (14.4 ± 4.7 days) than it was in the vaccinated animals (24.0 ± 10.7 days). This failure of protection is likely to relate specifically to the particular recombinant protein used, as well as to other more down-stream issues such as delivery systems and routes, dose rates, and choice of adjuvants used.

Rosey *et al.* (1996) selectively disrupted the *flaA1* and *flaB1* genes of *B. hyodysenteriae* using allelic exchange. Western blot analysis indicated that expression of the gene products had been successfully abolished. Electron microscopy showed that the dual mutation had not affected the ability of the spirochaete to assemble its periplasmic flagella. The virulence of the mutants was assessed using a murine model of SD. The dual *flaA1 flaB1* mutant of *B. hyodysenteriae* showed severe attenuation compared to the wild-type. This study showed that the FlaA1 and FlaB1 proteins were dispensable for flagella assembly but critical for normal flagella function and subsequently influence the virulence of the spirochaete.

### 1.8.3.3. Surface-exposed proteins

The outer surface of *B. hyodysenteriae* can be extracted using procedures based on its solubility in detergent and the gravitational fractionation of proteins in a density gradient. Using the appropriate characterised markers, proteins associated with the outer and inner membranes can then be identified (Chatfield *et al.*, 1988a; Chatfield *et al.*, 1988b; Sellwood *et al.*, 1989; Smith *et al.*, 1990; Thomas *et al.*, 1992; Joens *et al.*, 1993; Plaza *et al.*, 1997; Trott *et al.*, 2001). Sarkosyl-insoluble fractions of *B. hyodysenteriae* were examined by Joens *et al.* (1993) using SDS-PAGE and Western blotting with convalescent pig serum against *B. hyodysenteriae*. Seven major protein bands were observed. Six of these were periplasmic flagella proteins in the molecular mass range
of 32 to 42 kDa, and the other was a 16 kDa band. Acute pig serum against SD only recognized
some of these proteins (Joens et al., 1993). Smith et al. (1990) compared Sarkosyl-insoluble
fractions of *B. hyodysenteriae* and *B. innocens*, and found a distinct band with a molecular mass of
approximately 39 kDa specific to *B. hyodysenteriae*. Plaza et al. (1997) also identified a
predominant protein with an approximate molecular mass of 39 kDa in the outer membranes of *B.
hyodysenteriae* purified using discontinuous sucrose density gradients. This protein has since been
identified as a major cell surface protein, and the eight linked gene copies, designated *vsp*ABCD
and *vsp*EFGH, encoding variable surface proteins homologous to it have been cloned and
sequenced (Gabe et al., 1998; McCaman et al., 1999; McCaman et al., 2003). These eight genes
are present on two non-adjacent loci and do not appear to have arisen from a gene duplication
event. Gene products of the two unlinked four-member gene cluster share between 41% and 93%
similarity, and expression of the individual open-reading frames appear to be regulated by a set of
similar but distinct regulatory elements (McCaman et al., 2003). Hydrophobicity plots of VspA-D
and VspE-H indicated that their highly variable hydrophilic regions would allow these proteins to
span the outer membrane and be present on the outer surface of the spirochaete (Gabe et al., 1998;
McCaman et al., 1999; McCaman et al., 2003). Although this 39 kDa protein may contain regions
of variability, conserved regions which exist could be useful for detection of antibody specific for
*B. hyodysenteriae*. The cell surface location of this protein also makes it potentially useful as the
basis of a subunit vaccine. The *vsp*ABCD and *vsp*EFGH loci has recently been renamed
*bhmp39abcddefgh* in light of the new nomenclature (Witchell et al., 2006)

Sodium dodecyl sulfate (SDS)-soluble proteins were extracted from *B. hyodysenteriae* and
analysed by Chatfield et al. (1988a, b). Porcine hyperimmune serum to *B. hyodysenteriae* detected
polypeptide antigens of molecular mass within the range 30 and 36 kDa. When the cell envelope
from *B. hyodysenteriae* was extracted using Triton X-100, several major immunogenic
polypeptides with molecular mass between 24 and 45 kDa were detected using serum from a pig
vaccinated with whole cells of *B. hyodysenteriae* (Chatfield *et al*., 1988a). A 36 kDa antigen associated with the cell envelope was immunologically distinct in *B. hyodysenteriae*, and antibody against this antigen was not removed by absorption with whole *B. innocens* cells. This 36 kDa protein may be related to the 39 kDa protein observed by Smith *et al.* (1990) and Plaza *et al.*, (1997).

Sellwood *et al.* (1989) found a prominent 16 kDa protein of *B. hyodysenteriae* which was soluble in SDS and reacted strongly with hyperimmune and convalescent pig sera. Subsequently, this protein was cloned and characterised as a 16 kDa membrane-associated lipoprotein common to many strains of *B. hyodysenteriae*. The identified and sequenced gene encoding the 16 kDa lipoprotein was designated *smpA* (Thomas and Sellwood, 1992; Thomas *et al*., 1992; Thomas and Sellwood, 1993). A monoclonal antibody specific for the SmpA lipoprotein was used to develop an ELISA for detecting SmpA in the faeces of pigs infected with *B. hyodysenteriae* (Sellwood *et al.*, 1995). When pigs were experimentally challenged with *B. hyodysenteriae*, SmpA was detected only during the initial post-challenge period and failed to be detected after the onset of clinical SD. When the inoculated strain of *B. hyodysenteriae* was isolated and re-cultured *in vitro*, SmpA was detected. This loss of *in vivo* expression was proposed to be due to the down-regulated expression of the gene encoding SmpA during colonisation. The low antibody titres which developed against SmpA were believed to be a result of its repressed *in vivo* expression, and this prevented its use as the basis of a serologic assay. The lack of *in vivo* expression of *smpA* also limits its usefulness as a subunit vaccine for *B. hyodysenteriae*.

A gene encoding an outer membrane protein of similar size to SmpA was recently identified and designated *smpB* (Holden *et al*., 2006). Although SmpB shares no detectable similarity with SmpA, both are similar in size, SmpB being slightly larger at 17.6 kDa, and they possess an identical 10 residue leader peptide sequence. SmpB was shown to be specific to *B. hyodysenteriae*
using Southern blotting and reactivity with rabbit serum raised against *B. hyodysenteriae* whole cells. Southern blot analysis also revealed that *smpA* and *smpB* reside at the same locus, however strains possess either one or the other gene. There was no evidence suggesting both genes were present in the same strain. Western blotting using polyclonal sera also indicated that no cross-reactivity was present between a strain which possessed *smpA* and a strain which possessed *smpB*.

Li *et al.* (1995) reported that *B. hyodysenteriae* expressed at least three iron-regulated proteins with approximate molecular masses of 109, 134 and greater than 200 kDa when grown under iron-limited conditions. Western blotting with serum from an experimentally challenged pig showed that the 109 kDa major iron-regulated protein was expressed *in vivo* and was conserved amongst all *B. hyodysenteriae* strains tested. However, the iron-regulated proteins are presumably not antigenically specific for *B. hyodysenteriae* since they are also expressed in *B. innocens*.

Recently, a locus encoding four paralogous outer membrane lipoproteins of *B. hyodysenteriae* was cloned, characterised and designated *blp*GFEA (Cullen *et al.*, 2003). Since the *blpA* member of the gene cluster is identical to the gene under study in this thesis, further review of the *blp*GFEA loci will be presented in a later chapter.

### 1.8.3.4. DNA Vaccination

To date, only one study using DNA for vaccination against SD has been reported. In this study, the *B. hyodysenteriae* *ftnA* gene, encoding a putative ferritin, was cloned into an *E. coli* plasmid and the plasmid DNA used to coat gold beads for ballistic vaccination. A murine model for SD was used to determine the protective nature of vaccination with DNA and/or recombinant protein. Vaccination with recombinant protein induced a good systemic response against ferritin, however vaccination with DNA induced a barely detectable serological response. Vaccination with DNA followed by a boost with recombinant protein induced a systemic immune response to ferritin only
after boosting with protein. However, none of the vaccination regimes tested was able to provide mice with protection against *B. hyodysenteriae* colonisation and the associated lesions. Interestingly, vaccination of the mice with DNA alone also resulted in significant exacerbation of disease (Davis et al., 2005).

1.9. AIMS OF THE PROJECT

In order to minimise the economic losses caused by SD, extensive studies have been performed to develop a vaccine against SD. When studies described in this thesis began, no subunit vaccine had been identified with certainty, and published experimental vaccines gave only partial protection. Little was known of the molecular biology of *B. hyodysenteriae*, and *Brachyspira* spp. in general. Genetic approaches to elucidate the pathogenesis of *B. hyodysenteriae* infections had been hampered by a lack of sufficient genomic data for the spirochaete. A monoclonal antibody (BJL/SH1) specific for *B. hyodysenteriae* had been produced and characterised (Lee and Hampson, 1996). A genomic library for *B. hyodysenteriae*, produced in bacteriophage lambda, had been screened using this monoclonal antibody. Phage clones possessing fragments of the *B. hyodysenteriae* genome and reacting with the monoclonal antibody had been isolated, although the DNA sequence encoding the protein responsible for this reactivity had not been identified (Lee, 1996).

Therefore, the aim of the work presented in this thesis was to assess the usefulness of the protein, responsible for interacting with the monoclonal antibody BJL/SH1, as a subunit for vaccination and/or serological diagnosis of SD. To achieve this, it was necessary to identify and characterise the gene encoding this protein of interest. At this stage, it may be useful to mention that the work presented in this thesis was started in 1998, and its presentation has been delayed until now due to the need to preserve its commercial confidentiality.
Consequently, to achieve the desired aims, the specific objectives of this study were as follows:

1) To confirm the reactivity of the monoclonal antibody BJL/SH1 with the identified reactive phage clone.

2) To determine the nucleotide sequence of the *B. hyodysenteriae* genomic DNA fragment which was incorporated into the genome of the lambda bacteriophage clone.

3) To determine the gene sequence encoding the protein responsible for the interaction with the monoclonal antibody (BJL/SH1) and to determine its likely identity.

4) To confirm that the protein was a lipoprotein by radioactive labelling with tritiated palmitate.

5) To check the distribution of the gene in all *Brachyspira* species.

6) To clone the identified gene into an *E. coli* expression system and purify the recombinant protein in preparative-scale and large-scale.

7) To assess the usefulness of the recombinant protein as an antigen for an ELISA test.

8) To evaluate the immunogenicity of the recombinant protein when used to vaccinate mice.

9) To evaluate the efficacy of vaccination with the recombinant protein in pigs experimental challenged with *B. hyodysenteriae*. 

CHAPTER 2: IDENTIFICATION AND CHARACTERISATION OF A 29.7 KDA OUTER MEMBRANE LIPOPROTEIN OF *BRACHYSPIRA HYODYSENTERIAE*

2.1. INTRODUCTION

This chapter describes the screening of a *Brachyspira hyodysenteriae* genomic library prepared in bacteriophage λ to identify and subsequently characterise the gene encoding a 29.7 kDa outer membrane lipoprotein. Since the work was completed in 1999, the general approach has been somewhat superceded. For that reason, more recent approaches will be briefly mentioned here. Recent advances in recombinant DNA technology, in combination with robotic automation of molecular processes, now have allowed sequencing of whole bacterial genomes to be accomplished in relatively short time-frames. Bioinformatics has emerged as a hot-spot area of research, with several hundred bacterial genomes sequenced to completion since the sequencing of the first bacterial genome (*Haemophilus influenzae*) (Fleischmann *et al.*, 1995). Prior to the feasibility of whole genome sequencing and large-scale annotation of gene sequences, identifying and characterising individual genes or gene clusters involved screening of genomic libraries using antibodies or labelled probes, as was done in the work described in this thesis.

Genomic libraries generally are constructed by cloning random fragments of genomic DNA into either plasmid vectors or λ bacteriophage. The fragments of genomic DNA to be cloned are generated by mechanically shearing the DNA followed by enzyme-mediated fill-in of protruding terminal ends, or by partial digestion with a frequent-cutting restriction endonuclease. Generally, random fragments of a specified length are selected for cloning by centrifugation through a density gradient prior to cloning into the bacteriophage vector (Sambrook *et al.*, 1989). Although genomic libraries generated in plasmid vectors are not uncommon, λ bacteriophage vectors are preferred
due to their capacity to ligate larger-sized insert DNA, and their suitability for screening with either antibodies or probes (Young and Davis, 1983a; Young and Davis, 1983b). Bacteriophage \( \lambda \) has a double stranded DNA genome and adsors to receptors on the outer membrane of \( E. coli \), from which it is transported into the cell. The virus replicates and accumulates in the cell until lysis is induced. In the case of many \( \lambda \) vectors, lysis is facilitated by a temperature sensitive transcription repressor so that at high temperatures, the mutated repressor is unable to maintain lysogeny (Johnson et al., 1979). Lambda vectors are commonly supplied as insertion vectors with a multiple cloning site located in the N-terminal portion of the \( lacZ \) gene such that if the cloned DNA is in-frame with the \( lacZ \) sequence, a fusion protein may be expressed and detected by antibody screening (Frischauf et al., 1983). A further advantage of some \( \lambda \) vectors is the ability to be excised \textit{in vivo} and converted to plasmid (phagemid) in the presence of M13 helper phage. Combined with blue-white (colour) identification when plated on \( lac^- \) hosts in the presence of isopropyl-\( \beta \)-D-thio-galacto-pyranoside (IPTG) and 5-bromo-4-chloro-3-indoyl-\( \beta \)-D-galactopyranoside (X-gal), the bacteriophage \( \lambda \) vectors allow efficient construction and screening of the genomic library.

Screening of the \( \lambda \) genomic library usually involves plaque-lifts whereby the DNA or expressed protein from single bacteriophage plaques are passively transferred onto nitrocellulose and used for probe hybridisation or immuno-blotting. Infection of \( E. coli \) cells with \( \lambda \) bacteriophage results in the lysis of the cell. As a result, spread of the bacteriophage infection leads to the formation of cleared-zones on a bacterial lawn, termed plaques. These plaques contain not only the DNA of the bacteriophage clone but also the proteins expressed by the phage. Screening of the plaque-lift membranes can be done using DNA hybridisation probes, monoclonal antibodies or polyclonal serum. The choice of screening method largely depends on availability of a probe/antibody, the sensitivity of detection required, and the intended purpose of the identified gene. For example, if a
gene being identified is intended for a potential vaccine antigen, screening the genomic library with convalescent polyclonal serum may be preferred.

Once a bacteriophage clone has been isolated, the recombinant region of the phage genome can be sequenced and potential open reading frames identified. Various computational analyses of the identified gene sequence can be performed to provide further information as to the possible function of the gene or gene product. These annotation tools are based largely on homology with other genes present in the cross-referenced databases. Ultimately, the true function of the identified gene sequence needs to be determined using experimental molecular and biochemical techniques.

Identification of bacterial genes using bacteriophage genomic libraries has been a common method in the “pre-bioinformatics” era. Notably, very little has been published for the *Brachyspira* genus. Of the few genes described, most have been identified by the screening of genomic libraries constructed in bacteriophage $\lambda$ (Thomas and Sellwood, 1993; Gabe *et al.*, 1998; Rayment *et al.*, 1998; Cullen *et al.*, 2003; McCaman *et al.*, 2003). In this chapter, the identification, characterisation and immuno-reactivity of a ~30 kDa outer membrane protein of *B. hyodysenteriae* is described. The generation of a genomic library for *B. hyodysenteriae* in bacteriophage $\lambda$, and the subsequent screening of this library with the monoclonal antibody BJL/SH1 have been described in a previous PhD thesis (Lee, 1996). Parts of the work were repeated here, and the study was extended to fully sequence and identify the gene encoding the membrane protein and analyse its distribution in *Brachyspira* spp. strains.
2.2. MATERIALS AND METHODS

Abbreviations used in this chapter, and those to follow, are listed on pages xxiv-xxv. Manufacturers of key reagents and consumables are listed in appendix A.1 together with the place of manufacture. Details of major buffers and solutions used are listed in appendix A.2, and will therefore not be given in the body of the text.

2.2.1. Production of monoclonal antibody BJL/SH1

The monoclonal antibody (Mab) BJL/SH1 used in this experiment was prepared and characterised previously (Lee and Hampson, 1996). It has been shown to react with a species-specific 30 kDa outer envelope protein of *B. hyodysenteriae*. The hybridomas were stored in liquid nitrogen in Dulbecco's Modified Eagle Medium (DMEM; Gibco-BRL Life Sciences) containing 10% (v/v) foetal calf serum (FCS; Commonwealth Serum Laboratories) and 10% (v/v) dimethyl sulfoxide (DMSO). Frozen stocks of hybridoma were taken from liquid nitrogen and immediately thawed in a 37°C waterbath. The thawed cells were transferred to a 10 ml centrifuge tube containing 9 ml pre-warmed DMEM and centrifuged at 1,000 \( \times g \) for 5 min. The supernatant was discarded and the cell pellet resuspended with 2 ml DMEM supplemented with 100 \( \mu M \) hypoxanthine, 0.4 \( \mu M \) aminopterin, 16 \( \mu M \) thymidine, 0.58 mg/ml glutamine, 0.22 mg/ml sodium pyruvate, 200 IU/ml penicillin, 200 \( \mu g/ml \) streptomycin, 0.05 mg/ml gentamycin (DMEM-HAT) and containing 20% (v/v) FCS. The hybridomas were first recovered in 96-well flat bottom tissue culture plates, then in 24-well tissue culture plates, and finally in 25 cm\(^2\) tissue culture flasks. During flask culture, the DMEM-HAT medium was supplemented with 10% (v/v) FCS. At this stage, the hybridoma cells were allowed to grow until the cells were at the point of death and then the culture supernatant was clarified by centrifugation at 2,500 \( \times g \) for 5 min. Prior to storage at -20°C, tissue culture supernatant was tested by Western blot analysis (section 2.2.5) against a whole-cell preparation of *B. hyodysenteriae* strain WA1. Undiluted tissue culture supernatant containing the Mab was used in all plaque-lift assays and Western blotting, unless otherwise stated.
2.2.2. Screening of the genomic library

A Lambda ZAP II bacteriophage library (Stratagene) prepared from *B. hyodysenteriae* strain P18A was screened for expression of the 29.7 kDa antigen by B. J. Lee (Lee, 1996) using Mab BJL/SH1 in a plaque-lift method. Five phagemid clones (designated pSHA, pSHB, pSHC, pSHD, and pSHE) were excised by combining the Mab-screened phage isolate with ExAssist helper phage (Stratagene) and transfected into *E. coli* XLOLR cells (Stratagene). The cells were stored at -80°C in Luria-Bertani (LB) broth containing 20% (v/v) glycerol (Lee, 1996).

2.2.3. Expression of the gene encoding the 30 kDa protein in *E. coli*

The *E. coli* XLOLR clones harbouring the recombinant phagemids were streaked out onto LB agar plates supplemented with 50 mg/L kanamycin and incubated at 37°C overnight. A single colony was used to inoculate 10 ml of LB broth supplemented with 50 mg/l kanamycin, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1 mM IPTG. The broth culture was incubated at 37°C for 12 h with shaking. One ml of each culture was centrifuged at 2,500 × g for 15 min and washed three times. The washing process involved resuspension of the cell pellet with 1 ml phosphate buffered saline (PBS) and centrifugation at 2,500 × g. The washed cell pellets were resuspended in 100 μl of PBS ready for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

2.2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

All SDS-PAGE analysis of protein involved electrophoretic separation using the discontinuous Tris-glycine buffer system of Laemmli (Laemmli, 1970). Thirty μl of protein sample were mixed with 10 μl of 4× sample treatment buffer. Samples were boiled for 5 min immediately prior to loading 10 μl of the sample into wells in the gel. The gel comprised a stacking gel and a separating gel. These gels were polymerised by the addition of 0.1% (v/v) N,N,N′,N′-Tetramethyl-ethylenediamine (TEMED) and 0.05% (w/v) freshly prepared ammonium sulphate solution and
cast into the mini-Protean dual slab cell (Bio-Rad Laboratories). Samples were electrophoresed at 150 V at room temperature (RT) until the bromophenol blue dye-front reached the bottom of the gel. Pre-stained molecular mass standards were electrophoresed in parallel with the samples in order to allow molecular mass estimations. After electrophoresis, the gel was immediately subjected to electro-transfer onto a nitrocellulose membrane for Western blotting.

2.2.5. Western Blot Analysis

Electrophoretic transfer of separated proteins from the SDS-PAGE gel to a nitrocellulose membrane was performed using the method of Towbin (Towbin et al., 1979). After electrophoresis, the gel was equilibrated in transfer buffer for 15 min. The proteins in the gel were transferred to a nitrocellulose membrane (Schleicher & Schuell) using the Mini-Protean Trans-Blot apparatus (Bio-Rad Laboratories) and electrophoretic transfer was performed at 30 V overnight at 4°C. The freshly transferred nitrocellulose membrane containing the separated proteins was blocked with 10 ml of Tris buffered saline (TBS) containing 5% (w/v) skim milk powder for 1 h at RT. The membrane was washed with TBS containing 0.05% (v/v) Tween 20 (TBST) and then incubated with 10 ml BJL/SH1 (diluted 10-fold with TBST) for 1 h at RT. After washing three times for 5 min with TBST, the membrane was incubated with 10 ml goat anti-mouse IgG-HRP (Sigma Chemical Company) diluted 2,000-fold in TBST for 1 h at RT. The membrane was developed with 10 ml 3-3’ diaminobenzidinetetrahydrochloride (DAB) substrate solution (Sigma Chemical Company). The development reaction was stopped by washing the membrane with distilled water. The membrane was then dried and scanned for presentation.

2.2.6. Sequencing of the B. hyodysenteriae insert

The phagemid pSHA was chosen for direct sequencing of the B. hyodysenteriae genomic insert using the ABI 373A DNA Sequencer (PE Applied Biosystems). Previously it had not been successfully sequenced (Lee, 1996). The phagemid was purified from the E. coli XLOLR cells
using the QIAprep Spin Miniprep Kit (Qiagen). Briefly, 5 ml of an overnight culture was pelleted by centrifugation at 5,000 × g for 10 min. The cell pellet was thoroughly resuspended in 250 μl of buffer P1 by repeated aspiration, before adding 250 μl of buffer P2. The tube was mixed thoroughly by vortexing. Three hundred and fifty μl of buffer N3 was added to the slurry and the tube was gently inverted to mix. Cellular debris was removed by centrifugation at 20,000 × g for 5 min. The supernatant was transferred to a spin column and centrifuged at 6,000 × g for 1 min. The flow-through was discarded and 500 μl of buffer PE was added to the column before centrifuging at 6,000 × g for 1 min. The column was dried by centrifugation at 20,000 × g before 50 μl of buffer AE was added directly to the column filter. To improve elution, the column was incubated at RT for 2 min before centrifugation at 10,000 × g for 1 min. The eluted purified plasmid DNA was stored at -20°C.

The initial insert sequences were obtained using commercially available T3 and T7 oligonucleotides (Stratagene) which annealed to the vector regions flanking either ends of the insert. The remaining oligonucleotides were designed based on the 3'-OH end of the upstream insert sequences (Table 2.1). Each sequencing reaction was performed in a 10 μl volume consisting of 300 ng of phagemid, 4 pmol of primer, and 4 μl of the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Mix (PE Applied Biosystems). Cycling conditions involved a 2 min denaturing step at 96°C, followed by 25 cycles of denaturation at 96°C for 10s, annealing at the primer’s melting temperature (Table 2.1) for 5s, and primer extension at 60°C for 4 min. Residual dye terminators were removed from the sequencing products by precipitation with 95% (v/v) ethanol containing 120 mM sodium acetate (pH 4.6), and vacuum dried. The sequencing products were analysed using an ABI 373A DNA Sequencer (PE Applied Biosystems).
Table 2.1. Oligonucleotide primers used for PCR and sequencing of \textit{bhlp29.7}.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>T&lt;sub&gt;A&lt;/sub&gt; (°C)</th>
<th>Sequence (5'-3')</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3</td>
<td>60</td>
<td>TAACCCTCACTAAAGGGAAC</td>
<td>Phagemid sequencing</td>
</tr>
<tr>
<td>T7</td>
<td>60</td>
<td>GTAATACGACTCACTATAGGAC</td>
<td>Phagemid sequencing</td>
</tr>
<tr>
<td>SHA-R1</td>
<td>50</td>
<td>GCTGA ACTTATATATATCTGC</td>
<td>Phagemid sequencing</td>
</tr>
<tr>
<td>SHA-R2</td>
<td>55</td>
<td>GTTCTTATCTTCTCTACTTGG</td>
<td>Phagemid sequencing</td>
</tr>
<tr>
<td>SHA-F3</td>
<td>55</td>
<td>GCGGAGAAGTTCTAGACTCC</td>
<td>Phagemid sequencing</td>
</tr>
<tr>
<td>SHA-F2</td>
<td>50</td>
<td>TCTATGTGGAATAAAGCGGC</td>
<td>Phagemid sequencing</td>
</tr>
<tr>
<td>SHA-F1</td>
<td>55</td>
<td>AGGCGCAGAAGATTCAAAGAG</td>
<td>Phagemid sequencing</td>
</tr>
<tr>
<td>Bhlp29.7-US</td>
<td>50</td>
<td>AAAAGAGGTTGTAGAATCAG</td>
<td>\textit{bhlp29.7} sequencing</td>
</tr>
<tr>
<td>Bhlp29.7-DS</td>
<td>50</td>
<td>TGAAGAAGATGATGATATCG</td>
<td>\textit{bhlp29.7} sequencing</td>
</tr>
<tr>
<td>Bhlp29.7-F58</td>
<td>60</td>
<td>TGCGGAAATACTTCTTCTGAG</td>
<td>\textit{bhlp29.7} PCR and sequencing</td>
</tr>
<tr>
<td>Bhlp29.7-R630</td>
<td>60</td>
<td>AGAACCGGATCAAAACCGAA</td>
<td>\textit{bhlp29.7} PCR and sequencing</td>
</tr>
<tr>
<td>Bhlp29.7-F13</td>
<td>55</td>
<td>TTATTATTGTGTATCATCAGC</td>
<td>Probe for hybridisation</td>
</tr>
<tr>
<td>Bhlp29.7-R809</td>
<td>55</td>
<td>CAAGTAGGAAGATAAAGACC</td>
<td>Probe for hybridisation</td>
</tr>
<tr>
<td>H1</td>
<td>52</td>
<td>ACTAAAGATCCTGATGTTATTTG</td>
<td>NADH oxidase (\textit{nox}) PCR</td>
</tr>
<tr>
<td>H2</td>
<td>52</td>
<td>CTAATAAACGTCTGCTGCTG</td>
<td>NADH oxidase (\textit{nox}) PCR</td>
</tr>
</tbody>
</table>
2.2.7. *In silico* analysis of the hypothetical open reading frame

Sequence results were edited and compiled using SeqEd v1.0.3 (PE Applied Biosystems). The nucleotide sequences were analysed using Vector NTI version 6 (InforMax) and the University of Wisconsin Genetics Computer Group program. The deduced hypothetical open reading frame (ORF) was used to search for homology against all sequence databases available at the National Center of Bioinformatics (NCBI). In order to determine the possible subcellular localisation of the protein, the translated ORF was analysed using the HMMTOP Prediction of Transmembrane Helices and Topology of Proteins Version 2.0 (Tusnady and Simon, 1998; 2001) and PSORTb Version 2.0 (Gardy et al., 2005) software. The Antigenic program (Kolaskar and Tongaonkar, 1990) was used to determine the most antigenic sites of the protein. The ORF was initially designated *bmpB* and the encoded lipoprotein BmpB, however this gene was later renamed as *bhlp29.7* and the lipoprotein Bhlp29.7 (Hampson *et al.*, 2006b).

2.2.8. Western blot reactivity of convalescent pig sera with *B. hyodysenteriae* cell envelope proteins

Five serum samples were obtained from pigs suffering from SD in a naturally infected herd. Serum samples from two pigs from a high-health status herd free of SD were used as normal pig serum controls. Ten μg of a Triton X-114 cell envelope preparation of the fully characterised Western Australian *B. hyodysenteriae* strain WA1, prepared by I.W.M. Tenaya (Tenaya, 1997), were loaded into a 7 cm preparative well, separated by SDS-PAGE (section 2.2.4) and electro-transferred to a nitrocellulose membrane (section 2.2.5). The membrane was assembled into the Deca-Probe apparatus (Hoefer). Individual pig serums were diluted 100-fold in TBST and reacted with the immobilised protein in separate lanes of the Deca-Probe. Mab BJL/SH1 diluted 10-fold was also included in a separate lane as a positive control. The Western blot was performed as described in section 2.2.5. Bound antibodies were detected using goat anti-pig IgG-horse-radish peroxidise (HRP) (Southern Biotechnology) diluted 2,000-fold.
2.2.9. Spirochaete culture

2.2.9.1. Preparation of culture media

Pure isolates of intestinal spirochaetes were grown in modified pre-reduced Kunkle’s broth (Kunkle et al., 1986). This pre-reduced anaerobic broth contained 30 g/l Trypticase Soy Broth (TSB; Oxoid), 10 g/l yeast extract (Oxoid), 5 g/l glucose and 4 g/l sodium hydrogen carbonate. These ingredients were mixed with distilled water and adjusted to pH 6.85 with hydrochloric acid before heating and adding 0.5% (v/v) newborn calf serum (Gibco-BRL Laboratories), 2% (v/v) FCS (Commonwealth Serum Laboratories), 1 g/l L-cysteine, 0.2 g/l cholesterol and 0.1% (v/v) resazurin (Sigma Chemical Company). The mixture was boiled and 10 ml volumes were aliquoted into 20 ml test tubes, or 300 ml volumes were poured into 500 ml glass medical flat bottles under a stream of deoxygenated N₂, and the gas was then bubbled through the media until the colour of the resazurin indicated that the oxygen had been eliminated. Rubber bungs were inserted into the necks of the glassware, and the tubes or bottles were clamped into metal racks to prevent the bungs being expelled. The media were autoclaved at 121°C for 30 min before use. After the glassware was cool, small strips of parafilm were wrapped around the interface between the necks of the glassware and the bungs to help prevent the bungs from being ejected during storage. Media were stored at RT for up to 2 weeks before use.

2.2.9.2. Growth conditions

Two ml volumes of the pure isolates stored at -80°C were revived by inoculating the entire 2 ml volume of thawed culture into 10 ml of anaerobic broth in a test tube. The bungs were flooded with 70% (v/v) ethanol before using a 20 gauge needle and syringe to inject the cells into the test tube, whilst keeping the medium in the tube anaerobic. The cultures were incubated at 37°C on a rocking platform for 72-96 h. Once cultures reached approximately 10⁵-10⁶ cells per ml, as determined by counting in a haemocytometer chamber under a phase-contrast microscope, they were transferred into the 300 ml volumes of anaerobic broth in bottles and incubated under the
same conditions for 72-96 h or until mid log phase growth (10⁹ cells per ml) was observed. The spirochaete cells were harvested by centrifugation at 10,000 × g for 20 min and the cell pellet stored at -80°C until required.

2.2.10. Spirochaetal strains and chromosomal DNA extraction

Fully characterised pure cultures of *Brachyspira* spp. strains (n=102) were obtained from the culture collection held at the Reference Centre for Intestinal Spirochaetes at Murdoch University. These included 48 strains of *B. hyodysenteriae*, 18 strains of *B. pilosicoli*, 12 strains of *B. innocens*, 12 strains of *B. intermedia*, eight strains of *B. murdochii*, two strains of “*Brachyspira canis*”, 2 strains of *Brachyspira alvinipulli* and one strain of *Brachyspira aalborgi*. The strain names are listed in Appendix A.3. These strains were propagated at 37°C in Kunkle's pre-reduced anaerobic broth containing 2% (v/v) FCS and a 1% (v/v) ethanolic cholesterol solution (section 2.2.9).

Chromosomal DNA was prepared from these *Brachyspira* spp. strains using the DNeasy Tissue Kit (Qiagen). Briefly, 400 mg (wet weight) of cell pellet was thoroughly resuspended in 400 μl of PBS by repeated aspiration, before adding 40 μl of proteinase K (20 mg/ml; Sigma Chemical Company) and 400 μl of buffer AL. The tube was vortexed to mix and incubated at 70°C for 15 min. Four hundred μl of absolute ethanol was added to the suspension and the tube was gently inverted to mix. The entire contents of the tube was divided into two spin columns and centrifuged at 6,000 × g for 1 min. The flow-through was discarded and the column washed with 500 μl of buffer AW1 followed by 500 μl of buffer AW2. The column was centrifuged at 6,000 × g for 1 min between wash buffers and the flow-through discarded. The column was dried by centrifugation at 20,000 × g before 100 μl of buffer AE was added directly to the column filter. To improve elution, the column was incubated at 70°C for 2 min before centrifugation at 10,000 × g.
for 1 min. The eluted purified DNA from both columns were pooled into a fresh tube and stored at -20°C.

2.2.11. Polymerase chain reactions

PCR assays were performed as follows, unless otherwise stated. The amplification mixture consisted of 1× PCR buffer (containing 1.5 mM of MgCl₂), 0.5 U of Taq DNA polymerase (Biotech International), 0.05 U Pfu DNA polymerase (Promega), 0.2 mM of each dNTP (Promega), 0.5 μM of the primer set, and 50-100 ng of chromosomal template DNA in a total volume of 50 μl. Cycling conditions involved an initial template denaturation step of 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and primer extension at 72°C for 2 min. The PCR products were separated by electrophoresis in 1.5% (w/v) agarose in 1× Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer, stained by emersion in a 1 μg/ml ethidium bromide solution and viewed over ultraviolet (UV) light.

All oligonucleotide primer sequences and annealing temperatures used for PCR analysis in this chapter are listed in Table 2.1.

2.2.12. PCR amplification and sequencing of bhlp29.7 in Brachyspira spp.

2.2.12.1. PCR amplification of bhlp29.7

Two pairs of primers that annealed to regions internal and external to the bhlp29.7 coding sequence were designed and optimised for PCR detection of bhlp29.7 using chromosomal DNA from the 102 strains of Brachyspira species (section 2.2.10). The external primer set used consisted of Bhlp29.7-US and Bhlp29.7-DS (Table 2.1) that anneal to complementary sequences adjacent to the coding region of bhlp29.7. The internal primer set used consisted of Bhlp29.7-F58 and Bhlp29.7-R630 (Table 2.1) that amplified a 572 bp region within the coding region of bhlp29.7. PCR reactions were performed as outlined in section 2.2.11.
2.2.12.2. Purification of PCR products

Products generated by PCR were purified using the UltraClean PCR Clean-up Kit (Mo Bio Laboratories). Briefly, 50 μl of PCR product was mixed with 250 μl of SpinBind buffer. The mixture was transferred to a spin column and centrifuged at 6,000 × g for 1 min. The flow-through was discarded and 250 μl of SpinClean buffer was added to the column before centrifuging at 6,000 × g for 1 min. The column was dried by centrifugation at 20,000 × g for 2 min. Fifty μl of elution buffer was added directly to the filter of the column and centrifuged at 10,000 × g for 1 min. The purified PCR product was stored at -20°C.

2.2.12.3. Sequencing of bhlp29.7

Sequencing of the PCR product from 11 strains of *B. hyodysenteriae* (B78\(^T\), B169, B204, P18A, B/Q02, WA1, WA4, WA5, WA6, WA15 and WA16) and *B. innocens* strain B256\(^T\) was performed using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Mix (PE Applied Biosystems), using the same sets of primers as the PCR (section 2.2.12.1). The sequencing method is outlined in section 2.2.6. Sequence results were edited and compiled using SeqEd v1.0.3 (PE Applied Biosystems) before being translated into amino acid sequence using the BioEdit Sequence Alignment Editor (North Carolina State University). The nucleotide and translated protein sequences for all the *bhlp29.7* genes detected were compared to generate a pair-wise identity matrix.

2.2.13. Slot blotting and Southern hybridisation

2.2.13.1. *Brachyspira* strains

A subset of 25 spirochaete strains was randomly selected to provide a numerically representative selection of the whole collection, and tested for *bhlp29.7* by PCR. These included 11 strains of *B. hyodysenteriae* (B78\(^T\), P18A, B169, B204, B/Q02, WA1, WA4, WA5, WA6, WA15 and WA16),
four strains of *B. innocens* (B256^T^, 4/71, WestA and Q91.1530.1), four strains of *B. pilosicoli* (P43/6/78^T^, 95/1000, 1331-6 and B155A), three strains of *B. intermedia* (PWS/A^T^, P280-1 and 889), and three strains of *B. murdochii* (56/150^T^, 155/20 and 155/21). To ensure that the *Brachyspira* strains were not contaminated with *B. hyodysenteriae*, the NADH oxidase (*nox*) PCR, which targets a 354-bp region of the *nox* gene of *B. hyodysenteriae*, was performed on all cultures prior to Southern blot analysis. The PCR was performed as outlined in section 2.2.11 but without *Pfu* DNA polymerase. The primers H1 and H2 (Table 2.1) were used and *B. hyodysenteriae* strain B78^T^ was the positive control for the assay.

### 2.2.13.2. Probe preparation

The *bhlp29.7* probe was constructed to represent nearly the entire gene. DNA from positions 13 to 816 of the *bhlp29.7* coding sequence was amplified using the primers Bhlp29.7-F13 and Bhlp29.7-R809 (Table 2.1) and the PCR product was purified using the UltraClean PCR Clean-up Kit (section 2.2.12.2). The purified PCR product was denatured for 10 min and immediately chilled on ice. Twelve μl of DIG-High Prime Probe Labelling reagent (Roche) was added to 48 μl of denatured PCR product, mixed well and briefly centrifuged to collect the mixture at the bottom of the tube. The labelling reaction was incubated at 37°C overnight. The following day, the probe labelling reaction was stopped by incubating the reaction at 65°C for 10 min. The DIG-labelled DNA probe was stored at -20°C.

### 2.2.13.3. Slot blot preparation

Chromosomal DNA (0.5 μg) from the 25 *Brachyspira* strains was digested to completion at 37°C overnight with 20U of *Hin*III (Promega) in 1× *Hind*III buffer and transferred onto Hybond N+ membranes (Amersham Pharmacia Biotech) using the Bio-Dot SF apparatus (Bio-Rad Laboratories). The membrane was then semi-dried and cross-linked at 150 mJ using the GS Gene
Linker™ (Bio-Rad Laboratories) and stored between two sheets of filter paper at RT until required.

2.2.13.4. Southern hybridisation preparation
Chromosomal DNA (0.5 μg) from *B. pilosicoli* strain 95/1000, *B. innocens* strain B256T, *B. intermedia* strain P280-1, *B. murdochii* strain 155/20 and *B. hyodysenteriae* strains WA1 and B78T were digested to completion at 37°C overnight with 20U of *Hind*III (Promega) in 1× *Hind*III buffer and the restriction fragments were separated by electrophoresis through a 1% (w/v) agarose gel in 1× TAE buffer. The separated restriction fragments were transferred from the agarose gel to the Hybond N+ membrane (Amersham Pharmacia Biotech) using vacuum-assisted transfer. The gel was de-purinated in 250 mM HCl with gentle shaking for 15 min. The HCl solution was then removed and the gel was rinsed twice with distilled water. The gel was then denatured in 500 mM NaOH with gentle shaking for 30 min. The vacuum transfer unit (Bio-Rad Laboratories) was used to transfer the restricted DNA fragments from the agarose gel to the membrane buffered in 10× SSC for 1.5 hours at 5 inches Hg pressure. Once the transfer was completed the gel was discarded and the membrane incubated in 2× SSC for 5 min. The membrane was then semi-dried and cross-linked at 150 mJ using the GS Gene Linker™ (Bio-Rad Laboratories) and stored between two sheets of filter paper at RT until required.

2.2.13.5. Probe hybridisation
Hybridisation of the *bhlp29.7* probe onto the slot blot and Southern blot membranes was performed under the same conditions. Ten ml of DIG Easy Hyb (Roche) was pre-heated at 50°C and used to pre-hybridise the membrane for 30 min with gentle agitation. The DIG-labelled DNA probe was diluted to 25 ng/ml in a second 10 ml of DIG Easy Hyb, denatured by boiling for 5 min and immediately cooled in ice. The denatured probe was added to another 3.5 ml of pre-heated
DIG Easy Hyb solution and mixed gently to avoiding foaming. The pre-hybridisation solution on the membrane was discarded and the probe/hybridisation mixture was added to the membrane. The membrane was incubated with gentle shaking at 50°C overnight.

After the overnight hybridisation, the membrane was washed twice under the low stringency conditions of 2× SSC containing 0.1% (w/v) sodium dodecyl sulfate (SDS) at 37°C with constant agitation for 5 min. This was then followed by a further two identical washes.

After the membrane was washed, it was briefly rinsed in washing buffer. The membrane was then incubated for 30 min at RT in 100 ml of 1× Blotto (Roche) solution. Following the blocking, the membrane was incubated in 20 ml of 1× Blotto solution containing a 10,000-fold dilution of Anti-Digoxigenin-AP (Roche). The membrane was then washed three times with 100 ml washing buffer for 15 min, then equilibrated in 20 ml detection buffer for 5 min. The membrane was placed in a hybridisation bag (DNA side facing up) and 1 ml of ready-to-use chemiluminescent substrate for alkaline phosphatase development (CSPD; Roche) was applied to the membrane. The membrane was immediately covered with another sheet of hybridisation bag to spread the substrate evenly and air bubbles smoothed out of the bag. The hybridisation bag was heat-sealed and incubated at 37°C for 10 min to enhance the luminescent reaction. An autoradiograph was obtained by incubating the membrane in a film cassette with pre-flashed Hyperfilm-MP (Amersham Pharmacia Biotech) at RT overnight. The autoradiograph was developed and scanned for presentation.

2.2.14. Radioactive palmitate incorporation assay

Ten ml of Kunkle's pre-reduced anaerobic broth containing 2% (v/v) FCS and a 1% (v/v) ethanolic cholesterol solution was inoculated with a 2 ml pure culture of *B. hyodysenteriae* strain WA1 and cultured at 37°C until a cell density of 10⁶ cells per ml was obtained. Two hundred μCi of ³[H]
palmitic acid (Amersham Pharmacia Biotech) was then added to the tube and the cells returned for further growth at 37°C. When a cell density of approximately $10^9$ cells per ml was obtained (72 h), the cells were harvested by centrifugation at $10,000 \times g$ for 20 min. All centrifugation procedures were performed at 4°C unless otherwise stated. The cells were washed three times by resuspending with 10 ml of sterile PBS and centrifuging at $10,000 \times g$ for 20 min. The washed cell pellet was resuspended in 1 ml of PBS and lysed by three 30s bursts of sonication on ice. The cells were placed on ice for 2 min between sonication bursts. Cellular debris was then removed by centrifugation at $20,000 \times g$ for 20 min. SDS-PAGE was used to separate 15 μl of the sample (section 2.2.4) and the separated proteins were electro-transferred to nitrocellulose membrane for Western blotting with Mab BJL/SH1 (section 2.2.5). After Western blotting, the developed membrane was hydrated with Amplify solution (Amersham) and wrapped in plastic food wrap. An autoradiograph was obtained by incubating the membrane in a film cassette with pre-flashed Hyperfilm-MP (Amersham Pharmacia Biotech) at −80°C for 14 days. The autoradiograph was developed and superimposed onto the developed Western blot membrane to identify corresponding bands. Both the autoradiograph and Western blot membrane were scanned for comparative presentation.
2.3. RESULTS

2.3.1. Isolation and characterisation of recombinant phagemids encoding an approximately 30 kDa protein band in *E. coli*

Mab BJL/SHI reacted strongly with a band of approximately 30 kDa in the whole-cell lysate of all *E. coli* cells containing each of the five phagemids, as well as with two additional bands of approximately 31 kDa in cells containing the pSHC clone. In the outer envelope preparation of *B. hyodysenteriae* WA1, Mab BJL/SHI reacted with a band with an apparent molecular mass of approximately 30 kDa (Fig. 2.1).

![Figure 2.1](image)

**Figure 2.1.** Western blot reactivity of Mab BJL/SH1 against whole-cell extracts of the *E. coli* cells expressing phagemids pSHA, pSHB, pSHC, pSHD and pSHE. Lane 1, *B. hyodysenteriae* strain WA1 outer membrane proteins; lanes 3-5, whole cell lysates of recombinant *E. coli* clones containing the phagemids: lane 2, pSHA; lane 3, pSHB; lane 4, pSHC; lane 5, pSHD; lane 6, pSHE. The arrow indicates the position of the native Bhlp29.7 lipoprotein.
2.3.2. Reactivity of serum from naturally infected pigs with the native *B. hyodysenteriae* lipoprotein and the recombinant *E.coli* expressed protein

The sera from pigs with natural SD recognised a range of proteins in the Triton X-114 protein preparation of *B. hyodysenteriae* strain WA1. All five serum samples reacted with a protein of similar size to the approximately 30 kDa protein that reacted with Mab BJL/SH1. The normal pig sera showed no reactivity with proteins of this size (Fig. 2.2).

![Western blot reactivity of serum from pigs naturally infected with SD against Triton X-114 preparation of *B. hyodysenteriae* WA1. The arrow indicates the reaction of an approximately 30 kDa protein with both the Mab BJL/SH1 and the serum from the infected pigs. Lane 1, Mab BJL/SH1; lanes 2-6, individual infected pig serum; lanes 7-8, normal pig serum.](image)

**Figure 2.2.** Western blot reactivity of serum from pigs naturally infected with SD against Triton X-114 preparation of *B. hyodysenteriae* WA1. The arrow indicates the reaction of an approximately 30 kDa protein with both the Mab BJL/SH1 and the serum from the infected pigs. Lane 1, Mab BJL/SH1; lanes 2-6, individual infected pig serum; lanes 7-8, normal pig serum.

2.3.3. Sequence analysis of the open reading frame

2.3.3.1. Motifs and conserved domains

Sequencing of the pSHA phagemid revealed a 3,698 bp insert of *B. hyodysenteriae* genomic DNA. Sequence analysis of the insert DNA revealed a potential ORF (designated *bhlp29.7*) of 816 bp
from bases 242 to 1057, with a putative ATG start codon and a TAA stop codon (Fig. 2.3). A potential Shine-Dalgarno (Shine and Dalgarno, 1974) ribosome binding site (AGGAG), and putative –10 (TATAAT) and –35 (TTGAAA) promoter regions were identified upstream from the ATG start codon. A 12 bp region with dyad symmetry was present downstream from the TAA stop codon. Analysis of the amino acid sequence showed the presence of a 19 residue lipoprotein precursor signal peptide (MKKFLLLVSSAILSMILS) at the N-terminal of the predicted polypeptide. A Kyte-Doolittle hydropathy plot (Kyte and Doolittle, 1982) of the deduced amino acid sequence showed this N-terminal to be highly hydrophobic (Fig. 2.4). The theoretical 271 amino acid (aa) pro-lipoprotein and mature 253 aa lipoprotein had predicted molecular masses of 29.7 kDa and 27.6 kDa, respectively. These deduced sizes differed slightly from those seen in the Western blots. The gene encoding the lipoprotein was designated bhlp29.7, and the lipoprotein was designated Bhlp29.7.

```
-35  -10
CATCAATTATTATTAGTTTTTATTTGAAATTATTCAAAAATAATTATAATC - 50
S/D
TAAATAAAAATTTTATTAAATTTTTATTAAAGGAGCGAAAATGAAAAA - 100
  M K K
TTTTTTATTATTGCTATCATCAGCCATATTATCTATTTATGATATTATCATG - 150
  F L L L V S S A I L S L M I L S
CGGAAATTCCTCTCTGTTGATCAAAAAAGATAGTTAAAGTTGTTTGTCTG - 200
  G N T S S G D Q K I V K V G F A
GAGAGCTCCTATTAAATTTGGATCTATAGCTAAATTAGCCTGAA - 250
  G E S D Y Q I W D P I V A K L A E
GAAGGAAATAAAAGTAGAGGCAGTATCTCTTTCTGATTATATACCTATACCTAA - 300
  E G I K V E L V S F S D Y T I P N
TCAGGCTCTTGAATGACGGGAATTTGACTTGAAGCTTTTTTCAGCATTATG - 350
  Q A L N D G E I D L N A F Q H Y
CATACTTTATGATGAATATCATAAATATAAAGATAGTGAATTTAATGCTATT - 400
  A Y F N D E V S N K G Y D L T A I
GCTGATCTTTATATCTCTGATGAATATTATTTATATCACATTATCTACTG - 450
  A D T Y I S A M N I Y S T N I T D
TGAAAAAGAATTTAAAAATGGCGATAAAATAGCTTTATACCTATTAGCCTT - 500
  V K E L K N G D K I A I P N D P
CTAAATGAGGAAGGCTTAAAGTTTCTCAGGCTTGAGGAATCTTTAAA - 550
  S N G G R A L K V L Q A A G I K
```
2.3.3.2. Subcellular localisation, topology and antigenicity

The analysis of the Bhlp29.7 translated amino acid sequence using PSORTb v2.0 predicted that the lipoprotein was localised on the outer membrane of the spirochaete. The HMMTOP analysis revealed that Bhlp29.7 possessed three hydrophobic helices which were preceded and followed by hydrophilic regions. The first hydrophobic helix (residues 20 to 23) immediately followed the GTAAAACCTGAAGCAGGAGATACTCCTAGCGTAAGCGATATAATAAAAA = 600

V K P E A G D T P S V S D I I K N

TCCTCTAAATTTAGAAATGATGAAATGGCTAGGCTGCTATTTGCTATATAC = 650

P L N I E I V E M D A G A I Y G

TTCTTTCTGTGCTTGTGCGCTTTATCAATGAAAACATGCTATATAC = 700

V L F D V A C A V I N G N Y A I Y

TTGGGTGGATCTGTTTCGATTATATTTCAATGATGTTTCTCTATATAC = 750

F G L N P G S D Y I F K D D P S I

TTACAGCGGAAAAATCTTTTGTAAATTATTAGCTGAAGAATGATAAAAT = 800

Y S G K S F V N L I A A R T K D

AAGATAATGAAATATACAAAAAAGTTGAGAAAACCTTATCAATGCAAATA = 850

K D N E L Y K K V V E T Y Q S E I

GTAGAAAGTTTATAATGAAAATTTTCTTAGGCTTTATCTCCTACTTG = 900

V E K V Y N E N F L G S Y L P T W

GAAATAATATAAAAATATTTAGAATAATATTTGAATTATAAAAAAGAAAAG = 950

K *

CATGAGATTTCTTATTATCTCATGCTTTTTTTTTTTTTTTTTATATCAT = 1000

Figure 2.3. Nucleotide sequence and the deduced amino acid sequence of the ORF bhlp29.7 encoding a putative lipoprotein designated Bhlp29.7. The ribosome-binding site and putative promoter regions are underlined. The region of dyad symmetry, which forms a possible ρ-independent transcriptional terminator, is underlined with arrows. The potential signal peptide is in bold, and the possible signal peptidase II cleavage site is shown by the arrow.

2.3.3.2. Subcellular localisation, topology and antigenicity

The analysis of the Bhlp29.7 translated amino acid sequence using PSORTb v2.0 predicted that the lipoprotein was localised on the outer membrane of the spirochaete. The HMMTOP analysis revealed that Bhlp29.7 possessed three hydrophobic helices which were preceded and followed by hydrophilic regions. The first hydrophobic helix (residues 20 to 23) immediately followed the...
signal peptidase II cleavage site and possibly indicated the lipid attachment site. The second (residues 100 to 119) and third (residues 182 to 206) hydrophobic helices probably indicated transmembrane regions. Three hydrophilic regions were predicted, two of which were on the external side of the membrane (residues 24 to 99 and residues 207-271) and one which was on the internal side of the membrane (residues 120 to 181). A diagrammatic representation combining the PSORTb and HMMTOP predictions is shown in Fig. 2.5.

The most antigenic regions of the Bhlp29.7 lipoprotein were predicted to reside at the C-terminal between residues 201 to 268.

**Figure 2.4.** Kyte-Doolittle hydropathy plot of the deduced amino acid sequence of the Bhlp29.7 polypeptide. Hydrophobic regions are shown above the line. The potential signal peptide is indicated by the arrow. The amino acid positions in the ORF are shown on the horizontal axis, and the hydrophobic index is shown on the vertical axis.
2.3.3.3. Homology

The translated-BlastP homology search of bhlp29.7 against the SWISS-PROT protein database identified 33.9-39.9% homology between this protein and D-methionine-binding lipoproteins (MetQ) of other bacteria including *E. coli*, *Haemophilus influenzae*, *Pasteurella multocida*, *Salmonella enterica* serovar Typhimurium, *S. enterica* serovar Typhi, *Vibrio cholera* and *Yersinia pestis* (Table 2.2). Similarity (32.1-38.4%) was also seen between Bhlp29.7 and the gene products (PlpABC) of a tandem multiple gene loci encoding 30 kDa membrane lipoproteins of *Pasteurella haemolytica* (Table 2.2). Comparison of the bhlp29.7 nucleotide sequence with the GenBank nucleotide database did not reveal any strong homology with other bacterial genes.

![Figure 2.5. The subcellular localisation and topology of the Bhlp29.7 lipoprotein predicted using PSORTb v2.0 and HMMTOP v2.0. OM, outer membrane; A, lipid attachment region; B and F, extracellular regions; C and E, transmembrane regions; D, periplasmic region.](image-url)
Table 2.2. Sequence similarity of translated Bhlp29.7 (271 amino acids) with the amino acid sequence of bacterial lipoproteins obtained from the SWISS-PROT database.

<table>
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<th>Organism</th>
<th>Protein</th>
<th>Size (aa)</th>
<th>Identity (aa)</th>
<th>Similarity (%)</th>
<th>Accession Number</th>
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<td>MetQ</td>
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<td>108</td>
<td>39.9</td>
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<td>P28635</td>
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<tr>
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<td>39.5</td>
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<tr>
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<td>106</td>
<td>39.1</td>
<td>Q8X8V9</td>
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<tr>
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<td>105</td>
<td>38.7</td>
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</tr>
<tr>
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<tr>
<td></td>
<td>PlpC</td>
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<td>38.4</td>
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<tr>
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</table>

2.3.4. Sequencing of the *bhlp29.7* gene present in *Brachyspira* spp.

Amongst the 102 *Brachyspira* spp. analysed for *bhlp29.7* by PCR, the gene was identified in all 48 strains of *B. hyodysenteriae*, and in *B. innocens* B256\(^T\), but was not present in any strains of *B. pilosicoli*, *B. intermedia*, *B. murdochii*, “*B. canis*”, *B. alvinipulli* or *B. aalborgi*, or in the other strains of *B. innocens*. The level of identity between the Bhlp29.7 gene of the *B. hyodysenteriae* strains and *B. innocens* B256\(^T\) that were sequenced are summarised in Tables 2.3 and 2.4. The Bhlp29.7 gene of the 11 *B. hyodysenteriae* strains shared 97.9-100% identity at the nucleotide level (Table 2.3), and 98.5-100% at the protein level (Table 2.4). Western Australian isolates WA4
and WA5 were identical at the nucleotide level, as were WA1, WA6, WA15 and WA16. The Bhlp29.7 gene of *B. innocens* B256<sup>T</sup> showed slightly more variation, having between 97.5-99.3% similarity with the Bhlp29.7 gene of the different *B. hyodysenteriae* strains at the nucleotide level, and 97.5-99.5% at the protein level. Comparison of the amino acid sequences of Bhlp29.7 for the *B. hyodysenteriae* strains with that of *B. innocens* B256<sup>T</sup> identified only one consistent difference between the protein in the two species. This was an amino acid dissimilarity at position 210, which occurred only in *B. innocens* B256<sup>T</sup>, where the serine residue for *B. hyodysenteriae* was replaced by a tyrosine residue for *B. innocens*. This will be discussed more fully in Chapter 3.

**Table 2.3.** Nucleotide sequence homology (%) of *bhlp29.7* from 11 different *B. hyodysenteriae* strains and *B. innocens* strain B256<sup>T</sup>. All strains posses an 816 bp coding sequence. The *B. innocens* strain is indicated with bold typeface.

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<th>B204</th>
<th>B/Q02</th>
<th>WA4, WA5</th>
<th>WA1, WA6, WA15, WA16</th>
<th>B256&lt;sup&gt;T&lt;/sup&gt;</th>
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</tbody>
</table>
Sequence data for *bhlp29.7* in *B. hyodysenteriae* strains P18A, B78<sup>T</sup>, B169, B204, B/Q02, WA1, WA4, WA5, WA6, WA15 and WA16 were deposited in GenBank under the respective accession numbers AY512343, and AY706759–AY706768. The *bhlp29.7* sequence in *B. innocens* B256<sup>T</sup> was deposited under accession number AY512344.

**Table 2.4.** Amino acid sequence homology (%) of translated *bhlp29.7* from 11 different *B. hyodysenteriae* strains and *B. innocens* strain B256<sup>T</sup>. All strains posses a 271 amino acid protein. The *B. innocens* strain is indicated with bold typeface.

<table>
<thead>
<tr>
<th></th>
<th>P18A</th>
<th>B78&lt;sup&gt;T&lt;/sup&gt;</th>
<th>B169</th>
<th>B204</th>
<th>B/Q02</th>
<th>WA4, WA5</th>
<th>WA1, WA6, WA15, WA16</th>
<th>B256&lt;sup&gt;T&lt;/sup&gt;</th>
</tr>
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<td>P18A</td>
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<td>99.1</td>
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2.3.5. Southern Blot of bhlp29.7

The low stringency slot-blot DNA hybridisation of a selection of 25 of the *Brachyspira* spp. strains demonstrated that the *bhlp29.7* probe hybridised only with the *B. hyodysenteriae* strains and the *B. innocens* strain B256T. One representative strain from each *Brachyspira* spp. analysed by slot-blot hybridisation was selected for Southern blot analysis. On Southern blotting, the *bhlp29.7* probe reacted strongly with a 1.9 kb *Hind*III fragment of the *B. hyodysenteriae* genome, and with a 3.1 kb fragment of the genome from *B. innocens* strain B256T, and again not with restriction fragments of the other *Brachyspira* spp. genomic DNA (Fig. 2.6).

![Image](image_url)

**Figure 2.6.** Chemiluminograph of chromosomal DNA from *Brachyspira* spp. digested with *Hind*III, separated by electrophoresis and hybridised with the *bhlp29.7* probe under low stringency conditions. Lane 1, *bhlp29.7* PCR product; lane 2, herring sperm DNA; lane 3, *B. pilosicoli* strain 95/1000; lane 4, *B. innocens* strain B256T; lane 5, *B. intermedia* strain P280-1; lane 6, *B. murdochii* strain155/20; lane 7, *B. hyodysenteriae* strain WA1; lane 8, *B. hyodysenteriae* strain B78T. The arrow shows the position of the hybridisation with the two *B. hyodysenteriae* strains.
2.3.6. Lipidation of Bhlp29.7

The tritiated palmitate incorporation study indicated the presence of a range of lipidated proteins in the *B. hyodysenteriae* cell lysate in the range of 15 to 40 kDa. The majority of these lipoproteins were concentrated in the range of 15 to 25 kDa. A lipidated protein with an apparent molecular mass of 30 kDa was visibly dominant in the cell lysate of *B. hyodysenteriae*. This same lipoprotein also was the only lipoprotein cell lysate that reacted with the Mab BJL/SH1 (Fig. 2.7).

![Figure 2.7](image)

**Figure 2.7.** Autoradiograph of cell-lysates of *B. hyodysenteriae* grown in the presence of $^3$H]palmitic acid. A whole-cell preparation of the *B. hyodysenteriae* cells was electrophoresed and transferred to nitrocellulose membrane. The membrane was exposed to x-ray film (lane 1) and then immunoblotted with Mab BJL/SH1 (lane 2). The arrow indicates the native Bhlp29.7 lipoprotein.
In the present study an 816 bp ORF encoding a 29.7 kDa putative lipoprotein of *B. hyodysenteriae*, reactive with the monoclonal antibody BJL/SH1, was isolated and sequenced. In previous studies BJL/SH1 was found to be reactive with *B. hyodysenteriae*, and not with other species in the genus *Brachyspira* (Lee and Hampson, 1996). The recombinant *E. coli* clones expressed proteins which were of similar apparent molecular mass to the native *B. hyodysenteriae* protein, and were antigenically related to it. In one of the *E. coli* clones (pSHC), three bands were reactive with BJL/SH1, one of which was similar in apparent molecular mass to the native *B. hyodysenteriae* protein. The intensities of the other two bands were much weaker. Differences in molecular mass between the native and recombinant lipoprotein may be due to differences in post-translational modifications of *E. coli* and *B. hyodysenteriae* (Wu et al., 1983). It has been suggested that processing of such pro-lipoproteins may not be as effective in *E. coli* as in *B. hyodysenteriae* (Thomas and Sellwood, 1993). Thomas and Sellwood (1993) also proposed that the efficiency of these modifications may be associated with the growth phase at which the cultures were harvested.

Differences in molecular mass between the deduced amino acid sequence of the 29.7 kDa lipoprotein and the apparent molecular mass seen in the Western blot may also be due to minor post-translational modifications not accounted for during *in silico* translation, with SDS-PAGE only providing an estimate of the true molecular mass of the protein.

It was initially proposed that the 29.7 kDa outer membrane lipoprotein of *B. hyodysenteriae* be designated BmpB (*Brachyspira* membrane protein B), and that the encoding gene be designated *bmpB*. This would be in accordance with the nomenclature of the other *B. hyodysenteriae* outer membrane lipoprotein (SmpA) characterised by Thomas and Sellwood (1993), the name of which should be amended from SmpA (*Serpulina* membrane protein A) to BmpA to be consistent with the new genus name *Brachyspira*. However, the nomenclature of *Brachyspira* outer membrane proteins and lipoproteins has since been re-defined to account for the originating species, the class.
of membrane protein, and the predicted molecular mass of the translated gene. As a result, BmpB has been renamed as Bhlp29.7 (29.7 kDa lipoprotein of \textit{B. hyodysenteriae}) and the gene encoding it changed from \textit{bmpB} to \textit{bhlp29.7} (Hampson \textit{et al.}, 2006b).

Analysis of the N-terminal amino acid sequence suggests that \textit{bhlp29.7} encodes a potential lipoprotein. Lipoproteins generally share common features including a short hydrophobic signal peptide containing a 4 residue consensus signal peptidase II cleavage site. Upon cleavage of the signal peptide, the cysteine residue in this motif becomes the N-terminal and is also the position of lipid attachment (Wu \textit{et al.}, 1983). Examination of the Bhlp29.7 polypeptide indicated the presence of a cleavage site (I\(_3\)-L\(_2\)-S\(_1\)-C\(_{+1}\)) at the proper positions. The signal peptidase II recognition site for Bhlp29.7 is similar to, but does not exactly match the consensus sequence determined by the alignment of 26 spirochaetal lipoproteins for which there was experimental evidence of lipid modification. The consensus spirochaete signal peptidase II recognition site was defined as L(A,S)\(_4\)-L(V,F,I)\(_3\)-I(V,G)\(_2\)-A(S,G)\(_1\)-C\(_{+1}\) (Haake, 2000). The signal peptidase II recognition site of Bhlp29.7 differed from this consensus by the presence of a methionine (M) at position -4 and leucine (L) at position -2. The signal peptidase II recognition site of the \textit{B. hyodysenteriae} outer membrane lipoprotein SmpA (Thomas and Sellwood, 1993) and \textit{B. pilosicoli} outer membrane lipoprotein BmpC (Trott \textit{et al.}, 2004) also differed from the spirochaetal consensus site. It is likely that during the gradual evolutionary divergence of the spirochaetes, enzyme recognition sites and sites of cleavage have also evolved. The Kyte-Doolittle hydropathy plot of the deduced Bhlp29.7 protein revealed the hydrophobic nature of the precursor signal peptide as well as a hydrophilic N-terminal region adjacent to the predicted lipid attachment site, which is similar to that of other bacterial lipoproteins (Sharma \textit{et al.}, 1998).

Since Bhlp29.7 was expressed \textit{in vitro}, it was possible to demonstrate its lipidation by tritiated palmitate incorporation, thus confirming that Bhlp29.7 is a lipoprotein. The lipidation assay also
indicated that Bhlp29.7 was a dominant lipoprotein on the membrane of *B. hyodysenteriae*, making it an even stronger candidate antigen for use in serological diagnosis of SD and vaccination against SD.

Nakai and Kanehisa (1991) developed a scheme for determining the localisation of lipoproteins in Gram-negative bacteria. The expert system makes use of various kinds of knowledge, based on experimental observations, for theoretically sorting a protein into one or more of four cellular compartments: cytoplasm; cytoplasmic (inner) membrane; periplasm; and outer membrane. The system was developed for Gram-negative bacteria, but there is reason to believe that spirochaetal lipoproteins may be exported to the outer membrane by a unique pathway, which is still not fully understood (Haake, 2000). The second and third amino acids of the Bhlp29.7 lipoprotein were glycine (G) and asparagine (N), respectively. Both these amino acids are uncharged, therefore according to the system of Nakai and Kanehisa, the Bhlp29.7 lipoprotein is possibly localised on the outer membrane of *B. hyodysenteriae*. This was further substantiated by the *in silico* analysis performed using HMMTOP and PSORTb. HMMTOP uses a hidden Markov model-based method to identify transmembrane alpha helices, which can then be used to predict proteins which spanning the cytoplasmic or outer membranes (Tusnady and Simon, 1998; 2001). The PSORTb tool is frequently used for prediction of prokaryotic localisation sites and is especially useful for Gram-negative bacteria. It consists of multiple analytical modules, each of which analyses one biological feature known to influence or be characteristic of subcellular localisation (Gardy et al., 2005). When used in combination, these analyses indicated a high probability of Bhlp29.7 being localised on the *B. hyodysenteriae* outer membrane. In addition, the predictions also indicated that the Bhlp29.7 polypeptide contained two regions (residues 24 to 99 and 207 to 271) which are exposed to the outside of the cell.
Although different theoretical subcellular localisation prediction systems were used, computations based on amino acid sequence still require experimental evidence before the true localisation of lipoproteins can be determined. Detergent-phase partitioning extraction of native Bhlp29.7 and immunogold-labeling of \textit{B. hyodysenteriae} using BJL/SH1 provided further support for an outer membrane location of Bhlp29.7 (Lee and Hampson, 1996).

Interestingly, the same gene sequence was independently identified and published four years after the identification of \textit{bhlp29.7}, as described in this chapter, and was designated \textit{blpA} (Cullen \textit{et al.}, 2003). The \textit{bhlp29.7} and \textit{blpA} sequence refer to the same gene and share 99.6% similarity at the nucleotide level, since \textit{bhlp29.7} and \textit{blpA} were initially sequenced from the \textit{B. hyodysenteriae} stains P18A and B204, respectively. Further data presented by Cullen \textit{et al.} (2003) indicate that \textit{bhlp29.7/blpA} is a member of a locus encoding four paralogous outer membrane proteins (BlpGFEA). However, of these four proteins, only Bhlp29.7/BlpA is transcribed and translated \textit{in vivo}. The palmitate lipidation studies described here and independently undertaken by Cullen \textit{et al.} (2003) indicate that Bhlp29.7/BlpA is a lipoprotein. A possible role for the paralogs could be in the evasion of the immune system by antigenic variation of surface exposed proteins, as seen in \textit{Borrelia burgdorferi} (Burkot \textit{et al.}, 1994; Schwan \textit{et al.}, 1995; Montgomery \textit{et al.}, 1996), the relapsing fever spirochaete \textit{Borellia hermsii} (Barbour \textit{et al.}, 1991; Hinnebusch \textit{et al.}, 1998) and \textit{Leptospira kirschneri} (Barnett \textit{et al.}, 1999). Another two loci encoding eight linked gene copies of a predominant 39 kDa surface protein (\textit{vspABCD} and \textit{vspEFGH}) have been identified for \textit{B. hyodysenteriae}. Of these multi-gene loci, only one gene (\textit{vspH}) encodes the 39 kDa protein, although 83-90% amino acid similarity is shared amongst all the gene products (Gabe \textit{et al.}, 1998; McCaman \textit{et al.}, 2003; Witchell \textit{et al.}, 2006).

Annotation of the translated \textit{bhlp29.7} gene sequence indicated that its highest homology (33.9-39.9%) was with a D-methionine binding lipoprotein of various bacteria including \textit{E. coli},
**Haemophilus influenzae**, *Pasteurella multocida*, *Salmonella enterica* serovar Typhimurium, *Salmonella typhi*, *Vibrio cholera* and *Yersinia pestis*. Similar homology (32.1-38.4%) was seen with the 30 kDa membrane proteins of *Mannheimia haemolytica* (PlpABC) encoded by tandem multiple genes sharing 55-60% nucleotide sequence identity. These homology values were calculated based on the number of identically matching amino acids. Direct comparison of the Bhlp29.7 nucleotide sequence with available GenBank sequences did not return any significant matches.

D-methionine is an effective source of methionine in many enteric bacteria (Cooper, 1966; Kuhn and Somerville, 1971; Kadner, 1977). There are currently 48 members of the methionine uptake transporter (MUT) family and these include members from both Gram-negative and Gram-positive bacteria. The MUT system and its regulation have been extensively investigated in *E. coli* and *Salmonella enterica* serovar Typhimurium. Recently, this system was fully characterised for *E. coli*. The MUT system is an ATP-binding cassette (ABC) transporter consisting of a transmembrane protein, a cytoplasmic ATP-hydrolysing protein, and at least one specific substrate-binding receptor. Regulation of the MUT is facilitated by repressor (MetJ) binding which negatively controls the expression of the MUT-encoding operon (Zhang *et al.*, 2003). The MUT system has also been found to be sensitive to osmotic shock (Cottam and Ayling, 1989). The genes encoding the MUT components are located on the *metD* locus (Kadner and Watson, 1974; Kadner, 1977) which has since been renamed as the *metNIQ* gene cluster (Gal *et al.*, 2002; Merlin *et al.*, 2002). MetD is believed to be a high-affinity methionine transport system which involves an ATPase (MetN), permease (MetI) as well as a D-methionine binding protein (MetQ), and is capable of transporting both the D- and L-methionine stereoisomers. A low-affinity transport system (MetP) operates in parallel, but can only transport the L-methionine stereoisomer. Once D-methionine has been transported into the cell, it is converted to L-methionine. Deletion of the MetD transport system in mutants leads to impaired cell growth on D-methionine as the sole
source of sulphur, although they will still grow on L-methionine. A corresponding system with the same uptake properties has also been described in *S. enterica* serovar Typhimurium (Cottam and Ayling, 1989; Grundy and Ayling, 1992). In addition, a similar MUT system has been predicted from the genome sequence of the spirochaete *Treponema pallidum*. The MetQ homologue (TpN32) in this spirochaete has 25.5% (69 out of 271 aa) homology with Bhlp29.7. A BlastP sequence comparison of the translated *blpGFE* genes, shown to be paralogous to *bhlp29.7/blpA* (Cullen *et al.*, 2003), with the SWISS-PROT database identified large regions of conserved domains reminiscent of ABC constituents. The combined annotation of *bhlp29.7* and the homologous *blpGFEA* loci strongly suggest that these genes belong to the MUT family.

ABC transporters constitute a large superfamily of permeases that are involved in the ATP-dependent transport of solutes across biological membranes. They are present in both prokaryotes and eukaryotes and have relative specificity for a given substrate. Many ABC transporters have been identified for the transport of bio-molecules required for catabolism. They are also important during adaptation of pathogens to osmotic shock, allowing the accumulation of important substrates. These physiological processes are widespread in nature, and play essential roles in the virulence of both prokaryotic and eukaryotic pathogens such as entero-aggregative *E. coli* (Nishi *et al.*, 2003), *S. enterica* (Kingsley *et al.*, 1999), *S. enterica* serovar Typhimurium (Parra-Lopez *et al.*, 1993; Janakiraman and Slauch, 2000; Liao and Maloy, 2001), and *Yersinia ruckeri* (Fernandez *et al.*, 2002). MUT proteins are no exception, and have been found to influence the virulence of certain bacteria. One example is the 30 kDa membrane proteins (PlpABC) of *M. haemolytica*, the causative agent of bovine pneumonic pasteurellosis in feedlot cattle, which have been shown to be important in generating immunity to the disease. Serological analyses indicate high antibody responses to several surface antigens, including the 30 kDa protein, correlate with resistance to pneumonic pasteurellosis. Vaccination of cattle with the surface antigens enhances resistance to experimental challenge (Mosier *et al.*, 1989). Annotation of PlpABC revealed a high degree of
homology with a 28 kDa membrane protein of *H. influenzae*, which may be associated with invasion (Murphy and Whitworth, 1993). In these studies, mutant strains of *H. influenzae* expressing insertion-inactivated 28 kDa protein were less virulent than wild-type strains expressing the intact 28 kDa protein, as determined by the ability to cause bacteraemia after intranasal inoculation of mice. The role of the protein in virulence is related to the initial invasion of the mucosal epithelium, since both wild-type and mutant strains were able to cause bacteraemia in animals inoculated intraperitoneally (Chanyangam *et al.*, 1991). The 28 kDa membrane protein of *H. influenzae* has since been re-annotated as a D-methionine binding lipoprotein (Fleischmann *et al.*, 1995). Although the pig gastrointestinal tract differs from the respiratory epithelium, the suggestion that inactivation of the D-methionine binding lipoprotein homologue can prevent initial invasion of mucosal epithelium indicates that immunity to Bhlp29.7 potentially could offer protection against lesion development in SD.

Previously, it has been shown that pigs recovering from experimental challenge with *B. hyodysenteriae* develop a humoral immune response to a range of outer membrane proteins ranging in size from 30 kDa to 45 kDa (Wannemuehler *et al.*, 1988; Smith *et al.*, 1990; Sellwood *et al.*, 1995). Similarly, serum from a pig vaccinated with a *B. hyodysenteriae* bacterin, which was then resistant to SD, reacted with a series of *B. hyodysenteriae* cell envelope proteins with molecular masses between 30 and 36 kDa (Chatfield *et al.*, 1988b). It is presumed that Bhlp29.7 was represented amongst the cocktail of surface proteins of similar molecular mass that were recognised by the pigs. In the current study sera from pigs in a herd with SD recognised a protein of similar molecular mass to Bhlp29.7, suggesting that Bhlp29.7 is both immunogenic and expressed *in vivo*. The results of immunofluorescent-antibody tests (IFAT) using Mab BJL/SH1, on faeces also support both the outer surface location of Bhlp29.7, and its *in vivo* expression.
Immunisation of mice with the recombinant *E. coli* cells provided the first evidence that recombinant Bhlp29.7 expressed in *E. coli* was antigenically related to the native *B. hyodysenteriae* lipoprotein (Lee, 1996). The mouse sera showed some cross-reactivity with proteins of similar molecular mass in other *Brachyspira* spp., but this was expected. Structural proteins in related organisms are likely to share epitopes which would stimulate cross-reacting antibodies when they are used as immunogens. The fact that BJL/SH1 did not react with *Brachyspira* spp. other than *B. hyodysenteriae* confirmed that the epitope reacting with this Mab was unique to *B. hyodysenteriae* (Lee and Hampson, 1996). However, in the current study the Bhlp29.7 gene was found to be present in *B. innocens* B256<sup>T</sup> and the sequence was almost identical to the bhlp29.7 of the 11 *B. hyodysenteriae* strains analysed. Assuming Bhlp29.7 is a D-methionine binding lipoprotein homologue, and the D-methionine binding lipoprotein may be associated with virulence, it is possible that the Bhlp29.7 lipoprotein is not required for the survival of non-pathogenic *B. innocens*, and consequently the expression of bhlp29.7 has been silenced during the course of its evolutionary divergence.

The bhlp29.7 gene appeared to be ubiquitous amongst *B. hyodysenteriae* strains. Its location at a single site on a 1.9 kb fragment of the *B. hyodysenteriae* strains tested suggests that the sequence is present as a single copy. The high level of identity between the bhlp29.7 genes of all the *B. hyodysenteriae* strains studied also indicates the highly conserved nature of the Bhlp29.7 lipoprotein. *B. innocens* strain B256<sup>T</sup> also possessed the bhlp29.7 gene, assessed by PCR and low stringency hybridisation, and the nucleotide sequence shared 97.5-99.3% similarity with the *B. hyodysenteriae* sequences. Interestingly, Cullen *et al.* (2003) also identified blpA in *B. innocens* B256<sup>T</sup>, although they did not investigate other *B. innocens* strains. The presence of the gene in strains of B256<sup>T</sup> held by different laboratories helps to verify that the culture was not contaminated with *B. hyodysenteriae*. This was further confirmed in the current study by the negative result when the culture was amplified using a *B. hyodysenteriae*-specific PCR based on the NADH
oxidase gene. The \textit{bhlp29.7} gene also mapped to a different genomic restriction fragment in B256\textsuperscript{T} than it did in the \textit{B. hyodysenteriae} strains. The presence of \textit{bhlp29.7} in a single strain of \textit{B. innocens} that was originally isolated in the 1970s (Kinyon and Harris, 1979) could also be interpreted as arising from a relatively recent strain-specific lateral transfer of the gene from \textit{B. hyodysenteriae} to \textit{B. innocens} strain B256\textsuperscript{T}. Such transfer could perhaps involve VSH-1, a generalised transducing bacteriophage present in \textit{B. hyodysenteriae} and other \textit{Brachyspira} species (Humphrey \textit{et al}., 1995; Stanton \textit{et al}., 2003).

Generally, lipoproteins are believed to be highly immunogenic due to the attached fatty acid moiety and its surface exposure. The significance of this reactivity in relation to protection in animals remains uncertain, however the Bhlp29.7 lipoprotein is a potential candidate for use in vaccine development. This is particularly so as pigs immunised with \textit{E. coli} expressing recombinant Bhlp29.7 developed serum antibody that reacted with Bhlp29.7 in the outer envelope of a range of different strains of \textit{B. hyodysenteriae} (Lee, 1996). Annotation of Bhlp29.7 suggests that this protein may be a homologue of the D-methionine binding lipoprotein associated with the ATP-dependent uptake system for methionine. The fact that MUT systems in other bacteria may be involved in the virulence of these organisms further supports the potential of Bhlp29.7 as a vaccine candidate.

Recombinant Bhlp29.7 may also be useful as an antigen for serological diagnosis of SD, as infected pigs recognised Bhlp29.7, whilst healthy uninfected pigs did not. However, the presence of a nearly identical Bhlp29.7 gene in a strain of \textit{B. innocens} may pose a problem to the specificity of a Bhlp29.7-based serological assay, as \textit{B. innocens} is a commensal commonly found amongst the porcine gastrointestinal flora. However, if expression of Bhlp29.7 in \textit{B. innocens} is cryptic, then the likelihood of animals colonised with \textit{B. innocens} having antibody levels against Bhlp29.7 may be small.
Further work is required to determine whether recombinant Bhlp29.7 can induce protective immunity, and whether it can be adapted for use in the serological diagnosis of SD. Studies on these aspects will be presented in the chapters to follow.
CHAPTER 3: RECOMBINANT EXPRESSION AND PURIFICATION OF BHLP29.7 IN *ESCHERICHIA COLI*

3.1. INTRODUCTION

Recombinant proteins are produced by the transcription and translation of a gene of interest in a heterologous host. The foreign DNA encoding the protein of interest is cloned into an appropriate plasmid expression vector that will allow its expression in a desired heterologous host. The successful expression of recombinant proteins in sufficient quantities is a vital prerequisite to any detailed downstream application.

Over the last twenty years recombinant protein expression has become a routine tool in most molecular biology research laboratories. The relatively recent revolution resulting from the development of molecular techniques allowing the genetic manipulation of micro-organisms has made a plethora of choices available for *in vitro* production of proteins in many host systems. Selection of the appropriate expression system and host cell-type greatly depends on the quality and quantity of the desired protein. Quality encompasses the requirement for post-translational protein modifications, as well as the purity of the final protein. Quantity is a measure of the yield of purified protein required for the intended application. In general terms, the higher the degree of post-translational modification required, the smaller the expected yield of protein. Eukaryotic recombinant gene expression systems are primarily used for the production of high quality proteins where conformation and post-translational modifications are desired, but at the expense of quantity. Prokaryotic recombinant protein expression systems generally provide low quality proteins in considerably higher quantity. These expression systems are ideal for studies which are not dependent on activity or conformation of the recombinant protein, such as serological antigens (Gray and Subramanian, 2000). The production of recombinant Bhlp29.7 for this study was achieved using a prokaryotic expression system (*E. coli*).

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Two distinct generations of prokaryotic expression systems have evolved since the early 1980s when genetic engineering was first utilised for heterologous protein production. The first generation prokaryotic expression systems involved cloning genes of interest into plasmid vectors which provided controlled gene expression inside the bacterial host cell. These early systems were controlled by well-known bacterial operon systems (such as lacZ and trpE) where promoters and factors facilitating expression had been well characterised (reviewed in Makrides, 1996; Baneyx, 1999). Purification of recombinant proteins expressed by the first generation systems involved traditional chromatography or antibody-based affinity chromatography. Inclusion body formation by the host cell made purification problematic due to the insoluble state of the aggregates (Coligan et al., 2002).

A second generation of prokaryotic expression systems emerged which improved the efficiency of protein purification and allowed universal detection of the expressed protein. These upgraded systems included the addition of fusion-proteins or peptides to the gene of interest, and often incorporated adjacent protease cleavage sites for removal of the fusion after purification. Co-translation of the fusion-protein with the protein of interest allowed specific high-affinity purification procedures to be performed followed by removal of the fusion using specific protease cleavage, if required. Examples of such fusion-proteins include maltose-binding protein (MBP, Maina et al., 1988), glutathione S-transferase (GST, Smith and Johnson, 1988) and thioredoxin (Trx, LaVallie et al., 1993). Many commercial expression systems are supplied with specialised strains of bacteria that provide tolerance of the elevated level of foreign protein expression, tight expression control, and reduced proteolytic degradation of the expressed protein (Wingfield, 2002).

A commonly used modification of protein fusions are fusion-tags. These tags are small stretches of amino acids added to the terminal end of the protein of interest. Although the fusion-tags do not usually improve expression levels or protein solubility, they assist with purification and detection of the expressed protein. A popular example is the polyhistidine
(His$_6$) tag used in the current study. The His$_6$-tag consists of six consecutive histidine residues added to the N-terminal of the expressed protein and provides the capability of high-affinity purification using metal-chelate resins. In addition, affinity of the His$_6$-tag for the metal-chelate resin is independent of conformation, thus purification is not affected after treatment with denaturants (Janknecht et al., 1991; Schmitt et al., 1993).

Purification of proteins using metal-chelate affinity chromatography (MCAC, also known as immobilised-metal affinity chromatography) was first described in 1975 (Porath et al., 1975) and later verified with a variety of different proteins (Sułkowski, 1985). The technology is based on the ability of certain amino acids to bind reversibly to transition-metal ions that have been immobilised onto a chelating matrix. The amino acids (histidine, tryptophan, tyrosine and phenylalanine) act as electron donors on the surface of the proteins. Histidine is the most important of these amino acids as it has the highest affinity for the immobilised metal ions, and will bind selectively to them even in the presence of excess free-form metal ions in solution. Nickel and copper are the transition-metal ions which have the highest affinity for histidine (Yip et al., 1989). The initial application of MCAC used iminodiacetic acid (IDA) as the chelating adsorbent and was found to be associated with problematic yields and purity. The development of nitrilotriacetic acid (NTA) significantly overcame these problems. IDA has only three metal-chelating sites compared to six metal-chelating sites in NTA. This means that NTA binds metal ions more stably thus allowing purification to be performed under more stringent conditions (Hochuli et al., 1987). A commonly used MCAC resin is Ni-NTA which consists of nickel ions immobilised onto a NTA solid support. Disassociation of the bound and washed proteins from the immobilised nickel ion can be achieved by lowering the buffer pH or using buffers containing high concentrations of a histidine analogue (imidazole) to displace the His$_6$-tag.

Production of recombinant proteins using E. coli expression systems have been achieved for a wide range of Gram-positive and Gram-negative bacteria. Cytoplasmic and membrane-
associated spirochaetal proteins have been produced in *E. coli* for *Borrelia* spp. (Dunn *et al.*, 1990; Hansson *et al.*, 1995; Shang *et al.*, 1997; Jauris-Heipke *et al.*, 1999; Skare *et al.*, 1999), *Treponema* spp. (Schouls *et al.*, 1989; Blanco *et al.*, 1996; Fenno *et al.*, 1996; Gerber *et al.*, 1996; Champion *et al.*, 1997; Xu *et al.*, 2001), *Leptospira* spp. (Shang *et al.*, 1995; Shang *et al.*, 1996; Haake *et al.*, 1999) and *Brachyspira* spp. (Koopman *et al.*, 1992; Gabe *et al.*, 1995; Dugourd *et al.*, 1999; Hsu *et al.*, 2001). Recombinant production of surface-exposed proteins has been of increased interest due to their potential as immuno-stimulating antigens for vaccination. Amongst the recombinant proteins from *Brachyspira* that have been studied, only three membrane proteins from *Brachyspira* species have been cloned and expressed. These include a 16 kDa outer membrane lipoprotein (SmpA) (Thomas and Sellwood, 1993) and a 39 kDa surface-exposed protein (VspH) (McCaman *et al.*, 2003; Witchell *et al.*, 2006) for *B. hyodysenteriae*, and a 36 kDa glucose-galactose MglB lipoprotein of *B. pilosicoli* (Zhang *et al.*, 2000).

This chapter describes the cloning, over-expression and large-scale purification of the 29.7 kDa outer-membrane lipoprotein (Bhlp29.7) of *B. hyodysenteriae* using an *E. coli* expression system. The ability to produce a sufficient quantity of the recombinant protein was an essential requirement for subsequent studies. The Bhlp29.7 gene was also sub-cloned and expressed in the same expression system in order to identify the approximate location of the epitope for the BJL/SH1 monoclonal antibody.
3.2. MATERIALS AND METHODS

3.2.1. Cloning strategy

Cloning of Bhlp29.7 into the pTrcHis Escherichia coli expression system had a two-fold purpose. Firstly, it allowed full-length Bhlp29.7 polypeptide to be expressed in E. coli and subsequently purified for further immunological studies. Secondly, it allowed portions of the Bhlp29.7 polypeptide to be sequentially truncated without the need to use protease digestion followed by complex fractionation procedures. The truncated Bhlp29.7 polypeptides were then used to determine the approximate binding location of the Mab BJL/SH1, which had initially allowed the Bhlp29.7 gene to be isolated.

3.2.2. Plasmid Extraction

E. coli JM109 clones harbouring the pTrcHisA plasmid (Invitrogen) were streaked out from glycerol stock storage onto LB agar plates supplemented with 100 mg/l ampicillin and incubated at 37°C for 16 h. A single colony was used to inoculate 10 ml of LB broth supplemented with 100 mg/l ampicillin and the broth culture was incubated at 37°C for 12 h with shaking. The entire overnight culture was centrifuged at 5,000 \( \times g \) for 10 min and the plasmid contained in the cells extracted using the QIAprep Spin Miniprep Kit (Qiagen) as described in Chapter 2 (Section 2.2.6). The purified plasmid was quantified using a Dynaquant DNA fluorometer (Hoefer), using pUC18 plasmid (Promega) as the standard, and the DNA concentration adjusted to 100 µg/ml by dilution with Tris-ethylenediaminetetraacetic acid (TE) buffer. The purified pTrcHisA plasmid was stored at -20°C.

3.2.3. Vector Preparation

One µg of the purified pTrcHisA plasmid was digested at 37°C overnight in a total volume of 100µl containing 5 U of EcoR1 (Promega) and 5 U of Xho1 (Promega) in 100 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM MgCl₂, dithiothreitol (DTT) and 100 µg/ml bovine serum albumin (BSA). The restricted vector was verified by electrophoresing 2 µl of the digestion
reaction through a 1% (w/v) agarose gel in 1× TAE buffer at 90V for 1 h. The electrophoresed DNA was stained with 1 μg/ml ethidium bromide and viewed over UV light.

Linearised pTrcHisA vector was purified using the UltraClean PCR Clean-up Kit (Mo Bio Laboratories) as described in Chapter 2 (Section 2.2.12.2). Purified linear vector was quantified using the fluorometer (Hoefer), using calf thymus DNA (Hoefer) as the molecular mass standard, and the DNA concentration adjusted to 50 μg/ml by dilution with TE buffer. The purified restricted vector was stored at -20°C.

3.2.4. Insert Preparation

3.2.4.1. Primer design

The primers used for PCR amplification and cloning in this chapter are listed in Table 3.1. All cloning primers included a restriction enzyme recognition site at the 5’-OH end to enable cohesive-end ligation of the resultant amplicon into the linearised pTrcHisA vector. All upstream (forward) primers contained a terminal XhoI recognition site and all downstream (reverse) primers contained a terminal EcoRI recognition site. The primers were tested *in silico* using Amplify 1.2 (University of Wisconsin) and the theoretical amplicon sequence was inserted into the appropriate position in the pTrcHisA vector sequence. Deduced translation of the chimeric pTrcHisA expression cassette was performed using Vector NTI version 6 (InforMax) to determine the expected molecular mass of the recombinant polypeptides, and to confirm that the Bhlp29.7 inserts were in the correct reading frame.

Two primers (Bhlp29.7-F13-XhoI and Bhlp29.7-R809-EcoRI) were designed to amplify an internal 796 bp coding region of Bhlp29.7 such that upon translation, the lipoprotein signal peptide was modified in the polypeptide. Three reverse primers (Bhlp29.7-R195-XhoI, Bhlp29.7-R411-EcoRI, Bhlp29.7-R613-EcoRI) were designed which annealed to the Bhlp29.7 gene at approximately 25%, 50% and 75% of the gene, respectively. The
combination of the Bhlp29.7-F13-Xho1 primer and each of the above reverse primers generated amplicons whereby the expressed Bhlp29.7 polypeptide would be sequentially truncated by 25% with each reverse primer. That is, Bhlp29.7-F13-Xho1 with Bhlp29.7-R195-EcoR1 produced the N-terminal 25% portion of Bhlp29.7 (residues 5 to 65), Bhlp29.7-F13-Xho1 with Bhlp29.7-R411-EcoR1 produced the N-terminal 50% portion of Bhlp29.7 (residues 5 to 137), and Bhlp29.7-F13-Xho1 with Bhlp29.7-R613-EcoR1 produced the N-terminal 75% portion of Bhlp29.7 (residues 5 to 204). One forward primer (Bhlp29.7-F604-Xho1) was designed to anneal to the Bhlp29.7 gene at approximately 75% along the length of the gene. When used in combination with Bhlp29.7-R809-EcoR1, this allowed the C-terminal 25% (residue 202 to 269) to be cloned. All truncates of Bhlp29.7 overlapped such that every part of Bhlp29.7 was represented in at least one of the sub-clones. A schematic representation of the cloning and sub-cloning of Bhlp29.7 is shown in Fig. 3.1.

Table 3.1. Oligonucleotide primers used for the cloning of bhlp29.7 into pTrcHis.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>T_A (°C)</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bhlp29.7-F13-Xho1</td>
<td>60</td>
<td>A A ACTCGAGTTATTATTGGTATCATCAGC</td>
</tr>
<tr>
<td>Bhlp29.7-R809-EcoR1</td>
<td>60</td>
<td>TATGAATTCCAAGTAGGAAGATAAGAACC</td>
</tr>
<tr>
<td>Bhlp29.7-R195-EcoR1</td>
<td>58</td>
<td>TATGAATTCACTCAGAAAAGATACTGCTC</td>
</tr>
<tr>
<td>Bhlp29.7-R411-EcoR1</td>
<td>57</td>
<td>TCCGAATTCAGAAGGTCATTAGGTATAGC</td>
</tr>
<tr>
<td>Bhlp29.7-R613-EcoR1</td>
<td>60</td>
<td>GATGAATTCCGAAGTTATAGCATAGTTTTC</td>
</tr>
<tr>
<td>Bhlp29.7-F604-Xho1</td>
<td>57</td>
<td>AACCTCGAGATATCATCCGTTTGAATCTCG</td>
</tr>
<tr>
<td>pTrcHis-F</td>
<td>60</td>
<td>CAATTTATCAGACAATCTGTGTG</td>
</tr>
<tr>
<td>pTrcHis-R</td>
<td>60</td>
<td>TGCCTGCGAGTTTCCCTACTCTCG</td>
</tr>
</tbody>
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3.2.4.2. Amplification of the Bhlp29.7 inserts

The Bhlp29.7 inserts were amplified by PCR in a 100 μl total volume using Taq DNA polymerase (Biotech International) and Pfu DNA polymerase (Promega). Briefly, the amplification mixture consisted of 1× PCR buffer (containing 1.5 mM of MgCl₂), 1 U of Taq DNA polymerase, 0.1 U Pfu DNA polymerase, 0.2 mM of each deoxynucleotide triphosphate (dNTP; Promega), 0.5 μM of the primer pair (Bhlp29.7-F13-Xho1 and Bhlp29.7-R195-EcoR1; Bhlp29.7-F13-Xho1 and Bhlp29.7-R411-EcoR1; Bhlp29.7-F13-Xho1 and Bhlp29.7-R613-EcoR1; Bhlp29.7-F13-Xho1 and Bhlp29.7-R809-EcoR1; Bhlp29.7-F604-Xho1 and Bhlp29.7-R809-EcoR1), and 2.5 μl chromosomal template DNA from *B. hyodysenteriae* strain WA1 (a
Western Australian field strain isolated from a pig) that was prepared in Chapter 2 (section 2.2.1). Cycling conditions involved an initial template denaturation step of 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 15 sec, and primer extension at 68°C for 2 min. The PCR products were subjected to electrophoresis in 1.5% (w/v) agarose gels in 1× TAE buffer, stained with a 1 μg/ml ethidium bromide solution and viewed over UV light. After verifying the presence of the correct size PCR product, the PCR reaction was purified using the UltraClean PCR Clean-up Kit as described in Chapter 2 (section 2.2.12.2). The purified PCR product was eluted from the clean-up column using 100 μl of TE buffer.

3.2.4.3. Restriction enzyme digestion of the Bhlp29.7 inserts

Fifty μl of each purified PCR product was digested in a 100 μl total volume with 1 U of EcoR1 and 1 U of XhoI in 100 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM MgCl2, 1 mM DTT and 100 μg/ml BSA at 37°C overnight. The digested insert DNA was purified using the UltraClean PCR Clean-up Kit (section 2.2.12.2). Purified digested insert DNA was eluted from the clean-up column using 50μl of TE buffer and was quantified using a fluorometer (Hoefer), with calf thymus DNA (Hoefer) as the molecular mass standard. The DNA concentration was adjusted to 20 μg/ml by dilution with TE buffer. The purified restricted insert DNA was used immediately for vector ligation.

3.2.4.4. Ligation of the Bhlp29.7 inserts into the pTrcHisA vector

Ligation reactions were all performed in a total volume of 20 μl. One hundred ng of XhoI/EcoR1-linearised pTrcHisA was incubated with 20 ng of XhoI/EcoR1-restricted Bhlp29.7 insert at 14°C for 16 h in 30 mM Tris-HCl (pH 7.8), 10 mM MgCl2, 10 mM DTT and 1 mM adenosine triphosphate (ATP) containing 1 U of T4 DNA ligase (Promega). An identical ligation reaction containing no Bhlp29.7 insert DNA was also included as a vector re-circularisation negative control.
3.2.5. Transformation of pTrc-Bhlp29.7 into *E. coli* cells

Competent *E. coli* BL21 Star™ (DE3) pLys One Shot® (Invitrogen) cells were thawed from -80°C storage on ice and then 50 μl of the cells were transferred into ice-cold 1.5 ml microfuge tubes containing 5 μl of the overnight ligation reactions (equivalent to 25 ng of pTrcHisA vector). The tubes were mixed by gently tapping the bottom of each tube on the bench and left on ice for 30 min. The cells were then heat-shocked by placing the tubes into a 42°C water-bath for 45s before returning the tube to ice for 2 min. The transformed cells were recovered in 1 ml LB broth for 1 h at 37°C with gentle mixing. The recovered cells were harvested at 2,500 × g for 5 min and the cells resuspended in 50 μl of fresh LB broth. The entire 50 μl of resuspended cells were spread evenly onto a LB agar plate containing 100 mg/l ampicillin using a sterile glass rod. Plates were incubated at 37°C for 16 h.

3.2.6. Detection of the pTrc-Bhlp29.7 constructs in *E. coli* by PCR

For each transformation reaction, 12 single transformant colonies were streaked onto fresh LB agar plates containing 100 mg/l ampicillin and incubated at 37°C for 16 h. A single colony from each transformation event was resuspended in 50 μl of TE buffer and boiled for 1 min. Two μl of boiled cells were used as template for PCR. The amplification mixture consisted of 1× PCR buffer (containing 1.5 mM of MgCl₂), 1 U of *Taq* DNA polymerase, 0.2 mM of each dNTP, 0.5 μM of the pTrcHis-F primer and 0.5 μM of the pTrcHis-R primer. Cycling conditions involved an initial template denaturation step of 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 15 sec, and a primer extension at 72°C for 30s. The PCR products were subjected to electrophoresis in 1.5% (w/v) agarose gels in 1× TAE buffer, stained with a 1 μg/ml ethidium bromide solution and viewed over UV light.
3.2.7. Verification of the pTrc-Bhlp29.7 constructs by direct sequence analysis

Two transformant clones which produced the correct sized PCR products for each construct were inoculated into 10 ml LB broth containing 100 mg/l ampicillin and incubated at 37°C for 12 h with shaking. The overnight cultures were centrifuged at 5,000 × g for 10 min and the plasmid contained in the cells extracted using the QIAprep Spin Miniprep Kit as described in chapter 2 (section 2.2.6). The purified plasmid was quantified using a fluorometer (Hoefer), and pUC18 plasmid (Promega) as the standard. Both purified plasmids for each construct were subjected to automated direct sequencing of the pTrcHisA expression cassette using the pTrcHis-F and pTrcHis-R primers. Each sequencing reaction was performed in a 10 μl volume consisting of 200 ng of plasmid DNA, 2 pmol of primer, and 4 μl of the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Mix (PE Applied Biosystems). Cycling conditions involved a 2 min denaturing step at 96°C, followed by 25 cycles of denaturation at 96°C for 10s and a combined primer annealing and extension step at 60°C for 4 min. Residual dye terminators were removed from the sequencing products by precipitation with 95% (v/v) ethanol containing 120 mM sodium acetate (pH 4.6), and vacuum dried. The plasmids were sequenced in duplicate using each primer. Sequencing products were analysed using an ABI 373A DNA Sequencer (PE Applied Biosystems). Successfully ligated plasmids were designated pTrc-Bhlp29.7, pTrc-Bhlp29.7-R195, pTrc-Bhlp29.7-R411, pTrc-Bhlp29.7-R613 and pTrc-Bhlp29.7-F604.

3.2.8. Pilot expression of recombinant Bhlp29.7 proteins

A single colony of each Bhlp29.7 construct in E. coli BL21 was inoculated into 10 ml LB broth containing 100 mg/l ampicillin and incubated at 37°C for 16 h with shaking. Fifty ml of LB broth in a 250 ml conical flask containing 100 mg/l ampicillin was inoculated with 1 ml of the overnight culture and incubated at 37°C until the optical density of the cells at 600 nm was 0.5 (approximately 3 h). IPTG was then added to the cells to a final concentration of 1 mM and the cells returned to 37°C with shaking for 6 h. The cells were harvested by centrifugation at
5,000 \times g for 10 min before resuspending each cell pellet with 2 ml of nickel-nitrilotriacetic acid (Ni-NTA) denaturing lysis buffer. All cell lysates were incubated at -20°C overnight. Cell debris was clarified from the thawed lysates by centrifuging at 20,000 \times g for 15 min at 4°C. The supernatant from each construct was individually collected and used for SDS-PAGE (chapter 2, section 2.2.4) and Western blotting (chapter 2, section 2.2.5) with Mab BJL/SH1 diluted 10-fold. To confirm that the recombinant his-tagged proteins had been expressed, mouse anti-polyhistidine (Sigma Chemical Company) diluted 5,000-fold was also used for Western blotting. Goat anti-mouse IgG-HRP (Sigma Chemical Company) was used to detect antibody binding.

3.2.9. Large-scale expression of recombinant His$_6$-Bhlp29.7

The His$_6$-Bhlp29.7 protein was needed in high concentration for development of an indirect antibody test (see chapter 4) and for vaccination of pigs (see chapter 5). For these reasons, His$_6$-Bhlp29.7 was expressed and purified in large-scale. A single colony of pTrc-Bhlp29.7 in E. coli BL21 was inoculated into 50 ml LB broth in a 250 ml conical flask containing 100 mg/l ampicillin and incubated at 37°C for 16 h with shaking. A 2 litre conical flask containing 1 l of LB broth supplemented with 100 mg/l ampicillin was then inoculated with 10 ml of the overnight culture and incubated at 37°C until the optical density of the cells at 600 nm was 0.5 (approximately 3-4 h). Expression was induced by adding IPTG to a final concentration of 1 mM and the cells returned to 37°C with shaking. After 6 h of induction, the culture was transferred to 250 ml centrifuge bottles and the bottles were centrifuged at 5,000 \times g for 20 min at 4°C. The supernatant was discarded and each pellet was resuspended with 10 ml Ni-NTA denaturing lysis buffer (i.e. 40 ml lysis buffer per 1 l cell culture). The lysates were incubated at -20°C overnight.
3.2.10. Large-scale purification of recombinant His6-Bhlp29.7

The cell lysates were removed from -20°C and thawed on ice. To reduce the viscosity of the lysate (due to E. coli DNA), the lysates were sonicated on ice three times for 30s with 2 min incubation on ice between sonication rounds. The lysed cells were clarified by centrifugation at 20,000 × g for 10 min at 4°C and the supernatants transferred to 15 ml columns containing a 1 ml bed volume of Ni-NTA agarose resin (Qiagen). The columns were securely capped and the his-tagged proteins were allowed to bind to the resin for 1 h at 4°C with end-over-end mixing. The resin was then washed five times with 10 ml of Ni-NTA denaturing wash buffer before elution with Ni-NTA denaturing elution buffer. Three 10 ml fractions of the eluate were collected and stored at 4°C. The purified proteins were subjected to SDS-PAGE (chapter 2, section 2.2.4) and stained with Coomassie Brilliant Blue G250 (CBB-G250; Sigma Chemical Company). The electrophoresed proteins in the gel were fixed for 30 min in CBB-G250 fixing solution before being transferred to the CBB-G250 staining solution for 1 h. The staining solution was decanted and the gel was de-stained for 3 h with three changes of CBB-G250 destaining solution. The de-stained gel was equilibrated in distilled water for 1 h and dried between two sheets of cellulose overnight at RT.

3.2.11. Dialysis and lyophilisation of the purified recombinant His6-Bhlp29.7

The eluted fractions containing purified his-tagged proteins were pooled and transferred into a hydrated dialysis tube (Spectrum Laboratories) with a molecular weight cut-off (MWCO) of 3,500 Da. A 200 µl aliquot of the pooled eluate was taken and quantified using the Bio-Rad Protein Assay (Bio-rad Laboratories) (see below). The proteins were dialysed against 2 l of distilled water at 4°C with stirring. The distilled water was changed eight times at 12-hourly intervals. The dialysed proteins were transferred from the dialysis tube into a 50 ml centrifuge tubes (40 ml maximum volume) and the tubes were placed at -80°C overnight before being transferred to a MAXI freeze-drier (Heto-Holten) and lyophilised to dryness. The lyophilised
protein was then re-hydrated with physiological saline to a concentration of 2 mg/ml and stored at -20°C in 1 ml aliquots.

3.2.12. Western blot analysis of re-hydrated, purified His$_6$-Bhlp29.7 with infected pig serum

Ten μg of the purified His$_6$-Bhlp29.7 antigen was loaded into a 7cm preparative well and subjected to SDS-PAGE (described in chapter 2, section 2.2.4) and Western blotting (described in chapter 2, section 2.2.5) using 6 pig serum samples obtained from a herd in which SD was observed. Serum from a pig hyper-immunised with a bacterin made with $B.\ hyodysenteriae$ strain B78$^T$ (see chapter 4, section 4.2.2) was used as a positive control. The pig serum was diluted 50-fold in TBST and goat anti-pig IgG-HRP conjugate (diluted 5,000-fold) (Southern Biotechnology) was used for the blot. The hyper-immunised pig serum raised against $B.\ hyodysenteriae$ strain B78$^T$ (diluted 100-fold) was included as a positive control.

3.2.13. Quantification of the purified recombinant His$_6$-Bhlp29.7

A commercial BSA protein standard (Sigma Chemical Company) was serially diluted with Ni-NTA native elution buffer in 2-fold serial dilutions from 6.25 μg/ml to 100 μg/ml. The purified recombinant His$_6$-Bhlp29.7 was also diluted with the same buffer in 2-fold serial dilutions from 2-fold to 32-fold. Twenty μl of the Protein Assay Reagent (Bio-rad Laboratories) was added to the wells of a 96-well microtitre plate. Eighty μl of the corresponding protein standards or purified protein was added to the appropriate wells and the microtitre plate was gently vortexed to mix. The BSA standards were assayed in triplicate and the diluted purified his-tagged proteins were tested in duplicate. The microtitre plate was then placed into a microtitre plate reader (Model 3550-UV; Bio-rad Laboratories) and the optical density at 600 nm recorded. The standard curve generated from the optical density of the protein standards was used to interpolate the concentration of the His$_6$-Bhlp29.7 sample and subsequently the yield of protein obtained from the 1 litre culture.
3.3. RESULTS

3.3.1. Construction of the pTrc-Bhlp29.7 recombinant vector

Cloning of the full-length bhlp29.7 insert into the pTrcHisA expression vector produced a recombinant construct, designated pTrc-Bhlp29.7, which was 5,168 bp in size. A complete plasmid map of pTrc-Bhlp29.7 expression vector is shown in Fig. 3.2. Sub-cloning of bhlp29.7 to yield truncates of the gene produced four constructs. These constructs were essentially identical to pTrc-Bhlp29.7 but had shorter inserted sequences following the XhoI-EcoRI cloning site. The constructs were designated pTrc-Bhlp29.7-R195 (4,554 bp), pTrc-Bhlp29.7-R411 (4,770 bp), pTrc-Bhlp29.7-R613 (4,972 bp) and pTrc-Bhlp29.7-F604 (5,577 bp). Nucleotide sequencing of the pTrc-Bhlp29.7 constructs verified that the expression cassette was in the correct frame for all five plasmids. Predicted translation of the pTrc-Bhlp29.7 constructs indicated the recombinant Bhlp29.7 proteins were identical to the deduced amino acid sequence of corresponding portions of native Bhlp29.7.

3.3.2. Expression and purification of recombinant His$_6$-Bhlp29.7

Expression of the pTrc-Bhlp29.7 construct produced a recombinant his-tagged Bhlp29.7 protein with an apparent molecular mass of 34 kDa. The anti-histidine and BJL/SH1 monoclonal antibodies reacted strongly with the recombinant His$_6$-Bhlp29.7 (Fig. 3.3 and Fig. 3.4). Purification of the His$_6$-Bhlp29.7 recombinant protein by affinity chromatography under denaturing conditions successfully yielded his-tagged Bhlp29.7 at a level of purity at which CBB-G250 staining was unable to detect contaminating co-elution products (Fig. 3.5). Recombinant protein yields of 4 mg/L were consistently obtained. Following dialysis and lyophilisation, a stable recombinant His$_6$-Bhlp29.7 antigen was successfully produced. Expression of Bhlp29.7 in E. coli as a his-tagged protein, and the purification of His$_6$-Bhlp29.7 under denaturing conditions did not alter its reactivity with infected pig serum and hyper-immunised pig serum (Fig. 3.6).
Figure 3.2. Complete plasmid map of the pTrc-Bhlp29.7 expression vector showing features of the vector and the restriction sites used in its construction. The plasmid map for the truncated Bhlp29.7 constructs (pTrc-Bhlp29.7-R195, pTrc-Bhlp29.7-R411, pTrc-Bhlp29.7-R611 and pTrc-Bhlp29.7-F604) is essentially identical except for shorter coding sequences between the Xho1 and EcoR1 enzyme recognition sites.
Figure 3.3. Western blot analysis of recombinant his-tagged Bhlp29.7 truncates expressed in *E. coli*. The expressed proteins were detected using the anti-histidine monoclonal antibody. Lane 1, “full-length” Bhlp29.7; lane 2, Bhlp29.7-R195 (N-terminal 25% portion); lane 3, Bhlp29.7-R411 (N-terminal 50% portion); lane 4, Bhlp29.7-R613 (N-terminal 75% portion). The arrow indicates the position of the “full-length” his-tagged Bhlp29.7 protein.

Figure 3.4. Western blot analysis of recombinant his-tagged Bhlp29.7 truncates expressed in *E. coli*. The expressed proteins were detected using the monoclonal antibody BJL/SH1. Lane 1, “full-length” Bhlp29.7; lane 2, Bhlp29.7-R195 (N-terminal 25% portion); lane 3, Bhlp29.7-R411 (N-terminal 50% portion); lane 4, Bhlp29.7-R613 (N-terminal 75% portion). The arrow indicates the position of the “full-length his-tagged Bhlp29.7 protein.
Figure 3.5. Coomassie Brilliant Blue G250 stain of recombinant his-tagged Bhlp29.7 purified by affinity chromatography using Ni-NTA agarose. Lane 1, first elution fraction; lane 2, second elution fraction; lane 3, third elution fraction.

Figure 3.6. Reactivity of recombinant his-tagged Bhlp29.7 against convalescent pig serum. The pig serum was obtained from a herd in which SD had been observed. The anti-histidine monoclonal antibody was included as an internal control. Lane 1, hyper-immunised pig serum raised against *B. hyodysenteriae* strain B78\(^T\); lanes 2-7, infected pig sera. The arrow indicates the position of the his-tagged Bhlp29.7 protein.
3.3.3. Reactivity of monoclonal antibody BJL/SH1 with truncated Bhlp29.7 proteins

Expression of the pTrc-Bhlp29.7 sub-cloning constructs produced four recombinant his-tagged proteins with an apparent molecular mass of 11.6 kDa, 20.1 kDa, 26.7 kDa and 12 kDa for His<sub>6</sub>-Bhlp29.7-R195, His<sub>6</sub>-Bhlp29.7-R411, His<sub>6</sub>-Bhlp29.7-R613 and His<sub>6</sub>-Bhlp29.7-F604, respectively. The anti-histidine Mab reacted strongly with the all the recombinant Bhlp29.7 truncates indicating that all these truncates were expressed with an intact his-tag (Fig. 3.3 and Fig. 3.7). However, only the His<sub>6</sub>-Bhlp29.7-F604 protein (C-terminal 25% polypeptide) reacted with Mab BJL/SH1 (Fig. 3.4 and Fig. 3.7). Nucleotide sequencing of all the sub-cloning constructs indicated that there were no discrepancies between the deduced amino acid sequence of the recombinant truncates and the native Bhlp29.7 protein. The lack of reactivity of Mab BJL/SH1 with all Bhlp29.7 truncates except the Bhlp29.7-F604 truncate indicates that the epitope for Mab BJL/SH1 is located on the C-terminal 25% portion of Bhlp29.7 between residues 202 and 269.

![Figure 3.7](image)

**Figure 3.7.** Western blot analysis of recombinant his-tagged Bhlp29.7-F604 (C-terminal 25% portion) using the anti-histidine and BJL/SH1 monoclonal antibodies. Lane 1, “full-length” his-tagged Bhlp29.7 protein reacting with Mab BJL/SH1; lane 2, his-tagged Bhlp29.7-F604 protein reacting with Mab BJL/SH1; lane 3, his-tagged Bhlp29.7-F604 protein reacting with the anti-histidine Mab.
Comparative analysis of the translated amino acid sequence of Bhlp29.7 for the 11 *B. hyodysenteriae* strains and *B. innocens* strain B256^T^ (chapter 2, section 2.3.4) identified an amino acid dissimilarity at position 210 which occurred only in *B. innocens* strain B256^T^, where the serine for *B. hyodysenteriae* was replaced by a tyrosine for *B. innocens* strain B256^T^ (Fig. 3.8).

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<th>205</th>
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<th>225</th>
<th>235</th>
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<tr>
<td>B256^T^</td>
<td>AIDFGLNPGY DYIFKDDPSI YSGKFVNLN AARTKDKDNE</td>
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</tbody>
</table>

**Figure 3.8.** Comparative analysis of the translated amino acid sequence of Bhlp29.7 for the 11 *B. hyodysenteriae* strains and *B. innocens* strain B256^T^.

The polypeptide between residues 202 and 269 possibly contains the epitope for Mab BJL/SH1. The amino acid dissimilarity present only in *B. innocens* strain B256^T^ is indicated in bold type-face. Sequence between residues 202 to 199, and 241 to 269 were identical for all strains. The *bhlp29.7* sequence for WA4 and WA5 were identical, as were the sequences of WA1, WA6, WA15 and WA16.
3.4. DISCUSSION

The Bhlp29.7 gene of *B. hyodysenteriae* was amplified by PCR and cloned into the bacterial expression vector pTrcHis. Oligonucleotide primers were designed to replicate the Bhlp29.7 ORF such that upon translation, the pro-lipoprotein would have amino acids 1 to 4 of the native Bhlp29.7 replaced with the His$_6$-tag. The remaining amino acid sequence was left unchanged. PCR amplification was performed using *Taq* DNA polymerase and *Pfu* DNA polymerase. *Pfu* DNA polymerase is responsible for DNA repair of mis-matched bases (Barnes, 1994). The use of the high-fidelity *Pfu* DNA polymerase for PCR reduces the high error rate characteristic of *Taq* DNA polymerase activity (Flaman *et al.*, 1994; Cline *et al.*, 1996). In addition, the Bhlp29.7 gene was sequentially sub-cloned into the pTrcHis vector such that the expressed recombinant proteins represented four truncates of the Bhlp29.7 protein. These included an N-terminal 25% portion (Bhlp29.7-R195), an N-terminal 50% portion (Bhlp29.7-R411), an N-terminal 75% portion (Bhlp29.7-R613) and a C-terminal 25% portion (Bhlp29.7-F604). Sequence analysis of the cloned Bhlp29.7 ORF indicated that no mutations were introduced by the DNA polymerase during PCR amplification for cloning. The PCR-amplified Bhlp29.7 products were ligated into the pTrcHis expression cassette in the correct reading frame and the deduced translation of the cloned inserts were identical to the deduced translation of the original Bhlp29.7 gene.

The purpose for sub-cloning Bhlp29.7 was to determine the approximate location of the epitope for Mab BJL/SH1. Western blot analysis with an anti-histidine Mab indicated that all Bhlp29.7 truncates had been expressed. Western blotting with Mab BJL/SH1 showed reactivity with the full recombinant Bhlp29.7 protein and the Bhlp29.7-F604 truncate, but not with the other truncates. This indicated that the epitope for the Mab BJL/SH1 was located at the C-terminal end of Bhlp29.7 between amino acid residues 202 and 269. Comparative analysis of the amino acid sequence of Bhlp29.7 for 11 *B. hyodysenteriae* strains and *B. innocens* strain B256$^T$ identified an amino acid substitution at residue 210 which occurred only in the *B. innocens* strain, where the serine for *B. hyodysenteriae* was replaced by a tyrosine for
B256T. The Mab BJL/SH1 is specific for *B. hyodysenteriae*, and does not bind to *B. innocens* strain B256T even though it possess a homologue of Bhlp29.7. Based on the presence of this single consistent difference in the amino acid sequences of Bhlp29.7 between the *B. hyodysenteriae* strains and *B. innocens* strain B256T, it is likely that the binding site of Mab BJL/SH1 is localised around position 210 (serine) of Bhlp29.7, and that the tyrosine residue contained on the Bhlp29.7 of *B. innocens* B256T at position 210 prevents cross-reactivity. Identification of the epitope for this antibody could provide a suitable peptide antigen for serological diagnosis of SD. Although an estimation of the epitope location has been suggested, further work is needed to confirm this hypothesis.

The remaining emphasis of this chapter was to generate recombinant Bhlp29.7 proteins in high concentration and purity without the need for complex methodologies such as high performance liquid chromatography and fast performance liquid chromatography. Following the outcome from sub-cloning *bhlp29.7*, His6-Bhlp29.7 and His6-Bhlp29.7-F604 were expressed and purified for subsequent studies.

Over-expression of foreign proteins in *E. coli* frequently results in the sequestering of these proteins into insoluble inclusion bodies (Schein, 1989). The inclusion bodies can be solubilised easily with denaturants such as guanidine hydrochloride and urea, or with a variety of detergents, however, all tertiary structure is lost and refolding of the denatured protein can be complex with no guarantee of restoring the native structure. In addition, proteins in inclusion bodies are usually resistant to proteolytic degradation. Unfortunately the solubilisation process results in denaturation of the recombinant protein which is a serious disadvantage for intended applications requiring correct conformation and presentation. For applications in which activity of the expressed protein is not essential (such as antibody production) inclusion body formation is the most common strategy for high-level expression of recombinant protein in *E. coli* (Coligan *et al.*, 2002). In this project, the recombinant Bhlp29.7 protein was expressed at high levels in insoluble form and subsequently solubilised using 8 M urea. The
immunogenicity of the recombinant His6-Bhlp29.7 protein did not appear to affect by
denaturation as six different sera taken from pigs from a herd with SD reacted strongly with
the denatured form of the recombinant protein.

The native Bhlp29.7 lipoprotein has been found to be membrane-associated and shares
homology with the D-methionine transport proteins of various bacteria, including E. coli
(Chapter 2). Expression of recombinant membrane proteins in E. coli can be toxic, resulting in
the inhibition of cell growth following induction. This is most likely due to the association of
hydrophobic regions of the protein with or incorporation into vital membrane systems of the
host (Coligan et al., 2002). It seems likely that interference of the E. coli D-methionine
transport system with a possible homologue from B. hyodysenteriae would hinder cell growth,
and hence be toxic to E. coli during recombinant protein expression, but fortunately this did
not occur. Although the signal peptidase cleavage site of Bhlp29.7 is similar to that of E. coli
(Chapter 2), the cellular processes involved in post-translational modifications leading to the
ultimate attachment of the lipoprotein to the cell membrane may be incompatible with the E.
coli system - hence recombinant His6-Bhlp29.7 may not be recognised by E. coli as a
membrane destined protein. In addition, amino acid residues 1 to 4 of the native Bhlp29.7 were
replaced with the His6-tag during the cloning process. If the signal peptides of E. coli and B.
hyodysenteriae were compatible, the modifications to the Bhlp29.7 signal peptide during
cloning may have caused the signal peptide to be unrecognisable to the E. coli cellular
machinery, hence accounting for the unexpected non-toxicity of recombinant His6-Bhlp29.7.
Furthermore, the over-expression of recombinant His6-Bhlp29.7 as inclusion bodies renders
the protein inactive, thus preventing it from associating with the membrane of the host.

Purification of the His6-tagged proteins using nickel-nitrilotriacetic acid (Ni-NTA) metal-
affinity chromatography is independent of conformation, and exploits the high affinity of
histidine for nickel ions (Ni²⁺). Due to this characteristic, purification of the recombinant
Bhlp29.7 proteins were performed under denaturing conditions allowing easy solubilisation of
inclusion bodies which had formed. The proteins after affinity purification were highly pure and any co-purification contaminants were of insufficient quantity to be detected by Coomassie Brilliant Blue staining. The pooled fractions containing the purified His$_6$-Bhlp29.7 and His$_6$-Bhlp29.7-F604 were dialysed against distilled water to remove the urea present in the eluate. Re-folding of the purified protein was not considered worthwhile as the activity and conformation of His$_6$-Bhlp29.7 was not believed to be essential for downstream applications.

In this study, the expression conditions for His$_6$-Bhlp29.7 were chosen to favour high-yield over solubility. Following solubilisation and purification by Ni-NTA metal-affinity chromatography under denaturing conditions, yields of at least 4 mg per litre of culture were consistently achieved. This high-level expression and purification of the Bhlp29.7 protein in recombinant form was a crucial component of the subsequent studies described in this thesis. The recombinant Bhlp29.7 protein produced was used for serological analysis of blood samples taken from pigs in Australian herds, and for vaccination of experimentally challenged pigs, as described in the following chapters.
CHAPTER 4: EVALUATION OF RECOMBINANT BHLP29.7 AS AN ELISA ANTIGEN FOR SD SEROLOGY

4.1. INTRODUCTION

In the field, diagnosis of SD primarily relies on the herd history and observation of typical clinical signs including the presence of diarrhoea containing fresh blood and/or mucus, usually restricted to pigs in the grower/finisher phase. However, these clinical signs can be similar to those of other diseases including salmonellosis, swine fever and colibacillosis (Harris et al., 1999). Furthermore, in some herds typical signs may not be observed – particularly if the herd is receiving routine antimicrobial therapy. A definitive diagnosis of SD still requires the isolation and identification of the causative agent. For many years, the identification of *B. hyodysenteriae* has been dependant on observing its phenotypic characteristics, however, the development of PCR now allows a molecular-based approach to *B. hyodysenteriae*-specific diagnosis, supplementing microbiological culture and biochemical testing. Antigen-based detection methods also have been used to help identify *B. hyodysenteriae*, however these methods have a tendency to lack sensitivity and specificity. To date, reliable serological assay to detect antibody to *B. hyodysenteriae*, and hence provide indirect evidence of exposure, are not available.

Enzyme immunoassays (such as enzyme-linked immunosorbent assay or ELISA) are based on two important biological phenomena: (i) the extraordinary discriminatory power of antibodies, based on the ability of the immune system of vertebrates to produce a virtually unlimited array of antibodies, each with an affinity for a specific foreign antigen; and (ii) the extremely high catalytic power and specificity of enzymes, which quite often may be detectable with great ease. ELISA consists of a two-pronged strategy: the reaction between the immuno-reactants (antibody with the corresponding antigen) and the detection of that reaction using enzymes,
labelled to the reactants, as indicators. The specificity of the assay resides with the particular antigen molecule used to capture reactive antibody.

Colonisation by *B. hyodysenteriae* elicits a strong immunological response against the spirochaete (Harris *et al.*, 1999), and hence indirect evidence of exposure to *B. hyodysenteriae* can be obtained by measuring circulating antibody titres in the blood of infected animals. Several serological tests have been developed that detect antibodies to *B. hyodysenteriae* in the serum of infected pigs (reviewed in chapter 1). These tests have not been based on species-specific antigens, and, as a consequence, lack specificity and/or sensitivity. Of the serological tests available, ELISA appears to be the most promising, potentially providing the greatest sensitivity and the ability to analyse large numbers of samples simultaneously. Although classical ELISA tests for SD (based on lipopolysaccharides or sonicated cells) have shown some potential, there is an underlying need for a more specific plate-coating antigen which is applicable to all serogroups of *B. hyodysenteriae*. A number of proteins associated with the outer surface of *B. hyodysenteriae* have already been identified and characterised (reviewed in chapter 1), however the suitability of these components for serological detection remains unclear. Ideally, a suitable assay component would involve a single, cell membrane-associated protein, preferably recombinant, which is specific for all strains of *B. hyodysenteriae* and is known to be highly immunogenic in experimentally and naturally infected pigs.

This chapter describes the development and evaluation of an ELISA test for the serological detection of SD using recombinant His<sub>6</sub>-Bhlp29.7 as the discriminatory antigen. The test was aimed at detection of SD at the herd level, and used sera collected from pigs at slaughter in abattoirs – since this is a convenient source of sera for screening the health status of herds. The practical limitations of obtaining well-defined serum precluded a thorough evaluation of the usefulness of the test for detecting individual infected animals, although a set of sera from a group of acutely affected animals was examined.
4.2. MATERIALS AND METHODS

4.2.1. Preparation of ELISA antigen

Purified recombinant His6-Bhlp29.7 antigen was prepared as described in Chapter 3 (section 3.2.10). The lyophilised antigen was re-hydrated and diluted to a working concentration of 200 μg/ml using sterile PBS.

4.2.2. Preparation of reference sera

The reference sera used for the development of the Bhlp29.7-ELISA were obtained from pigs which had been hyper-immunised with bacterin preparations of *B. hyodysenteriae* strain B78T and *B. pilosicoli* strain 1648. The hyper-immunised pig sera were prepared by I.W.M. Tenaya (Tenaya, 1997), as described below.

Spirochaete cells were grown in 500 ml of modified pre-reduced Kunkle’s broth at 37°C as described in chapter 2 (section 2.2.9) The mid-log phase cultures were harvested at 10,000 × g at 4°C and washing twice in PBS. The washed cells were resuspended in 0.3% formaldehyde in PBS and inactivated for 24 h with constant stirring at room temperature. The bacterin suspension was adjusted to a cell density of approximately 10⁹ cells/ml and stored in aliquots at -20°C.

Two Large White pigs of approximately 5 weeks of age were obtained from an SD-free herd in Western Australia. These pigs were housed in a pen in an isolation house, and offered an antibiotic-free weaner diet and water on an *ad libitum* basis. The pigs were injected intramuscularly in the neck with 3 ml of a 1:1 emulsion of Freund’s Incomplete Adjuvant (FIA; Sigma Chemical Company) and thawed bacterin. Each pig was vaccinated with a different spirochaete bacterin. After two weeks, the intramuscular injection was repeated weekly for four weeks using the same vaccine. Finally, 1.5 ml of bacterin without adjuvant was given intravenously twice at weekly intervals. Blood was collected by jugular puncture.
one week after the final immunisation and the sera was separated and stored at -20°C until required.

4.2.3. ELISA conditions

Assaying conditions involved overnight antigen coating in 100 μl carbonate buffer at 4°C onto 96-well microtitre plates (Immulon 4HBX, Dynex Technologies) followed by blocking of unbound sites with 150 μl PBS containing 1% (w/v) BSA (Sigma Chemical Company). The wells were washed three times with 150 μl of PBST before the addition of diluted pig serum (200-fold) and incubation for 2 h. The wells were washed (as above) and the bound serum antibodies were detected with goat anti-swine IgG-HRP (Southern Biotechnology) diluted 5,000-fold. All antibodies were diluted in 100 μl of PBST containing 0.1% (w/v) BSA and incubations occurred at RT with mixing for 1 h unless stated otherwise. Colour development occurred after the addition of 100 μl of the K-Blue Tetramethylbenzidine (TMB) substrate (ELISA Systems) and was allowed to intensify for 15 minutes at RT. The colour reaction was stopped by the addition of 50 μl of 1 M sulphuric acid and the absorbance of the coloured substrate reaction was recorded at 450 nm using a microtitre plate reader (Bio-Rad Model 3550-UV).

4.2.4. Optimisation of the immunoassay

To optimise the Bhlp29.7-ELISA, a checkerboard titration involving serial dilutions of plate coating antigen and primary antibody (reference sera) was performed under the ELISA conditions described above. A checkerboard titration of plate coating antigen and primary antibody dilution was performed with a constant 5,000-fold dilution of the HRP-conjugated goat anti-pig IgG antibody. The procedure was optimised to give the greatest distinction between the positive signal (absorbance) and the background noise. Immunoassay plates were coated with serial 2-fold dilutions of antigen from 750 ng/ml to 6,000 ng/ml. Reference sera were serially diluted in 2-fold increments from 50-fold to 800-fold. The optimal concentration
for antigen coating was chosen as the concentration that gave a signal greater than 1.0 and allowed the highest signal-to-noise ratio. The optimal coating concentration of His6-Bhlp29.7 was used for all subsequent ELISA tests.

4.2.5. Collection of pig serum
Sera from 1121 finisher pigs were obtained from 19 Australian piggeries with differing health-status located in Queensland, Victoria and Western Australia (see Tables 4.2 to 4.4 in Results section). These samples were provided by consultant veterinarians who were familiar with the health-status of the herds, and were collected at slaughter from the abattoir. These herds included five in which SD had never been observed (group A, n=464), six in which SD was confirmed to be present (group B, n=337), and eight herds with a history of diarrhoea and where it was thought that SD might be present (group C, n=320), although it had not been confirmed by laboratory testing. In addition, 25 sera from 12 to 13 week old pigs with acute signs of SD in a 20th herd (group D, n=25) were also tested. Apart from the pigs in group D, the health status of the individual pigs from which the serum was obtained in the other herds was not known.

4.2.6. Strategy used for the development and evaluation of the ELISA test
The approach taken for the standardisation of the ELISA test consisted of three steps. In the first step, the 464 serum samples from the five herds in which SD had never been observed (group A) were used to set a suitable cut-off value for the test. The threshold value was set as three standard deviations above the mean of all individuals in this population in order to have the ELISA result of all individuals in this group below the cut-off value. Due to the stringency of the threshold value, herds were considered to be diseased when one or more individuals returned an ELISA value greater than the set cut-off. A moderate stringency cut-off calculated as two standard deviations above the mean was also determined for comparison.
In the second step, the ELISA test was then applied to 337 serum samples from six herds in which SD had been confirmed (group B), to check that it was capable of detecting infected herds. Using the outcomes of the assay from the healthy and SD affected herds, the sensitivity and specificity of the assay was determined. The sensitivity was defined as the proportion of diseased herds identified by the test as having at least one serum sample with an ELISA value above the set cut-off. Sensitivity provides an indication of the ability of the test to correctly identify herds containing animals with SD. The specificity was defined as the proportion of healthy herds identified by the test as having all 40 animals with an ELISA value below the set cut-off. Specificity provides an indication of the ability of the test to correctly identify herds containing animals which are healthy.

The third step of the ELISA test development involved the application of the assay to 320 serum samples from pigs in eight herds in which diarrhoea had been observed but SD had not been confirmed. For the analysis of these herds, a sample size of 40 was chosen since it would give a confidence level of approximately 95% of determining that SD was present in the herds tested. This sample size was selected in accordance with a similar study in Australia which evaluated the use of a LPS-based ELISA test for the diagnosis of SD, where it was assumed that the minimum prevalence of disease in most infected herds was 10% (Mhoma, 1992).

In addition, the opportunity was taken to apply the ELISA to sera from a group of pigs with acute SD (group D) in another herd. This would test whether the time of sample collection, in relation to the progression of disease, would affect the outcome of the test. These pigs were ear-tagged with the intention to obtain convalescent sera when the pigs were sent to the abattoir. Unfortunately, these pigs were sent to slaughter without informing the investigator, and the second set of samples was missed.
4.2.7. Application of immunoassay to field samples

Microtitre plates were coated with the optimal concentration of His<sub>6</sub>-Bhlp29.7 determined in section 4.2.4 and assayed in triplicate according to section 4.2.3. Immunised pig sera raised against *B. hyodysenteriae* strain B78<sup>T</sup> was used as the positive control reference (A<sub>P</sub>) and immunised pig sera raised against *B. pilosicoli* strain 1648 was used as the negative control reference (A<sub>N</sub>). These controls were included in all tests. The raw absorbance values measured at 450 nm (A<sub>X</sub>) were manipulated by the equation below to attain cross-plate standardisation and normalisation.

\[
\chi = 100 \left( \frac{A_X - A_N}{A_P - A_N} \right)
\]

where,

- \( \chi \) – ELISA value
- \( A_X \) – absorbance of test sample
- \( A_P \) – absorbance of positive control antiserum
- \( A_N \) – absorbance of negative control antiserum

4.2.8. Analysis of *B. hyodysenteriae* isolates from group D

The Bhlp29.7-ELISA detects antibody directed against the Bhlp29.7 protein. One Western Australian farm (group D) gave negative ELISA results using serum samples obtained from 25 pigs of 12 to 13 weeks of age that were showing acute signs of SD. Since *B. hyodysenteriae* isolates were cultured from pigs kept on this farm at the time of blood collection, it was possible to investigate the presence and expression of the Bhlp29.7 gene in these isolates.

4.2.8.1. Western blot analysis of pig serum reactivity to the ELISA antigen

Ten μg of the purified His<sub>6</sub>-Bhlp29.7 antigen was loaded into a 7cm preparative well and subjected to SDS-PAGE (described in chapter 2, section 2.2.4) and Western blotting (described in chapter 2, section 2.2.5) using six pig serum samples from herd B1 and 14 pig sera from group D. Each pig serum was diluted 50-fold in TBST and the same goat anti-pig IgG-HRP conjugate (diluted 5,000-fold) (Southern Biotechnology) as used in the ELISA was used for the blot. The hyper-immunised pig serum raised against *B. hyodysenteriae* strain B78<sup>T</sup> (diluted 100-fold) was included as a positive control.
4.2.8.2. PCR and sequencing of the *B. hyodysenteriae* isolates for the Bhlp29.7 gene

Three *B. hyodysenteriae* isolates were obtained from pigs in group D. Bacteriology swabs taken from the pigs were streaked onto selective agar plates consisting of TSA (Becton Dickinson Microbiology Systems) containing 5% (v/v) defibrinated sheep blood, 400 μg/ml spectinomycin and 25 μg/ml each of colistin and vancomycin (Sigma Chemical Company). The plates were incubated for 7 days at 37°C in an anaerobic environment of 94% H₂ and 6% CO₂, generated using anaerobic Gaspak plus sachets (Becton Dickinson Microbiology Systems), before being examined. The presence of low flat spreading growth of spirochaetes on the plate, and any haemolysis around the growth was recorded. Spirochaetes were confirmed by picking off areas of suspected growth and sub-culturing until a pure culture of the isolate was obtained. The isolate was re-suspended in PBS, used as template in a PCR for the Bhlp29.7 gene, and the amplified gene was subsequently sequenced (described in chapter 2, section 2.2.12).

4.2.8.3. Western blot analysis of the *B. hyodysenteriae* isolates for Bhlp29.7 expression

The three isolates obtained from group D were grown in 10 ml broth culture as described in chapter 2 (section 2.2.9). The cells were harvested by centrifugation at 10,000 × g for 20 min and the pellet resuspended with 100 μl of PBS. The resuspended cells were subjected to SDS-PAGE (described in chapter 2, section 2.2.4) and Western blotting (described in section 2.2.5) using the Mab BJL/SH1.
4.3. RESULTS

4.3.1. Immunoassay development

Optimised results were obtained with a 200-fold dilution of positive serum and 300 ng/ml of antigen (Table 4.1). The reactivity of the serum from the pig immunised against *B. hyodysenteriae* B78<sup>T</sup> gave an ELISA value of 94.1, which was approximately 2.6-fold greater than the serum raised against *B. pilosicoli* 1648 (ELISA value of 36.3).

**Table 4.1.** Ratio of SD-positive (*B. hyodysenteriae* immunised pig) serum to SD-negative (*B. pilosicoli* immunised pig) serum for the optimisation of assay conditions for ELISA against recombinant His<sub>6</sub>-Bhlp29.7 antigen. Optimum values with the best signal-to-noise ratio are highlighted.

<table>
<thead>
<tr>
<th>Antigen concentration (ng/ml)</th>
<th>Reciprocal of serum dilution (50)</th>
<th>100</th>
<th>200</th>
<th>400</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>5.8</td>
<td>6.4</td>
<td>6.8</td>
<td>5.7</td>
</tr>
<tr>
<td>150</td>
<td>5.9</td>
<td>7.0</td>
<td>7.2</td>
<td>5.6</td>
</tr>
<tr>
<td>300</td>
<td>5.9</td>
<td>7.5</td>
<td>7.6</td>
<td>6.5</td>
</tr>
<tr>
<td>600</td>
<td>4.6</td>
<td>6.4</td>
<td>6.9</td>
<td>6.0</td>
</tr>
</tbody>
</table>
4.3.2. Determination of the cut-off value for the ELISA test

The mean and standard deviation of the normalised ELISA absorbance value of sera from pigs in group A (SD not observed) were 16.9 and 9.4, respectively. The cut-off value for the assay was determined to be 45.0 (mean + three standard deviations) for high stringency, and 35.7 (mean + two standard deviations) for moderate stringency.

4.3.3. Specificity and sensitivity of the ELISA test

Diagrammatic representations of the ELISA results for pigs in groups A and B are shown in Fig. 4.1 and 4.2, respectively. The mean ELISA values for the serum from the five healthy herds (group A) and six infected herds (group B) are shown in Tables 4.2 and 4.3. Using the criterion that any serum from a herd that exceeded the cut-off value indicated that the herd was infected, and using the two standard deviations cut-off, the test had zero specificity (all five negative herds scored positive) and a sensitivity of 100% (detecting all 6 positive herds correctly; group B). Using a cut-off value of three standard deviations above the mean, the ELISA test had a specificity of 100% (correctly identifying 5 of 5 negative herds; group A) and the specificity remained unchanged at 100% (detecting all 6 positive herds, group B). Since the ELISA test showed zero sensitivity using a cut-off value two standard deviations above the mean, use of this value was not continued for the remainder of the study.

4.3.4. Application of the Bhlp29.7-ELISA to field samples

Four of the eight (50%) herds in the farms where SD was suspected (group C) were determined to be diseased according to the test. Prevalence within these four confirmed diseased herds ranged from 2.5% (1 of 40 samples) to 15% (6 of 40 samples), with a mean of 8.75%. The remaining four herds in this group did not contain any individuals with ELISA values above the three standard deviation cut-off criterion (Table 4.4). The actual disease status of these four herds was unclear, as there was no laboratory evidence to show that SD was present on these farms. The consulting veterinarians had only suspected that it would be present.
Figure 4.1. Serological reactivity of pigs (n=464) from five herds where SD was not present (group A). Immunological response towards recombinant His$_r$-Bhlp29.7 was determined by ELISA. The different symbols correspond to different pig herds: -●- herd A1 and -○- herd A2, -▼- herd A3, -▽- herd A4, and -■- herd A5. The dotted line indicates the cut-off value ($P<0.05$) for the immunoassay, two standard deviations above the mean. The dashed line indicates the cut-off value ($P<0.01$) three standard deviations above the mean.
Figure 4.2. Serological reactivity of pigs (n=337) from six herds where SD was present (group B) and confirmed by laboratory-based methods. Immunological response towards recombinant His<sub>6</sub>-Bhlp29.7 was determined by ELISA. The different symbols correspond to different pig herds (n=2): - ▨ herd B1 and - □ herd B2, - ▼ herd B3, - ▽ herd B4, and - ▣ herd B5, - △ herd B6. The dotted line indicates the cut-off value (P<0.05) for the immunoassay, two standard deviations above the mean. The dashed line indicates the cut-off value (P<0.01) three standard deviations above the mean. Both cut-off values were set using sera from uninfected farms (Fig. 4.1).
Table 4.2. Summary of normalised ELISA absorbance values for the detection of specific antibody against recombinant His<sub>6</sub>-Bhlp29.7 in sera taken from five Australian pig herds in which SD was not present (group A). The two cut-off values were obtained from the means and standard deviations of the serum samples from this group. Qld, Queensland; Vic, Victoria; WA, Western Australia.

<table>
<thead>
<tr>
<th>Herd</th>
<th>Origin</th>
<th>No. of samples</th>
<th>Mean ± standard deviation</th>
<th>Seropositive pigs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cut-off 1*</td>
</tr>
<tr>
<td>A1</td>
<td>WA</td>
<td>132</td>
<td>13.5 ± 9.4</td>
<td>4 (3.0)</td>
</tr>
<tr>
<td>A2</td>
<td>Vic</td>
<td>236</td>
<td>18.0 ± 8.7</td>
<td>4 (1.7)</td>
</tr>
<tr>
<td>A3</td>
<td>WA</td>
<td>55</td>
<td>12.6 ± 6.9</td>
<td>1 (1.8)</td>
</tr>
<tr>
<td>A4</td>
<td>Vic</td>
<td>20</td>
<td>24.5 ± 5.3</td>
<td>1 (5.0)</td>
</tr>
<tr>
<td>A5</td>
<td>Vic</td>
<td>21</td>
<td>29.9 ± 5.7</td>
<td>1 (4.8)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>464</td>
<td>16.9 ± 9.4</td>
<td>11 (2.4)</td>
</tr>
</tbody>
</table>

* mean plus two standard deviations
** mean plus three standard deviations
Table 4.3. Summary of normalised ELISA absorbance values for the detection of specific antibody against recombinant His<sub>6</sub>-Bhlp29.7 in sera taken from six Australian pig herds which were confirmed to have SD present (group B). The two cut-off values were obtained from the mean and standard deviation of the serum samples obtained for five uninfected herds (group A). Qld, Queensland; Vic, Victoria.

<table>
<thead>
<tr>
<th>Herd</th>
<th>Origin</th>
<th>No. of samples</th>
<th>Mean ± standard deviation</th>
<th>Seropositive pigs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cut-off 1*</td>
</tr>
<tr>
<td>B1</td>
<td>Vic</td>
<td>140</td>
<td>24.6 ± 13.2</td>
<td>22 (15.7)</td>
</tr>
<tr>
<td>B2</td>
<td>Vic</td>
<td>38</td>
<td>32.5 ± 11.1</td>
<td>11 (28.9)</td>
</tr>
<tr>
<td>B3</td>
<td>Qld</td>
<td>39</td>
<td>31.1 ± 6.7</td>
<td>8 (20.5)</td>
</tr>
<tr>
<td>B4</td>
<td>Qld</td>
<td>40</td>
<td>29.9 ± .5</td>
<td>9 (22.5)</td>
</tr>
<tr>
<td>B5</td>
<td>Qld</td>
<td>40</td>
<td>34.4 ± 5.8</td>
<td>17 (42.5)</td>
</tr>
<tr>
<td>B6</td>
<td>Qld</td>
<td>40</td>
<td>17.7 ± 8.8</td>
<td>3 (7.5)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>337</td>
<td>27.2 ± 11.6</td>
<td>70 (20.8)</td>
</tr>
</tbody>
</table>

* mean plus two standard deviations

** mean plus three standard deviations
Table 4.4. Summary of normalised ELISA absorbance values for the detection of specific antibody against recombinant His<sub>6</sub>-Bhlp29.7 in sera taken from eight pig herds obtained from different farms in Queensland, Australia (group C). All herds were suspected to have SD, but had not been confirmed positive by laboratory methods.

<table>
<thead>
<tr>
<th>Herd</th>
<th>No. of samples</th>
<th>Mean ± standard deviation</th>
<th>Seropositive pigs* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>40</td>
<td>33.2 ± 11.9</td>
<td>6 (15.0)</td>
</tr>
<tr>
<td>C2</td>
<td>40</td>
<td>30.6 ± 8.9</td>
<td>4 (10.0)</td>
</tr>
<tr>
<td>C3</td>
<td>40</td>
<td>24.2 ± 6.3</td>
<td>0 (0)</td>
</tr>
<tr>
<td>C4</td>
<td>40</td>
<td>18.9 ± 9.4</td>
<td>0 (0)</td>
</tr>
<tr>
<td>C5</td>
<td>40</td>
<td>27.9 ± 7.3</td>
<td>3 (7.5)</td>
</tr>
<tr>
<td>C6</td>
<td>40</td>
<td>32.5 ± 6.6</td>
<td>0 (0)</td>
</tr>
<tr>
<td>C7</td>
<td>40</td>
<td>20.4 ± 6.9</td>
<td>0 (0)</td>
</tr>
<tr>
<td>C8</td>
<td>40</td>
<td>14.3 ± 5.5</td>
<td>1 (2.5)</td>
</tr>
<tr>
<td>Total</td>
<td>320</td>
<td>25.3 ± 10.3</td>
<td>17 (4.7)</td>
</tr>
</tbody>
</table>
4.3.5. Application of the Bhlp29.7-ELISA to samples from group D

The ELISA result of all 25 pigs from group D (acute SD observed) did not exceed the mean plus two standard deviations cut-off value or the mean plus three standard deviations value (Fig. 4.3). The mean and standard deviation of the samples in this group were 18.8 and 5.5, respectively.

![Normalized ELISA Absorbance](image)

**Figure 4.3.** Serological reactivity of 25 pigs from the herd where acute SD was observed (group D). Immunological response towards recombinant His<sub>6</sub>-Bhlp29.7 was determined by ELISA. The dashed line indicates the cut-off value ($p<0.01$) for the immunoassay and the dotted line indicates the cut-off value ($P<0.01$) three standard deviations above the mean. Both cut-off values were set using sera from uninfected farms (Fig. 4.1).

4.3.6. Analysis of Bhlp29.7 expression in *B. hyodysenteriae* isolates from group D

PCR and sequencing of the three *B. hyodysenteriae* isolates cultured from faeces obtained from the same farm which housed group D indicated that the Bhlp29.7 gene was present in all
three isolates and that they shared 97.4 to 99.2% homology to the protein in the WA1 strain of
*B. hyodysenteriae*.

Western blotting of whole-cell extracts prepared from the three isolates showed strong
reactivity with the Mab BJL/SH1 (Fig. 4.4). When 14 serum samples from group D were used
in a Western blot against the recombinant Bhlp29.7 antigen, weak or no reactivity was seen
(Fig. 4.5). This was compared to the strong reactivity exhibited by serum taken from a pig
hyper-immunised with a bacterin prepared with *B. hyodysenteriae* strain B78<sup>T</sup>, and by six
serum samples taken from herd B1 (chapter 3, Fig. 3.6).

**Figure 4.4.** Western blot reactivity of three *B. hyodysenteriae* isolates with Mab
BJL/SH1. The isolates were cultured from faeces obtained from pigs in group D
(acute SD observed). Lane 1, isolate 1; lane 2, isolate 2; lane 3, isolate 3. The arrow
indicates the position of the Bhlp29.7 protein.
Figure 4.5. Reactivity of pig serum from group D (acute SD observed) against the recombinant His$_6$-Bhlp29.7 protein that was used as plate-coating antigen for the Bhlp29.7-ELISA. Serum from a pig hyper-immunised with a bacterin preparation of *B. hyodysenteriae* strain B78$^T$ was used as a positive control. Lane 1, hyper-immunised pig serum raised against *B. hyodysenteriae* strain B78$^T$; lane 2-15, individual serum samples of 14 pigs from group D. The arrow indicates the position of the His$_6$-Bhlp29.7 protein.
4.4. DISCUSSION

Since its first description in 1971 (Engvall and Perlman, 1971), ELISA has become the system of choice when assaying soluble antigens and antibodies. Factors which have contributed to the success of the method include high sensitivity, long shelf-life of the reagents, ease of reagent preparation, and the speed and reproducibility of the assay.

In this chapter, an indirect ELISA test was established and standardised for the detection of antibodies to Bhlp29.7, an outer membrane lipoprotein of *B. hyodysenteriae*. This lipoprotein is believed to be surface exposed and immunogenic in infected pigs. In order to evaluate the usefulness of the ELISA test, sera from herds of pigs observed to be SD-free (group A) were used to determine a cut-off value for the assay. The cut-off value was then applied to sera from herds which had been observed to have SD (group B) to determine the sensitivity and specificity of the test. The ELISA then was used on “test” sera from herds in which diarrhoea had been observed, but had not been confirmed to be SD by laboratory methods (group C). Finally the ELISA was used on sera from younger pigs with acute SD at the time of sampling (group D).

4.4.1. Sensitivity and specificity of the ELISA

The criterion used for determining the disease status of the different herds according to the current ELISA was determined statistically using the mean and distribution of the serum reactivity of pigs from the healthy farms (group A). The actual cut-off value that was selected for use can be easily manipulated to either increase the sensitivity of the assay at the expense of the specificity, or vice-versa. In this study, two stringency levels were applied in establishing the cut-off value – one value which was two standard deviations above the mean of the healthy herds, and another which was three standard deviations above the mean. When the lower stringency cut-off value was applied to the healthy and diseased herds, the test lacked any sensitivity and so this clearly was not appropriate. False-positive diagnosis would
lead to the implementation of costly antibiotic treatment of an entire herd, or implementation of other control or quarantine measures.

When the higher stringency cut-off value was applied, all samples from the five healthy herds scored below the cut-off values, indicating the test was 100% sensitive. Relatively large numbers of sera were used in this step (464), which increased confidence in the results. Subsequently, all six diseased herds (group B) were successfully confirmed by the ELISA as being infected (100% specificity), although 94.4% of the animals (318 out of 337) had ELISA values below the cut-off value. Pigs in these herds were of slaughter age (22 weeks) and some had suffered clinical SD approximately four to six weeks prior to sampling. In a study using LPS as the plate-coating antigen for ELISA, it was reported that within infected herds, only a minority of individuals have antibody levels exceeding the cut-off value at a given time of sampling (Mhoma et al., 1992). Since only 19 of the 337 (5.6%) serum samples tested gave an ELISA result above the cut-off value, and a herd is considered infected when one sample returns a positive ELISA result, the number of samples assayed per herd is critical to the test. A sample size of 40 was chosen for testing of herds with unconfirmed health status, since this would give a confidence level of approximately 95% of identifying SD in an infected herd. In choosing the sample size, a within-herd prevalence of 10% was assumed in accordance with a previous Australian serological study (Mhoma, 1992). Based on the current ELISA test using the serum samples for group C, the within-herd serological prevalence of the infected herds was found to be between 2.5 (1 out of 40 pigs) and 15% (6 out of 40 pigs). Although the true prevalence would need to be confirmed by culture and PCR on individual pigs, it nevertheless indicates that a sample size of 40 would be appropriate for the ELISA test.

4.4.2. Application of the ELISA to samples from herds with uncertain health status

The use of the ELISA test on serum samples from the eight herds with a history of diarrhoea but which had not been confirmed to have SD indicated that, according to the criterion of the test, 50% (4 out of 8 herds) were seropositive. Within these herds, the seroprevalence ranged
from 2.5% (1 out of 40 samples) to 15% (6 out of 40 samples). When SD first enters a herd, the pigs are usually exposed to only low infectious doses of *B. hyodysenteriae* and, as a consequence, the disease is generally observed in only a few pigs. As infectious faeces accumulate, SD spreads to other animals in the exposed herd. The rate of its spread within the herd depends on the level of contact, and may take several weeks (Hampson *et al*., 1997). Since the ELISA test was intended for detecting SD at the herd level and not at the individual animal level, all samples did not necessarily need to be seropositive for the herd to be diagnosed as diseased. During the standardisation of the ELISA test, a within-herd prevalence of 10% was assumed, hence with 40 serum samples tested for each herd, it would be expected that up to four samples would be seropositive. Consistent with this, for the herds in which SD was present (group B), 2.5% (1 out of 40 samples) to 10.5% (4 out of 38 samples) of the animals tested were seropositive.

Infection with *B. hyodysenteriae* induces a systemic immune response which can last up to 17 weeks after the initial exposure (Fisher and Olander, 1981; Fernie *et al*., 1983; Joens *et al*., 1981b). For many of the herds in which SD had been observed (group B) or was suspected (group C), there was a high standard deviation in the ELISA test values (Table 4.3 and 4.4). Since the pigs in these herds were of a similar age, and SD had been observed four to six weeks prior to collection of serum, the scatter seen in the ELISA values for a herd suggests that some pigs had been more recently exposed to the spirochaete than others. If this was the case, investigation of different antibody subclasses, such as IgM, which is induced during the primary response following exposure, may provide more information.

4.4.3. Antibody response in acutely affected pigs

When the ELISA was applied to the samples obtained from pigs suffering acute disease, the ELISA values did not exceed the cut-off value, despite the observation of clinical SD in these pigs at the time of sampling. When a subset of these samples was tested in a Western blot using the plate-coating antigen, little or no reactivity was seen. In contrast, serum samples
from another infected herd (herd B1) reacted strongly in a Western blot using the same antigen. This indicated that the lack of reactivity seen in this herd was due to serum-specificity and not due to problems with the ELISA test. The lack of systemic response may have been because these acutely affected pigs had insufficient time to mount an antibody response. It is also possible, although not likely, that the younger age of these pigs meant that they were less able to mount an immune response. Had these animals been sampled after a few weeks, they may have become seropositive – although seroconversion has been detected in the pigs as early as four to seven days after the onset of clinical SD (Joens et al., 1985). This seroconversion was directed towards the whole cell of *B. hyodysenteriae*, which also includes cellular components and LPS. The current ELISA only detects antibodies against Bhlp29.7 and it is possible that the antibody response towards Bhlp29.7 occurs some time after the appearance of clinical signs of SD. As will be seen in the following chapter (chapter 5), experimentally infected pigs with acute signs of disease also do not have significant antibody titres towards Bhlp29.7, compared to the good local antibody titres seen in the colon. Nevertheless, on the basis of these results, it is recommended that the ELISA test only be used on pigs of slaughter age – since it has been standardised for these animals. More work is needed to investigate the dynamics of the systemic immune response to Bhlp29.7 before the test can be applied to serum samples collected in acutely affected pigs, or in animals tested before slaughter age.

To further investigate the lack of ELISA reactivity in the pigs of group D, isolates of *B. hyodysenteriae* from this farm were examined. They were shown to possess the Bhlp29.7 gene, and it was expressed *in vitro*, hence there was nothing unusual about the strain of *B. hyodysenteriae* present on the farm. Cullen *et al.* (2003) looked at the genome sequence surrounding Bhlp29.7 and found that the *bhlp29.7* locus (referred to as *blpGFEA*) contained a number of paralogous genes which were not expressed simultaneously. In their work, they further substantiated that Bhlp29.7 (referred to by them as BlpA) was the gene member which was expressed in the strain they studied. It is possible that in different *B. hyodysenteriae*
isolates, different members of the Bhlp29.7 gene set are expressed under different environmental conditions, as may be the case for the \textit{B. hyodysenteriae} variable surface exposed protein Bhmp39 (Witchell \textit{et al.}, 2006). In the current work, the presence of the Bhlp29.7 gene in a large number of \textit{B. hyodysenteriae} strains has been examined, but the expression of Bhlp29.7 in these strains has not been studied. Future work is required to analyse \textit{in vitro} and \textit{in vivo} expression of Bhlp29.7, and the other paralogous genes, in different \textit{B. hyodysenteriae} strains.

4.4.4. False-positives

Early ELISA tests developed for SD using crude whole cell preparations of \textit{B. hyodysenteriae} were hampered by false-positive readings associated with the cross-reactivity of antibodies produced against intestinal spirochaetes other than \textit{B. hyodysenteriae}. The use of a recombinant protein, purified by affinity chromatography, as the coating antigen in the current ELISA test reduces the possibility of cross-reactivity by providing only one \textit{B. hyodysenteriae} protein for antibodies to interact with. In this ELISA test, the wide distribution of ELISA values (indicated by the standard deviations from the means) seen for the healthy herds (group A), suggest that cross-reactivities probably still occurred. These cross-reactivities could have been due to \textit{E. coli} proteins which co-purified with the His\textsubscript{6}-Bhlp29.7 and were subsequently coated onto the ELISA plate. Another source of cross-reactivity may have been with the six histidine fusion present at the N-terminal of the recombinant Bhlp29.7 protein, however this was unlikely since histidine-tags show low immunogenicity (Coligan \textit{et al.}, 1995). Other sources of cross-reactivity could reside with the Bhlp29.7 protein itself. It was shown in chapter 2 that Bhlp29.7 probably belongs to the family of methionine substrate-binding proteins (MetQ), which are found in a wide range of Gram-negative bacteria. As such, there are possibly epitopes present on Bhlp29.7 which would be similar to those of MetQ proteins in other bacteria. Exposure of pigs to these bacteria could induce antibody responses which would result in cross-reactivity with His\textsubscript{6}-Bhlp29.7. Another possible source of cross-reactivity would be due to exposure of the pigs to certain strains of \textit{B. innocens}, a common commensal
species colonising the pig colon (Stanton et al., 1991). In chapter 3, PCR and sequencing of different strains of *Brachyspira* species revealed that the Bhlp29.7 gene was present in *B. innocens* strain B256T. This gene was nearly identical to that of all the *B. hyodysenteriae* strains tested. Although in this study Bhlp29.7 was only found in one of the four *B. innocens* strains tested, it is possible that other *B. innocens* strains may possess the gene, and hence be a source of cross-reactivity if this is expressed.

Despite the possibility of cross-reactivities associated with the His6-Bhlp29.7 antigen, the standardised ELISA test showed a good sensitivity and specificity at the herd level. Reducing the likelihood of cross-reactivities, and hence false-positives, may enable a better discrimination between positive and negative samples, and especially samples with titres close to the cut-off value.

### 4.4.5. Practical limitations

The major limitation in the development of this ELISA test was the practical problems associated with obtaining a reliable well-defined set of sera from pigs/herds with confirmed health status. The definitive diagnosis of SD in herds requires a combination of clinical observation and microbiological culture and PCR of *B. hyodysenteriae* from a large number of clinical samples. For most of the herds in this study, the health status was determined from the clinical history of the herd, and the sera from these herds were collected from slaughter-aged animals at the abattoir. Although this method of collection does not provide a truly random representation of the pigs in a herd, and misidentification of herds or individuals is possible, it was far less difficult than collecting blood directly from the pigs – especially since most of the farms in this study were located in a different State. Additionally, obtaining diagnostic samples for follow-up studies on certain herds (such as those in groups C and D) was virtually impossible due to the herds having been depopulated or sold, or the consulting veterinarian having relocated. Moreover, issues concerning confidentiality also hindered the communication of information. Besides the limitations associated with sample collection,
estimation of the prevalence of SD in the herds being tested may be problematic. Prevalence studies are labour intensive and require the co-ordination of a large number of professionals. Due to this, prevalence studies are infrequently performed, even though the internal prevalence is an important parameter required to establish the necessary number of test samples. All these practical problems were encountered in this study, and made a definitive evaluation of the ELISA test difficult.

4.4.5. Future improvements

It remains unclear why the pigs in group D did not show seroconversion, even though clinical SD was observed at the time of serum collection and *B. hyodysenteriae* was present on the farm. An obvious difference between the pigs in this herd and the pigs in the remaining herds was the age of the pigs and the acuteness of the clinical signs. Further investigation may be necessary to ascertain whether parameters such as age and disease progression will affect the effectiveness of the ELISA test. In addition, it is not known whether Bhlp29.7 is expressed *in vivo* in all strains of *B. hyodysenteriae*. Although the presence of Bhlp29.7-specific antibody titres produced in response to *B. hyodysenteriae* exposure has been confirmed in a number of Australian herds, it is still unclear whether this response is universal for all strains/serogroups. Application of the ELISA test to serum samples collected from a larger number of herds of known disease status in the major pig producing countries would be desirable, however this may be difficult given the current state of international biosecurity.

It is possible that the use of the entire Bhlp29.7 protein as the ELISA antigen may lead to cross-reactivities which reduce the capacity of the assay to discriminate between seropositive and seronegative samples. The *in silico* analyses revealed that the most antigenic epitopes of the Bhlp29.7 protein lie at the C-terminal portion (Chapter 2, section 2.3.3.2). It is also within this C-terminal region that the epitope for the *B. hyodysenteriae*-specific monoclonal antibody (BJL/SH1) is found. Knowing this, it may be possible to use the monoclonal antibody as a blocking agent in a competitive ELISA test. In such an assay, the Bhlp29.7 antigen is bound to
the microtitre plate and the test serum allowed to bind. The HRP-labelled monoclonal antibody is then applied and allowed to bind and an ELISA reading is obtained after washing away all unbound material after. The ELISA reading for the blocked antigen is compared with that for the unblocked antigen. A sample with a lower reading for the blocked antigen compared to the unblocked antigen indicates a seropositive sample. Unfortunately the hybridoma cell-line producing the monoclonal antibody BJL/SH1 has expired, and the remaining BJL/SH1 stocks were insufficient to evaluate this modification. It would be useful to raise new hybridomas expressing monoclonal antibodies reacting with the C-terminal of Bhlp29.7, and to test these in blocking ELISAs.

Another alternative method of utilising the C-terminal region of Bhlp29.7 would be to use this polypeptide as the ELISA coating antigen. A polypeptide (Bhlp29.7-F604) containing residues 202 to 271 of the 271 amino acid Bhlp29.7 protein has been cloned and expressed in *E. coli* (chapter 3). Due to the possible hydrophilicity and small size of Bhlp29.7-F604, expression levels of this protein in *E. coli* were extremely low, making it difficult to generate sufficient protein for use in the ELISA test. The Bhlp29.7-F604 gene region was subsequently cloned and expressed as a MBP fusion-protein (MBP-Bhlp29.7-F604) for vaccination of pigs (chapter 5). The MBP fusion-tag allows the Bhlp29.7-F604 polypeptide to be expressed abundantly, however the MBP component is highly immunogenic in pigs. Cleavage and purification of the MBP fusion would be necessary to further explore the possibility of using the Bhlp29.7-F604 protein as the basis for an alternative ELISA test.

4.4.6. Conclusions

Overall, the ELISA that was developed and standardised appears useful as an indirect test for determining exposure of herds to *B. hyodysenteriae*. It does not appear to be particularly useful at the individual pig level, but this was not properly investigated in this study since, in nearly all cases, the disease history of individual animals was not recorded. The timing of sample collection appeared to be important, and it is recommended that the ELISA should be used for
slaughter-age pigs to avoid possible false-negative results. Although it may require follow-up culture and PCR to confirm the test result, the ELISA still provides another convenient method of diagnosis other than directly isolating the causative agent. The ELISA test was 100% specific and 100% sensitive at the herd level – although it was recognised that relatively few herds were tested. In a previous assessment of an ELISA test for Australian herds using LPS as the plate-coating antigen, a specificity of 81.8% and a sensitivity of 77.3% were achieved (Mhoma et al., 1992). Compared to this LPS-ELISA, the current ELISA shows both better specificity and sensitivity. Furthermore, in the event that a subunit vaccine using recombinant His$_6$-Bhlp29.7 becomes available (Chapter 5), this ELISA test should prove useful for monitoring the efficacy of vaccination during its development and application in the field.
CHAPTER 5: EVALUATION OF IMMUNISATION WITH
RECOMBINANT BHLP29.7 FOR PROTECTION AGAINST
BRACHYSPIRA HYODYSENTERIAE COLONISATION
IN MICE AND PIGS

5.1. INTRODUCTION
In infected herds SD can be controlled using antimicrobials, although strains of *Brachyspira hyodysenteriae* with reduced antimicrobial susceptibility are increasingly being encountered (Karlsson *et al.*, 2002; Karlsson *et al.*, 2003; Karlsson *et al.*, 2004). Pigs with SD show a strong circulating antibody response to *B. hyodysenteriae*, and a proportion of recovered animals are protected from re-infection (Olson, 1974; Joens *et al.*, 1979). Past attempts to develop bacterin and live vaccines to control SD have met with limited success (reviewed in chapter 1). The use of recombinant subunit vaccines for the control of SD is highly appealing, since the products would be well-defined (essential for registration purposes), and relatively easy to produce on a large scale. Several attempts have been made to identify outer envelope proteins from *B. hyodysenteriae* that could be used as vaccine components (reviewed in chapter 1), but to date attempts to use recombinant proteins from *B. hyodysenteriae* as vaccine subunits have failed to prevent colonisation in pigs or mice (Gabe *et al.*, 1995; Davis *et al.*, 2005).

Earlier chapters have described the identification and recombinant production of the 29.7 kDa outer membrane lipoprotein of *B. hyodysenteriae*, designated Bhlp29.7. Mice and pigs immunised with an *E. coli* clone expressing recombinant Bhlp29.7 generated antibodies recognising the native lipoprotein of *B. hyodysenteriae*, and pigs naturally infected with *B. hyodysenteriae* also recognise both recombinant and native Bhlp29.7.
This chapter contains two sections. The first section primarily evaluates the immunogenicity of recombinant histidine-tagged Bhlp29.7 in mice. To achieve this, a recombinant lipitated histidine-tagged homologue of Bhlp29.7 was cloned and expressed in \textit{E. coli}. The lipilated and non-lipilated recombinant histidine-tagged Bhlp29.7 were used to immunise mice and the effect of the lipidation on immunogenicity was evaluated. The more immunogenic recombinant Bhlp29.7 homologue was subsequently evaluated for its ability to provide protection against \textit{B. hyodysenteriae} colonisation in experimentally challenged mice.

The second section of this chapter describes production in \textit{E. coli} of recombinant histidine-tagged Bhlp29.7 and an approximately 8 kDa polypeptide from the carboxy-terminal end of Bhlp29.7, fused to maltose binding protein, and designated MBP-Bhlp29.7-F604, and the use of these purified proteins to vaccinate pigs. The carboxy-terminal portion of Bhlp29.7 was chosen for use as a vaccine component since computer-assisted immunogenicity analysis of the Bhlp29.7 amino acid sequence indicated that the most antigenic portion of the lipoprotein was at the carboxy-terminal end (chapter 2). Hence it was considered that it would be useful to compare protection achieved with the carboxy-terminal end and the whole molecule.

The aim of the studies in this chapter was to investigate whether immunisation with these recombinant Bhlp29.7 proteins would protect mice and pigs from SD following experimental challenge with \textit{B. hyodysenteriae}. 
5.2. MATERIALS AND METHODS

5.2.1. Preparation of recombinant His$_6$-Bhlp29.7

Recombinant histidine-tagged Bhlp29.7 was expressed and purified using the methods outlined in chapter 3. Purified, dialysed and concentrated His$_6$-Bhlp29.7 protein (2 mg/ml) was prepared as described in chapter 3 (sections 3.2.9-3.2.11) and stored at -20°C.

5.2.2. Preparation of recombinant lipidated His$_6$-Bhlp29.7

5.2.2.1. Construction of the pLIP-Bhlp29.7 expression vector

The lipidation cassette containing the conserved *E. coli* lipoprotein signal peptidase recognition site [L, V, I][A, S, T, G][G, A]C (von Heijne, 1989) followed by a six histidine motif was constructed and used to replace the six histidine expression cassette of the pTrc-Bhlp29.7. The lipidation cassette was constructed by directional ligation of three synthetic linkers (lpp1-3). The lpp-1 linker was generated with oligonucleotides lpp-1A and lpp-1B, the lpp-2 linker was generated with oligonucleotides lpp-2A and lpp-2B, and the lpp-3 linker was generated with oligonucleotides lpp-3A and lpp-3B (Table 5.1). All the linkers were individually generated by combining 50 μM of each oligonucleotide in a 0.2 ml PCR tube and heating the tube to 96°C for 5 min in a thermocycler. The tubes were removed from the thermocycler and cooled slowly at RT to allow the oligonucleotides to anneal. The three linkers were combined with 5 U of T4 DNA ligase (Promega) in 30 mM Tris-HCl (pH 7.8), 10 mM MgCl$_2$, 10 mM DTT and 1 mM ATP. Ligation was allowed to occur at 14°C for 16 h. Following ligation, the resulting DNA fragment was purified using the UltraClean PCR Clean-up kit as described in chapter 2, section 2.2.12.2.

Cloning of the lipidation cassette into pTrc-Bhlp29.7 was carried out in a similar manner to that described for the initial construction of pTrc-Bhlp29.7 (chapter 3, sections 3.2.4-3.2.7). The pTrc-Bhlp29.7 plasmid was restricted with 1 U of *NcoI* (Promega) and 1 U of *BamHI* (Promega) in 6 mM Tris-HCl (pH 7.5), 100 mM NaCl, 6 mM MgCl$_2$, 1 mM DTT and 100 μg/ml BSA at 37°C overnight. The restricted plasmid was purified using the UltraClean PCR
Clean-up kit. Since the DNA fragment comprising the lipidation cassette was designed with terminal \textit{NcoI}- and \textit{BamHI}-compatible cohesive ends, equimolar amounts of this DNA fragment and the linearised pTrc-Bhlp29.7 plasmid were ligated at 14°C for 16 h in 30 mM Tris-HCl (pH 7.8), 10 mM MgCl$_2$, 10 mM DTT and 1 mM ATP containing 1 U of T4 DNA ligase (Promega). The ligation products were transformed into chemically competent \textit{E. coli} BL21 Star (DE3) pLys One Shot (Invitrogen) cells by heat-shock. Transformed cells were plated onto LB agar plates containing 100 mg/l ampicillin and incubated at 37°C for 16 h.

Table 5.1. Oligonucleotide sequences used to produce synthetic linkers for the generation of the lipidation cassette for expression in \textit{E. coli}.

<table>
<thead>
<tr>
<th>Oligonucleotide name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lpp-1A</td>
<td>CATGAAAGCTACTAAACTGGTACTGGGGCGCGGTAATCCTGGTTTCTACTCTGCTGG</td>
</tr>
<tr>
<td>lpp-1B</td>
<td>AGAGTGAGAAAAACCGATTACCACGGCCAGTACCAGTTTTAGCTAGCTTT</td>
</tr>
<tr>
<td>lpp-2A</td>
<td>CAGGTTGCTCCAGCAACCATCATCATCATCATATGGGTATGGCTAAGCATGACTGG</td>
</tr>
<tr>
<td>lpp-2B</td>
<td>CATGCTAGCCATACCATGATGATGATGATGATGTTGCTGGACACAGATGCCAGC</td>
</tr>
<tr>
<td>lpp-3A</td>
<td>TGGACAGCAAATGGGTGACGATGACGATAAGGATCGATGGGGATC</td>
</tr>
<tr>
<td>lpp-3B</td>
<td>CCCATCGATCCTATTATCGTCATCGTACCCCATTGGCTGCTCCACCAGT</td>
</tr>
</tbody>
</table>

Ten single transformant colonies were streaked onto fresh LB agar plates containing 100 mg/l ampicillin and incubated at 37°C for 16 h. A single colony from each transformation event was resuspended in 50 μl of TE buffer and boiled for 1 min. Two μl of boiled cells were used as template for PCR with the pTrcHis-F and pTrcHis-R primers. Two transformant clones which produced the correct sized PCR products were inoculated into 10 ml LB broth containing 100 mg/l ampicillin and incubated at 37°C for 12 h with shaking. The entire overnight cultures were centrifuged at 5,000 × g for 10 min and the plasmid contained in the cells extracted using
the QIAprep Spin Miniprep Kit as described previously (chapter 2, section 2.2.6). Both purified plasmids were subjected to automated direct sequencing of the cloned lipidation cassette using the pTrcHis-F primer. Successfully ligated plasmids were designated pLIP-Bhlp29.7.

5.2.2.2. Verification of His$_6$-Bhlp29.7 lipidation

A single colony of the pLIP-Bhlp29.7 construct in *E. coli* BL21 was inoculated into 10 ml LB broth containing 100 mg/l ampicillin and incubated at 37°C for 16 h with shaking. Fifty ml of LB broth in a 250 ml conical flask containing 100 mg/l ampicillin was inoculated with 1 ml of the overnight culture and incubated at 37°C until the optical density of the cells at 600 nm was 0.5 (approximately 3 h). The culture was spiked with 200 μCi of $^3$H] palmitic acid (Amersham Pharmacia Biotech) and IPTG was added to a final concentration of 1 mM. The cells were returned to 37°C with shaking for 6 h. The cells were harvested by centrifugation at 5,000 × g for 10 min before resuspending the pellet with 1 ml of Ni-NTA denaturing lysis buffer. The cell lysate was incubated at -20°C overnight. Cell debris was clarified from the thawed lysate by centrifuging at 20,000 × g for 15 min at 4°C. SDS-PAGE (chapter 2, section 2.2.4) was used to separate 15μl of the purified lipidated His$_6$-Bhlp29.7 in two duplicate gels. One polyacrylamide gel containing the separated proteins was stained with Coomassie Brilliant Blue G250 (chapter 3, section 3.2.10). The other polyacrylamide gel was equilibrated in Amplify solution (Amersham Pharmacia Biotech) for 15 min before the gel was placed into a plastic bag and heat sealed. An autoradiograph was obtained by incubating the sealed gel with pre-flashed Hyperfilm-MP (Amersham Pharmacia Biotech) in a film cassette at −80°C for 14 days. The autoradiograph was developed and compared with the stained gel to identify corresponding bands. Both autoradiograph and stained gel were scanned for comparative presentation.
5.2.2.3. Expression and purification of recombinant lipidated His$_6$-Bhlp29.7

Recombinant lipidated His$_6$-Bhlp29.7 was expressed and purified using the methods outlined in chapter 3 (sections 3.2.9-3.2.10). Purified, dialysed and concentrated His$_6$-Bhlp29.7 protein (2 mg/ml) was prepared as described in chapter 3 (sections 3.2.11 and 3.2.13) and stored at -20°C.

5.2.3. Preparation of recombinant MBP-Bhlp29.7-F604

The pMAL-c2x Expression System (New England Biolabs) was chosen for the recombinant expression of the carboxy-terminal portion of Bhlp29.7 in *E. coli*, as the pTrcHis Expression System was unable to express proteins smaller than 12 kDa at high levels (Coligan *et al.*, 2002).

5.2.3.1. Cloning of the carboxyl-terminus of Bhlp29.7 for recombinant expression

Cloning of the carboxyl-terminus of Bhlp29.7 into the pMAL-c2x Expression System (New England Biolabs) was carried out in a similar manner to that described for pTrc-Bhlp29.7 (chapter 3, sections 3.2.4-3.2.5). The gene sequence encoding the carboxyl-terminus of the Bhlp29.7 polypeptide between amino acid residues 201 to 271 was amplified from *B. hyodysenteriae* strain WA1 (Western Australian field strain isolated from a pig) chromosomal DNA that was prepared in the work described in chapter 2 (section 2.2.1) using oligonucleotide primers Bhlp29.7-F604-BamH1 (5’-GTAGGATCCATATACTTCGGTTTGAATCCTG-3’) and Bhlp29.7-R809-HinDIII (5’-TATAAGCTTCAAGTAGGAAGATAAGAACC-3’). The pMAL-c2x vector and the amplified PCR product was restricted separately with 1 U of *BamH1* (Promega) and 1 U of *HinDIII* (Promega) in 6 mM Tris-HCl (pH 7.5), 100 mM NaCl, 6 mM MgCl$_2$, 1 mM DTT and 100 µg/ml BSA at 37°C overnight. Restricted DNA was purified and equimolar amounts of the linearised pMAL-c2x vector and the Bhlp29.7-F604 insert was ligated at 14°C for 16 h in 30 mM Tris-HCl (pH 7.8), 10 mM MgCl$_2$, 10 mM DTT and 1 mM ATP containing 1 U of T4 DNA ligase (Promega). Ligation products were transformed into chemically competent *E. coli* BL21 Star (DE3) pLys One Shot (Invitrogen)
cells by heat-shock. Transformed cells were plated onto LB agar plates containing 100 mg/l ampicillin and incubated at 37°C for 16 h.

5.2.3.2. Detection and verification of pMAL-Bhlp29.7-F604 transformants

Twelve single transformant colonies were streaked onto fresh LB agar plates containing 100 mg/l ampicillin and incubated at 37°C for 16 h. A single colony from each transformation event was resuspended in 50 μl of TE buffer and boiled for 1 min. Two μl of boiled cells were used as template for PCR. The amplification mixture consisted of 1× PCR buffer (containing 1.5 mM of MgCl₂), 1 U of Taq DNA polymerase, 0.2 mM of each dNTP (Promega), 0.5 μM of the pMAL-F primer (5’-GGTCGTCAGACTGTCGATGAAGCC-3’) and 0.5 μM of the pMAL-R primer (5’-CGCCAGGGTTTCCCAGTCACGAC-3’). Cycling conditions involved an initial template denaturation step of 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 15 sec, and a primer extension at 72°C for 30 s. The PCR products were subjected to electrophoresis in 1% (w/v) agarose gels in 1× TAE buffer, stained by emersion in 1 μg/ml ethidium bromide solution for 15 min and viewed over UV light.

Two transformant clones which produced the correct sized PCR products were inoculated into 10 ml LB broth containing 100 mg/l ampicillin and incubated at 37°C for 12 h with shaking. The entire overnight cultures were centrifuged at 5,000 × g for 10 min and the plasmid contained in the cells was extracted using the QIAprep Spin Miniprep Kit as described previously (chapter 2, section 2.2.6). The purified plasmid was quantified with a Dynaquant DNA fluorometer (Hoefer), using pUC18 plasmid (Promega) as the standard, and the DNA concentration adjusted to 100 μg/ml by dilution with TE buffer. Both purified plasmids were subjected to automated direct sequencing of the pMAL-c2x expression cassette using the pMAL-F and pMAL-R primers. Each sequencing reaction was performed in a 10 μl volume consisting of 200 ng of plasmid DNA, 2 pmol of primer, and 4 μl of the ABI PRISM Dye
Terminator Cycle Sequencing Ready Reaction Mix (PE Applied Biosystems). Cycling conditions involved a 2 min denaturing step at 96°C, followed by 25 cycles of denaturation at 96°C for 10 s, annealing at 55°C for 5 s and extension at 60°C for 4 min. Residual dye terminators were removed from the sequencing products by precipitation with 95% (v/v) ethanol containing 120 mM sodium acetate (pH 4.6), and vacuum dried. The plasmids were sequenced in duplicate with each primer, using an ABI 373A DNA Sequencer (PE Applied Biosystems). The successfully ligated plasmid was designated pMAL-Bhlp29.7-F604.

5.2.3.3. Expression and purification of recombinant MBP-Bhlp29.7-F604

A single colony of pMAL-Bhlp29.7-F604 in *E. coli* BL21 was inoculated into 50 ml LB broth in a 250 ml conical flask containing 100 mg/l ampicillin and incubated at 37°C for 16 h with shaking. A 2 litre conical flask containing 1 l of LB broth supplemented with 2 g/l glucose and 100 mg/l ampicillin was inoculated with 10 ml of the overnight culture and incubated at 37°C until the optical density of the cells at 600 nm was approximately 0.5. The culture was then induced by adding IPTG to a final concentration of 0.3 mM and the cells returned to 37°C with shaking. After 3 h of induction, the culture was transferred to 250 ml centrifuge bottles and the bottles were centrifuged at 5,000 \( \times g \) for 10 min at 4°C. The cell pellets were combined and resuspended with 50 ml of MBP column buffer. All subsequent protein purification was performed at 4°C. The resuspended cells were frozen in liquid nitrogen and immediately thawed in ice-water. The cell lysate was then sonicated on ice three times for 30 s with 1 min incubation on ice between sonication rounds. The lysed cells were cleared by centrifugation at 20,000 \( \times g \) for 15 min at 4°C and the supernatant diluted by adding 150 ml of MBP column buffer. A 100 ml syringe containing glass wool at the bottom was loaded with 15 ml of amylose resin (New England Biolabs) and equilibrated by washing with 400 ml of MBP column buffer. The diluted cell lysate was then applied to the amylose column in 100 ml amounts and the protein allowed to bind at a flow rate of approximately 2 ml/min. The column was washed 10 times with 100 ml of MBP column buffer before eluting with 200 ml of MBP
column buffer containing 10 mM maltose. Twenty 10 ml fractions of the eluate were collected and stored at 4°C. The samples were subjected to SDS-PAGE (chapter 2, section 2.2.4) and stained with Coomassie Brilliant Blue G250 (chapter 3, section 3.2.10). The fractions containing the purified MBP-Bhlp29.7-F604 protein were pooled, quantified, dialysed and lyophilised to dryness (chapter 2, section 3.2.11). The lyophilised protein was resuspended with PBS to a concentration of 2 mg/ml and stored at -20°C.

5.2.4. Preparation of spirochaete inoculum

A full description of spirochaete growth is provided in Chapter 2 (section 2.2.9). A pure culture of Australian B. hyodysenteriae strain B/Q02 was obtained as frozen stock from the culture collection held at the Reference Centre for Intestinal Spirochaetes, Murdoch University. The cells were propagated on a rocking platform at 37°C in Kunkle's pre-reduced anaerobic broth containing 2% (v/v) FBS and a 1% (v/v) ethanolic cholesterol solution. Once cultures reached approximately 10⁹ cells per ml (after approximately 3-5 days), as determined by counting in a haemocytometer chamber under a phase-contrast microscope, they were removed from 37°C and used immediately for oral challenge.

5.2.5. Animals and experimental protocols

Four animal experiments were conducted to evaluate the usefulness of recombinant His₆-Bhlp29.7 as a vaccine subunit for SD. Two mouse experiments were carried out, the first of which was performed to compare the immunogenicity of non-lipidated and lipidated forms of His₆-Bhlp29.7. The second mouse experiment evaluated the usefulness of His₆-Bhlp29.7 as a vaccine subunit for SD in mice. Two pig experiments were then conducted to evaluate the efficacy of vaccination with recombinant His₆-Bhlp29.7 and MBP-Bhlp29.7-F604 (C-terminal portion of Bhlp29.7) in pigs experimentally challenged with B. hyodysenteriae.

All animal experiments were performed with the permission of the Animal Ethics Committee of Murdoch University.
5.2.5.1. Experiment 1 – immunogenicity of lipidated His$_6$-Bhlp29.7 in mice

Twenty C3H/HeJARC weanling female mice (5 weeks of age) were purchased from a specific pathogen free (SPF) facility. The mice were randomly assigned to two groups of 10, numbered by punching the ears and blood taken from the tail vein. Each group of mice was housed in a separate cage kept in the same room of the animal house. The mice were fed *ad libidum* on a standard balanced mouse diet that did not contain antibiotics. Mice in group M1 were vaccinated with non-lipidated recombinant His$_6$-Bhlp29.7 and mice in group M2 were vaccinated with lipidated recombinant His$_6$-Bhlp29.7.

Both groups of mice initially received either 100 µg recombinant His$_6$-Bhlp29.7 or 100 µg recombinant lipidated His$_6$-Bhlp29.7 emulsified with VSA3 adjuvant (MVP Laboratories) in a 50 µl volume administered intramuscularly (im) into the quadriceps muscle of the right hind leg. Three weeks later, the mice received a similar im vaccination. Two weeks after the second vaccination, all mice were removed for post-mortem examination. Euthanasia was by gassing in methoxyfluorane followed by cervical dislocation. Blood was collected from the heart of each mouse and the serum was collected following overnight coagulation at 14°C.

5.2.5.2. Experiment 2 – His$_6$-Bhlp29.7 vaccination of mice

Twenty C3H/HeJARC weanling female mice of the same age and from the same source as those in experiment 1 were used. The mice were randomly assigned to two groups of 10 (groups M3 and M4), numbered by punching the ears and blood taken from the tail vein. The mice were fed *ad libidum* on a standard balanced mouse diet that did not contain antibiotics. Mice in group M3 acted as unvaccinated controls, whilst mice in group M4 were vaccinated with 100 µg recombinant His$_6$-Bhlp29.7 emulsified with VSA3 adjuvant (MVP Laboratories) in a 50 µl volume administered intramuscularly (im) into the quadriceps muscle of the right hind leg. Three weeks later, mice in group M4 received 100 µg recombinant His$_6$-Bhlp29.7 in PBS administered by gastric intubation. Ten days prior to challenge with *B. hyodysenteriae*, all mice were placed on a diet formulation which contained approximately 63% (w/w) glucose.
This diet composition has been shown to improve \textit{B. hyodysenteriae} colonisation in mice (Nibbelink and Wannemuehler 1992). Two weeks after the second vaccination, all mice in both groups were given 0.8 ml of exponential log-phase \textit{B. hyodysenteriae} strain B/Q02 culture (approximately $10^9$ cells/ml) by gastric intubation. Experimental challenge was repeated over three consecutive days using freshly cultured inoculum for each day. Faeces were collected from individual mice twice weekly and cultured for spirochaetes. All mice were removed for necropsy 13 days after the last day of experimental challenge. Euthanasia was as in experiment 1. The presence and nature of lesions in the caecum and large intestine were recorded, and swabs were taken from the wall of the caecum for spirochaete culture. Blood was collected from the heart of each mouse and the serum was collected following overnight coagulation at 14°C.

\textbf{5.2.5.3. Experiment 3 – His}_6\text{-Bhlp29.7 vaccination of pigs}

Thirty female pigs (Large White x Landrace x Duroc) which had just been weaned at 21 days of age were purchased from a commercial piggery that was regularly monitored to ensure it remained SD-free. The animals were weighed and ear-tagged, then randomly assigned to three groups of ten, with each group housed in a single pen with open wire-mesh partitions. The three pens were adjacent to each other, and were located in one room of an isolation animal house. The animals were fed \textit{ad libidum} on a commercial pelleted weaner diet that did not contain antibiotics (20% crude protein, 14.7% MJ/kg digestible energy, Wesfeeds Ltd). The animals were weighed weekly throughout the experiment. Group 1A acted as unvaccinated controls, whilst groups 1B and 1C were vaccinated with His$_6$-Bhlp29.7 by different routes.

Both vaccinated groups (1B and 1C) initially received 1 mg recombinant His$_6$-Bhlp29.7 emulsified in FIA (Sigma Chemical Company), in a 2 ml volume administered intramuscularly (im) into the neck muscles behind the left ear. Three weeks later, pigs in group 1B received a solution containing 1 mg recombinant His$_6$-Bhlp29.7 (without adjuvant) in 10 ml PBS by gastric intubation, whilst pigs in group 1C received their second vaccination with adjuvant im
behind the right ear. Two weeks after the second vaccination, all 30 pigs were given 50 ml of exponential log-phase \textit{B. hyodysenteriae} strain B/Q02 culture (approximately $10^9$ cells/ml) by gastric intubation. Experimental challenge was repeated over five consecutive days using freshly cultured inoculum for each day. Serum was obtained by bleeding from the anterior vena cava prior to the first vaccination (“pre-bleed”), just prior to the second vaccination (“pre-boost”), prior to the first day of challenge (“pre-infection”), and at post-mortem. Rectal swabs for spirochaete culture were taken from all pigs at arrival to the facility, just prior to inoculation, and thereafter three times per week. The pigs were examined daily, and were removed for post-mortem examination within 24 h of diarrhoea containing mucus and/or fresh blood being observed. Such pigs were recorded as having clinical signs of SD. All other pigs were removed for necropsy between 20 and 23 days after the last day of experimental challenge. Euthanasia was by captive bolt stunning, followed by exsanguination. The presence, distribution and nature of gross lesions in the large intestine were recorded, and swabs were taken from the wall of the proximal colon for spirochaete culture. Ten centimetre long sections of the proximal colon were collected and the mucosa scraped off as described in section 5.2.7.2. This was subsequently used to test for specific immunoglobulin content by ELISA and Western blot analysis. Sections of the colon also were removed and fixed in 10% formalin for subsequent histological examination.

\textbf{5.2.5.4. Experiment 4 – MBP-Bhlp29.7-F604 vaccination of pigs}

Thirty six female weaner pigs of the same age and from the same source as in experiment 4 were used. They were randomly assigned to three groups of twelve. Group 2A acted as unvaccinated controls. Group 2B received 1 mg recombinant His$_6$-Bhlp29.7 emulsified with VSA3 adjuvant (MVP Laboratories) administered im in a 2ml volume, followed 3 weeks later by 1 mg recombinant His$_6$-Bhlp29.7 in 10 ml PBS by gastric intubation. Group 2C received 1 mg recombinant MBP-Bhlp29.7-F604 with VSA3 adjuvant im, followed 3 weeks later by 1 mg recombinant MBP-Bhlp29.7-F604 in 10 ml PBS by gastric intubation. The control pigs in group 2A were housed in a pen in a separate room from the two pens of vaccinated pigs.
(groups 2B and 2C). The pigs were observed and sampled exactly as in experiment 1, except that animals that did not develop disease were not removed for post-mortem examination until 51 days after experimental challenge. The same strain of *B. hyodysenteriae* was used for oral challenge.

5.2.6. Microbiological analysis

5.2.6.1. Culture of faecal swabs

Bacteriology swabs taken from rectal faeces or the colonic walls of experimentally infected mice and pigs were streaked onto selective agar plates (Jenkinson and Wingar, 1981), consisting of TSA (Becton Dickinson Microbiology Systems) containing 5% (v/v) defibrinated sheep blood, 400 μg/ml spectinomycin and 25 μg/ml each of colistin and vancomycin (Sigma Chemical Company). The plates were incubated for 5 to 7 days at 37°C in an anaerobic environment of 94% H₂ and 6% CO₂, generated using anaerobic Gaspak plus sachets (Becton Dickinson Microbiology Systems), before being examined. The presence of low flat spreading growth of spirochaetes on the plate, and any haemolysis around the growth was recorded. Spirochaetes were confirmed by picking off areas of suspected growth, re-suspending this in PBS, and examining the suspension under a phase contrast microscope at 400× magnification.

5.2.6.2. Detection of *B. hyodysenteriae* by PCR

Spirochaetes were identified as *B. hyodysenteriae* on the basis of strong β-haemolysis, microscopic morphology and results of a NADH oxidase (nox) PCR of cell growth on the plates. The PCR primers and conditions used have been described previously (chapter 2, section 2.2.13.1). A cell-pick method was used to obtain spirochaetal DNA from the plates. The tip of a sterile wooden toothpick was used to stab through the area of strong haemolysis, and the material adhering to the tip was resuspended in 50 μL of ultra-pure water before being boiled for 30 s. A 2.5 μL volume of boiled cells was used as template for the nox PCR. The
PCR products were subjected to electrophoresis through a 1% (w/v) agarose gel in 1× TAE buffer, stained with ethidium bromide and viewed over UV light.

5.2.7. Serological analysis

5.2.7.1. ELISA for systemic antibodies in pigs and mice

Ninety-six well microtitre plates (Immulon 4HBX, Dynex Technologies) were coated with 100 μl per well of purified lipidated His6-Bhlp29.7 (0.5 μg/ml), His6-Bhlp29.7 (0.5 μg/ml), MBP-Bhlp29.7-F604 (0.5 μg/ml) or a whole-cell preparation of *B. hyodysenteriae* strain Q/B02 (1 μg/ml) in 0.1 M carbonate buffer (pH 9.6). The whole-cell preparation was prepared by harvesting 50 ml of mid-log phase *B. hyodysenteriae* strain Q/B02 cells at 5,000 × g for 15 min and resuspending the pellet in 15 ml PBS. The cells were sonicated three times for 30 s with 1 min incubation on ice between sonication rounds. The lysed cells were cleared by centrifugation at 20,000 × g for 15 min at 4°C and the proteins in the supernatant quantified using the Bio-Rad Protein Assay (Bio-Rad Laboratories) (chapter 2, section 3.2.13). Plate coating was allowed to occur at 4°C overnight. Plates were blocked with 150 μl of PBS containing 1% (w/v) BSA for 1 h at 25°C with mixing, and then washed three times with 150 μl of PBST. Mice and pig sera were diluted 200-fold in 100 μl of PBST containing 0.1% (w/v) BSA and incubated at 25°C for 2 h with mixing. Plates were washed as above before adding 100 μl of goat anti-mouse IgG-HRP (Sigma Chemical Company) or goat anti-swine IgG-HRP (Southern Biotechnology) diluted 2,000-fold in PBST containing 0.1% (w/v) BSA. After incubating for 1 h at 25°C, the plates were washed and 100 μl of K-Blue substrate (ELISA Systems) added. Colour development was allowed to occur for 10 min before being stopped with the addition of 50 μl of 1 M sulphuric acid. The optical density of each well was read at 450 nm using a microtitre plate reader (Bio-Rad Model 3550-UV). For experiment 1, only His6-Bhlp29.7 and whole cell ELISAs were undertaken on mouse sera whilst for experiment 2, His6-Bhlp29.7 and whole cell ELISAs were undertaken on mouse sera and colonic extracts. Only His6-Bhlp29.7 and whole cell ELISAs were undertaken on pig sera and colonic extracts.
from experiment 3, whilst His6-Bhlp29.7, MBP-Bhlp29.7-F604 and whole cell ELISAs were used on samples from experiment 4.

5.2.7.2. ELISA for porcine colonic IgA

Pig colonic extracts were made from a 15 cm² section of the proximal colon. The epithelium was briefly rinsed with sterile PBS to remove digesta, stripped off with a scalpel blade and resuspended in PBS containing 1% (w/v) BSA, 2 mM PMSF, 1 mM EDTA and 0.2% (w/v) sodium azide to a concentration of approximately 250 mg/ml. The suspensions were vortexed thoroughly and centrifuged at 20,000 × g for 10 min at 4°C to remove the particulate material. The supernatant was removed and 100 μl used directly for the ELISA. The mucosal ELISA was performed as for the serum ELISA (chapter 4, section 4.2.3), except goat anti-swine IgA-HRP (Southern Biotechnology) was used to detect the reactive mucosal antibody.

5.2.7.3. Western blot analysis of porcine antibodies

Fifteen μg of the *B. hyodysenteriae* strain Q/B02 whole-cell preparation was loaded into a 7cm preparative well and subjected to SDS-PAGE (chapter 2, section 2.2.4) and the separated proteins electro-transferred to nitrocellulose membrane (chapter 2, section 2.2.5). After transfer, the membrane was removed from the transfer apparatus, washed briefly with distilled water and blocked with TBS containing 5% (w/v) skim milk powder. The membrane was then assembled into a Multi-Screen Apparatus (Bio-Rad Laboratories) that allowed the simultaneous analysis of 20 samples using the identical antigen. Each well of the Multi-Screen Apparatus was loaded with 100 μl of diluted serum pooled from two pigs (100-fold) or mucosal supernatant (2-fold) and incubated for 2 h at 25°C with gently mixing. Serum from a pig hyperimmunised with formalinised *B. hyodysenteriae* strain B78T (chapter 4, section 4.2.2) was diluted 500-fold and used as a reaction control. The wells were washed three times with TBST then incubated for 1 h at 25°C with gentle mixing with 100 μl of either goat anti-swine IgG-HRP (Southern Biotechnology) for serum samples, or goat anti-swine IgA-HRP (Southern
Biotechnology) for colon samples. Both secondary antibodies were diluted 2,000-fold. The membrane was removed and washed three times with TBST and twice with distilled water. Colour development occurred with the addition of 10 ml of DAB substrate (Sigma Chemical Company), and the membrane was washed with tap water when sufficient development had occurred.

5.2.8. Statistical Analysis

In the two experiments using mice, serological titres between corresponding groups of mice were compared using either Student’s paired or unpaired t-tests, as appropriate, and statistical significance was accepted at the $P \geq 0.05$ level. Within each of the two pig experiments, comparisons were made between the groups. The data were analysed using GraphPad InStat, version 3.0 for Macintosh (GraphPad Software Inc.). For faecal excretion of *B. hyodysenteriae*, for each group, the number of faecal samples taken that were positive and the number negative over the experimental period were recorded. Ratios for each group were then compared using a Chi-squared test for independence. Where a significant value was obtained, the results for sequential pairs of groups were compared using Fisher’s exact test. The disease incidence over the experiment (number developing SD/number remaining healthy) was similarly examined. For the purposes of measuring and comparing disease incidence, animals were only recorded as having SD if they had both clinical signs of SD and lesions of SD in their colons (whether mild or severe). Pigs that had lesions in the colon at post-mortem, but remained healthy and did not have clinical SD, were scored as being healthy for the purpose of this analysis. Weekly body weights and ELISA titres were compared using one-way ANOVA, with the Tukey-Kramer Multiple Comparisons Test used to identify significant between-group differences. Where only two sets of data were available, these were compared using Student’s unpaired t-tests.
5.3. RESULTS

5.3.1. Recombinant proteins

5.3.1.1. Lipidated His\textsubscript{6}-Bhlp29.7

Cloning of the lipidation cassette, containing the conserved \textit{E. coli} lipoprotein signal peptidase recognition site (L-A-G-C) and the six histidine fusion, into the pTrc-Bhlp29.7 plasmid produced a recombinant construct, designated pLIP-Bhlp29.7, which was 5,150 bp in size. A complete plasmid map of pTrc-Bhlp29.7 expression vector is shown in Fig. 5.1. Nucleotide sequencing of the pLIP-Bhlp29.7 construct verified that the lipidation cassette was in the correct frame for protein translation. Predicted translation of the pLIP-Bhlp29.7 construct indicated the recombinant His\textsubscript{6}-Bhlp29.7 lipoprotein was identical to the deduced amino acid sequence of the corresponding portion of native Bhlp29.7. Expression of the pLIP-Bhlp29.7 construct produced a recombinant his-tagged Bhlp29.7 lipoprotein with an apparent molecular mass of 34.1 kDa (Fig. 5.2, lane 3). The radioactive palmitate incorporation assay indicated that recombinant His\textsubscript{6}-Bhlp29.7 was expressed as a lipoprotein in \textit{E. coli} (Fig. 5.2, lane 1). Purification of the His\textsubscript{6}-Bhlp29.7 recombinant lipoprotein by affinity chromatography under denaturing conditions successfully yielded his-tagged Bhlp29.7 without affecting its lipidation (Fig. 5.2, lane 2). Following dialysis and lyophilisation, a stable recombinant His\textsubscript{6}-Bhlp29.7 lipoprotein was successfully produced.
**Figure 5.1.** Complete plasmid map of the pLIP-Bhlp29.7 expression vector showing features of the vector and the restriction sites used in its construction. SP, conserved *E. coli* signal peptidase recognition site; HIS, six histidine tag.
Analysis of the *E. coli* expressed and purified recombinant His\(_6\)-Bhlp29.7 was shown in chapter 3 (section 3.3.2). The His\(_6\)-Bhlp29.7 protein had an apparent molecular mass of 34 kDa (chapter 3, Fig. 3.5). The gene region encoding the carboxyl-terminal portion (Bhlp29.7-F604) of the Bhlp29.7 lipoprotein was successfully cloned into the pMAL-c2x Expression System. Sequencing of the pMAL-c2x expression cassette indicated that the cloned insert DNA was in-frame with the pMAL-c2x start codon and that the expressed Bhlp29.7 protein fragment had an amino acid sequence identical to the *B. hyodysenteriae* Bhlp29.7 lipoprotein. The carboxyl-
terminal portion was expressed in *E. coli* BL21 as a maltose-binding protein fusion (MBP-
Bhlp29.7-F604) and purified by amylose-facilitated chromatography to produce a protein with
an apparent molecular mass of 50 kDa of which approximately 42 kDa was contributed by the
fusion with maltose binding protein (Fig. 5.3). Both recombinant His<sub>c</sub>-Bhlp29.7 and MBP-
Bhlp29.7-F604 remained stable following dialysis and lyophilisation.

![SDS-PAGE of the amylose-facilitated affinity chromatography purified recombinant maltose-binding protein fusion of the carboxy-terminal portion of the Bhlp29.7 lipoprotein (MBP-Bhlp29.7-F604) used for the vaccination of pigs. Lanes 1-2, MBP-Bhlp29.7-F604.](image)

**Figure 5.3.** SDS-PAGE of the amylose-facilitated affinity chromatography purified recombinant maltose-binding protein fusion of the carboxy-terminal portion of the Bhlp29.7 lipoprotein (MBP-Bhlp29.7-F604) used for the vaccination of pigs. Lanes 1-2, MBP-Bhlp29.7-F604.

5.3.2. Mouse experiments

5.3.2.1. Experiment 1

The mean serum antibody responses of the mice to recombinant His<sub>c</sub>-Bhlp29.7 and to whole cell antigen of *B. hyodysenteriae* are shown in Table 5.2a and Table 5.2b, respectively. In this experiment, vaccination with both non-lipidated and lipidated His<sub>c</sub>-Bhlp29.7 resulted in significant increases in serum antibody titres to both Bhlp29.7 and *B. hyodysenteriae* whole cell antigens. No significant differences (*P*>0.05) were observed between the immunogenicity of the two vaccine antigens in either ELISA.
**Table 5.2a.** Means ± standard deviation of group ELISA titres before and after vaccination with non-lipidated and lipidated recombinant His$_{6}$-Bhlp29.7 in experiment 1. Mouse serum was used in ELISA using His$_{6}$-Bhlp29.7 as the plate coating antigen.

<table>
<thead>
<tr>
<th>Vaccine antigen</th>
<th>Sample time</th>
<th>Non-lipidated</th>
<th>Lipidated</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>His$_{6}$-Bhlp29.7</td>
<td>His$_{6}$-Bhlp29.7</td>
<td></td>
</tr>
<tr>
<td>Pre-vaccination</td>
<td>0.041 ± 0.028</td>
<td>0.052 ± 0.025</td>
<td>$P=0.1021$</td>
<td></td>
</tr>
<tr>
<td>Post-vaccination</td>
<td>0.876 ± 0.118</td>
<td>0.799 ± 0.083</td>
<td>$P=0.2886$</td>
<td></td>
</tr>
<tr>
<td>$P$-value</td>
<td>$P=0.0011$</td>
<td>$P&lt;0.0001$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 5.2b.** Means ± standard deviation of group ELISA titres before and after vaccination with non-lipidated and lipidated recombinant His$_{6}$-Bhlp29.7 in experiment 1. Mouse serum was used in ELISA using a sonicated whole cell preparation of *B. hyodysenteriae* as the plate coating antigen.

<table>
<thead>
<tr>
<th>Vaccine antigen</th>
<th>Sample time</th>
<th>Non-lipidated</th>
<th>Lipidated</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>His$_{6}$-Bhlp29.7</td>
<td>His$_{6}$-Bhlp29.7</td>
<td></td>
</tr>
<tr>
<td>Pre-vaccination</td>
<td>0.073 ± 0.059</td>
<td>0.049 ± 0.044</td>
<td>$P=0.5966$</td>
<td></td>
</tr>
<tr>
<td>Post-vaccination</td>
<td>0.832 ± 0.124</td>
<td>0.816 ± 0.089</td>
<td>$P=0.4113$</td>
<td></td>
</tr>
<tr>
<td>$P$-value</td>
<td>$P=0.0008$</td>
<td>$P&lt;0.0001$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.3.2.2. Experiment 2

5.3.2.2.1. Colonisation of mice with *B. hyodysenteriae*

The summarised results for microbiological culture of *B. hyodysenteriae* from the faeces of unvaccinated mice (group M3) and the His$_6$-Bhlp29.7 vaccinated mice (group M4) are shown in Table 5.3. Apart from the first sampling four days post infection, when four of the vaccinated mice (group M4) were excreting *B. hyodysenteriae* in the faeces, none of the vaccinated animals showed evidence of colonisation up to 13 days post-infection. At necropsy (13 days post-infection), *B. hyodysenteriae* was not detected in the caecum of any vaccinated mice. Four of the remaining seven (57%) unvaccinated mice were excreting *B. hyodysenteriae* in their faeces on sampling days four, six and 11 post-infection. At the time of necropsy, three of these unvaccinated mice were positive for *B. hyodysenteriae* in their faeces, however *B. hyodysenteriae* was detected in the caecum of all seven (100%). Three of the 10 unvaccinated mice died prior to experimental infection. The cause of death was not established.

<table>
<thead>
<tr>
<th>Days post-infection</th>
<th>-3</th>
<th>4</th>
<th>6</th>
<th>11</th>
<th>13 *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site for culture</td>
<td>Faeces</td>
<td>Faeces</td>
<td>Faeces</td>
<td>Faeces</td>
<td>Caecum</td>
</tr>
<tr>
<td>Non-vaccinated</td>
<td>0/7 (0%)</td>
<td>4/7 (57%)</td>
<td>4/7 (57%)</td>
<td>4/7 (57%)</td>
<td>3/7 (43%)</td>
</tr>
<tr>
<td>His$_6$-Bhlp29.7 vaccinated</td>
<td>0/10 (0%)</td>
<td>4/10 (40%)</td>
<td>0/10 (0%)</td>
<td>0/10 (0%)</td>
<td>0/10 (0%)</td>
</tr>
</tbody>
</table>

* samples taken at necropsy

**Table 5.3.** Number and percentage of unvaccinated and His$_6$-Bhlp29.7 vaccinated mice that were orally challenged with strain Q/01 having *B. hyodysenteriae* in their faeces, or in their caecum at post-mortem. Each challenge regime initially consisted of 10 mice. Three unvaccinated mice died of unknown causes unrelated to vaccination prior to experimental challenge.
5.3.2.2.2. Antibody response

The systemic antibody response of the mice at necropsy, 13 days after experimental challenge with *B. hyodysenteriae*, is shown in Fig. 5.4 for the ELISA using His$_6$-Bhlp29.7 as the antigen, and Fig. 5.5 for the ELISA using *B. hyodysenteriae* whole cells as the antigen. In the non-vaccinated mice (group M3), little or no systemic antibody response to recombinant His$_6$-Bhlp29.7 was detected before or after challenge with *B. hyodysenteriae* (Fig. 5.4). All the mice in this group showed some response to the *B. hyodysenteriae* whole-cell preparation, although the response of some mice (mice 1, 3 and 4) was higher than others (Fig. 5.5).

All vaccinated mice (group M4) showed a strong systemic antibody response to both the recombinant His$_6$-Bhlp29.7 protein (Fig. 5.4) and the *B. hyodysenteriae* whole-cell preparation (Fig. 5.5). In some mice (mice 8, 9, 10 and 12), a higher systemic response was directed at the *B. hyodysenteriae* whole-cell compared to the recombinant His$_6$-Bhlp29.7.

5.3.2.2.3. Macroscopic lesions

No macroscopic lesions were observed in the caecum or colon of mice in either group.
Figure 5.4. Systemic antibody titres against recombinant His$_6$-Bhlp29.7 following vaccination with recombinant His$_6$-Bhlp29.7 and experimental challenge of the mice with *B. hyodysenteriae*. Mice 1-7 were not vaccinated and mice 8-17 were vaccinated prior to challenge with *B. hyodysenteriae*. Circulating antibodies prior to vaccination and at necropsy were detected by ELISA using recombinant His$_6$-Bhlp29.7 as the coating antigen.
Figure 5.5. Systemic antibody titres against *B. hyodysenteriae* whole cells following vaccination with recombinant His<sub>6</sub>-Bhlp29.7 and experimental challenge of the mice with *B. hyodysenteriae*. Mice 1-7 were not vaccinated and mice 8-17 were vaccinated prior to challenge with *B. hyodysenteriae*. Circulating antibodies prior to vaccination and at necropsy were detected by ELISA using a whole cell preparation of *B. hyodysenteriae* as the coating antigen.
5.3.3. Pig experiments

5.3.3.1. Body weights

In both pig experiments no significant differences were found in body weights of the groups of pigs at any weighing (Table 5.4 and 5.5). Individual pigs rapidly lost weight when they developed clinical signs of SD, but because they were then removed from the experiment, their weights were not subsequently recorded in the group data.

Table 5.4. Mean ± standard deviations of body weights (kg) of the pigs in experiment 3 recorded at the start of the experiment before the first vaccination (pre-vaccination), prior to spirochaetal challenge after the second vaccination (pre-infection) and at necropsy (post-mortem). Post-mortem weights were calculated on the day when most of the non-vaccinated pigs were taken for necropsy. im, intramuscularly vaccinated.

<table>
<thead>
<tr>
<th>Time of sampling</th>
<th>Group</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-vaccinated</td>
<td>im/oral</td>
<td>im/im</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(1A, n=10)</td>
<td>(1B, n=10)</td>
<td>(1C, n=10)</td>
<td></td>
</tr>
<tr>
<td>Pre-vaccination</td>
<td>6.1 ± 0.6</td>
<td>6.3 ± 0.8</td>
<td>6.3 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Pre-infection</td>
<td>19.6 ± 3.6</td>
<td>20.3 ± 1.6</td>
<td>20.1 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>Post-mortem</td>
<td>38.5 ± 5.2</td>
<td>43.6 ± 2.8</td>
<td>43.7 ± 4.5</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.5. Mean ± standard deviation of body weights (kg) of the pigs in experiment 4 recorded at the start of the experiment before the first vaccination (pre-vaccination), prior to spirochaetal challenge after the second vaccination (pre-infection) and at necropsy (post-mortem).

<table>
<thead>
<tr>
<th>Time of sampling</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-vaccinated (2A, n=12)</td>
</tr>
<tr>
<td>Pre-vaccination</td>
<td>5.8 ± 0.4</td>
</tr>
<tr>
<td>Pre-infection</td>
<td>12.0 ± 1.5</td>
</tr>
<tr>
<td>Post-mortem</td>
<td>28.9 ± 3.9</td>
</tr>
</tbody>
</table>

5.3.4. Experiment 3

5.3.4.1. Antibody response

The serum and colonic antibody responses of the pigs in experiment 3 to recombinant His\textsubscript{66}-Bhlp29.7 and whole cell antigen are summarised in Table 5.6. In this experiment, vaccination by the im/oral (group 1B) and im/im (group 1C) routes both resulted in good primary and secondary serum antibody responses to Bhlp29.7, with the im/im route resulting in the highest titres. Infection of the unvaccinated control pigs did not result in an increase in serum antibody. No differences were observed between the three groups in colonic IgA titres. Using a whole cell preparation as ELISA antigen, neither vaccination with His\textsubscript{66}-Bhlp29.7 nor experimental infection with \textit{B. hyodysenteriae} resulted in increases in serum antibodies, and no differences were observed between any of the groups in the colon. Western blot analysis of serum from the vaccinated pigs confirmed that the systemic antibody response induced by both the vaccination regimens was primarily directed against the native Bhlp29.7 protein of \textit{B. hyodysenteriae} (Fig. 5.6 and 5.7). The non-vaccinated pigs failed to show any significant
antibody titres specific for Bhlp29.7 following infection and at necropsy (when acute SD was observed), although they did recognise other proteins in the *B. hyodysenteriae* whole-cell extract. Western blot analysis of the colonic IgA indicated that the local response in experiment 3 was directed at the native Bhlp29.7 lipoprotein (Fig. 5.8).

**Table 5.6.** Means ± standard deviation of group ELISA titres at four sampling times in experiment 3. Each pig was sampled prior to the first vaccination (pre-bleed), prior to the second vaccination (pre-boost), prior to spirochaetal challenge (pre-infection) and at necropsy (post-mortem). Within a row, mean values with different superscripts differ at least at the 5% level of significance. ND denotes sample not taken.

<table>
<thead>
<tr>
<th>ELISA antigen</th>
<th>Sample type* and time</th>
<th>Experimental group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control (1A)</td>
<td>im/oral (1B)</td>
</tr>
<tr>
<td>Bhlp29.7</td>
<td>S: pre-bleed</td>
<td>0.039 ± 0.040</td>
<td>0.048 ± 0.021</td>
</tr>
<tr>
<td></td>
<td>S: pre-boost</td>
<td>ND</td>
<td>0.932 ± 0.271</td>
</tr>
<tr>
<td>Bhlp29.7</td>
<td>S: pre-infection</td>
<td>0.038ᵃ ± 0.016</td>
<td>1.280ᵇ ± 0.396</td>
</tr>
<tr>
<td>Bhlp29.7</td>
<td>S: post-mortem</td>
<td>0.069ᵃ ± 0.022</td>
<td>1.152ᵇ ± 0.283</td>
</tr>
<tr>
<td>Bhlp29.7</td>
<td>C: post-mortem</td>
<td>0.315 ± 0.304</td>
<td>0.382 ± 0.200</td>
</tr>
<tr>
<td>Whole cell</td>
<td>S: pre-bleed</td>
<td>0.051 ± 0.038</td>
<td>0.040 ± 0.034</td>
</tr>
<tr>
<td>Whole cell</td>
<td>S: pre-boost</td>
<td>ND</td>
<td>0.083 ± 0.030</td>
</tr>
<tr>
<td>Whole cell</td>
<td>S: pre-infection</td>
<td>0.101 ± 0.054</td>
<td>0.131 ± 0.042</td>
</tr>
<tr>
<td>Whole cell</td>
<td>S: post-mortem</td>
<td>0.307 ± 0.130</td>
<td>0.392 ± 0.134</td>
</tr>
<tr>
<td>Whole cell</td>
<td>C: post-mortem</td>
<td>0.231 ± 0.300</td>
<td>0.157 ± 0.230</td>
</tr>
</tbody>
</table>

* S, serum IgG; C, colonic IgA
Figure 5.6. Western blot analysis of pooled serum from the pigs in experiment 3 vaccinated intramuscularly and orally with recombinant His<sub>6</sub>-Bhlp29.7 (group 1B). Sera from two pigs were pooled for each sampling time. The antigen used was a whole-cell extract of the homologous *B. hyodsenteriae* strain used for challenge. Lane 1, serum from a pig hyper-immunised with a *B. hyodsenteriae* B78<sup>T</sup> (positive control), lanes 2-6, serum taken pre-vaccination; lanes 7-11, serum taken pre-challenge, lanes 12-16, serum taken at post-mortem. Each set of three sampling times includes serum taken from pigs 21-22 (lanes 2, 7 and 12), pigs 23-24 (lanes 3, 8 and 13), pigs 25-26 (lanes 4, 9 and 14), pigs 27-28 (lanes 5, 10 and 15) and pigs 29-30 (lanes 6, 11 and 16). The native Bhlp29.7 protein of *B. hyodsenteriae* is indicated with the arrow.
Figure 5.7. Western blot analysis of pooled serum from the pigs in experiment 3 vaccinated twice intramuscularly with recombinant His<sub>6</sub>-Bhlp29.7 (group 1C). Sera from two pigs were pooled for each sampling time. The antigen used was a whole-cell extract of the homologous *B. hyodysenteriae* strain used for challenge. Lane 1, serum from a pig hyper-immunised with a *B. hyodysenteriae* B78<sup>T</sup> (positive control), lanes 2-6, serum taken pre-vaccination; lanes 7-11, serum taken pre-challenge, lanes 12-16, serum taken at post-mortem. Each set of three sampling times includes serum taken from pigs 31-32 (lanes 2, 7 and 12), pigs 33-34 (lanes 3, 8 and 13), pigs 35-36 (lanes 4, 9 and 14), pigs 37-38 (lanes 5, 10 and 15) and pigs 39-40 (lanes 6, 11 and 16). The native Bhlp29.7 protein of *B. hyodysenteriae* is indicated with the arrow.
Figure 5.8. Western blot analysis of mucosal IgA in the colon of selected pigs in experiment 3 which showed reactivity to recombinant His₆-Bhlp29.7 in ELISA. The antigen used was a whole-cell extract of the *B. hyodysenteriae* strain used for challenge. Lane 1, pig 1; lane 2, pig 7; lane 3, pig 8; lane 4, pig 12; lane 5, pig 13; lane 6, pig 19; lane 7, pig 20; lane 8, pig 21; lane 9, pig 23; lane 10, pig 24; lane 11, pig 29. Lanes 1-3 were from the non-vaccinated pigs (group 1A). Lanes 4-7 were from the group of pigs vaccinated intramuscularly and orally with recombinant His₆-Bhlp29.7 (group 1B). Lanes 8-11 were from the pigs vaccinated intramuscularly with recombinant His₆-Bhlp29.7 (group 1C). The position of the native Bhlp29.7 protein is indicated with the arrow.
5.3.4.2. Faecal excretion of *B. hyodysenteriae*

For the non-vaccinated control pigs (group 1A), excretion of *B. hyodysenteriae* was first detected in two pigs six days after the end of experimental infection (Table 5.7). Pig number 4 was killed before the end of the experiment, when diarrhoea was observed, although it was culture negative and did not have lesions consistent with SD at post-mortem. Eight of the remaining nine pigs excreted *B. hyodysenteriae* in their faeces prior to development of clinical signs. In contrast, the first faecal sample positive for *B. hyodysenteriae* in pigs vaccinated im/oral (group 1B) was 14 days after experimental infection (Table 5.8). One pig was removed due to lameness. Five of the remaining nine pigs were faecal positive at some point during the experimental period. For the pigs vaccinated im/im (Group 1C), the first pig became culture positive six days after experimental infection (Table 5.9). Overall, seven of the ten pigs were faecal culture positive at some point during the experiment. The number of positive faecal samples compared to the total number of faecal samples examined for the three groups were 19/73, 9/87 and 14/82 (groups 1A, 1B and 1C, respectively). Overall there was a significant difference in these rates ($\chi^2=6.813; df=2; P=0.033$), but the only significant difference between individual pairs of groups was between the unvaccinated animals (group 1A) and the pigs vaccinated im/oral (group 1B), with the faeces of the latter being less commonly positive ($\chi^2=6.762; df=1; P=0.009$).

5.3.4.3. Development of clinical signs, and lesions at post-mortem

A summary of the clinical signs and severity of colonic lesions in the pigs at post-mortem is presented in Table 5.10. The appearance of clinical signs of SD was always preceded by the presence of positive faecal cultures, although amongst vaccinates positive cultures were not always followed by disease. Seven of the ten non-vaccinated pigs (70%) developed clinical signs of SD and had lesions of severe mucohaemorrhagic colitis at post-mortem examination (Fig. 5.9). Three of the ten pigs (30%) that were vaccinated im/oral (group 1B) developed diarrhoea. Two had severe lesions of mucohaemorrhagic colitis at post-mortem, whilst the
Table 5.7. Culture results for faecal swabs taken from individual pigs in the non-vaccinated group of experiment 3 (group 1A) after experimental challenge with *B. hyodysenteriae*. Pigs were removed for post-mortem when clinical SD was observed, or else at the end of the experimental period. Culture at post-mortem was taken from the caecum. Dpi denotes days post-infection. The arrow indicates that no culture result was available as the pig had been removed for post-mortem. Positive (+) and negative (-) culture for *B. hyodysenteriae* are indicated.

<table>
<thead>
<tr>
<th>dpi</th>
<th>-9</th>
<th>3</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>14</th>
<th>16</th>
<th>20</th>
<th>22</th>
<th>23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig 11</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Pig 12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Pig 13</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Pig 14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↓</td>
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<td>Pig 15</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pig 16</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Pig 17</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>↓</td>
</tr>
<tr>
<td>Pig 18</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Pig 19</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Pig 20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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Table 5.8. Culture results for faecal swabs taken from individual pigs in experiment 3 which were vaccinated intramuscularly and orally with His$_6$-Bhlp29.7 (group 1B). The swabs were taken after the experimental challenge with *B. hyodysenteriae*. Pigs were removed for post-mortem when clinical SD was observed, or else at the end of the experimental period. Culture at post-mortem was taken from the caecum. Dpi denotes days post-infection. The arrow indicates that no culture result was available as the pig had been removed for post-mortem. Pig 30 was taken to PM due to physical injury unrelated to SD. Positive (+) and negative (-) culture for *B. hyodysenteriae* are indicated.

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Table 5.9. Culture results for faecal swabs taken from individual pigs in experiment 3 which were vaccinated twice intramuscularly with His6-Bhlp29.7 (group 1C). The swabs were taken after the experimental challenge with *B. hyodysenteriae*. Pigs were removed for post-mortem when clinical SD was observed, or else at the end of the experimental period. Culture at post-mortem was taken from the caecum. Dpi denotes days post-infection. The arrow indicates that no culture result was available as the pig had been removed for post-mortem. Positive (+) and negative (-) culture for *B. hyodysenteriae* are indicated.

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Table 5.10. Occurrence of clinical SD, presence of *B. hyodysenteriae* in the colon and severity of colonic lesions in the pigs of experiment 3, including controls and two groups vaccinated with His<sub>e</sub>-Bhlp29.7 by different routes. Pig 4 had watery diarrhoea which was not caused by SD.

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<tr>
<th>Pig</th>
<th>Clinical SD</th>
<th>PM Culture</th>
<th>Lesion severity</th>
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<td>30</td>
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third only had mild localised lesions (Fig. 5.10). One pig (10%) was removed due to physical injury, and had no lesions in the colon. The remaining six pigs (60%) stayed healthy throughout the experimental period, and did not develop diarrhoea. Two of these healthy pigs had mild lesions limited to the proximal colon at post-mortem. One of these two pigs was culture negative from the colon, although previously it had been culture positive from the faeces. The other pig was culture positive from the colon, and had been faecal positive on several samplings before post-mortem. For the pigs that were vaccinated im/im (group 1C), four (40%) developed diarrhoea and were culture positive from the colon at post-mortem examination. One of these four pigs had severe mucohaemorrhagic lesions in the colon, whilst the other three had mild and/or localised lesions. The other six pigs (60%) remained healthy. Three of these six were culture positive from the colon and also were faecal culture positive prior to slaughter, although none had colonic lesions at post-mortem. The incidences of disease in the three groups 1A, 1B and 1C were 7/10, 3/9 and 4/10 respectively, and these differences were not significant ($\chi^2=2.97; \text{df}=2; P=0.227$).

Figure 5.9. Colon showing the severe lesions of mucohaemorrhagic colitis seen in seven out of ten non-vaccinated pigs.
5.3.5. Experiment 4

5.3.5.1. Antibody response

The serum and colonic antibody responses of the pigs in experiment 4 to recombinant His<sub>6</sub>-Bhlp29.7, whole cell antigen and MBP-Bhlp29.7-F604 are summarised in Table 5.11. Vaccination with His<sub>6</sub>-Bhlp29.7 (group 2B) induced good primary and secondary serum antibody responses to His<sub>6</sub>-Bhlp29.7. Titres also tended to be higher in the colon of these vaccinated pigs than in the other pigs. Pigs vaccinated with MBP-Bhlp29.7-F604 (group 2C) showed little or no reactivity to His<sub>6</sub>-Bhlp29.7. Using a whole cell preparation as ELISA antigen, the pigs vaccinated with MBP-Bhlp29.7-F604 (group 2C) showed a slight increase in serum titres compared to the control pigs. With the MBP-Bhlp29.7-F604 antigen, serum titres were significantly elevated in the animals vaccinated with this antigen (group 2C), but not in the other groups. Little colonic antibody was detected. The results of Western Blot analysis of the serum from the vaccinated pigs, using whole-cell preparations as antigen, were similar to those in experiment 3. The systemic response induced by vaccination with recombinant

Figure 5.10. Colon showing the mild localised lesions seen in some of the vaccinated pigs.
His$_6$-Bhlp29.7 was directed predominately at the native Bhlp29.7 lipoprotein of *B. hyodysenteriae*, although reactivity with other spirochaetal proteins also was observed.

**Table 5.11.** Mean ± standard deviation of group ELISA titres at four sampling times in experiment 4. Each pig was sampled prior to the first vaccination (pre-bleed), prior to second vaccination (pre-boost), prior to spirochaetal challenge (pre-infection) and at necropsy (post-mortem). Within a row, mean values with different superscripts differ at least at the 5% level of significance. ND denotes sample not taken.

<table>
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<th>ELISA antigen$^\text{y}$</th>
<th>Sample type* and time</th>
<th>Experimental group</th>
<th>$P$-value</th>
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<tr>
<td></td>
<td></td>
<td>Control (2A)</td>
<td>His$_6$-Bhlp29.7 vaccine (2B)</td>
</tr>
<tr>
<td>Bhlp29.7</td>
<td>S: pre-bleed</td>
<td>ND</td>
<td>0.036 ± 0.021</td>
</tr>
<tr>
<td>Bhlp29.7</td>
<td>S: pre-boost</td>
<td>0.036$^a$ ± 0.014</td>
<td>0.725$^b$ ± 0.313</td>
</tr>
<tr>
<td>Bhlp29.7</td>
<td>S: pre-infection</td>
<td>0.091$^a$ ± 0.035</td>
<td>1.879$^b$ ± 0.260</td>
</tr>
<tr>
<td>Bhlp29.7</td>
<td>S: post-mortem</td>
<td>0.118$^b$ ± 0.090</td>
<td>1.710$^b$ ± 0.545</td>
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<tr>
<td>Bhlp29.7</td>
<td>C: post-mortem</td>
<td>1.264$^{ab}$ ± 0.414</td>
<td>1.837$^a$ ± 0.451</td>
</tr>
<tr>
<td>Whole cell</td>
<td>S: pre-bleed</td>
<td>ND</td>
<td>0.151 ± 0.122</td>
</tr>
<tr>
<td>Whole cell</td>
<td>S: pre-boost</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Whole cell</td>
<td>S: pre-infection</td>
<td>0.333$^{ab}$ ± 0.437</td>
<td>0.578$^a$ ± 0.406</td>
</tr>
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<td>S: post-mortem</td>
<td>0.361 ± 0.302</td>
<td>0.687 ± 0.400</td>
</tr>
<tr>
<td>Whole cell</td>
<td>C: post-mortem</td>
<td>0.140 ± 0.156</td>
<td>0.135 ± 0.171</td>
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<tr>
<td>F604</td>
<td>S: pre-bleed</td>
<td>ND</td>
<td>0.116 ± 0.095</td>
</tr>
<tr>
<td>F604</td>
<td>S: pre-boost</td>
<td>0.071$^a$ ± 0.087</td>
<td>0.101$^a$ ± 0.097</td>
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<tr>
<td>F604</td>
<td>S: pre-infection</td>
<td>0.049$^a$ ± 0.033</td>
<td>0.091$^a$ ± 0.029</td>
</tr>
<tr>
<td>F604</td>
<td>S: post-mortem</td>
<td>0.027$^b$ ± 0.030</td>
<td>0.039$^a$ ± 0.028</td>
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<td>F604</td>
<td>C: post-mortem</td>
<td>0.440$^{ab}$ ± 0.269</td>
<td>0.606$^a$ ± 0.243</td>
</tr>
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</table>

* S, serum IgG; C, colonic IgA

$^\text{y}$ F604=MBP-Bhlp29.7-F604
5.3.5.2. Faecal excretion of *B. hyodysenteriae*

For the non-vaccinated control pigs (group 2A), excretion of *B. hyodysenteriae* was first detected in the faeces eight days after the last day of experimental infection (Table 5.12). All the non-vaccinated pigs were culture positive from the faeces at some point during the experimental period. For the pigs vaccinated with recombinant His<sub>6</sub>-Bhlp29.7 (group 2B), the first faecal positive pig was detected ten days after experimental infection (Table 5.13). Eleven of these 12 pigs were culture positive from the faeces at some point in the experiment. For the pigs vaccinated with MBP-Bhlp29.7-F604 (group 2C), the first pig became culture positive six days after experimental infection (Table 5.14). All but one of these vaccinated pigs was culture positive from the faeces at some point during the experimental period. One pig died due to an unknown cause not related to SD (as established at post-mortem examination by a veterinary pathologist). The number of positive faecal samples compared to the total number of faecal samples examined for the three groups were 30/174, 51/191 and 32/129 (groups 2A, 2B and 2C, respectively). Overall there were no significant differences in these rates, but a trend was present ($\chi^2=4.99; df=2; P=0.083$). Pairwise comparisons revealed that the control pigs had a significantly smaller proportion of faecal positive days than the vaccinated pigs in group 2B ($\chi^2=4.72; df=1; P=0.030$).

5.3.5.3. Development of clinical signs, and lesions at post-mortem

The clinical signs and lesions in the colon obtained from the pigs in experiment 4 are summarised in Table 5.15. For the unvaccinated pigs (group 2A), six (50%) developed clinical signs of SD. Five of these had severe mucohaemorrhagic colitis at post-mortem, whilst only one had mild localised lesions. Two of the 12 pigs (17%) did not develop clinical signs of SD, however they had severe lesions in the colon at post-mortem. The remaining four pigs (33%) stayed healthy and were culture negative at post-mortem. For the 12 pigs vaccinated with recombinant His<sub>6</sub>-Bhlp29.7, two (17%) developed clinical signs of SD and both had mild localised lesions in the colon at post-mortem. The remaining ten pigs (83%) remained healthy, and did not have diarrhoea, but two had severe mucohaemorrhagic lesions in the colon at post-
Table 5.12. Culture results for faecal swabs taken from individual pigs in the non-vaccinated group of experiment 4 (group 2A) after experimental challenge with *B. hyodysenteriae*. Pigs were removed for post-mortem when clinical SD was observed, or else at the end of the experimental period. Culture at post-mortem was taken from the caecum. Dpi denotes days post-infection. The arrow indicates that no culture result was available as the pig had been removed for post-mortem. Positive (+) and negative (-) culture for *B. hyodysenteriae* are indicated.

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**Table 5.13.** Culture results for faecal swabs taken from individual pigs in experiment 4 which were vaccinated His6-Bhlp29.7 (group 2B). The swabs were taken after the experimental challenge with *B. hyodysenteriae*. Pigs were removed for post-mortem when clinical SD was observed, or else at the end of the experimental period. Culture at post-mortem was taken from the caecum. Dpi denotes days post-infection. The arrow indicates that no culture result was available as the pig had been removed for post-mortem. Positive (+) and negative (-) culture for *B. hyodysenteriae* are indicated.

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Table 5.14. Culture results for faecal swabs taken from individual pigs in experiment 4 which were vaccinated MBP-Bhlp29.7-F604 (group 2C). The swabs were taken after the experimental challenge with *B. hyodysenteriae*. Pigs were removed for post-mortem when clinical SD was observed, or else at the end of the experimental period. Culture at post-mortem was taken from the caecum. Dpi denotes days post-infection. The arrow indicates that no culture result was available as the pig had been removed for post-mortem. Pig 33 was taken to PM due an unknown cause unrelated to SD. Positive (+) and negative (-) culture for *B. hyodysenteriae* are indicated.

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Table 5.15. Clinical signs and severity of colonic lesions in the pigs of experiment 4. Pig 63 died of an unknown cause not related to SD.

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<th>Lesion severity</th>
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mortem, and one had mild localised lesions limited to the proximal colon. The other seven pigs did not show any evidence of colitis, although six of them had been culture positive some time during the experimental period. Two of the six pigs also were culture positive from the caecum at post-mortem. For the 12 pigs vaccinated with MBP-Bhlp29.7-F604 (group 2C), nine (75%) developed clinical signs of SD and had severe mucohaemorrhagic lesions in the colon. These pigs were culture positive from the colon. One pig (8%) died during the experimental period due to an unknown cause not related to SD. The remaining two pigs stayed healthy throughout the experiment, although one had severe mucohaemorrhagic colitis in the distal colon at post-mortem. The other pig remained culture negative from the faeces for the duration of the experiment. Both pigs were culture negative at post-mortem. Disease incidence in the three groups (6/12, 2/12 and 9/11 respectively for groups 2A, 2B and 2C) showed a significant effect ($\chi^2=10.167; \text{df}=2; P=0.006$). Pair-wise comparisons showed a significantly lower incidence in the group vaccinated with His$_6$-Bhlp29.7 compared to those vaccinated with MBP-Bhlp29.7-F604 ($P=0.003$).

When results for the three groups vaccinated with His$_6$-Bhlp29.7 in the two experiments (3 and 4) were combined, regardless of route of vaccination, 9/31 developed SD. In comparison 13/22 pigs in the two unvaccinated control groups developed SD. This difference, equivalent to a 51% reduction in disease incidence attributable to vaccination, was statistically significant ($P=0.047$).
5.4. DISCUSSION

In this chapter, murine and porcine models for SD were used to evaluate the efficacy of vaccination with recombinant his-tagged Bhlp29.7. The outcomes for these models will be discussed here, with the key focus being on the experiments in pigs, the target animal species.

5.4.1. Mouse experiments

Immunisation of mice with non-lipidated and lipidated His6-Bhlp29.7 in experiment 1 induced good systemic antibody titres which were not significantly different. These antibodies were cross-reactive with both the recombinant non-lipidated His6-Bhlp29.7 as well as with the native Bhlp29.7 lipoprotein represented in the whole cell preparation of *B. hyodysenteriae*. Since lipidation did not appear to affect the immunogenicity of His6-Bhlp29.7, the non-lipidated variant was chosen as the vaccine subunit for the subsequent protection study in mice.

In the second mouse experiment, good systemic antibody titres were induced in all the vaccinated mice and these titres were similar to those seen in the immunogenicity experiment. In this experiment, all mice were fed a defined diet (consisting of 63% w/w glucose) 10 days before experimental challenge. This diet has been shown to increase the susceptibility of the mice to *B. hyodysenteriae*, possibly by altering the microflora in the caecum (Suenaga and Yamazaki, 1984; Nibbelink and Wannemuehler, 1992; Hutto *et al.*, 1998). Oral inoculation with *B. hyodysenteriae* resulted in reasonably good levels of *B. hyodysenteriae* excretion in the faeces of the unvaccinated mice, although it was apparent that examining the caecum at post-mortem was a better measure of colonisation than was faecal culture. At necropsy, all the unvaccinated mice were colonised at the caecum whilst none of the vaccinated mice were colonised. This represents clear evidence that His6-Bhlp29.7 has potential as a vaccine component for SD.
Gross lesions were not observed in the caecum of any of the infected mice, even though other authors have reported mouse experiments reported that gross lesions occur in the murine model (Suenaga and Yamazaki, 1984; Mysore et al., 1992; Nibbelink and Wannemuehler, 1992; Hutto et al., 1998). The reason for this discrepancy remains unknown since both the C3H/HeJ and C3H/HeN mouse strains have been shown to display lesions in the caecum (Nibbelink and Wannemuehler, 1992), and the C3H/HeJARC mouse strain used in this study was a subline of the C3H/HeJ strain with a LPS responsiveness reversion to the same level as its predecessor C3H/HeJ (Silvia and Urosevic, 1999). It is possible that the lack of lesions seen in the infected mice was due to the particular *B. hyodysenteriae* strain used for inoculation.

The mechanism(s) that provided the vaccinated mice with protection from colonisation with *B. hyodysenteriae* was not determined. Unfortunately, local antibody and/or cell-mediated responses were not investigated. In pigs, neither systemic nor local antibody responses have strong correlation with protection from the development of SD (Joens et al. 1982; Fernie et al. 1983; Rees et al. 1989). Limited information is available about the systemic and local immune responses of mice colonised with *B. hyodysenteriae*. One study in mice found that antigen-specific IgG1 and IgG2 responses to commensal microflora increased following colonisation with *B. hyodysenteriae* (Jergens et al., 2006). Also, no significant changes in T-cell populations in the murine lamina propria have been observed following infection, and changes in the mucosal mast cell population numbers have not correlated with the development of lesions associated with *B. hyodysenteriae* infection (Nibbelink and Wannemuehler, 1992). Further interrogation of these aspects of immunity would be beneficial for future protection studies in mice.

Nevertheless, vaccination with recombinant Bhlp29.7 induced serum antibody titres and reduced and/or prevented *B. hyodysenteriae* colonisation in mice. These findings reinforce the likelihood that recombinant Bhlp29.7 is a good vaccine candidate for controlling *B.
hyodysenteriae infections, and also demonstrated its potential usefulness as a subunit vaccine for protection of pigs from SD.

5.4.2. Pig experiments

5.4.2.1. The infection model used

The two pig experiments conducted in this study followed a somewhat simple infection model for inducing SD. In both experiments, pigs were inoculated by gastric intubation with approximately $10^{10}$ cfu of *B. hyodysenteriae* at exponential log-phase on five consecutive days. The inoculum was cultured from a low-passaged isolate which showed strong β-haemolysis on blood agar plates and high motility under phase contrast microscopy. The pigs were approximately nine weeks of age at the time of inoculation and were confirmed to be free of *B. hyodysenteriae* based on faecal culture and PCR. During the experiment, pigs receiving the same vaccination regime were housed in group pens with open wire-mesh partitions and raised wire-mesh floors. In experiment 3, all three groups were housed in adjacent pens in the same room of the isolation house. In experiment 4, pigs in the two vaccinated groups were housed in adjacent pens in one room of the isolation house, and unvaccinated control pigs were housed in another room. The walls and ceilings of the rooms were made of solid concrete and the only form of supplementary heating provided was a heat-lamp in each pen.

Using this infection model, the incidence of disease in the unvaccinated groups was 70% (7/10) for experiment 3 and 50% (6/12) for experiment 4. Due to the larger group sizes used in experiment 4, the unvaccinated control pigs were housed in a separate room from the vaccinated pigs. The reduced density of pigs in this separate room may help explain the lower incidence of disease seen in the control group compared to experiment 3, since circulation of faeces from diseased animals has been shown to be a major predisposing factor for SD (Jacobson *et al.*, 2004). However, in the room which housed two groups of 12 pigs, the incidence of disease in the pigs vaccinated with MBP-Bhlp29.7-F604 was 75% (9/12), indicating that a good level of colonisation could be achieved. Previous experiments using
similar infection models have reported incidences of 82-94% in control groups (Hampson et al., 1993; Joens et al., 1979; Rees et al., 1989), suggesting that the incidence in the first experiment was acceptable. Although an incidence of 100% would be desirable, this may not be achievable since it has been reported that some experimentally challenged pigs may be recalcitrant to clinical SD even after exposure to large numbers of *B. hyodysenteriae* (Albassam et al., 1985; Jacobson et al., 2004).

Factors such as the age of the animal being challenged, the *B. hyodysenteriae* strain used for inoculation, the method of inoculation, the number of consecutive doses, the housing temperatures, types of flooring used in pens, stocking density, and stress levels of the pigs have been suggested to influence the development of disease in SD models (Hampson et al., 2006a). More recently, interest has been focused on the influence of feed composition on the incidence of disease. A highly-digestible diet was found to confer pigs with a better protection from developing SD than a highly-fermentable diet (Pluske et al., 1996; Siba et al., 1996; Durmic et al., 1998; Pluske et al., 1998). A recent study investigating the influence of factors such as age, diet, housing and administration of stress-inducing drugs on the development of SD in experimental pigs concluded that a diet based on soybean meal and group-housing of pigs were the major factors influencing the experimental induction of SD (Jacobson et al., 2004). Using this infection model, nine out of nine pigs developed SD compared with four out of nine when the pigs were individually housed.

In future experiments, feeding of large quantities of soybean meal may improve the incidence of SD in experimental animals. Where possible, pigs should be group-housed with all experimental groups in the same room. In experiments requiring larger group numbers, representative subsets of each group should be housed together in an attempt to reduce room effects.
5.4.2.2. Monitoring and defining disease

In the present study, daily examination of the experimental animals mainly focused on observation of the consistency and colour of faeces on the pen floor. SD was defined as the occurrence of mucous-containing haemorrhagic diarrhoea connected with consistent shedding of *B. hyodysenteriae* over several sampling times. Pens in which SD was observed were further monitored to identify the diseased pig(s). Although this method of monitoring was somewhat accurate, suggested by the fact near all pigs taken to necropsy due to the observation of suspected SD presented severe lesions upon post-mortem examination, it was only qualitative and was subjective. An earlier study by Stanton and Jensen (1993) used a commercial faecal occult blood test kit to monitor individual pigs which had been experimentally infected with *B. hyodysenteriae*. The study concluded that the faecal occult blood test was a reliable adjunct to bacteriology for monitoring individual animals with experimental SD. Another study looked at the dynamics of acute-phase protein (APP) levels in response to experimental SD (Jacobsen *et al.*, 2004). This study found that there was a several-fold increase in the levels of haptoglobin and serum acute amyloid (SAA) in response to haemorrhagic diarrhoea in infected pigs, but no associated response in pigs with non-haemorrhagic diarrhoea. Both studies indicate that quantitative methods may be applicable for the objective monitoring of experimental SD in individual pigs. A foreseeable problem could be the misrepresentation of occult blood or APP levels caused by inflammatory effects associated with other intestinal disorders of swine, such as proliferative enteritis, salmonellosis, trichuriasis and gastric ulcers (Hampson *et al.*, 2006). However, since pigs are normally kept in controlled conditions during the experimental period, treatment with antibiotics at the time of acquiring the animals may prevent the occurrence of inflammations associated with bacterial infections other than SD.

In future experiments, assaying for these disease biomarkers may enable the quantitative assessment of the dynamics of disease development to be performed, and hence provide a clearer judgement of the animal’s health.
5.4.2.3. Vaccination regimes

The vaccination regimes examined in this study included both parenteral (intramuscular) and parenteral/mucosal (oral) routes of immunisation. Both routes are known to be capable of stimulating systemic and local antibody responses (Galvin et al., 1997). In both pig experiments, an antigen load of 1 mg of protein per pig was given, both for the intramuscular and oral routes. This amount of antigen successfully induced good antibody titres in all individuals (section 5.4.2.4), although it is possible that the antigen load could have been reduced without affecting the antibody response. Given more time and resources, a titration of antigen load in pigs would have provided this information. For the intramuscular vaccinations, the His\textsubscript{6}-Bhlp29.7 antigen was prepared using Freund’s Incomplete Adjuvant (FIA) for experiment 3, and another oil-based adjuvant (VSA3) in experiment 4. FIA is commonly used in vaccine preparation and has been shown to induce strong antibody titres in a large range of animals, including pigs (Bennett et al., 1992). Since the vaccine experiments were supported by a commercial partner, in-house efficacy experiments indicated that subunit vaccines prepared using the VSA3 adjuvant generated antibody responses superior to FIA. For this reason the VSA3 adjuvant was used in experiment 4.

In the oral vaccinations, the vaccine antigen was solubilised in PBS and administered to the back of the pig’s palate using a syringe. Since the antigen was not administered with any form of protection, exposure to the acidic conditions of the stomach may potentially have resulted in the proteolysis of the antigen before it arrived at the intestinal mucosa. Although this did not appear to have occurred, as determined by the fact that antibody levels increased after the oral boost, the effect of this gastric exposure on the stability of the vaccine antigen is not known. Various strategies using alternative means for the mucosal delivery of vaccines, such as the use of bacterial vectors, DNA vaccines, plant vector delivery systems, immune stimulating complexes (ISCOMs) and liposomes, are all available. In addition, mucosal adjuvants, such as bacterial toxins and CpG oligonucleotides, have also been shown to enhance immunity at the mucosal surfaces. These strategies for improved mucosal delivery will be discussed in more
detail in chapter 6. Adaptation of vaccination regimes to deliver antigens to the mucosal surfaces may be useful for future studies involving oral vaccination of animals with Bhlp29.7.

5.4.2.4. Experimental outcomes

5.4.2.4.1. Serological response in experiment 3

In experiment 3, the im/im vaccination route induced slightly higher serum antibody titres to Bhlp29.7 than the im/oral route. In the unvaccinated control pigs, infection with *B. hyodysenteriae* failed to induce a systemic immune response to His<sub>6</sub>-Bhlp29.7 even though the pigs presented acute signs of SD, although an increase in systemic titres against the *B. hyodysenteriae* whole cell preparation was seen. As suggested in chapter 4, the lack of systemic response against His<sub>6</sub>-Bhlp29.7 may have been because these acutely affected pigs had insufficient time to mount an antibody response, and the increased systemic response detected against the *B. hyodysenteriae* whole cell was due to cumulative antibody titres induced towards all components of the spirochaete, including other lipoproteins and LPS. Neither vaccination route enhanced colonic IgA responses over that seen in the infected control pigs, but it was unclear to what extent the experimental infection contributed to the colonic IgA levels. To get a better understanding of the capacity of the vaccines to induce local colonic antibody, it would have been necessary to include a group of uninfected and non-vaccinated control animals for comparison. The results of the whole cell ELISA and Western blots confirmed the specificity of the vaccine-induced response towards Bhlp29.7. The im/oral route also significantly reduced the amount of faecal excretion of *B. hyodysenteriae* compared to the control group. The explanation for this is not known, but it could reside with the activation of CD4+ T-cells present in the colonic lymph nodes as a result of the antigen exposure due to the oral immunisation (Waters *et al*., 2000; Hontecillas *et al*., 2005). Further studies are required to elucidate the mechanisms involved. Both vaccination routes similarly reduced the incidence of disease compared to the controls, but the difference was not significantly, probably due to the small group sizes and the fact that the incidence of disease amongst the control group was only 70%. Increased disease incidence, possibly using methods
discussed earlier (section 5.4.2.1), or larger group sizes may have resulted in statistically significant differences.

5.4.2.4.2. Serological response in experiment 4

As a result of the lower faecal excretion of *B. hyodysenteriae* and the marginally lower incidence of disease in the pigs vaccinated im/oral compared to im/im in the first experiment, the im/oral route was used for His₆-Bhlp29.7 immunisation in experiment 4. A difference was the use of another oil-based adjuvant (VSA3 rather than FIA) for the primary immunisation in the second experiment. The im/oral vaccination regimen again produced good primary and secondary serum antibody responses directed at Bhlp29.7. A local colonic IgA response to Bhlp29.7 that was significantly greater than that of the group immunised with the carboxy-terminal fusion of Bhlp29.7 also was generated. Again, fewer of the His₆-Bhlp29.7 immunised pigs developed disease than did the controls (2/12 compared to 6/12), although, unexpectedly, the immunised pigs shed *B. hyodysenteriae* in their faeces significantly more frequently than did the control animals. This may be explained by the fact that more samples were taken from the vaccinated pigs because, unlike the control pigs, they remained healthy and were not removed even though they were excreting *B. hyodysenteriae* in their faeces. The immunised pigs were housed in a separate room from the control animals, and the level of environmental challenge in the room containing vaccinated pigs was much higher than in the control room. Only 50% of control pigs developed SD, whereas 82% (9/11) of the pigs in group 2C (immunised with MBP-Bhlp29.7-F604), housed in the room with the pigs vaccinated with His₆-Bhlp29.7, developed SD. Hence this greater exposure may also have lead to the increased duration of faecal excretion compared to the control pigs.

The MBP-Bhlp29.7-F604 protein was immunogenic, inducing good serum antibody titres, but there was no cross-reactivity with the native or his-tagged Bhlp29.7. Pig serum taken from animals vaccinated with MBP-Bhlp2.7-F604 reacted strongly with the immunising antigen but not with the whole cell of *B. hyodysenteriae* or with the his-tagged Bhlp29.7. This indicated
that most of the immune response generated towards MBP-Bhlp29.7-F604 was directed at the
dominant 42 kDa MBP portion of the fusion. In addition, this vaccine offered no protection
against SD, and, indeed, significantly more pigs vaccinated with MBP-Bhlp29.7-F604 than
pigs in the same room vaccinated with His<sub>s</sub>-Bhlp29.7 developed SD. The failure of MBP-
Bhlp29.7-F604 to protect pigs may relate to the relatively large size of MBP compared to the
carboxy-terminal end of Bhlp29.7. In future work it would be instructive to try other fusions to
determine whether a protective immune response could be generated using the carboxy-
terminal end of the protein.

Generally there was no clear relationship between the titres of serum or colonic antibodies
present and the occurrence or severity of SD. Colonic IgA has been shown to be produced in
pigs resistant to SD, but is believed to indicate recent exposure of the animal to <i>B. hyodysenteriae</i>
rather than protective immunity (Rees et al., 1989). Besides antibodies, it is
likely that the His<sub>s</sub>-Bhlp29.7 vaccine induced other cell-mediated immune mechanisms that
may contributed to the protection observed (Waters et al., 1999a; Hontecillas et al., 2005).
These cellular responses may involve monocytes, neutrophils, CD8α+ CD8β- CD4-
lymphocytes and CD45RA- lymphocytes, which have been observed at elevated levels during
SD, and neutrophils and γδ T-cells, which have been seen in increased levels during recovery
(Jonasson et al., 2006). Unfortunately, cellular responses were not investigated in the present
study.

5.4.2.4.3. Gross lesions

Most pigs that developed mucoid or haemorrhagic diarrhoea had severe and extensive lesions
in their large intestines at post-mortem. Furthermore, these clinically affected animals tended
to excrete spirochaetes in their faeces between two to four sampling times prior to the
development of clinical signs of disease. This was consistent with there being a slow
accumulation of spirochaete numbers in the large intestine, and progressive development of
lesions along the colon to a point where diarrhoea and SD developed. Other situations also
occurred. In group 1A, control pig 2 did not have diarrhoea or colonic lesions, but spirochaetes were isolated from its colon. It is possible that this pig may have gone on to develop SD if it had been kept for longer. In most cases where vaccinated pigs developed diarrhoea, their colonic lesions were milder and less extensive than in control animals. The mild lesions seen in these pigs were localised in the proximal colon and extended only one-third of the way down towards the distal colon. It is believed that disease development involves a synergistic effect between *B. hyodysenteriae* and certain members of the normal intestinal microflora of the pig (Neef *et al*., 1994; Whipp *et al*., 1979). The development of lesions is thought to be accompanied by the translocation of luminal bacteria into the lamina propria following exposure to the haemolysin of *B. hyodysenteriae*. It has been proposed that this loss of an epithelial cell barrier causes exposure of the underlying mucosa to antigen in the lumen and triggers a severe inflammatory response which manifests as gross lesions (Hutto and Wannemuehler, 1999). The reduced faecal shedding observed in the im/oral vaccinated pigs may indicate lower *B. hyodysenteriae* numbers in the colon, thus exposing the mucosa to less haemolysin and consequently reducing epithelial damage. In vaccinated group 1B, of three pigs with mild colitis, pig 13 had no diarrhoea and was spirochaete negative, pig 17 was culture positive but did not have diarrhoea, and pig 18 was culture positive and did have diarrhoea. Even in these pigs, some degree of vaccine-induced protection seemed to be occurring.

In experiment 4, pigs 31 and 39 in control group 2A, pigs 44 and 49 in vaccinated group 2B and pig 61 in vaccinated group 2C did not have diarrhoea, but had severe colonic lesions. In these animals it would appear that colonic function was still sufficient to maintain a normal stool consistency, although it is likely that they would have gone on to develop SD. Surprisingly, animals 31 and 61 were also culture negative at post-mortem. Animals that have colonic lesions but appear healthy are particularly problematic from a diagnostic perspective. Their growth performance would also probably be affected. Unfortunately the small group sizes and the need to remove animals from the groups as SD appeared meant that no
meaningful data on body weights could be collected. Larger scale field trials would be required to obtain this sort of production data.

In experiment 4, all pigs were kept for 51 days after infection, compared to 20-23 days in the experiment 3. The purpose of this was to determine whether asymptomatic vaccinated animals would ultimately develop disease. By 24 days after infection, one pig vaccinated with His$_6$-Bhlp29.7 had developed SD, and it had mild localised lesions in the colon. Only one additional vaccinated pig developed diarrhoea between 25 days after infection and the end of the experiment, and this animal also only had mild lesions in the colon. Other pigs had colonic lesions at post-mortem, but no diarrhoea. These results suggest that the protection provided by vaccination lasts at least two months, and generally may be sufficient both to prevent the development of severe and/or extensive colonic lesions, and keep the pig asymptomatic. Vaccination also tended to delay the onset of faecal excretion of spirochaetes, but did not necessarily stop it occurring. It is not clear whether the presence of low-grade exposure to \textit{B. hyodysenteriae} on naturally infected piggeries would provide sufficient immunological boosting to maintain protection through the grower phase.

5.4.2.4.4. Efficacy of the vaccination

Taken together, the results of the mouse and pig experiments indicate that Bhlp29.7 has promise as a candidate for use in vaccines for SD. The vaccination of mice with His$_6$-Bhlp29.7 provided complete protection from \textit{B. hyodysenteriae} colonisation of the caecum, and this prompted the subsequent experiments in pigs. Although the complete protection seen in the mice was not achieved in either of the two pig experiments, a significant level of protection was seen. This level of protection was generally better than that achieved with whole-cell \textit{B. hyodysenteriae} bacterins (Glock \textit{et al.}, 1978; Fernie \textit{et al.}, 1983; Diego \textit{et al.}, 1995; Waters \textit{et al.}, 1999a, Waters \textit{et al.}, 1999b), and in addition bacterins may exacerbate the disease in some animals (Olsen \textit{et al.}, 1994). This is the first time that a recombinant protein has been shown to have promise as a potential subunit vaccine component for the control of SD in pigs.
5.4.4. Conclusions

In an earlier study it was demonstrated that immunisation of pigs with formalinised *E. coli* expressing recombinant Bhlp29.7 induced antibodies to the native Bhlp29.7 lipoprotein of *B. hyodysenteriae* (Lee, 1996). The present study aimed to assess the protection afforded by a recombinant Bhlp29.7 subunit vaccine and to investigate the effect of the immunisation on colonisation and SD development. Although microbiological sterility would be the best long-term approach for the control of SD, an economically produced vaccine capable of reducing disease incidence and severity, coupled with proper management practices, should prove an attractive means of control for many herds with SD. The results of the experiments described in this chapter confirmed the immunogenicity of recombinant His6-Bhlp29.7, which induced good primary and secondary serum antibody responses, and demonstrated that pigs immunised with His6-Bhlp29.7 show a reduced susceptibility to SD. Although some pigs vaccinated with Bhlp29.7 did develop SD (17-40%), this was significantly less than amongst the unvaccinated control pigs (50-70%) when results for the two experiments were pooled. A similar approximately 50% reduction in incidence of SD in infected vaccinated herds would confer a considerable cost-benefit. The level of protection achieved was broadly similar to that reported for *B. hyodysenteriae* bacterin vaccines in some pen trials (Parizek et al., 1985; Hampson et al., 1993). Unlike bacterins, recombinant Bhlp29.7 should provide protection across all serovars of *B. hyodysenteriae*, although this requires further investigation. The vaccine also should be cheaper and easier to produce and standardise on a commercial scale than *B. hyodysenteriae* bacterins.
CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

6.1. INTRODUCTION

SD is one of the major economic diseases of pigs worldwide. There is currently no subunit vaccine available for SD and those based on LPS or sonicated cells have provided partial protection at best. Detection of the causative agent of SD has become more accurate partly due to the use of molecular methods, although indirect detection of exposure to *B. hyodysenteriae* within herds by serological means still remain undeveloped. The aims of this PhD was to interrogate the properties of a 29.7 kDa outer membrane protein previously described as being reactive with a monoclonal antibody specific for *B. hyodysenteriae*, and investigate the efficacy of the recombinant protein as a reagent for serological assays, and as a subunit for vaccination against SD. The aims were successfully achieved, and the data generated suggest that the protein has potential applications in serology and vaccination. Nevertheless, more work is required to develop these areas to the point where commercial products become available.

6.2. CHARACTERISATION OF BHLP29.7

When the study began little was known about the properties of the 29.7 kDa protein of *B. hyodysenteriae*, except that it was possibly surface-exposed; it reacted with a monoclonal antibody which was specific for *B. hyodysenteriae*; and that a formalinised *E. coli* clone expressing the protein was immunogenic in pigs following intramuscular immunisation (Lee, 1996). The first stage of this project was the revival of the *E. coli* clones which had been stored at -80°C. Little information was known about the properties of the plasmid vector which harboured the gene of interest at that time, and after some unsuccessful attempts, the first fragment of *B. hyodysenteriae* sequence was acquired which quickly lead to the assembly of the complete contiguous sequence. Bioinformatics analysis of this sequence identified a gene, which has since been designated *bhlp29.7*, encoding a 29.7 kDa outer membrane lipoprotein.
The Bhlp29.7 protein was found to be a putative lipoprotein indicated by the presence of a signal peptide containing a consensus signal peptidase II cleavage site at the N-terminal. Annotation of the Bhlp29.7 polypeptide amino acid sequence indicated highest similarity with the D-methionine binding lipoprotein (MetQ) of various pathogenic bacteria. Subcellular localisation prediction software (PSORTb and HMMTOP) combined with IFAT using a monoclonal antibody specific for *B. hyodysenteriae* indicated that Bhlp29.7 was present on the cell surface of *B. hyodysenteriae*. Radioactive palmitate incorporation assays confirmed that Bhlp29.7 was a lipoprotein.

The possible outer membrane localisation and lipoprotein nature of Bhlp29.7 made it a potential candidate for use in a serological test for SD, as well as a subunit for a SD vaccine. Outer membrane proteins (OMPs) exposed to the cell surface are in a position to interact with the environment and subsequently, contribute to the pathogenesis of the bacteria by interacting with the host (Haake, 2000). The OMPs of a range of bacterial pathogens, including spirochaetes, have diverse roles, including adhesion and invasion (Palaniappa et al., 2002; Matsunaga et al., 2003; Cameron et al., 2004; Li et al., 2006; Seshu et al., 2006). The lipoproteins are one subclass of OMPs and have been implicated to participate directly in the pathogenesis of the spirochaete (Cullen et al., 2004). For example, the outer surface protein A (OspA) of the Lyme disease agent *Borrelia burgdorferi* is involved in the colonisation of the tick midgut and has been used in a transmission-blocking vaccine for humans (Pal et al., 2004). Similarly, leptospiral immunoglobulin-like (Lig) proteins, a family of surface-exposed lipoproteins which have similarity to bacterial adhesions, are believed to contribute to the pathogenicity of *Leptospira interrogans* since the loss of *lig* expression has been correlated to the loss of virulence during culture attenuation of pathogenic strains (Palaniappa et al., 2002; Matsunaga et al., 2003).

Spirochaetal lipoproteins are highly immunogenic molecules and have been the subject of intense investigation due to their suggested role in immunity. For spirochaetes such as *B.*
*B. burgdorferi* and *L. interrogans*, a substantial number of immunogens have been identified and their potential functions characterised (Haake, 2000; Cullen *et al.*, 2004). Little is known about the importance of lipoproteins in relation to immunity to *B. hyodysenteriae* infections or about the role of lipoproteins in *B. hyodysenteriae* pathogenicity, however it can be assumed that the roles lipoproteins play in the biology of different spirochaetes are similar. Lipoproteins which are prominent immunogens during disease are likely targets for serological detection, and for generating protection. With this in mind, the Bhlp29.7 outer membrane lipoprotein was cloned and expressed in *E. coli* as a histidine fusion-protein. The recombinant His<sub>6</sub>-Bhlp29.7 was expressed and purified in denatured form since this allowed the protein to be produced in high quantities with few purification artefacts. At the same time subclones representing progressive one-quarter truncations of the Bhlp29.7 polypeptide were also cloned and expressed. Reactivity of these truncates with the *B. hyodysenteriae*-specific monoclonal antibody (BJL/SH1) revealed that the *B. hyodysenteriae*-specific epitope was contained at the C-terminal quarter (Bhlp-29.7-F604) of the Bhlp29.7. It was the intention that this subclone would be used for the development of an indirect serological test for detection of SD. Unfortunately, due to the inefficient expression of smaller size proteins in the *E. coli* expression system, the recombinant expression of Bhlp29.7-F604 in *E. coli* was lost and could not be recovered. Consequently, the full-length His<sub>6</sub>-Bhlp29.7 protein was used as the coating antigen for development of the ELISA test for SD. The Bhlp29.7-F604 C-terminal fragment was subsequently cloned and expressed as a MBP fusion for use as a vaccine subunit. The full-length His<sub>6</sub>-Bhlp29.7 was also used for vaccination of mice and pigs.

### 6.3. DEVELOPMENT OF THE ELISA TEST

The pig serum collected for the standardisation of the ELISA test were obtained from Australian herds which had no prior history of SD, as well as herds in which SD had been observed and the animals treated with antibiotics. The serum samples were provided by consultant veterinarians who were familiar with the clinical signs of SD. The samples were collected from slaughter-aged animals (~22 weeks) at the abattoir. Serum samples were also
obtained from several herds in which there was a history of diarrhoea but not laboratory-based diagnosis of SD had been confirmed. These samples acted as unknown samples for the application of the ELISA test once it had been standardised. In another herd, serum samples were taken from 12 to 13 week old pigs with acute clinical SD. Due to the nature of the samples obtained, the ELISA test was pitched at the herd level rather than the individual pig level. Had each serum sample been accompanied with culture data for *B. hyodysenteriae*, the ELISA test could have been evaluated for diagnosing individual infected animals.

Standardisation of the ELISA test involved two steps. Serum samples from the healthy herds (SD not observed) were tested with the ELISA and the mean and standard deviation of the ELISA results from these herds used to establish a cut-off value for distinguishing negative and positive samples. The ELISA was then tested on the infected herds (SD observed) and the relevance of the cut-off value assessed.

When a cut-off value was defined as two standard deviations above the mean ELISA value of the healthy herds (95% confidence), none of the healthy herds were deemed negative and all six (100%) infected herds were positive. Since this cut-off value gave no sensitivity, it was decided that a cut-off value of three standard deviations above the mean would be used. This gave the ELISA test a sensitivity of 100% and the specificity remained the same at 100%. For the infected herds, serum samples were collected four to six weeks after the observation of clinical signs. Antibody titres can be detected in pigs as early as four to seven days after exposure to *B. hyodysenteriae* (Joens *et al.*, 1985) and these titres can persist for up to 17 weeks (Fisher and Olander, 1981; Fernie *et al.*, 1983; Joens *et al.*, 1981b). This indicates that the timing of the sample collection may play an important role in the accurate diagnosis of SD using the ELISA test.

Application of the standardised ELISA test to the herds of unconfirmed disease status indicated that four of the eight herds had been exposed to *B. hyodysenteriae*. Regardless of the
diagnosis, confirmation of the disease status by bacteriology and PCR on faeces is still necessary. This was a major practical limitation for evaluating the ELISA test, since follow-up samples are difficult to obtain due to the herds having been depopulated or sold, or the consulting veterinarian changed. Unfortunately, it was not possible to confirm the accuracy of the ELISA test for the unknown herds by using culture and/or PCR. Future studies should include this important step.

6.3. VACCINATION WITH BHLP29.7

To elicit protective immunity to infection, it is necessary for the putative protective antigens to be immunogenic. Since Bhlp29.7 was believed to be a lipoprotein, an *E. coli* lipidation signal was cloned into the construct expressing recombinant His<sub>6</sub>-Bhlp29.7. The resulting construct successfully expressed His<sub>6</sub>-Bhlp29.7 in lipidated form. Mice vaccinated with the recombinant non-lipidated His<sub>6</sub>-Bhlp29.7 showed no significant difference in systemic antibody response to the mice vaccinated with lipidated His<sub>6</sub>-Bhlp29.7. Vaccination with non-lipidated His<sub>6</sub>-Bhlp29.7 provided mice with complete protection from *B. hyodysenteriae* colonisation. Vaccination of pigs with non-lipidated His<sub>6</sub>-Bhlp29.7 significantly improved previously reported attempts to provide protection from SD using vaccines made from whole spirochaete cells. The mechanism of the protection provided by the vaccination remains unknown and does not appear to correlate with systemic or mucosal antibody specific for *B. hyodysenteriae*. The vaccination regimes examined in this study provided some degree of protection against colonisation and disease, although neither provided complete protection. There were less total days of colonisation with the vaccinated groups than with the unvaccinated controls and colonisation tended to occur later, especially with the pigs given a parenteral and mucosal immunisation. There was also less diarrhoea in the vaccinated pigs and fewer animals had severe lesions in the colon. The vaccinated pigs which suffered diarrhoea had localised and mild lesions in the proximal colon, but were robust and clinically healthy. These pigs appeared to be showing early signs of SD, and this delay in disease onset may have been due to the immunological responses induced by the vaccination. It is possible that in the pigs vaccinated
intramuscularly and orally, the local immunity provided by the oral immunisation was unable to extend past the small intestine, hence leaving the colon relatively unprotected. However, this may also have been the case in the pigs vaccinated twice intramuscularly, since a similar severity of disease was seen. Unfortunately, no clear correlation could be made between the antibody response and protection, and it is not known whether the local antibodies detected against Bhlp29.7 in the colon of the vaccinated pigs were induced by the vaccination and/or by the experimental challenge. Further studies are required to investigate this.

Although the present protection studies shows promising efficacy, the mechanisms involved in the immunity has not been elucidated. In future studies involving the immunisation of pigs with recombinant Bhlp29.7, a more detailed investigation of the immune response of the animal to the infection may be necessary to provide more insight into the mechanisms governing the protection provided by vaccination. In particular, elucidation of cell-mediated immunity stimulated by vaccination may provide a clearer understanding of the responses involved beyond the production of antibodies. Colonisation with *B. hyodysenteriae* rarely results in invasion beyond the lamina propria, therefore it can be assumed that phagocytic activity would be of little benefit for the elimination of the invading spirochaete. However, the cells involved in cell-mediated responses (such as mucosal mast cells, neutrophils, macrophages and T-lymphocytes) have been shown to release mediators which contribute to the mucosal inflammatory processes associated with SD (Hontecillas *et al*., 2005; Jonasson *et al*., 2006). Similar damaging responses have been induced by vaccination (Olsen *et al*., 1994). In some cases, immunity appears to be antibody-mediated, since passive transfer of convalescent serum into colonic loops provides protection from *B. hyodysenteriae* infection (Joens *et al*., 1985), however antibodies detected during infection have not correlated with the occurrence of disease (Rees *et al*., 1989; Wright *et al*., 1989). The relative significance of the cell-mediated and antibody-mediated responses for recovery from infection with *B. hyodysenteriae* remains unclear.
The likely influence of the non-lipidated state of the Bhlp29.7 subunit on the immunity observed is also not known. In studies involving the *B. burgdorferi* outer surface lipoprotein (OspA), immunogenicity and immunoprotection are enhanced by lipidation (Fikrig et al., 1990; Erdile et al., 1993; Weis et al., 1994). In light of this, and despite the comparable immunogenicity of lipidated and non-lipidated Bhlp29.7 in mice, the current protection afforded by vaccination of pigs with Bhlp29.7 might be further enhanced by the immunisation with the lipidated form of the immunogen.

### 6.4. FUTURE WORK

The search for novel immunogenic and protective antigens from *B. hyodysenteriae* continues to gain interest. The application of biotechnology has resulted in the discovery of antigenic molecules for many pathogens, and these have been shown to induce protective immunity in laboratory animal models and natural hosts. Research on subunit vaccines has resulted in the design and development of some very successful vaccines. One such example is the LYMErix vaccine for Lyme disease which used the OspA outer membrane lipoprotein of *B. burgdorferi*. On-going developments for many bacterial subunit vaccines have relied on mining of complete genome sequences. The future availability of a complete or partial genome sequence for *B. hyodysenteriae* will allow similar technology to be applied to the search for an effective vaccine for SD. It is possible that additional immunogenic subunits could be used in combination with Bhlp29.7 to improve vaccine protection. The route of delivery is also extremely important, and potential methods that could result in improved protection will be summarised.

#### 6.4.1. Methods for identifying potential vaccine candidates

##### 6.4.1.1. Genomics and reverse-vaccinology

Since the commencement of the study presented in this thesis, major advances have been made in the field of vaccine development and design. Classical approaches, such as those used in this
thesis, are less common now following the technological advances of computational (in silico) analysis typical of the bioinformatics era. The high-throughput capacity of reverse-vaccinology now allows the entire genome of the organism to be mined for potential vaccine candidates. One of the forces motivating the new approach to vaccine discovery is the availability of a diverse array of full-length bacterial genomes in the public databases. As of September 2006, 976 bacterial genomes had been sequenced to completion or were currently being completed (GOLD; http://www.genomesonline.org/). Although the genome sequence for B. hyodysenteriae is currently unavailable, it soon may be given the speed at which sequencing of bacterial genomes is being undertaken. The “reverse vaccinology approach” to vaccine development takes advantage of the genome sequence of the pathogen. The genome represents a list of all the potential protein-encoding genes that the pathogen can express at any time. It becomes possible to choose any target protein in a reverse manner, starting from the genome rather than from the whole microorganism (Rappuoli, 2000; Rappuoli, 2001). Vaccine candidates can now be rapidly identified by mining the pathogen's genome with in silico tools that predict secretory signal peptides (Menne et al., 2000), transmembrane domains (Suhan and Hovde, 1998), lipoprotein attachment sites (Falquet et al., 2002) and T-cell stimulating proteins (De Groot et al., 2002). In the field of molecular biology and immunology, genomics provides a vast reservoir of genes that can be screened and tested as putative immunogens for serological assays and vaccine subunits.

6.4.1.2. DNA microarrays

Whole-genome sequence data can be further exploited to assemble DNA microarrays printed with representatives of all the predicted open-reading frames of a sequenced reference strain. Microarray technology provides a means of selecting proteins from genomic sequences that are highly relevant to immunity (Zagursky et al., 2003; Capecchi et al., 2004). Microarray-based expression studies can be used to identify genes that are differentially expressed in response to an alteration in environmental parameters and provide a strong contribution to understanding of how a pathogen orchestrates its’ responses to the host environment (Behr et
Avirulent strains of *B. hyodysenteriae* have previously been reported (Milner and Sellwood, 1994; Achacha et al., 1996). The use of differential microarrays to compare the gene content and transcriptome of virulent and avirulent strains of *B. hyodysenteriae* would be a useful first step that would limit the search to candidate immunogenic proteins of virulent strains. Gene expression studies may also offer insight into the suite of genes up-regulated (or down-regulated) during infection. Targeting the products of these genes may provide a neat method of preventing colonisation.

6.4.1.3. Proteomics

Traditionally, protein analysis has been performed by assaying one protein at a time, with very little parallel analysis. Proteomics is the large-scale study of proteins and will contribute greatly to the understanding of gene function in the post-genomic era (Grandi, 2001). In proteomic analysis, protein preparations are separated into their individual components typically using two-dimensional gel electrophoresis. Using protease digestion coupled with mass spectrometry, the identity of a protein can be determined by comparing the peptide fragmentation profile with an *in silico* predicted profile. Recently, advances in protein-separation technologies have allowed the elucidation of total protein components of a defined subcellular localisation, such as the outer membrane (Scarselli et al., 2005). Studies into the purification of proteins present on the cell surface of *Brachyspira* spp. have previously been reported (Thomas et al., 1992; Lee and Hampson, 1996; Plaza et al., 1997; Tenaya et al., 1998; Trott et al., 2001; Trott et al., 2004). The characterization of outer membrane and surface-associated proteins, in conjunction with proteomic analysis and reactivity with convalescent serum, may play a particularly important role in identifying the “antigenome” for *B. hyodysenteriae*.

6.4.2. Methods for modulating mucosal immune responses

A number of new methods have become available to improve the effectiveness of subunit vaccines for intestinal infections, and these are briefly described below.
6.4.2.1. Antigen delivery systems

An ideal vaccine should be stable and confer long lasting immunity with minimal adverse side effects in the vaccinated animal. Immunisation strategies have been developed to enhance the immunogenicity of vaccine candidates. SD is a disease of the mucosal surface of the pig colon. Traditional vaccine strategies that involve parenteral immunisation do little to prevent the initial interaction between pathogen and the mucosal surfaces (Ogra et al., 2001; Yuki and Kiyono, 2003). A great deal of attention has focused on mucosal immunisation as a means of inducing specific secretory IgA antibodies directed against the mucosal pathogen. In addition, mucosal immunisation can be an effective means of inducing not only secretory IgA, but also systemic and cell-mediated immunity. Mucosal adjuvants, bacterial vectors, bacterial ghosts DNA vaccines, and plant-derived vaccines are among the most promising antigen delivery systems for enteric diseases (Shams, 2005).

6.4.2.2. Mucosal adjuvants

The induction of immune responses following mucosal immunisation is usually dependent upon the co-administration of appropriate adjuvants that can initiate and support the transition from innate to adaptive immunity. While a number of substances of bacterial origin have been tested as mucosal adjuvants, the three bacterial products with the greatest potential to function as mucosal adjuvants are the ADP-ribosylating enterotoxins (cholera toxin (CT), produced by various strains of Vibrio cholerae, and the heat-labile enterotoxin (LT) produced by some enterotoxigenic strains of E. coli (Clements et al., 1988; Elson, 1989; Lycke et al., 1992; Xu-Amano et al., 1993; Yamamoto et al., 1996), synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides (Krieg et al., 1998), and monophosphoryl lipid A (Carozzi et al., 1989; Henricson et al., 1993; Myers et al., 1995). CT and LT both enhance the systemic and mucosal antibody responses to the co-administered antigen, resulting in a long-term memory to the antigen (Clements et al., 1988; Vajdy and Lycke, 1995). Binding of CT and LT to B-cells favours the induction of the Th2-dominated responses. The induced mucosal immune response is concentrated at the mucosal site directly exposed to the adjuvant and...
antigen (Pierce and Cray, 1982). Unfortunately the toxic effects of CT and LT means that doses used in pigs need to be evaluated since high doses can cause severe inflammation (Cox et al., 1991). Removal of the toxic subunit of CT and LT reduces the toxicity of the adjuvants at the expense of consistent results (Rappuoli et al., 1999; Yamamoto et al., 1999).

Vaccine delivery vehicles mainly assist the uptake, processing and presentation of antigens by antigen-presenting-cells, however immuno-stimulatory molecules are mostly derived from pathogens and also activate the innate immune responses. Unfortunately, in practice mucosal vaccination has often proved inefficient to stimulate strong mucosal IgA immune responses and protection (Holmgren et al., 2003a; Holmgren et al., 2003b).

6.4.2.3. Bacterial vectors
Live bacterial vectors are commensal bacteria (such as Lactobacilli and certain Streptococci and Staphylococci) or genetically attenuated pathogens (such as Salmonellae, Shigellae and Listeria species) which have been engineered to deliver antigen into mucosal inductive sites to induce antigen-specific immune responses. This can be achieved by insertion of one or more genes expressing protective antigens from heterologous pathogens into the genome of the bacterial vector (Wernerus and Stahl, 2004). Mycobacterium bovis BCG and Salmonellae have been studied as vectors for several years, and can induce solid immunity following oral administration (Barletta et al., 2000). BCG has great potential to express a large number of heterologous antigens and induce cytotoxic T-lymphocyte and mucosal immunity. Salmonella is a facultative intracellular bacteria and is able to induce secretory IgA following oral administration (Stover et al., 1991). Live attenuated bacteria are also an attractive vaccine strategy since they can mimic natural infection and have intrinsic adjuvant properties (Detmer and Glenting 2006). Examples of live bacterial vaccines available include Enterisol®Ileitis against porcine proliferative enteropathy (Lawsonia intracellularis), Vivotif® against typhoid fever (Salmonella typhi), Orochol® against cholera (Vibrio cholerae) and M-NinevaxÂ®-C against avian pasteurellosis (Pasteurella multocida). In respect to SD, live attenuated B.
hyodysenteriae strains, such as one described by Hudson et al. (1976), may be useful in conjunction with other antigen delivery systems. Unfortunately, the molecular biology of gene transfer in B. hyodysenteriae remains unclear, and no system for targeted gene insertion has been described. In addition, the major concern regarding the use of bacterial vectors derived from attenuated strains is the potential reversion to full virulence (Medina and Guzman, 2001).

6.4.2.4. Bacterial ghosts

The bacterial ghost system is a vaccine delivery system which combines targeting of the antigen components to antigen-presenting-cells with adjuvant activity without the need for complex formulation. The intrinsic adjuvant properties of the bacterial ghost enhances systemic, mucosal and cellular immunity to the target antigens (Mayr et al., 2005). Bacterial ghosts are produced by expression of cloned gene E from bacteriophage PhiX174 resulting in the cell lysis of Gram-negative bacteria. Gene E encodes a membrane protein which is able to fuse to the inner and outer membranes of Gram-negative bacteria, forming a lysis tunnel through which the cytoplasm of the cell is expelled. The empty cytoplasmic space have intact cell envelope structures devoid of cytoplasmic contents (Witte et al., 1990a; 1990b; Witte et al., 1992). Foreign target antigens can be attached to the outer membrane of bacterial ghost systems by modifying the genes encoding the outer membrane proteins to incorporate the target antigen (Hobom et al., 1995). The resulting bacterial ghosts can be used for different routes of mucosal immunisations and are not limited to parenteral modes of delivery. Bacterial ghosts have been developed for a range of Gram-negative pathogens including E. coli, Mannheimia haemolytica, Actinobacillus pleuropneumoniae, Salmonella Typhimurium, and Vibrio cholerae (Mayr et al., 2005). The exceptional adjuvant properties of bacterial ghosts makes them a desirable delivery system for B. hyodysenteriae cell surface.

6.4.2.5. Particulate delivery systems

In general, killed vaccines and subunit antigens are poorly immunogenic when administered orally. This has been attributed to antigen degradation or inefficient presentation of the antigen
at the mucosal surfaces. Studies in laboratory animals have clearly demonstrated that particulate delivery systems, such as immunostimulating complexes (ISCOMs) and liposomes, can significantly improve the mucosal antigenicity of administered antigens (Gerds et al., 2006).

ISCOMs are nanoparticles composed of saponin adjuvant, lipids and antigen which have been demonstrated to be effective antigen delivery systems due to their adjuvant activity and ability to target antigen-presenting-cells (Morein et al., 2004). The underlying mechanisms supporting the effectiveness of ISCOMs in inducing immunity are related to its ability to stimulate the innate responses and modulate antigen uptake by recruitment of antigen-presenting-cells (Smith et al., 1999; Furrie et al., 2002). When used to deliver antigens to the mucosa, ISCOMs administered orally can induce systemic, mucosal IgA, Th1 and Th2 T-cell responses (Maloy et al., 1995; Mowat et al., 1999). Studies using ISCOMs for antigen delivery have been focused on inducing immune responses in the upper respiratory tract via intranasal immunisation (Carol and Nieto, 1998; Hu et al., 1998; Abusugra et al., 1999), and little is known about their effectiveness in the proximal colon. Nonetheless, ISCOMs are potentially an attractive mucosal delivery system due to their adjuvant properties and cell targeting.

Liposomes are membrane vesicles which enhance immune responses by increasing antigen uptake and presentation (Gregoriadis, 1990). Co-administration of liposomes with adjuvants such as CT, can improve mucosal responses by improving the uptake of antigen at the mucosa. In mice, the conjugation of liposomes with CT significantly enhanced mucosal IgA and systemic IgG responses after oral immunisation (Harokopakis et al., 1998). Liposomes have also been successfully used for the oral and intranasal immunisation of chickens (Fukutome et al., 2001; Li et al., 2003), fish (Irie et al., 2003), guinea pigs (Chambers et al., 2004; Kita et al., 2005) and monkeys (Kita et al., 2005).
Particulate delivery systems appear to be a useful way of improving the mucosal immune response to soluble antigens. Although synthesis of the particles can be technically challenging, it has the advantage that no complex genetic manipulation is required for its preparation.

### 6.4.2.6. Delivery routes

The compartmentalisation within the mucosal immune system places constraints on the choice of vaccination routes available for inducing effective immune responses at the desired sites. Oral immunisation induces substantial antibody responses in the small intestine and ascending colon (Kozlowski et al., 1997; Eriksson et al., 1998; Jertborn et al., 2001). Nasal immunisation results in antibody responses in the upper respiratory tract and nasal and salivary secretions (Johansson et al., 2001; 2004). Rectal immunisation evokes strong local antibody responses in the proximal colon and rectum (Quiding et al., 1991; Kozlowski et al., 1997; Eriksson et al., 1998). Given that *B. hyodysenteriae* colonises the large intestine, rectal vaccination with putative immunogens such as Bhlp29.7 may provide better mucosal immunity than the intramuscular route.

For the vaccination of large numbers of animals on the farms, a needle-less route of vaccination may be preferred. Incorporation of the vaccine antigen in the drinking water or feed may be an alternative. Oral vaccination provided by the drinking water may not induce immunity at the proximal colon, however, and degradation of the antigen may reduce the effectiveness of the vaccine. Combining the vaccine with animal feed may provide the antigen with protection from gastric proteolysis in the stomach, however ensuring the correct dosage is consumed by each pig would be difficult to control. An alternative for both these delivery methods could be the expression of the vaccine subunit in grain.
6.4.2.7. Plant-derived vaccines

The potential applications for plants as vaccine expression vectors are immense (Streatfield and Howard, 2003a; Streatfield and Howard, 2003b; Streatfield et al., 2003; Warzecha and Mason, 2003) and they will change the fundamentals of the way antigens are generated and delivered. In veterinary vaccinology, transgenic plants can produce and deliver immunogenic antigens as animal feed, and therefore eliminate the need for trained staff for the administration of vaccines, and cold chain storage and transportation of vaccines. These benefits along with the low cost of production make plant-derived vaccines a highly attractive system for vaccine delivery. Like all other innovations, plant vaccines need to overcome certain challenges. The efficacy of orally administered antigens may not be as strong as parenteral and intranasal routes, and the effects of digestive enzymes and adsorption processes on edible antigens will need to be addressed adequately. In addition to efficacy, other issues such as the calculation of the exact dose to administer, the risk of transgenic plants entering the food chain, and the induction of immunological tolerance have highlighted unique safety concerns for plant vaccines. However, it seems that the advantages of plant vaccines surpass their disadvantages, and resolving these issues should secure commercialisation of plant vaccines as new products in the relatively near future (Dus Santos and Wigdorovitz, 2005; Koprowski, 2005; Tacket, 2005).

6.4.2.8. DNA vaccines

DNA can be injected into a host using either conventional syringes or by gene guns to inoculate DNA-coated gold beads into the muscle. DNA vaccines are very stable, and unlike most conventional vaccines they do not require a cold chain. This is of particular importance in delivering vaccines in the veterinary field. Direct immunisation with naked DNA containing genes encoding antigens leads to the uptake of the DNA and expression of the antigens. Therefore, actual production of the immunogenic antigens will take place in the host cells (Robinson and Torres, 1997). A heterologous prime-boost immunisation approach has been shown to induce strong immunity in several different animal disease models (McShane, 2002).
This strategy is based on the use of multiple delivery systems to induce immunity against the same antigen. In most cases, priming is done with the DNA vaccine and the protein subunit is used for boosting (Shams, 2005). The single reported use of DNA vaccination for SD was unsuccessful, and vaccinated animals appeared to have exacerbated disease compared to unvaccinated controls (Davis et al., 2005). The failure of this DNA vaccine to provide protection may have been due to the use of a gene encoding a non-protective antigen, and not due to the mode of delivery. Perhaps with the use of a gene encoding a more potent antigen, such as Bhlp29.7, naked DNA immunisation may prove to be a potentially useful mode of vaccination.

6.5. CONCLUDING REMARKS

In this thesis, the traditional method of small-scale proteome analysis coupled with monoclonal antibody production has enabled the identification of a potential antigen for the diagnosis and vaccination of pigs against SD. Evaluation of an ELISA tested using the recombinant Bhlp29.7 antigen suggested that this test had potential as a herd-level assay. The vaccination studies reported in this thesis indicated a reasonable degree of protection against colonisation and disease, however complete protection was not achieved. Further studies into the delivery of the antigen to the mucosal surfaces of the pig colon may improve the efficacy of the vaccine. It is possible that an effective vaccine for SD may contain a cocktail of subunits working synergistically. The identification of virulence factors and immunogenic antigens are important future steps towards designing informative diagnostic assays and potent vaccines. With explosive advances in genome sequencing, bioinformatics, and the availability of new molecular approaches such as reverse-vaccinology, proteomic analysis and DNA microarrays, it is expected that the number of putative immunogens for use in vaccines against SD will increase rapidly in the next few years.
REFERENCES


Burch, D. G. (1982). Tiamulin feed premix in the prevention and control of swine dysentery under farm conditions in the UK. *Veterinary Record* **110**: 244-246.


## APPENDIX

### A.1. Suppliers of instruments, chemicals and reagents.

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## A.2. Composition of the buffers and solutions used.

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<td>8 M urea</td>
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<td><strong>1× PCR buffer (Biotech International)</strong></td>
<td>67 mM Tris-HCl (pH 8.8)</td>
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<td>16.6 mM (NH₄)₂SO₄</td>
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<td>0.45% (v/v) Triton X-100</td>
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<td>0.2% (w/v) of gelatin</td>
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<tr>
<td><strong>PBS</strong></td>
<td>137 mM sodium chloride</td>
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<td>2.68 mM potassium chloride</td>
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<td>10 mM disodium hydrogen phosphate</td>
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<tr>
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<td>1.76 mM monobasic potassium phosphate</td>
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### A.2. Composition of the buffers and solutions used (continued).

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| PBST                       | 137 mM sodium chloride  
2.68 mM potassium chloride  
10 mM disodium hydrogen phosphate  
1.76 mM monobasic potassium phosphate  
0.05% (v/v) Tween 20  
pH 7.4 |
| 4× SDS-PAGE sample treatment buffer | 250 mM Tris-HCl (pH 6.0)  
8% (w/v) SDS  
200 mM DTT  
40% (v/v) glycerol  
0.04% (w/v) bromophenol blue |
| SDS-PAGE Separating gel    | 375 mM Tris-HCl (pH 8.8)  
12% (w/v) acrylamide  
0.31% (w/v) bis-acrylamide  
0.1% (w/v) SDS |
| SDS-PAGE Stacking gel      | 125 mM Tris-HCl (pH 6.8)  
4% (w/v) acrylamide  
0.15% (w/v) bis-acrylamide  
0.1% (w/v) SDS |
| 2× SSC                     | 30 mM tri-sodium citrate  
300 mM NaCl |
| 10× SSC                    | 150 mM tri-sodium citrate  
1.5 M NaCl |
| 1× TAE                     | 40 mM Tris-acetate  
1 mM EDTA (pH 8.0) |
### A.2. Composition of the buffers and solutions used (continued).

<table>
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<th>Buffer</th>
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| TBS    | 20 mM Tris base  
        | 500 mM sodium chloride  
        | pH 7.5 |
| TBST   | 20 mM Tris base  
        | 500 mM sodium chloride  
        | 0.05% (v/v) Tween 20  
        | pH 7.5 |
| TE     | 10 mM Tris-HCl  
        | 1 mM EDTA  
        | pH 8.0 |
| Transfer buffer (Electro-transfer) | 25 mM Tris base  
| | 192 mM glycine  
| | 20% v/v methanol  
| | pH 8.3 |
| Tris-glycine buffer | 25 mM Tris  
| | 192 mM glycine  
| | 0.1% (w/v) SDS  
| | pH 8.3 |
| Washing buffer (Southern blot) | 100 mM maleic acid  
| | 150 mM NaCl  
| | 0.3% (v/v) Tween 20  
| | pH 7.5 |
A.3. Strains of *Brachyspira* spp. used for the distribution analysis of *bhlp29.7*.

<table>
<thead>
<tr>
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A.3. Strains of *Brachyspira* spp. used for the distribution analysis of *bhlp29.7* (continued).

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A.3. Strains of *Brachyspira* spp. used for the distribution analysis of *bhlp*29.7 (continued).

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A.3. Strains of *Brachyspira* spp. used for the distribution analysis of *bhlp29.7* (continued).

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A.3. Strains of *Brachyspira* spp. used for the distribution analysis of *bhlp29.7* (continued).

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<td>Stanton <em>et al.</em>, 1998</td>
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<td>C2</td>
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<td><em>B. aalborgi</em></td>
<td>513&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Human</td>
<td>Denmark</td>
<td>Hovind-Hougen <em>et al.</em>, 1982</td>
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<td><em>“B. canis”</em></td>
<td>D15</td>
<td>Dog</td>
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<td>Duhamel <em>et al.</em>, 1998</td>
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