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Characterisation of *in vivo* expressed proteins of *Pasteurella multocida*

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SUMMARY

An *in vivo* expression technology (IVET) system was previously developed and used to identify *Pasteurella multocida* genes which are up-regulated during infection of the host. Of the many genes identified, two encoded products which showed similarity to the *Haemophilus influenzae* lipoproteins, protein D and PCP, while another gene encoded a putative zinc finger protein.

In *H. influenzae*, protein D and PCP have been shown to stimulate heterologous immunity against infection with *H. influenzae*. Therefore, in this study, the lipoprotein homologues in *P. multocida*, designated GlpQ and PCP, were investigated. Using real-time reverse transcription PCR, it was found that *glpQ* was not significantly up-regulated in *P. multocida* during growth in mice. However, when *P. multocida* is grown in chickens, *glpQ* expression was shown to vary in individual animals; in *P. multocida* grown in one chicken, *glpQ* was not up-regulated, while in another chicken, *glpQ* was up-regulated 1.43-fold. Using the same technique, *pcp* was found to be down-regulated when *P. multocida* was grown in chickens. GlpQ and PCP were shown to be lipoproteins by demonstrating that post-translational processing of the proteins was inhibited by globomycin. The *P. multocida* GlpQ homologue showed glycerophosphodiester phosphodiesterase enzyme activity, indicating that it is a functional homologue of other characterised GlpQ enzymes. Using surface immunoprecipitation, PCP was found to be surface exposed, but GlpQ was not. Non-lipidated forms of GlpQ and PCP were expressed and purified from *E. coli* and used to vaccinate mice. However, mice were not protected from challenge with live *P. multocida*. The lipoproteins were then expressed in *E. coli* in the lipidated form and used to vaccinate mice and chickens. Protection against challenge with live *P. multocida* was not observed. Attempts to insertionally inactivate *glpQ* were unsuccessful and therefore, the role of GlpQ in virulence could not be elucidated.

The gene encoding the putative zinc finger protein (designated *pzfA*) was confirmed to be up-regulated *in vivo* using real-time reverse transcription PCR. Analysis of the PzfA amino acid sequence revealed that PzfA had similarity to the DNA-binding region of topoisomerase I from a variety of organisms. Zinc finger proteins are often involved in transcriptional regulation and therefore, PzfA was

overexpressed in *P. multocida* and whole genome microarray analysis was performed to determine the effects on global transcription. 10 genes were found to be up-regulated while 22 were down-regulated. Due to time constraints, it was not determined whether the differentially expressed genes were due to a direct or indirect effect of PzfA overexpression. Attempts to inactivate *pzfA* by allelic exchange were unsuccessful, and therefore, the function of PzfA has yet to be determined.

STATEMENT

I, Miranda Lo, declare that this thesis contains no material which has been accepted for the award of any degree or diploma in any other University and, to the best of my knowledge and belief, contains no material which has been previously published or written by another person, except where due reference is made in the text of the thesis.

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(Miranda Lo)

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PUBLICATIONS

Cullen, P.A., **Lo, M.**, Bulach, D.M., Cordwell, S.J., and Adler B. (2003). Construction and evaluation of a plasmid vector for the expression of recombinant lipoproteins in *Escherichia coli*. *Plasmid* **49**, 18-29.

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Lo, M., Doughty, S., and Adler, B. (1999). Cloning and characterisation of a putative regulatory zinc finger protein in *Pasteurella multocida*. *ACIAR meeting – Pasteurella and pasteurellosis, University of Queensland, Brisbane, Queensland, Australia.*

Lo, M., Boyce, J. D., and Adler, B. (1999). An *in vivo* expressed lipoprotein in *Pasteurella multocida*. *Bacterial Pathogenesis Conference, Department of Microbiology, Monash University, Victoria, Australia.*

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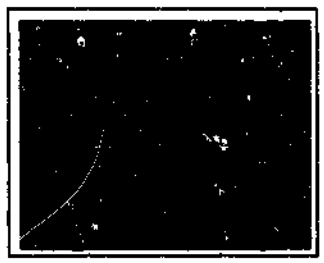
Adler, B., Boyce, J. D., Hunt, M., Boucher, D., Harper, M., **Lo, M.**, Wilkie, I., Townsend, K., Kapur, V., and Paustian, M. (2002). Molecular approaches to understanding pathogenesis of *Pasteurella multocida*. *International Pasteurellaceae Conference, Banff, Canada.*

CHAPTER ONE

INTRODUCTION



Cross-section of *P. multocida*
X-73, × 100,000 magnification.

PASTEURELLA MULTOCIDA

THE ORGANISM

P. multocida is an important animal pathogen of major economical significance in the poultry, cattle and swine industries. It is often found as part of the normal oral flora in a range of animal species. Many types of animals can be infected with the organism without severe consequences, and thus, may serve as the source of infection when they come into contact with livestock. In susceptible animals, *P. multocida* can spread rapidly, causing high mortality in a population of livestock (Matsumoto & Strain, 1993).

Taxonomy

P. multocida is a member of the family *Pasteurellaceae*, which also includes bacteria of the genera *Haemophilus*, *Actinobacillus*, *Lonepinella* (Osawa *et al.*, 1995), *Mannheimia*, following the reclassification of *Pasteurella haemolytica* (Angen *et al.*, 1999) and the recently proposed *Gallibacterium* (Christensen *et al.*, 2003). *P. multocida* is the type species for the genus *Pasteurella sensu stricto*. Three subspecies (subsp.) have been identified based on DNA hybridisation studies (Mutters *et al.*, 1985). These are *P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica*, and *P. multocida* subsp. *gallicida*.

Genome sequence

The *P. multocida* A:1 genome is approximately 2.35 Mbp in length (Hunt *et al.*, 1998). Mannheim (1984) has reported that the G+C content falls in the range of 38-47%, while a similar estimate of 40-45% has also been made (Lo & Shewen, 1992). The complete genome sequence of the *P. multocida* strain PM70 was recently determined and was found to consist of a single circular chromosome of 2,257,487 bp (May *et al.*, 2001). The overall G+C content was 41%. The sequence has been annotated to encode 2,014 potential open reading frames (ORFs), accounting for 89% of the entire genome, 6 rRNA operons and 57 tRNAs. Sequence comparisons

identified 200 (10%) coding sequences which were unique to *P. multocida* (May *et al.*, 2001).

Morphology

The organism is a Gram negative, non-sporulating, non-motile, facultatively anaerobic and fermentative coccobacillus. Grown under aerobic conditions at 37°C on enriched agar media, three colony variants of *P. multocida* can develop, depending on the amount of capsule produced: mucoid, rough and smooth (Carter, 1967). The colonies range from approximately 1.0-3.0 mm in diameter. Mucoid colonies are produced by capsulated strains which belong primarily to serogroups A and D. Rough colonies are formed by noncapsulated, filamentous cells (Carter, 1967; Rimler & Rhoades, 1989). Using obliquely transmitted light, smooth colonies can be iridescent or noniridescent, with the latter consisting of noncapsulated cells. *P. multocida* is generally non-haemolytic under aerobic conditions.

Biochemical characteristics

Strains of *P. multocida* are indole, catalase, oxidase and ornithine decarboxylase positive, but are methyl red and Voges-Proskauer negative. They do not possess urease or gelatinase. *P. multocida* can ferment carbohydrates, resulting in production of acid, but not gas. Sugars which are commonly fermented include glucose, mannose, galactose, fructose and sucrose. The organism is unable to ferment lactose, and cannot grow on MacConkey agar (Namioka, 1978; Rimler & Rhoades, 1989).

Growth requirements

P. multocida can be cultured at temperatures ranging from 22-44°C, with 35-37°C being optimal for growth under aerobic conditions. A number of growth factors, nicotinamide, pantothenic acid and thiamine, is required (Jordan, 1952, cited by Rimler and Rhoades, 1989), while cysteine is the only essential amino acid. Oleic acid added to chemically defined media increases the rate and amount of growth. Addition of yeast extract to various chemically undefined media provides essential nutrients for growth. Media enriched with blood or 5% inactivated serum can further enhance growth.

Blood agar is used in the initial isolation of the organism, but colonial variants are not distinguishable (Carter, 1955). Difco-tryptose agar, Gibco-dextrose agar, or YPC (yeast extract, proteose-peptone, glucose and cysteine) agar can be used to differentiate between colonial variants (Namioka & Murata, 1961a; Rimler & Rhoades, 1989). Moore *et al.* (1994) used selective enrichment media which increased sensitivity for detection of low numbers of *P. multocida* from wild birds and environmental samples.

TYPING OF *P. MULTOCIDA*

P. multocida causes a range of diseases in various animal hosts. Therefore, there was a need for a typing system to correlate *P. multocida* isolates with host predilection and specific disease syndromes. Methods which have been used include biotyping, serological typing and molecular typing.

Biotyping

Investigators have made attempts to classify *P. multocida* isolates according to their biochemical characteristics such as fermentation of carbohydrates and polyhydric alcohols (Carter, 1976; Fegan *et al.*, 1995; Rimler & Rhoades, 1989). Australian poultry and porcine isolates have been classified using this method (Blackall *et al.*, 1997; Fegan *et al.*, 1995). However, this approach does not seem adequate to provide a correlation between biotypes and virulence or clinical disease, although it may be useful for epidemiological tracing purposes.

Serological typing

Several serological tests have been developed for *P. multocida*, but not all are currently in use. Little and Lyon (1943) used a slide agglutination test and passive mouse protection test (PMPT) which identified three serotypes, 1, 2 and 3. Using the PMPT method, Roberts (1947) grouped 37 isolates into four serotypes, I, II, III and IV.

A capsular typing system based on indirect haemagglutination tests was first developed by Carter (1955; 1961). In this typing system, surface antigen extracts are

adsorbed onto human group O erythrocytes, which are then tested for agglutination with rabbit antisera. As a result, Carter (1955; 1961) identified four serogroups, A, B, D and E. Using the same system, a fifth serogroup, designated F, was later identified by Rimler and Rhoades (1987). However, there are limitations to this type of system, such as the difficulty in inducing antibodies to capsular antigens, where inert capsule material, for example, hyaluronic acid, may interfere with antigen recognition. Therefore, non-serological tests were devised for some strains. For serogroup A, the test was based on depolymerisation of the capsule by a hyaluronidase-producing strain of *Staphylococcus aureus* (Carter & Rundell, 1975) while serotype D can be identified by flocculation with acriflavine (Carter & Subronto, 1973).

The two most commonly used somatic serotyping systems are the tube agglutination test (Namioka & Murata, 1961b) and the gel diffusion precipitin test (Heddleston *et al.*, 1972). The method devised by Namioka and Murata (1961b) used rabbit antisera and HCl-treated bacterial suspensions. Eleven somatic serotypes (1-11) were identified. However, this method had problems due to autoagglutination arising from HCl treatment of the cells.

The somatic antigen typing system developed by Heddleston *et al.* (1972) used heat stable antigens and antisera raised in chickens. This method identified sixteen somatic serotypes (1-16). The major component of the heat stable antigen responsible for the type specificity was shown to be lipopolysaccharide (LPS) (Brogden & Rebers, 1978; Ramdani & Adler, 1991; Rimler *et al.*, 1984).

Due to the complexity and nature of the antigens involved in the serotyping tests, the serotypes of one system do not correlate with those of other systems, although there is some overlap (Brogden & Packer, 1979). To standardise classification of isolates and use of the serotyping systems, it was recommended that *P. multocida* be identified by the Carter capsular serogroups (A, B, D, E and F) followed by the Heddleston somatic serotypes (1-16) (Carter & Chengappa, 1981). This method of classification has been used to correlate serotypes with specific disease, for example, fowl cholera (FC) is primarily caused by serotypes A:1, A:3 and A:4 (Carter & Chengappa, 1981; Hofacre & Glisson, 1986). Of less frequent usage is the classification system using the Namioka somatic antigens followed by the Carter serogroups. Using this system, the common FC causing strains are designated 5:A,

8:A and 9:A (Namioka, 1978). However, some strains have been found to be untypeable using serological methods (Manning, 1982).

Molecular typing

Molecular based typing techniques have been developed which overcome many of the limitations of phenotype- and serotype-based typing schemes. Methods such as restriction endonuclease analysis (REA), ribotyping, pulsed-field gel electrophoresis (PFGE) and polymerase chain reaction (PCR) fingerprinting are highly discriminatory and reproducible and have been useful in epidemiological tracing and the identification of field isolates.

REA, used on its own or in conjunction with ribotyping, has shown a high level of diversity among *P. multocida*, and isolates of similar serotypes can be differentiated due to distinctive banding profiles (Blackall *et al.*, 1995; Snipes *et al.*, 1989; Wilson *et al.*, 1992). PFGE and repetitive extragenic palindromic sequence based PCR (REP-PCR) are comparable in their ability to differentiate between avian and swine isolates of *P. multocida* (Gunarwardana *et al.*, 2000; Townsend *et al.*, 1997; Townsend *et al.*, 1998b; Townsend *et al.*, 2000). Both PFGE and REP-PCR show a high level of differentiation between isolates, with the latter being a less laborious procedure.

PCR based assays have facilitated rapid detection and identification of *P. multocida* directly from clinical samples. A PCR assay has been developed using 23S rRNA-specific primers which was able to correctly identify 144 avian and porcine isolates of *P. multocida* tested (Miflin & Blackall, 2001). Phylogenetically unrelated avian and porcine organisms tested negative with this assay. By using genomic subtractive hybridisation, Townsend *et al.* (1998a) identified a DNA sequence which is unique to *P. multocida* and used this as the basis of a *P. multocida* specific PCR test. Strains of *P. multocida* can also be differentiated using PCR technology. Serogroup B-specific sequences have been identified and used in the development of a type B-specific PCR assay (Brickell *et al.*, 1998; Townsend *et al.*, 1998a). Nucleotide sequence analysis of the capsule biosynthetic loci from each of the five capsular groups revealed serogroup-specific regions, allowing development of a multiplex PCR capsular typing system (Townsend *et al.*, 2001).

Other molecular typing methods include multi-locus enzyme electrophoresis (MLEE), multi-locus sequence typing (MLST) and amplified fragment length polymorphism (AFLP) (Amonsin *et al.*, 2002; Blackall & Miflin, 2000). MLEE is actually not a genotypic method but a phenotypic one which looks at the variation in the electrophoretic mobility of water-soluble enzymes (Blackall & Miflin, 2000). MLST is the genotypic equivalent of MLEE as the DNA sequences of the proteins are used to determine the genetic variation between the enzymes (Blackall & Miflin, 2000). AFLP has been shown to be rapid with a high level of discrimination and reproducibility (Amonsin *et al.*, 2002).

A comparison of five typing methods (REA, ribotyping, PFGE, REP-PCR and MLEE) showed strong correlation, but there was a lack of total agreement indicating that at least two different typing methods should be used for epidemiological studies (Blackall & Miflin, 2000).

IN SITU DETECTION OF *P. MULTOCIDA*

A *P. multocida* species-specific probe which targets 16S rRNA has been designed, enabling detection of *P. multocida* in the lung tissues of infected chickens and pigs by fluorescent in situ hybridisation (FISH) (Mbuthia *et al.*, 2001). The FISH test was shown to be a rapid and reproducible method for specific detection of *P. multocida* in histological formalin-fixed tissues and could be used as a supplementary test for diagnosis or as a tool in studying the pathogenesis of *P. multocida*. The major advantage of this test is that there is no need to obtain pure cultures.

DISEASES CAUSED BY *P. MULTOCIDA*

P. multocida is one of the most important bacterial pathogens of domestic animals, which can cause major economic losses for livestock producers globally (Donachie, 1992). Host specificity and pathogenicity depend on the strain of *P. multocida*. In cattle, haemorrhagic septicaemia (HS) is predominantly caused by

serotypes B:2 and E:2 (Bain *et al.*, 1982; Penn & Nagy, 1976), while respiratory diseases are generally associated with serogroup A (De Alwis, 1992). Atrophic rhinitis (AR) in swine is caused by toxigenic strains of *P. multocida* belonging to serogroups A and D (De Alwis, 1992). As stated previously, isolates of *P. multocida* which commonly cause FC in domestic and wild birds are serogroup A, serotypes 1, 3 and 4 (designated A:1, A:3 and A:4).

P. multocida is a ubiquitous organism and can be found as part of the normal oral or nasopharyngeal flora of many domestic animals (Lo & Shewen, 1992; Rimler & Rhoades, 1989; Smith, 1955). Humans usually become infected with the organism following animal bites or scratches (Weber *et al.*, 1984). *P. multocida* can cause a range of infections in humans, including cellulitis, septicaemia, osteomyelitis, pneumonia, endocarditis, necrotising fasciitis, and meningitis (Boerlin *et al.*, 2000; Griego *et al.*, 1995; Hamamoto *et al.*, 1995; Hombal & Dincsoy, 1992). Urinary tract infections have also been reported although these are extremely rare (Liu *et al.*, 2003).

FOWL CHOLERA

Epidemiology

FC is a disease of domestic and wild birds which has been recognised since the late 1700s (Carpenter *et al.*, 1988). *P. multocida* was first identified as the causative agent of fowl cholera in the early 1880s and a laboratory attenuated strain was used as the first live bacterial vaccine (Pasteur, 1880; Pasteur, 1881). High morbidity and mortality rates (Carter, 1967) have made FC an economically significant disease. In 1985, FC was ranked as one of the top two diseases affecting the US turkey industry (Carpenter *et al.*, 1988). The disease has a worldwide distribution, with most countries experiencing sporadic outbreaks which vary in location and prevalence (Rhoades & Rimler, 1989). The degree of susceptibility to FC has been found to vary depending on the age and type of bird. Free range chickens, which are often subclinically infected with *Ascaridia galli*, may have an increased risk of FC outbreaks (Dahl *et al.*, 2002). All commercially important poultry (turkeys, chickens, ducks and geese) are affected, but wild birds can also suffer outbreaks of FC (Rhoades & Rimler, 1989).

Transmission

The transmission of FC between and within flocks is not completely understood. It has been proposed that rodents, dogs, cats, wild birds and humans may act as reservoirs, facilitating the spread of the organism (Bickford, 1986). Contaminated water and food could serve as a source of infection, as it has been found that toxigenic strains of *P. multocida* are able to survive in water (Glisson, 1992; Thomson *et al.*, 1992). Cannibalism of dead birds may also lead to dissemination of *P. multocida* within a flock (Bickford, 1986).

Pathogenesis and symptoms

The mechanisms involved in the pathogenesis of FC are poorly understood. It is believed that *P. multocida* enters via the upper respiratory tract and multiplies, gradually spreading to the lower respiratory tract. Colonisation of the respiratory tract by invasive *P. multocida* is thought to be the first step in the disease (Lee *et al.*, 1994). In the majority of turkeys infected with *P. multocida*, the organisms from the respiratory tract invade the circulatory system and multiply rapidly in the liver and spleen (Matsumoto *et al.*, 1991). This may aid in the spread of the organism into the bloodstream, leading to septicaemia.

FC can occur as either a chronic or acute infection. High mortality rates are associated with acute FC, which is characterised by debilitating pneumonia followed by spleen and liver necrosis (Lee *et al.*, 1994). Other symptoms include anorexia, ruffled feathers, depression, diarrhoea, and nasal or oral discharge of mucus (Bickford, 1986; Rhoades & Rimler, 1991; Rimler & Rhoades, 1989). Acute septicaemia, which is characterised by disseminated intravascular coagulation, can also occur (Confer, 1993).

Chronic FC is manifested as localised infections in the wattles, sinuses, air sacs, footpads and joints. A fibrinopurulent exudate and necrosis can be seen at these sites. Lesions can occur in almost any organ, but most often in the lungs, wattles and air sacs (Rhoades & Rimler, 1991; Rimler & Rhoades, 1989). Oesophageal abscesses have been noted (Morishita *et al.*, 1997). Birds may also have pharyngitis and conjunctivitis. Chronic FC usually has a low mortality rate, but can lead to the persistence of the pathogen within flocks, creating a potential reservoir for future outbreaks.

TREATMENT AND PREVENTION OF FOWL CHOLERA

Use of antibiotics

Antibiotics are used in the management of *P. multocida* infections to reduce mortality rates, but subsequent outbreaks of FC can occur following termination of antimicrobial treatment. Use of antibiotics may have undesirable consequences such as side effects and in the longer term, emergence of resistance. A study by Reece and Coloe (1985) showed that 44% of avian isolates were resistant to tetracycline. Sulphonamides and antibiotics such as penicillins and streptomycin have also been used to treat FC. However, Kehrenberg and Schwarz (2001) found that genes encoding sulphonamide and streptomycin resistance were widely distributed among epidemiologically unrelated isolates of the genera *Pasteurella* and *Mannheimia*. Poultry isolates of *P. multocida* have been shown to be susceptible to danofloxacin, a novel fluoroquinolone, which may be an efficacious alternative in the control and treatment of FC (Raemdonck *et al.*, 1992). However, as with any drug, the stage of disease, bird species and side effects must be taken into account before proceeding with treatment (Rhoades & Rimler, 1989).

Vaccines

Despite many years of research, traditional vaccine and medication practices have had little success in controlling *P. multocida* infections in poultry. A safe and effective vaccine would be ideal, especially with the rise in antibiotic resistance. There are currently two types of vaccines available, inactivated (or killed) and live.

Inactivated vaccines, or bacterins, for FC contain *P. multocida* serotypes 1, 3 or 4, the serotypes which commonly cause the disease (Glisson, 1992). However, bacterins provide only homologous protection against the serotypes contained in the bacterin. In addition, the benefit of bacterin vaccines in reducing the incidence of fowl cholera outbreaks is dubious (Carpenter *et al.*, 1988). An epidemiological study by Carpenter *et al.* (1988) found that flocks which were vaccinated with bacterins had an increased risk of developing a subsequent fowl cholera outbreak. Therefore, bacterin vaccination alone does not appear to offer protection against fowl cholera outbreaks. Furthermore, in experimental infections, bacterin vaccines were shown to

be of no value in stimulating immunity to infection (Ireland and Adler, unpublished data; Wilkie and Frost, personal communication).

Live vaccines have an advantage over bacterins in that some degree of cross-protection against heterologous serotypes can be induced (Confer, 1993). Chemically altered strains of *P. multocida* have been generated, which were sufficiently attenuated for use as live vaccines, and elicited a high degree of protection (Kucera *et al.*, 1981; Wei & Carter, 1978). The Clemson University (CU) strain of *P. multocida* is a naturally occurring strain of relatively low virulence. It has been widely used as a live vaccine in the poultry industry (Hofacre & Glisson, 1986). However, the CU strain has the tendency to cause side effects such as swollen wattles, swollen joints and mortality (Glisson, 1992). Vaccinating previously unvaccinated breeder hens with the CU strain has been linked with a decrease in egg production, and cold temperatures can increase the susceptibility of laying breeder hens to infection by the CU strain (Schlink & Olson, 1987). More attenuated strains of CU have been developed for use as vaccines, for example, M-9 and PM-1 (Glisson, 1992). However, it was later found that the PM-1 vaccine was no safer than the CU strain (Hopkins & Olson, 1997). It has also been shown that vaccination with live avirulent FC vaccines can be detrimental to body weight gain (Hopkins & Olson, 1997).

Of major concern is the fact that an increased incidence of FC has been associated with increased use of vaccines derived from the CU strain (Hofacre & Glisson, 1986). Since the genetic basis of attenuation is undefined, reversion to virulence is not uncommon with CU-derived vaccine strains, and outbreaks of FC have occasionally followed the use of these live vaccines. Therefore, attempts have been made to develop genetically defined attenuated vaccines. Examples include *aroA* and *galE* mutants.

Biosynthetic genes, such as *aroA*, are essential for the bacteria to survive and grow *in vivo* and thus cause disease. The *aroA* gene is required for the synthesis of aromatic amino acids, which are found at low levels *in vivo*. As a result, *aroA* mutant strains of *P. multocida* have a decreased ability to survive and/or multiply in the host and are therefore less virulent (Homchampa *et al.*, 1992). Mice immunised with *aroA* mutants of *P. multocida* were protected against infection by heterologous strains (Homchampa *et al.*, 1997), thus emphasising the potential of these mutants as vaccine candidates. Two *aroA* mutants, designated PMP1 (derived from X-73, an A:1 strain)

and PMP3 (derived from P-1059, an A:3 strain), have been tested as vaccine candidates in chickens (Scott *et al.*, 1999). Both were shown to be significantly attenuated and provided cross-protection against challenge with PM206, a serotype 4 strain. Low, but protective, dose levels of the mutant strains were shown to be safe, as there were no clinical signs or lesions, and the strains were unable to be re-isolated 24 hours after vaccination. Further work is being undertaken to evaluate the extent of cross-protection and durability of the vaccine (Scott *et al.*, 1999). The *aroA* gene has also been inactivated in a bovine strain of *P. multocida* (Tabatabaei *et al.*, 2002). These mutants were found to be highly attenuated for virulence in a mouse model of HS. Mice which were vaccinated intraperitoneally or intranasally with the *aroA* mutants were completely protected against challenge with a live B:2 strain of *P. multocida* (Tabatabaei *et al.*, 2002).

The *galE* gene product is required for the metabolism of galactose. Fernandez de Henestrosa *et al.* (1997) showed that *galE* mutants were highly attenuated and thus may also be potential candidates for a vaccine.

IMMUNITY TO *P. MULTOCIDA* IN FOWL CHOLERA

Immunity is generally serotype specific (Adler *et al.*, 1996) and somatic antigens, in particular lipopolysaccharide (LPS), appear to play a major role in immunity (Confer, 1993). However, birds which survive a *P. multocida* infection develop a protective immune response against heterologous serotypes (Glisson & Cheng, 1991). The precise mechanisms of immunity are as yet unknown and the specific antigens involved in immunity are also not clearly identified.

Humoral immunity appears to be of major importance in pasteurellosis. Antisera against *in vivo* grown *P. multocida* were shown to provide passive protection against heterologous serotypes in turkeys (Rimler, 1987). Other studies have shown that anti-LPS antibodies were able to opsonise homologous *P. multocida* for phagocytosis by mouse macrophages (Collins *et al.*, 1983; Ramdani & Adler, 1991), but no solid protection was observed. This may be due to *P. multocida* surviving inside avian mononuclear phagocytic cells. The organism also appears to be resistant to intracellular killing mechanisms (Truscott *et al.*, 1990). Truscott *et al.* (1990)

suggested that the inhibition of phagocytosis and killing by phagocytes was due to a 50 kDa toxic outer membrane protein, as these effects were almost completely neutralised by an antibody specific for the protein. The mechanisms used by *P. multocida* for intracellular survival are as yet unknown.

Complement itself may play a role in the initial clearance of *P. multocida* from the circulation by the initiation of the alternative complement pathway (Snipes & Hirsh, 1986). Complement alone seems to be adequate for opsonisation and killing of less virulent strains, while complement and specific antibodies are required for macrophage bactericidal activity against the more virulent, heavily encapsulated strains (Harmon *et al.*, 1992). Wijewardana and Sutherland (1990) found that both complement and specific antibodies were essential components of protective antisera, thus indicating that bactericidal activity was due to the classical complement pathway or perhaps complement-enhanced opsonophagocytosis.

Cell-mediated immunity may also play a role in immunity against *P. multocida* in fowl cholera. It has been shown that activation of macrophages by T cells results in enhanced bactericidal activity, leading to protection against fowl cholera in chickens (Baba, 1984). Thus, T lymphocytes may play a role in immunity to *P. multocida*. However, the fact that immunity can be transferred by immune serum suggests that humoral immunity is of prime importance.

ANTIGENS INVOLVED IN PATHOGENESIS AND IMMUNITY

Lipopolysaccharide

It is known that LPS is a major factor responsible for the pathogenesis of severe Gram-negative infections and inflammation (Jacques, 1996). The LPS of *P. multocida* has been described as being similar to the semi-rough LPS of *Enterobacteriaceae* (Manning *et al.*, 1986). The role of LPS in immunity to FC remains uncertain, especially due to the difficulty in purifying LPS which is free of contaminating protein or polysaccharide. LPS alone appears to have a limited role in immunity. A monoclonal antibody specific for serotype A LPS was shown to completely protect mice against homologous and heterologous challenge, by promoting complement-mediated killing (Wijewardana *et al.*, 1990). However, this

finding was not supported by other studies. Ramdani and Adler (1991) found that mice immunised with LPS alone (from an HS-causing B:2 strain) were not completely protected from infection. Another study showed that mice were not protected against *P. multocida* infection by passive transfer of affinity-purified rabbit anti-LPS serum (Lu *et al.*, 1991a). Tsuji and Matsumoto (1988) found that an LPS-protein complex was essential for inducing immunity to *P. multocida* in turkeys. Ficken *et al.* (1992) found that cell free culture filtrate which did not contain LPS was unable to protect turkeys from FC. Therefore, it seems that LPS may play only a minor role in immunity to FC, and other antigens are also important. As LPS is the basis upon which isolates can be grouped into the 16 Heddleston somatic serotypes, it is doubtful that LPS would contribute to cross protective immunity.

However, LPS may contribute to pathogenesis in FC. In turkeys, cell free culture filtrates containing LPS were found to induce pathological changes in the airsacs, which were similar to changes caused by *P. multocida* cultures (Ficken & Barnes, 1989; Ficken *et al.*, 1991). Other studies found that LPS caused localised inflammatory responses, microscopic lesions, vasculitis and thrombosis in turkeys (Mendes *et al.*, 1994; Rhoades & Rimler, 1987). Therefore, LPS may initiate inflammatory responses in FC. Recently, using signature-tagged mutagenesis (see page 24), a mutant was identified with an insertional inactivation of a gene encoding a heptosyl transferase which is required for the addition of heptose to LPS (Harper *et al.*, 2003). The mutant was found to be attenuated for virulence in chickens. Further investigations are required to ascertain what role(s) LPS may play in pathogenesis and immunity to *P. multocida*.

Capsule

The polysaccharide capsule of *P. multocida* has long been implicated in virulence. In serogroup A strains, the capsule is composed of hyaluronic acid (Carter, 1967), while the capsules of serogroup B and E strains consist of as yet unidentified anionic polysaccharides (Rimler & Rhoades, 1989). Serogroup D and F have been found to contain heparin and chondroitin respectively (DeAngelis & Padgett-McCue, 2000; DeAngelis *et al.*, 2002; Rimler, 1994). The capsule forms the outermost surface of the bacteria, and inhibits the bactericidal and opsonic activity of normal serum (Inzana, 1990). Heddleston *et al.* (1964) found that encapsulated avian strains

are generally more virulent and resistant to phagocytosis. Harmon *et al.* (1991) suggested that poor phagocytic uptake of *P. multocida* may be due to inhibition of opsonisation or physical interference of receptor-ligand binding between opsonised bacteria and phagocytes. Removal of the capsule renders *P. multocida* more sensitive to complement (Snipes & Hirsh, 1986) and bactericidal activity of chicken macrophages (Poermadjaja & Frost, 2000). However, although the loss in ability to form a capsule results in loss of virulence, immunogenicity is retained. This suggests that antibodies to capsular material may play a limited role in protection against pasteurellosis.

The capsule biosynthetic locus of *P. multocida* A:1 has been cloned and sequenced (Chung *et al.*, 1998). The locus consists of three regions. Region 2, which is bounded by two regions (1 and 3), encodes enzymes which synthesise and assemble the hyaluronic acid capsule. Region 1 genes encode proteins involved in exportation of the capsule to the cell surface, while Region 3 encodes products which may be involved in phospholipid substitution and anchoring of the capsule to the outer membrane. A genetically defined acapsular mutant has been constructed in a serogroup A strain which has helped clarify the role of the serogroup A capsule in virulence and immunity to FC (Chung *et al.*, 2001). The *hexA* gene, predicted to be involved in capsule transport, was insertionally inactivated and the resulting mutants were shown to be attenuated in both mice and chickens. The acapsular mutant was also susceptible to the bactericidal activity of avian serum whereas the parent was resistant.

The serogroup B capsule locus has also been cloned and sequenced (Boyce *et al.*, 2000). Its organisation was found to differ from that of the serogroup A locus, and there is also relatively low nucleotide sequence similarity between some of the orthologues. The serogroup B capsule also has been found to play a significant role in virulence. The *cexA* gene, which encodes a capsule transport protein, was targeted for mutagenesis. The resulting acapsular *cexA* mutants were readily cleared from the blood, spleen and liver of mice, while wild-type bacteria multiplied rapidly (Boyce & Adler, 2000). The acapsular mutants were also highly susceptible to phagocytosis compared to the wild-type bacteria. Another serogroup B acapsular mutant was constructed by insertional inactivation of the *bcbH* gene which encodes a product predicted to be involved in polysaccharide biosynthesis (Boyce & Adler, 2001).

Immunisation of mice with live *bcbH* mutants conferred significant protection against wild-type challenge. Therefore, the capsule is an important virulence determinant in pasteurellosis, but protection against wild-type challenge can be afforded in the absence of capsular polysaccharide. However, avirulent capsulated avian strains have been isolated by Brogden and Rebers (1978), and some acapsular strains have been found to be highly virulent (Matsumoto & Strain, 1993) which suggests that there are other virulence determinants.

Outer membrane proteins (OMPs)

Various OMPs of *P. multocida* have been implicated as potential protective antigens. Several *P. multocida* OMPs have been shown to be immunogenic in rabbits. Passive immunisation using antisera against these OMPs was shown to protect against subsequent homologous challenge with *P. multocida* (Lu *et al.*, 1988a). OMPs may play roles such as adhesion, iron or nutrient uptake and evasion of the host immune response. Therefore, due to their role in virulence and/or immunity, their potential as vaccine candidates warrants further investigation. Several *P. multocida* OMPs have been characterised.

A 50 kDa protein was identified which inhibited the ability of the phagocytes to ingest and kill the bacteria (Truscott & Hirsh, 1988). Antibodies specific for this protein neutralised the anti-phagocytic effects of *P. multocida*, and passive immunisation with these antibodies protected turkeys against a lethal challenge with *P. multocida.*

A 16 kDa protein with sequence homology to the P6 protein of *H. influenzae* has also been isolated and there is evidence that this protein induces a protective immune response in animal models (Kasten *et al.*, 1995). The P6 homologue may have potential as part of a vaccine, as this protein is conserved and expressed by all somatic serotypes of *P. multocida.*

An outer membrane protein, which has been designated Oma87 (87 kDa outer membrane antigen), has been identified, which is conserved amongst all 16 serotypes of *P. multocida* (Ruffolo & Adler, 1996). This protein appears to be expressed during growth in chickens, and is exposed on the cell surface. Rabbit antiserum against Oma87 was shown to passively protect mice against homologous lethal *P. multocida* challenge, suggesting that Oma87 is a protective outer membrane antigen. Oma87 has

high similarity to the D15 OMP of *H. influenzae* (Loosmore *et al.*, 1997). The N-terminus of D15 was found to bear the protective epitope(s) (Yang *et al.*, 1998). However, the N-terminal region of a serogroup D Oma87 (expressed as a GST fusion protein) was unable to protect chickens against challenge with a serotype A:1 strain, although there is greater than 95% sequence identity between Oma87 from both strains (Mitchison *et al.*, 2000).

An immunogenic cell surface protein which had porin-like properties was identified in AR strains of *P. multocida* (Lugtenberg *et al.*, 1986). This 37-41.8 kDa protein, named OmpH, was later purified by Chevalier (1993) and its N-terminal sequence determined. The *ompH* gene was cloned from an A:1 strain, X-73, and porin activity of OmpH was demonstrated (Luo *et al.*, 1997). It was shown that monoclonal antibodies against OmpH were able to protect mice against lethal challenge with homologous *P. multocida* strains (Vasfi Marandi & Mittal, 1997). Antibodies against OmpH also inhibit the binding of *P. multocida* organisms to respiratory mucosal surfaces (Lubke *et al.*, 1994). OmpH is highly conserved among different serotypes, and a synthetic peptide derived from the predicted secondary structure of OmpH was shown to induce partial homologous protection in chickens (Luo *et al.*, 1999). OmpH was demonstrated to be negatively regulated by iron and glucose (Bosch *et al.*, 2001).

A 37.5 kDa OMP has been identified in a rabbit isolate of *P. multocida* (Lu *et al.*, 1988b). Monoclonal antibodies against this protein provided passive heterologous protection in mice and rabbits against other strains which possessed the 37.5 kDa OMP (Lu *et al.*, 1991b). It is unknown whether this protein is also present in avian strains of *P. multocida* or whether it plays a role in FC.

In a serogroup A strain, expression of a 40 kDa surface exposed lipoprotein, Plp-40, has been found to be correlated with the degree of capsulation suggesting that biosynthesis of hyaluronic acid and the lipoprotein may be co-ordinately regulated (Champlin *et al.*, 1999; Ryals *et al.*, 1998). The role of the 40 kDa lipoprotein in virulence and/or immunity has yet to be determined.

Adhesins

Many pathogenic species of bacteria produce adhesins such as fimbriae which allow the organisms to colonise and/or invade the host. The ability to attach to host

surfaces is usually correlated with virulence. Pili-like structures have been observed on avian strains of *P. multocida* (Glorioso *et al.*, 1982; Rebers *et al.*, 1988). Glorioso *et al.* (1982) found that fimbriae on serogroup A strains were responsible for attachment of bacteria to mucosal epithelium. Type 4 fimbriae have been identified on serogroup A, B and D strains of *P. multocida* (Ruffolo *et al.*, 1997). Type 4 fimbriae are long structures on the surface of the cell which are composed of single repeating protein subunits, ranging in molecular mass from 15 to 20 kDa in different bacterial species. In *P. multocida*, the subunit protein has been termed PtfA. Fimbriae play a role in pathogenesis by mediating the colonisation of host surfaces (Funnell & Robinson, 1993). The gene encoding the fimbrial subunit has been cloned and characterised, but the role of PtfA in immunity has yet to be determined (Doughty *et al.*, 2000).

The capsular hyaluronic acid of serogroup A strains seems to mediate adhesion of the bacteria to turkey air sac macrophages, and an isoform of CD44 has been proposed as the receptor for adherence (Pruimboom *et al.*, 1999; Pruimboom *et al.*, 1996). OmpH may also play a role in adhesion due to the fact that antibodies directed against the protein have been shown to inhibit binding of *P. multocida* to the respiratory mucosa (Lubke *et al.*, 1994).

Enzymes

Various enzymes have been studied in *P. multocida*. These may contribute to pathogenesis by facilitating invasion of cells and evasion of the host immune response.

Neuraminidase was originally isolated from a type A:3 strain of *P. multocida* present in the bovine respiratory tract (Ifeanyi & Bailie, 1992). The neuraminidase from this A:3 strain has been characterised and was shown to be expressed *in vivo* (White *et al.*, 1995). Neuraminidase is an enzyme which removes sialic acid from glycoprotein and glycolipid compounds and this activity has been detected in serogroups A, B, D and E strains (Drzeniek *et al.*, 1972; Scharmann *et al.*, 1970). Its production by *P. multocida* may contribute to the disease process. Passive immunisation of mice using rabbit antiserum to *P. multocida* neuraminidase resulted in protection against subsequent homologous challenge (Ifeanyi & Bailie, 1992). This may have been due to *in vivo* neutralisation of the enzyme. The role of neuraminidase

in FC is unclear, but it has been speculated that neuraminidase may aid invasion of avian respiratory epithelial cells by exposing carbohydrate residues which could be used as receptors (Lee *et al.*, 1994).

Hyaluronidase production by *P. multocida* has been reported, and seems to be limited to the serotype B:2 HS-causing strains (Carter & Chengappa, 1980; Rimler & Rhoades, 1994). It is thought that hyaluronidase is associated with invasion mechanisms of bacteria, but the role of hyaluronidase in the pathogenesis of HS has not been investigated.

Production of IgA proteases is a common characteristic of bacteria which colonise the mucosa. Two freshly isolated strains of *P. multocida* from humans were shown to produce proteases which cleaved human IgA and IgG (Pouedras *et al.*, 1992). However, the protease activity was readily lost with repeated passaging. IgG degradation by secreted metalloproteases has been observed in chicken, bovine, pig and sheep isolates of *P. multocida* (Negrete-Abascal *et al.*, 1999). This activity was also readily lost upon repeated subculture. It is likely that production of such proteases evolved as a means of evading the host immune response. However, a search of the published *P. multocida* strain PM70 genome failed to identify a gene encoding a specific Ig protease.

The superoxide dismutase gene, *sodC*, in *P. multocida* was identified by Kroll *et al.* (1995). This enzyme has been found in the periplasm of many bacterial pathogens. When bacteria are phagocytosed, they are killed by the release of superoxide radical anions. Superoxide dismutase is thought to protect bacteria from the harmful effects of the anions, enabling them to survive longer thereby promoting infection.

Toxins and tissue damaging agents

Certain *P. multocida* strains produce a protein toxin of approximately 143 kDa. The *P. multocida* toxin (PMT), produced mainly by type D strains, is a virulence factor in atrophic rhinitis of pigs (Foged *et al.*, 1987). The toxin induces bone resorption of the turbinates and other bone structures in the nasal cavity without causing inflammation. The bone resorption results from enhanced osteoclast activity and impaired osteoblastic bone formation. This is thought to be responsible for the clinical and pathological signs of atrophic rhinitis in pigs. It has been found that

pregnant pigs vaccinated with the purified inactivated toxin produced progeny which were highly protected against atrophic rhinitis (Foged *et al.*, 1989). Although the presence of PMT in serogroup A strains occurs infrequently, production of PMT may play a role in avian infections with serogroup D strains (Christensen & Bisgaard, 1997).

Although *P. multocida* is classified as non-haemolytic, Lee *et al.* (1991) found avian FC isolates which lysed turkey red blood cells. It was suggested that the haemolysis may be due to a secreted haemolysin, cell wall component or metabolite. A haemolytic extract isolated from many avian and other *P. multocida* isolates was able to lyse erythrocytes of various species (Diallo & Frost, 2000). The haemolytic agent(s) in the extract has not been identified, but has been shown to be between 100-300 kDa in size, and may be composed of lipid and protein. The genes *mesA*, which encodes an esterase enzyme (Hunt *et al.*, 2000), and *ahpA* (Cox *et al.*, 2000) from a *P. multocida* A:1 strain have been shown to confer a haemolytic phenotype in *Escherichia coli* when grown under anaerobic conditions, although this was due to activation of a latent *E. coli* haemolysin gene, *sheA*. The exact function of MesA and AhpA in *P. multocida* is still unclear.

Invasion of various epithelial cell lines by avian isolates of *P. multocida* has been observed (Lee *et al.*, 1994; Rabier *et al.*, 1997). Adherence to the epithelial cells may be facilitated by an epithelial cell glycoprotein or protein receptor (Lee *et al.*, 1994; Rabier *et al.*, 1997). Rabier *et al.* (1997) showed that invasion of MDCK epithelial cell monolayers was dependent on polymerisation of F-actin by the epithelial cells. This may aid in pulmonary damage, leading to vascular migration and eventual spread to the liver and spleen (Prantner *et al.*, 1990; Rabier *et al.*, 1997; Snipes *et al.*, 1987).

FirA, Skp and RecA

The *firA* and *skp* genes from *P. multocida* have been cloned (Delamarche *et al.*, 1995). These genes are part of an operon involved in the first steps of lipid A synthesis. The Skp homologue from *H. influenzae* was found to induce protective immunity in an infant rat model (El-Adhami *et al.*, 1999). Therefore, the possibility of the *P. multocida* Skp as a protective antigen was investigated. However, it was found that recombinant Skp was unable to protect mice from *P. multocida* infection

(Doughty *et al.*, unpublished data). The *recA* gene has been cloned from a serogroup D strain of *P. multocida* (Cardenas *et al.*, 2001). The RecA protein plays an important role in homologous recombination in bacteria. *P. multocida recA* mutants were shown to have a slight reduction in virulence which the authors attributed to a longer lag phase which may result in a delay in the infection process (Cardenas *et al.*, 2001).

Iron regulated proteins and iron acquisition systems

A low concentration of free iron is an environmental signal which often controls the expression of genes involved in iron acquisition as well as other virulence genes (Heithoff *et al.*, 1997; Mekalanos, 1992). It has been speculated that the *in vivo* expression of cross-protective antigens in *P. multocida* may be induced by the low iron concentration (Ikeda & Hirsh, 1988). Ikeda and Hirsh (1988) demonstrated that 15 out of 16 Heddleston serotypes expressed antigenically related high molecular weight proteins under iron limiting conditions. Thus, these proteins may be involved in heterologous protection. Glisson *et al.* (1993) found that bacterins prepared from *P. multocida* grown in low iron media were able to induce heterologous protection in chickens and turkeys. However, the cross-protection was not induced consistently. Therefore, it seems that some but not all cross-protective antigens are expressed under low iron conditions.

Various iron-regulated outer membrane proteins (IROMPs) of *P. multocida* have been identified which appear to play a role in virulence and/or immunity against pasteurellosis. *P. multocida* grown under conditions of iron deprivation secreted a growth enhancing factor which was able to reduce significantly the amount of iron binding to transferrin (Hu *et al.*, 1986). This growth factor, which functions as a siderophore, was termed multocidin and is produced in high amounts when the iron concentration is low. However, its production was inhibited in a high iron medium. Three IROMPs of 76 kDa, 84 kDa and 94 kDa were detected which are expressed during growth in iron-limiting medium (Choi-Kim *et al.*, 1991). These three proteins have the ability to bind to the iron-multocidin complex. However, binding is inhibited by pre-incubation with convalescent turkey antisera, which indicates that these proteins are expressed *in vivo*.

Other iron sequestering systems have been reported. Mutants have been constructed in the functionally related iron uptake genes, *tonB*, *exbD* and *exbB*, and

each mutant was shown to be attenuated in mice (Bosch *et al.*, 2002). An 82 kDa IROMP has been identified in some serotype B strains, which specifically binds bovine transferrin (Veken *et al.*, 1994). HS-associated strains which do not bind transferrin were shown to bind strongly to haemoglobin, while lactoferrin binding activity was not detected (Veken *et al.*, 1996). Ogunnariwo *et al.* (2001; 1991) also identified an 82 kDa IROMP, designated TbpA, in a serotype A bovine strain which could bind transferrin, but this activity was not detected in avian strains. A haemoglobin (Hb) binding protein, HgbB, from an A:1 avian strain of *P. multocida* has been characterised (Cox *et al.*, 2003). The gene encoding HgbB was insertionally inactivated but *hgbB* mutants showed no loss of Hb binding activity, nor loss of virulence suggesting that other Hb binding homologues may be present in *P. multocida* A:1. Analysis of the *P. multocida* strain PM70 genome sequence revealed 53 genes encoding proteins predicted to be involved in iron uptake or aquisition (May *et al.*, 2001). As iron is essential for bacterial growth and conditions within host tissues are generally iron-limiting, it would not be unusual for a pathogen to evolve numerous iron uptake systems in order to acquire iron from the host during infection.

Outer membrane proteins of high molecular weights have been identified which are expressed only under iron limiting conditions (Ruffolo *et al.*, 1998; Snipes *et al.*, 1988). These were expressed only *in vivo* and induced a humoral response in chickens. These proteins may be homologues of iron-regulated OMPs, and could be involved in the uptake of iron. It has been shown that under low iron conditions, the surface of *P. multocida* is markedly reduced in the amount of capsular material (Jacques *et al.*, 1994). This may allow detection of proteins by the host immune system, which would usually be masked by a thicker capsule when the cells are grown *in vitro*.

in vivo expressed proteins

There is evidence that virulence genes of many bacterial species are expressed exclusively *in vivo*. For example, a previous study found that mutations in all *in vivo*-induced genes in *Salmonella typhimurium* resulted in a decrease in virulence (Mahan *et al.*, 1993). Thus, the mutation of *in vivo*-induced genes may have potential applications in both vaccine and antimicrobial drug development.

Past studies have suggested that cross-protective antigens in *P. multocida* are expressed *in vivo*. This is supported by the finding that bacterins prepared from *in vitro* grown cells do not induce cross-immunity. However, bacterins prepared from *P. multocida* isolated from the tissues of turkeys, which had died of acute FC, induced heterologous immunity in turkeys (Heddleston & Rebers, 1974).

Glisson and Cheng (1991) found antigens in the membranes of *in vivo* propagated bacteria that were not detected in membrane preparations of the same *P. multocida* strain grown *in vitro*. These *in vivo* expressed proteins covered a range of sizes, from 27-94 kDa (Choi *et al.*, 1989). Furthermore, it was demonstrated that when *P. multocida* was propagated within the avian host, a protective immune response was induced against heterologous somatic serotypes (Glisson & Cheng, 1991). This was considered to be due to the expression of unique antigens under *in vivo* conditions (Choi-Kim *et al.*, 1991; Wang & Glisson, 1994a). These antigens have been given the term cross-protective factors (CPFs). CPFs may be involved in the induction of heterologous immunity, as indicated by the finding that antisera obtained from chickens which had survived an infection by live *P. multocida* provided passive heterologous protection (Wang & Glisson, 1994b). To date, only one CPF has been isolated but not characterised (Rimler, 2001; Rimler & Brogden, 2001).

The expression of CPFs is most likely due to unique environmental factors found in the animal host. As CPFs appear to be preferentially expressed *in vivo*, their importance in virulence and/or immunity requires further study.

GENOME-SCALE ANALYSIS OF GENE EXPRESSION AND IDENTIFICATION OF VIRULENCE-ASSOCIATED GENES

The development of DNA microarray technology has enabled the ability to analyse gene expression on a genomic scale. This technique has been used to identify genes which are differentially expressed under a range of conditions such as bacterial growth in different media (Paustian *et al.*, 2002a), low iron conditions (May *et al.*, 2001; Paustian *et al.*, 2001; Paustian *et al.*, 2002b), and within the natural host (Boyce *et al.*, 2002).

Using microarray technology, Paustian *et al.* (2002b) demonstrated that unique subsets of *P. multocida* genes are expressed in response to different iron sources. Genes encoding amino acid biosynthesis and transport systems, outer membrane proteins and heat shock proteins were identified to be up-regulated when *P. multocida* is cultured in media with limited nutrients (Paustian *et al.*, 2002a). When *P. multocida* is grown in chickens, genes encoding amino acid biosynthesis and transport systems were also up-regulated as well as those involved in energy production and conversion (Boyce *et al.*, 2002). This showed that bacteria need to alter their biosynthetic and energy production pathways in order to survive in the host environment where availability of available nutrients is presumably low.

Analysis of the *P. multocida* PM70 genome sequence identified 104 putative virulence-associated genes (May *et al.*, 2001). In particular, two putative filamentous haemagglutinins, PfhB1 and PfhB2, were identified based on amino acid identity to characterised haemagglutinins from other species. PfhB1 and PfhB2 were predicted to be involved in adherence of *P. multocida* to host cells. Although the genome sequence is a valuable resource in identifying genes potentially involved in pathogenesis, information as to the actual roles of the genes is limited and further studies are required to determine the function of individual genes and whether they are involved in virulence or induction of host immunity.

GENETIC ANALYSIS OF *IN VIVO* EXPRESSED GENES

In the past, identification of virulence factors has largely been hampered by the inability of laboratory conditions to mimic adequately the host environmental factors required for the expression of virulence associated genes. To overcome this, genetic methods such as signature-tagged mutagenesis (STM) and *in vivo* expression technology (IVET) have been developed. These systems allow the identification of previously undetected virulence factors, as the host is used as the selective medium.

Signature-tagged mutagenesis (STM)

STM is a genetic technique which has been used to identify genes required for virulence or *in vivo* survival. Mutants are generated by random insertion of a

transposon (which has a unique DNA sequence tag) into various sites of the genome (Hensel *et al.*, 1995). Pools of mutants are then used to infect a host, and those with a reduction in growth are selected for further analysis to identify which gene has been disrupted by the transposon. These genes are most likely required for *in vivo* growth and/or virulence. Mutants are distinguished from each other by virtue of the unique tags on the transposons. STM technology has identified a number of virulence associated genes in a range of pathogens including *Staphylococcus aureus* (Mei *et al.*, 1997), *Streptococcus pneumoniae* (Polissi *et al.*, 1998), *Mycobacterium tuberculosis* (Camacho *et al.*, 1999), *Streptococcus agalactiae* (Jones *et al.*, 2000), and *Brucella suis* (Foulongne *et al.*, 2000). Transposon mutagenesis using Tn*10* has been demonstrated in *P. multocida* (Lee & Henk, 1996). Using Tn*10*, STM was been applied to a serogroup A isolate of *P. multocida* (Fuller *et al.*, 2000). The disrupted genes identified from attenuated mutants are listed in Table 1.1.

Table 1.1. Genes identified from *P. multocida* using STM (adapted from Fuller *et al.* 2000). Genes were assigned putative identities based on their homology to known sequences.

Gene	Putative or known functions
purF	Purine biosynthesis
guaB	Guanine synthesis
atpG	ATP synthase
HI0719	Isoleucine biosynthesis
yjgF	
HI0145	N-acetylmannosamine-6-phosphate to N-acetylglucosamine-6-phosphate
yhcJ	epimerase
dsbB	Re-oxidation of *dsbA*
has	Hyaluronic acid synthetase
HI0258	Glycosyl transferase
lgtC	
lspA1	Filamentous haemagglutinin
fhaB1	
lspA2	Filamentous haemagglutinin
fhaB2	
lspB	Haemolysin/secretion accessory protein
fhaC	
exbB	Accessory protein – Ton dependent transport of iron compounds
HI1567	TonB dependent receptor or haemoglobin binding protein
HI1565	
tadD	Adherence
HI1424	Shufflon
rci	
pnp	Polynucleotide phosphorylase
mreB	Rod shape determination
mioC	Unknown role in chromosomal replication

HI1020 *yabK*	ABC transporter
HI1046 *yiaO*	Unknown
HI0019 *yleA*	Unknown
HI0687	Unknown
HI1246	Unknown

in vivo expression technology (IVET)

Mahan *et al.* (1993) developed an *in vivo* expression technology (IVET) system to identify bacterial genes that are upregulated *in vivo*, but minimally expressed *in vitro*. IVET methods use promoterless reporter genes conferring phenotypes which can be selected positively in the host. A genetic system based on that described by Mahan *et al.* (1993) was developed and used by Hunt and Zhang (unpublished data) to identify *in vivo* expressed genes in *P. multocida*.

Development of the IVET-I system was based on the knowledge that *aroA* mutants are unable to synthesise their own aromatic amino acids. As a result, these mutants survive poorly *in vivo*, where there are very low levels of aromatic amino acids. However, *aroA* mutants can be restored to full virulence by complementation with an intact *aroA* gene (Homchampa *et al.*, 1992).

In the IVET-I system, fragments of *P. multocida* genomic DNA were inserted into a plasmid with a unique cloning site upstream of a promoterless *aroA* gene. The resulting plasmids were transformed into *aroA* mutants, which were then injected into mice. Fragments containing an active promoter would switch on the *aroA* gene and allow the organism to survive more readily *in vivo*. After an appropriate incubation period, the mice were bled and *P. multocida* isolated. Isolates were then patched onto minimal medium and medium supplemented with 'aromix' (a mixture of aromatic amino acids). Isolates which could grow on both media possessed a gene fragment with a promoter that was active constitutively, and thus was able to drive the expression of the *aroA* gene both *in vitro* and *in vivo*. However, the isolates which grew only on supplemented medium possessed a gene fragment with a promoter that was switched on only *in vivo*. It was hypothesised that these *in vivo* expressed genes are required for virulence and survival in the host. Sequence analysis of the putative *in vivo* expressed genes led to the identification of an incomplete gene sequence,

which had high similarity to a hypothetical zinc finger protein in *H. influenzae* annotated as HI0656.

However, it was later found that the IVET-I system identified a high proportion of false positives. It is likely that the *aroA* mutants were able to remain in mice despite poor survival at low levels of aromatic amino acids. Therefore, a second system, PmIVET, was developed, which used kanamycin resistance as a reporter (Hunt *et al.*, 2001). In the PmIVET system, fragments of *P. multocida* genomic DNA were inserted into a plasmid which had a unique cloning site upstream of a promoterless kanamycin resistance gene. The resulting plasmids were then transformed into the X-73 (A:1) strain, and the transformants were passaged in kanamycin treated mice Genes identified by the PmIVET system are listed in Table 1.2. An advantage of the PmIVET over the IVET-I system is that any wild-type strain of *P. multocida* can be used. Isolates which were unable to grow on medium containing 50 µg/ml of kanamycin were subjected to further analysis. Sequence analysis of two of the putative *in vivo* expressed genes from the PmIVET system led to the identification of two partial gene sequences, each encoding a different lipoprotein.

Table 1.2. Genes identified from *P. multocida* X-73 using the PmIVET system (adapted from Hunt *et al.* 2001).

Gene identified as *in vivo* expressed	PM70 no.[a]	Function or role of homologue
hpd	PM1444	Glycerol metabolism, surface-exposed lipoprotein
pcp	PM0554	Outer membrane-associated lipoprotein
dcd	PM0951	Deoxycytidine deaminase
dsbD	PM0221	Thiol-disulfide interchange protein
speF	PM0806	Inducible ornithine decarboxylase, maintenance of cellular polyamines
ackA-pta	PM0704 and PM0705	Fermentation of acetyl-CoA; regulation of virulence gene expression
srlD	PM1968	Sorbitol-6-phosphate dehydrogenase
nrfE	PM0027	Formate-dependent nitrite reduction protein
yiaK	PM1256	Putative dehydrogenase
ycbK	PM0271	Unknown
ycbL	PM0272	Unknown
ychN	PM0514	Unknown
orfX	No match	Unknown
HI0894	PM1135	Putative membrane protein
yeeX	PM0836	Putative alpha-helix protein

[a] Gene designation scheme for the *P. multocida* PM70 genome sequence (http://ww.cbc.umn.edu/ResearchProjects/AGAC/Pm/pmhome.html).

The putative zinc finger protein identified by the IVET I system warranted further characterisation as such proteins are generally involved in gene expression (see below). The identification of two potentially *in vivo* expressed lipoproteins was of interest due to their likely membrane location and therefore, the possibility that they may be potential vaccine candidates.

ZINC FINGER PROTEINS

Zinc finger proteins make up a class of metalloproteins which generally have structural and catalytic roles in gene expression (O'Halloran, 1993). Genes under the control of metalloproteins tend to be involved in metabolism or stress-response systems. Metalloproteins detect changes in metal ion concentrations and induce rapid physiological responses. An example is iron-uptake and storage, which is particularly relevant to pathogens, as the *in vivo* concentration of free iron is generally low. Other metalloproteins, such as zinc finger proteins, are generally involved in gene expression.

The most common role of zinc finger proteins is to provide DNA binding domains within transcription factors (Klug & Schwabe, 1995). Zinc finger proteins have diverse sets of motifs with no common structural elements except for conserved cysteine-rich regions which are involved in the binding of metal ions (Hagman *et al.*, 1995). They may be classified as C4, C3H or C2H2 depending on the number of cysteine or histidine residues interacting with the zinc ion (Hagman *et al.*, 1995). Zinc finger proteins of the C2H2 class are generally sequence-specific transcription factors.

Most zinc finger proteins act by binding to specific DNA sequences upstream of the promoter region of the target gene and interact with the RNA-polymerase holoenzyme (Pountney *et al.*, 1997). It has been proposed that the zinc finger motif may induce a conformational change in the target DNA to enhance initiation of transcription (Pountney *et al.*, 1997). Zinc finger proteins do not seem to recognise DNA sequences *per se*, but rather a sequence-specific DNA structure (McBryant *et al.*, 1996).

The classical zinc finger consists of a short polypeptide loop folded back on itself, held together by disulphide bonds with the aid of a zinc ion. The coordinated

zinc ion anchors the loop by binding to the cysteine and/or histidine residues, and stabilises a conformation necessary for the recognition of a specific DNA sequence (Klug & Schwabe, 1995). Removal of the coordinated zinc ion generally results in loss of binding (O'Halloran, 1993). The zinc finger structure is small and would presumably become unstable without the zinc ion. In particular, the disulphide bridges would probably not be able to withstand the reducing conditions inside cells.

The zinc-binding domain, or zinc finger, forms a small rigid domain which is ideal for DNA-binding as it can be easily inserted into the major groove of the DNA double helix, thus enabling sequence-specific contact (O'Halloran, 1993). In all proteins containing classical zinc fingers, the zinc-binding motif is repeated, with the number of repetitions varying from 2 to 37 in different proteins (Klug & Schwabe, 1995). There is no interaction between adjacent zinc-finger domains, and nuclear magnetic resonance (NMR) studies have shown that each finger acts as an independent module.

Zinc finger proteins are major transcriptional activators in eukaryotes, but seem to be rarely found in prokaryotes (Pountney *et al.*, 1997). However, some prokaryotic zinc finger proteins and proteins with zinc-binding motifs have been identified.

The *Bacillus subtilis* RecR protein plays a role in DNA repair and recombination *in vivo*. This protein has an N-terminal zinc finger motif, Cys-X_2-Cys-X_n-Cys-X_2-Cys, which is associated with the C4 superfamily (Ayora *et al.*, 1997). The RecR zinc finger motif does not recognise specific nucleotide sequences, but a structure or distortion of the helical axis of DNA. Such zinc-containing repair proteins are ubiquitous in prokaryotes. Another example is the UvrA protein in *E. coli* which plays an important role in the repair of UV-damaged DNA. This repair protein possesses two zinc finger motifs which are required for excision repair (Wang & Grossman, 1993).

Rhizobium leguminosarum, a nitrogen-fixing species, possesses a protein, HypB, which has two Cys-X_2-Cys-X_8-Cys-X_2-Cys motifs (Rey *et al.*, 1993). This protein has the capacity to form zinc finger-like structures, and may be involved in the metabolism of nickel.

Pathogens need the ability to sense and respond rapidly to different environmental signals in order to survive in the host. Therefore, it is not surprising

that a proportion of *in vivo* induced genes encode regulatory proteins (Heithoff *et al.*, 1997). Controlling the expression of one regulatory gene by another gene or environmental factor provides a means of fine-tuning and/or amplifying the response to host signals encountered by the pathogen during an infection (Heithoff *et al.*, 1997).

LIPOPROTEINS

All prokaryotic lipoproteins possess an N-terminal hydrophobic signal peptide ending with the four-residue signal peptidase II (SPaseII) recognition sequence, (L,V,I)(A,S,T,G)(G,A)C, in positions –3 to +1 of the prolipoprotein (von Heijne, 1989). The cysteine residue at the +1 position appears to be an absolute requirement. Upon cleavage of the signal sequence by SPaseII, the mature protein undergoes lipid modification where a fatty acid, usually palmitate, is covalently linked to the cysteine residue (Hayashi & Wu, 1990; Tokunaga *et al.*, 1982). The lipid moiety at the N-terminus anchors the protein to the surface membrane (Hayashi & Wu, 1990). The amino acid directly after the cysteine of the SPaseII recognition sequence is thought to determine the localisation of the lipoprotein, possibly via its interaction with the lipoprotein shuttle, LolA (Seydel *et al.*, 1999).

It seems likely that the lipid moiety may be responsible for the immunogenic property of lipoproteins (Nardini *et al.*, 1998). OspA in *Borrelia burgdorferi*, for example, has been shown to be unable to stimulate immunity without the attached lipid. Lipoproteins have been shown to play a role in virulence and immunity. Zhang *et al.* (1997) demonstrated that bacterial lipoproteins can induce lethal shock and *in vivo* production of TNF-α and IL-6 in mice. Lipoproteins can also be released by growing or lysed bacteria, and the released lipoproteins may be associated with pathogenesis of gram-negative bacterial infections, as levels are high enough to induce cytokine production in macrophages (Zhang *et al.*, 1998). *Borrelia burgdorferi* lipoproteins have also been shown to directly stimulate inflammatory responses (Giambartolomei *et al.*, 1999). Lipoproteins have been identified and characterised from *P. haemolytica* A1 (Cooney & Lo, 1993; Nardini *et al.*, 1998; Pandher *et al.*, 1998). One of these lipoproteins, PlpE, has been found to be important in

complement-mediated killing, and antibodies against PlpE may contribute to host defence against *P. haemolytica* in cattle (Pardher *et al.*, 1998).

OUTLINE OF THESIS

The research presented in **Chapter 2** focuses on the characterisation of GlpQ. To corroborate the PmIVET result, real-time RT-PCR was performed to determine if *glpQ* is indeed *in vivo* up-regulated. Studies were undertaken to elucidate the function of GlpQ and also to determine if the protein is lipidated. The protective capacity of GlpQ was investigated by expressing and purifying recombinant GlpQ from *E. coli* for vaccine trials in mice and chickens. **Chapter 3** presents data on the characterisation of PCP. As for *glpQ*, transcriptional studies were performed to determine if *pcp* is up-regulated *in vivo*. Vaccination studies were undertaken in mice and chickens to determine whether PCP can stimulate protective immunity. The data presented in **Chapter 4** include transcriptional studies to confirm if *pzfA* is also *in vivo* up-regulated. As PzfA is a putative zinc finger protein and possibly involved in transcriptional regulation, the protein was overexpressed in *P. multocida* and the subsequent effects on global transcription were analysed by whole genome microarrays.

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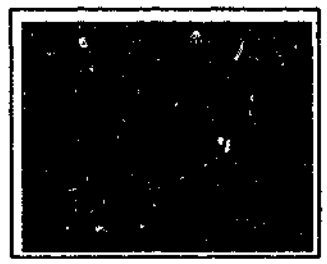
CHAPTER TWO

CLONING AND CHARACTERISATION OF GlpQ - A LIPOPROTEIN IDENTIFIED BY PmIVET



Based on:
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INTRODUCTION

Pasteurella multocida is a ubiquitous animal pathogen that is associated with a range of diseases including haemorrhagic septicaemia in ungulates, atrophic rhinitis in swine, and fowl cholera in wild and domestic birds. Fowl cholera is a highly contagious disease and causes significant economic losses to poultry industries worldwide. Current vaccines for the control of fowl cholera are unsafe and ineffective (Carpenter *et al.*, 1988; Glisson, 1992; Hopkins & Olson, 1997), and our understanding of the molecular mechanisms of pathogenesis is still limited.

Previous studies have shown that outer membrane protein preparations from *in vivo*-grown *P. multocida* were able to protect birds from challenge with heterologous serotypes, while *in vitro*-grown bacteria were able to protect only against the homologous serotype (Glisson & Cheng, 1991; Heddleston & Rebers, 1972; Heddleston *et al.*, 1970). The *in vivo*-expressed proteins responsible for heterologous protection have been termed "cross-protective factors", and currently only one has been identified, but not characterised (Rimler, 2001; Rimler & Brogden, 2001).

An *in vivo* expression technology (IVET) system was first described by Mahan *et al.* (1993a; 1993b) as a means of identifying *in vivo*-expressed genes. IVET systems have been developed and used in various organisms (reviewed in Chiang *et al.*, 1999; Handfield & Levesque, 1999; Heithoff *et al.*, 1997) to identify genes required for virulence and survival in the host. Such genes may potentially be useful as targets for attenuating mutations, or their products may be used as vaccine candidates or targets for antimicrobial agents.

In order to identify genes up-regulated during pasteurellosis and therefore, possible cross-protective factors, an IVET system, termed PmIVET, was developed for use in *P. multocida* (Hunt *et al.*, 2001). One of the genes identified by PmIVET encoded a protein with similarity to protein D, a surface exposed lipoprotein in *Haemophilus influenzae* (Ruan *et al.*, 1990). Protein D has been shown to elicit cross-protection against virulent heterologous strains of *H. influenzae* in rats (Akkoyunlu *et al.*, 1996). Therefore, the protective capacity of the *P. multocida* homologue was investigated.

This report describes the cloning and sequencing of the gene encoding the protein D homologue of *P. multocida* (designated *glpQ*), its characterisation, and expression in *Escherichia coli* for vaccine trials in mice and chickens.

MATERIALS AND METHODS

Oligonucleotides, bacterial strains and plasmids

Oligonucleotides used in this study are listed in Table 2.1 while bacterial strains and plasmids are shown in Table 2.2.

Table 2.1. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5'-3')	Target
BAP974	TCTAGAATCCAAAGCCCTCG	Outward facing inverse-PCR primer for *glpQ*.
BAP1035	ATGCTTGAACTTGAACAGGC	Outward facing inverse-PCR primer for *glpQ*.
BAP1099	CCTTTTTTCTTTCTCATCGCC	Forward primer for *glpQ*.
BAP1100	TTGTTGACCAAAAGCGAGG	Reverse primer for *glpQ*.
BAP1224	CTTAGGATCCTGTTCAAGTTCAAGC ATG	Forward primer for *glpQ*. Incorporates a *Bam*HI site (underlined).
BAP1226	TTTGGAATTCCTTGTTTAATCAAGA CAACG	Reverse primer for *glpQ*. Incorporates an *Eco*RI site (underlined).
BAP1489	TGCCTGCCATGGTTCAAGCATGAT GAAAAATGATG	Forward primer for *glpQ*. Incorporates an *Nco*I site (underlined).
BAP1490	TCTTTGGGATCCCTTGTTTAATCAA GACAACG	Reverse primer for *glpQ*. Incorporates a *Bam*HI site (underlined).
BAP2098	ACAAGATCTCGCAATGACGAAAG	Forward primer for real-time RT-PCR
BAP2099	CTCGCTCGATTTGGGAATTTT	Reverse primer for real-time RT-PCR

Table 2.2. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference or source
P. multocida strain		
X-73	Serotype A:1 chicken isolate; reference strain	(Heddleston & Rebers, 1972)
PBA917	X-73 harbouring pMKΩ	(Hunt *et al.*, 2001)
MHS25	X-73 PmIVET clone containing 186 bp of the start of *glpQ* and 165 bp upstream	(Hunt *et al.*, 2001)
PBA100	Serotype A:1 chicken isolate	(Ireland *et al.*, 1991)
VP161	Serotype A:1, Vietnamese chicken isolate	(Wilkie *et al.*, 2000)
P-1059	Serotype A:3 turkey isolate; reference strain	John R. Glisson[a]
P-1662	Serotype A:4 turkey isolate; reference strain	John R. Glisson[a]
M1404	Serotype B:2 buffalo isolate; reference strain	K.R. Rhoades[b]
E. coli strains		
DH5α	*F endA1 hsdR17* ($r_k^- m_k^+$) *supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA-arg F*) U169 Ø80d*lac*ZΔM15	Bethesda Research Laboratories
BL21(DE3)pLysS	F $^-$ *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm* (DE3) pLysS (Cm^R)	Invitrogen, Groningen, The Netherlands
PBA1468	BL21(DE3)pLysS harbouring pRSET-A	Laboratory strain
AL120	BL21(DE3)pLysS harbouring pDUMP	(Cullen *et al.*, 2003)
AL262	BL21(DE3)pLysS harbouring pPBA1664	This study
AL264	BL21(DE3)pLysS harbouring pAL78	This study
AL303	Clone with full length *glpQ*	This study
AL317	Clone with *tet*(M) disrupted *glpQ*	This study
Plasmids		
pMKΩ	6.5 kb, Spe^R and Str^R *in vitro*, unique *Bam*HI site upstream of a promoterless *kan* gene, *P. multocida* origin	(Hunt *et al.*, 2001)
pWSK29	Amp^R, *lacZ'*, pSC101 origin, f1 origin	(Wang & Kushner, 1991)
pRSET-A	Amp^R, f1 origin, T7 promoter	Invitrogen
pDUMP	*E. coli* lipoprotein expression vector. Section encoding the signal peptide from the *E. coli* lipoprotein Lpp cloned into pET-9c (Novagen, Madison, WI.)	(Cullen *et al.*, 2003)
pPBA1664	Section of *glpQ* encoding the mature length protein cloned into pRSET-A	This study
pAL78	Section of *glpQ* encoding the mature length protein cloned into pDUMP	This study
pVB101	*tet*(M) gene from Tn*916* cloned into pBR322, Tet^R, Amp^R	Vickers Burdett[c]
pAL174	Plasmid with full length *glpQ*	This study
pAL183	Plasmid with *tet*(M) disrupted *glpQ*	This study

[a] University of Georgia, Athens, GA, USA
[b] National Animal Disease Center, Ames, Iowa
[c] Duke University, Durham, N.C.

Media

P. multocida X-73 was cultured at 37°C in nutrient broth (NB) or brain heart infusion broth (BHI; Oxoid) with constant aeration, or on nutrient agar (NA). *E. coli* strains were grown at 37°C in 2YT broth with constant aeration or on 2YT agar. For

protein expression, *E. coli* strains were grown in Super Broth (3.2% tryptone, 2.0% yeast extract, 0.5% NaCl) at 37°C with constant aeration. Antibiotics were added when required at the following concentrations: ampicillin (Amp), 100 μg/ml; kanamycin (Kan), 5, 10, 20, 30, 40 or 50 μg/ml; chloramphenicol (Cm), 40 μg/ml.

Recombinant DNA techniques

Plasmid DNA from *E. coli* and *P. multocida* strains was prepared as described by Le Gouill *et al.* (1994), while *P. multocida* genomic DNA was prepared using the cetyltrimethylammonium bromide (CTAB) precipitation method (Ausubel *et al.*, 1987). Standard restriction digestion, dephosphorylation, and ligation of DNA were performed using DNA-modifying enzymes supplied by Roche Molecular Biochemicals or New England Biolabs Inc. (NEB) under conditions recommended by the manufacturer. Plasmids were introduced by transformation into RbCl-treated *E. coli*. After heat shock, the transformed cells were recovered in 1 ml of SOC (2.0% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose) for 1 hr at 37°C, prior to plating on 2YT agar containing Amp or Kan.

Sequence analysis

Nucleotide sequences were determined using the *Taq* Big DyeDeoxy terminator kit (Applied Biosystems Inc.). Individual sequences were aligned and assembled using the Sequencher 3.0 program (Gene Codes Corp.). Comparison of sequences with those in GenBank and EMBL was performed using the BLAST (Altschul *et al.*, 1990) program through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Comparison of sequences with the published *P. multocida* PM70 genome was performed via the University of Minnesota Microbial Genome Project website (http://www.cbc.umn.edu/Research Projects/AGAC/Pm/). Computer analysis of the deduced protein sequence was performed via the Australian National Genomic Information Service (ANGIS) (http://www.angis.org.au) using programs within the Genetics Computer Group sequencing analysis software package (GCG, Inc.).

RNA purification

Bacteria were grown in BHI until mid-late log phase and then added to 0.5 volumes of RNA stabilising reagent (RNALater, QIAGEN). The cells were incubated at room temperature for 5 min prior to pelleting by centrifugation at 12,000 × *g* at 4°C for 10 min. Cells were resuspended in Trizol (Gibco/BRL) and the RNA was purified as described previously (Simms *et al.*, 1993). The resuspended cells were incubated at 65°C for 15 min and 0.2 ml of chloroform was then added. This mixture was shaken for 15 sec, incubated at room temperature for 15 min and then centrifuged at 12,000 × *g* for 15 min at 4°C. The resulting aqueous phase was transferred to a fresh tube and extracted with an equal volume of phenol/chloroform followed by an extraction with an equal volume of chloroform-isoamyl alcohol (24:1). The resulting aqueous phase was precipitated by adding 300 µl of isopropanol and 1 µl of RNAsin (400 U, Promega) and the mixture incubated at room temperature for 10 min. The RNA/DNA was pelleted by centrifugation at 4°C for 10 min at 12,000 × *g*, and washed in 70% ethanol. The pellet was then air dried and resuspended in 100 µl DEPC H_2O by pipetting at room temperature. The RNA/DNA was precipitated again by addition of 1/10 volume of 3 M sodium acetate (pH 5.2) and three volumes of 99% ethanol. The mixture was incubated at -70°C for 30 min then centrifuged for 15 min at 12,000 × *g* at 4°C. The resulting pellet was washed in 70% ethanol, then air-dried and resuspended in DEPC H_2O. To remove contaminating DNA, the purified RNA (100 µg/ml) was treated with DNase (15 U for 10 min at 37°C) and the RNA further purified using RNeasy minicolumns (QIAGEN). RNA quality was determined by agarose gel electrophoresis while RNA concentration was measured spectrophotometrically at 260 nm.

Extraction of RNA from *in vivo*-grown *P. multocida*

For the isolation of in-vivo grown bacteria, mice (female 6 to 8 week-old BALB/c outbred) were infected with 4×10^6 cfu of *P. multocida* strain X-73 by intraperitoneal (ip) injection. Mice were monitored for clinical symptoms and when considered moribund, were anaesthetised by injecting 40 µg of Nembutal (Boehringer Ingelheim) per gram body weight. Blood was recovered from the orbital plexus and by heart puncture and the mice were then euthanased in accordance with animal ethics requirements. Heparin (20 U/ml) and sodium azide (1 mM/ml) were added

immediately to the blood samples to prevent clotting of blood and to prevent further transcription by the bacteria. Bacteria were then harvested by layering the blood (2 ml total) over 15 ml of 30% sucrose in 20 mM Tris-HCl (pH 7.5). The sample was centrifuged at 3,400 × *g* for 10 min at 4°C using a swing-out rotor. The top layer containing plasma and bacteria was then aspirated and the cells pelleted by centrifugation at 24,500 × *g* for 15 min at 4°C. Cells were resuspended in Trizol and the RNA purified as specified above. RNA from bacteria grown in chickens was obtained from Boyce *et al.* (2002).

Real-time reverse transcription (RT)-PCR

Real-time RT-PCR was performed using a Roche Molecular Biochemicals LightCycler in accordance with the instructions of the manufacturer. The *lpxA* gene which encodes UDP-N-acetylglucosamine acetyltransferase, an enzyme involved in the first step of lipid A biosynthesis (Anderson & Raetz, 1987), was used as the normaliser for all reactions. RT reactions contained 10-20 µg of total RNA, 50 nM of BAP1666 and BAP2009 (*lpxA* and *glpQ* reverse primers respectively), 10 U Superscript II reverse transcriptase (Gibco/BRL), and 500 µM each of dATP, dCTP, dGTP and dTTP. RNA was first heated with the primers at 70°C for 10 min then chilled on ice for 10 min. The remaining components were then added and the samples incubated at 42°C for 2.5 hr, followed by 15 min at 70°C. The synthesised cDNA was then diluted 1/10, 1/100, 1/500 and 1/1000 prior to use as template in real-time RT-PCR. Reactions were performed in triplicate. Each 20 µl reaction contained 2 µl of cDNA, 0.5 µM of each gene-specific primer (Table 2.1), 3 mM or 4 mM $MgCl_2$ (for *glpQ* and *lpxA* reactions respectively) and 2 µl of LightCycler FastStart DNA Master SYBR Green I mi (Roche Molecular Biochemicals). Known concentrations of *lpxA* PCR product generated from *P. multocida* strain X-73 genomic DNA were used to construct a standard curve so that the concentration of template in each reaction could be determined. Analysis was performed using the LightCycler software. Melting curve analysis confirmed that all RT-PCRs amplified a single product.

Later real-time RT-PCR experiments were conducted using an ABI PRISM model 7700 sequence detection system. Primer Express software (ABI) was used to design primers for real-time RT-PCR. A 30 µl RT reaction contained 10-20 µg of

total RNA, 30 µg of random hexamers, 10 U Superscript II reverse transcriptase (Gibco/BRL), and 500 µM each of dATP, dCTP, dGTP and dTTP. RNA was first heated with the random hexamers at 70°C for 10 min then chilled on ice for 10 min. cDNA was synthesised as specified above. The synthesised cDNA was diluted 1/80 prior to use in real-time RT-PCR. Reactions were performed in triplicate. Each 20 µl reaction contained 2 µl of cDNA, 50 nM of each gene-specific primer (Table 2.1) and 10 µl of SYBR Green PCR master mix (ABI). Known concentrations of *P. multocida* strain X-73 genomic DNA were used to construct a gene-specific standard curve so that the concentration of template in each reaction could be determined. The gene encoding DNA gyrase subunit B, *gyrB*, was used as the normaliser for all reactions. Melting curve analysis confirmed that all RT-PCRs amplified a single product. Student's *t* test with a two-tailed hypothesis was used to determine the statistical significance between mean values of *in vitro* and *in vivo* expression levels of *glpQ*. *P* values of <0.05 indicated statistical significance at the 95% confidence level.

Expression and purification of recombinant protein

For expression of non-lipidated, T7/hexa-histidine-tagged proteins in *E. coli* BL21(DE3)pLysS, the section of the gene encoding the mature length protein was amplified using the Expand™ High Fidelity PCR system (Roche) as specified by the manufacturer. PCR reaction mixtures typically contained 0.1-0.75 µg of plasmid DNA in a final volume of 50 µl containing 200 µM of each dNTP, 300 nM of each primer, 2.6 units of Expand™ High Fidelity PCR system enzyme mix, and 5 µl of 10x Expand™ High Fidelity buffer with 15 mM magnesium chloride. The samples were subjected to denaturation (94°C, 2 minutes) and then an initial 10 cycles of denaturation (94°C, 15 seconds), annealing (60°C, 30 seconds) and extension (68°C, 4.5 minutes). This was followed by 20 cycles of denaturation (94°C, 15 seconds), annealing (60°C, 30 seconds), extension (68°C, 1 minute, plus an additional 5 seconds for each successive cycle), and a final prolonged extension time (72°C, 7 minutes). The PCR product was then cloned into the pRSET-A expression vector (Invitrogen). Sequence analysis was used to confirm that the gene had been cloned in-frame with the T7/hexa-histidine tag before transforming the plasmid into the *E. coli* strain BL21(DE3)pLysS. BL21(DE3)pLysS transformants were grown in Super Broth in the presence of Amp and Cm to mid-log phase, and induced with a final concentration

of 1 mM isopropylthio-β-D-galactoside (IPTG) (Sigma Chemical Co.) for 2 hr before harvesting the cells. Cells were washed twice with phosphate-buffered saline pH 7.4 (PBS) and resuspended in PBS at 1/10th of the original culture volume. Samples were then analysed by Western immunoblot (see below) using anti-T7 tag monoclonal antibodies (Novagen) at 1/10,000 dilution to detect expression of T7/hexa-histidine tagged recombinant proteins. To purify T7/hexa-histidine tagged proteins, cells were disrupted using a French pressure cell (Aminco) at 9,000-10,000 MPa, and the fusion proteins purified using TALON™ metal affinity resin (Clontech) as specified by the manufacturer. The concentration of purified protein was determined by the Bradford assay (Bio-Rad) with bovine serum albumin (BSA) as a standard.

For expression of lipidated recombinant protein, the section of the gene encoding mature length protein was amplified using Expand™ High Fidelity PCR system (Roche) and cloned in-frame with the Lpp signal peptide in the lipoprotein expression vector pDUMP. Sequence analysis was used to confirm that the gene had been cloned in-frame with the Lpp signal peptide before transforming the plasmid into the *E. coli* strain BL21(DE3)pLysS. BL21(DE3)pLysS transformants were induced for protein expression as described above and lipidated proteins were purified in the form of membrane fractions (see below).

Preparation of membrane fractions

Membrane fractions were purified by stepwise sucrose gradient centrifugation as reported previously (Ruffolo & Adler, 1996). Overnight cultures of *E. coli* were pelleted by centrifugation and washed once with PBS. Cell pellets were resuspended in 6 ml of 20% sucrose in 10 mM Tris-HCl (pH 8.0) containing 40 μg/ml of DNase, and incubated at room temperature for 15 min. Cells were disrupted by two passages through a French pressure cell at 9,000-10,000 MPa. Cell debris and unbroken cells were removed by centrifugation at 12,000 × *g* for 15 min and 3 ml of supernatant were applied to a stepwise gradient of 25% and 70% sucrose containing 3 mM EDTA and centrifuged at 230,500 × *g* for 2 hr at 4°C. The cytoplasmic fraction was collected above the 25% sucrose step and stored at –20°C. The total membrane fraction was collected above the 70% sucrose step, diluted in 3 mM EDTA, and pelleted at 230,500 × *g* for 2 hr at 4°C. Pelleted membranes were resuspended in

sterile distilled water and stored at -20°C. The protein concentration of the samples was determined by Bradford assay.

Immunisation trials

Female 6 to 8 week-old BALB/c mice were injected ip with either 50 μg of purified protein in incomplete Freund's adjuvant (IFA), 50 μg of total membrane preparation in IFA, or 10^6 cfu of live *E. coli*. The mice were boosted at 2 weeks following the primary immunisation and test bled from the orbital plexus 2 weeks after the boost to determine the reactivity of the sera to the required protein. At 4 weeks after the initial vaccination, mice were challenged ip with 10^2 cfu of *P. multocida* X-73 (ID_{50}<10 cells). Mice were monitored for clinical symptoms and if considered moribund, were euthanased in accordance with animal ethics requirements.

For vaccine trials in chickens, Leghorn cross commercial layers were injected in the breast muscle with 50-100 μg of total membrane preparation in Alhydrogel™. Chickens were boosted at 2 weeks following the primary immunisation, then test bled 2 weeks later before challenge with 10^3 cfu of live *P. multocida* X-73. Chickens were monitored and euthanased if considered moribund.

SDS-PAGE and Western immunoblot analysis

Samples were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Laemmli, 1970) using a Bio-Rad Protean I or II apparatus. SDS-polyacrylamide gels were stained with Coomassie brilliant blue or transferred electrophoretically onto Immobilon-P polyvinylidene fluoride (PVDF) membranes (0.45 μm) (Millipore) in a Bio-Rad TransBlot cell for Western immunoblot analysis. Primary antisera were used at a dilution of 1/1,000 and the immobilised immune complexes were detected with a 1/500 dilution of peroxidase-conjugated sheep anti-mouse immunoglobulin (Silenus Laboratories) or peroxidase-conjugated rabbit anti-chicken immunoglobulin (Sigma Chemical Co.).

Measurement of antibody levels

Antibody levels were measured by Western immunoblot analysis using PVDF membrane strips with 100 ng of purified T7/hexa-histidine tagged recombinant protein. Antisera from vaccinated animals were diluted serially from 1/500 to

1/16,000, while antisera from control animals were tested at 1/500. Sonicated BL21(DE3)pLysS cells expressing T7/hexa-histidine tagged MesA, an esterase enzyme from *P. multocida* A:1 (Hunt *et al.*, 2000), were added to the diluted primary antisera at 5 μg/ml to remove any reactivity to the T7/hexa-histidine tag.

Absorption of antiserum with *E. coli* whole cell lysate

Cells from a 200 ml overnight culture of *E. coli* BL21(DE3)pLysS were harvested by centrifugation at 12,000 × *g* for 10 min, washed once with an equal volume of PBS and resuspended in 1 ml PBS. The cells were disrupted by sonication and 0.33 ml of sonicate was added to 1 ml of pooled serum from the mouse vaccine trials. The mixture was incubated at 37°C for 20 min before another 0.33 ml of sonicate was added and incubated for another 20 min at 37°C. The remaining sonicate was then added and the mixture incubated overnight at 4°C. The serum was centrifuged at 12,000 × *g* for 20 min and the supernatant was filtered first through a 0.45 μm filter, then a 0.22 μm filter.

Globomycin treatment

P. multocida or *E. coli* cultures were grown to mid-log phase and then treated with 50 μg/ml final concentration of globomycin for 2.5 hr. Whole cell lysates were then analysed by SDS-PAGE and Western immunoblot using anti-GlpQ serum that had been absorbed with *E. coli* BL21(DE3)pLysS whole cell lysate.

Assay for glycerophosphodiester phosphodiesterase activity

Glycerophosphodiester phosphodiesterase (GlpQ) activity was determined by a coupled spectrophotometric assay as reported previously (Larson & van Loo-Bhattacharya, 1988; Larson *et al.*, 1983). Briefly, each 0.5 ml reaction contained 0.45 ml of 1 M hydrazine/glycine buffer, 0.5 mM NAD (Sigma Chemical Co.), 10 mM $CaCl_2$, 10 units of glycerol-3-phosphate dehydrogenase (Roche Molecular Biochemicals), 0.5 mM substrate (glycerophosphorylcholine) (Sigma Chemical Co.) and 0.5 to 4.5 μg of total protein. The rate of accumulation of NADH was measured at 340 nm over 5 min. One unit of activity is defined as the reduction of 1 μmol of NAD per minute. Reported values are the average of three biological replicates. Student's *t* test with a two-tailed hypothesis was used to determine the statistical significance between mean enzyme activity values of non-GlpQ-expressing and

GlpQ-expressing strains of *E. coli*. *P* values of <0.05 indicated statistical significance at the 95% confidence level.

Determination of surface exposure

To determine surface exposure of the protein of interest, surface immunoprecipitation was performed according to the method of Shang *et al.* (1996), but using $1/10^{th}$ of the volumes reported. A 3 ml culture containing 10^9 cfu/ml of *P. multocida* strain X-73 was pelleted at 12,000 × *g* for 10 min, washed once with an equal volume of PBS and resuspended in 3 ml PBS. The suspension was incubated with 150 µl of heat-inactivated (56°C for 30 min) specific mouse anti-serum which had been absorbed previously with *E. coli* whole cell lysate. The mixture was shaken gently on a rotating wheel for 1 hr at room temperature. Cells were harvested by centrifugation at 12,000 × *g* for 10 min, washed once with 5 mM $MgCl_2$ in PBS, pelleted again at 12,000 × *g* for 10 min and then resuspended in 0.9 ml of buffer 1 (10 mM Tris HCl (pH 8.0), 2 mM EDTA, 1 mM PMSF). To this mixture, 100 µl of 10% protein grade Triton X-100 were added and the suspension was incubated on a rotating wheel for 30 min at 4°C. Insoluble material was removed by centrifugation at 12,000 × *g* for 20 min. To the supernatant were added 100 µl of 2% deoxycholate (DOC), 10 µl of 10% SDS and 60 µl of staphylococcal protein A-sepharose CL-4B (Amersham Life Sciences) slurry. The mixture was agitated on a rotating wheel for 30 min at 4°C. The protein A-sepharose-antibody-antigen complexes were washed twice in buffer 2 (0.01% Triton X-100 in 10 mM Tris HCL (pH 8.0)) by centrifugation at 1,000 × *g* for 5 min then resuspended in an equal volume of 2× sample buffer (SB; 0.2 M Tris-HCl with 2% SDS (pH 6.8), 20% glycerol, 2.5% SDS, 10% β-mercaptoethanol, 0.1% aqueous bromophenol blue) and analysed by Western immunoblot with mouse anti-GlpQ serum. Sonicated cells were used as a positive control for immunoprecipitation which was carried out in parallel with the following exception: after the lysed cells had been incubated with heat inactivated antiserum for 30 min, protein A-sepharose slurry was added to the mixture and incubated for a further 30 min at room temperature. The protein A-sepharose-antibody-antigen complexes were pelleted, then resuspended in 0.9 ml of buffer 1 and 100 µl of 10% Triton X-100 were added. After the suspension was incubated on a rotating wheel for 30 min at 4°C, 100 µl of 2% DOC and 10 µl of 10% SDS were added. The mixture

was agitated on a rotating wheel for another 30 min at 4°C, then the protein A-sepharose-antibody-antigen complexes were washed twice in buffer 2 and resuspended in an equal volume of 2×SB.

Preparation of electrocompetent *P. multocida* cells

P. multocida cells were prepared according to the method of Smith *et al.* (1990) with the addition of hyaluronidase (Roche Molecular Biochemicals). 250 ml of BHI were inoculated with 2.5 ml of an overnight culture of the desired *P. multocida* strain and grown at 37°C with aeration to an absorbance at 550 nm of 0.6. Hyaluronidase was then added at 37 U per ml of culture and incubated for another hour at 37°C with occasional shaking. Cells were pelleted by centrifugation at 12,000 × *g* for 10 min at 4°C and washed twice in an equal volume of ice-cold 10% (v/v) glycerol. Excess glycerol was removed from the pellet which was then resuspended in 1 ml 10% (v/v) glycerol and 100 µl aliquots were snap-frozen in a dry ice/ethanol bath and stored at -70°C until required.

Electroporation

Electroporation of *P. multocida* was performed using a BTX Electrocell Manipulator with the following conditions; 2 kV, 350 Ω and 25 µF. Electrocompetent cells (100 µl) were thawed on ice and mixed with ≤10 µl of DNA which had been cleaned by ethanol precipitation, dialysis, or using the QIAquick PCR purification kit (QIAGEN) as per the manufacturer's instructions. The DNA was pipetted into chilled BTX 1 mm electroporation cuvettes and after electric pulse, the cells were immediately resuspended in 1 ml of BHI containing 0.3% sucrose and incubated aerobically at 37°C for 2 hr without shaking. Transformants were then plated onto appropriate selective media.

Natural transformation

Both methods detailed below were obtained from Ruffolo *et al.* (personal communication).

Liquid transformation

P. multocida strain X-73 was grown aerobically in 5 ml BHI broth in 20 ml vials for 4 days at 37°C, static. After incubation, 4.6 ml of broth were removed

without disturbing the bacterial film which had formed on the bottom of the vial and 2-3 μg of mutated construct DNA (either plasmid or PCR product in linear or circularised form) was added to the vial. Blunt-ended linear DNA was generated by PCR using *Pwo* DNA polymerase (Roche Molecular Biochemicals) according to the instructions of the manufacturer and when necessary, the PCR product was circularised using T4 DNA ligase. The culture was incubated static for another 2 days at 37°C and then treated with DNase (1 U/μg of DNA) for 15 min at 37°C. The culture was then plated onto BHI agar supplemented with Kan at 30 or 50 μg/ml and grown at 37°C for at least 3 days. An alternative method involved inoculating 350 μl of an overnight *P. multocida* culture into 50 ml BHI. This was grown to an absorbance at 600 nm of 0.3 or 0.9. When the desired cell density was reached, a 1 ml volume of culture was taken out and placed into a fresh vial. The cells were washed twice with an equal volume of PBS, then resuspended in 1 ml of PBS. The suspension was put into a 15 ml tube and incubated for 100 min at 37°C with shaking before adding 1 μg of linear mutated construct DNA. Cells were incubated for another 2 hr at 37°C with slow shaking (100 rpm) after which the entire mix was plated onto BHI agar supplemented with Kan at 60 μg/ml. Plates were incubated aerobically at 37°C for at least 4 days.

Plate transformation

The mutated construct DNA (2-3 μg, either plasmid or PCR product in linear or circular form) was spread onto BHI agar supplemented with Kan at 30 or 50 μg/ml and allowed to dry for 10 min at room temperature. A suspension of 10^4 cfu/ml of *P. multocida* strain X-73 was prepared in 500 μl of BHI from a freshly grown plate. The cell suspension was spread plated onto the DNA treated plates and allowed to dry. The plates were incubated aerobically for at least 3 days at 37°C.

An alternative method involved emulsifying one *P. multocida* colony (approximately 10^6 cfu) from a fresh plate in 100 μl PBS. Linear mutated construct DNA (1 μg) was pipetted onto BHI (supplemented with Kan at 60 μg/ml) on a section of a quartered agar plate and the emulsified colony then added onto the DNA. The mixture was then spread and the plates incubated aerobically at 37°C for at least 4 days.

RESULTS

Cloning and sequence analysis

A PmIVET clone, MHS25, had previously been identified which contained a section of a gene encoding a protein with high similarity to protein D from *H. influenzae*. As MHS25 contained only partial sequence of the gene encoding the *P. multocida* protein D homologue, the entire gene needed to be cloned from *P. multocida* X-73 and its nucleotide sequence determined. At the time of commencement of this work, the sequencing of the *P. multocida* PM70 genome (May *et al.*, 2001) had not yet been completed. Using the existing DNA sequence from MHS25, a comparison with the incomplete PM70 genome database was performed. The nucleotide sequence of the protein D homologue was not present in the PM70 genomic database, so to clone the gene encoding the *P. multocida* protein D homologue, designated *glpQ*, Southern hybridisation was first carried out. *P. multocida* X-73 genomic DNA was digested with various restriction enzymes and *glpQ* was detected on a 2.5 kb *Kpn*I fragment using a DIG-labelled probe generated with the primers BAP1099 and BAP1100, designed from the nucleotide sequence of the PmIVET clone (Figure 2.1). Outward facing primers, BAP974 and BAP1035,

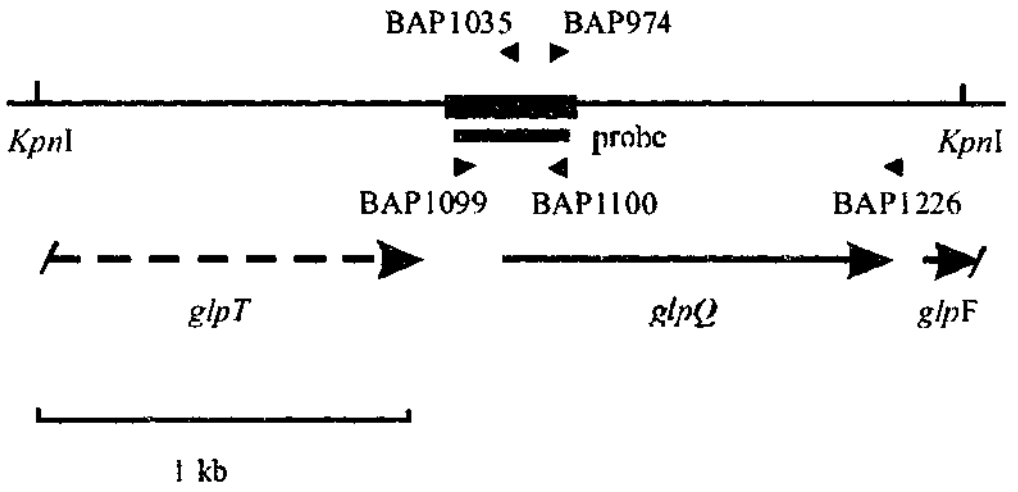

Figure 2.1. Schematic diagram of the 2.5 kb *Kpn*I fragment on which *glpQ* (blue) is located. Known sequence from MHS25 is shown in green while the probe used for Southern hybridisation is shown in red. Positions of primers are denoted by the arrow heads. Incomplete coding sequences flanking *glpQ* are also shown.

situated within *glpQ* (Figure 2.1), were then designed from the known sequence to amplify an inverse-PCR product from *P. multocida* X-73 genomic DNA which had

been digested with *Kpn*I and religated. The sequence obtained from the inverse-PCR product was combined with the sequence already available from MHS25. The primers BAP1226 (designed from the combined nucleotide sequence) and BAP1099 (Figure 2.1) were then used to amplify the entire *glpQ* gene from the *P. multocida* X-73 genome and the PCR product was cloned into pWSK29 giving rise to the plasmid pAL174.

The entire *glpQ* gene (Figure 2.2) consisted of 1,074 bp and encoded a protein of 358 amino acids with a predicted molecular mass of 41.2 kDa. The deduced amino acid sequence contained a 17-amino-acid stretch at the N-terminus ending in a putative signal peptidase II (SPaseII) cleavage site, LTAC (von Heijne, 1989), suggesting that the encoded protein was post-translationally modified and lipidated at the cysteine residue. Kyte-Doolittle hydropathy plot analysis of the amino acid sequence showed that GlpQ is mostly hydrophilic with the exception of the putative signal peptide (Figure 2.3). The processed mature length protein was predicted to have a molecular mass of 39.4 kDa and a calculated pI of 6.7. Putative -35 and -10 promoter sequences were not identified. A consensus ribosomal binding sequence was identified 8 bp upstream of the start codon (Figure 2.2). GlpQ had 90% similarity to protein D of *H. influenzae* and 81% similarity to GlpQ of *E. coli*. GlpQ also showed high similarity to the GlpQ homologues from *Bacillus subtilis*, *Treponema pallidum* and *Borrelia hermsii* (68%, 76% and 71% similarity respectively). The *P. multocida* X-73 *glpQ* gene had 98% nucleotide identity to the homologue in the published *P. multocida* PM70 genome (May *et al.*, 2001).

The nucleotide sequence of the *P. multocida* strain X-73 *glpQ* gene has been deposited in the GenBank database under the accession number AY218825.

Identification and sequence analysis of flanking genes

The complete nucleotide sequence of the 2.5 kb *Kpn*I fragment was determined in order to identify genes surrounding *glpQ*. Immediately upstream of *glpQ*, an ORF was identified encoding a protein with 94% and 72% similarity to GlpT, a glycerol-3-phosphatase transporter, in *H. influenzae* and *E. coli* respectively. The deduced protein of the ORF downstream of *glpQ* showed similarity to GlpF, a glycerol uptake facilitator protein, in *H. influenzae*, *Salmonella enterica* serovar Typhimurium, and *E. coli* (88%, 84% and 84% respectively).

```
       10                  30                  50
CAAATAAAAAATGAATAAAAAGGAAGAATCACTATTCTTCCTTTTTTCTTTCTCATCGCA
       70                  90                 110
TTGCAATGAAATCATTTTACACTTCATTCTGTTTAACACAGCGGTACTGGTGACACAACA
      130                 150                 170
GGATGCCTATTCACTCATTCAAAAGGATGCTTTCTATGAAACTAAAAACCCTTGTGGCAA
                                    M  K  L  K  T  L  V  A  I
      190                 210                 230
TATTAGCCCTATCGACCTTAACTGCCTGTTCAAGTTCAAGCATGATGAAAAATGATGATA
  L  A  L  S  T  L  T  A  C  S  S  S  S  M  M  K  N  D  D  K
      250                 270                 290
AATTGATTATTGCACACCGTGGTGCAAGTGGTTACTTACCCGAACATACTCTAGAATCCA
  L  I  I  A  H  R  G  A  S  G  Y  L  P  E  H  T  L  E  S  K
      310                 330                 350
AAGCCCTCGCTTTTGGTCAACAAGCTGATTATTTAGAACAAGATCTCGCAATGACGAAAG
  A  L  A  F  G  Q  Q  A  D  Y  L  E  Q  D  L  A  M  T  K  D
      370                 390                 410
ACAACCGCCTGATTGTAATTCATGATCATTTCTTAGACGGCTTAACTGATGTAGCAAAAA
  N  R  L  I  V  I  H  D  H  F  L  D  G  L  T  D  V  A  K  K
      430                 450                 470
AATTCCCAAATCGAGCGAGAAAAGATGGACGCTATTATGTTGTGGATTTCACCTTAAAAG
  F  P  N  R  A  R  K  D  G  R  Y  Y  V  V  D  F  T  L  K  E
      490                 510                 530
AAATCCAAAGTTTAGAGATGACAGAAAACTTCAAAGTTGAAAACGGCAAACAAGTACAAG
  I  Q  S  L  E  M  T  E  N  F  K  V  E  N  G  K  Q  V  Q  V
      550                 570                 590
TTTACCCAAACCGTTTCCCCCTTTGGCAATCCCATTTCCGTATTCACACCTTTGAAGATG
  Y  P  N  R  F  P  L  W  Q  S  H  F  R  I  H  T  F  E  D  E
      610                 630                 650
AAATTGAATTTATTCAAGGTTTAGAAAAATCCACTGGCAAAAAAATTGGTATCTATCCTG
  I  E  F  I  Q  G  L  E  K  S  T  G  K  K  I  G  I  Y  P  E
      670                 690                 710
AAATCAAAGCCCCTTGGTTACACCATAAAGAAGGAAAAGACATCGCGGCAGAAACCTTAA
  I  K  A  P  W  L  H  H  K  E  G  K  D  I  A  A  E  T  L  K
      730                 750                 770
AAGTGCTGAAAAAATATGGCTATGACACAAAAGACGCGCGGGTTTATTTACAAACCTTCG
  V  L  K  K  Y  G  Y  D  T  K  D  A  R  V  Y  L  Q  T  F  D
      790                 810                 830
ACTTTAATGAGCTAAAACGGATTAAAACTGAATTGTTGCCAAAAATGGGCATGGATATCA
  F  N  E  L  K  R  I  K  T  E  L  L  P  K  M  G  M  D  I  K
      850                 870                 890
AGTTAGTTCAATTAGTCGCATACACTGATTGGCATGAAACAGAAGAGAAAAATGCACAAG
  L  V  Q  L  V  A  Y  T  D  W  H  E  T  E  E  K  N  A  Q  G
      910                 930                 950
GTAAATGGGTCAACTATGATTATGACTGGATGTTCAAACCGGGTGCGATGGCGGAAGTCG
  K  W  V  N  Y  D  Y  D  W  M  F  K  P  G  A  M  A  E  V  A
      970                 990                1010
CCAAATATGCCGATGGTGTAGGACCGGGCTGGTATATGCTCATCGACAACGAAAAATCGA
  K  Y  A  D  G  V  G  P  G  W  Y  M  L  I  D  N  E  K  S  K
     1030                1050                1070
AAGTGGGCAATATCGTTTATACCCCATTAGTCAAACAGCTCGCCAACTACAAGCTAGAAT
  V  G  N  I  V  Y  T  P  L  V  K  E  L  A  N  Y  K  L  E  L
     1090                1110                1130
TACATCCTTATACTGTCCGTAAAGATGCGTTACCTGCCTTCTTCACTGATGTGGATCAAA
  H  P  Y  T  V  R  K  D  A  L  P  A  F  F  T  D  V  D  Q  M
     1150                1170                1190
TGTACGATGCCCTGTTAAATAAACCTGGTGCAACAGGCGTCTTCACAGACTTCCCAGATA
  Y  D  A  L  L  N  K  A  G  A  T  G  V  F  T  D  F  P  D  T
     1210                1230                1250
CAGGTGTTGAGTTCTTAAAAAGAAACAAATAATCAAAGAATACGTTGTCTTGATTAAACA
  G  V  E  F  L  K  R  N  K  *
     1270                1290                1310
AGGAGGTACAAAGAGCGGTCAATTTTCACAATGTTGTTTATTGTCGGAAATGACCGCTTT
     1330                1350                1370
TCACATGATAAAAACGTTAACCAATCTCATACATTCACATTTATTTTCTACTACAATCCT
```

Figure 2.2. Nucleotide sequence of *P. multocida* X-73 *glpQ* and the deduced amino acid sequence for the ORF. The putative ribosome binding site is double-underlined and the asterisk denotes the TAA stop codon. The predicted SPaseII recognition sequence is boxed.

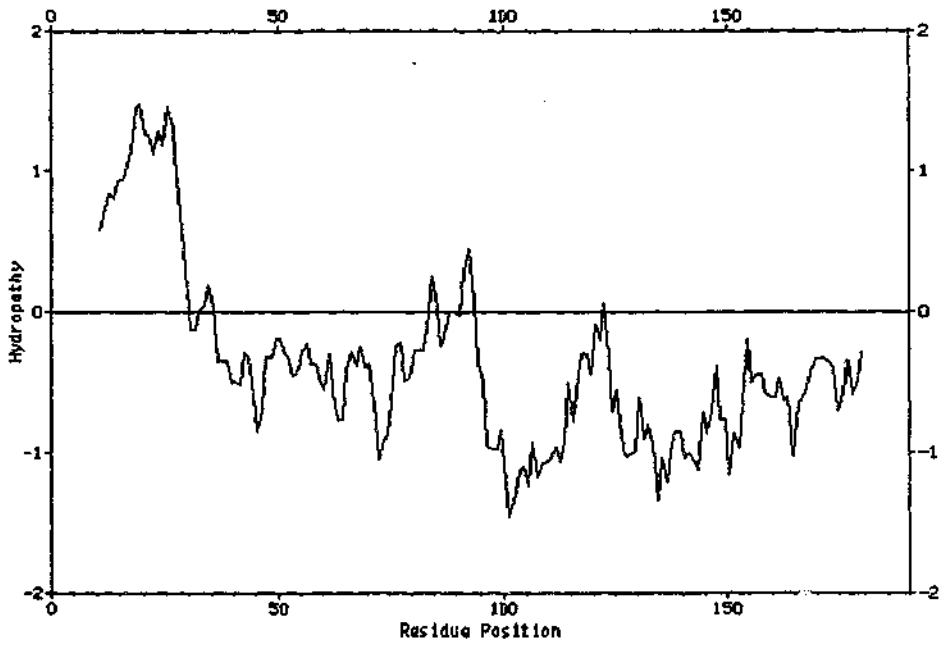

Figure 2.3. Kyte-Doolittle hydropathy profile of the deduced amino acid sequence of GlpQ. The hydrophobic peak at the N-terminus corresponds to the putative signal peptide. Window size of 20.

Transcriptional analysis of *glpQ*

To determine the *in vitro* activity of the *glpQ* promoter, the PmIVET clone, MHS25, was tested for sensitivity to Kan *in vitro* by plating onto BHI containing 0, 5, 10, 20, 30, 40 or 50 μg/ml of Kan. PBA917, the Kan sensitive control, was also plated onto varying concentrations of Kan. PBA917 grew only weakly at 5 μg/ml of Kan while the PmIVET clone grew at 30 μg/ml of Kan, indicating that the *glpQ* promoter has low activity *in vitro*. In order to corroborate the PmIVET result that *glpQ* is up-regulated *in vivo* when *P. multocida* X-73 is grown in mice, *in vivo* and *in vitro* transcription levels were compared by using real-time RT-PCR. Bacteria were grown in 10 mice and blood recovered from moribund mice was pooled before harvesting the bacteria and purifying the RNA. *lpxA* (which encodes UDP-N-acetylglucosamine acetyltransferase, an enzyme involved in the first step of lipid A biosynthesis) was used as the normaliser. *glpQ* was not significantly up-regulated in *P. multocida* X-73 grown in mice (Table 2.3).

Table 2.3. Comparison of *glpQ* expression levels between bacteria grown *in vitro* (in BHI) and bacteria recovered from mice. *lpxA* was used as the normaliser.

Sample	Normalised expression value (average *glpQ*/average *lpxA*) ± 1 standard deviation	*P* value
in vitro	1.367 ± 0.622	
Mice	4.627 ± 3.116	0.3803 (not significant)

To determine if *glpQ* is up-regulated when *P. multocida* X-73 is grown in chickens, the natural host, real-time RT-PCR was repeated using RNA isolated from bacteria grown in two individual chickens and using the gene encoding DNA gyrase subunit B, *gyrB*, as the normaliser. *gyrB* was used as the normaliser in subsequent real-time RT-PCR experiments in place of *lpxA*, as microarray analysis of *P. multocida* X-73 grown in chickens showed that there was less variation in the expression of *gyrB* compared to *lpxA*, and thus was a more suitable control for normalisation (J. Boyce, personal communication). The expression level of *glpQ* varied between bacteria grown in individual hosts; in *P. multocida* grown in chicken 10, *glpQ* was expressed at slightly lower levels than *in vitro*, while bacteria grown in chicken 32 expressed *glpQ* at 1.43-fold higher than bacteria grown *in vitro* (Table 2.4).

Table 2.4. Comparison of *glpQ* expression levels between bacteria grown *in vitro* (in BHI) and bacteria grown in two individual chickens. *gyrB* was used as the normaliser.

Sample	Normalised expression value (average *glpQ*/average *gyrB*) ± 1 standard deviation	*P* value
in vitro	0.099 ± 0.004	
Chicken 10	0.094 ± 0.002	0.1924 (not significant)
Chicken 32	0.141 ± 0.001	0.0032 (very significant)

Expression and purification of non-lipidated recombinant GlpQ

The section of *glpQ* encoding the predicted mature length protein was amplified by PCR from *P. multocida* X-73 genomic DNA using the primers BAP1224 and BAP1226 which had restriction enzyme sites engineered at their 5' ends. The PCR product was then cloned into pRSET-A and sequence analysis confirmed that *glpQ* was in-frame with the T7/hexa-histidine tag. The resulting plasmid, pPBA1664, was transformed into the *E. coli* strain BL21(DE3)pLysS to express the GlpQ fusion protein. Induction of the BL21(DE3)pLysS strain, AL262, with IPTG resulted in overexpression of a 43 kDa protein consistent with the estimated size of the T7/hexa-histidine tag (4 kDa) and mature length GlpQ (39.4 kDa). Western immunoblot analysis using an anti-T7 monoclonal antibody confirmed expression of recombinant GlpQ in *E. coli* (Figure 2.4, lane A) and the protein was then purified from disrupted cells using metal affinity resin (Figure 2.4, lane B).

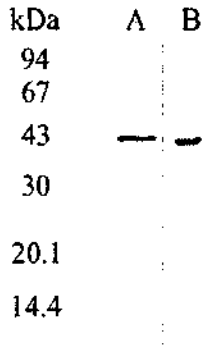

Figure 2.4. Expression and purification of T7/hexa-histidine tagged GlpQ. (A) Western immunoblot using the anti-T7 tag monoclonal antibody against AL264 whole cell lysate. (B) Purified T7-His tagged GlpQ stained with Coomassie brilliant blue. The positions of standard molecular mass markers (kDa) are shown on the left.

Immunisation of mice with non-lipidated recombinant GlpQ

To determine whether non-lipidated GlpQ would be able to stimulate immunity against challenge with live *P. multocida*, 10 mice were vaccinated ip with 50 µg of purified GlpQ emulsified in IFA. Another 10 mice received PBS in IFA, while 5 remained unvaccinated. None of the animals survived challenge with 10^2 cfu of live *P. multocida* X-73. Serum from each mouse was tested for reactivity to recombinant GlpQ. All vaccinated mice showed a reaction at 1/100 dilution of antiserum. Serum from two vaccinated mice was then selected to measure the antibody titre against GlpQ. To discount the possibility of antiserum reacting against the T7/hexa-histidine tag, a whole cell lysate (WCL) of *E. coli* expressing T7/hexa-histidine tagged MesA was added to the diluted antisera, which proved to be sufficient in removing reactivity to the tag (Figure 2.5). Antisera were able to detect 100 ng of GlpQ at a dilution of 1/16,000 (Figure 2.6); therefore, the antibody titres were ≥16,000.

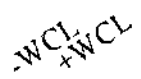

Figure 2.5. Removal of antibody reactivity to the T7/hexa-histidine tag. Each membrane strip contained T7 positive control cell extract which was included with the anti-T7 tag monoclonal antibody (Novagen). In the absence of WCL, anti-GlpQ serum recognised the T7 control, but when WCL was added to the serum, reactivity was removed.

The antisera raised against the non-lipidated recombinant GlpQ were absorbed with *E. coli* whole cell lysate and used in further characterisation of GlpQ.

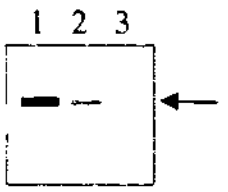

Figure 2.6. Reactivity of diluted GlpQ antiserum with 100 ng of purified GlpQ. Lane 1; membrane strip incubated with 1/500 dilution of antiserum. Lane 2; membrane strip incubated with 1/16,000 dilution of antiserum. Lane 3; membrane strip incubated with 1/500 dilution of pre-immunisation serum. GlpQ is indicated by the arrow.

Globomycin treatment

To confirm that GlpQ was subject to post-translational processing by SPaseII, mid-log phase *P. multocida* X-73 cultures were treated with globomycin. Globomycin specifically inhibits SPaseII activity resulting in accumulation of the larger, unprocessed prelipoprotein (Inukai *et al.*, 1984). Whole cell lysates were then analysed by Western immunoblot using anti-GlpQ serum. Globomycin was found to inhibit the processing of GlpQ (Figure 2.7). In the absence of globomycin, only one protein band was present, corresponding to mature length GlpQ. However, in the presence of globomycin, two protein bands were detected, corresponding to the processed mature form lipoprotein and the larger, unprocessed prelipoprotein. These results suggested that GlpQ was processed via the lipoprotein export pathway.

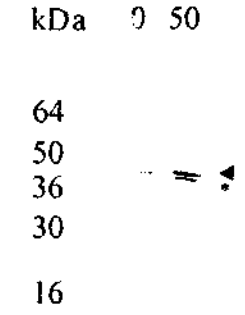

Figure 2.7. Western immunoblot showing the effect of globomycin on the processing of GlpQ in *P. multocida* X-73. Concentrations of globomycin used (μg/ml) are shown at the top of each lane. The processed lipoprotein is indicated by the asterisk and the prelipoprotein is indicated by the arrow. The positions of standard molecular mass markers (kDa) are shown on the left.

Expression and purification of lipidated recombinant GlpQ

To express large amounts of lipidated recombinant GlpQ, the gene, lacking the sequence encoding the N-terminal amino acids up to and including the cysteine residue of the SPaseII recognition site, was amplified by PCR from *P. multocida* X-73 genomic DNA using the primers BAP1489 and BAP1490 which had restriction enzyme sites engineered at their 5' ends. The PCR product was cloned into pDUMP and sequence analysis confirmed that *glpQ* was in-frame with the *E. coli* Lpp signal sequence and SPaseII recognition site. The resulting plasmid, pAL78, was transformed into the *E. coli* strain BL21(DE3)pLyS for expression of the recombinant protein. Induction of the BL21(DE3)pLysS strain, AL264, with IPTG resulted in overexpression of a 39 kDa protein. Western immunoblot analysis using anti-GlpQ antiserum obtained from the mouse vaccine trial confirmed expression of recombinant GlpQ in *E. coli* (Figure 2.8). Western immunoblot analysis of whole cell lysates from globomycin-treated AL264 cells showed that recombinant GlpQ was processed post-translationally by the *E. coli* SPaseII (Figure 2.9). Use of pDUMP has previously

been shown to result in expression of large quantities of lipidated recombinant proteins (Cullen *et al.*, 2003) and GlpQ (designated MlpA in that paper) was found to be 100% lipidated.

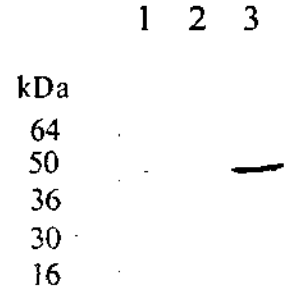

Figure 2.8. Western immunoblot showing expression of recombinant GlpQ in *E. coli*. Lane 1; *P. multocida* X-73 WCL. Lane 2; AL120 WCL. Lane 3; AL264 WCL. The positions of standard molecular mass markers (kDa) are shown on the left.

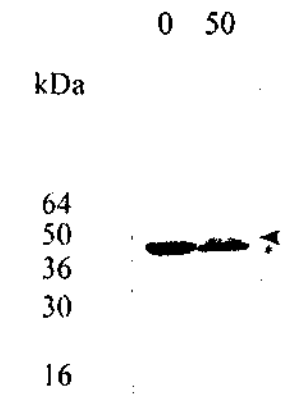

Figure 2.9. Western immunoblot showing the effect of globomycin on processing of recombinant GlpQ in *E. coli* BL21(DE3)pLysS. Concentrations of globomycin used (μg/ml) are shown at the top of each lane. The processed lipoprotein is indicated by the asterisk and the prelipoprotein is indicated by the arrow. The positions of standard molecular mass markers (kDa) are shown on the left.

Immunisation of mice and chickens with lipidated recombinant GlpQ

In the BL21(DE3)pLysS strain expressing lipidated GlpQ, the recombinant protein was found to be localised mainly in the *E. coli* membrane by Western immunoblot analysis of cytoplasmic and total membrane fractions separated using sucrose gradients (Figure 2.10). Therefore, live AL264 as well as purified AL264 total membranes was used in a vaccine trial to determine if lipidated GlpQ could protect mice against challenge with live *P. multocida*. From Coomassie brilliant blue stained

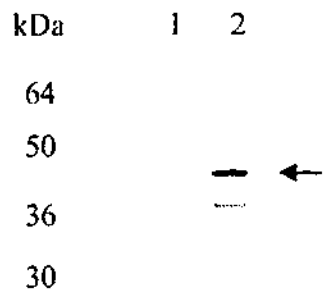

Figure 2.10. Western immunoblot using anti-GlpQ serum. Lane 1 contains cytoplasmic material from *P. multocida* X-73 while lane 2 contains the total membrane fraction. GlpQ, which appears to have electrophoresed further in the cytoplasmic fraction, is indicated by the arrow. A smaller band is seen in the membrane fraction which is most likely a cross-reacting *E. coli* protein. The positions of standard molecular mass markers (kDa) are shown on the left.

SDS-PAGE gels, it was estimated that recombinant GlpQ comprised approximately 50% of the total membrane fraction. 10 mice were vaccinated with live AL264, while 10 were vaccinated with AL264 membranes in IFA. As negative controls for reactivity to *E. coli* proteins, 8 mice received live AL120 and 8 were injected with AL120 membranes. 7 mice remained unvaccinated. All mice were challenged with 10^2 cfu of live *P. multocida* X-73. None of the animals survived challenge, although the vaccinated mice had strong antibody responses (antibody titres of ≥16,000) to GlpQ, similar to the result shown in Figure 2.6.

The total membrane fraction from AL264 expressing lipidated GlpQ was also used to vaccinate chickens. 5 chickens were vaccinated with AL264 membranes, 5 received AL120 membranes, while 5 remained unvaccinated. Chickens were challenged with 10^2 cfu of live *P. multocida* X-73. Again, no protection was observed (100% mortality) although vaccinated chickens also showed strong antibody responses to GlpQ. Anti-GlpQ sera were able to react with 100 ng of purified GlpQ at a dilution of 1/16,000, similar to the result shown in Figure 2.6.

Assay for GlpQ function

To determine if the *P. multocida* GlpQ homologue possessed glycerophosphodiester phosphodiesterase enzyme activity, a coupled spectrophotometric assay which had previously been optimised for the *E. coli* enzyme (Larson & van Loo-Bhattacharya, 1988; Larson *et al.*, 1983) was used. Three biological replicates of each strain or sample were tested. A high level of activity (13.4 ± 1.6 units/mg) was recorded for purified non-lipidated GlpQ (Table 2.5).

Table 2.5. GlpQ enzyme activity of strains expressing GlpQ

Sample	Form of GlpQ expressed	GlpQ activity[a] (Units/mg)	*P* value[b]
P. multocida X-73 sonicated extract	Native GlpQ	0.17 ± 0.01	
E. coli PBA1468 sonicated extract	GlpQ$^-$	0.22 ± 0.04	
E. coli AL262 sonicated extract	Non-lipidated GlpQ$^+$	0.36 ± 0.006	0.024
E. coli AL120 sonicated extract	GlpQ$^-$	0.05 ± 0.01	
E. coli AL264 sonicated extract	Lipidated GlpQ$^+$	3.35 ± 0.31	0.04
Purified GlpQ	Non-lipidated	13.4 ± 1.6	

[a] One unit of enzyme activity is defined as the reduction of 1 µmol of NAD per minute. Results are expressed as units per mg of total protein ± 1 standard deviation.
[b] Comparison of mean activity values between *E. coli* expressing GlpQ and *E. coli* harbouring the appropriate empty vector

Activity was also readily demonstrated in the sonicated extracts of *P. multocida* X-73 (0.17 ± 0.01 units/mg) and the *E. coli* strains, PBA1468 (harbouring the empty expression vector, pRSET-A) and AL120 (harbouring empty expression vector, pDUMP) (0.22 ± 0.04 and 0.05 ± 0.01 units/mg respectively). As *E. coli* possesses its own GlpQ enzyme (Larson *et al.*, 1983; Tommassen *et al.*, 1991) a background level of activity was observed for all *E. coli* strains. The sonicated extracts of the *E. coli* BL21(DE3)pLysS strains overexpressing non-lipidated and lipidated GlpQ (AL262 and AL264 respectively) were found to have higher enzyme activities (0.36 ± 0.006 and 3.35 ± 0.31 units/mg respectively) than those observed in extracts of *E. coli* not expressing recombinant GlpQ (Table 2.5). These results indicated that the *P. multocida* GlpQ homologue possessed glycerophosphodiester phosphodiesterase activity and that activity may be influenced by, but was not dependent on, N-terminal lipidation.

Localisation of GlpQ

Surface immunoprecipitation was used to determine if GlpQ was surface exposed. Antiserum raised against purified recombinant GlpQ was incubated with live, intact organisms. Unbound antibodies were removed prior to lysis and solubilisation of the whole cells. Protein A-Sepharose was then added to bind any antibody-antigen complexes. Material immunoprecipitated from intact cells and whole cell lysate controls was then analysed by Western immunoblot using GlpQ specific serum. Untreated whole cell lysate was included to indicate the size of GlpQ (Figure 2.11). GlpQ was immunoprecipitated from lysed cells but not intact cells, indicating that GlpQ was not accessible to specific antibodies and therefore not exposed on the surface of *P. multocida* X-73.

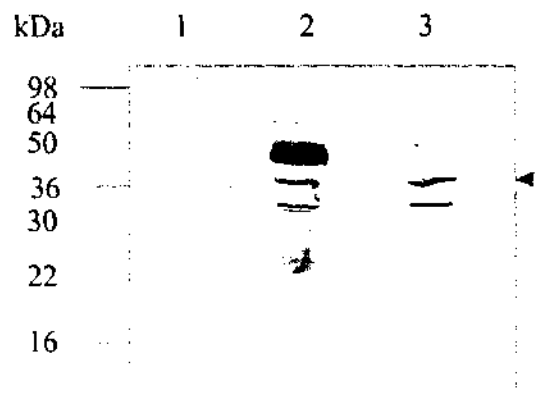

Figure 2.11. Western immunoblot showing immunoprecipitation of GlpQ. Lane 1 contains material immunoprecipitated from whole *P. multocida* X-73 cells and lane 2 contains material immunoprecipitated from *P. multocida* X-73 WCL. Lane 3 contains *P. multocida* X-73 WCL which has been untreated. The arrow indicates GlpQ. Where immunoprecipitation has occurred, the immunoglobulin heavy and light chains can be seen at ~50 kDa and ~25 kDa respectively. The positions of standard molecular mass markers (kDa) are shown on the left.

Distribution of GlpQ in *P. multocida* strains

Western immunoblot analysis was performed on WCLs from various strains of *P. multocida* to determine if GlpQ was present in strains other than X-73. Anti-GlpQ serum detected a 39 kDa band in each lane indicating that all strains of *P. multocida* tested possess GlpQ (Figure 2.12).

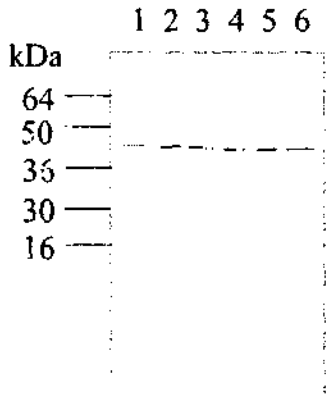

Figure 2.12. Western immunoblot using anti-GlpQ serum against WCLs from different strains of *P. multocida*, PBA100 (lane 1), X-73 (lane 2), VP161 (lane 3), P-1059 (lane 4), P-1662 (lane 5) and M1404 (lane 6). The positions of standard molecular mass markers (kDa) are shown on the left.

Attempted mutant construction

In order to assess the role of GlpQ in pasteurellosis, attempts were made to inactivate GlpQ in *P. multocida* X-73. The cloned copy of *glpQ* in pAL174 was disrupted by digesting pAL174 with *Bgl*II (a unique site within *glpQ*) and inserting a 3.2 kb *Bam*HI fragment containing the *tet*(M) gene, giving rise to the plasmid pAL183. However, attempts to introduce this mutated copy of *glpQ* by allelic exchange into the *P. multocida* X-73 genome were unsuccessful. Electroporation of 2 µg of pAL183 on four separate occasions yielded no transformants. A search of the *P. multocida* PM70 genome indicated the possible presence of a restriction modification system. Therefore, to prevent restriction digest of the plasmid, pAL183 was treated with Dam methylase prior to electroporation. Two attempts were made to electroporate 1 µg of Dam-methylase treated pAL183 into *P. multocida* X-73, but this strategy also yielded no transformants.

A *P. multocida* X-73 mutant in the *invA* gene had successfully been constructed by natural transformation (C. Ruffolo, unpublished data). Therefore, liquid and plate transformation methods were attempted to obtain a *glpQ* mutant in *P. multocida* X-73. In liquid transformation, 1.3 µg of linear PCR product (amplified by the *glpQ* flanking primers, BAP1224 and BAP1226) was added to a *P. multocida* X-73 culture grown to A_{600} of 0.3 and 0.9. In plate transformation, the linear PCR

product was circularised using ligase and 1 μg was added to the plate. Each method was attempted twice, but no transformants were obtained.

DISCUSSION

The *P. multocida* GlpQ homologue was originally identified using the PmIVET system, indicating that it is expressed during pasteurellosis in mice. However, transcriptional analysis by real-time RT-PCR showed that *glpQ* was not significantly up-regulated during growth in mice (Table 2.3). In the PmIVET system, clones were considered to possess a promoter active *in vivo*, but not *in vitro*, if they survived passaging in Kan-treated mice but were unable to grow *in vitro* in the presence of 50 μg/ml of Kan. The PmIVET clone, MHS25, was found to grow *in vitro* at 30 μg/ml of Kan compared with the wild-type X-73 resistance level of 5 μg/ml, indicating that *glpQ* is constitutively expressed. Real-time RT-PCR was performed to determine if *glpQ* was up-regulated during infection in chickens, the natural host. The results showed that the expression level of *glpQ* varied in *P. multocida* grown in two individual chickens (Table 2.4). Variability in gene expression between *P. multocida* populations grown in different chickens has previously been shown by Boyce *et al.* (2002). In chicken 10, the level of expression of *glpQ* was slightly lower than the expression level *in vitro*, but in chicken 32, *glpQ* was expressed at 1.43-fold higher than the *in vitro* level. Differences in gene expression between the different bacterial populations are not surprising given that individual host variation would have an impact on gene expression in the pathogen. However, a 1.43-fold level of over-expression is lower than the minimum level considered to be biologically significant (≥1.5-fold) (Hughes *et al.*, 2000; Smoot *et al.*, 2001). Although *glpQ* was not significantly up-regulated in *P. multocida* during growth *in vivo*, it is possible that *glpQ* may be differentially expressed at different stages of infection or under different environmental conditions. For example, it has been found that in *B. subtilis* GlpQ expression was strongly induced under phosphate starvation (Antelmann *et al.*, 2000), but expression was repressed under anaerobic conditions (Ye *et al.*, 2000).

The *P. multocida* GlpQ homologue showed the highest degree of similarity to protein D from *H. influenzae*, which has also been shown to possess GlpQ activity

(Munson & Sasaki, 1993). GlpQ homologues have been reported in many species of bacteria including *E. coli* (the first to be identified and characterised) (Larson *et al.*, 1983), *B. hermsii* (Shang *et al.*, 1997), *B. subtilis* (Nilsson *et al.*, 1994), *T. pallidum* (Shevchenko *et al.*, 1999) and *B. recurrentis* (Porcella *et al.*, 2000). The homologues are highly conserved between phylogenetically divergent bacteria (Shang *et al.*, 1997). GlpQ was also found to be present in all *P. multocida* strains tested (Figure 2.12).

The deduced amino acid sequences of the *P. multocida* GlpQ revealed a putative signal sequence followed by a consensus L-X-Y-C SPaseII cleavage site. Maturation of GlpQ was shown to be inhibited by globomycin and therefore indicated that *P. multocida* GlpQ is a lipoprotein (Figure 2.7). The deduced amino acid sequence of *P. multocida* GlpQ showed a serine residue in the +2 position, the first amino acid after the cysteine residue in the mature protein. Based on the '+2 rule' (Seydel *et al.*, 1999), GlpQ would be predicted to be exported to the outer membrane. However, this prediction does not indicate whether an outer membrane lipoprotein faces in or out of the membrane. In terms of immunity, it was more relevant to determine if the protein was exposed on the surface of live *P. multocida* and therefore readily accessible to specific antibodies. Surface immunoprecipitation has previously been used to demonstrate surface exposure of the leptospiral proteins OmpL1 and LipL41 (Shang *et al.*, 1996). Using this approach, it was found that the *P. multocida* GlpQ was not surface-exposed (Figure 2.10). However, analysis of *P. multocida* outer membrane fractions by 2-D gel electrophoresis and subsequent matrix-assisted laser desorption ionisation-time-of-flight (MALDI-TOF) mass spectroscopy indicated that GlpQ is present in the outer membrane (V. Nguyen, unpublished data).

The GlpQ homologue of *P. multocida* X-73 was shown to have GlpQ enzyme activity (Table 2.5). In *E. coli*, GlpQ is not a lipoprotein and is located in the periplasm (Larson & van Loo-Bhattacharya, 1988; Larson *et al.*, 1983). Given that GlpQ is a lipoprotein that appears not to be surface-exposed, it is likely that the protein is attached to the outer membrane but is exposed on the periplasmic face. Thus, its location and activity may be the same as in *E. coli*. GlpQ hydrolyses glycerophosphodiesters (G3POR) entering the periplasmic space to glycerol-3-phosphate (G3P) which is then actively transported into the cytoplasm by GlpT, a transporter protein or permease. G3P is then utilised in lipid biosynthesis or further processed for use in the glycolytic pathway (Figure 2.13). In *E. coli* and *B. subtilis*,

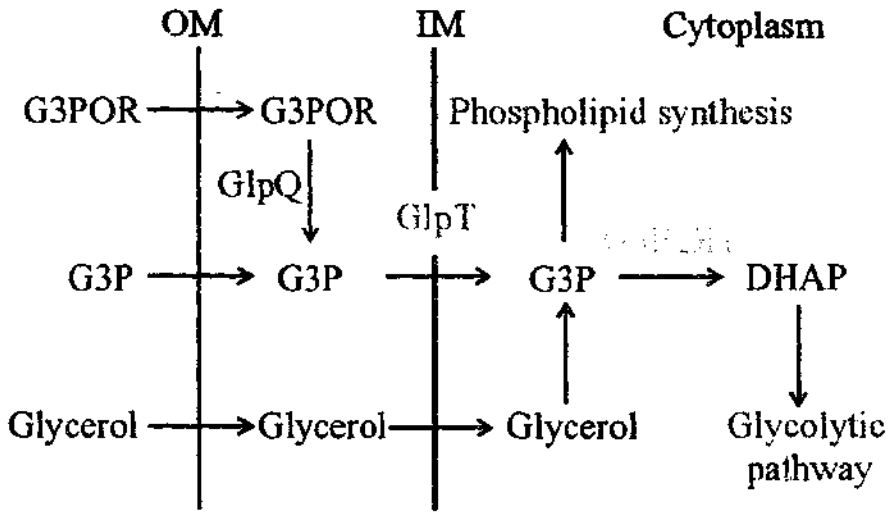

Figure 2.13. Schematic diagram of glycerol metabolism in *E. coli*. Glycerol, G3P, and glycerophosphodiester (G3POR) enter the periplasm via the outer membrane (OM) and G3POR is converted to G3P by GlpQ. G3P is then transported across the inner membrane (IM) by GlpT. G3P can then be used in phospholipid synthesis or converted into dihydroxyacetone phosphate (DHAP) by G3P dehydrogenase (G3P DH) which can then be used in the glycolytic pathway. Diagram modified from Shang *et al.* (1997).

GlpT and GlpQ are located in an operon (Larson *et al.*, 1982; Nilsson *et al.*, 1994). There is evidence to suggest that in *B. hermsii*, the GlpT and GlpQ homologues are not arranged in an operon (Shang *et al.*, 1997) although the genes have a similar arrangement. Protein D of *H. influenzae* was found to be expressed in *E. coli* from an endogenous promoter (Janson *et al.*, 1991). Interestingly, a sequence with similarity to that recognised by RpoS (a stationary phase sigma factor) was identified upstream of the *P. multocida glpQ* and an integration host factor (IF)-like binding site was also identified. This suggests that the *P. multocida glpT* and *glpQ* may not be arranged in an operon.

Despite the fact that *glpQ* does not appear to be surface-exposed nor up-regulated *in vivo*, the protective capacity of *P. multocida* GlpQ was investigated, as outer membrane proteins commonly play a role in stimulating host immunity. Previous studies showed that sera raised against protein D possessed bactericidal activity against heterologous strains of *H. influenzae* (Akkoyunlu *et al.*, 1996). However, vaccine trials using purified non-lipidated T7/hexa-histidine tagged recombinant GlpQ showed that *P. multocida* GlpQ was unable to stimulate protective immunity against live *P. multocida* X-73, even though mice had high antibody titres against the protein. We initially reasoned that this lack of protection may have been due to absence of the N-terminal lipid. In other species, lipidation has been shown to be important for immunogenicity. Bacterial lipoprotein gene fusions to non-lipoprotein antigens can enhance immunity as the lipoprotein can act as an adjuvant.

This approach was shown to stimulate a protective immune response against *Leishmania major* in mice (Cote-Sierra *et al.*, 2002). In *B. burgdorferi*, the lipidated form of the OspA lipoprotein stimulated protective immunity, whereas the non-lipidated form could not (Erdile *et al.*, 1993). Protein D in *H. influenzae* has also been shown to be more immunogenic in its lipidated form (Akkoyunlu *et al.*, 1997). Sera raised against lipidated protein D exhibited higher bactercidal activities against type b and nontypeable strains of *H. influenzae*. Therefore, lipidated *P. multocida* X-73 GlpQ was expressed in *E. coli* by cloning the gene into the lipoprotein expression vector, pDUMP. Total membrane preparations were used to vaccinate mice and chickens as the recombinant protein delivered in a membrane conformation would probably be more similar to the native conformation presented to the host during natural infection. Haake *et al.* (1999) found that recombinant OmpL1 and LipL41 administered in *E. coli* membrane fractions stimulated protective immunity against leptospirosis in hamsters, but purified recombinant hexa-histidine tagged proteins could not.

However, recombinant lipidated GlpQ also failed to stimulate protective immunity in mice and chickens, even though vaccinated animals again showed high antibody titres against GlpQ. This could possibly be due to inaccessibility of GlpQ-specific antibodies to GlpQ which was shown to be not present on the surface of the cell by lack of surface immunoprecipitation from intact cells (Figure 2.11). GlpQ in *T. pallidum* has also been shown to be a lipoprotein which is associated with the outer membrane, but is not surface-exposed (Shevchenko *et al.*, 1999). Rabbits which were immunised with recombinant *T. pallidum* GlpQ were not protected against intradermal challenge with virulent treponemes (Shevchenko *et al.*, 1999). It is also possible that the recombinant GlpQ may be folded differently from the native protein such that different epitopes are presented, but without loss of enzyme activity. Luo *et al.* (1997) had shown previously that the native form of the major outer membrane protein, OmpH, of *P. multocida* could stimulate homologous protection in chickens whereas the recombinant form purified from *E. coli* could not, even though high levels of antibodies towards recombinant OmpH were detected. Synthetic peptides which mimicked the conformational epitopes of the native OmpH were also able to induce homologous protection in chickens (Luo *et al.*, 1999). Therefore, future work could involve vaccine trials using native GlpQ from *P. multocida* X-73. It is interesting to note that although mouse antiserum raised against recombinant GlpQ

recognised native GlpQ from *P. multocida* X-73, convalescent antiserum (at a dilution of 1/00) from one chicken was unable to recognise recombinant GlpQ (data not shown). The real-time RT-PCR results showed that *glpQ* was not significantly up-regulated in *P. multocida* X-73 grown in two individual chickens so it is possible that there are insufficient quantities of GlpQ to generate detectable levels of anti-GlpQ antibodies. However, antiserum from more chickens and perhaps at different stages of infection would need to be tested before any conclusions can be drawn.

Although the *P. multocida* GlpQ does not appear to play a role in immunity, it may be important in pathogenesis. Protein D, the GlpQ homologue in *H. influenzae*, has been shown to be a virulence determinant in otitis media. A protein D deficient mutant of nontypeable *H. influenzae* was found to be 100-fold less virulent than the wild-type strain in experimental otitis media in rats and it was suggested that protein D may be required to cause ciliary damage (Janson *et al.*, 1994). Protein D has also been shown to bind IgD, which may allow *H. influenzae* to evade the host immune response (Ruan *et al.*, 1990; Sasaki & Munson, 1993). The *P. multocida* GlpQ may also possess IgD-binding capability, but this is irrelevant in the pathogenesis of fowl cholera as chickens do not have IgD (I. Wilkie, personal communication). However, it would be interesting to see if GlpQ has the ability to bind immunoglobulins. It has been speculated that the GlpQ activity of protein D may be important for the use of deacylated phosphlipids in mucosal secretions, thus enabling *H. influenzae* to multiply in the mucus layer (Janson *et al.*, 1999). In *Borrelia* species, GlpQ activity has been detected in relapsing-fever isolates, but not Lyme disease isolates, which may allow the organisms to attain higher cell densities in blood compared to Lyme disease spirochetes (Schwan *et al.*, 2003). The surface-exposed GlpQ in *H. influenzae* has recently been shown to mediate the acquisition of choline directly from the membranes of epithelial cells in culture and incorporate it into its own lipopolysaccharide, which may allow the organism to mimic host cells (Fan *et al.*, 2001). Although the *P. multocida* homologue was found to be non-surface exposed and therefore, may not interact directly with host choline, it may be required for acquiring extra nutrients for growth in the host.

Unfortunately, despite numerous attempts, and for reasons unknown, a GlpQ deficient mutant of *P. multocida* was not obtained and therefore the role of GlpQ in virulence could not be assessed. It does not seem likely that *glpQ* is an essential gene as G3P can probably be readily obtained from sources other than G3POR when the

organism is grown *in vitro*. Furthermore, as protein D mutants have been constructed in the closely related organism *H. influenzae*, inactivation of *glpQ* in *P. multocida* would probably not be lethal. Mutant construction in *P. multocida* has been difficult, and to date, only 7 defined mutants have been constructed in our laboratory (see Chapter 4). Perhaps more attempts at electroporation or natural transformation could be performed to increase the chances of obtaining a *glpQ* mutant by insertional inactivation. Another strategy such as conjugation could also be employed (see Chapter 4). Further study is thus needed to determine whether the *P. multocida* GlpQ plays a role in the pathogenesis of fowl cholera.

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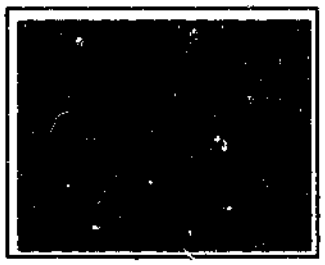
CHAPTER THREE

CLONING AND CHARACTERISATION OF PCP - A LIPOPROTEIN IDENTIFIED BY PmIVET



Based on:
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INTRODUCTION

An *in vivo* expression technology (IVET) system was previously developed and used to identify genes in *Pasteurella multocida* which are upregulated during infection of the host. This system, termed PmIVET, identified many genes (Hunt *et al.*, 2001), one of which encoded a protein with 88% similarity to PAL (peptidoglycan-associated lipoprotein) cross-reacting protein, or PCP. PCP is a membrane-associated lipoprotein in *Haemophilus influenzae* and anti-PCP serum has previously been shown to possess bactericidal activity against several clinical isolates of type b and nontypeable *H. influenzae* (Deich *et al.*, 1988; Deich *et al.*, 1990). Therefore, the protective capacity of the *P. multocida* homologue was investigated. This report describes the cloning and sequencing of the gene encoding the PCP homologue of *P. multocida* (designated *pcp*), its characterisation, and expression in *Escherichia coli* for vaccine trials in mice and chickens.

MATERIALS AND METHODS

Unless specified below, all materials and methods were as described in Chapter 2.

Oligonucleotides, bacterial strains and plasmids

Oligonucleotides used in this study are listed in Table 3.1 while bacterial strains and plasmids are shown in Table 3.2.

Table 3.1. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5'-3')	Target
BAP1308	AATAGGATCCAACTTTGGTTGTCG GTGC	Forward primer for *pcp*.
BAP1309	TCATGAATTCAAGAAAATTTAATG AATCAC	Reverse primer for *pcp*. Incorporates an *Eco*RI site (underlined).
BAP1406	CTTAGGATCCTGCGCTAACACCGA CATTTAC	Forward primer for *pcp*. Incorporates a *Bam*HI site (underlined).
BAP1491	CTTAGCCCATGGCGCTAACACCGA CATTTACAGTG	Forward primer for *pcp*. Incorporates an *Nco*I site (underlined).
BAP1492	TTTCATGGATCCAAGAAAATTTAAT GAATCAC	Reverse primer for *pcp*. Incorporates a *Bam*HI site (underlined).
BAP2100	AAGCTGATAGCCAAG GAGTTCTTG	Forward primer for real-time RT-PCR
BAP2101	CGTTGCAATCATTTGACCAGTAC	Reverse primer for real-time RT-PCR

Table 3.2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
P. multocida strain		
X-73	Serotype A:1 chicken isolate; reference strain	(Heddleston & Rebers, 1972)
MHS30	X-73 PmIVET clone containing 344 bp of *pcp* and 37 bp upstream	(Hunt *et al.*, 2001)
E. coli strains		
DH5α	*F endA1 hsdR17* ($r_k^- m_k^+$) *supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA-arg F*) U169 Ø80d*lacZ*ΔM15	Bethesda Research Laboratories
BL21(DE3)pLysS	F *ompT hsd*S_B($r_B^- m_B^-$) *gal dcm* (DE3) pLysS (Cm^R)	Invitrogen, Groningen, The Netherlands
PBA1468	BL21(DE3)pLysS harbouring pRSET-A	Laboratory strain
PBA1678	DH5α harbouring pPBA1678	This study
AL120	BL21(DE3)pLysS harbouring pDUMP	(Cullen *et al.*, 2003)
AL263	BL21(DE3)pLysS harbouring pAL19	This study
AL265	BL21(DE3)pLysS harbouring pAL79	This study
Plasmids		
pWSK29	Amp^R, *lacZ'*, pSC101 origin, f1 origin	(Wang & Kushner, 1991)
pPBA1678	Full length *pcp* gene cloned into pWSK29	This study
pRSET-A	Amp^R, f1 origin, T7 promoter	Invitrogen
pDUMP	*E. coli* lipoprotein expression vector. Section encoding the signal peptide from the *E. coli* lipoprotein Lpp cloned into pET-9c (Novagen, Madison, WI.)	(Cullen *et al.*, 2003)
pAL19	Section of *pcp* encoding the mature length protein cloned into pRSET-A	This study
pAL79	Section of *pcp* encoding the mature length protein cloned into pDUMP	This study

RESULTS

Cloning and sequence analysis of *pcp*

As the PmIVET clones identified previously (Hunt *et al.*, 2001) did not contain the complete sequence of the lipoprotein gene, the entire gene had to be cloned from *P. multocida* X-73 and its nucleotide sequence determined. At the time of commencement of this work, the sequencing of the *P. multocida* PM70 genome (May *et al.*, 2001) had not yet been completed. Using the existing DNA sequence from the PmIVET clone, a comparison with the incomplete PM70 genome database was carried out. The nucleotide sequence of the *P. multocida* PCP homologue was available on the *P. multocida* PM70 genome database. In PM70, the gene encoding the PCP homologue has been annotated as *lpp* or PM0554, but in this study, the gene is designated *pcp* due to its similarity with the *H. influenzae* homologue. The primers

BAP1308 and BAP1309 were designed from the PM70 sequence to amplify the entire *pcp* gene from the X-73 genome. The PCR product was then cloned into pWSK29 giving rise to the plasmid pPBA1678. The entire *pcp* gene (Figure 3.1) comprised 462 bp and encoded a protein of 154 amino acids with a predicted molecular mass of 15.6 kDa. The N-terminus of the deduced amino acid sequence contained an 18-amino-acid stretch ending in a putative signal peptidase II (SPaseII) recognition site, LAGC, indicating that PCP was most likely a lipoprotein (von Heijne, 1989). A putative -10 promoter sequence was identified with the upstream region of *pcp* and a consensus ribosomal binding site was located 5 bp upstream of the start codon (Figure 3.1). A putative -35 promoter sequence was not identified. Kyte-Doolittle

```
        10                  30                  50
GATCACCTTTATAATAATTCTATTTTTAAAGTAGGAGTTCTATGAAAAAAGTAACATTTG
          -10                                 M  K  K  V  T  F  A
        70                  90                  110
CCTTAGCTATTTTAATGAGTTTAGGCTTAGCAGGTTGCGCTAACACCGACATTTACAGTG
  L  A  I  L  M  S  L  G  L  A  G  C  A  N  T  D  I  Y  S  G
      130                 150                 170
GTAATGTGTACGAAGGTAATCAAGCAAAAGAAGTACGTTCAATTTCTTATGGTACGATTG
  N  V  Y  E  G  N  Q  A  K  E  V  R  S  I  S  Y  G  T  I  V
      190                 210                 230
TTTCTAGTCGTCCTGTCAAAATTCAAGCTGATAGCCAAGGAGTTCTTGGTGGCTTTGGTG
  S  S  R  P  V  K  I  Q  A  D  S  Q  G  V  L  G  G  F  G  G
      250                 270                 290
GCGGTGCGCTAGGGGGTATCGTAGGTTCTGGTATCGGTGGTGGTACTGGTCAAATGATTG
  G  A  L  G  G  I  V  G  S  G  I  G  G  G  T  G  Q  M  I  A
      310                 330                 350
CAACGACAGTTGGGGCAATTGCTGGTGCAGTTATTGGTGCGAAAGCGGAAGAAAAATTGA
  T  T  V  G  A  I  A  G  A  V  I  G  A  K  A  E  E  K  L  N
      370                 390                 410
ACCAAGTAGATTCTTTAGAATTAGTGATCAAAAAAGACAACGGACAAGAAATCGTTGTTG
  Q  V  D  S  L  E  L  V  I  K  K  D  N  G  Q  E  I  V  V  V
      430                 450                 470
TACAAAAATATGATCAAAGCTTAGTTCCAGGTGCGCGTGTGCGTATCGTAGGTGGTTCTA
  Q  K  Y  D  Q  S  L  V  P  G  A  R  V  R  I  V  G  G  S  K
      490                 510                 530
AAGTGAATGTATCTGTGATTCATTAAATTTTCTTTTATTCATGAAAAGGCTTCGAGTAAC
  V  N  V  S  V  I  H  *
```

Figure 3.1. Nucleotide sequence of the *P. multocida* X-73 *pcp* gene and the deduced amino acid sequence for the encoded protein. The putative -10 promoter sequence is underlined and the putative ribosome binding site is double-underlined. The asterisk denotes the TAA stop codon and the predicted SPaseII recognition site is boxed.

hydropathy plot analysis of the amino acid sequence showed that the N-terminal region corresponding to the putative signal peptide is hydrophobic (Figure 3.2). There is also a hydrophobic region in the middle of the protein which may allow PCP to interact with other proteins in the cell membrane. The hydrophobic central region is unlikely to be a transmembrane domain given that the PEPTIDESTRUCTURE program on ANGIS did not identify any alpha-helical domains in that region. The

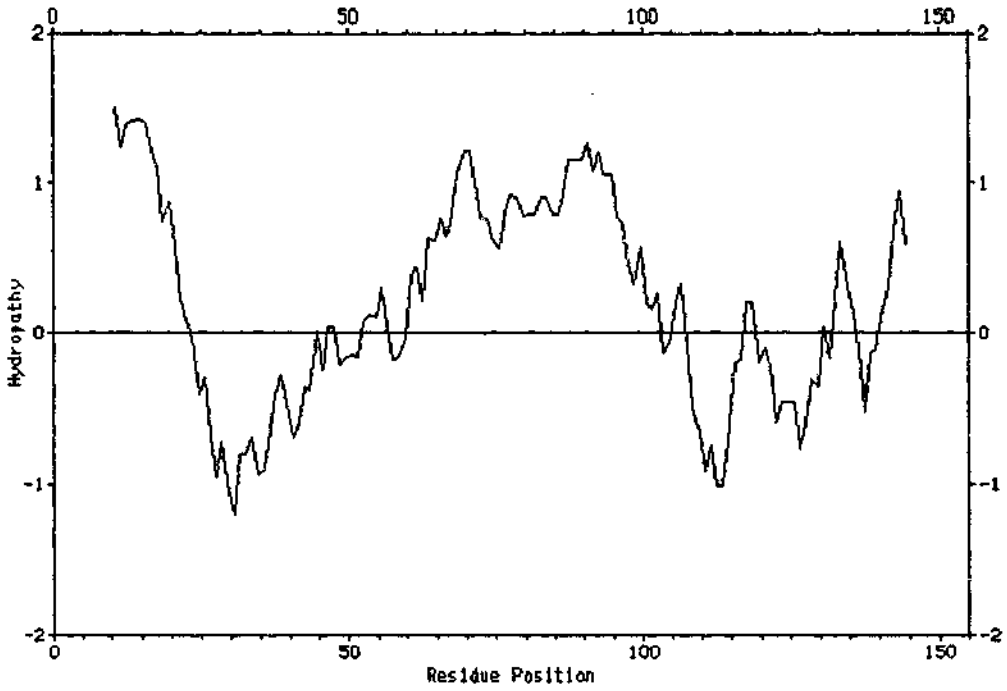

Figure 3.2. Kyte-Doolittle hydrophobicity plot of the deduced amino acid sequence of PCP. Window size of 20. The hydrophobic peak at the N-terminus corresponds to the putative signal peptide.

processed mature length protein had a predicted molecular mass of 13.7 kDa and a calculated pI of 8.4. PCP showed 80% similarity to the PAL (peptidoglycan associated lipoprotein) cross-reacting protein of *H. influenzae*. It also had similarity to the outer membrane lipoproteins SlyB from *Salmonella enterica* serovar Typhimurium (69%), SlyB from *E. coli* (67%) and PCP or PCPY from *Yersinia enterocolitica* (67%). The *P. multocida* X-73 *pcp* gene had 99% nucleotide identity to the homologue *lpp* in the published *P. multocida* PM70 genome (May *et al.*, 2001).

The nucleotide sequence of the *P. multocida* strain X-73 *pcp* gene has been deposited in the GenBank database under the accession number AY218826.

Transcriptional analysis of *pcp*

The PmIVET clone, MHS30, was tested for sensitivity to Kan *in vitro* by plating onto BHI containing 0, 5, 10, 20, 30, 40 or 50 µg/ml of Kan. PBA917, the Kan sensitive control, was also plated onto varying concentrations of Kan. The PBA917 control grew only weakly at 5 µg/ml of Kan while the PmIVET clone grew at 10 µg/ml of Kan, indicating that the *pcp* promoter is very weakly active *in vitro*. *In vivo* and *in vitro* transcription levels were then compared by using real-time RT-PCR. Blood from 10 mice in the late stages of *P. multocida* infection was pooled before harvesting the bacteria and purifying the *in vivo*-expressed RNA. *lpxA* (which encodes UDP-N-acetylglucosamine acetyltransferase, an enzyme involved in the first

step of lipid A biosynthesis (Anderson & Raetz, 1987)) was used as the normaliser. *pcp* was found to be up-regulated when the bacteria were grown in mice (Table 3.3). However, the result from the mouse sample was not statistically significant as there was only sufficient RNA available to do one real time RT-PCR experiment. Due to difficulties in obtaining adequate quantities of blood (and therefore bacteria) from moribund mice, the real-time RT-PCR experiment on *P. multocida* grown in mice was not repeated.

Table 3.3. Comparison of *pcp* expression levels between bacteria grown *in vitro* (in BHI) and bacteria recovered from mice. *lpxA* was used as the normaliser.

Sample	Normalised expression value (average *pcp*/average *lpxA*) ± 1 standard deviation	*P* value
in vitro	7.266 ± 6.729	
Mice	44.89	No value calculated as only one sample tested

To determine if *pcp* was up-regulated in chickens, the natural host, real-time RT-PCR was repeated using RNA isolated from *P. multocida* X-73 grown in two individual chickens and using the gene encoding DNA gyrase subunit B, *gyrB* (see Chapter 2), as the normaliser. *pcp* appeared to be significantly down-regulated in *P. multocida* X-73 grown in chickens (Table 3.4).

Table 3.4. Comparison of *pcp* expression levels between bacteria grown *in vitro* (in BHI) and bacteria grown in two individual chickens. *gyrB* was used as the normaliser.

Sample	Normalised expression value (average *pcp*/average *gyrB*) ± 1 standard deviation	*P* value
in vitro	3.455 ± 0.082	
Chicken 10	2.017 ± 0.009	0.0011 (very significant)
Chicken 32	3.024 ± 0.015	0.0122 (significant)

Expression and purification of non-lipidated recombinant PCP

For expression of non-lipidated, T7-hexa-histidine-tagged proteins in *E. coli* BL21(DE3)pLysS, the section of the gene encoding the predicted mature length PCP was amplified by PCR from *P. multocida* X-73 genomic DNA using the primers BAP1406 and BAP1309 which had restriction enzyme sites engineered at their 5' ends. The PCR product was then cloned into pRSET-A and mature length PCP was

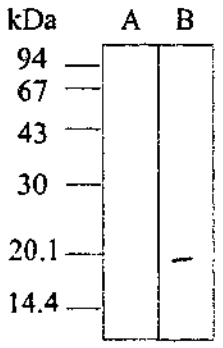

Figure 3.3. Expression and purification of T7/hexa-histidine tagged PCP. (A) Western immunoblot using the anti-T7 tag monoclonal antibody against AL263 whole cell lysate. (B) Purified T7/hexa-histidine tagged PCP stained with Coomassie brilliant blue. The positions of standard molecular mass markers (kDa) are shown on the left.

expressed as a T7/hexa-histidine tagged fusion protein. The BL21(DE3)pLysS strain, AL263, was then induced with IPTG which resulted in overexpression of a 17 kDa PCP fusion protein. Western immunoblot analysis using an anti-T7 monoclonal antibody confirmed expression of recombinant PCP in *E. coli* (Figure 3.3, lane A) and the protein was then purified from disrupted cells using metal affinity resin (Figure 3.3, lane B).

Immunisation of mice with non-lipidated recombinant PCP

To determine whether non-lipidated PCP would be able to stimulate immunity against challenge with live *P. multocida*, 10 mice were vaccinated ip with 50 μg of protein emulsified in IFA, another 5 received PBS in IFA, while 2 remained unvaccinated. None of the animals survived challenge with 10^2 cfu of live X-73. Serum from each mouse was tested for reactivity to recombinant PCP. All vaccinated mice showed a reaction at 1/100 dilution of antiserum. Serum from two vaccinated mice was then selected to measure the antibody titre against PCP. To absorb any reactivity against the T7/hexa-histidine tag, a whole cell lysate of *E. coli* expressing T7/hexa-histidine tagged MesA was added to the diluted antisera, which proved to be sufficient in removing reactivity to the tag (Figure 2.5, Chapter 2). Antisera were able to detect 100 ng of PCP at a dilution of 1/16,000 (Figure 3.4); therefore, the antibody titres were ≥16,000.

The antisera raised against the non-lipidated recombinant PCP were absorbed with *E. coli* whole cell lysate and used in further characterisation of PCP.

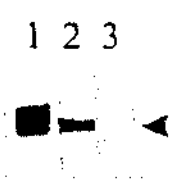

Figure 3.4. Reactivity of diluted PCP antiserum to 100 ng of purified PCP. Lane 1; membrane strip incubated with 1/500 dilution of antiserum. Lane 2; membrane strip incubated with 1/16,000 dilution of antiserum. Lane 3; membrane strip incubated with 1/500 dilution of pre-immunisation serum. PCP is indicated by the arrow.

Globomycin treatment

To confirm that PCP was subject to post-translational processing by SPaseII, mid-log phase *P. multocida* X-73 cultures were treated with globomycin, a specific inhibitor of SPaseII activity. Globomycin was found to inhibit the processing of PCP (Figure 3.5). In the absence of globomycin, only one protein band was present, corresponding to mature length PCP. However, in the presence of globomycin, two protein bands were detected, corresponding to the processed mature form lipoprotein and the larger, unprocessed prelipoprotein. These results suggested that PCP was processed via the lipoprotein export pathway.

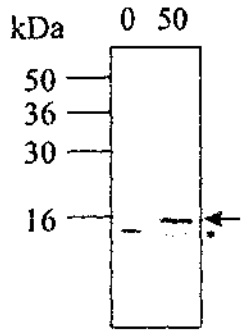

Figure 3.5. Western immunoblot showing the effect of globomycin on the processing of PCP in *P. multocida* X-73. Concentrations of globomycin used (μg/ml) are shown at the top of each lane. The processed lipoprotein is indicated by the asterisk and the prelipoprotein is indicated by the arrow. The positions of standard molecular mass markers (kDa) are shown on the left.

Expression and purification of lipidated recombinant PCP

To express lipidated recombinant PCP in *E. coli*, the gene, lacking the sequence encoding the N-terminal amino acids up to and including the cysteine residue of the SPaseII recognition site, was amplified by PCR from *P. multocida* X-73 genomic DNA using the primers BAP1491 and BAP1492 which had restriction enzyme sites engineered at their 5' ends. The PCR product was cloned into pDUMP and sequence analysis confirmed that *pcp* was in-frame with the *E. coli* Lpp signal sequence and SPaseII recognition site. The resulting plasmid, pAL79, was transformed into the *E. coli* strain BL21(DE3)pLyS for expression of the recombinant protein. Induction of the resulting BL21(DE3)pLysS strain, AL265, with IPTG resulted in overexpression of PCP. Western immunoblot analysis using anti-PCP antiserum obtained from the mouse vaccine trial confirmed expression of recombinant PCP in *E. coli*

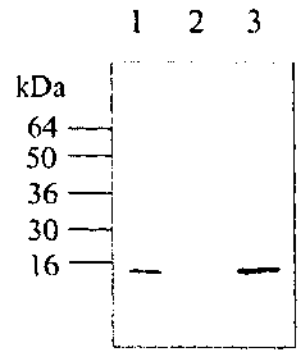

Figure 3.6. Western immunoblot showing expression of recombinant PCP in *E. coli*. Lane 1; *P. multocida* X-73 WCL. Lane 2; AL120 WCL. Lane 3; AL265 WCL. The positions of standard molecular mass markers (kDa) are shown on the left.

(Figure 3.6). Western immunoblot analysis of whole cell lysates from globomycin-treated AL265 cells showed that PCP was processed post-translationally by the *E. coli* SPaseII (Figure 3.7). Use of pDUMP has previously been shown to result in expression of large quantities of lipidated recombinant proteins (Cullen *et al.*, 2003).

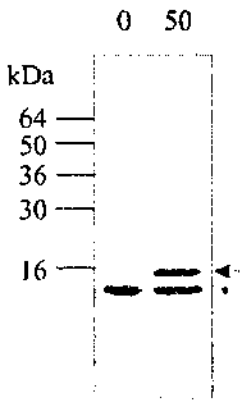

Figure 3.7. Western immunoblot showing the effect of globomycin on processing of recombinant PCP in *E. coli* BL21(DE3)pLysS. Concentrations of globomycin used (μg/ml) are shown at the top of each lane. The processed lipoprotein is indicated by the asterisk and the prelipoprotein is indicated by the arrow. The positions of standard molecular mass markers (kDa) are shown on the left.

Immunisation of mice and chickens with lipidated recombinant PCP

In the BL21(DE3)pLysS strain expressing lipidated PCP, AL265, the recombinant proteins were found to be localised in the *E. coli* membrane by Western immunoblot analysis of cytoplasmic and total membrane fractions separated using sucrose gradients (Figure 3.8). Therefore, live AL265 as well as purified AL265 total membranes were used in a vaccine trial to determine if lipidated PCP could protect mice against challenge with live *P. multocida*. From Coomassie brilliant blue stained SDS-PAGE gels, it was estimated that recombinant PCP comprised approximately 30% of the total membrane fraction. 10 mice were vaccinated with live AL265, while 10 were vaccinated with AL265 membranes in IFA. As negative controls for reactivity to *E. coli* proteins, 8 mice received live AL120 (BL21(DE3)pLysS harbouring empty pDUMP) and 8 were injected with AL120 membranes. 7 mice remained unvaccinated. No protection was observed although the vaccinated mice had strong antibody responses

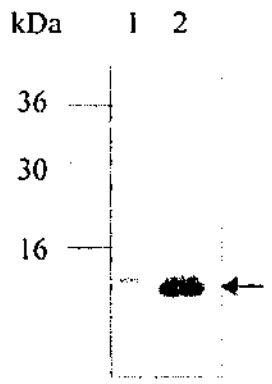

Figure 3.8. Western immunoblot using anti-PCP serum. Lane 1 contains cytoplasmic material from *P. multocida* X-73 while lane 2 contains the total membrane fraction. The two bands most likely represent the lipidated and unprocessed, non-lipidated forms of PCP. PCP is indicated by the arrow. The positions of standard molecular mass markers (kDa) are shown on the left.

(antibody titres of ≥16,000) to PCP, similar to the result shown in Figure 3.4.

The total membrane fractions from AL265 expressing lipidated PCP were also used to vaccinate chickens. 5 chickens were vaccinated with AL265 membranes, 5 received AL120 membranes, while 5 remained unvaccinated. Chickens were challenged with 10^2 cfu of live *P. multocida* X-73. Again, no protection was observed although vaccinated chickens also showed strong antibody responses. Serum from vaccinated chickens were able to react with 100 ng of purified PCP at a dilution of 1/16,000, similar to the result shown in Figure 3.4.

Localisation of PCP

Surface immunoprecipitation was used to determine if PCP was surface exposed. Antiserum raised against purified recombinant PCP was incubated with live, intact *P. multocida* cells. Unbound antibodies were removed prior to lysis and solubilisation of the whole cells and protein A-sepharose was then added to bind any antibody-antigen complexes. Material immunoprecipitated from intact cells and whole cell lysate controls was then analysed by Western immunoblot using PCP specific sera. Untreated whole cell lysate was included to show the size of PCP (Figure 3.9). PCP was immunoprecipitated from intact cells as well as lysed cells, consistent with surface exposure.

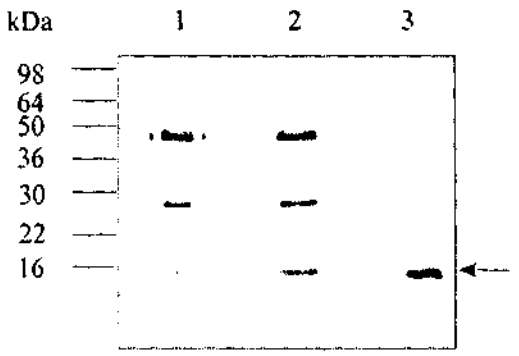

Figure 3.9. Immunoprecipitation of PCP. Western immunoblot was probed with anti-PCP antiserum. Lane 1 contains material immunoprecipitated from whole *P. multocida* X-73 cells and lane 2 contains material immunoprecipitated from *P. multocida* X-73 WCL. Lane 3 contains *P. multocida* X-73 WCL which has been untreated. The arrow indicates PCP. Where immunoprecipitation has occurred, the immunoglobulin heavy and light chains can be seen at ~50 kDa and ~25 kDa respectively. The positions of standard molecular mass markers (kDa) are shown on the left.

Distribution of PCP in *P. multocida* strains

Western immunoblot analysis was performed on WCLs from various strains of *P. multocida* to determine if PCP was present in strains other than X-73. Anti-PCP serum detected a 14 kDa band in each lane indicating that all strains of *P. multocida* tested possess PCP (Figure 3.10).

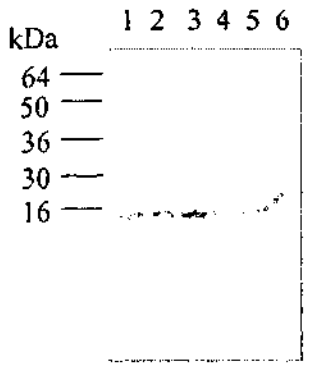

Figure 3.10. Western immunoblot using anti-PCP serum against WCLs from different strains of *P. multocida*, PBA100 (lane 1), X-73 (lane 2), VP161 (lane 3), P-1059 (lane 4), P-1662 (lane 5) and M1404 (lane 6). The positions of standard molecular mass markers (kDa) are shown on the left.

Reactivity of convalescent chicken antiserum to PCP

To determine if PCP was recognised by the host immune response during pasteurellosis, Western immunoblot analysis was performed on purified non-lipidated recombinant PCP using convalescent chicken antiserum. The serum was able to detect PCP (Figure 3.11).

Figure 3.11. Reactivity of convalescent chicken antiserum to purified non-lipidated recombinant PCP. The arrow denotes PCP. The larger band is a contaminating *E. coli* protein which is occasionally co-purified with the T7/hexa-histidine tagged PCP. The positions of standard molecular mass markers (kDa) are shown on the left.

DISCUSSION

Outer membrane proteins commonly play a role in stimulating host immunity. The *P. multocida* PCP homologue was originally identified using the PmIVET system, indicating that it is expressed during pasteurellosis in mice. The PmIVET clone, MHS30, grew very weakly on BHI plates containing >5 μg/ml of Kan, indicating that the *pcp* promoter is not active or very weakly active *in vitro*. However, *pcp* appeared to be down-regulated when *P. multocida* was grown in chickens. This result contradicts the fact that *pcp* was identified by an IVET system which aims to select for promoters that are active *in vivo*. However, it is possible that *pcp* is expressed differently during growth in chickens compared with growth in mice. The

one real-time RT-PCR result obtained with RNA from mouse-propagated *P. multocida* seemed to indicate that *pcp* is up-regulated *in vivo* (Table 3.3), consistent with the PmIVET result (Hunt *et al.*, 2001). However, the result was statistically insignificant as there was only sufficient RNA to perform the experiment once. Unfortunately, this experiment was not repeated due to difficulties involved in obtaining enough blood from moribund mice, and therefore, there were inadequate numbers of bacteria from which to purify RNA. It is also possible that *pcp* may be expressed at different levels during different stages of infection. In the PmIVET system, clones were recovered over a 13 hr period during infection (Hunt *et al.*, 2001) whereas *in vivo* RNA samples were obtained from bacteria late in the infection process (≥24 hr) when the animals were moribund. Boyce *et al.* (2002) showed that gene expression can vary between *P. multocida* populations grown in different chickens, so real-time RT-PCR could perhaps be performed on *P. multocida* isolated from more chickens. Nonetheless, the fact that PCP is a putative outer membrane lipoprotein warranted further studies into its role in immunity. In *H. influenzae*, previous studies showed that sera raised against PCP possessed bactericidal activity against heterologous strains (Deich *et al.*, 1990). Therefore, the protective capacity of *P. multocida* PCP was investigated.

The deduced amino acid sequence of PCP revealed a signal peptide followed by a consensus L-X-Y-C SPaseII cleavage site. Maturation of PCP was shown to be inhibited by globomycin and therefore indicated that *P. multocida* PCP is indeed a lipoprotein (Figure 3.5). The deduced amino acid sequence of *P. multocida* PCP showed an alanine residue in the +2 position, the first amino acid after the cysteine residue in the mature protein. Based on the '+2 rule' (Seydel *et al.*, 1999), PCP was predicted to be exported to the outer membrane. However, this prediction does not indicate whether an outer membrane lipoprotein faces in or out of the membrane. In terms of immunity, it was more relevant to determine if the protein was exposed on the surface of live *P. multocida* and therefore readily accessible to specific antibodies. Surface immunoprecipitation has previously been used to demonstrate surface exposure of the leptospiral proteins OmpL1 and LipL41 (Shang *et al.*, 1996). Using this approach, it was found that the *P. multocida* PCP was surface-exposed (Figure 3.9). Analysis of *P. multocida* outer membrane preparations by 2-D gel electrophoresis and subsequent matrix-assisted laser desorption ionisation-time-of-

flight (MALDI-TOF) mass spectroscopy confirmed that PCP is present in the outer membrane (V. Nguyen, unpublished data).

The *P. multocida* PCP homologue showed highest similarity to PCP (PAL (peptidoglycan associated lipoprotein) cross-reacting protein) of *H. influenzae.* PCP in *H. influenzae* was originally identified due to cross-reactivity with antiserum against a 15 kDa peptidoglycan-associated lipoprotein (PAL) located in the outer membrane (Deich *et al.*, 1988). The *P. multocida* homologue also had similarity to the outer membrane lipoproteins SlyB from *S. enterica* serovar Typhimurium, SlyB from *E. coli* and PCP or PCPY from *Y. enterocolitica*.

We were unable to assign function to the *P. multocida* PCP homologue. SlyB from *S. enterica*, when expressed in *E. coli*, has been implicated in non-specifically enhancing secretion of a haemolytic protein (Ludwig *et al.*, 1995). The homologue in *Y. enterocolitica* was initially thought to play a role in ferrioxamine B uptake, but this was attributed to changes in membrane permeability due to overexpression of an outer membrane lipoprotein rather than active uptake by PCP (Baumler & Hantke, 1992).

Vaccine trials using purified, non-lipidated, T7/hexa-histidine tagged recombinant PCP showed that the protein was unable to stimulate protective immunity, although mice had high antibody titres to PCP. This may have been due to absence of the N-terminal lipid. In other species, lipidation has been shown to be important for immunogenicity. In *B. burgdorferi*, the lipidated form of the OspA lipoprotein stimulated protective immunity, whereas the non-lipidated form could not (Erdile *et al.*, 1993). Therefore, lipidated PCP was expressed in *E. coli* by cloning the gene into the lipoprotein expression vector, pDUMP. As with lipidated GlpQ, total membrane preparations from *E. coli* expressing lipidated PCP were used to vaccinate mice and chickens. Total membrane preparations were used as the recombinant protein delivered in a membrane conformation would probably be more similar to the native protein presented to the host during natural infection. Haake *et al.* (1999) found that recombinant OmpL1 and LipL41 administered in *E. coli* membrane fractions stimulated protective immunity against leptospirosis in hamsters, but purified recombinant hexa-histidine tagged proteins could not.

However, recombinant lipidated PCP also failed to stimulate protective immunity in mice and chickens, even though vaccinated animals had high antibody titres. As with recombinant GlpQ (Chapter 2), lack of protection against pasteurellosis after vaccination with recombinant PCP may have been due to the

recombinant protein adopting a conformation different from that of the native protein. The major outer membrane protein of *P. multocida*, OmpH, has been shown to stimulate homologous immunity when chickens were immunised with the native protein, but recombinant OmpH purified from *E. coli* could not (Luo *et al.*, 1999). Therefore, future work could involve vaccine trials using native PCP purified from *P. multocida*.

PCP was recognised by convalescent chicken antiserum (Figure 3.11). It is possible that PCP may be involved in immunity in conjunction with other antigens. In *H. influenzae*, PCP has been shown to be antigenically conserved in 30 tested strains and bactericidal activity of polyclonal anti-PCP serum was enhanced in combination with anti-PAL serum, indicating that PCP may be a valuable component in a subunit vaccine, especially in combination with PAL (Deich *et al.*, 1990). PAL in *H. influenzae* was formerly designated P6 (Deich *et al.*, 1990; Green *et al.*, 1987) and a P6 homologue has been identified in *P. multocida* (Kasten *et al.*, 1995). However, vaccination of turkeys with recombinant P6 failed to stimulate protective immunity against fowl cholera (Kasten *et al.*, 1997). Although neither *P. multocida* PCP nor P6, when administered alone, were able to stimulate protective immunity, they may still be useful in a combination vaccine. For example, the leptospiral outer membrane proteins OmpL1 and LipL41 when administered together, stimulated protection in immunised hamsters (Haake *et al.*, 1999), but neither protein was protective when administered alone. Future studies are necessary to determine if PCP will stimulate immunity when administered in combination with other *P. multocida* membrane antigens.

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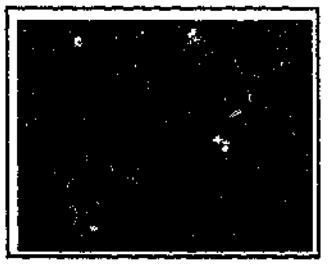
CHAPTER FOUR

CHARACTERISATION OF A PUTATIVE ZINC FINGER PROTEIN



INTRODUCTION

Mahan *et al.* (1993) had previously developed an *in vivo* expression technology (IVET) system to identify bacterial genes that are up-regulated and/or highly expressed *in vivo*, but minimally expressed *in vitro*. Such genes are of interest as many virulence genes have been found to be transcriptionally induced during the course of infection. These *in vivo* expressed genes may potentially be used as targets for attenuating mutation or the gene products could be possible vaccine candidates. Indeed, when some of the *in vivo*-induced genes identified in *Salmonella typhimurium* by Mahan *et al.* (1993) were inactivated, there was a decrease in virulence (Mahan *et al.*, 1993).

Findings from previous studies have provided evidence that cross-protective antigens in *P. multocida* are expressed *in vivo* (Choi-Kim *et al.*, 1991; Glisson & Cheng, 1991; Wang & Glisson, 1994). In order to identify genes encoding these cross-protective antigens and potential virulence associated genes, an IVET system, IVET-I, was developed and used by Hunt and Zhang (unpublished data) to identify genes in *P. multocida* which were up-regulated or expressed only when the organism was propagated *in vivo* (see IVET section, page 26). It was hypothesised that these *in vivo* expressed genes encode proteins required for virulence and/or survival in the host. Using the IVET-I system, part of a gene with high similarity to a hypothetical zinc finger protein in *H. influenzae* was identified from *P. multocida* strain VP161. As zinc finger proteins are commonly transcriptional activators (O'Halloran, 1993), it was of interest to characterise the putative zinc finger protein in *P. multocida* in order to determine if it is involved in transcriptional regulation and to assess its role in virulence.

Previous work on PzfA involved cloning the entire *pzfA* gene from *P. multocida* strain VP161, expression and purification of recombinant T7/hexa-histidine tagged PzfA for DNA binding studies, and attempts at mutant construction by allelic exchange with an insertionally inactivated copy of *pzfA* (Lo, 1998). This present study was aimed at confirming if *pzfA* is indeed more highly expressed *in vivo*, determining if PzfA has any effect on transcription, and constructing a *pzfA* mutant.

MATERIALS AND METHODS

Oligonucleotides, bacterial strains and plasmids

Oligonucleotides used in this study are listed in Table 4.1 while bacterial strains and plasmids are shown in Table 4.2.

Media

P. multocida strains were cultured at 37°C in nutrient broth (NB) or brain heart infusion broth (BHI, Oxoid) with constant aeration, or on nutrient agar (NA) or BHI agar. *E. coli* strains were grown at 37°C in 2YT broth with constant aeration or on 2YT agar. When required, antibiotics were added at the following concentrations: ampicillin (Amp), 100 μg/ml; kanamycin (Kan), 5, 10, 20, 30, 40 or 50 μg/ml; chloramphenicol (Cm), 40 μg/ml; streptomycin (Str), 25 μg/ml; spectinomycin (Spe), 25 or 75 μg/ml; rifampicin (Rif), 100 μg/ml; nalidixic acid (Nal), 15 μg/ml; erythromycin (Em), 100 or 200 μg/ml.

Recombinant DNA techniques

Plasmid DNA from *E. coli* and *P. multocida* strains was prepared as described by Le Gouill (1994) while *P. multocida* genomic DNA was prepared using the cetyltrimethylammonium bromide (CTAB) precipitation method (Ausubel, 1995). Standard PCR, restriction digestion, dephosphorylation, ligation and methylation of DNA were performed using DNA-modifying enzymes supplied by Roche Molecular Biochemicals or New England Biolabs Inc. under conditions recommended by the manufacturer. Plasmids were introduced by transformation into RbCl-treated *E. coli*. After heat shock, the transformed cells were recovered in 1 ml of SOC (2.0% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose) for 1 hr at 37°C, prior to plating on 2YT agar containing ampicillin or kanamycin. For transformation of *P. multocida*, plasmids were introduced into electrocompetent cells by electroporation (see below).

Table 4.1. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5'-3')	Target/purpose
YZ3555	TTGAGAGTAATAGCGGTCGC	Reverse primer for *aroA* to amplify insert from pPBA839
BAP386	GGTTTTTCGGCGTATCCAGC	Forward internal primer for *pzfA*
BAP452	GAGGGGATCCATGAGCGAACGCCTTTTTC	Forward *pzfA* primer. Incorporates a *Bam*HI site (underlined).
BAP453	TTGAGAATTCATTAAGATGCGATTTCCGAG	Reverse *pzfA* primer. Incorporates an *Eco*RI site (underlined).
BAP503	ACCGAATAGCCTCTCCAC	Reverse primer in the Tn*5* *kan*^{R} gene
BAP685	CACAACATGGATAAGCGGC	To amplify a 6.1 kb fragment from *P. multocida* (Figure 4.6)
BAP906	GCGTTGGTTTAGAGATGCG	To amplify a 6.1 kb fragment from *P. multocida* (Figure 4.6)
BAP1056	ATCTAGCGAGGGCTTTAC	Ω cassette primer
BAP1109	ATTCTCCCCACATCACCAGC	pPBA839 vector primer upstream of *pzfA* to amplify insert
BAP1665	CCTACCTCAATTGTGGAGGC	Forward *lpxA* primer for real-time RT-PCR
BAP1666	TCAACAAAGAAGCTAATCGC	Reverse *lpxA* primer for real-time RT-PCR
BAP1827	TTGAAAGCTTATTAAGATGCGATTTCCGAG	Reverse *pzfA* primer. Incorporates a *Hin*dIII site (underlined).
BAP2128	GAAGAAACGATCCCTTGTCCC	Forward *pzfA* primer for real-time RT-PCR
BAP2129	AGGAAAGCGATCACAGCCAT	Reverse *pzfA* primer for real-time RT-PCR
BAP2234	CGGCGATCACCGCTTCCC	Reverse primer for *aadA* of pUA826 (Cardenas *et al.*, 2001)
BAP2235	ACACGCGACCAAATAGGGC	Reverse *pzfA* primer to generate an internal fragment for mutagenesis by conjugation
BAP2335	CGGGTCCAGTTTTGATCGATA	Forward *ilvG* primer for real-time RT-PCR
BAP2336	TTGTGAAAGTGCGGTGGGTT	Reverse *ilvG* primer for real-time RT-PCR
BAP2337	AACGTCCTGAAACCCTAGAACG	Forward *ilvM* primer for real-time RT-PCR
BAP2338	AATGTTGGCTTGTTCGCCTT	Reverse *ilvM* primer for real-time RT-PCR
BAP2339	CCATTGCCCGCCATATTAAG	Forward *leuA* primer for real-time RT-PCR
BAP2340	TGCCTCCGCTACTTTCAACG	Reverse *leuA* primer for real-time RT-PCR
BAP2341	TAGACAAAGATGCGAAAGCTCG	Forward *ompW* primer for real-time RT-PCR
BAP2342	TTTCAGATCAGTCACGCCATTT	Reverse *ompW* primer for real-time RT-PCR
T7	TAATACGACTCACTATAGGG	Universal vector primer
RP	GGAAACAGCTATGACCATG	Universal vector primer
primerML	AAGTGCGGTGAGGGGATCCATGAGCGAACGCCTTTTTC	BAP452 with 9 bp uptake sequence (double underlined)
KMT1SP6	GCTGTAAACGAACTCGCCAC	*P. multocida* specific primer (Townsend *et al.*, 1998)
KMT1T7	ATCCGCTATTTACCCAGTGG	*P. multocida* specific primer (Townsend *et al.*, 1998)

Table 4.2. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics	Reference or source
P. multocida strains		
X-73	Serotype A:1 chicken isolate; reference strain	(Heddleston & Rebers, 1972)
VP161	Serotype A:1, Vietnamese chicken isolate	(Wilkie *et al.*, 2000)
PM70	Serotype A:3, common avian isolate	(May *et al.*, 2001)
PBA100	Serotype A:1, Australian chicken isolate	(Ireland *et al.*, 1991)
PBA839	*P. multocida* strain PBA100 harbouring pPBA839	Zhang (unpublished data)
PBA907	X-73 harbouring pPBA906	This study
PBA917	X-73 harbouring pMKΩ	(Hunt *et al.*, 2001)
AL186	Pm70 harbouring pAL99	This laboratory
AL241	Pm70 harbouring pAL119	This study
AL291	Rif^R strain of X-73	This study
AL338	X-73 harbouring pGH9:ISS1	This laboratory
AL348	Rif^R, Nal^R strain of X-73	This study
E. coli strains		
DH5α	*F'endA1 hsdR17* ($r_k^- m_k^+$) *supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA-arg F*) U169 Ø80d*lacZ*ΔM15	Bethesda Research Laboratories
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2Tc::Mu [Km^R] [λ*pir*]	(Simon *et al.*, 1983)
AL344	SM10λ*pir* harbouring pAL189	This study
Plasmids		
pAL99	Km^R, 240bp *Eco*R1 fragment containing *tpiA* promoter region cloned into the *Eco*R1 site of the *E. coli*/*P. multocida* shuttle vector pPBA1100	This laboratory
pAL119	*pzfA* cloned into pAL99	This study
pAL189	345 bp internal fragment of *pzfA* cloned into pUA826	This study
pBZ	Derived from pBluescript SK (Invitrogen Corporation) and pZero (Stratagene), Amp^R	Doughty (unpublished data)
pGh9:ISS1	Temperature sensitive broad host range vector, Em^R	(Biswas *et al.*, 1993)
pMKΩ	6.5 kb, Spe^R and Str^R, unique *Bam*HI site upstream of a promoterless *kan* gene, *P. multocida* origin	(Hunt *et al.*, 2001)
pPBA839	IVET-I construct consisting of pMK7 with the promoter region and first 315 bp of *pzfA*	Zhang (unpublished data)
pPBA906	pMKΩ with the promoter region and first 315 bp of *pzfA*	This study
pPBA1460	Mutated construct (1.6 kb *Hin*dIII Km^R cassette from pUC4KIXX (Beck *et al.*, 1982; Vieira & Messing, 1982) inserted into *pzfA*) in pBZ	(Lo, 1998)
pPBA1553	Mutated construct from pPBA1460 cloned into pWSK29	This study
pPBA1554	Mutated construct from pPBA1460 cloned into pSU2719	This study
pSU2719	Derived from pACYC184. Cm^R, *lacZ'*, P15A origin	(Martinez *et al.*, 1988)
pUA826	Mob^+, R6K replicon, Ap^R, Str^R and Spe^R	(Cardenas *et al.*, 2001)
pWSK29	Amp^R, *lacZ'*, pSC101 origin, f1 origin	(Wang & Kushner, 1991)
RSF1010	Broad host range conjugative vector, Str^R, Mob^+	(Scherzinger *et al.*, 1984)

Preparation of electrocompetent *P. multocida* cells

See Chapter 2.

Electroporation

See Chapter 2.

Sequence analysis

Nucleotide sequence was determined using the *Taq* Big DyeDeoxy terminator kit. Individual sequences were aligned and assembled using the Sequencher 3.0 program. Comparison of sequences with those in GenBank and EMBL was performed using the BLAST (Altschul *et al.*, 1990) program through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Computer analysis of deduced protein sequences was carried out via the Australian National Genomic Information Service (ANGIS) (http://www.angis.org.au) using programs within the Genetics Computer Group sequencing analysis software package. Searches of the published *P. multocida* strain PM70 genome were done using the University of Minnesota Microbial Genome Project website (http://www.cbc.umn.edu/Research Projects/AGAC/Pm/).

RNA purification

See Chapter 2.

Extraction of RNA from *in-vivo* grown *P. multocida*

See Chapter 2. RNA from bacteria grown in chickens was obtained from Boyce *et al.* (2002).

Transcriptional analysis

Determining in vivo activity of the pzfA promoter using the PmIVET system

The primers BAP1109 and YZ3555 were used to amplify the start of the *pzfA* gene and its promoter region from pPBA839 by PCR. The PCR product was then digested with *Sau*3A, ligated into *Bam*HI-digested pMKΩ and introduced into electrocompetent *P. multocida* strain PBA100. The cloning was performed in PBA100 as this strain does not contain resident plasmids. The resulting plasmid,

pPBA906, contained the first 315 bp of *pzfA* and 283 bp upstream of the start codon, with the predicted *pzfA* promoter in the same orientation as the promoterless *kan* gene. The plasmid, pPBA906, was then introduced into *P. multocida* strain X-73 giving rise to the strain PBA907. PBA907 was then tested for *in vivo* survival by passaging through Kan-treated mice as described previously (Hunt *et al.*, 2001). Each of the 4 mice (outbred female 6 to 8 week old BALB/c) was injected ip with a mix of 10^6 cfu of PBA907 and the PBA917 control in equal numbers. At 2 hr after infection, mice were bled from the orbital plexus to recover bacteria and then treated with Kan (100 µg per g body weight). Mice were again treated with Kan at 4 hr after infection and bled from the orbital plexus at 8 hr and 24 hr after infection. Mice were then euthanased in accordance with animal ethics requirements. Heparin (20 U/ml) was immediately added to the blood samples which were then plated onto NA containing Str and Spe. Plates were incubated overnight and bacterial colonies were identified by colony PCR.

Determination of in vivo versus in vitro transcription levels using real-time reverse transcription (RT)-PCR

Real-time RT-PCR was carried out using a Roche Molecular Biochemicals LightCycler in accordance with the instructions of the manufacturer. RT reactions contained 10-20 µg of total RNA, 50 nM each of BAP1666 and BAP453 (*lpxA* and *pzfA* reverse primers respectively), 10 U Superscript II reverse transcriptase (Gibco/BRL), and 500 µM each of dATP, dCTP, dGTP and dTTP. Samples were incubated at 42°C for 2.5 hr, followed by 15 min at 70°C. The synthesised cDNA was then diluted 1/10, 1/100, 1/500 and 1/1000 prior to use in real-time RT-PCR. Reactions were performed in triplicate. Each 20 µl reaction contained 2 µl of cDNA, 0.5 µM of each gene-specific primer (Table 4.1), 3 mM or 4 mM $MgCl_2$ (for *pzfA* and *lpxA* reactions respectively) and 2 µl of LightCycler FastStart DNA Master SYBR Green I mix (Roche Molecular Biochemicals). Known concentrations of *lpxA* PCR product generated from *P. multocida* strain X-73 genomic DNA were used to construct a standard curve so that the concentration of template in each reaction could be determined. *lpxA* was used as the normaliser for all reactions. Analysis was done using the LightCycler software. Melting curve analysis confirmed that all RT-PCRs amplified a single product. Student's *t* test with a two-tailed hypothesis was used to

determine the statistical significance between mean values of *in vitro* and *in vivo* expression levels of *pzfA*. *P* values of <0.05 indicated statistical significance at the 95% confidence level.

Colony PCR

Colonies picked from plates were resuspended in 20 µl of ultra-pure H_2O and 2 µl of the suspension were subsequently used as template in a 20 µl PCR mixture containing PCR buffer (Roche Molecular Biochemicals), 1.25 mM each of dATP, dTTP, dCTP and dGTP, 1 µM concentrations each of forward and reverse primers and 0.5 U of *Taq* DNA polymerase (Roche Molecular Biochemicals). Reactions were carried out in 0.2 ml thin-walled tubes using a Perkin-Elmer GeneAmp PCR system 2400 thermocycler. Thermocycling conditions were an initial denaturation step at 94°C for 8 min, followed by 30 cycles of 94°C for 15 s, 50°C for 30 s, and 72°C for 2 min. PCR products were analysed by agarose gel electrophoresis.

Preparation of probes for microarray hybridisations

P. multocida strain PM70 arrays were obtained from Paustian *et al.* (2001) consisting of triplicate spots of each of the 2,015 annotated *P. multocida* strain PM70 genes (Boyce *et al.*, 2002). Labeled cDNA for microarray hybridisation was prepared using the indirect amino-allyl labeling method as described previously (Smoot *et al.*, 2001). Amino-allyl dUTP (Sigma) was incorporated into cDNA during reverse transcription (RT). A 30 µl RT reaction contained 10-20 µg of total RNA, 30 µg of random hexamers, 10 U Superscript II reverse transcriptase (Gibco/BRL), 500 µM each of dATP, dCTP and dGTP, 200 µM dTTP and 300 µM amino-allyl dUTP. RNA was first heated with the random hexamers at 70°C for 10 min then chilled on ice for 10 min. The remaining components were then added and the samples incubated at 42°C for 2.5 hr, followed by 15 min at 70°C. RNA was hydrolysed by adding 10 µl of 1 M NaOH and 10 µl of 0.5 M EDTA and heated at 65°C for 15 min. 25 µl of 1 M Tris-HCl (pH 7.4) was then added to neutralise the solution. The Tris was then removed by repeated washing using a Microcon YM-30 column (Millipore). The cDNA was dried and resuspended in 9 µl of 0.1 M $NaHCO_3$ (pH 9.0) then coupled to either cy3 or cy5 monofunctional dyes (Amersham) by incubation at 20°C in the dark for 1 hr. The coupling reaction was stopped by incubation with 4.5 µl of 4 M

hydroxylamine for 15 min at 20°C. Experimental and control probes, each labelled with a different dye, were combined and unincorporated dye was removed using a QIAquick PCR Purification kit (QIAGEN). The probe was then stored at 4°C until required.

Microarray hybridisations

The microarray was pre-hybridised using a solution containing 25% formamide, 5 × SSC (1 × SSC consists of 0.15 M NaCl with 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), 10 mg/ml bovine serum albumin (BSA) and 1 mg/ml sheared salmon sperm DNA. The solution was placed on the microarray under a coverslip and incubation was carried out for 45 min in a humidified slide chamber placed in a 42°C water bath after which the slides were washed in ultra-pure H_2O and dried by centrifugation at 1,000 × *g* for 3 min. The fluorescently labelled cDNA probe was mixed with 25% formamide, 5 × SSC, 0.1% SDS, 0.5 mg/ml of yeast tRNA and 1 mg/ml of sheared salmon sperm DNA. The probe was denatured by heating at 95°C for 5 min, centrifuged for 2 min at 12,000 × *g* and placed on the microarray under a coverslip. Hybridisation was carried out for 16 hr in a humidified slide chamber placed in a 42°C water bath. Slides were given one wash with 2 × SSC-0.1% SDS for 5 min at 42°C, one in 0.1 × SSC-0.1% SDS for 10 min at room temperature, four 1-min washes in 0.1% SDS at room temperature, then rinsed in dH_2O and 95% ethanol. Slides were dried by centrifugation at 1,000 × *g* for 3 min and scanned immediately.

Analysis of microarray images

Microarray hybridisations were scanned using a GMS 418 Array Scanner (Genetic MicroSystems, Inc.). The cy3 and cy5 images were aligned then overlaid with a grid using ImaGene version 4.1 (Biodiscovery) to allow accurate gene identification and quantification of fluorescent intensity. To calculate expression values for each gene, GeneSight version 3.2.2 (Biodiscovery) was used, which standardised cy3 and cy5 fluorescent intensities for each spot by subtracting local background and omitting all negative and very low positive values (<100). The resultant data were then transformed such that the global mean expression values for the cy3 and cy5 channels had a log ratio of zero. RNA isolated from two separate

AL241 clones (each overexpressing *pzfA*) was used for two comparative hybridisations with RNA derived from AL186 (empty vector control). Each pair of hybridisations, corresponding to RNA derived from a single biological replicate, was analysed separately and the replicate spots were combined and the average log ratios used for further analysis. Identification of differentially expressed genes was performed with GeneSight. Genes were considered to be differentially expressed if there was a 95% chance that their average expression value was above a $\log_2$ of 0.6 or below a $\log_2$ of -0.6. These values correspond to approximately 1.5-fold differences in expression for up- and down-regulated genes respectively. GeneSpring version 5.1 (Silicon Genetics) was used to identify possible regulatory sequences of differentially expressed genes.

Validation of microarray data by real-time RT-PCR

Primer Express software (ABI) was used to design primers for real-time RT-PCR. RT reactions were carried out as described for microarray hybridisations but using 500 µM of each dNTP. Samples were incubated at 42°C for 2.5 hr, followed by 15 min at 70°C. The synthesised cDNA was then diluted 1/80 prior to use in real-time RT-PCR. Reactions were done in triplicate. Each 20 µl reaction contained 2.4 µl of cDNA, 50 nM of each gene-specific primer (Table 4.1) and 10 µl of SYBR Green PCR master mix (ABI). Real-time RT-PCR was carried out using an ABI PRISM model 7700 sequence detection system. Known concentrations of *P. multocida* strain X-73 genomic DNA was used to construct a gene-specific standard curve so that the concentration of template in each reaction could be determined. The gene encoding DNA gyrase subunit B, *gyrB*, was used as the normaliser for all reactions. Melting curve analysis confirmed that all RT-PCRs amplified a single product.

Screening of putative transformants by rapid nitrocefin test

Beta-lactamase activity was detected using the chromogenic substrate, nitrocefin (Glaxo Research), as described by O'Callaghan *et al.* (1972). Bacterial colonies to be tested were emulsified in 50 µl of 250 µg/ml nitrocefin and incubated at room temperature for 30 minutes. A colour change from pale orange to red was recorded as a positive result.

Natural transformation

See Chapter 2.

Conjugation

The suicide plasmid, pUA826 (Cardenas *et al.*, 2001), was used for conjugation into *P. multocida*. A blunt-ended 345 bp internal fragment of *pzfA* was amplified by PCR using the primers BAP386 and BAP2235 and cloned into *Sma*I-digested pUA826 giving rise to the plasmid pAL189. Conjugation was performed by mixing 1 ml aliquots of early stationary phase cultures of the donor (*E. coli* strain AL344) with the *P. multocida* recipient (strains AL291, AL338 or AL348) and the mixture was filtered through a sterile filter unit with a 0.45 μM Millipore filter. The filter was then removed, placed onto BHI agar and incubated at 30°C overnight. Colonies were then recovered by placing the filter in 3 ml of BHI and vortexing. Dilutions of the suspension were plated onto BHI agar supplemented with the appropriate antibiotics. The plates were incubated aerobically at 37°C.

An alternative plate conjugation method was used where strains were grown to early stationary phase and 100 μl of donor were mixed with 900 μl of recipient. The mixture was then pelleted by centrifugation at 12,000 × *g* and resuspended in 100 μl of BHI. The cells were spread onto a BHI plate and incubated at 30°C overnight. Colonies were scraped off the plate in 2 ml of BHI and dilutions were plated onto BHI agar supplemented with the appropriate antibiotics. The plates were incubated aerobically at 37°C.

RESULTS

Transcriptional analysis of *pzfA*

pzfA was originally identified using IVET-I, the first IVET system developed for *P. multocida*. To confirm that *pzfA* was indeed up-regulated *in vivo*, part of the gene and the promoter region were cloned into pMKΩ for use in the PmIVET system. Mice were injected ip with PBA907 (10^6 cfu) along with PBA917 (10^6 cfu) which harbours empty pMKΩ and was therefore used as a control for *in vivo* clearance of Kan sensitive organisms. Mice were treated with Kan (100 μg/g of body weight) at 2

Table 4.3. Numbers of *P. multocida* colonies recovered from Kan-treated mice at 2, 8 and 24 hr after infection.

Mouse	cfu/ml bacteria recovered		
	1st bleed (2 hr)	2nd bleed (8 hr)	3rd bleed (24 hr)
1	6	0	-
2	8.2×10^3	0	-
3	5.1×10^3	0	8×10^7
4	3.7×10^4	1	8×10^7

and 4 hr after infection. It was found that blood Kan levels of 80 to 100 µg/ml of blood were achieved 15 min after each injection of Kan (Hunt *et al.*, 2001). Bacteria were recovered from blood at 2, 8 and 24 hr after infection (Table 4.3). Surprisingly, only 1 colony was recovered from a single mouse (mouse 4) at 8 hr after infection. Mice 1 and 2 were dead by 24 hr after infection but bacteria were recovered from the remaining mice before they were euthanased. Colony PCR using BAP503 and BAP1056 was performed to

Table 4.4. Results of colony PCR. Percentage of PBA907 (*pzfA* clone) recovered from each mouse at each bleed.

Bleed	Colonies screened	% of AL907 recovered
1st	2 from each mouse	50%
2nd	Single colony from mouse 5	100%
3rd	10 from mouse 4; 10 from mouse 5	75%

differentiate between PBA907 (expected PCR product of ~700 bp) and PBA917 (expected product of ~150 bp). Results of colony PCR are shown in Table 4.4. At 2 hr after infection, 2 colonies from each mouse were screened by colony PCR and 50% of the colonies were PBA907. The single colony, recovered from mouse 4 at 8 h after infection was shown to be PBA907. At 24 h after infection, bacteria were recovered from mouse 3 and 4 only. Of the 20 colonies tested (10 from each mouse), 75% were PBA907. Therefore, from these results, it could not be concluded if the *pzfA* promoter was active *in vivo*.

PBA907 was also tested for sensitivity to Kan *in vitro*. PBA907 and PBA917 were plated onto BHI containing 0, 5, 10, 20, 30, 40 and 50 µg/ml of Kan. Both strains grew in the absence of Kan and only weakly at 5 µg/ml of Kan indicating that the *pzfA* promoter is not very active *in vitro*.

As the PmIVET results were ambiguous and did not confirm that *pzfA* was up-regulated *in vivo*, real-time RT-PCR was performed to directly measure the levels of *pzfA* expression. For all reactions, *lpxA* was used as the normaliser. The *lpxA* gene was expected to be constitutively expressed as the gene encodes UDP-N-

acetylglucosamine acetyltransferase, an essential enzyme involved in the first step of lipid A biosynthesis (Anderson & Raetz, 1987). Control (*in vitro*) RNA was purified from *P. multocida* strain X-73 grown in BHI aerobically at 37°C, while *in vivo* RNA was purified from *P. multocida* strain X-73 recovered from infected mice or chickens. Real-time RT-PCR showed that when *P. multocida* was grown in chickens and mice, *pzfA* was much more highly expressed than when the cells were grown *in vitro* (Table 4.5).

Table 4.5. Comparison of *pzfA* expression levels between *in vitro* and *in vivo* (bacteria recovered from mice or chickens) RNA samples.

Sample	Expression value (average *pzfA*/average *lpxA*) ± 1 standard deviation	*P* value
in vitro	0.720 ± 0.316	
Chicken	7.043 ± 4.104	0.0034 (very significant)
Mouse	4.506 ± 0.491	0.0062 (very significant)

Sequence analysis of *pzfA*

The *pzfA* gene was previously cloned from *P. multocida* strain VP161 and the nucleotide sequence determined (Lo, 1998). The promoter possessed a –10 box TTTAAT with 1 bp mismatch to the *E. coli* consensus. A –35 box was not identified. A consensus ribosome binding site, AGGA, was identified 5 bp upstream of the ATG start codon of the putative zinc finger protein gene. The complete nucleotide sequence is shown in Figure 4.1. The gene was found to be 558 bp in length, encoding a protein of 185 amino acids. Analysis of the deduced amino acid sequence showed that PzfA was cysteine-rich with 9.2% of the protein consisting of cysteine residues. The isoelectric point of PzfA was predicted by PSORT to be 8.05. Using the Conserved Domain Architecture Retrieval Tool (CDART) on the NCBI website, two putative zinc binding motifs were identified which matched the pfam01396 family (topoisomerase DNA binding C4 zinc finger; $E = 2 \times 10^{-6}$ and 0.002 respectively). Two other putative zinc binding motifs were identified by eye based on the presence of four cysteine residues which also appear to be possible zinc coordination sites (Figure 4.1). Other domains identified by CDART include TopA

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TCAACAGCAGTATTGTTGTGGGCAAGGATGGAAAACCGGAATGCAAACCGGGTGATAACCAAAACGGAGCCCAACGTGGCGTTTTAAAGT   90

GCTGGACGGAAAAGGAAATTCAAGGGTAACATAGCCACTTGGTGAGCCGATGGATAAAGTTGGGTGCCTATGGTATTCAACAACTTGGTG   180

GCGTTTTTGTTGAACACATTGAGGGCAGTTTTAGCGGGGTGATGGGGTTGCCGGTGTGTGAAACGGTGGCATTGTTGAAAGCCTTTGGGG   270

              -10                       M  S  E  R  L  F  Q  H  K  K  Q  Q  E  H  C  P  R  C  D  A
TAGAATTGTTTTAATTTCATTGAGGAACACCATGAGCGAACGCCTTTTTCAACACAAAAAACAACAAGAACATTGCCCTCGTTGCGATGC   360

 V  T  L  I  K  Q  G  F  K  G  I  F  I  Q  C  F  A  Y  P  A  C  D  Y  I  K  P  L  S  F  Q
TGTATTACAGCTGAAGCAAGGCAAAAAAGGGTTGTTTTTGGCTGTTCGGCGTATCCAGCATGTGATTATATTAAGCCACTTTCATTTCA   450

 S  E  S  K  I  I  K  V  L  E  E  T  C  P  E  C  R  H  P  L  V  L  K  Q  G  H  F  G  M  F
ATCAGAAAGTAAAATCATTAAAGTCTTAGAGGAAACCTGTCCTGAATGCCGGCATCCTTTGGTATTGAAACAAGGGCATTTTGGCATGTT   540

 I  G  C  S  H  Y  P  E  C  H  F  V  V  H  D  E  P  E  E  Q  A  E  E  T  I  P  C  P  D  C
TATTGGTTGTAGCCACTATCCAGAATGCCATTTTGTGGTGCATGACGAACCCGAGGAACAAGCAGAAGAAACGATCCCTTGTCCCGATTG   630

 Q  Q  G  G  L  V  A  R  R  G  R  Q  G  K  V  F  Y  G  C  D  R  F  P  H  C  K  F  T  L  A
CCAACAAGGCGGATTGGTGGCTCGTCGTGGTCGTCAGGGCAAAGTCTTTTATGGCTGTGATCGCTTTCCTCATTGCAAATTTACCTTAGC   720

 A  K  P  Y  L  V  A  T  P  N  C  G  L  R  C  T  L  Y  K  K  I  P  T  Q  P  T  F  Q  C
GGCTAAGCCCTATTTGGTCGCGTGTCCAGCGTGTGGGAGTTCGGTTTGTACCTTGAAAAAGAAACGGACACTCAGCGCACTTTTCAATG   810

 A  R  K  R  C  R  H  I  F  N  S  E  I  A  S  *
TGCGAACAAACGTTGTCGCCATATTTTTAACTCGGAAATCGCATCTTAATGAACATTCAACAAATCGTCGAACAACTTAAACAAAATGAA   900

GTCGTCGCCTATCCGACCGAAGCGGTCTTTGGTTTAGGTTGTAATCCCA   949
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Figure 4.1. Nucleotide sequence of *P. multocida* VP161 *pzfA* and the deduced amino acid sequence. The putative -10 box is underlined and the ribosome binding site is double-underlined. The asterisk denotes the TAA stop codon and the start codon of the next ORF is boxed. The putative zinc finger domains identified by CDART are shown in red, while those identified by eye due to the presence of four cysteine residues are shown in green.

(zinc finger domain associated with topoisomerase type I; E = 10^{-23}) and COG1754 (uncharacterised C-terminal domain of topoisomerase IA; E = 0.001) (Figure 4.2).

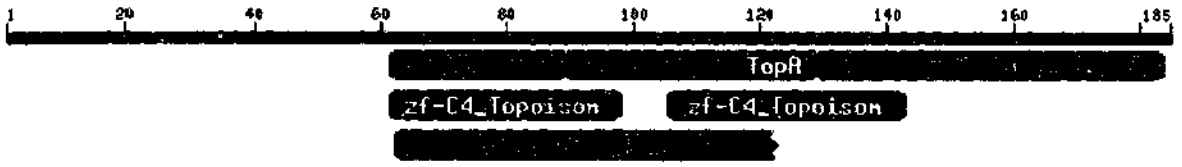

Figure. 4 2. Output from CDART showing the identified conserved motifs in PzfA. Sections in blue indicate regions of PzfA which show similarity to DNA binding C4 zinc finger motifs found in topoisomerases. The region with similarity to TopA (zinc finger domain associated with topoisomerase type I ... shown in red, while the green section represents COG174, an uncharacterised C-termi... domain of topoisomerase IA.

PzfA showed 58% - 65% similarity to many putative topoisomerase-like proteins from various bacteria including *Yersinia pestis*, *E. coli*, *Shigella flexneri*, *Salmonella enterica* serovar Typhimurium and *Vibrio cholerae*. PzfA also showed similarity to sections of the DNA topoisomerase I from *Coxiella burnetii*, *Helicobacter pylori* and *Neisseria meningitidis* (60%, 50%, and 48% respectively) although the DNA topoisomerase I proteins are much larger proteins than PzfA. Indeed, the similarity to the DNA topoisomerases is limited to the putative zinc binding motifs; when PzfA was compared with the DNA topoisomerase I characterised from *H. pylori*, it was found that PzfA matched only the C-terminal region which contains four zinc finger motifs (Figure 4.3). Although PzfA did not show any significant similarity to the *E. coli* topoisomerase, a diagram of the protein is also shown in Figure 4.3 for comparison as the functional domains of the *E. coli*

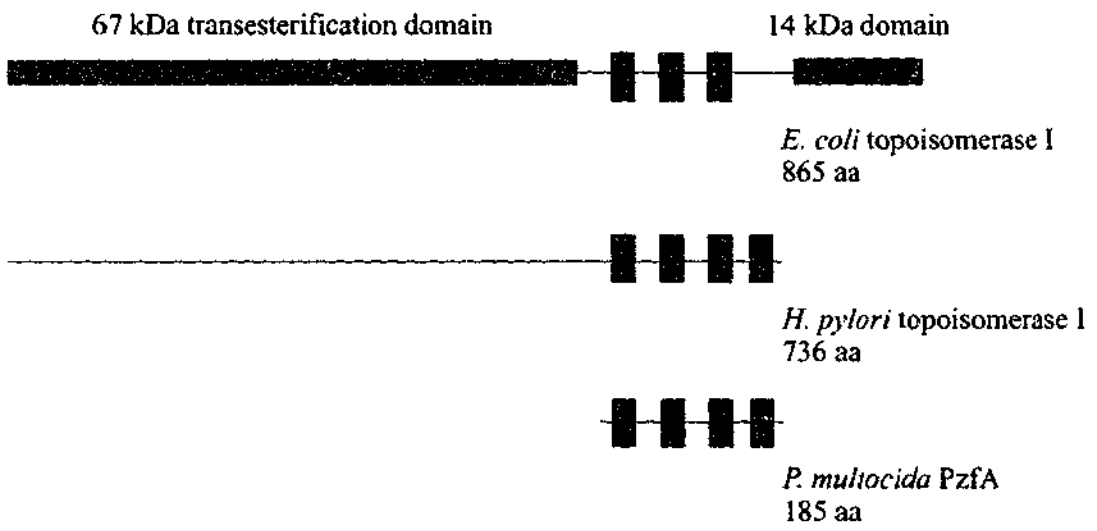

Figure 4.3. PzfA and its alignment with topoisomerase I from *E. coli* and *H. pylori*. The zinc finger motifs are indicated by the red bars.

enzyme have been characterised. The *P. multocida* strain VP161 *pzfA* gene had 99% nucleotide identity to the homologue in the published *P. multocida* PM70 genome which is designated *pm1269* (May *et al.*, 2001).

Effects of overexpression of *pzfA* on global transcription

In order to determine if *pzfA* may be a potential transcriptional regulator, the gene was overexpressed in *P. multocida* PM70. The *P. multocida* PM70 strain was chosen as its genome sequence has been obtained and microarray slides are available which contain all of the *P. multocida* PM70 gene sequences. *pzfA* was amplified from the *P. multocida* VP161 genome by PCR using the primers BAP452 and BAP1827. The resulting PCR product was then cloned into pAL99 downstream of the highly active *tpiA* promoter, giving rise to the plasmid pAL119. The plasmid was then introduced into electrocompetent *P. multocida* PM70, generating the *P. multocida* strain AL241. Whole genome microarray analysis was then performed to examine what effects overexpression of *pzfA* would have on global transcription. RNA was purified from two biological replicates of *P. multocida* strain AL241, while control RNA was isolated from *P. multocida* strain AL186. Both strains were grown aerobically at 37°C to OD_{650} of 0.6-0.65 which corresponds to 10^9 cfu/ml. cDNA was then synthesised from the RNA samples and labeled with either cy3 or cy5. Microarrays containing all 2,015 of the annotated *P. multocida* strain PM70 genes were then hybridised with control and experimental cDNA. For each biological replicate, two hybridisations were carried out, one with the experimental sample labeled with cy3 and the other with the experimental sample labeled cy5. Hybridisations from each biological replicate were analysed separately. Genes which were found to be ≥1.5-fold up or down in both biological replicates are shown in Tables 4.6 and 4.7 respectively. *pzfA* was overexpressed in both biological replicates (3.2-fold and 2.7-fold respectively). Of the 32 differentially expressed genes, 10 were up-regulated and 22 were down-regulated. Most of the up-regulated genes (7 of 10) were of unknown or poorly defined function. The other highly up-regulated genes, *ilvG*, *ilvM* and *leuA*, are involved in amino acid biosynthesis (Umbarger, 1996). With the exception of *ompW*, which has no homologue in *E. coli*, all genes down-regulated in both biological replicates appear to play a role in metabolism. The majority of down-regulated genes (9 of 22) were involved in energy production and conversion. Seven genes were of unknown or poorly characterised

Table 4.6. Genes up-regulated in *P. multocida* strain PM70 carrying the *pzfA* overexpression plasmid, pAL119. *pzfA* is highlighted in red.

Gene	Precise or predicted function (COGs category[a])	Expression value[b]		Function of homologue in *E. coli*
		Replicate 1	Replicate 2	
pm0297	Unknown (-)	1.04 ± 0.24	0.86 ± 0.31	*c*
pm0718	Sodium-dependent transporter, putative (R)	1.34 ± 0.12	0.81 ± 0.09	*c*
pm1232	Superoxide inducible protein, putative (H)	2.32 ± 0.66	0.82 ± 0.18	*c*
pm1378	Ribose ABC transporter, permease protein (R)	0.85 ± 0.1	0.74 ± 0.09	*c*
pm1522	4-hydroxyphenylacetic acid hydroxylase, putative (R)	0.79 ± 0.12	0.91 ± 0.1	*c*
pm1626	Putative resistance protein (M)	0.86 ± 0.28	0.81 ± 0.31	*c*
pm1944	Arsenate reductase, putative (P)	1.10 ± 0.29	0.77 ± 0.1	*c*
ilvG	Acetolactate synthase large subunit (E)	0.89 ± 0.31	0.76 ± 0.33	Converts pyruvate to α-acetolactate (first step in valine biosynthesis). Also converts pyruvate and α-ketobutyrate to α-aceto-α-hydroxybutyrate (second step in isoleucine biosynthesis) (Umbarger, 1996).
ilvM	Acetolactate synthase small subunit (S)	0.86 ± 0.05	1.03 ± 0.16	Converts pyruvate to α-acetolactate (first step in valine biosynthesis). Also converts pyruvate and α-ketobutyrate to α-aceto-α-hydroxybutyrate (second step in isoleucine biosynthesis) (Umbarger, 1996).
leuA	2-isopropylmalate synthase (E)	1.19 ± 0.08	0.83 ± 0.16	Converts α-ketoisovalerate to α-isopropylmalate, the first step in leucine biosynthesis (Umbarger, 1996).
pm1269/pzfA	DNA topoisomerase I-like protein, putative (L)	1.66 ± 0.48	1.45 ± 0.53	*c*

[a] Clusters-of-orthologous-groups (COGs) functional categories (Tatusov, 1997). Categories are as follows: J, translation, ribosomal structure, and biogenesis; K, transcription; L, DNA replication, recombination and repair; D, cell division and chromosome partitioning; O, posttranslational modification, protein turn-over, and chaperone functions; M, cell envelope and outer membrane biogenesis; N, cell motility and secretion; P, inorganic ion transport and metabolism; T, signal transduction mechanisms; C, energy production and conversion; G, carbohydrate transport and metabolism; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; H, coenzyme metabolism; I, lipid metabolism; Q, secondary metabolite biosynthesis, transport, and catabolism; S, unknown function; R, general function prediction only, and category -, not in COGs.

[b] Expression values are expressed as the $\log_2$ of (average experimental intensity/average control intensity) ± 1 standard deviation.

[c] No homologue in *E. coli*.

Table 4.7. Genes down-regulated in *P. multocida* strain PM70 carrying the *pzfA* overexpression plasmid, pAL119.

Gene	Precise or predicted function (COGs category[a])	Expression value[b]		Function of homologue in *E. coli*
		Replicate 1	Replicate 2	
pm0074	Formate transporter, putative (P)	-1.06 ± 0.46	-1.31 ± 0.24	*c*
pm0688	Unknown (S)	-0.94 ± 0.17	-1.04 ± 0.21	*c*
pm0834	Phosphotransferase system enzyme II, mannose-specific, factor IIAB (G)	-1.00 ± 0.34	-1.19 ± 0.12	*c*
pm0948	Sulfate permease, putative (P)	-1.21 ± 0.07	-0.99 ± 0.11	*c*
pm1299	C_4-dicarboxylate anaerobic carrier, putative (C)	-1.17 ± 0.17	-1.31 ± 0.13	*c*
pm1300	Aminobenzoyl-glutamate utilisation protein, putative (R)	-0.79 ± 0.16	-0.92 ± 0.17	*c*
pm1792	Pentahemic c-type cytochrome (C)	-1.40 ± 0.28	-1.40 ± 0.25	*c*
adhC	Alcohol dehydrogenase 2 (C)	-2.12 ± 0.22	-1.74 ± 0.37	Reduces acetyl CoA to ethanol via acetaldehyde (Bock & Sawers, 1996). Expression in *E. coli* induced by anaerobiosis.
arcA	Aerobic respiration control protein (T)	-1.34 ± 0.23	-1.37 ± 0.28	Response regulator component of the ArcAB two-component signal transduction system (Lynch & Lin, 1996). Expression in *E. coli* increases four-fold under anaerobic growth.
bioD1	Dethiobiotin synthase (H)	-1.18 ± 0.13	-0.91 ± 0.26	Converts 7,8-diaminopelargonic acid to dethiobiotin, the fourth step in biotin synthesis (DeMoll, 1996).
ccmH_2	Cytochrome C-type biogenesis (O)	-1.05 ± 0.14	-0.94 ± 0.2	Cytochrome *c* maturation (Berlyn *et al.*, 1996).
cdd	Cytidine deaminase (F)	-1.57 ± 0.33	-1.57 ± 0.24	Converts cytidine and deoxycytidine to uridine and deoxyuridine (Lin, 1996).
dcuC	C_4-dicarboxylate anaerobic carrier (C)	-0.90 ± 0.13	-1.02 ± 0.26	Transport of C_4-dicarboxylates during anaerobic growth and glucose fermentation (Zientz *et al.*, 1996; Zientz *et al.*, 1999).
era	Phosphoglycerate dehydrogenase (E)	-0.80 ± 0.28	-0.79 ± 0.14	Essential GTP-binding protein localised throughout the cell poles and cell midpoint (Schmid & von Freiesleben, 1996). Involved in nucleoid segregation.
fumC	Fumarate hydratase class II (C)	-0.87 ± 0.14	-0.78 ± 0.2	Converts fumarate to malate (Cronan Jr & Laporte, 1996).
napA	Periplasmic nitrate reductase precursor (C)	-1.32 ± 0.46	-1.46 ± 0.11	Periplasmic molybdoprotein; catalytic subunit of the periplasmic nitrate reductase complex involved in nitrate reduction (Potter *et al.*, 2001).
napC	Cytochrome C-type protein (C)	-0.90 ± 0.49	-0.91 ± 0.19	Putative quinol oxidase; transfers electrons from benzoquinols and napthoquinols to the periplasmic nitrate reductase complex, NapAB (Potter *et al.*, 2001).
napF	Ferredoxin-type protein (C)	-2.23 ± 0.38	-2.02 ± 0.37	Periplasmic nitrate reductase, ferredoxin homolog (Berlyn *et al.*, 1996).

nrfC	Nitrite reductase, Fe-S protein (C)	-0.88 ± 0.22	-0.93 ± 0.19	Cytoplasmic iron-sulfur protein involved in nitrite reduction (Gennis & Stewart, 1996).
ompW	Outer membrane protein *ompW* precursor (M)	-2.18 ± 0.18	-1.83 ± 0.13	*c*
pckA	Phosphoenolpyruvate carboxykinase (C)	-1.15 ± 0.36	-1.00 ± 0.2	Involved in the glycolytic pathway (Fraenkel, 1996). Decarboxylates oxalacetate to phosphoenolpyruvate.
torA	Trimethylamine-n-oxide reductase precursor (C)	-1.14 ± 0.22	-0.90 ± 0.16	Catalytic subunit of trimethylamine oxide (TMAO) reductase (Kadner, 1996). Induced by TMAO under anaerobic conditions.

[a] COGs functional categories (Tatusov, 1997). Categories associated with each COGs letter designation are shown in the legend to Table 4.6.

[b] Expression values are expressed as the $\log_2$ of (average experimental intensity/average control intensity) ± 1 standard deviation.

[c] No homologue in *E. coli*.

function, although 2 of these were predicted to also be involved in energy production and conversion.

Four genes from the up-regulated list, including *pzfA*, and one gene from the down-regulated list were selected for real-time RT-PCR to confirm the microarray results. Microarray expression values were then plotted against real-time RT-PCR expression values (Figure 4.4). An r^2 value of 0.86 indicated a strong correlation between the two methods.

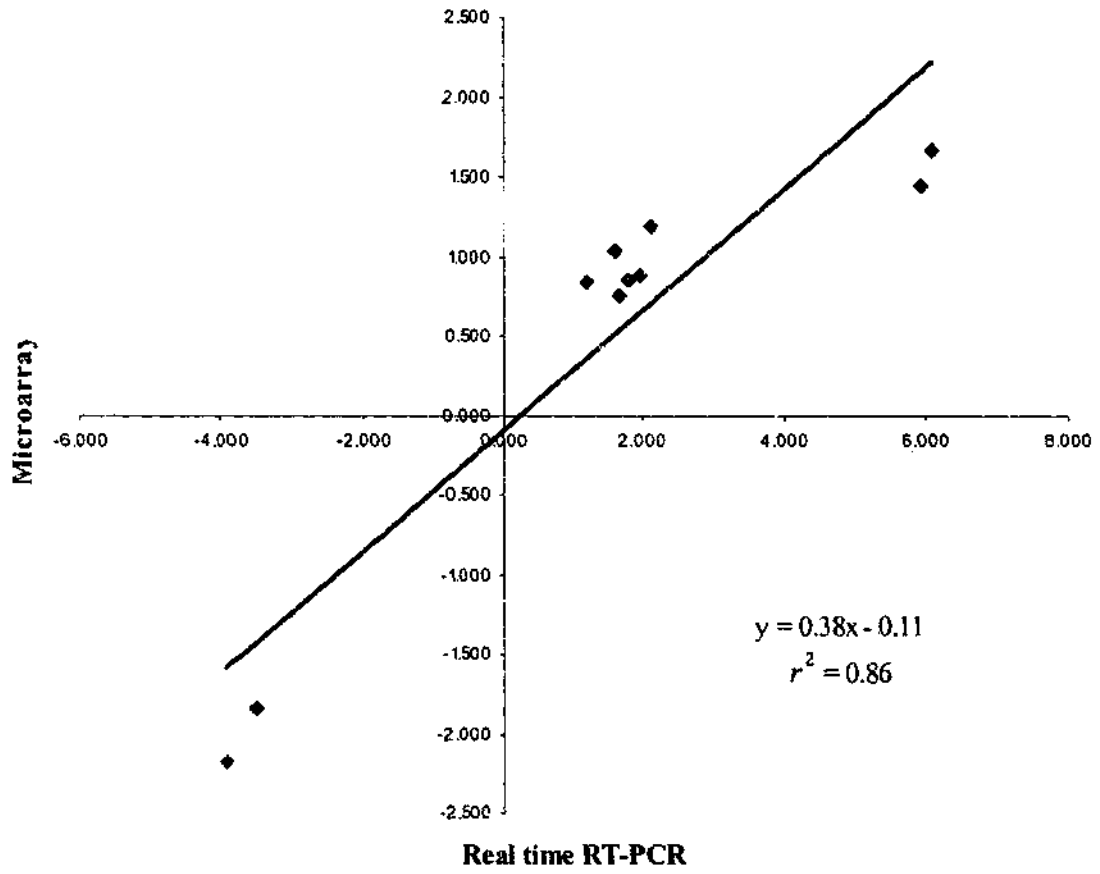

Figure 4.4. Correlation between real-time RT-PCR and microarray expression values. Log_2 expression values for four up-regulated genes (including *pzfA*) and one down-regulated gene were compared. The best-fit linear regression line is shown along with its calculated equation and the r^2 value.

Attempted mutant construction

Following the hypothesis that *pzfA* may be required for *in vivo* survival or virulence, numerous attempts were made to inactivate *pzfA* so that a mutant could be assessed for altered virulence in mice or chickens. Several methods of introducing a disrupted copy of *pzfA* into *P. multocida* were attempted including electroporation, natural transformation, and conjugation (Tables 4.8, 4.9 and 4.10 respectively).

Previously, *pzfA* was cloned from *P. multocida* strain VP161 on a 7.5 kb *Hin*dIII fragment (Lo, 1998). The 4.5 kb *Bam*HI fragment within the 7.5 kb *Hin*dII fragment was then cloned into pBZ (which has no *Hin*dIII site) and a *Hin*dIII site introduced into *pzfA* by PCR for insertion of a 1.6 kb Kan cassette from Tn*5*, giving rise to the plasmid pPBA1460 (Figure 4.5) (Lo, 1998). The mutated construct was

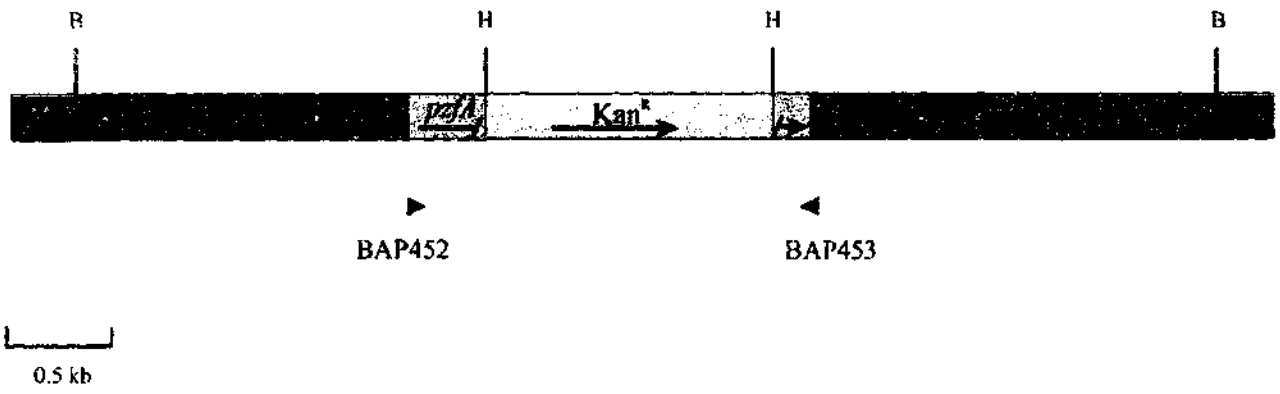

Figure 4.5. The mutated construct in pPBA1460. Blue regions represent the vector, pBZ, and the red regions indicate DNA flanking *pzfA* from *P. multocida* (VP161). Arrow heads denote the positions of the primers used to amplify the truncated mutated construct. Restriction sites shown are B: *Bam*HI and H: *Hin*dIII.

introduced into electrocompetent *P. multocida* strain VP161 in circular (plasmid) form or linear from (amplified by PCR from the plasmid using the universal vector primers T7 and RP). A putative single cross-over mutant was obtained which was confirmed by PCR using *pzfA* flanking primers which yielded two fragments corresponding to the wild type gene and *pzfA* disrupted with the 1.6 kb Kan^R cassette. Plasmid was not isolated from the putative single cross-over mutant and PCR using vector primers did not generate a product indicating absence of free plasmid. After several rounds of passaging, colonies were screened using nitrocefin to identify whether the single cross-over had resolved to a double cross-over. Homologous recombination of inactivated *pzfA* into the genome by double cross-over would result in loss of the plasmid (which confers ampicillin resistance), and therefore, loss of beta-lactamase activity. Ampicillin resistance could not be used as a selectable marker in this case, as *P. multocida* remains sensitive to ampicillin, possibly due to inadequate expression of beta-lactamase (M. Hunt, personal communication). A double cross-over mutant was not identified. To exclude the possibility of freely replicating plasmid or the integration of the plasmid into the *P. multocida* (VP161) genome as a single crossover event, the mutated construct was amplified from

pPBA1460 by PCR, and transformed into *P. multocida*. However, no transformants were obtained.

The mutated construct was also cloned into the vectors, pWSK29 and pSU2719, and the resulting plasmids (pPBA1553 and pPBA1554) were introduced into *P. multocida* strain VP161. However, pSU2719 was found to replicate in *P. multocida* as intact pPBA1554 was recovered from transformed cells, and attempts at introducing pPBA1553 into *P. multocida* by electroporation yielded only spontaneous Kan^R mutants or no transformants. The *P. multocida* strains PBA100 and X-73 were shown to have a similar genetic organisation to VP161 in the region surrounding *pzfA* (Figure 4.6) by amplification of a 6.1 kb PCR product using BAP685 and BAP906 and subsequent digestion of the PCR product by *Bam*HI to yield two fragments of 1.6 kb and 4.5 kb. Therefore, introduction of the mutated

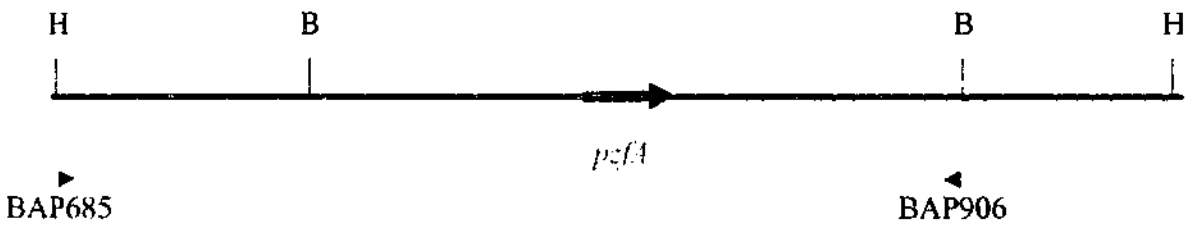

Figure 4.6. Position of *pzfA* on the 7.5 kb *Hin*dIII fragment cloned from *P. multocida* strain VP161. Restriction sites shown are B: *Bam*HI and H: *Hin*dIII. Positions of primers are indicated by the arrow heads. *P. multocida* strains PBA100 and X-73 were shown to have a similar genetic organisation in this region by PCR using BAP685 and BAP906 and subsequent restriction digest of the PCR product.

construct, pPBA1553, by electroporation was also attempted using *P. multocida* strains PBA100 and X-73 to overcome any possible differences in transformation and/or recombination efficiencies. Due to the presence of a gene encoding a DNA adenine methylase (annotated as PM1222; May *et al.*, 2001), pPBA1553 was also treated with Dam methylase before electroporation into *P. multocida* strain X-73 to reduce the chance of restriction endonuclease digestion of incoming plasmid. All attempts to introduce the mutated copy of *pzfA* by marker exchange into the *P. multocida* chromosome were unsuccessful. Attempts at mutagenesis by introduction of a mutated construct using electroporation are listed in Table 4.8.

Ruffolo *et al.* were successful in obtaining a defined mutant in the gene *invA* in *P. multocida* strain X-73 by natural transformation (unpublished data). Therefore, different methods of natural transformation were attempted in *P. multocida* strains VP161 and X-73 using various forms of the *pzfA* mutated construct: PCR product generated with vector primers (full length mutated construct), PCR product generated

with *pzfA* flanking primers (truncated mutated construct) and circularised truncated mutated construct. However, this was also unsuccessful. Attempts are listed in Table 4.9.

Several single cross-over mutants in a serotype D strain of *P. multocida* have been constructed via conjugation (Bosch *et al.*, 2002a; Bosch *et al.*, 2002b; Bosch *et al.*, 2001; Cardenas *et al.*, 2001; Fernandez de Henestrosa *et al.*, 1997). The base plasmid used to obtain the single cross-over mutants, pUA826, was acquired and attempts were made to introduce this plasmid into X-73 by electroporation. No transformants were obtained, confirming that pUA826 was unable to replicate in *P. multocida* strain X-73. The broad host range plasmid, RSF1010, was successfully used as a control to confirm that plasmid could be conjugated from the *E. coli* strain SM10λ*pir* to *P. multocida* strain X-73. An internal fragment of *pzfA*, generated by PCR using the primers BAP386 and BAP2235, was then cloned into pUA826 giving rise to the plasmid pAL189. The *E. coli* strain SM10λ*pir* harbouring pAL189 was used as the donor in several conjugation experiments using various strains of *P. multocida*, AL291, AL338 and AL348, as recipients. AL338 was used as a recipient strain with the idea being that the Em^R encoded on the temperature-sensitive plasmid, pGh9:IS*S1*, could be used for counterselection, then if a single cross-over mutant was obtained, the cells could be cured of pGh9:IS*S1* by incubation at 43°C. Colonies obtained on all of the conjugation plates were patched onto MacConkey agar and BHI plates with the appropriate antibiotics in order to differentiate between *E. coli* and *P. multocida*. Screening was performed using *pzfA* flanking primers (BAP452 and BAP453) as well as primers for the construct (BAP386 and BAP2234). A schematic diagram of the predicted single cross-over event is shown in Figure 4.7. Conjugation attempts are listed in Table 4.10. Different combinations and levels of antibiotics were used, but in each attempt there were break-through *E. coli* and *P. multocida* colonies despite lack of growth when the strains were plated individually onto agar supplemented with the antibiotics which were to be used for selection of conjugants. This method clearly requires further optimisation, although mutants were obtained in genes unrelated to *pzfA* using a different strain of *P. multocida*, VP161 (J. Boyce, personal communication).

Thus, despite numerous attempts using different mutagenesis strategies and different strains of *P. multocida*, a mutant was not obtained.

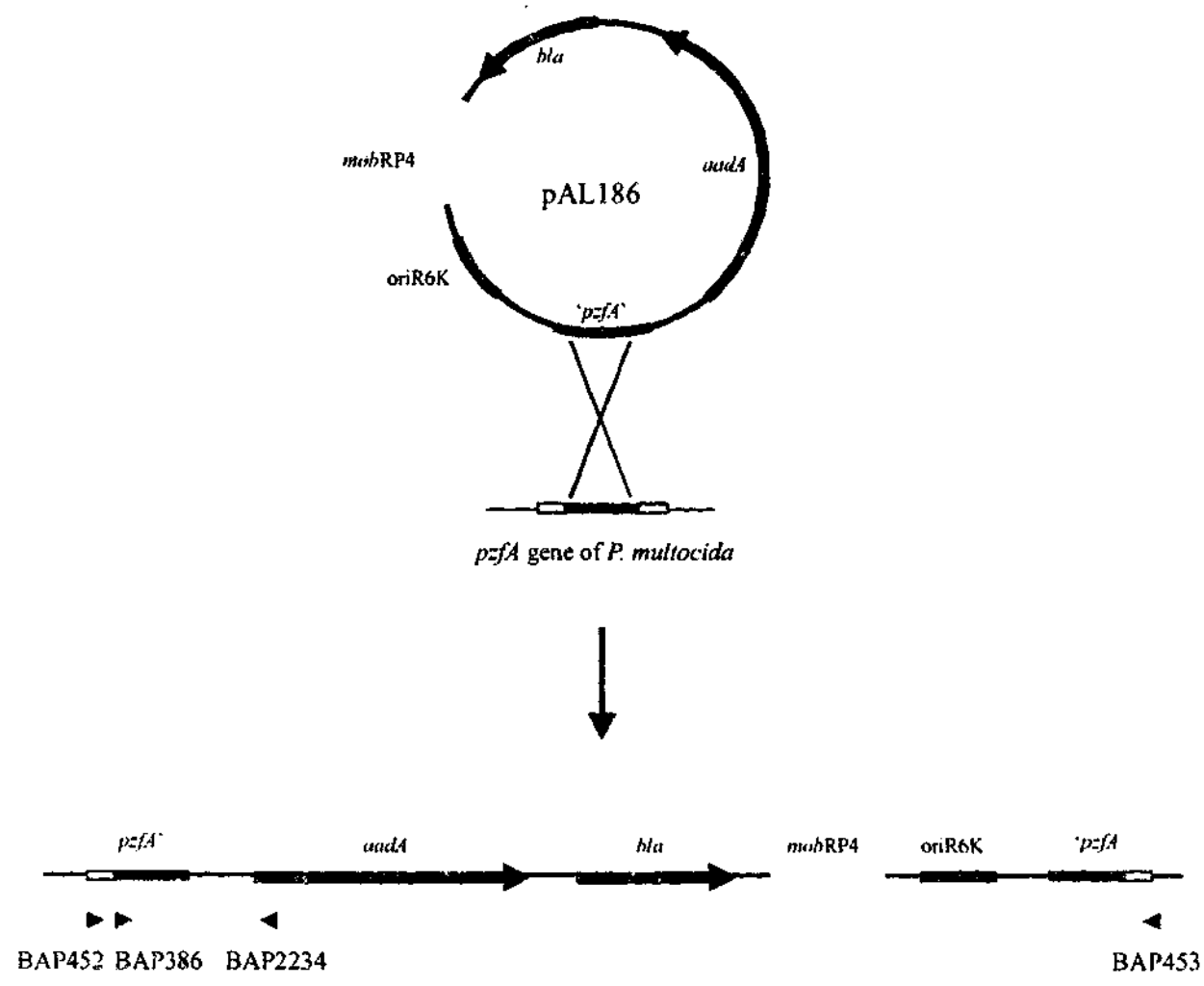

Figure 4.7. Schematic representation of predicted single cross-over event between the internal 345 bp fragment of *pzfA* in pAL189 and the *P. multocida* chromosomal *pzfA*. Primers used for screening are indicated by the arrow heads.

Table 4.8. Attempts at *pzfA* mutant construction by electroporation.

Mutagenesis method	Attempts	Results
Electroporation of mutated construct generated by PCR	Mutated construct amplified from pPBA1460 (Figure 4.5) using vector primers T7 and RP. Electroporation of PCR product attempted twice in VP161 using 18 µg of DNA.	No transformants.
Electroporation of plasmid containing mutated construct	Transformant previously obtained after electroporation with pPBA1460. Transformant appeared to be a single cross-over mutant based on PCR using *pzfA* flanking primers which yielded 2 fragments corresponding to wild type gene and *pzfA* disrupted with the 1.6 kb Kan^R cassette. Plasmid was not isolated from this clone and PCR using vector primers generated no product indicating absence of replicating plasmid. Clone was passaged several times in an attempt to resolve the single cross-over and obtain a double cross-over mutant.	94 putative single cross-over clones (derived from original transformant) screened with nitrocefin to detect any colonies which had lost beta-lactamase activity encoded by the Amp^R gene borne on the base plasmid. All colonies were nitrocefin positive indicating presence of plasmid. Plasmid was not isolated and colony PCR using *pzfA* flanking primers still yielded 2 products corresponding to wild type gene and *pzfA* disrupted with the 1.6 kb Kan^R cassette. No double cross-over mutants obtained.
	Mutated construct from pPBA1460 cloned into pWSK29 giving rise to the plasmid pPBA1553. pPBA1553 was electroporated three times into VP161 (using 3.5-10 µg of DNA), once into PBA100 (using 3.5 µg of DNA), and twice into X-73 (using 5 µg of DNA).	One colony was obtained after electroporation of 10 µg of pPBA1553 into VP161. However, colony PCR screening showed that the colony was a spontaneous antibiotic resistant mutant as only one fragment was amplified corresponding to the wild type gene. No transformants were obtained with the other attempts using pPBA1553.
	Mutated construct from pPBA1460 cloned into pSU2719, giving rise to the plasmid pPBA1554. pPBA1554 electroporated once into VP161 using 2 µg of DNA.	pSU2719 was found to replicate in *P. multocida* strain VP161 as pPBA1554 was recovered after electroporation.
Electroporation of Dam methylase treated pPBA1553	Four times in X-73 using 5 µg of Dam methylase treated plasmid (once) or 8 µg of treated plasmid (3 times).	No transformants.

Table 4.9. Attempts at *pzfA* mutant construction by natural transformation.

Mutagenesis method	Attempts	Results
Natural transformation – liquid transformation	Six times in VP161. Mutated construct or the truncated mutated construct was amplified by PCR and 0.5 µg and 2 µg of DNA respectively was used. 1.6 µg of the truncated mutated construct was also circularised before using for transformation. Liquid transformation was carried out in small plastic petri dishes as well as in 20 ml glass bottles.	Colonies obtained from VP161 transformed with linear mutated construct. 60 colonies obtained and 24 screened by colony PCR using *pzfA* flanking primers. All were shown to be spontaneous antibiotic resistant mutants as only one fragment corresponding to the wild type gene was amplified.
	Six times in X-73. Mutated construct was amplified by PCR and 0.5 µg and 2 µg of DNA was used. 1.6 µg of the PCR product was circularised before using for transformation. Liquid transformation was carried out in small plastic petri dishes as well as in 20 ml glass bottles.	No transformants.
	Twice in X-73 grown to OD_{600} of 0.3. 1 µg of linear PCR product containing the 9 bp uptake sequence used.	No transformants.
	Twice in X-73 grown to OD_{600} of 0.9. 1 µg of linear PCR product containing the 9 bp uptake sequence used.	Plates were almost confluent. 20 colonies were screened by colony PCR using *pzfA* flanking primers. All were shown to be spontaneous antibiotic resistant mutants as only one fragment corresponding to the wild type gene was amplified.
Natural transformation – plate transformation	Three times using *P. multocida* strain X-73. 2 µg of circularised mutated construct used (PCR product generated with blunt ends and ligated)	One colony obtained which upon colony PCR screening with *pzfA* flanking primers, was shown to be a spontaneous mutant as only the wild type gene was amplified.
	Three times using *P. multocida* strain X-73. 1 µg of circularised mutated construct containing 9 bp uptake sequence used (PCR product generated with blunt ends and ligated)	No transformants.

Table 4.10. Attempts at *pzfA* mutant construction by conjugation.

Mutagenesis method	Attempts	Results
Conjugation – filter conjugation	Using AL291 (Rif^R strain of X-73) as recipient and selecting for conjugants on Rif100, Spe25 and Str25.	Mixture of large (presumably *E. coli*) colonies and small (presumably *P. multocida*) colonies grew on Rif100, Spe25 and Str25. Six colonies were screened with KMT primers to see if they were *P. multocida*. Two colonies (the smaller ones) were confirmed to be *P. multocida*. Five more of the small colonies were tested with KMT primers and confirmed to be *P. multocida*. These five colonies were further tested by colony PCR to see if *pzfA* had been disrupted (using the *pzfA* flanking primers BAP452 and BAP453) and whether the construct, pAL189, was present in the cell (using BAP386 and BAP2234). A single fragment was amplified by BAP452 and BAP453 corresponding to wild-type *pzfA*, and absence of a product using BAP386 and BAP2234 showed that pAL189 was not present either as replicating plasmid nor inserted into the genome. Therefore, a single-crossover event had not taken place.
	Using AL348 ($Rif^R Nal^R$ strain of X-73) as recipient and selecting for conjugants on Rif100, Nal15, Spe25 and Str25.	No conjugants.
	Using AL338 (X-73 harbouring pGH9:ISS1) as recipient and selecting for conjugants on Em100, Spe25 and Str25.	300 colonies from filter and plate conjugation patched onto BHI (supplemented with Em100, Spe25 and Str25) and MacConkey agar. 103 colonies (which grew on BHI but not MacConkey) were confirmed to be *P. multocida* by colony PCR using KMT primers. Colony PCR using *pzfA* flanking primers, BAP452 and BAP453, showed that *pzfA* was not disrupted.
	Using AL338 (X-73 harbouring pGH9:ISS1) as recipient and selecting for conjugants on Em200 and Spe75.	300 colonies from filter and plate conjugation patched onto BHI (supplemented with Em200 and Spe75) and MacConkey agar. 96 colonies (which grew on BHI but not MacConkey) were confirmed to be *P. multocida* by colony PCR using KMT primers. Colony PCR using *pzfA* flanking primers, BAP452

		and BAP453, showed that *pzfA* was not disrupted. Colony PCR using primers BAP386 and BAP2234 showed absence of the construct.
Conjugation – plate conjugation	Using AL348 ($Rif^R Nal^R$ strain of X-73) as recipient and selecting for conjugants on Rif100, Nal15, Spe25 and Str25.	No conjugants.
	Using AL338 (X-73 harbouring pGH9:ISS1) as recipient and selecting for conjugants on Em100, Spe25 and Str25.	300 colonies from filter and plate conjugation patched onto BHI (supplemented with Em100, Spe25 and Str25) and MacConkey agar. 103 colonies (which grew on BHI but not MacConkey) were confirmed to be *P. multocida* by colony PCR using KMT primers. Colony PCR using *pzfA* flanking primers, BAP452 and BAP453, showed that *pzfA* was not disrupted.
	Using AL338 (X-73 harbouring pGH9:ISS1) as recipient and selecting for conjugants on Em200 and Spe75.	300 colonies from filter and plate conjugation patched onto BHI (supplemented with Em200 and Spe75) and MacConkey agar. 96 colonies (which grew on BHI but not MacConkey) were confirmed to be *P. multocida* by colony PCR using KMT primers. Colony PCR using *pzfA* flanking primers, BAP452 and BAP453, showed that *pzfA* was not disrupted. Colony PCR using primers BAP386 and BAP2234 showed absence of the construct.

DISCUSSION

The *pzfA* gene was previously identified using the IVET-I system (Hunt and Zhang, unpublished data). However, it was later found that the IVET-I system was inefficient. Although *P. multocida aroA* mutants have been shown to be highly attenuated in mice (Homchampa *et al.*, 1992), it appeared that the *aroA* mutants were able to linger in the host, leading to the identification of false positives. Therefore, a second system, PmIVET, was developed, which used kanamycin resistance as a reporter (Hunt *et al.*, 2001). An advantage of the PmIVET over the IVET-I system is that any wild-type strain of *P. multocida* can be used. The PmIVET system did not identify *pzfA*, probably because only approximately 46% of the genome was covered (Hunt *et al.*, 2001). Alternatively, it is possible that *pzfA* is not expressed at high enough levels *in vivo* to be detected by the PmIVET system. Therefore, to determine if *pzfA* possessed a promoter which was active *in vivo*, the promoter region and start of *pzfA* were cloned into pMKΩ and the resulting clone, PBA907, passaged through Kan-treated mice.

pzfA is up-regulated *in vivo*

Using the PmIVET system, 50% of the bacteria screened from the first bleed (2 hr after infection) were found to be the negative control, PBA917 (Table 4.4). This was expected as equal amounts of PBA907 and the PBA917 control were injected into the mice which had not yet been treated with Kan. At 8 hr after infection, only one colony was isolated from a single mouse. The colony was shown to be PBA907, which indicated that the Kan^S control strain, PBA917, had been cleared by Kan. However, at 24 hr after infection, 75% of the colonies tested by colony PCR were found to be the *pzfA* clone, PBA907 (Table 4.4). It is possible that the bacteria were sequestered in the liver or spleen resulting in very low levels of bacteria in the blood at 8 hr after infection. This may also explain why some of the colonies recovered at 24 hr after infection were found to be the PBA917 control strain. By 24 hr, the Kan would have been cleared from the mice, allowing any sequestered Kan^S bacteria to grow in the blood. The results appeared to indicate that the *pzfA* promoter may be only weakly active *in vivo*. However, as the Kan^S strain was not cleared from the Kan-treated mice, no conclusions could be drawn regarding whether *pzfA* is up-

regulated *in vivo*. It is possible that in this particular experiment, the levels of Kan in the mice may not have been as high as reported previously by Hunt *et al.* (2001) or that the presence of Kan was of short duration. The *pzfA* promoter seemed to be inactive or very weakly active *in vitro* based on lack of growth of PBA907 on BHI plates containing >5 µg/ml of Kan. Therefore, real-time RT-PCR was conducted to compare the levels of *pzfA* transcripts between *in vivo* and *in vitro* RNA samples. The results showed that *pzfA* was more highly expressed when *P. multocida* is grown in the host than *in vitro* (Table 4.5). If PzfA is indeed a protein involved in regulation of genes required for virulence or *in vivo*-growth, it is not surprising that the gene would be more highly expressed *in vivo*. Heithoff *et al.* (1997) identified a number of *in vivo* induced genes in *S. typhimurium* which encoded regulatory proteins.

Sequence analysis of PzfA and its comparison with known proteins

The deduced amino acid sequence of the protein, PzfA, was extremely cysteine-rich, consistent with previously characterised zinc finger proteins. In all classical zinc finger proteins, the zinc finger motif is repeated from 2 to 37 times, depending on the protein (Klug & Schwabe, 1995). In PzfA, four putative zinc-binding motifs each comprising Cys-X_2-Cys-X_{16-20}-Cys-X_{4-5}-Cys were identified (Figure 4.1), which is a common motif in the C4 superfamily of zinc finger proteins (Klug & Schwabe, 1995). The isoelectric point of PzfA was predicted by PSORT to be 8.05. At physiological pH, PzfA would become positively charged which would facilitate binding to the negatively charged DNA.

Comparison of the deduced amino acid sequence of PzfA with Genbank database sequences indicated similarity to topoisomerases and topoisomerase-like proteins from a variety of organisms but not *E. coli*, as the *E. coli* topoisomerase has only three zinc binding domains (Tse-Dinh & Beran-Steed, 1988). In *E. coli*, DNA topoisomerase I plays a role in controlling supercoiling of DNA to maintain an optimal DNA conformation (Drlica, 1990). The enzyme changes the topological state of DNA by breaking and rejoining DNA strands. The 67 kDa N-terminal transesterification domain is responsible for cleavage and religation of DNA (Tse-Dinh, 1998) while the C-terminal domain contains three zinc binding domains which are part of a high affinity DNA binding domain critical for relaxation of negatively supercoiled DNA (Ahumada & Tse-Dinh, 1998; Tse-Dinh & Beran-Steed, 1988). The C-terminal domain also contains a 14 kDa region which possesses ssDNA

binding activity and contributes to enzyme processivity (Zhu *et al.*, 1995). The *H. pylori* topoisomerase I described by Suerbaum *et al.* (1998) is 129 amino acids shorter than the *E. coli* topoisomerase I enzyme and appears to lack the 14 kDa C-terminal domain described in the *E. coli* protein, although this was not addressed by the authors. However, the *H. pylori* topoisomerase I was able to complement an *E. coli* strain with a defective *topA* gene (Suerbaum *et al.*, 1998). PzfA aligns only with the zinc finger DNA-binding region of the *H. pylori* topoisomerase and lacks the other functional domains of topoisomerase I (Figure 4.3), suggesting that PzfA functions purely as a non-topoisomerase DNA-binding protein.

Changes in DNA topology can occur as bacteria adapt to environmental stress conditions (Dorman *et al.*, 1988; Higgins *et al.*, 1988). A study by Marshall *et al.* (2000) found that *Salmonella* DNA becomes more relaxed upon entry into macrophages, and genes encoding proteins involved in regulation of DNA topology (DNA gyrase, nucleoid-associated protein and integration host factor (IHF), but not topoisomerase I) were induced. It has been proposed that IHF, a histone-like protein, may maintain a DNA topology in a form favourable to some promoters under conditions which cause relaxation of DNA (Porter & Dorman, 1997). It is possible that PzfA may play a similar role to IHF and is therefore induced *in vivo* due to changes in DNA topology when *P. multocida* enters the host.

Overexpression of PzfA alters global transcription patterns

Most zinc finger proteins act by binding to specific DNA sequences upstream of the promoter region of the target gene and presumably induce a conformational change in the DNA (Pountney *et al.*, 1997). The zinc finger protein then interacts with the RNA-polymerase holoenzyme to initiate transcription. If PzfA is indeed a transcriptional regulator, the DNA binding activity may result in a conformational change in the target DNA thus facilitating initiation of transcription.

PzfA was previously shown to be able to bind DNA by spotting intact genomic DNA from *P. multocida* onto a membrane and probing with recombinant T7/hexa-histidine tagged PzfA (Lo, 1998). To elucidate the function of PzfA and to identify a possible target gene(s), *pzfA* was overexpressed in *P. multocida* strain PM70 and whole genome microarray analysis was performed to determine how the transcription of other genes would be affected. Genes which were up- or down-regulated by at least 1.5-fold compared to the control were considered to be

differentially expressed. A threshold of 1.5-fold was chosen as other studies have found that a 1.5-fold difference in expression may be biologically significant (Hughes *et al.*, 2000; Smoot *et al.*, 2001).

In both biological replicates, *pzfA* was overexpressed by 3.2- and 2.7-fold (Table 4.6). Therefore, any differences in gene transcription can most likely be attributed to overexpression of *pzfA*. Previously in our laboratory, other *P. multocida* genes, *pm0092* (putative σ^{54} modulation protein), *pm0209* (putative sigma factor), and *pm1460* (phosphoglycerate transport system activator protein which has a σ^{54} interaction domain), have been overexpressed in *P. multocida* PM70 and whole genome microarray analyses have been carried out on these clones. When *pm0092* and *pm1460* were overexpressed, there did not appear to be significant differences in the transcription of other genes between the experimental and control samples, while overexpression of *pm0209* resulted in the up-regulation of a gene set completely distinct from the genes induced by *pzfA* overexpression (J. Boyce, personal communication). Therefore, the genes which were identified to be differentially expressed when *pzfA* is overexpressed appear to be unique to this system. However, without further experimentation, conclusions cannot be drawn as to whether differential gene transcription was due directly or indirectly to overexpression of *pzfA*. Differences in transcriptional levels of metabolic and biosynthetic genes may have arisen due to slight differences in growth conditions, although every effort was made to keep conditions identical between strains.

Most of the genes which were up-regulated when *pzfA* was overexpressed were of unknown or poorly characterised function (Table 4.6). However, 3 of the 10 up-regulated genes encode proteins involved in branched-chain amino acid biosynthesis. In *E. coli*, enzymes involved in isoleucine and valine synthesis are encoded by the *ilvGMEDA* operon (Umbarger, 1996). When *pzfA* was overexpressed, *ilvG* and *ilvM* were also up-regulated (Table 4.6). *pm1626* was also up-regulated in both biological replicates and may be part of the *P. multocida ilv* operon. Although *pm1626* does not have similarity to *ilvE*, it is immediately downstream of *ilvM* followed by *ilvD* and *ilvA*. Both *ilvD* and *ilvA* were slightly up-regulated in both biological replicates. IHF has been found to affect the expression of a broad spectrum of genes (Friedman, 1988) and can activate transcription of the *ilv* operon by bending

the DNA helix (Pagel *et al.*, 1992). It is possible that PzfA may also be able to stimulate expression of the *ilv* operon by altering DNA topology.

The *leuA* gene was also up-regulated in both biological replicates (Table 4.6), while the downstream genes in the operon, *leuB*, *leuC*, and *leuD*, were also slightly up-regulated. A previous study found that when a leucine rich protein, methionyl bovine somatostatin (which consists of 14% leucine), was overexpressed in *E. coli*, the drain on the leucine pool resulted in derepression of the *leu* operon and insufficient leucine to exert negative feedback on the leucine synthesis pathway (Bogosian *et al.*, 1989; Umbarger, 1996). Although PzfA consists of only 7% leucine, the levels of overexpression may have been sufficient to drain the leucine pool leading to up-regulation of the *leu* operon. As with many biosynthetic pathways, the *ilv* operon is also subject to negative feedback regulation (Umbarger, 1996). Therefore, up-regulation of the *ilv* operon in both biological replicates may also have been a result of overexpression of PzfA leading to depletion of the amino acid pool.

The genes *pm0297*, *pm0718*, *pm1378*, *pm1626*, *pm1944*, *ilvG*, and *ilvM* were also found to be significantly up-regulated in three individual chickens during infection (Boyce *et al.*, 2002), although the regulatory mechanisms were not elucidated.

Most of the genes which were down-regulated in both biological replicates overexpressing PzfA appear to be metabolic or biosynthetic genes (Table 4.7). Surprisingly, a number of these genes (*adh2*, *arcA*, *dcuC* and *torA*) are induced under anaerobic conditions. Expression of the *arcA* gene increases four-fold under anaerobic conditions and induction is dependent on Fnr (Lynch & Lin, 1996). The *fnr* gene was found to be slightly up-regulated in both biological replicates of AL241. The *nap* and *nrf* operons are also important for anaerobic growth (Potter *et al.*, 2001), and *pckA* is a glycolytic pathway enzyme. All the genes in the *nap* operon were down-regulated, while only *nrfC* was significantly down-regulated in the *nrf* operon. Interestingly, *nrfE* was up-regulated and there is evidence that *nrfE* has its own promoter (D. Boucher, unpublished data). The gene, *pm1299*, is also predicted to encode a putative C_4-dicarboxylate anaerobic carrier. These results are puzzling, as they suggest that the control strain, AL186, was growing under more anaerobic conditions than both biological replicates of the experimental strain, AL241. However, it is possible that PzfA may repress transcription of these genes.

The genes *pm0688*, *pm0834*, *pm1299*, *adh2*, *arcA*, *bioD1*, *dcuC*, *era*, *nrfC*, *ompW*, and *pckA* were also found to be significantly down-regulated in three individual chickens during infection (Boyce *et al.*, 2002). However, the *nap* operon was found to be significantly up-regulated in chickens, but down-regulated when PzfA was overexpressed. This could perhaps be attributed to differences in growth conditions or the growth phase at which RNA was isolated. Nitrate sources may have been limited in the chickens, but plentiful *in vitro*.

Attempts were made to identify common potential regulatory sequences upstream of the differentially expressed genes using the GeneSpring program, but this failed to identify any conserved sequences which may serve as possible binding sites for PzfA. However, lack of obvious regulatory sequences in target genes is not unusual; for example, a consensus DNA-binding site has not been defined for ArcA target operons (Lynch & Lin, 1996). A microarray-based approach has been used to identify the DNA-binding site of a mouse zinc finger protein (Bulyk *et al.*, 2001). In this method, a DNA microarray was probed with a phage display library. Binding of phage was then detected by using a primary antibody against the phage coat protein and a fluorescent-labeled conjugate. A similar method could perhaps be used in future to identify a possible specific PzfA binding sequence(s).

Attempted insertional inactivation of *pzfA*

Zinc finger proteins activate gene transcription by binding to specific DNA sequences via the zinc binding domains (Pountney *et al.*, 1997). Past studies have shown that mutations in zinc finger proteins, particularly in the zinc binding domains, resulted in decreased DNA binding activity or a change in binding specificity (Cheng & Young, 1995; Zang *et al.*, 1995). Therefore, attempts were made to inactivate *pzfA* in *P. multocida* to determine whether PzfA may be involved in virulence. However, despite using numerous methods and different strains of *P. multocida*, a *pzfA* null mutant was not obtained. Attempts to introduce an insertionally inactivated copy of *pzfA* by allelic exchange were unsuccessful, either by electroporation or natural transformation. A single cross-over mutant was previously constructed, but unfortunately, *pzfA* was not disrupted (Lo, 1998). A search of the *P. multocida* strain PM70 genome showed that there may be a restriction modification system (May *et al.*, 2001). A methyltransferase, type III DNA modification enzyme (PM0698) and a corresponding type III restriction endonuclease (PM0699) have been identified as

well as a putative DNA adenine specific methylase (PM0390). Although high-copy plasmids such as pPBA1100, the base plasmid of pAL99 (Table 4.2), can be used to transform *P. multocida* via electroporation with high efficiency (10^5 – 10^6 cfu/μg of DNA), it is possible that DNA may be cleaved by intracellular restriction endonucleases as it is entering the cell. Therefore, plasmid DNA was treated with Dam methylase prior to electroporation in order to prevent its degradation by the possible presence of restriction endonucleases. However, electroporation of Dam methylase treated plasmid DNA also failed to generate a mutant. Conjugation has previously been successful in construction of single cross-over mutants in a type D strain of *P. multocida*, PM25 (Bosch *et al.*, 2002a; Bosch *et al.*, 2002b; Bosch *et al.*, 2001; Cardenas *et al.*, 2001; Fernandez de Henestrosa *et al.*, 1997). This system has been used in our laboratory to inactivate genes unrelated to *pzfA* in *P. multocida* strain VP161. It appears that the conjugation method needs to be optimised if *P. multocida* strain X-73 is to be used as the recipient. Alternatively, disruption of *pzfA* via conjugation could be attempted using different strains of *P. multocida*. Mutant construction in *P. multocida* has been difficult, and to date, only 7 defined mutants have been constructed in our laboratory. The genes which were inactivated include *aroA* (Homchampa *et al.*, 1992), *mesA* (Hunt *et al.*, 2000), *hexA* (Chung *et al.*, 2001), *cexA* (Boyce & Adler, 2000), *bcbH* (Boyce & Adler, 2001), *hgbB* (Cox *et al.*, 2003) and *nrfE* (D. Boucher, unpublished data). Suerbaum *et al.* (1998) were unable to insertionally inactivate the *topA* gene in *H. pylori* as the mutation would cause disruption of the fourth zinc finger. Therefore, it is also possible that PzfA is functionally important and a mutation in any of the zinc fingers may be lethal.

Overexpression of *pzfA* in *P. multocida* strain PM70 appeared to affect transcription of a wide range of genes. Histone-like proteins such as IHF are able to affect expression of many different genes (Friedman, 1988). Although PzfA did not show any similarity to histone-like proteins, it may play a similar role in gene expression. It would have been of interest to determine if *pzfA* has a direct effect on any of the up- or down-regulated genes. However, due to time constraints, gel shift assays were not attempted on the 32 differentially expressed genes to determine if PzfA can bind to any of them. Once a target gene(s) is identified, a PzfA-specific binding sequence could then be elucidated using DNaseI footprinting. As PzfA is up-regulated *in vivo*, it would be interesting to determine the factors which activate its

transcription. *P. multocida* could be grown under different conditions (for example, varying iron concentrations) and the levels of mRNA transcripts quantitated by real-time RT-PCR to identify the environmental signal or signals which activate the expression of PzfA. Southwestern analysis could be used to identify any proteins which bind to, and interact with, *pzfA*. In this method, whole cell lysates of *P. multocida* would be separated by SDS-PAGE and transferred onto a membrane. Labelled *pzfA* can then be used to probe for proteins which interact with the *pzfA* gene. Classical zinc finger proteins require coordinated zinc ions in order to be functional (O'Halloran, 1993). Therefore, experiments could be done to determine if removal of zinc ions from PzfA will impair its DNA-binding activity.

Unfortunately, the role of PzfA in virulence could not be assessed as a mutant was not obtained. Therefore, the definitive function of PzfA awaits further characterisation.

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APPENDIX

List of Abbreviations and Symbols

Amp	Ampicillin
ATP	Adenosine triphosphate
BHI	Brain heart infusion
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary DNA
cfu	Colony forming units
Cm	Chloramphenicol
°C	Degrees Celsius
CTAB	Cetyltrimethylammonium bromide (hexadecyltrimethylammonium bromide)
dNTP	Deoxynucleotide triphosphate
dUTP	Deoxyuridine triphosphate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Em	Erythromycin
g	Grams
× *g*	Relative centrifugal force, expressed as units of gravitational force
HCl	Hydrochloric acid
hr	Hour
IFA	Incomplete Freund's adjuvant
Inc.	Incorporated
ip	Intraperitoneal
IPTG	Isopropyl-B-D-thiogalactopyranoside
kb	Kilobase
kDa	Kilodalton
Kan	Kanamycin
Kan^R	Kanamycin resistance
kPa	Kilopascals

LB	Luria-Bertani
LPS	Lipopolysaccharide
Ltd	Limited
µF	Microfarad
µg	Microgram
µl	Microlitre
µM	Micromolar
µm	Micrometres
M	Molar
MCS	Multiple cloning site
Mg^{2+}	Magnesium ions
mA	Milliamps
mg	Milligram
ml	Millilitre
mM	Millimolar
MPa	Megapascals
mRNA	Messenger RNA
Nal	Nalidixic acid
NB	Nutrient broth
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
nm	Nanometres
Ω	Ohms
OD	Optical density
ORF	Open reading frame
%	Percentage
PAGE	Polyacrylamide gel elecrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	$\log_{10}[H^+]$
pmol	Picomol
PMSF	Phenylmethylsulfonyl fluoride
Pty	Proprietary

Rif	Rifampicin
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
Spe	Spectinomycin
SSC	Sodium citrate salt
Str	Streptomycin
σ	Sigma
TAE	Tris-acetate-ethylenediaminetetraacetic acid
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with tween 20
TEMED	N,N,N',N'-Tetramthylethylenediamine
Tet	Tetracycline
Tris	Tris (hydroxymethyl) aminomethane
U	Units
UV	Ultraviolet
V	Volts
(v/v)	Volume per volume
WCL	Whole cell lysate
(w/v)	Weight per volume

AMENDMENTS

	Replace:	With:
p30, para 3, line 12	*P. haemolytica*	*M. haemolytica*
p31, line 2	*P. haemolytica*	*M. haemolytica*
p82, para 2, line 14	Figure 2.10	Figure 2.11
p83, para 1, line 7	IF	IHF
p122, table 4.4	AL907	PBA907

Chapter 1

p6. Replace "Molecular typing" with "Molecular identification and typing"

p6 para 4, line 2. Insert after "from clinical samples.": "The first *P. multocida*-specific PCR was first used by Kasten *et al.* (1997) to detect *P. multocida* in turkey flocks. This PCR assay used primers which amplified the P6-like protein (Psl) of *P. multocida*." Add reference: Kasten, R. W., Carpenter, T. E., Snipes, K. P., and Hirsch, D. C. (1997). Detection of *Pasteurella multocida*-specific DNA in turkey flocks by use of the polymerase chain reaction. Avian Diseases 41, 676-682.

p6 para 4, line 7. "unique to *P. multocida*" should read "unique to all *P. multocida* isolates analysed".

p6 para 4, line 8. Insert after "differentiated using PCR technology.": "However, the methods described by Townsend *et al.* (1998a) and Miflin and Blackall (2001) were not specific to *P. multocida* as amplification from *P. canis* biotype 2 was observed ."

p7 para 1, line 3. Delete "Blackall & Miflin, 2000" and insert "Blackall *et al.* 1998". Add reference: Blackall, P. J., Fegan, N., Chew, G. T., and Hampson, D. J. (1998). Population structure and diversity of avian isolates of *Pasteurella multocida* from Australia. Microbiology 144, 279-289.

p7 para 1, line 7. Delete "Blackall & Miflin, 2000" and read "Spratt, 1999". Add reference: Spratt, B. G. (1999). Multilocus sequence typing: molecular typing of bacterial pathogens in an era of rapid DNA sequencing and the internet. Current Opinions in Microbiology 2, 312-316.

p10 para 1, line 12. Insert after "Raemdonck *et al.*, 1992)": "but fluoroquinolones are not registered for use in Australia in food animals (Barton *et al.*, 2003)". Add reference: Barton, M. D, Pratt, R., and Hart, W. S. (2003). Antibiotic resistance in animals. Communicable Diseases Intelligence 27 Supplement, S121-S126.

p10 para 3, line 8. "does not appear to offer protection" should read "does not appear to offer complete protection".

p10 para 3, line 9. Delete "Furthermore, in experimental infections, bacterin vaccines were shown to be of no value in stimulating immunity to infection (Ireland and Adler, unpublished data; Wilkie and Frost, personal communication)."

p20 para 1, line 4. Delete "(Christensen & Bisgaard, 1997)" and insert "(Rhoades & Rimler, 1990)". Add reference: Rhoades, K. R., and Rimler, R. B. (1990). Virulence and toxicity of capsular serogroup D *Pasteurella multocida* strains isolated from avian hosts. Avian Diseases 34, 384-388.

p26 table. Insert before the table:
Table 1.1 (continued)

Gene	Putative or known functions

p32 para 1, line 1. Insert after "characterisation of GlpQ": "(designated *hpd* in Table 1.2)".

p32, para 1, line 10. Insert after "to confirm if *pzfA*": "(the putative zinc finger protein identified by the IVET I system)"

Chapter 2

p57 para 1. "Current vaccines for the control of fowl cholera are unsafe and ineffective" should read "Current vaccines for the control of fowl cholera are generally ineffective and sometimes unsafe"

p59 para , line 3. Insert after "in 2YT broth": "(1.6% tryptone, 1% yeast extract, 0.5% NaCl)"

p60 para 2, line 7. Insert after "RbCl-treated *E. coli*": "(Glover, 1985)." Add reference: Glover, D. M. (1985). DNA Cloning: A Practical Approach. Oxford: IRL Press Limited.

p61 para 1, line 15. "The pellet was then air dried and resuspended in 100 µl DEPC" should read "The pellet was then air dried and resuspended in 100 µl diethylpyranocarbonate (DEPC) treated H_2O"

p85 para 1, line 1. Insert after "convalescent antiserum (at a dilution of 1/100) from one chicken": "experimentally infected with *P. multocida*"

Chapter 3

p104 para 1, line 3. Insert after "convalescent chicken antiserum": "(at a dilution of 1/100) obtained from one chicken which had been experimentally infected with *P. multocida*"

Chapter 4

p113 para 3, line 2. "Le Gouill (1994)" should read "Le Gouill *et al.* (1994).

p113 para 3, line 3. "(Ausubel, 1995)" should read "(Ausubel *et al.*, 1995)"

p120 para 1, line 11. Insert after "sequences of differentially expressed genes": "using the following search settings: length of oligonucleotides 5-8 bases; search 10-500 bases upstream of ORF."

p122 para 2, line 4. "indicating that the *pzfA* promoter is not very active *in vitro*" should read "indicating that the *pzfA* promoter is not active *in vitro*"

p145 para 1, line 12. "inactivate genes unrelated to *pzfA* in *P. multocida* VP161" should read "inactivate genes unrelated to *pzfA* in *P. multocida* VP161, for example, *pm1982* (J. Boyce, personal communication)."

p151. The reference "Lo, M. (1998). Cloning and characterisation of a gene encoding a putative zinc finger protein in *Pasteurella multocida*. In *Department of Microbiology*, pp. 59. Melbourne: Monash University" should read "Lo, M. (1998). Cloning and characterisation of a gene encoding a putative zinc finger protein in *Pasteurella multocida*. B. Sc. (Biomed) (Hons) thesis, Bacterial Pathogenesis Research Group, Microbiology Department, Monash University, Melbourne, Australia."