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Virulence determinants of *Pasteurella multocida*

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Summary

Pasteurella multocida is the causative agent of fowl cholera in birds. Signature-tagged mutagenesis was used to identify potential virulence factors in a mouse septicaemia disease model and a chicken fowl cholera model. A library of *P. multocida* mutants was constructed using a modified Tn*916* and screened for attenuation in both animal models. Mutants identified by the STM screening were confirmed as attenuated by competitive growth assays in both chickens and mice. Of the fifteen mutants identified in the chicken model, only five were also attenuated in mice, showing for the first time the presence of host-specific virulence factors and indicating the importance of screening for attenuation in the natural host. Attenuated mutants were identified with insertions in metabolic genes, cell envelope genes and in two genes encoding enzymes that are involved in LPS biosynthesis. One attenuated mutant was also identified with an insertion in the *flp1* gene, part of a new pilin gene subfamily and may be involved in adhesion during colonization.

One of the LPS mutants identified, AL251, which was attenuated for virulence in mice and in the natural chicken host, was chosen for further study. Sequence analysis indicated that AL251 had an insertional inactivation of the gene $waaQ_{PM}$, encoding a putative heptosyl transferase, required for the addition of heptose to lipopolysaccharide (LPS). Using mass spectrometry and nuclear magnetic resonance, the enzyme encoded by $waaQ_{PM}$ was confirmed as a heptosyl transferase III and the predominant LPS glycoforms isolated from AL251 were severely truncated. Complementation experiments demonstrated that providing a functional $waaQ_{PM}$ gene *in trans*, can restore both the LPS to its full length and growth in mice to wild type levels. Furthermore, it was shown that the mutant AL251 was unable to cause fowl cholera in chickens and that the attenuation observed was not due to increased serum sensitivity.

Statement

I, Marina Harper, declare that this thesis contains no material that 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.

Marina Harper

Publications

Publications arising from research presented in this thesis

Harper, M., Cox, A.D., St. Michael, F., Wilkie, I.W., Boyce, J.D. & Adler, B. **2003.** A heptosyltransferase mutant of *Pasteurella multocida* produces a truncated lipopolysaccharide structure and is attenuated in virulence. *Infect Immun* Submitted.

Harper, M., Boyce, J. D., Wilkie, I. W. & Adler, B. **2003.** Signature-tagged mutagenesis of *Pasteurella multocida* identifies mutants displaying differential virulence characteristics in mice and chickens. *Infect Immun*, **71**:5440-5446.

Other Publications

Boyce, J. D., Wilkie, I., **Harper, M.**, Paustian, M. L., Kapur, V. & Adler, B. **2003.** Genomic-scale analysis of *Pasteurella multocida* gene expression during growth within liver tissue of chickens with fowl cholera. *Microbes Infect*, submitted.

Boyce, J. D., Wilkie, I.W., **Harper, M.**, Paustian, M. L., Kapur, V. & Adler, B. **2002.** Genomic scale analysis of *Pasteurella multocida* gene expression during growth within the natural chicken host. *Infect Immun* **70:** 6871-6879.

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Mitchison, M., Bulach, D. M., Vinh, T., Rajakumar, K., Faine, S. & Adler, B. **1997.** Identification and characterization of the dTDP-rhamnose biosynthesis and transfer genes of the lipopolysaccharide-related rfb locus in *Leptospira interrogans* serovar Copenhageni. *J Bacteriol* **179:** 1262-1267.

Mitchison M., Rood J.I., Faine S. & Adler B. **1991.** Molecular analysis of a *Leptospira borgpetersenii* gene encoding an endoflagellar subunit protein. *J Gen Microbiol* **137:** 1529-1536.

Billington S. J, **Mitchison M.**, Adler B. & Rood J. I. **1990.** Identification and genetic mapping of proteins encoded in the fimbrial subunit gene region of *Bacteroides nodosus*. *Vet Microbiol* **22:** 53-68.

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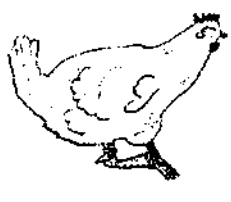
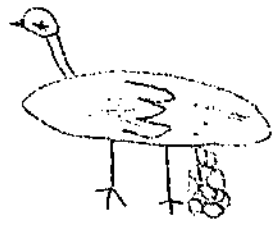
Finally, I need to clarify a few things for my children. Madeleine, I don't think you should have told your Grade 1 teacher that, "Mummy's gone to Brisbane for a week to kill baby chicks." And besides, they were adult birds and that wasn't the objective of the experiment. Simon, it isn't really true that "as a scientist Mum knows everything".....but almost, so eat your veggies.





Chapter 1

Introduction

INTRODUCTION

In 1880, Louis Pasteur reported the identification of an organism, later named *Pasteurella multocida*, as the causative agent of fowl cholera. He also observed that repeated passaging of the bacteria in chicken broth gradually decreased virulence to the extent that chickens innoculated with the passaged cultures no longer developed fowl cholera (Pasteur, 1880). Following these experiments, Pasteur engineered the first vaccine by inoculating chickens with the attenuated *P. multocida*. He demonstrated that vaccinated birds that were later challenged with a virulent culture of *P. multocida* survived and developed no signs of fowl cholera (Pasteur, 1881). In 1887, in recognition of the initial aetiological studies by Pasteur, Trevisan proposed the genus name *Pasteurella* (Mutters, 1989). Initially bacteria belonging to the genus were named according to which host they infected but in 1939, after years of nomenclature changes, Rosenbach and Merchant proposed grouping all the typical Pasteurellae into the type species *Pasteurella multocida* based on their biochemistry, serology and epidemiology. Presently, within the family *Pasteurellaceae* there are six genera: *Pasteurella*, *Haemophilus* and *Actinobacillus* (Mutters, 1989) and the recently added genera *Mannheimia*, *Lonepinella* and *Gallibacterium* (Angen *et al.*, 1999; Christensen *et al.*, 2003; Osawa *et al.*, 1995).

MORPHOLOGY, GROWTH AND NUTRITION

Bacteria of the species *P. multocida* are non-motile, non-spore forming Gram negative coccobacilli. Bipolar staining of freshly isolated bacterial cells is frequently observed when using Giemsa, methylene blue or Gram stains (Mannheim, 1984). Most strains possess a capsule that varies in thickness and composition and which may be lost after repeated subculturing of the bacteria (Namioka, 1978). Fimbriae have also been observed by electron microscopy on the surface of avian strains of *P. multocida* (Rebers *et al.*, 1988) and structures observed on the surface of *P. multocida* have recently been identified and characterized as type 4 fimbriae (Ruffolo *et al.*, 1997). Fimbrial structures resembling Type 1 fimbriae and curli-like pili have also been identified in serotype D strains of *P. multocida* (Isaacson & Trigo, 1995).

The colony morphology of *P. multocida* when grown aerobically at 37° C on enriched media varies according to the presence or absence of capsule. Strains

belonging to serotype A typically give rise to large, sticky, mucoid colonies with flowing margins. This morphology is due to the thick hyaluronic acid capsule that the *P. multocida* scrotype A bacteria produce (Carter, 1967). Both mucoid and smooth colonies are irridescent under oblique light, but smooth colonies are smaller and more regular in shape. Un-encapsulated variants are uncommon and produce rough colonies that are non-irridescent (Carter, 1967).

P. multocida is a facultative anaerobe that grows optimally at 35 to 37 °C in air with or without 5% carbon dioxide (Carter & Chengappa, 1981). Isolation from clinical specimens is usually made on enriched agar media supplemented with blood or serum. However, once isolated, cultures can be maintained on a variety of chemically undefined media with or without serum supplements (Rimler & Rhoades, 1989). *P. multocida* requires nicotinamide, pantothenic acid, thiamine, cystine and iron for growth and can utilise lactic acid or sucrose as a carbon source but glucose, maltose and galactose inhibit aerobic growth (reviewed by Rimler & Rhoades, 1989).

TYPING AND IDENTIFICATION OF *P. MULTOCIDA*

Many serological typing methods have been reported. The first was a serum plate agglutination test, but the majority of strains did not agglutinate using this method because of the presence of capsule (Lyon and Little, 1943 cited in (Brogden & Packer, 1979)). A capsule serotyping method using a passive haemmagglutination test with erythrocytes sensitized with capsular antigen was developed by Carter and is currently still in use. Using this method, five serogroups, A, B, D, E and F, can be distinguished, but the system is limited by the varying ability to produce capsular antibodies against the different serogroups (Carter, 1955).

Somatic (LPS) typing can also be used for the identification of strains and there have been two main systems reported. The Namioka system is based on a tube agglutination test and is able to recognise 11 serotypes, whereas the Heddleston system using a gel diffusion precipitation test can recognise 16 serotypes and is currently the preferred method (Heddleston *et al.*, 1972; Namioka, 1978).

In 1981 a standard system for the identification of *P. multocida* serotypes was recommended that utilized both the Carter capsular typing identified by the letters A, B,

D, E and F, and the Heddleston somatic typing system identified by numbers (Carter & Chengappa, 1981).

New-generation tests for the identification and typing of *P. multocida* have recently been developed using restriction endonuclease analysis (REA), ribotyping, pulsed field gel electrophoresis (PFGE), polymerase chain reaction (PCR), repetitive extragenic palindromic-PCR (REP-PCR) and multi-locus enzyme electrophoresis (MLEE). Utilization of these new tests has proved invaluable for the accurate identification of isolates from several outbreaks of fowl cholera including those in vaccinated turkey flocks where the vaccine strain was suspected to have caused the outbreak of the disease (Wilson *et al.*, 1993). REA or DNA fingerprinting of *P. multocida* field isolates from infected turkeys, and comparison with the vaccine strains M9 and CU, demonstrated that there were differences among isolates that could not be detected using conventional serotyping methods (Snipes *et al.*, 1990). The use of REA has also allowed the differentiation of the two closely related vaccine strains, M9 and CU, and in future, could help to determine if any new outbreaks are caused by either of the vaccine strains (Kim & Nagaraja, 1990). DNA fingerprinting of *P. multocida* isolates using REA has been successful in distinguishing between the 16 Heddleston serotype reference strains, each producing a unique profile using the restriction enzyme *Hha*I (Wilson *et al.*, 1992; Wilson *et al.*, 1993). REA has also been used successfully in the investigation of outbreaks of fowl cholera in Australian turkeys and in determining the prevalence of plasmids in 45 strains isolated from cases of fowl cholera (Blackall *et al.*, 1995; Diallo *et al.*, 1995).

Ribotyping has been used in conjunction with REA to differentiate strains isolated from swine (Gardner *et al.*, 1994; Zhao *et al.*, 1992) and poultry (Blackall *et al.*, 1995; Carpenter *et al.*, 1991; Snipes *et al.*, 1989). The analysis of *P. multocida* genomic DNA by PFGE and palindromic sequence based PCR (REP-PCR) has also been used successfully to differentiate avian, swine and bovine *P. multocida* isolates from Australia and Vietnam (Gunawardana *et al.*, 2000; Townsend *et al.*, 1997).

Multi-locus enzyme electrophoresis (MLEE) differs from other recent typing methods in that it examines differences in the electrophoretic mobility of water-soluble enzymes within native starch gels. Sections of the electrophoresed gel are each stained for a specific enzyme and the distance the enzyme migrates within the gel is used to designate an electromorph number. Any variation in the amino acid sequence of

enzymes from different strains is detected as a difference in electrophoretic mobility (Blackall & Miflin, 2000).

Several PCR tests have been developed for identification of *P. multocida*. The first that was developed amplified a region of DNA within the gene (currently annotated in the PM70 genome as PM0966) encoding the protein PSL that has significant similarity to the P6 lipoprotein of *Haemophilus influenzae* (Kasten *et al.*, 1995). The PCR test could detect all reference strains representing the Heddleston somatic serotypes and two other strains, although it was not tested extensively on other *Pastereulla* species. It also amplified the same region from the *H. influenzae* type b genome but this was not considered a problem for routine testing of poultry flocks, as *H. influenzae* is not normally associated with poultry. However, the PCR test lacked the sensitivity required to consistently detect *P. multocida* in infected turkey flocks (Kasten *et al.*, 1997a). The second PCR test was developed after genomic subtractive hybridization was used to try to identify regions of the *P. multocida* genome unique to haemorrhagic septicemia-causing type B strains. One of the clones identified, KMT1, contained a fragment of DNA that unexpectedly hybridised to all *P. multocida* serotypes. Internal primers generated from within the cloned region of KMT1 were able to amplify DNA from a wide range of *P. multocida* field isolates and three *P. multocida* subspecies reference strains (Townsend *et al.*, 1998). A false positive reaction with the type strain of *Pasteurella canis* biotype 2 was not considered a major drawback for this PCR test when used for avian bacteriology (Blackall & Miflin, 2000). The sensitivity of the PCR test was reported to be high in experiments where *P. multocida* infected avian tissues or intestinal contents were enriched overnight in broth prior to the PCR amplification (Lee *et al.*, 2000), but there have been no reports on its sensitivity when used directly on tissues without prior enrichment.

Finally, a rapid multiplex PCR has been developed that has the potential to replace the Carter capsular serotyping method. Serogroup specific primers were developed from within the unique regions of the capsule biosynthetic loci from each serogroup A, B, D, E and F. The assay was highly specific and the results correlated well with conventional methods with the exception of three of the five strains serologically classified as serotype F. Using multiplex PCR the three were designated as serotype A and sequence analysis of the unique capsule regions amplified from the strains confirmed their new classification (Townsend *et al.*, 2001).

THE DISEASES

P. multocida is a commensal of a wide range of animals including mammals and is the causative agent of many serious, economically important diseases, including avian fowl cholera, bovine haemorrhagic septicaemia and swine atrophic rhinitis (De Alwis, 1992). Haemorrhagic septicaemia, predominantly caused by serotypes B and E strains of *P. multocida*, is an acute disease that affects cattle and buffalo and is characterized by oedematous swelling of the head and neck and swollen, haemorrhagic lymph nodes (Carter & De Alwis, 1989). Atrophic rhinitis in pigs is caused by toxigenic strains of *P. multocida* which typically belong to serotype D. In pigs, the most obvious symptoms include the shortening and twisting of the snout, dark tear staining and pneumonia (Chanter & Rutter, 1989). This chronic wasting disease rarely causes death but it is considered economically important as it significantly reduces the growth rate of the infected pigs.

P. multocida can also be a primary or secondary agent involved in pneumonia in cattle (predominantly caused by serotype A:1), pigs and occasionally sheep (Chanter & Rutter, 1989; Frank, 1989; Gilmour, 1978). *P. multocida* can also infect rabbits resulting in rhinitis ('snuffles") and pneumonia (Manning *et al.*, 1989). Although relatively uncommon, human diseases have also been caused by *P. multocida*, including infections of the skin (commonly following cat or dog bites), bone and joint, cardiovascular system, central nervous system and gastrointestinal tract (Weber, 1984).

Fowl Cholera

Fowl cholera can present in either acute or chronic forms. The chronic form of the disease may develop in birds recovering from an acute form of fowl cholera or develop from an infection with a strain of low virulence. Signs of chronic infection include swellings of wattles, sinuses, periorbital subcutaneous tissues, leg or wing joints, sternal bursea and footpads. Conjunctivitis, pharyngitis, emaciation and lethargy have also been observed in chronically infected birds (Rhoades & Rimler, 1989). Birds suffering from a chronic form of fowl cholera can act as a reservoir for the disease and may later cause acute fowl cholera outbreaks.

The majority of acute fowl cholera cases is caused by serogroup A strains of *P. multocida*, although in some instances outbreaks are attributed to serogroup D and possibly other strains. It is a serious disease of poultry and in the U.S.A. it is estimated

to cost the turkey industry millions of dollars annually due to death, condemnation losses, vaccination and treatment (Carpenter *et al.*, 1988). There are several vaccines available, but these have variable efficacy and do not always prevent of fowl cholera outbreaks. Although *P. multocida* is the causative agent of fowl cholera, other factors such as stress and illness can affect the susceptibility of a flock. For example, there is a statistically significant correlation between colibacillosis and fowl cholera outbreaks where it is thought that the stress associated with the colibacillosis increased the susceptibility of the flock to fowl cholera (Carpenter *et al.*, 1988).

Obvious clinical signs of fowl cholera may not occur until very late in the infection; often the first evidence of the disease may be a number of dead birds present in the flock (Rhoades & Rimler, 1989). Birds suffering acute fowl cholera commonly have depression, ruffled feathers, fever, anorexia, mucous discharge from the mouth, diarrhoea and an increased respiratory rate (Rhoades & Rimler, 1989).

The process by which a flock initially becomes infected with *P. multocida* is not known, but it is widely believed that outbreaks occur after the introduction of chronic carriers. *P. multocida* has been isolated from mucosal samples taken from apparently healthy birds in flocks previously infected with fowl cholera and in flocks with no previous history of the disease (Muhairwa *et al.*, 2000). Transmission may also be possible via wild birds, mammals, insects or exposure to contaminated carcasses (Christensen & Bisgaard, 2000). Environmental exposure is also possible and it has been demonstrated that fowl cholera within a flock can be transmitted by exposure to contaminated water (Pabs-Garnon & Soltys, 1971a).

The progression of fowl cholera is not clearly understood, but it is widely believed that *P. multocida* enters the host through tissues of the respiratory tract and adhesion of *P. multocida* to turkey air sac macrophages has been demonstrated (Pruimboom *et al.*, 1999; Pruimboom *et al.*, 1996). Invasion of turkey epithelial cells has also been demonstrated *in vitro* and this invasive ability could be an important component of pathogenicity (Lee *et al.*, 1994). Virulent *P. multocida* inoculated into the upper respiratory tract of turkeys can be subsequently detected in internal organs between 6 and 12 h post-inoculation (Rhoades & Rimler, 1990). It has also been shown that intratracheal challenge results in rapid multiplication of the bacteria on the surface of the trachea followed by invasion of the circulatory system within 6 h and in some cases the intratracheal inoculation resulted in immediate localisation to the liver by some unknown mechanism (Matsumoto *et al.*, 1991).

It has been generally accepted that fowl cholera is a septicaemic infection, but bacteria can only be isolated in large numbers from the blood of birds very late in infection and it has been proposed that this late re-emergence of blood-borne bacteria is due to the rupture of liver and spleen phagocytes caused by intracellular multiplication of the bacteria (Pabs-Garnon & Soltys, 1971b), although, to date, there is no direct evidence that *P. multocida* can multiply within host cells. Bacteraemia in turkeys can be induced by inoculation of the lungs and air sacs despite the infiltration into the lungs of large numbers of heterophils (Ficken & Barnes, 1989). Clearance of *P. multocida* from turkey blood has been shown to be mediated rapidly by reticuloendothelial phagocytes in the liver and spleen (Snipes *et al.*, 1987; Tsuji & Matsumoto, 1989).

After intravascular clearance, the bacteria localise and multiply in the liver and to a lesser extent the spleen (Pabs-Garnon & Soltys, 1971b; Tsuji & Matsumoto, 1989). In an immune bird, both encapsulated and un-encapsulated bacteria are quickly killed inside hepatic phagocytes. However, in naïve birds, only the virulent, encapsulated bacteria survive and presumably multiply (Tsuji & Matsumoto, 1989). In the same study by Tsuji and Matsumoto, in immune birds, bacterial inactivation was only observed in the liver, not the spleen, suggesting a difference in the bactericidal ability of each macrophage population (Tsuji & Matsumoto, 1989). In the terminal stages of fowl cholera, death is probably caused by massive bacteraemia and endotoxic shock (Carter, 1967; Rhoades & Rimler, 1984).

The first systemic host defence mechanism against bacterial infection involves the innate immune system and includes phagocytosis and bactericidal properties of serum components such as complement. However, *P. multocida* has mechanisms to evade this system, including the presence of a capsule (Chung *et al.*, 2001). Active immunity against the invading bacteria can either be humoral or cell-mediated and in the case of *P. multocida* infection immunity is mainly humoral. Vaccination with heat killed *P. multocida* stimulates full protection against homologous challenge and opsonization studies in mice have shown that bactericidal antibodies are produced (Wijewardana & Sutherland, 1990).

The first fowl cholera vaccine was developed by Pasteur over 120 years ago and there have been many vaccines produced subsequently, including both live and killed (bacterin) preparations (Rhoades & Rimler, 1991). Bacterin vaccination and cross challenge experiments have demonstrated that there are multiple immunotypes of *P. multocida* and therefore killed vaccines need to consist of multiple immunotypes in

order to be effective. Most commercial vaccines for fowl cholera are bacterins made from a pool of killed *in vitro* grown *P. multocida* representing serotypes A:1, A:2 and A:4 (Bairey, 1975, Rhoades, 1991 #1016; Rhoades & Rimler, 1991). Several live attenuated vaccines have been developed from a strain first isolated from chronic carriers, designated the Clemson University (CU) strain, that has the serotype A:3,4 (Bierer & Derieux, 1972). This strain produces excellent humoral and cell mediated cross-protective immunity in turkeys when given orally but it must be innoculated via the wing-web to afford any protection in chickens (Dua & Maheswaran, 1978). Since its introduction, the CU vaccine has caused some disease outbreaks and other complications that suggest its use is limited to young turkeys (Schlink & Olson, 1987). To make a safer live vaccine the CU strain was mutagenized and temperature sensitive mutants were selected that were able to grow at 37 °C, but not at 42 °C. The strain could be grown *in vitro* for preparation of the vaccine but once *in vivo* the strain is unable to grow because the normal body temperature of birds is 42 °C (Hertman *et al.*, 1979; Hofacre & Glisson, 1986). One of the mutants, M9, is in commercial use, but it is not as efficacious as the CU strain and much higher doses are required for protection against disease (Friedlander *et al.*, 1991). Unfortunately M9 has also been implicated in outbreaks of fowl cholera as it is believed that the vaccine is capable of causing disease in birds subjected to stressful conditions (Snipes *et al.*, 1990).

A genetically defined mutant of *P. multocida* has been made for potential use as a commercial live vaccine. Two *aroA* mutants that confer homologous protection in mice were constructed in both *P. multocida* and *P. haemolytica* by insertional inactivation of the *aroA* gene using a kanamycin-resistance cassette (Homchampa *et al.*, 1992, Homchampa, 1994 #575; Homchampa *et al.*, 1994). Subsequently, a marker-free *aroA* mutant of *P. multocida* was developed in both an A:1 and an A:3 strain and mice immunized with the A:3 attenuated mutant were protected against homologous and heterologous challenge (Homchampa *et al.*, 1997). Vaccination trials in chickens using the *aroA* mutants have given mixed results, with 100% protection afforded after intramuscular vaccination but no protection afforded by oral administration, the most economical route for large-scale vaccination (Scott *et al.*, 1999).

VIRULENCE FACTORS

Capsule

Serogroup A strains of *P. multocida* possess a capsule that is composed primarily of hyaluronic acid (Pandit & Smith, 1993). In general, fowl cholera isolates that possess a capsule are more virulent than their acapsular variants (Heddleston *et al.*, 1964; Snipes *et al.*, 1987; Tsuji & Matsumoto, 1989). The important role of capsule in the pathogenesis of fowl cholera was recently demonstrated using a genetically defined acapsular mutant. The mutant was attenuated in both mice and chickens and was unable to establish growth in chicken muscle (Chung *et al.*, 2001).

It is widely believed that capsule plays a significant role in resistance to phagocytosis and this has been demonstrated *in vitro* by Harmon *et al* (1991) and others who have correlated sensitivity to phagocytosis with the presence and thickness of the bacterial capsule (Harmon *et al.*, 1991; Pruimboom *et al.*, 1996; Truscott & Hirsh, 1988). However, it has also been shown in turkeys that the intravascular clearance rate and phagocytic uptake of spontaneously derived, un-encapsulated mutants was no different from the encapsulated strains (Snipes *et al.*, 1987; Tsuji & Matsumoto, 1989). Fully encapsulated *P. multocida* cells adhere to, but are not internalised by, turkey air sac macrophages, adherence probably being mediated by capsular hyaluronic acid (Pruimboom *et al.*, 1996).

Resistance to complement-mediated lysis is considered an important virulence factor and experiments on *P. multocida* type A strains have shown that serum resistance correlates with the possession of a type A capsule (Hansen & Hirsh, 1989; Snipes & Hirsh, 1986). A genetically defined acapsular mutant was no longer serum resistant in normal avian serum compared to the serotype A wild-type parent and complemented mutant (Chung *et al.*, 2001). The capsule, rather than masking the components of the cell wall that may activate the complement pathway, probably shields the outer membrane from the membrane attack complex formed by the complement pathway, since serum incubated with capsulated strains was depleted of complement activity (Hansen & Hirsh, 1989).

A genetically defined capsule mutant of a serotype B strain has also been constructed and although capsular polysaccharide is produced in this mutant it remains inside the cell as the bacteria no longer have a functional export mechanism (Boyce & Adler, 2000). In contrast to the findings using the genetically defined, serotype A

acapsular mutant, studies with the serotype B acapsular mutant and its wild-type parent found there was no difference with respect to serum resistance, with both being equally resistant to the bactericidal effects of bovine or murine serum (Boyce & Adler, 2000). Despite serum resistance, the bacteria were cleared rapidly *in vivo* and were only capable of lethal infection in mice at very high doses. The mutant did have increased sensitivity to phagocytosis by murine macrophages and it is likely this was the main mechanism for clearance of the acapsular bacteria (Boyce & Adler, 2000).

The entire capsule biosynthetic locus has been cloned and sequenced from two strains of *P. multocida*, a serogroup A:1 strain X-73 (Chung *et al.*, 1998) and a serogroup B:2 strain, M1404 (Boyce *et al.*, 2000). The capsule region has also been identified and partially sequenced in the remaining serotypes D, E and F (Townsend *et al.*, 2001). From the analysis of the loci it was determined that there were three distinct regions.

In all serogroups, regions 1 and 3 comprise a total of six genes that are involved in polysaccharide assembly and transport to the cell surface. Region 2, the biosynthetic region of the capsule loci, encodes the genes involved in determining the composition of the capsule and therefore shows the most variation between serotypes.

In serogroups A, D and F, three of the four genes in region 2 are highly conserved, the remaining gene encoding a different glycosyltransferase, depending on the serotype. In serotype A strains the unique region 2 gene encodes hyaluronic acid synthase and is required for the polymerisation of hyaluronic acid, the principle component of type A capsule (DeAngelis, 1996; DeAngelis *et al.*, 1998). In serotype F the glycosyltransferase has been identified as chondroitin synthase and is responsible for polymerisation of the serotype F capsule (DeAngelis & Padgett-McCue, 2000; Rimler, 1994). Finally, in serotype D, the gene product was found to be similar to the *Escherichia coli* glycosyltransferase, KfiC, which is required for the synthesis of heparin, likely to be a major component of type D capsule since heparinase III is able to decapsulate serotype D strains (Rimler, 1994; Townsend *et al.*, 2001).

The structure and composition of serotype B capsule is unknown but mannose has been identified as a major component (Muniandy *et al.*, 1992). The entire capsule locus of a serotype B strain has been sequenced and analysed and only two of the region 2 genes, *bcbA* and *bcbB* are similar to known genes and encode enzymes that catalyze the conversion of UDP-*N*-acetylglucosamine to *N*-acetyl-D-mannosaminuronic acid (Boyce *et al.*, 2000).

Whilst there is unequivocal evidence that the capsule is a virulence factor and therefore plays a large role in pathogenesis, there is no clear evidence that it can function independently as an immunogen. This is unsurprising as the composition of capsular material found in *P. multocida* serotypes A, D and F strains is very similar to host glycosaminoglycans such as chondroitin, heparin and hyaluronan. This may be an example of molecular mimicry whereby the bacteria coated in these materials are recognised by the host immune system as "self" rather than foreign material (DeAngelis & Padgett-McCue, 2000).

Lipopolysaccharide (LPS)

P. multocida LPS is considered to play an important role in the pathogenicity of disease. LPS from many bacteria has been shown to be endotoxic, but there are conflicting reports as to the endotoxic properties of LPS isolated from *P. multocida*. Intravenous inoculation of LPS from Serotype B: 2 stains could reproduce clinical signs of haemorrhagic septicemia in buffalo, indicating strong endotoxic properties (Horadagoda *et al.*, 2002). However, the endotoxic capability of LPS from serotype A strains is less clear, with some studies indicating that chicken embryos and mice were highly susceptible, but that turkey poults were relatively resistant despite an inflammatory response and microscopic hepatic lesions (Ganfield *et al.*, 1976; Mendes *et al.*, 1994; Rhoades & Rimler, 1987). Although some of the variation observed with the toxicity of LPS may be due to differences in host responses, it may also be due to the method of LPS preparation. For example, it has been reported that LPS cannot be extracted from *P. multocida* serotype A strain X-73 using the classic phenol-water Whestphal procedure unless the cells are killed using formalin prior to extraction (Rebers & Rimler, 1984). However, although there was no apparent loss of antigenicity, LPS prepared from formalin killed cells using the Whestphal procedure was nearly 100-fold less toxic to chicken embryos than LPS from non-formalin killed cells, extracted using the phenol-chloroform-petroleum ether method (Rebers & Rimler, 1984). It was suggested that formalin killing of *P. multocida* prior to LPS extraction could partially detoxify LPS and, observations of the LPS samples using electron microscopy, revealed morphological differences between the two LPS sample types (Rebers & Rimler, 1984).

LPS, along with other potential virulence factors such as fimbriae, may help to mediate adherence to host cells and studies have shown that *P. multocida* can invade

epithelial cell layers and adhere to rabbit and porcine respiratory tract epithelium (Lee *et al.*, 1994). Recently it has been shown in the bovine model that LPS from *P. multocida* assists in adhesion to neutrophils and transmigration through endothelial cells (Galdiero *et al.*, 2000).

The LPS of *P. multocida* stimulates humoral immunity and is considered to be a protective antigen. *P. multocida* strains are classified into Heddleston serotypes based on the antibody responses to LPS and antibodies raised as a response to heat killed *P. multocida* vaccines are primarily directed against LPS and protect the host against strains within the same serotype (Brogden & Rebers, 1978). Early studies demonstrated that LPS purified using the Westphal method and injected into mice and rabbits stimulated a poor antibody response and no protection against *P. multocida* infection was observed. In contrast, phenol-extracted LPS injected into chickens induced a good antibody response and passively protected recipients against disease (Rebers *et al.*, 1980). Furthermore LPS coupled to ribosomal protein was able to induce active immunity in chickens and to passively protect against challenge with *P. multocida* (Phillips & Rimler, 1984; Rimler & Phillips, 1986). Monoclonal antibodies raised against the LPS from a serotype A strain were shown to be bactericidal and to completely protect mice against homologous challenge (Wijewardana *et al.*, 1990). In addition, an opsonic monoclonal antibody was raised against a serotype B strain of *P. multocida* LPS and was shown to partially protect mice against *P. multocida* infection (Ramdani & Adler, 1991).

Little is known about the structure and composition of *P. multocida* LPS. There are presently only published data on the LPS isolated from two serotype A strains of *P. multocida* and these contain a tri-heptose unit linked to a 2-keto-3-deoxyoctulosonic acid (Kdo) residue (Erler *et al.*, 1988; Erler *et al.*, 1986; Erler *et al.*, 1981).

Since the release of the *P. multocida* PM70 genome sequence many genes have been identified that have significant similarity with genes that are known to be involved in LPS biosynthesis in other bacteria (May *et al.*, 2001). These genes are located throughout the genome with some clustered in small operons. There have been two LPS related genes cloned and characterised; both are located within the Lipid A biosynthesis operon (Delamarche *et al.*, 1995; Manoha *et al.*, 1994). The deduced amino acid sequence of *firA* has strong similarity to proteins that are Lipid A acyl transferases involved in LPS synthesis (Delamarche *et al.*, 1995). The other characterised gene, *skp*,

encodes a protein with similarity to the Skp protein of *E. coli* which is a molecular chaperone that assists in the folding of outer membrane proteins in the periplasmic space (Schafer *et al.*, 1999). Recombinant Skp protein from *P. multocida* did not elicit a protective immune response in mice (S.Doughty, personal communication) and it is not clear if this protein has any role in LPS biosynthesis, despite being located in the same operon as *firA*.

A *galE* mutant has been constructed in *P. multocida* and has significantly reduced viability in mice (Fernandez de Henestrosa *et al.*, 1997). Included in the role of *galE* in bacteria is the epimerization of UDP-glucose to UDP-galactose prior to LPS assembly and this mutant probably expresses an altered LPS, although the structural analysis of the LPS was not reported (Fernandez de Henestrosa *et al.*, 1997).

Fimbriae and Adhesins.

Fimbriae have frequently been observed on the surface of *P. multocida* cells. In particular, fimbrial structures have been observed on the surface of *P. multocida* serotype A strains that were able to adhere to mucosal epithelium, but not on the surface of those strains unable to adhere (Glorioso *et al.*, 1982; Isaacson & Trigo, 1995; Rebers *et al.*, 1988; Ruffolo *et al.*, 1997). Type 4 fimbriae have been isolated and characterized from *P. multocida* serotypes A, B and D. Antisera raised against the purified subunit protein recognized type 4 fimbrial subunits from *Moraxella bovis* and *Dichelobacter nodosus* (Ruffolo *et al.*, 1997). Fimbriae belonging to the type 4 family are filamentous, flexible surface appendages composed of identical monomeric protein subunits and have been identified on many Gram-negative organisms. They are often associated with virulence because of their ability to attach to host cell surfaces. However, the role of fimbrial structures in *P. multocida* virulence is still unproven and attempts to clone the entire type 4 subunit gene, *ptfA* from *P. multocida* into *E. coli* were unsuccessful (Doughty *et al.*, 2000).

There are several other genes identified on the *P. multocida* genome that have significant similarity to known fimbriae or fibril genes including *fimA*, *hsf_1*, *hsf_2*, *pm0855*, and a previously unannotated gene adjacent to *pm0855*. *fimA* is predicted to encode a fimbrial subunit, *hsf_1* and *hsf_2* surface fibrils and *pm0855* and the adjacent gene are predicted to encode Flp pili, a new type IV pilin subfamily (Kachlany *et al.*, 2001b; May *et al.*, 2001). In addition, a *P. multocida* STM mutant, significantly

attenuated for growth in mice, had a transposon insertion in *tadD*, a gene downstream from the *flp* homologues and possibly within the same operon. The protein, TadD, has shared identity to an adherence protein associated with the Flp pilin subunits of *Actinobacillus actinomycetemcomitans* and may form a part of a secretion apparatus required for Flp pilin assembly (Fuller *et al.*, 2000; Kachlany *et al.*, 2001a; Kachlany *et al.*, 2001b; May *et al.*, 2001).

Two *P. multocida* genes, *pfhaB1* and *pfhaB2*, share significant similarity with a class of genes that encode filamentous haemagglutinins, which in *Bordetella pertussis* play a major role in the colonisation of the upper respiratory tract (Kimura *et al.*, 1990; Mooi *et al.*, 1992). Mutation of these genes in *P. multocida* resulted in significantly reduced virulence in mice (Fuller *et al.*, 2000).

Toxins

In general, most *P. multocida* strains that cause fowl cholera, haemorrhagic septicaemia and pneumonias do not have any known toxins. The only toxin identified in *P. multocida* is the dermonecrotic *Pasteurella multocida* toxin (PMT) produced by strains that cause atrophic rhinitis in pigs. PMT is present in strains belonging mainly to serogroup D and occasionally serotype A and is responsible for the clinical and pathological signs of atrophic rhinitis (Foged *et al.*, 1987; Rimler & Rhoades, 1989). The toxin is not highly similar to any other known proteins and is a potent mitogen inducing many cellular effects including rearrangements in the actin cytoskeleton (Zywietz *et al.*, 2001). The cloned *toxA* gene encoding PMT has been sequenced and it is was proposed that it was transcriptionally regulated by a repressor protein TxaR, encoded upstream of the *toxA* gene(Petersen, 1990), although more recent studies contradict this finding and suggest that TxaR is constitutively expressed (Hoskins & Lax, 1995; Hoskins & Lax, 1996). A toxoid was developed by deletion mutagenesis of the cloned PMT toxin gene and it was found to protect mice and their offspring against challenge with purified PMT (Petersen *et al.*, 1991).

Iron Regulated and Iron Acquisition Proteins

Iron is an essential element required for growth and it must be acquired by bacteria in order to survive. Because of its inherent toxicity, the level of free iron available *in vivo* is very limited and *P. multocida*, like other bacterial species, has

developed mechanisms by which iron can be sequestered into the cell. Comparisons of *P. multocida* grown in iron rich, iron depleted media and *in vivo* has demonstrated that many high molecular weight outer membrane proteins are regulated by iron levels and have therefore been called iron-regulated outer membrane proteins (IROMPs)(Snipes *et al.*, 1988) (Choi-Kim *et al.*, 1991). *P. multocida* grown under iron limited conditions also induces a stronger protective response in mice compared to the same strain grown under iron replete conditions (Kennett *et al.*, 1993). IROMPs may therefore play a significant role in cross-protective immunity (Glisson *et al.*, 1993; Ruffolo *et al.*, 1998).

It has not been fully established what iron acquisition systems are used by *P. multocida*, but an early study found that *P. multocida* expressed a siderophore-like substance named multocidin under iron-limited conditions (Hu *et al.*, 1986). In contrast, in a study by Ogunnariwo (1991), siderophores were not detected in several *P. multocida* strains representing both bovine isolates and two of the same avian strains previously analysed by Hu (1986). However, the bovine isolates could utilize iron bound to bovine transferrin, but not iron bound to other transferrin species, suggesting that the bovine strains tested had a transferrin receptor-mediated mechanism of iron acquisition (Ogunnariwo *et al.*, 1991). Interestingly, the avian isolates tested could not utilize iron bound to any transferrin species, including avian derived transferrin (Ogunnariwo *et al.*, 1991). Transferrin receptors utilized by bacterial species in the *Pasteurellaceae* and *Neisseriaceae* family usually consist of two iron binding receptors TbpA and TbpB (Gray-Owen & Schryvers, 1996), but recent evidence suggests that the transferrin receptor in bovine strains of *P. multocida* is composed of only a single protein TbpA (Ogunnariwo & Schryvers, 2001). In *Mannheimia haemolytica*, antibodies that were raised against TbpB, and to some extent TpbA, afforded good protection against experimental *M. haemolytica* infection (Potter *et al.*, 1999). This indicates that other proteins required for iron acquisition and transport may also be good vaccine candidates because they are often essential for growth *in vivo* and many are also located in the outer membrane. Mutants, attenuated in mice, have been identified with insertional inactivations in genes encoding the proteins ExbB and HgbA, that are both either directly or indirectly involved in iron acquisition (Fuller *et al.*, 2000). ExbB is part of the TonB transport complex, required to transport and provide energy for iron sequestration and *hgbA* is predicted to encode a haemoglobin-binding protein required for the acquisition of iron from host proteins (Bosch *et al.*, 2002).

Recently whole genome expression profiling of *P. multocida* PM70 grown under iron limited conditions identified a number of iron-related genes that were up-regulated, including those encoding proteins involved in iron transport such as YfeABCD, FbcABC, FecBCD and those that provide energy for iron transport, TonB and ExbBD (Paustian *et al.*, 2002b). Sequence analysis of *P. multocida* PM70 has revealed that a relatively large proportion of the genome (over 2.5%) encodes 53 proteins with shared identity to proteins involved in iron uptake or acquisition (May *et al.*, 2001).

Sialidase (Neuraminidase)

Sialidases are produced by some bacterial species to remove sialic acid from host glycosylated proteins and lipids for use as a carbon source. They are also thought to enhance bacterial virulence by unmasking key host receptors and/or reducing the effectiveness of host defences such as mucin. Gottschalk in 1960 demonstrated that the removal of sialic acid from salivary glycoproteins inhibited the protective capacity of these secretions, suggesting that exogenous neuraminidase would help an invading organism survive against host defences (cited in Straus *et al.*, 1993). More recently, sialidase expressed by *Streptococcus pneumoniae* has been shown to desialylate LPS from *Neisseria meningitidis* and *H. influenzae*, a process which *in vivo* could render them more susceptible to host defences such as complement (Shakhnovich *et al.*, 2002). It was proposed that this ability is an example of inter-species competition, as *N. meningitidis*, *H. influenzae* and *S. pneumoniae* all colonize the human upper respiratory tract (Shakhnovich *et al.*, 2002).

Most *P. multocida* and *M. haemolytica* strains produce sialidase and both cell bound and extracellular sialidases have been reported in *P. multocida* (Drzeniek *et al.*, 1972; Scharmann *et al.*, 1970; White *et al.*, 1995). Bacterial sialidase is produced *in vivo* in goats after transthoracic challenge with either *P. multocida* or *M. haemolytica* (Straus & Purdy, 1994; Straus *et al.*, 1996). Interestingly, antibodies raised against *M. haemolytica* A:1 sialidase were unable to neutralize the *P. multocida* A:3 sialidase as measured by enzyme activity, suggesting that the two enzymes are not antigenically related (White *et al.*, 1995). Passive protection of mice against *P. multocida* A:3 homologous challenge has been demonstrated using rabbit antisera raised against

partially purified sialidase, although there was some difficulty in removing anti-LPS antibodies from the serum prior to administration into mice (Ifeanyi & Bailie, 1992).

Two sialidases, NanH and NanB, have recently been cloned and characterized from a fowl cholera isolate of *P. multocida* (Mizan *et al.*, 2000). These sialyldases differed in their specificity, with both able to utilize 2-3' sialyl lactose but only NanB could fully utilize 2-6' sialyl lactose. It was proposed that the presence of two sialidases with slightly different specificities would enhance the metabolic capacity of *P. multocida* in the host (Mizan *et al.*, 2000).

Other Enzymes

The secretion of proteases from seven *P. multocida* strains, freshly isolated from a range of animals and humans, has been reported (Negrete-Abascal *et al.*, 1999; Pouedras *et al.*, 1992). Two strains isolated from human infections were shown to produce proteases that cleaved human IgA and IgG, but not IgM (Pouedras *et al.*, 1992). The proteases isolated from animal isolates varied in molecular mass, were able to cleave IgG and were biochemically similar to known neutral metalloproteases (Negrete-Abascal *et al.*, 1999). The role that these enzymes play in virulence and colonization has not been determined.

Superoxide dismutases, containing either copper or zinc, have recently been reported in a number of Gram-negative bacteria including *Haemophilus* sp., *Actinobacillus* sp., *P. multocida* and *N. meningitidis* (Kroll *et al.*, 1995). These enzymes, once considered exclusively eukaryotic, are located in the periplasm of the bacteria where they function to protect the cell from damage by exogenously derived, superoxide radicals.

Although the role of hyaluronidase in the pathogenicity of infection has not been determined, it is present in many of the serotype B strains of *P. multocida* that cause bovine haemorrhagic septicaemia. A study of 74 *P. multocida* strains representing all capsular serotypes found that only the type B strains, isolated from haemorrhagic septicaemia infections, produced hyaluronidase (Carter & Chengappa, 1980). Another study of 176 strains of *P. multocida* representing different serotypes also found hyaluronidase activity confined to serotype B, but more specifically B:2 and it was suggested that a test for hyaluronidase activity could be used to presumptively identify B:2 strains (Rimler & Rhoades, 1994). However, because other serotypes can cause

disease with similar symptoms it should not be used as a substitute for capsular and somatic typing methods. Presumptive typing of type B strains by measuring hyaluronidase activity has also been used in conjunction with REA using *Hha*I and *Hpa*II to discriminate successfully among type B strains that cause haemorrhagic septicaemia (Rimler, 2000).

Antigen P6

The gene encoding a 16-kDa outer membrane protein from *P. multocida* was cloned and sequenced and found to have extensive sequence similarity to the gene encoding the P6 protein of *H. influenzae* (Kasten *et al.*, 1995). Human antibodies against the *H. influenzae* P6 protein are bactericidal for *H. influenzae* and the antisera can provide passive protection against *H. influenzae* type b-induced meningitis in infant rats (Munson & Granoff, 1985; Murphy *et al.*, 1986).

The P6 homologue in *P. multocida* was shown by Western blotting to be expressed by all 16 somatic serotypes and therefore a likely candidate for vaccine studies (Kasten *et al.*, 1995). However, a subsequent report in 1997 found that vaccination with a recombinant P6 protein failed to protect turkeys against *P. multocida* challenge (Kasten *et al.*, 1997b).

OmpH

OmpH, a major polypeptide of the outer membrane of *P. multocida*, was shown to be a porin using planar lipid bilayers, and crystal array studies showed that the pore was formed using a trimeric arrangement of the protein (Chevalier *et al.*, 1993). Monoclonal antibodies specific for OmpH were able to protect mice against *P. multocida* challenge (Marandi & Mittal, 1997).

The gene encoding OmpH has been cloned from a *P. multocida* serotype A strain, X-73 and used to express recombinant OmpH in *E. coli*. The recombinant OmpH protein was used in a vaccine trial in chickens but failed to protect against challenge (Luo *et al.*, 1997). In contrast, vaccination with the native OmpH protein did elicit a protective immune response that was able to protect the birds against subsequent homologous challenge (Luo *et al.*, 1997). It was hypothesised that the recombinant OmpH protein was in a denatured state that could not induce protective antibodies (Luo *et al.*, 1997). Several peptides were made that encompassed two hypervariable regions

of the OmpH protein that were predicted to contain serotype specific epitopes and the antibodies raised to one of these synthetic peptides, Cyclic-L2 induced 70% protection in chickens against homologous challenge. (Luo *et al.*, 1999).

37.5 kDa Omp

A 37 kDa protein from *P. multocida* was among five identified as possible protective immunogens based firstly on radioimmunoprecipitation results using protective immune rabbit sera, and secondly on their location in the outer membrane (Lu *et al.*, 1988). Monoclonal antibodies raised against the 37 kDa protein were able to passively protect rabbits and mice against infection with *P. multocida* (Lu *et al.*, 1991). Good protection was afforded against homologous strains, and some protection was observed against heterologous strains that possessed the antigenic determinant recognized by the monoclonal antibody. Animals challenged with *P. multocida* strains lacking the determinant were not protected against infection (Lu *et al.*, 1991).

It should be noted that the 37.5 kDa protein is similar in size to the outer membrane proteins OmpH and OmpA and due to the limited data available on the properties of this protein it is unclear whether it is a unique protein or one of these better characterized proteins.

50 kDa Toxic Omp

Cell extracts and culture filtrates of a *P. multocida*, serotype A strain, were analysed for substances that prevent the phagocytosis of *Candida albicans* by mononuclear phagocytes. From cell extracts, a 50 kDa outer membrane protein was identified as having anti-phagocytic activity and inhibition of this protein with specific antibodies reversed this effect (Truscott & Hirsh, 1988; Truscott *et al.*, 1990). It was hypothesized that the protein may down regulate receptors for other bacterial toxins and hormones, allowing *P. multocida* to enter the phagocytes in sufficient numbers to allow intracellular growth to occur (Truscott *et al.*, 1990), although at this stage there is no experimental evidence that *P. multocida* multiplies within macrophages.

Oma87

Oma87 is an outer membrane protein of *P. multocida* with significant similarity to the D15 protein of *H. influenzae*. It was first identified by colony

immunoblots of a *P. multocida* A:1 recombinant library using a rabbit antiserum prepared against whole membrane fractions from the homologous strain (Ruffolo & Adler, 1996).

The gene for the *H. influenzae* D15 antigen has been cloned and sequenced and is highly conserved among all *H. influenzae* serotypes, including non-typeable strains (Flack, 1995; Loosmore *et al.*, 1997; Thomas, 1990). In addition, antibodies raised to the D15 antigen and specifically the 20 kDa N-terminal end, tD15, were shown to protect infant rats from live *H. influenzae* challenge (Thomas, 1990; Yang *et al.*, 1998). Eight synthetic peptides representing the entire tD15 region were used in competitive inhibition studies to identify the protective epitopes and it was found that both tD15 and a mixture of tD15-derived peptides (spanning amino acids 93 to 209) were capable of inhibition (Yang *et al.*, 1998).

Antisera raised in rabbits against recombinant Oma87 protein provided passive protection in mice against homologous *P. multocida* challenge (Ruffolo & Adler, 1996), and convalescent chicken serum reacted with recombinant Oma87 protein but more specifically, only the N-terminal F1 fragment, representing amino acids 18 through to 130 (Mitchison *et al.*, 2000). Unfortunately, vaccination of chickens with the recombinant F1 fragment fused to GST failed to protect chickens against challenge with a virulent serotype A strain of *P. multocida* (Mitchison *et al.*, 2000). Subsequent vaccine trials in mice using the F1 fragment have yielded inconsistent protection results suggesting that Oma87 may at best offer only partial protection against disease (Harper, unpublished).

THE GENOME AND WHOLE GENOME EXPRESSION PROFILING

The genome of a *P. multocida* serotype A:3 turkey strain, PM70, has been completely sequenced and found to contain over 2000 predicted coding regions, 6 ribosomal RNA operons and 57 tRNAs (May *et al.*, 2001). Features of the genome include 53 genes with predicted involvment in iron acquisition and uptake, 104 putative virulence-associated genes and 200 genes with no similarity to known genes. Evolutionary analysis of the genome also confirmed the close relationship between *P. multocida* and *H. influenzae*, suggesting that they diverged from *E. coli* approximately 270 million years ago (May *et al.*, 2001).

Sequencing and public availability of the PM70 genome has allowed the use of whole-genome expression profiling using DNA microarray technology to determine the genes that are expressed under different environmental conditions. When *P. multocida* was grown under iron-limiting conditions, expression of 135 genes changed significantly compared to their expression during growth in the control medium. Those genes with increased expression included many required for iron acquisition and transport, whilst in general, expression of genes required for energy metabolism and electron transport decreased (Paustian *et al.*, 2001). Interestingly, a large number of genes that displayed altered expression levels encoded uncharacterised hypothetical proteins, highlighting the current lack of knowledge about bacterial metabolic processes. It has been shown that *P. multocida* grown in minimal medium with defined iron sources up-regulates a different set of genes depending on the iron source (Paustian *et al.*, 2002b). Genomic analysis using microarray has also been employed to compare growth of *P. multocida* in a rich medium, BHI, with growth in a minimal medium. In rich media, many of the genes up-regulated were involved in energy-intensive biosynthetic pathways, whereas many of the up-regulated genes in minimal medium were involved in amino acid biosynthesis and transport (Paustian *et al.*, 2002a). Again it was observed that a large number of the genes showing altered expression levels had no known function.

Recently genomic-scale analysis has been used to determine which *P. multocida* genes show altered expression levels during infections in chickens, a natural host of *P. multocida* (Boyce *et al.*, 2002). The expression profiles of *P. multocida* isolated from the blood of three infected chickens were compared independently with the profile from *P. multocida* grown in rich medium. Of the forty genes that showed altered gene expression in all three chicken infections, most that were up-regulated were involved in amino acid transport and metabolism and energy production and conversion. Down regulated genes were largely unknown or of poorly characterized function. Although there was a core set of genes differentially expressed, there were also significant differences in the gene expression profile from each of the infections, highlighting the point that host variation should be noted when determining gene expression profiles (Boyce *et al.*, 2002).

In Vivo Expression Technology (IVET)

An alternative method to microarray technology for determining *in vivo* expressed genes is *in vivo* expression technology (IVET). The method assumes that virulence genes that are required for infection are transcriptionally activated in the host and therefore the active promoters can be detected using a reporter gene system (Angelichio & Camilli, 2002). IVET was first used to detect *in vivo* expressed genes in *Salmonella enterica* serovar Typhimurium (Mahan *et al.*, 1993). Small, random fragments of the *Salmonella* genome were cloned upstream of a promoterless *pur*A reporter gene in a suicide plasmid and the library transferred by conjugation into a *S. enterica* serovar Typhimurium Δ *pur*A strain and allowed to integrate into the genome by homologous recombination. The recombinant bacteria were then screened in mice for their ability to survive; as expression of *purA* is necessary for survival *in vivo*, only those bacteria that had received an active promoter fused to *purA* were able to survive. The surviving bacteria were recovered from the mice and the promoters of several virulence genes were identified that were active *in vivo* but showed no promoter activity *in vitro* (Mahan *et al.*, 1993).

There have been several variations of the IVET system, including the use of promoterless antibiotic reporter genes, as used in *P. multocida* (Hunt *et al.*, 2001). The plasmid-based, promoterless kanamycin gene was used to screen for *in vivo* active promoters from the genome of a *P. multocida* serotype A strain X-73. Those bacteria that had a functional promoter fused to the kanamycin gene survived in infected mice treated with kanamycin. Many fragments with putative *in vivo* expressed promoters were identified and some of the downstream genes included two lipoproteins with strong similarity to the *H. influenzae* lipoproteins PCP and Protein D and two genes with similarity to the pyrimidine synthesis and salvage genes *udk* and *dcd*. A gene encoding a DsbD homologue was also identified; this protein in *E. coli* is involved in disulphide bond formation in periplasmic proteins. Interestingly, the *dsbB* gene from *P. multocida* was also identified in a separate study as required for *in vivo* growth (Fuller *et al.*, 2000).

Although IVET has been effective in the identification of many *in vivo* expressed promoters, there are several disadvantages of the IVET system. Low level gene expression or genes expressed only for short periods of time *in vivo* may not be

detected because the bacteria harbouring these fusions would not survive long enough in the host to be recovered. In addition, some *in vivo* activated promoters identified in IVET may be required for *in vivo* metabolic or growth requirements but may not be essential for virulence (Angelichio & Camilli, 2002).

Signature Tagged Mutagenesis

Signature tagged mutagenesis (STM) is a powerful method for screening a large number of mutants for attenuation using a single host and can be used to identify genes required for specific host niches. Attenuated mutants are most commonly screened *in vivo* but can be screened using other models such as in cell culture or other *in vitro* assays. In STM, a comprehensive screening of the bacterial genome can occur because screening for attenuation in an animal model can be done using relatively large pools. Each mutant in the pool is tagged with a unique sequence of DNA, allowing for the identification of individual mutants from the original input pool after screening for attenuation.

STM was first described in 1995(Hensel *et al.*, 1995); Figure 1 outlines the most commonly used STM screening method. Each plasmid, harbouring a uniquely tagged transposon, is introduced separately into the bacteria under investigation and, after growth with appropriate selection, an individual mutant is selected at random for construction of the pool. In this way, multiple pools, each consisting of a bank of individually tagged mutants, can be constructed. The input pool is then used to infect a single host and the infection allowed to proceed until established. The bacteria are then harvested from the infected host (output) and total genomic DNA is extracted from both the output pool and a reserved sample of the input pool (input). To determine which mutants were attenuated *in vivo*, the tags present in the input and output DNA pools are amplified by PCR from the genomic DNA and labelled. The labelled probes are then used in dot blot hybridisations with the target DNA, containing the tag sequence, arrayed on nylon membranes. Mutants that give significantly reduced signal from the output pool compared with the signal from the input pool are deemed attenuated as they are unable to compete efficiently *in vivo* (Hensel *et al.*, 1995). *Salmonella enterica* serovar Typhimurium was the first bacterial species to be studied using STM, and this study identified a number of virulence genes required for growth in a mouse infection model (Hensel *et al.*, 1995). In total, 1152 mutants were screened and 40 were found to

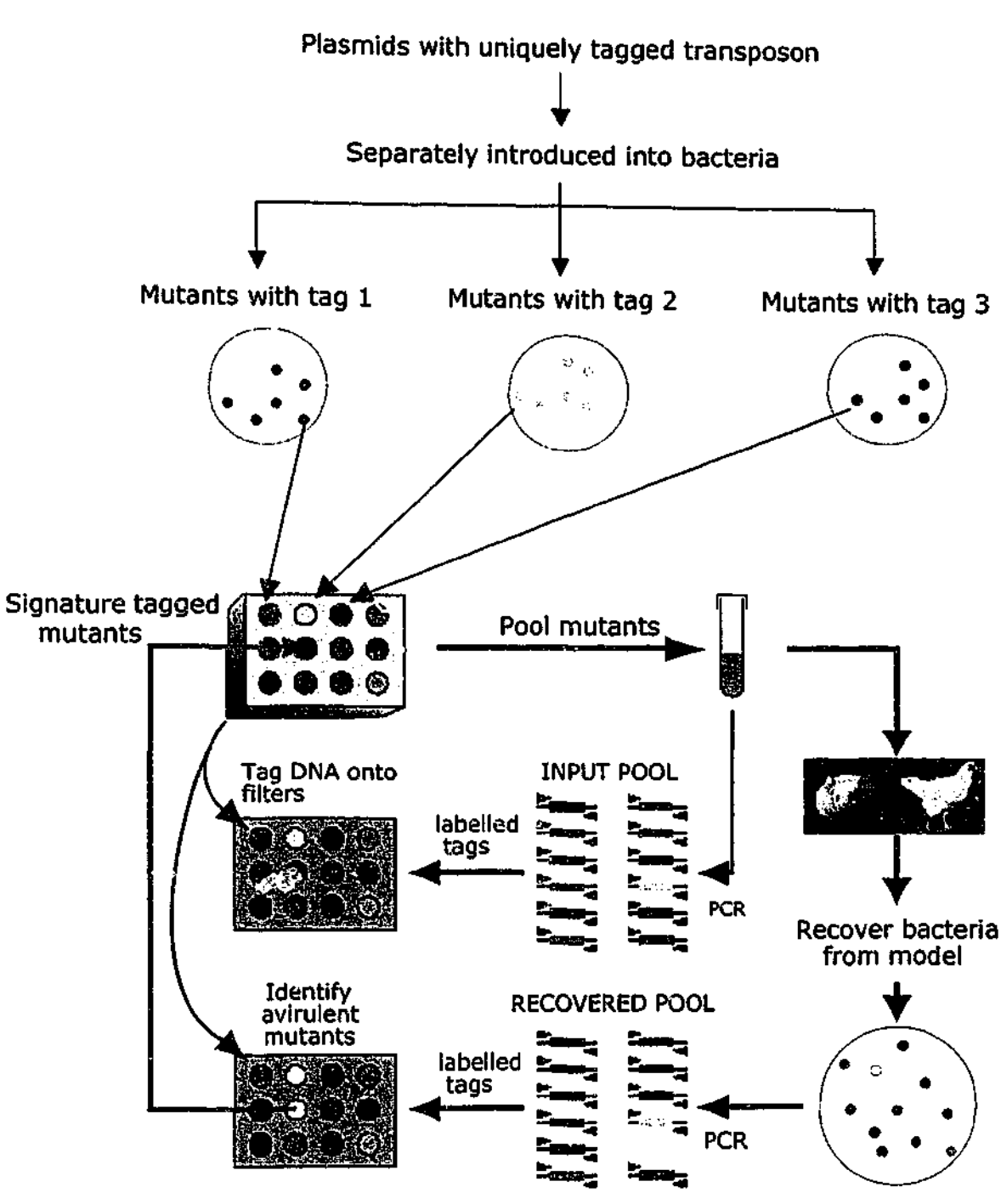


FIG 1. Diagram outlining the screening of signature-tagged mutants for reduced virulence. See p 24 for detailed description. Adapted from www.v-max.co.uk.

have reduced signal from the output pool. The DNA flanking the transposon was sequenced from 28 of the attenuated mutants. Of these mutants, 13 had insertions within genes previously identified as virulence genes in *S.* Typhimurium; six insertions were in genes with shared identity to known bacterial genes and nine insertions were in genes previously unidentified with no known homologues (Hensel *et al.*, 1995).

Since the first study, STM has been used to identify virulence genes in a large number of bacterial species. Of interest are the extensive studies of *Staphylococcus aureus* mutants using a number of different animal infection models and tissue environments, including abscess, wound and bacteraemia infections in mice and endocarditis infections in rabbits (Coulter *et al.*, 1998; Mei *et al.*, 1997). These studies demonstrated that a different set of genes is required for each of the host infection models with only a small subset common to all of the infections. Many studies have successfully used *in vitro* tissue culture cell lines to screen for attenuated mutants, including the use of human brain microvascular endothelial cells to detect *E. coli* K1 mutants attenuated for invasion (Martindale *et al.*, 2000), and human macrophages to screen *Brucella suis* STM mutants for reduced intracellular survival (Foulongne *et al.*, 2000). STM has also been used to identify genes involved in *Pseudomonas aeruginosa* infection in rats where the screening method for attenuated mutants involved PCR amplification instead of dot blot hybridizations (Lehoux *et al.*, 1999). This variation of the STM method has since been used to screen over 15,000 mutants from 11 different bacterial species and 323 attenuated mutants were identified (Lehoux & Levesque, 2000).

STM has recently been used to identify virulence genes in *P. multocida* (Fuller *et al.*, 2000). A *P. multocida* serotype A strain isolated from a clinical case of bovine pneumoniae was mutagenized using a mini-Tn*10* tagged transposon to generate a bank of 1400 mutants. The mutants were screened in mice in pools of 96 and the spleens harvested after 2 days to recover the output bacteria. Using dot blot hybridisation and chemiluminescent detection the input and output pools were compared and 62 mutants were identified as having reduced output signal. To confirm the attenuation, all identified mutants were used in separate challenge experiments in mice and approximately half were significantly attenuated. However, over one third of the identified mutants contained multiple transposons and in these, the insertion responsible for the disrupted phenotype could not be readily identified. The regions flanking the transposon in the remaining 41 mutants were sequenced and 25 unique genes were

identified, including homologues of virulence-associated biosynthetic genes *purF*, *guaB*, *atpG* and two biosynthetic genes *yjgF* and *yhcJ* that had known homologues but had not been previously associated with virulence. Several classical virulence or virulence-associated genes were also identified, including *dsbB* that encodes a protein involved in disulphide bond formation, *has*, encoding hyaluronan synthase, required for serotype A capsule formation, and *lgt*C that encodes a glycosyl transferase involved in LPS biosynthesis. Mutants were also identified with inactivations in genes likely to be required for adhesion and/or colonization. The gene, *tadD*, was identified as a virulence associated gene with significant similarity to *tadD* in *A. actinomycetemcomitans*, that encodes a protein involved in the assembly of Flp pilin, structures recently shown to be essential for virulence (Fuller *et al.*, 2000; Kachlany *et al.*, 2001a; Schreiner *et al.*, 200_). Genes that encode the putative filamentous haemagglutinins, PfhaB1 and PfhaB2, and the putative outer membrane transport protein required for export of these proteins, encoded by *pfhaC*, were also identified as important in virulence (Fuller *et al.*, 2000).

The authors acknowledged that there were limitations to the STM study in *P. multocida*, including doubt over the suitability of the mouse septicaemic model of infection to identify genes that may be required in a bovine pneumonic infection (Fuller *et al.*, 2000). Although it has been demonstrated previously that Tn*10* can transpose into *P. multocida* in a random manner (Lee & Henk, 1996), in the Fuller STM study (Fuller *et al.*, 2000) over one third of the Tn*10* mutants identified contained multiple transposon insertions and in these instances it was not possible to determine the gene responsible for the attenuated phenotype. Preliminary studies by Fuller and co-workers determined that there was an 8% multiple insertion rate but in the STM studies that followed on *P. multocida* and *A. pleuropneumoniae* using Tn*10*, the multiple insertion rate in the attenuated mutants was found to be significantly higher, perhaps due to a higher probability of multiple insertions resulting in an attenuating phenotype (Fuller *et al.*, 2000).

Although the Fuller STM study identified a significant number of genes, it would have been unlikely to identify all the virulence associated genes encoded by *P. multocida*. However, the construction of an exhaustive library using transposon mutagenesis would be extremely difficult to obtain due to the number of mutants required. For example, the *N. meningitidis* genome encodes approximately 2100 open reading frames, a number similar to that found in the *P. multocida* PM70 genome (May

et al., 2001; Tettelin *et al.*, 2000), and it has been determined that a library of over 4500 *Himar1 mariner* random mutants of *N. meningitidis* is required to reach near-saturation coverage of the genome. Sequence analysis of the random mutants revealed that there were insertions in 80-90% of open reading frames in the genome (Geoffroy *et al.*, 2003; Pelicic *et al.*, 2000).

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Outline of Thesis

This project aimed to use signature-tagged mutagenesis (STM) to identify some of the genes required for *P. multocida* infection. The *P. multocida* STM-library was screened in mice, because it is an established animal model for *P. multocida* infection, and in chickens, one of the natural hosts for fowl cholera infection, to determine if there was any difference in the virulence genes required in each host. The transposon Tn*916* was chosen for use in this STM study because it has been shown previously to insert into the *P. multocida* genome in a quasi-random manner (DeAngelis, 1998). However, the inherent instability of the transposon in the base plasmid made it difficult to construct a library of tagged transposons required for STM. Chapter 2 describes the synthesis of the variable DNA tags and the construction of a smaller version of Tn*916*, designated Tn*916*EΔC, that is stable for manipulation in *E. coli* and, when required for mutagenesis in *P. multocida*, can be easily be restored to an active transposon.

Chapter 3 describes the construction of the *P. multocida* library of STM mutants using the modified Tn*916* transposon and the subsequent screening of the library in both mice and chickens. Mutants identified by the STM screening were confirmed as attenuated by competitive growth assays in both chickens and mice. Of the fifteen mutants identified in the chicken model, only five were also attenuated in mice, showing for the first time the presence of host-specific virulence factors and indicating the importance of screening for attenuation in the natural host.

Finally, Chapter 4 describes the identification of an LPS mutant with an insertional inactivation of the gene encoding the heptosyl transferase III enzyme that is required for the addition of the third heptose to the LPS molecule. Complementation experiments in mice and virulence data in chickens show that possession of a truncated LPS molecule significantly reduces the virulence of the mutant. The core composition of the wild type LPS and the mutant LPS are presented and the significance of modifications to the wild type LPS, such as the addition of phosphocholine, sialic acid and phosphoethanolamine is discussed.

Chapter 2

Construction of DNA tags and modification of Tn*916* for use in signature-tagged mutagesis.

INTRODUCTION

STM is a powerful method for identifying potential virulence factors in bacteria, but its use requires consideration of a number of factors including the careful choice of transposon and bacterial strain. The *P. multocida* strain, VP161, was chosen for this STM study because it is a highly virulent strain that when used to infect chickens, reproducibly produces fowl cholera. Some transposons have been tested to determine their suitability for quasi-random mutagenesis of the *P. multocida* genome. Both Tn*5*, which was used in the original STM method for mutagenesis of the *S.* Typhimurium genome (Hensel, 1998) and another transposon, Tn*7*, were shown to be unsuitable for *P. multocida* mutagenesis; Tn*5* because it failed to integrate sufficiently, and Tn*7*, because it preferentially inserted into a single site in the genome (Nnalue, 1990; Nnalue & Stocker, 1989). Random mutagenesis of the *P. multocida* genome has been achieved using Tn*10*, although Tn*10* STM libraries in *P. multocida* and *A. actinomycetemcomitans* had a high number of mutants with multiple genomic insertions (DeAngelis, 1998; Fuller *et al.*, 2000a; Fuller *et al.*, 2000b; Lee & Henk, 1996). Tn*916* has been shown to successfully transpose into the *P. multocida* genome in a quasi-random fashion (DeAngelis, 1998). In addition, tetracycline resistance, encoded by the *tet*(M) gene on the transposon, was stable in the absence of selection over many generations and the transposon could be introduced into *P. multocida* by electroporation rather than conjugation, avoiding the need for a recipient strain carrying a counter selectable marker and thus minimizing the number of manipulations required on the wild-type strain (DeAngelis, 1998).

The transposon Tn*916* is able to conjugate into a wide range of bacteria by transferring a single strand of transposon DNA from donor to recipient (Scott *et al.*, 1994). Although there is no specific site required for the transposon to integrate into host DNA, it has been determined that Tn*916* preferentially inserts into target sites that have a static bend and a common AT rich sequence (Lu & Churchward, 1995). Recombination of the transposon is thought to occur by staggered double stranded cleavages at the end of the transposon whereby one strand of the transposon DNA is cut 6 bases from the end (coupling region), whilst the other strand is cut immediately adjacent to the end (Fig. 1) (Caparon & Scott, 1989). The cleaved transposon then forms a circular intermediate with a six base pair heteroduplex region (Caparon & Scott, 1989; Scott *et al.*, 1988). For integration into the host DNA, the transposon and target DNA

are cleaved in the same staggered fashion, resulting in the insertion of the transposon flanked by heteroduplex regions that are resolved by replication or mismatch repair (Fig. 1) (Scott & Churchward, 1995).

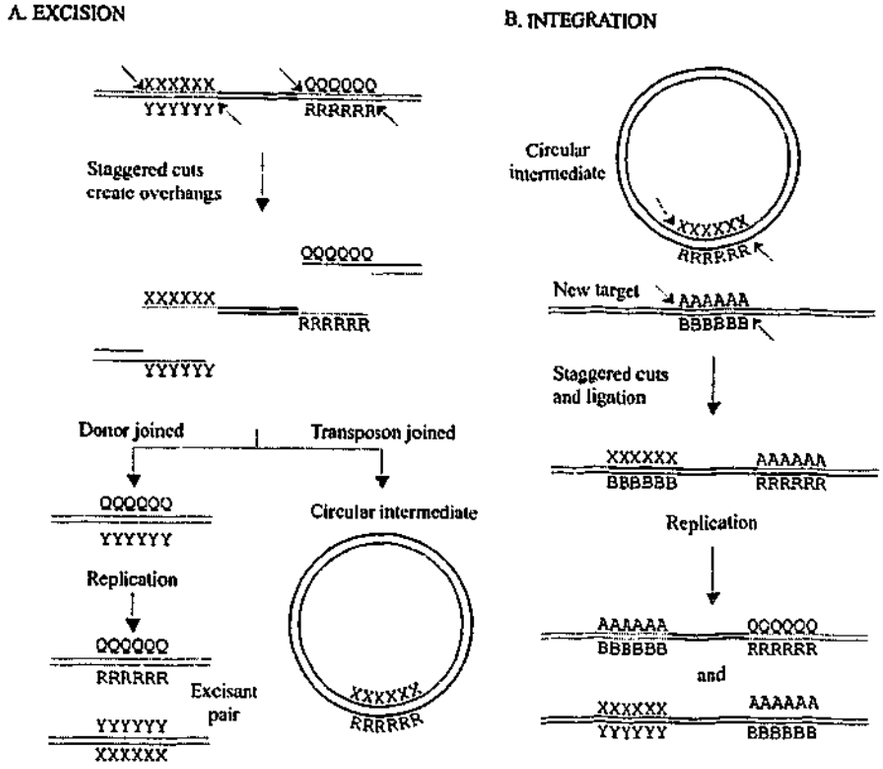

Fig. 1. Model of Tn*916* excision and integration. Taken from Scott & Churchward (1995). Bold lines represent transposon DNA. Thin lines donor/recipient DNA. Complementary base pairs in the coupling region are represented by the letters X-Y, Q-R and A-B. A) Excision. Small arrows represent staggered cleavage sites. The cleaved ends with 5' overhangs are then joined to generate the donor excisant molecule with a heteroduplex region (resolved by semi-conservative replication to create an excisant pair of donor molecules) and the circular intermediate of the transposon. B) Integration. Small arrows represent staggered cleavage sites on both new target sequence and the circular intermediate of the transposon. Heteroduplex regions are formed at each end of the transposon after integration into the target DNA that are later resolved during semi-conservative replication.

Two enzymes encoded by genes *int* and *xis* are involved in excision and integration of the transposon and are located near the left junction of Tn*916* (Poyart-Salmeron *et al.*, 1989; Senghas *et al.*, 1988; Su & Clewell, 1993). The site-specific

recombinase, Int, is required to cleave the DNA at the ends of the transposon and the Xis protein is believed to regulate transposon excision in a manner similar to that described for the Xis protein in λ (Hinerfeld & Churchward, 2001; Marra & Scott, 1999; Rudy *et al.*, 1997; Taylor & Churchward, 1997). It has been proposed that binding of Xis to the left end of the transposon, bends the DNA sufficiently to allow Int to bind and subsequent excision events to occur, whereas the lower affinity binding of Xis to the right end of the transposon prevents excision, by preventing access of Int to a specific DNA binding site, an interaction required for excision to occur (Hinerfeld & Churchward, 2001). As there are different binding affinities of Xis to each end of the transposon, excision is likely to occur when levels of the Xis protein in the cell fall below some threshold value (Hinerfeld & Churchward, 2001).

In *E. coli*, the excision of the transposon Tn*916* can occur from base plasmids at a high rate, but this does not negatively affect transposition, as the excised transposon can act as a transposition intermediate (Scott *et al.*, 1988). In this study, construction of an STM library of tagged transposons was initially attempted in *E. coli* by cloning a library of unique DNA tags into Tn*916*. However, because of the inherent instability of Tn*916* in the base plasmid pAD1, an inadequate number of plasmid-integrated tagged transposons was generated. To overcome this problem, a smaller version of the transposon was constructed, designated Tn*916* EΔC, by removing the conjugation genes and inserting a removable *erm*(B) cassette, encoding erythromycin resistance, into the *int* gene of the transposon thereby preventing excision of the transposon from the base plasmid. This construct was stable for manipulation in *E. coli* and, when required for transposition into *P. multocida*, could be restored to an active transposon by removal of the *erm*(B) cassette from the *int* gene.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *E. coli* was grown routinely in Luria-Bertani broth. *P. multocida* strains were grown in either brain heart infusion (BHI) (Oxoid) or nutrient broth (NB) supplemented with yeast extract (3%). When required, the media were supplemented with tetracycline (2.5μg/ml for *P. multocida* and 10μg/ml for *E. coli*) and/or 1.5% agar.

DNA manipulations. Restriction digests, ligations and polymerase reactions were performed according to the manufacturers' instructions using enzymes obtained from NEB (Beverley, MA) or Roche Diagnostics GmbH (Mannheim, Germany). Plasmid DNA was prepared using alkaline lysis (Birnboim & Doly, 1979) and further purified using Qiagen columns (QIAGEN GmbH, Germany) or by PEG precipitation (Ausubel *et al.*, 1995). Genomic DNA was prepared using the CTAB method (Ausubel *et al.*, 1995).

PCR amplification of DNA was performed using Taq DNA polymerase or Expand High Fidelity PCR System (Roche Diagnostics) and purified using the Qiagen PCR Purification Kit. The oligonucleotides used in this study are listed in Table 2.

TABLE 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant genotype or phenotype	Source or reference
P. multocida		
VP161	Serotype A:1, Vietnamese isolate from chickens.	(Wilkie *et al.*, 2000)
E. coli		
DH5α	*deoR*, *endA*1, *gyrA*96, *hsdR*17(r_k^- m_k^+), *recA*1, *relA*1, *supE*44, *thi*-1, (*lacZYA-argFV*169), Φ80*lacZ* ΔM15, F-	Bethesda Research Laboratories
Plasmids		
pAM120	Harbours Tn*916*. Suicidal in *P. multocida*.	(Gawron-Burke & Clewell, 1984)
pPBA1638	Harbours Tn*916*EΔC. Tn*916* conjugative genes deleted.	This Study
pJIR599	Source of e*rm*(B) resistance gene.	(Berryman & Rood, 1995)

Electroporation. Electrocompetent *P. multocida* VP161 cells were prepared as described by DeAngelis (1998). Electroporation of *P. multocida* was performed using a Bio-Rad Gene Pulser™ apparatus with a 2.0 kV field strength, 25 μF capacitance and 600 Ω resistance. Immediately after electroporation, 1ml of NB was added to the cells and equal amounts of the mix were immediately poured onto four selective plates

(nutrient agar with 2.5 µg/ml tetracycline) and distributed gently to avoid damage to the cells. For growth of transformants, plates were incubated at 37 °C for 2 days.

Preparation of competent *E. coli* cells for electroporation, and electroporation conditions, were as described previously (Ausubel *et al.*, 1995).

Southern hybridisations. Enzyme digested genomic DNA was loaded onto a 0.6 % agarose TAE gel and subjected to electrophoresis prior to Southern transfer onto a positively charged nylon membrane (Roche) using an alkaline buffer as described previously (Ausubel *et al.*, 1995). DNA probes were labelled by incorporating Digoxigenin-11-dUTP (Roche Diagnostics) during PCR in accordance with the manufacturer's instructions. High stringency hybridisation and CDP-Star™ detection were performed according to the manufacturer's instructions (Roche Diagnostics).

TABLE 2. Oligonucleotides used in this study

Primer	Sequence (5'- 3')	Description
BAP1126	TCG CTG CTC GAG TTT ATC CTC GCC AG	Reverse primer at position 1,737 on pAM120[a]. Located near right junction of Tn*916*.
BAP1127	AGC AGT TCT AGA TGA TGA TAC TGT CCC	Forward primer in *int* gene. At position 18,517 on pAM120[a] sequence.
BAP1128	TCA TCA TCT AGA ACT GCT TTC AGT TG	Reverse primer in *int* gene. At position 18,510 on pAM120[a] sequence.
BAP1129	TTT GGT ACT CGA GAA GAA CGG GAG	Forward primer upstream of the *Tet*(M) gene. At position 13,044 on pAM120[a] sequence.
BAP1298	GTA CAA CCT CAA GCT $(NK)_{20}$ G AAT TCG GTT AGA ATG	Central region of STM tag.
BAP1299	ATA AAC CCG GGT ACA ACC TCA AGC T	Forward extension primer for amplification of central STM tag.
BAP1300	GCA TCC CGG GCA TTC TAA CCG AAT TC	Reverse extension primer for amplification of central STM tag.
BAP1414	GTG CAT GAA ATA ATA TAC GAG T	Reverse primer in *Tet*(M) gene. At position 13,789 on pAM120[a] sequence.
BAP2252	ACA TAG AAT AAG GCT TTA CGA GC	Forward primer located near left junction in Tn*916*. At position 19,308 on pAM120[a] sequence.
BAP2459	CTA CTA AGC AAC AAG ACG CTC CTG	Reverse primer located near left junction in Tn*916*. At position 19,181 on pAM120[a] sequence.

[a] pAM120 Accession No.U49939.

RESULTS AND DISCUSSION

Integration of Tn*916* into the *P. multocida* VP161 genome. Southern analysis was performed on VP161 transformants to determine if Tn*916* had inserted into the strain VP161 in a quasi-random manner as described previously with *P. multocida* strain P-1059 (DeAngelis, 1998). Electrocompetent *P. multocida* VP161 cells were transformed with pAM120 harbouring Tn*916*. Genomic DNA was prepared from 18 of the tetracycline resistant transformants, digested with *Hin*dII, and analysed by Southern hybridisation using a probe specific for the left junction of the transposon. The majority of transformants contained a single transposon (Fig. 1) and after stripping and re-probing the membrane with a probe specific for pAD1, only two of the 18 transformants were found to contain base plasmid DNA (data not shown).

It was therefore concluded that transposition of Tn*916* into the VP161 genome was quasi-random and demonstrated that persistence of the base plasmid, pAD1, in *P. multocida* did not occur at a high frequency.

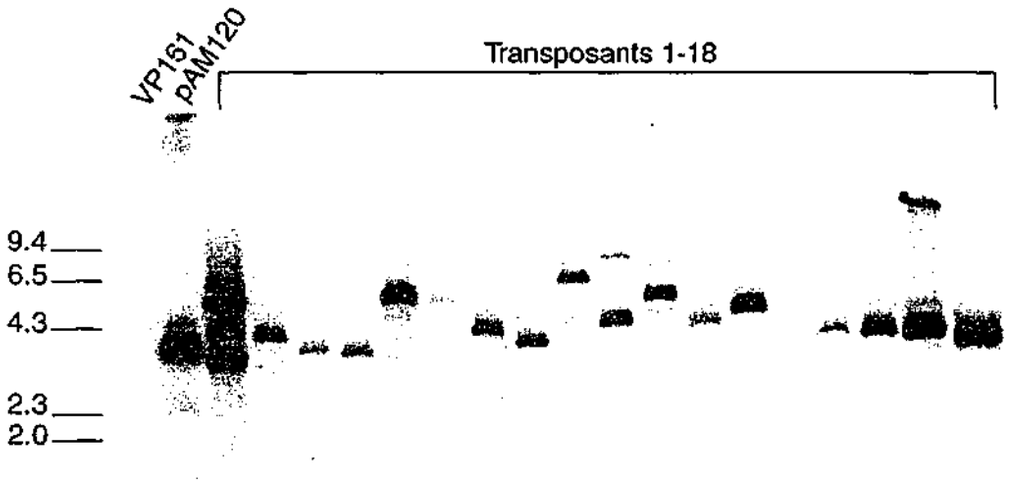

Fig. 1. High stringency Southern blot showing quasi-random insertion of Tn*916* into *P. multocida*. VP161genomic DNA, pAM120 plasmid DNA and genomic DNA from 18 transformants was digested with *Hin*dII, separated by gel electrophoresis and transferred to a nylon membrane by Southern blotting. The membrane was hybridised with a probe specific for the left junction of the transposon. The positions of the *Hin*dIII-digested lambda DNA size markers (kb) is shown on the left.

Construction of a modified Tn*916*. In this study the inherent instability of the transposon carried by the base plasmid pAM120, resulted in an inadequate number of tagged transposons being generated for the construction of the library. To overcome this

problem, a smaller version of the transposon was constructed, designated Tn*916* EΔC, by deletion of the conjugative genes of Tn*916* and the insertional inactivation of the *int* gene using an *erm*(B) cassette (Fig. 2). The inactivation of the *int* gene prevented excision of the transposon from the base plasmid, allowing DNA manipulations to be carried out in *E. coli* (such as the insertion of the variable DNA tags). To generate an active transposon, the *erm*(B) cassette could be removed by enzyme digestion and religation.

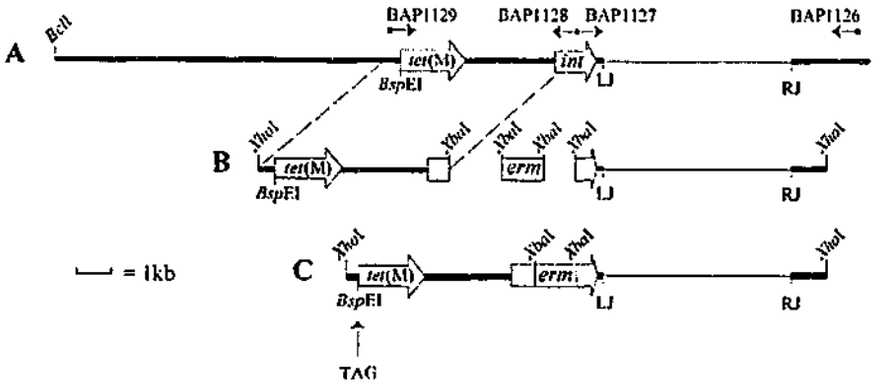

Fig. 2. Schematic representation of the strategy used to construct Tn*916*EΔC. (A) Linear diagram of pAM120. Bold lines represent the transposon Tn*916* and the fine line represents the base plasmid. Only genes of relevance are shown. *tet*(M); tetracycline resistance gene and *int*, the integrase gene required for excision and integration of the transposon. Arrows above the line indicate the direction of amplification from each oligonucleotide (not to scale). RJ; right junction of transposon. LJ; left junction. (B) Fragments generated for construction of Tn*916*EΔC. The erythromycin resistance gene (*erm*(B)) was obtained by digestion of the plasmid pJIR599 with *Xba*I. The remaining fragments and the restriction sites *Xho*I and *Xba*I were generated by PCR amplification of pAM120 using the oligonucleotides shown in (A). (C) Diagrammatic representation of the plasmid pPBA1638 containing the transposon Tn*916*EΔC. The vertical arrow labeled TAG indicates the site of insertion (*Bsp*E1) of the variable DNA tags.

To construct the modified transposon, two PCR products were generated from pAM120. The first fragment was generated using primers BAP1127 and BAP1126 (Fig. 2, Table 2) and included the right and left junctions of the transposon, the 3' end of the *int* gene and the entire base plasmid. The second fragment was amplified using BAP1128 and BAP1129 and included the 5' end of the *int* gene and the entire

tetracycline resistance gene, *tet*(M). Two new restriction enzyme sites were introduced into the fragment by incorporating silent nucleotide changes into the primers, an *Xho*I site near the right junction of the transposon and an *Xba*I site located at the 3' end of the *int* gene. A third fragment of DNA containing an *erm*(B) gene conferring erythromycin resistance was purified after digestion of the plasmid pJIR599 (Table 1) with *Xba*I and inserted into the *Xba*I site engineered within the *int* gene. All three fragments were then ligated and the subsequent *E. coli* transformants screened for the correct plasmid profile. The *erm*(B) insertion resulted in the inactivation of transposon excision during DNA manipulations and the *Xba*I site located at either end of the *erm*(B) gene allowed for the removal of the cassette when transposition was required. The modified transposon was designated Tn*916*EΔC as it conferred erythromycin resistance (E) and no longer contained the conjugation genes (ΔC).

Stability of Tn*916*EΔC *in vivo*. To determine the stability of Tn*916*EΔC in the genome, a transposant was chosen at random, injected into a single mouse and the infection allowed to proceed for six hours before blood was recovered and plated onto BHI with tetracycline. Genomic DNA was isolated from 12 colonies recovered from the blood and from an *in vitro* grown culture of the same transposant. The DNA was digested with *Hin*dII, and analysed by Southern hybridisation using a probe specific for the left junction of the transposon Although the transposon in this transposant had inserted into two sites within the genome, there was no change in the position of the transposon Tn*916*EΔC *in vivo* compared with that *in vitro*, confirming that the transposon was unlikely to transpose to another site within the genome during the course of an infection (Fig. 3).

Construction and cloning of DNA tags. The variable oligonucleotide used for construction of the tagged Tn*916*EΔC was essentially as described previously (Hensel *et al.*, 1995), with the following modifications. Initially the tag was synthesized as a large single oligonucleotide, but a significant number of nucleotide sequence errors was detected. Therefore, to improve tag fidelity the tag was constructed from three smaller oligonucleotides (Fig. 4). The first (designated BAP1298: Table 2) was a 72 bp oligonucleotide, which included the 40 bp variable region. The remaining bases required for cloning into the Tn*916*EΔC were synthesized on separate oligonucleotides BAP1299 and BAP1300 (Table 2) and each included an *Xma*I site for insertion into the *Bsp*EI site of Tn*916*. Double-stranded tags were generated by annealing BAP1298 and BAP1300

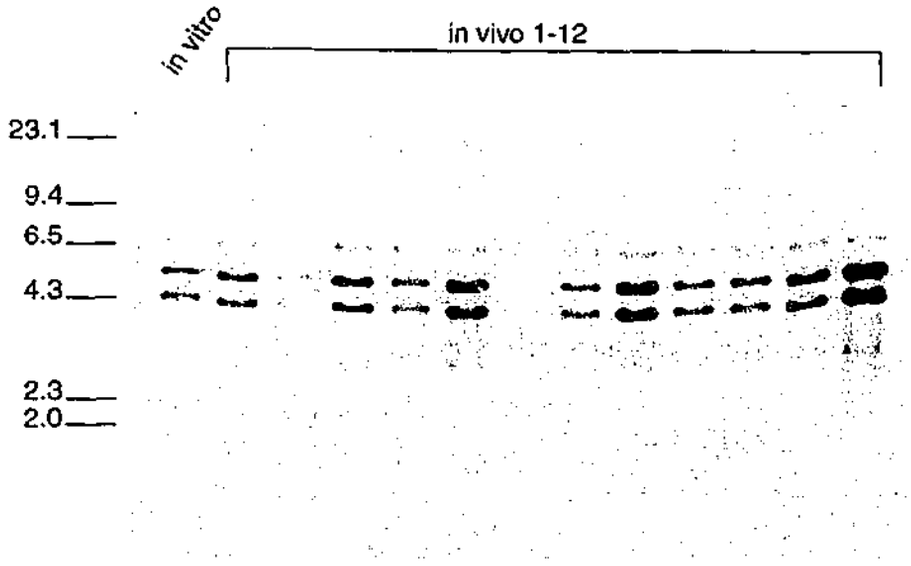

Fig. 3. High stringency Southern blot showing *in vivo* stability of Tn*916*EΔC in *P. multocida*. Genomic DNA isolated from 12 colonies recovered from the blood of an infected mouse, and from an *in vitro* grown culture of the same transposant, was digested with *Hin*dII, separated by gel electrophoresis and transferred to a nylon membrane by Southern blotting. The membrane was hybridised with a probe specific for the left junction of the transposon. The positions of the *Hin*dIII-digested lambda DNA size markers (kb) is shown on the left.

and allowing T4 polymerase to fill the 5' single stranded extensions. The resulting double-stranded molecule was further extended by PCR amplification using oligonucleotides BAP1299 and BAP1300 (Fig. 4). For construction of the tagged transposon library, the plasmid pPBA1638 (Table 1) containing Tn*916*EΔC was digested with *Bsp*E1 (Fig. 2) and ligated to *Xma*I-digested DNA tags, generating plasmids that could no longer be digested by either enzyme. Prior to electroporation into competent *E. coli* DH5α cells, the ligated products were digested with *Bsp*EI to digest any plasmid not containing a DNA tag. Following transformation, the tag region of ten recombinants was sequenced to confirm that the constant region was correct and that the central 40bp region was unique. One hundred and twenty four transformants were checked for single tag insertions by restriction digest; two thirds were found to contain multiple tag insertions and were discarded. The remaining transformants were stored at –20 °C in glycerol until required.

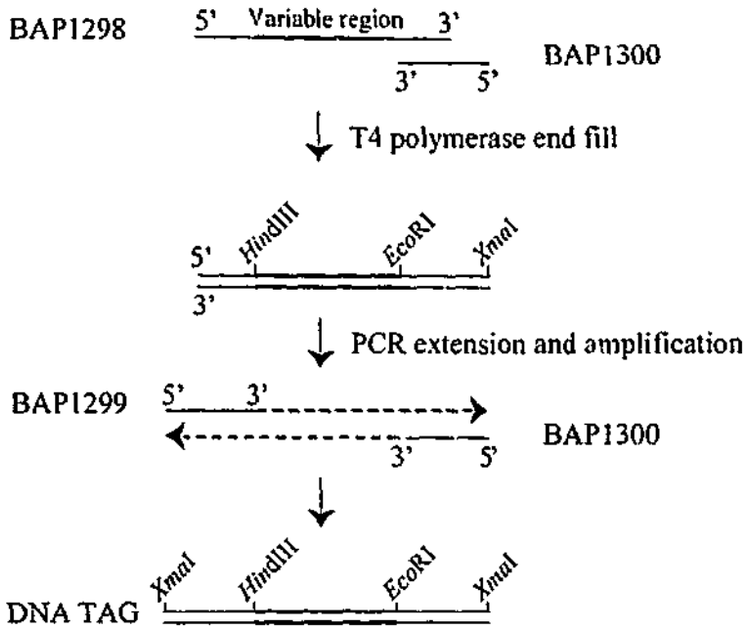

Fig. 4. Construction of the variable DNA tags. Figure is not drawn to scale. The oligonucleotide BAP1298, 72 bp in length including the 40 bp variable region, was used as a template for the full-length tag construction. Double-stranded tags were generated by annealing the oligonucleotides, BAP1298 and BAP1300, and allowing T4 polymerase to fill the 5' single stranded extensions. The resulting double-stranded molecule was further extended by PCR amplification using oligonucleotides BAP1299 and BAP1300.

Concluding remarks. The transposon Tn*916*EΔC was constructed by deletion of the conjugation genes from Tn*916* and reversible inactivation of the *int* gene. When introduced by electroporation, the smaller transposon was stable at the original insertion point in the *P. multocida* genome over the course of a 6 h infection in mice. Moreover, the presence of a reversible *erm* cassette within the *int* gene prevented spontaneous excision of the transposon from the base plasmid, an inherent problem for manipulations in *E. coli*. This stability allowed for genetic manipulation of the transposon, such as insertion of the random tag DNA required for STM experiments and when an active transposon was required, the *erm* cassette could be removed by simple restriction digestion and re-ligation.

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Chapter 3

Signature-tagged mutagenesis of *Pasteurella multocida* identifies mutants displaying differential virulence characteristics in mice and chickens.

Based on: **Harper, M., Boyce, J. D., Wilkie, I. W. & Adler, B. (2003).** Signature-tagged mutagenesis of *Pasteurella multocida* identifies mutants displaying differential virulence characteristics in mice and chickens. *Infect. Immun* **71,** 5440-5446.

INTRODUCTION

Pasteurella multocida is a capsulated, gram-negative coccobacillus and is the causative agent of avian fowl cholera, bovine hemorrhagic septicemia, atrophic rhinitis in pigs and snuffles in rabbits (Mannheim, 1984). To date, few virulence factors of *P. multocida* have been identified; they include the capsule in serogroups A and B (Boyce & Adler, 2000; Chung *et al.*, 2001), PMT toxin in strains causing atrophic rhinitis (Foged *et al.*, 1989), putative filamentous hemagglutinins PfhB1 and PfhB2 (Fuller *et al.*, 2000) and several iron acquisition proteins such as TonB, ExbD and ExbB (Bosch *et al.*, 2002; Fuller *et al.*, 2000; May *et al.*, 2001).

Recently several studies have been undertaken to identify the genes involved in the pathogenesis of pasteurellosis, including *in vivo* expression technology (IVET)(Hunt *et al.*, 2001), signature-tagged mutagenesis (STM)(Fuller *et al.*, 2000) and whole genome expression profiling (Boyce *et al.*, 2002). These genome-scale methods have identified some true virulence factors and virulence-associated genes, including those involved in iron transport and metabolism as well as nucleotide and amino acid biosynthesis. However, many genes identified by these analyses have no known function and neither IVET nor whole genome expression profiling give direct information about the importance of genes in virulence.

Signature-tagged mutagenesis (STM) allows the large-scale screening of mutants for reduced survival *in vivo*, colonization and adhesion defects and decreased invasive ability (Badger *et al.*, 2000; Chiang & Mekalanos, 1998; Hensel *et al.*, 1995). The method has been applied to many bacterial species, including a previous study on a bovine isolate of *P. multocida* using a tagged mini-Tn*10* (Fuller *et al.*, 2000). This previous study used a septicemic mouse model of infection, and identified twenty-five *P. multocida* genes that were important for survival and growth *in vivo*. However, one third of the 62 mutants identified in that study contained multiple transposon insertions and in these instances it was not possible to determine the gene responsible for the attenuated phenotype (Fuller *et al.*, 2000).

In this chapter, an STM bank of *P. multocida* mutants was constructed and screened in two animal models to identify, for the first time, potential host-specific virulence factors. The mutant library was screened in mice, the most widely used *P. multocida* animal model, and in chickens, the natural host. For random mutagenesis

of the *P. multocida* genome, Tn*916* EΔC was used, a modified Tn*916* transposon, constructed as described in Chapter 2.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions. The *P. multocida* strain and plasmids used in this study are shown in Table 1. *P. multocida* strains were grown in either brain heart infusion (Oxoid) or nutrient broth (NB) supplemented with yeast extract (3%). When required, the media were supplemented with tetracycline (2.5μg/ml for *P. multocida*) and/or 1.5% agar.

TABLE 1. Bacterial strains, plasmids and oligonucleotides (oligo) used in this study.

Strain, plasmid or oligo	Relevant description	Source or reference
Strains		
P. multocida		
VP161	Serotype A:1, Vietnamese isolate from chickens.	(Wilkie *et al.*, 2000)
Plasmids		
pPBA1638	Harbours Tn*916*EΔC. Tn*916* conjugative genes deleted.	(Chapter 2)
Oligonucleotides		
BAP1128	TCA TCA TCT AGA ACT GCT TTC AGT TG	Reverse primer in *int* gene. At position 18510 on pAM120[a] sequence.
BAP1129	TTT GGT ACT CGA GAA GAA CGG GAG	Forward primer upstream of the *Tet*(M) gene. At position 13044 on pAM120[a] sequence.
BAP1414	GTG CAT GAA ATA ATA TAC GAG T	Reverse primer in *Tet*(M) gene. At position 13789 on pAM120[a] sequence.
BAP2252	ACA TAG AAT AAG GCT TTA CGA GC	Forward primer located near left junction in Tn*916*. At position 19308 on pAM120[a] sequence.

[a] pAM120 Accession No.U49939.

DNA manipulations. Restriction digests, ligations and polymerase reactions were performed according to the manufacturers' instructions using enzymes obtained from NEB (Beverley, MA) or Roche Diagnostics GmbH (Mannheim, Germany).

Plasmid DNA was prepared using alkaline lysis (Birnboim & Doly, 1979) and purified using Qiagen columns (QIAGEN GmbH, Germany) or by PEG precipitation (Ausubel *et al.*, 1995). Genomic DNA was prepared using the CTAB method (Ausubel *et al.*, 1995). PCR amplification of DNA was performed using Taq DNA polymerase (Roche Diagnostics) and purified using the Qiagen PCR Purification Kit. The oligonucleotides used in this study are listed in Table 1.

Generation of *P. multocida* signature-tagged Tn*916*EΔC library. The library of 42 tagged Tn*916*EΔC transposons in the plasmid pPBA1638 was constructed by selecting those transposons that would act as efficient templates for PCR and hybridize specifically in DNA dot blot hybridizations (data not shown). In order to activate the excision and integration function of the transposon prior to transposition into *P. multocida*, the *erm*(B) cassette was removed from the plasmids by digestion with *Xba*I and self-ligation. To obtain sufficient DNA for transforming into *P. multocida*, the ligation mix was used to transform *E. coli* and plasmid DNA was isolated from a single transformant (containing the active transposon). Each plasmid harboring a uniquely tagged transposon was then separately introduced into *P. multocida* strain VP161 by electroporation. Each pool of 42 mutants was assembled by selecting a single *P. multocida* colony from each transformation plate and the procedure repeated until ten pools were assembled.

***In vivo* screening of the STM library in two animal models.** For each pool, the mutants were grown separately overnight in BHI with shaking at 37 °C. For preparation of the input pool, an aliquot of 100µl from each overnight culture was transferred to a fresh tube. A 100µl sample of the mix was then spread onto BHI plates with tetracycline and grown overnight. Colonies were washed from the plate and used to prepare genomic DNA representing the input pool. For screening in mice, female inbred Balb/c mice, aged 8-10 weeks, were infected intraperitoneally with 100µl of the input pool diluted 1000-fold in BHI (total 10^5 CFU). A blood sample was taken 6 h post-infection and the animals were euthanized in accordance with animal ethics requirements. For screening in chickens, commercially obtained Leghorn cross chickens were infected with 100µl of diluted input pool by injection into breast muscle (total 10^5 CFU). Blood samples were obtained 12 h post-infection, and the birds were euthanized in accordance with animal ethics requirements. Blood collected from either host was diluted 5-fold in BHI and spread onto BHI plates containing tetracycline. A

minimum of 5,000 colonies were washed from these plates and used to prepare genomic DNA representing the output pool.

Digoxigenin labeling of unique tags and hybridizations. For preparation of the labeled probe, genomic DNA isolated from each pool was used as a template for PCR amplification with the primers BAP1129 and BAP1414 (Table 1), generating an 800-bp fragment that contained the unique tag region. The amplified products were digested with *Hin*dIII and *Eco*RI (located at the end of the variable tag region) followed by end-filling with Klenow fragment. The digested DNA was then separated on a 20% polyacrylamide gel and the fragment containing the DNA-tags excised from the gel. The DNA fragment was eluted overnight in 0.5 M ammonium acetate and 1mM EDTA, precipitated in ethanol and resuspended in 10 µl H_2O (Ausubel *et al.*, 1995). The purified DNA was then labeled using the DIG oligonucleotide 3' end labeling kit (Roche Diagnostics) according to the manufacturer's instructions. For preparation of the target DNA, each tag was amplified separately by PCR using the primers BAP1414 and BAP1129 (Table 2). The concentration of the amplified DNA was adjusted to 4 ng/µl and 1 µl was spotted onto nylon filters. DIG dot blot hybridizations were then performed under high stringency conditions according to the manufacturer's instructions (Roche Diagnostics).

Competitive growth assays. Competitive growth assays were used to quantify the relative growth rates of putative attenuated mutants. Log phase cultures of mutant and wild-type were mixed at a ratio of 1:1 and serial 10-fold dilutions were prepared. To determine the input ratio of wild-type to mutant, 100µl of the appropriately diluted culture were plated immediately onto NB agar in order to obtain single colonies representing the input pool. For the *in vivo* growth assays, animals were infected with approximately 10^5 CFU and blood samples obtained after 6 (mice) or 12 h (chickens). Blood was diluted two-fold in BHI containing heparin and plated onto NB agar. For the *in vitro* growth assay, a 100-fold dilution of the mixed bacteria was grown in BHI at 37 C with shaking for the same length of time as the animal infections, diluted appropriately and spread onto NB agar.

After growth on non-selective plates, a minimum of 100 individual colonies was patched onto NB agar with tetracycline and NB agar to determine the percentage of tetracycline resistant bacteria (mutant) for each sample. The relative competitive index (rCI), which measures the difference between growth *in vivo* and growth *in vitro*, was determined by dividing the percentage of tetracycline resistant colonies (transposon

mutants) obtained *in vivo* by the percentage of tetracycline colonies obtained *in vitro*. Mutants were identified as attenuated if the rCI value was significantly less than 1.0 as determined by statistical analysis using the one sided z-test ($p<0.05$).

Identification of the site of transposon insertion. To confirm single transposon insertions, genomic DNA was prepared from each of the attenuated mutants, digested with *Sau*3A, *Eco*R1 or *Hin*dIII, and analyzed by Southern hybridization with a probe amplified from the left junction of Tn*916*EΔC. To identify the exact site of transposon insertion in each mutant, inverse PCR was performed using *Sau*3A-digested and ligated genomic DNA as template and oligonucleotides BAP1128 and BAP2252 (Table 1)(Ochman *et al.*, 1988). The amplified products were sequenced directly using BAP2252 (Table 2) and the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). DNA Sequence was determined on a Model 373A DNA Sequencing System (Applied Biosystems) and analysed with Sequencher Version 3.1.1 (GenCodes, Ann Arbor, MI). *P. multocida* DNA sequence adjoining the Tn*916* sequence was searched against the GenBank database using the BLASTX search algorithm (Altschul *et al.*, 1990).

RESULTS

A bank of 420 *P. multocida* mutants was constructed and arranged in ten pools of 42 uniquely tagged Tn*916*EΔC mutants. The pools of mutants were then screened for reduced growth rate *in vivo* in two animal models, mice and chickens.

Screening of the *P. multocida* mutant library in mice. The *P. multocida* strain chosen for this study, VP161, was a highly virulent fowl cholera isolate from Vietnam (Wilkie *et al.*, 2000) and preliminary studies demonstrated that the ID_{50} in mice was <10 CFU and at high doses (>10^5 CFU) the mice became moribund within 8 h (data not shown). To ensure that all mutants were well represented, mice were injected with approximately 10^5 CFU (approx. $2x10^2$ CFU/mutant). This dose gave reproducible disease progression and all mice showed visible signs of disease within 6 h, when blood was recovered. Each pool of mutants was tested in two mice and the relative abundance of each tag in the input and output pool was determined using dot blot hybridizations. Genomic DNA from bacteria isolated from both animals was pooled prior to generation of the output probe and therefore represented the average output profile from two animals. Mutants were identified as displaying reduced growth *in vivo* if the

hybridization with the corresponding target DNA was stronger with the input probe than with the output probe. Mutants identified in the first screening were collected into a new pool, injected into a mouse, and used in a second STM screen. After the second STM screening 15 mutants were identified that were potentially attenuated.

Each mutant that was identified in dot blot hybridizations as having a reduced output (*in vivo*) hybridization signal was tested in a competitive growth assay together with the wild-type strain VP161 (see Materials and Methods). Of the fifteen mutants identified as showing reduced hybridization signals with the output pool, only five were significantly attenuated as determined by the competitive growth assay in mice (Table 2). Subsequent competitive growth assays in chickens showed that all five mutants were also attenuated in chickens, although the level of attenuation varied between the two hosts (see below).

STM screening and competitive growth assays in chickens. For each pool of 42 mutants, duplicate chickens were infected by injection of approximately 10^5 CFU of the pool into the breast muscle. Infections were allowed to proceed until the onset of clinical signs of infection (12-16 h) whereupon blood was recovered. An output probe was generated from the bacteria recovered from each bird and used separately in dot blot hybridizations to determine the relative abundance of each tagged mutant.

Thirty-five mutants were identified as having reduced output hybridization signals and subsequently tested individually for reduced growth *in vivo* using competitive growth assays in chickens. Fifteen mutants were identified that showed significantly reduced growth. Each of these was also tested in competitive growth assays in mice to assess if there was a difference in the level of attenuation between the two animal models (Table 2). Only 5 of the 15 mutants were also attenuated in mice; these were the same mutants identified following the mouse STM screening.

Characterization of attenuated strains identified by STM. Southern blot analysis indicated that all of the attenuated mutants had a single transposon inserted into the chromosome. The DNA flanking the transposon insertion site was amplified by inverse PCR and the nucleotide sequence determined. This sequence was then used to search the *P. multocida* PM70 genome (May *et al.*, 2001) and the GenBank database for homologous genes. The identities of the disrupted genes are listed in Table 2 and discussed below.

TABLE 2. Characterization of the *P. multocida* mutants.

Strain	Disrupted gene or region	Predicted function or homologue	Competitive Growth Assay Values			
			Mouse		Chicken	
			rCI[a]	P value[b]	rCI[a]	P value[b]
Mutants attenuated in chickens and mice.						
AL249	*dcaA* (*lpt-3*)	Phosphoethanolamine transferase.	0.33	<0.01	0.34	<0.01
			0.41	<0.01	0.66	<0.01
			0.12	<0.01	0.35	<0.01
AL250	*pm0855* (*flp*1)	Surface adhesion	0.62	<0.01	0.39	<0.01
			0.67	<0.01	0.49	<0.01
			0.50	<0.01	0.89	0.18
					0.79	0.02
					0.71	0.01
AL251	*pm1294*	Heptosyl transferase	0.62	<0.01	<0.01	<0.01
			0.44	<0.01	0.01	<0.01
			0.77	0.03	0.01	<0.01
			0.64	<0.01		
AL252	*purN*	*de novo* purine biosynthesis	0.20	<0.01	0.41	<0.01
			0.16	<0.01	0.83	0.15
			0.40	<0.01	0.28	<0.01
			0.39	<0.01		
AL396	Upstream of *pm0529*	sodium / alanine symport protein.	0.42	<0.01	0.16	<0.01
					0.13	<0.01
					0.20	<0.01
Mutants attenuated only in chickens.						
AL383	Between *rpl20* and *pm0606*	unknown	1.13	0.90	0.65	0.01
AL386	*pm1069*	Homology with *H. influenzae* P1 surface protein.	0.86	0.15	0.26	<0.01
			1.29	0.89		
AL387	*deoC*	Deoxyribose synthesis.	1.02	0.56	0.69	0.01
AL388	*pyrF*	Pyrimidine biosynthesis	1.48	0.99	0.58	<0.01
			0.90	0.19		
AL389	*ponC*	Penicillin binding, murein synthesis	0.97	0.44	0.57	<0.01
AL390	*pm1294*	Heptosyl transferase. Transposon in opposite orientation to AL251.	0.72	0.04	0.47	<0.01
			0.83	0.15		
			1.13	0.20		
AL391	*pm1294*	Heptosyl transferase. Transposon in same orientation as AL251.	0.85	0.08	<0.01	<0.01
			1.08	0.69	0.04	<0.01
			1.07	0.24		
AL392	*pm0081*	Homology with HI0902. Probable integral membrane protein.	1.18	0.84	0.42	<0.01
AL393	Between *purD* and *glyA*	Probable effect on *glyA* that encodes glycine hydroxymethyltransferase.	1.00	0.50	0.73	<0.01
AL407	295bp Upstream of *tfoX*	Between *tfoX* and a 16S RNA gene.	3.15	<0.99	0.33	<0.01

[a] Values represent the relative competitive index (rCI) calculated for individual animals, determined by dividing the *in vivo* competitive index (CI) by the *in vitro* CI. Each CI value was determined by dividing the output mutant/wild-type ratio by the input mutant/wild-type ratio.

[b] P value was calculated using the one-tailed z test and was performed to determine if the ratio of mutant to wild-type bacteria *in vivo* was significantly different from the ratio obtained *in vitro*.

DISCUSSION

Other studies have indicated that the animal model chosen for STM studies can influence the genes identified, as some virulence genes will be host-specific or involved in host adaptation (Tsolis *et al.*, 1999). Two animal models were chosen, the mouse, which has been used regularly as a model for fowl cholera, and the chicken, a natural host for *P. multocida*. Fifteen mutants were identified that were all attenuated in chickens, but only five of the fifteen were attenuated in mice.

Metabolic genes identified. Like previous global screenings in bacteria such as *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Yersinia enterocolitica*, certain biosynthetic genes were found to be important for growth *in vivo* (Coulter *et al.*, 1998; Darwin & Miller, 1999; Lau *et al.*, 2001). A number of mutants was identified that had transposon insertions affecting genes encoding biosynthetic enzymes including *purN*, *pyrF* and *deoC*. A mutant was identified in this study with an insertion in the *purN* gene, which was attenuated in both mice and chickens. This gene encodes the enzyme 5'-phosphoribosylglycinamide transformalase N, that catalyzes the fourth step of *de novo* purine biosynthesis (Zalkin & Nygaard, 1996). The ability to synthesize purine nucleotides from simple precursors has been identified as important for bacterial growth *in vivo* and it has been reported recently that several of the genes involved in purine biosynthesis in *P. multocida* are up-regulated in bacteria harvested from the blood of infected chickens (Boyce *et al.*, 2002). In addition, in a previous STM study on a bovine isolate of *P. multocida* in mice a *purF* mutant was identified, whose product is also involved in the *de novo* purine biosynthesis pathway (Fuller *et al.*, 2000). Another biosynthetic mutant identified in this study was attenuated only in chickens and had an insertion in the *pyrF* gene, the homologue of which in *E. coli* encodes orotidine 5'-monophosphate decarboxylase that is involved in pyrimidine biosynthesis (Neuhard & Kellin, 1996).

A mutant was identified that had an insertion upstream of *deoC* and *deoR*, which was attenuated in chickens, but not in mice. These genes encode a deoxyribose phosphate aldolase and a repressor protein respectively and are predicted to be part of the nucleoside catabolism pathway. In *E. coli*, purine and pyrimidine nucleosides and deoxynucleosides can be utilized in the nucleoside catabolic pathway to extract pentose or deoxypentose for use as a carbon and energy source. DeoC converts a deoxyribose intermediate to glylceraldehyde-3-P and acetaldehyde, which can be utilized in glycolysis and the TCA cycle respectively. In *E. coli* four genes encoding the deoxynucleoside enzymes are located in two adjacent operons and the DeoR repressor protein, encoded elsewhere, binds to two DNA binding sites upstream of the *deoC*, *A*, *B* and *D* genes, thereby generating a loop in the DNA and preventing initiation of transcription (Mortensen *et al.*, 1989). In *P. multocida*, the gene arrangement is different, with only *deoC* and *deoR* adjacent to each other. Without a functional deoxyribose aldolase enzyme the mutant will be unable to convert the deoxyribose intermediates to the final products in the pathway. In a rich *in vitro* medium this pathway may not be essential for the mutant to grow normally, but in chickens the bacteria may need to utilize nucleosides as an alternative source of carbon and energy. It is also possible that in the mutant the transposon insertion upstream of *deoC* and *deoR* affects the transcriptional regulation of the *deo* genes, as the DeoR repressor protein may no longer be synthesized.

Cell envelope genes. Three genes predicted to be involved in cell envelope biosynthesis were identified: *ponC*, *pm1294* and *dcaA*. The mutant AL389 had a transposon insertion in *ponC*, a homologue of the *E. coli* gene *pbpC*, which encodes the protein PBP 1C that has a penicillin-binding domain and functions as a transglycosylase for murein polymerization (Schiffer & Holtje, 1999). Murein is required for the biosynthesis of the murein sacculus, the scaffolding structure of the bacterial envelope. Mutants in the *pbp*C gene in *E. coli* are viable *in vitro*, but the cross-linkage of the murein structure is different from that of wild-type, with mainly nascent murein being used for the biosynthesis of the sacculus instead of a combination of pre-existing murein strands with newly synthesized ones (Park, 1996; Schiffer & Holtje, 1999). The *P. multocida ponC* mutant was also viable *in vitro*, but showed significant attenuation in chickens, perhaps indicating that the changed structure of the murein sacculus affects the integrity of the cell wall and *in vivo*, the mutant is more susceptible to osmotic stress.

Three mutants were identified with insertions in the gene *pm1294*. This gene is predicted to encode a heptosyl transferase, as the amino acid sequence displayed 58% identity to the heptosyltransferases II and III from *Haemophilus ducreyi*. These *H. ducreyi* proteins are glycosyltransferases responsible for the addition of heptose to lipooligosaccharide (Filiatrault *et al.*, 2000). The three *P. multocida* mutants had the transposon inserted at the same site, but in one, AL391, the transposon was in the opposite orientation. Interestingly, each mutant showed different levels of attenuation in mice and chickens. Strain AL251 was significantly attenuated in mice whereas AL390 and AL391 were not. In chickens, all three strains were attenuated, but AL251 and AL391 more so than AL390. Although the mutant AL251 was significantly attenuated in mice with an average rCI value of 0.62, it was very highly attenuated in chickens with an average rCI of less than 0.01. Interestingly, the strain AL391 had the same insertion site and transposon orientation as AL251 and was also highly attenuated in chickens, but in contrast showed no attenuation in mice. Preliminary data on the virulence of strains AL251 and AL391 indicate that they are both unable to cause disease in chickens.

The mutant AL249 had an insertion in the gene previously annotated as *dcaA*, but based on recent data this gene has now been renamed *lpt-3* because of its similarity to the gene *lpt-3* from *Neisseria meningitidis* (Mackinnon *et al.*, 2002; May *et al.*, 2001). In *N. meningitidis lpt-3* is required for the addition of phosphoethanolamine (PEtn) to the inner core of lipopolysaccharide and is predicted to encode a phosphoethanolamine transferase (Mackinnon *et al.*, 2002). Its inactivation in *N. meningitidis* resulted in bacteria that were lacking PEtn on the LPS structure and the mutants were less resistant to bactericidal killing and *in vitro* opsonophagocytosis (Mackinnon *et al.*, 2002). This gene has also been identified as significantly up-regulated in *P. multocida* during infections in chickens (Boyce *et al.*, 2002). Preliminary structural information on the *P. multocida* LPS purified from the wild-type VP161 indicates that a PEtn molecule is attached to the LPS (A.D. Cox, unpublished observations). Moreover, based on the position of PEtn in *N. meningitidis* (Mackinnon *et al.*, 2002) and *Haemophilus influenzae* (Phillips *et al.*, 1992; Phillips *et al.*, 1993), it is unlikely that the lack of the PEtn in the mutant has any significant effect on the LPS structure that would result in major structural changes in the outer membrane. Therefore, the attenuation observed in the *P. multocida* mutant AL249 is likely to be a direct effect of an altered LPS. Taken together, these data are consistent with a more

important role for lipopolysaccharide (LPS) in the progression of fowl cholera in chickens than in the progression of pasteurellosis in mice.

Notably, in the previous STM study of a bovine pneumonia isolate of *P. multocida* in a septicemic mouse model, another LPS mutant was identified with an insertion in *lgtC*, encoding a galactosyltransferase, the homologue of which in *H. influenzae* is responsible for the addition of galactose to the LPS oligosaccharide (Fuller *et al.*, 2000; Hood *et al.*, 1996).

Outer membrane proteins and surface structures. Fimbriae have previously been identified on the surface of *P. multocida* cells and genes encoding putative fimbriae and fibrils are present in the *P. multocida* genome (Isaacson & Trigo, 1995; May *et al.*, 2001; Rebers *et al.*, 1988; Ruffolo *et al.*, 1997). However, it has not been determined which of the fimbrial proteins are present within the observed structures and what role, if any, they play in the pathogenesis of disease. In this study one attenuated mutant AL250, was identified with the transposon inserted between the start codon and the putative promoter of the gene *pm0855*. The deduced amino acid sequence of *pm0855* has 43% identity with Flp1 from *Actinobacillus actinomycetemcomitans* and is located at the start of a potential operon containing fourteen genes. This putative *P. multocida* operon also contains other genes with similarities to those from the *flp* operon of *A. actinomycetemcomitans*, including genes encoding tight adherence proteins (Tad) that are thought to be involved in the synthesis of long filamentous fimbriae (Kachlany *et al.*, 2000). A second *flp* homologue, *flp2* was identified adjacent to *flp1*, but this putative gene has not been annotated in the published *P. multocida* genome sequence (May *et al.*, 2001). These two *P. multocida* genes contain a Flp motif that has been predicted as characteristic of a new pilin gene subfamily (Kachlany *et al.*, 2001).

In a previous STM study on *P. multocida*, a *tadD* mutant was shown to be attenuated. The *tadD* gene is positioned downstream of the *flp* genes, but potentially within the same operon (Fuller *et al.*, 2000). The *tadD* DNA sequence from VP161 has not been determined, a requirement for the construction of real-time PCR primers. Instead, to determine if the *tad* operon was affected by the insertional inactivation of *flp1* in the mutant AL250, the levels of RNA expressed from *flp1* and *orf4*, located downstream of *flp1* but upstream of *tadD*, were determined from both wild-type VP161 and the mutant AL250. Quantitative analysis of mRNA levels from *flp1* and *orf4* indicated that the mRNA transcription levels from *flp1* were, on average, four-fold

reduced in the mutant compared with the wild-type, whilst the mRNA expression levels from *orf4* were almost unchanged in the mutant compared to wild-type (ratio of *orf4* expression in mutant/wild type = 0.8). This finding indicates that there may be a promoter between the end of *flp1* and *orf4*. However, given that the transposon was inserted between the putative promoter and the start codon, it was surprising that there was any mRNA expression from the *flp1* gene in the mutant.

In competitive growth assays the *flp* mutant was consistently attenuated in mice. However, in chickens there was a large variation in the level of attenuation between individual birds, with the *flp* mutant able to grow at levels similar to wild-type in one of the four chickens tested. This may indicate real variability in the growth of the strain in different birds or may indicate that there is some instability of the mutant.

Attempts were made to produce antisera against several types of recombinant fusion proteins containing the mature length region of Flp1. However, in all cases antibodies were directed against only the carrier region of the protein and failed to recognise any *P. multocida* proteins (data not shown). Attempts to synthesize a portion of the Flp protein were unsuccessful due to the highly hydrophobic nature of the peptide. Thus, whether Flp proteins are part of a specific structure on the surface of the cell is yet to be determined.

Finally, a mutant was identified with an insertion in *pm1069*, the amino acid sequence of which has significant similarity to the *E. coli* protein FadL and the P1 outer membrane protein from *H. influenzae*. In *E. coli* FadL has been identified as an outer membrane porin required for the uptake of long chain fatty acids (Black *et al.*, 1987; Maloy *et al.*, 1981). Mutants lacking FadL are unable to grow on medium containing long chain fatty acids as a sole carbon source (Nunn & Simons, 1978). A search of the PM70 genome sequence showed that homologues of two other proteins required for the uptake of long chain fatty acids, FadR and FadL, are also present. The *H. influenzae* homologue P1 has been considered a potential vaccine candidate for both type b and NTHI strains of *H. influenzae*, as both polyclonal and monoclonal antibodies raised against P1 protect against bacteremia in the infant rat model (Hansen *et al.*, 1982; Loeb, 1987). In *H. influenzae* type b, a P1 mutant was shown to grow at the same rate as wild-type *H. influenzae in vitro* and could still induce bacteremia in the infant rat model (Munson & Hunt, 1989). However, analysis of a P1 mutant in *H. influenzae* biogroup aegyptius demonstrated that although both wild-type and mutant grew at the same rate under anaerobic conditions, the expression of P1 was up-regulated in the wild-type

strain (Segada *et al.*, 2000). The *P. multocida* mutant AL386 was attenuated only in chickens, suggesting that the uptake of long chain fatty acids as an energy source is only required during infections in chickens but not *in vitro* nor during infections in mice.

Concluding remarks. In summary, 420 *P. multocida* STM mutants were screened in both mice and chickens and fifteen attenuated mutants and thirteen disrupted genes/regions were identified. Ten of the insertions caused attenuation in chickens, but not in mice, indicating for the first time *P. multocida* virulence genes that may be host-specific. Five mutants were attenuated in both hosts and no mutants were identified that were attenuated in mice, but not in chickens.

Significantly, in this study none of the genes identified in a previous STM study that used a bovine pneumonia isolate of *P. multocida* in a mouse infection model were detected. However, several genes were identified that encoded proteins involved in similar pathways (Fuller *et al.*, 2000). These differences are unsurprising as the studies utilized different transposons, the *P. multocida* strains were isolated from two very different diseases (fowl cholera and bovine pneumonia), and each strain exhibited different infection kinetics in the mouse model. Furthermore, it is likely that neither study approached saturation coverage of the genome.

Mutant pools were introduced into the intraperitoneal cavity of mice and via the breast muscle of chickens. Whilst these inoculation routes reliably cause disease in both animal models, these infection routes would not allow the detection of bacteria with mutations in genes involved in the initial attachment and colonization of mucosal surfaces. Previous studies have indicated that birds can be infected via the respiratory tract and it has been demonstrated that *P. multocida* can adhere to turkey air sac macrophages (Pruimboom *et al.*, 1999; Pruimboom *et al.*, 1996; Rhoades & Rimler, 1990). Future studies will aim to detect mutants unable to attach and colonize by using intratracheal inoculation of the STM pools into chickens.

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Chapter 4

A heptosyltransferase mutant of *Pasteurella multocida* produces a truncated lipopolysaccharide structure and is attenuated in virulence.

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INTRODUCTION

During infections by gram-negative bacteria, the presence of lipopolysaccharide (LPS) stimulates the innate immune system whereby the inflammatory response plays a critical role in helping to clear the bacteria and prevent infection. This initial response to gram-negative bacteria can be elicited by a number of bacterial components, the most potent being lipid A, a component in the core structure of LPS. If the inflammatory response in the host is unable to clear the bacteria, and the infection is allowed to proceed, the presence of large amounts of systemic LPS can result in endotoxic shock in which an over-production of inflammatory mediators causes damage to tissues, septic shock, organ failure and death (Raetz & Whitfield, 2002).

In *P. multocida*, LPS is considered to play an important role in the pathogenesis of disease. Recently it has been shown that LPS from *P. multocida* assists in adhesion to neutrophils and transmigration through endothelial cells (Galdiero *et al.*, 2000). However, there are conflicting reports as to the endotoxic properties of LPS isolated from *P. multocida*. LPS isolated from a serotype B: 2 strain was shown to be endotoxic and intravenously administered LPS could reproduce clinical signs of hemorrhagic septicemia in buffalo (Horadagoda *et al.*, 2002). However, turkey poults were found to be relatively resistant to the lethal effects of LPS isolated from serogroup A strains of *P. multocida*, although the inflammatory response and microscopic hepatic lesions were similar to those observed in mammalian hosts (Mendes *et al.*, 1994; Rhoades & Rimler, 1987). In contrast, chicken embryos and mice were found to be highly susceptible to the toxic effects of *P. multocida* LPS (Ganfield *et al.*, 1976).

It is clear that the LPS of *P. multocida* stimulates humoral immunity and is considered to be a protective antigen. *P. multocida* strains are classified into Heddleston serotypes based on the antibody responses to LPS, whilst antibodies raised against heat-killed *P. multocida* vaccines are primarily directed against LPS and protect the host against strains within the same serotype (Brogden & Rebers, 1978). Early studies demonstrated that LPS purified using the Westphal method and injected into mice and rabbits resulted in a poor antibody response and no protection against *P. multocida* infection. In contrast, LPS injected into chickens induced a good antibody response which passively protected recipients against disease (Rebers *et al.*, 1980). Monoclonal antibodies raised against the LPS from a serotype A strain were shown to

be bactericidal and to completely protect mice against homologous challenge (Wijewardana *et al.*, 1990). In addition, an opsonic monoclonal antibody against a serotype B strain of *P. multocida* LPS was shown to partially protect mice against *P. multocida* infection (Ramdani & Adler, 1991).

A modified LPS structure clearly affects the viability of *P. multocida in vivo*. Results reported in Chapter 3 identified three strongly attenuated mutants that each had a single transposon insertion in the gene *pm1294* (designated $wcaQ_{PM}$). This gene is predicted to encode a heptosyltransferase, based on 72 % amino acid similarity to the heptosyltransferase III from *Haemophilus ducreyi*, a member of the *Pasteurellaceae* family (Filiatrault *et al.*, 2000). Furthermore, a *P. multocida galE* mutant has been constructed previously and was attenuated in mice (Fernandez de Henestrosa *et al.*, 1997). *GalE* encodes an enzyme required for the epimerization of UDP-glucose to UDP-galactose prior to LPS assembly and this mutant probably expresses an altered LPS although no structural analysis of the LPS was reported (Fernandez de Henestrosa *et al.*, 1997).

The sugar composition of LPS isolated from two serotype A strains of *P. multocida* has been analyzed previously and a partial structure proposed that included the identification of a tri-heptose unit linked to a 2-keto-3-deoxyoctulosonic acid (Kdo) residue (Erler *et al.*, 1988; Erler *et al.*, 1986; Erler *et al.*, 1981). In this chapter, the core structure of LPS from a highly virulent *P. multocida* serotype A: 1 strain, VP161, that causes fowl cholera is presented and compared with the LPS structure from a transposon mutant deficient in heptosyltransferase III. Virulence data is also presented that demonstrate a significant role for LPS in *P. multocida* disease progression.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *Escherichia coli* was grown routinely in Luria-Bertani broth. *P. multocida* strains were grown in either brain heart infusion (BHI) or nutrient broth (NB) supplemented with yeast extract (3%) (Oxoid, Basingstoke, United Kingdom). Solid media were obtained with the addition of 1.5% agar. When required, the media were supplemented with tetracycline at 2.5

μg/ml. For structural studies, *P. multocida* strains VP161 and AL251 were grown in a 28L fermenter in 24L of BHI broth for 18 h at 37 °C with 20% DO saturation. The cells were killed by addition of phenol to 2%, and 3 h post-phenol 1 g of hyaluronidase (Roche Chemicals) was added and stirred for 1 h before harvesting cells by using a Sharples continuous flow centrifuge.

Transposon stability studies. *P. multocida* AL251 was grown in 10 ml of NB, shaken at 37 °C. After approximately 10, 34 and 58 generations, samples of the culture were taken, diluted appropriately, and plated onto NB agar. After overnight incubation 100 colonies were patched onto NB with tetracycline and incubated overnight at 37 °C. Transposon loss was expressed as the percentage of tetracycline sensitive colonies.

TABLE 1. Bacterial strains, plasmids and oligonucleotides (oligo) used in this study.

Strain, plasmid or oligo	Relevant description	Source or reference
Strains		
P. multocida		
VP161	Serotype A:1, Vietnamese isolate from chickens.	(Wilkie *et al.*, 2000)
AL251	VP161 Tn*916*EΔC $waaQ_{PM}$ mutant	(Chapter 3)
AL298	AL251 with plasmid pAL170	This study
AL438	AL251 with plasmid pAL99	This study
E. coli		
DH5α	*deoR*, *endA*1, *gyrA*96, *hsdR*17($r_k^- m_k^+$), *recA*1, *relA*1, *supE*44, *thi*-1, (*lacZYA-argF*V169), Φ80*lacZ* ΔM15, F-	Bethesda Research Laboratories
Plasmids		
pPBA1100	*P. multocida* / *E. coli* shuttle vector.	(Homchampa *et al.*, 1997)
pAL99	240bp *Eco*R1 fragment containing *P. multocida tpiA* promoter region cloned into pPBA1100 *Eco*R1 site.	This study
pAL170	pAL99 containing $waaQ_{PM}$ gene.	This study
Oligonucleotides		
BAP2146	Forward primer for $waaQ_{PM}$ amplification; has *Bam*HI site for cloning. GAGTAGGATCCTGAAACATGTTCCC	This study
BAP2147	Reverse primer for $waaQ_{PM}$ amplification, has *Sal*I site for cloning. GGTTGGGTCGACCAAGCCACATTACTG	This study

PAGE and silver staining. Analysis of LPS was performed with a Bio-Rad mini protein gel apparatus using SDS-PAGE as described previously (Laemmli, 1970). LPS was then visualized by silver stain (Tsai & Frasch, 1982).

DNA manipulations. Restriction digests, ligations and polymerase reactions were performed according to the manufacturers' instructions using enzymes obtained from NEB (Beverley, MA) or Roche Diagnostics GmbH (Mannheim, Germany). Plasmid DNA was prepared using alkaline lysis (Birnboim & Doly, 1979) and purified using Qiagen columns (QIAGEN GmbH, Germany) or by PEG precipitation (Ausubel *et al.*, 1995). Genomic DNA was prepared using the CTAB method (Ausubel *et al.*, 1995). PCR amplification of DNA was performed using Taq DNA polymerase or Expand High Fidelity PCR System (Roche Diagnostics) and purified using the Qiagen PCR Purification Kit. The oligonucleotides used in this study are listed in Table 1. DNA Sequence was determined on a Model 373A DNA Sequencing System (Applied Biosystems) and analyzed with Sequencher Version 3.1.1 (GenCodes, Ann Arbor, MI).

***In trans* complementation of *waaQ*$_{PM}$.** The complete *waaQ*$_{PM}$ gene was amplified from *P. multocida* VP161 genomic DNA using oligonucleotides BAP2146 and BAP2147 (Table 1). The amplified 1.1 kb DNA fragment was ligated to *Sal*I and *Bam*HI-digested vector pAL99 (Table 1), such that transcription was driven by the *P. multocida tpiA* promoter. *E. coli* transformants were screened for the presence of the correct plasmid and one, designated pAL170, was used to transform *P. multocida* AL251, generating the strain AL298. As a control, the vector pAL99 was transformed separately into AL251 generating strain AL438 (Table 1).

Competitive growth assays. Competitive growth assays were performed as described previously (Chapter 3) and were used to quantify the relative growth rates of the *P. multocida* LPS mutant AL251 and the complemented mutant AL298. The competitive index (CI) was determined by dividing the percentage of tetracycline resistant colonies obtained from the output culture (*in vitro* or *in vivo*) by the percentage of tetracycline colonies obtained from the input culture. The relative competitive index (rCI), which measures the difference between growth *in vivo* and growth *in vitro*, was determined by dividing the *in vivo* CI by the *in vitro* CI. Mutants were identified as attenuated if the rCI value was significantly less than 1.0 as determined by statistical analysis using the one sided z-test ($p < 0.05$).

Virulence trials. Groups of ten commercially obtained Leghorn-cross chickens aged 12 weeks were infected with *P. multocida* VP161 or AL251 at two

different doses by injection of 100 µl into breast muscle. Blood samples were obtained at various time points after infection with AL251, and the birds deemed incapable of survival were euthanized in accordance with animal ethics requirements. Blood samples were diluted two-fold in BHI containing heparin and plated onto BHI plates. *P. multocida* colonies isolated from the blood were patched onto NB agar and NB agar with tetracycline.

Serum sensitivity assays. The sensitivity of *P. multocida* and *E. coli* to fresh chicken serum was determined as described previously (Chung *et al.*, 2001).

LPS STRUCTURAL STUDIES.

Andrew D. Cox and Frank St Michael at the Institute for Biological Sciences, National Research Council, Ottawa, Canada, performed the following; purification of lipopolysaccharide, analytical methods, mass spectrometry and nuclear magnetic resonance.

Purification of lipopolysaccharide. *P. multocida* cells (210g,VP161; 254g AL251) were freeze-dried, yielding 56g of VP161 and 52g of AL251. Freeze-dried cells were washed with organic solvents to remove lipids and other lipophilic components to enhance the efficiency of the LPS extraction (Masoud *et al.*, 1997). Washed cells (42g, VP161; 50g, AL251) were extracted by the hot-phenol / water method (Westphal & Jann, 1965) and the aqueous phases combined and dialysed against running water for 48 h. The retentate was freeze-dried, made up to a 2% solution in water and treated with DNase and RNase at 37 °C for 4 h followed by proteinase K treatment at 37 °C for 4 h. Small peptides were removed by dialysis. After freeze-drying, the retentate was made up to a 2 % solution in water, centrifuged at 8,000 g for 15 min followed by further centrifugation of the supernatant at 100,000 g for 5 h. The pellet, containing purified LPS, was redissolved and freeze-dried. The core oligosaccharide (OS) was isolated by treating the purified LPS with 1 % acetic acid (10 mg/ml, 100 °C, 1.5 h) with subsequent removal of the insoluble lipid A by centrifugation (5,000 x *g*).

Analytical methods. Sugars were determined by their alditol acetate derivatives (Sawardeker *et al.*, 1965) by GLC-MS. LPS was hydrolyzed for 4 h using 4 M trifluoroacetic acid at 100 °C, reduced overnight with $NaBD_4$ in H_2O and then acetylated with acetic anhydride at 100 °C for 2 h using residual sodium acetate as

catalyst. The GLC-MS was equipped with a 30 M DB-17 capillary column (180 °C to 260 °C at 3.5 °C/min) and MS was performed in the electron impact mode on a Varian Saturn II mass spectrometer. Methylation analysis was carried out by the NaOH / DMSO /methyl iodide procedure (Ciucanu & Kerek, 1984) and analyzed by GLC-MS as above.

Mass spectrometry. CE-ESI-MS was performed on a crystal Model 310 capillary electrophoresis (CE) instrument (AYI Unicam) coupled to an API 3000 mass spectrometer (Perkin-Elmer/Sciex) via a microIonspray interface (Cox *et al.*, 2001). A sheath solution (isopropanol-methanol, 2:1) was delivered at a flow rate of 1 µL/min to a low dead volume tee (250 µm i.d., Chromatographic Specialties). All aqueous solutions were filtered through a 0.45-µm filter (Millipore) before use.

Nuclear Magnetic Resonance. NMR spectra were acquired on a Varian Inova 500 MHz spectrometer using a 3 mm triple resonance (^{1}H, ^{13}C, ^{31}P) probe. The lyophilized sugar sample was dissolved in 140 µL (3mm) of 99% D_2O. The experiments were performed at 25 °C with suppression of the HOD (deuterated H_2O) signal at 4.78 ppm. The methyl resonance of acetone was used as an internal or external reference at 2.225 ppm for ^{1}H spectra and 31.07 ppm for ^{13}C spectra. Standard homo and heteronuclear correlated 2D pulse sequences from Varian, COSY, TOCSY, NOESY, ^{13}C-^{1}H HSQC, ^{13}C-^{1}H HSQC-TOCSY and ^{13}C-^{1}H HMBC, were used for general assignments.

RESULTS

An attenuated *P. multocida* STM mutant produces a truncated LPS that is restored to full-length LPS by complementation with a functional *waaQ$_{PM}$* gene. Signature-tagged mutagenesis was used to identify mutants attenuated for growth in mice and chickens (Chapter 3). During this previous analysis a mutant was identified (designated AL251) that was attenuated in both chickens and mice. Sequence analysis of the mutant revealed a single transposon insertion within the gene *waaQ$_{PM}$* that is predicted to encode a heptosyltransferase, a glycosyltransferase responsible for the addition of heptose to LPS (Filiatrault *et al.*, 2000; Harper *et al.*, 2003).

The LPS profile of AL251 was compared with that of its wild-type parent VP161, and the complemented mutant AL298, using polyacrylamide gel

electrophoresis followed by silver staining. The LPS from AL251 migrated further within the gel compared to wild-type LPS indicating that the LPS produced by the mutant was significantly truncated (Fig. 1a). Furthermore, the LPS profile of the complemented mutant AL298, was identical to that observed for the wild-type indicating that complementation of the mutant AL251 with an intact $waaQ_{PM}$ gene was able to restore the synthesis of wild-type LPS (Fig. 1a).

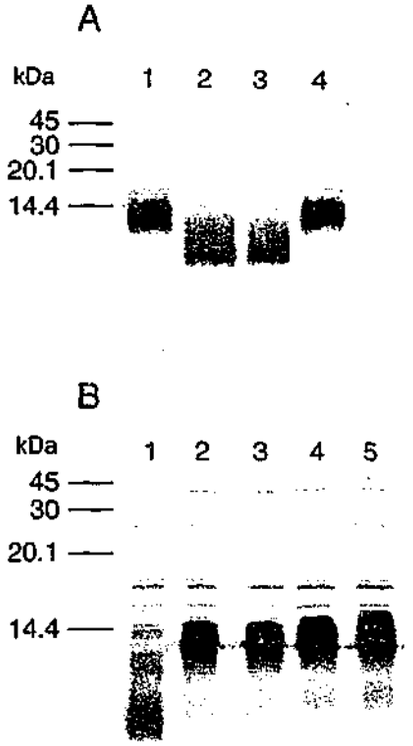

FIG. 1. Analysis of *P. multocida* LPS by SDS-PAGE and silver staining of whole cell lysates. (A) Comparison of *P. multocida* LPS profiles from wild-type VP161 (lane 1); heptosyltransferase mutant AL251 (lane 2); control strain AL438 (AL251 containing vector plasmid pAL99) (lane 3) and the complemented mutant strain AL298 (lane 4).

(B) Comparison of LPS profiles of *P. multocida* heptosyltransferase mutant AL251 (lane 1); wild-type VP161 (lane 2) and *P. multocida* wild-type revertants isolated from three different chickens inoculated with AL251 (lanes 3,4 and 5).

The positions of standard molecular mass markers are shown on the left (kDa).

Complementation of AL251 with $waaQ_{PM}$ also restores *in vivo* growth to wild-type levels. As complementation of AL251 with $waaQ_{PM}$ restored production of wild-type LPS it was of interest to determine if complementing the inactivated $waaQ_{PM}$ also restored the mutant AL251 to wild-type levels of growth *in vivo*. Initial studies with the complemented mutant AL298 indicated that there was significant loss of the complementing plasmid pAL170 once antibiotic selection for the plasmid was removed (44% retention after 6 h). For this reason, mice were chosen for the competitive growth assay instead of chickens, as studies in Chapter 3 had demonstrated that the infection time required to harvest bacteria from mice was only 6 h compared with more than 12 h for infections in chickens. Three mice were injected with an equal mix of VP161 and the complemented strain AL298. As controls, two mice were injected with an equal

mix of wild-type VP161, and the control strain AL438 (AL251 with vector pAL99). The complemented mutant AL298 was able to compete equally with wild-type VP161 with an average rCI value of 1.0 while the control strain AL438 had an average rCI value of 0.57 ($p = 0.03$), similar to the rCI values previously reported for AL251 in mice (Chapter 3). These results demonstrate that *waaQ*$_{PM}$ is required for both production of full-length LPS and for normal growth during infection.

The *P. multocida waaQ*$_{PM}$ mutant is unable to cause disease in chickens. It had been shown previously that strain AL251 displayed a profoundly reduced growth rate in chickens (Chapter 3); therefore, it was of interest to determine whether the mutant was still capable of causing disease in these hosts. Chickens were challenged with either VP161 or AL251 at two different doses (Table 2). All of the chickens challenged with wild-type VP161 died within 20 h. In contrast, most chickens challenged with AL251 remained well over the first twenty hours, but within four days all of the chickens inoculated with AL251, irrespective of dose, succumbed to fowl cholera infection. *P. multocida* was isolated from the blood of AL251-infected chickens in the late or terminal stages of the disease and it was found that all of the isolated *P. multocida* colonies were tetracycline sensitive indicating that the transposon was no longer present in the bacteria. Sequence analysis of *waaQ*$_{PM}$ from the recovered colonies indicated that in all cases the transposon had excised, thereby reconstituting a functional *waaQ*$_{PM}$ gene. Interestingly, for all but one isolate, the sequence analysis also revealed the presence of nucleotide substitutions at the point of transposon

TABLE 2. Virulence of VP161 and AL251 in groups of 10 chickens. CFU = colony forming units, h = hours, N.D = not determined.

Strain	Dose (CFU)	Mean time to death (h)	Range (h)
VP161	$1.5x10^2$	<20	ND
	$1.5x10^3$	<20	ND
AL251	70	65	33-120
	$7x10^2$	30	23-42

excision resulting in two amino acid changes within *waaQ*$_{PM}$ (amino acids 88,89; Ser to Leu, and Asp to Cys respectively). These amino acid changes did not affect the function of *waaQ*$_{PM}$, as the LPS profiles of the *P. multocida* isolates recovered from the chickens challenged with AL251 were all identical to wild type (Figure 1b). Taken together, these results indicate that the later onset of fowl cholera observed in the chickens inoculated with AL251 was due entirely to wild-type revertants of AL251 and therefore strains with an inactivated *waaQ*$_{PM}$ gene are incapable of causing disease.

Attenuation of the *waaQ*$_{PM}$ mutant in chickens is not due to increased sensitivity to chicken serum. To determine the relative sensitivity of the wild-type *P. multocida* strain VP161 and the LPS mutant AL251 to complement-mediated killing, mid-log phase cells of each strain and a control strain, *E. coli* DH5α, were incubated in either normal or heat-treated chicken serum for 3 h. Wild-type *P. multocida*, strain VP161 was able to grow at the same rate in either the heated or un-heated serum, indicating that it is fully serum resistant as previously reported for other *P. multocida* serotype A strains (Diallo & Frost, 2000). The bactericidal activity of the chicken serum was confirmed using the *E. coli* control strain, which multiplied 19-fold in heat-treated serum, whilst in untreated serum its viability was reduced approximately 9-fold. Interestingly, for the mutant AL251, there was no difference in growth observed between the heat-treated and normal serum with approximately 160-fold increase in growth in the heat-treated serum and an identical growth rate in the normal serum. These results indicate that the attenuation observed for the LPS mutant in chickens was not due to increased sensitivity to complement.

Structural analysis of the LPS from *P. multocida* VP161 and AL251. Andrew D. Cox and Frank St Michael at the Institute for Biological Sciences, National Research Council, Ottawa, Canada, interpreted all LPS structural data and contributed the following section.

Sugar analysis of the LPS from the parent strain VP161 revealed glucose (Glc), galactose (Gal), N-acetyl glucosamine (GlcNAc) and L-*glycero*-D-*manno*-heptose (LD-Hep) in the ratio of 2 : 1 : 1 : 3 respectively. In contrast, the LPS from mutant strain AL251 revealed only Glc and LD-Hep in the ratio of 1 : 3 with traces of Gal and GlcNAc. It is possible that the presence of GlcNAc could be contributed from residual amounts of the hyaluronic acid capsule known for this serogroup in addition to the anticipated two residues in the lipid A region of each LPS molecule. CE-ES-MS analyses of the core OS sample derived from the parent VP161 LPS revealed a simple

mass spectrum (Appendix Fig. 1a) corresponding to two glycoforms of mass 1805 and 1967 Da, the smaller glycoform being consistent with a composition of 2*P*Cho, 3Hex, 4Hep, Kdo, where Kdo is the unique LPS sugar 2-keto-3-deoxy-octulosonic acid and *P*Cho is the phosphate moiety phosphocholine. The larger glycoform contains an additional hexose species (Table 3, Appendix Fig. 1a). Consistent with SDS-PAGE analysis of the mutant AL251 LPS, CE-ES-MS analyses of the core OS sample derived from the mutant AL251 LPS revealed a simple mass spectrum of a highly truncated molecule of mass 605 Da (Fig. 2b). This mass corresponds to a composition of 2Hep, Kdo. A larger glycoform was observed consistent with the presence of an additional hexose residue (767 Da). Trace amounts of a more extended glycoform of 1448 Da (*P*Cho, 3Hep, 3Hex, Kdo) were observed, but no glycoforms were observed that contained the full parental complement of 4 Hep residues (Table 3). In order to characterise completely the nature of the truncation in the core OS of the mutant strain, ^{1}H-NMR experiments were performed on the parent and mutant derived core OS. As MS data had suggested that a Hep residue was missing in the mutant OS and amino acid homology comparisons had suggested that $WaaQ_{PM}$ was a heptosyltransferase, close attention was paid to the resonances from the heptose residues of the molecule. Examination of the ^{1}H-NMR spectra revealed some heterogeneity for the heptose residues closest to Kdo as is often observed for core oligosaccharides due to rearrangement of the Kdo residue under the acidic hydrolysis conditions employed to obtain the core OS (Appendix Fig. 2). For the core OS derived from the parent strain, chemical shifts for the Hep II anomeric proton were identified at ~ 5.7 ppm, consistent with 2-substitution of this residue (Appendix Fig. 2a) (Cox *et al.*, 2001). However, in the AL251 mutant OS ^{1}H-NMR spectrum (Appendix Fig. 2b) the anomeric resonance for the Hep II residue had shifted up-field about 0.5 ppm, consistent with this residue no longer being 2-substituted. The Hep II residue is now a terminal moiety as observed previously for a *H. somnus* strain, and the chemical shifts to the H-4 resonance were consistent with this assignment (Cox *et al.*, 1998). Definitive evidence for the structural nature of the mutation was obtained from 2D NOESY experiments (Appendix Fig. 3). Characteristic NOEs were observed for VP161 core OS between the Hep III and Hep II anomeric protons that are diagnostic for the α-1-2 linkage between the Hep III and Hep II residues (Appendix Fig. 3)(Cox *et al.*, 2001). The NOESY spectrum of the AL251 mutant core OS confirmed the lack of 2-substitution of Hep II as the characteristic

TABLE 3. Negative ion CE-MS data and proposed compositions for core OS from P. multocida strains VP161 (parent) and AL251 (mutant). Average mass units were used for calculation of molecular weight based on proposed composition as follows: Hex, 162.15; Hep, 192.17; Kdo, 220.18; PCho, 165.05. Relative intensity is expressed as relative height of either doubly or singly charged ions.

Strain	$[M-H]^-$	$[M-2H]^{2-}$	Observed Molecular Ion	Calculated Molecular Ion	Relative Intensity	Proposed Composition[a]
VP161 CORE OS	1804.4	901.8	1805.6	1805.4	0.2	2PCho, 4Hep, 3Hex, aKdo[b]
	1822.4	910.9	1823.1	1823.4	1.0	2PCho, 4Hep, 3Hex, Kdo
	-	921.8	1845.1	1845.4	0.3	2PCho, 4Hep, 3Hex, Kdo, Na
	1966.3	982.9	1967.8	1967.6	0.9	2PCho, 4Hep, 4Hex, aKdo
	1988.4	993.9	1989.1	1989.6	0.3	2PCho, 4Hep, 4Hex, aKdo, Na
AL251 CORE OS	603.5	-	604.5	604.5	1.0	2Hep, aKdo
	621.5	-	622.5	622.5	0.9	2Hep, Kdo
	765.5	-	766.5	766.7	0.8	Hex, 2Hep, aKdo
	1447.5	-	1448.5	1448.2	0.1	PCho, 3Hep, 3Hex, aKdo

[a] PCho, phosphocholine; Hep, heptose; Hex, hexose; Kdo, 2-keto-3-deoxyoctulosonic acid.
[b] aKdo refers to *anhydro*-Kdo derivative.

NOEs described above were absent, confirming that the Hep III residue is no longer present in the mutant OS. Chemical shift and NOE data for the Hep II and Hep III residues for the parent OS and Hep II residue for the mutant OS are summarised in Appendix Table 1. Structural techniques have therefore demonstrated that the effect on the LPS structure of mutating gene *waaQ*$_{PM}$ is to preclude addition of Hep III to Hep II (Fig. 2), and this function is consistent with strong similarity of the encoded protein to known heptosyltransferases.

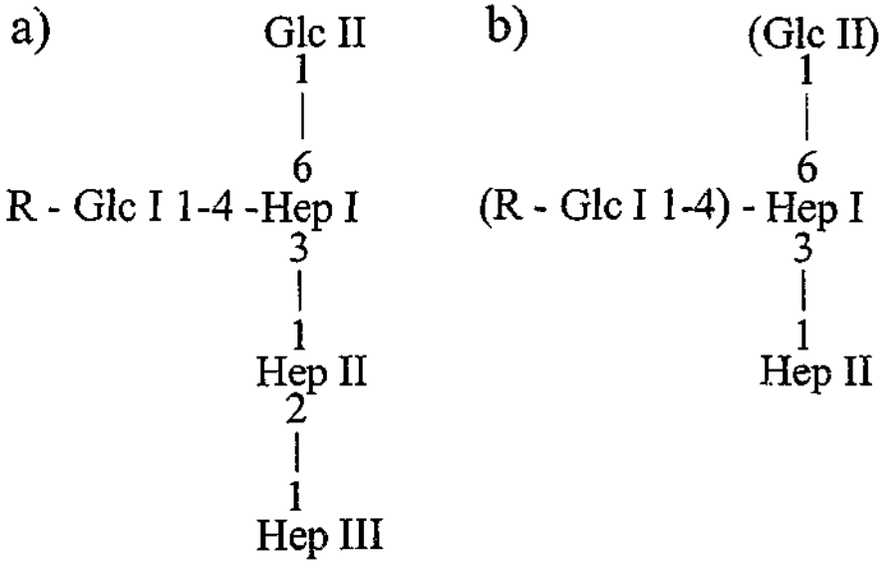

Fig. 2. Proposed structures of inner core LPS of *P. multocida* from (A) parent strain VP161 and (B) mutant strain AL251, where R is oligosaccharide chain extension beyond Glc.

DISCUSSION

The *P. multocida* LPS mutant AL251, first identified using STM in mice and chickens, was shown to have a transposon insertion in a predicted heptosyltransferase gene *waaQ*$_{PM}$ and was significantly attenuated in chickens and mice (Chapter 3). Silver stained polyacrylamide gels of cell lysates from wild-type VP161 and AL251 showed that the LPS from the mutant was significantly truncated (Fig. 1a), consistent with

waaQ$_{PM}$ encoding a heptosyltransferase responsible for the addition of a heptose molecule in the core region of the LPS structure.

Analysis of the *P. multocida* Pm70 genome revealed that the gene *waaQ*$_{PM}$ was probably transcribed independently and therefore the truncated LPS structure and reduced growth *in vivo* in chickens and mice was due directly to the inactivation of *waaQ*$_{PM}$ and not due to polar effects on downstream genes. This was confirmed by complementation, as introduction of a wild-type *waaQ*$_{PM}$ gene *in trans* restored both the LPS structure (Fig. 1a) and wild-type levels of growth in mice.

Virulence trials in chickens using the LPS mutant AL251 resulted in a delayed onset of fowl cholera symptoms (Table 2) and *P. multocida* isolated from chickens with disease symptoms were tetracycline sensitive, indicating that they no longer carried the transposon. Nucleotide sequence data obtained from *P. multocida* DNA isolated from infected birds confirmed that the gene *waaQ*$_{PM}$ was intact and in most cases, had nucleotide base changes at the point where the transposon had previously been inserted, resulting in two amino acid changes. The LPS from the *P. multocida* isolates recovered from the chickens challenged with AL251 showed that they produced wild-type LPS, confirming that the *waaQ*$_{PM}$ gene was functional despite the amino acid changes (Fig 1b). Moreover, these results suggest that the amino acids serine and aspartate, at amino acid positions 88 and 89 respectively in WaaQ$_{PM}$, are not essential for full enzyme activity as non-conservative changes at these positions did not prevent the formation of full-length LPS. Taken together, these data indicate that the infection observed in chickens inoculated with AL251 was due only to revertant strains that had lost the transposon insertion. The wild-type *P. multocida* strain VP161 is a highly virulent organism, with less than 50 bacteria causing fowl cholera in chickens (Wilkie *et al.*, 2000). Our observations measured the transposon excision rate from AL251 cells at 1% after the first ten generations (overnight *in vitro* growth) rising to 4% after 58 generations (data not shown). This rate of excision would result in the generation and selection of wild-type revertants in chickens at a rate sufficient to cause lethal fowl cholera infection during the course of the trial. It is therefore probable that a stable inactivation of *waaQ*$_{PM}$ would result in a *P. multocida* strain incapable of causing fowl cholera. However, a reliable method of constructing defined mutants in *P. multocida* has not yet been established.

Analysis of the *P. multocida* VP161 wild-type LPS indicated a "rough" LPS, similar to the LPS or lipooligosaccharide (LOS) isolated from Gram-negative mucosal

pathogens such as *H influenzae*, *H. ducreyi*, *N. meningitidis* and *N. gonorrhoeae*, with only a short non-repeating polysaccharide unit attached to the lipid A (Erridge *et al.*, 2002). The inner core structure of *P. multocida* LPS is similar to that described for *H. influenzae*, *M. haemolytica* and *H. ducreyi*, with a tri-heptose unit linked via a Kdo residue to lipid A (Appendix Fig. 3). (Cox *et al.*, 1998; Melaugh *et al.*, 1994; Phillips *et al.*, 1992). In the mutant AL251, inactivation of $waaQ_{PM}$ resulted in expression of a highly truncated LPS that lacked the third heptose molecule (Hep III) in the inner core region (Appendix Fig. 1b). The most abundant glycoforms of LPS in the mutant also lacked all sugars distal to the first heptose, suggesting that the inactivation of $waaQ_{PM}$ prevented further sugar additions (Table 3). It is therefore probable that conformational changes in the LPS intermediates due to the lack of the third heptose for the most part prevented the action of subsequent transferases.

The loss of a full length LPS molecule clearly affects the ability of the mutant AL251 to grow in mice and to cause disease in chickens. The specific reasons for this attenuation are not clear. However, in wild-type *P. multocida* VP161 LPS, two *P*Cho groups were identified while the mutant AL251 contained only a single *P*Cho group in a very minor glycoform (Table 3). The presence of more than one *P*Cho residue in the VP161 LPS is unusual; bacteria with *P*Cho-decorated LPS usually have only a single residue attached, although the position of attachment onto the LPS structure varies. Only one other bacterium, a non-typeable *H. influenzae* strain, is known to have two *P*Cho residues attached to the LPS (Landerholm *et al.*, unpublished). Interestingly, there are no *P*Cho groups on the LPS of the serotype A turkey isolate, PM70 (Cox, unpublished observations), and this strain is not virulent for chickens (Wilkie, unpublished observations).

*P*Cho groups are frequently attached to various bacterial structures on the surface of mucosal pathogens such as *H. influenzae*, *Actinobacillus actinomycetemcomitans*, *Streptococcus pneumoniae* and *Neisseria* spp., and play a key role in adhesion and invasion of epithelial and endothelial host cells by binding to the platelet-activating receptor (PAF) (Cundell *et al.*, 1995; Schenkein *et al.*, 2000; Serino & Virji, 2002; Swords *et al.*, 2000). Non-typeable *H. influenzae* that has *P*Cho positive glycoforms of LPS can attach and invade human bronchial epithelial cells via a series of signalling events (Swords *et al.*, 2001; Swords *et al.*, 2000). However, in both *H. influenzae* and *Neisseria* spp., although expression of *P*Cho on LPS was required for adhesion and invasion of human epithelial cells, its presence reduced survival in some

host niches, as strains expressing *P*Cho were more serum sensitive, mediated by binding of *P*Cho to C-reactive protein and subsequent activation of the complement system (Serino & Virji, 2002; Weiser *et al.*, 1998). Interestingly, this study has demonstrated that the LPS mutant AL251 is still highly resistant to the bactericidal action of complement in chicken serum, indicating that a complete wild-type LPS structure is not required for the bacteria to have full serum resistance. This is not surprising, as the $waaQ_{PM}$ mutant is still encapsulated and the hyaluronic acid capsule of *P. multocida* serotype A strains confers serum resistance (Chung *et al.*, 2001).

Preliminary studies identified a terminal sialic acid residue in the O-deacylated LPS of the wild-type strain VP161 (A.D. Cox, unpublished), but it is unlikely that sialic acid would be present in significant amounts on the attenuated LPS mutant, AL251, as most glycoforms lack the oligosaccharide chain extension beyond the first hexose residue that is required for sialic acid incorporation. Sialyltransferase activity in *P. multocida* has been previously reported and it was predicted that, based on this activity, *P. multocida* was likely to sialylate surface components (Vimr & Lichtensteiger, 2002). Moreover, a gene, *pm0508*, predicted to encode an LPS sialyltransferase and required for the addition of sialic acid to LPS, is present and transcribed in VP161 (data not shown). Preliminary results from whole genome expression profiling of *P. multocida* during growth in the liver tissue of chickens indicated that *pm0508* is always up-regulated during *in vivo* growth in liver (Boyce *et al.*, unpublished), but is not up-regulated in *P. multocida* isolated from the blood of infected chickens (Boyce *et al.*, 2002). In the host, sialic acid is present in large quantities in the mucin covering epithelial cells and is a component of glycoconjugates present on most host cells and serum proteins where it plays a role in specific recognition processes including complement regulation (Vimr & Lichtensteiger, 2002). Decoration of bacterial surface components with sialic acid is a classic example of molecular mimicry and sialic acid is commonly expressed on the surface of many mucosal pathogens, allowing the bacteria to elude host defence mechanisms. In *N. gonorrhoeae* it has been demonstrated that sialylation of the LPS prevents complement-mediated killing by non-immune and immune sera by masking LPS polysaccharide (Estabrook *et al.*, 1997; Inzana *et al.*, 2002; Wetzler *et al.*, 1992). Most clinical, but not commensal, isolates of *H. somnus* are also capable of sialylating their LPS, resulting in reduced antibody binding and enhanced resistance to serum (Inzana *et al.*, 2002) In contrast, in *N. meningitidis* it was demonstrated that sialylation of LPS

was unlikely to enhance serum resistance but did inhibit endocytosis by human dendritic cells (Unkmeir *et al.*, 2002; Vogel *et al.*, 1999). Similarly, it has been shown that the LPS mutant AL251 is as serum resistant as the parent strain, VP161.

P. multocida also produces two sialidases with specificities that indicate that they may be capable of de-sialylating host receptors (Mizan *et al.*, 2000). *P. multocida* may use these sialidases to sequester sialic acid from the host and it is tempting to speculate that *P. multocida* through sialylation of LPS is able to masquerade as "self" and then, by using specific sialidases, unmask key receptors on host cells thereby facilitating attachment and/or invasion. A search of the non-redundant Genbank database indicated that, to our knowledge, this is the first example of a bacterial species that has both sialidase activity and a sialyltransferase required for the attachment of sialic acid to LPS.

Although it is likely that the observed attenuation of the $waaQ_{PM}$ mutant was due directly to the truncated LPS, it cannot be completely excluded that pleiotropic effects, such as changes to the outer membrane structure, may play a role. However, a similar LPS mutant in *E. coli* retained near wild-type levels of outer membrane stability, as assessed by sensitivity to sodium dodecyl sulphate (SDS) and novobiocin (Yethon *et al.*, 1998).

Concluding remarks. A *P. multocida* $waaQ_{PM}$ mutant that expresses a severely truncated LPS has been characterised. The inner core structure for both the wild-type *P. multocida* LPS molecule and the $waaQ_{PM}$ mutant has been determined and it has been demonstrated that a functional $waaQ_{PM}$ gene is required for the addition of the third heptose residue to the inner core of the LPS. Thus $waaQ_{PM}$ has been identified as a heptosyltransferase III and through virulence trials it has been demonstrated that its activity is required for bacterial virulence in chickens, the natural host for this *P. multocida* strain. The inactivation of $waaQ_{PM}$ leads to the complete absence of the third heptose in the inner core and, as a result, the full wild-type glycoforms. The majority of glycoforms identified from the mutant LPS lack PCho and, based on the degree of truncation, is predicted to lack sialic acid, residues that in LPS of other Gram negative mucosal pathogens play key roles in bacterial virulence. Future work will focus on identifying which, if any, of these specific residues are required for bacterial virulence.

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Appendix

TABLE 1. ^{1}H-NMR chemical shifts for the Hep II and Hep III residues from the core OS derived from strains of P. multocida VP161 (parent) and AL251 (mutant). Recorded at 25 °C, in D_2O. Chemical shifts referenced to internal acetone at 2.225 ppm. nd, not determined. Two resonances were observed for each residue due to heterogeneity of Kdo molecule following core hydrolysis.

	H-1	H-2	H-3	H-4	H-5	H-6	H-7	NOEs	
								Inter	Intra
VP161									
Hep-II	5.76	4.17	3.85	3.83	3.60	4.05	3.76 3.65	5.11 Hep III H-1 4.04 Hep *I* H-3	4.17 H-2
Hep-II	5.70	4.19	3.86	3.84	3.55	4.05	3.76 3.65	5.14 Hep III H-1 3.96 Hep I H-3	4.19 H-2
Hep-III	5.14	4.02	3.87	3.83	3.78	4.05	3.77 3.65	5.70 Hep II H-1 4.19 Hep II H-2	4.02 H-2
Hep-III	5.11	4.01	3.87	3.83	3.78	4.05	3.77 3.65	5.76 Hep II H-1 4.17 Hep II H-2	4.01 H-2
AL251									
Hep II	5.22	4.07	3.89	3.72	nd	nd	nd	4.03 Hep I H-3	
Hep II	5.17	4.06	3.88	3.65	nd	nd	nd	nd	

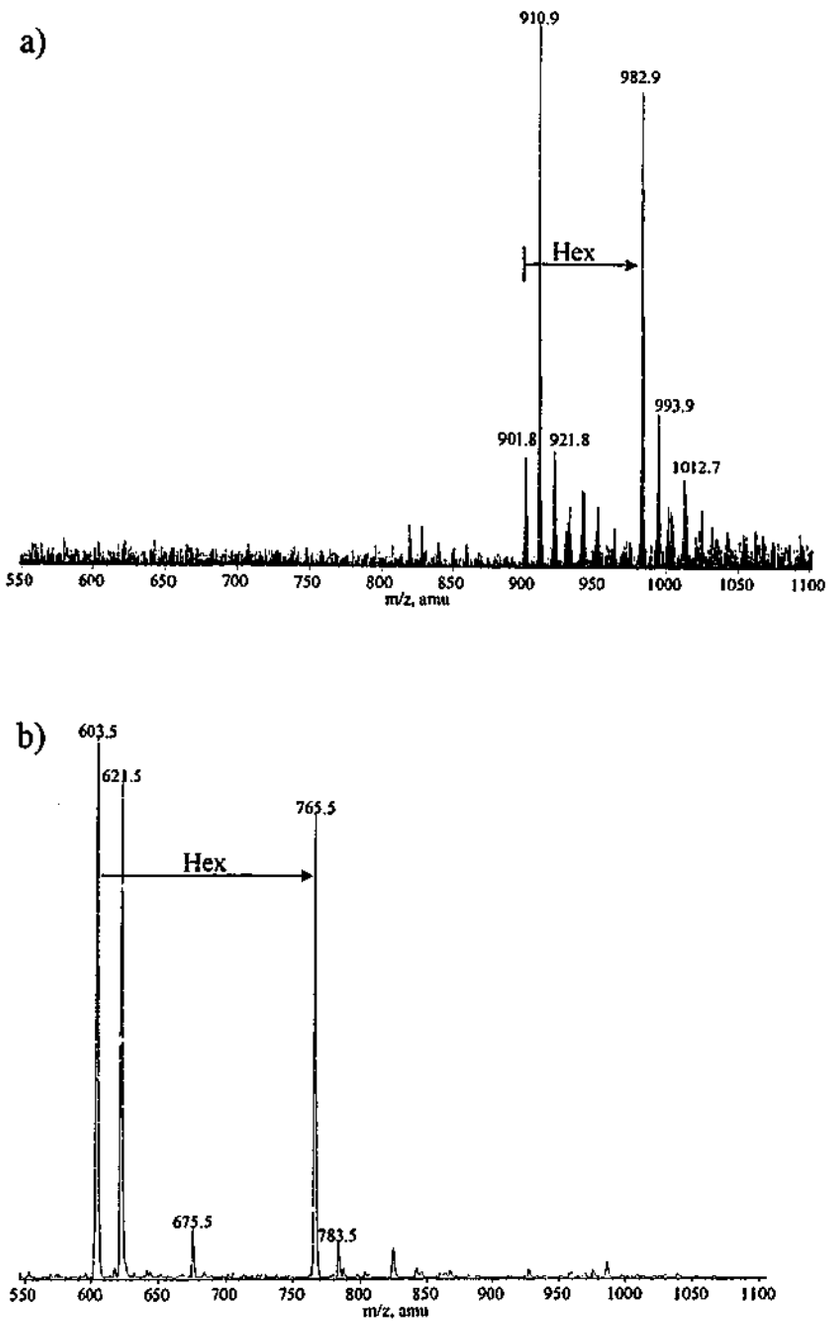

Fig. 1. Negative ion capillary electrophoresis electrospray mass spectra of *P. multocida* core OS. (A) Doubly charged region of core OS from parent strain VP161; (B) Singly charged region of core OS from mutant strain AL251.

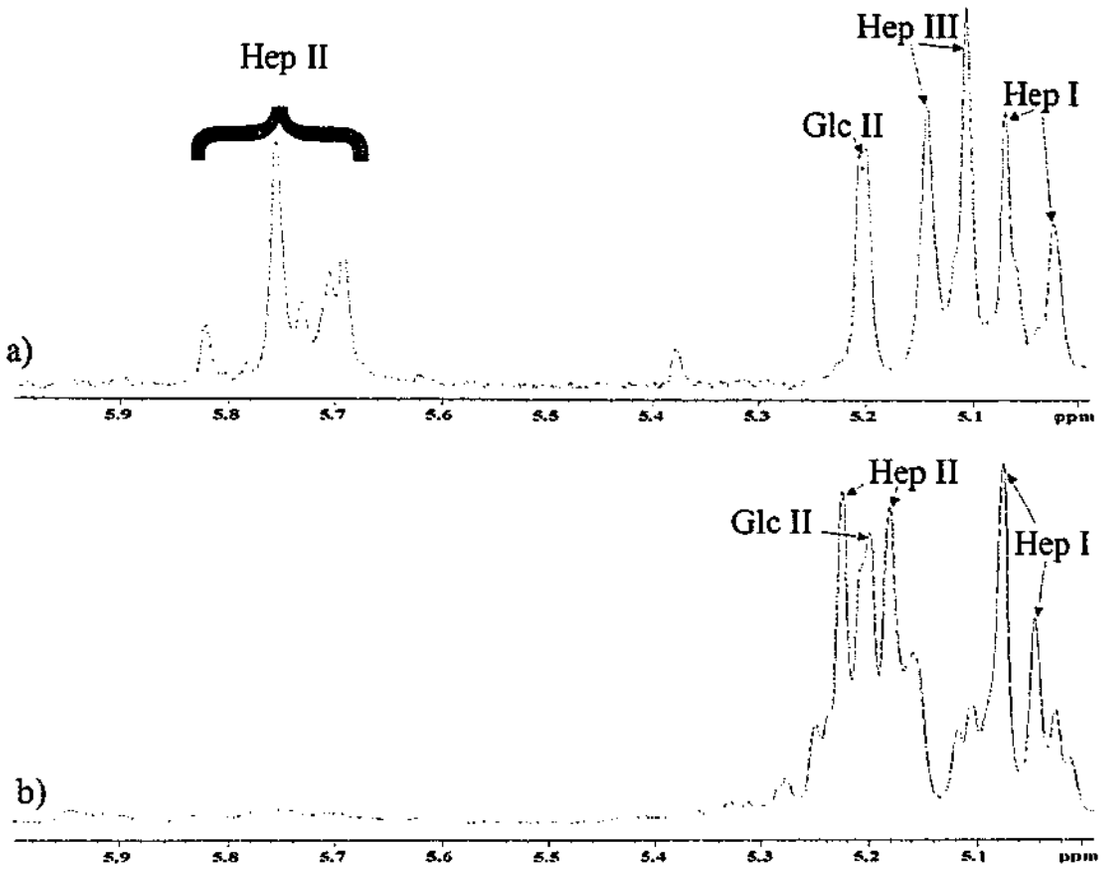

Fig. 2. Region of the ^{1}H-NMR spectrum of the core OS derived from the LPS of (A) *P. multocida* parent strain VP161 and (B) *P. multocida* mutant strain AL251. The spectra were recorded at 25 °C and referenced against internal acetone at 2.225 ppm.

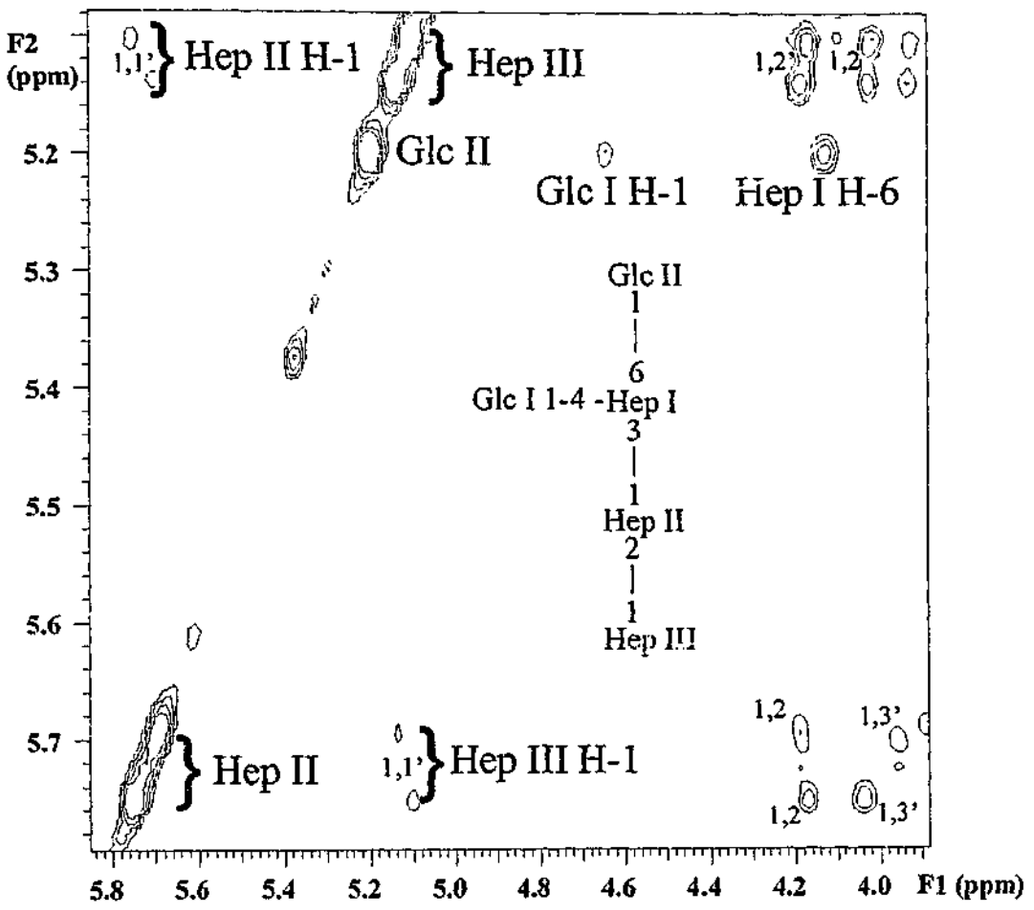

Fig. 3. Region of the NOESY spectrum of *P. multocida* VP161 core OS. NOE connectivities are as indicated. Inset; structure of the inner core OS from VP161. The spectrum was recorded at 25 °C and referenced against internal acetone at 2.225 ppm.

Amendments

Table of Contents:
Line 32, replace "mutagesis" with "mutagenesis".

Chapter 1:
p7 para 1, line 3. Remove "of".

p9 para 2, line 5. Remove "Homchampa, 1994 #575".

p26 para1, line 3. Delete "*S.* Typhimurium" and insert "*S. enterica* serovar Typhimurium".

Outline of Thesis:
P39 para 1, line 12. Remove "be".

Chapter 2:
p41 para 1, line 8. Delete "*S.* Typhimurium" and insert "*S. enterica* serovar Typhimurium".

p44 para 2 line 1. The word "Taq" should be italicised.

Chapter 3:
p56 para 3, line 8, insert the sentence; "Whilst intraperitoneal inoculation is not a natural route of *P. multocida* infection, inoculation via this route gave rise to consistent and reproducible infections."

p56 para 3, line 12, insert the sentence; "*P. multocida* was introduced into the chickens via breast tissue to ensure consistent and reproducible infections."

p55 para 3, line 1. Replace "polymerase reactions" with "polymerase chain reactions".
p56 para 1, line 5. The word "Taq" should be italicised.

p64 para 2, line 3: Delete "……insertion in *lgtC*, encoding a galactosyltransferase, the homologue of which in *H. influenzae* is responsible for the addition of galactose to the LPS oligosaccharide (Fuller *et al.*, 2000 ; Hood *et al.*, 1996)" and insert "insertion in *lgtC*, encoding a protein with similarity to LgtC, a galactose transferase from *H. influenzae* that is responsible for the addition of galactose to the LPS oligosaccharide (Fuller *et al.*, 2000 ; Hood *et al.*, 1996)."

Chapter 4.

Replace $waaQ_{PM}$ with $waaQ_{Pm}$ throughout entire chapter.

p74 para 2, line 7. The word "Taq" should be italicised.

p82 Figure 2 Legend. Insert end of line 2. "Based on Negative ion CE-MS data shown in Table 3, extension of the mutant LPS molecule to include the structures shown in brackets occurs at only low frequency (less than 4%)."

p83 para 3, line 23. Delete "However, a reliable method of constructing defined mutants in *P. multocida* has not yet been established" and insert "However, construction of stable mutants by allelic exchange is time consuming and not always successful and, despite considerable efforts by a number of laboratories, few allelic exchange mutants have been constructed in *P. multocida* (Fernandez de Henestrosa *et al.* 1997, Homchampa *et al.*, 1997, Bosch *et al.*, 2002, Boyce & Adler, 2000)."

p85, para 2, line 20. Insert references Inzana *et al.*, 2002, Estabrook *et al.* and Kim *et al.*, 1992.

p85 para 2, line 23. Remove references Inzana *et al.*, 2002 and Estabrook *et al.*, 1997.

p87. Insert the following into the references section of Chapter 4.

Bosch, M., Garrido, E., Llagostera, M., Perez de Rozas, A. M., Badiola, I. & Barbe, J. (2002). Pasteurella multocida exbB, exbD and tonB genes are physically linked but independently transcribed. *FEMS Microbiol Lett* 210, 201-208.

Boyce, J. D. & Adler, B. (2000). The capsule is a virulence determinant in the pathogenesis of *Pasteurella multocida* M1404 (B:2). *Infect Immun* 68, 3463-3468.