No Restrictions

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MONASH UNIVERSITY

THESIS ACCEPTED IN SATISFACTION OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

ON..... 2 June 2004.....

.....[redacted].....

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Amendment to thesis

I confirm that I did indeed contribute to at least 80% of the research in **Chapter 2-*In vivo* expressed genes of *Pasteurella multocida***, and at least 50% of the paper which forms part of this chapter (Hunt, M.L., Boucher, D.J., Boyce, J.D., Adler, B., (2001), *In vivo* expressed genes of *Pasteurella multocida*," **Infection and Immunity**, Vol. 69 (5), pp 3004-12).

I also confirm that carried out all the electroporations described in **Chapter 3-Generation of defined mutants in the *P. multocida* A:1 strain X-73**.

[redacted]

David James Boucher

Identification and characterisation of *in vivo* expressed genes of *Pasteurella multocida*

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Thesis submitted for the degree of Doctor of Philosophy at Monash University,
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Summary

Pasteurella multocida is the causative agent of infectious diseases of economic importance, such as fowl cholera, bovine haemorrhagic septicaemia and porcine atrophic rhinitis. However, knowledge of the molecular mechanisms and determinants that *P. multocida* requires for pathogenesis is still limited. To address this issue, a genetic expression system, based on the *in vivo* expression technology (IVET) approach first described by Mahan *et al.* (Science 259:686-688, 1993), was developed to identify *in vivo* expressed genes of *P. multocida*. Numerous genes, including those encoding outer membrane lipoproteins, metabolic and biosynthetic enzymes and a number of hypothetical proteins were identified.

To investigate the function of two of these *in vivo* expressed genes, an attempt was made to inactivate them by allelic exchange mutagenesis. The ability to construct defined mutants is invaluable in assigning function to putative pathogenic and virulence factors, and also provides a source of novel vaccine strains, but directed mutagenesis is not routine for *P. multocida*. Several commonly used methods for constructing defined mutants in *P. multocida* were evaluated. One of these methods, using a *dam* methylated mutagenesis construct, resulted in the generation of a defined *nrfE* mutant, and another based on screening a *P. multocida* Tn*916* library proved to be a useful technique that warrants further investigation

nrfE is part of the Nrf (formate dependent nitrite reductase) system involved in utilising available nitrite in the host as an alternative electron accepter during anaerobic conditions. Analysis of the inactivated *nrfE* mutant indicated that *P. multocida* has an Nrf system that is active both aerobically and anaerobically. It was also found that, similar to the situation in *Escherichia coli*, *nrfE* is essential for Nrf activity. However, the inactivation of *nrfE* did not result in attenuation of *P. multocida* in mice. Additionally, *nrfE* was found to potentially be transcribed from a previously unknown promoter between *nrfD* and *nrfE* that is most likely expressed *in vivo*.

Statement

I, David Boucher, 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.

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David Boucher

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Publications

Publications arising from research presented in this thesis

-Hunt, M.L., Boucher, D.J., Boyce, J.D., Adler B.,(2001), "*In vivo* expressed genes of *Pasteurella multocida*," Infection and Immunity, Vol 69 (5), pp 3004-12 (Joint first authorship).

-Boucher, D.J., Boyce, J.D., Adler, B "Characterisation of the *nrfE* gene of *Pasteurella multocida"* (in preparation).

Selected Conference presentations of research presented in this thesis

2002- *In vivo* expressed genes of *P. multocida,*" D.J. Boucher, J.D. Boyce, B. Adler, *Bacterial pathogenesis research group conference*, Clayton, Australia

2002- *In vivo* expressed genes of *P. multocida,* D.J. Boucher, M.L. Hunt, J.D.Boyce, B. Adler, *6th International Pasteurellaceae society conference*, Banff, Alberta, Canada

2001- *In vivo* expressed genes of *P. multocida,* D.J. Boucher, M.L. Hunt, J.D.Boyce, B. Adler, *6th Australian conference on molecular analysis of bacterial pathogens*, Marysville, Victoria, Australia.

1999-Close encounters of the *In vivo* kind, D.J. Boucher, M.L. Hunt, J.D.Boyce, B. Adler, *Bacterial pathogenesis research group conference*, Clayton, Victoria, Australia.

Chapter 1

Introduction

Chapter 1-Introduction

Characteristics of Pasteurella multocida

Nomenclature and taxonomy

Pasteurella multocida is a member of the family *Pasteurellaceae* which comprises the genera, *Pasteurella, Haemophilus,* and *Actinobacillus*, (Phol, 1981) and three recently proposed additions *Mannheimia* (Angen *et al.*, 1999), *Gallibacterium* (Christensen *et al.*, 2003) and *Lonepinella* (Osawa *et al.*, 1995). *Pasterellaceae*, together with *Enterobacteriaceae* and *Vibrionaceae* make up the gamma division of Proteobacteria (Dewhirst *et al.*, 1992).

The species name *multocida* was proposed in 1939 by Rosenbusch and Merchant (cited by (Rimler *et al.*, 1989)), where previously it had been *P. septica*. Three subspecies have been identified (Christensen *et al.*, 2003) based on DNA hybridisation; *P. multocida* subsp. *multocida*, *P. multocida* subsp. *gallicida,* and *P. multocida* subsp. *septica.*

Cell and colony morphology

Pasteurella multocida is a Gram-negative coccobacillus that is nonmotile and non spore forming. It is facultatively anaerobic, and has been shown to be pleomorphic when isolated initially or grown under unfavourable conditions such as reduced oxygen tension or in the presence of homologous antibody (Carter, 1967). Old cultures have also been shown to produce long filamentous forms. *P. multocida* possesses a capsule that differs in composition and size between strains and the presence of capsule has been demonstrated on a number of them (Bain *et al.*, 1982, Jasmin, 1945, Möller, 1951). The capsule is usually present on freshly isolated strains; however, it can be lost after repeated *in vitro* subculture (Namioka, 1978, Watt *et al.*, 2003).

Colonies tend to be described in three forms; mucoid, smooth, and rough (Carter, 1967). Mucoid colonies tend to be the largest, and are of a sticky consistency due to the presence of capsule, whereas the appearance of the rough colonies is due to the absence of capsule. Smooth colonies that fluoresce under obliquely transmitted light have been shown to possess capsules, whereas non-fluorescent smooth colonies tend to have little or no capsular material (Rimler *et al.*, 1989).

Growth & biochemical characteristics

P. multocida is a facultative anaerobe that grows best at temperatures of 35-37°C in air (Carter, 1981). Nicotinamide, pantothenic acid, and thiamine have been found to be essential for growth (Rimler *et al.*, 1989). Clinical specimens are usually grown on blood agar, but colonies may be difficult to distinguish (Carter, 1955). Media such as Difco-tryptose, and Gibco-dextrose can be used to isolate and maintain cultures (Rimler *et al.*, 1989).

P. multocida is catalase, indole and oxidase positive, and does not grow on MacConkey agar (Namioka, 1978, Rimler *et al.*, 1989). It is positive for the production of H_2S and ferments glucose and mannose, producing acid but not gas (Carter, 1967). *P. multocida* is gelatinase, urease negative, and does not ferment lactose (Rimler *et al.*, 1989).

Detection and typing methods

Serological typing schemes

P. multocida has been classified into several serotypes based on different types of antigens recognised by the assays. Based on haemagglutination tests that involve the adsorption of specific capsular substances to erythrocytes, five different serotypes viz., A, B, D, E, and F have been identified (Carter, 1967, Rimler *et al.*, 1987). Somatic serotyping carried out using the agar gel precipitin test method (AGPT) has yielded 16 serotypes (Heddleston *et al.*, 1972a). The lipopolysaccharide present in the outer membrane was demonstrated to be the antigen recognised in the somatic serotyping method (Manning, 1984). An agglutination test carried out using heat-treated cells (and also based on somatic serotyping) was utilised by Namioka and Murata (1961) that yielded 11 serotypes. Most typing is now accomplished by a combination of the capsular serotyping method of Carter (1967), with the somatic serotyping method of Heddleston *et al.* (1972a), and this is referred to as the Carter-Heddleston serotyping (eg A:16, Carter-Heddleston). Occasionally Namioka and Murata's typing method is combined with that of Carter's typing method, particularly when the organism is type B, and is referred to as Namioka-Carter typing (eg. serotype 6:B, Namioka-Carter) (De Alwis, 1993). Designation of serotypes according to these two methods are shown in Table 1 and Table 2 respectively.

Enzyme-linked immunosorbent assays (ELISA) have been used extensively in detecting and quantifying antibodies in vaccine trials, and they could also potentially be

used to type many serotypes of *P. multocida*. At present only an ELISA for detection of HS strains has been described (Dawkins *et al.*, 1990).

Table 1-Designation of serotypes of *P. multocida* by the Carter-Heddleston method

Capsular	Somatic type	Serotype	Disease association
A	1, 3, 4	A:1	Fowl cholera
		A:3	Fowl cholera
		A:4	Fowl cholera
	5, 6	A:5	Fowl cholera
		A:6	Fowl cholera
	7-10	A:7, A:8, A:9	Fowl cholera
	12-15	A:10, A:12, A:13	Fowl cholera
		A:14, A:15	Fowl cholera
	16	A:16	Fowl cholera (turkeys)
B	2	B:2	Haemorrhagic septicaemia
D	11	D:11	Atrophic rhinitis (pigs)
E	2	E:2	Haemorrhagic septicaemia
F	1,3, 7,12	F:1, F:3, F:7, F:12	Fowl cholera (turkeys)

Adapted from De Alwis (1993)

Table 2- Designation of serotypes of *P. multocida* by th Namioka-Carter method

Capsular type	Somatic type	Serotype	Disease association
A	1, 3, 5, 7 , 8 & 9	1:A	Pneumonia (cattle, sheep, pigs)
		3:A	Pneumonia (pigs)
		5:A	Fowl cholera
		7:A	Septicaemia (cattle)
		8:A, 9:A	Fowl cholera
B	6 & 11	6:B	Haemorrhagic septicaemia (bovine)
		11:B	Wound infection (bovine)
D	1, 2, 3, 4 & 10	1:D, 2:D, 10:D	Pneumonia (pigs)
		3:D	Pneumonia (cats)
		4:D	Pneumonia (sheep and pigs)
E	6	6:E	Haemorrhagic septicaemia (bovine)

Adapted from De Alwis (1993)

Non serological typing schemes

Biotyping has been shown to have limited ability in distinguishing strains and hence other methods are usually utilised (Blackall *et al.*, 1997, Fegan *et al.*, 1995).

Non-serological techniques involve the detection of serotype A strains by growing these in the presence of hyaluronidase. These serotypes have capsules that consist primarily of hyaluronic acid. Thus, growth in the presence of hyaluronidase affects the production of hyaluronic acid in the capsule, and causes the colonies to be blue and reduced in size (Rimler, 1993). Flocculation of broth culture by using acriflavidine dye is a useful method for recognising serotype D (Rimler, 1993). Serotype B:2 strains produce enzymes with hyaluronidase and chondroitinase activity, whereas other serotypes do not, and this can be used as a distinguishing feature.

Apart from biochemical and physical characteristics, molecular methods have increasingly been used to identify *P. multocida*. Species specific polymerase chain reaction (PCR) assays have been developed for detection of *P. multocida* in mixed cultures and clinical samples. Kasten *et al.* (1997a) have used oligonucleotide primers based on a *psl* gene from *P. multocida*. Studies using these in PCR demonstrated that they were effective in detecting *P. multocida* in avian species.

A rapid, highly sensitive colony lift-hybridisation assay was developed using a commercially available multicolour detection kit that can detect toxigenic *P. multocida* and *Bordetella bronchiseptica* (Register *et al.*, 1998). The assay can screen the primary plate culture from clinical samples, allowing quick and easy identification of colonies.

Townsend *et al.* (1998) have developed PCR based assays for identification of specific species and serotypes of *P. multocida* based on a DNA sequence unique to *P. multocida* (KMT1). This technique does not require the extraction and purification of genomic DNA and can be used directly on colonies and could provide rapid species identification, even in mixed cultures. This method is rapid and reliable, with high sensitivity; however, it does falsely identify *Pasteurella canis*. In addition, two PCR assays specific for serogroup B *P. multocida* have been utilised and developed independently (Brickell *et al.*, 1998, Townsend *et al.*, 1998). Both assays were based on sequences unique to haemorrhagic septicaemia causing strains, and were within 1 kb of each other. The technique developed by Townsend *et al.* (1998) is specific for serogroup B only; however, the assay used by Brickell *et al.* (1998) was shown to also detect serogroup E.

More recently a multiplex PCR system based on differences between the genes involved in capsule biosynth. in *P. multocida* was described by Townsend *et al.* (2001). The system correlates with the conventional capsular serotyping system, and has corrected the ambiguous typing of some serogroup F strains which were previously typed as A strains. The system has proved useful in providing a rapid definitive classification of *P. multocida* capsular types.

The use of restriction endonuclease analysis (REA) has been investigated and it was found that *Hha*I and *Hpa*II are the most useful restriction enzymes for producing easily distinguished profiles (Blackall *et al.*, 1995, Diallo *et al.*, 1995, Wilson *et al.*, 1992). In particular, *Hha*I gave profiles that could distinguish between the 16 Heddleston serotypes (Wilson *et al.*, 1992).

Ribotyping has been used in conjunction with REA to differentiate between porcine and avian strains of *P. multocida* (Blackall *et al.*, 1995, Gardner *et al.*, 1994, Snipes *et al.*, 1989), while ribotyping of Australian and U.S. strains has demonstrated considerable genomic differences between local strains (Blackall *et al.*, 1995, Carpenter *et al.*, 1991).

Pasteurellosis

Diseases

P. multocida causes disease in most production animals and is associated with many conditions collectively referred to as pasteurellosis. *P. multocida* is an opportunistic pathogen that is responsible for secondary infections in many animals including humans; however, it can also act as a primary pathogen (De Alwis, 1984).

Haemorrhagic septicaemia

Bovine haemorrhagic septicaemia (HS) is a form of primary pasteurellosis that affects most ungulates, in the form of an acute septicaemia (De Alwis, 1984). It is prevalent in many countries, particularly in Southern and South-East Asia, the Middle-East, Southern Europe, Africa, and in bison in the U.S.A. (De Alwis, 1984). HS is caused by serotypes 6:B or 6:E (Namioka-Carter) or B:2 or E:2 (Carter-Heddleston).

The clinical signs of HS as described by De Alwis (1984) are initially an elevated temperature, and then submandibular oedema that spreads to the brisket region. This is then followed by respiratory distress, shortly after which the animal becomes prostrate and dies. This illness lasts 1-3 days, and the first sign may be the sudden death

of the animal. HS causes widespread haemorrhaging throughout the animal's body particularly in the heart, lungs, and the intestines.

Bovine chronic respiratory disease (BCRD)

A number of different microorganisms including bacteria and viruses may be involved in the aetiology of the BCRD syndrome. Pneumonic pasteurellosis or shipping fever is one of the significant components of this disease syndrome.

Pasteurella species may be involved as primary or secondary invaders in BCRD and the infections can be caused by different *Pasteurellaceae* species (eg *P. multocida* and *M. haemolytica*). The most common serotype of *P. multocida* involved in BCRD syndrome is capsular serotype A (De Alwis, 1993). There have been reports of toxigenic strains of *P. multocida* (capsular serotype D) also being involved in causing pneumonic pasteurellosis (Erler *et al.*, 1993). The losses due to BCRD in the U.S.A. are believed to be greater than all the other diseases of cattle put together (De Alwis, 1993). Viruses involved in the etiology of BCRD syndrome may include parainfluenza virus-3, infectious bovine rhinotracheitis virus (IBR), bovine virus diarrhoea (BVD), and bovine respiratory syncytial virus (BRSV) (Radostits *et al.*, 1994). The BCRD syndrome manifests when animals are transported over long distances in overcrowded conditions as encountered in the movement of animals from saleyards to feedlots. The close proximity of the animals results in a rapid transmission of *Pasteurella sp.* from animal to animal.

The clinical signs of BCRD include unexpected dyspnoea (shortness of breath), coughing, nasal discharge, depression and anorexia, followed frequently by death due to pneumonic pasteurellosis (Radostits *et al.*, 1994). An elevation of body temperature ranging from 40-41.5°C is the first sign seen in feedlots. There also is usually evidence of pneumonia that may be accompanied by pleuritis (inflammation of the pleural membranes).

Swine Pasteurellosis

There are three types of swine pasteurellosis, viz.; atrophic rhinitis, pneumonic pasteurellosis, and septicaemic pasteurellosis. Pneumonic pasteurellosis of swine is usually caused by capsular serotypes A and D of *P. multocida* (White *et al.*, 1993). This microorganism also plays an important role as a secondary invader in the aetiology of epizootic pneumonia of pigs caused by *Mycoplasma hyopneumoniae,* and causes

significant mortality and morbidity (White *et al.*; 1993). Clinical manifestations of pneumonic pasteurellosis include a fever of up to 41°C, disinclination to move, and significant respiratory distress (laboured respiration) (Radostits *et al.*, 1994). In chronic disease there may be reduced weight gain, and frequent relapses. Pleuritis and inflammation around the heart are typical features found on post mortem examination.

Septicaemic pasteurellosis is found occasionally in pigs and its clinical manifestations are fever, dyspnoea, and oedema of the throat and lower jaw (Radostits *et al.*, 1994). In piglets death can occur within 12 hours without any signs of pneumonia.

Atrophic rhinitis is caused primarily by capsular serotype D, and in particular is associated with the *Pasteurella multocida* toxin (PMT) (De Alwis, 1993). Clinical signs are low weight gain, upper respiratory distress (sneezing), turbinate lesions, and in severe cases twisting of the snout due to atrophy (Confer, 1993). The turbinate atrophy has been attributed to PMT. This disease may also occur in association with *Bordetella bronchiseptica* (De Alwis, 1993).

Other diseases

P. multocida also causes disease in other animals and humans. In particular it is well known for causing "snuffles" (purulent rhinitis) in laboratory rabbits, which often leads to other sequelae such as conjunctivitis, otitis media, and pulmonary abscesses (Manning, 1984).

Fowl cholera

Significance

Fowl cholera is a highly contagious disease, and this combined with high mortality and morbidity has resulted in it being listed as a disease of economic importance in the poultry industry. Fowl cholera in the U.S. was determined to be one of the top two infectious diseases affecting the turkey industry (Carpenter *et al.*, 1988). Fowl cholera is prominent in Australia and was identified as a key area for research due to losses estimated between $3-6 million per year (Australian Chicken Meat Research and Development Corporation).

Epidemiology

Fowl cholera has a worldwide distribution and occurs in all poultry producing countries. The occurrence of fowl cholera can be sporadic, varying in location and prevalence (Rhoades *et al.*, 1989). Most reported cases involve domestic poultry. However, all types of wild birds are susceptible, with susceptibility varying with different species (Rhoades *et al.*, 1989).

The Disease and pathogenesis

Fowl cholera occurs in acute, subacute, and chronic forms, and affects poultry (chickens, ducks, turkeys etc.) (Adlakha, 1967). The first two forms are associated with high mortality, whereas the latter is associated with localised infections of wattles, sinuses, and joints (Adlakha, 1967, Rhoades *et al.*, 1989). *P. multocida* is considered to be the primary pathogen of fowl cholera, with serogroup A, serotypes 1, 3 and 4 being isolated most frequently (Adlakha, 1967, Rhoades *et al.*, 1989). Clinical signs in acute septicaemia include disseminated intravascular coagulation, haemorrhages, hepatic and splenic necrosis, and fibrinous pneumonia, whereas chronic cholera is characterised by necrosis of the wattles, lungs, air sacs, foot pads, bones and joints (Confer, 1993).

Chemotherapy & treatment

Most isolates are highly sensitive to penicillin, ampicillin, cephalothin, enroflaxin, choramphenicol, streptomycin, and nitrofurantoin (Abeynayake *et al.*, 1993). Fusadin, sulphamethaxole, spiramycin, and clindamycin resistance was present in many of the isolates tested and use of these has been discouraged. Ceftiofur a newly developed cephalosporin, and cefquinone have also shown to be effective in treatment of pasteurellosis (Chandrasekaran, 1993) . Semjen, Magyar, and Laczay (1998) have shown that doxycycline is effective in cases of fowl cholera.

Virulence determinants and protective antigens

Capsular polysaccharide (CPS)

A capsular polysaccharide is produced by many strains of *P. multocida* and is the basis for the Carter serotyping scheme. The capsule has been indicated as a major virulence factor, and the loss of capsule has been shown to cause *in vivo* attenuation in a turkey strain during infection (Snipes *et al.*, 1987). All serotypes have been shown to

produce CPS, however serotype A strains tend to produce the most capsular material which accounts for the mucoid appearance of colonies (Carter, 1967).

The polysaccharide component of serotype A strains has been identified as hyaluronic acid (HA) which has been shown to be non-immunogenic in a purified form (Confer, 1993), consistent with the fact that HA is a normal component of vertebrate tissues (DeAngelis *et al.*, 1998). Thus the CPS of type A strains would act as a camouflage to host immune defences. The CPS is closely associated with many proteins, polysaccharides, and lipids including LPS (Carter, 1967), and the antigenicity of CPS extracts injected into animals has been attributed to the presence of these (Knox *et al.*, 1960).

Capsules are known to possess antiphagocytic properties and studies have shown that the HA in type A strains indeed demonstrates this property (Harmon *et al.*, 1991, Maheswaran *et al.*, 1979). It has been suggested by Snipes *et al.* (1987) that encapsulated strains also have resistance to intracellular bactericidal mechanisms of hepatic phagocytes, thus allowing them to survive within liver tissue. Significant protection from complement was also suggested in capsulated strains (Snipes *et al.*, 1987). HA has also been proposed to act as an adhesin during infection, and hyaluronidase treated serotype A *P. multocida* showed markedly less adhesion to HeLa cells than untreated *P. multocida* (Esslinger *et al.*, 1994). More recently adhesion to turkey macrophages without internalisation was shown to be mediated by HA via an isoform of CD44 (Pruimboom *et al.*, 1999).

The composition of other capsular serotypes of *P. multocida* has remained elusive, and studies have so far only investigated the composition of serotypes A, B, D, and F. The composition of type D was found to be composed of heparin, while the composition of F was found to be chondroitin (DeAngelis *et al.*, 2002). Both of these monomers are commonly found in the vertebrate host and are non-immunogenic which makes these effective virulence factors. Purified capsule of serogroup B has been shown to be comprised of arabinose, mannose, and galactose, but no linkage of these sugars was determined (Muniandy *et al.*, 1992).

The complete nucleotide sequence of the capsule biosynthetic locus of *P. multocida* strain X-73 (A:1), was determined by Chung *et al* (1998) and was shown to have 11 ORFs organised into three regions typical of Gram-negative bacteria (Boulnois *et al.*, 1990). The first region was composed of four genes with homology to proteins involved in transport in several bacteria including *Haemophilus influenzae*

(Chung *et al.*, 1998). Region two comprised five genes designated *hyaABCDE* that were proposed to encode proteins involved in the synthesis of hyaluronic acid. HyaD has been shown previously to be the hyaluronic synthase from *P. multocida* by De Angelis (1998). Region three comprised two genes proposed to be involved in the anchorage of the capsule in the outer membrane.

The capsule locus of serotype B has also recently been cloned and has the same three regions as serogroup A (Boyce *et al.*, 2000b). However there are nine genes that are proposed to be involved in the formation of the serogroup B capsular polysaccharide and these have little or no identity with serogroup A region two genes (Boyce *et al.*, 2000c). This is consistent with the different capsular polysaccharides produced by the two strains. The flanking regions one and three are proposed to perform similar functions to those in serogroup A and have moderate identity to those found in serogroup A. Interestingly, the sequences flanking both capsule loci share 99% identity, indicating that the chromosomal position of the capsule locus is conserved.

A defined acapsular mutant in *P. multocida* (serogroup A:1) was constructed by insertionally inactivating the *hexA* gene with a *tet* (M) antibiotic cassette (Chung *et al.*, 2001). The *hexA*::*tet*(M) strain was shown to be attenuated in both mice and the natural chicken host, and sensitive to chicken serum whereas the wild-type was not. A vector containing an intact copy of *hexAB* was used to complement the *hexA*::*tet*(M) strain and was shown to restore virulence to that of the wild-type strain in mice but not in chickens. The complemented strain was also shown to be resistant to chicken serum, indicating restoration to the wild-type phenotype. The attenuation of the complemented mutant in chickens but not in chicken serum was proposed to be due to slow growth at 42°C (normal chicken temperature) and possibly abnormal presentation of the capsule (Chung *et al.*, 2001). The *cexA* gene in serogroup B:2 was inactivated in a similar manner to *hexA* by insertion of *tet*(M) and was shown to also be attenuated in mice and susceptible to phagocytosis by murine peritoneal macrophages (Boyce *et al.*, 2000a). In contrast to the *hexA*::*tet*(M) strain, the *cexA*:: *tet*(M) strain was resistant to bovine and murine serum. An additional gene *bcbH* was also inactivated by *tet*(M) insertion and the resultant strain was also attenuated in mice (Boyce *et al.*, 2001). Both serogroup B:2 mutant strains were shown to stimulate protection to subsequent challenges with wild-type B:2. The generation of these three mutant strains has further elucidated the role of CPS in immunity and definitively shown that immunity can be stimulated in its absence.

Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) is found in Gram-negative bacteria and has toxic properties, hence the term endotoxin. The LPS of *P. multocida* has been shown to contain lipid A, KDO, heptose and glucose, which is similar to LPS of other Gram-negative bacteria (Rimler *et al.*, 1984). However the KDO content is much lower than in other bacteria (Manning, 1984, Rimler *et al.*, 1984). The toxicity of LPS is due to the lipid A portion of the molecule, and the pyrogenic potency of *P. multocida* is comparable to that of some *Salmonella* species (Manning, 1984). The LPS is also the basis for somatic typing of *P. multocida*.

The role of LPS in immunity remains controversial as it has proven difficult to obtain pure LPS without contaminating polysaccharide or proteins. LPS has been shown to be protective against both homologous and heterologous challenge in passive immunisation experiments (Wijewardana *et al.*, 1990). This was due to a mechanism associated with complement-dependant, antibody-mediated bacterial killing. In contrast, Ramdani *et al.* (1991) using active immunisation experiments, reported that purified LPS afforded only partial immunity in mice that was serotype specific. However, Ramdani *et al.* (1991) used a B:2 serotype whereas Wijeewardana *et al.* (1990), used capsular serotype A. Muniandy *et al.* (1998) demonstrated that the protective potential of LPS in serogroup B was due to its associated proteins, more than the LPS itself. This is consistent with the varying degrees of protection shown by various extracts, as the amount and type of protein contamination may affect the immunogenicity.

Outer membrane proteins (OMPs)

Outer membrane proteins (OMPs) are commonly investigated in bacteria due their location on the outer surface of bacteria and hence exposure to the extracellular host environment. Thus they are potential targets of the host immune system. They frequently have a role in virulence such as involvement in adhesion, nutrient uptake, iron acquisition and resistance to the host environment. A number of OMPs have been characterised from *P. multocida,* but until recently the genes encoding them have been unknown, and hence many have not been cloned or expressed.

OmpH

A protein designated OmpH was described as a major porin of *P. multocida* and was estimated to be 36 kDa in size and accessible at the cell surface (Chevalier *et al.*,

1993, Lugtenberg *et al.*, 1986). Amino acid sequencing of OmpH from the N-terminal region identified it as a homologue of the P2 porin from *Haemophilus influenzae*, which has a trimeric arrangement in the membrane (Chevalier *et al.*, 1993). The *ompH* gene was cloned and OmpH was shown experimentally to have porin activity (Luo *et al.*, 1997). A monoclonal antibody against OmpH was shown to passively protect mice against infection (Vasfi Marandi *et al.*, 1997b) and immunisation of chickens with recombinant OmpH provided immunity against homologous challenge (Luo *et al.*, 1997). Analysis of the OmpH protein sequences from fifteen strains of *P. multocida* was indicated that variability in the protein was confined to two loops (Luo *et al.*, 1999). These variable regions were synthesised and it was found that the peptide mimicking loop 2 provided 70% protection in chickens against the homologous X-73 strain (Luo *et al.*, 1999). Recently it was found that *ompH* expression is controlled by the iron uptake regulator *fur* and is inhibited in the presence of glucose (Bosch *et al.*, 2001). In light of this, Bosch *et al.* (2001) suggested that bacteria for use in vaccines should be grown in the absence of glucose to maximise the presence of OmpH.

32 kDa OMP

A 32 kDa protein with N-terminal amino acid sequence identity to OmpH has also been identified, and was shown to be located in the outer membrane (Vasfi Marandi *et al.*, 1996). Polyclonal antibodies raised against the 32 kDa Omp showed reactivity with both the 37 kDa OmpH and the 32 kDa protein. However, monoclonal antibodies showed reactivity with only the serogroup D strains bearing the 32 kDa, but not the 37 kDa OMPs. Due to the specificity of this monoclonal antibody it was subsequently used in an ELISA assay for rapid identification of serogroup D strains (Vasfi Marandi *et al.*, 1997a).

Oma87

An 87 kDa OMP designated Oma87 was cloned, sequenced and expressed in *E. coli* (Ruffolo *et al.*, 1996). Oma87 showed high similarity to the D15 OMP of *H. influenzae* which was shown to be protective in rodents (Loosmore *et al.*, 1997). Oma87/D15 homologues have been identified in several Gram-negative species and protective epitopes localised to the N-terminus region of the protein (Yang *et al.*, 1998). Subsequently the recombinant N-terminal 25% of Oma87 from serogroup D was

overexpressed and purified (Mitchison *et al.*, 2000). Chickens immunised with this protein were afforded no protection against challenge by an A:1 strain despite >95% identity of Oma87 between the two strains. Since Oma87 has been shown to be protective in a closely related species of bacteria, further investigation of its role in immunity to *P. multocida* may be useful.

50 kDa OMP

A 50 kDa OMP has been described that was identified as inhibiting phagocytic activity of avian mononuclear cells (Truscott *et al.*, 1988). Turkeys given antibodies specific for the 50 kDa outer membrane protein were shown to be protected against lethal challenge with *P. multocida*. The OMP was suggested to be totally or partly responsible for the survival of *P. multocida* intracellularly.

P6-like protein

The gene encoding a 16kDa protein found in all 16 somatic serotypes was cloned, sequenced and found to have extensive sequence homology with a gene encoding the P6 protein of *H. influenzae* (Kasten *et al.*, 1995). Although the P6 protein from *H. influenzae* was protective, immunisation of turkeys with recombinant P6 failed to protect them against challenge by *P. multocida* (Kasten *et al.*, 1997b).

37.5 kDa OMP

A 37.5 kDa protein was found to be the target of a monoclonal antibody against *P. multocida* (Lu *et al.*, 1988, Lu *et al.*, 1991). Homologous protection in rabbits was stimulated by the protein, and the monoclonal antibody raised against it conferred homologous and heterologous protection in mice and rabbits (Lu *et al.*, 1991). However, not surprisingly heterologous protection was shown only against the strains which produce the 37.5 kDa protein (Abdullahi *et al.*, 1990).

Omp28 (A) & P1

N-terminal amino acid sequencing of a heat labile 35 kDa OMP identified a protein with 80% similarity to OmpA from *E. coli* and P5 from *H. influenzae* (Dabo *et al.*, 1997). The main role of OmpA has been shown to be the maintenance of structural

integrity of the outer membrane (Dabo *et al.*, 1997). The protein was also shown to be recognised by antibodies raised in cattle against *P. multocida* A:3, by Western blot and ELISA, demonstrating its antigenicity (Confer *et al.*, 1996). OmpA was recently renamed Omp28 and studies indicated that although it was antigenic, it failed to provide protection in mice against homologous challenge

N-terminal amino acid sequencing of another heat labile 46 kDa OMP showed it to have 96% identity with *H. influenzae* P1 (Dabo *et al.*, 1997). The role of P1 is unknown, but antibodies raised against *P. multocida* A:3 in cattle also reacted with this protein indicating that it was also immunogenic and expressed *in vivo* (Confer *et al.*, 1996).

The role of both these proteins in pathogenesis and immunity is currently under investigation (Dabo *et al.*, 1997).

Iron acquisition and iron regulated OMPs (IROMPs)

Studies of *P. multocida* growth under iron restricted conditions have identified a number of proteins that are expressed under iron-limited growth conditions. Low iron often induces expression of bacterial genes (Mekalanos, 1992), and it has been proposed that cross-protective antigens may be induced by low iron conditions (Ikeda *et al.*, 1988). Iron is important for the growth and survival of bacteria, but iron in the host is usually unavailable as it is bound to iron binding proteins such as lactoferrin or transferrin (Payne, 1988). Due to this, bacteria must utilise systems capable of scavenging iron while growing in the host. Siderophores are low MW compounds that remove iron from host glycoproteins and in conjunction with specific OMPs import iron into the bacterial cell. *P. multocida* serogroup A strains were shown to release a siderophore (termed multocidin) into culture supernatants during growth under iron limited conditions (Hu *et al.*, 1986). Three OMPs (70 kDa, 84 kDa, and 94 kDa in size) from *P. multocida* expressed during growth in iron-restricted conditions (IROMPs), were shown to bind to the multocidin-iron complex (Choi-Kim *et al.*, 1991). They were also expressed by *in vivo* grown *P. multocida*, thus supporting a role for their involvement in iron acquisition during infection.

An 84 kDa OMP was shown to be expressed under iron-limited conditions by a serotype 3 turkey strain of *P. multocida* (Ikeda *et al.*, 1988). Antigenically related proteins were found in all other somatic serotypes except possibly 12, indicating its

potential cross protective ability. It was also shown to be expressed with proteins 96 kDa and 80 kDa in size when grown in turkey plasma, indicating possible expression *in vivo* (Snipes *et al.*, 1988).

Kennett *et al.* (1993) found that a type 6:B *P. multocida* strain produced high MW OMPs under iron-limited conditions that, when used to immunise, provided homologous protection. Glisson *et al.* (1993) found that cross protection could be induced in chickens and turkeys by bacterins produced from *P. multocida* grown under iron-limited conditions; however, this protection appeared inconsistent. More recently, Ruffolo *et al.* (1998) achieved heterologous protection against a low challenge dose in mice, using OM fractions from *P. multocida* grown under iron limited conditions.

Serogroup B strains have been shown to produce an OM transferrin binding protein (Tbp) that binds directly to host transferrin, and removes iron from it (Veken *et al.*, 1996). Furthermore, serogroups B and E both bound haemoglobin, and growth was restored in iron depleted conditions by the addition of transferrin or haemoglobin (Veken *et al.*, 1996). Ogunnariwo *et al.* (1991) identified an 82 kDa protein that was produced under iron-limited conditions and bound to bovine transferrin. However, they found that avian strains neither produced this IROMP, nor showed an ability to bind transferrin. The 82 kDa protein was affinity purified from bovine isolates of *P. multocida* using immobilised bovine transferrin and was found to be TbpA. However, amino acid sequencing showed that it was substantially different from other members of the TbpA family and was not in *tbpAB* operon arrangement as seen previously in other bacteria (Ogunnariwo *et al.*, 2001). For this reason it was proposed that in *P. multocida* TbpA is capable of mediating iron acquisition without the involvement of TbpB (Ogunnariwo *et al.*, 2001).

A haemoglobin binding protein was cloned and characterised from a type D serotype of *P. multocida* and designated HgbA (Bosch *et al.*, 2002b). The *hgbA* gene was disrupted by insertional inactivation and the resultant strain was found to bind haemoglobin to the same extent as the wild-type strain indicating that there were alternative haemoglobin receptors in *P. multocida* (Bosch *et al.*, 2002b). In agreement with this, the *hgbA* mutant was still virulent in mice. *hgbA* was found to be iron regulated and a recombinase expression system showed that its expression was triggered within two hours in a mouse infection model. *hgbA* was also shown to be present in all serotypes of *P. multocida* analysed indicating it may be useful as a protective antigen against all *P. multocida* strains. The *hgbB* gene was recently cloned and characterised

from a *P. multocida* type A strain (Cox *et al.*, 2003). Recombinant HgbB was found to bind haemoglobin but a truncated form did not. Insertional inactivation of *hgbB* did not affect binding of haemoglobin nor its virulence in mice. This is consistent with the work involving *hgbA* and identifies a redundancy in the haemoglobin binding ability in *P. multocida*.

Iron specific receptors present on the surface of bacteria, such as TbpA and HbpAB, require the presence of the TonB protein which acts as a pore for the iron to pass through, and the ExbB/D proteins which stabilise TonB (Braun, 1995, Higgs *et al.*, 1998). The *tonB*, *exbB*, and *exbD* genes from *P. multocida* were cloned and found to be physically linked but independently transcribed (Bosch *et al.*, 2002a). All three genes were found to be regulated by iron, as was expected, and inactivation of any of the genes resulted in attenuation of virulence, indicating they are all required for virulence.

In *E. coli* the *fur* gene controls iron uptake by regulating IROMPs, and is regulated by Fe^{2+}-dependent DNA binding activity (Escolar *et al.*, 1999). The *fur* gene of *P. multocida* was cloned and was found to be regulated by its own product as well as iron (Bosch *et al.*, 2001). A *fur* mutant was constructed, but not found to be attenuated. However, it caused increased expression of the OMP gene *ompH*, as discussed previously.

Cross protective factors (CPFs)

The ability of various *in vivo* expressed OMPs to provide heterologous protection against different somatic strains has been discussed briefly above. *In vivo* expressed antigens with this ability have been termed cross protective factors (CPFs). Heddleston *et al.* (1974) found that bacterins made from infected embryonated turkey eggs stimulated cross-protective immunity against *P. multocida*. Various *in vivo* OMP preparations have been shown to protect poultry against heterologous serotypes, whereas the same strains grown *in vitro* provided only homologous protection (Wang *et al.*, 1994a, Glisson *et al.*, 1991, Heddleston *et al.*, 1972a). This is most likely due to artificial media not providing stimuli to prompt expression of CPFs. The exclusive expression of these CPFs *in vivo* indicates their possible role in virulence (Choi-Kim *et al.*, 1991, Glisson *et al.*, 1991, Ikeda *et al.*, 1988, Snipes *et al.*, 1988). A 39 kDa protein and proteins of 59-65 kDa were found that were proposed to provide cross protective immunity, although this was also suspected to be due to contaminating carbohydrates

(Rimler, 1994). Recently, the 39 kDa protein was confirmed to stimulate both active and passive cross protection in turkeys, and was localised to the outer membrane (Rimler, 2001, Rimler *et al.*, 2001). High molecular weight membrane proteins between 153-204 kDa in size were proposed to be responsible for cross protection, and were shown to be common to serotypes 1 and 3 (Wang *et al.*, 1994a, Wang *et al.*, 1994b). Although membrane proteins were identified that were expressed by *in vivo* propagated bacteria but not by *in vitro* propagated bacteria, it was not determined if these were cross-protective (Glisson *et al.*, 1991). Thus, the precise identity of individual CPFs awaits further study.

Type IV fimbriae

The presence of fimbriae in *P. multocida* was described by Glorioso *et al.* (1982) and by Rebers *et al.* (1988), and was proposed to be responsible for attachment of serogroup A strains to mucosal epithelium. Type 4 fimbriae were identified by Ruffolo *et al.* (1997) in *P. multocida* and the fimbrial subunit protein was designated Ptfa. The *ptfA* gene encoding the fimbrial subunit has been cloned, and the role of fimbriae in infection by *P. multocida* is currently under investigation (Doughty *et al.*, 2000).

Enzymes

Neuraminidase

Scharmann *et al.* (1970) first described the production of neuraminidase by *P. multocida*. It was identified in 102 of 104 strains tested and was shown to be bacterial cells. Neuraminidase acts by removing sialic acid from glycoproteins and this is thought to be useful for protection during infection of the host (Gottschalk 1960, as cited by (Straus *et al.*, 1996b)). The activity of neuraminidase has been shown to be inducible by sialic acid, and N-acetyl-D-mannosamine (Drzeniek *et al.*, 1972). Furthermore the neuraminidase produced by *P. multocida* type A:3 was found to degrade N-acetylneuramin lactose, human α-1-acid glycoprotein, fetuin, colominic acid, and bovine submaxillary mucin (White *et al.*, 1995). *P. multocida* neuraminidase is heat labile and approximately 75% of its activity is lost after incubation at 50°C for 30 minutes (White *et al.*, 1995).

Neuraminidase from *P. multocida* was reported to be approximately 500,000 kDa in size (Straus *et al.*, 1996b). This was in disagreement with that reported by

Drzeniek *et al.* (1972), who estimated it to be around 250,000 kDa. The apparent discrepancy was resolved recently when two neuraminidases (NanH, NanB) were described in a type A strain of *P. multocida* Mizan *et al.* (2000). NanH was 80 kDa in size and demonstrated sialidase activity on 2-3' sialyl lactose, while NanB was 120 kDa and could degrade both 2-3' and 2-6' sialyl lactose. Both NanH and NanB were proposed to have a nutritional function, as recombinant NanH and NanB were able to use several sialoconjugants as a sole carbon source.

P. multocida neuraminidase is not serologically related to *M. haemolytica* neuraminidase (White *et al.*, 1995). Straus *et al.* (1996a) recently demonstrated that neuraminidase was produced *in vivo* during active *P. multocida* infections, thus reinforcing its involvement in pathogenesis. Immunisation with *P. multocida* neuraminidase was able to provide protection against homologous challenge and the antibodies produced against neuraminidase effectively neutralised its enzymatic activity (Straus *et al.*, 1996a). Antibodies raised against one serotype were effective in reducing the neuraminidase activity of other serotypes; antiserum raised against neuraminidase from serotype A:3 caused a 30.8% reduction in neuraminidase activity from serotype 7, and a 59.6% reduction in neuraminidase activity from serotype 8 (Straus *et al.*, 1996a).

P. multocida toxin (PMT)

P. multocida serogroups A and D have been shown to produce a dermonecrotic toxin, that has been called *P. multocida* toxin (PMT), which has been shown to be a principle agent in causing atrophic rhinitis in pigs. The *toxA* gene was cloned and modified to produce variant derivatives of the toxin, one of which was shown to induce protective immunity in mice (Petersen *et al.*, 1991). A proposed regulator gene *txaR* was found upstream of *toxA*, and deletion of *txaR* resulted in a ten-fold increase in toxin production (Petersen, 1990). However, a later study found no effect on toxin production after *txaR* was deleted (Hoskins *et al.*, 1996), indicating that PMT may be constitutively expressed. The production of PMT has been suggested to play a role in some avian infections in serogroup A strains (Christensen *et al.*, 1997); however, the presence of PMT in avian serogroup A strains is limited.

Proteases

The presence of proteases has been reported in *P. multocida* from both human and animal isolates (Negrete-Abascal *et al.*, 1999, Poucdras *et al.*, 1992). Human

isolates were shown to produce proteases that cleaved human immunoglobulins IgA and IgG (Pouedras *et al.*, 1992). Secreted metalloproteases were demonstrated to be present in chicken, pig, bovine, and sheep isolates of *P. multocida* and IgG degradation by these proteases was also observed (Negrete-Abascal *et al.*, 1999).

Prevention

Commercial vaccines

Several types of commercial vaccines have been used for prevention of pasteurellosis; however, these are relatively ineffective. Hence there is need for better alternatives. The broth bacterin is the most widely used vaccine, due to its ease of production, and cost effectiveness (De Alwis, 1984). Broth bacterins have been used for prevention of HS, fowl cholera, and in prevention of BCRD. However, the level of immunity is poor (De Alwis, 1993). The broth bacterin stimulates immunity for only approximately 2-9 months against HS (De Alwis, 1984). BCRD vaccines composed of bacterin and viral vaccines have been used with limited success (Radostits *et al.*, 1994). Alum-precipitated vaccine (APV) is used extensively in Asian countries and has been claimed to provide immunity for 5-6 months against serotype B strains (De Alwis, 1984). This is similar to earlier reports by De Alwis *et al.* (1978) who found that it induced immunity that lasted for 3-4 months. Aluminium hydroxide gel vaccine (AV or Al-gel) is another vaccine that is in use mainly in Thailand against capsular serotype B, and has been reported to induce immunity for up to 4 months against HS (De Alwis, 1984).

Oil adjuvant vaccine (OAV) is the most potent of the commercially available vaccines and is used mainly for prevention of HS. This vaccine typically combines concentrated bacteria, light oil/s, and an emulsifier (De Alwis, 1984). It is popular in Malaysia, Indonesia, Sri Lanka, Egypt, Iraq, and to a smaller extent in India, and Bangladesh (De Alwis, 1984). Its duration of immunity is variable, with trials in India and Sri Lanka indicating 6-9 months, and up to 1 year in Malaysia (De Alwis, 1984). However, these trials were conducted in areas free from haemorrhagic septicaemia, and areas enzootic with HS showed immunity for up to 14 months (De Alwis, 1984). OAV suffers from problems with administration due to its high viscosity and the objective of many studies has been to reduce the viscosity without compromising immunity. OAVs can also cause adverse reactions at the point of injection such as inflammation and local necrosis (Radostits *et al.*, 1994).

A vaccine consisting of an attenuated strain of *P. multocida* called the Clemson University vaccine (CU) has been used for the prevention of fowl cholera (Derieux, 1984). The duration of immunity via the stickwing method was reported to be from at least 20 weeks to 80 weeks (Derieux, 1984). However there have been reports of reversion to virulence (Hopkins *et al.*, 1997).

Currently there are no satisfactory commercially available vaccines suitable for providing protection against the disease syndromes associated with *P. multocida* type A strains. Hence there is a need to develop suitable vaccines.

Experimental vaccines

Much work has been devoted to producing effective vaccines that will give a longer duration of immunity and provide cross-protection against all serotypes of *P. multocida*, as alternatives to broth bacterins, AVs and OAVs.

A new form of bacterin was recently developed by Purdy *et al.* (1997), using *P. multocida* type A:3 (Carter-Heddleston). This work showed that the protection afforded by the bacterin was equal to that of live organisms. However, the duration of immunity was not tested. A bacterin based on the cross-protective features of IROMPs was produced by Glisson *et al.* (1993), but it did not provide consistent protection.

Reddy *et al.* (1996) showed that OAV prepared using montanicide ISA 206 induced protective antibody titres lasting over 12 months, compared to AV where titres lasted up to 6 months. An OAV using Mercol 52 as the mineral oil and Tween 85 and Span 85 as the emulsifiers provided protection for at least 250 days (Shah *et al.*, 1997). This vaccine was more protective than the broth bacterins, and did not require any boosters.

Several vaccines have been produced by using attenuated or 'non-pathogenic' strains of *P. multocida*. One of these, the CU strain, has already been described. Recently Hopkins *et al.* (1997) have described another vaccine tentatively called PM-1 that is composed of another avirulent strain of *P. multocida*. The PM-1 vaccine was evaluated, and found to offer no better protection than the CU vaccine in the prevention of fowl cholera in turkeys. However both strains have been shown to revert to virulence under some circumstances (Hopkins *et al.*, 1997). The growth of the infected turkeys tested was also impaired leading to the conclusion that this vaccine is not particularly useful.

An HS vaccine consisting of a strain of *P. multocida* that was obtained from Fallow deer has been tested for effectiveness for the prevention of haemorrhagic septicaemia (Myint *et al.*, 1987). The strain (B:3, 4 (Carter-Heddleston)), was shown to be less virulent than the B:2 serotypes that cause haemorrhagic septicaemia. It was shown to be protective against *P. multocida* serotype B:2, and the protection lasted for six months in five out of the six calves tested, and for a further three months in two of these animals. The protection was also shown to be more efficacious than that induced by APV. The immunity was proposed to be due to capsular antigens that are common between capsular type B *P. multocida*. This vaccine is easier to inject than OAV due to lower viscosity, but has a shorter length of immunity (6 months).

The inherent risk of using these natural mutants is that there is always a chance of reversion to virulence. This has been illustrated with the CU vaccine and construction of defined attenuated mutants could significantly reduce this risk. Directed attenuated mutants have been constructed in many species by inactivation of genes involved in aromatic amino acid synthesis. The *aroA, D, E, C & F* genes encode products involved in converting shikimic acid to chorismic acid via the common aromatic biosynthetic pathway. Chorismic acid is the precursor of several aromatic compounds of which two (p-aminobenzoic acid and dihydroxybenzoic acid) are not available in the vertebrate host (CSIRO, n.d.). Organisms unable to synthesise these compounds can only survive when cultured in media supplemented with both of these components. This means that the organism can only survive for short periods in the vertebrate host. An *aroA* mutant of *P. multocida* has been constructed (Homchampa *et al.*, 1992, Homchampa *et al.*, 1997) and shown to stimulate cross-protective immunity against a heterologous strain (Scott *et al.*, 1999). Further investigations on the longevity and extent of the cross-protection are currently being undertaken.

Other protective antigens include LPS (Adler *et al.*, 1996, Manning, 1984, Wijewardana *et al.*, 1990) and IROMPs (Adler *et al.*, 1999, Adler *et al.*, 1996, Confer *et al.*, 1995). However, effective fully evaluated vaccines containing these antigens have not been described, and studies are still at the developmental stage.

Genomics and whole genome expression profiling

Until recently the genetics of *P. multocida* was limited to gene homology searches of the closely related *H. influenzae* genome (Fleischmann *et al.*, 1995) the *E. coli* genome (Blattner *et al.*, 1997), and a *P. multocida* genome restriction map (Hunt *et*

al., 1998). The recent determination of the genome of a *P. multocida* strain (May *et al.*, 2001) has allowed *P. multocida* research to expand into previously unavailable areas such as microarray and proteomics work. Furthermore, bioinformatic analysis has identified new putative virulence factors.

Pm70 genome

As mentioned above, the *P. multocida* genome was recently sequenced in the strain Pm70 (May *et al.*, 2001). The Pm70 strain was chosen as it was considered to be responsible for a large proportion of infections in avian hosts. The Pm70 genome consists of a single chromosome of 2,257,487 bp and was predicted to encode 2,015 coding sequences (CDS), 6 ribosomal RNA operons and 57 tRNAs. Sequence comparisons between Pm70, *H influenzae* and *E. coli* revealed 200 CDSs (10%) unique to *P. multocida*, and 1,197 orthologs in both *H. influenzae* and *E. coli*. Of interest, 531 (26%) genes were homologous to hypothetical proteins of unknown function, indicating that a substantial portion of *P. multocida*'s biochemistry and biology is still unknown. Putative virulence factors accounted for 7 percent of the CDS, and two novel genes (*pfhB1, pfhB2*) with strong homology to the filamentous haemagglutinin (FhaB) of *Bordetella pertussis* were found. FhaB is a known virulence factor involved in adherence to host cells and is a major component of the acellular vaccine against whooping cough (Sato *et al.*, 1999). For this reason, further research into the roles of pfhB1 and pfh2 is warranted as they may be useful vaccine candidates.

DNA Microarrays

Since the release of the Pm70 genome there have been several studies using microarray technology to investigate whole genome transcription under various conditions. The first involved identifying differences from Pm70 grown under iron limiting conditions versus normal media. It was found that of the 174 CDS that had a significant change in expression, 53% decreased in expression under iron limitation and 47% increased (May *et al.*, 2001, Paustian *et al.*, 2001). Of these, the *frd* and *fdx* operons had reduced expression, both of which require iron to function. Additionally, 11 homologues involved in glycolysis or electron transport were also decreased in expression. Of the CDS that increased in expression 16 were found to be homologous to iron-specific transport proteins, and the known iron regulated gene families of *fec, yfe, fbp, exb* and *tonB* were also upregulated (May *et al.*, 2001, Paustian *et al.*, 2001).

Another iron related microarray study investigated the response of Pm70 grown in iron-free media supplemented with either: haemoglobin, transferrin, ferritin or ferric citrate. In brief, 12% of genes were differentially expressed and the majority of these were involved in either binding or transport or were hypothetical (Paustian *et al.*, 2002a). Only two genes were upregulated during growth under all the conditions; *ptsN* a putative regulatory protein and *sapD* an ATP binding protein. In general, genes that were upregulated in the presence of haemoglobin did not respond to transferrin or ferritin as iron sources and vice versa (Paustian *et al.*, 2002a). Additionally, genes most responsive to the presence of ferric citrate did not follow a trend similar to that of the other iron sources. This was suggested to be due to different pathways being used to respond to organic and inorganic sources of iron (Paustian *et al.*, 2002a).

A microarray comparing transcription differences between BHI and CDM found that 439 genes were upregulated in the former and 230 in the latter (Paustian *et al.*, 2002b). A large proportion of genes upregulated in BHI compared to CDM were involved in energy metabolism, transport, protein synthesis and binding, including the ATP synthase operon *atpABCDFGH*, and the host cell adherence operon *tadABCEG*. Additionally, a large proportion of genes involved in protein, lipid and nucleotide synthesis were also upregulated in BHI which the authors suggested was due to *P. multocida* switching off energy-intensive pathways upon exposure to a nutrient limited environment (Paustian *et al.*, 2002b). In CDM, genes involved in amino acid synthesis of tryptophan, tyrosine, isoleucine and valine were found to be upregulated compared to *P. multocida* grown in BHI.

Recently a study was conducted that investigated genomic transcription of *P. multocida* during a natural infection (Boyce *et al.*, 2002). This study, was the first to measure gene expression during growth in the host, and utilised the previously mentioned Pm70 arrays hybridised with RNA purified from the blood of three infected chickens. 17 genes were found to be upregulated during growth within all 3 chickens, with the majority of these involved in amino acid transport and metabolism, and energy production and conversion. In contrast most of the downregulated genes (23) were unknown or of poorly characterised function (Boyce *et al.*, 2002). Of particular interest in this study was the variability between the three infections, indicating the effect of individual hosts on determining pathogen gene expression.

In vivo expression systems

The importance of *in vivo* expressed antigens and CPFs in *P. multocida* has been discussed already. In addition, the molecular mechanisms of *P. multocida* pathogenesis are still largely unknown. Therefore, a number of methods have been devised for analysing bacterial gene expression during growth *in vivo*. These have proved useful for identifying unknown virulence factors that are not seen using conventional *in vitro* models.

Signature Tagged mutagenesis (STM)

A transposon mutagenesis system has been developed which identifies genes critical for growth *in vivo* or under particular environmental conditions. (Handfield *et al.*, 1999). Signature-tagged mutagenesis (STM) involves using transposons that are uniquely labelled with DNA sequences or "tags." These uniquely labelled transposons are used to generate mutants in the desired bacteria. The mutants are pooled, and then introduced into the host. Bacteria capable of surviving within the host are collected and screened by colony blots using the unique tag on each mutant, and then compared to the original pool of mutants to detect mutants that exhibit reduced survival in the host (Quinn *et al.*, 1997). These attenuated mutants are a result of the transposon insertion affecting genes that are essential for survival in the host, and are therefore putative virulence factors.

STM has been used to search for virulence genes in a number of organisms. Twenty-eight mutants were obtained using this system with *Salmonella enterica* serovar Typhimurium, thirteen of which were known virulence genes, six were homologues of known genes, and nine were unknown (Hensel *et al.*, 1995). Eighteen mutants were obtained from a murine infection model using *Brucella melitensis*, none of which had been previously described as involved in virulence (Lestrate *et al.*, 2000). Most were involved in biosynthetic activities, but five showed no similarity to characterised proteins.

The use of STM in *Vibrio cholerae* showed that mutations of the toxin-coregulated pilus caused severe defects in colonisation, indicating its critical role in virulence (Chiang *et al.*, 1998). Insertions in LPS, purine and biotin biosynthetic genes also had similar effects. The detection of these virulence factors reinforced the ability of STM to identify genes critical for survival within a suitable infection model.

In *Streptococcus pneumoniae* 126 mutants were identified by STM with insertions in genes involved in virulence, metabolic pathways, DNA repair, genes encoding proteases or transporters, and genes with no known functions (Polissi *et al.*, 1998). Many of the genes were similar to those found using STM in other bacteria such as S. *typhimurium*, indicating that some virulence determinants may be common between different bacteria (Polissi *et al.*, 1998).

STM was applied to *Yersinia enterocolitca* and revealed fifty-five mutants, twenty-eight of which carried insertions in genes in the virulence plasmid, supporting the validity of the system (Darwin *et al.*, 1999). Interestingly, two insertions in the virulence plasmid were in previously uncharacterised regions, and caused severe attenuation. Several insertions were in a gene cluster affecting O-antigen synthesis, while other insertions were identified in genes coding for OMPs, stress response proteins, and proteins involved in nutrient acquisition.

STM has recently been applied to both *P. multocida* and the closely related species *Actinobacillus pleuropneumoniae*. Twenty-five unique mutants were identified in *P. multocida*, with the disrupted genes coding for biosynthetic enzymes, virulence factors, regulatory functions, and a number of genes with no known function (Fuller *et al.*, 2000a). Twenty mutants were found in *A. pleuropneumoniae* and the disrupted genes included biosynthetic, virulence and regulatory genes, and of which four had no homology to characterised genes (Fuller *et al.*, 2000b). Three biosynthetic genes were identified in both *P. multocida* and *A. pleuropneumoniae* indicating common genes required for virulence. Seven of the *A. pleuropneumoniae* mutants were tested for efficacy as live-attenuated vaccines, and although all afforded partial protection to homologous challenge, only four strains caused no mortality (Fuller *et al.*, 2000b).

In vivo expression technology (IVET)

A genetic system, termed *in vivo* expression technology (IVET), was developed for the identification of bacterial genes that are upregulated or highly expressed *in vivo*, but minimally expressed *in vitro* (Mahan *et al.*, 1993a). Genes identified by this method are preferentially expressed *in vivo* and may encode proteins required for virulence.

IVET-auxotrophic selection

The original IVET system was developed in *S. typhimurium* and involved using a suicide plasmid with a promoterless *purA* gene fused to a promoterless *lacZ* gene (Mahan *et al.*, 1993a). Random fragments of genomic DNA were cloned upstream of the *purA* gene, and the recombinant plasmids were then transformed into a *purA* deletion strain of *S. typhimurium*. In this background, expression of *purA* is required for viability. The plasmids integrated into the chromosome by homologous recombination and these resultant fusion strains were used to infect mice. Bacteria were recovered from the spleens of infected mice; thus only strains that had an active *in vivo* promoter fused to *pur*A-*lac*Z survived. The strains were grown on MacConkey indicator medium and Lac$^-$ colonies were chosen for further study as these indicated genes that are poorly expressed *in vitro,* but highly expressed in animal tissues (Mahan *et al.*, 1993a). Five different genes were found driving *purA-lacZ*; two were involved in biosynthetic functions, one in the synthesis of O-antigen, and two did not show homology with characterised genes. Thus, the results were broadly similar to those observed for STM.

A similar method was adapted for use in *Pseudomonas aeruginosa* using a vector carrying a promoterless *purEK* gene (Wang *et al.*, 1996b). The system was employed *in vivo* in mice and *in vitro* in respiratory mucus from cystic fibrosis (CF) patients (Wang *et al.*, 1996a). Fifteen of the genes identified *in vivo* in the mouse had homology to either biosynthetic genes, or genes coding for proteins involved in gene regulation (Wang *et al.*, 1996b). The remaining six showed no homology with known genes, indicating possible unknown virulence factors. The *in vitro* CF mucus study identified three genes, two of which were also found in the *in vivo* study, these being an iron siderophore, and a possible transcriptional regulator (Wang *et al.*, 1996a). The third showed homology to a glycosyltransferase, involved in the synthesis of LPS.

A more recent study employing the original IVET plasmid was used for testing *P. aeruginosa* in a rat lung model, and a mouse model, and also found three genes involved in iron uptake, membrane biogenesis, and adhesion, whilst a further six genes had no known functions (Handfield *et al.*, 2000).

Another modified IVET system was used to analyse gene expression of *A. pleuropneumoniae* during growth in pigs. This system used a vector with a promoterless *ribBAH* gene fused to a promoterless *luxAB* (luciferase) (Fuller *et al.*, 1999). The *rib*

genes are involved in riboflavin synthesis and are essential in a Rib⁻ strain in the absence of riboflavin. A library of *A. pleuropneumoniae* genomic DNA was cloned into the vector, transformed into a Rib⁻ *A. pleuropneumoniae* mutant and used to infect pigs. Ten clones were recovered from the lungs that showed minimal luciferase activity *in vitro*; four of these matched to known proteins, whilst the remaining six had no known homologues.

IVET-antibiotic selection

Another version of IVET that was first used in *S. typhimurium* utilised an antibiotic resistance gene (choramphenicol (*cat*)) in place of *pur*A. The vector pIVET8 had a promoterless *cat-lac*ZY fusion similar to the original IVET vector, and fusions with random genomic fragments were constructed as before (Mahan *et al.*, 1995). Bacteria were selected for the ability to survive in mice treated with chloramphenicol (Cm). The system was also used to select strains able to survive in cultured macrophages grown in media containing choramphenicol. These systems, combined with earlier results obtained from the *pur*A-IVET, have resulted in over one hundred *in vivo* expressed genes being identified in *S. typhimurium,* half of which do not show homology with genes of known function.

A chloramphenicol based IVET vector was also used successfully to detect *in vivo* expressed genes of *Yersinia enterocolitica.* Selection was performed in the Peyer's patches of chloramphenicol-treated mice, and analysis of 48 genes showed that approximately half had similarity to various biosynthetic, regulatory, and iron transport genes (Young *et al.*, 1997). Four showed homology to proteins with no known function, and 18 had no similarity to sequences in public databases.

IVET-Recombination

Another variant on the IVET system has been developed that is useful for detecting promoters that are active only briefly *in vivo* rather than throughout the infection as is required for auxotrophic and antibiotic selection. The system used a promoterless recombinase γδ resolvase gene, that acts to permanently excise regions surrounded by its recognition sites *res* (Camilli *et al.*, 1994). This system was used in *Vibrio cholerae* and a tetracycline gene (*tet*) was inserted between the *res* sites. As before, genomic DNA was ligated into a site before the promoterless reporter gene (γδ resolvase) and the plasmid incorporated into the chromosome. The bacteria were

injected IP into mice and subsequent promoter activity was detected by excision of the *tet* gene, and the resultant inability to grow on media containing Tet. Thirteen *in vivo* expressed genes were detected, of which five performed metabolic and biosynthetic functions, one encoded a secreted lipase, five had unknown functions, and two were proposed to code for antisense genes involved in motility (Camilli *et al.*, 1995). Insertion mutants of thirteen of these genes were tested in infant mouse competition assays, and three demonstrated moderate colonisation defects.

A similar recombination system was employed in *Staphylococcus aureus* in a murine renal abscess model; only six of the genes identified were previously known, eleven had homology to proteins from other species, and the remaining 28 had no similarity to known proteins (Lowe *et al.*, 1998). Eleven of these genes were then mutated, and seven of the mutants showed reduced virulence. Interestingly, six of those seven were genes with no homologues, reinforcing the usefulness of IVET in detecting previously uncharacterised virulence genes.

Differential fluorescence induction (DFI)

A system designated differential fluorescence induction (DFI) employed the use of the green fluorescent protein (GFP) and a fluorescence activated cell sorter (FACS) as an IVET. Fragments of *S.* Typhimurium genomic DNA were cloned upstream of a promoterless *gfp* gene, the recombinant plasmids transformed into *S.* Typhimurium and the library used to infect macrophages (Valdivia *et al.*, 1997). After FACS sorting, macrophages with active *gfp* fusions were retained. The isolated bacteria were then grown in tissue culture medium and sorted by FACS again to obtain clones with low fluorescence. Fourteen genes were identified; of those eight had homologues with known function, and six had homology to uncharacterised proteins. Two of the six unknown genes were disrupted by insertional inactivation and were shown to contribute to virulence as determined by competitive growth assays in mice.

PmIVET 1

An IVET system was developed for *P. multocida* to detect *in vivo* expressed genes (Hunt, Zhang & Adler, unpublished data). The system involved cloning random fragments of *P. multocida* DNA upstream of a promoterless *aroA* gene. These plasmids were transformed into an attenuated *aroA*$^-$ mutant described earlier (Homchampa *et al.*,

1997), that cannot survive *in vivo*. The mutant library was used to infect mice and clones were recovered that were unable to grow on media in the absence of exogenous aromatic acids. Four clones were identified as *in vivo* expressed with this system. However, problems were encountered with many false positives.

Outline of thesis

This project aimed to use an *in vivo* expression system (IVET) to identify potential virulence factors in *P. multocida*. Chapter 2 describes the development and use of a *P. multocida* based IVET system (PmIVET) which was based on the antibiotic selection method utilised by Mahan *et al.* (1995). This system relied on cloned fragments driving the expression of a promoterless kanamycin resistance gene (*kan*). A plasmid with a promoterless *kan* gene was constructed and random *P. multocida* fragments cloned upstream of the *kan* gene. The resultant clones were transformed into mouse-virulent *P. multocida* X-73, pooled, and used to infect mice treated with kanamycin. Genes preferentially *in vivo* expressed were identified by selecting strains that survived *in vivo* selection but showed low Kan resistance *in vitro*. Clones recovered were sequenced and their fragments identified by comparison with public sequence databases. Clones were then repassaged individually in mice under more stringent kanamycin selection conditions and also tested for *in vitro* Kan resistance at various levels and under various environmental conditions.

Chapter 3 describes the methods used to inactivate two genes (*pta* and *nrfE*) in *P. multocida* that were identified in the PmIVET system in order to investigate their functions and determine their role in virulence. Inactivation of genes in *P. multocida* (particularly X-73) is currently difficult and far from routine. A number of commonly used methods and unique methods was evaluated. Of these, one method resulted in a *tet*(M)-disrupted *nrfE* mutant in *P. multocida* X-73, and another method proved useful for further studies.

Finally, in chapter 4, the formate dependent nitrite reductase (Nrf) system in *P. multocida* was characterised using the *nrfE* mutant. It was found that the Nrf system was active in *P. multocida* X-73 and that the *nrfE* gene was essential for its activity. It was also found that the Nrf system in *P. multocida* is active both aerobically and anaerobically. However, the inactivated *nrfE* mutant was not found to be attenuated in mice compared to wild-type *P. multocida* X-73. Finally it was found that *nrfE* is most

likely expressed from a previously unknown promoter that is potentially expressed *in vivo*.

Chapter 2

In vivo expressed genes of *Pasteurella multocida*

Based on Hunt *et al.*(2001) with additional data included

Chapter 2- *In vivo* expressed genes of *Pasteurella multocida*

Abstract

Pasteurella multocida is the causative agent of infectious diseases of economic importance such as fowl cholera, bovine haemorrhagic septicaemia and porcine atrophic rhinitis. However, knowledge of the molecular mechanisms and determinants that *P. multocida* requires for virulence and pathogenicity is still limited. To address this issue, we developed a genetic expression system, based on the *in vivo* expression technology (IVET) approach first described by Mahan *et al.* (Science 259:686-688, 1993), to identify *in vivo* expressed genes of *P. multocida*. Numerous genes, including those encoding outer membrane lipoproteins, metabolic and biosynthetic enzymes and a number of hypothetical proteins were identified. These may prove to be useful targets for attenuating mutation and/or warrant further investigation of their role in immunity and/or pathogenesis.

Introduction

P. multocida is an opportunistic veterinary and human pathogen with worldwide distribution. Certain serotypes are the etiologic agents of severe pasteurellosis such as fowl cholera in avian species, haemorrhagic septicaemia in cattle and buffalo and atrophic rhinitis in swine. Despite considerable research into the mechanisms of immunity, virulence and pathogenesis, safe and effective vaccines against pasteurellosis are still lacking and little is known of the molecular mechanisms of pathogenesis.

Mahan *et al.* (1993a) first described a system to identify *in vivo* expressed genes and termed this *in vivo* expression technology (IVET). Various IVET systems have since been designed and used in a number of different organisms (Reviewed in references (Chiang *et al.*, 1999, Heithoff *et al.*, 1997a, Heithoff *et al.*, 1997b)). Information gained from these research efforts has identified a number of known virulence factors, metabolic and biosynthetic genes and, interestingly, many genes with no known function. IVET systems provide an insight into the genes which are required for survival and multiplication *in vivo* and the gene products identified may represent new targets for attenuating mutations, antimicrobial agents or recombinant vaccines. The inactivation of genes identified by IVET systems has, in many cases, resulted in the attenuation of virulence, indicating an important role for these *in vivo* expressed genes in pathogenesis (Lowe *et al.*, 1998, Wang *et al.*, 1996b). In addition, the *in vivo* promoters themselves could be utilised for heterologous antigen expression *in vivo*.

Outer membrane protein preparations from *in vivo* grown *P. multocida* cells protect birds from heterologous serotypes, whereas *in vitro* grown bacteria provide protection only against the homologous somatic serotype (Glisson *et al.*, 1991, Heddleston *et al.*, 1970, Heddleston *et al.*, 1972b). The *in vivo* expressed antigens involved in providing heterologous protection have been termed the cross protective factors (CPFs). Much interest has been focused on identifying the CPFs of *P. multocida* fowl cholera strains, yet none to date has been isolated and characterised. The IVET system provides a new approach for identifying such genes and overcomes the limitations of using *in vitro* media and conditions to mimic the host factors responsible for triggering bacterial gene expression *in vivo*. The IVET approach is designed to identify simultaneously a number of genes expressed *in vivo*.

This report describes the construction and use of a *P. multocida* IVET system termed PmIVET, to identify genes that are expressed exclusively or preferentially

during infection. A plasmid based promoter-probe system was designed and constructed for use in *P. multocida*, which relied on the expression of an antibiotic resistance marker, *kan*. A plasmid carrying a promoterless *kan* gene, pMKΩ, was constructed and random genomic fragments were cloned upstream of this gene to generate transcriptional fusions. The plasmid library was introduced into the virulent *P. multocida* strain X-73. After infection with *P. multocida*, mice were treated with kanamycin and *in vivo* expressed genes were identified by analysing the bacteria that survived *in vivo*, but were kanamycin sensitive (Kan^S) *in vitro*.

Materials and methods

Media

P. multocida strains were cultured at 37°C on nutrient agar (NA) or in nutrient broth (NB). *E. coli* strains were grown in Luria Bertani broth (LB) or on Luria Bertani agar (LA) at 37°C. Antibiotics were added when required at the following concentrations: ampicillin (Amp) 100 μg/ml, kanamycin (Kan) 5, 10, 20, 30, 40 or 50 μg/ml, streptomycin (Str) 25 μg/ml, spectinomycin (Spe) 25 μg/ml.

Individual *P. multocida* strains for mouse *in vivo* selection assays were grown overnight in NB, diluted 1:100 in 10 ml of fresh NB and incubated with shaking at 37°C for 4 to 6 h. The absorbance at 600 nm of the cultures was determined, after which the cultures were diluted in sterile phosphate buffered saline pH 7.2 to provide the required number of colony forming units (cfu).

Bacterial strains and plasmids.

Bacterial strains and plasmids used in this study are listed in Table 1.

DNA manipulations.

P. multocida genomic DNA was prepared using the method of Ausubel *et al.* (1987) and plasmid DNA from *E. coli* or *P. multocida* strains was prepared as described by Le Gouill *et al.* (1994). DNA was digested using restriction endonucleases supplied by Roche Molecular Biochemicals (Basel, Switzerland) or New England Biolabs Inc. (NEB, Beverly, MA) using conditions recommended by the manufacturer.

Library construction.

P. multocida strain X-73 genomic DNA was digested to completion with *Sau*3AI and the resulting fragments purified using a Qiaex II gel extraction kit (Qiagen, Hilden, Germany) before ligation into *Bam*HI-digested, dephosphorylated pMKΩ (Table 1). The ligation mix was used to transform cells of *P. multocida* strain X-73 (Smith *et al.*, 1990), which were allowed to recover at 37°C for 2 h and then spread onto NA containing 25 μg/ml of both Spe and Str to select transformants containing pMKΩ. Transformants were used in mouse infection experiments to select for recombinant clones expressing Kan resistance (Kan^r) *in vivo*.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
P. multocida X-73	serogroup A: serotype 1 chicken isolate, reference strain	(Heddleston *et al.*, 1972b)
PmIVET clones		
MHS6	X-73 harbouring three non-contiguous X-73 genomic *Sau*3AI fragments, 355, 253 and 127 bp in pMKΩ	This study
MHS7	X-73 harbouring three non-contiguous X-73 genomic *Sau*3AI fragments, 143, 149, 967 bp in pMKΩ	This study
MHS17	X-73 harbouring two non-contiguous X-73 genomic *Sau*3AI fragments, 339 and 191 bp in pMKΩ	This study
MHS23	X-73 harbouring a 604 bp X-73 genomic *Sau*3AI fragment in pMKΩ	This study
MHS25	X-73 harbouring four non-contiguous X-73 genomic *Sau*3AI fragments, 94, 98, 351 and 144 bp in pMKΩ	This study
MHS27	X-73 harbouring a 465 bp X-73 genomic *Sau*3AI fragment in pMKΩ	This study
MHS30	X-73 harbouring three non-contiguous X-73 genomic *Sau*3AI fragments, 123, 276 and 387 bp in pMKΩ	This study
MHT6	X-73 harbouring a 267 bp X-73 genomic *Sau*3AI fragment in pMKΩ	This study
MHW32	X-73 harbouring three non-contiguous X-73 genomic *Sau*3AI fragments, 666, 267 and 129 bp in pMKΩ	This study
MHW39	X-73 harbouring two non-contiguous X-73 genomic *Sau*3AI fragments, 198 and 909 bp in pMKΩ	This study
MHX6	X-73 harbouring two non-contiguous X-73 genomic *Sau*3AI fragments, 431 and 549 bp in pMKΩ	This study
MHY6	X-73 harbouring four non-contiguous X-73 genomic *Sau*3AI fragments, 171, 1111, 158, 876 bp in pMKΩ	This study
MHY29	X-73 harbouring two non-contiguous X-73 genomic *Sau*3AI fragments, 119 and 261 bp in pMKΩ	This study
MHY40	X-73 harbouring six non-contiguous X-73 genomic *Sau*3AI fragments, 444, 295, 465, 092, 021, 123 bp in pMKΩ	This study
MHZ12	X-73 harbouring a 66 bp X-73 genomic *Sau*3AI fragment in pMKΩ	This study
Plasmids		
pUC4-KIXX	Amp^r, Kan^r, ColE1 origin	(Vieira *et al.*, 1982)
pUCΩ	Amp^r, Spe^r, Str^r, ColE1 origin	(Prentki *et al.*, 1984)
pPMK1	4.9 kb Kan^r, *P. multocida* origin	(Bills *et al.*, 1993)
pPBA844	2.0 kb *Bam*HI Spe/Str cassette of pUCΩ cloned into *Bgl*II digested pPMK1, removing *kan* gene promoter	This study
pMKΩ	Spe/Str selection *in vitro*. 6.5 kb Expand™ PCR product generated using primers BAP-03 and BAP-04 from pPBA844 template, digested with *Bam*HI and religated on itself. Used for selection of *in vivo* expressed genes in PmIVET.	This study

Estimation of the *in vivo* kanamycin level.

Female Balb/c mice were weighed and Kan doses of 50, 100 or 200 μg/g of bodyweight were injected IP. To measure the circulating Kan concentration, 100 μl volumes of heparinised blood obtained from the orbital plexus were added to 5 mm wells cut in diagnostic sensitivity test agar plates which had been seeded with sufficient *E. coli* to produce a confluent lawn after subsequent overnight incubation at 37°C. Zones of inhibition of *E. coli* growth were then measured and the circulating

concentration of Kan in the mouse determined by comparison with Kan standards. All measurements were performed at least in triplicate.

In vivo library selection.

A *P. multocida* strain X-73 *Sau*3AI genomic DNA library in the vector pMKΩ was expressed in X-73 cells grown in NB containing 25 μg/ml Spe and Str *in vitro*. Female 6-8 week old Balb/c outbred mice were injected intraperitoneally (IP) with 2.9×10^6 cfu of the library, and the infection allowed to progress for a minimum of 2 h before an appropriate volume of Kan, calculated by determining the mouse weight, was injected IP to give an approximate *in vivo* blood level of 100 μg/ml. Mice were bled from the orbital plexus immediately prior to the injection of Kan and then at periodic intervals over 13 h. Blood was plated onto NA containing 25 μg/ml Spe and Str and incubated at 37°C overnight. Bacterial colonies appearing after overnight incubation were patched or replica plated onto NA containing 50 μg/ml Kan. Plasmids purified from Kan sensitive (Kan^S) clones were analysed further by restriction digestion, and the nucleotide sequence of the cloned inserts determined to identify putative *in vivo* expressed genes.

Nucleotide sequence analysis of the PmlVET clones.

Synthetic oligonucleotides were designed that were homologous to the 5' and 3' ends (BAP-1056, 5'-ATCTAGCGAGGGCTTTAC-3' and BAP-503, 5'-ACCGAATAGCCTCTCCAC-3' respectively) of the *Bam*HI cloning site of pMKΩ. These were used in either *Taq* DyeDeoxy Terminator Cycle kit or *Taq* Big DyeDeoxy Terminator kit (Applied Biosystems Inc., Foster City, CA) sequencing reactions, that typically contained 200-500 ng of purified template DNA, 6-8 μl of *Taq* DyeDeoxy or *Taq* BigDyeDeoxy Terminator mix, and 3.2 pmol of the required oligonucleotide in a 15-20 μl volume. Cycle sequencing was performed on a Perkin-Elmer GeneAmp PCR System 2400 thermocycler and the DNA products were purified according to the manufacturer's instructions and analysed with an Applied Biosystems Inc. Automated DNA sequencer model 373. The Sequencher 3.0 program (Gene Codes Corp., Ann Arbor. MI) was used to align and assemble individual sequences. Comparison of sequences with those in GenBank, EMBL and unfinished genome databases was performed using the BLAST (Altschul *et al.*, 1990) and FASTA (Pearson *et al.*, 1988) programs through the Australian National Genomic Information Service (ANGIS)

(http://www.angis.org.au) and the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Computer analysis of deduced protein sequences was carried out through ANGIS using programs within the Genetics Computer Group Sequencing Analysis Software Package (GCG, Inc., Madison, WI).

Colony PCR.

Colonies picked from a plate were resuspended in 50 µl distilled water, boiled for 10 minutes, then centrifuged at 12,000g for 10 minutes. 2 µl was then used as template DNA in a 20µl PCR reaction containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3, 0.01% (w/v) gelatin, 200 µM each of dATP, dCTP, dGTP and dTTP, 1 µM each of forward and reverse oligonucleotide primers, and 1 µl *Taq* DNA polymerase (Roche Molecular Biochemicals). Reactions were performed using a Perkin-Elmer GeneAmp PCR System 2400 thermocycler and 0.6 ml thin-walled tubes. Thermocycling conditions were 25 cycles of 94°C for 15sec, 50°C for 30sec, 72°C for 2 min.

Results

Construction of the promoter probe vector pMKΩ, for selection of *in vivo* expressed genes.

The 4.9 kb plasmid pPMK1 (Table 1, Fig.1), was constructed by cloning the kanamycin resistance cartridge from pUC4KIXX (Vieira *et al.*, 1982) into a native cryptic *P. multocida* plasmid (Bills *et al.*, 1993). This plasmid was used to generate the promoterless *kan* gene promoter-probe vector for use in PmIVET. A putative ribosome binding site (RBS), AGGA, was present 10 bp upstream of the ATG start codon of the *kan* gene. The native *kan* promoter was identified in the region 100 to 160 bp upstream of the start codon.

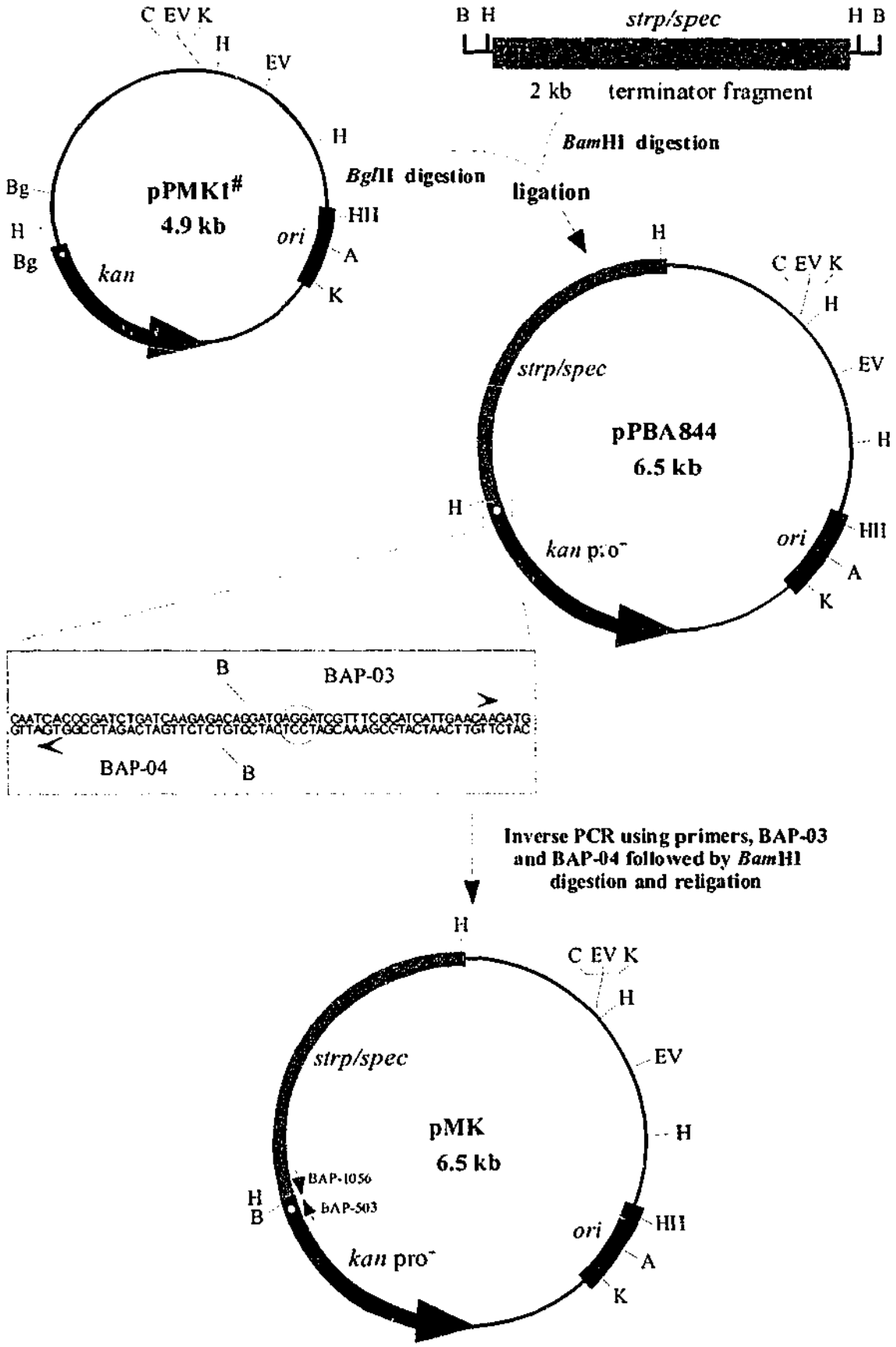

Fig. 1. Construction of the PmlVET *in vivo* promoter-probe vector pMKΩ. The *P. multocida* plasmid origin of replication region (*ori*) is shown on each plasmid as a black box. The open circle at the 5' end of the *kan* gene indicates the ribosome binding site, AGGA. The oligonucleotide primers BAP-03 and BAP-04, used for inverse PCR to introduce a unique *Bam*HI restriction site, are shown as bent arrows above and below the sequence in the expanded boxed section. The ribosome binding site preceeding the start of the *kan* gene is circled. The positions of two sequencing primers, BAP-503 and BAP-1056 are denoted by the small arrows. The restriction site abbreviations used are A: *Ava*I, B: *Bam*HI, Bg: *Bgl*II, C: *Cla*I, EV: *Eco*RV, H: *Hin*dIII, HII: *Hin*dII, K: *Kpn*I. '#' The approximate 4.5 kb size of pPMK1 as published by Bills *et al.* (1993) has been subsequently re-estimated to be 4.9 kb as shown here.

Two *Bgl*II sites were identified in pPMK1 that could be used to remove the *kan* gene promoter. The 2 kb *Bam*HI Omega (Ω)-terminator fragment (Prentki *et al.*, 1984), which carries two transcriptional terminators (TT) and translational stop codons in all three frames at either end of the element, in addition to encoding streptomycin (Str) and spectinomycin (Spe) resistance, was cloned into *Bgl*II-digested pPMK1 upstream of the promoterless *kan* gene, resulting in the 6.5 kb plasmid pPBA844 (Fig. 1).

To allow the cloning of *P. multocida* genomic DNA *Sau*3AI fragments upstream of the promoterless *kan* gene in pPBA844, inverse PCR was employed, using outward facing primers carrying *Bam*HI sites at their 5' ends, to introduce a unique *Bam*HI cloning site (Fig. 1). The resulting vector carrying a promoterless *kan* gene, Ω terminator fragment, *P. multocida* plasmid origin of replication and a unique *Bam*HI cloning site upstream of the *kan* gene, was designated pMKΩ (Fig. 1).

P. multocida carrying pMKΩ was unable to grow in NB or on NA plates containing >5 μg/ml Kan, whereas *P. multocida* carrying the vector pPMK1 grew in NB containing up to 400 μg ml^{-1} Kan.

Survival of *P. multocida* harbouring either pPMK1 or pMKΩ in Kan-treated mice

A kanamycin-treated mouse infection model was established that selected for bacteria expressing Kan^r *in vivo* but did not permit the survival of detectable levels of Kan^s bacteria. A Kan dose of 100 μg/g of bodyweight was found to remove Kan^s organisms *in vivo* while allowing Kan^r organisms to multiply and infect mice. Pairs of mice were given either 1×10^7 cfu of X-73(pPMK1) or 2×10^7 cfu of X-73(pMKΩ) and then given a Kan dose (100 μg/g of bodyweight) after 4 and 6.5 h (Fig. 2). The effectiveness of even a single Kan dose was demonstrated by the significant reduction in bacterial numbers observed in the mice infected with X-73(pMKΩ), where within 1 h of the first Kan dose, the numbers had decreased from 6×10^6 cfu ml^{-1} to 20-50 cfu/ml (Fig. 2). No reduction in numbers was seen for the mice infected with X-73(pPMK1) after the first Kan dose. A second Kan dose was given after 2.5 h and the circulating bacteria were enumerated after a further 2 h and 4 h. No X-73(pMKΩ) bacteria were detected, whereas X-73(pPMK1) continued to be detected in high numbers (Fig. 2).

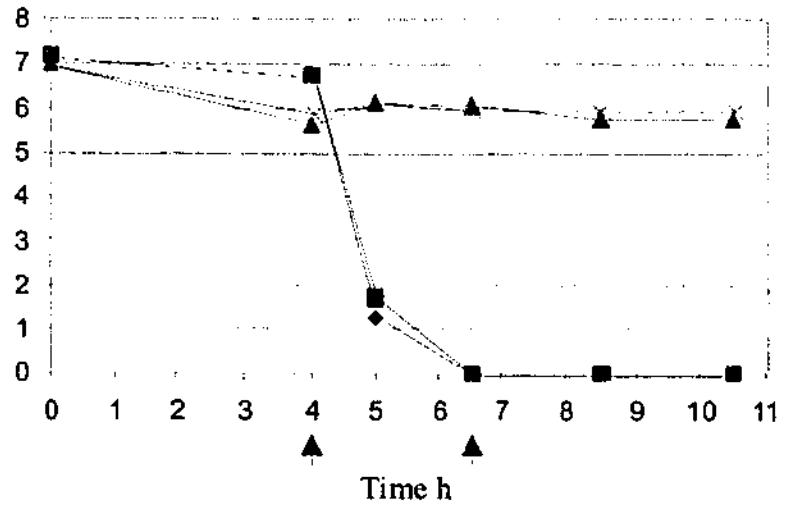

Fig. 2. Survival of *P. multocida* strain X-73 [pPMK1] (▲, ×) and X-73 [pMKΩ] (■, ◆) in kanamycin treated mice. The arrows indicate the times at which a kanamycin dose of 100 μg g^{-1} of bodyweight was injected. The value given on the Y-axis at 0 h is the total cfu administered to each mouse to initiate infection. All other values plotted are cfu ml^{-1} of blood. Where a Y value of 10^0 is given, no bacteria were recovered.

Generation of an X-73 genomic DNA *Sau*3AI library in pMKΩ.

The genomic library contained a total of 10,300 transformants, of which approximately 61% (6,300) contained insert DNA with an average size of 0.8 kb. Using the Clarke and Carbon (Clarke *et al.*, 1976) formula and the approximate genome size of 2,350 kb of another A:1 strain (the size of the X-73 genome was not known), this library was estimated to cover 46% of the genome. Replica plating of 500 colonies onto NA plates containing 50 μg/ml Kan (NA-Kan) demonstrated that the library contained approximately 4.4% *in vitro* Kan^r bacteria prior to *in vivo* selection. This experiment also confirmed the function of the *kan* gene in pMKΩ.

Selection of *in vivo* active promoters.

The transformants were scraped off plates and pooled into 200 ml of NB containing Spe and Str before incubation for 1.5 h at 37°C. Aliquots of this suspension (representing 3×10^6 cfu) were injected IP into four pairs of mice for *in vivo* selection of clones carrying promoter elements expressed during infection. A fifth pair of mice was injected IP with 1×10^5 cfu of X-73(pMKΩ) as a control for the *in vivo* clearance of Kan^s bacteria. Kan was administered to this control group at 2 h post infection. The four pairs of mice, used for the *in vivo* selection of the X-73 library, were treated with Kan at various time points in an attempt to maximise the recovery of *in vivo* expressed genes by allowing the infection to progress to different stages before Kan treatment.

Blood was taken from the mice at various times over a 13 h period and bacteria present were selected on NA-Spe/Str plates at 37°C. Of the resulting colonies recovered

from the mice 2,900 were transferred to NA plates containing 50 μg/ml Kan to determine the *in vitro* Kan-phenotype. Of these 292 (10%) were found to be Kan^s, indicating that a promoter functioning *in vitro* was not present upstream of the promoterless kan gen of pMKΩ in these clones.

Plasmid DNA was prepared from the 292 Kan^s clones and 159 of these (54.5%) were shown to contain insert DNA in pMKΩ. The nucleotide sequence of insert DNA was obtained using the two oligonucleotide primers, BAP-503 and BAP-1056. Analysis of the individual nucleotide sequences and comparison with sequences in GenBank and other databases identified 76 clones which could potentially contain promoter regions driving the expression of *kan in vivo*.

Re-passaging potential *in vivo* expressed clones through Kan-treated mice.

To confirm the specificity of *in vivo* selection, clones were re-passaged individually using pairs of mice. In these experiments each clone (10^5-10^6 cfu) was injected IP into pairs of mice together with X-73(pMKΩ) (10^5-10^6 cfu) as an internal control for *in vivo* clearance of Kan^s organisms. The mice were treated with Kan (100 μg/g of bodyweight) at 2 h and 4 h post infection. Bacteria were recovered from blood at 8 h and Kan^r bacteria plated on NA containing 25 μg/ml Spe and Str. Measurement of blood Kan levels using this regime indicated that concentrations of 80-100 μg/ml of blood were achieved 15 minutes after each Kan injection in mice. Random colonies were patched onto NA containing 50 μg/ml Kan to check *in vitro* sensitivity. Where possible 12 colonies were screened by PCR from each plate using the primers BAP503 and BAP1056 to differentiate between clones containing plasmids with inserts and those containing pMKΩ vector only. Plasmids were selected as potentially carrying an *in vivo* expressed promoter only if they were recovered without co-recovery of bacteria carrying pMKΩ. Re-passaging the clones through mice with the internal pMKΩ control greatly improved the specificity of the PmIVET system.

Of the 76 clones with potential *in vivo* promoters, only 43 contained unique fragments (Table 2) and one representative for each of these gene fusions was used for re-passaging. After re-passaging, 17 of the 43 clones were recovered from mice without co-isolation of X-73 carrying pMKΩ. These clones were designated as carrying an *in vivo* expressed promoter element.

Table 2. ***Pasteurella multocida*** **genes identified as** ***in vivo*** **expressed**

Category	Strain[a]	cfu recovered after repassage *in vivo*[b]	*in vitro* MIC level of kanamycin µg/ml	Gene identified as *in vivo* expressed	% Amino acid identity (similarity)[c]	Function/ role of homolog	Pm70[d] #
Lipoproteins	MHS25[3]	+++	30	*hpd*	65 (78) to Protein D	Glycerol metabolism, surface exposed lipoprotein	PM1444
	MHS30[6]	++	10	*pcp*	73 (88) to PCP	Outer membrane associated lipoprotein	PM0554
Pyrimidine synthesis and salvage	MHS17[1]	+++	20	*dcd*	91 (97) to Dcd	Deoxycytidine deaminase	PM0951
Biosynthetic and metabolic	MHS23[6]	+++	10	*dsbD*	43 (58) to DsbD	Thiol-disulphide interchange protein	PM0221
	MHT6	+	10	*speF*	56 (77) to SpeF	Inducible ornithine decarboxylase, maintenance of cellular polyamines	PM0806
	MHZ12	+	20	*ackA-pta*	Only putative promoter region of pta present	Fermentation of acetyl coenzyme A/ regulation of virulence gene expression	PM0704-705
	MHW40	+	10	*srlD*	81 (87) to SrlD of *Ea*	Sorbitol-6-phosphate dehydrogenase	PM1968
	MHS7[2]	++	10	*nrfE*	Only putative promoter region present	Formate dependent nitrite reduction protein	PM0027
	MHX6	++	10	*yiaK*	71 (83) to YiaK	Putative dehydrogenase	PM1256
Hypothetical or unknown proteins	MHS6	+	40	*ycbK*	64 (82) to YcbK	Unknown	PM0271
	MHS27[10]	+	10	*ycbL*	90 (100) to YcbL	Unknown	PM0272
	MHW39	+	5	*ychN*	44 (76) to YchN of *Ec*	Unknown	PM0514
	MHW32	+	0	*orfX*	No database match	Unknown	-
	MHY6	++	10	-	No database match	Unknown	-
	MHY29	+	5	*HI0894*	Only putative promoter region present	Putative membrane protein	PM1134
	MHY40	+++	20	*yeeX*	80 (89) to YeeX of *Ec*	Putative alpha helix protein	PM0836

[a] Strain isolated multiple times from different mice during primary *in vivo* selection. The superscript number indicates the number of other identical clones found.

[b] Approximate numbers recovered after repassaging strains through mice. "+" denotes 10-10^2 cfu, "++" denotes 10^2-10^3 cfu and "+++" denotes >10^3 cfu.

[c] *Ec and Ea* denote *Escherichia coli* and *Erwinia amylovora*. All other database matches were to proteins of *Haemophilus influenzae*.

[d] Denotes gene designation scheme for the *P. multocida* Pm70 genome sequence (http://www.cbc.umn.edu/ResearchProjects/AGAC/Pm/pmhome.html)

As a number of these clones contained non-contiguous *Sau*3AI fragments, definite assignment of some *in vivo* expressed genes is not yet possible. However, from nucleotide sequence analysis of the individual *Sau*3AI fragments of each clone the promoter region of the gene most likely to be driving *kan* expression *in vivo* was predicted. The analysis of these clones is detailed in Table 2 and in Discussion. Of the remaining 25 re-passaged clones, 14 were not recovered and 11 clones were re-isolated, but with the concurrent isolation of X-73(pMKΩ), indicating that Kan selection in these mice was insufficient to remove Kan^s bacteria.

In vitro kanamycin MIC.

Clones containing *in vivo* expressed promoter regions upstream of the pMKΩ *kan* gene were grown *in vitro* on NA plates containing 5, 10, 20, 30, or 40 μg/ml kanamycin to determine if the clones demonstrated any detectable Kan resistance *in vitro*. Individual clones displayed various levels of kanamycin resistance *in vitro*, indicating different basal levels of expression from the cloned regions upstream of the promoterless *kan* gene of pMKΩ (Table 2). *In vitro* Kan resistance levels appeared to have no relation to bacterial numbers recovered from mice, indicating that survival *in vivo* was independent of the *in vitro* Kan resistance. The *in vitro* Kan resistance levels were also investigated under various growth conditions (Table 3). This was to determine whether the cloned promoter elements were activated when grown under anaerobic or low iron conditions or at differing temperatures.

Table 3 Kan resistance of PmIVET clones under various *in vitro* environmental conditions

Category	Strain/plasmid	Gene identified as *in vivo* expressed	Pm70 no.	*In vitro* Kan MIC (5-40μg/ml)			
				37°C	42°C	Anaerobic	Iron restricted
Pyrimidine synthesis and salvage functions	MHS17	*dcd*	PM0951	30	30	30	20
Biosynthetic and metabolic functions	MHS7	*nrfE*	PM0027	10	10	10	10
	MHS23	*dsbD*	PM0221	20	5	40	20
	MHT6	*speF*	PM0806	10	10	40	10
	MHW40	*srlD*	PM1968	10	10	10	10
	MHX6	*yiaK*	PM1256	10	10	30	10
	MHZ12	*ackA-pta*	PM0704/5	20	0	30	30
Hypothetical or unknown proteins	MHS6	*ycbK*	PM0271	40	40	0	40
	MHS27	*ycbL*	PM0272	20	5	40	20
	MHW32	*orfX*		0	0	0	0
	MHW39	*ychN*	PM0514	5	5	5	5
	MHY6	none		10	10	40	10
	MHY29	*HI0894*	PM1134	5	0	20	5
	MHY40	*yeeX*	PM0836	20	10	40	20
Controls	pMKΩ	N/A		0	0	0	0
	pMK1	N/A		40	40	40	40
	X-73	N/A		0	0	0	0

Discussion

A system for the identification of *in vivo* expressed genes of *P. multocida* was constructed and tested in mice. As this PmIVET system is plasmid based, it is readily transferable to any *P. multocida* strain provided that the plasmid can be maintained and kanamycin resistance expressed. The method of selection is straightforward as it does not require complex media or the generation of *P. multocida* mutants. Importantly, the inherent problems of variability between individual animals were effectively addressed for PmIVET by the use of an internal Kan^s X-73(pMKΩ) control during re-passaging.

Using various IVET systems, researchers have noted significant *in vivo* selection in animal models with approximately 86-95% of the recovered population expressing the phenotype required for survival *in vivo* (Mahan *et al.*, 1993a, Wang *et al.*, 1996b, Young *et al.*, 1997). Significant selective pressure for Kan^r bacteria appears to have been exerted *in vivo* when the pre-passaged Kan^r level in the library, measured at 4.4%, was compared to the 90% Kan^r bacteria recovered from mice after the first *in vivo* selection of the library pool. However, of the 292 Kan^s clones recovered after the initial *in vivo* library selection, 45.5% contained pMKΩ without any insert DNA. Variability in the circulating Kan concentrations between mice may play a role in the survival of some Kan^s bacteria *in vivo*. Measurement of the blood Kan levels showed that the concentration of Kan reached the required level 15 min after injection. To address the problem of variability, re-passaging was performed using duplicate Kan doses 2 h apart to maintain Kan selection for a longer period of time. In addition, mice were injected simultaneously with both the clone to be tested and a similar dose of X-73 (pMKΩ). Clones were then only selected as potentially carrying an *in vivo* expressed gene if after *in vivo* Kan selection the clone demonstrated an *in vitro* Kan^s phenotype and was recovered without co-recovery of bacteria carrying pMKΩ. Multiple isolations of identical clones from mice with different treatment regimes and the occurrence of identical clones isolated from more than one mouse during the first round of *in vivo* selection also provided confidence in the PmIVET selection procedure. Of the 46 clones that were chosen for re-passage through mice, 8 were isolated a number of times. Six of these were recovered after a second round of PmIVET selection. The other two clones were recovered after re-passage in mice, but in conjunction with X-73(pMKΩ) indicating that selection in these mice was inadequate. Thus, the classification of these clones as potentially expressing Kan^r *in vivo* awaits further testing.

In order to identify the conditions responsible for activating promoter activity in the *in vivo* expressed promoter elements, clones recovered from the second round of PmIVET selection were grown at varying Kan concentrations (5-40μg/ml) *in vitro* under various environmental conditions (Table 3). Conditions included growth under anaerobic and low iron conditions and growth during elevated temperatures (42°C). The outcomes of the *in vitro* Kan studies are discussed later in the relevant sections.

In vivo expressed genes identified.

Although a number of clones carried non-contiguous genomic *Sau*3AI fragments, and thus we cannot rule out the possibility that some *Sau*3AI fusions have created fortuitous *in vivo* active promoters, we propose that the promoters of the forward pointing ORFs in these clones were responsible for the expression of *kan*. In the following discussion, PM number refers to the gene designation scheme for the *P. multocida* Pm70 genome sequence (May *et al.*, 2001).

Lipoproteins.

The identification of potentially *in vivo* expressed putative membrane proteins may reflect the change in the bacterial surface properties required during infection. Two clones were identified that contained the forward pointing ORFs *pcp* (PM0554) and *hpd* (PM1444). The deduced amino acid sequences of these ORFs showed 88% and 78% respectively to two membrane lipoproteins of *H. influenzae*, PCP and Protein D, encoded by the *pcp* and *hpd* genes respectively. Multiple isolations of both clones were obtained (Table 2). In nontypeable *H. influenzae,* PCP was found to be antigenically conserved and has shown promise for use in recombinant subunit vaccines (Deich *et al.*, 1990, Green *et al.*, 1991, Green *et al.*, 1993). The immunogenic 42 kDa surface-exposed protein D is widely distributed among *H. influenzae* strains (Akkoyunlu *et al.*, 1991) and has been shown to mediate binding to IgD, which may represent a form of immune evasion by the bacteria (Ruan *et al.*, 1990, Sasaki *et al.*, 1993). Protein D is also involved in the metabolism of glycerol, mediating glycerophosphodiesterase phosphodiesterase activity (Munson *et al.*, 1993) and has been shown to cause damage to epithelial cell cilia in a nasopharyngeal tissue culture model (Janson *et al.*, 1999). Additionally, a *H. influenzae hpd* mutant was found to be 100 times less virulent than the wild-type strain (Janson *et al.*, 1994). The identification of an *in vivo* expressed *P. multocida*

homologue of protein D raises the possibility that it may play a similar role in immunity and virulence.

Pyrimidine synthesis and salvage.

IVET systems from a number of organisms have identified genes involved in pyrimidine and purine biosynthesis and nucleotide recycling *in vivo* (Heithoff *et al.*, 1997a, Heithoff *et al.*, 1997b, Mahan *et al.*, 1993b). Mammalian blood contains very low levels of purines and pyrimidines and thus blood borne pathogenic bacteria must synthesise nucleotides *de novo*. The ORF fused to *kan* on MHS17 showed sequence similarity to a dCTP deaminase (Dcd) of *H. influenzae* Rd (Fleischmann *et al.*, 1995). Upstream of the *P. multocida dcd* (PM0951), a gene encoding the enzyme uridine kinase (*udk*) involved in pyrimidine salvage was identified (Table 2). A similar arrangement occurs in *E. coli* and *H. influenzae* Rd (Blattner *et al.*, 1997, Fleischmann *et al.*, 1995). The *E. coli* and *S.* Typhimurium *dcd* genes are involved in the formation of dUTP, a precursor for the *de novo* synthesis of thymidylate (Neuhard *et al.*, 1987, Weiss *et al.*, 1994). Although the *udk* and *dcd* genes on MHS17 were separated by only 20 bp, suggesting that they are part of an operon and thus transcribed from a promoter preceding *udk*, the possibility exists that sequences upstream and within the *udk* gene could allow transcription of *dcd in vivo*.

Biosynthetic and metabolic genes.

Many *in vivo* expressed genes identified to date have been involved in biosynthesis, metabolism or nutrient acquisition. The identification of such genes reveals information about the environmental stimuli within the host during infection that may act as signals for the induction of bacterial genes to complement nutrient limiting conditions and signal the induction of virulence genes required for immediate survival and spread to other anatomical sites of infection (Heithoff *et al.*, 1997a).

The *P. multocida dsbD* homologue (PM0221) was fused to *kan* on clone MHS23. After the first PmIVET *in vivo* selection, six clones identical to MHS23 were recovered independently (Tables 1 and 2). In *E. coli* the integral membrane protein, DsbD, is essential for growth above 42°C, with *dsbD* transcripts still detectable at 50°C (Missiakas *et al.*, 1995). This may contribute to the virulence of X-73 given that the normal body temperature of poultry ranges between 39-43°C. However, when grown *in vitro* at 42°C, the MHS23 clone showed diminished growth compared to growth at 37°C (Table 3). This may indicate that in *P. multocida dsdD* is regulated differently at

higher temperatures than in *E. coli* or that the cloned promoter element may have interfered with normal regulation of *dsdD*. The *dsbD* gene in *E. coli* is transcribed from its own promoter and is involved in disulphide bond formation in periplasmic proteins. As disulphide bonds are often essential for the proper folding, stability and activity of many extracellular proteins, the expression of such a gene *in vivo* may have implications in bacterial pathogenesis for the secretion of toxins or other virulence factors. Recently Fuller *et al.* (2000a) found that mutation of *dsbB*, also involved in disulphide bond formation, caused attenuation of virulence in a *P. multocida* septicemic mouse model. Additionally, DsbD is required for C-type cytochrome biogenesis under anaerobic conditions and plays a role in copper (Cu^{2+}) tolerance in bacteria such as *E. coli*, *S.* Typhimurium and *P. aeruginosa* (Crooke *et al.*, 1995, Gupta *et al.*, 1997, Page *et al.*, 1997). Other genes involved in Cu^{2+} homeostasis have been identified, using IVET systems, from *S.* Typhimurium (Heithoff *et al.*, 1997a) and *Staphylococcus aureus* (Lowe *et al.*, 1998). MHS23 showed increased growth on Kan under anaerobic conditions, which is in agreement with its predicted role in cytochrome biogenesis. Furthermore, a putative ArcA box (TTTTATTAT), a known anaerobic regulator (Chattopadhyay *et al.*, 1997), was found 33 bp upstream of the ATG of *dsbD*, suggesting that the *in vivo* induced promoter in MHS23 may indeed be induced anaerobically.

A *P. multocida* homolog of the *H. influenzae speF* gene (PM0806), that encodes an ornithine decarboxlyase, was found to potentially drive *kan* expression *in vivo* (MHT6; Table 2). Similarity to *E. coli* SpeF, a biodegradative enzyme for the production of putrescine that is inducible at low pH in the presence of ornithine, was also found (Kashiwagi *et al.*, 1991). In *E. coli* and *H. influenzae, speF* is the first gene of the *speF-potE* operon which is involved in the maintenance of cellular polyamines required for normal cell growth (Tabor *et al.*, 1984) and transport of putrescine (Kashiwagi *et al.*, 1991). Induction of this operon is believed to neutralise the extracellular medium via the excretion of putrescine (Kashiwagi *et al.*, 1994). Other genes involved in biosynthesis of the polyamines cadaverine, spermidine and putrescine have been isolated from *S.* Typhimurium, *S. pneumoniae* and *V. cholerae* using IVET and signature tagged mutagenesis systems (Heithoff *et al.*, 1997a, Merrell *et al.*, 1999, Polissi *et al.*, 1998). These genes are also induced at low pH and are believed to play a role in acid tolerance (Merrell *et al.*, 1999, Park *et al.*, 1996). However, Merrell *et al.* (1999) did not find a role for SpeF in acid tolerance of *V. cholerae*. MHT6 was found to

be upregulated under anaerobic conditions (Table 3) grown *in vitro* on Kan, but no putative anaerobic regulatory sequences were identified upstream of the gene. Anaerobic regulation of *speF* may indicate a previously unknown characteristic of the operon in *P. multocida*. Further work is required to determine the role *speF* plays in pathogenesis of pasteurellosis and why this gene is up-regulated *in vivo*.

A single 66 bp *Sau*3AI fragment was present in MHZ12. Sequence analysis of this short fragment indicated the presence of a truncated ORF, in the same orientation as *kan*, with similarity to the acetate kinase, AckA, of *H. influenzae* Rd (Fleischmann *et al.*, 1995). Only 5 bp are present downstream of the *P. multocida ackA* stop codon on this fragment. Analysis of the *P. multocida* genome sequence identified a homolog of the *H. influenzae* Rd phosphotransacetylase gene, *pta* (Fleischmann *et al.*, 1995), 78 bp downstream of *ackA*. Pta (PM0705) together with the acetate kinase AckA (PM0704), is involved in the fermentation of acetyl coenzyme A (acetyl-CoA) to generate ATP and acetate and the reverse process utilising acetate to produce acetyl-CoA. A potential role for acetyl phosphate, the intermediate of the acetyl-CoA⇔acetate pathway, as an effector of gene regulation through interaction with two component response regulators such as the phosphate regulon of *E. coli* has been proposed (McCleary *et al.*, 1994, Wanner *et al.*, 1992). Recently, Chiang *et al.* (1998) identified an attenuated *pta* mutant of *V. cholerae* using signature-tagged mutagenesis. The expression of cholera toxin and the toxin co-regulated pilus was also affected by the *pta* mutation, further suggesting a regulatory relationship between the Pta-AckA metabolic pathway and virulence gene expression (Chiang *et al.*, 1998). MHZ12 was found to be slightly up regulated on Kan *in vitro* under both iron limited and anaerobic conditions. Although AckA/Pta are not reported to be regulated directly by either of these conditions, they may be indirectly regulated due to their central role in metabolism or global gene regulation.

A gene (PM1968) with deduced amino acid sequence similarity to SrlD (also named GutD) of *Erwinia amylovora* and *E. coli* (Aldridge *et al.*, 1997, Blattner *et al.*, 1997) involved in the conversion of sorbitol-6-phosphate to fructose-6-phosphate was fused to *kan* on MHW40 (Table 2). The *srlD* gene is the third and fourth gene of an operon in *E. coli* and *E. amylovora* respectively (Aldridge *et al.*, 1997, Yamada *et al.*, 1987). Transcription from a promoter upstream of the first gene in the operon, *srlA*, was found to be sensitive to catabolite repression by glucose and also dependent on

repressor and activator proteins (Aldridge *et al.*, 1997, Fratamico *et al.*, 1993, Lin, 1987). However, until further genetic experiments are performed, a promoter present immediately upstream of the *srlD* gene cannot be ruled out.

An ORF (PM1256) with similarity to a hypothetical protein, HI1031, of *H. influenzae* Rd (Fleischmann *et al.*, 1995) and the putative dehydrogenase, YiaK, of *E. coli* (Badia *et a* 8) was identified in the clone MHX6. In *E. coli yiaK* is the first of 9 genes in an ...on involved in carbohydrate utilisation (Badia *et al.*, 1998). A divergently transc...d repressor of the *E. coli yiaK-S* operon, *yiaJ* was found upstream (Badia *et al.*, 1998). Analysis of the Pm70 genome sequence also indicated the presence of a *yiaJ* homologue (PM1257) 217 bp upstream of the *P. multocida yiaK* gene. The up-regulation of genes involved in the use of alternative carbohydrates may indicate different nutritional requirements *in vivo*. The MHX6 clone was upregulated anaerobically when grown *in vitro* (Table 3), however, no known anaerobic regulatory sequences were found upstream of *yiaK*. This may indicate regulation by unknown anaerobic regulators, or indirect effects on carbohydrate me'abolism during anaerobic growth.

Two ORFs with sequence similarity to the formate dependent nitrite reducing proteins from *E. coli* and *H. influenzae nrfD* and *nrfE* respectively were identified in the clone MHS7. Comparison of MHS7 with the Pm70 genome indicated that the start of *nrfD* was not present on this clone. However, the *nrfE* start codon was found 71 bp downstream of *nrfD* on MHS7, preceeding the *kan* gene. Therefore, a promoter in the intergenic region between *nrfD* and *nrfE* may be expressed *in vivo*. The *nrfE-G* gene products have been implicated as part of a heme lyase that is responsible for attaching heme to cytochrome c_{552} (*nrfA*) at a motif that has been shown to be the site of nitrite reduction (Eaves *et al.*, 1998). These data are consistent with previous reports that *nrfE-G* were essential for nitrite reduction (Grove *et al.*, 1996). The Nrf operon in *E coli* has been shown to be upregulated anaerobically (Darwin *et al.*, 1993); however, MHS7 showed no change in Kan resistance when grown *in vitro* under anaerobic conditions. This may indicate that the putative *in vivo* promoter within MHS7 is regulated independently from that of *nrfABCD* or is not expressed under these conditions.

Hypothetical proteins.

A number of PmIVET isolated clones contained ORFs that were similar to hypothetical proteins and which appeared to be driving *kan* expression *in vivo*. Why

these gene products are required and what cellular function they perform *in vivo* is unknown. It is of interest that two clones contained ORFs not present in the *P. multocida* Pm70 database (W32, Y6), and hence may represent genes unique to X-73.

The only forward pointing ORF of clone MHW32 spanned the entire 129 bp *Sau*3AI fragment and displayed similarity to an internal region of a hypothetical protein, Sll1723, of *Synechocystis* sp. strain PCC6803. Amino acid sequence similarity was also seen to similar regions of a number of bacterial glycosyl transferases. Comparison to the Pm70 genome did not reveal any sequence similar to that of MHW32. Thus this ORF may represent a gene unique to X-73 (Table 2).

A forward pointing 377 bp ORF was found on a *Sau*3AI fragment in the clone MHY6 that displayed no nucleotide or amino acid sequence similarity to any sequences stored in the databases searched. This ORF may represent a gene unique to the *P. multocida* X-73 strain. Several putative ArcA boxes (TTTTATTA, TCTTATTAT, and AGTTAATAA) were found 360 bp, 500 bp and 530 bp respectively upstream of *kan* but downstream of the unknown ORF. In addition an Fnr binding region (TTGTATCGA) was found 430 bp upstream of *kan*. Both of these are known anaerobic regulators and may be responsible for the increased Kan resistance observed when clone MHY6 was grown anaerobically *in vitro* (Table 3).

A single *Sau*3AI fragment with the start of *ycbL* (a hypothetical *E. coli* protein) fused to *kan* (MHS27) was isolated from three different mice a total of 10 times after the first *in vivo* mouse selection experiment. A homologue of *ycbK* was found upstream of *ycbL*. One of the *Sau*3AI fragments of another clone, MHS6, was found to contain a region further upstream in the *P. multocida* genome than that found in MHS27. In MHS6, an N-terminal coding region of the *ycbK* gene was found in the same direction as *kan*, upstream of which the C-terminal coding region of *ycbB* was identified. A putative transcriptional terminator downstream of *ycbB* was indicated by an inverted repeat sequence, suggesting that these two genes do not form an operon and that transcription of *ycbK* may initiate from its own promoter. MHS27 was upregulated anaerobically *in vitro* compared to MHS6 which was downregulated (Table 3). No known anaerobic regulatory sequences were found in MHS27 or MHS6 and this difference in expression between two closely located genes supports the prediction that *ycbK* and *ycbL* may be transcribed independently.

A clone designated MHY29 contained a 64 bp sequence fused to *kan* that appeared to be part of the intergenic region between the hypothetical *H. influenzae HI0893* and *HI0894* ORFs. HI0894 is a putative membrane protein, and because it is downstream of a potential *in vivo* active promoter in *P. multocida,* it is possible that it is up-regulated *in vivo*. MHY29 was also found to be slightly upregulated when grown *in vitro* under anaerobic conditions (Table 3), despite the absence of any known regulatory sequences. Further study of the *P. multocida* HI0894 homologue is warranted to investigate the possibility that it may represent one of the *P. multocida* cross protective factors, due to its putative membrane location and likely upregulation *in vivo*.

An 80 bp forward pointing ORF with deduced amino acid sequence similarity to the first 27 amino acids of the hypothetical *E. coli* protein YeeX and the *H. influenzae* homolog HI1168 was identified on one of the *Sau*3AI fragments in the clone MHY40. *yeeX* is proposed to encode a 15.1 kDa putative alpha helix protein in the *cobU-smbC* intergenic region in *E. coli*. A partial ORF 196 bp upstream on the complementary strand displayed deduced amino acid sequence similarity to a phosphoserine aminotransferase (PSAT) in *H. influenzae*. Analysis downstream in the PM70 genome indicated a hypothetical metabolite transport protein HI1104 in *H. influenzae* on the complementary strands, indicating that this ORF is not part of an operon. A potential ribosome binding site (GAGA) was found 10 bp upstream from the start codon. However, no promoter-like sequences were found upstream between the ORFs. MHY40 was also found to be upregulated during anaerobic growth *in vitro*, but no known anaerobic regulatory sequences were found. Further cloning of the 196 bp region between these two ORFs is needed to identify definitively the promoter element in this clone.

This study represents the first application of the IVET approach to *P. multocida* for the identification of genes that are preferentially expressed *in vivo*. Using a mouse model of infection, a number of genes were isolated that represent *in vivo* expressed loci from the fowl cholera causing isolate, X-73. These may prove to be useful targets for attenuating mutation and/or warrant further investigation of their gene products' role in immunity and pathogenesis. Additionally, the promoter regions of these *in vivo* expressed genes will be useful for the delivery of recombinant antigens *in vivo* by placing a heterologous gene under the control of an "*in vivo*" promoter for expression in an attenuated *P. multocida* strain.

Chapter 3

Generation of defined mutants in the *Pasteurella multocida* A:1 strain X-73

Chapter 3 Generation of defined mutants in the *P. multocida* A:1 strain X-73

Abstract

Pasteurella multocida is the causative agent of many diseases in production animals. Despite the release of the *P. multocida* Pm70 genome by May *et al.* (2001), the function of many genes and their role in pathogenesis and virulence is still largely unknown. The ability to construct defined mutants is invaluable in assigning function to putative pathogenic and virulence factors, but is not routine for *P. multocida*. It also provides a source of novel vaccine strains. This work describes the evaluation of commonly used methods of constructing defined mutants in *P. multocida*. One of these methods, using a *dam* methylated mutagenesis construct, resulted in the generation of a defined mutant in the *in vivo* upregulated gene of *nrfE*, and another method based on screening a *P. multocida* Tn*916* library proved to be a useful technique that warrants further investigation.

Introduction

Pasteurella multocida is a Gram-negative organism that is the causative agent of disease in many species of animals. Of these, fowl cholera in birds and haemorrhagic septicaemia in ungulates cause significant economic losses worldwide (Carpenter *et al.*, 1988, De Alwis, 1984). Despite substantial research, the pathogenesis, immunity and the molecular mechanisms of *P. multocida* are still largely unknown and therefore require further elucidation. Current vaccines comprise either empirically derived attenuated mutants that are not genetically defined and in which reversion to virulence has been observed (Hopkins *et al.*, 1997), or bacterins that provide limited protection (De Alwis, 1984).

The ability to construct defined mutants in pathogenic bacteria is useful for characterisation of unknown genes, the development of novel vaccine strains and for identifying new antimicrobial targets. Although a number of defined mutants have been made in *P. multocida* using various techniques, the ability to construct mutants in *P. multocida* is not routine. Homchampa *et al.* (1992) first described an *aroA* mutant in *P. multocida* in the strains X-73 and P-1059. The mutants were constructed by allelic exchange of an insertionally inactivated *aroA* gene cloned into a suicide vector and electroporated into *P. multocida*. The procedure was facilitated markedly by the use of penicillin enrichment to select for the auxotrophic *aroA* mutants. Similar methods have also been repeated with the *P. multocida* type A strains X-73 (Chung *et al.*, 2001), PBA100 (Hunt *et al.*, 2000), and VP161 (Cox *et al.*, 2003), and the type B strain M1404 (Boyce *et al.*, 2000a). The aforementioned method involved double crossover events occurring in all strains except for VP161 in which the mutant was the result of a single crossover event (Cox *et al.*, 2003). A similar approach utilising conjugation of a suicide construct has been successfully applied to the *P. multocida* type D strain PM25 a number of times to produce defined mutants. (Bosch *et al.*, 2002a, Bosch *et al.*, 2002b, Bosch *et al.*, 2001, Fernandez de Henestrosa *et al.*, 1997), and to the type A strain P-1059 (Mizan *et al.*, 2000). All of the mutants obtained using the conjugation method involved single crossover events except for *galE* (Fernandez de Henestrosa *et al.*, 1997). In a non directed method, the transposons Tn*10* and Tn*916* have been used to randomly disrupt genes in *P. multocida* (Harper *et al.*, 2003, Fuller *et al.*, 2000a, DeAngelis, 1998, Lee *et al.*, 1996).

Several *P. multocida* genes were found to be upregulated *in vivo* during infection in a previous study (Chapter 2), and of these *pta* and *nrfE* were selected for further study. Pta (phosphotransacetylase), together with AckA (acetate kinase), converts acetyl coenzyme A into ATP and acetate and is thus involved in energy metabolism. An attenuated *pta* mutant was identified by Chiang *et al.* (1998) using signature-tagged mutagenesis (STM) in *Vibrio cholera*. The mutant showed reduced expression of the cholera toxin and the toxin coregulated pilus. Both AckA and Pta have also been proposed to play a role in gene regulation in *Escherichia coli* (McCleary *et al.*, 1994). For these reasons, inactivation of *pta* in *P. multocida* could potentially result in an attenuated vaccine strain, and also may help to determine the role of *pta* during infection. *nrfE* is part of a seven gene operon in. *E. coli* that is involved in nitrite reduction (Grove *et al.*, 1996). *nrfE* specifically has been shown to be essential for nitrite reduction (Eaves *et al.*, 1998) and has been shown to be upregulated *in vivo* (Chapter 2). Inactivation of *nrfE* would be useful in determining its function in *P. multocida in vivo* and if the mutant strain is attenuated, it may be useful as a novel vaccine.

Several methods of constructing genetically defined *P. multocida* mutants have recently been tested in our laboratory. Of these methods, two proved to be potentially useful in generating mutants. One of these involved screening a *P. multocida* Tn*916* library by PCR and although it was unsuccessful in gaining either of the two genes of interest, it may be useful on a larger scale in *P. multocida* and other bacteria. The second method involved methylating the mutagenesis construct DNA before transformation into *P. multocida*. This method proved successful in gaining an *nrfE* mutant which will be invaluable for determining the role of *nrfE* in virulence in *P. multocida*.

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Materials and Methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are shown in Table 1. *P. multocida* and *E. coli* were grown with aeration at 37°C or 34°C in brain heart infusion (BHI) or 2YT (Oxoid, Hampshire, England) respectively. Kanamycin (50 μg/ml) and tetracycline (5 μg/ml for *P. multocida*, 10 μg/ml for *E .coli*) were added to solid and liquid media when required.

Table 1 Bacterial strains and plasmids used in this study

Strain or Plasmid	Relevant characteristics	Reference or source
Strains		
E. coli DH5α	F⁻ *endA1 hsdR17*(r_k^- m_k^+) *thi-1* λ⁻ *recA1 gyrA96 relA1* Φ80d*lac*ZΔM15	Bethesda Research Laboratories, Rockville, Md.
P. multocida		
X-73	Serotype A:1 wild-type strain	(Heddleston *et al.*, 1972b)
AL 362	X-73 *nrfE*::*tet*(M) mutant	This study
Plasmids		
pACP1002	pWSK29 containing *pnhA*::*tet*(M) cassette, Amp^r, Tet^r	Carmel Ruffolo, University of Wisconsin-Parkside
pAL109	2 kb insert containing *pta* cloned into pWSK129, Kan^r	This study
pAL159	pWSK129 containing the *pta*::*tet* (M) cassette, Kan^r Tet^r	This study
pAL209	2 kb insert containing *nrfE* cloned into pWSK129, Kan^r	This study
pAL211	pWSK129 containing the *nrfE*::*tet* (M) cassette, Kan^r Tet^r	This study
pVB101	*Tet*(M) gene from Tn*916* cloned into pBR322, Tet^r Amp^r	Vickers Burdett, Duke University, Durham, N.C.
pWSK29	Low-copy-number *E. coli* shuttle vector, Amp^r	(Wang *et al.*, 1991)
pWSK129	Low-copy-number *E. coli* shuttle vector, Kan^r	(Wang *et al.*, 1991)

Recombinant DNA techniques

Genomic DNA was purified by using the cetyltrimethylammonium bromide method (Ausubel *et al.*, 1987). Plasmid DNA was purified by either the alkaline lysis method (Birnboim *et al.*, 1979) or by Qiagen anion exchange columns (Hilden, Germany). PCR amplification of DNA was carried out with *Taq* or *Pwo* polymerases using the reaction conditions as specified by the manufacturer (Roche). End repair of DNA using the Klenow fragment of DNA polymerase was conducted as per manufacturer's instructions (Roche). DNA *dam* methylation was conducted as specified

by the manufacturer (Roche). DNA was introduced into *E. coli* by the chemical transformation method of Hanahan (1985), and electroporated into *P. multocida* as described previously (Jablonski *et al.*, 1992). DNA sequencing was carried out using the BigDye Ready reaction DyeDeoxy Terminator cycle sequencing kits (Perkin-Elmer, Foster City, CA.), and the reactions analysed with a 373A DNA sequencing system. Oligonucleotides used in this study are shown in Table 2. Prior to sequencing or cloning, PCR fragments were either purified by polyethylene glycol precipitation or passage through a Qiagen PCR purification kit (Hilden, Germany).

Southern hybridisation

DNA was digested with appropriate restriction enzymes and separated on 0.8% agarose in 1x TAE buffer and transferred to a nylon membrane (Roche Molecular Biochemicals) by capillary blotting. DNA was fixed to the membrane by baking for 1 hr at 80°C. DIG labelled probes were amplified by PCR with incorporation of DIG-labelled dUTP (Roche Molecular Biochemicals). Hybridisation was carried out at 68°C for 16 h and the membrane was washed twice at room temperature with 2 x SSC, 0.1% SDS and twice at 68°C with 0.5 x SSC, 0.1% SDS. CDP-star (Roche Molecular Biochemicals) was used for detection of DIG-labelled hybridising fragments.

Table 2 Oligonucleotides used in this study

Name	Sequence[a]	Position[b]
BAP683	5'- CAATTTCAATCAATGCTCCCAC-3'	n/a
BAP1127	5'-AGCAGTTCTAGATGATGATACTGTCCC-3'	n/a
BAP1414	5'-GTGCATGAAATAATATACGAGT-3'	n/a
BAP1700	5'-AACAATTCTAGAGTCGTGGTGGAAGGTTTAATC-3'	821478-821510
BAP1701	5'-TAATCGGGATCCCTTTGCCTAATTGTATGCC-3'	822555-822525
BAP1702	5'-CAAGGCGGATCCTTAGGCAAAGGGATTACG-3'	822522-822551
BAP1749	5'-AGCTGCGAATTCGATTGCCAGTAAGCGTGG-3'	823546-823517
BAP1914	5'-GCACATGTCGACTGGATTGGGGTAG-3'	23284-23308
BAP1915	5'-GGTAAATCTAGACATAAGCTTGGTTTTGC-3'	25218-25190
BAP1996	5'-CACGGGTGGTATTGGTGA-3'	820896-820913
BAP2147	5'-GGTTGGGTCGACCAAGCCACATTACTG-3'	1488832-1488858
BAP2277	5'-GCCGGTAGTGTTGATGGA-3'	23172-23189
BAP2278	5'-AGCAAGGCGCCTAGTGCC-3'	25411-25394
CR1	5'-GACAAGACCGCCACCGATACC-3'	108571-108591
CR2	5'-GGCTACCGTCCGAATGTAGG-3'	109643-109624

[a] Underlined bases indicate engineered restriction sites
[b] Position in PM70 genome, accession number: NC_002663

Results

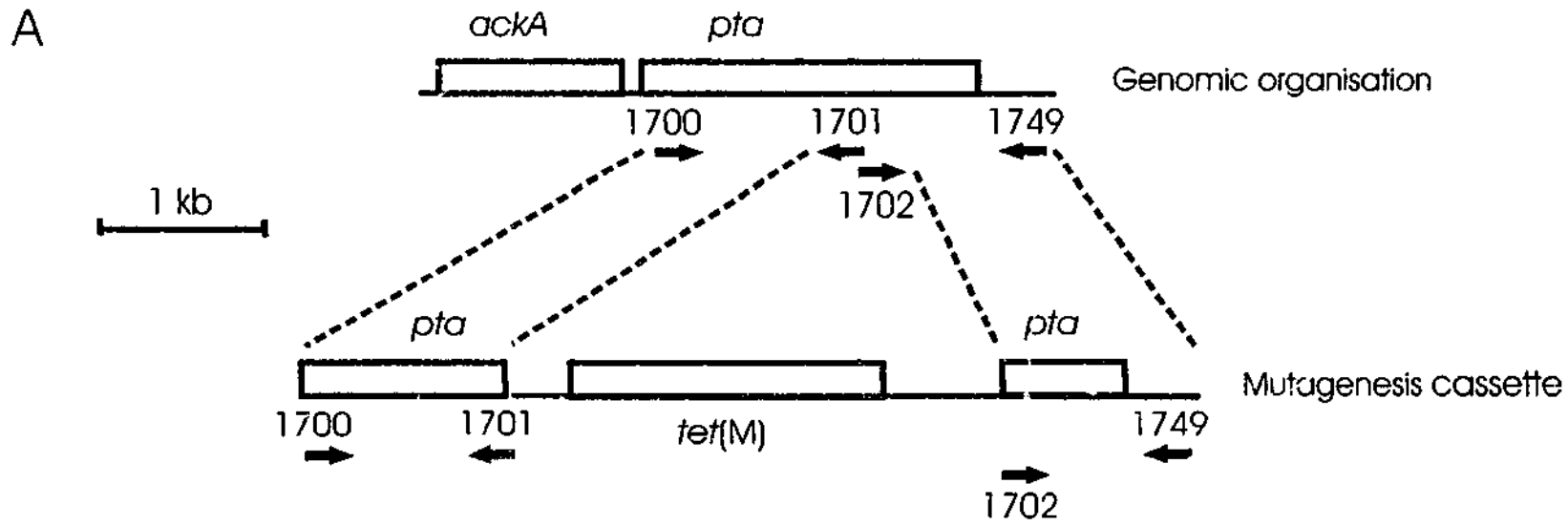
Construction of *pta* & *nrfE* mutagenesis plasmids

A mutagenesis construct with a *tet*(M) disrupted *pta* gene was made by ligating two PCR-generated DNA fragments to the *tet*(M) gene from pVB101 (Fig. 1A). The oligonucleotides BAP1700 and BAP1701, and BAP1702 and BAP1749 (Table 2) were used to amplify two contiguous 1 kb sections of *P. multocida* X-73 *pta* gene by PCR. These fragments were ligated together to form a unique *Bam*HI site and were cloned into pWSK129 to generate pAL109 (Table 1). A 3.2 kb *Bam*HI-digested fragment of pVB101 containing *tet*(M) was cloned into the *Bam*HI site of pAL109 generating pAL159. In a manner similar to that used for *pta*, a construct was made containing *nrfE* disrupted by *tet*(M) (Fig. 1B). BAP1914 and BAP1915 (Table 2) were used to amplify a 2.1 kb DNA fragment by PCR that was then ligated into pWSK129 to make pAL209 (Table 1). A 3.2 kb *Bam*HI-digested fragment of pVB101 was cloned into a unique *Bam*HI site in pAL209 to form pAL211.

Methods of mutation

Electroporation of *P. multocida*

A number of approaches were used in an attempt to generate defined mutants in the *P. multocida* strain X-73 using the pAL159 and pAL211 constructs (Table 3). Both were electroporated into X-73 in both native plasmid form, and as amplified PCR fragments generated using the primers BAP1700/1749 and BAP1914/1915 from pAL159 and pAL211 respectively. However, despite numerous attempts with varying amounts of DNA (Table 3), no mutants were recovered using these methods. Hinds *et al.* (1999) found that homologous recombination was increased in *Mycobacterium* sp. when using DNA denatured by sodium hydroxide. pAL159 and PCR fragments amplified from this construct were treated according to the method of Hinds *et al.* (1999) and electroporated into X-73. However, again no mutants were recovered using this technique (Table 3). An analysis of the Pm70 genome revealed the presence of a *dam* methylase and adenine methylase homologue indicating the presence of a potential modification/restriction system in *P. multocida*. Both pAL159 and pAL211 and PCR products amplified from these products (as described previously) were treated with *dam* methylase and electroporated into X-73.



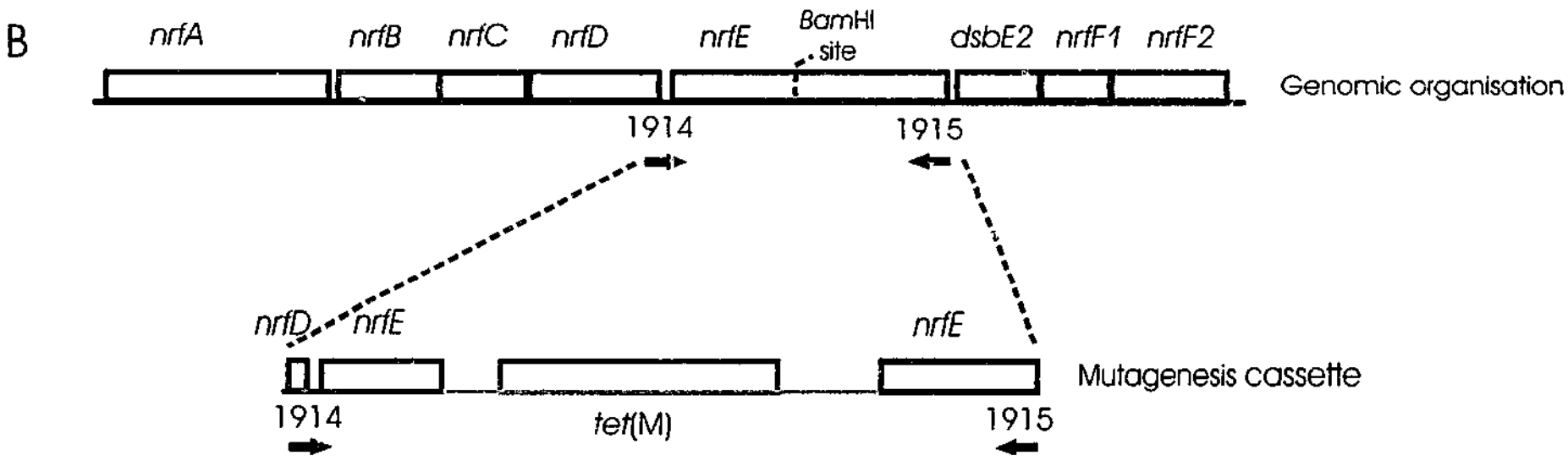


Fig. 1 Schematic representation of construction of the *P. multocida pta* and *nrfE* mutagenesis constructs. (A) Two 1 kb fragments containing *pta* were amplified by PCR using the primers BAP1700 and BAP1701 (designated by the arrows 1700/1701), and BAP1702 and BAP1749 respectively (arrows 1702/1749). These fragments were ligated to either end of the *tet*(M) gene to obtain linear DNA (mutagenesis cassette) used for mutagenesis by allelic exchange. (B) A single 1.8kb fragment containing *nrfE* was amplified by PCR using the primers BAP1914 and BAP1915 (designated by the arrows 1914/1915). This fragment was digested by *Bam*HI to yield two 900bp fragments that were then ligated to either end of the *tet*(M) to produce the mutagenesis cassette used for allelic exchange.

Table 3 Summary of transformation methods tested for production of *pta* and *nrfE* mutants

Construct used	DNA form	Method of transformation		
		Electroporation[a]	Natural[ab]	
			Klenow[c]	Pwo[d]
pta	Plasmid	5^{2}	n/a	n/a
		3^{n}		
		5^{n}		
		3.6^{m}		
	PCR product	4^{2}	1^{2}	0.3
		5^{2}		1^{2}
		6		
		2^{m}		
		4^{m}		
		4^{nm}		
nrfE	Plasmid	$5^{m(3)}$	n/a	n/a
	PCR product	3	-	-
		5		
		5^{m}		

[a] Amount of DNA (µg) used in transformation; superscript numbers indicate number of times attempted; superscript number in brackets () indicates number of colonies recovered where present.
[b] Natural transformation method as described in this chapter.
[c] End filling by Klenow as described in this chapter.
[d] Pwo PCR products used as described in this chapter.
[m] Methylated by dam methylase.
[n] Treated with NaOH as described in this chapter
n/a Procedure cannot be performed on plasmids.

Despite repeated attempts (Table 3) no colonies were recovered from the *pta* construct pA159, nor from PCR products amplified from this construct. However, three colonies were recovered from the *dam* methylated pAL211, but none from methylated PCR products amplified from pAL211.

Natural transformation of *P. multocida*

A method of natural transformation was used as described by Ruffolo (Personal communication. 2002). Briefly, overnight cultures of *P. multocida* X-73 grown at 34°C were used to inoculate 250 ml BHI in a 2 L flask and incubated at 34°C to an OD_{550} of 0.75. Amplified PCR products generated from pAL159 (as described previously) were either end-filled using Klenow fragments or amplified with *Pwo* instead of *Taq* to yield blunt-ended fragments. These fragments were then ligated and 100 µl of DNA (containing between 300 ng and 1 µg) was spread onto ¼ BHI plates. 100 µl of bacterial culture was then spread onto the DNA plates and incubated at 34°C for a minimum of 36 h. A PCR product amplified using CR1 and CR2 from the plasmid pACP1002 was

used a positive control. pACP1002 contains the *P. multocida pnhA* gene disrupted by *tet*(M), and was previously used via the natural transformation method to obtain *pnhA* mutants disrupted by *tet*(M) (Ruffolo, Personal communication, 2002). Despite several attempts (Table 3) no colonies were recovered from either the positive control or the *pta* construct.

Screening of a Signature tagged mutagenesis library for *nrfE* and *pta* mutants

A PCR-based system was designed to screen a previously constructed *P. multocida* STM library (Harper *et al.*, 2003) for existing *pta* or *nrfE* mutants. The system utilised outward firing primers from within the Tn*916* transposon (BAP1414 and BAP1127) in conjunction with the corresponding gene specific primers BAP1996 or BAP1914 for *pta* and *nrfE* respectively (Fig. 2). These primers were then used to screen the *P. multocida* STM library in 10 genomic DNA pools consisting of 42 unique transposon mutants in each pool (420 mutants in total). A known *P. multocida* STM mutant (Pm1294) was used as a positive control by using the transposon primer BAP1127 and the Pm1294 specific primer BAP2147. No PCR products were observed for any of the *pta* or *nrfE* primer combinations. However, a product of the expected size was amplified for the positive control (Pm1294). These results indicated that neither *pta* nor *nrfE* were disrupted in any of the screened Tn*916* mutants.

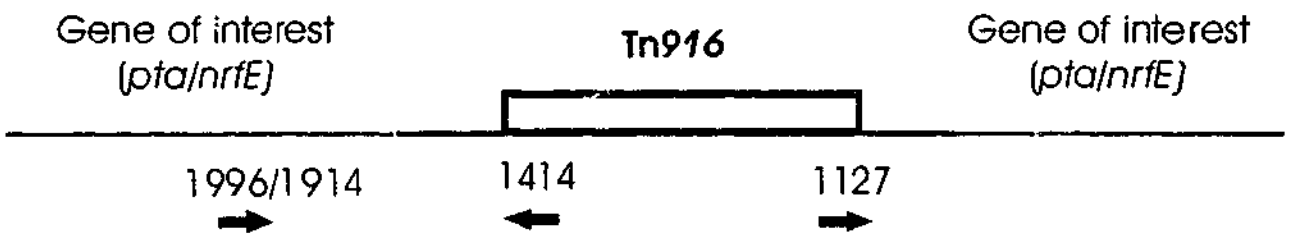


Fig. 2 Experimental design for the PCR screening of the *P. multocida* STM library for either *pta* or *nrfE* mutants. The library was screened by PCR using the outward firing Tn*916* primers BAP1414 and 1127 (denoted by the arrows 1414 and 1127 respectively). The Tn*916* primers were paired with the gene specific primers BAP1996 or BAP1914 for *pta* and *nrfE* respectively (denoted by the arrows and 1996 and 1914)

Confirmation of *nrfE* mutant by Southern hybridisation

Three putative *nrfE* mutants were recovered from the electroporation of *dam* methylated pAL211 into *P. multocida* X-73. All three were tetracycline resistant, but two were also kanamycin resistant, and all three were analysed by Southern hybridisation (Fig. 3). Genomic DNA was isolated from the wild-type strain X-73 and the three mutant strains (M1-3) and digested with either *Pvu*II or *Hin*cII. As a control, the mutagenesis plasmid pAL211 was also digested with either *Pvu*II or *Hin*cII. The restriction digests were separated by agarose gel electrophoresis and transferred to a nylon membrane which was hybridised with a DIG-labelled probe homologous to the *nrfE* gene. The *nrfE* probe was synthesised by PCR from pAL211 using the primers BAP1914 and BAP1915 (Fig. 1). The *nrfE* probe hybridised to a single 2.5 kb fragment in the wild-type X-73 genomic DNA digested with *Pvu*II (Fig. 3, lane 4) and to a 5.7 kb fragment in the *Pvu*II digested M1, M2, M3 DNA (Fig. 3, lanes 6, 8, and 10 respectively). These data indicate that strains M1, M2 and M3 all contain a 3.2 kb insertion within *nrfE* and this is likely the result of a double crossover with the *tet*(M) disrupted *nrfE* from pAL211. This was also confirmed in the *Hin*cII digests, where the *nrfE* probe hybridised to 2.1 and 2.2 kb fragments in the X-73 digested DNA (Fig. 3, lane 5), and to 2.1 kb and 5.3 kb fragments in the M1, M2 and M3 digested DNA (Fig. 3, lanes 7, 9 and 11). Thus the mutants always show a 3.2 kb increase in size compared to the wild-type fragments which corresponds with the insertion of the 3.2 kb *tet*(M) fragment. pAL211 digested with *Pvu*II yielded a single 5kb fragment (Fig. 3, lane 2) which was also seen in the Tet/Kan resistant strains M1 and M3 (Fig. 3, lanes 6 and 10). Similarly, *Hin*cII digested pAL211 yielded a 4.4 kb fragment and a 7.5 kb fragment (Fig. 3. lane 3) which was seen in both Kan/Tet resistant strains (M1 and M3, Fig. 3, lanes 7 and 11) The presence of these identical bands is consistent with the retention of pAL211 in the strains M1 and M3.

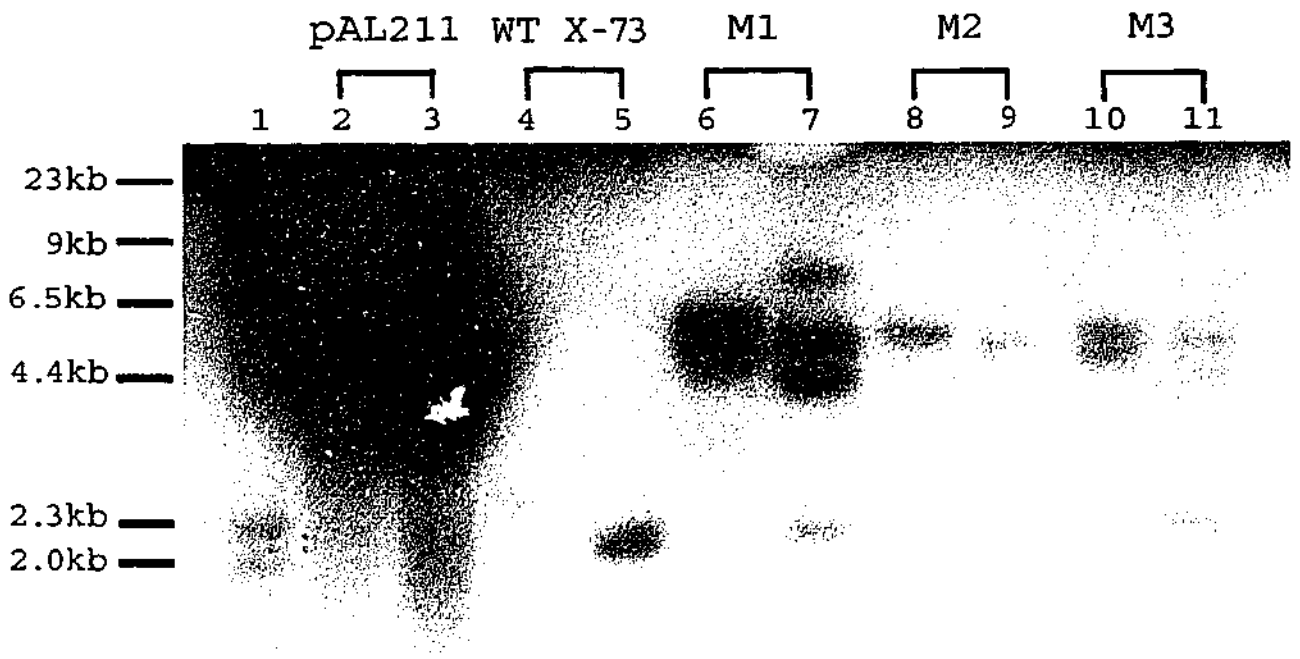
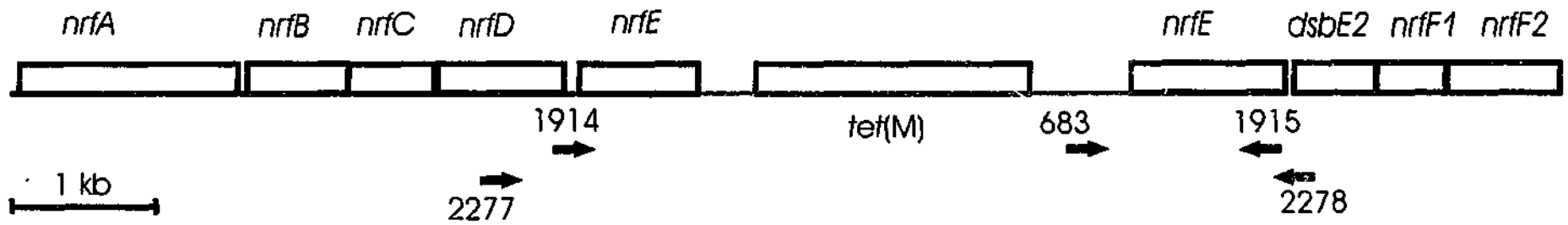


Fig. 3. Southern analysis of the potential *nrfE*::*tet*(M) transformants (M1, M2 and M3), wild-type X-73 and the mutagenesis plasmid pAL211. Total genomic DNA was digested with either *Pvu*II or *Hin*cII separated on a 0.8% agarose gel and transferred onto a nylon membrane. This was then probed with a DIG labelled PCR product homologous to *nrfE*. The probe was synthesised by PCR from pAL211 using the primers BAP1914 and BAP1915. Lane1, λ *Hin*dIII size markers; lane 2, pAL211 digested with *Pvu*II; lane 3, pAL211 digested with *Hin*cII; lane 4, parent strain X-73 DNA digested with *Pvu*II; lane 5, parent strain X-73 DNA digested with *Hin*cII; lane 6, M1 DNA digested with *Pvu*II; lane 7, M1 DNA digested with *Hin*cII; lane 8, M2 DNA digested with *Pvu*II; lane 9, M2 DNA digested with *Hin*cII; lane 10, M3 DNA digested with *Pvu*II; lane 11, M3 DNA digested with *Hin*cII.

Confirmation of the *nrfE* mutant by PCR

Mutant M2 was designated AL362 and the presence of the *tet*(M) cassette in this strain was confirmed by PCR (Fig. 4). Wild-type X-73 and AL362 genomic DNA was used as the template for PCR with the primers BAP1914 and BAP1915. A 1.8 kb fragment was amplified from X-73 genomic DNA while a 5 kb fragment was amplified from AL362 DNA (Fig. 4, lanes 2 and 3 respectivcly). Using the oligonucleotides BAP2277 and BAP2278, (both of which are located outside of the region cloned in the mutagenesis construct pAL211) a 2.2 kb fragment was amplified from X-73 and a 5.4 kb fragment was amplified from AL362 (Fig. 4, lanes 4 and 5 respectively). In addition, using the primers BAP2278 and BAP683 (a primer specific for *tet*(M)) no product was observed from the wild-type X-73 DNA, but a product of 1.4 kb was identified in the AL362 DNA (Fig. 4., lanes 6 and 7 respectively). These data indicated that the *tet*(M) gene was inserted within the *nrfE* gene present in the genome and that the *tet*(M) gene was in the same orientation as the *nrfE* operon.

A



B

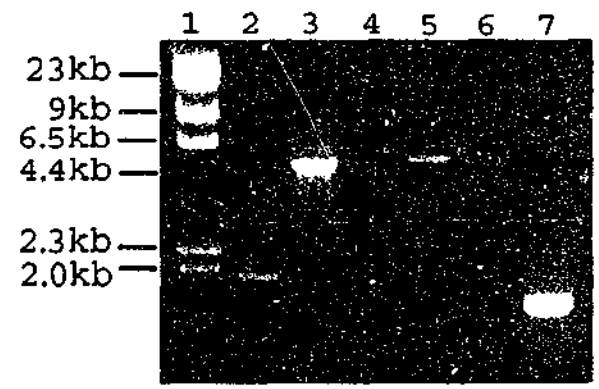


Fig. 4 PCR confirmation of *tet*(M) insertion within *nrfE* (A) Schematic representation of the genome organisation around *nrfE* after insertion of the *tet*(M) cassette. Labelled arrows indicate primers used for PCR as shown in part B. (B) The genotype of AL362 was investigated by PCR. Genomic DNA from AL362 (lanes 3, 5, 7) was compared to wild-type X-73 (lanes 2, 4, 6). Lanes 2 and 3 were amplified using BAP1914 and 1915, lanes 4 and 5 were amplified with BAP2277 and 2278. Lanes 6 and 7 were amplified using the *tet*(M) primer BAP683 together with the genomic primer BAP2278). Lane 1contains λ DNA digested with *Hin*dIII.

Discussion

The inactivation of genes in a targeted manner has proved to be difficult in *P. multocida*. Certainly gene expression inactivation by homologous recombination in *P. multocida* is possible. This is evidenced by the small but growing number of mutants in the literature, but progress has been very slow. It is likely that problems are due to a number of factors including low transformation efficiency, low recombination efficiency and the possible presence of restriction/modification barriers. There also appears to be a bias in the literature towards gene inactivation by single crossover events, particularly using conjugation as the DNA mobilisation method. Indeed, Cárdenas *et al.* (2001) and Bosch *et al.* (2001) observed homologous recombination (HR) by single crossover events, but not by double cross over events. This is not unexpected, as the frequency of single crossover events would be expected to be higher than double crossover events. However, it may also indicate that the efficiency of either recombination and/or transformation is very low in *P. multocida*.

Several methods of obtaining a defined *pta* and *nrfE* mutant in *P. multocida* were attempted; however, only one was successful, and only for *nrfE*. Both plasmid and PCR amplified DNA have been used successfully in previous experiments (Chung *et al.*, 2001, Boyce *et al.*, 2000a). In this study, DNA in the native form proved unsuccessful in constructing defined mutants in either *pta* or *nrfE*. DNA denatured by sodium hydroxide was shown by Hinds *et al.* (1999) to increase the rate of HR in *Mycobacterium smegmatis*. This was proposed to be due to stimulation of *recA* by single DNA strands or other DNA repair systems. However, it is yet to be determined whether it increases HR in *P. multocida* as no mutants were ever recovered using this method in X-73.

A method of natural transformation has been used by Ruffolo *et al.* (Personal communication, 2002) to obtain a number of defined mutants by HR. However, attempts in using this method were unsuccessful either with the *pta* construct pAL159 or the positive control pACP1002, which had been previously used to disrupt *pnhA* (Ruffolo, Personal communication, 2002). The mechanisms underlying the natural transformation method in *P. multocida* are still unknown, and it is therefore difficult to speculate why this method was unsuccessful, although it is likely that natural competence is dependent on very specific media and growth conditions. The positive

control construct pACP1002 had previously been used to inactivate *pnhA* by HR using naturally competent X-73 in another laboratory, but failed on many attempts in this work and in other work from our laboratory (data not shown). This was unexpected and may be due to specific conditions for natural competence not being met, either by slight technical variations, or possibly due to minor strain variation within the laboratory strains due to culture adaptation.

The screening of the *P. multocida* STM library by PCR failed to identify either *pta* or *nrfE* Tn*916* mutants. However, the library consisted of a pool of only 420 STM mutants (some of these known duplicates) and there are 2015 proposed open reading frames in the Pm70 genome. The method reliably detected a known mutant in the pool (Pm1294), and therefore would be of further use in a larger library. Tn*916* has previously been shown to transpose in a quasirandom fashion in *P. multocida* (Harper *et al.*, 2003), and therefore if a library is large enough it should be possible to detect an insertion in a gene of interest, unless the transposition causes lethal effects. It would also be possible to use this system with other bacteria where it is difficult to inactivate genes by HR but where transposons are available.

Analysis of the Pm70 genome revealed that there was both a proposed *dam* methylase gene and an adenine methylase homologue (May *et al.*, 2001), suggesting that there may be an active *dam* modification/restriction system present in *P. multocida*. Several putative type I restriction modification genes were also identified in Pm70 (*hsdM*, *hsdR*, *hsdA*). Therefore, in order to overcome a possible restriction barrier, pAL211, pAL159 and the PCR products amplified from pAL211 and pAL159 were *dam* methylated before electroporation into X-73. Although the plasmids should have been methylated by the native *dam* and *hsd* methylases in DH5α, they were treated to ensure *dam* methylation had occurred to maximise the efficiency of transformation. Despite several attempts using this method, no *pta* mutants were recovered. However, three *nrfE* mutants were recovered, two of which also appeared to carry free pAL211 plasmid. pAL211 is based on pWSK129 which has been detected replicating in *P. multocida* in our laboratory (Adler, Personal communication, 2003) but the occurrence is rare. The plasmid carriage may have also been due to the plasmid being *dam* methylated and hence the replication of pAL211 was affected by this, although this has not been reported in pWSK129. *dam* methylation may also have increased the efficiency of pAL211 transformation into X-73, causing the normally rare event of free replicating plasmid to occur more frequently.

The inability to construct a *pta* mutant may be due to a number of reasons. AckA and Pta are both central enzymes in metabolism (Fox *et al.*, 1986) and have been proposed to be involved in gene regulation (McCleary *et al.*, 1994, Wanner *et al.*, 1992). They have also been proposed as regulators of virulence gene expression in *V. cholerae* (Chiang *et al.*, 1998). Therefore it is possible that inactivation of *pta* in *P. multocida* has lethal effects due to the loss of essential functions. Notably, *pta* and *ackA* mutants in *E. coli* and *S.* Typhimurium have been constructed, but they exhibited slower growth rates *in vitro* (LeVine *et al.*, 1980) Furthermore, a *pta* mutant was obtained in *Vibrio cholerae* by STM and was shown to be attenuated in mice (Chiang *et al.*, 1998). Therefore, if inactivation of *pta* in *P. multocida* is not lethal, the mutant could potentially be very weak growing *in vitro* and difficult to recover, particularly with the low efficiency of directed mutations in *P. multocida*. It is possible that the transformation methods used were too inefficient to generate a *pta* mutant and further optimisation is needed, or an alternative method such as conjugation should be used. One possible method of improving the transformation would be methylating the mutagenesis construct using the *hsd* system. The *hsd* system involves identifying "self" DNA from foreign DNA by methylating specific positions (Russell *et al.*, 1989) and homologues of this system have been identified in the Pm70 genome (May *et al.*, 2001). Native methylation in DH5α by the *hsd* system most likely occurs in a different position from the native *P. multocida hsd* system. For this reason it may be useful to clone and express the *P. multocida* Hsd methylase in order to methylate foreign DNA such as the *pta* construct correctly.

The data presented here describe a number of methods tested for generating defined mutants in *P. multocida*. Of these, methylating DNA prior to electroporation of X-73 resulted in generation of an *nrfE* mutant, and the characterisation of this mutant will be described further in Chapter 4. The success of the DNA methylation strategy suggests that this may be a useful method for generating mutants by homologous recombination in *P. multocida*. Screening of transposon libraries using a PCR based system as outlined here may also be useful for identifying mutants with specific gene disruptions, as well as in other bacteria that have proved difficult hosts for targeted mutagenesis.

Chapter 4

Characterisation of the *nrfE* gene of *Pasteurella multocida*

Chapter 4 Characterisation of the *nrfE* gene of *P. multocida*

Abstract

Pasteurella multocida is the causative agent of many diseases in production animals. Many systems have been employed to identify virulence factors of *P. multocida* including *in vivo* expression technology (IVET), signature-tagged mutagenesis, and whole genome expression profiling. In a previous study using IVET, *nrfE* was identified as being expressed *in vivo*. In *Escherichia coli, nrfE* is part of the Nrf (formate dependent nitrite reductase) system involved in utilising available nitrite in the host as an electron accepter during growth under anaerobic conditions. In this study, we found that *P. multocida* has an active Nrf system that functions during growth under aerobic and anaerobic conditions. Furthermore, similar to *E. coli*, *nrfE* is essential for Nrf activity. Real time RT-PCR analysis indicated that *nrfE* was regulated independently of *nrfABCD* by an independent promoter that is likely to be upregulated *in vivo*.

Introduction

Pasteurella multocida is a Gram-negative bacterial pathogen responsible for a number of diseases prevalent worldwide including bovine haemorrhagic septicaemia, avian fowl cholera, atrophic rhinitis in swine and snuffles in rabbits. The worldwide economic cost of these diseases in production animals is significant, but despite considerable research, safe and effective vaccines against pasteurellosis are still lacking. The molecular mechanisms of *P. multocida* pathogenesis are still largely unknown and only a few virulence factors have been identified, including; toxins (Chanter, 1990), capsule (Boyce *et al.*, 2000a, Chung *et al.*, 2001), iron acquisition proteins (Bosch *et al.*, 2002a, Fuller *et al.*, 2000a, May *et al.*, 2001), and haemagglutinins (May *et al.*, 2001). As it is likely that many virulence factors remain uncharacterised, there is a need to utilise new screening methods to identify virulence factors that might be useful as vaccine antigens, or as targets for antimicrobial compounds.

A number of methods have been utilised to identify genes expressed during the pathogenesis of pasteurellosis, including *in vivo* expression technology (IVET) ((Hunt *et al.*, 2001), (Chapter 2)), signature-tagged mutagenesis (Fuller *et al.*, 2000a, Harper *et al.*, 2003) and whole genome expression profiling (Boyce *et al.*, 2002). The *P. multocida* IVET study identified a number of genes upregulated *in vivo* in mice (Chapter 2). One of these genes (*nrfE*) was selected for further characterisation and subsequently disrupted by insertional inactivation (Chapter 3).

The formate dependent nitrite reductase (Nrf) system is present in a number of enteric bacteria, including *Escherichia coli* and *Salmonella* species and in the species closely related to *P. multocida*; *Haemophilus influenzae* and *Actinobacillus actinomycetemcomitans* (Potter *et al.*, 2001). The Nrf system in *E. coli* is encoded by a seven gene operon (*nrfABCDEFG*) and functions to use nitrite as an alternate electron acceptor to oxygen during anaerobic growth. *nrfA* encodes a 50 kDa cytochrome that utilises nitrite as an electron acceptor and *nrfBCD* encode proteins that are essential for electron transfer to the catalytic subunit, NrfA (Hussain *et al.*, 1994). *nrfEFG* have been proposed to encode proteins that form a haem lyase required for attachment of a haem group to the site of catalysis of NrfA (Eaves *et al.*, 1998, Grove *et al.*, 1996, Hussain *et al.*, 1994). *The nrf* operon has been shown to be regulated in *E. coli* by FNR in response to anaerobic conditions, and to NarL and NarP when in the presence of nitrate and

nitrite (Darwin *et al.*, 1993, Potter *et al.*, 2001). *nrfE* has been shown to be essential for formate-dependent nitrite reduction in *E. coli* (Eaves *et al.*, 1998) and has been identified in *P. multocida* as being upregulated *in vivo* during infection (Chapter 2). For this reason it was of interest to determine what role *nrfE* plays in the metabolism of *P. multocida* during growth *in vitro* and during infection of the host.

In this study, the function of the *nrfE* gene of *P. multocida* was characterised using an *nrfE* inactivated strain of X-73 and a preliminary analysis of the transcriptional regulation of the *P. multocida nrf* operon was conducted.

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are shown in Table 1. *P. multocida* and *E. coli* were grown with aeration at 37°C in brain heart infusion (BHI) or 2YT (Oxoid, Hampshire, England) respectively. Kanamycin (50 µg/ml) and tetracycline (5 µg/ml for *P. multocida*, 10 µg/ml for *E. coli*) were added to solid and liquid media when required.

Table 1 Bacterial strains and plasmids used in this study

Strain or Plasmid	Relevant characteristics	Reference or source
Strains		
E. coli DH5α	F⁻ *endA1 hsdR17*($r_k^- m_k^+$) *thi-1* λ⁻ *recA1 gyrA96 relA1* Φ80d*lacZ*ΔM15	Bethesda Research Laboratories, Rockville, Md.
P. multocida		
X-73	Serotype A:1 wild-type strain	(Heddleston *et al.*, 1972b)
AL362	X-73 *nrfE*::*tet*(M) mutant	This study
AL464	*E. coli* DH5α harbouring pAL263	This study
AL465	AL362 harbouring pAL99	This study
AL466	AL362 harbouring pAL263	This study
Plasmids		
pAL99	40 bp *Eco*R1 fragment containing *P. multocida tpiA* promoter region cloned into pPBA1100 *Eco*R1 site	(Harper *et al.*, 2004)
pAL263	2 kb insert containing full *nrfE* gene in pAL99 (*nrfE* expression driven by *tpiA* promoter)	This study
pBA1100	*Pasteurella-E. coli* shuttle vector, pUC18 derivative	(Homchampa *et al.*, 1997)
pWSK129	Low-copy-number *E. coli* shuttle vector, Kan^r	(Wang *et al.*, 1991)

Modified chemically defined media (CDM) for nitrite reduction studies

A modified version of the chemically defined medium (CDM) described by Jablonski *et al.* (1996) was used for nitrite reduction studies. Unless specifically mentioned, concentrations of solutions were as per Jablonski *et al.* (1996). Briefly the CDM was made to a 5 X stock devoid of L-Arginine, L-serine, L-glutamic acid, L-phenylalanine, L-leucine, L-isoleucine, L-aspartic acid, L-tyrosine, $MgSO_4$ and glucose but supplemented with 50 g/L casamino acids (Sigma). The 5 X stock was filter sterilised and diluted to a 1 X working concentration using one of three different filter sterilised 2.5 X base solutions. The first 2.5 X base solution (Normal) contained; L-

aspartic acid, L-tyrosine and $MgSO_4$, while the second was identical but supplemented to give a 1 X CDM working concentration of 0.4% glycerol and 40 mM sodium fumarate (glycerol/fumarate CDM). The third solution was the same as the glycerol/fumarate CDM, but additionally supplemented to give a final 1 X CDM concentration with 2 mM sodium nitrite and 1 mM sodium nitrate (nitrite/nitrate CDM).

Recombinant DNA techniques

Genomic DNA was purified by using the cetyltrimethylammonium bromide method (Ausubel *et al.*, 1987). Plasmid DNA was purified by either the alkaline lysis method (Birnboim *et al.*, 1979) or by Qiagen anion exchange columns (Hilden, Germany). PCR amplification of DNA was carried out with *Taq* polymerase using the reaction conditions as specified by the manufacturer (Roche Molecular Biochemicals, Basel, Switzerland). DNA was introduced into *E. coli* by the chemical transformation method of Hanahan (1985), and electroporated into *P. multocida* as described previously (Jablonski *et al.*, 1992). DNA sequencing was carried out using the BigDye Ready reaction DyeDeoxy Terminator cycle sequencing kits (Perkin-Elmer, Foster City, CA), and the reactions analysed with a 373A DNA sequencing system. Oligonucleotides used in this study are shown in Table 2. Prior to sequencing or cloning, PCR fragments were either purified by polyethylene glycol precipitation or passage through a Qiagen PCR purification kit (Hilden, Germany).

Table 2 Oligonucleotides used in this study

Name	Sequence[a]	Position[b]
BAP2106	5'-GCCCTTTCCGATAAATTGCAA-3'	1670646-1670626
BAP2107	5'- ATCGCGGCTAATGGTGCTT-3'	1670549-1670567
BAP2123	5'-AATGGCATGTTGGTCTTGTAAAAG-3'	20017-20040
BAP2124	5'-CCACCACTTGCCCATGTTG-3'	20112-20094
BAP2283	5'-TTGATAGAGCTCTTCAAATTT-3'	23510-23530
BAP2284	5'-CATACTCATGAGCTCACCTGT-3'	25494-25474
BAP2363	5'-CCATCGCCGTTTATTTGTTCTT-3'	22553-22574
BAP2364	5'-TGCCGTACAACGTATCACATGA-3'	22677-22656
BAP2398	5'-GATTTTTGGCTTTACTGATCGCTT-3'	23560-23583
BAP2399	5'-CGCAGTACCAATCATATACGGC-3'	23660-23639
BAP2403	5'-GGACAAATGACGCTAGCATAAATG-3'	23449-23472
BAP2404	5'-ACATCGCAAAGGCTTTATGCTAC-3'	23367-23389
BAP2405	5'-AGCGTCATTTGTCCTGCATAGA-3'	23462-23441
BAP2407	5'-AAGCCAAAAAACCAAGTTCTGG-3'	23571-23550
BAP2442	5'-CACTATGATCCCAGAACTTGGTTTT-3'	23540-23564

[a] Underlined bases indicate engineered restriction sites
[b] Position in Pm70 genome, accession number: NC_002663

RNA isolation

Bacteria were harvested from four replicate cultures at an OD_{600} of 0.5 (4 x 10^9 CFU/ml), added to 0.1 volume of ice cold killing buffer (0.05 M Tris-HCl pH 7.5, 15 mg/ml of sodium azide, 0.6 mg/ml chloramphenicol), and pelleted by centrifugation. RNA was isolated from the bacteria using Trizol (Gibco/BRL) as described by the manufacturer. Purified RNA was treated with DNase (20 U for 20 min at 37°C), and the RNA was further purified using RNeasy columns (Qiagen).

Reverse transcription (RT) and real-time RT-PCR

Primers for real-time RT-PCR were designed with Primer Express software (ABI) (Table 2). Reverse transcription reactions were preformed at 42°C for 2 h and contained 10 µg of total RNA, 15 µg of random hexamers, 5 U of Superscript II (Gibco/BRL) reverse transcriptase, and 200 µM (each) of dATP, dGTP, dCTP and dTTP. Synthesised cDNA samples were diluted 80-fold prior to real-time RT-PCR, which was carried out using an ABI PRISM model 7700 sequence detector with product accumulation quantified by incorporation of the fluorescent dye SYBR Green. Triplicate real-time RT-PCRs were performed using 2.4 µl of cDNA with SYBR Green PCR mix (ABI) and 50 nM concentrations of each gene-specific primer. Gene-specific standard curves were constructed from known concentrations of *P. multocida* X-73 genomic DNA and used to determine relative template concentrations in each reaction. *gyrB* was used as a normaliser for all reactions using the primers BAP2106 and BAP2107. Relative expression was determined by normalising data against *gyrB*. All RT-PCRs amplified a single product as determined by melting curve analysis.

Nitrite reduction assay

The nitrite reduction assay was based on the method used in *E. coli* by Hussain *et al.* (1994). For aerobic studies, 1 ml of CDM (Normal) was inoculated with *P. multocida* and grown at 37°C overnight. 200 µl of this was used to inoculate 1 ml of CDM (nitrate/nitrite) in triplicate cultures which were then incubated at 37°C overnight. The overnight cultures were diluted 1/10 in fresh CDM (glycerol/fumarate) and incubated for 24 h or until an OD_{600} of > 0.5 was reached. Aliquots (50 µl) of each test culture were mixed with 0.5 ml of 1% (w/v) of sulphanilamide in 1 M HCl and 0.5 ml of 0.02% *N*-1-napthylethylenediamine dihydrochloride. The formation of an intense

pink colour after 30 s indicated the presence of nitrite, and the optical density of the samples was measured in a spectrophotometer at 530nm.

Disk diffusion *S*-nitroso-*N*-acetyl-penicillamine (SNAP) susceptibility assay

Agar suspensions of the *P. multocida* test strains were made by adding approximately 1 ml of 10^{-8} cfu/ml bacteria to 3 ml of CDM (Nitrite/Nitrate) containing 3% (wt/vol) Noble Agar (Oxoid) and pouring onto plates. Whatmann paper disks (4 mm) were then soaked in 15 µl of a 250 mM solution of SNAP dissolved in methanol and placed in the centre of each plate. Disks soaked in methanol were used as controls and the plates were incubated overnight under either aerobic or anaerobic growth conditions.

Competition growth assays in mice

Log-phase cultures of mutant and wild-type *P. multocida* were mixed at a 1:1 ratio and prepared in a series of tenfold dilutions. 100 µl of an appropriate dilution was plated onto NB agar to obtain single colonies representing the input pool. For the *in vivo* assays, approximately 10^6 CFU were injected into mice, and blood was recovered at 6 h. Blood was diluted two-fold in BHI containing heparin and plated onto NB agar. For the *in vitro* assay, a hundred-fold dilution of the mixed bacteria was grown for 6 h at 37°C, diluted appropriately and plated onto NB agar. A minimum of 100 colonies grown on non-selective plates were patched onto NB agar with and without tetracycline. Relative competitive index (rCI) was determined by dividing the percentage of tetracycline-resistant colonies (AL362) obtained *in vivo* by the percentage of tetracycline-resistant colonies obtained *in vitro*. Significance was determined by calculating a *P* value from an approximate *z*-test for the difference between two proportions.

Sequence analysis and statistical programs

Sequences were aligned using the lalign algorithm (Huang *et al.*, 1991). Statistical analyses were conducted using the InStat program (Graphpad Software Inc.)

Results

Genetic organisation of the *nrf* operon of *P. multocida*

The organisation of the *nrf* operon in the *P. multocida* Pm70 strain (May *et al.*, 2001) was compared to that of the *E. coli* K12 (Blattner *et al.*, 1997) and *Haemophilus influenzae* Rd (Fleischmann *et al.*, 1995) strains (Fig. 1). The Pm70 *P. multocida nrf* operon comprised eight open reading frames (ORFs), namely *nrfABCDE, nrfF_1, nrfF_2* and *dsbE_2*. In contrast to *E. coli*, *P. multocida* had two *nrfF* genes, and a *dsbE_2* gene (Fig. 1). However, the *P. multocida nrfF_2* gene had a higher identity to the *nrfG* gene of *E. coli* than to the *nrfF* gene indicating that it is most likely an orthologue of *nrfG*. *H. influenzae* was also lacking the *nrfG* gene but had a *dsbE* gene and a single *nrfF* gene. However, in *H. influenzae, nrfEF* and *dsbE* were 134 kb downstream of *nrfABCD* indicating that the *nrf* operon may be either non-functional in *H. influenzae*, or that the groups of genes are transcribed independently. The *nrfF* gene in *H. influenzae* showed partial homology to *nrfF_1* (first 150 bp) and partial homology to *nrfF_2* (150-270 bp) indicating that the NrfF protein in *H. influenzae* may have two domains that perform the function of *nrfF* and *nrfG*. *The P. multocida nrfABCDEF* genes showed highest identity to the *nrf* genes of *H. influenzae*. This is not unexpected as *H. influenzae* and *P. multocida* are closely related and the presence and similar position of the *dsbE* gene indicates that they may have originally shared a similar *nrf* operon arrangement.

Complementation of the *nrfE* mutant strain AL362

The construction of an isogenic *nrfE* mutant in *P. multocida* was described in Chapter 3. A plasmid containing an uninterrupted copy of *nrfE* was constructed to complement AL362 by cloning *nrfE* into the vector pAL99 (which contains the *P. multocida tpiA* promoter upstream of the cloning site). The complete *nrfE* encoding fragment was amplified by PCR from *P. multocida* X-73 genomic DNA using the primers BAP2283 and BAP2284 (Table 2), digested with *Sac*I and cloned into the *Sac*I site of pAL99 to generate pAL263 (Table 1). The *nrfE* insert was verified by DNA sequencing to be identical to wild-type *nrfE* and was in the same orientation as the *tpiA* promoter in pAL99. The plasmid pAL263 was transformed into strain AL362 to produce the complemented strain AL466. pAL99 was also transformed into AL362 as a vector control to produce the strain AL465.

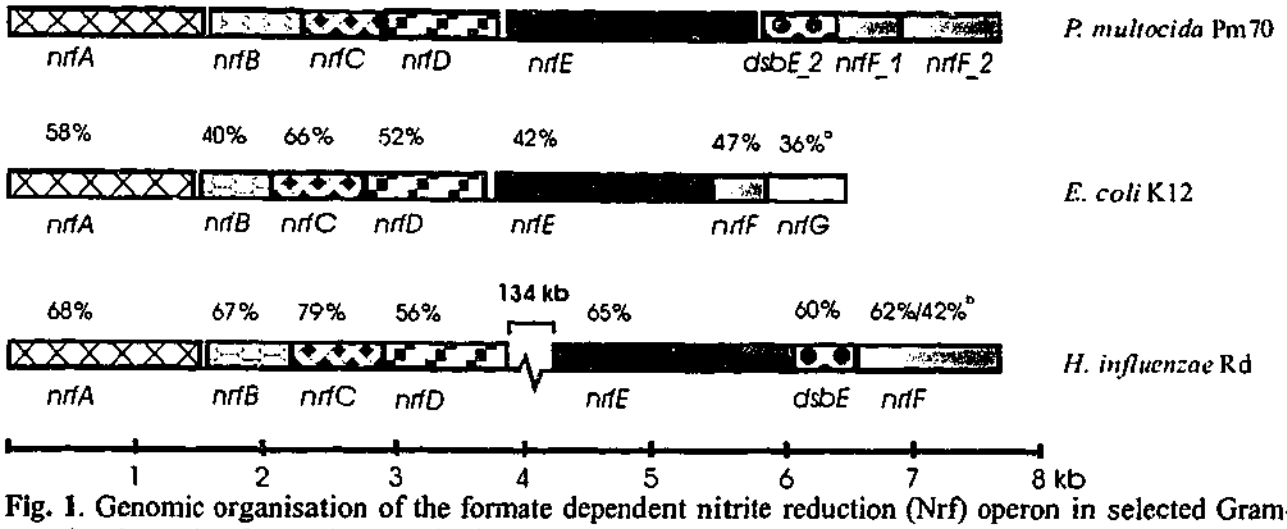


Fig. 1. Genomic organisation of the formate dependent nitrite reduction (Nrf) operon in selected Gram-negative bacteria. Genomic organisations shown are from the following strains; *P. multocida* Pm70 (GenBank accession number NC_002663), *Haemophilus influenzae* Rd (GenBank accession number NC_000907) and *Escherichia coli* K12 (GenBank accession number NC_000913). ORFs are indicated by the named boxes. Hatched boxes indicate predicted orthologues and percentages above each box indicate the level of protein sequence identity to the *P. multocida* Pm70 protein.[a] Homology to *nrfF_2*.
[b] Homology to *nrfF_1* and *nrfF_2* respectively.

Nitrite reduction studies of the *P. multocida nrfE* gene

A test based on the method of Hussain *et al.* (1994) was used to evaluate the function of *nrfE* in nitrite reduction in *P. multocida*. Briefly, triplicate cultures of wild-type X-73, the *nrfE* mutant AL362, the complemented mutant AL466 and the vector control AL465 were grown in CDM (nitrate/nitrite) and tested for the reduction of nitrite when grown either aerobically or anaerobically. The presence of nitrite is indicated by the formation of a red azo dye which is measured spectrophotometrically at 530 nm. An uninoculated medium control was also included to identify whether nitrite breakdown occurs during incubation at 37°C. When grown aerobically, wild-type X-73 was shown to reduce nitrite (Nrf^+ phenotype) whereas the mutant strain AL362 was unable to do so (Nrf^- phenotype) (Fig. 2). The difference in reduction of nitrite between wild-type X-73 and AL362 was highly significant (Tukey-Kramer multiple-comparison test; $P < 0.001$), and indicated that inactivation of *nrfE* caused a loss of the ability of *P. multocida* to reduce nitrite (Nrf^- phenotype). The *nrfE* complemented strain AL466 was able to reduce nitrite at a level that was not significantly different from the nitrite reduction of wild-type X-73 ($P > 0.05$), but was significantly different from AL362 and AL465 indicating restoration of the Nrf^+ phenotype (Fig. 2). The vector control AL465 and the medium control were significantly different to all of the strains ($P < 0.001$) except each other. The difference between the level of nitrite present in the AL362 culture and the uninoculated medium indicated that there was a higher amount of nitrite present in the AL362 strains sampled than the CDM medium originally contained. This

probably indicates that during growth additional nitrite is produced by the *P. multocida* strains. This most likely due to the actions of nitrate reductases such as the *nap* operon (periplasmic nitrate reductase) which has been shown to be present in *P. multocida* Pm70 strain (May *et al.*, 2001).

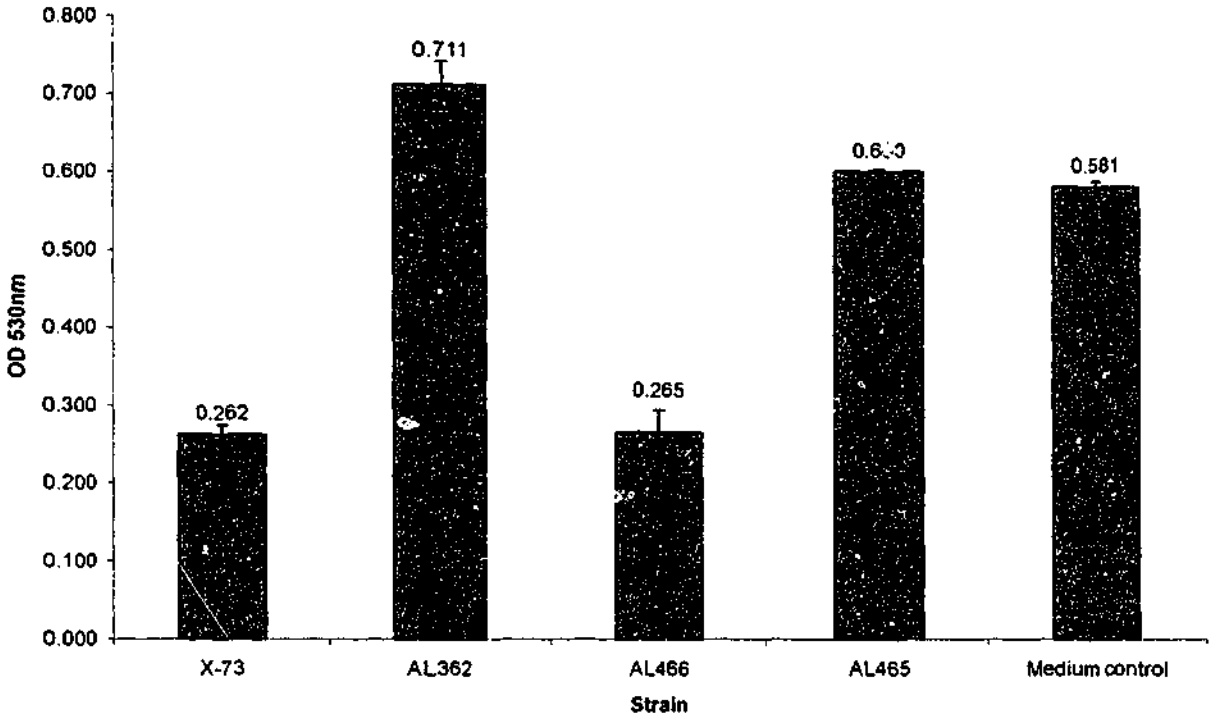


Fig. 2. Nitrite reduction by *P. multocida* strains grown aerobically. Strains were grown in modified CDM (nitrate/nitrite) and samples from cultures were tested for the presence of nitrite after 18 h. Values shown are the means of triplicate cultures ± one standard deviation.

The ability of *P. multocida* strains to reduce nitrite under anaerobic growth conditions was also assessed and there was a significant difference between the levels of nitrite in the wild-type X-73 and AL362 cultures ($P < 0.01$), but not between AL466 and wild-type X-73 cultures (Fig. 3). Also there was a significant difference in nitrite between the levels of nitrite measured in the AL362 and AL466 cultures ($P < 0.05$) indicating restoration of the Nrf^+ phenotype (Fig. 3). The levels of nitrite in the AL362 and the vector control AL465 cultures were not significantly different from each other ($P > 0.05$) but the level of nitrite in the AL465 cultures was significantly different from that in the AL466 cultures ($P < 0.01$) indicating that restoration of the Nrf^+ phenotype was due to the copy of *nrfE*. Thus the *nrfE* gene is required for nitrite reduction under both aerobic and anaerobic conditions. The medium control had lower amounts of nitrite than seen during the aerobic nitrite assays and was not significantly different

from the wild-type X-73, AL362 and AL466 cultures ($P > 0.05$), but was significantly different from AL465 cultures ($P < 0.01$).

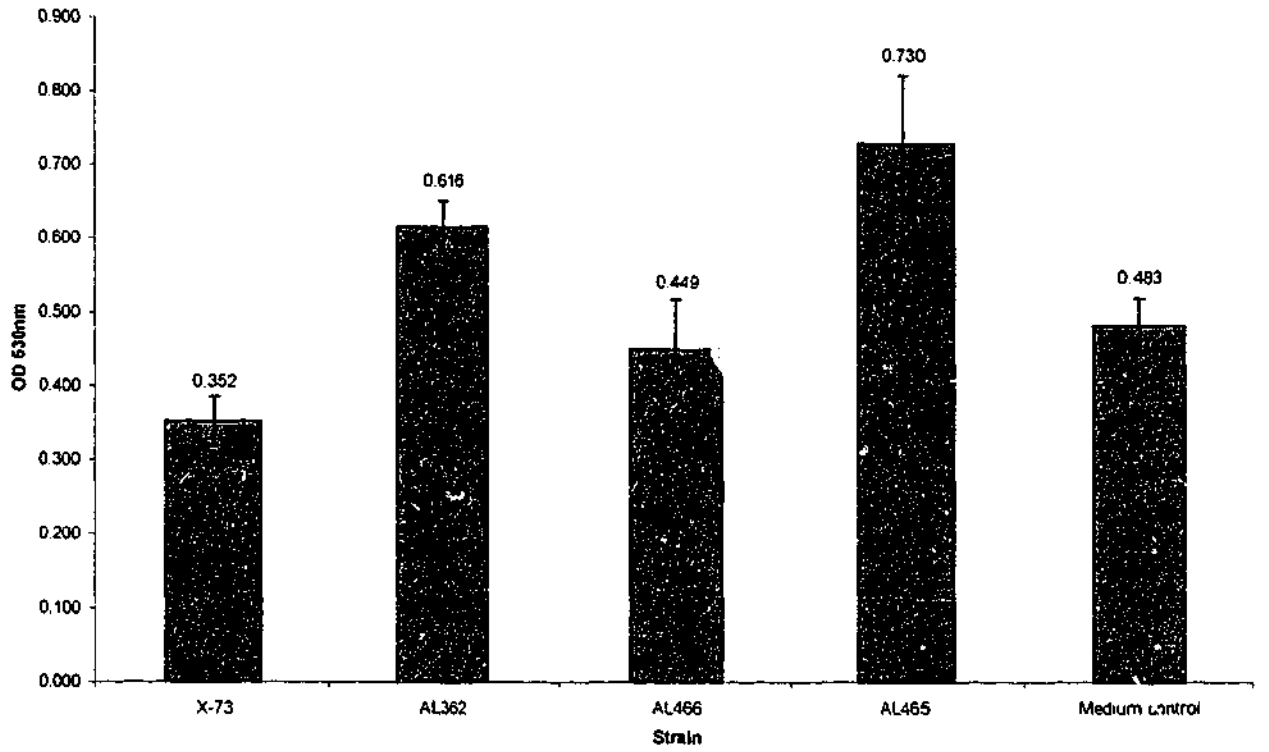


Fig. 3. Nitrite reduction by *P. multocida* strains grown anaerobically. Strains were grown in modified CDM (nitrate/nitrite) and samples from cultures were tested for the presence of nitrite after 72 h. Values shown are the means of triplicate cultures ± one standard deviation.

Nitric oxide reduction tests

Disk diffusion sensitivity assays were used to compare the growth sensitivity of the strains AL362, AL465, AL466 and wild-type X-73 to SNAP under both aerobic and anaerobic conditions. The assay (Poock *et al.*, 2002) measures the ability of strains of *E. coli* to reduce nitric oxide released by SNAP. None of the *P. multocida* strains tested showed zones of growth inhibition by SNAP (data not shown). This indicated that inactivation of *nrfE* in *P. multocida* did not affect reduction of nitric oxide released by SNAP.

Virulence in mice

To determine the virulence of the *nrfE* mutant AL362, various doses of *P. multocida* X-73 and AL362 were injected intraperitoneally (IP) into mice. Inactivation of *nrfE* did not result in a decrease in virulence and there were no survivors from any of the test groups (Table 3).

Table 3 Survival of BALB/c mice infected with *P. multocida* strains

Strain	Dose (CFU)	No. of survivors/group size
X-73	$1X\ 10^2$	0/5
AL362	$2\ X\ 10^2$	0/5
	$2\ X\ 10^3$	0/5
	$2\ X\ 10^4$	0/5
	$2\ X\ 10^5$	0/5

To quantitatively assess the growth rate of the mutant *in vivo*, competitive growth assays were used to compare the relative survival of AL362 and X-73 *in vitro* and *in vivo* in mice. Using this method, mutants are identified as attenuated if the ratio of mutant to wild-type bacteria recovered after *in vivo* growth is significantly less than the ratio of mutants to wild-type bacteria recovered after *in vitro* growth. The relative competitive index (rCI) (Table 4) is determined by dividing the percentage of tetracycline resistant mutants obtained *in vivo* by the percentage of tetracycline resistant mutants obtained *in vitro* and a low rCI is indicative of attenuation. Significance was determined by calculating a *P* value by an approximate *z* test for the difference in two proportions (Table 4). The mutant AL362 was found not to be significantly attenuated for growth *in vivo* compared to X-73 in any of the mice tested (Table 4), in agreement with the virulence results described previously (Table 3).

Table 4 Competitive growth assay of AL362 and X-73 in mice

Mouse	Competitive growth assay results	
	rCI[a]	*P* value[b]
M1	0.98	0.56
M2	0.70	0.98
M3	0.86	0.84
M4	0.76	0.96
M5	0.74	0.97

[a] Values represent the rCI 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* values were calculated by using an approximate *z* test for the difference in two proportions which determines whether the proportion of mutants *in vivo* was significantly less than the proportion *in vitro*.

Transcriptional analysis of *nrfE*

The transcriptional regulation of the Nrf operon under various growth conditions was investigated using quantitative real-time reverse transcription PCR (real-time RT-PCR). Cultures of X-73 and AL362 were grown either aerobically or anaerobically in the same media used for the nitrite reduction experiments (CDM-nitrate/nitrite). Real-time RT-PCR was carried out using primers that amplified internal regions within *nrfA* (BAP2123 and BAP2124), *nrfD* (BAP2363 and BAP2364) and *nrfE* (BAP2398 and BAP2399, located upstream of the site of *tet* (M) insertion in AL362) from X-73 and AL362 cDNA. *gyrB* was used as a normaliser for all reactions as previously described (Boyce *et al.*, 2002) to determine relative expression from triplicate data sets. The expression of *nrfA*, *nrfD* and *nrfE* was significantly higher (at least nine-fold) (Tukey-Kramer multiple-comparison test; $P < 0.001$) during anaerobic growth than during aerobic growth in both X-73 and AL362 (Fig. 4, Table 5). Thus *nrfA*, *nrfD* and *nrfE* are expressed at low levels during growth under aerobic conditions but are significantly upregulated during growth under anaerobic conditions. However, *nrfE* had a sixty six-fold increase in expression in AL362 when grown anaerobically compared to

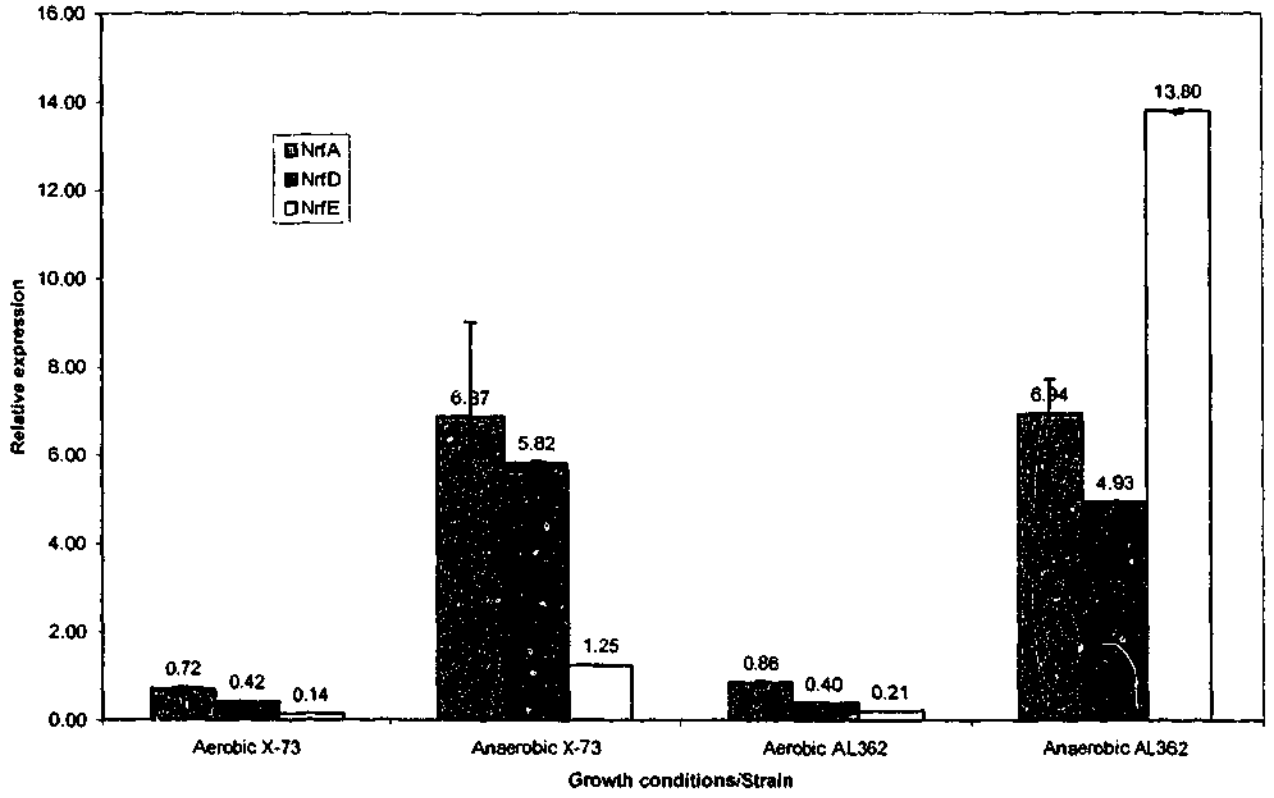


Fig 4. Relative level of *nrfA*, *nrfD*, and *nrfE* expression (normalised against *gyrB*) during anaerobic growth in X-73 and AL362. Cultures of X-73 and AL362 were grown simultaneously in conditions optimal for nitrite reduction. Values indicate the mean relative expression determined from a minimum of three reactions (± 1 Standard deviation).

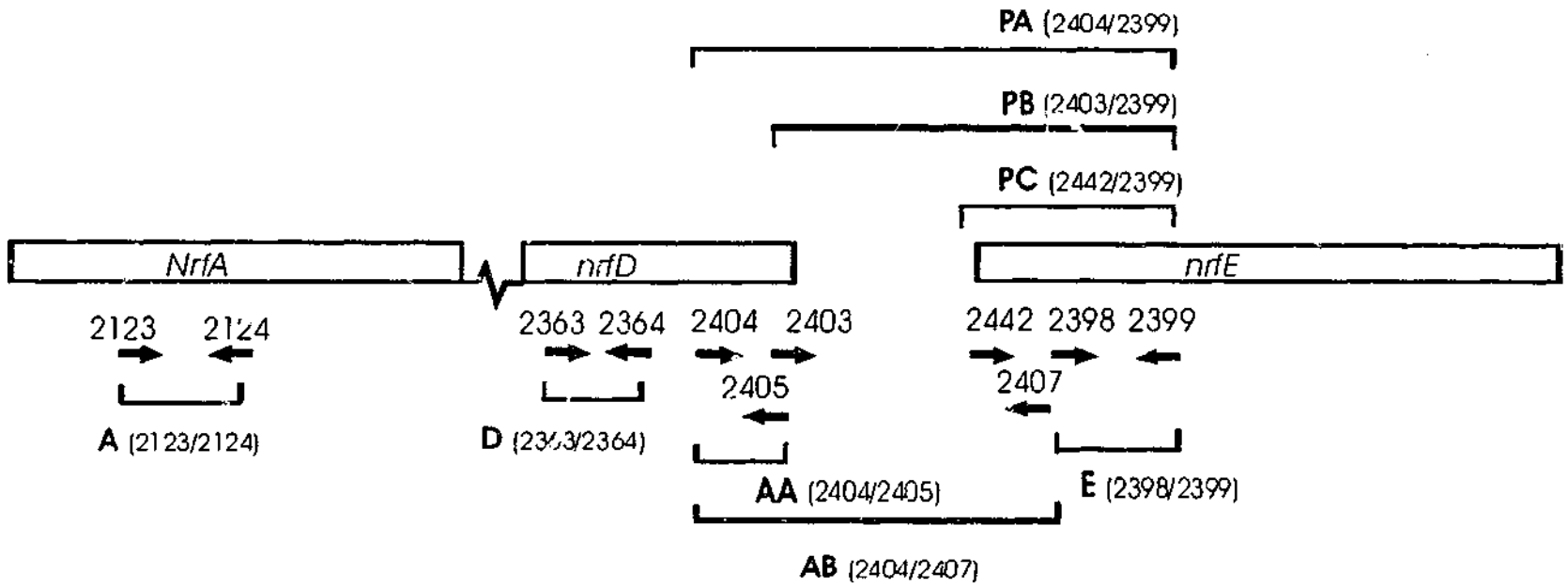
Table 5 Ratio of anaerobic/aerobic expression of the *nrf* genes of *P. multocida*

Gene	*P. multocida* strain X-73	*P. multocida* strain AL362
nrfA	9.5 ± 3.1	8.0 ± 1.0
nrfD	13.8 ± 0.2	12.3 ± 0.1
nrfE	8.9 ± 0.1	66.2 ± 1.2

aerobically that was significantly different ($P < 0.001$) to the increase in *nrfE* expression seen in X-73 (Fig. 4, Table 5). Furthermore, during anaerobic growth, the expression of *nrfE* was significantly different in both X-73 and AL362 to that of *nrfA* and *nrfD* ($P < 0.001$), whereas expression of *nrfA, nrfD,* and *nrfE* during aerobic growth was not significantly different ($P > 0.05$) (Figure 4). In addition, there were no statistically significant differences between the expression of *nrfA* and *nrfD* when either X-73 or AL362 were grown anaerobically. Taken together, these data indicated that *nrfA*, *nrfD* and *nrfE* were all upregulated during anaerobic growth in both strains, but *nrfE* was expressed at much higher levels in the *nrfE* inactivated strain AL362, probably from an uncharacterised promoter downstream of *nrfD*. The level of expression of *nrfE* in X-73 grown anaerobically was also significantly lower than that of *nrfA* and *nrfD* indicating the presence of a transcriptional terminator or transcriptional attenuation sequence within this region. In *E. coli* grown anaerobically, *nrfA* is under the control of FNR (Darwin *et al.*, 1993). A putative FNR recognition sequence (5'-TTGATCAAGCGCAA-3') was identified 128 bp upstream of *nrfA* in the Pm70 genome. However, no potential FNR recognition site was located upstream of *nrfE,* indicating that, unlike *nrfA*, the unknown *nrfE* promoter may not be regulated by FNR.

Real-time PCR was used to further investigate the transcriptional regulation of *nrfE* in both the wild-type X-73 and the *nrfE* mutant AL362 grown under anaerobic conditions. This was conducted using a series of primer sets designated **A** (BAP2123 and BAP2124), **D** (BAP2363 and BAP2364), **E** (BAP2398 and BAP2399), **PA** (BAP2399 & BAP2404), **PB** (BAP2399 & BAP2403), **PC** (BAP2399 & BAP2442), **AA** (BAP2404/2405), and **AB** (BAP2404/2407) (Fig. 5A and 5B). The relative level of transcript dropped significantly ($P < 0.001$) between primer sets **D** and **AA** (Fig. 5A) in both X-73 and in AL362. Thus there appears to be some transcriptional attenuation

within *nrfD*. The relative level of transcript dropped even further between the primer sets **AA** and **AB** (Fig. 5A) indicating further transcript attenuation in the intergenic region between *nrfD* and *nrfE* that is independent of expression of *nrfE*. Importantly, there was an increase in relative level of transcript between primer sets **PB** and **PC** (Fig. 5A) that was significantly higher ($P < 0.001$) in AL362 than in X-73. This data indicated that there is likely to be a promoter between BAP2403 and BAP2442 which is regulated either directly or indirectly by the level of active *nrfE*. Thus, it is expressed at low levels in X-73 but at much higher levels in AL362 where no active NrfE is produced. Furthermore, these data indicated that a low level of transcription of *nrfE* probably occurs from the *nrfA* promoter, however most *nrfE* expression is a result of the *nrfE* promoter and this promoter is itself regulated by expression levels of *nrfE*.



P. multocida strain	Relative expression of each primer set							
	A	D	AA	AB	PA	PB	PC	E
X-73	6.9 ±2.14	5.8 ±0.04	0.9 ±0.01	0.3 ±0.01	0.3 ±0.004	0.2 ±0.01	1.2 ±0.01	1.4 ±0.01
AL362	6.9 ±0.77	4.9 ±0.03	0.8 ±0.01	0.3 ±0.004	0.3 ±0.003	0.3 ±0.003	10.8 ±0.11	13.3 ±0.14

Fig.5A. Transcriptional regulation of expression of the *nrf* operon of *P. multocida*. Primer sets were used to determine levels of transcripts at different points between *nrfA-nrfE* and were designated **A, D, E, AA, AB, PA, PB, PC**. The arrows and numbers 2123, 2124, 2363, 2364, 2404, 2405, 2403, 2442, 2407, 2398, and 2399 denote the primers BAP2123, BAP2124, BAP2363, BAP2364, BAP2404, BAP2405, BAP2403, BAP2442, BAP2407, BAP2398 and BAP2399 respectively. Values in the table are the mean relative expression determined from triplicate reactions ±1 standard deviation.

ATGaacagtccatttcatttcccgtctttagtatgggatcataccatcgccgtttatttgttcttattggggatctctgcc[illegible]gtgcaacca
nrfD

BAP2363

tgttagccgttttgttaaaacgcgcagggctagctggcgcagaaccgtcaaacaatcatgtgatacgttgtacggcatttttg

BAP2364

gcgccattaagtctgattattggcttggtgattttgattttccatttaactaagccatggacctttggtacttgatgtttaattacagtaccacc
tctattatgtcgatgggggttatgctattccaggcctacatgttgttttactggtctggttagcgattattttccatcagcttgtggcaacttta
attgagcaatatgccaaaccgttacgatttgtgaataaattgattgcttttgctgccaaattcactggattaattgaaattattttgataattctc
tctgtgttattgggcgcctatacgggcttcttattatcggctttgattagctacccaatgttgaataacccagtgttaccaattttattcttggc
ctcagggacatcctcaggtattgcggcattaattttctgtaccttgctctttactaaagaagacacccattcacctgcgttaggcttctaca
taagtttgaattgccggtagtgttgatggaaatcttttacttgccgccttctttattggcttgtatttaggggggcggtcaaaaaacggtggc
atttgagaccgcacttttaggtggattctgggcacatgtcttctggattggggtagttggtattggtttagtcttgccattactcctcaatgg
catagccactccgcagttaaaacatcgcaaaggctttatgctactggtggcttgcttaggcttaattggcgtgttatgtttgcgtttctttatc

BAP2404

ct atgcaggacaaatgacgctagca**TAA**atgcttcatttatgagaaggcgaacctaagttcgccttttgatacagcttttcaaattt

BAP2405

BAP2403

gtttatattcact**ATG**atcccagaacttggtttttggctttactgatcgcttctttgtgtgcgttcttgcttgcgctagtgccacaagtcgg

nrfE

BAP2398

BAP2442

BAP2407

catttttttgcgtaagccgtatatgattggtactgcgtggaatttgagttatctctttgggatatttacctgtatagcgttattctgccttgcctat

BAP2399

gcctttgcactcgatgatttttctgtgcaatatgtggcacatcattctaattcacaactgccaatttttcttaaagtgtctgctacttggggtgg
acatgaagggtcgatgctatttttggttgtttgctttagcactctggttgatgctctttgcgttttccagtcgtcataaagatccgattatttgtg
cgcgtgcactttcagtgcttgggctcatttgtttggttttttgtctctttatttattttttatcaaatccatttgaacgcagttttccacaagcatttg
aagggcgtgatttaaatccgatgttgcaagatgtgggggctgatttttcatccgcctttattgtatttgggctatgtgggctttgccgtcaattt
tgctattaccatttctgctttagtgagtgggcatttagatgccgccgtggctcgcatgatgcgcccttgggttttgctatcttggttatttttaa
ctttagggattgtgttagggtcttggtgggcgtattacgaactaggctggggcggctggtggttttgggatccggtcgaaaacgcctca
ttgatgccttggttattaggtttggcattattacatagtctatctatcacagaacagcgtggcatttttagttactggacgctgctatttttcactt
ttggcatttttctttcagtttactgggtaccttcatcgtgcgctcgggcgtcttaacttctgtgcacgcgtttgcagtagatggtgatcgggga
attgccttactgattttattctttttgttgactgtgagtgcattcactttatttgcattaaaggttaacttgccacaaagtgcggtacgtttccgtt
taatttcgaaggaaaatgccctgttgcttctcaatatttattgacgattgccacggtcagtgtctttttaggcacttttatccgattgtcttta
ccgcattacaatggggatctatttcagtcggcgcgccgtattttaatacaatttttctgccactgtttgtgatggcaatgcttgctatgatttt
ggtgttgtctttacgttggaaaacactgaataaaaaaccgtttattcaacgtacgttattgttatttcccgcgcttatattggcttatggattaa
tccgttattatgtagatcataaccaagggttgcctttcaatttcagcgcttatggcttgttaagcttgtcgatttggttaattctggcgacgtta
tggctcccatggacgcagctgaaactgaaacagctcgccatggtgatcgcgcatttaggtgtagcgataacggcaattggggcgatc
atgagtagctattacagcagtgagattggcgtgcgcttggcaccgcaacaacgccaaaaactcggtgattacgagtttcactatcagg
gttttcagcatgtgttaggtccgaattacaccagtgaaaaagcccattttgtggtgacaaagcaaaaccaagcttatgctgagatttaccc
agaacgccgttattatgatgtgcgtactatgaacatgagcgaagttgggattgattggggatggcttggtgatgtctatattgtaatgggc
gataaattgggacaaggcgaatttactttccgcttgcaatataaaccttttatccgttggttatggtttggcgggcttttaatggcactaggc
gccttgcttgccatattttccttgcgcaatagcaataaactaaaaccacgtcgtcaatcagat**TGA**

Fig. 5B. Location of real-time RT-PCR primers within *nrfD* and *nrfE* from Pm70 (Genbank accession number NC_002663). Primer sites and direction are marked by arrows, and translational start and stop codons are capitalised and marked in bold.

Discussion

The *P. multocida* formate dependent nitrite reduction (*nrf*) operon has not been previously characterised nor has its role in virulence been assessed. The organisation of the *nrf* operon in *P. multocida* is somewhat different from that of other members of the Enterobacteriaceae family (Fig. 1) with the presence of two *nrfF* homologues, no *nrfG* and an additional gene *dsbE_2*, although *nrfF_2* is most likely the orthologue of *nrfG*. In *E. coli nrfF* and *nrfG* (together with *nrfE*) are proposed to be essential for nitrite reduction (Eaves *et al.*, 1998), although their role in *P. multocida* is still uncertain. Indeed nitrite reduction is certainly active in *P. multocida* indicating that the operon is functional. The closest related bacterium, *H. influenzae* Rd, had a similar *nrf* organisation to *P. multocida* although it had a single *nrfF* homologue and *nrfE*, *nrfF*, and *dsbE* were clustered 134 kb away from *nrfA*, *nrfB*, *nrfC*, *nrfD*. The *nrfF* gene in *H. influenzae* had two domains matching to both *nrfF_1* and *nrfF_2* in *P. multocida* and (*nrfF* and *nrfG* in *E. coli*), indicating that it may perform the function of both proteins. If the *nrf* system in *H. influenzae* is active, it is clear that *nrfE*, *nrfF* and *dsbE* must be transcribed from their own promoter due to their isolation from the rest of the *nrf* operon. As shown in this study, *nrfE*, *nrfF_1*, *nrfF_2* and *dsbE_2* in *P. multocida* are also regulated separately from *nrfABCD*.

The functions of *dsbE_2* in the *nrf* operon of *P. multocida* and *dsbE* in *H. influenzae* are unknown, although *dsb* genes (*dsbA*, *dsbD*, and *dsbB*) have been shown to be essential for Nrf activity and maturation of c-type cytochromes in *E. coli* (Metheringham *et al.*, 1996). There is also a second *dsbE* gene (*dsbE_1*) present in the Pm70 *P. multocida* genome and therefore it is possible that *dsbE_2* may play a specialised role in maturation of *nrfA* (c_{552}) in *P. multocida*.

In *E. coli*, formate dependent nitrite reduction has been shown to be repressed under aerobic growth conditions and upregulated anaerobically (Darwin *et al.*, 1993). In this study we have demonstrated that *P. multocida* has the ability to reduce nitrite both aerobically and anaerobically. Unlike *E. coli*, *P. multocida* lacks any of the aerobic nitrate reductases such as the *nir* family so it is possible that the *nrf* operon may have a dual function during both aerobic and anaerobic growth. Potter *et al.* (2001) proposed that pathogenic bacteria such as *H. influenzae* and *P. multocida* may rely on the *nrf* and the closely regulated *nap* (periplasmic nitrate reductase) operon for scavenging nitrate

and nitrite from the low levels present in the host. In this context, it is likely that the *nrf* operon plays a role in *P. multocida* (and possibly in *H. influenzae*) by using nitrite/nitrate as a source of energy during infection of the host.

The nitrite reduction studies indicated that there was an increased amount of nitrite present in the mutant strain AL362 and in the vector control (derived from AL362) than was originally present in the uninoculated medium control. This was not unexpected, as the conditions under which optimal Nrf activity is attained are also optimal for *nap* activity. Therefore it is likely that the *nap* system is indeed active in *P. multocida* and that it acts to convert the added nitrate in the medium to nitrite. Furthermore, as there was no active Nrf system in the AL362 mutant it was unable to utilise this nitrite.

In this work, *nrfE* was shown to be critical for nitrite reduction, which correlates with previous work in *E. coli* (Eaves *et al.*, 1998). The *nrfE* mutant AL362 was unable to reduce nitrite either aerobically or anaerobically, whereas the wild-type X-73 was able to reduce nitrite under both conditions. Additionally, the complemented mutant (strain AL466) was able to reduce nitrite at levels indistinguishable from the wild-type strain both aerobically and anaerobically. Both the complemented mutant AL466 and the vector control strain AL465 grew more slowly than AL362 and X-73 and reduction of nitrite in AL466 anaerobically was slower compared to that during aerobic growth. The increase in culture nitrite levels after growth of AL465 was also less than that observed for AL362, indicating that the presence of the pBA1100 derived plasmid in these strains was most likely affecting growth and hence affecting nitrite reduction. It is also possible that the expression of *nrfE* from the *tpiA* promoter in the complementing plasmid affected normal regulation. The media controls in the anaerobic studies showed a minor breakdown in nitrite, but as AL362 and AL465 both showed increased nitrite levels (most probably caused by *nap* activity) and were significantly different from both X-73 and AL466, the effect of the nitrite breakdown was negligible.

The reduction of nitric oxide (NO) by *E. coli* was investigated recently by Poock *et al.* (2002) and it was found that strains deficient in Nrf activity were no longer able to reduce the nitric oxide released from SNAP. This property was investigated in the *nrfE* inactivated strain of *P. multocida* (AL362) as the reduction of NO in the host might be an important virulence factor. However, AL362 was identical to wild-type X-73 in its ability to reduce SNAP. Therefore it is probable that *P. multocida* has alternative pathways for detoxifying NO that were not present in the *E. coli* strains.

The inactivation of *nrfE* in *P. multocida* did not result in a significant attenuation in mice either in virulence studies or in a competitive growth assay. This result indicates that although *nrfE* is essential for nitrite reduction in *P. multocida*, it is not essential for virulence despite being upregulated during mouse infection (Chapter 2). *P. multocida* has a number of alternate electron acceptor systems and it is likely that when one is unavailable (such as Nrf), the other systems are utilised and hence *P. multocida* survival *in vivo* is not adversely affected in virulence.

The expression of the Nrf operon in *E. coli* has been demonstrated to be regulated by FNR in response to anaerobic conditions, and activated by the NarL or NarP proteins in response to nitrate or nitrite (Browning *et al.*, 2002). A putative FNR recognition sequence was identified upstream of the *P. multocida nrfA* gene indicating that the operon in *P. multocida* may also be regulated by FNR. Transcriptional studies of the *nrf* operon in *P. multocida* X-73 and AL362 revealed that the expression of *nrfA-E* was indeed upregulated during anaerobic growth in the appropriate medium, although there was also low level expression aerobically.

The expression of the *nrf* operon in *E. coli* has been proposed to be driven solely by a promoter upstream of *nrfA* (Browning *et al.*, 2002). In this study, when X-73 was grown anaerobically, *nrfE* was expressed at lower levels than *nrfA* and *nrfD* (Fig. 4/5A). However, when the mutant AL362 was grown under anaerobic conditions, *nrfE* was expressed six-fold more than *nrfA* and *nrfD*. Despite the differences in *nrfE* expression between X-73 and AL362 when grown anaerobically, the levels of transcription of *nrfA* and *nrfD* were not significantly different between the two strains. These results indicate that expression of *nrfE* is not under the control of the *nrfA* promoter (*pnrfA*). The most likely explanation is that there is an independent promoter present between *nrfD* and *nrfE* and that there is attenuation of the *pnrfA* transcript occurring within the same region. This is consistent with our previous work which identified *nrfE* as being downstream of an *in vivo* active promoter (Chapter 2). To address this issue, further transcriptional studies investigated the presence of a potential *nrfE* promoter (*pnrfE*), and found that there was most likely an internal promoter located within the intergenic region of *nrfD-E*. It was also found that the promoter was expressed at significantly higher levels in AL362 than in X-73. As mentioned previously, *nrfE* has been proposed to form part of a haem lyase that is responsible for attaching a haem group to the active site of NrfA (c_{552}) (Grove *et al.*, 1996). It is therefore likely that in AL362, *nrfE* is upregulated because its protein form is non-functional. This upregulation is probably

due to an, as yet, uncharacterised regulatory feedback loop. Therefore, further work is warranted to analyse this regulatory network in more detail.

The data presented here indicate that *P. multocida* X-73 has a functional Nrf system that, is active both aerobically and anaerobically. This work has also demonstrated that *nrfE* in *P. multocida* is essential for Nrf activity but is not required for virulence in mice. *nrfE* has also been shown to be upregulated independently of the *pnrfA* promoter by an unknown promoter that is potentially expressed *in vivo*. This unknown promoter warrants further investigation as it may be useful for expressing heterologous vaccine antigens *in vivo*.

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Appendix A1-List of Abbreviations and Symbols

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

µF	Microfarad
µg	Microgram
µl	Microlitre
µM	Micromolar
µm	Micrometres
M	Molar
MCS	Multiple cloning site
Mg^{2+}	Magnesium ions
mA	Milliamps
m/min	Minutes
mg	Milligram
ml	Millilitre
mM	Millimolar
MPa	Megapascals
mRNA	Messenger RNA
Nal	Nalidixic acid
NB	Nutrient broth
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
nm	Nanometres
NO	Nitric oxide
Ω	Ohms
OD	Optical density
ORF	Open reading frame
%	Percentage
PAGE	Polyacrylamide gel elecrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	$\log_{10}[H^+]$
pmol	Picomol
PMSF	Phenylmethylsulfonyl fluoride
Pty	Proprietary
Rif	Rifampicin

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