DEVELOPMENT OF A CLONING SYSTEM FOR GENE EXPRESSION IN *PASTEURELLA MULTOCIDA*

by

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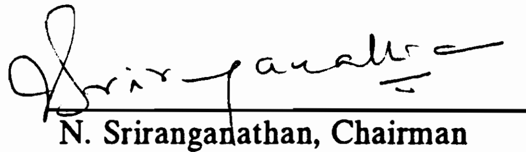

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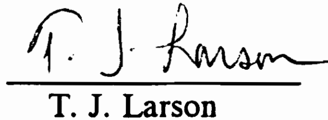

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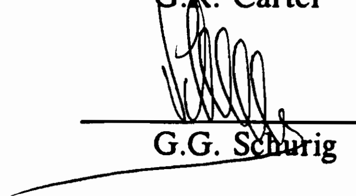

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Veterinary Medical Sciences

(ABSTRACT)

To identify antigens unique to live *Pasteurella multocida* P1059, 10 week old specific pathogen-free (SPF) chickens were vaccinated three times with one of the following: viable cells from *P. multocida* P1059, 3865, 3866, or cells from formalin-killed strain P1059 or formalin-killed strain P1059 that were opsonized with antiserum directed against killed strain P1059 prior to immunization. Vaccinated birds were challenged with 1.5 x 10^7 CFU of live strain P1059. Eight, 71, 86, and 50% of the birds that received live strains P1059, 3865, 3866 and killed strain P1059 (respectively), exhibited clinical signs of fowl cholera. Antisera directed against live strain P1059 recognized 23 proteins ranging from 14- to 92-kilodaltons (kDa); 20 of which were adsorbed by strain 3865. The molecular masses of the three remaining proteins were 25-, 30- and 43-kDa.

A genomic library of strain P1059 was constructed using the plasmid vector pUC-19 and screened with antisera against live strain P1059; 12 out of 4,100 clones were recognized. The inserts of the plasmids from these clones ranged from 0.48- to 6.8-kilobases (kb) in length. Five of the 12 clones expressed proteins with

molecular masses of 34-, 37-, 42-, 46- and 55-kDa. *Escherichia coli* CSR603(pOP43-2G) and CSR603(pOP33-8F) expressed proteins recognized by antisera directed against live strain P1059. *E. coli* CSR603(pOP43-2G) expressed an epitope(s) which was recognized by antisera directed against strains 3865 and 3866.

Conditions for transformation were optimized and attempts were made to create a shuttle vector in order to establish a cloning system for gene expression in *P. multocida*. The highest efficiency of transformation (1.25 x 10^7 CFU/μg DNA) was obtained when 7.6 x 10^{10} cells of *P. multocida* R473 were electroporated at 12.5 kV cm^{-1} for 10 ms with 5 ng of the plasmid, pVM109. Of the six strains tested, representing serogroups A, B, D and E, all were transformed successfully. Vectors including pBR322, pUC19, pJFF224-NX and pSP329 were unable to transform *P. multocida*. To create a shuttle vector for gene expression in *P. multocida*, a *Pasteurella* plasmid (pLAR-1) was cloned in both orientations into the *Bam*H I site of pBR322 . These plasmids, pLRBR-21 and pLRBR-67, had a transformation efficiency of 4.5 to 8 x 10^4 CFU/μg of DNA in strain R473. Chromosomal DNA containing the *Brucella abortus* copper-zinc superoxide dismutase gene was cloned into the *Cla* I site of pLRBR-21. The 1.8-kb fragment encoding a 42-kDa *Pasteurella* protein was cloned into an additional unique site (*Nru* 1) of pLRBR-21 to determine if this plasmid was a viable shuttle vector for gene expression in *P. multocida*.

This thesis is dedicated to

my husband and my family.

I am forever grateful for their love and support.

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Table 1. Abbreviations and their explanations [a].

Antibiotics:

Amp^r - ampicillin resistance
Tet^r - tetracycline resistance
Cam^r - chloramphenicol resistance
Sulfa^r - sulfonamide resistance
Strep^r - streptomycin resistance

Measurements:

kb - kilobases of DNA
bp - basepairs of DNA
Da - daltons
kDa - kilodaltons
Mdal - megadaltons
CFU - colony forming unit(s)

Genes:

phoA - encodes alkaline phosphatase. In this study, alkaline phosphatase was used as a reporter gene in the transcriptional vector pCB267.

lacZ - encodes ß-galactosidase. *LacZ* was located on the pUC19 vector used to create a genomic library of *P. multocida* P1059. *LacZ* was also a reporter gene in the transcriptional fusion vector pCB267.

xylE - encodes catechol deoxygenase. Catechol deoxygenase was a marker located on the broad host range shuttle vector pJFF224-NX.

uvrA6 - encodes an enzyme associated with excision repair of thymine dimers formed by ultraviolet irradiation (UV).

recA1- encodes an enzyme responsible for recombination of DNA within the host. Cells possessing this genotype exhibit an increased sensitivity and DNA degradation in the presence of UV light (173). *RecA* cells are unable to repair the thymine dimers.

phr-1 - encodes photoreactivating enzymes which are inducible by visible light. Cells deficient in this phenotype are unable to repair thymidine dimers formed by irradiation with UV light.

Techniques:

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis, also known as denaturing gel electrophoresis

[a] - according to IUPAC-IUB (*Biochemical Nomenclature and Related Documents*, 1978)

Table 2. Bacterial strains of *Pasteurella multocida*

Strain	Source	PMT[a]	Serotype[b]
P. multocida:			
P1059	Avian	negative	A:3
X-73	Avian	negative	A:1
1062	Bovine	negative	A:3
P86-338	Avian	negative	A:3
R473	Bovine	negative	B:2
Bunia II	Bovine	negative	E:2
3865	Porcine	negative	D[c]
3866	Porcine	positive	D[c]
3867	Porcine	negative	D[c]

[a] - *Pasteurella multocida* toxin

[b] - These strains were serotyped according to the methods of Carter (26) and Heddleston (64).

[c] - The capsular type of these strains is as designated, however the serotype (or somatic antigens) have not been determined.

Table 3. Bacterial strains of *Escherichia coli*

Strain	Description
DH5α	- F^- *endA1 hsdR17*$(r^-_K m^+_K)$ *supE44 thi-1 recA1 gyrA relA1* Φ*80lacZ M15 (lacZYA - argF)*$_{U169}$ - important properties include mutations in *recA1* and *lacZ* - allows for the cloning of foreign DNA without cleavage by endogenous endonucleases - host for pUC and other α complementation vectors - host for pBR322 derivatives - used as a host for the genomic library of *P. multocida* strain P1059 - also used for the host in the transcriptional fusion study
CSR603	- *recA, phr-6, uvrA6* - used for maxicell analysis
O1:K1	- wild-type strain - chromosomal DNA isolated from this strains was used as a positive control for the transcriptional fusion study

Table 4. Plasmids

Plasmid	Description
pUC19	2.6-kb, ColE1 origin of replication, confers Amp^r, encodes the α peptide of ß - galactosidase (116)
pBR322	4.36-kb, ColE1 origin of replication, confers Amp^r, Tet^r (13,168)
pLAR-1	4.0-kb, cryptic plasmid isolated from *P. multocida* Larsen (101)
pLRBR-21	8.4-kb, pLAR-1 cloned into *Bam* HI site of pBR322 (101)
pLRBR-67	same as pLRBR-21 but in the opposite orientation (101)
pVMRL-100	kb, pLAR-1 cloned into the *Bam* HI site of pBR325 (this study)
pCB267	4.0-kb, transcriptional fusion vector, confers Amp^r; the promoters for *lac*Z and *pho*A are deleted (162)
pVM109	8.0-kb, confers $Strep^r$, $Sulfa^r$, isolated from *P. multocida* P1085 (71)
pJFF224-NX	confers Cam^r. *xylE*, carries an RSF1010 origin of replication (53)
pSP329	confers Tet^r, carries an OriV and OriT origins of replication

LITERATURE REVIEW

PASTEURELLA NOMENCLATURE AND CLASSIFICATION

Classification of *Pasteurella* spp.

DNA:DNA hybridization studies suggest there are 11 species belonging to the genus *Pasteurella* (109,110) including *P. multocida*, *P. dagmatis*, *P. gallinarum*, *P. volantium*, *P.* species A, *P. canis*, *P. stomatis*, *P. avium*, *P.* species B, *P. langaa*, and *P. anatis*. Other members traditionally included in this genus, such as *P. haemolytica* and *P. ureae*, were transferred to the genus *Actinobacillus* (109). Accordingly, the species *P. multocida* was divided into 3 subspecies: subsp. *multocida*, subsp. *septica* and subsp. *gallicida* (110).

16S ribosomal RNA sequence homology has also been used to classify *Pasteurella*. Dewhirst *et al.* (44) described 15 species belonging to this genus including *P. aerogenes, P. anatis, P. avium, P. canis, P. dagmatis, P. gallinarum, P. haemolytica, P. trehalosi, P. langaa, P. multocida, P. pneumotropica, P. stomatis, P. volantium*, *Pasteurella species* A and *Pasteurella* species B.

Since the classification of *Pasteurella* spp. is continually being revised due to the heterogeneity of the genus; the conventional nomenclature presented by Carter in *Bergey's Manual of Systematic Bacteriology* (23) was used in this dissertation.

P. multocida - A General Description

P. multocida was first isolated in 1879 (58); the genus *Pasteurella* was not suggested until 1887 (110). The species name, *P. multocida* was not proposed until 1939 by Rosenbuch and Merchant (149). *P. multocida* is a small, Gram negative coccobacillus belonging to the bacterial family *Pasteurellaceae*. Other members of this family include *Haemophilus* and *Actinobacillus* (85).

P. multocida is a normal commensal localized in the upper respiratory tract of most mammalian and avian species (144). It is the causative agent of fowl cholera in poultry, hemorraghic septicemia in cattle, bison and water buffalo, snuffles in rabbits, and is a secondary pathogen associated with atrophic rhinitis in swine (144). Colonization of the nasal turbinates by *P. multocida* is dependent upon prior infection with toxigenic strains of *Bordetella bronchiseptica* (29,114,139,152). It has been suggested that *B. bronchiseptica* damages the epithelial lining thereby releasing nutrients required for growth of *P. multocida* (154). *B. bronchiseptica* secretes a cytotoxin which increases adherence of virulent strains of *P. multocida* to the nasopharyngeal mucosa of the swine. Simultaneously, *P. multocida* may produce an unknown substance which increases survivability of *B. bronchiseptica* (29). Prior infection with *B. bronchiseptica* may expose previously hidden attachment or receptor sites for the toxigenic strains of *P. multocida* (29).

Serologic grouping of *P. multocida*

P. multocida is divided into five serogroups based on differences in capsular antigens. These serogroups are designated A, B, D, E, and F (26,146). Members belonging to capsular serogroup A possess a hyaluronic acid capsule consisting of repeating units of *N*-acetyl D-glucosamine in a ß-1,4 linkage to D-glucuronic acid (144). Serogroups B and D also produce a polysaccharide capsule, however, unlike those belonging to serogroup A, the composition of type B capsule consists of varying ratios of fructose and glucosamine associated with protein (84). The composition of the capsules of serogroups D, E, and F have yet to be characterized. Encapsulated strains of *P. multocida* appear as mucoid, gray to white irredescent colonies while unencapsulated strains are nonmucoid, blue-gray to gray (144).

P. multocida is further subdivided based on the somatic antigens (O-antigens) present on the individual isolates (25,26,64,115). The capsular antigen is designated with a capital letter, followed by a numerical value which represents the serotype of the particular strain (e.g. *P. multocida* P1059 is serotype A:3).

VIRULENCE FACTORS OF *P. MULTOCIDA*

Definition

Mekalanos (102) defined virulence factors as those factors synthesized by a bacterium that contributed to both infection and disease including factors which were relevant to the invasion of host cells, resistance to host defense mechanisms

and/or production of host specific toxins. This definition does not include those processes that are required for multiplication on nonliving substrates. This definition was used in this dissertation to differentiate virulence factors from other cellular components of *P. multocida*.

Capsule

Snipes *et al.* (164) suggested that type A capsule may play a role in the ability of *P. multocida* to avoid phagocytosis by the macrophages located in the spleen. Studies comparing an encapsulated strain with a capsule-deficient mutant showed that the encapsulated bacterium replicated more rapidly *in vivo* than the nonencapsulated variant. In contrast, Collins *et al.* (39) suggested that growth differences between encapsulated and nonencapsulated strains were due to an increased phagocytosis of the nonencapsulated strains thus resulting in more efficient removal from the bloodstream. Hansen and Hirsh (63) hypothesized that the presence of capsule inhibits the formation of a stable membrane attack complex with the outer membrane based on the finding that removal of the capsule with hyaluronidase increased sensitivity of the strain to killing by the activation of complement (63).

The role of capsule from serogroup B in protection is unknown (84,111). Purified capsule from strains belonging to serogroup B is immunogenic but not protective (84). According to Knox and Bain (84), the difference in pathogenicity

among strains of serogroup B results from the ability of the capsule to remain tightly associated with the outer membrane in virulent strains, unlike avirulent strains which "lose" capsule into the media. Nagy and Penn (111) report that antiserum directed against whole cells of *P. multocida* serogroup B protected cattle and mice against homologous challenge. Protection was lost when the same sera was adsorbed with purified capsule prior to immunization suggesting that this factor may play an important role in protection.

Endotoxin

Endotoxin of *P. multocida* is composed of lipopolysaccharide (LPS) and protein (66,134). Endotoxin is thought to be the ultimate cause of death in cases of fowl cholera (91). *Pasteurella* LPS is composed of lipid A, linked to an O-side chain through a core of 2-keto-3-deoxyoctonic acid (KDO), similar to other Gram negative bacteria (144). There are two forms of LPS associated with strains of *P. multocida* whose chemical compositions are indistinguishable. The first form is intergrated into the outer membrane. The second form, termed free endotoxin (FET), is released into the surrounding media (66). FET is composed of 4.1% nitrogen, 25-27% protein, 10-11% carbohydrate, and 13-18% phospholipid and has a buoyant density similar to LPS found in *E. coli* (66). FET is highly toxic to mice, rabbits, chickens, turkeys, and calves, and affords cross-protection upon challenge (66). In contrast LPS affords protection which is serotype specific (132).

Lee *et al.* (91) reported a direct correlation between the release of endotoxin from *P. multocida* and the presence of complement. A virulent strain of *P. multocida* (strain 86-1913) released more endotoxin in the presence of complement proteins than did a moderately virulent vaccine strain (CU strain). The continual release of endotoxin may cause the immune system of the host to become depleted of complement thereby allowing the remaining cells to continue to replicate in the bloodstream.

Siderophores and Iron Binding Proteins

P. multocida is capable of binding available iron through siderophore and non-siderophore-mediated iron acquisition. Hu *et al.* (75) isolated a highly polar, nonaromatic siderophore, designated multocidin, that was capable of sequestering iron from transferrin. Ogunnariwo *et al.* (117) reported an additional method of binding iron that is non-siderophore-mediated. Bovine isolates of *P. multocida* produced an 84-kDa protein capable of chelating iron from bovine transferrin. Since this protein is unable to bind transferrin from other sources, it was suggested that the 84-kDa protein:transferrin complex is host specific.

Snipes *et al.* (165) reported the synthesis of 80-, 84- and 96-kDa proteins when cells of *P. multocida* are grown under iron-limiting conditions. Choi-Kim *et al.* (35) described proteins of a similar molecular mass that are capable of binding an iron-multocidin complex. Ikeda and Hirsh (76) demonstrated that outer

membrane preparations from 94% of the strains surveyed contained a protein that shared epitopes with the 84-kDa iron-regulated outer membrane protein reported by Snipes *et al.* (165).

Outer Membrane Proteins (OMPs) with Antiphagocytic Activity

Truscott and Hirsh (171) reported the isolation of a 50-kDa OMP from *P. multocida* P1059 that had antiphagocytic activity. A substance possessing similar activity with a mass of approximately 300-kDa has been isolated from *P. multocida* group B (155).

Adhesins, Hemagglutinins, and Fimbriae

Glorioso *et al.* (57) demonstrated that strains belonging to serogroup A produce fimbriae. Fimbriae were not produced by strains belonging to serogroup D. Adhesin-producing strains were able to bind to rabbit pharyngeal cells and porcine tracheal epithelial cells, unlike isolates belonging to serogroup D.

Fortin and Jacques (52) found hemagglutinins in 44% of the virulent strains tested. Most of the strains producing hemagglutinin belonged to serogroup A. Pestana de Castro *et al.* (124) found hemagglutinins in 31 isolates surveyed. These hemagglutinins were mannose-resistant and their agglutination activity was lost when heated at 100°C for 10 min. There was no correlation between the ability of *P. multocida* to produce atrophic rhinitis *in vivo* and the presence of the hemagglutinin

(28).

Antibiotic Resistance and Plasmids

Antibiotic resistant strains of *P. multocida* have been isolated from both avian and bovine species (9,27,70,71,127,163). Silver *et al.* (163) reported the presence of a 3.0 Mdal antibiotic resistance plasmid (R-plasmid) in *P. multocida* that encoded tetracycline resistance. There was an additional 2.7 Mdal plasmid that encoded resistance to both streptomycin and sulfonamides (163). Berman and Hirsh (9) isolated two R-plasmids; a 3.4-kDa plasmid which encoded resistance to streptomycin and sulfonamide and a 4.4-kDa plasmid that encoded tetracycline, streptomycin, and sulfonamide resistance. Livrelli *et al.* (93) reported a 4.4-kb plasmid that encoded resistance to penicillin.

R-plasmids constituted a small percentage of the total plasmids found in *P. multocida* overall (61,71,166). Hirsh *et al.* (71,72) isolated plasmids from 77-96% of the strains surveyed. Only 13 of the 206 strains surveyed contained R-plasmids (71,72). Of the 163 strains surveyed by Haghour *et al.* (61), 21.5% contained plasmids; none of which encoded antibiotic resistance. Similar results were obtained by Sriranganathan *et al.* (166). The function(s) of these plasmids is unknown.

In 1989, Hirsh *et al.* (70) reported the isolation of a 70 Mdal plasmid from *P. multocida*. This conjugal plasmid was responsible for the transfer of smaller, nonconjugal R-plasmids that encoded resistance to kanamycin, streptomycin,

sulfonamides, and/or tetracycline.

P. multocida Toxin

P. multocida toxin (PMT) is produced by virulent strains belonging to serogroups A and D that are associated with atrophic rhinitis (30,51,154). The size of PMT has been estimated to be between 112- and 160-kDa as determined by gel filtration and SDS-PAGE (51). It is possible that the higher molecular mass forms reported are precursors of the mature protein (51). Rimler and Brogden (140) reported two forms of PMT, one of which was cell-associated and one which was exported to the cell surface. These proteins were thought to be distinct entities synthesized as single polypeptides.

PMT is a heat labile protein (113) with an isoelectric point of 4.6-4.8 (140). The amino acid composition of PMT consists of a high percentage of glutamine, aspartic acid, glycine, proline, alanine, and leucine residues (51). In addition, there are 6 cysteines that allow for the formation of 3 disulfide bonds (125). Originally it was hypothesized that PMT may be an exotoxin since it was isolated from the supernatant of 30 day old cultures. Later studies showed that PMT is actually released upon cell death (30).

The mode of action of PMT is not well understood. It is composed of three subunits entitled *a*, *b*, and *c* with molecular masses of 23-, 67- and 74-kDa, respectively (112). These subunits combine in a 1:1:1 ratio to form an active toxin.

Subunit *a* is associated with dermonecrosis observed in the guinea pig assay. Subunits *b* and/or *c* possess cytotoxic activity. PMT may have two distinct regions (regions A and B), each with an independent function. Region A may function as a ligand in binding of the toxin to receptors on epithelial cells, while region B is cleaved from the intact protein, transported across the cell membrane, and released into the host's cytoplasm.

The gene encoding for PMT (*toxA*) was cloned and expressed in *E. coli* (90,125,126,158). Recombinant PMT has an activity comparable to purified PMT, as determined by the guinea pig assay (126), cytotoxicity on embryonic bovine lung cells (153), and minimal lethal dose in mice (152). The mass of recombinant PMT is 143-kDa. The deduced amino acid sequence has very little identity with other known toxins and/or proteins (90,120). DNA probes directed *toxA* have been found to be specific to toxigenic strains for *P. multocida* (82). These DNA probes may be valuable for diagnostic purposes for differentiating toxigenic from nontoxigenic strains of *P. multocida*.

ADDITIONAL CELLULAR COMPONENTS OF *P. MULTOCIDA*

Characteristics assocaisted with PMT that are not virulence factors

Rozengurt *et al.* (151) report that PMT is the most potent mitogen known to date. This mitogen also has a role in signal transduction and cell division. PMT induces cell division in Swiss 3T3 cells, mouse embryo and human fibroblast cell

lines. Staddon *et al.* (167) hypothesized that PMT enters the cell and induces DNA replication thereby causing the cells to enter the *S* phase of mitosis. Unlike other mitogens, PMT stimulates inositol phosphate turnover instead of cAMP (151). An increase in inositol tri-phosphate is part of a signal transduction mechanism leading to cell growth (150).

Bacteriocin

Of the 33 bovine and bison strains of *Pasteurella* surveyed by Chengappa and Carter (31), 14 were shown to produce a bacteriocin. Six strains produced bacteriocin that was active against 3 or more strains of *P. multocida*. Seventeen of the 33 strains surveyed were susceptible to *Pasteurella* bacteriocin. In this study (31), bacteriocin was activated by UV light in 15 of 33 strains of *P. multocida* surveyed. Iordache and Ungureanu (77) reported that *P. haemolytica* was susceptible to bacteriocin from *P. multocida* and *vice versa*. Mushin reported 85 of 111 strains of *P. multocida* surveyed produced a bacteriocin which was named multocin (108).

Neuraminidase

Neuraminidase from *P. multocida* was first isolated in 1970 (158). Drzeniek *et al.* (49) reported neuraminidase in 103 of 104 of the strains encompassing serogroups A, B, D, and E. This enzyme was membrane-bound and had a mass of approximately 250-kDa. *In vitro*, neuraminidase is able to cleave human transferrin into 5 subunits (157); whether the enzyme is capable of similar cleavage *in vivo* has yet to be established. Strains belonging to serogroups A and D have the highest

level of neuraminidase activity, while those belonging to serogroup B possessed the lowest activity (49).

Outer Membrane Proteins (OMPs)

In an attempt to see if OMP profiles could be used to distinguish vaccinated from nonvaccinated birds, Choi *et al.* (33) surveyed OMPs from reference strains of *P. multocida* representing sixteen serotypes. Fourteen of these strains produced similar OMP profiles.

Cross Protection Factors (CPFs)

Bacterial pathogens such as *E. coli* and *Vibrio cholera* undergo phenotypic changes when they multiply *in vivo* during infection (60,81). These bacteria express novel surface antigens which are not present on bacteria grown *in vitro* on defined or enriched culture media. There are reports of novel antigens expressed by avian strains of *P. multocida* grown *in vivo* which are not present on the same strains grown *in vitro* (33,56). Novel surface antigens such as those reported by Choi *et al.* (33) and Glisson and Cheng (56) were also reported by Rimler and Rhoades (143). These surface antigens, located in the detergent soluble fraction of cell lysates, induced protective immunity against both homologous and heterologous challenge by *P. multocida* strains P1059 and X-73. These proteins were subsequently termed cross protection factors (CPFs) (142,143).

There has been a great deal of interest in the identification of CPFs. Choi *et al.* (33) described OMPs ranging from 27- to 94-kDa expressed strictly *in vivo*. Glisson and Cheng (56) identified 6 *in vivo* antigens ranging from 70- to 138-kDa that were not recognized by antisera against formalin-killed *Pasteurella* P1059. It is uncertain whether these OMPs are considered CPFs since the antigens were isolated from the detergent insoluble phase (142).

FOWL CHOLERA

Incidence and Distribution

Clinical signs of fowl cholera (also known as avian cholera or avian pasteurellosis) were first reported in the 1600's by Androvandus (58). Reports of the disease continued throughout Europe during the 17th and 18th centuries (136). Toussaint (135) correlated the occurrence of fowl cholera with a bacterium in 1879. In 1880 Pasteur (123) isolated and cultivated the causative agent of fowl cholera; subsequently named *Pasteurella multocida* (149). In 1881, Pasteur (122) used an avirulent strain of *P. multocida* to immunize chickens against fowl cholera. This was the first demonstration of vaccination with a live bacterium.

Economic Losses

Estimates of the losses attributed to fowl cholera range from 200 million in 1986 (131) to over 500 million U.S. dollars in 1987 (92) worldwide. Carpenter *et al.* (22) calculated losses to the poultry industry of over 1 million U.S. dollars annually in Missouri alone. Fowl cholera was considered one of the two most important infectious diseases affecting the U.S. turkey industry as recently as 1985 (136). Outbreaks of this disease have been reported in Africa, North, South and Central America, Asia and Europe (136).

Fowl cholera has periodically devastated entire flocks of both domestic and wild bird populations. Alberts and Graham (2) reported losses of up to 68% of a flock of 22 week old turkeys in less than 6 days. In 1902, 80% of a population of domesticated geese were lost due to fowl cholera (40). Over 50% of the duck population on Long Island succumbed to fowl cholera (47). Twenty-three of the 64 flocks surveyed in Northern California (encompassing over 3.25 million birds) contracted fowl cholera in 1985 (22). In 1949, approximately 40,000 waterfowl died in the San Francisco Bay (148). Over 60,000 waterfowl died in the Muleshoe National Wildlife Refuge in Texas (80). In the spring of 1980, over 72,000 birds were lost in the Rainwater Basin of central Nebraska (16). During a three month period, an estimated 2,500 waterfowl succumbed to fowl cholera again in the Rainwater Basin of central Nebraska (174).

Clinical Signs of Fowl Cholera

The clinical signs of fowl cholera include lethargy, swollen joints, conjunctivitis, diarrhea, mucous discharges, cyanosis of the combs and wattles, anorexia, and dehydration (Fig. 1). Necropsy reveals a marked increase in peritoneal and pericardial fluid, small focal lesions of the liver, lungs and kidneys, and inflammatory exudate from the joints (Fig. 2) (135,136).

Treatment and Prevention of Fowl Cholera

Fowl cholera is treated with sulphonamides, streptomycin, penicillin, oxytetracycline, novobiocin and erythromycin (135,136,144). Since *P. multocida* is a normal respiratory commensal of many animal species, it is difficult if not impossible to eradicate. Once introduced to a flock, the spread of infection to other flocks must be controlled in order to prevent significant mortality (21,36,37,138). The reservoir for *P. multocida* include wild birds (e.g. sparrows, finches, and crows), rats, feral cats, and dogs in the vicinity of breeding flocks (21,34,36,37,46,59,65,83,121).

FIG. 1. Clinical signs of fowl cholera I. These chickens are 10 week old specific pathogen-free (SPF) white leghorns, four days postinoculation with 6.4×10^5 CFU of live *P. multocida* P1059. The clinical symptoms include lethargy, anorexia, diarrhea, cyanosis of the combs and wattles, and ruffled feathers.

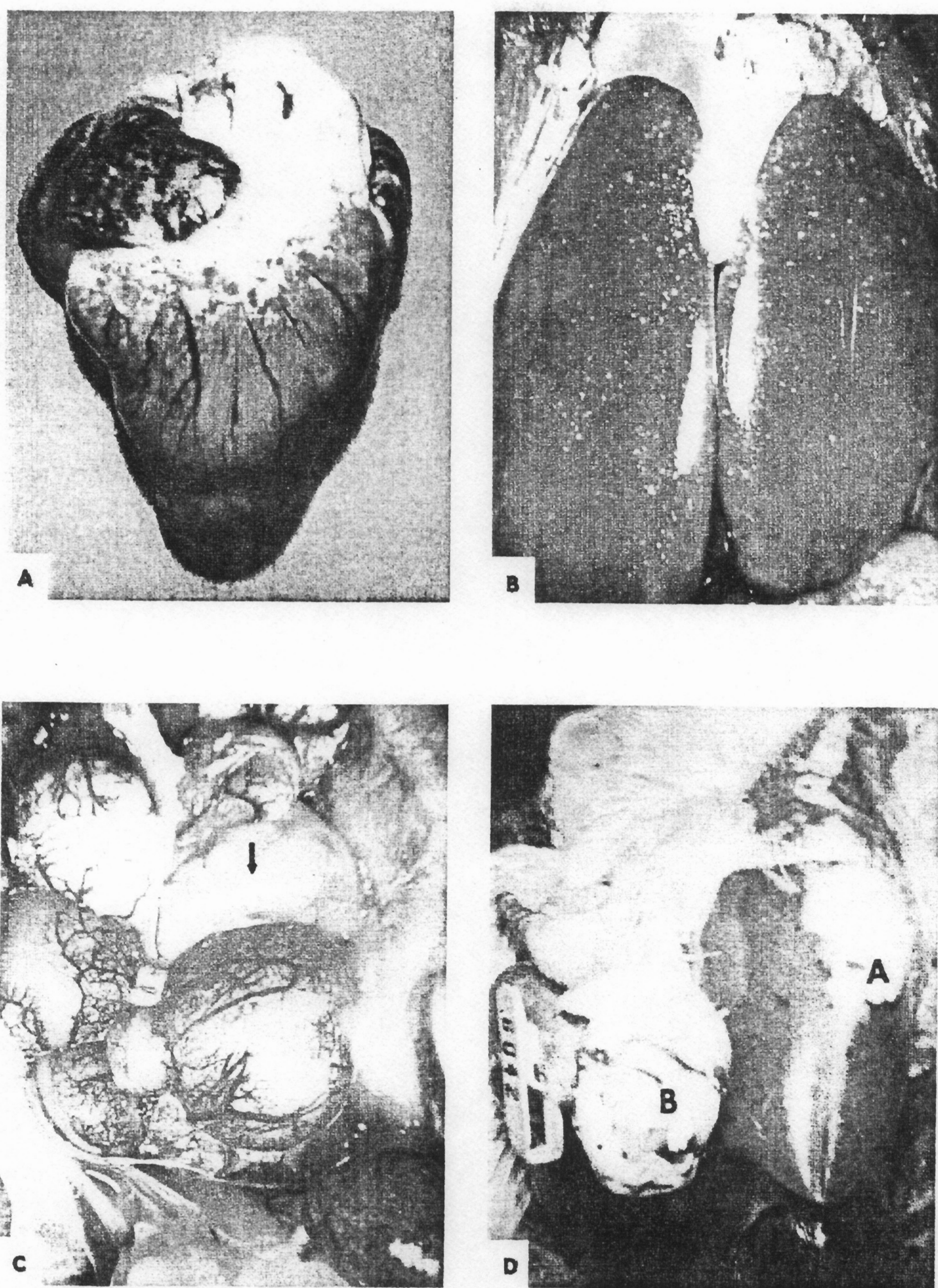


FIG. 2. Gross lesions associated with fowl cholera. (A) Subepicardial hemorrhages in a chicken. (B) Multiple necrotic foci in chicken liver. (C) Flaccid ovarian follicle (arrow) with thecal blood vessels less evident than normal. (D) Caseous exudate in sternal bursa (A) and hock joint (B) of turkey. This figure was reproduced with permission from Pasteurellosis by K.R. Rhoades and R.B. Rimler in Diseases of Poultry, Copyright 1991. 9th Ed. Iowa State University Press, Ames, Iowa.

General Introduction to the Dissertation

Vaccines using modified live strains of *P. multocida* have been developed to prevent fowl cholera (69,73,97,103,119,130). Examples of modified live vaccines include strain CU (11,12) and its temperature sensitive derivative, M-9 (69). The effectiveness of these strains as vaccines is variable. Schlink and Olson (160) reported losses due to decreased egg production and increased tortillosis, and 29% mortality following vaccination with the CU strain. Similar losses have been reported elsewhere (22,36,41,58,122).

Reports suggest that immunization with either CU or M-9 vaccines is risky since evidence indicates that under certain conditions, they may actually cause fowl cholera in vaccinated birds (36,37,41,92,159). Isolates of *P. multocida* recovered from birds suffering from fowl cholera possessed the same serotype, restriction fragment length polymorphisms, and subspecies identification as strains CU or M-9 (36,37). These isolates were recovered only from flocks previously immunized with the vaccine strains. Based on the morbidity and mortality associated with immunization with either CU or M-9, a new, more effective vaccine needs to be developed.

To create an effective vaccine against fowl cholera it is necessary to understand the interaction between the pathogen and its avian host. Clarification of this interaction will aid in the identification of antigens and immune responses that are important for protection against diseases such as fowl cholera.

When studying the interaction between a pathogen and its host, the following question arises: what is expressed by the pathogen that elicits an immune response in the host? Capsular group (39,63,164), serotype (17-19,78), the production of specific outer membrane proteins (33,35,63,79,94,142,145,171) and endotoxin (66,91,132) are just a few factors that may play a role in the ability of certain strains of *P. multocida* to produce fowl cholera.

The focus of this dissertation was to: i) examine the humoral immune response of chickens to three strains of *P. multocida*; ii) clone and express genes in *E. coli* encoding *Pasteurella* proteins that are recognized by the antisera developed against *P. multocida* P1059; and iii) develop a method to move genes encoding these antigens into strains of *P. multocida*.

The factors involved in the protection of poultry against fowl cholera are numerous. This is demonstrated by the failure of investigators to identify factors (e.g. siderophores, capsule, and LPS) which are protective by themselves. A method to clone and express potential virulence factors in an avirulent strain of *P. multocida* would be beneficial to identify elements that contribute to the pathogenesis of *P. multocida*. Expression of the genes encoding these factors in their native host would alleviate problems such as methylation of DNA, codon usage, and protein folding often associated with expression of foreign genes. Most importantly, such a system would allow an investigator to study the effect of a specific factor in its "true" background. For example, an investigator would be able to complement a

siderophore-negative mutant with the gene encoding multocidin to assess the role of this virulence factor more accurately. Finally, it would be beneficial to have a better understanding of gene expression in *P. multocida* in general. Investigation into the regulation of expression of genes that are crucial to protection, would increase our understanding of the pathogenesis of this species. Subsequently, if we increase our understanding of *P. multocida* then we may be able to develop a more effective vaccine against fowl cholera.

Chapter 1

Identification of Antigens from Three Strains of *Pasteurella multocida* that are Recognized by the Humoral Immune Response of Chickens

RATIONALE

There have been numerous studies monitoring the humoral response produced by poultry to highly virulent strains of *P. multocida* (4-7,10,54,100). This chapter of the disseration focused on proteins present in strain P1059 that induce a humoral response in chickens. By observing differences in the humoral response to strain P1059 compared to two potentially avirulent strains (3865 and 3866), one may be able to identify antigens that protect against fowl cholera. In addition, if strains 3865 and/or 3866 induce a strong humoral response in chickens and are of low virulence, they could act as potential hosts for the expression of these antigens.

MATERIALS AND METHODS

Chickens. Ten week old specific pathogen-free (SPF) (SPAFAS, Norwich, Conn.) white leghorn chickens were divided into five groups for treatment with immunogen (Table 1.1). Each groups was kept in isolation; however, within a group, birds were free to interact for the duration of the experiment. Birds were kept at constant temperature (20°C) while given feed and water *ad lib.*

Table 1.1. Assignment of individual birds for the identification of immunogenic proteins expressed by *P. multocida* P1059.

Group	Identification Number	Sex[a]	Antigen
A_1	851	F	Live *P. multocida* P1059
	852	M	
	853	M	
A_2	854	F	
	856	F	
	857	F	
A_3	858	F	
	859	M	
	860	M	
A_4	861	F	
	862	F	
	797	M	
A_5	863	F	PBS
	864	M	
B_1	865	F	Live *P. multocida* 3865
	866	M	
	867	M	
B_2	868	F	
	869	F	
	870	F	
	871	M	
B_3	872	M	PBS
	873	F	

[a] F = female, M = male

Table 1.1. (continued). Assignment of individual birds for the identification of immunogenic proteins expressed by *P. multocida* P1059.

Group	Identification Number	Sex[a]	Antigen
C_1	875	F	Live *P. multocida* 3866
	876	M	
	877	F	
C_2	878	F	
	879	F	
	880	F	
	881	F	
C_3	882	F	PBS
	883	M	
D_1	777	M	Formalin-killed *P. multocida* P1059
	778	F	
	779	M	
D_2	780	F	
	781	F	
	782	M	
E_1	789	F	Adsorbed formalin-killed *P. multocida* P1059
	790	M	
	791	M	
E_2	792	F	
	793	F	
	794	F	
E_3	795	F	PBS
	796	M	

[a] F= female, M = male

Determination of the lethal dose$_{50}$ (LD_{50}) of *P. multocida* P1059. An 0.1 ml aliquot of an overnight culture grown in Brain Heart Infusion broth (BHIB, Difco Laboratories, Detroit) was used to inoculate 10 ml of fresh BHIB. The culture was incubated in a rotary shaker at 37°C at 150 rpm until late-log phase (approximately 425 Klett units [Klett-Summerson photoelectric colorimeter, green filter]). Cells were harvested by centrifugation at 7,000 × *g*, 22°C, 10 min, washed twice in phosphate-buffered saline (10 mM potassium phosphate [pH 7.0], 10 mM NaCl [PBS]), and resuspended in 10 ml of PBS. Inocula were made by serial dilutions. To approximate the LD_{50}, 22 birds were divided into four groups each of which received one of the following dose: 6.4×10^5 CFU (Group 1); 6.4×10^3 CFU (Group 2); 6.4×10^1 CFU, (Group 3); 6.4×10^0 CFU (Group 4); PBS controls (Group 5). Birds were inoculated intravenously (i.v.) through the wing vein and observed for 10 days. The LD_{50} was then determined according to Reed and Muench (133). Once the approximate LD_{50} was determined, the experiment was repeated using the following dosages: 1.7×10^8 CFU (Group 1); 1.7×10^7 CFU (Group 2); 1.7×10^6 CFU (Group 3); and 1.7×10^5 CFU (Group 4).

Preparation of live antigens. *P. multocida* P1059, 3865, and 3866 were grown in BHIB as described above. Bacterial cells were recovered by centrifugation as described above, resuspended in 10 ml of PBS and serially diluted for quantification. A 200 μl inoculum containing approximately 10^4 CFU/ml was used to immunize the chickens as described above. The actual cell concentration of the inoculum was

determined by plate counts.

Preparation of killed antigens. Formalin-killed strain P1059 was prepared as follows. An overnight culture was harvested as above and the cell pellet was washed twice with PBS. The pellet was resuspended in 25 ml of PBS containing 0.1% formalin and then incubated overnight at 4°C. The cells were harvested, washed twice in PBS (to remove the remaining formalin), and resuspended to a final concentration of 10^8 CFU/ml. To assess whether there were viable cells remaining in the antigen preparation, 100 μl of the suspension was inoculated onto a BHI agar plate and incubated overnight at 37°C. A 200 μl inoculum [1.9 x 10^6 CFU, determined prior to the addition of formalin] was used to immunize the chickens as described below.

Adsorbed formalin-killed P1059 was prepared as follows. Formalin-killed P1059 was diluted to give an absorbance of A_{595} of 0.92. The cells were adsorbed for 18 hrs at 37°C with antisera prepared in chickens directed against formalin-killed P1059 (1:9 v:v of sera to diluted cell suspension), washed once, and resuspended in PBS to approximately 10^7 CFU/ml. A 200 μl inoculum (1.9 x 10^6 CFU) was used to immunize the chickens as described below.

Experimental design. Each chicken was immunized in the wing vein 3 times (Table 2). Fresh cultures of live antigen were administered 10 days apart. Chickens immunized with live *P. multocida* received an average dose of 3.6 x 10^3 CFU. Similarly, birds immunized with killed antigen received an average dose of 1.9 x 10^6

CFU. The individual doses given to each group of birds are shown in Table 1.2. Two birds in each group received 200 μl of PBS as a negative control. Ten days after the final immunization, the birds were challenged with 1.5 x 10^7 CFU of strain P1059. Mortality in each group was assessed during 10 days postchallenge at which time remaining birds were sacrificed by cervical dislocation. Immediately prior to death, blood samples were obtained from the birds by cardiac puncture. To validate infection by *P. multocida*, samples were taken aseptically from the liver, heart, and pericardial fluid. To validate septicemia, samples were taken from the bone marrow of the right femur. The presence of *P. multocida* was confirmed by Gram staining, characteristic colonial morphology on 5% blood agar and lack of growth on MacConkey agar.

Collection of sera. For immunoblot analysis, serum was collected from each chicken prior to the first immunization (prebleed), prior to challenge (prechallenge), and postchallenge. To separate the sera from other components of the blood, the samples were centrifuged at 3,000 × *g* for 25 min at 4°C. Sera was removed, aliquoted, and stored at -80°C. Once thawed, the sera was kept at 4°C. To simplify analysis, sera from 3 birds was pooled (Table 1.1).

Preparation of cell lysates for analysis. Cultures of *P. multocida* P1059 were grown for 12-18 h with shaking at 37°C in BHIB in the presence of 2 μg/ml hyaluronidase (Sigma Chemical Co., St. Louis) to remove the hyaluronic acid capsule (24).

Table 1.2. Experimental design. Immunization schedule and antigens doses for identification of proteins that may play a role in protection against fowl cholera. Chickens were challenged on Day 30 of the experiment.

Group	Antigen	Day Immunized	Dosage (CFU)	Challenge Dose (CFU)
	Live strains:			
A	P1059 [a]	1 10 20	1.3×10^3 1.9×10^3 8.0×10^3	1.5×10^7
B	3865 [b]	1 10 20	1.1×10^3 6.4×10^3 6.5×10^3	1.5×10^7
C	3866 [b]	1 10 20	7.3×10^2 2.2×10^3 4.0×10^3	1.5×10^7
	Killed strain:			
D	P1059 [c]	1 10 20	1.9×10^6	1.5×10^7
E	Adsorbed killed P1059 [c]	1 10 20	1.9×10^6	1.5×10^7

[a] $n = 12$
[b] $n = 7$
[c] $n = 6$

The cells were pelleted by centrifugation at 7000 × *g* for 10 min at 4°C. The pellets were washed twice and resuspended in 10 mM Tris (pH 8.0) to one-fifth the original culture volume. Cell suspensions were sonicated 4 times for 15 s at 35% power using a model 300 Dismembrator fitted with a microtip (Fisher Scientific, Pittsburgh). Lysozyme (2 mg/ml) and 100 μg/ml of DNase I were added to the sonicate and samples were incubated at 37°C for 18 h. Lysed cells were centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatant was removed and stored at -20°C. Prior to electrophoresis, samples were diluted in 2X loading buffer (625 mM Tris-base [pH 6.8], 2% SDS, 10% glycerol, 5% beta-mercaptoethanol, 0.001% bromphenol blue), boiled for 5 min, and centrifuged at 10,000 × *g* at room temperature for 15 min.

Polyacrylamide gel electrophoresis (PAGE). Protein concentration was determined using the Bradford assay (15) with the Bio-Rad protein dye reagent (Bio-Rad Laboratories, Richmond, Calif.) and bovine serum albumin as a standard. Denaturing polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemelli (86). Three to 5 μg of total protein per lane and 3 μg of high molecular weight standards (ferritin, catalase, aldolase, bovine serum albumin) (Bethesda Research Laboratories [BRL], Gaithersburg, Md.) were loaded onto a 12.5% polyacrylamide minigel (Mighty Small II, Hoeffer Scientific, San Fransisco, Calif.), and resolved at constant current (25 mA/gel) at 10°C until the dye front reached the bottom of the gel (approximately 1.5-2 h). Proteins were stained with

Coomassie Brilliant Blue (0.025% Coomassie Brilliant Blue R-250, 50% methanol, and 10% acetic acid).

Immunoblot analysis. Immunoblot analysis was performed according to Towbin *et al.* (170). Following electrophoresis, gels were equilibrated in Bjerrum and Schafer-Nielsen transfer buffer (48 mM Tris-base, 39 mM glycine, 20% methanol, 0.00375% SDS, [pH 9.2]) for 30 min. Membranes (0.22 μm Nitro ME [Fisher]) and blotting pads (BRL) were equilibrated in transfer buffer for approximately 30 min. The proteins were electroblotted at constant voltage (12 V) for 20-25 min (Bio-Rad Semidry Transfer Unit, [Bio-Rad]). After the transfer was completed, the lane containing the molecular weight markers was cut from the membrane and stained with Amido Black (0.1% Amido Black 10B, 25% isopropanol, 10% acetic acid). The remaining blot was blocked for at least 1 h in 1% powdered milk dissolved in Tris-buffered saline (0.02 M Tris [pH 7.5], 0.5 M NaCl [TBS]). The membranes were then rinsed in TBST (TBS containing 0.3% Tween 20) for 10 min, and incubated in antisera diluted 1:50 in TBS for a minimum of 3 h. Following incubation with primary antibodies, the membranes were rinsed in TBST for 10 min. The membranes were then incubated for 1 h in the secondary antibody (anti-chicken IgG prepared in rabbits and conjugated with alkaline phosphatase [Sigma]), diluted 1:400 in TBS, and then rinsed in TBST for 10 min. The immunoblots were developed in 7.5 ml of 50 mM Tris buffer [pH 9.0] containing 10 mM $MgCl_2$, 25 μl of 75 mg/ml nitroblue tetrazolium (NBT) and 33 μl

of 50 mg/ml 5-bromo-3-chloro-indoyl phosphate (BCIP). Color reactions were stopped by transferring the membranes to TE (10 mM Tris buffer [pH 8.0], 1 mM EDTA).

Preparation of adsorbed sera. Four hundred ml of an overnight culture of strain P1059 grown in BHIB containing 2 μg/ml hyaluronidase was divided into 4-100 ml aliquots and centrifuged at 5000 $\times$ *g* for 20 min at 4°C. The cell pellets were washed twice with PBS. To adsorb antibodies to surface antigens, 1 pellet was resuspended in diluted antisera (1:49 v:v of antisera to TBS) and incubated in a shaking incubator for 1 h at 22°C. The sample was centrifuged as above and the supernatant containing the adsorbed diluted serum, was readsorbed using a second cell pellet and clarified by centrifugation as described above.

The 2 remaining pellets were resuspended in 5 ml of TBS per pellet and sonicated with 4 bursts of 45 s as described above except at 60% power. These sonicated cells were used to adsorb antibodies reacting to intracellular and extracellular antigens that remained in the diluted antiserum. The antibodies were adsorbed as described above.

RESULTS

LD_{50} determination and collection of antisera directed against 3 strains of *P. multocida* [1]. The LD_{50} of *P. multocida* P1059 for 10 week old white leghorn chickens was 5.2 x 10^5 CFU (Table 3). Only 1 out of 12 (or 92%) of the birds immunized with two logs below the LD_{50} of live *P. multocida* P1059 exhibited detectable clinical symptoms after challenge (Table 1.4). In contrast, 71% of the chickens receiving strain 3865 and 85.8% receiving strain 3866 as immunogens exhibited severe symptoms of fowl cholera between 4-7 days postchallenge (Table 1.4). Fifty percent of the birds that received either formalin-killed P1059 or opsonized strain P1059 exhibited clinical symptoms of fowl cholera after challenge (Table 1.4).

Analysis of antisera. Immunoblot analysis using prebleed antisera from groups A_{1-4}, B_1, and C_1 recognized proteins with molecular masses of 68-, 39-, 26-, 18-kDa in strain P1059 in addition to one protein less than 14-kDa (Fig. 1.1). Prebleed antiserum from group A_2 recognized an additional 30-kDa protein. Prebleed antiserum from birds belonging to group B_1 reacted with 3 proteins smaller than 14-kDa, in addition to those mentioned. Prebleed antisera from birds belonging to group C_1 reacted with a 22-kDa protein in addition to those proteins

[1] The study presented here was used to generate antisera directed against 3 strains of *P. multocida*. This antisera was subsequently used to identify antigens that are unique to strain P1059. This was not meant to be a challenge study as determine by the USDA and the data should be interpreted accordingly.

Inoculum (CFU)	Mortality ratio	Total dead	Total survived	Mortality ratio (overall)	% Mortality (overall)
1.7×10^8	4/4	12	0	12/12	100
1.7×10^7	4/4	8	0	8/8	100
1.7×10^6	3/4	4	1	4/5	80
1.7×10^5	1/4	1	4	1/4	25

$$\log LD_{50} = A + h \times \frac{(B - 50\ \%)}{(B-C)}$$

A = log of total dilution step above 50% value
B = % animals affected at dilution below 50% mortality
C = % animals affected at dilution above 50% mortality
h = log dilution step

example:

A = if the starting CFU was 1.69×10^9 then the log of the dilution step above the 50% value was -3
B = 80%
C = 25%
h = 1/10

$\log LD_{50} = -3.5$

3.5 LD_{50} units were contained in 1.7×10^6 CFU
therefore the LD_{50} was 5.2×10^5 CFU

Table 1.3. Calculation of the LD_{50} determination. Ten week old SPF white leghorn chickens were immunized through the wing vein with increasing doses of strain P1059 afterwhich clinical symptoms were monitored for 10 day post inoculation. The LD_{50} was calculated according to Reed and Muench (133).

Table 1.4. Results from study to generate antisera directed against strains of *P. multocida*. White leghorn chickens were immunized with *P. multocida* P1059, 3865, 3866 or killed strain P1059 and then challenged with live strain P1059. Birds were monitored for clinical symptoms throughout the course of the experiment. The controls in groups A, B and C represent transmission controls, since they were not separated from those immunized with the live strains of *P. multocida*.

Group	Immunogen	down[a]	died
	Live:		
A	P1059 (n=12)	0	1
	Controls (n=2)	1	1
B	3865 (n=7)	4[b]	1
	Controls (n=2)	2	0
C	3866 (n=7)	6[b]	0
	Controls (n=2)	1	0
	Killed:		
D	P1059 (n=6)	2	1
E	Adsorbed (n=6)	3	0
	controls (n=2)	1	0

[a] Down was defined as chickens that exhibited signs of anorexia, diarrhea, cyanosis of the combs and wattles, conjunctivitis and could not walk.

[b] these birds were sacrificed on days 6 and 7 postchallenge due to the severity of their symptoms

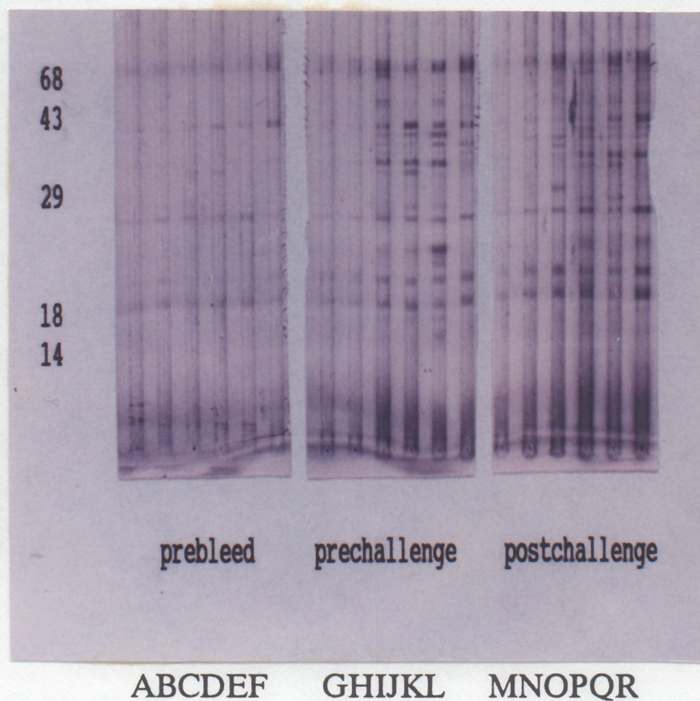


Fig. 1.1. Immunoblot analysis to examine pooled sera reacted with *P. multocida* P1059. Lanes A, G, and M represent sera from group B_1. Lanes B, H, and N represent sera from group C_1. Lanes C through F, I through L, and O through R represent sera from groups A_1 through A_4. Molecular weight standards are expressed in kDa.

in common with groups A_{1-4} and B_1.

Birds immunized with live strain P1059 responded to 23 proteins between 12- and 70-kDa (Fig. 1.2). There was variation among chickens in group A in response to proteins of 22-, 23-, 24-, 29-, 33- and 55-kDa. Prechallenge sera from groups B_1 and C_1 did not recognize proteins in cell lysates of *P. multocida* P1059 other than those in prebleed sera for the same groups.

Antisera directed against strain 3865 reacted strongly with 16- and 60-kDa proteins in both strains 3865 and 3866 (Fig. 1.2). There were weak responses with 4 additional proteins with molecular masses between 20- and 60-kDa. In contrast, the same antisera reacted with approximately 20 smaller proteins in strain P1059 (molecular masses $<$14-kDa to 68-kDa).

Antisera against strain 3866 recognized 3 proteins of 16-, 28-, and 60-kDa present in strains 3865 and 3866 (Fig. 1.2). Again, there were more proteins recognized on strain P1059 than either strains 3865 or 3866. Antisera directed against 3866 reacted with 19 proteins expressed by P1059 with molecular masses of less than 14-kDa to approximately 70-kDa. A 42- kDa protein was recognized by antisera directed against strains 3865 and 3866 but not by antisera against P1059.

Antisera from birds immunized with *P. multocida* P1059 recognized 10 proteins with molecular masses ranging from less than 14-kDa to approximately 70-kDa in strains 3865 and 3866 (Fig. 1.2). This antisera reacted with 23 proteins (M_r $<$14-kDa to 72-kDa) in P1059.

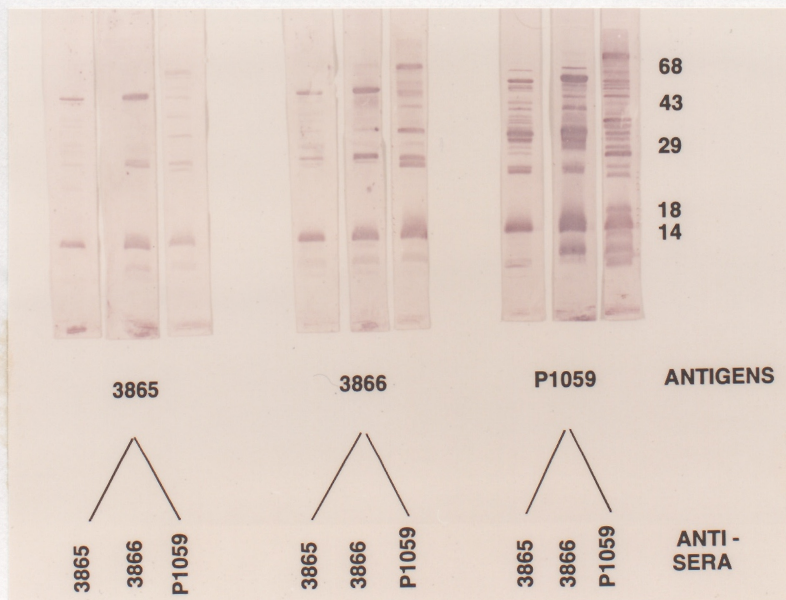


Fig. 1.2. Reactivity of homologous and heterologous antisera with proteins from three strains of *P. multocida*. Antisera from chickens immunized with live strains 3865, 3866, and P1059 were analyzed to determine antigens that are unique to P1059. The antigens and antisera used in this study are labeled. Molecular weight standards are expressed in kDa.

Antisera from chickens immunized with viable strain P1059 that had been adsorbed with whole-cells from strain P1059 failed to recognize proteins of 28-, 30-, 41- and 60-kDa (Fig. 1.3). Adsorption of this same antisera with sonicated strain P1059 removed reactivity with the remaining proteins.

To identify proteins expressed by strain P1059 but not strain 3865, antisera directed against strain P1059 was adsorbed with both whole-cell and sonicated strain 3865. Most, if not all antibodies that recognized proteins present in strain P1059 remained after absorption with whole-cell strain 3865 (Fig 1.4). Antibodies against 3 proteins remained after adsorption with sonicated strain 3865. These proteins had molecular masses of 25-, 34- and 43-kDa.

To compare proteins expressed by strain P1059 and other type A strains, antiserum directed against strain P1059 was reacted with another highly virulent strain; *in vitro* grown strain X-73. The only discernable difference between *P. multocida* strains P1059 and X-73 was in the region less than 18-kDa. In this region, antisera directed against strain P1059 reacted with the homologous cell lysate. This reaction was not seen with strain X-73 (Fig. 1.5).

To examine if the immunogenicity of killed strain P1059 cells elicited the same response as live strain P1059, the humoral response of chickens immunized with formalin-killed P1059 was compared to the response directed against live P1059. Proteins of 24-, 26-, 37- and 39-kDa identified by antisera directed against

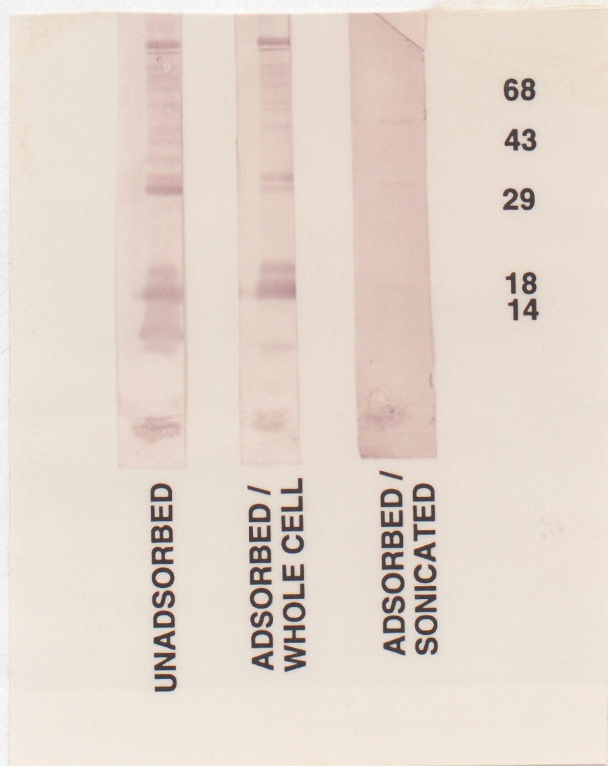


Fig. 1.3. The localization of antigens. Antiserum directed against *P. multocida* P1059 was adsorbed with whole-cell and sonicated strain P1059 to localize the antigens recognized by this serum. Cell lysates of strain P1059 were analyzed by immunoblot using the adsorbed serum (as designated). Molecular weight standards are expressed in kDa.

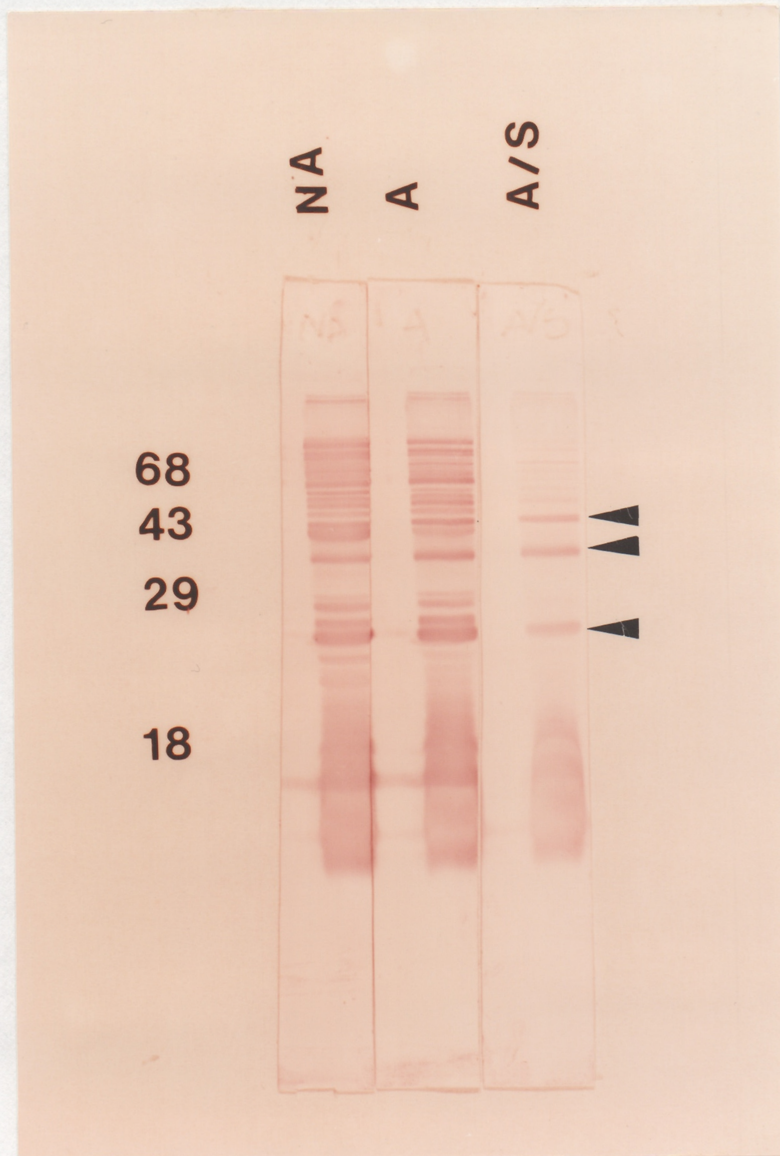


Fig. 1.4. Antigens expressed by *P. multocida* P1059 which differ from strain 3865. To identify antigens unique to strain P1059, antiserum directed against strain P1059 was adsorbed with whole-cell and sonicated strain 3865 cell lysates. The adsorbed antiserum was analyzed by immunoblot analysis. Lane NA represents unabsorbed antiserum. Lane A represents the same sera adsorbed with whole cell strain 3865. Lane A-S represents the same sera adsorbed with both whole-cell and sonicated 3865. Molecular weight standards are expressed in kDa. Proteins remaining after reaction with the sera that had been adsorbed with both whole-cell and sonicated strain 3865 are designated with arrows.

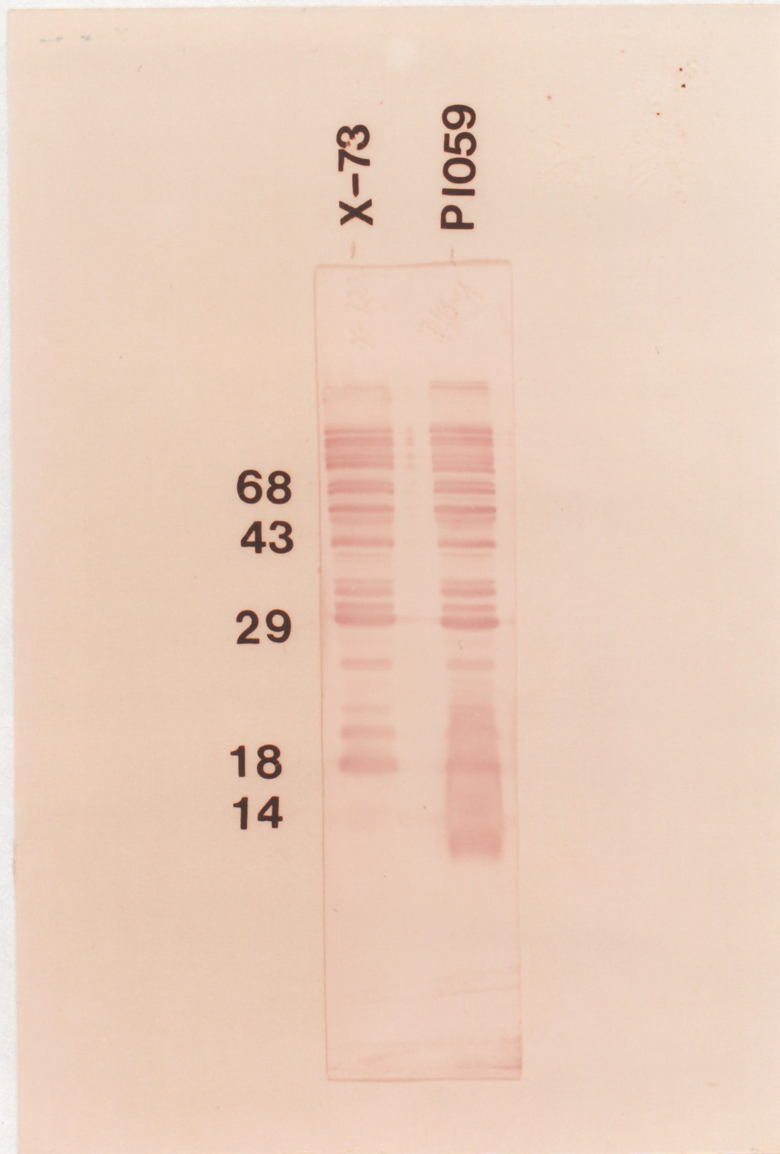


Fig. 1.5. Immunoblot analysis of *P. multocida* P1059 and X-73. Antiserum directed against strain P1059 was reacted with cell lysates of strains P1059 and X-73 to identify similarities and differences in protein recognition between two highly virulent strains of *P. multocida*. The antigens used in immunoblot analysis are designated. Molecular weight standards are expressed in kDa.

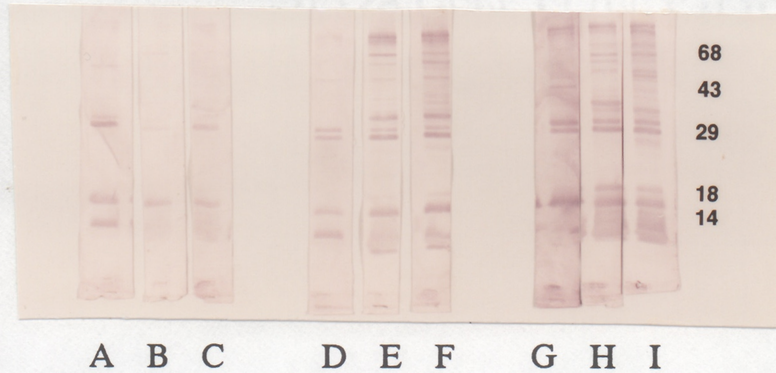


Fig. 1.6. Immunoblot analysis of antisera directed against formalin-killed *P. multocida* P1059. To contrast proteins recognized by antiserum against live strain P1059 with those recognized by formalin-killed strain P1059, antiserum against formalin-killed strain P1059 was used for immunoblot analysis. The antiserum was reacted with cell lysates of strain P1059. Lanes A through C, prebleed serum; D through F, prechallenge serum; and G through I, postchallenge serum. Lanes A, D and G represent serum from the control group, E_3. Lanes B, E, H and C, F, I represent serum from birds immunized with formalin-killed strain P1059, (groups D_1 and D_2 respectively). Molecular weight standards are expressed in kDa.

live strain P1059 (Fig. 1.2) were not recognized by antisera against formalin-killed strain P1059 (Fig. 1.6).

To determine if the immune response to opsonized antigens would differ from non-opsonized antigens, formalin-killed strain P1059 was adsorbed with antisera directed against formalin-killed strain P1059 and used to immunize chickens. Few differences were discernable between proteins recognized by antisera from chickens immunized with opsonized antigens and to those in which the antigens had not been opsonized. There were strong reactions to 66-, 44-, 27-, and 20-kDa proteins (Fig. 1.7).

DISCUSSION

To create an effective vaccine, one needs to evaluate the interaction between a host and a pathogen. This interaction can be observed from two perspectives: i) the response of the host to the pathogen, and ii) the response of the pathogen to the host.

> " Survival and multiplication are clearly the priorities for the microbe, while disease is simply a manifestation of the complex interactions required to accomplish these two goals within the milieu of host tissues" (102).

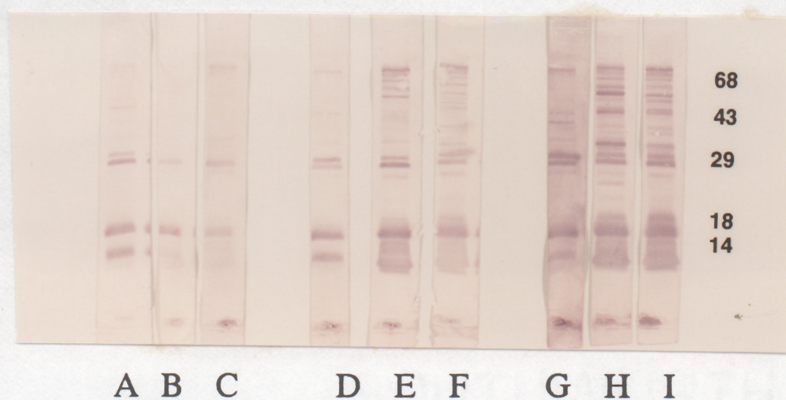


Fig. 1.7. Analysis of sera from chickens immunized with opsonized formalin - killed *P. multocida* P1059. Antiserum from chickens immunized with opsonized strainP1059 was analyzed to determine if opsonization elicited a different response than immunization with nonopsonized strain P1059. Lanes A through C, prebleed serum; D through F, prechallenge serum; G though I, postchallenge serum. Lanes A, D and G represent serum from the control group (E_3). Lanes B, E, H and C, F, I represent sera from groups immunized with opsonized strain P1059 (E_1 and E_2 respectively).

The complexity of the interaction of a pathogen and its host have been well documented for many bacteria including *Yersinia pestis* (129), *Bordetella pertussis* (45,105), and *Haemophilus influenzae* (172) in addition to *P. multocida*. Host specific responses, antigenic variation and virulence factors are examples of the interaction of *P. multocida* with its host (33,67,68,142,143,147).

Host specific variation is seen not only in the response of the same hosts to different strains of *P. multocida* but also the converse (42,68). For example, the LD_{50} of *P. multocida* P1059 for chickens was 3.7 x 10^5 CFU (as determined in this study). This was approximately two logs higher than the LD_{50} for the same strain in turkeys reported by Snipes *et al.* (164). Other strains of *P. multocida* exhibit the opposite effect. For example, strain X-73 is more virulent for chickens than turkeys (33,147). Young poults are more susceptible to fowl cholera than adult turkeys (14), however, older chickens are more susceptible than younger chickens (142). Increased hormone levels of older birds during production, specifically estrogens and androgens, may be responsible for immunosuppressive effects that make these birds more susceptible to infection by *P. multocida* (161). In addition, attempts to increase production have caused domesticated poultry to become highly inbred. This loss of "hybrid vigor" contributes to their increased susceptibility to infection by pathogens such as *P. multocida*.

Chickens immunized with 10^3 CFU of strains 3865 or 3866 did not develop clinical signs of fowl cholera. Since these strains were highly immunogenic and did

not produce clinical symptoms in the chickens, it is possible that they may serve as potential hosts for the delivery of protective antigens. Strains 3865 and 3866 belong to serogroup D and do not possess a hyaluronic acid-based capsule typical of the type A strains isolated from cases of fowl cholera. Studies to determine the LD_{50} for the strains 3865 and 3866, their ability to produce specific virulence factors, replicate *in vivo*, and colonize the respiratory tract of poultry would be important to assess their usefulness in the development of a vaccine.

The antigens recognized by antisera directed against strains 3865 and 3866 were fewer in number than those recognized by antiserum directed against strain P1059. In fact, prechallenge sera from the birds immunized with strains 3865 and 3866 resembles prebleed sera from these same animals.[2] This observation initially suggested that strains 3865 and 3866 may not be immunogenic in chickens. However, reaction of antisera directed against strains 3865 or 3866 with strain P1059 suggests that these strains are indeed immunogenic. Antisera against either of the type D strains recognized more antigens expressed by strain P1059 than with itself. It is possible that strains 3865 and 3866 express additional immunogenic antigens *in vivo*. Examples of antigenic variation expressed by a bacterium when it is grown *in vivo* have been reported (33,56,60,81). Bacterial pathogens such as *E. coli*, *Vibrio cholera* and *P. multocida* undergo phenotypic changes as exhibited by the expression

[2] The reaction of the prebleed sera with *P. multocida* suggests that these birds have been exposed to an organism (probably a Gram negative bacterium) that elicited a humoral response that crossed react with antigens present on *P. multocida*.

of novel surface antigens that are not expressed when they are grown *in vitro*.

The lack of recognition by antiserum directed against strains 3865 or 3866 with cell lysates from strains 3865 or 3866 may result from the type D strains being cleared from the chickens too rapidly to induce an immune response. However, reaction of this same antisera with strain P1059 suggests that even if strains 3865 and 3866 were cleared more rapidly than strain P1059, they remained in the host long enough to induce a strong immune response. Since LPS contamination is difficult to remove from protein preparations, it is possible that there is significant contamination that is blocking the recognition of proteins by the homologous antisera (eg. 3865 reacted with antisera directed against 3865, or 3866 with antisera against strain 3866). However, reaction with heterologous antisera (eg. antisera directed against strain P1059) recognizes additional antigens in preparations of strains 3865 and 3866. Determination of the serotype of strains 3865 and 3866 would help to answer this problem. It is also possible that the antigens present in strains 3865 and 3866 that are recognized by antiserum directed against either of these strains are insoluable under the conditions used in this study.

Much of the literature on *P. multocida* focuses on the humoral immune response to extracellular components including OMPs (33,75,76,117,155,165,171), LPS (66,91,137,141) and capsule (63,164). Based on the data presented here, most of the protein antigens recognized by the humoral immune system of the chickens were located intracellularly. Adsorption of antisera directed against live strain

P1059 with whole-cells of strain P1059 removed few, if any, of the antibodies that recognized proteins with masses between 14- and 68-kDa. Adsorption of the same sera with both whole-cells and lysates from sonicated strain P1059 removed most of the antigens recognized within this same range. The identification of these proteins and their association with either virulence or protection is not known.

Antibodies to 3 proteins (25-, 30-, and 43-kDa) were not removed by adsorption of strain P1059 antiserum with strain 3865. Ireland *et al.* (78) reported proteins of a similar mass that were expressed by another highly virulent strain of *P. multocida*, strain X-73. These proteins were also recognized by antiserum directed against strain X-73. The only discernable difference in reaction of anti-P1059 antiserum to either strains P1059 or X-73 was against proteins in the mass region <14-kDa (this study). This is the region of the LPS (78). The variation in this region is not surprising since strains P1059 and X-73 are of different serotypes (A:3 and A:1 respectively). The identity, function and role in protection (if any) of the proteins described in these studies have yet to be determined.

Chapter 2

The Cloning and Expression of Proteins Recognized by Antisera Isolated from Chickens Surviving Severe Challenge with *Pasteurella multocida* P1059

RATIONALE

The molecular basis of the pathogenesis of *Pasteurella multocida* is not well understood. Many putative virulence factors such as capsule (63,164), outer membrane proteins (94,95,139,171), lipopolysaccharide (43,66,91,94,137,173), siderophores (33,76,79), and toxins (75,165) have been reported. Only the genes for adenylate cyclase (106), *P. multocida* toxin (*toxA*) (90,125,126,158), and a repressor of *tox*A termed *Txa*R (125) have been cloned. Chevalier *et al.* (32) have determined the N-terminal sequence of the OMP, protein H.

The purpose of this study was to clone and express antigenic proteins of *P. multocida* P1059. Such a study would allow an analysis of protein expression at a molecular level, as well as increase our understanding of the pathogenesis of *P. multocida*.

MATERIALS AND METHODS

Isolation of genomic DNA. Genomic DNA was isolated from *P. multocida* P1059 and *Escherichia coli* O1:K1 according to Murray and Thompson (107). Twenty-five milliters of overnight broth cultures strains O1:K1 and P1059 (grown in

either Luria Bertani broth (LB, [99]) or Brain Heart Infusion broth (BHIB, Difco Laboratories, Detroit) were centrifuged 9000 × *g* for 7 min at 4°C. Cell pellets were resuspended in 2.4 ml of TE (10 mM Tris [pH 8.0], 1 mM EDTA) containing 0.5% SDS and 0.01 mg/ml Proteinase K (Sigma Chemical Co., St. Louis), and incubated at 37°C for 60 min. After incubation, a solution containing 450 μl of 5 M NaCl and 375 μl of CTAB/NaCl (10% wt/vol hexadecyltrimethyl ammonium bromide [CTAB], 0.7 M NaCl) was added, incubated at 65°C for 20 min, and extracted with an equal volume of chloroform/isoamyl alcohol (24:1) (vol/vol). The nucleic acids in the aqueous phase were precipitated with 0.6 volumes of isopropanol for 18 h at -20°C. The precipitates were recovered by centrifugation for 30 min at 10,000 × *g*, (4°C) and resuspended in 0.5 ml of 1/10 TE.

Digestion and ligation conditions for the construction of the transcriptional fusions. Partial fragmentation of the genomic DNA from both *P. multocida* P1059 and *E. coli* O1:K1 was created by digestion with the restriction endonuclease *Sau*3A I for 15 min at 37°C. The enzyme was heat inactivated at 65°C for 10 min. The plasmid vector, pCB267 (162), was digested to completion with *Bam*H I, dephosphorylated with calf intestinal alkaline phosphatase (CIP [GIBCO/BRL, Gaithersburg, Md.]) and ligated to the *Sau*3A I digested genomic DNA mixture at a molar ratio of 1:1 (T4 DNA ligase [GIBCO/BRL]). Ligation reactions were performed overnight at 23°C. Recombinant plasmids were used to transform competent cells of *E. coli* DH5α (98). Transformed cells were allowed to recover

for 60 min in LB at 37°C prior to plating on LB plates containing 100 μg/ml ampicillin (LB-amp). Plates were spread with 100 μl of 10 mg/ml Bluo-gal solution (GIBCO/BRL) in dimethylformamide (DMF). *E. coli* CB806 transformed with the plasmids pCLL and pCO2L were used as controls (169). The plasmid pCLL has the *lac*Z promoter cloned into the multiple cloning site of pCB267, while pCO2L has an insert with no promoter activity. For this analysis, the production of ß-galactosidase detected by the presence of blue colonies was used as an indicator of promoter activity (Fig. 2.1).

Preparation of competent cells. Competent cells were prepared according to Mandel and Higa (98). An overnight culture of *E. coli* DH5α was used as a 0.5% inoculum into 50 ml of fresh LB broth (99). Cells were incubated in a shaking incubator at 37°C until early-log phase (approximately 37-40 Klett units), chilled on ice for 15 min and centrifuged at 9000 $\times$ *g* for 7 min at 4°C. Cells were resuspended in 12.5 ml of $CaCl_2$ buffer (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid [HEPES] [pH 7.0], 60 mM $CaCl_2$, 15% glycerol), incubated on ice for 2 min and centrifuged as above. The resulting cell pellets were resuspended in 6 ml of $CaCl_2$ buffer, incubated on ice for 30 min, centrifuged, resuspended in 2 ml of $CaCl_2$ buffer and frozen using dry ice and ethanol. Competent cells were stored at -80°C.

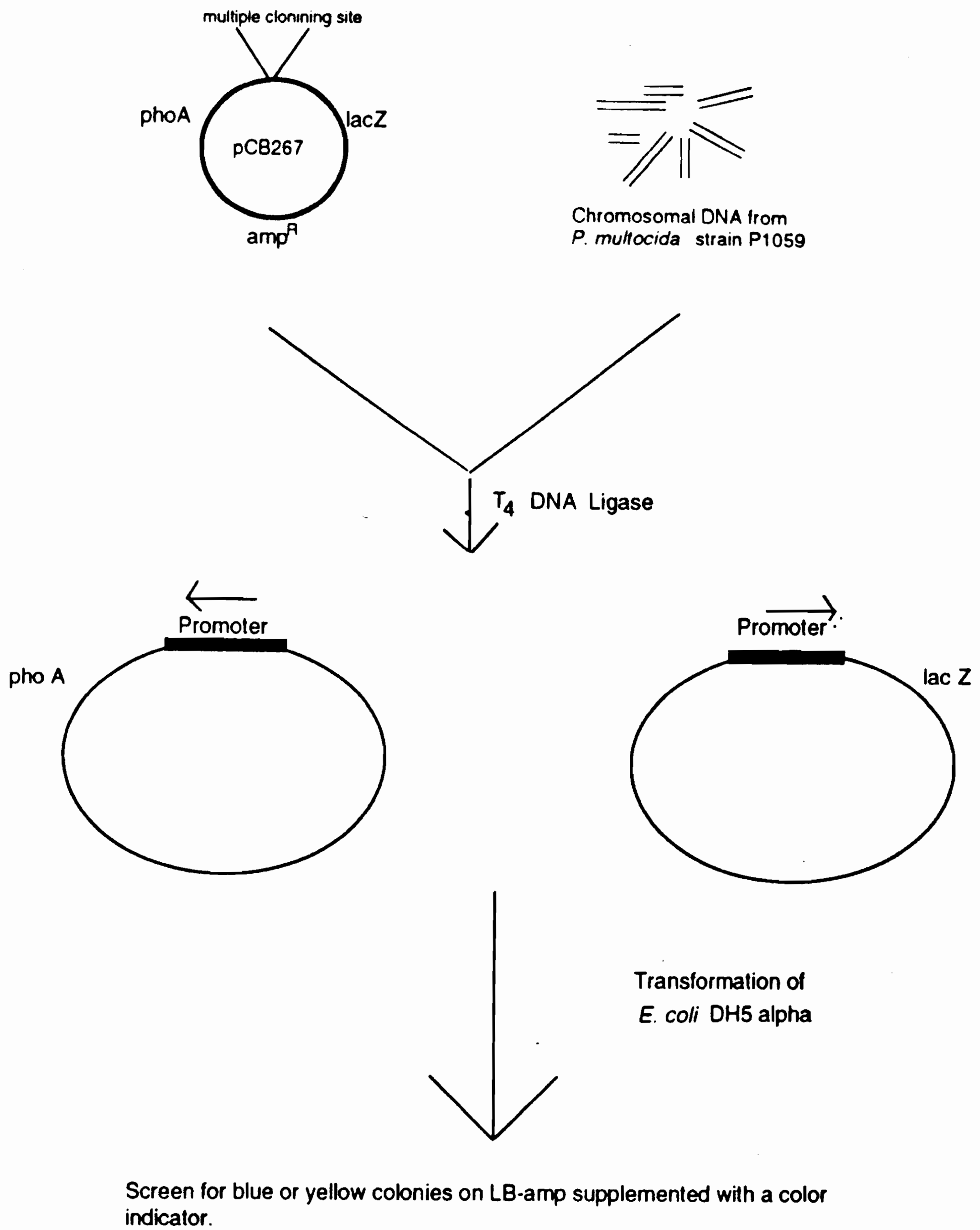


Fig. 2.1. Construction of transcriptional fusions to determine promoter activity of *P. multocida* P1059 DNA in *E. coli* DH5α.

Construction of a genomic library of *P. multocida* P1059. *Sau*3A I - digested genomic DNA from strain P1059, was resolved by electrophoresis through a 0.7% Sea-Plaque® low-melt agarose gel (FMC Bioproducts, Rockland, Maine) using Tris-Acetate EDTA (40 mM Tris-acetate, 2 mM EDTA [TAE]) as the running buffer. The agarose containing DNA fragments ranging in size from 1- to 10-kb was excised from the gel bed, heated for 15 min at 65°C and mixed with linearized, dephosporylated pUC19 (vector:insert ratio of 1:1). The mixture was incubated at 37°C for 15 min after which T_4 DNA ligase added. The ligation was allowed to continue overnight at 22°C (3). The ligation mixture was used to transform *E. coli* DH5α utilizing the Hanahan procedure (62). Clones were screened on LB-amp plates containing Bluo-gal as described. Four thousand one hundred clones were selected; each clone contained an average insert size of 4-kb. The genomic library represents over 99% of the genome (based on a genome size of 1.2 to 2.2 x 10^6 bp) (128) (Fig. 2.2). Individual clones were grown overnight at 37°C in Terrific Broth (TB) (3), and stored at -80°C in TB containing 15% glycerol. The genomic library was also stored as a pooled plasmid preparation at -20°C.

Screening of clones that express *P. multocida* antigens. The genomic library was screened with antisera by colony blot analysis. Briefly, clones were transferred to LB and LB-amp plates using a colony transfer apparatus and incubated overnight at 37°C. Colonies were then transferred to 0.22 μM nylon membranes (Nitro ME, Fisher) by colony lift. The membranes were incubated in chloroform for 15 min to

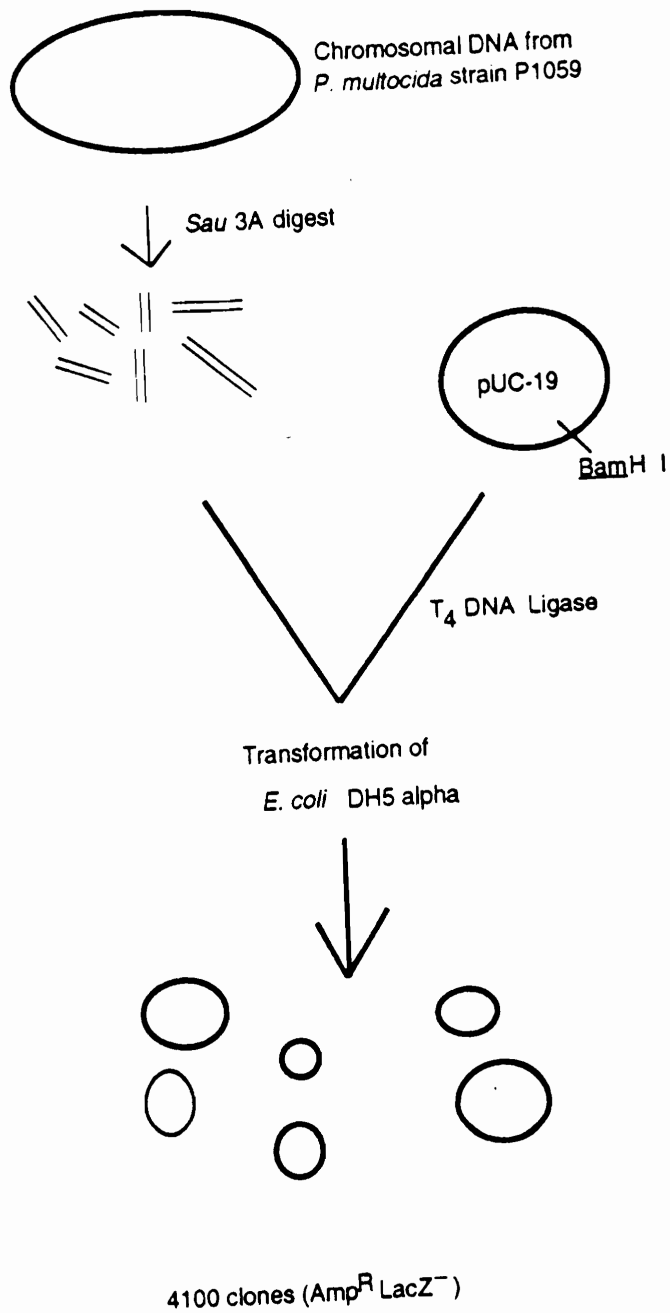


Fig. 2.2. Construction of the genomic library of *P. multocida* P1059.

lyse the cells and then dried for 15 min. To prevent nonspecific binding of the antibodies, membranes were blocked with Tris-buffered saline (0.02 M Tris [pH 7.5], 0.15 M NaCl, 0.005 M $MgCl_2$ [TBS]) containing 3% bovine serum albumin (BSA) for 45 min. After blocking, the membranes were washed for 10 min in TBS supplemented with 0.03% Tween 20 (TBST) and incubated with pooled antiserum directed against live strain P1059 (1:50 dilution in TBS) for 60 min on a rotary shaker[3]. Membranes were washed in TBST for 10 min and incubated for 60 min in anti-chicken IgG conjugated with horseradish peroxidase (Sigma) (1:400 dilution in TBS). After a final rinse in TBST, membranes were developed in a solution containing 0.6% hydrogen peroxide in 100 ml of TBS and 0.06 gms of 4-chloro-1-naphthol dissolved in 10 ml methanol. Clones which reacted repeatedly (3 times) were confirmed to be positive for the expression of antigens from *P. multocida* (Fig. 2.3).

Plasmid isolation and restriction enzyme analysis. Plasmids were isolated from antibody-reactive clones by the method of Ish-Horowitz (99). Briefly, cells from overnight cultures grown in TB were harvested by centrifugation at 9,000 × *g* for 7 min at 4°C. Cell pellets were resuspended in solution I (25 mM Tris-Cl [pH 8.0], 50 mM glucose, 10 mM EDTA, 5 mg/ml lysozyme) to 1/50 of their original volume and incubated at room temperature for 10 min. After incubation, 1/25 of

[3] Serum was adsorbed with both whole-cells and sonicated *E. coli* DH5α to eliminate background prior to screening the library.

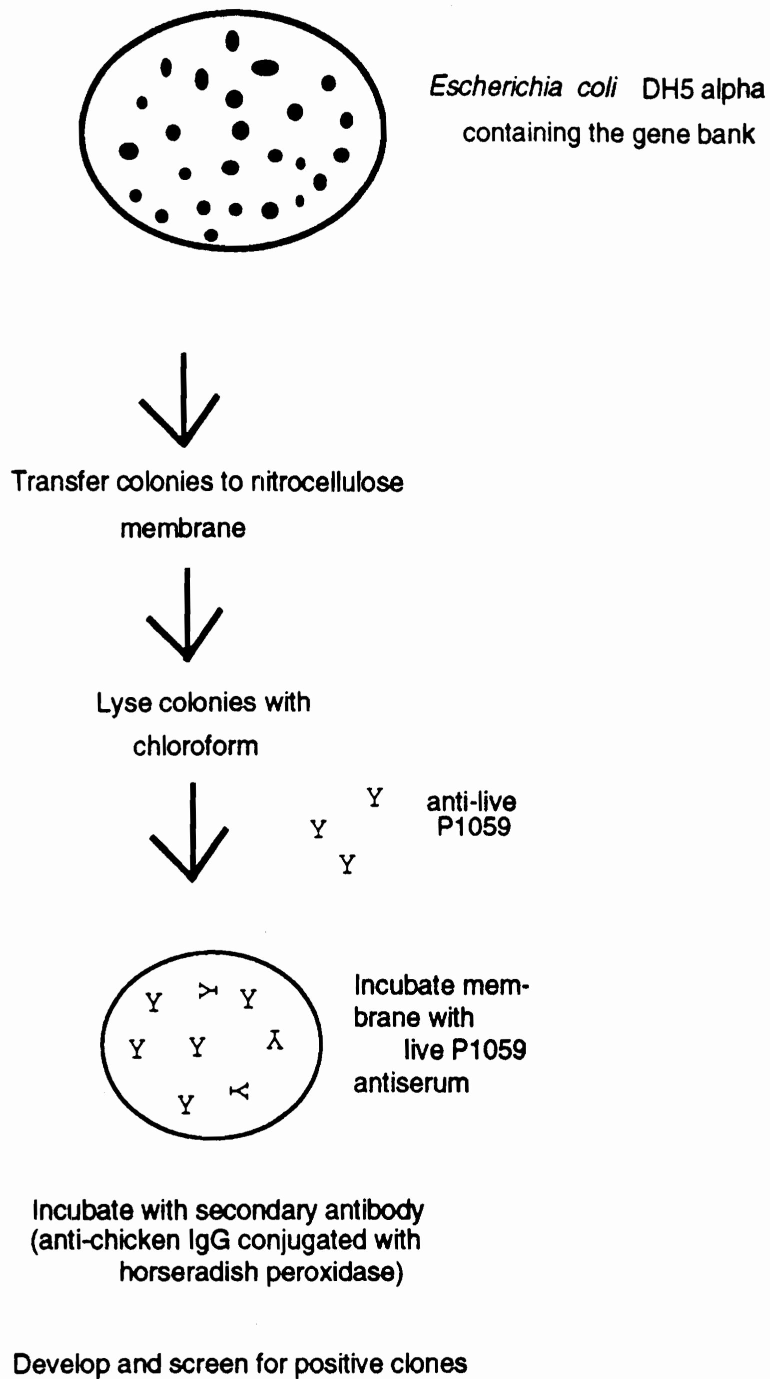


Fig. 2.3. Screening of the genomic library with antisera directed against *P. multocida* P1059.

the original culture volume of solution II (1% SDS, 0.2 N NaOH) was added and incubated on ice for 10 min. Contaminating chromosomal DNA and protein were precipitated by the addition of solution III (3 M potassium acetate) and the slurry was incubated on ice for 10 min. Plasmid DNA was separated from the chromosomal DNA/protein mixture by centrifugation at 9000 × *g* for 20 min at 4°C.

Polyacrylamide gel electrophoresis and immunoblot analysis. See Materials and Methods of Chapter 1 for details of these protocols.

Analysis of maxicells containing plasmids from the genomic library. The rationale for the use of maxicells (*E. coli* CSR603) was as follows: When *uvrA*, *recA* deficient cells are irradiated with UV light, DNA synthesis stops and chromosomal DNA is degraded (74). Simultaneously, plasmids such as pUC19 which have not been irradiated are able to increase their numbers of copies/cell by approximately 10-fold (156). This situation allows for labeling of plasmid encoded proteins over chromosomally encoded proteins.

E. coli CSR603 (98) was transformed with the plasmids isolated from the antibody-reactive clones and analyzed according to Sancar *et al.* (156). An overnight culture of each strain was used to inoculate minimal medium 63 (M63) (Difco Laboratories, Detroit) supplemented with 1% Casamino acids (Difco) and ampicillin (100 μg/ml). Cultures were incubated at 37°C until mid-log phase (approximately 100-110 Klett Units), transferred to a 20 ml Petri plate (10 mm x 150 mm) and irradiated using a Stratalinker 1800 (Stratagene, La Jolla, Calif.) with a

total of 5300 microjoules of UV energy. Irradiated cultures were allowed to recover at 37°C for 30 min prior to adding 40 μl of cycloserine (50 mg/ml stock, [Sigma]). The following morning, cells were rinsed twice with M63 and resuspended in 2 ml of M63 lacking Casamino acids. Cells were starved of amino acids for 60 min and then pulsed for 30 min with 0.6 μCi of ^{35}S-methionine (Dupont/New England Nuclear Corp., Boston) per ml of sample. After 30 min cells were pelleted by centrifugation for 5 min at 11,000 × *g*. Unincorporated label was removed by rinsing the cell pellets with an equal volume of M63. After the final rinse, cell pellets were resuspended in 100 μl of 2X loading buffer (625 mM Tris-Cl [pH 6.8], 2% SDS, 10% glycerol, 5% beta-mercaptoethanol, 0.001% bromphenol blue) and boiled for 5 min. Protein samples containing approximately 1.5 x 10^5 dpm were resolved by electrophoresis through a 12.5% polyacrylamide gel. Gels were dried for 90-120 min at 60°C and visualized by autoradiography using diagnostic film (X-Omat, Eastman Kodak Co., Rochester, NY) (24-48 h at -80°C).

RESULTS

Analysis of transcriptional fusions. To determine the relative promoter activity, genomic DNA from *P. multocida* P1059 was cloned randomly into promoterless *lacZ* or *phoA* genes on the vector pCB267. The transcription of β-galactosidase or alkaline phosphatase is dependent upon supplying an active promoter. Colonies containing these enzymes are easily identified on a plate

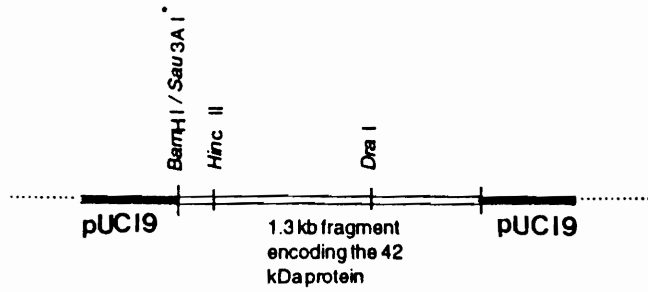
containing the appropriate chromogenic substrate. Genomic DNA from *E. coli* O1:K1 cloned into pCB267 was used as a positive control. The results obtained from Table 2.1 suggest that many promoters of *P. multocida* are able to function in *E. coli*.

Analysis of the genomic library. Of the 4,100 clones surveyed, 12 reacted with antiserum directed against *P. multocida* P1059 in colony blot analysis. DNA inserts of the recombinant plasmids contained in these twelve clones ranged in size from 0.48- to approximately 6.8-kb. Restriction enzyme analysis performed on pOP-9, pOP-10, and pOP43-2G revealed that pOP-9 and pOP-10 were similar and may encode for the same protein (data not shown). As seen in Fig 2.4, the third plasmid, pOP43-2G, contained an insert of 1.3-kb that differed from either pOP-9 and pOP-10. *E. coli* harboring these 3 plasmids were analyzed by native PAGE, SDS-PAGE and immunoblot analysis. Using these methods, *Pasteurella* proteins were not expressed in detectable quantities.

Maxicell analysis. To enhance the detection of recombinant *Pasteurella* proteins, the 12 plasmids were used to transform *E. coli* CSR603. These recombinants would that allow for the specific labeling of plasmid encoded proteins (Table 2.1). Two recombinant plasmids, pOP33-8F and pOP43-2G expressed *Pasteurella* proteins of 50- and 42-kDa respectively. These proteins were detectable

Table 2.1. Promoter activity of chromosomal DNA from *P. multocida* P1059 relative to *E. coli* O1:K1. Genomic DNA from strain P1059 and *E. coli* O1:K1 were cloned into the transcriptional fusion vector, pCB267, and expressed in *E. coli* DH5α. Promoter activity was determined by the production of ß-galactosidase. The percent promoter activity was calculated as the percentage of postive clones of the total number of clones surveyed. The average insert size was 2.8 kb.

	Number of clones expressing ß-galactosidase acivity		
Source of DNA	Positive	Negative	Promoter Activity (%)
O1:K1	54	237	18.6
P1059	67	113	37.2



Restriction endonucleases which do not cleave the 1.3 kb insert of pOP43-2G	
*Bam*H I	*Pst* I
Eco RI	*Pvu* II
Hind III	*Sma* I

* designated site of ligation of *Pasteurella* DNA

Fig. 2.4. Restriction enzyme analysis of the plasmid pOP43-2G.

by Coomassie blue staining and/or immunoblot analysis (Figs. 2.5-7). *Pasteurella* proteins of 37-, 48-, and 55-kDa were detected in CSR603(pOP47-5C), CSR603(pOP37-7A) and CSR603(pOP65-3A) respectively by autoradiography of cell extracts of ^{35}S-methionine labeled maxicells (Fig. 2.8).

Analysis of pOP43-2G. *E. coli* CSR603(pOP43-2G) expresses a 42-kDa protein that was recognized by antisera directed against live *P. multocida* strains 3865 or 3866 (Fig. 2.9). There were no proteins expressed by *in vitro* grown *P. multocida* P1059 which correspond to this size (Fig. 2.10A). Antisera directed against either live *P. multocida* P1059 or formalin-killed strain P1059 both recognized the 42-kDa protein encoded by CSR603(pOP43-2G) (Figs. 2.10A and B). A protein of similar size expressed by *in vivo* grown strain P1059 is also recognized by antisera directed against live *P. multocida* strain P1059 (Fig. 2.10B).

DISCUSSION

There have been only a limited number of reports concerning the cloning of genes from *P. multocida* (90,106,125,126,158). Therefore, it was important to ascertain whether *E. coli* could express other genes of *Pasteurella*. The purpose of this study was to determine if *E. coli* DH5α could serve as a host for the

Table 2.2. Maxicell analysis. A summary of the results from restriction enzyme analysis and autoradiographs of the positive clones recognized by P1059 antisera expressed in maxicells.

Plasmid	Size of insert (kb)	Size of proteins on SDS-PAGE[a] (kDa)
pUC19	None	
pOP-9	2.8	
pOP-10	2.8	
pOP29-8A	2.2	
pOP33-8F	0.9	50
pOP36-7A	4.0	37,48
pOP37-5E	4.5	
pOP43-2G	1.3	42
pOP43-6D	0.5	
pOP47-5B	0.5	
pOP47-5C	2.2	37
pOP65-3A	2.4	55
pOP65-6C	6.8	

[a] proteins produced in addition to the 28- and 31-kDa proteins of ß-lactamase encoded on the vector, pUC19 (100).

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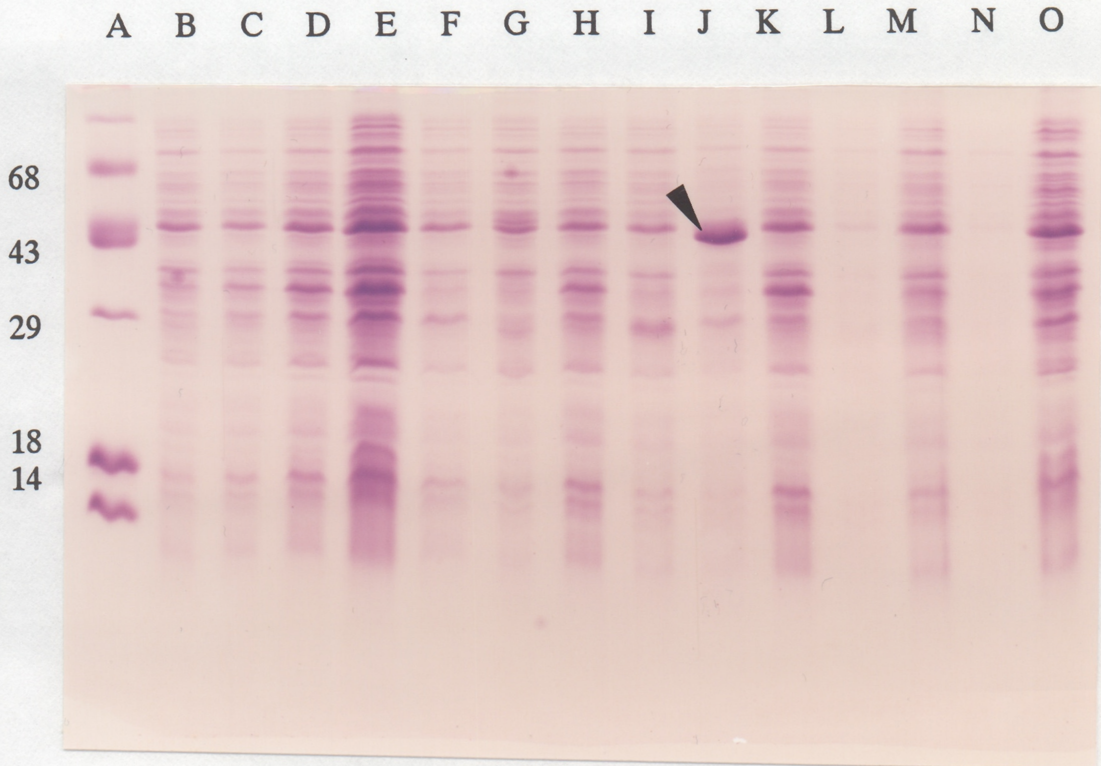


Fig. 2.5. Coomassie blue staining of maxicell lysates resovled by denaturing gel electrophoresis. Lanes A, molecular weight markers; B, CSR603; C, CSR603(pUC19); D, CSR603(pOP-9); E, CSR603(pOP-10); F, CSR603(pOP29-8A); G, CSR603(pOP33-8F); H, CSR603(pOP36-7A); I, CSR603(pOP37-5E); J, CSR603(pOP43-2G); K, CSR603(pOP43-6D); L, CSR603(pOP47-5B); M, CSR603(pOP47-5C); N, CSR603(pOP65-3A); O, CSR603(pOP65-6C). Molecular weight standards are designated in kDa. The arrow in lane J designates expression of a 42-kDa *Pasteurella* protein by strain CSR603(pOP43-2G).

Fig. 2.6. Immunoblot analysis of proteins expressed by maxicells (strain CSR603) containing the selected plasmids. Cell lysates of 12 clones containing plasmids selected from a genomic library of *P. multocida* P1059 were reacted with antiserum directed against strain P1059 that had been adsorbed with *E. coli* DH5α. Lane A, CSR603; B, CSR603(pUC19; C, CSR603(pOP-9); D, CSR603(pOP-10); E, CSR603(pOP29-8A); F, CSR603(pOP33-8F); G, CSR603(pOP36-7A); H, CSR603(pOP37-5E); I, CSR603(pOP43-2G); J, CSR603(pOP43-6D); K, CSR603(pOP47-5B); L, CSR603(pOP47-5C); M, CSR603(pOP65-3A); N, CSR603(pOP65-6C). Molecular weight standards are designated in kDa. The arrows in lanes F and I designate expression of *Pasteurella* proteins.

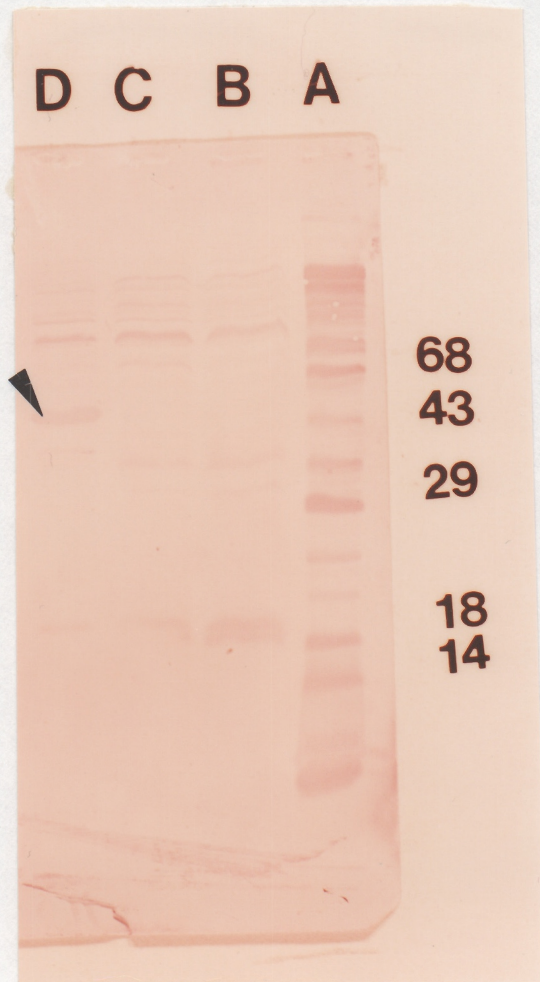


Fig. 2.7. Expression of a 42-kDa *Pasteurella* protein by *E. coli* CSR603(pOP43-2G). Cell lysates containing the *Pasteurella* protein produced by CSR603(pOP43-2G) was reacted with antisera directed against strain P1059. Lane A, *in vitro P. multocida* P1059; B, *E. coli* CSR603; C, *E. coli* CSR603(pUC19); D, *E. coli* CSR603(pOP43-2G). The arrow in lane D designates expression of the 42-kDa *Pasteurella* protein by strain CSR603(pOP43-2G). Molecular weight standards are expressed in kDa.

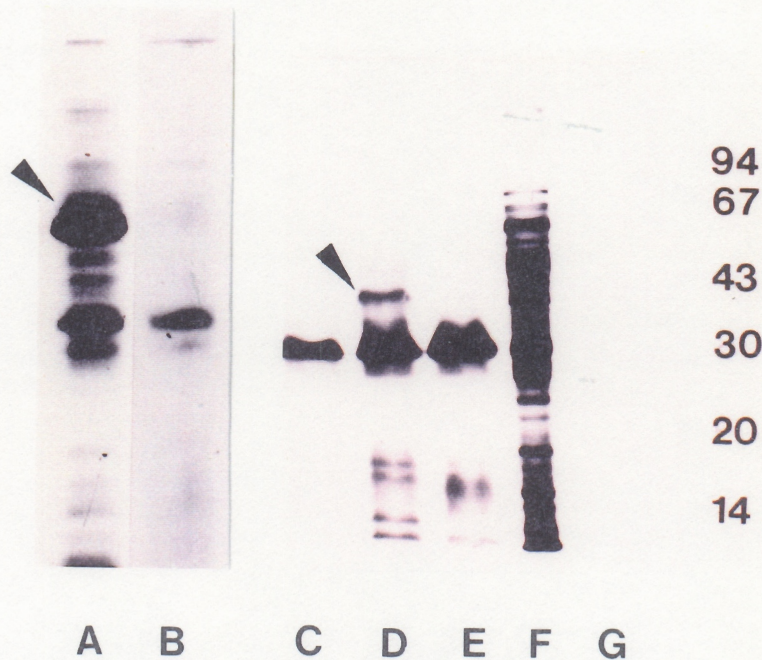


Fig. 2.8. Representative autoradiographs of maxicell analysis. *Pasteurella* proteins encoded on plasmid from a genomic library of *P. multocida* P1059 were radiolabled in maxicells. Lane A, *E. coli* CSR603(pOP43-2G); B, *E. coli* CSR603(pUC19); C, *E. coli* CSR603(pOP65-3A); D, *E. coli* CSR603(pOP47-5C); E, *E. coli* CSR603(pOP47-5B); F, *E. coli* CSR603(pUC19); G, *E. coli* CSR603. Molecular weight standards are expressed in kDa. The arrows in lanes A and E designate expression of *Pasteurella* proteins.

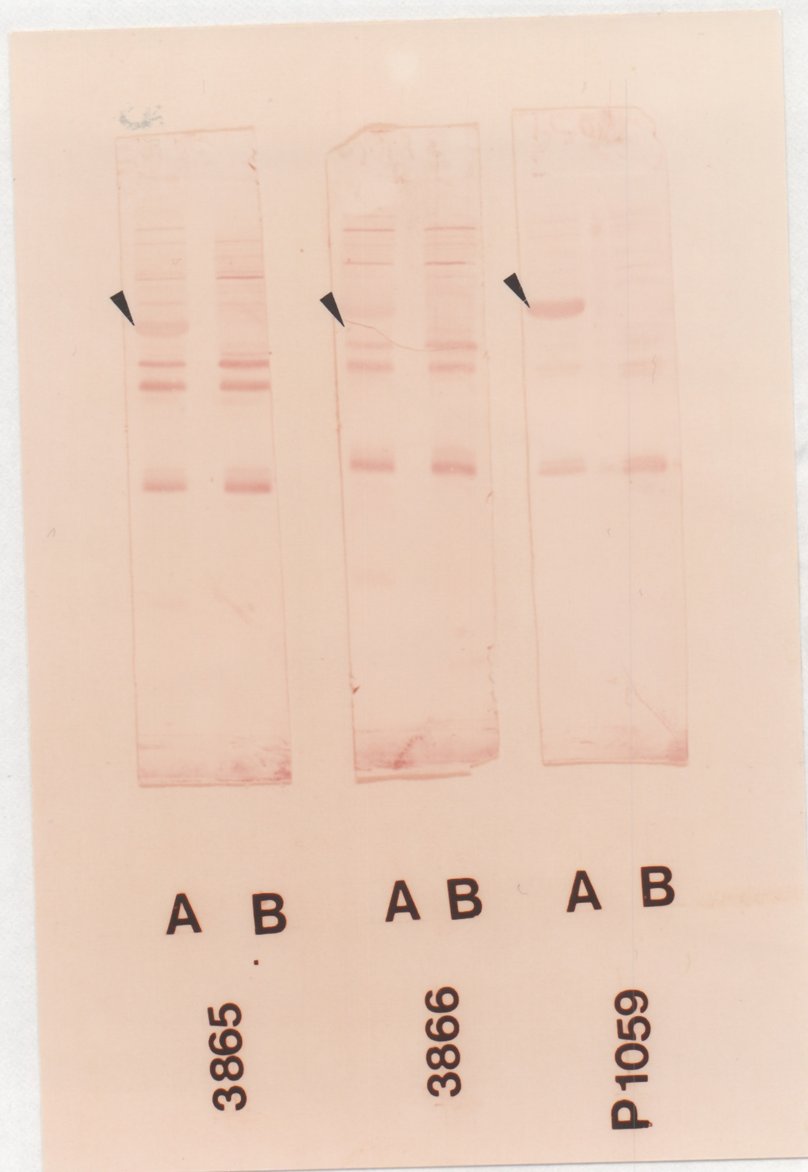


Fig. 2.9. Immunoblot analysis of *E. coli* CSR603(pOP43-2G). To determine if strains 3865 and 3866 also express the 42-kDa protein, antisera against these strains were adsorbed with sonicated CSR603 and then reacted with cell lysates of CSR603(pOP43-2G) and CSR603(pUC19). The antigens are presented in lanes A, CSR603(pOP43-2G); B,CSR603(pUC19). The antisera used is designated. Arrows designate expression of the 42-kDa *Pasteurella* protein.

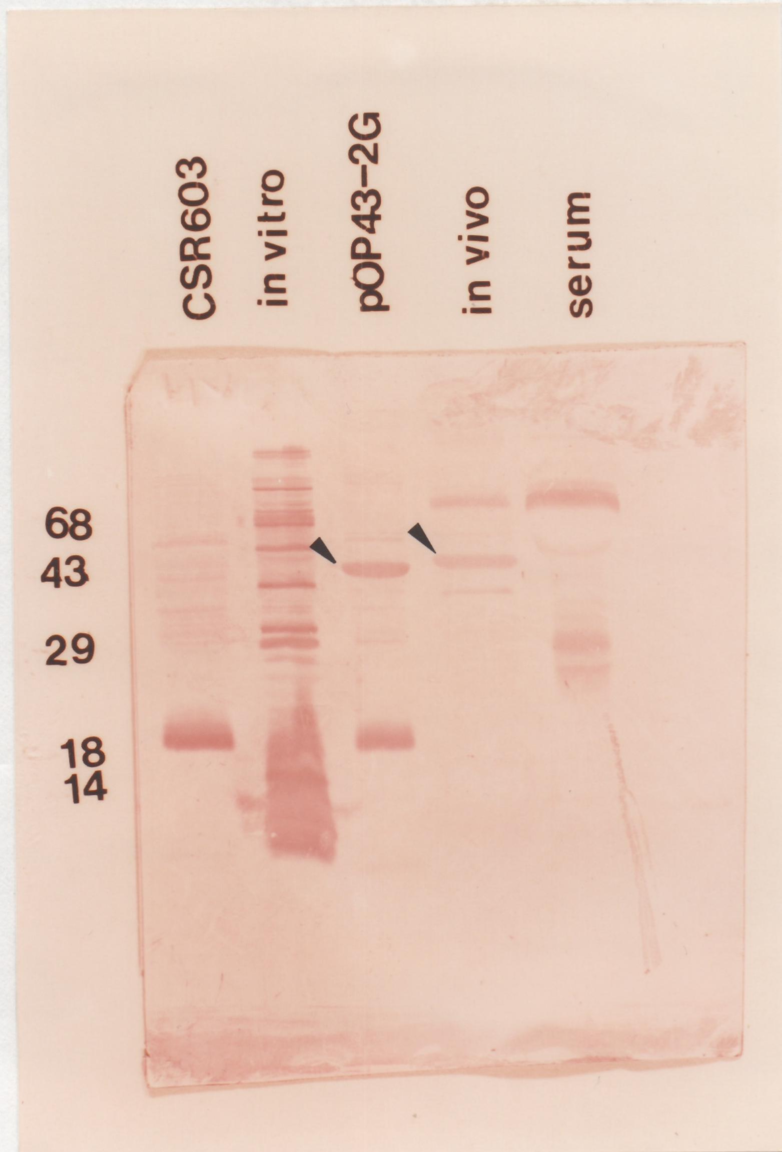


Fig. 2.10 A. Comparison of *in vivo* grown and *in vitro* grown *P. multocida* P1059 with *E. coli* CSR603(pOP43-2G). Lanes are strain CSR603; *in vitro* grown strain P1059; strain CSR603(pOP43-2G); *in vivo* grown strain P1059; normal chicken sera. The membrane was developed with antisera against live strain P1059. The larger arrows designate the 42-kDa *Pasteurella* protein expression by strain CSR603(pOP43-2G) and the 43-kDa protein expressed by *in vivo* grown strain P1059. The molecular weight standards are expressed in kDa.

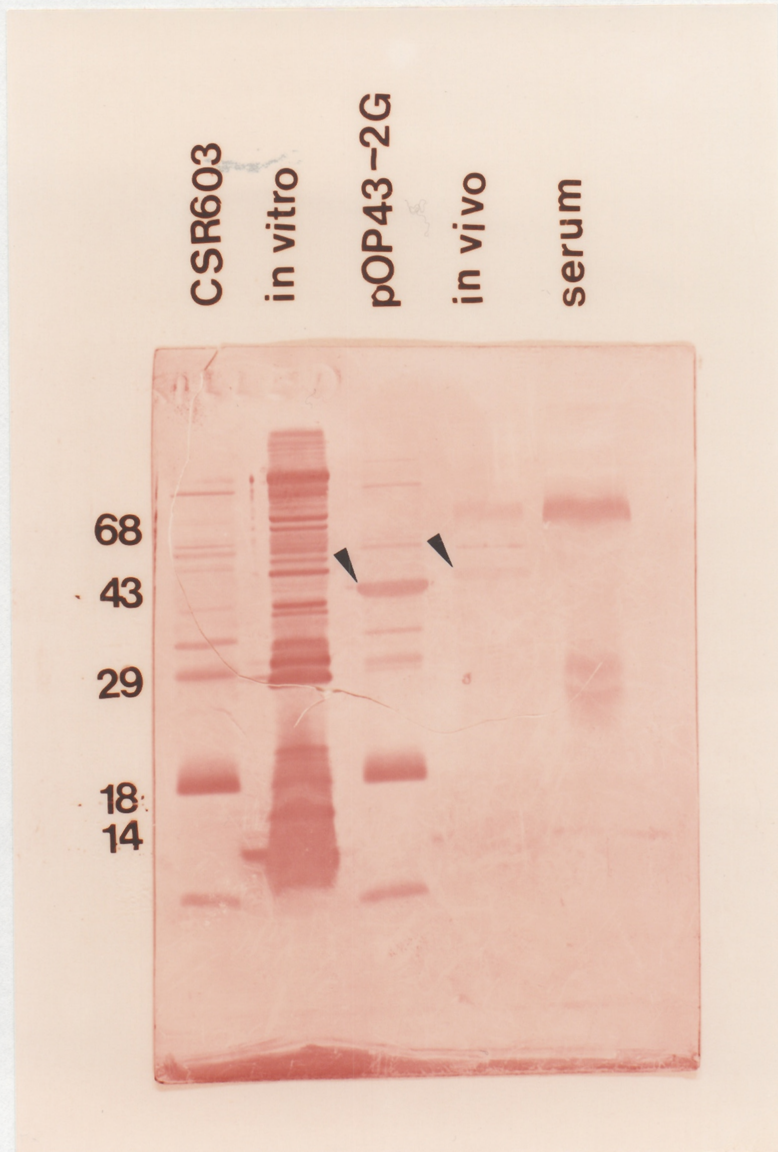


Fig. 2.10 B. Comparison of *in vivo* grown and *in vitro* grown *P. multocida* P1059 with *E. coli* CSR603(pOP43-2G). Lanes are strain CSR603; *in vitro* grown strain P1059; strain CSR603(pOP43-2G); *in vivo* grown strain P1059; normal chicken sera. The membrane was developed with antisera directed against formalin-killed strain P1059. Molecular weight standards are expressed in kDa. The larger arrows designates the 42-kDa *Pasteurella* protein expressed by strain CSR603(pOP43-2G) and the 43-kDa protein expressed by *in vivo* grown strain P1059.

maintenance and expression of a genomic library of strain P1059.

The results of the transcriptional fusion study suggest that DNA containing promoters from *P. multocida* are recognized by *E. coli* RNA polymerase. In fact, *Pasteurella* DNA cloned into the transcriptional fusion vector, pCB267 contained twice as many promoters as the *E. coli* DNA cloned into the same vector; these results were unexpected. A number of explanations could account for the unexpected difference in promoter activity: i) The reporter gene encoding for alkaline phosphatase (*phoA*) was not used in this analysis because of the difficulty distinguishing clones expressing alkaline phosphatase from those not expressing the enzyme. Since the orientation of the insertion of chromosomal DNA into the vector was random, there could have been significant promoter activity in the opposite orientation that remained undetected; ii) it is possible that there were more active promoters in the *Pasteurella* DNA than in the *E. coli* DNA; and iii) the size of the inserts in the plasmids containing *Pasteurella* DNA and those containing *E. coli* DNA may have been different. Regardless of the explanation, promoters of *P. multocida* were active in *E. coli*; therefore strain DH5α was considered a satisfactory host for expression of the genomic library of *P. multocida*.

Several explanations can be offered to explain the low number of *Pasteurella* proteins that were detected by maxicell analysis: i) M63 medium may inhibit the production of specific *Pasteurella* proteins though this is unlikely; ii) the plasmid encoded proteins may be unstable in *E. coli* CSR603; iii) the proteins identified by

colony blot analysis may not incorporate enough radiolabelled methionine to be detected due to their low methionine composition; iv) the antibodies may have been directed against conformational epitopes that would be lost under the denaturing conditions used in this analysis; v) the antibodies may be against antigens that were SDS insoluble and vi) the remaining clones may have been false positives. This last explanation seems unlikely since the colony reactions were confirmed by retesting in the presence of positive and negative controls.

Five of the clones expressed immunoreactive proteins that were 37-, 42-, 48-, 50- and 55-kDa. OMPs of a similar molecular mass have been reported by Ikeda and Hirsh (76) and Snipes *et al.* (165). Choi-Kim *et al.* (35) showed that 4 outer membrane proteins smaller than 45-kDa were recognized by convalescent antisera directed against strain P1059. Since the antisera used to screen the genomic library was directed against strain P1059, one or more of the proteins expressed by the recombinant clones may be an OMP. Similar sized proteins from whole-cell extracts of strain X-73 (serotype A:1) were recognized by antisera directed against live strain X-73 (78). Lutenburg (96) reported a 36-kDa protein (protein H) isolated from a swine isolate (serogroup D) that was immunogenic, however there was no correlation between the presence of protein H and virulence. Amino acid sequencing and immunoblot analysis with serum directed against OMPs of strain P1059 would answer whether the proteins identified in this study are the same as those reported in the literature.

Antisera directed against strain P1059 detected a 50-kDa *Pasteurella* protein. The insert of pOP33-8F is only 0.9-kb; an insert of this size can encode for a protein no larger than 30.6-kDa. Thus the plasmid pOP33-8F appears to encode a fusion protein of the N-terminus of the α peptide of ß-galactosidase and a *Pasteurella* protein that has retained one or more epitopes recognized by the antisera.

The last portion of this study focused on a protein encoded by the plasmid pOP43-2G. This protein was easily detectable by Coomassie blue staining and immunoblot analysis. It was difficult to determine the identity of the protein encoded on pOP43-2G since the antisera used in these experiments was directed against both intracellular and extracellular antigens present on strain P1059.

There was no direct correlation between proteins expressed by strain P1059 grown *in vitro* and the 42-kDa *Pasteurella* protein encoded by pOP43-2G. This protein was recognized by antisera directed against *P. multocida* 3865, 3866 and P1059. Antisera against strains 3865 and 3866 recognized a protein of similar molecular weight present in strain P1059 but not in strains 3865 or 3866 (Chapter 1, Fig. 2).

There was a 43-kDa protein expressed by *in vivo* grown strain P1059 that is recognized by homologous antisera. Antisera directed against formalin-killed strain P1059 also recognized the same 43-kDa protein, however, the recognition was much weaker. The 42-kDa protein expressed by CSR603(pOP43-2G) was recognized

strongly by antisera directed against both live and formalin-killed strain P1059 suggesting that CSR603(pOP43-2G) does not encode the 43-kDa protein expressed by *in vivo* grown strain P1059. However, it is possible that the 43-kDa protein is expressed at low levels in *in vitro* grown strain P1059 and would therefore be present in the formalin-killed antigen used to generate antisera against formalin-killed strain P1059. The strong reaction seen in Fig. 10B may be due to the high concentration of the 42-kDa protein present in the sample.

Alternatively, the 42-kDa protein may be an OMP reported in the literature. Choi *et al.* (33) describe a 38-kDa protein which was present across serotypes of group A strains, a member of serogroup B and 18 field isolates of serotype A:3 as well as the vaccines strains CU and M-9 (33). Ireland *et al.* (78) reported the presence of 4 major antigens between 30- and 43-kDa. Whether the protein reported by Choi *et al.* (33) and the protein encoded by pOP43-2G are the same is not known. Since the 42-kDa protein reacted with antisera directed against strains belonging to type A and type D strains of *P. multocida*, it is unlikely that it plays a direct role in the virulence of strain P1059. A final possibility is that the protein encoded by pOP43-2G is a truncated version of a larger protein which still possesses the epitope recognized by the antisera. Immunoblot analysis using antibodies directed specifically against the 42-kDa protein would be necessary to determine if this possibility were true.

Chapter 3

The Development of a Cloning System for Gene Expression in *Pasteurella multocida*

RATIONALE

The final aspect of this dissertation was to optimize a transformation system and develop a shuttle vector for gene expression in *Pasteurella multocida*. Although several genes from *P. multocida* have been expressed in *Escherichia coli* (90,106,125,126,158), there is no shuttle vector available that would allow for the transfer of DNA between these species. On a broader scale, a cloning system could enable the study of the genes encoding cellular components such as outer membrane proteins and *P. multocida* toxin within their "true" environment.

Since traditional methods of transformation have been unsuccessful with *P. multocida*, attempts to intoduce plasmids into *P. multocida* by electroporation were undertaken. To determine if available shuttle vectors were stable in *P. multocida* R473, cells were electroporated with limited-host range as well as broad-host range vectors. Since these vectors were unable to transform *P. multocida*, attempts were made to develop a shuttle vector using the *Pasteurella* plasmid, pLAR-1, and the commercially available vector, pBR322.

MATERIALS AND METHODS

Strains. *P. multocida* R473, Bunia II, 1062, P86-338, 3865 and 3867 were obtained from G.R. Carter (Dept. of Pathobiology, Va. Md. Regional College of Veterinary Medicine, VPI and SU, Blacksburg, Va.). *P. multocida* P1085 was obtained from R. Rhoades (U.S.D.A., Ames, Iowa). *E. coli* DH5α was purchased from Bethesda Research Laboratories (GIBCO/BRL, Gaithersburg, Md). Hyaluronidase (2μg/ml) was added at the time of inoculation to remove the capsule from type A strains of *P. multocida*. Cultures of *P. multocida* were grown in Brain Heart Infusion Broth (Difco Laboratories, Detroit [BHIB]). *E. coli* cultures were grown in Luria Bertani (LB) broth (99) unless stated otherwise.

Plasmids and plasmid isolation. The streptomycin and sulfonamide resistance plasmid, pVM109, was isolated from *P. multocida* P1085 (71). The plasmids, pBR322, pBR325 and pUC19 were purchased from GIBCO/BRL. Broad host range vectors pSP329 and pJFF224-NX (53) were obtained from T. Inzana (Dept. of Pathobiology, Va. Md. Regional College of Veterinary Medicine, VPI and SU, Blacksburg, Va.) (Fig. 3.1). The *Pasteurella* plasmid, pLAR-1 (Fig. 3.2) was ligated into the *Bam*H I site of pBR322 in both orientations to create pLRBR-21 and pLRBR-67 (101) (Fig. 3.3). These hybrid plasmids contain two origins of replication; the ColE1 origin located on pBR322 and the uncharacterized *Pasteurella* origin located on pLAR-1. *E. coli* DH5α(pBAII-3), encoding *Brucella abortus*

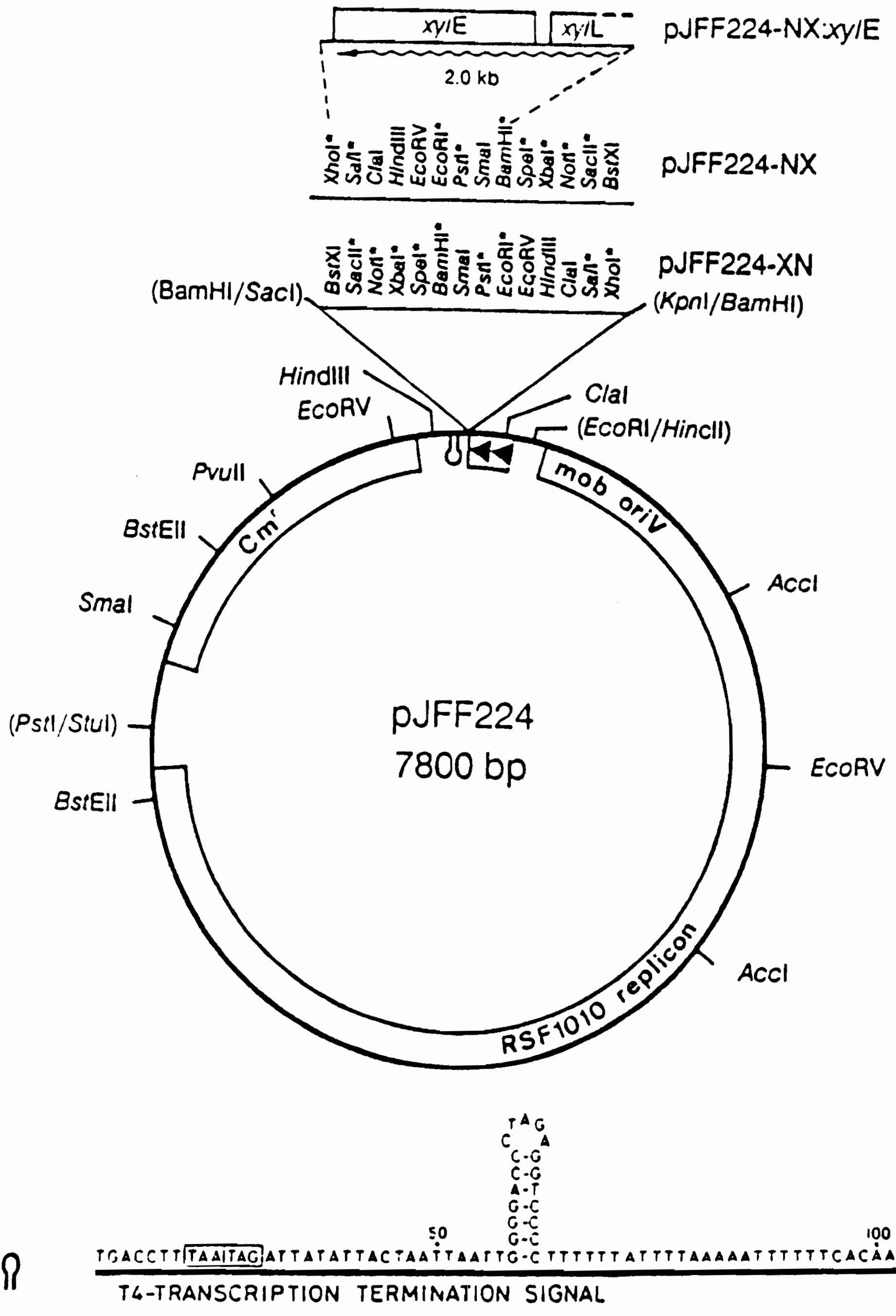


Fig. 3.1. A restriction map of the broad host range shuttle vector pJFF224-NX (53).

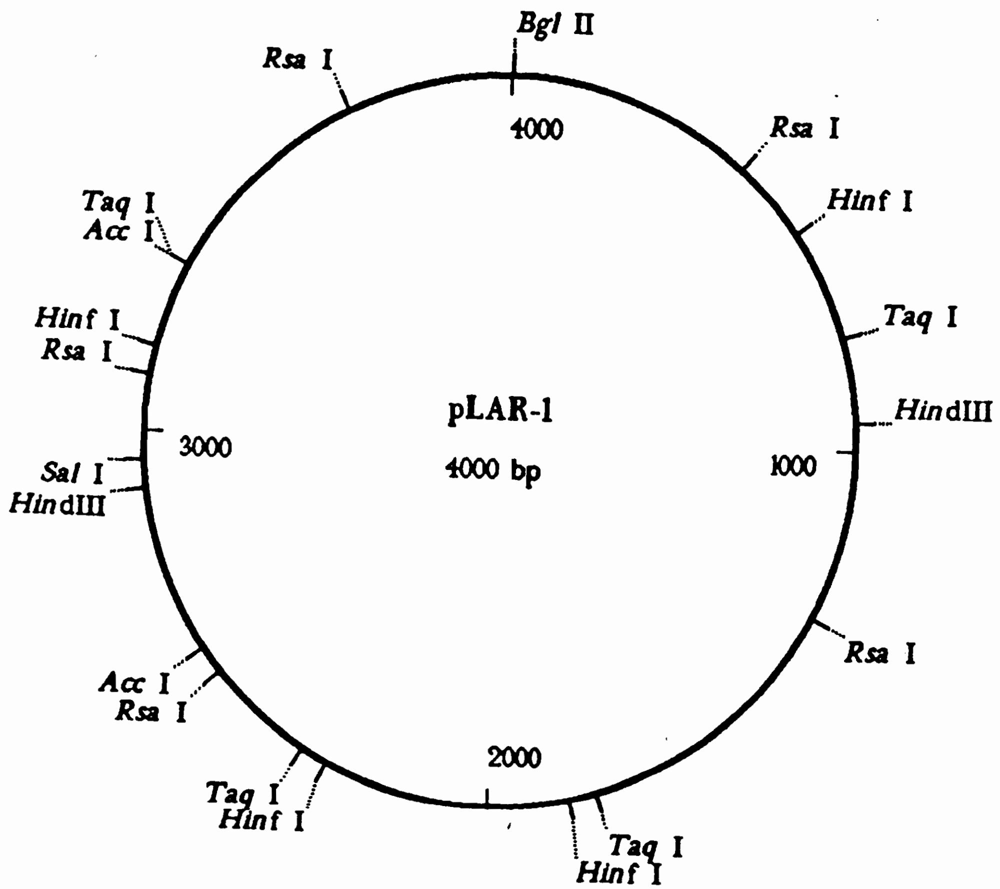
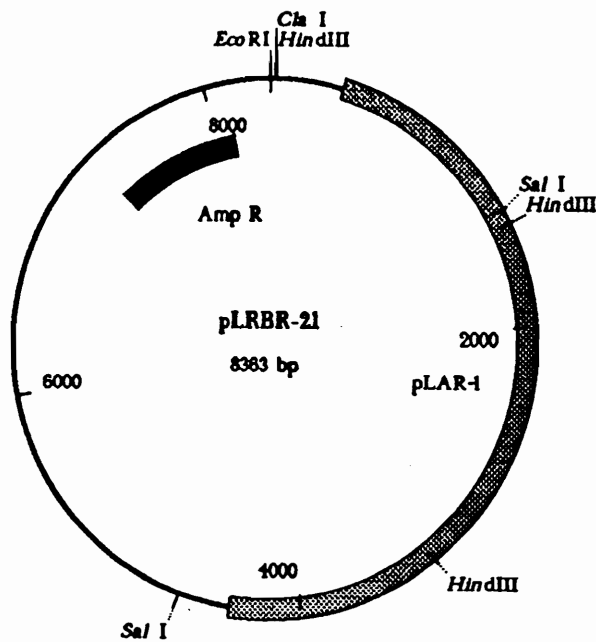


Fig. 3.2. A restriction map of the *Pasteurella* plasmid pLAR-1.



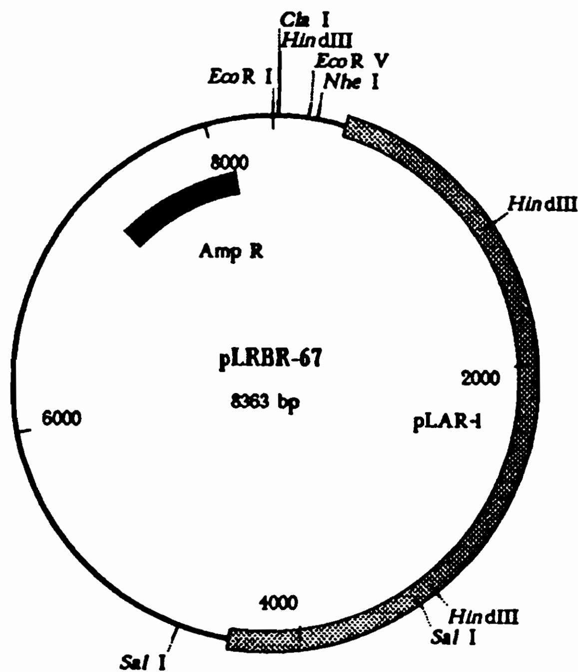


Fig. 3.3. Restriction maps of pLRBR-21 and pLRBR-67. The naturally occurring *Pasteurella* plasmid, pLAR-1 was ligated into the *Bam*H I site of pBR322 to create pLRBR-21 and pLRBR-67 (101). Basepairs 352 through approximately 4352 are derived from pLAR-1, the remaining bases are pBR322.

superoxide dismutase (Cu/Zn SOD), was obtained from S.M. Boyle (89). Creation and characterization of the ampicillin resistant plasmid pOP43-2G encoding a 42-kDa protein of *P. multocida* was described in Chapter 2.

For plasmid isolations, *E. coli* transformants were grown overnight in Terrific Broth (TB)(3) containing the appropriate antibiotic. Plasmid containing strains of *P. multocida* were grown overnight in BHIB supplemented with the appropriate antibiotic. The Ish-Horowitz alkaline lysis procedure as outlined by Maniatis *et al.* (99) was used for isolation of the plasmids from both *E. coli* and *P. multocida*.

Ligation conditions. Ligations were performed according to Ausebel *et al.* (3). Briefly, approximately 100 ng of vector was digested according to the manufacturer. The vector and insert were mixed at a molar ratio of 1:1 (vector:insert), in the presence of T4 DNA Ligase (GIBCO/BRL), and allowed to incubate overnight at 22°C. Ligation mixtures were used to transform *E. coli* DH5α (98). Transformants were selected on LB agar plates containing the appropriate antibiotics.

Transformation of *E. coli* DH5α. Competent cells were prepared according to Mandel and Higa (98). An overnight culture of *E. coli* DH5α was used as an 0.5% inoculum into 50 ml of LB (99). Cells were incubated in a shaking incubator at 37°C until early-log phase (approximately 37-40 Klett units), placed on ice for 15 min, and then centrifuged at 9000 $\times$ g for 7 min at 4°C. Cell pellets were resuspended in 12.5 ml of HEPES buffer containing $CaCl_2$ ($CaCl_2$ buffer) (10 mM

N-2-hydroxy- ethylpiperazine-*N'*-2-ethane-sulfonic acid [HEPES] [pH 7.0], 60 mM $CaCl_2$, 15% glycerol), incubated on ice for 2 min, and centrifuged as above. Resulting cell pellets were resuspended in 6 ml of $CaCl_2$ buffer, incubated on ice for 30 min, and centrifuged. After the final centrifugation the cell pellets were resuspended in 2 ml of $CaCl_2$ buffer, frozen on dry ice and ethanol, and stored at -80°C.

Transformation of *P. multocida*. Cells were electroporated using the following modifications of Dower *et al.* (48). A 1% inoculum of an overnight culture was used to inoculate BHIB. Cultures were grown to late-log phase (approximately 240 Klett units), incubated on ice for 15 min and centrifuged at 5000 $\times$ *g* for 15 min at 4°C. Cell pellets were rinsed twice in 1 mM HEPES [pH 7.0] and resuspended in 10% glycerol to 1/12 their original culture volume. The cell suspensions were centrifuged at 5,000 $\times$ *g* for 10 min at 4°C and resuspended in the glycerol solution remaining after the supernatant was decanted. The cell concentration in the final suspension was approximately 10^{10} colony forming units (CFU)/ml. Cells were aliquoted, frozen by immersion in dry ice and ethanol, and stored at -80°C. Prior to electroporation, 50 μl of thawed cell suspension was incubated with plasmid DNA for 5 min on ice. Cells were electroporated at 12.5 kV cm^{-1} with a model 100 BTX Transfector equipped with electrode 474, 0.5 mm gap (Biotechnologies and Experimental Research, Inc. San Diego, Calif.). A pulse time of 10 ms was used throughout these experiments unless indicated. Prepared cells were electroporated

in the absence of plasmid as a negative control. Electroporated cells were allowed to recover in 950 μl of BHI broth for 2 h. Transformants were selected on BHI agar containing the appropriate antibiotics. The efficiencies reported are the averages of three independent electroporations. Plasmids were extracted from suspected transformants to verify that they were the appropriate size.

RESULTS

Calcium chloride transformation. Transformation of *P. multocida* R473 with pBR322 and pVM109 was unsuccessful using the calcium chloride procedure. *E. coli* DH5α was used as a positive control for transformation (data not shown).

Optimization of electroporation conditions. The maximum transformation frequency of 4.5 x 10^7 transformants/μg of DNA was obtained at a field strength of 12.5 kV cm^{-1} corresponding to 625 kV (Fig. 3.4). Field strength is defined as the voltage applied multiplied by a factor of 0.02 (BTX Operating Manual).

To determine the effect of pulse time on electroporation efficiency, cells were electroporated at a field strength of 12.5 kV cm^{-1} while varying the pulse time. The highest transformation efficiency of 5.25 x 10^7 transformants/μg of DNA was achieved at 10 ms (Fig. 3.5).

There was a direct correlation between cell concentration and electroporation efficiency (Table 3.1). The highest efficiency of 1.25 x 10^7 transformants/μg DNA

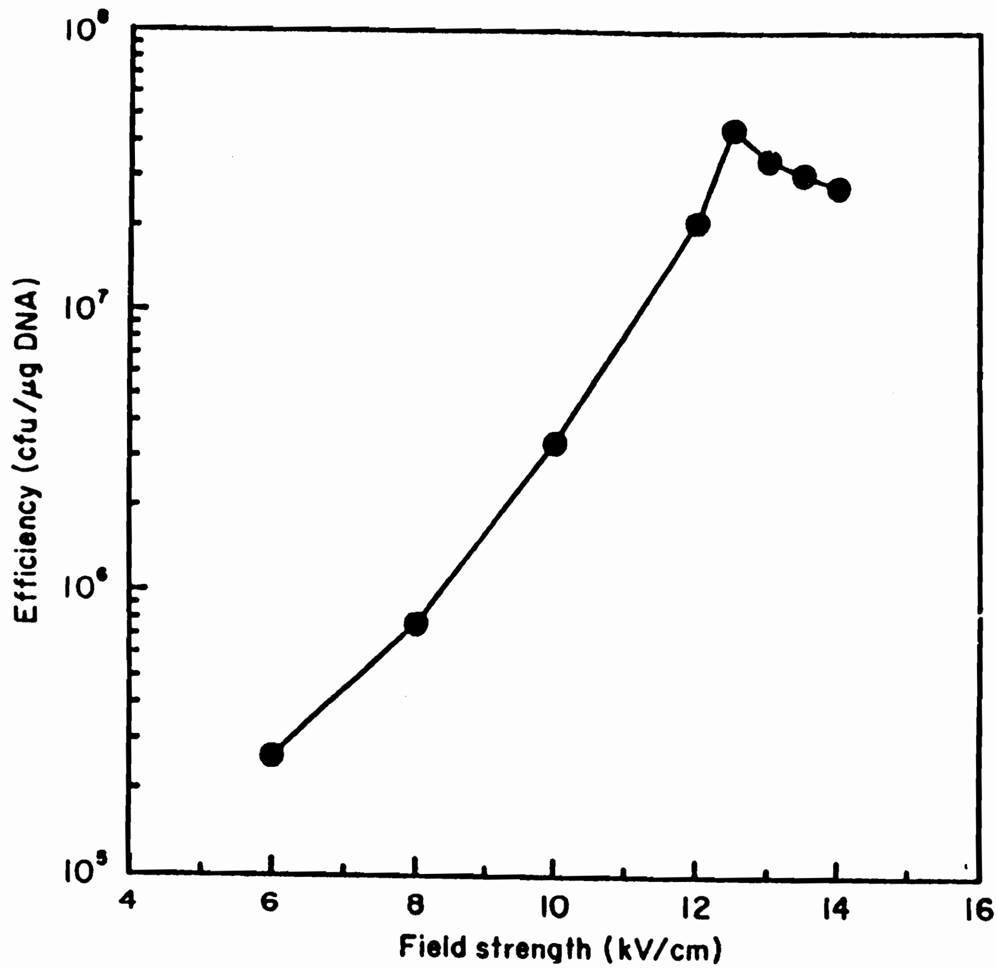


Fig. 3.4. The effect of field strength on the electroporation efficiency. Competent cells of *P. multocida* R473 were electroporated with 5 ng of the *Pasteurella* R-plasmid pVM109.

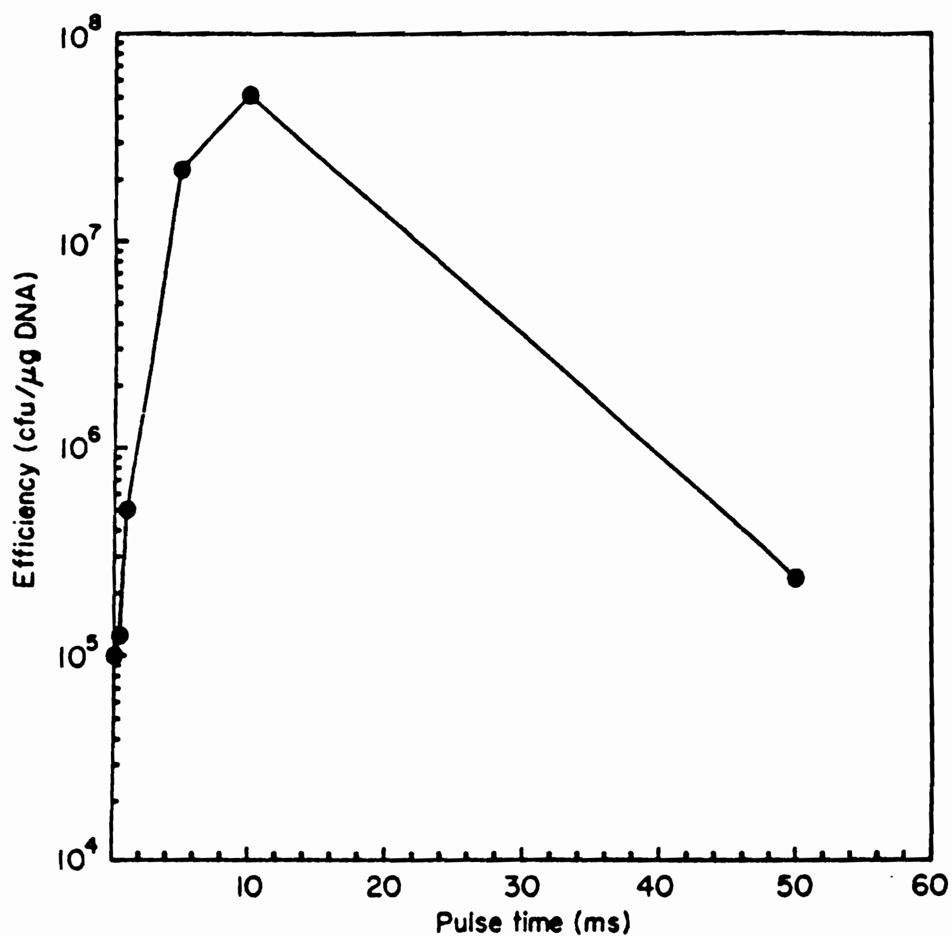


Fig. 3.5. Effect of pulse time on the electroporation efficiency. *P. multocida* R473 was electroporated at 625 kV with 5 ng of pVM109 for the pulse times indicated.

Table 3.1. Effect of cell concentration on transformation efficiency. Preparations of 7.6 x 10^{10} cells/ml were serially diluted 1:9 in sterile distilled water prior to incubation with DNA. Cells from *P. multocida* R473 were electroporated at 12.5 Kv cm^{-1} for 10 ms with 5 ng pVM109.

Cell Concentration (CFU/ml)	Transformation Efficiency (CFU/μg DNA)
7.6 x 10^{10}	1.25 x 10^{7}
7.2 x 10^{9}	1.56 x 10^{6}
5.7 x 10^{8}	1.01 x 10^{4}
9.8 x 10^{7}	< 200
2.5 x 10^{5}	< 200

Transformation efficiency was calculated using the following formula:

$$\text{Efficiency} = \frac{\text{Total CFU per ml of transformation mixture}}{\text{amount of DNA added (ng)}} \times 10^{3\,a}$$

[a] factor necessary to convert from nanograms to μg of DNA

was achieved at a cell concentration of 7.6×10^{10} CFU/ml. Transformants were not detected when cell concentrations were below 9.8×10^{7} CFU/ml. Cells prepared from mid-log phase had and electroporation efficiency that was approximately 1/2 $\log_{10}$ less efficient than cells prepared from early-log, late-log or stationary phases (Table 3.2).

Six plasmidless strains of *P. multocida* (R473, 1062, P86-338, Bunia II, 3865, and 3867) were transformed with pVM109. Transformation efficiencies for these strains ranged from 1.1×10^{5} to 5.8×10^{6} transformants/μg DNA. The average transformation efficiencies of type B and E strains were one $\log_{10}$ higher than either strains belonging to serogroups A or D (Table 3.3).

Plasmid origin of replication played a significant role in efficiency of transformation (Table 3.4). The *Pasteurella* plasmid, pVM109, gave the highest transformation efficiency of 1.1×10^{6} CFU/μg DNA. Plasmids with limited host ranges (28), for example pBR322 and pUC-19, were unable to transform *P. multocida* R473 even when DNA concentrations were increased to 1 μg. Broad host range vectors such as pSP329 and pJFF224-NX were also unable to transform *P. multocida* R473. Hybrid plasmids (pLRBR-21 and 67) containing pBR322 ligated into a naturally occurring *Pasteurella* plasmid (pLAR-1) had transformation efficiencies of 3.5×10^{4} and 8.0×10^{3} transformants/μg DNA.

Creation of a potential shuttle vector, pVMRL-100. Since the vectors tested were unable to transform *P. multocida*, attempts were made to create a shuttle

Table 3.2. Effect of growth phase on electroporation efficiency. Competent cells were prepared from 200 ml aliquots of *P. multocida* R473 were harvested at 80, 160, 240, and 350 Klett units and electroporated at 12.5 Kv cm^{-1} for 10 ms with 5 ng pVM109. Cell concentrations were approximately 1 x 10^{10} CFU/ml.

Klett Units	Growth Phase	Transformation Efficiency (CFU/μg DNA)
80	early-log	3.15 x 10^7
160	mid-log	5.50 x 10^6
240	late-log	3.07 x 10^7
350	stationary	1.37 x 10^7

Table 3.3. Electroporation efficiency of various strains of *P. multocida*. Competent cells were prepared from six strains of *P. multocida* and electroporated at 12.5 kV cm^{-1} for 10 ms with 5 ng of pVM109.

Strain	Serotype	Source	Transformation Efficiency (CFU/μg)
1062	A:3	Bovine	4.9×10^5
P86-338	A:3	Avian	3.2×10^5
R473	B:2	Bovine	4.9×10^6
Bunia II	E:2	Bovine	5.8×10^6
3865	D	Swine	3.4×10^5
3867	D	Swine	1.1×10^5

Table 3.4. Electroporation of various plasmids into *P. multocida* R473. Competent cells were electroporated with the concentrations of plasmid DNA that are designated.

Plasmid	Origin of Replication	DNA Concentration (ng/50 μl cells)	Transformation Efficiency (CFU/μg DNA)
pVM109	Unknown	5	1.1×10^6
pBR322	ColE1	5	< 200
		100	< 10
		1000	0
pUC19	ColE1	5	< 200
		100	< 10
		1000	0
pSP329	OriV/OriT	5	< 200
		300	< 3
		1000	0
pJFF224-NX	RSF1010	5	< 200
		100	< 10
		1000	0
pLRBR-21	ColE1, Unknown	5	0
		100	3.5×10^4
		500	1.2×10^3
pLRBR-67	ColE1, Unknown	5	0
		100	8.0×10^3
		250	4.5×10^2

vector using an endogenous *Pasteurella* plasmid, pLAR-1 and the commercially available plasmid pBR325. One criteria for a shuttle vector is the presence of two or more genetic markers (8) thereby allowing for selection of recombinant plasmids through insertional gene inactivation of one of the markers. The constructs pLRBR-21 and pLRBR-67 are ampicillin-resistant and tetracycline-sensitive, thereby, leaving only one gene for use as a selection marker to distinguish transformed cells. To introduce an additional marker, pLAR-1 was cloned into the *Bam*H I site of pBR325. This plasmid, named pVMRL-100, had two antibiotic resistance markers (Amp^r, Cam^r) in addition to two origins of replication: i) the ColE1 origin of replication located on pBR325, and ii) the *Pasteurella* origin of replication located on pLAR-1. Attempts to electroporate pVMRL-100 into strain R473 were unsuccessful.

Heterologous gene expression in pLRBR-21: An additional feature of a useful shuttle vector is the presence of unique restriction sites (8). Based on the restriction map of pLAR-1 and the DNA sequence of pBR322 there were a few unique sites remaining in these plasmids. To determine if exogenous DNA could be cloned and expressed in pLRBR-21, attempts were made to ligate the gene encoding the *B. abortus* Cu/Zn SOD into the *Cla* I site of pLRBR-21. Restriction enzyme analysis of plasmids recovered from transformed *E. coli* DH5α revealed restriction patterns unlike pBAII-3, pLRBR-21, or pLRBR-21 containing the gene encoding the *B. abortus* Cu/Zn SOD; instead there were deletions of the original

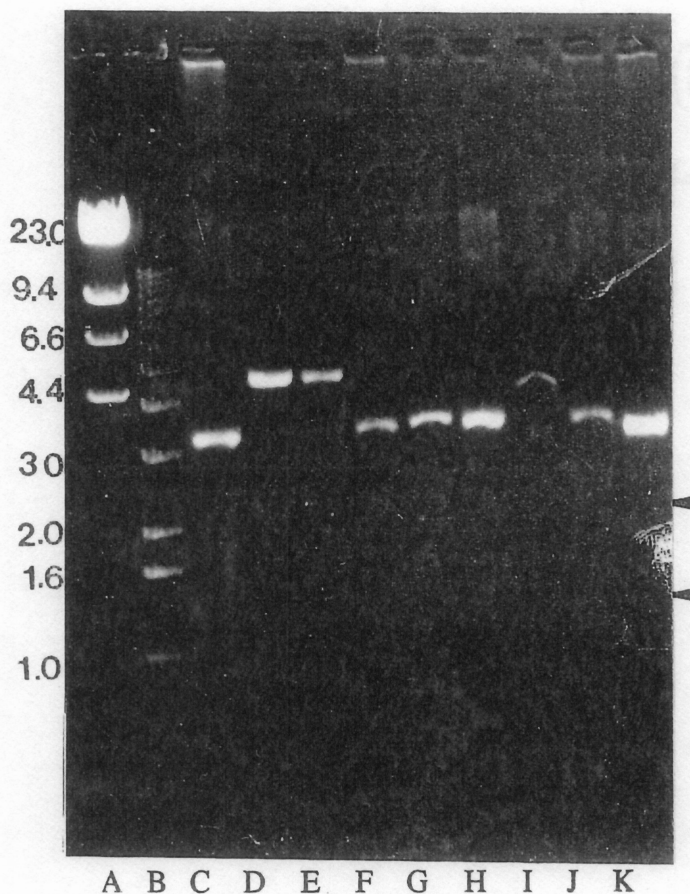


Fig. 3.6. Restriction enzyme fragments of plasmids digested with *Hin*d III isolated from transformed cells. The gene encoding *B. abortus* Cu/Zn SOD was ligated into the *Cla* I site of pLRBR-21. Lanes A, B and S, molecular weight standards. *Hin*d III digests of lanes C - N, clones 3, 8, 9, 12, 14-16, 24 and 25. The location of the restriction fragments of pLRBR-21/ *Hin*d III digests are designated with arrows for comparison with the clones. Molecular weight markers are expressed in kb.

vector, pLRBR-21 (Fig. 3.6).

It is possible that the deletions observed were due to an interaction between the insert (Cu/Zn SOD gene) and proteins encoded by *Pasteurella* plasmid (pLAR-1) present on the shuttle vector. To address this issue, the 1.8-kb fragment encoding a 42-kDa protein of *P. multocida* strain P1059 and 0.5 kb of pUC19 were cloned into the *Nru* I site of pLRBR-21. Plasmid analysis of transformants from this ligation mixture also revealed deletions in the hybrid plasmid. Thus it appears that the deletions are not a function of the source of DNA but a property of the pLRBR-21 itself.

Localization of the origin of replication of pLAR-1 using pLRBR-21 and pLRBR-67. Attempts were made to identify the origin of replication and regions of pLRBR-21 and pLRBR-67 which were pertinent to plasmid stability in *P. multocida*. These plasmids were digested with the restriction enzyme *Sal* I, religated and used to transform *E. coli* DH5α. Digestion of pLRBR-21 and pLRBR-67 with *Sal* I deleted reciprocal portions of the *Pasteurella* plasmid pLAR-1 as shown in Figs. 3.7A and 3.8. The plasmids which encompassed approximately 1 kb of pLAR-1, (bases 3000-4000), were stable in *E. coli*. This plasmid was designated pLRBR-21S. An additional deletion clone was made using a *Hin*d III digestion of pLRBR-21 to create pLRBR-21H as demonstrated in Figs. 3.7B and 3.8. This clone represents bases 1-900 of the original plasmid pLAR-1. Attempts to subclone the remaining 2-kb region were unsuccessful. Preliminary attempts to electroporate these plasmids

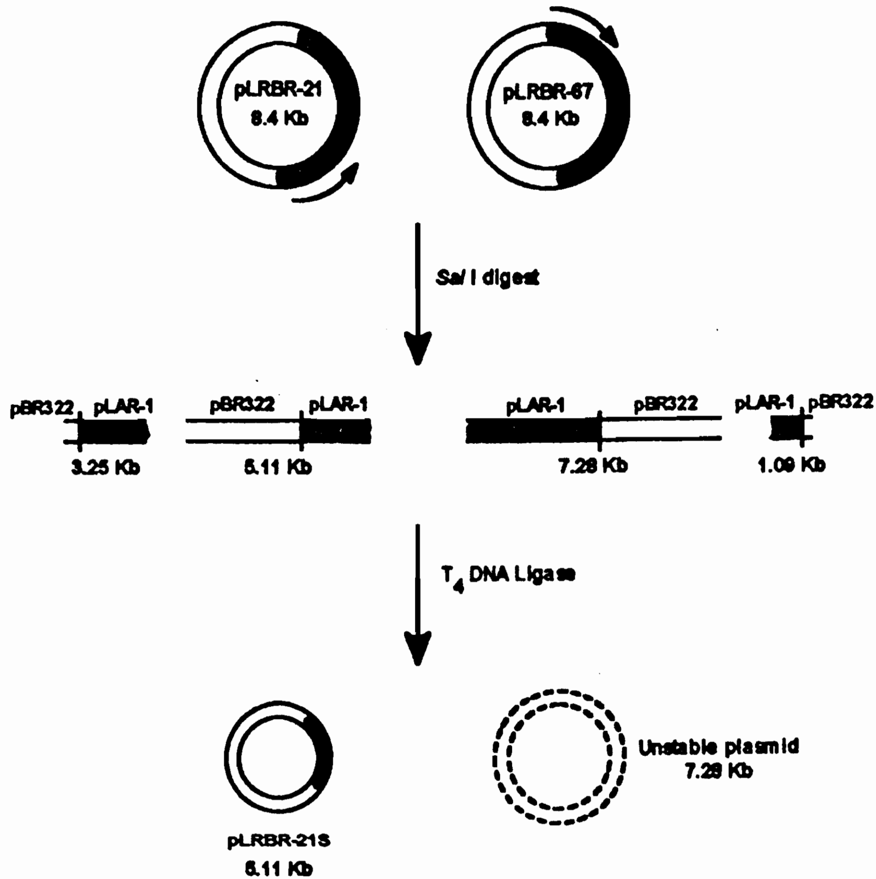


Fig. 3.7A. *Sal* I digestion of pLRBR-21 and pLRBR-67. A schematic diagram of the method to isolate the origin of replication and regions responsible for stability of the plasmids pLRBR-21 and pLRBR-67 when they are introduced into *P. multocida* R473. See figs. 2 and 3 for a detailed restriction map of these plasmids.

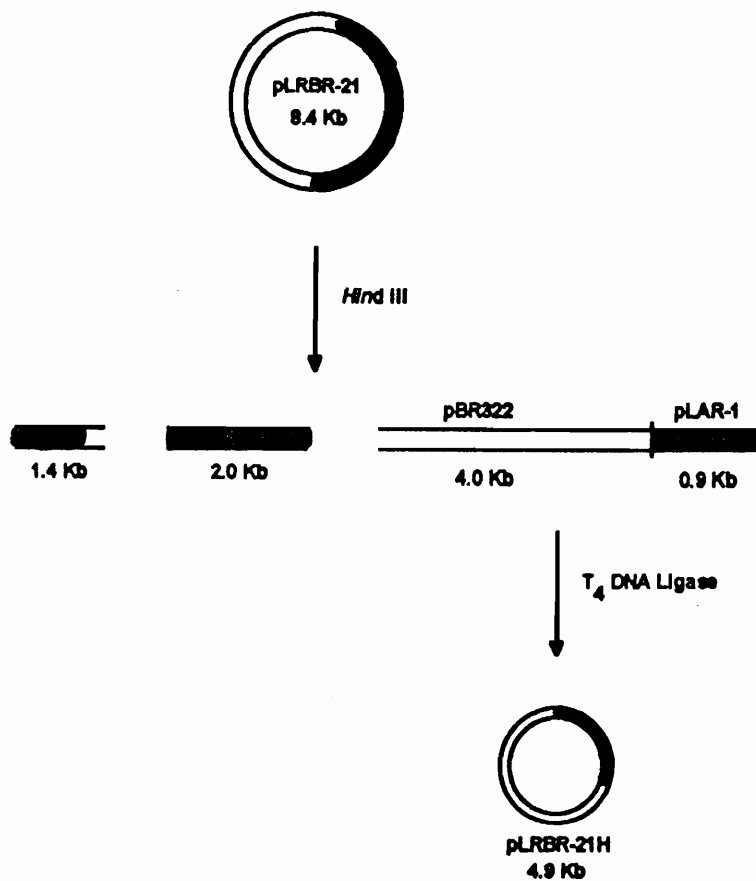


Fig. 3.7B. Hind III digests of pLRBR-21. A schematic diagram of the method to isolate the origin of replication and regions responsible for stability of the plasmid pLRBR-21 when introduced into *P. multocida* R473.

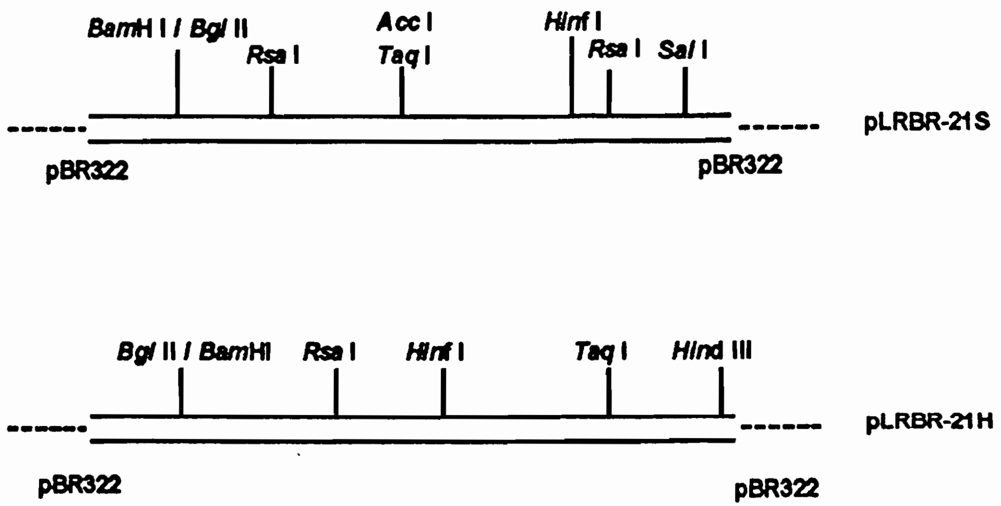


Fig. 3.8. Restriction maps of the plasmids pLRBR-21S and pLRBR-21H.

P. multocida R473 were unsucessful.

DISCUSSION

This is the first report of the introduction of DNA into *P. multocida* by means other than conjugation (70). The optimum conditions for electroporation of *P. multocida* were determined. Although these conditions typically yield 10^7 transformants/μg of plasmid DNA, there was a large standard error (sometimes as large as one log) between individual experiments and replicates within each experiment.

Unlike *B. abortus* (87) or *Actinobacillus pleuropneumoniae* (88), growth phase had a less significant effect on the transformation efficiency compared to either field strength or pulse time. A similar phenomenon has been reported for *Campylobacter jejuni* (104).

The difference in transformation efficiencies among serotypes of *P. multocida* could be attributed to variation in the strain's ability to be transformed or the percent survival of the bacteria after electroporation. Since the latter was not calculated, no conclusions can be drawn as to the number of survivors influencing the calculated transformation efficiency. Successful transformation of serotypes A, B, D and E should allow for the application of this technique to study diseases such as fowl cholera, atrophic rhinitis, snuffles and hemorraghic septicemia.

The lack of successful transformation of *P. multocida* by pBR322 or pUC19

was not due to an inability to express ß-lactamase or plasmid instability since the pLRBR constructs containing pBR322 were able to confer ampicillin resistance to strain R473. It is likely that the ColE1 origin of replication found in both pBR322 and pUC19 is not recognized by the replication enzymes in strain R473. In contrast, the stability of the pLRBR constructs in *Pasteurella* are presumable due to recognition of the origin of replication in pLAR-1.

The RSF1010 and OriV/OriT origins present on the plasmids pJFF224-NX and pSP329 respectively may not be recognized by *P. multocida*. This result was surprising considering that pJFF224-NX is able to transform other species closely related to *P. multocida* including *A. pleuropnemoniae* and *H. influenzae*, and *P. haemolytica* (53). The electroporation efficiency of these bacteria with pJFF224-NX was approximately two $logs_{10}$ lower than the efficiency presented here (53).

The actual function of the *Pasteurella* plasmid, pLAR-1, remains unknown (101). Southern blot analysis revealed no correlation between the presence of pLAR-1 and siderophore production, antibiotic resistance, biochemical/ metabolic profiles, host specificity, capsular and/or somatic antigens. Northern analysis of total RNA from *Pasteurella* revealed two transcripts (1.5- and 2-kb) that hybridize with pLAR-1 (data not shown). These transcripts could encode for proteins of 48- and 72-kDa, respectively. Shuttle vectors have been developed using plasmids with no known function for gene expression in other systems (50,118). The shuttle vector pCS41 (a derivative of the cryptic plasmid SMP-2) is stable in both *E. coli* and

Serratia marcescens (50). pRO1614 (developed from a cryptic plasmid isolated from *Pseudomonas aeruginosa*) is stable in *E. coli* and *Klebsiella pneumoniae* (118).

Initially, pVMRL-100, was tested as a candidate shuttle vector. This construct proved to be inadequate due to its inability to transform *P. multocida* R473. The lack of successful transformation could result from a trans-acting factor interacting between the chloramphenicol acetyl transferase (*cam*) gene and pLAR-1. This factor may be activated when the plasmid is electroporated into *P. multocida* R473. It is also possible that there is a restriction site within the *cam* gene that is recognized by a endogenous endonuclease found in *P. multocida* strain R473.

The plasmids pLRBR-21 and pLRBR-67 or their derivatives may serve as potential shuttle vectors based on the following analysis: i) they are stable in *P. multocida*; ii) Southern blot analysis of strain R473 containing these constructs revealed that neither pLRBR-21 nor pLRBR-67 had integrated into the chromosome of the host (data not shown); iii) these plasmids are capable of replicating in both *P. multocida* and *E. coli*; and iv) these plasmids are easily extracted from either *E. coli* or *P. multocida*. Due to the relaxed mode of replication present on pBR322, the plasmid can be amplified in the presence of low levels of chloramphenicol, thereby increasing its yield (38).

Even though pLBR-21 was unable to function as a shuttle vector, the plasmids pLRBR-21S and/or pLRBR-21H may serve as suitable shuttle vectors. If there is a region responsible for the plasmid instability observed when pLRBR-21

was used as a shuttle vector, then this region(s) may have been deleted during the creation of pLRBR-21S or pLRBR-21H. The other positive attributes of the vector pLRBR-21 discussed above still remain in pLRBR-21S and pLRBR-21H.

The deletions observed in this study may be independent of pLAR-1 and due to pBR322 (1,20,55). Brunel *et al.* (20) reported intramolecular rearrangement within the gene encoding tetracycline resistance when attempts were made to clone into pBR322. Garaev *et al.* (55) also reported a relatively high frequency of *recA* independent deletions occurred when eukaryotic DNA was cloned into the *Bam*H I site of pBR322. The nicking activity of *Bam*H I was thought to activate a region between basepairs 1666 and 1670 that was responsible for the recombination of pBR322.

SUMMARY AND DISCUSSION

Chapters 1 and 2: The identification, cloning and expression of antigens recognized by antisera against *Pasteurella multocida* P1059.

Antisera directed against *P. multocida* P1059 reacted with approximately 23 proteins ranging in mass between 14- and 68-kDa. Antigens of a similar molecular mass have been identified from cell lysates of another highly virulent strain of *P. multocida*, strain X-73 (78). The results of this study revealed that proteins profiles produced by strains X-73 and P1059 were almost identical. Since the antisera used in both of these studies were directed against whole cells and not subcellular fractions (eg. OMPs), it is not possible to determine whether the proteins identified in this study were the same as those reported elsewhere (32,33,78,165).

Antisera directed against strains 3865 and 3866 reacted with more antigens present in strain P1059 than with homologous antigen preparations. The explanation for this is unclear. It is possible that there is significant LPS contamination that is effecting the ability of the proteins in prepartions of strains 3865 or 3866 to react with homologous antiserum. In addition, the antigens of strains 3865 and 3866 may have been insoluble under the conditions used for immunoblot anlaysis and therefore, were not detected by the antiserum. It is also possible that many of the proteins recognized by 3865 and 3866 specific antisera may represent *in vivo* antigens since they were not present in strains 3865 and/or 3866

when they were grown *in vitro*. Though not protective against heterologous challenge, strains 3865 and 3866 were immunogenic as demonstrated by reaction with antiserum directed against strain P1059.

To clone the antigens expressed by strain P1059, a genomic library was created and screened with antisera directed against live strain P1059. Twelve clones were identified as positive by colony blot analysis. Plasmids from these clones were used to transform *E. coli* CSR603. Five clones expressed *Pasteurella* proteins of 37-, 42-, 48-, 50-, and 55-kDa. Reports in the *Pasteurella* literature have identified outer membrane proteins from *P. multocida* strain P1059 that are within this size range (35,165). Choi *et al.* described a 38 kDa protein which is present across serotypes of group A strains, a member of serogroup B and 18 field isolates of serotype A:3 (33). Chevalier et al (32) report the purification and N-terminal sequence of a porin protein (protein H) which was composed of three subunits, each with a molecular mass of 37 kDa. Comparison of DNA sequences, amino terminal sequences, and/or immunoblot analysis would determine if the 42-kDa protein encoded by pOP43-2G were the same as those reported in the literature.

Chapter 3: Development of a cloning system for gene expression in *Pasteurella multocida*.

The optimal conditions for transformation of *P. multocida* by electroporation have been established. The highest efficiency of transformation (1.25 x 10^7 CFU/μg

DNA) was obtained when 7.6 x 10^{10} cells of *P. multocida* strain R473 were electroporated at 12.5 kV cm^{-1} (10 ms, 5 ng of pVM109). Six strains of *P. multocida*, representing serogroups A, B, D and E, were transformed successfully. This is the first report of the introduction of DNA into *P. multocida* by means other than conjugation (70).

Vectors such as pBR322, pUC19, pJFF224-NX and pSP329 were unable to transformed *P. multocida* R473. This was surprising since pJFF224-NX was able to transform *Actinobacillus pleuropnemoniae*, *Haemophilus influenzae*, and *P. haemolytica* (53).

A potential shuttle vector for gene expression in *P. multocida* was developed using the criteria for establishing a plasmid vector according to Balbas *et al.* (8). These criteria include:

i. A plasmid vector should be small, with little or no extraneous genetic information. Because the efficiency of transformation of many host cells decreases as the size of the plasmid approaches 15 kb, the vector should contribute as little as possible to the overall size of the recombinant molecule.

ii. Vectors should be well characterized with respect to gene number and location, restriction enzyme cleavage sites, and nucleotide sequence.

iii. The vector should be easily propagated in the desired host so that large quantities of vector and recombinant DNA molecules can be obtained.

iv. The vector should have a selectable marker (gene) to allow cells harboring the vector to be distinguished from nontransformed cells.

v. An ideal vector should have an additional genetic marker that can be activated or inactivated by the insertion of foreign DNA fragments. The marker gene will allow cells harboring recombinant molecules to be distinguished from nonrecombinant molecules on the basis of altered phenotype.

vi. Finally, the vector should possess the maximum number of unique restriction enzyme cleavage sites located in one or the other genetic marker. This provides maximum flexibility for cloning different kinds of restriction fragments [4].

The proposed shuttle vectors developed in this study (pLRBR-21, pLRBR-21S, and pLRBR-21H) fulfill many of the criteria listed above. These vectors are relatively small, ranging between 5.0 and 8.3 kb. A significant portion of these plasmids has been sequenced, restriction sites have been enumerated and regulatory

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segments identified (13). The regions of the *Pasteurella* plasmid pLAR-1, used in the creation of these shuttle vectors have yet to be sequenced.

The vectors pLRBR-21 and pLRBR-67 were easily isolated from both *E. coli* and *P. multocida*. The presence of the ColE1 origin of replication allows for amplification through the addition of low levels of chloramphenicol to the culture prior to isolation of the plasmids (38). Plasmid amplification by chloramphenicol can increase plasmid yields by as much as 10% (38,99). In addition, the relaxed mode of replication present on pBR322 allows for approximately 20 copies/cell even in the absence of chloramphenicol (8,1311).

Investigation into the cloning, expression and gene regulation of *P. multocida* would increase our understanding of this economically important bacterial pathogen. Subsequently, we may be able to develop more effective vaccines to eradicate diseases such as fowl cholera, snuffles and atrophic rhinitis.

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Education: Ph.D. candidate. Department of Veterinary Medical Sciences, Virginia Polytechnic Institute and State University, Blacksburg, Virginia. Advisor: Dr. N. Sriranganathan. Dissertation: The Development of a Cloning System for Gene Expression in *Pasteurella multocida*.

M.Sc. 1989. Department of Veterinary Medical Sciences. Virginia Polytechnic Institute and State University, Blacksburg, Virginia. Advisor: Dr. N. Sriranganathan Thesis: Characterization of a 4.0 Kilobase Plasmid from *Pasteurella multocida*.

B.Sc. 1985. Hobart/William Smith Colleges, Geneva, N.Y.

Teaching Experience:
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Virginia Academy of Science
American Society for Microbiology

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McGonagle, L., N. Sriranganathan, S.M. Boyle, and G.R. Carter. Characterization of a plasmid, pLAR-1, from *Pasteurella multocida*. 70 Annual Meeting of the Conference of Research Workers in Animal Disease. Chicago, Ill. Nov. 1989.

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Lynn McGonagle Jablonski