Molecular cloning, nucleotide sequencing, and construction of a *Pasteurella haemolytica* biotype A serotype 2 *aroA* mutant

by

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ABSTRACT

We have cloned and sequenced the *aroA* gene of *Pasteurella haemolytica* serotype (ST) 2, the principal etiologic agent of pneumonic pasteurellosis of sheep. The *aroA* gene is 1,296 nucleotides long, has a %G+C content of 42%, and encodes a polypeptide of 432 amino acids. The *P. haemolytica* ST 2 AroA protein has a deduced molecular weight of 47,070 daltons and an isoelectric point of 4.88. In addition, we have constructed an *aroA* mutant of *P. haemolytica* ST 2 and demonstrated its diminished ability to grow in Wessman defined media lacking the amino acid tryptophan. Previous studies utilizing avirulent *aroA* mutants of the *Pasteurella* spp. have shown promise as live attenuated vaccine candidates in cattle. We hope to use this mutant in future research as a vaccine candidate against pneumonic pasteurellosis in sheep. Furthermore, incorporation of this mutant as part of a multivalent vaccine containing *aroA* mutants of *P. haemolytica* A1 and *P. multocida* A3 as well as *Haemophilus somnus* may be desirable for increased control of pasteurellosis in cattle.

GENERAL INTRODUCTION

Hypothesis and Rational

Pasteurella haemolytica is a prominent pathogen of ruminant species such as cattle and sheep (25, 98). It is estimated that the loss to the North American cattle industry due to this pathogen is between $750 million to $4 billion annually (98, 71). In the United States, Bighorn Sheep are the latest to succumb to both the pneumonic and septicemic forms of pasteurellosis (98). In the United Kingdom, pasteurellosis of sheep is also a serious economic problem (39). Moreover, due to the questionable efficacy of current vaccination programs, this disease still poses a threat to the cattle and sheep industries (34, 39).

Pasteurella haemolytica serotype (ST) 2 is the predominant etiologic agent of pneumonic pasteurellosis of lambs and sheep (39). It is a commensal of the upper respiratory tract and tonsils of both sheep and cattle (10, 34, 98). During periods of stress, bacterial or viral infection, climate changes, and seasonal changes, outbreaks of pasteurellosis are often observed (10, 34, 39, 98). The spread of *Pasteurella* through a flock of sheep is not always uniform and researchers are not sure as to the primary mechanism of pathogenesis or infectious spread. In cattle, however, the speculation is that aerosolized particles from *P. haemolytica* ST 1 infected calves are inspired by neighboring calves (34). In animals experiencing stress, the pathogen proliferates on upper respiratory tract mucosal surfaces (34). Colonization of these mucosal surfaces by *P. haemolytica* results in no adverse clinical effects except transient fever, but is presumed to be a necessary precursor to the development of pneumonia.

The use of live attenuated pathogens as vaccines has been well studied. In the 1950s, Bacon demonstrated attenuation of *Salmonella typhi* by disruption of the aromatic amino acid biosynthetic pathway (6). During infection, the *S. typhi* mutant exhibited a diminished ability to grow yet remained immunogenic (6, 12, 21, 27, 44-46, 71, 92, 93). Further studies yielded information about the aromatic amino acid biosynthetic pathway of which the *aroA* gene product, 5-enolpyruvylshikimate 3-phosphate synthase is a critical enzyme. This pathway yields the amino acids tryptophan, tyrosine, and phenylalanine as well as folate, vitamin K, and 2,3-dihydroxybenzoate (38, 101). Many studies have utilized *aroA* mutants as avirulent attenuated live vaccine candidates. For example, pathogens such as *Salmonella typhimurium* (46), *Salmonella enteritidis* (27), *Shigella flexneri* (93), *Yersinia enterocolitica* (12), *Yersinia pestis* (70), *Neisseria gonorrhoeae* (21), *Pasteurella multocida* (44), and *Pasteurella haemolytica* ST 1 (45) were shown to be attenuated when the *aroA* gene was mutated. Moreover, further experiments have demonstrated that they were good live vaccine candidates. Based on these examples, the goal of this study was to clone, sequence and construct an *aroA* mutant of *P. haemolytica* ST 2 for use as an attenuated live vaccine candidate to protect against pneumonic pasteurellosis in sheep.

Thesis Organization

This thesis is organized in a standard format consisting of four chapters, a General Introduction with Literature Review, Materials and Methods, Results and Discussion and Conclusions. This thesis follows the convention of the American Society for Microbiology.

Literature Review

> In the early days of bacteriology the only criteria for classifying bacteria were their microscopic and cultural morphological features and the circumstances, loyalties or personalities associated with their first recognition.
>
> **- K. Zinneman, 1980**

General history

The history of *Pasteurella* spp. is vast. Much of the early classification of this group of bacteria was based on three criteria: the animal that was infected, biochemical reactions and serological reactions (33, 76). The latter two were often contradictory therefore, on many occasions the names or classifications of *Pasteurella* spp. were changed to fit new, emerging criteria and ideology (33, 76).

As early as the 1600s, before the advanced techniques of bacterial isolation and identification had been developed, Androvandus of Italy had reported an epizootic disease of fowls that proved to be deadly. This seems to be the earliest report of what was later called fowl cholera (76). In the 1880s, Toussaint was the first to isolate and describe *P. multocida* from blood, cultivate it in neutralized urine and prove that it was the causative agent of fowl cholera (76).

It was, however, Louis Pasteur who isolated and cultivated *P. multocida* in pure cultures using chicken broth in the 1880s (76). He studied the many aspects of this organism and was able to show that an attenuated form of the bacteria protected chickens from subsequent infections with the parental strain. From his work came three important biological principles: 1) recovery from infection would protect against subsequent challenge, 2) attenuation of the organism would affect virulence, and 3) immunity would be overwhelmed by a large challenge dose (17, 52).

Pasteurellosis of sheep was first described in 1923 by Spray where he reported a non-septicemic form of pneumonia in lambs at slaughter (33). Upon examination of diseased lamb lungs varying areas of consolidation or hepatization were observed. Furthermore Spray isolated *Pasteurella*-like organisms associated with the pneumonic lesions and demonstrated a narrow ring of partial hemolysis on culture. In 1931, Dungal reported an outbreak of pneumonia in housed sheep. He reproduced the disease by inoculating healthy sheep intratracheally with cultivated organisms collected from infected sheep (39). Although there is skepticism based on his description of the causal organism, there seems to be little doubt that Dungal was describing *P. haemolytica* (39). The first observation that clinical symptoms may be induced by stress was described in 1938 by Montgomerie et al. (39). They noticed that rapid environmental changes such as climate precipitated outbreaks of pneumonia in sheep. In 1955 the septicemic form of the disease was first described in the South of Scottland by Stamp et al. (39). There they reported a rapidly fatal disease of lambs and found the causitive agent to be *P. haemolytica*.

Taxonomy of the family *Pasteurellaceae*

The family *Pasteurellaceae* includes a wide variety of Gram negative, facultatively anaerobic, chemoorganotrophic, and fermentative bacteria (67). Table 1 lists some common features of the family *Pasteurellaceae* (67). Based on phenotypic and genetic analysis, approximately twenty different species of *Pasteurella* have been identified (25). The *Pasteurella* spp. are differentiated on the basis of beta hemolysis, growth on MacConkey's agar, indole production, urease activity, gas from carbohydrates, acid production from lactose or mannitol, and 2-dimensional protein electrophoresis (19, 69, 100). However, due to the

Table 1. Some common features of the family *Pasteurellaceae*

Small Gram-negative rods or coccobacilli	
Facultatively anaerobic or microaerophilic	
Growth temperature	30°C-40°C
Motility	-
Spores	-
Oxidase and/or alkaline phosphatase	+
Glucose catabolism (0-F test)	F*
Nitrate reduction	+
Simmons' citrate (growth)	-
Arginine dihydrolase	-
Adonitol	-
L-Sorbose	-

*Exceptions: *H.aegyptius, H. ducreyi*
Adapted from: Mutters, R., Mannheim, W., Bisgaard, M., 1989. Taxonomy of the group, In: C. Adlam and J.M. Rutter (Editors), *Pasteurella and Pasteurellosis*. Academic Press, London, pp. 3-34.

problems inherent with classifying genera based on phenotypic properties alone, i.e., incomplete expression of the genome, techniques such as DNA/DNA hybridization (66) and rRNA analysis (28) are used to help establish taxonomic relationships.

Pasteurella haemolytica was first described in 1921 by Jones and referred to as *Pasteurella boviseptica* group I by Lingieres in his zoological classification of the *Pasteurella* spp. (33). However, as identification of infectious organisms improved, it became clear that the classification of bovine and ovine isolates of the family *Pasteurellaceae* into one species, *P. boviseptica*, was not sufficient. Lingieres thought that the family should be broken up into host specific groups such as *P. avicida* (fowl), *P. boviseptica* (cattle), *P suiseptica* (swine), and *P. oviseptica* (ovine) (66, 67). Furthermore, in 1932, Newsome and Cross separated *P. haemolytica* from *P. multocida* giving rise to the two independent species of the *Pasteurellae* (52, 66, 67).

The taxonomy of the *Pasteurellaceae* has undergone several modifications as classification techniques have become more precise based on new technologies. Through the use of techniques such as capsular antigen typing and DNA/DNA hybridization many of the members of this vast family of organisms have been reclassified or eliminated. For example, a new classification scheme has excluded several traditional species from *P. sensu stricto* including *P. haemolytica*, and rRNA evidence has even brought into question their classification as *Pasteurella* (25, 28). Table 2 illustrates the taxa eliminated from the family *Pasteurellaceae* as of 1989 (67).

Table 2. Taxa eliminated from the family *Pasteurellaceae*

Previous designation	New description or location	Criteria used
Haemophilus equigenitalis	*Taylorella equigenitalis*	Phenotype
	rRNA superfamily III	Ribosomal cistrons
H. piscium	Genus *Aeromonas*	Phenotype
H. vaginalis	*Gardnerella vaginalis*	Phenotype
Morazella (Pasteurella) anatipestifer	*Flavobacterium/Cytophaga* group	DNA binding
Pasteurella-like Bovine-lymphangitis group	*Enterobacteriaceae*	Ribosomal cistrons
P. piscicida	*Vibrionaceae*	Ribosomal cistrons
Pasteurella-like CDC group EF-4	rRNA superfamily III	Ribosomal cistrons

Adapted from: Mutters, R., Mannheim, W., Bisgaard, M., 1989. Taxonomy of the group, In: C. Adlam and J.M. Rutter (Editors), *Pasteurella and Pasteurellosis*. Academic Press, London, pp. 3-34

Currently, *Pasteurella haemolytica* is classified under the family *Pasteurella Pohl 1981* on the basis of genetic relatedness to the other members of this family as determined by DNA renaturation (67). This family consists of what's known as the HAP complex of organisms which are the genera ***H**aemophilus*, ***A**ctinobacillus*, and ***P**asteurella*. These organisms encompass a wide tropism of hosts but share genetic, phenotypic, and to some extent,

pathologic similarities. For example, hosts of the HAP complex include most mammals such as man, cattle, sheep, pigs, dogs, rats, mice, and various birds including chickens and pigeons (67). Moreover, the pathology can be pneumonic or septicemic with preference for mucosal surfaces (67). A thorough review of the HAP complex is beyond the scope of this paper. Therefore, the reader is referred to reference 67 for an in-depth discussion of the genera *Haemophilus* and *Actinobacillus*.

Pasteurella haemolytica

P. haemolytica has been divided into two groups based on fermentation of the sugars trehalose and arabinose. The former comprise the T biotypes whereas the latter comprise the A biotypes. Recently, however, a move has been made toward reclassifying the T biotypes into a separate species, *P. trehalosi* (25). *P. haemolytica* can be further typed according to capsular antigens. Sixteen serotypes (12 are biotype A and 4 are biotype T) have been distinguished by indirect hemagglutination developed by Biberstein (4, 34) or rapid plate agglutination developed by Frank (34, 36). Biotype A consists of serotypes 1, 2, 5, 6, 7, 8, 9, 11, 12, 13, 14, and 16. Biotype T includes serotypes 3, 4, 10, and 15 (34). Moreover, many strains fall into an 'untypable' category. Indirect hemagglutination of these untypable strains fails, presumably due to a lack of soluble antigen. They can be subgrouped by the rapid plate agglutination procedure, however (34). The disease produced by the A biotypes and the T biotypes are markedly different, and only the A biotypes are the causitive agents of the pneumonic form of the disease.

Pasteurellosis: the disease

P. haemolytica and *P. multocida* are the most prominent pathogens in domestic animals causing severe diseases and major economic losses to the cattle, swine, sheep and poultry industries. It has been estimated that the cost to the North American cattle industry ranges from $750 million to $4 billion annually (71, 98). Recently, *P. haemolytica* has been identified in the Rocky Mountain bighorn sheep population resulting in a loss of bighorn sheep due to the pneumonic and septicemic forms of pasteurellosis (98). Moreover, *Pasteurella* is not an isolated problem to North America. As recently as 1991, Bloch conducted a serosurvey on 1,474 small ruminants in four districts of Niger (11). Of eight diseases studied, pasteurellosis was found to be the main health problem (11). Therefore, efforts to control the progression and propagation of pasteurellosis have been of importance to researchers. A full understanding of the pathology of these organisms and their host preference is critical to a successful eradication program.

Pasteurella haemolytica is a member of the HAP complex of pathogens, which infect a wide variety of hosts. Many of the diseases produced by these organisms are associated with the upper respiratory tract (URT) or the lower genital tract (LGT) (73). The HAP complex of diseases are characterized by (i) the clinical symptoms manifested, (ii) the host infected, (iii) and the serogroup and/or serotype isolated from the site of infection (99). Diseases produced by the HAP complex have been placed into three categories based on clinical symptoms: 1) Pneumonia/septicemia which include: hemorrhagic septicemia of cattle, *P. multocida*; fowl cholera of turkeys, water fowl, etc., *P. multocida*; septicemic pasteurellosis of lambs and goats, *P. haemolytica*; pasteurellosis of rabbits, *P. multocida*; shipping fever of cattle, *P. haemolytica*, *P. multocida*, *H. somnus*; enzootic pneumonia of sheep, *P. haemolytica*. 2)

Upper respiratory infections which include: infectious coryza, conjunctivitis, sinusitis of chickens and other fowl, *H. (para)gallinarum*; atrophic rhinitus of swine, *P. multocida*; and conjunctivitis of humans, *Haemophilus spp.* 3) Local/traumatic infections which include: Infected bite wounds of humans and carnivores, *P. multocida, P. dagmatis, P. stomatis, P. canis*; serosal empyemas (e.g. pyothorax) of cats and dogs, *P. multocida* and others; periodontal disease of humans, *Actinobacillus actinomycetecomitans*; subcutaneous granulomas of cattle, *P. granulomatis*; and cervicovaginitis of cattle, *H. somnus* (73). This list illustrates the wide range of hosts the HAP complex infects and underscores a common attraction for mucosal surfaces.

Pasteurella spp. survive as commensals on the mucus membranes of the URT and LGT of healthy animals. *P. haemolytica* ST2 , and to a lesser extent ST1, can be recovered from healthy cattle, however, in calves that show clinical manifestations of pneumonic pasteurellosis, ST1 is almost exclusively recovered from the lung (34, 39). Sheep shed and succumb to a number of serotypes of *P. haemolytica*, although serotype 2 is most often recovered from pneumonic lung (10, 34, 39, 67). In cattle and sheep, clinical infection with *P. haemolytica* is commonly characterized as an acute, fulminating, fibrinonecrotic pleuropneumonic infection of the respiratory tract most frequently associated with "shipping fever" (34). The stress associated with the movement of cattle to feedlots allows *Pasteurellae* to proliferate in the URT by an unknown mechanism and spread to the lower respiratory tract (LRT) where colonization of the lung occurs. Much focus has been spent on elucidating the mechanism of passage of *P. haemolytica* from the URT to the LRT where clinical pneumonia results (41). The diagram in Figure 1 shows the steps involved in infection of a host by members of the HAP complex (81).

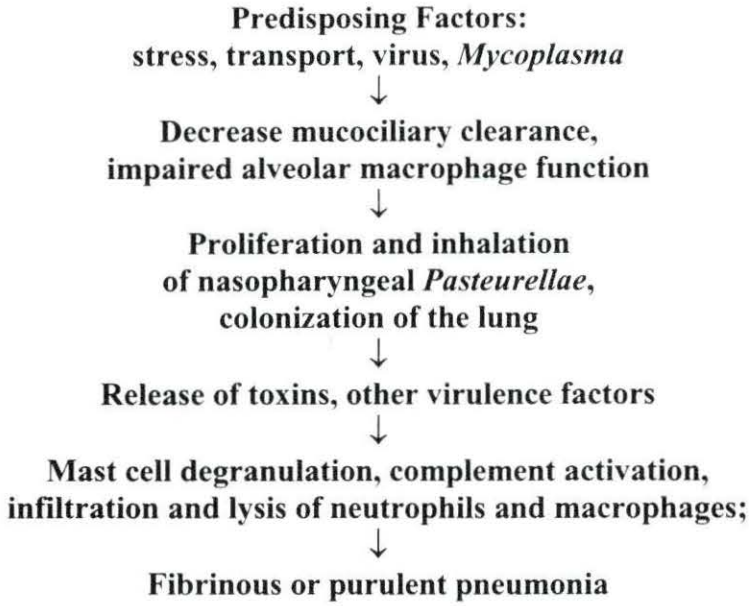


Figure 1. Flow chart of the steps of pasteurellosis infection.

It has been hypothesized that several factors may be involved in mucosal colonization of *P. haemolytica* including fimbriae, *P. haemolytica* A1 serotype-specific agglutination antigen (PHA1SSA), polysaccharide capsule (glycocalyx), lipopolysaccharide (LPS), neuraminidase and glycoprotease. Some of these factors directly associate with host cell membranes whereas others modify the microenvironment to promote proliferation of the pathogen. *P. haemolytica* colonizes the lung and evades the host immune response by secreting enzymes and toxins (discussed below). Moreover, capsule, capsular antigens, cytotoxins and lipopolysaccharides prevent the host immune system from mounting an effective attack against the invading pathogen. For example, the capsule is biologically inert, and therefore, has a low affinity for opsonization and fixation of immune modulators to the bacterial cell membrane, e.g. nonspecific antibody and C3b of the complement cascade respectively.

To more fully understand the disease progression of *P. haemolytica* and to develop strategies to eliminate this pathogen, knowledge of its virulence factors and metabolic pathways are required. For example, it is understood that the *Pasteurellae* share many of their virulence factors. There are exceptions, however, and perhaps these exceptions allow for the host specificity found with each species. An example of this idea is demonstrated between *P. haemolytica* and *P. multocida.* These two organisms present a relatively similar mucoid morphology when cultured on blood agar media containing 5% bovine erythrocytes (34). However, the clinical disease often described for each species is different; a fibrinous disease is usually seen with *P. haemolytica* where a purulent pneumonia is normally associated with *P. multocida* (34, 77). Both species use the capsular polysaccharide for protection against the host immune response, while each species produces different toxins, i.e. leukotoxin and dermonecrotic toxin, respectively, that aid in tissue damage (34, 77). Therefore, a better understanding of the immunogens of *P. haemolytica* and elucidation of mechanisms involved in mucosal colonization and destruction of host tissues may aid in the design of vaccines to protect cattle and sheep from clinical infection with this pathogen.

Immunogens of *Pasteurella haemolytica*

Prokaryotes have evolved mechanisms that allow them to survive in specific hosts. For example, facultative gram negative bacteria like *Salmonella*, *Brucella* and *Mycobacterium* have developed the ability to survive intracellularly in macrophages and neutrophils after release from the phagolysosomal vacuole (100). Other gram negative bacteria, for example *Escherichia coli*, can survive through mimicry of host proteins on their cell surface or antigenic shift of cell membrane proteins (100). Still others, such as members of the family

Pasteurellaceae, produce toxins that can kill host tissues or immune cells (4, 10, 34). Many pathogenic bacteria utilize a combination of these factors in order to aid their survival within the host. The virulence factors associated with *P. haemolytica* that may play a role in pathogenesis are the adhesins, capsule (glycocalyx), LPS, cytotoxin (exotoxin), and secreted enzymes such as neuraminidase or glycoprotease. The list is not comprehensive, but it includes some of the more important virulence factors that contribute to colonization and pathogenesis of the respiratory tract.

Adhesins

Adhesins are cell associated moieties or appendages that facilitate attachment to host cells via a cell surface receptor interaction (26). This interaction is important in establishing colonization and allowing microcolony formation. These attached colonies resist host clearance mechanisms such as the mucociliary escalator, and gain a valuable advantage over the host defenses. This advantage may be one of the key points in the pathogenesis of *P. haemolytica* in the ruminant respiratory tract (26). The most common adhesins of prokaryotes are fimbriae or pili which are small filamentous appendages on the surface of bacterial cells. Their role in infection is thought to be one of adherence to mucosal surfaces in the initial stages of infection (62). In addition to fimbriae however, Gram negative pathogens express adhesive factors that are often distinct and independent from fimbriae or pili. For example, *Bordatella pertussis* utilizes a filamentous hemagglutinin to colonize the upper respiratory tract (72). The main adhesin utilized by *Bordatella bronchiseptica* is the bovine erythrocyte hemagglutinin (72). In both of these examples the presence of fimbriae does not appear critical for adhesion to cell surfaces.

In the late 1980s, Morck et al. demonstrated capsule and fimbriae were present on *P. haemolytica* isolated from cattle showing clinical signs of pneumonic pasteurellosis (64). Typical *P. haemolytica* cells were evident with clearly visible radial glycocalyces suggesting the presence of fimbriae. Two types of fimbriae were identified, a large 12 nm rigid structure and a smaller 5 nm more flexible structure. Furthermore, Potter et al., have reported isolation and purification of fimbrial subunits, although several laboratories have failed to repeat these findings using the same techniques (74). The presence of fimbriae in *P. haemolytica* has not been investigated fully, nor have their existence been confirmed by other investigators (26).

The serotype-specific capsular antigen of *P. haemolytica* A1, PHA1SSA, was cloned and described as a fimbrial structural unit by Lo et al. (58). Immunoelectron microscopy studies of the cloned PHA1SSA confirmed its cell surface location, but failed to reveal a cellular morphology consistent with the presence of fimbrial structures (41). The role of PHA1SSA in the pathogenesis of *P. haemolytica* is still uncertain, however, Gonzalez et al. speculate that the ST1-specific agglutinating antigen could promote adhesion of the pathogen to host tissues through a lectin binding interaction with cell surface molecules (41).

Capsule

The capsule (glycocalyx) of *P. haemolytica* is composed of polyanionic, acidic, high molecular weight, heterogeneous polysaccharides, which are useful in serotyping (49). Encapsulated strains are thought to be more pathogenic, although the presence of a capsule does not always correlate with virulence (68). Non-encapsulated isogenic mutants of other species are generally avirulent and are quickly removed from the host (49). Bacterial capsule has been purified and found to be both biologically inert and poorly immunogenic (49).

Purified capsule behaves immunologically like a hapten, and has been shown to interfere with phagocytosis and complement-mediated bacteriolysis (10, 20, 25, 49). Serum resistance may be due to the capsule's ability to evade complement fixation by C3b by either the alternate or the classical pathways (49). *P. haemolytica* ST1 and *Haemophilus influenza* type B are good examples of evading complement lysis (10, 49). However, specific immune serum is bactericidal through either of the complement pathways. In contrast, *Actinobacillus pleuropneumonia* ST 5, a member of the HAP complex of organisms, is completely resistant to cell lysis in the presence of capsule-specific antibody and complement (49). Furthermore, an isogenic nonencapsulated mutant of *A. pleuropneumonia* ST 5 is susceptible to killing by complement alone thereby demonstrating that the capsule forms an important barrier between the pathogen and the host immune response (10, 49). Inzana hypothesized that encapsulation interferes with C3b fixation of the cellular membrane, thereby thwarting the early stages of the membrane attack complex (49). Although the cascade is activated, the capsule prevents the membrane attack complex from forming on the cellular membrane, thereby rendering the organism resistant to complement.

P. haemolytica is resistant to phagocytosis in normal serum, and therefore is not cleared from the bloodstream or tissues of the host (10, 25, 75). Phagocytosis is thought to occur via a receptor mediated pathway whereby C3b or antibody opsonize the bacterium and receptors for C3b or the Fc region of the antibody allow recognition of the opsonized complex by the macrophage (10, 51, 75). Therefore, *Pasteurellae* which are able to evade opsonization tend to evade phagocytosis by alveolar macrophages and neutrophils. In addition to evading host immune responses, Morck has shown that *P. haemolytica* uses it's capsule to attach to alveolar epithelium (15, 64). Some reports have shown that fimbriae may act to support this

adhesion and therefore resist clearance by alveolar macrophages, however the mechanism by which this occurs is not fully understood. Furthermore the existence of fimbriae is still under much debate thereby making elucidation of the mechanism of adhesion of *Pasteurellae* more difficult to study.

Lipopolysaccharide

Lipopolysaccharide produced by *Pasteurella* spp. is similar to LPS of other gram negative bacteria (49). It is composed of lipid A, a core oligosaccharide, and an antigenic polysaccharide chain referred to as O antigen (49). LPS has been studied extensively in *Pasteurellae* and other organisms. Its chemical composition has been determined and its role in pathogenicity is briefly summarized below.

P. haemolytica LPS is intimately associated with the capsule and capsular antigens. Hence, purification of LPS has been difficult. Nevertheless, LPS has been shown to be associated with immune protection of animals; the extent of the immune reaction depends on the animal species inoculated, LPS-type (either S or R types), route of inoculation and method of inoculation (96). Purified LPS has been determined to be antigenic and is found to be of the R-type. Pirosky isolated LPS-protein complexes and demonstrated that they were immunogenic and toxic (73). However, rabbit antiserum against the LPS antigen was shown to weakly protect mice after challenge with LPS (22). Conversely, in some avian strains of *Pasteurella* anti-LPS antibody seems to be of importance in immunity (25, 78). Therefore, the efficacy of anti-LPS antibody against pasteurellosis is still somewhat unresolved.

The effects of endotoxin occur through a two-fold mechanism (26, 49). During the first hour of exposure to LPS, a neutrophil independent event occurs whereby pulmonary

hypertension is observed without increased vascular permeability. This response is primarily due to increased plasma cyclooxygenase-dependent arachidonic acid metabolites, especially thromboxane A_2 and prostaglandin F_2. In addition, cAMP levels in plasma elevate to a peak in 1 hour and cGMP elevate more slowly to a maximum at 12 hour. Emau et al. have suggested that the effects of endotoxin at the cellular level are mediated by the cyclic nucleotides (29, 30). The second phase of LPS toxicity occurs within an hour and is neutrophil dependent. Neutrophils infiltrate the lung, and cause an increase in vascular permeability due to secretion of mediators, e.g. leukotrienes, oxygen radicals, and proteolytic enzymes. Slocombe et al. (85) and Breider et al. (13) have found that neutrophils play an important role in the development of the severe lesions of bovine pneumonic pasteurellosis, especially fibrinous exudation and thrombosis.

LPS delivered by bronchoscopy to sheep lung led to a variety of clinical and pathological effects (16). A marked decline in circulating leukocytes was followed by mild leukocytosis. An increase in neutrophils was noted, and LPS evoked local inflammation, edema, hyperemia and hemorrhage in the lung (16, 85). The response to LPS in that experiment was comparable to acute pneumonia elicited by live *P. haemolytica*.

There is evidence that LPS toxicity may be enhanced by complexing with lung surfactant (15, 85). Brogden et al. found that after incubation of LPS with surfactant, surfactant vesicles formed and stabilized the LPS. This complex was significantly more dense than surfactant alone (15). They found that toxicity in mice with the complex was greatly enhanced over LPS alone. Goto and Rylander noted the same results using *E. coli* LPS (4).

Cytotoxins

Many pathogenic bacteria make use of cytotoxins that target substrates found on the host cells to inactivate host cell defenses. The classic example is that of the RTX toxin α–hemolysin (HlyA) produced by the non-hemorrhagic strains of *E. coli* and the enterohemorrhagic toxin (EhxA) produced by hemorrhagic strains of *E. coli* (4, 7). The HlyA and EhxA toxins associate with the target cell and disrupt cellular membranes by forming pores and thereby lyse the cell (7). Similarly, leukotoxin, produced by *P. haemolytica* disrupts cell membrane structures ultimately lysing the cell.

Classically, two exotoxins have been associated with *P. haemolytica*, hemolysin, which gave rise to the *P. haemolytica* species name, and leukotoxin. However, recently, Murphy et al. constructed a leukotoxin mutant by allelic exchange and demonstrated typical β-hemolytic activity on sheep and rabbit blood agar was abolished. Similar results were confirmed in our lab and therefore suggest that hemolysin and leukotoxin are one in the same (65).

Leukotoxin (LKT) has received much attention due to its ability to destroy cells involved with adherence and immunity. In the late 1970s and early 1980s studies of *P. haemolytica* culture supernatants or washed cells demonstrated toxicity for bovine alveolar macrophages (8, 60). These studies yielded evidence that LKT was involved in the destruction of macrophages and neutrophils, and lent credence to the thesis that much of the pathological cellular damage of the host was due to destruction of host granulocytes. Hence, isolation, identification and elucidation of the role of LKT on the pathogenesis of *P. haemolytica* have been investigated extensively.

Leukotoxin is produced by all known serotypes of *P. haemolytica* (25) with the exception of four untypable strains (4). LKT, a RTX toxin, shares a high degree of homology to the

E.coli α-hemolysin at the nucleotide level (4). Researchers have postulated that LKT plays a role in virulence by causing cytolysis of alveolar macrophages and neutrophils. Furthermore, LKT induces platelet destruction and release of fibrinogen and vasoactive compounds as additive affects in the infected lung lesion (25). It has been suggested that LKT has a duel action dependent on concentration of the toxin in the microenvironment and the cells present (4, 86). That is, ruminant leukocytes exposed to low levels of LKT have reduced phagocytic and oxygen radical generation capabilities. Conversely, high levels of LKT lyse leukocytes and platelets (25, 86). In an attempt to explain the activation-inhibition paradox, Steven and Czuprynski determined that cell death was in part mediated by apoptosis (86). They noted marked cytoplasmic membrane blebbing (zeiosis) and chromatin condensation and margination, the hallmarks of apoptosis. In another study, however, LKT effects on bovine neutrophils included ruptured membranes, numerous vesicles within the cytoplasm and pyknotic nuclei, all hallmarks of necrosis (4). These studies, however, are not universally accepted, though they do demonstrate the detrimental effects of LKT on bovine leukocytes and may, in part, explain the contrast between commensal or pathogenic *P. haemolytica*.

Leukotoxin has shown specificity for ruminant leukocytes whereas nonruminant leukocytes and other cell types such as kidney cells, fibroblasts, and endothelium are resistant (25, 83). This specificity may define the different forms of pneumonia associated with *P. haemolytica* and *P. multocida*. In the former, a fibrinous pneumonia is most often described whereas the latter usually produces a purulent pneumonia (34). This striking difference may be attributed to the production of dermonecrotic toxin (DMT) by *P. multocida* that seems to have affinity for porcine osteoclasts (32). The DMT has little if any effect on ruminant leukocytes whereas LKT will lyse them (32). It has been postulated that

the release of toxic intermediates and inflammatory mediators from lysed and activated leukocytes contributes to the cellular damage and inflammation in the lung resulting in a fibrinous pneumonia (25, 34). Analysis of the lung shows focal arrays of cellular damage where toxic intermediates have killed host tissue (34).

Characterization of LKT at the molecular level has revealed that the toxin is a protein with a molecular mass of 105 kDa. Purified leukotoxin has an isoelectric point between 4.1 and 4.6 and retains activity at pH 4.5 through pH 9 (4). The gene coding for LKT, *lkt*, has been cloned and sequenced (23, 56, 57). More recently, studies have focused on the *lkt* operon and construction of *lkt* mutants to further investigate the role of LKT in disease (43). The *lkt* operon has the typical RTX family genetic arrangement. It is comprised of four genes designated *lktC-lktA-lktB-lktD*, where the *lktC* gene product, 19.9 kDa, is required for activation of the proleukotoxin to the mature leukotoxin by posttranslational acylation of the LKT peptide (7, 31). The *lktA* gene encodes the structural toxin and *lktB,* 79.6 kDa, and *lktD*, 54.7 kDa, genes are associated with exportation of the mature toxin to the extracellular environment (9).

Previous studies have correlated high serum antibody responses to LKT with resistance to experimental challenge of cattle (37). Furthermore, recent studies by Lainson et al. confirmed that LKT was immunogenic when a *lkt*A:*lacZ* fusion reacted with a strongly neutralizing monoclonal antibody localized to a 32 amino-acid region near the C-terminus of the leukotoxin A molecule (53). These studies provide the knowledge needed to construct LKT mutants targeting operon sequences other than the *lkt*A gene sequences. Based on these studies, one can speculate that mutants designed to retain immunogenicity while abolishing toxicity may be of great advantage.

Secreted enzymes

Prokaryotic organisms under harsh conditions have developed modalities of survival that involve the production of enzymes that can be secreted into the microenvironment to neutralize deleterious molecules present on the host cell surface (47, 87, 88, 90, 94). Some of these enzymes target cell surfaces and cleave acidic residues to make the microenvironment more habitable. Neuraminidase and glycoprotease (generally referred to as sialidases) cleave N-linked and O-linked sialoglycoconjugates, respectively, into free sialic acid residues (63). Pathogens such as *Vibrio cholera* (94), *Bacteroides fragilis* (47), *Salmonella typhimurium* LT2 (47), *P. haemolytica* (87, 90), and *P. multocida* (88) contain sialidases. These organisms also contain genes that allow them to ingest and metabolize the released sialic acid residues (47, 87, 88, 90, 94, 95). Other organisms such as *E. coli* don't contain sialidase genes, but they may contain systems for uptake and degradation of scavenged free sialic acid residues (95). In this fashion host cell surface defenses are used to propagate the invading pathogen by neutralizing their protective capacity. Gottschalk demonstrated this phenomenon by removing the sialic acid from salivary glycoproteins and noted their reduced protective affect against pathogens (42). In these cases the pathogen strips away the sialic acid and allows attachment and formation of microcolonies (89).

The first report of *Pasteurella* spp. producing neuraminidase was by Scharmann et al. in 1970 (80). In that report, 3 of 5 strains of *P. haemolytica* and 102 of 104 strains of *P. multocida* were shown to produce neuraminidase. In 1981 Frank and Tabatabai examined *P. haemolytica* and found that biotype A serotypes 1, 5, 6, 7, 9, and 12 and no biotype T strains produced neuraminidase (35). However, a later report by Straus et al. demonstrated that all serotypes except serotype 11 of *P. haemolytica* biotype A produced neuraminidase (87).

These neuraminidases were characterized with neutralizing antibody from *P. haemolytica* A1, by substrate specificity, and by molecular weight and found they were similar.

The role neuraminidase may play in the virulence of *P. haemolytica in vivo* is still unclear. *In vivo* production of anti-neuraminidase antibodies occurs during experimental infection with *P. haemolytica* suggesting that neuraminidase is induced during infection, and based on previous reports, one may hypothesize that neuraminidase may play a role in mucosal colonization and survival of the pathogen (82, 84, 89, 48). Presponse^{tm}, a vaccine developed in the late 1980s by Shewen and colleagues, utilized the soluble antigens; leukotoxin, a serotype-specific outer membrane protein, neuraminidase, and sialoglycoprotease harvested during logarithmic phase of *P. haemolytica* (82, 84) to protect cattle against *P. haemolytica* ST 1. They demonstrated in both field and laboratory trials that Presponse^{tm} was effective in preventing disease in cattle (54, 82, 84).

Otulakowski was the first to describe the glycoprotease of *P. haemolytica* in the early 1980s (4). He demonstrated the formation of a protease specific for sialoglycoproteins of human erythrocytes and cytotoxic for bovine pulmonary macrophages. In 1990 Abdullah et al. showed that glycoprotease was found in culture supernatants of *P. haemolytica* ST 1, 2, 5, 6, 7, 8, 9, and 12 by degradation of glycophorin A (1). However, no activity was found in ST 3, 4, 10, and 11. In 1991 the same group reported they had cloned the *P. haemolytica* ST1 glycoprotease gene and expressed it in *E.coli* (2). Furthermore, in 1992 they characterized the enzyme and reported it as a neutral metalloprotease that specifically cleaves O-linked sialoglycoconjugates, in effect confirming the work of Otulakowski (3). Lee et al. demonstrated that *in vivo* production of glycoprotease results in antiglycoprotease antibody production in calves suggesting the presence of the enzyme during pathogenesis of *P.*

haemolytica (54). The role of this enzyme seems to parallel that of neuraminidase, but relatively little work has been done to conclusively demonstrate this point (63)

The role of secreted enzymes has not been clearly shown in the pathogenesis of *P. haemolytica*. However, work toward producing mutant *P. haemolytica* ST1 devoid of neuraminidase or glycoprotease is underway and may provide the evidence needed to link these enzymes to virulence (91).

Vaccination

Vaccination has been an important strategy in controlling the incidence of pasteurellosis. Multiple types of vaccines have been used to vaccinate cattle and sheep against *P. haemolytica* including subunit vaccines, bacterins, supernatant fractions, and live attenuated vaccines strains (2, 5, 18, 22, 24, 25, 34, 39, 40, 45, 49, 50). Commercial multicomponent vaccines are currently available utilizing antigens from *P. haemolytica* biotypes T3, T4, T10, and T15 that are involved in the septicemic form of pasteurellosis (50). However, few effective vaccination protocols are currently available to protect sheep against pneumonic pasteurellosis, and those that are available show marginal efficacy (34). Several cattle vaccines are available which confer moderate protection, but these are ineffective in sheep due to serotype specificity and a lack of cross protection (34).

As of 1989, inactivated bacterial preparations, bacterial extracts and some live preparations have been used as vaccines in cattle and sheep without much success (34, 39). For example, Rodger et al. used a *parainfluenza 3* (PI3) virus attenuated live vaccine (originally licensed for use in cattle) to vaccinate sheep intranasally based on the premise that PI3 is a predisposing factor to pasteurellosis in cattle (79). Ewes that seroconverted to PI3

were either immune to the disease or showed reduced incidence of pasteurellosis (79). However, this study also demonstrated that this vaccine protected sheep from pneumonic pasteurellosis temporarily, for one season.

Vaccine failures have led to increased interest in the role of mucosal immunity in protection. Early experiments by Wells in the late 1970s using sodium salicylate extracts of *P. haemolytica* A1 to immunize sheep followed by inoculation with PI3 virus (to predispose the animals to disease) showed that animals that were challenged 7 days after vaccination with *P. haemolytica* A1 succumbed to infection (96). This led Wells to conclude that humoral immunity was not sufficient to provide protection. He speculated that cell-mediated immunity must play a key role in protection against pneumonic pasteurellosis (96). In a separate study, however, passive transfer of purified and nonpurified convalescent serum from either conventionally reared sheep that presented with pneumonia or experimentally-infected sheep immunized with a *P. haemolytica* A2 vaccine to specific pathogen free lambs, protected 94-100% of the animals (50). The authors concluded that humoral immunity was sufficient to prevent pasteurellosis. This apparent contradiction illustrates the complexity associated with assessing immunological resistance to pneumonic pasteurellosis.

Vaccination studies continued, however, despite the lack of understanding of mucosal immunity. For example, Chandrasekaran compared an oil adjuvant vaccine consisting of locally isolated *P. haemolytica* ST 7 and *P. multocida* serotypes A and D from infected sheep to the commercially available Carovax vaccine (Wellcome Laboratories) in imported cross-bred lambs (22). Although the oil adjuvant vaccine reduced lung lesions as compared to controls when *P. haemolytica* was used for challenge, lambs challenged with both *P. haemolytica* and *P. multocida* were not protected from pneumonic lesions. Carovax was also

found to be ineffective at reducing lesions in lambs infected with both *Pasteurellae* (22, 30). In another study, a sodium salicylate extract of *P. haemolytica* ST2 grown in iron replete media to induce the expression of iron regulated proteins was used to vaccinate specific pathogen free lambs (40). In this study some protection was conferred to SPF lambs after aerosol challenge with *P. haemolytica* A2. Another example is the vaccine Presponse^{tm} developed by Shewen and colleagues in the late 1980s. It is comprised of soluble antigens evolved from logarithmic growth phase *P. haemolytica* ST1 and both field and laboratory trials have shown promise in protection against pneumonic pasteurellosis in cattle (54, 82, 84).

These vaccines are all examples of either bacterins or supernatants used to elicit protective antibodies in the immunized animals. A number of live vaccines of *P. haemolytica* have been used and none required transformation of the bacterium. However, current technologies now allow genetic manipulation of *P. haemolytica* to be possible, and for the first time yield site directed mutants for the purpose of vaccine development. However, work to refine and evolve bacterins, extracts with new adjuvants, etc. is ongoing, and as yet, efficacy in the field has not met with expectations of the user. Therefore rationally attenuated live vaccines have become more desirable.

The use of rationally attenuated live vaccines in the control of pasteurellosis in cattle and sheep is a relatively new field (91). Construction of live attenuated vaccines has become feasible since the genes involved in restriction-modification of exogenous DNA have been characterized (14). Construction of attenuated mutant strains may prove to be better vaccine candidates then their predecessors. Previous studies by Collins et al. noted that live vaccines appear to protect better than bacterins because they elicit a stronger cell-mediated immune

response (24). In addition, humoral immunity is enhanced in live preparations due to the induction of genes during *in vivo* growth (92). Therefore, with the stronger immunity elicited by live vaccines, attenuated live *P. haemolytica* may prove to be of great importance in controlling pneumonic pasteurellosis (24, 92).

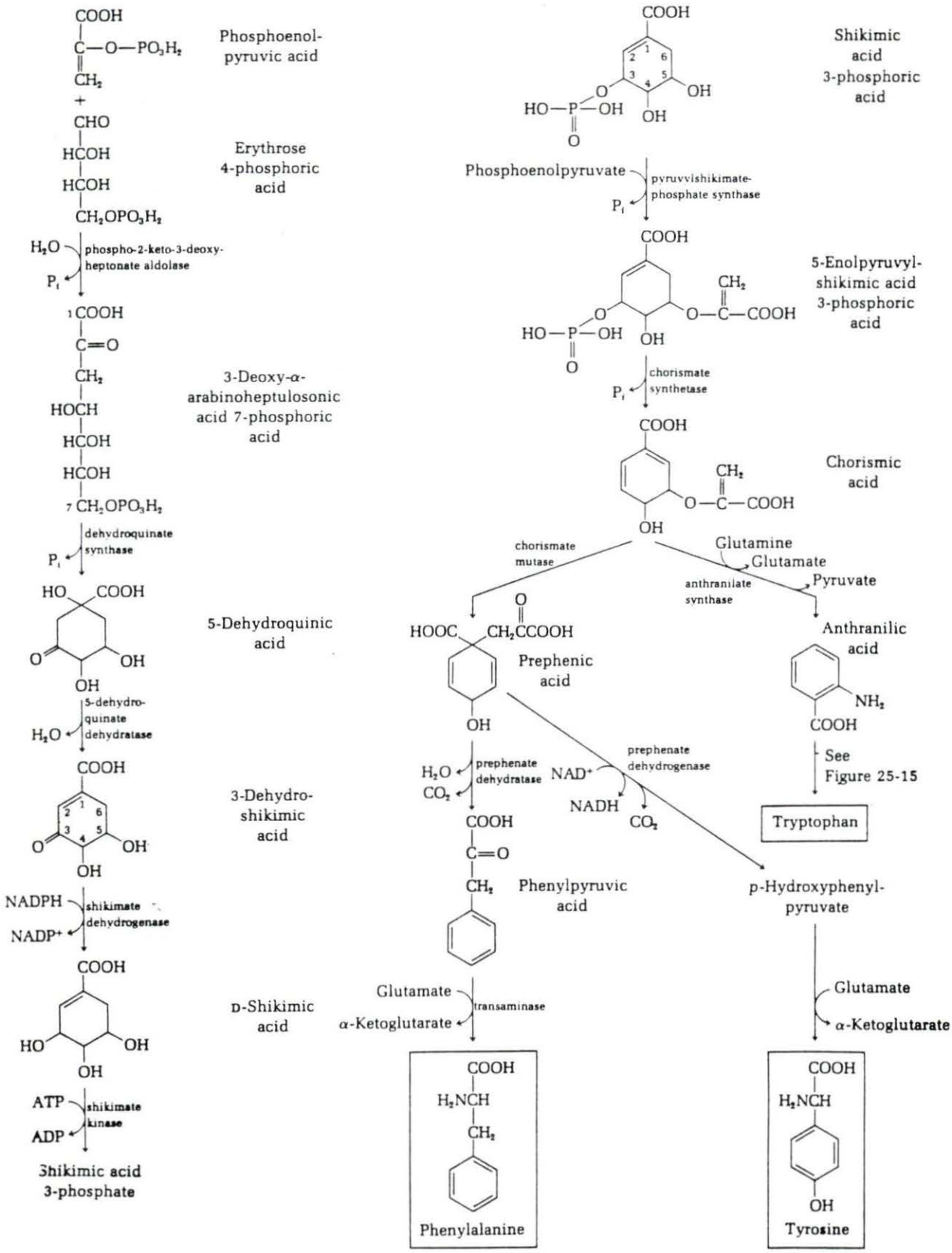
With these arguments in mind, our aim in this study was to develop a rationally attenuated strain of *P. haemolytica* ST 2 for use as a live vaccine candidate. Previous studies have demonstrated that the attenuation of virulent organisms through mutation of the *aroA* gene has produced effective auxotrophic mutant strains (6, 12, 21, 27, 44-46, 70, 93, 101).

Use of auxotrophic *aroA* mutants as attenuated live vaccines

The aromatic amino acids phenylalanine, tyrosine and tryptophan are essential for growth of all known life forms. Elucidation of the biosynthetic pathway(s) of each of the aromatic amino acids has been reported (38, 101). Moreover, the pathways consist of an elegantly arranged cascade of reactions containing molecules that converge into a branch point reaction whereby all of the aromatic amino acids are derived. Elimination of the precursor reactions, therefore, truncates the pathway and the organism cannot synthesize these amino acids.

Figure 2 shows the pathway(s) involved and the common intermediates required for the synthesis of the aromatic amino acids (101). Briefly, glucose is catabolized to phosphoenol pyruvate and erythrose 4-phosphate through the glycolysis pathway (38). These two molecules then proceed through a common "shikimate" pathway to yield chorismate (38). Chorismate is know as a "common branchpoint" molecule from which all three aromatic amino acids are synthesized as well as folate, vitamin K, ubiquinone, and 2,3-dihydroxybenzoate (38). The enzyme 5-enolpyruvylshikimate 3-phosphate synthase (*aroA*)

Figure 2. Biosynthetic pathway of the amino acids tyrosine, phenylalanine, and tryptophan. (Biochemistry third edition, Zubay, 1993) The enzyme 5-enolpyruvylshikimate 3-phosphate synthatse (*aroA*) catalizes the reaction that converts shikimate 3-phosphate to 5-enolpyruvylshikimate 3-phosphate. Mutation of the *aroA* gene blocks this pathway thereby starving the organism of these essential amino acids.


Phosphoenol-pyruvic acid
Erythrose 4-phosphoric acid
phospho-2-keto-3-deoxy-heptonate aldolase
3-Deoxy-α-arabinoheptulosonic acid 7-phosphoric acid
dehydroquinate synthase
5-Dehydroquinic acid
5-dehydro-quinate dehydratase
3-Dehydro-shikimic acid
NADPH
NADP+
shikimate dehydrogenase
D-Shikimic acid
ATP
ADP
shikimate kinase
Shikimic acid 3-phosphate
Shikimic acid 3-phosphoric acid
Phosphoenolpyruvate
pyruvylshikimate-phosphate synthase
5-Enolpyruvyl-shikimic acid 3-phosphoric acid
chorismate synthetase
Chorismic acid
chorismate mutase
Glutamine
Glutamate
anthranilate synthase
Pyruvate
Prephenic acid
Anthranilic acid
prephenate dehydratase
prephenate dehydrogenase
See Figure 25-15
Tryptophan
Phenylpyruvic acid
p-Hydroxyphenyl-pyruvate
Glutamate
transaminase
α-Ketoglutarate
Phenylalanine
Tyrosine

is responsible for catalyzing the reaction that converts shikimate 3-phosphate to 5-enolpyruvylshikimate 3-phosphate. The product is a precursor to the common branchpoint molecule chorismate from which all three aromatic amino acids are derived. Mutation in the *aroA* gene eliminates the precursors necessary for biosynthesis of all three amino acids thereby creating an auxotrophic mutant phenotype that relies on exogenous aromatic amino acids for proper protein synthesis. Mutants of this nature should lack the ability to survive *in vivo* for long periods of time, therefore making them good vaccine candidates. Organisms mutated in the *aroA* gene locus have been well characterized in the literature for use as rationally attenuated live vaccines (6, 12, 21, 27, 44-46, 70, 93, 101).

In 1950, Bacon et al. were the first to demonstrate attenuation of *Salmonella typhi* after mutation in the aromatic amino acid biosynthetic pathway (6). Subsequent efforts with pathogens such as *Salmonella typhimurium* (46), *S. enteritidis* (27), *Shigella flexneri* (93), *Yersinia enterocolitica* (12), *Y. pestis* (70), *Neisseria gonorrhea* (21), *Pasteurella multocida* (44), and *P. haemolytica* ST 1 (45, 92) have produced additional *aroA* mutants as vaccines candidates.

Mutants of the aromatic amino acid family have shown some promise in *Pasteurella* spp. Homphampa et al. observed that a live *aroA P. multocida* mutant completely protected mice that were immunized intraperitoneally twice and subsequently challenged with the parental strain (44). Two years later the same researchers demonstrated the efficacy of an *aroA* mutant of *P. haemolytica* ST1 in mice that were immunized intraperitoneally twice and subsequently challenged with a lethal dose of the parental strain (45). Moreover, Adlar recently demonstrated attenuation after observing a 6-log increase in infectious dose 50 in mice exposed to *aroA P. multocida* ST 1 and 3 mutants (5). Furthermore, virulence was

restored by complementation of the mutant with a copy of wild type *aroA* in *trans*. These studies provide evidence that supports the contention that inactivation of the *aroA* gene effectively attenuates *Pasteurella* spp. and may be a useful means to produce an effective live vaccine in sheep against *P. haemolytica* A2.

MATERIALS AND METHODS

Bacterial strains and growth

Luria broth (LB) and 2xYT were prepared as described (59). Columbia broth and Columbia agar were prepared according to the manufacturer's directions (Difco, Detroit, Mich.). Wessman defined media (97) lacks the amino acid tryptophan, therefore, organisms harboring a mutation in the aromatic amino acid biosynthetic pathway cannot grow on this media. A protocol is supplied in Appendix A. Ampicillin (10 μg per ml) and kanamycin (50 μg per ml) were used for selection with *P. haemolytica* NADC-D153 ST1 and NADC-D171 ST2. For *E. coli* strains, ampicillin (100 μg per ml), kanamycin (50 μg per ml), and tetracycline (35 μg per ml) were used for appropriate selection. All strains were grown at 37°C unless otherwise stated. A summary of the bacterial strains and plasmids appears in Table 3.

Cloning of the *P. haemolytica* serotype 2 *aroA* gene

Polymerase chain reaction (PCR) primers (Integrated DNA Technologies [IDT], Corralville, Ia.) were designed based on the ST1 *aroA* DNA sequence (92) and used to amplify the ST 2 *aroA* from *P. haemolytica* NADC-D171. A logarithmic phase *P. haemolytica* culture grown in Columbia broth at 37°C for 3 h with shaking were used as the template source. The forward primer, 5'-TATGAGGCATTACTGCGTGAAGG-3', designated D171#1 hybridized to nucleotides 1 through 23 of the minus strand, the reverse primer, 5'-AGCGGTTGGGCTTAGTCTGCCAC-3', designated D171#2 hybridized to the plus strand at nucleotides 1534 through 1556. PCR was carried out on a thermocycler (Perkin-Elmer 9600 Thermocycler, Perkin-Elmer Inc., Branchburg, N.J.) according to the

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
E.coli		
AB2829	K-12 strain harboring a mutation in the *aroA* gene	92
DH10B	Standard cloning strain used in this study	Gibco-BRL
*Pha*IMtase	Recombinant DH10B harboring the *m Pha* I methyltransferase gene	14,92
P. haemolytica		
NADC-D153	Serotype 1, plasmidless	NADC[a] (R. Briggs)
NADC-D171	Serotype 2, plasmidless	NADC (R. Briggs)
Plasmids		
pSK	Cloning vector (Amp^r)	Stratagene
pD70	3.5 kb plasmid encoding Kan^r	NADC (R. Briggs)
pD80	4.2 kb plasmid encoding Amp^r	NADC (R.Briggs)
pPh*aroA* 1	3.2 kb *Hind*III clone of *P. haemolytica aroA* (pSK)	92
pPh*aroA* 2	2.2kb *Hind*III-*Cla* I digested pPh*aroA* 1 *aroA* fragment (pSK)	92
pPh*aroA* 3	Same fragment as pPh*aroA* 2 cloned on pBC SK	92
pPh*aroA* 3.1	200 bp *Nde* I-*BstEI* I deletion of *aroA* from pPh*aroA* 3	This study
pPCRII*aroA*	Serotype 2 *aroA* PCR product cloned onto pPCRII TA cloning vector	This study
$pPhAmp^r$	2.2 kb *Sau* 3AI fragment of pD80 cloned onto pBC SK	92
pPh*aroA* $^-amp^r$	pPh*aroA* 3.1 with Amp^r gene from $pPhAmp^r$ cloned into the *Nde* I-*Bst* EII site	This study
pBB192C	Broadhost range temperature sensitive	This study
pBB192CΔ*aroA* $^-amp^r$	*Bss* HI Δ*aroA* $^-amp^r$ cassette from pPh*aroA* $^-amp^r$ cloned on pBB192C	This study

[a] NADC, National Animal Disease Center, Ames, Iowa.

manufacturer's directions in a total volume of 50 µl with 1 µl of cells using the ampliTaq Gold system (Perkin-Elmer). The cycle program was as follows: 10 seconds at 95°C; 1 minute at 95°C; 2 minutes at 55°C; 3 minutes at 72°C; 7 minutes at 72°C; hold at 4°C. Steps 2-4 were cycled 30 times.

The ST 2 *aroA* PCR product was ligated into the pPCRII TA cloning vector (Invitrogen Inc, San Diego, Calif.) using T4 DNA ligase (Bethesda Research Laboratories [BRL], Gaithersburg, Md.). The ligation mix was diluted 1:10 in sterile dH_20 and used to transform electrocompetent *E. coli* DH-10B (BRL) cells (Gene Pulser, Bio-Rad Laboratories, Richmond, Calif.). Cells were recovered for 1 h at 37°C in 1 ml of SOC broth (2% Bacto-Tryptone, 0.5% Bacto Yeast Extract, 8 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 20 mM glucose) and then spread onto B-Agar plates (1% Bacto-Tryptone, 0.8% NaCl, 2% Difco agar, 10 µg per ml thiamine) containing 0.002% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 100 µM isopropyl-β-D-thiogalactopyranoside (IPTG) and ampicillin and grown overnight at 37°C.

Seven white colonies were picked and each was grown in 2 ml of 2x YT (59) broth containing ampicillin. Double stranded plasmid DNA was prepared by a standard alkaline lysis method (59). Plasmid DNA was digested with *Eco*RI (Bethesda Research Laboratories), and electrophoresed on a 1% agarose gel (data not shown). One isolate containing a 1.5 kb insert was chosen for further study was designated pPCRII*aroA*.

Nucleotide sequencing of the *P. haemolytica* serotype 2 *aroA* gene

Fourteen primers were designed and synthesized (IDT) utilizing the ST 1 *aroA* published sequence for both the plus and minus strands (92). Cesium chloride purified (59) double

stranded pPCRII*aroA* plasmid was used as template for sequencing reactions (Iowa State University Nucleic Acid Facility, Ames, IA.), and the nucleotide sequence (Automated Sequencer ABI Prism Model 377 Version 3.0 ABI200 Version 2.1.1.) of *aroA* was determined (MacDnasis Pro, Hitachi Software Ltd., San Bruno, Calif. and MacVector, Kodak Co., New Haven, Conn.) for both strands.

Plasmid and *P. haemolytica* serotype 2 *aroA* mutant constructions

To construct an *aroA* mutant of *P. haemolytica* ST2, we used an existing replacement plasmid containing the *P. haemolytica* ST 1 *aroA* as described by Tatum et al. (92). Briefly, the ST 1 *aroA* was cloned in the following way. A 1.3 kb PCR amplified *E. coli aroA* fragment was used to probe *Hin*dIII digested *P. haemolytica* ST1 genomic DNA revealing a 3.2 kb band. *Hin*dIII digested *P. haemolytica* ST 1 genomic DNA was then fractionated by agarose gel electrophoresis, and the 3.0 kb-3.4 kb fragments were isolated and cloned into *Hin*dIII digested alkaline phosphatase-treated pBlueskript SK cloning vector (Stratagene, La Jolla, Calif.). The resulting plasmid was designated pPh*aroA*1. Functional *aroA* was confirmed by complementation of an *aroA*$^-$ strain of *E. coli* K-12, AB2829. Restriction enzyme analysis of pPh*aroA*1 revealed that an insert of 3.2 kb had been cloned. Another plasmid, pPh*aroA*2, was constructed by excision of an internal 2.2 kb *Cla*I-*Hin*dIII *aroA* fragment from pPh*aroA*1 and cloned into the *Acc*I-*Hin*dIII site of pBluescript SK. To permit engineering of a replacement plasmid, the 2.2 kb *Cla*I-*Hin*dIII *aroA* fragment was ligated into the *Acc*I-*Hin*dIII site of pBC SK (Stratagene). This plasmid was designated pPh*aroA*3.

In order to construct a vector for allelic exchange, the *aroA* gene in pPh*aroA*3 was disrupted by inserting a gel-purified ampicillin resistance cassette from the plasmid pPh*amp*^{R} (92) into a unique *Nde*I site of *aroA*. The resulting plasmid, pPh*aroA*$^-$amp^r was amplified in

E. coli DH 10B *Pha*IMtase harboring the *mPha*I methylase gene to protect the DNA against *Pha*I cleavage (14). *P. haemolytica* ST1-NADC-D153 and ST2-NADC-D171 were prepared for electroporation by the following protocol. A 25 ml Columbia broth culture was incubated for 3 h at 37°C, the cells were centrifuged at 5,000 x *g* for 15 min, washed twice in 25 ml of 272 mM sucrose at 0°C and resuspended in 272 mM sucrose to an equal volume of pelleted cells. One to five micrograms of DNA were introduced into 100 µl of competent *P. haemolytica* by electroporation (Gene Pulser, Bio-Rad) using 0.1 cm cuvettes (Bio-Rad) at 15 KV/cm, 800 Ω and 25 µF. Time constants ranged from 9.0 to 10.5 ms. Additional controls consisted of an endogenous *P. haemolytica* plasmid from the NADC-D80 strain, pD80, bearing an ampicillin resistance cassette as the positive control, and a no DNA control. Cells were recovered in 1 ml of Columbia broth on ice for 10 min and then transferred to 37°C for 3 h with shaking. After 3 h, 10 Columbia agar plates containing ampicillin were spread with 100 µl of transformed cells and incubated overnight at 37°C. The positive and negative controls were plated on Columbia agar containing ampicillin. Since no ampicillin resistant *P. haemolytica* ST2 were recovered after several efforts with pPh*aroA*::amp^r, it was desirable to construct and utilize a non-suicidal replacement plasmid.

A deletion plasmid was constructed using the *aroA* gene from pPh*aroA*3 and a novel plasmid, pBB192C. Plasmid pBB192C contains a temperature sensitive origin of replication cloned from an endogenous plasmid of *P. haemolytica* ST1-NADC-D70, pD70, a kanamycin (kan) resistance cassette from the transposon Tn*903* (ClonTech, Palo Alto, Calif), and the *Col*EI origin of replication for amplification in *E. coli* (unpublished data). A 200 bp deletion was created in the *aroA* gene of pPh*aroA*3 by double digestion with *Nde*I and *Bst*EII restriction endonucleases. The resultant ends were filled in using deoxynucleoside

triphosphates and the Klenow fragment of *E. coli* polymerase I. A 2.2 kb β-lactamase gene was isolated from plasmid pPh*Amp*^{r} (92) by double digestion using the restriction endonucleases *Hin*dIII and *Xba*I. The resultant ends were filled in as described above and the fragments was separated on a 1% agarose gel and purified using a spin column (Supelco 5-5600A GenElute Agarose Spin Column, Supelco Inc, Bellefonte, Pa.). The purified β-lactamase gene was mixed with the pPh*aroA*3 vector and ligated with T4 DNA ligase. The resulting plasmid was designated pPh*aroA*3.1.

The Δ*aroA*$^-$amp^r fragment was excised from p*PharoA*3.1 by digestion with *Bss*HI and cloned into the *Eco*RI site of pBB192C after filling the ends to make each fragment blunt. The resultant replacement plasmid, pBB192CΔ*aroA*$^-$amp^r, was methylated by passage through *E. coli Pha*IMtase as described above. Approximately 1 μg of methylated pBB192Δ*aroA*$^-$amp^r was used to transform *P. haemolytica* ST1-NADC-D153 and *P. haemolytica* ST2-NADC-D171 as described above. Electroporated cells were transferred to 1 ml of cold Columbia broth and incubated at 4°C for 10 min. The cells were then recovered at the permissive temperature of 30°C for 1 h. After recovery, the cells were spread onto Columbia agar plates containing kanamycin and incubated 24 h at 30°C.

Six kanamycin resistant colonies were grown in 5 ml Columbia broth containing kanamycin overnight at 30°C with shaking. To ensure that the *P. haemolytica* isolates contained the replacement plasmid and to determine that the plasmid did not rearrange, double stranded plasmid DNA was extracted by a standard alkaline lysis method (59), digested with *Eco*RI restriction endonuclease and separated on a 1% agarose gel. One isolate containing plasmid DNA consistent with pBB192CΔ*aroA*$^-$amp^r was chosen for further work.

In order to select for recombinant *P. haemolytica*, cells containing pBB192Δ*aroA*$^{-}$amp^{r} were incubated at the nonpermissive temperature of 40°C on Columbia agar containing ampicillin. Resultant colonies were passed to 2 ml of Columbia broth containing ampicillin and incubated overnight at 40°C to confirm ampicillin resistance. The cultures were struck onto Columbia agar plates without selection and grown overnight at 30°C to ensure the single crossover mutants were cured of plasmid. Individual colonies then were scored for ampicillin and kanamycin resistance and tryptophan auxotrophy by inoculation into 96 well microtiter plates containing Columbia broth with ampicillin, Columbia broth with kanamycin, and Wessman defined media lacking the amino acid tryptophan and grown at 37°C (97).

Several isolates were found to be ampicillin and kanamycin resistant and tryptophan negative. One such ST 2 isolate designated E11, was chosen for further study. To generate the desired *aroA* mutant via excision of the replacement plasmid from the chromosome, E11 was passed at 40°C in 2 ml Columbia broth with ampicillin selection for 5 h. Resultant growth was inoculated into 200 ml of Columbia broth without selection and incubated overnight at 30°C. After passage the culture was struck for isolation onto Columbia agar without antibiotic and incubated overnight at 40°C. Resultant colonies were patched onto Columbia agar containing ampicillin and replica-patched onto Columbia agar containing kanamycin and were grown at 37°C overnight. Colonies which were both ampicillin resistant and kanamycin sensitive were passed in 2 ml Columbia broth containing ampicillin and incubated at 37°C overnight to confirm antibiotic resistance. Four colonies confirmed the appropriate antibiotic phenotype were inoculated into 2 ml of Wessman broth. All failed to grow in the defined media.

Southern blots were performed on the putative *aroA* mutants to determine if double crossover mutant were generated. Chromosomal DNAs were obtained from all 4 putative double crossover mutants in the following way. Cell were grown overnight in 25 ml Columbia broth containing ampicillin at 37°C. The cells were pelleted by centrifugation for 15 min at 5,000 x g, resuspended in lysis buffer (1% sodium dodecyl sulfate (SDS), 15 mM EDTA, 60 mM Tris-HCl pH 7.5, 50 mM NaCl), and incubated for 30 min at room temperature. The resultant DNA was extracted 3 times with an equal volume of chloroform and precipitated with 2 volumes 95% ethanol. The DNA was pelleted by centrifugation for 15 min at 10,000 x g and washed in cold 70% ethanol. The DNAs were resuspended in 2 ml TE buffer, and samples were quantitated spectrophotometrically as described by Maniatis (59).

One microgram of genomic DNA from putative mutant and wild type ST2-NADC-D171 were digested with *Hin*dIII. Plasmid pBB192CΔ*aroA*::amp^r was digested with *Ssp*I. The DNAs were fractionated on 0.8% agarose gels. The gels were subsequently denatured in 1.0 M NaCl and 0.5 M NaOH for 30 min, neutralized with 3.0 M NaCl and 1.5 M NaOH for 30 min, and DNAs were transferred to a nylon membrane (Hybond-N; Amersham Corp., Arlington Heights, Ill.) by capillaryphoresis according to the procedure of Maniatis (59). Filters were baked for 1 h at 80°C followed by incubation with prehybridization solution [5X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate), 5X Denhardt's solution (59), 0.1% SDS, 10 μg per ml sonicated salmon sperm DNA, and 50% formamide] at 42°C for 30 min. The prehybridization solution was removed and replaced with hybridization solution (prehybridization solution without Denhardt's solution) containing 10^7 cpm of ^{32}P-labeled probe. Probes were prepared from a gel purified 2.2 kb fragment containing the ST1 *aroA*

gene or linearized plasmid pBB192C using a random hexanucleotide radiolabeling kit (Boehringer Manneheim) and [^{32}α-P]dCTP. Filters were hybridized at 42°C for 18 h and washed twice with wash solution (1X SSC and 0.1% SDS) at 42°C for 15 min and once at 65°C for 15 min. Membranes were exposed to X-AR (Eastman Kodak Co., Rochester, N.Y.) film at -70°C for 2 h. One isolate designated, F9.80, showed genetic characteristics consistent with allelic exchange of the host chromosomal *aroA* gene and the Δ*aroA*::amp^r replacement cassette as demonstrated when probed with gel-purified radiolabeled *aroA*. Moreover, F9.80 genomic DNA was shown to be devoid of vector sequences when probed with radiolabeled pBB192C vector.

To conclusively demonstrate that F9.80 was an *aroA* deletion mutant, Southern blot analysis was repeated. One microgram of *Hin*dIII digested genomic DNA of parental *P. haemolytica* NADC-D171, *P. haemolytica* NADC-D171 Δ*aroA*::amp^r mutant F9.80, and 15 ng of *Sty*I digested pBB192CΔ*aroA*::amp^r linearized plasmid were fractionated on an agarose gel as described above. Southern blots were performed as described above and the filters were probed with *P. haemolytica* NADC-D60 (ST1) *aroA*; linearized pBB192C plasmid, and the 200 bp *Nde*I-*Bst*EII fragment from ST1 *aroA* respectively. Furthermore, tryptophan auxotrophy confirmed that F9.80 was a mutant in the aromatic amino acid biosynthetic pathway. Inoculation of the mutant into Columbia broth containing ampicillin, Wessman defined media lacking tryptophan, and Wessman defined media containing tryptophan (0.5 μg per ml) and monitoring growth was performed to demonstrate auxotrophy.

In addition to the genomic endonuclease restriction pattern observed above, final confirmation that F9.80 was *P. haemolytica* serotype 2 was performed when F9.80 was struck onto blood agar plates containing 5% sheep erythrocytes and incubated overnight at

37°C for observation of β-hemolysis (34). In addition, rapid plate agglutination using ST 1 and ST 2 specific antiserum was performed by mixing a sample of cells harvested from 5 % sheep blood agar plates with the specific antisera and observing agglutination (36) to confirm the authenticity of the *P. haemolytica aroA* mutant serotype.

Nucleotide sequence accession number

The *P. haemolytica* ST2 *aroA* sequence reported here was deposited in Genbank under the accession number U89948 and a copy is supplied in Appendix B.

RESULTS AND DISCUSSION

Cloning of the *P. haemolytica* serotype 2 *aroA* gene

Two specific primers corresponding to *P. haemolytica* ST1 *aroA* gene sequences were used to synthesize by PCR a 1.5 kb *aroA* product using *P. haemolytica* ST 2 genomic DNA as template. Previous studies indicate that the nucleotide sequence of *aroA*, an essential housekeeping gene in bacteria, is highly conserved across many genera of prokaryotes (92, 101). For instance, the *aroA* gene of *P. haemolytica* ST1 shows 75, 70, 69, and 68% identity with the *aroA* gene sequences of *P. multocida*, *Klebsiella pneumonia*, *Y. enterocolitica*, and *E. coli*, respectively (92). Therefore, it appeared likely that the *P. haemolytica* ST2 *aroA* nucleotide sequence would have a high degree of similarity to the ST1 *aroA* nucleotide sequence. As expected, the primers amplified a 1.5 kb product from ST2 equal in size to the *aroA* from serotype 1 (data not shown). The PCR product was cloned into PCRII TA to generate plasmid pPCRII*aroA* (Table 3) and the DNA sequence of the insert was determined.

Nucleotide sequencing of the *P. haemolytica* serotype 2 *aroA* gene

The *P. haemolytica* ST2 *aroA* sequence was obtained using fourteen different primers designed from *P. haemolytica* ST1 *aroA* (92). The complete nucleotide sequence of the ST2 *aroA* and the deduced amino acid sequence are shown in Figure 3. An open reading frame of 1,296 nucleotides encoded a 432 amino acid residue polypeptide. Serotype 2 *aroA* has a deduced molecular weight of 47,070 daltons, a %G+C content of 42% and an isoelectric point of 4.88. The gene had 99.1% identity to serotype 1 *aroA* suggesting a close relationship between these serotypes of *P. haemolytica*. An erratum was detected in the original ST 1 *aroA* sequence, discrepancies between ST1 and ST2 *aroA* were confirmed by analysis of the

```
      *         *         *         *         *         *         *         *

GTGATTGTTCGCTCGATAGCAGGTTATGGAATGCCGAATCATTTACGCATTAGTATCGGTTTACCGCAAGAAAACGAGAG      80

                                                                              M
ATTTTTTACTGCCTTATTGAAAGTGTTAGCTTAACAAGCGGTTACCTTTTATGAAAATTTTACAAATTTAAAGAGAAAAA     160

  E  K  L  T  L  T  P  I  S  R  V  E  G  E  I  N  L  P  G  S  K  S  L  S  N  R>
TGGAAAAACTAACTTTAACCCCGATTTCCCGAGTAGAAGGCGAGATCAATTTACCTGGTTCTAAAAGCCTGTCTAACCGA     240

A  L  L  L  A  A  L  A  T  G  T  T  Q  V  T  N  L  L  D  S  D  D  I  R  H  M  L>
GCCTTATTATTAGCCGCCTTAGCCACCGGTACGACTCAAGTGACCAATTTATTAGATAGTGATGATATTCGACATATGCT     320

 N  A  L  K  A  L  G  V  K  Y  E  L  S  D  D  K  T  V  C  V  V  E  G  I  G  G  A>
CAATGCCTTAAAAGCGTTAGGCGTGAAATATGAGCTATCGGACGATAAAACCGTCTGTGTAGTTGAAGGGATTGGTGGAG     400

  F  K  V  Q  N  G  L  S  L  F  L  G  N  A  G  T  A  M  R  P  L  A  A  A  L  C>
CTTTTAAGGTTCAAAACGGCTTATCACTGTTTCTCGGCAATGCAGGCACGGCAATGCGACCACTTGCAGCAGCATTGTGT     480

L  K  G  A  E  T  A  Q  I  I  L  T  G  E  P  R  M  K  E  R  P  I  K  H  L  V  D>
TTAAAAGGGGCGGAAACAGCTCAAATCATTCTTACCGGTGAGCCAAGAATGAAAGAACGCCCGATTAAACATTTAGTCGA     560

 A  L  R  Q  V  G  A  E  V  Q  Y  L  E  N  E  G  Y  P  P  L  A  I  S  N  S  G  L>
TGCTTTACGCCAAGTAGGGGCAGAGGTACAGTATTTAGAAAATGAAGGCTATCCGCCGTTAGCAATTAGCAATAGCGGTT     640

  Q  G  G  K  V  Q  I  D  G  S  I  S  S  Q  F  L  T  A  L  L  M  S  A  P  L  A>
TGCAAGGTGGAAAAGTGCAAATTGACGGCTCGATTTCCAGCCAATTTCTAACCGCATTGCTGATGTCTGCCCCATTAGCG     720

E  S  D  M  E  I  E  I  I  G  D  L  V  S  K  P  Y  I  D  I  T  L  S  M  M  N  D>
GAAAGCGATATGGAAATTGAGATTATCGGTGATCTGGTATCAAAACCTTATATTGATATTACCCTTTCGATGATGAACGA     800

 F  G  I  T  V  E  N  R  D  Y  K  T  F  L  V  K  G  K  Q  G  Y  V  A  P  Q  G  N>
TTTTGGTATTACGGTTGAAAATCGAGATTACAAAACCTTTTTAGTTAAAGGTAAACAAGGCTATGTTGCTCCACAAGGTA     880

 Y  L  V  E  G  D  A  S  S  A  S  Y  F  L  A  S  G  A  I  K  G  K  V  K  V  T>
ATTATTTGGTGGAGGGAGATGCCTCTTCTGCCTCTTATTTCTTAGCCTCCGGTGCGATTAAAGGCAAGGTAAAAGTAACG     960

G  I  G  K  K  S  I  Q  G  D  R  L  F  A  D  V  L  E  K  M  G  A  K  I  T  W  G>
GGCATTGGTAAAAAATCGATCCAAGGCGACCGCTTGTTTGCCGATGTGTTGGAAAAAATGGGGGCAAAAATCACTTGGGG    1040

 E  D  F  I  Q  A  E  Q  S  P  L  K  G  V  D  M  D  M  N  H  I  P  D  A  A  M  T>
AGAGGATTTTATTCAAGCCGAGCAATCCCCGCTAAAAGGCGTAGATATGGATATGAATCATATTCCTGATGCGGCAATGA    1120

  I  A  T  T  A  L  F  A  E  G  E  T  V  I  R  N  I  Y  N  W  R  V  K  E  T  D>
CGATTGCAACAACCGCTTTATTTGCCGAAGGAGAAACAGTTATCCGCAATATTTATAACTGGCGGGTAAAAGAAACCGAC    1200

R  L  T  A  M  A  T  E  L  R  K  V  G  A  E  V  E  E  G  E  D  F  I  R  I  Q  P>
CGCTTGACAGCAATGGCAACCGAATTGCGTAAAGTCGGGGCAGAGGTAGAAGAAGGGGAAGATTTTATTCGGATTCAACC    1280

 L  A  L  E  N  F  Q  H  A  E  I  E  T  Y  N  D  H  R  M  A  M  C  F  S  L  I  A>
GCTTGCGTTAGAAAACTTCCAGCACGCTGAAATTGAAACCTATAACGATCACCGTATGGCAATGTGTTTTTCATTAATTG    1360

  L  S  N  T  E  V  T  I  L  D  P  N  C  T  A  K  T  F  P  T  Y  F  R  E  L  E>
CGTTATCGAATACAGAAGTGACGATCTTAGATCCAAATTGTACCGCTAAAACGTTCCCGACTTACTTTAGGGAGTTGGAA    1440

K  L  S  V  R
AAATTATCGGTCAGATAAAAGTAAAAAAGGATTCAGAAAACTGAATCCTTTTTACGTTTTATTGTGGCAGACTAAGCCCA    1520
```

Figure 3. Nucleotide sequence and deduced amino acid sequence of *P. haemolytica* NADC-D171 ST2 *aroA*

original sequence autoradiographs of ST1 *aroA* and the automated sequence results of ST2 *aroA*. Corrections were made to the ST1 *aroA* sequence where the autoradiograph of both the plus and minus strands agreed, and a homology comparison of the ST2 *aroA* to ST1 *aroA* was based on corrected sequence (Figure 4).

Previous studies by Tatum et al. demonstrated that *P. haemolytica* ST1 *aroA*, like *P. multocida*, was transcribed by its own promoter (44, 92). These observations were based on experiments demonstrating that the cloned *aroA* was capable of complementing a mutant strain of *E. coli* K-12 AB2829 independent of orientation in the plasmid (92). Furthermore, *P. haemolytica* ST1 *aroA* was found to differ in genetic arrangement to other gram negative bacteria in which *aroA* and *serC* constitute an operon with *aroA* downstream of the *serC* promoter (92). The *serC* gene encodes a biosynthetic enzyme leading to the synthesis of the non-aromatic amino acid serine. Studies by Lim et al. utilizing a *serC-aroA*::*lacZ* translational fusion, demonstrated that the *E. coli aroA* gene product is coordinately expressed by the *serC* promoter in a cAMP dependent manner (55). A comparison of the sequences upstream of the *P. haemolytica* ST1 *aroA* gene on plasmid pPh*aroA*2 demonstrated no homology to *serC* genes thereby suggesting that *aroA* and *serC* do not constitute an operon in *P. haemolytica*. It remains unclear as to why *Pasteurella aroA* possesses its own promoter. Other respiratory pathogens such as *Haemophilus influenzae* contain an *aroA* under the control of the upstream *purN* promoter (61). In this example, the *purN* (glycinamide ribonucleotide transformylase) stop codon overlaps the start codon of *aroA* suggesting that they may be translationally coupled. These studies indicate a regulation of *aroA* in response to environmental factors aside from synthesis of aromatic amino acids

```
ST1 aroA  GTGATTGTTCGCTCGATAGCAGGTTATGGAATGCCGAATCATTTACGCATTAGTATCGGT
          ************************************************************
ST2 aroA  GTGATTGTTCGCTCGATAGCAGGTTATGGAATGCCGAATCATTTACGCATTAGTATCGGT

ST1 aroA  TTACCGCAAGAAAACGAGAGATTTTTTACTGCCTTATTGAAAGTGTTAGCTTAACAAGCG
          ************************************************************
ST2 aroA  TTACCGCAAGAAAACGAGAGATTTTTTACTGCCTTATTGAAAGTGTTAGCTTAACAAGCG

ST1 aroA  GTTACCTTTTATGAAAATTTTACAAATTTAAAGAGAAAAATGGAAAAACTAACTTTAACC
          ************************************************************
ST2 aroA  GTTACCTTTTATGAAAATTTTACAAATTTAAAGAGAAAAATGGAAAAACTAACTTTAACC

ST1 aroA  CCGATTTCCCGAGTAGAAGGCGAGATCAATTTACCTGGTTCTAAAAGCCTGTCTAACCGA
          ************************************************************
ST2 aroA  CCGATTTCCCGAGTAGAAGGCGAGATCAATTTACCTGGTTCTAAAAGCCTGTCTAACCGA

ST1 aroA  GCCTTATTATTAGCCGCCTTAGCCACCGGTACGACTCAAGTGACCAATTTATTAGATAGT
          ************************************************************
ST2 aroA  GCCTTATTATTAGCCGCCTTAGCCACCGGTACGACTCAAGTGACCAATTTATTAGATAGT

ST1 aroA  GATGATATTCGACATATGCTCAATGCCTTAAAAGCGTTAGGCGTGAAATATGAGCTATCG
          ************************************************************
ST2 aroA  GATGATATTCGACATATGCTCAATGCCTTAAAAGCGTTAGGCGTGAAATATGAGCTATCG

ST1 aroA  GACGATAAAACCGTCTGTGTAGTTGAAGGGATTGGTGGAGCTTTTAAGGTTCAAAACGGC
          ************************************************************
ST2 aroA  GACGATAAAACCGTCTGTGTAGTTGAAGGGATTGGTGGAGCTTTTAAGGTTCAAAACGGC

ST1 aroA  TTATCACTGTTTCTCGGCAATGCAGGCACGGCAATGCGACCACTTGCAGCAGCATTGTGT
          ************************************************************
ST2 aroA  TTATCACTGTTTCTCGGCAATGCAGGCACGGCAATGCGACCACTTGCAGCAGCATTGTGT

ST1 aroA  TTAAAAGGTGAGGAAAAATCCCAAATCATTCTTACCGGTGAACCAAGAATGAAAGAACGC
          ********|*|*****|*|*|********************|******************
ST2 aroA  TTAAAAGGGGCGGAAACAGCTCAAATCATTCTTACCGGTGAGCCAAGAATGAAAGAACGC

ST1 aroA  CCGATTAAACACTTAGTCGATGCTTTACGCCAAGTAGGGGCAGAGGTACAGTATTTAGAA
          ***********|************************************************
ST2 aroA  CCGATTAAACATTTAGTCGATGCTTTACGCCAAGTAGGGGCAGAGGTACAGTATTTAGAA

ST1 aroA  AATGAAGGCTATCCACCGTTGGCAATTAGCAATAGCGGTTTGCAGGGCGGAAAAGTGCAA
          **************|*****|***********************|**|************
ST2 aroA  AATGAAGGCTATCCGCCGTTAGCAATTAGCAATAGCGGTTTGCAAGGTGGAAAAGTGCAA
```

Figure 4. Homology comparison between *P. haemolytica* NADC-D60 ST1 and *P. haemolytica* NADC-D171 ST2 *aroA*. The two genes display a 99.1% identical match. The ATG start codon and TAA stop codon are in bold and mismatches are denoted by vertical lines.

```
ST1 aroA  ATTGACGGCTCGATTTCCAGCCAATTTCTAACCGCATTGCTGATGTCTGCCCCATTAGCG
          ************************************************************
ST2 aroA  ATTGACGGCTCGATTTCCAGCCAATTTCTAACCGCATTGCTGATGTCTGCCCCATTAGCG

ST1 aroA  GAAGGCGATATGGAAATTGAGATTATCGGTGATCTGGTATCAAAACCTTATATTGATATT
          ***|********************************************************
ST2 aroA  GAAAGCGATATGGAAATTGAGATTATCGGTGATCTGGTATCAAAACCTTATATTGATATT

ST1 aroA  ACCCTTTCGATGATGAACGATTTTGGTATTACGGTTGAAAATCGAGATTACAAAACCTTT
          ************************************************************
ST2 aroA  ACCCTTTCGATGATGAACGATTTTGGTATTACGGTTGAAAATCGAGATTACAAAACCTTT

ST1 aroA  TTAGTTAAAGGTAAACAAGGCTATGTTGCTCCACAAGGTAATTATTTGGTGGAGGGAGAT
          ************************************************************
ST2 aroA  TTAGTTAAAGGTAAACAAGGCTATGTTGCTCCACAAGGTAATTATTTGGTGGAGGGAGAT

ST1 aroA  GCCTCTTCTGCCTCTTATTTCTTAGCCTCCGGTGCGATTAAAGGCAAGGTAAAAGTAACG
          ************************************************************
ST2 aroA  GCCTCTTCTGCCTCTTATTTCTTAGCCTCCGGTGCGATTAAAGGCAAGGTAAAAGTAACG

ST1 aroA  GGCATTGGTAAAAAATCGATCCAAGGCGACCGCTTGTTTGCCGATGTGTTGGAAAAAATG
          ************************************************************
ST2 aroA  GGCATTGGTAAAAAATCGATCCAAGGCGACCGCTTGTTTGCCGATGTGTTGGAAAAAATG

ST1 aroA  GGGGCAAAAATCACTTGGGGAGAGGATTTTATTCAAGCCGAGCAATCCCCGCTAAAAGGC
          ************************************************************
ST2 aroA  GGGGCAAAAATCACTTGGGGAGAGGATTTTATTCAAGCCGAGCAATCCCCGCTAAAAGGC

ST1 aroA  GTAGATATGGATATGAATCATATTCCTGATGCGGCAATGACGATTGCAACAACCGCTTTA
          ************************************************************
ST2 aroA  GTAGATATGGATATGAATCATATTCCTGATGCGGCAATGACGATTGCAACAACCGCTTTA

ST1 aroA  TTTGCCGAAGGAGAAACAGTTATCCGCAATATTTATAACTGGCGGGTAAAAGAAACCGAC
          ************************************************************
ST2 aroA  TTTGCCGAAGGAGAAACAGTTATCCGCAATATTTATAACTGGCGGGTAAAAGAAACCGAC

ST1 aroA  CGCTTGACAGCAATGGCAACCGAATTGCGTAAAGTCGGGGCAGAGGTAGAAGAAGGGGAA
          ************************************************************
ST2 aroA  CGCTTGACAGCAATGGCAACCGAATTGCGTAAAGTCGGGGCAGAGGTAGAAGAAGGGGAA

ST1 aroA  GATTTTATTCGGATTCAACCGCTTGCGTTAGAAAACTTCCAGCACGCTGAAATTGAAACC
          ************************************************************
ST2 aroA  GATTTTATTCGGATTCAACCGCTTGCGTTAGAAAACTTCCAGCACGCTGAAATTGAAACC

ST1 aroA  TATAACGATCACCGTATGGCAATGTGTTTTTCATTAATTGCGTTATCGAATACAGAAGTG
          ************************************************************
ST2 aroA  TATAACGATCACCGTATGGCAATGTGTTTTTCATTAATTGCGTTATCGAATACAGAAGTG
```

Figure 4 (continued).

```
ST1 aroA  ACGATCTTAGATCCAAATTGTACCGCTAAAACGTTCCCGACTTACTTTAGGGACTTGGAA
          ************************************************|******
ST2 aroA  ACGATCTTAGATCCAAATTGTACCGCTAAAACGTTCCCGACTTACTTTAGGGAGTTGGAA

ST1 aroA  AAATTATCGGTCAGATAAAAGTAAAAAAGGATTCAGAAAACTGAATCCTTTTTACGTTTT
          ************************************************************
ST2 aroA  AAATTATCGGTCAGATAAAAGTAAAAAAGGATTCAGAAAACTGAATCCTTTTTACGTTTT

ST1 aroA  ATTGTGGCAGACTAAGCCCAACCGCT
          *************************
ST2 aroA  ATTGTGGCAGACTAAGCCCAACCGCT
```

Figure 4 (continued).

alone. However, regulation of the *P. haemolytica aroA* gene remains to be examined. Constitutive expression of *aroA*, however, may explain *de novo* synthesis of aromatic amino acids to support growth of *P. haemolytica* on Wessman defined media.

A homology comparison between *P. haemolytica* ST1 and ST2 *aroA* revealed 99.1% overall sequence identity (Figure 4). Sequences upstream of the structural gene, however, were shown to be 100% identical suggesting that ST2 *aroA* does not constitute an operon with *serC*.

P. haemolytica mutant construction and characterization

The purpose of this study was to construct an *aroA* mutant of *P. haemolytica* ST2 and demonstrate its attenuation *in vitro*. Towards that end, several replacement plasmids were developed and introduced into *P. haemolytica* using new technology to protect the exogenous DNA from endogenous restriction barriers. Each plasmid developed was considered superior to the previous plasmid, which failed to produce an *aroA* mutant. First, plasmid pPh*aroA*3 containing the *P. haemolytica* ST1 *aroA* gene disrupted by an ampicillin resistance cassette was methylated by passing the plasmid through *E. coli* containing *PhaI*Mtase on a cosmid and subsequently using the plasmid to transform logarithmic phase *P. haemolytica* ST1 and

ST2. Despite repeated attempts no transformants were obtained by using up to 5 μg of pPh*aroA*3. Positive control pD80 plasmids from *E. coli Pha*IMtase transformed with a frequency of approximately 6 x 10^3 transformants per μg supercoiled DNA. These results indicated that *ColE*1-based plasmids do not replicate in NADC-D171. The relatively low efficiency of transformation with pD80 implies that the larger pPh*aroA*3 may have been introduced at levels too low for generating a mutant by allelic exchange (Figure 5).

Previous studies by Tatum et al. employing a chimeric hybrid plasmid which contained both the *P. haemolytica* pD70 origin of replication (an endogenous plasmid of *Pasteurella*) and the *Col*E1 origin of replication were successful in generating deletion mutations (92). This shuttle vector could replicate both in *Pasteurella* and in *E. coli* thereby circumventing the low transformation frequency observed with the introduction of suicide plasmids into *Pasteurella* while allowing amplification of plasmid in *E. coli*. After its introduction into *P. haemolytica* the hybrid plasmid generated single crossover products. The authors found that the single crossover products were unstable, presumably due to the plasmid origin of replication destabilizing chromosomal replication (92). Since antibiotic selection was maintained, the unstable single crossover products would be eliminated if they resolved to wild type during passage at 40°C due to loss of the excised hybrid plasmid. Only mutants arising by excision of the plasmid to produce a deletion mutant exhibited robust growth under antibiotic selection.

To permit more control over the plasmid system, a temperature sensitive shuttle vector was developed utilizing the *Col*EI origin of replication and a mutagenized *P. haemolytica* origin of replication derived from the streptomycin resistant plasmid pD70. This plasmid,

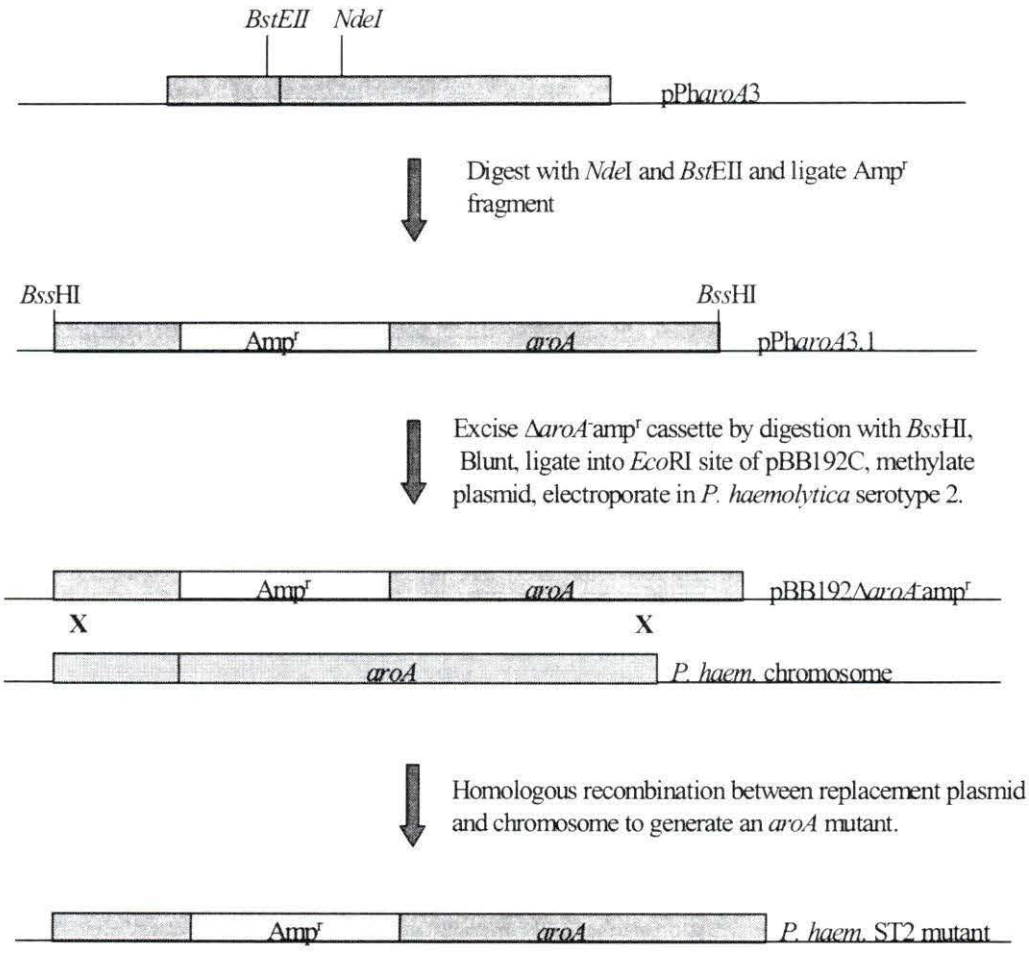


Figure 5. Construction of an *aroA* mutant of *P. haemolytica* NADC-D171 ST 2. The temperature conditional broad host range plasmid pBB192C carrying the $\Delta aroA^{-}amp^{r}$ cassette was employed to successfully develop an *aroA* mutant.

pBB192C, contained a kanamycin resistance cassette and was approximately 3.5 kb in size. In the current study, a deletion was introduced into *P. haemolytica aroA* and into the deletion site a 2.2 kb *P. haemolytica* ampicillin resistance cassette was inserted. The mutated *aroA* gene containing amp^r was cloned into pBB192C giving rise to the plasmid pBB192CΔ*aroA*$^-$ amp^r. This construct was able to overcome problems associated with the suicide vector described previously. The steps involved in constructing the replacement plasmid pBB192CΔ*aroA*$^-$amp^r and generating a gene replacement mutant via allelic exchange are depicted in Figure 5.

Since pBB192C separates transformation and recombination steps, it was anticipated that transformation of *P. haemolytica* could be accomplished. Plasmid pBB192CΔ*aroA*$^-$amp^r was methylated by passage through *E. coli Pha*IMtase and used to transform *P. haemolytica* ST1 and ST2 by electroporation. Double stranded plasmid DNA was isolated from six kanamycin resistant colonies and all plasmid DNAs analyzed by restriction enzyme digestion were shown to be pBB192CΔ*aroA*$^-$amp^r.

Since recombination is a rare event, amplification of a single transformant would result in the generation of large numbers of bacteria harboring the replacement plasmid and thus increase the likelihood that specific integration of the replacement plasmid into the host chromosome would occur. After growth at 30°C, the temperature was shifted to the nonpermissive temperature of 40°C or 42°C in ampicillin selection to prevent plasmid replication and promote integration of the plasmid into the host chromosome. It was found that passage of NADC-D171 at >40°C resulted in unacceptably short duration of viability. Passage at 40°C resulted in acceptable viability and several colonies were selected for further study. These colonies, in theory should contain recombination products with integrated

plasmids or double crossover mutants, however, our previous experience with *P. haemolytica* demonstrated that ampicillin selection was weak giving rise to several spontaneously resistant colonies on solid media which failed to grow in broth containing ampicillin. Therefore, colonies from the plates grown at 40°C were passed in broth containing ampicillin and grown at 40°C to collect viable clones. To induce a second recombination event that resulted in resolution of the replacement plasmid and to cure the cells of the remaining plasmids, the overnight culture was struck for isolation onto Columbia agar plates containing no antibiotic and grown at 30°C. Individual colonies were then scored for antibiotic resistance and auxotrophy. Construction of the replacement cassette yielded a mutated *aroA* gene in which 200 bp of the N-terminal region of the *aroA* gene product was deleted. Therefore, a single recombination event could produce one of two products based on the site of homologous recombination. If the integration of the plasmid resulted from a recombination event occurring upstream of the deletion, an inactivated *aroA* gene would result. However, if the integration of the plasmid resulted from a recombination event occurring downstream of the deletion, a functional *aroA* gene would result. Therefore, by screening for *P. haemolytica* mutants unable to support growth in Wessman defined media, one would isolate single crossover mutants occurring on the upstream arm of the replacement plasmid or products of double crossover events (Figure 6). As expected, several ampicillin and kanamycin resistant isolates failed to grow in Wessman defined media. Southern blot analysis of *Hind*III digested genomic DNAs from these isolates demonstrated the presence of integrated plasmid DNA as compared to wild type DNAs when probed with the gel-purified 2.2 kb *aroA* gene from pPh*aroA*3 and the plasmid vector pBB192C (data not shown). The isolates yielded two bands of 4 kb and 5.5 kb when probed with *aroA* sequences whereas

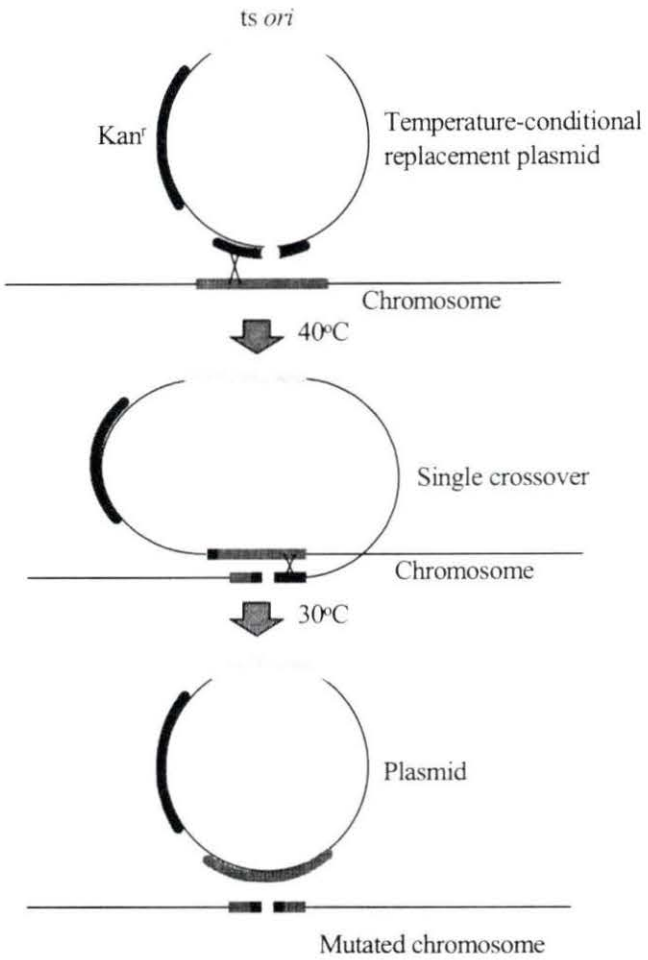


Figure 6. Illustration of the events required to yield a single crossover integrant and excision of the plasmid to generate a gene replacement mutant.

wild type *aroA* yielded a 3 kb band. Furthermore plasmid sequences were identified in each isolate when probed with vector pBB192C sequences. These results are consistent with integration of the replacement plasmid into the chromosomal *aroA* gene. No mutants resulting from double crossover events were isolated.

To screen for mutants that have undergone a second crossover event the E11 isolate, a single crossover mutant unable to grow in defined media, was passed at the permissive temperature of 30°C for several generations. Growth at this temperature will yield one of two events; 1) the plasmid may resolve out of the chromosome yielding a wild type genotype, or 2) an allelic exchange event may occur yielding a mutated chromosomal *aroA* gene. Furthermore, resolved plasmid is cured from the when the organism is grown without antibiotic selection at the permissive temperature. Each passage was struck onto a master plate of Columbia agar without antibiotic and grown overnight at 40°C to stabilize remaining single crossover integrants and to generate isolated colonies for screening. Isolated colonies were scored for ampicillin and kanamycin resistance.

Four isolates were identified that were ampicillin resistant, kanamycin sensitive, and failed to grow in Wessman defined medium. Southern blot analysis of *Hind*III digested genomic DNAs of the four isolates and wild-type NADC-D171 revealed that three isolates contained vector sequences and the $\Delta aroA^{-}amp^{r}$ cassette when probed with radiolabeled pBB192C. This was consistent with the molecular profile of a single crossover product (data not shown). Also Southern blotting confirmed the presence of the kanamycin resistance gene in the chromosome which implied that a mutation had occurred in the kanamycin gene resulting in the antibiotic sensitive phenotype. The fourth isolate, F9.80, revealed that no vector sequences were retained in its genome when probed with radiolabeled pBB192C and

was further analyzed as a putative *aroA* mutant Southern blot analysis (Figure 7) comparing *Hin*dIII-digested genomic DNA of wild-type *P. haemolytica* NADC-D171 to the putative *aroA* mutant F9.80 using radiolabeled ST1 *aroA* confirmed earlier Southern blot data demonstrating a 2.0 kb increase in molecular size of *aroA* containing fragment in the mutant as compared to wild-type *aroA*. The increase in size is equal to the β-lactamase gene inserted into the deletion site of *aroA*. Furthermore, radiolabeled plasmid pBB192C confirmed that no vector sequences were present in the genome of F9.80. Linearized plasmid pBB192CΔ*aroA*::*amp*^{R} was used as a control. Finally, the 200 bp *Nde*I-*BstE*II *aroA* deleted fragment was radiolabeled and hybridized to wildtype, F9.80 DNAs, and linearized pBB192CΔ*aroA*::*amp*^{R} replacement plasmid. The mutant DNA failed to hybridize to the 200 bp fragment whereas wildtype DNA did so. The events that transpired to produce the *aroA* mutant are depicted in Figure 6.

To confirm tryptophan auxotrophy, *aroA* mutant F9.80 was inoculated into Columbia broth containing ampicillin and into Wessman defined media with or without tryptophan. Growth in the Columbia broth and in the Wessman broth supplemented with tryptophan were comparable, but F9.80 failed to grow in Wessman broth lacking tryptophan. This, taken together with Southern blot data demonstrated that F9.80 was an *aroA* mutant.

Finally, it was important to confirm that F9.80 was *P. haemolytica* ST2. Therefore, the mutant was struck onto blood agar plates containing 5% sheep erythrocytes for observation of β-hemolysis (34). After overnight incubation at 37°C, noticeable clearing had occurred under the mucoid colonies showing erythrocyte lysis consistent with *P. haemolytica* morphology. Furthermore, isolates were chosen from these plates for confirmation of

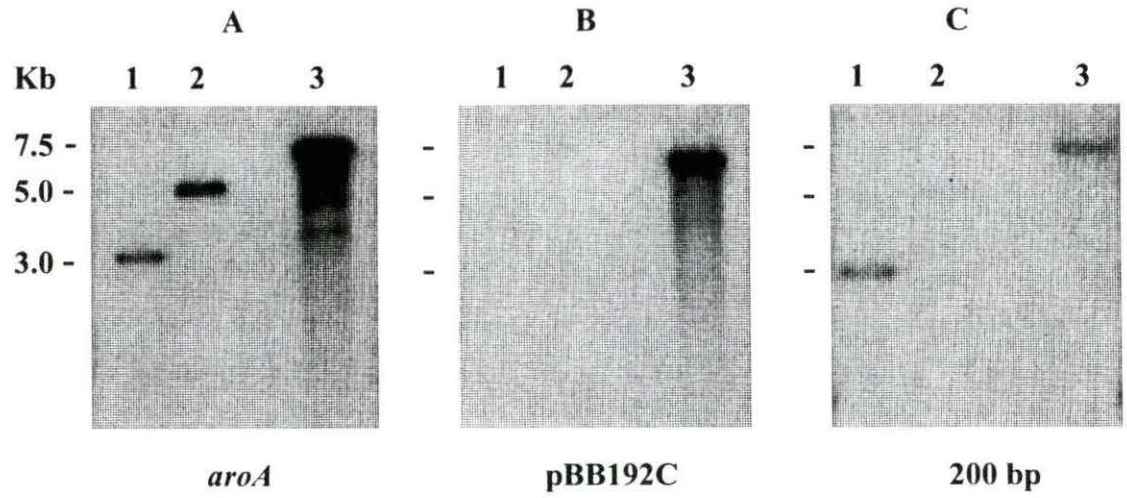


Figure 7. Southern hybridization of genomic DNA from the parental *P. haemolytica* NADC-D171 ST 2 (lanes 1), the *aroA* mutant (lanes 2), and plasmid pBB192CD*aroA*$^{-}$*amp*^{r} (lanes 3). One microgram of parental and mutant DNA were digested with *Hind*III whereas 15 ng of plasmid DNA was digested with *Sty*I to linearize it. (A) Probed with *P. haemolytica* ST1 *aroA*; (B) probed with pBB192C; (C) Probed with *Nde*I and *Bst*EII double digested and isolated 200bp *aroA* fragment. A slight band in lane 3 of panel (C) indicates that the probe may have been contaminated with some vector sequences.

serotype. The rapid plate agglutination assay using ST 1 and ST 2 specific antiserum was also confirmatory; no agglutination was observed with ST1 antiserum, but marked agglutination was observed using ST2 antiserum demonstrating that F9.80 was *P. haemolytica* ST2 (36).

CONCLUSIONS

Summary

Pneumonic pasteurellosis is a devastating disease affecting a wide variety of animals. The principal etiologic agents in cattle and sheep are *P. haemolytica* ST1 and ST2, respectively. To effectively control these pathogens, vaccines that induce a strong immunity to the organism are desirable. One attractive means of achieving this goal is through construction of attenuated live vaccines. The aromatic amino acid biosynthetic pathway of prokaryotes yields the amino acids tyrosine, tryptophan, and phenylalanine. Many pathogenic bacteria defective in this pathway have been shown to be attenuated with a diminished ability to replicate *in vivo*.

In this study we have cloned and sequenced the *P. haemolytica* ST2 *aroA* gene and demonstrated that it has 99.1% identity with *P. haemolytica* ST1 *aroA*. Furthermore, we have constructed a *P. haemolytica* ST2 *aroA* mutant by allelic exchange and demonstrated it required tryptophan for growth in chemically defined media. Previous studies have shown that *aroA* mutants are good attenuated live vaccine candidates (21, 46, 70, 92). A combination of ST1 and ST2 vaccines may cross protect against the other *P. haemolytica* (biotype A) important to disease.

Future Research

A combined multivalent vaccine consisting of an *aroA* mutant of *P. haemolytica* ST1 and ST2, *P. multocida* A3, and *Haemophilus somnus* may provide cattle and sheep protection from field infections with the wild-type of the respective organisms better than the existing

vaccines. This hypothesis needs to be further investigated in large scale animal studies. We will be further testing the *P. haemolytica* ST2 *aroA* mutant in a small scale sheep study to determine the efficacy of this mutant as a potential vaccine candidate.

APPENDIX A

WESSMAN DEFINED MINIMAL MEDIA

For use with *Pasteurella haemolytica, P. multocida,* and *P. ureae.*

Salt solution is mixed in a total volume of 50 ml dH_2O:

Citric acid	1.05 g
K_2HPO_4	2.18 g
$FeSO_4$	27.8 mg
$MnSO_4$	1.6 mg

pH to 6.5 using xN NaOH

Amino acid solution is mixed in a total volume of 400 ml dH2O

Alanine	290 mg	Leucine	830 mg
Arginine	370 mg	Lysine HCl	740 mg
Aspartic acid	640 mg	Phenylalanine	450 mg
Glutamic acid	2.03 g	Proline	960 mg
Glycine	180 mg	Valine	650 mg
Histidine HCl	280 mg	Threonine	440 mg
Isoleucine	550 mg	Tyrosine	150 mg

pH to 7.5 with Na_2CO_3

Mix the Salt solution and the Amino acid solution and re-pH to 7.4 using Na_2CO_3

Add to the resulting solution:

Cysteine HCl	0.16 g
$MgSO_4$	2.23 g
Ca^{+2} Pantothenate	0.0025 g
Nicotinamide	0.005 g
Thiamine HCl	0.0001 g

pH to 7.4 using Na_2CO_3 and add the following to the resulting solution:

Galactose	5 g
Glucose	0.5 g

Filter sterilize and store at 4°C

APPENDIX B

GENBANK DEPOSIT OF *P. HAEMOLYTICA* ST2 *AROA* NUCLEOTIDE AND AMINO ACID SEQUENCES.

```
LOCUS       PHU89948     1579 bp    DNA  BCT       16-MAR-1997
DEFINITION  Pasteurella haemolytica serotype 2 aroA gene,
    complete cds.
ACCESSION   U89948
NID         g1890138
KEYWORDS    .
SOURCE      Pasteurella haemolytica.
  ORGANISM  Pasteurella haemolytica
            Eubacteria; Proteobacteria; gamma subdivision;
            Pasteurellaceae; Pasteurella.
REFERENCE   1  (bases 1 to 1579)
  AUTHORS   Hellrung,D.J., Tatum,F.M. and Briggs,R.E.
  TITLE     Molecular Cloning, Nucleotide Sequencing, and
            Construction of a Pasteurella haemolytica Serotype
            2 aroA Mutant
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1579)
  AUTHORS   Hellrung,D.J., Tatum,F.M. and Briggs,R.E.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-FEB-1997) RDNRU, National Animal
            Disease Center, Agricultural Research Service,
            United States Department of Agriculture, 2300
            Dayton Ave., Ames, IA 50010, USA
FEATURES             Location/Qualifiers
     source          1..1579
                     /organism="Pasteurella haemolytica"
                     /strain="NADC-D171"
                     /serotype="2"
                     /db_xref="taxon:746"
     gene            160..1458
                     /gene="aroA"
     CDS             160..1458
                     /gene="aroA"
                     /codon_start=1
                     /product="AroA"
                     /db_xref="PID:g1890139"
                     /transl_table=11
```

/translation="MEKLTLTPISRVEGEINLPGSKSLSNRALLLAALATGTTQVT
NLLDSDDIRHMLNALKALGVKYELSDDKTVCVVEGIGGAFKVQNGLSLFLGN
AGTAMRPLAAALCLKGAETAQIILTGEPRMKERPIKHLVDALRQVGAEVQYL
ENEGYPPLAISNSGLQGGKVQIDGSISSQFLTALLMSAPLAESDMEIEIIGD
LVSKPYIDITLSMMNDFGITVENRDYKTFLVKGKQGYVAPQGNYLVEGDASS
ASYFLASGAIKGKVKVTGIGKKSIQGDRLFADVLEKMGAKITWGEDFIQAEQ
SPLKGVDMDMNHIPDAAMTIATTALFAEGETVIRNIYNWRVKETDRLTAMAT
ELRKVGAEVEEGEDFIRIQPLALENFQHAEIETYNDHRMAMCFSLIALSNTE
VTILDPNCTAKTFPTYFRELEKLSVR"

BASE COUNT 488 a 301 c 364 g 426 t

ORIGIN

```
   1 gtgattgttc gctcgatagc aggttatgga atgccgaatc atttacgcat tagtatcggt
  61 ttaccgcaag aaaacgagag atttttact gccttattga aagtgttagc ttaacaagcg
 121 gttacctttt atgaaaattt tacaaattta aagagaaaaa tggaaaaact aactttaacc
 181 ccgatttccc gagtagaagg cgagatcaat ttacctggtt ctaaaagcct gtctaaccga
 241 gccttattat tagccgcctt agccaccggt acgactcaag tgaccaattt attagatagt
 301 gatgatattc gacatatgct caatgcctta aaagcgttag gcgtgaaata tgagctatcg
 361 gacgataaaa ccgtctgtgt agttgaaggg attggtggag cttttaaggt tcaaaacggc
 421 ttatcactgt ttctcggcaa tgcaggcacg gcaatgcgac cacttgcagc agcattgtgt
 481 ttaaaagggg cggaaacagc tcaaatcatt cttaccggtg agccaagaat gaaagaacgc
 541 ccgattaaac atttagtcga tgctttacgc caagtagggg cagaggtaca gtatttagaa
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 721 gaaagcgata tggaaattga gattatcggt gatctggtat caaaacctta tattgatatt
 781 acccttttcga tgatgaacga ttttggtatt acggttgaaa atcgagatta caaaaccttt
 841 ttagttaaag gtaaacaagg ctatgttgct ccacaaggta attatttggt ggagggagat
 901 gcctcttctg cctcttattt cttagcctcc ggtgcgatta aaggcaaggt aaaagtaacg
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1441 aaattatcgg tcagataaaa gtaaaaaagg attcagaaaa ctgaatcctt tttacgtttt
1501 attgtggcag actaagccca accgctaagc cgaattctgc agatatccat cacactggcg
1561 gccgctcgag catgcatct
```

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