

**THE CAPSULAR POLYSACCHARIDE OF *ACTINOBACILLUS*
PLEUROPNEUMONIAE SEROTYPE 5A: ROLE IN SERUM RESISTANCE
AND CHARACTERIZATION OF THE GENETIC BASIS FOR EXPRESSION**

by

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**THE CAPSULAR POLYSACCHARIDE OF *ACTINOBACILLUS*
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(ABSTRACT)

Actinobacillus pleuropneumoniae synthesizes a serotype-specific capsular polysaccharide (CP) that protects this bacterium from host defenses. In the presence of anti-CP IgG, encapsulated *A. pleuropneumoniae* K17 was killed in precolostral calf serum (PCS) but not in normal serum used as a complement source. In contrast, two capsule-deficient mutants were killed in normal serum. The CP of *A. pleuropneumoniae* contributed to serum-resistance by limiting the amount of C9, a component of the membrane attack complex, but not C3, that bound to the bacteria in PCS. A second mechanism of serum resistance was due to a lipopolysaccharide (LPS)-specific antibody present in the IgG fractions of normal swine serum, swine anti-K17 serum, and guinea pig anti-K17 LPS serum that blocked anti-CP IgG complement-mediated killing of *A. pleuropneumoniae*. This LPS-specific antibody prevented complement-mediated killing of K17 in the presence of potentially bactericidal anti-CP IgG by reducing the deposition of C9 onto *A. pleuropneumoniae*, and by directing the deposition of C9 to sites on the bacteria where the bound C9 was easily eluted. Thus, CP and anti-LPS antibody may act synergistically or at different stages of infection to limit the ability of complement to eliminate *A. pleuropneumoniae*.

Two overlapping regions of the *A. pleuropneumoniae* J45 capsulation locus were cloned and partially sequenced. One region was conserved among *A. pleuropneumoniae* serotypes and contained four open reading frames, *cpxDCBA*, that were highly homologous at both the nucleotide and amino acid levels to genes involved in the export of the CP of *H. influenzae* type b (*bexDCBA*), *Neisseria meningitidis* group B (*ctrABCD*), and to a lesser extent *Escherichia coli* K1 and K5 (*kpsED*, *kpsMT*). The J45 *cpxDCBA* gene cluster was able to partially complement *kpsM::Tnp ϕ A* or *kpsT::Tnp ϕ A* mutations within a plasmid-encoded *E. coli* K5 *kps* locus and restored sensitivity to a K5-specific bacteriophage, indicating that *cpxDCBA* functioned in capsular polysaccharide export. A DNA region adjacent to *A. pleuropneumoniae* J45 *cpxDCBA* was identified that was serotype-specific. This region contained two complete open reading frames (*cpsA* and *cpsB*), and a third partial open reading frame, *cpsC*. These genes may encode proteins involved in *A. pleuropneumoniae* J45 CP biosynthesis. A recombinant *A. pleuropneumoniae* J45 mutant in which the three serotype-specific genes, *cpsABC*, were partially or completely deleted was generated by allelic exchange. This mutant did not produce intracellular or extracellular CP, was serum-sensitive, and was attenuated in pigs. These studies demonstrated that CP contributed to the serum-resistance and virulence of *A. pleuropneumoniae*. This noncapsulated mutant will be evaluated as a potential live vaccine strain for the control of swine pleuropneumonia.

This dissertation is dedicated to Michael, my best friend and partner in life.

I am forever grateful for his friendship, love, and support.

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LIST OF ABBREVIATIONS

ABC	=	ATP-binding cassette
Apx	=	<i>Actinobacillus pleuropneumoniae</i> RTX toxin
BHI-N	=	brain-heart infusion medium supplemented with 5 μ g/ml NAD
BLAST	=	basic local alignment search tool
CAMP	=	Christie, Atkins, and Munch-Petersen
CFU	=	colony forming unit
CMP	=	cytidine 5'-monophosphate
CP	=	capsular polysaccharide
Da	=	Dalton
D-PBS	=	PBS diluted 1:5 in 5% dextrose
ELISA	=	enzyme-linked immunosorbent assay
GP	=	guinea pig
IL	=	interleukin
IPTG	=	isopropylthio- β -D-galactopyranoside
IS	=	insertion sequence
kb	=	kilobase
kDa	=	kilodalton
KDO	=	3-deoxy-D-manno-octulosonic acid
LD ₅₀	=	50% lethal dose
LPS	=	lipopolysaccharide
MAC	=	membrane attack complex of complement (C5b-9)
NAD	=	nicotinamide adenine dinucleotide
ND	=	not determined
NGPS	=	normal guinea pig serum

NHS	=	normal human serum
NSS	=	normal swine serum
OMP	=	outer membrane protein
ORF	=	open reading frame
PAGE	=	polyacrylamide gel electrophoresis
PBS	=	phosphate-buffered saline
PBS ⁺⁺	=	PBS containing 0.15 mM CaCl ₂ and 0.5 mM MgCl ₂
PCS	=	precolostral calf serum
PFU	=	plaque forming unit
PNHS	=	pooled normal human serum
RTX	=	repeats in toxin
SDS	=	sodium dodecyl sulfate
SRBC	=	sheep red blood cells
SSC	=	saline sodium citrate
TBS	=	Tris-buffered saline
Tn	=	transposon
TSY-N	=	tryptic soy broth containing 0.6% yeast extract and 5 μg/ml NAD
VBS	=	veronal-buffered saline
VBSG	=	VBS containing 0.1% gelatin
X-gal	=	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER 1

Introduction

Aims of the Work Presented in this Dissertation

Actinobacillus pleuropneumoniae causes swine pleuropneumonia, a disease of worldwide economic importance (Nicolet, 1992). The prevalence of *A. pleuropneumoniae* in the United States is increasing despite measures to prevent its spread (Fedorka-Cray et al., 1993a; Schultz, 1989). The failure to control disease caused by *A. pleuropneumoniae* can be attributed to a poor understanding of the bacterial antigens required for immunoprotection and the role of this bacterium's virulence factors in the pathogenesis of swine pleuropneumonia. The vaccines that are currently used to control swine pleuropneumonia consist of killed whole cell bacterins (Nicolet, 1992). Unfortunately, these vaccines provide only partial, serotype-specific protection against mortality and do not prevent the development of the lung lesions characteristic of this disease or the chronic form of the disease. Prevention of chronic pleuropneumonia is crucial to prevent the transmission of *A. pleuropneumoniae* to susceptible pigs. Whole cell bacterins are considered to be ineffective as vaccines because they do not contain the *A. pleuropneumoniae* RTX (Apx) toxins necessary for the development of protective immunity.

A considerable amount of research has focused on developing a more effective vaccine to control swine pleuropneumonia. An optimal vaccine should: a) provide all of the antigens required for complete protection without causing undesirable side-effects, b) be relatively easy to prepare, maintain, and administer, and c) be cost-effective. By studying the mechanisms *A. pleuropneumoniae* uses to evade host defenses, we will be

able to understand this disease and develop attenuated live vaccine strains to control swine pleuropneumonia.

A. pleuropneumoniae synthesizes a serotype-specific capsular polysaccharide that forms the outermost layer of this bacterium (Inzana and Mathison, 1987). The capsular polysaccharide of *A. pleuropneumoniae* protects this bacterium from host defense mechanisms and is recognized as an important virulence factor (Inzana, 1990). The studies presented in this dissertation have focused on examining *A. pleuropneumoniae* serotype 5 because this serotype accounts for more than 30% of the *A. pleuropneumoniae* strains isolated in the United States (Fedorka-Cray et al., 1993a). Encapsulated *A. pleuropneumoniae* is resistant to the complement-mediated bactericidal activity of serum even in the presence of specific antibody to capsular polysaccharide or whole cells (Inzana et al., 1988b; Rycroft and Cullen, 1990). In contrast, a spontaneous noncapsulated *A. pleuropneumoniae* mutant (K17-C) is easily killed by serum in the absence of specific antibody (Inzana et al., 1988b). These data indicate that the capsular polysaccharide of *A. pleuropneumoniae* protects this bacterium from complement-mediated killing (Inzana et al., 1988b). However, the precise mechanism by which capsular polysaccharide prevents complement-mediated killing of *A. pleuropneumoniae* is unknown. The first goal of the work presented in this dissertation was to determine the mechanism(s) by which *A. pleuropneumoniae* resists complement-mediated killing in both the presence and absence of specific antibody.

Resistance to the complement-mediated bactericidal activity of serum is recognized as an important virulence attribute of most invasive gram-negative bacteria (Joiner, 1988). Therefore, determining the mechanism of *A. pleuropneumoniae* serum-resistance may yield meaningful information about how this pathogen is able to avoid this important host defense mechanism and cause disease. Examining *A. pleuropneumoniae* serum-

resistance in the presence of specific antibody is particularly important because the serum-resistance of many pathogenic gram-negative bacteria is typically overcome by the host's production of specific antibodies that promote opsonization and complement-mediated killing of the pathogen (Cross, 1990; Joiner et al., 1988). I have focused on examining the contribution of the capsular polysaccharide to *A. pleuropneumoniae* serum-resistance because previous work with noncapsulated mutants (Inzana et al., 1988b) indicates that capsular polysaccharide may play a significant role in this resistance. The results of these studies should provide clues into the mechanism(s) by which capsular polysaccharide contributes to the virulence of *Actinobacillus pleuropneumoniae*.

Chemically-derived noncapsulated *A. pleuropneumoniae* mutants are avirulent in pigs, indicating the importance of capsular polysaccharide in virulence (Inzana et al., 1993a). Furthermore, these noncapsulated mutants are promising live vaccine candidates because they are capable of inducing protective immunity against challenge with homologous or heterologous serotypes (Inzana et al., 1993a). Chemically-derived noncapsulated mutants are undesirable as vaccine strains because they contain uncharacterized mutations and may contain mutations in a locus other than the capsulation locus. In addition, these strains may revert to the encapsulated phenotype and regain virulence. Therefore, another major goal of the research presented in this dissertation was to identify and characterize the locus required for *A. pleuropneumoniae* encapsulation so that a stable, genetically-defined, noncapsulated mutant may be created by targeted mutagenesis within the capsulation locus. Once a recombinant noncapsulated *A. pleuropneumoniae* mutant is generated, the precise role of the capsular polysaccharide in virulence may be determined. Furthermore, a recombinant noncapsulated mutant can be evaluated as a live vaccine strain for the control of swine pleuropneumonia.

Literature Review

Actinobacillus pleuropneumoniae

Description and history

A. pleuropneumoniae is recognized as an important pathogen of swine around the world. This bacterium is the causative agent of swine pleuropneumonia, a severe, highly contagious, and often fatal respiratory disease that causes substantial economic losses to the global swine industry (Nicolet, 1992). *A. pleuropneumoniae* is a gram-negative, facultatively anaerobic, nonmotile, nonspore-forming, encapsulated, and pleomorphic coccobacillus. This bacterium produces urease and is able to ferment fructose, mannose, xylose, ribose, sucrose, maltose, and mannitol (Kilian et al., 1978). *A. pleuropneumoniae* strains are divided into two biotypes based on requirement for V factor (nicotinamide adenine dinucleotide or NAD): biotype 1 strains require V factor for *in vitro* growth, whereas biotype 2 strains do not. Currently, twelve serotypes of biotype 1 strains and two serotypes of biotype 2 strains have been identified (Nicolet, 1992; Nielsen, 1986b; Fodor et al., 1989).

A. pleuropneumoniae is a member of the family *Pasteurellaceae*, which is comprised of the genera *Actinobacillus*, *Haemophilus*, and *Pasteurella* (Mannheim, 1984). The members of this family include many bacterial species that are important human and veterinary pathogens (Mannheim, 1984). *A. pleuropneumoniae* colonies are β -hemolytic on blood agar medium, and demonstrate the Christie, Atkins, and Munch-Petersen (CAMP) reaction which is the increased zone of hemolysis observed when colonies are grown near a β -toxigenic bacterium, such as *Staphylococcus aureus* (Kilian, 1976). *A. pleuropneumoniae* is encapsulated by a serotype-specific capsular polysaccharide (Inzana

and Mathison, 1987; Perry et al., 1990; White et al., 1964). The twelve biotype 1 serotypes (Nielsen 1986b) are comprised of thirteen distinct capsular types (Perry et al., 1990) of *A. pleuropneumoniae* have been characterized. Pigs are the only known natural host of *A. pleuropneumoniae* and this bacterium is not considered to be part of the normal respiratory tract flora of pigs (Nicolet, 1992; Olander, 1963). The mouse has been used as a laboratory model for examining several aspects of *A. pleuropneumoniae* infection (Idris et al., 1993; Inzana et al., 1988; Nakai et al., 1984; Sebunya and Saunders, 1982). However, the mouse may not be a good model for studying pathogenesis of the disease since mice exhibit post infection lung pathology different from that of pigs (Fenwick et al., 1986a, Sebunya and Saunders, 1982). Mice probably die after being exposed to *A. pleuropneumoniae* because of the toxins produced by this bacterium and not because a true infection has been established (Idris et al., 1993; Inzana, 1991; Inzana et al., 1995).

The first report of a *Haemophilus*-like organism isolated from a porcine pneumonic lung lesion was in 1957 in England (Pattison et al., 1957; Matthews and Pattison, 1961). Within a few years, porcine pleuropneumonia and the *Haemophilus*-like bacterium associated with it were also described in the United States (Olander, 1963), Argentina (Shope et al., 1964), and Switzerland (Nicolet, 1968). The name *Haemophilus pleuropneumoniae* was originally suggested for this bacterium, largely because of its strict requirement for V factor (Shope et al., 1964; White et al., 1964). This bacterium was also referred to as *Haemophilus parahaemolyticus* in some of the early literature (Olander, 1963; Nicolet, 1968). Kilian et al. (1978) clarified the confusion concerning the original classification by demonstrating that this bacterium differed biochemically from human isolates of *H. parahaemolyticus* and should be named *H. pleuropneumoniae*. In 1983, *Haemophilus pleuropneumoniae* was formally transferred to the genus

Actinobacillus because DNA:DNA hybridization studies demonstrated a much closer relationship to *Actinobacillus lignieresii* than to *Haemophilus influenzae* (Pohl et al., 1983). Comparison of 16S rRNA sequences has subsequently confirmed that *A. pleuropneumoniae* is most closely related to *A. lignieresii* and is much less related to *H. influenzae* (Dewhirst et al., 1992).

Clinical signs and pathology of the disease

A. pleuropneumoniae can infect pigs of all ages although young, fast growing pigs are most susceptible. *A. pleuropneumoniae* is transmitted from pig to pig by aerosols containing viable bacteria (Sebunya and Saunders, 1983; MacInnes and Rosendal, 1988). Outbreaks associated with high morbidity and mortality (affecting up to 40% of the animals in a herd) may occur in herds with no prior immunologic exposure to *A. pleuropneumoniae* when asymptomatic carrier pigs are introduced (Greenway, 1981; Nicolet, 1992). Swine pleuropneumonia may present itself in peracute, acute, and chronic forms. The clinical signs observed vary depending upon the size and virulence of the infecting inoculum, the immune status of the animal, and the presence of environmental stressors such as climate changes, poor ventilation, crowding, and transportation (Greenway, 1981; Nicolet, 1992). Commonly observed clinical signs include fever, lethargy, anorexia, respiratory distress, cyanosis, and sudden death (Nicolet, 1992). Acutely infected pigs may also vomit, develop diarrhea, and discharge a blood-tinged foam from the nostrils and mouth. Death may occur as early as 12 hours after clinical onset, but commonly occurs within 24-48 hours of clinical onset (Shope, 1964). In some cases, death occurs in the absence of previous clinical symptoms and may be the first clinical sign of infection observed by a caretaker (Nicolet, 1992; Sanford and Josephson, 1981; Shope, 1964). Many of the pigs that die are in good body

condition (Sebunya and Saunders 1983), indicating the fulminating nature of this disease. Many animals survive the acute infection and completely recover. However, chronic *A. pleuropneumoniae* infections may develop in some pigs following an acute infection (Sebunya et al., 1983, Nicolet, 1992; Nielsen and Mandrup, 1977), or in pigs that have been exposed to a small initial inoculum (Nicolet, 1992; Nielsen and Mandrup, 1977). Chronically infected pigs often do not exhibit overt clinical symptoms and can become subclinical carriers of the bacterium. Chronically infected pigs can harbor *A. pleuropneumoniae* in the tonsils, in the nasal cavity, or in resolving lung lesions (Kume et al., 1984, 1986; Sidibé et al., 1993), can exhibit a lower rate of average daily weight gain (Rohrbach et al., 1993), and increased susceptibility to secondary infections (Nicolet, 1992). Chronically infected pigs may also transmit *A. pleuropneumoniae* to susceptible pigs, causing an outbreak of swine pleuropneumonia (MacInnes and Rosendal, 1988; Nielsen and Mandrup, 1981).

The majority of the gross pathologic lesions observed upon necropsy of acutely infected animals are located within the respiratory tract and thoracic cavity, although fibrinous arthritis may also be observed (Didier et al., 1984; Nicolet, 1992). In the majority of acute cases a severe, hemorrhagic, necrotizing pneumonia with extensive fibrinous pleuritis is observed (Nicolet, 1992; Shope, 1964). Interstitial pulmonary edema, congestion, and vascular thrombosis are commonly observed. The trachea and bronchi often contain large amounts of a frothy, blood-tinged fluid (Nicolet, 1992; Shope, 1964), and the thoracic and pericardial spaces may contain large amounts of a fibrinous, hemorrhagic exudate (Nicolet, 1992; Shope, 1964). The pneumonia is usually bilateral and focal, frequently affecting the caudal lobes as well as the cardiac and apical lobes (Nicolet, 1992). Pneumonic areas are often dark red, firm, and consolidated, although some affected lobes may also exhibit diffuse petechial or ecchymotic

hemorrhages (Shope, 1964; Nicolet, 1992). Pleural adhesions to the thoracic wall are often present and reflect the extensive fibrin deposition that occurs on the pleural surface throughout the course of the disease (Didier et al., 1984; Nicolet, 1992; Shope, 1964).

Polymorphonuclear leukocytes (neutrophils), macrophages, red blood cells, aggregated platelets, and bacteria are microscopically observed in alveolar exudates and tissue sections (Liggett and Harrison, 1987). An intense inflammatory reaction characterized by the presence of many neutrophils and alveolar macrophages typically precedes the development of coagulative necrosis within pneumonic foci (Liggett and Harrison, 1987). However, neutrophils are observed less frequently in pulmonary exudates after 24 hours post infection (Liggett and Harrison, 1987). Liggett and Harrison (1987) have proposed that the release of toxic substances by neutrophils during infection substantially contributes to the development of necrotic lung lesions characteristic of swine pleuropneumonia. Commonly observed sequelae following the septicemic spread of *A. pleuropneumoniae* include pericarditis, arthritis, and fibrin deposition in the abdominal cavity (Didier et al., 1984). Macrophage infiltration, pleuritis, and fibrosis of necrotic areas are commonly observed in pigs that survive the acute phase of infection (Nicolet, 1992).

In cases of chronic pneumonia, sequestered necrotic lung lesions and fibrinous pleuritis are observed (Nicolet, 1992). These lesions are sometimes detected at slaughter in animals with no previous clinical history of pleuropneumonia (Falk and Lium, 1991; Nicolet, 1992). These chronic, pneumonic lesions frequently harbor viable *A. pleuropneumoniae* and can serve as a reservoir for future infection (Faulk and Lium, 1991).

Diagnosis and serotyping

A preliminary diagnosis of acute *A. pleuropneumoniae* swine pleuropneumonia is usually made based upon clinical history and necropsy findings of affected animals (Nicolet, 1992; Sebunya and Saunders, 1983). The preliminary diagnosis is typically confirmed by bacteriological culture of material from lung lesions. Samples are streaked onto blood agar plates along with a cross-streak of a V-factor producing *Staphylococcus* strain (Schultz, 1989). *A. pleuropneumoniae* typically appear as β -hemolytic satellite colonies around the *Staphylococcus* streak. Alternatively, supplemented chocolate agar may be used to culture *A. pleuropneumoniae* (T.J. Inzana, personal communication). Isolates are further characterized by biochemical testing to differentiate them from other genera commonly isolated from the porcine respiratory tract such as *Haemophilus parasuis*, and *Actinobacillus suis*. The coagglutination (Mittal et al., 1983) and indirect fluorescent antibody (Rosendal et al., 1981) tests may be used, as a complement to culture, for the rapid laboratory identification of *A. pleuropneumoniae* in lung tissue (Hoffman, 1989). A latex agglutination test has recently been developed for the rapid identification and serotyping of *A. pleuropneumoniae* in lung tissue and in nasal swabs (Inzana, 1995). This latex agglutination test may be useful in the future for rapid field diagnosis of swine pleuropneumonia outbreaks (Inzana, 1995).

Serotyping of disease isolates is an important diagnostic step for both epidemiological and pathogenic studies. Methods that have been developed for serotyping *A. pleuropneumoniae* isolates include slide agglutination (Nicolet, 1971; Rapp et al., 1985), tube agglutination (Gunnarsson et al., 1977; Mittal et al., 1984), coagglutination (Mittal et al., 1983), direct and indirect fluorescent antibody test (Nicolet, 1971; Rapp et al., 1985; Rosendal et al., 1981), immunodiffusion (Gunnarsson et al., 1978), latex agglutination (Inzana, 1995), and arbitrarily primed polymerase chain reaction (Hennessy

et al., 1993). Coagglutination is a method commonly used for routine serotyping (Mittal et al., 1987; Nicolet, 1988), although rapid slide agglutination is also used (Rapp et al., 1985). Serotyping of isolates can sometimes be difficult because of occasional nontypeable isolates and cross reactions between serotypes. In these cases, serotype confirmation can be achieved by the agar gel diffusion assay (Nicolet, 1992). Cross reactions between serotypes 3, 6, and 8, and 1, 9, and 11 are common (Mittal et al., 1988; Mittal, 1990; Mittal et al., 1992; Nielsen, 1985a; Nielsen, 1985b), and occur as a result of similar antigenic determinants present on the O-side chains located in the lipopolysaccharides (LPS) of these serotypes (Beynon et al., 1992; Perry et al., 1990). Therefore, serotyping methods based on capsular polysaccharide antigens are more desirable since these methods exhibit higher specificity and exhibit less cross-reactivity (Inzana et al., 1990; Inzana, 1995).

Diagnosis of animals chronically infected with *A. pleuropneumoniae* is more difficult because these animals typically do not exhibit clinical signs and are often culture-negative (Fedorka-Cray et al., 1993a; Hoffman, 1989). Serological methods have been developed and may be used to identify chronically infected pigs by detecting the presence of serum antibodies specific for *A. pleuropneumoniae*. Serological detection methods are more important as screening tools to identify chronically infected pigs and to study the epidemiology of *A. pleuropneumoniae* infection, than for the diagnosis of acute outbreaks of swine pleuropneumonia (Nicolet, 1992). Serological methods used for the detection of *A. pleuropneumoniae* specific antibodies in serum include the complement fixation assay (Lombin et al., 1982; Nielsen, 1979a), ELISA (Inzana and Mathison, 1987; Nicolet et al., 1981; Willson et al., 1987), indirect ELISA (Ma and Inzana, 1990), radioimmunoassay (Inzana et al., 1990), and the 2-mercaptoethanol tube agglutination test (Mittal et al., 1984). The performance, sensitivity and specificity of many of these

assays varies depending upon the source and purity of the antigens used. The complement fixation assay currently is the most commonly used method in the United States for serodiagnosis of *A. pleuropneumoniae* infections (Fedorka-Cray et al., 1993a). However, this assay is technically difficult to perform, may yield false-positive results, and cannot be performed with anticomplementary swine sera (Nicolet et al., 1981). In general, ELISAs appear to have the best potential for broad use in the serodiagnosis of *A. pleuropneumoniae* infection in pigs (Nielsen, 1988). Recently, a polymerase chain reaction assay that detects all twelve *A. pleuropneumoniae* serotypes has been described that may also be useful for the identification of chronically infected pigs (Sirois et al., 1991).

Treatment

During acute outbreaks of swine pleuropneumonia, pigs are typically treated with antibiotics at the first onset of clinical symptoms when parenteral administration of high doses of antibiotics can help reduce mortality (Nicolet, 1992). In general, *A. pleuropneumoniae* is sensitive to penicillin, ampicillin, cephalosporin, tetracycline, and gentamycin (Nicolet, 1992). However, it is useful to determine the antibiotic susceptibility of disease isolates since antibiotic resistant isolates of *A. pleuropneumoniae* are increasing in prevalence (Fedorka-Cray et al., 1993a; Gutiérrez et al., 1993; Hirsh et al., 1982; MacInnes and Rosendal, 1988). Unfortunately, antibiotic therapy does not always eliminate *A. pleuropneumoniae* infection in a herd. *A. pleuropneumoniae* may persist in chronic lung lesions or on the tonsils long after antibiotic treatment has ended. These chronically-infected animals are an important reservoir for future infections and outbreaks, and need to be identified by serological testing.

Epidemiology

A. pleuropneumoniae is found all over the world and is endemic to most swine producing countries (Nicolet, 1992, Sebunya and Saunders, 1983). The twelve serotypes of *A. pleuropneumoniae* are distributed differently around the world, although only a few serotypes are present in a particular geographic region (Sebunya and Saunders, 1983). Serotypes 1 and 5 are the most frequently isolated serotypes in United States, although serotypes 3 and 7 have also been isolated (Fedorka-Cray et al., 1993a; Hoffman, 1989; Rapp et al., 1985). In Canada, serotypes 1, 2, 3, 5, 6, 7, 8, 10, and 12 have been reported, but serotypes 1, 5, and 7 are most frequently isolated (Mittal et al., 1983; Mittal et al., 1992; Sidibé et al., 1993). Serotype 2 is the most frequently isolated serotype in Switzerland, Denmark, and Sweden (Nicolet, 1992; Nielsen, 1988), although serotypes 1, 5, 7, and 9 have also been isolated. Serotypes of *A. pleuropneumoniae* vary in their virulence for pigs (Brandreth and Smith, 1987; Komal and Mittal, 1990; Rogers et al., 1990; Rosendal et al., 1985). Serotypes 3, 6, 10, and 12 are generally less pathogenic than other serotypes (Nicolet, 1992; Rosendal et al., 1985; Desrosiers et al., 1984). The twelve *A. pleuropneumoniae* serotypes are genetically diverse and exhibit a clonal population structure (Møller et al., 1992; Musser et al., 1987).

A. pleuropneumoniae does not survive long outside of pigs (Nicolet, 1992). Consequently, *A. pleuropneumoniae* is mainly transmitted from pig to pig by direct contact or by aerosols that travel short distances (Nicolet, 1992; Nielsen and Mandrup, 1977; Shope, 1964). Therefore, subclinical carrier pigs that shed the bacterium from the nose, tonsils, or lungs are considered a major source of disease spread. Rosendal and Mitchell (1983) reported that the increased movement of pigs, particularly pigs with an unknown health status from sales barns, into a herd is associated with an increased risk for a pleuropneumonia outbreak. Once a herd is infected with *A. pleuropneumoniae*,

environmental factors frequently determine the clinical onset of disease. Outbreaks of swine pleuropneumonia frequently occur in operations that employ intense production techniques, indicating that stress is an important factor in disease outbreaks (Rosendal and Mitchell, 1983; Sanford and Josephson, 1981). Stressors such as crowding, insufficient ventilation, transportation, adverse weather conditions, and sudden changes in weather can precipitate a pleuropneumonia outbreak in an endemically infected herd (MacInnes and Rosendal, 1988; Nicolet, 1992; Rosendal and Mitchell, 1983; Sanford and Josephsen, 1981). The immune status of a herd also affects whether or not an outbreak will occur. The most severe pleuropneumonia outbreaks occur in pigs that have had no prior exposure to *A. pleuropneumoniae* (Nicolet, 1992; Sebunya and Saunders, 1983).

Prevention and control

Antibiotic therapy alone is insufficient to control *A. pleuropneumoniae* infection in a swine herd because treated animals may continue to carry the bacterium (Nicolet, 1992; Schultz, 1989). The best way to prevent swine pleuropneumonia is to maintain a closed, *A. pleuropneumoniae*-free herd (MacInnes and Rosendal, 1988; Rosendal and Mitchell, 1983). An alternate approach is to screen all animals entering an *A. pleuropneumoniae*-free herd for complement fixing antibodies specific for *A. pleuropneumoniae* and to eliminate seropositive animals (MacInnes and Rosendal, 1988). Periodic slaughterhouse checks for signs of disease should also be performed. For economic reasons, these are often difficult goals for many swine producers to achieve. Furthermore, these practices are not 100% effective, since some pigs may harbor *A. pleuropneumoniae* in the tonsils without demonstrating positive antibody titers (Sidibé et al., 1993). Therefore, other control and prevention programs are necessary. A considerable amount of attention is

currently being focused on developing a more efficient diagnostic test and an effective vaccine to prevent swine pleuropneumonia.

Pathogenesis

The pathogenesis of *A. pleuropneumoniae* infection is complex and not completely understood. The pathogenesis swine pleuropneumonia has been successfully studied under experimental conditions using an aerosol challenge model (Sebunya et al., 1983). When pigs are exposed to an experimental aerosol challenge with a virulent serotype, *A. pleuropneumoniae* is quickly deposited throughout all lung lobes (Sebunya et al., 1983), and rapidly colonizes the epithelium of the lower respiratory tract (Dom et al., 1994; Liggett et al., 1987; Sebunya et al., 1983). *A. pleuropneumoniae* appears to grow rapidly in the lungs of susceptible pigs during the peracute phase of infection, since large numbers of bacteria are observed in exudates early during the infection (Liggett et al., 1987; Sebunya et al., 1983). Rapid bacterial growth during the initial phase of infection may facilitate the delivery of large amounts of toxic substances that are involved in the development of the hemorrhagic and necrotic lung lesions characteristic of the disease.

Tissue injury occurs very quickly after an experimental challenge with *A. pleuropneumoniae*. Within 30 minutes post-infection, localized areas of edema can be observed microscopically, and the majority of bacteria observed (ca. 95%) adhere strongly to the alveolar epithelium or to the cilia of the terminal bronchioli (Dom et al., 1994). In contrast, *A. pleuropneumoniae* are only occasionally observed adhering to cilia and epithelial cells of the trachea and bronchi (Dom et al., 1994). At 90 minutes post-infection, multiple small focal lesions (1-2 mm) are observed microscopically in lung sections (Dom et al., 1994). Capillary congestion, hemorrhage, macrophage and neutrophil infiltration, and a fibrinous exudate are also observed at 90 minutes post-

infection (Dom et al., 1994).

Inflammatory processes appear to play a substantial role in the development of lung lesions (Liggett et al., 1987; Bertram, 1988). The development of coagulative necrosis in lung lesions is preceded by an intense inflammatory reaction characterized by the presence of many neutrophils (Liggett et al., 1987). Toxic products released by *A. pleuropneumoniae* during an infection may physically damage these neutrophils and cause them to release toxic substances that injure the local tissue. Further evidence for the contribution of inflammatory pathways to the development of pneumonic lung lesions is demonstrated by the presence of inflammatory cytokines during *A. pleuropneumoniae* infection and the protective effect of anti-inflammatory agents. Baarsch et al. (1995) have demonstrated that the inflammatory cytokines interleukin-1 (IL-1), IL-6, IL-8, and tumor necrosis factor are present at elevated levels in and around the periphery of pneumonic lung lesions, but not in unaffected areas of the same lungs, and speculate that these cytokines may contribute to disease severity. These cytokines are not detected in the lungs of pigs challenged with heat killed *A. pleuropneumoniae*, indicating that factors (other than LPS) expressed by live bacteria are necessary to stimulate the expression of inflammatory cytokines (Baarsch et al., 1995). Bertram (1988) reported that pigs given indomethacin (a drug that blocks cyclooxygenase-dependent inflammatory pathways) prior to an intratracheal exposure to *A. pleuropneumoniae* were protected from death and developed lung lesions that were significantly smaller than lesions in control pigs that did not receive indomethacin.

A. pleuropneumoniae produces several factors that substantially enhance its ability to cause disease in pigs. Many of these virulence factors have been well studied. A summary of the current knowledge about these factors including their role in the pathogenesis of swine pleuropneumonia is provided below.

Virulence factors

Apx toxins. *A. pleuropneumoniae* strains may secrete three different protein toxins that are involved in the development of the lung lesions characteristic of swine pleuropneumonia. These toxins are considered to be major *A. pleuropneumoniae* virulence determinants and a large amount of research has focused on characterizing these proteins and the genes involved in their expression over the last 15 years. Rosendal et al. (1980) were the first to demonstrate that lung lesions could be reproduced in pigs by the endobronchial administration of *A. pleuropneumoniae* sterile culture supernatant or sonicated cells. During early studies, there was much confusion about the proteins responsible for the hemolytic and cytotoxic activity of *A. pleuropneumoniae* whole cells and culture supernatants. Several investigators reported hemolytic activity associated with whole cells and culture supernatants of several *A. pleuropneumoniae* serotypes (Devendish and Rosendal, 1989; Frey and Nicolet, 1988a; Frey and Nicolet, 1990; Lalonde et al., 1989a; Martin et al., 1985; Nakai et al., 1983; Nakai et al., 1984; Rosendal et al., 1988). Other investigators reported cytotoxic activity only of *A. pleuropneumoniae* cells and culture supernatant for porcine alveolar macrophages, neutrophils, monocytes, endothelial cells, and testicular cells (Bendixen et al., 1981; Crujisen et al.,; Dom et al., 1992a; Dom et al., 1992b; Idris et al., 1992; Kume et al., 1986b; Rosendal et al., 1988; Serebrin and Rosendal, 1991; Udeze and Kadis, 1992a; Van Leengoed et al., 1989; Tarigan et al., 1994). With the assistance of genetic and immunologic techniques, several investigators determined that *A. pleuropneumoniae* strains produce three distinct proteins that have hemolytic and/or cytotoxic activity (Chang, et al., 1989; Frey and Nicolet, 1988a; Frey et al., 1991a; Frey et al., 1991b; Frey et al., 1992; Frey et al., 1993a; Gygi et al., 1992; Jansen et al., 1992; Jansen et al., 1993; Kamp et al., 1991; MacDonald and Rycroft, 1992; Rycroft et al., 1991a).

These three proteins have been designated ApxI, ApxII, and ApxIII (Apx for *Actinobacillus pleuropneumoniae* RTX-toxins), but ApxI was also referred to as HlyI and ClyI, ApxII was referred to as HlyII and ClyII, and ApxIII was referred to as ClyIII and Ptx in the early literature (Frey et al., 1993b). The Apx toxins belong to a family of proteins known as RTX (repeats in toxin) toxins that are pore-forming cytolysins expressed by many pathogenic gram-negative bacteria (Lalonde et al., 1989a; Welch, 1991). The Apx toxins are labile (easily inactivated by heat, formalin, and proteases) and are secreted into culture medium. All three Apx toxins contribute to the positive CAMP reaction characteristic of *A. pleuropneumoniae* strains (Jansen et al., 1995a). The biochemical properties and genetics of the Apx toxins of *A. pleuropneumoniae* have recently been reviewed (Frey, 1995a; Frey 1995b).

ApxI is a strongly hemolytic and strongly cytotoxic 105-110 kilodalton (kDa) protein (Frey and Nicolet, 1988a; Frey and Nicolet, 1990; Frey et al., 1991b; Gygi et al., 1992) that is similar (56% homology) to the *Escherichia coli* α -hemolysin, HlyA (Felmlee et al., 1985). ApxI is produced, with few exceptions, by strains of *A. pleuropneumoniae* serotypes 1, 5, 9, 10, and 11 (Beck et al., 1994; Frey and Nicolet, 1990; Kamp et al., 1994). The ApxI toxin is responsible for the strong hemolysis observed when strains are cultured on blood agar medium. Calcium (Ca^{2+}) induces ApxI synthesis and is required for the biological activity of the toxin (Gygi et al., 1992; Devendish and Rosendal, 1991; Frey and Nicolet, 1988b; Van Leengoed et al., 1992). *A. pleuropneumoniae* serotypes which express ApxI tend to be highly virulent (Beck et al., 1994; Frey and Nicolet, 1990).

ApxII is a weakly hemolytic and weakly cytotoxic 103-105 kDa protein (Chang et al., 1989; Frey et al., 1992; Kamp et al., 1991; Smits et al., 1991). The ApxII protein is more similar to the leukotoxin of *Pasteurella haemolytica* (78% homology) than to ApxI

(42% homology) or to the α -hemolysin of *E. coli* (Chang et al., 1989; Frey et al., 1991b; Smits et al., 1991; Strathdee and Lo, 1989). ApxII requires high levels of Ca^{2+} for its activity, but its expression is not inducible by Ca^{2+} (Frey and Nicolet, 1990; Frey et al., 1991a; Van Leengoed and Dickerson, 1992). ApxII is produced by strains of all *A. pleuropneumoniae* serotypes except serotype 10 (Beck et al., 1994; Frey et al., 1992; Kamp et al., 1991; Kamp et al., 1994). The precise contribution of ApxII to the virulence of *A. pleuropneumoniae* is unknown. However, serotype 3 strains that are less virulent are reported to secrete relatively little ApxII (Frey et al., 1994).

The third RTX toxin expressed by *A. pleuropneumoniae*, ApxIII, is a 120 kDa protein that is strongly cytotoxic but nonhemolytic (Cullen and Rycroft, 1994; Jansen et al., 1993; MacDonald and Rycroft, 1992; Rycroft et al., 1991a). ApxIII is 50% identical to ApxI and *E. coli* HlyI, and is 41% identical to ApxII (Jansen et al., 1993). ApxIII is produced by strains of *A. pleuropneumoniae* serotypes 2, 3, 4, 6, and 8. Relatively little is known of the role of ApxIII in the pathogenesis of swine pleuropneumonia. However, ApxIII is reported to rapidly kill alveolar macrophages and neutrophils *in vitro* (Rycroft et al., 1991a).

The pattern of toxin expression varies among *A. pleuropneumoniae* serotypes, but is constant within each serotype (Beck et al., 1994; Kamp et al., 1994). The following toxin secretion patterns have been reported for *A. pleuropneumoniae*: serotypes 1, 5, 9, and 11 secrete ApxI and ApxII; serotypes 2, 3, 4, 6, and 8 secrete ApxII and ApxIII; serotypes 7 and 12 secrete ApxII only; serotype 10 secretes ApxI only. One serotype 6 field strain was reported to secrete ApxIII only (Beck et al., 1994). In general, serotypes that secrete more than one Apx toxin (particularly those that secrete both ApxI and ApxII) are more virulent than those that secrete only one Apx toxin.

The importance of Apx toxins to the virulence of *A. pleuropneumoniae* and to the

pathogenesis of swine pleuropneumonia was demonstrated by studies using Apx toxin-minus mutants. *A. pleuropneumoniae* ApxI⁻ ApxII⁻ mutant strains of serotype 1 (generated by Tn10 mutagenesis) (Tascón et al., 1994), and serotype 5 (generated by chemical mutagenesis) (Inzana et al., 1991), that completely lack extracellular hemolytic and cytotoxic activity are avirulent in pigs and do not cause lung lesions. When ApxI and ApxII expression are restored to the serotype 5 ApxI⁻ ApxII⁻ mutant by genetic complementation, the resulting strain (ApxI⁺ ApxII⁺) is able to induce lethal pleuropneumonia in pigs (Reimer et al., 1995). When ApxII expression only is restored to the serotype 5 ApxI⁻ ApxII⁻ mutant, the resulting strain (ApxI⁻ ApxII⁺) is able to induce pleuropneumonia, although a larger challenge dose is required to observe clinical symptoms and lung pathology (Reimer et al., 1995). A serotype 2 ApxII⁻ ApxIII⁺ mutant strain causes severe pleuropneumonia in pigs, indicating that ApxIII is able to induce substantial lung lesions in the absence of the hemolytic and cytotoxic activity provided by ApxII (Rycroft et al., 1991b). Studies with Apx toxin-minus mutants have also verified the *in vitro* cytotoxicity exhibited by Apx toxins. Recombinant *A. pleuropneumoniae* serotype 1 mutants that secrete ApxI only (ApxI⁺ ApxII⁻) or ApxII only (ApxI⁻ ApxII⁺) provoke an oxidative burst and kill porcine neutrophils *in vitro*, whereas a mutant that secretes neither ApxI or ApxII (ApxI⁻ ApxII⁻) does not (Jansen et al., 1995b). Furthermore, Cullen and Rycroft (1994) reported that a serotype 2 mutant which does not secrete ApxII but secretes ApxIII only (ApxII⁻ ApxIII⁺) is still strongly cytotoxic for porcine alveolar macrophages.

A study of the effects of the endobronchial instillation of purified recombinant Apx toxins clearly demonstrated that these proteins are major virulence factors (Jansen, 1994). Apx toxins are able to cause substantial lung damage in the absence of *A. pleuropneumoniae* whole cells (Jansen, 1994). Purified recombinant toxins, rApxI and

rApxIII, cause intermediate to severe fibrinopurulent bronchopneumonia with moderate clinical symptoms when administered either individually or together to pigs (Jansen, 1994). In contrast, rApxII only causes mild lung lesions and symptoms when administered to pigs (Jansen, 1994). These studies indicate that ApxI and ApxIII are directly involved in lesion development. The role of ApxII in the development of lung lesions is less obvious. However, ApxII may contribute to lesion development by acting in conjunction with other Apx toxins or LPS to intensify the severity of the lesions.

Apx toxins probably contribute to the pathogenesis of swine pleuropneumonia by severely impairing the protective capabilities of pulmonary alveolar macrophages and neutrophils (Bendixen et al., 1981; Crujisen et al., 1992; Van Leengoed et al., 1989). Pulmonary alveolar macrophages are found in large numbers in porcine lungs and, in conjunction with neutrophils, are considered to be the main line of defense against infection in the lower respiratory tract (Pabst and Binns, 1994; Sibille and Reynolds, 1990). Apx toxins are likely to contribute to the lung pathology characteristic of swine pleuropneumonia by: 1) stimulating both alveolar macrophages and neutrophils to release toxic oxygen intermediates that cause substantial damage to the surrounding cells, or 2) by causing direct cellular necrosis of pulmonary tissues (Cullen and Rycroft, 1994; Dom et al., 1992a; Dom et al., 1992b; Tarigan et al., 1994). Apx toxins may also directly kill pulmonary alveolar macrophages and neutrophils, thereby preventing these cells from phagocytosing and eliminating *A. pleuropneumoniae*.

LPS. *A. pleuropneumoniae*, like all other gram-negative bacteria, contains LPS as a major constituent of its outer membrane. LPS is a high molecular weight molecule comprised of lipid A (a complex and unique lipid responsible for the toxic properties of LPS), a core region composed of sugars common to LPS, including 3-deoxy-D-manno-

octulosonic acid (KDO), and an O-polysaccharide side chain that is strongly antigenic (Raetz, 1990). LPS O-side chains vary in their sugar composition and in their length (Raetz, 1990). The structures of the LPS O-chains from all twelve serotypes of *A. pleuropneumoniae* have been determined (Perry et al., 1990). Each *A. pleuropneumoniae* serotype expresses a specific O-side chain, although different serotypes may express identical or similar O-side chains (Perry et al., 1990). Similarities among the O-side chains of different serotypes may cause the immunological cross-reactions typically observed between certain serotypes. The LPS O-side chains of serotypes 1 and 11 are identical and share a common linear trisaccharide backbone with the O-side chain of serotype 9 (Perry et al., 1990; Beynon et al., 1992). The LPS O-side chains of serotypes 3 and 8 are identical and share a linear pentasaccharide backbone with the O-side chain of serotype 6 (Perry et al., 1990). The O-side chains of serotypes 4 and 7 share a linear trisaccharide backbone (Perry et al., 1990). These structural similarities are responsible in part for the immunological cross-reactivity observed between serotypes a) 1, 9, and 11, b) 3, 6, and 8, and c) 4 and 7 (Mittal, 1990; Mittal et al., 1987; Mittal et al., 1988; Mittal and Bourdon, 1991; Nielsen, 1985b). The length of O-side chains varies among serotypes (Byrd and Kadis, 1989) and has also been demonstrated to vary among strains of serotype 5 (Altman et al., 1990). Serotypes 2, 4, and 7 are reported to express smooth LPS (long O-side chains), serotypes 3 and 6 express rough LPS (relatively short O-side chains), and serotypes 1 and 5 express a semirough LPS (medium length O-side chains composed of repeats of one sugar residue) (Altman et al., 1990; Byrd and Kadis, 1989).

A. pleuropneumoniae LPS exhibits biological activity similar to that reported for other bacterial endotoxins (Fenwick et al., 1986b; Maudsley et al., 1986). *A. pleuropneumoniae* LPS is pyrogenic for rabbits and pigs, clots *Limulus* amoebocyte

lysates, evokes a positive dermal Shwartzman reaction, is lethal for chicken embryos, and is mitogenic for porcine lymphocytes (Fenwick and Osburn, 1986; Fenwick et al., 1986b, Maudsley et al., 1986). *A. pleuropneumoniae* LPS has been postulated to be involved in the development of the lung lesions characteristic of swine pleuropneumonia, since some of the pathologic changes of this disease resemble those of endotoxic shock (Didier et al., 1984; Sebunya and Saunders, 1983; Udeze et al., 1987). Purified *A. pleuropneumoniae* LPS causes lung lesions in pigs along with inflammatory cell infiltration (Fenwick et al., 1986b; Udeze et al., 1987). However, these lesions differ from the lesions caused by whole bacteria since little necrosis and no hemorrhage are observed (Udeze et al., 1987). *A. pleuropneumoniae* LPS is likely to act in conjunction with Apx toxins to cause the tissue damage and inflammatory cell infiltration associated with swine pleuropneumonia (Inzana, 1991; Udeze et al., 1987).

LPS seems to be a major adhesin responsible for the binding of *A. pleuropneumoniae* to porcine tracheal epithelium, vascular endothelium, lung mesenchyme, and respiratory tract mucus (Bélanger et al., 1990; Bélanger et al., 1992; Paradis et al. 1994). In particular, smooth type LPS that protrude through the capsule layer and are exposed on the bacterial surface seem to play a predominant role in adherence (Paradis et al., 1994). Since adherence is an important prerequisite to the colonization of mucosal surfaces, LPS may play a significant role in the colonization of porcine respiratory tract epithelium.

Fimbriae. Fimbriae are non-flagellar, filamentous structures that are typically involved in the adherence to and colonization of mucosal surfaces by bacteria. *A. pleuropneumoniae* expresses fimbriae *in vivo* that are rapidly lost upon *in vitro* passage (Dom et al., 1994; Utrera and Pijoan, 1991). Fimbrial-like structures have also been observed on *A. pleuropneumoniae* located within the phagosome of a porcine neutrophil

(Inzana et al., 1988). Fimbriae are likely to be involved in the adherence of *A. pleuropneumoniae* to the mucosal surfaces of the respiratory tract. However, the precise role of fimbriae in the virulence of *A. pleuropneumoniae* remains to be determined.

Outer membrane proteins/transferrin binding proteins. *A. pleuropneumoniae* expresses three to five major outer membrane proteins (OMPs) and several minor OMPs (MacInnes and Rosendal, 1987; Rapp et al., 1986; Rycroft and Taylor, 1987). *A. pleuropneumoniae* OMP profiles are similar (but not identical) among serotypes and are similar to the OMP profiles of *A. suis*, *A. lignieresii*, and *P. haemolytica* (MacInnes and Rosendal, 1987; Rapp et al., 1986). OMPs have been associated with virulence in other gram-negative pathogens, but have not been definitively associated with virulence in *A. pleuropneumoniae*. *A. pleuropneumoniae* OMP profiles have been reported to change *in vitro* in response to iron-restriction (Deneer and Potter, 1989a; Gonzalez et al., 1990; Niven et al., 1989; Ricard et al., 1991), limitation of pyridine nucleotides (O'Reilly et al., 1991), and the addition of maltose (Deneer and Potter, 1989b). These changes in *in vitro* OMP profiles may reflect changes that occur *in vivo* and, therefore, may be associated with virulence.

A. pleuropneumoniae, like other pathogens, must effectively acquire iron from its host in order to multiply and establish an infection. Unfortunately (for the bacterium), most of the host's iron is sequestered in the form of transferrin (in serum), lactoferrin (in mucosal secretions), and in heme compounds and, thus, is not directly available for bacterial growth (Griffiths, 1987). Siderophores involved in iron acquisition have not been identified in *A. pleuropneumoniae* (Deneer and Potter, 1989; Niven et al., 1989). However, *A. pleuropneumoniae* is able to grow on iron-restricted medium in the presence of hemoglobin or porcine transferrin, but not bovine, ovine, avian or human transferrin

(D'Silva et al., 1995; Gonzalez et al., 1990; Niven et al., 1989). These findings indicate that *A. pleuropneumoniae* expresses a receptor or binding protein specific for porcine transferrin. This specificity for porcine transferrin may explain the host specificity of *A. pleuropneumoniae* (Schryvers and Gonzalez, 1990).

A. pleuropneumoniae produces two novel OMPs (of approximately 100 kDa and 65 kDa) in response to iron-restriction that have been purified and demonstrated to bind porcine transferrin (D'Silva et al., 1990; Gonzalez et al., 1990; Ricard et al., 1991). The genes encoding a 65 kDa transferrin-binding protein of *A. pleuropneumoniae* serotype 1 and a 60 kDa transferrin-binding protein of serotype 7 have been cloned and sequenced and are only 55% similar at the amino acid level (Gerlach et al., 1992a; Gerlach et al., 1992b). The precise role of these transferrin-binding proteins in the virulence of *A. pleuropneumoniae* remains to be determined.

Plasmids. Resistance to antimicrobial agents in *A. pleuropneumoniae* seems to be mediated predominantly by plasmids (Gilbride et al., 1989; Hirsh et al., 1982; Huether et al., 1987; Ishii et al., 1991; MacInnes et al., 1990). Plasmids have been isolated from *A. pleuropneumoniae* that mediate resistance to sulfonamides, streptomycin, ampicillin and chloramphenicol. However, plasmids have not been isolated from all strains that are antibiotic resistant (Huether et al., 1987; MacInnes et al., 1990). No virulence attributes other than antimicrobial resistance have been attributed to plasmids isolated from *A. pleuropneumoniae*.

Capsular polysaccharide. Most naturally occurring *A. pleuropneumoniae* strains are encapsulated by a negatively charged capsular polysaccharide that is the serotype-specific antigen (Inzana and Mathison, 1987). Thus, the twelve *A. pleuropneumoniae* serotype

each express a distinct capsular polysaccharide (Perry et al., 1990). Serotype 5 strains further are divided into two subtypes, 5a and 5b, that express similar, but structurally and immunologically distinct, capsular polysaccharides (Nielsen, 1986a; Perry et al., 1990). Encapsulated *A. pleuropneumoniae* colonies are iridescent when plated on a clear medium and viewed at an angle with a light source (Inzana, 1990). Large amounts of capsular polysaccharide can be obtained from late stationary phase culture supernatants (Inzana, 1987). The antigenic activity of purified *A. pleuropneumoniae* capsular polysaccharide is not destroyed by heating at 100°C (Inzana and Mathison, 1987).

The chemical structures of the thirteen different capsular polysaccharides expressed by *A. pleuropneumoniae* have been determined (Perry et al., 1990). The capsular polysaccharides of *A. pleuropneumoniae* are polymers comprised of either: a) teichoic acid type oligosaccharides in which repeating units are joined by phosphate diester linkages (e.g. the capsules of serotypes 2, 3, 6, 7, 8, 9, and 11), b) repeating oligosaccharide units joined by phosphate linkages (e.g. the capsules of serotypes 1, 4, and 12), or c) sequences of glycosidically linked sugar units (e.g. the capsules of serotypes 5a, 5b, and 10) (Perry et al., 1990). The capsular polysaccharides of *A. pleuropneumoniae* share several structural characteristics with the group II capsular polysaccharides of *E. coli* (Jann and Jann, 1990), including the presence of phosphate groups, amino sugars, and KDO residues (refer to the Bacterial Capsular Polysaccharides section below). However, *A. pleuropneumoniae* capsular polysaccharides may only be tentatively classified as group II capsular polysaccharides, since extensive phenotypic characterization (including CMP-KDO synthetase activity, and determination of expression below 20°C) has not yet been performed. Furthermore, the lipid moiety at the reducing end of the *A. pleuropneumoniae* capsular polysaccharide involved in anchoring the capsule to the outer membrane has not yet been characterized.

Capsular polysaccharide does not directly contribute to the lung damage associated with swine pleuropneumonia, since purified capsular polysaccharide from *A. pleuropneumoniae* does not cause clinical symptoms or pulmonary lesions when administered endobronchially to pigs (Fenwick et al., 1986b). Purified capsular polysaccharide is biologically inert, is not toxic for chicken embryos, is not pyrogenic, is not mitogenic for porcine lymphocytes, and is only weakly immunogenic (Fenwick et al., 1986b; Fenwick and Osburn, 1986; Inzana and Mathison, 1987).

The capsular polysaccharide seems to be important in protecting *A. pleuropneumoniae* from host defenses such as phagocytosis and complement-mediated killing (Inzana et al., 1988; Inzana, 1990; Rycroft and Cullen, 1990). Antibodies specific for the capsular polysaccharide opsonize *A. pleuropneumoniae* and promote phagocytosis but do not promote complement-mediated killing (Inzana et al., 1988, Rycroft and Cullen, 1990). A spontaneous noncapsulated mutant of *A. pleuropneumoniae* K17, K17-C, is killed by complement in the absence of specific antibody, suggesting that the capsular polysaccharide itself is responsible for the resistance of *A. pleuropneumoniae* to complement-mediated killing (Inzana et al., 1988). The precise mechanism by which the capsular polysaccharide prevents complement-mediated killing is unknown, although steric interference of host defenses is a strong possibility. However, it is possible that capsular polysaccharide sterically interferes with the deposition of complement proteins onto the bacterial surface.

The capsular polysaccharide appears to be a major virulence determinant since noncapsulated mutants isolated after chemical mutagenesis are avirulent in pigs and are rapidly cleared when administered intratracheally at a dose ten times the 50% lethal dose (LD₅₀) of the encapsulated parent strain (Inzana et al., 1993a). Differences in capsule content may contribute to differences in virulence among *A. pleuropneumoniae* isolates.

In general, isolates with larger capsules (examined by electron microscopy) appear to be more virulent. Jensen and Bertram (1986) reported that an avirulent serotype 5 isolate (B8) contained a fragile, less adherent capsule compared to another serotype 5 isolate (I200) which was virulent. Rosendal and MacInnes (1990) provided further evidence for the association between *A. pleuropneumoniae* virulence and capsule thickness by demonstrating that an avirulent strain (CM5A) derived after *in vitro* passage of a virulent serotype 1 strain (CM5) contained a thinner capsule. Jacques et al. (1988) and Steffens et al. (1990) also observed differences in capsule thickness and adherence among serotypes that may be associated with virulence. However, differences in virulence among serotypes are probably the result of multiple factors, particularly Apx toxin expression.

Vaccination and the development of protective immunity

Several early studies reported different levels of protective immunity in convalescent pigs, and in pigs immunized with live cells, killed cells, or cell extracts. Shope et al. (1964) demonstrated that pigs immunized subcutaneously with a virulent *A. pleuropneumoniae* strain do not develop pleuropneumonia and are fully protected from a subsequent intranasal challenge with the same strain. Nielsen (1979b) reported that pigs which survive *A. pleuropneumoniae* infection are substantially protected from future challenge with homologous or heterologous serotypes. In contrast, pigs vaccinated with killed *A. pleuropneumoniae* whole-cell bacterins or crude cell extracts are only partially protected (against death, but not from the development of lung lesions) from challenge with a homologous serotype (Nielsen, 1976; Rosendal et al., 1986), and are not protected from challenge with a heterologous serotype (Nielsen, 1984). These results suggest that pigs develop a fully protective immune response (against death and the development of

lung lesions) only after exposure to live but not killed *A. pleuropneumoniae*. These findings also indicate that vaccination to prevent swine pleuropneumonia is possible, but suggest that fully protective antigens may be provided only by live bacteria, or are inactivated during the preparation of killed whole cell vaccines (bacterins).

Subsequent studies have demonstrated that the best protection against death and the development of lung lesions from challenge with a homologous serotype is provided by vaccinating pigs with live virulent or attenuated strains (Bossé et al., 1992; Cruijssen et al., 1995; Hensel et al., 1995; Inzana et al., 1993a; Inzana et al. 1993b; MacInnes and Rosendal, 1988), or with preparations containing active Apx toxins (Beaudet et al., 1994; Byrd et al., 1992; Devendish et al., 1990; Fedorka-Cray et al., 1990; Fedorka-Cray et al., 1993b; Rossi-Campos et al., 1992). Inzana et al. (1991) provided further evidence for the importance of Apx toxins in the development of protective immunity. In this study, pigs immunized with a serotype 5 mutant which does not secrete ApxI or ApxII were not protected against challenge with the virulent parent strain.

Relatively few studies have examined the protection of vaccinated pigs against challenge with heterologous serotypes. These studies suggest that protection of pigs against challenge with heterologous serotypes depends on the Apx toxins present in the vaccine preparation. In general, pigs are substantially protected from challenge with heterologous serotypes that secrete the same Apx toxins provided in the vaccine (Cruijssen et al., 1995; Inzana et al. 1993a; Inzana et al., 1993b; Nielsen, 1979b; Rossi-Campos et al., 1992). In contrast, only limited protection is provided against challenge with heterologous serotypes that secrete Apx toxins not provided in the vaccine (Cruijssen et al., 1995; Rossi-Campos et al., 1992). These findings indicate that vaccines used to control swine pleuropneumoniae should contain forms of all three Apx toxins which promote protective immunity.

Pigs exposed to *A. pleuropneumoniae*, either naturally or experimentally, develop antibodies specific for many bacterial components including OMPs, capsular polysaccharide, LPS, and Apx toxins (Devendish et al., 1990; Fenwick and Osburn, 1986; Rapp and Ross, 1986). Antibodies specific for *A. pleuropneumoniae* can be detected in pigs by the complement fixation test as early as 10 days post-infection (Nicolet, 1992). Immune sows pass protective immunity through colostrum to their young, indicating that antibodies can confer protection against *A. pleuropneumoniae* infection (Nicolet, 1992). The importance of antibodies in protection against *A. pleuropneumoniae* is demonstrated by the protection conferred by convalescent pig serum passively transferred to pigs with no prior exposure to *A. pleuropneumoniae* (Bossé et al., 1992; Inzana et al., 1988b; Oishi et al., 1993). Antibodies of the IgG subclass, the predominant isotype present in the lower respiratory tract of pigs (Pabst and Binns, 1994), seem to be most important mediators of immunity against *A. pleuropneumoniae* infection and can be induced by parenteral immunization (Bossé et al., 1992; Byrd and Kadis, 1992; Byrd et al., 1992; Devendish et al., 1990; Fedorka-Cray et al., 1993b). Antibodies of the IgA subclass have not been demonstrated to confer protective immunity. Of all the antibody idiotypes generated in response to *A. pleuropneumoniae* infection, antibodies with Apx toxin neutralizing activity seem to be the most important to provide protective immunity (Byrd et al., 1992; Devendish et al., 1990). Furthermore, convalescent pigs develop high titers of antibodies with Apx toxin neutralizing activity (Rosendal et al., 1988).

Antibodies specific for capsular polysaccharide, LPS, and OMPs are also important in protection because these antibodies may serve as opsonins to promote the clearance of *A. pleuropneumoniae* from the porcine respiratory tract by phagocytic cells (Byrd and Kadis, 1992; Inzana et al., 1988b; Thwaites and Kadis, 1991). However, antibodies

specific for cellular components other than Apx toxins are not sufficient for complete protection, but enhance the protection provided by antibodies with Apx toxin neutralizing activity.

Currently, killed whole cell bacterins are the vaccines most widely used for the control of swine pleuropneumonia (Nicolet, 1992). However, the protection provided by these bacterins is incomplete, and does not prevent pigs from becoming asymptomatic *A. pleuropneumoniae* carriers. The serotype-specific partial protection induced by vaccination with killed whole cell bacterins is likely the result of the induction of antibodies specific for capsular polysaccharide but not Apx toxins (Fenwick and Osburn, 1986). It appears that the most effective vaccines to control swine pleuropneumonia are those that induce antibodies specific for Apx toxins (I, II, and III) as well as cellular components (such as capsular polysaccharide, LPS, and OMPs). The optimal vaccine to control swine pleuropneumonia should provide complete protection from challenge with homologous and heterologous serotypes against death and the development of lung lesions. Apx toxins are extremely labile proteins, and it may be difficult to maintain their immunogenicity when used as components of subunit vaccines. Subunit vaccines consisting of: 1) Apx toxin conjugated to LPS or to capsular polysaccharide (Byrd and Kadis, 1992; Byrd et al., 1992), 2) a fusion protein containing the carboxy-terminus of ApxII and a 60 kDa transferrin binding protein (Rossi-Campos et al., 1992), or 3) a purified 40 kDa OMP (Bunka et al., 1995; Gerlach et al., 1993) provide only partial protection against disease. However, a subunit vaccine containing both the ApxI toxin and a 42 kDa OMP expressed by all *A. pleuropneumoniae* serotypes is highly protective (Van den Bosch et al., 1995). Live vaccines consisting of attenuated *A. pleuropneumoniae* strains that produce a thinner capsule (Bossé et al., 1992) or strains that are capsule-deficient (Inzana et al., 1993) are also promising vaccine candidates.

These live vaccines are desirable because they provide all of the antigens necessary for protection without causing disease. However, the mutations in these strains have not been characterized, and the strain that produces a thinner capsule may revert to the virulent wild-type (Bossé et al., 1992). Furthermore, vaccines consisting of live attenuated strains may be difficult to produce and administer. The protective efficacy of both subunit vaccines and live attenuated strains against challenge with heterologous serotypes which exhibit different Apx toxin profiles needs to be further investigated.

Bacterial Capsular Polysaccharides

General properties

The outer membrane of gram-negative bacteria is a complex surface that contains proteins, lipoproteins, LPS, and phospholipids. Many bacterial species (both gram-negative and gram-positive) produce exopolysaccharides that can form distinct structures external to the outer membrane known as capsules. Bacterial capsules are polymers of acidic, negatively charged polysaccharides comprised of oligosaccharide repeating units (Jann and Jann, 1990). Capsules are different from slime layers produced by some bacteria because capsular polysaccharides are anchored into the outer membrane by a lipid moiety covalently attached to the reducing end of the carbohydrate (Arakere et al., 1994; Gotschlich et al., 1981; Kuo et al., 1985). Capsular polysaccharides are also distinct from LPS O-antigens. Capsules are highly hydrated structures that collapse during preparation for electron microscopy and, therefore, require special techniques for stabilization and visualization (Bayer, 1990). Capsules are important in the interaction of a bacterium with its environment, since these exopolysaccharides typically form the

outermost layer of the bacteria that produce them.

The capsular polysaccharides of *E. coli* have been the most extensively investigated of all the known gram-negative capsular polysaccharides. *E. coli* produces more than 70 chemically and immunologically distinct capsular polysaccharides (K antigens) that are divided into two groups based on several phenotypic and genotypic properties (Table 1.1) (Jann and Jann, 1990). The *E. coli* group I capsular polysaccharides are a relatively homogeneous group that resemble the capsular polysaccharides of *Klebsiella* and are LPS-like because they contain lipid A at their reducing end (Jann and Jann, 1990). The group II capsular polysaccharides of *E. coli* are a diverse group that are similar to the capsules of other medically important bacteria such as *H. influenzae* type b and *Neisseria meningitidis* group B. Group II capsular polysaccharides resemble teichoic acids from gram-positive bacteria because many contain ribitol or phosphate residues (Whitfield and Valvano, 1993). Despite the diversity of *E. coli* capsular polysaccharides, a single strain produces one particular capsular polysaccharide and does not switch to expressing another. However, different bacterial species may express structurally similar or identical polysaccharides (Whitfield and Valvano, 1993). For example, the *E. coli* K1 and the *N. meningitidis* group B capsular polysaccharides are identical polymers comprised of α -2,8 linked *N*-acetyl-neuraminic (sialic) acid residues (Boulnois and Roberts, 1990; Kasper et al., 1973).

Role in virulence

Many bacterial species which cause invasive diseases (such as septicemia, meningitis, and pneumonia) in man and animals are encapsulated. Noncapsulated variants of bacterial pathogens generally do not cause disease, indicating the importance of capsular polysaccharides as virulence factors (Timmis et al., 1985). Furthermore, a relationship

Table 1.1. Grouping of the capsular polysaccharide antigens of *E. coli* ^a

Property	Capsular Polysaccharide Group	
	I	II
Acidic component	Glucuronic acid Galacturonic acid Pyruvate	Glucuronic acid Sialic acid KDO ManNAcA ^b Phosphate
Expressed below 20°C	Yes	No
Coexpression with	O8, O9, O20	Many O-antigens
Lipid at the reducing end	Core-lipid A	Phosphatidic acid
Removal of lipid at pH 5-6/100°C	No	Yes
Chromosomal determination at (close to)	<i>rfb(his)</i> , <i>rfc(trp)</i>	<i>kpsA(serA)</i>
CMP-KDO synthetase activity elevated	No	Yes
Intergenic relationship with	<i>Klebsiella</i>	<i>H. influenzae</i> <i>N. meningitidis</i>

^a This table was taken from Jann and Jann, 1990

^b N-acetylmannosaminuronic acid

between the amount of capsular polysaccharide produced and the virulence of a strain seems to exist (Vermeulen, et al., 1988). Capsular polysaccharides protect bacteria from nonspecific host defense mechanisms such as phagocytosis and complement-mediated killing (Cross, 1990; Joiner, 1988; Moxon and Kroll, 1990; Timmis et al., 1985). These nonspecific defense mechanisms are important for the early clearance of potentially invasive bacteria before a specific immune response is generated. Many gram-negative bacteria that cause severe, invasive infections are resistant to the complement-mediated bactericidal activity of serum, indicating the importance of complement-mediated bacterial killing in host protection (Cross, 1990; Taylor, 1983).

Many capsular polysaccharides (particularly those that contain sialic acid) are poor activators of the complement cascade (Joiner, 1988). Some capsular polysaccharides prevent the activation of complement by subcapsular structures (Stevens et al., 1983), whereas others are permeable to complement components activated by subcapsular structures such as LPS (Joiner, 1988). Bacterial capsules may inhibit phagocytosis because they prevent the deposition of opsonic complement components (such as C3b) onto the bacterial surface, or because capsules prevent the access of opsonic complement components deposited onto the bacterial surface to complement receptors located on the surface of phagocytic cells (Joiner, 1988). The role of capsular polysaccharide in preventing direct complement-mediated bactericidal activity of serum (due to insertion of the late-acting complement components, C5b-9, into the outer membrane) is unknown. These investigations have been hampered because other gram-negative bacterial surface components (such as LPS and OMPs) also contribute to serum-resistance, and, therefore, it has been difficult to assess the role of the individual components (Timmis et al., 1985).

The protection provided by capsular polysaccharides to bacteria is often overcome by the host's production of antibodies specific for the capsular polysaccharide which

promote complement deposition and complement-mediated killing, and may also serve as opsonins (in conjunction with complement components) to promote phagocytosis. Many bacterial capsular polysaccharides are safe antigens to use for immunization (Robbins, 1978). Therefore, many bacterial polysaccharides are components of vaccines used to control disease caused by encapsulated bacteria (Jennings, 1990; Robbins, 1978). However, some capsular polysaccharides (particularly those that resemble host carbohydrate structures) are virtually non-immunogenic, rendering the bacteria that possess them particularly virulent. Other capsular polysaccharides, such as the polyribosyl ribitol phosphate capsule of *H. influenzae* type b, induce an age-dependent immune response, and must be covalently conjugated to proteins to render them immunogenic in children younger than 2 years of age (Jennings, 1990).

Biosynthesis and export

The production of capsular polysaccharides is a complex, sequential, and incompletely understood process requiring the participation of many gene products associated with different bacterial compartments. These processes have been extensively reviewed (Jann and Jann, 1990; Whitfield and Valvano, 1993) and will be summarized here. The initial step of capsular polysaccharide biosynthesis involves the formation of activated sugar precursors (nucleotide derivatives of sugars) in the cytoplasm, followed by the formation and polymerization of repeating units, and the export of the capsular polysaccharide to the cell surface (Whitfield and Valvano, 1993). The biosynthesis of capsular polysaccharides is linked to the carbohydrate metabolism of the bacterium since sugar precursors are required for capsule production. Polymerization of group II capsular polysaccharides by glycosyltransferases occurs on the cytoplasmic side of the cytoplasmic membrane. Sugar nucleotide precursors are not directly transferred to a growing

polymer chain, but are first assembled as oligosaccharides onto a lipid carrier. In many cases, this lipid carrier is undecaprenol phosphate, a polyisoprenyl phosphate coenzyme carrier. The undecaprenol-linked oligosaccharides are then polymerized at the non-reducing terminus into a polysaccharide chain. In contrast, no undecaprenol phosphate intermediate has been identified in the biosynthesis of the *E. coli* K5 capsular polysaccharide (Jann and Jann, 1990). However, there is evidence that KDO is involved in the polymerization of the K5 capsular polysaccharide (Finke et al., 1989; Finke et al., 1991).

Capsular polysaccharides are transported across the cytoplasmic membrane by a dedicated membrane transport system related to a family of transporters known as ATP-binding cassette (ABC) transporters (Fath and Kolter, 1993; Higgins, 1992; Reizer et al., 1992). Lipid modification of the reducing end of capsular polysaccharides appears to occur either during translocation across the cytoplasmic membrane or once the polysaccharide enters the periplasm (Whitfield and Valvano, 1993). Transport of capsular polysaccharide across the periplasm and through the outer membrane are the least studied steps in capsule expression. There is evidence that capsular polysaccharides are transported across Bayer's junctions (sites of close proximity of gram-negative inner and outer membranes) (Bayer, 1990) to the exterior of the cell, thus avoiding the difficulty of transport through the periplasm (Kröncke et al., 1990b; Whitfield and Valvano, 1993). There is evidence that transport of capsular polysaccharide across the outer membrane of gram-negative bacteria may involve OMPs (Whitfield and Valvano, 1993). In *Neisseria meningitidis*, and *E. coli*, outer membrane proteins, CtrA and protein K, respectively, have been identified that may be involved in capsular polysaccharide transport across the outer membrane (Frosch et al., 1991; Frosch et al., 1992; Whitfield and Valvano, 1993). The organization of extracellular capsular

polysaccharide into a capsule structure is one of the least characterized processes in capsule biogenesis. Once capsular polysaccharide is exported out of the cell, the lipid attached to the reducing end of the polysaccharide is thought to anchor the capsular polysaccharide in the outer membrane through hydrophobic interactions (Arakere et al., 1994; Gotschlich et al., 1981; Kuo et al., 1985). However, only 20-50% of all polysaccharide chains synthesized and exported contain a lipid moiety at the reducing end (Jann and Jann, 1990). Therefore, it is likely that other factors (such as ionic interactions) play a role in the formation of a capsule from extracellular capsular polysaccharide.

Genetics of expression

The genetics of several group II capsular polysaccharides have been well-studied. Capsular polysaccharides are generally chromosomally encoded (Jann and Jann, 1990). Genes encoding biosynthetic and export functions are linked together on the chromosome and are arranged in several transcriptional units. Regulatory genes are usually located at other loci. The capsule gene loci from gram-negative bacteria expressing group II capsules are organized in a similar but not identical fashion, and contain homologous genes involved in capsular polysaccharide export. These similarities indicate a common molecular origin for group II capsular polysaccharide expression (Frosch et al., 1991). The genetics of expression of the group II capsular polysaccharides of *E. coli*, *H. influenzae* type b, and *N. meningitidis* group B are described below.

Escherichia coli. The capsulation (*kps*) loci of *E. coli* K1 and K5 have been the most extensively studied of all the known gram-negative bacteria that express group II capsular polysaccharides. The *E. coli* *kps* locus is comprised of approximately 17 kilobases (kb)

of DNA and is divided into three functional regions that were identified by mutational studies (Boulnois et al., 1987; Echarti et al., 1983; Roberts et al., 1988; Silver et al., 1984; Vimr et al., 1989). Regions 1 and 3 of the *E. coli kps* locus flank the serotype-specific region 2, are conserved, and are required for capsular polysaccharide transport. Region 1 genes can function in the transport of heterologous *E. coli* capsular polysaccharides (Roberts et al., 1986; Roberts et al., 1988). Thus, the transport of structurally distinct *E. coli* group II capsular polysaccharides seems to be a common process.

Region 1 (7.5 kb) of the *E. coli kps* locus consists of at least five genes (*kpsEDUCS*, organized into a single transcriptional unit) that encode proteins involved at least in part with the translocation of capsular polysaccharide from the periplasm to the cell surface (Cieslewicz et al., 1993; Pazzani et al., 1993). KpsE is a 43 kDa cytoplasmic membrane protein with a large periplasmic domain (approximately 300 amino acids) (Cieslewicz et al., 1993; Pazzani et al., 1993; Rosenow et al., 1995a). The precise role of KpsE in capsular polysaccharide export is unknown, but a *kpsE* deletion mutant is known to accumulate polysaccharide in the periplasmic space (Boulnois et al., 1987; Bronner et al., 1993). KpsD is a 60 kDa periplasmic protein involved in the translocation of capsular polysaccharide from the periplasm to the cell surface (Bronner et al., 1993; Pazzani et al., 1993; Silver et al., 1987; Wunder et al., 1994). The *kpsU* gene encodes CMP-KDO synthetase, a 27 kDa cytoplasmic enzyme probably responsible for the elevated levels of this enzyme in bacteria expressing group II capsules (Pazzani et al., 1993; Rosenow et al., 1995b). It is possible that polymerization of group II capsular polysaccharides begins with KDO, since KDO is present at the reducing terminus of a number of group II capsular polysaccharides (even those that do not contain KDO as a component of the mature capsular polysaccharide) (Finke et al., 1989; Finke et al.,

1991). However, the precise role of KpsU in the expression of capsular polysaccharides is unknown. The function and location of KpsC (75.7 kDa) and KpsS (44 kDa) have not been determined. Studies on the effects of mutations within the region 1 gene cluster have been difficult to interpret because the possibility of polar effects on downstream genes cannot be ruled out.

Region 2 of the *E. coli kps* locus encodes proteins involved in the assembly and polymerization (glycosyltransferases) of a specific capsular polysaccharide, and, therefore, is serotype-specific (Boulnois and Roberts, 1990; Pazzani et al., 1991). The size of region 2 (in kb) reflects the complexity of the capsular polysaccharide it encodes (Boulnois and Roberts, 1990; Pazzani et al., 1991). Mutations in region 2 completely eliminate the production of both cytoplasmic and extracellular capsular polysaccharide (Boulnois et al., 1987; Kröncke et al., 1990a; Silver et al., 1984).

Region 3 (1.7 kb) of the *kps* locus encodes two genes, *kpsM* and *kpsT* (organized as a single transcriptional unit) involved in capsular polysaccharide transport across the cytoplasmic membrane (Pavelka et al., 1991; Pavelka et al., 1994; Pigeon and Silver, 1994; Smith et al., 1990). KpsM is a 29.6 kDa integral membrane protein (Pigeon and Silver, 1994) and KpsT is a 25 kDa ATP-binding peripheral cytoplasmic membrane protein (Pavelka et al., 1994). KpsM and KpsT belong to the ABC family of membrane transporters (Fath and Kolter, 1993; Higgins, 1992; Reizer et al., 1992). Mutations in either *kpsM* or *kpsT* result in the cytoplasmic accumulation of capsular polysaccharide that lacks a phospholipid moiety at the reducing end (Kröncke et al., 1990a; Whitfield and Valvano, 1993). Therefore, it appears that the lipid modification of *E. coli* capsular polysaccharides may occur either during transport of the polysaccharide across the cytoplasmic membrane or once the polysaccharide is in the periplasm.

Haemophilus influenzae. *H. influenzae* type b is an important pathogen of early childhood. Of the six serologically and chemically distinct capsular polysaccharides produced by *H. influenzae* strains (types a-f), strains that produce the type b capsular polysaccharide (a polymer of polyribosylribitol phosphate) account for approximately 95% of the severe invasive infections caused by *H. influenzae* in humans, such as meningitis and pneumonia. The genes required for the production of the type b capsular polysaccharide of *H. influenzae* are linked on a region of the chromosome known as the *cap* locus. Early studies indicated that the *cap* locus in type b strains may be as large as 50 kb (Catlin et al., 1972). Subsequent studies have demonstrated that in >98% of type b strains, the *cap* locus (approximately 36 kb) is comprised of an unstable 18 kb tandem duplication of genes involved in type b capsule expression separated by a 1.2 kb non-duplicated DNA fragment called the 'bridge' region (Hoiseith et al., 1985; Hoiseith et al., 1986; Moxon et al., 1984). This tandem duplication of capsule genes is observed in type b strains, but not in *H. influenzae* strains of other capsular types (Kroll et al., 1989). Each 18 kb DNA repeat contains three distinct regions of DNA (similar to the regions described for *E. coli*) in which a central serotype-specific DNA segment (region 2) is flanked by DNA conserved among serotypes (regions 1 and 3) (Kroll et al., 1989). The bridge region encodes a unique gene, *bexA*, essential for polysaccharide export (Kroll et al., 1988). *H. influenzae* strains that express the type b capsular polysaccharide have been identified which contain only one copy of the 18 kb repeat plus the bridge region (*bexA*) (Kroll and Moxon, 1988). Clinical type b isolates containing more than two and up to five copies of the 18 kb repeat have been identified that make substantially more capsular polysaccharide than isolates containing only one or two copies, although this multi-amplified state is relatively unstable *in vitro* (Corn et al., 1993). Each 18 kb repeat of the *H. influenzae* type b *cap* locus is flanked by direct repeats of the insertion

sequence (IS)-like element, *IS1016* (Kroll et al., 1991). This arrangement gives the *cap* locus the structure of a compound transposon, providing strains with the capacity to amplify or reduce the number of 18 kb repeats within the *cap* locus by homologous recombination. The tandem repeat configuration is maintained in the majority of *H. influenzae* type b strains because the duplication is not perfect: in one of the 18 kb repeats, a portion of the *bexA* gene is deleted. To express the encapsulated phenotype, the duplication of the 18 kb DNA segments must be maintained to preserve the intact copy of *bexA* located on the bridge region. This mutation has enhanced the pathogenicity of isolates with this arrangement by increasing the potential of these strains to amplify the genes required for capsule production (Kroll et al., 1993).

Nonetheless, *H. influenzae* type b isolates exhibit a spontaneous, relatively high frequency (0.1-0.3%) loss of capsular polysaccharide expression *in vitro* (Hoiseth et al., 1985). This high-frequency loss of capsular polysaccharide expression is associated with the loss of one of the 18 kb repeats along with the bridge region (*bexA*) by a *rec*-dependent recombination event (Hoiseth et al., 1986; Kroll et al., 1988; Kroll and Moxon, 1988). Alterations in capsular polysaccharide expression may confer a selective advantage to *H. influenzae* type b during different stages of infection.

Region 1 of the *cap* locus (including the bridge segment) contains four genes, *bexDCBA* involved in capsular polysaccharide export (Kroll et al., 1990). BexB and BexA appear to be components of an ABC-transporter involved in capsular polysaccharide transport across the cytoplasmic membrane and are homologous to KpsM and KpsT, respectively, of *E. coli* (Kroll et al., 1990). The precise functions of BexD and BexC are unknown. However, BexC appears to be associated with the cytoplasmic membrane and may function (in conjunction with BexB and BexA) as the third component of an ABC-2 type transporter (Reizer et al., 1992). BexD may be involved

in capsular polysaccharide transport across the outer membrane (Kroll et al., 1990).

Region 2 of the *cap* locus (5' to *bexD*) contains a serotype-specific gene cluster comprised of four open reading frames (ORFs 1-4) involved in the biosynthesis of the capsular polysaccharide of *H. influenzae* type b (Van Eldere et al., 1995). Transposon insertions in three of these genes (ORF1, ORF2, and ORF3) completely eliminate capsular polysaccharide production, verifying their role in capsular polysaccharide biosynthesis (Van Eldere et al., 1995). The role of region 3 of the *H. influenzae cap* locus has not been investigated.

Neisseria meningitidis. *N. meningitidis* group B is a major cause of bacterial septicemia and meningitis in humans. The capsular polysaccharide of group B strains is an acidic homopolymer of α -2,8-linked sialic acid. The genes required for biosynthesis and export of the *N. meningitidis* group B capsular polysaccharide (*cps* locus) are located on a 24 kb chromosomal DNA fragment that has been cloned and expressed in *E. coli* (Frosch et al., 1989). The *N. meningitidis* group B *cps* locus is divided into five functional regions (determined by deletion analyses) arranged in the following order (5' \rightarrow 3'): E, C, A, D, and B (Frosch et al., 1989). Region E (4 kb) seems to be involved in the regulation of group B capsular polysaccharide expression (Frosch et al., 1989), but has not yet been characterized. Region C (4 kb) of the *cps* locus contains four genes, *ctrABCD*, involved in the transport of phospholipid-substituted polysaccharide to the cell surface (Frosch et al., 1991). CtrA is an outer membrane protein conserved among serotypes of *N. meningitidis* (Frosch et al., 1991; Frosch et al., 1992). CtrB and CtrC appear to be cytoplasmic membrane proteins (Frosch et al., 1991), and CtrD contains an ATP-binding motif and appears to be a peripheral cytoplasmic membrane protein (Frosch et al., 1991). The nucleotide and predicted

protein sequences of *ctrABCD* are highly homologous to *bexDCBA* from *H. influenzae* type b. CtrB, CtrC, and CtrD may be components of an ABC-2 transporter involved in capsular polysaccharide export across the cytoplasmic membrane (Reizer et al., 1992), and CtrA may be involved in polysaccharide transport across the outer membrane. Region A (5 kb) encodes four genes, *siaABCD*, involved in the biosynthesis of the group B capsular polysaccharide (Edwards, et al., 1994). Region B (3 kb) encodes two genes, *lipA* and *lipB*, involved in capsular polysaccharide transport from the cytoplasm to the periplasm (Frosch et al., 1989). Frosch and Müller (1993) have demonstrated that both LipA (45.1 kDa) and LipB (48.7 kDa) are involved in modifying the reducing end of the *N. meningitidis* group B capsular polysaccharide with a phospholipid moiety. This phospholipid substitution of the capsular polysaccharide occurs within the cytoplasm, and is a strong requirement for translocation of capsular polysaccharide to the cell surface (Frosch and Müller, 1993). Both LipA and LipB are associated with the cytoplasmic face of the cytoplasmic membrane of *N. meningitidis* (Frosch and Müller, 1993). Region D (5 kb) of the *cps* locus encodes transcriptionally active genes (including *galE*, *rfbB*, *rfbC*, and *rfbD* homologs) involved in *N. meningitidis* lipooligosaccharide biosynthesis, but are not involved in capsular polysaccharide biosynthesis (Hammerschmidt et al., 1994). This is an interesting finding since LPS biosynthetic genes have not been found within the capsulation loci of *E. coli* or *H. influenzae*. It is likely that the region D genes were inserted into the *N. meningitidis* chromosome (within the *cps* locus) after the *cps* locus was acquired. Genes involved in *N. meningitidis* group B capsular polysaccharide transport are conserved among serotypes that express different capsules, similar to what has been observed for capsular polysaccharide transport genes of *E. coli* and *H. influenzae* (Frosch et al., 1989). In general, the genetics of *N. meningitidis* encapsulation appear to be more similar to *H. influenzae* type b than to *E. coli* K1 or K5.

CHAPTER 2

Resistance of *Actinobacillus pleuropneumoniae* to Bactericidal Antibody and Complement is Mediated by Capsular Polysaccharide and Blocking Antibody Specific for Lipopolysaccharide ¹

ABSTRACT

Actinobacillus pleuropneumoniae is resistant to complement-mediated killing, even in the presence of specific antibody. Our current studies focused on identifying the mechanism(s) responsible for this resistance. Encapsulated *A. pleuropneumoniae* was susceptible to killing in precolostral calf serum (PCS), but not in normal serum, as a complement source in the presence of anti-capsular polysaccharide IgG. In contrast, two capsule-deficient mutants were sensitive to killing in normal serum and one was sensitive to killing in PCS alone. Electron microscopy demonstrated that *A. pleuropneumoniae* serotype 5a synthesized a thick, adherent capsular polysaccharide that bound anti-capsular polysaccharide antibody distant from the outer membrane. The capsular polysaccharide of *A. pleuropneumoniae* did not prevent complement activation or the attachment of C3 to the cell surface. However, the capsular polysaccharide did limit the amount of C9, a component of the membrane attack complex, that bound to *A. pleuropneumoniae* in

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PCS. A second mechanism of serum resistance was due to an LPS-specific antibody present in the IgG fractions of normal swine serum, swine anti-K17 serum, and guinea pig anti-K17 LPS that blocked anti-capsular polysaccharide IgG complement-mediated killing of *A. pleuropneumoniae*. Incubation of swine anti-K17 IgG with purified K17 LPS depleted antibodies specific for K17 LPS, but not for K17 proteins, and removed all blocking activity. Immune swine serum containing this blocking antibody reduced the deposition of C9 on *A. pleuropneumoniae* in the presence of anti-capsular polysaccharide IgG, and also directed the deposition of C9 to sites on the bacteria in which the bound C9 was easily eluted. Thus, capsular polysaccharide and anti-LPS antibody may act synergistically or at different stages of infection to limit the ability of complement to eliminate *A. pleuropneumoniae*.

INTRODUCTION

Actinobacillus pleuropneumoniae is a gram-negative, encapsulated coccobacillus that causes swine pleuropneumonia. Pleuropneumonia is an acute to chronic respiratory disease of swine worldwide that is characterized by high mortality, fibrinous and hemorrhagic lesions, and pleural adhesions (Nicolet, 1992; Sebunya and Saunders, 1983). Vaccines consisting of killed whole cells provide some serotype-specific immunity and reduce mortality, but do not prevent chronic disease, lung lesions, or the subclinical carrier state (Henry and Marsteller, 1982; Higgins et al., 1985; Sebunya and Saunders, 1983;).

The capsular polysaccharide is the serotype-specific antigen of *A. pleuropneumoniae* (Inzana and Mathison, 1987). Twelve serotypes of *A. pleuropneumoniae* have been identified worldwide that differ in virulence and geographic distribution (Nicolet, 1992;

Sebunya and Saunders, 1983). The capsular polysaccharide may protect the bacteria from host humoral defense mechanisms and opsonophagocytosis (Inzana et al., 1988b), however, the exact role of capsular polysaccharide in the virulence of *A. pleuropneumoniae* is not known.

Many gram-negative bacterial pathogens are resistant to the complement-mediated bactericidal activity of serum. The mechanism of this resistance varies among bacterial pathogens, but may involve the expression of long O-side chains on LPS (Joiner et al., 1982a; Joiner et al., 1982b; Schiller et al., 1989), some outer membrane proteins (Moll et al., 1980; Vandenbosch et al., 1987), bacterial capsular polysaccharide (Joiner, 1988), or the binding of serum antibodies that block complement-mediated killing (Corbeil et al., 1988; Jarvis and Griffiss, 1991; Joiner et al., 1985). The role of capsular polysaccharide in contributing to serum resistance varies among gram-negative bacteria, and is not completely understood (Joiner, 1988). Several bacterial capsules, such as the sialic acid capsules of *Escherichia coli* K-1 and *Neisseria meningitidis* type B, do not activate the alternative complement pathway and therefore prevent complement activation by the bacteria (Joiner, 1988; Pluschke et al., 1983; Jarvis and Vedros, 1987). The purified polyribosylribitol phosphate capsule of *Haemophilus influenzae* type b fails to activate or covalently bind C3b via the alternative pathway (Levine et al., 1983). However, the type b capsule of *H. influenzae* does not limit the deposition of C3b onto the bacterial surface either in the presence or the absence of bactericidal anti-polyribosyl ribitol phosphate (Cates et al., 1992).

Encapsulated *A. pleuropneumoniae* is resistant to the bactericidal activity of normal and immune serum (Inzana et al., 1988b; Rycroft and Cullen, 1990). However, the exact mechanism of this resistance is unclear. A spontaneous, capsule-deficient mutant of *A. pleuropneumoniae* serotype 5a strain K17 (K17-C) is killed by normal serum,

indicating the importance of capsular polysaccharide in serum resistance (Inzana et al., 1988b). We report here that capsular polysaccharide limits the deposition of C9, a component of the MAC of complement, and that an antibody specific for LPS in immune and normal serum blocks antibody-dependent complement-mediated killing of *A. pleuropneumoniae*. This blocking antibody acted by reducing and directing the deposition of C9 to sites on the bacterial surface where it was easily eluted and, therefore, not inserted hydrophobically in the outer membrane.

MATERIALS AND METHODS

Buffers. Phosphate-buffered saline containing 0.15 mM CaCl₂ and 0.5 mM MgCl₂ (PBS⁺⁺) was used to wash bacteria and as a diluent for bactericidal assays. Veronal-buffered saline (VBS) was used for immunofixation electrophoresis, and VBS containing 0.1% gelatin (VBSG) was used in complement consumption assays.

Bacterial strains and culture conditions. *A. pleuropneumoniae* serotype 5a strain K17 and its spontaneous capsule-deficient mutant, K17-C, have been described previously (Inzana and Mathison, 1987). Strain J45-C is a capsule-deficient mutant derived from serotype 5a strain J45 by ethylmethanesulfonate mutagenesis (Murchison et al., 1981). Both mutants are identical to the parent in phenotypic characteristics, except for capsular polysaccharide production (Inzana et al., 1988b; Inzana et al., 1993). No capsular polysaccharide is detected on K17-C or J45-C by inhibition radioimmunoassay (limit of detection 4 ng/ml), whereas K17 contains 155 μg capsular polysaccharide/10⁹ CFU and J45 contains 130 μg capsular polysaccharide/10⁹ CFU (Inzana et al., 1990). *H. influenzae* type b strain Eag was obtained from Porter

Anderson, University of Rochester Medical Center, Rochester, N.Y.

Several bacterial colonies grown on brain-heart infusion agar containing 5 $\mu\text{g/ml}$ NAD (BHI-N) were inoculated into BHI-N broth and grown to midlogarithmic phase (approximately 10^9 CFU/ml, determined spectrophotometrically). For most experiments, bacteria were harvested by centrifugation at $7500 \times g$ for 10 minutes, washed once with PBS, then resuspended in PBS⁺⁺ to approximately 1×10^9 CFU/ml. *H. influenzae* type b was grown as described above in BHI-N supplemented with 10 $\mu\text{g/ml}$ of solubilized hemin.

Serum complement. Normal human serum (NHS) and normal guinea pig serum (NGPS) were obtained from Diamedix Corporation (Miami, Fla.). NHS was also obtained from 4 healthy adult volunteers and pooled (PNHS). Normal swine serum (NSS) was obtained from healthy pigs at the Virginia Polytechnic Institute and State University Swine Center. Precolostral calf serum (PCS) was obtained from healthy, newborn, colostrum-deprived Holstein calves at the Virginia Polytechnic Institute and State University Dairy Center. Some sera were heated at 56°C for 30 minutes to inactivate complement.

Purification of capsular polysaccharide and LPS. The serotype 5a capsular polysaccharide of *A. pleuropneumoniae* is a polymer of α -D-N-acetyl-glucosamine linked $1 \rightarrow 5$ to 3-deoxy-D-manno-2-octulosonic acid (KDO) (Altman et al., 1987), and was purified from *A. pleuropneumoniae* J45 as previously described (Inzana, 1987). The isolated capsular polysaccharide contained 0.17% protein, 0.20% nucleic acid, and 0.01% LPS (Inzana, 1987). LPS was extracted from *A. pleuropneumoniae* K17 with hot 45% phenol and purified as described by Inzana et al. (1988a).

Antisera. Hyperimmune swine antiserum to *A. pleuropneumoniae* K17 (swine anti-K17) was obtained by immunizing a healthy pig with live K17 in PBS as previously described (Inzana and Mathison, 1987). Guinea pig (GP) antiserum to *A. pleuropneumoniae* K17 LPS (GP anti-K17 LPS) was obtained by immunizing a healthy guinea pig intramuscularly with purified LPS in PBS until an enzyme-linked immunosorbent assay (ELISA) titer greater than 1:6400 to purified LPS was obtained. The IgG fractions from swine anti-K17 serum (anti-K17 IgG), NSS (NSS IgG), and GP anti-K17 LPS serum (GP anti-K17 LPS) were obtained by affinity chromatography on a hybrid protein A/G-Sepharose column as recommended by the manufacturer (Pharmacia Biotech, Inc., Piscataway, N.J.). The IgG fraction from swine anti-K17 serum was also obtained by affinity chromatography on protein G-Sepharose 4 Fast Flow (Pharmacia). The purity of the IgG preparations (>98%) was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 2-mercaptoethanol (Laemmli, 1970).

IgG specific for the serotype 5a capsular polysaccharide (anti-capsular polysaccharide IgG) was isolated from anti-K17 IgG by affinity chromatography. Ten milligrams of serotype 5a capsular polysaccharide was dissolved in 5 ml distilled water. *N*-hydroxysulfosuccinimide (Pierce, Rockford, Ill.) was then added to a final concentration of 0.02 M, and the pH adjusted to 4.5. Water soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (Polysciences, Inc., Warrington, Pa.) was dissolved separately in distilled water to a final concentration of 0.4 M, and the pH adjusted to 4.5. Ten milliliters of preswollen AH-Sepharose 4B (Pharmacia) was combined with the capsule and carbodiimide solutions, and mixed end-over-end overnight at room temperature to couple the KDO carboxyl groups in the capsular polysaccharide to the amino groups on the resin. The resin was washed, packed into a glass column, and the column

equilibrated with PBS, pH 7. Greater than 99% of the capsular polysaccharide was successfully coupled to the column, because less than 0.01 mg of the capsular polysaccharide remained in the post-coupling washes after dialysis against water, and lyophilization. Anti-K17 serum was diluted in buffer, passed through the column, and bound IgG was eluted with 3.5 M MgCl₂. Fractions containing protein (determined by A₂₆₀) were pooled, dialyzed against PBS, and concentrated by ultrafiltration. Protein concentrations were determined by BCA Protein Assay (Pierce). The specificity of the anti-capsular polysaccharide IgG was assessed by ELISA using purified capsular polysaccharide or K17-C as the antigen.

Antibody specific for K17 LPS was removed by incubating 2.5 mg of swine anti-K17 IgG with 175 μg of K17 LPS in PBS at room temperature for one hour, then overnight at 4°C with 100 μg K17 LPS in PBS. The mixture was then clarified by centrifugation at 15,000 x g.

Serum bactericidal assay. Bacteria were diluted to approximately 5 x 10⁴ CFU/ml in PBS⁺⁺. Assay tubes contained the following proportion of reagents: 10% diluted bacteria, 50% undiluted complement source, and 40% PBS⁺⁺ containing antiserum or IgG (where indicated). Samples (20 μl) were plated in duplicate on BHI-N agar before and after incubation at 37°C. Control tubes contained heat-inactivated serum. Plates were incubated overnight at 37°C and the percent viability was determined according to the equation: (number of colonies after incubation/number of colonies before incubation) X 100.

Immunoblotting. *A. pleuropneumoniae* K17 grown in BHI-N broth (5 x 10⁸ CFU/lane) were boiled in sample buffer and electrophoresed by discontinuous SDS-

PAGE through a 10% separating gel (Laemmli, 1970). The proteins were transferred to nitrocellulose (MSI, Westboro, Mass.) overnight at 25 V as described by Towbin (1979). The membrane was incubated one hour at room temperature in Tris-buffered saline, pH 7.5, containing 1.5% skim milk to block nonspecific binding. Strips were incubated 1 hour at room temperature with serum or IgG diluted in blocking buffer, washed with Tris-buffered saline containing 0.05% Tween-20, then incubated one hour at room temperature with a 1:2000 dilution of goat anti-swine IgG, goat anti-bovine IgG, or goat anti-GP IgG conjugated to horseradish peroxidase (heavy and light chains; Jackson ImmunoResearch, West Grove, Pa.) in blocking buffer. The blots were washed in Tris buffered saline, then developed with 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, Calif.) in Tris-buffered saline containing 0.02% H₂O₂.

For LPS dot blots, 4 μ g of purified K17 LPS in 2 μ l of PBS was spotted onto a nitrocellulose membrane (MSI) and allowed to air dry. The membrane was then blocked, incubated with serum or IgG, incubated with the appropriate horseradish peroxidase-conjugated antibody, and developed as described above.

Electron microscopy. Broth grown bacteria were gently washed, then resuspended in PBS to approximately 1×10^9 CFU/ml. The bacterial suspension was incubated with diluted antibody for 1 hour at 37°C, then centrifuged at 2000 x g to pellet the bacteria. The supernatant was discarded, and the pellet was resuspended in 0.5 ml of a 1:20 dilution of protein A-20 nm gold (Polysciences) and incubated for 1 hour at 37°C. The labeled bacteria were washed once in PBS, embedded in molten agar, fixed in 2.5% glutaraldehyde/0.1 M L-lysine for 25 minutes (Akin and Rigsby, 1990; Jacques and Graham, 1989), then fixed in 2.5% glutaraldehyde for 90 minutes at room temperature. The agar-embedded bacteria were washed in 0.1 M phosphate buffer, pH 7.4, dehydrated

in a graded series to 80% ethanol, and embedded in L.R. White resin (Polysciences). Thin sections on copper grids were stained with lead citrate and uranyl acetate and viewed with a JEOL 100 CX-II transmission electron microscope.

Immunofixation electrophoresis. A modification of the method described by Johnson was used (Johnson, 1982; Inzana et al., 1987). Bacteria were resuspended to approximately 10^9 CFU/ml in VBS. Aliquots (10 μ l) of bacteria, serotype 5a capsular polysaccharide (1 mg/ml), or K17 LPS (1 mg/ml) were incubated with 50 μ l NHS for 1 hour at 37°C. Two microliters of this activated serum or VBS only was loaded onto the cathode side of a 1.2% agarose gel prepared in barbital buffer (pH 8.6) containing 0.058% calcium lactate and cast on a mylar sheet. After the gel was electrophoresed at 40 mA for 3-4 hours, a 1:2 dilution of anti-human C3 (Atlantic Antibodies, Scarborough, Maine) in 0.85% NaCl was spread onto the gel, and the gel was incubated for 1 hour at room temperature in a humid chamber. The gel was pressed, washed in 0.85% NaCl for 2 hours, dried, then stained in Coomassie Blue R-250. The percentage of C3 conversion to split products was determined using a scanning densitometer (Molecular Dynamics, Sunnyvale, Calif.). For some experiments the classical pathway was inhibited by the addition of 10 mM EGTA/7 mM MgCl₂, or both the classical and alternative pathways were inhibited by the addition of 10 mM EDTA.

Complement consumption assay. A modification of methods described by Galanos and Lüderitz (1976), and Inzana et al. (1987) were used. Midlogarithmic phase bacteria were fixed in 1% formalin for 1 hour at room temperature to inactivate the Apx toxins of *A. pleuropneumoniae*, washed twice in PBS⁺⁺, then resuspended in PBS⁺⁺ to 1×10^9 CFU/ml. Serial, two-fold dilutions of the bacterial suspension (20 μ l) were incubated

with 100 μ l of a 1:10 dilution of NGPS for 30 minutes at 37°C. The samples were then diluted to 300 μ l with VBSG, 100 μ l added to 1.5 ml of 0.7% opsonized sheep red blood cells (SRBC), and the mixture incubated at 37°C for 1 hour. The mixtures were centrifuged at 750 x g to pellet unlysed SRBC and the absorbance of the supernatant at 546 nm was determined spectrophotometrically. The 0% lysis control contained heat-inactivated NGPS, and the 100% lysis control contained distilled water in place of buffer. Complement consumption was expressed as the percent decrease in hemolysis compared to a control containing buffer and no bacteria (which resulted in 87% lysis of SRBC).

Radiolabeling and binding of C3 and C9. Purified human C3 (Diamedix), and human C9 (Sigma Chemical Co., St. Louis, Mo.) were labeled with 125 I (Amersham, Arlington Heights, Ill.) using IODO-BEADS (Pierce). Specific activities of radiolabeled proteins were 4.28×10^5 - 1.75×10^6 cpm/ μ g for C3, and 2.9×10^5 - 1.37×10^6 cpm/ μ g for C9. Binding of radiolabeled components was determined using a modification of the method described by Joiner et al. (1982a). Briefly, approximately 1×10^8 CFU/ml were incubated at 37°C with 40% NHS or PNHS for C3 and C9 binding studies. For C9 binding in 40% PCS, approximately 1×10^7 CFU/ml were used. Labeled component was added to attain between 2.4×10^5 and 1.7×10^6 cpm/ml (0.73-0.95 μ g of 125 I-C3 or 125 I-C9/ml). Swine anti-capsular polysaccharide IgG or swine anti-K17 serum were added where indicated. At designated times, 200 μ l was removed from the reaction mixture, added to 1 ml ice-cold PBS, and centrifuged at 12,000 x g for 10 minutes. The supernatant was removed, and the pellet was washed with 1 ml PBS. The 125 I in the bacterial pellet was then counted in a gamma counter (Beckman, Palo Alto, Calif.). Control tubes to assess nonspecific binding of radiolabeled complement components

contained heat-inactivated PCS or NHS. Molecules of ^{125}I -C3 or ^{125}I -C9 per CFU were calculated from the specific cpm bound/pellet, the known original CFU/pellet, the molecular weight of the component, and the specific radioactivity of the labeled component. All measurements were performed in duplicate or triplicate in at least two separate experiments.

Elution of bound ^{125}I -C9. Bacteria were incubated at 37°C in PCS containing ^{125}I -C9 for 90 minutes (the time at which maximum C9 deposition occurred) as described above. Elution of bound ^{125}I -C9 was performed by modification of a previously described method (Joiner et al., 1982b). Aliquots (200 μl) were removed, and the bacteria washed twice at 12,000 x g in 1 ml of either: 1) PBS diluted 1:5 in 5% dextrose (D-PBS), 2) PBS, 3) 1 M NaCl, or 4) 0.1% trypsin (Sigma Chemical Co.) in PBS. After the second wash, the bacteria were resuspended in 500 μl of the same solution used to wash the bacterial pellet, and incubated at 37°C for 30 minutes. The bacteria were then washed once more with the same buffer used for the incubation, and the number of ^{125}I -C9 molecules per CFU was calculated as described above.

Statistics. Data were analyzed either by Student's *t*-test, or by analysis of variance followed by a Bonferroni test to determine *p* values with the use of InStat software (GraphPad Software, San Diego, Calif.).

RESULTS

Bactericidal activity of sera. NSS and NHS were not bactericidal for *A. pleuropneumoniae* K17 even when supplemented with 5% swine anti-K17 serum or 10, 100, or 200 $\mu\text{g/ml}$ anti-capsular polysaccharide IgG (Table 2.1). In addition, NGPS was not bactericidal for K17 (data not shown). In contrast, two serotype 5a capsule-deficient mutants, K17-C and J45-C, were efficiently killed by NSS (Table 2.1), NHS (Table 2.1), and NGPS (data not shown). The killing of K17-C and J45-C in NSS as a complement source was significantly ($p \leq 0.03$) decreased by the addition of 5% swine anti-K17 serum. However, killing of K17-C and J45-C in NHS as a complement source was not decreased by addition of 5% swine anti-K17 serum. These results indicated that capsular polysaccharide was necessary for resistance of *A. pleuropneumoniae* to complement-mediated killing in normal serum.

When PCS was used as a complement source, K17 was killed in the presence of 1, 10, or 100 $\mu\text{g/ml}$ of swine anti-capsular polysaccharide IgG, or 0.5% swine anti-K17 serum (Table 2.2). Up to 1 mg/ml of swine anti-capsular polysaccharide IgG was able to kill K17 in PCS (data not shown). We also performed a dose response experiment for the killing of K17 in various concentrations of PCS in the presence of 10 $\mu\text{g/ml}$ swine anti-capsular polysaccharide IgG and obtained a predictable response curve: no killing at 10% PCS, moderate killing at 25% PCS (11% viability), and nearly total killing at 50% PCS (less than 1% viability) (data not shown). Another encapsulated serotype 5a strain, J45, was also killed in PCS in the presence of 1 $\mu\text{g/ml}$ anti-capsular polysaccharide IgG (data not shown). Killing of K17 in PCS was inhibited when the concentration of swine anti-K17 serum was increased to 10% or more in the presence or absence of 10 $\mu\text{g/ml}$ swine anti-capsular polysaccharide IgG (Table 2.2). The killing of K17 achieved in the presence of 10 $\mu\text{g/ml}$ swine anti-capsular polysaccharide IgG and

Table 2.1. Percentage of viability^a of *A. pleuropneumoniae* K17, K17-C, and J45-C in NSS and NHS^b

Serum Source	Bacterial Strain	Added Antibody Source ^c		
		None	Swine anti-K17 Serum	Swine anti-CP IgG
NSS	K17	> 100	> 100	> 100
	K17-C	2.8 ± 4.0	16.5 ± 6.0	ND ^d
	J45-C	0	16.8 ± 7.4	ND
NHS	K17	> 100	> 100	> 100
	K17-C	0	0	ND
	J45-C	0.1 ± 0.2	0	ND

^a Percentage of viability is expressed as the mean ± standard deviation of at least three experiments performed in duplicate.

^b Bacteria were incubated with the complement source for 60 minutes at 37°C.

^c Swine anti-K17 serum was used at a concentration of 5%, and swine anti-capsular polysaccharide (CP) IgG was used at 10, 100, or 200 µg/ml.

^d Not determined.

Table 2.2. Percentage of viability^a of *A. pleuropneumoniae* K17, K17-C, and J45-C in PCS^b.

Added Antibody Source	Bacterial Strains		
	K17	K17-C	J45-C
None	> 100	96 ± 9.3 ^c	3.8 ± 5.1
Swine anti-K17			
0.5%	0.2 ± 0.3	0.1 ± 0.2	0.3 ± 0.2
10%	> 100	0.8 ± 0.8	0.2 ± 0.3
30%	> 100	ND	> 100
Swine anti-CP IgG:			
1, 10, or 100 µg/ml	0.7 ± 0.5	ND	ND
Swine anti-CP IgG (10 µg/ml):			
+ 30% NSS or NGPS	0.3 ± 0.5	ND	ND
+ 10% or 30% swine anti-K17	> 100	ND	ND
+ 30% GP anti-K17 LPS	> 100	ND	ND
+ 3, 5 or 8 mg/ml swine anti-K17 IgG	> 100	ND	ND
+ 3 mg/ml GP anti-K17 LPS IgG	> 100	ND	ND
+ 3 mg/ml swine anti-K17 IgG incubated with K17 LPS	1.8 ± 2.1	ND	ND
+ 25 mg/ml NSS IgG	> 100	ND	ND
Swine anti-K17 IgG:			
5 mg/ml	ND	ND	32.8 ± 1.1 ^d
8 mg/ml	ND	ND	> 100
GP anti-K17 LPS IgG (3 mg/ml)	ND	ND	> 100
NSS IgG (25 mg/ml)	ND	ND	2.5 ± 1.7

^a Percentage of viability is expressed as the mean ± standard deviation of at least three experiments performed in duplicate.

^b Bacteria were incubated with the complement source for 120 minutes at 37°C.

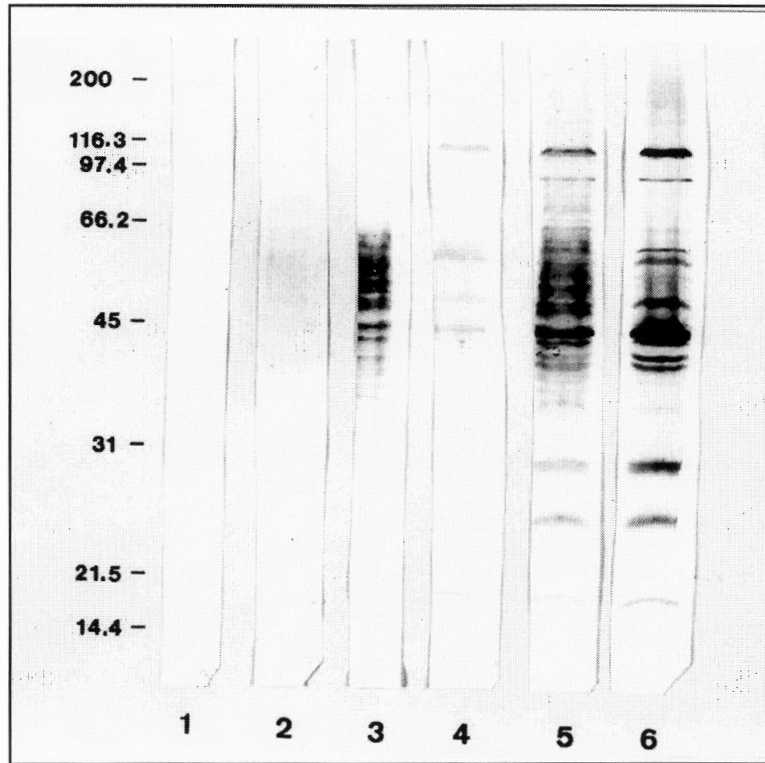
^c Some batches of PCS were able to kill K17-C, but this value reflects the killing of K17-C achieved with the majority of PCS used.

^d This value represents the mean ± standard deviation of 4 experiments.

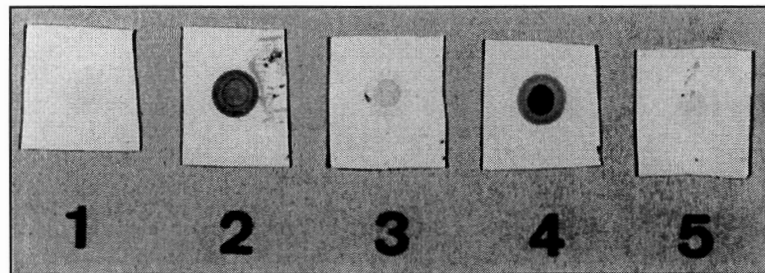
PCS was also inhibited by the addition of 3, 5, or 8 mg/ml swine anti-K17 IgG, 30% GP anti-K17 LPS serum, 3 mg/ml GP anti-K17 LPS IgG, or 25 mg/ml NSS IgG (Table 2.2). These results indicated that IgG present in normal and immune serum from several species blocked the specific anti-capsular polysaccharide complement-mediated killing of K17. This blocking antibody appeared to be specific for LPS because GP antiserum and IgG to purified LPS were very efficient at blocking killing (Table 2.2). Furthermore, all blocking activity was removed from swine anti-K17 IgG by incubating with purified K17 LPS (Table 2.2). No protein contamination of this LPS preparation was observed by BCA protein assay or SDS-PAGE followed by silver staining (data not shown). Therefore, incubation of swine anti-K17 IgG with LPS probably did not remove antibodies specific for protein components. This blocking antibody seemed to be present at a higher concentration in immune serum because smaller amounts of immune serum or immune IgG compared with normal serum or normal IgG were capable of blocking killing of K17.

Strain J45-C was killed by PCS alone, even when swine anti-K17 serum was added at a final concentration of 0.5% or 10% or when up to 25 mg/ml of NSS IgG was added (Table 2.2). The bactericidal action of PCS for J45-C was blocked by the addition of 30% swine anti-K17, 8 mg/ml swine anti-K17 IgG, or 3 mg/ml GP anti-K17 LPS IgG (Table 2.2). Therefore, it seemed that the blocking antibody specific for LPS in immune serum was also capable of blocking the killing of J45-C in PCS, but required at a higher concentration to block killing of J45-C than to block the killing of encapsulated strains.

Immunoblotting. PCS, as expected, did not contain antibodies reactive with any K17 antigens, determined by immunoblotting (Fig. 2.1A, lane 1). Antibodies in NSS and in swine anti-K17 serum reacted with ten or more K17 components ranging in molecular



A



B

Figure 2.1. Immunoblot of *A. pleuropneumoniae* K17 whole cells and dot blot of K17 LPS. A) Immunoblot of *A. pleuropneumoniae* K17 whole cells with a 1:100 dilution of PCS (lane 1), NGPS (lane 2), GP anti-K17 LPS (lane 3), NSS (lane 4), swine anti-K17 serum (lane 5), and swine anti-K17 IgG incubated with K17 LPS (lane 6). The molecular masses of markers (in kilodaltons) are indicated on the left. B) Dot blot of K17 LPS with a 1:100 dilution of NGPS (lane 1), GP anti-K17 LPS (lane 2), NSS (lane 3), swine anti-K17 IgG (lane 4), and swine anti-K17 IgG incubated with K17 LPS (lane 5).

mass from 17.8 to 191 kilodaltons (kDa) (Fig. 2.1A, lanes 4 and 5). Swine anti-K17 serum reacted more strongly and to more components than NSS. Antibodies in GP anti-K17 LPS serum and to a lesser extent NGPS, reacted with K17 components ranging in molecular mass from 33 to 66 kDa, most likely as a result of the hydrophobic association of LPS with membrane proteins (Fig. 2.1A, lanes 3 and 2, respectively). After swine anti-K17 IgG was incubated with K17 LPS, reactivity to the major components was still evident, although diminished reactivity was observed in the 33 to 66 kDa range (Fig. 2.1A, lane 6). This indicated that swine anti-K17 IgG incubated with LPS retained its reactivity with the majority of K17 whole cell proteins.

LPS dot blots demonstrated that both GP anti-K17 LPS and swine anti-K17 sera reacted strongly to K17 LPS (Fig. 2.1B, lanes 2 and 4). NGPS and NSS also reacted with K17 LPS, but to a lesser extent (Fig. 2.1B, lanes 1 and 3). Incubation of swine anti-K17 serum with LPS removed virtually all of the antibody specific for K17 LPS (Fig. 2.1B, lane 5).

A. pleuropneumoniae did not appear to express Fc receptors because colony blots did not react with peroxidase-labeled swine IgG (Fc fragment), whereas the same conjugate reacted strongly to colony blots of *Staphylococcus aureus* (data not shown). Therefore, the blocking effect observed was not caused by the nonspecific binding of IgG to *A. pleuropneumoniae* by Fc receptors.

Electron microscopy. The size and integrity of the capsular polysaccharide of *A. pleuropneumoniae* K17, and for comparison of *H. influenzae* type b, was examined by transmission electron microscopy. The capsular polysaccharide was only maintained after processing when L-lysine was used in the fixative. Immunogold electron microscopy demonstrated that antibody to capsular polysaccharide bound to *A.*

pleuropneumoniae K17 predominantly on or within the capsular polysaccharide and away from the outer membrane (Fig. 2.2A). Antibody to whole bacteria (swine anti-K17 serum) also bound most frequently in the domain of the capsular polysaccharide but was rarely observed to bind close to or on the outer membrane (Fig. 2.2B). Broth-grown K17 synthesized a thick, adherent capsule that extended 50 to 70 nm from the outer membrane. Virtually all *A. pleuropneumoniae* observed possessed this well-maintained capsule. In contrast, antibody to either the capsular polysaccharide or LPS of *H. influenzae* type b bound very close to the outer membrane and often in clumps (Fig. 2.2, C and D, respectively). The capsular polysaccharide of *H. influenzae* type b strain Eag was usually sparse, less adherent, and often not visible.

Complement activation. Immunofixation electrophoresis, was used to assess complement activation by *A. pleuropneumoniae* in NHS by measuring the conversion of C3 to split products (Table 2.3). Both K17 and K17-C converted significantly more C3 to split products than the VBS control ($p < 0.01$). Conversion of C3 occurred primarily by the alternative pathway, because NHS containing EGTA-Mg⁺⁺ did not significantly decrease conversion ($p = 0.22$ for K17, $p = 0.09$ for K17-C). Purified serotype 5a capsular polysaccharide did not convert significantly more C3 to split products than the VBS control ($p > 0.05$), whereas purified K17 LPS converted significantly more C3 to split products in both NHS and NHS EGTA-Mg⁺⁺ than the VBS control ($p < 0.001$).

Complement activation was also evaluated by measuring hemolytic complement consumption in NGPS by formalin-killed encapsulated and capsule-deficient *A. pleuropneumoniae*. In the absence of added specific antibody, both K17 and K17-C consumed similar levels of hemolytic complement over a range of bacterial concentrations (Fig. 2.3). Therefore, the capsular polysaccharide did not prevent

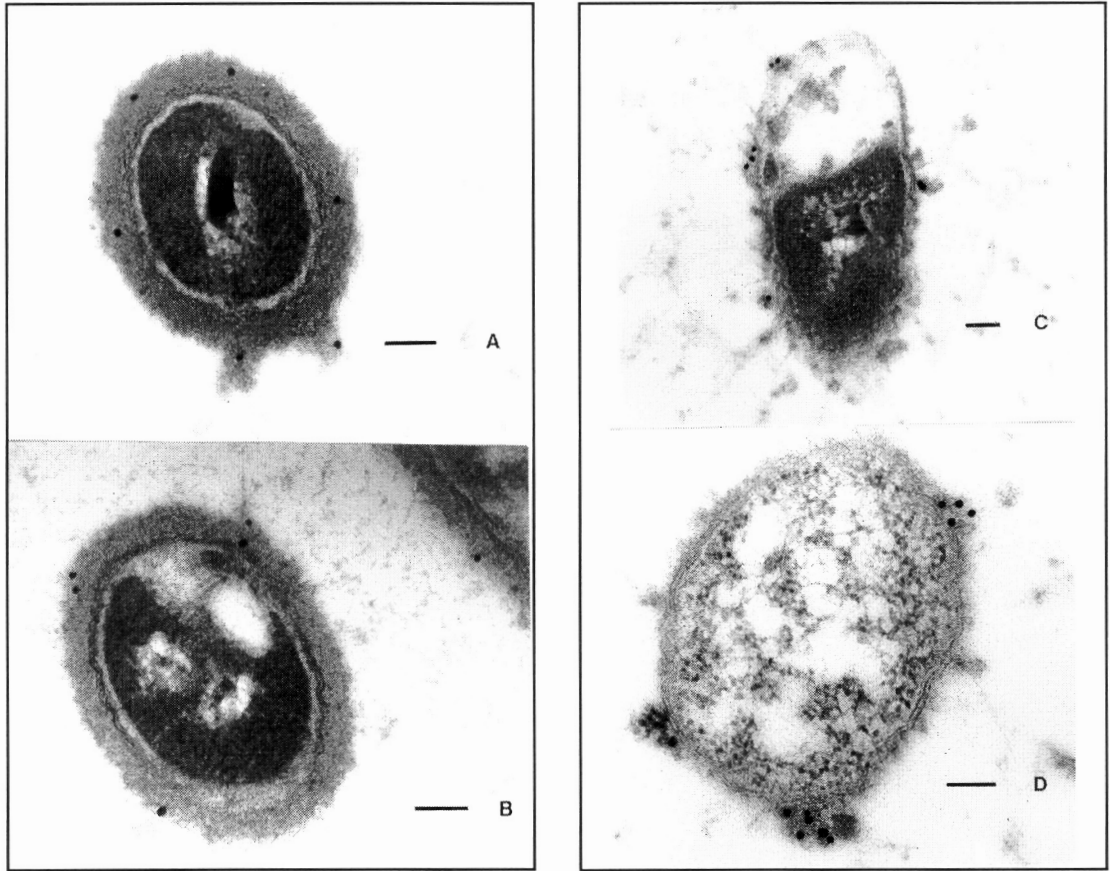


Figure 2.2. Transmission electron micrographs of *A. pleuropneumoniae* thin sections incubated with specific antibody followed by a protein A-20 nm gold conjugate then fixed in glutaraldehyde/L-lysine to preserve capsular structure. Bars represent 0.1 μm . A) *A. pleuropneumoniae* serotype 5a strain K17 incubated with antibody to capsular polysaccharide, B) *A. pleuropneumoniae* K17 incubated with swine anti-K17, C) *H. influenzae* type b strain Eag incubated with antibody to capsular polysaccharide, D) *H. influenzae* Eag incubated with antibody to LPS.

Table 2.3. Percentage of conversion of human C3 to split products by *A. pleuropneumoniae* cells and purified components in NHS^a

Sample	NHS	NHS + EGTA-Mg ⁺⁺
VBS	40.7 ± 2.1	ND
K17	67.8 ± 6.7	61.3 ± 6.8
K17-C	66.5 ± 7.5	57.3 ± 5.1
Purified CP ^b	45.9 ± 4.4	ND
Purified LPS ^b	75.7 ± 7.1	68.8 ± 4.1

^a Percent conversion was determined by scanning densitometry and is expressed as the mean ± standard deviation of three experiments.

^b Purified capsular polysaccharide (CP) and K17 LPS were used at a concentration of 1 mg/ml.

Percent Inhibition of Hemolytic Complement
by *A. pleuropneumoniae*

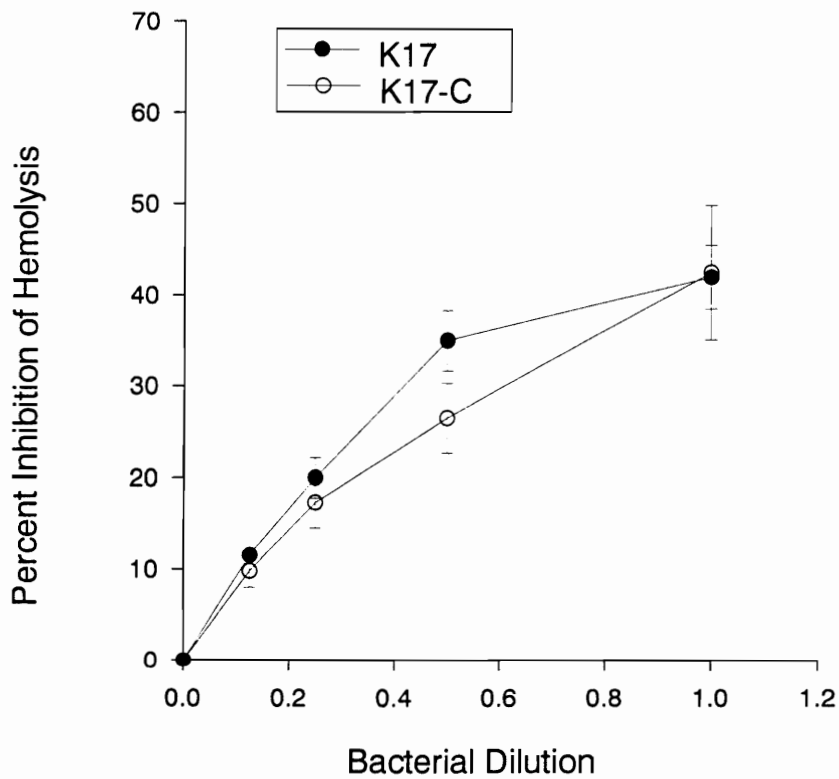


Figure 2.3. Consumption of hemolytic GP complement by *A. pleuropneumoniae* K17 and its capsule-deficient mutant, K17-C, compared with controls containing buffer and no bacteria. Bacterial concentration was expressed as the decimal value of serial two-fold dilutions of a 10^9 CFU/ml suspension. Each point represents the mean of at least six separate determinations. Error bars indicate the standard deviation of each point.

complement activation by subcapsular components of K17. However, 20 μ g of purified K17 LPS consumed 61% of the available hemolytic complement (data not shown).

Complement binding. The kinetics of ^{125}I -C3 binding to *A. pleuropneumoniae* was examined in NHS in the presence and absence of swine anti-capsular polysaccharide IgG over a period of 60 minutes (Fig. 2.4). After 30 minutes of incubation in NHS, K17-C bound significantly more ^{125}I -C3 than K17 whether or not swine anti-capsular polysaccharide IgG was present ($p < 0.001$). However, after 60 minutes incubation, K17 (in the absence of swine anti-capsular polysaccharide IgG) bound significantly more ^{125}I -C3 (429 ± 44 molecules of ^{125}I -C3/CFU) than either K17 in the presence of swine anti-capsular polysaccharide IgG (219 ± 53 molecules of ^{125}I -C3/CFU, $p < 0.001$) or K17-C (250 ± 49 molecules of ^{125}I -C3/CFU, $p < 0.01$). Because K17-C was killed in NHS after 60 minutes incubation at 37°C , it is possible that K17-C was lysed and an accurate estimation of the amount of ^{125}I -C3 bound to K17-C was not obtained. C3 was also determined to bind equally well to K17 and K17-C by immunoblotting of bacteria that had been incubated in NHS, then reacted with anti-human C3 polyclonal antibody (Cappel, Durham, N.C.) (data not shown). These results indicated that capsular polysaccharide did not prevent the deposition of C3 onto the bacterial surface and that the addition of swine anti-capsular polysaccharide IgG, which is nonbactericidal in NHS, reduced the amount of C3 bound to K17.

The kinetics of ^{125}I -C9 binding in NHS was measured to assess the binding of the MAC of complement to *A. pleuropneumoniae* (Fig. 2.5). After 60 minutes incubation, K17-C bound more ^{125}I -C9/CFU than K17 ($p < 0.001$): K17-C bound 2485 ± 45 molecules of ^{125}I -C9/CFU, whereas K17 bound 1592 ± 44 molecules of ^{125}I -C9 molecules/CFU. These data indicated that capsular polysaccharide significantly

Binding of ^{125}I -C3 to *A. pleuropneumoniae*
in Normal Human Serum

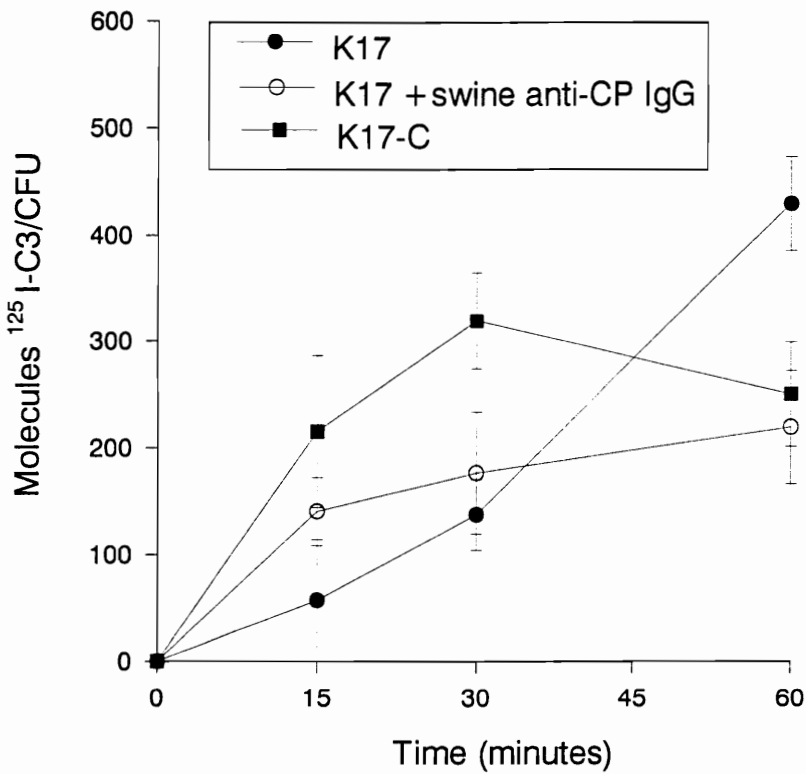


Figure 2.4. Binding of ^{125}I -C3 to *A. pleuropneumoniae* in 40% NHS. Anti-capsular polysaccharide IgG was added to a final concentration of $150\ \mu\text{g}/\text{ml}$. Of the conditions indicated in this figure, only K17-C was sensitive to killing. Total molecules of ^{125}I -C3 bound/CFU were calculated as described in *Materials and Methods* after subtracting the molecules bound/CFU in control tubes containing heat-inactivated serum.

Binding of ^{125}I -C9 to *A. pleuropneumoniae* in Normal Human Serum

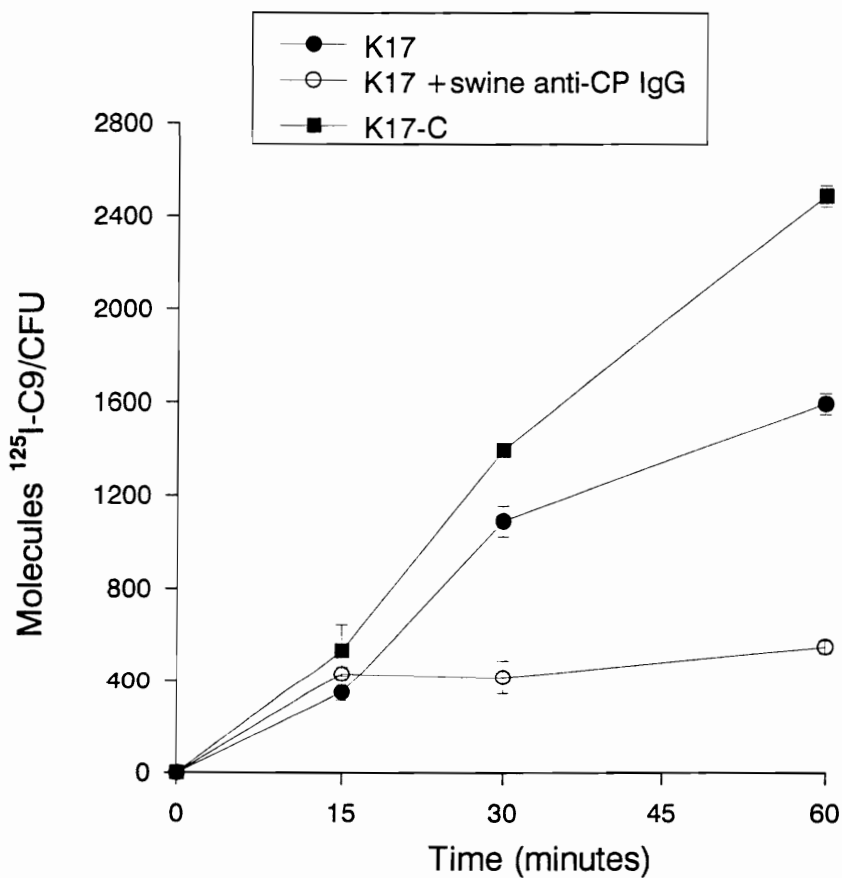


Figure 2.5. Binding of ^{125}I -C9 to *A. pleuropneumoniae* in 40% NHS. Anti-capsular polysaccharide IgG was added to a final concentration of 150 $\mu\text{g}/\text{ml}$. Of the conditions indicated in this figure, only K17-C was sensitive to killing. Total molecules of ^{125}I -C9 bound/CFU were calculated as outlined in the legend for Figure 2.4.

decreased but did not prevent the binding of ^{125}I -C9 to *A. pleuropneumoniae* in NHS. The amount of ^{125}I -C9 bound to K17 was further decreased to 553 ± 28 molecules of ^{125}I -C9/CFU when swine anti-capsular polysaccharide IgG, which is nonbactericidal in NHS, was added to a final concentration of $150 \mu\text{g/ml}$ ($p < 0.001$). This finding was similar to the reduction of ^{125}I -C3 bound to K17 when the same concentration of specific antibody to capsular polysaccharide was added.

The binding of ^{125}I -C9 was also examined in PCS to evaluate binding under conditions in which encapsulated *A. pleuropneumoniae* was killed (anti-capsular polysaccharide IgG added) or not killed (anti-capsular polysaccharide IgG absent) (Fig. 2.6). After 90 minutes of incubation, K17 in the absence of anti-capsular polysaccharide IgG bound relatively little ^{125}I -C9 compared to the capsule-deficient mutants K17-C and J45-C: K17 bound 297 ± 121 molecules of ^{125}I -C9/CFU, K17-C bound 771 ± 59 molecules of ^{125}I -C9/CFU, and J45-C bound 1021 ± 68 molecules of ^{125}I -C9/CFU. Therefore, capsular polysaccharide significantly limited the deposition of ^{125}I -C9 onto the bacterial surface ($p < 0.01$). When swine anti-capsular polysaccharide IgG was added at a concentration of $100 \mu\text{g/ml}$, the amount of ^{125}I -C9 bound to K17 was increased four-fold to 1299 ± 80 molecules of ^{125}I -C9/CFU ($p < 0.001$). This result was consistent with the bactericidal activity of PCS and swine anti-capsular polysaccharide IgG for strain K17 (Table 2.2). However, when swine anti-K17 serum was added (as a source of blocking antibody) to swine anti-capsular polysaccharide IgG and PCS, the amount of ^{125}I -C9 bound to K17 was limited to approximately 800 molecules of ^{125}I -C9/CFU after 30 minutes of incubation. After 90 of minutes incubation, the amount of bound ^{125}I -C9 decreased to approximately 650 molecules ^{125}I -C9/CFU ($p < 0.01$). These results indicated that although capsular polysaccharide was the predominant factor responsible for limiting C9 deposition in the absence of specific antibody, blocking antibody in

Binding of ^{125}I -C9 to *A. pleuropneumoniae* in Precolostral Calf Serum

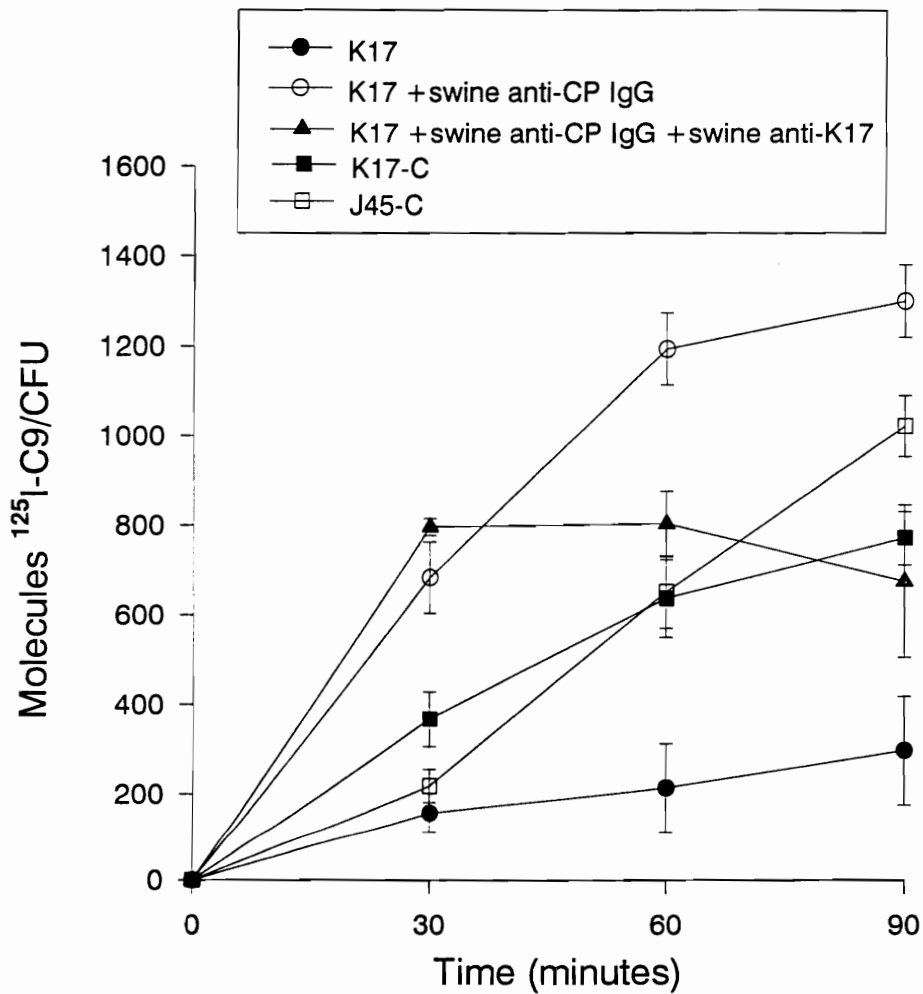


Figure 2.6. Binding of ^{125}I -C9 to *A. pleuropneumoniae* in 40% PCS. Anti-capsular polysaccharide IgG and swine anti-K17 were added to final concentrations of 100 $\mu\text{g}/\text{ml}$ and 30%, respectively. Of the conditions indicated in this figure, only K17 + swine anti-capsular polysaccharide IgG, and J45-C were sensitive to killing. Total molecules of ^{125}I -C9/CFU were calculated as outlined in the legend for Figure 2.4.

immune serum also reduced the amount of ^{125}I -C9 bound to *A. pleuropneumoniae*.

Elution of bound ^{125}I -C9. To evaluate the interaction of the MAC with the *A. pleuropneumoniae* membrane, we examined the elution of ^{125}I -C9 bound to the bacterial surface in PCS under bactericidal and nonbactericidal conditions by PBS, D-PBS, 1 M NaCl, or 0.1% trypsin (Fig. 2.7). After incubation in D-PBS, a low ionic strength buffer, less than 2% of bound ^{125}I -C9 was released from K17 in the presence of 100 $\mu\text{g}/\text{ml}$ anti-capsular polysaccharide IgG (bactericidal conditions), or K17 in the presence of 100 $\mu\text{g}/\text{ml}$ anti-capsular polysaccharide IgG and 30% anti-K17 serum as a source of blocking antibody (nonbactericidal conditions). More ^{125}I -C9 was released when the bacteria were incubated in PBS (a slightly higher ionic strength buffer), although the differences in eluted ^{125}I -C9 under bactericidal ($11 \pm 9.8\%$) and nonbactericidal conditions ($21 \pm 6\%$) were not significant. The ^{125}I -C9 bound to K17 in the presence of both anti-capsular polysaccharide IgG and swine anti-K17 serum (nonbactericidal conditions) was eluted significantly more by 1 M NaCl ($67 \pm 12\%$) or 0.1% trypsin ($69 \pm 4\%$) than ^{125}I -C9 bound to K17 in the presence of anti-capsular polysaccharide IgG (bactericidal conditions) by 1 M NaCl ($24 \pm 7.5\%$, $p < 0.01$) or 0.1% trypsin ($38 \pm 4.4\%$): $p < 0.001$. Furthermore, the ^{125}I -C9 bound to K17 in the presence of both anti-capsular polysaccharide IgG and swine anti-K17 (nonbactericidal conditions) serum was eluted significantly more by 1 M NaCl or 0.1% trypsin than by either PBS ($p < 0.01$) or D-PBS ($p < 0.001$). These data indicated that under conditions where encapsulated *A. pleuropneumoniae* was not killed (in the presence of blocking antibody), the ^{125}I -C9 bound to the bacterial surface was susceptible to elution by salt and trypsin.

Elution of ^{125}I -C9 Bound to *A. pleuropneumoniae* in Precolostral Calf Serum

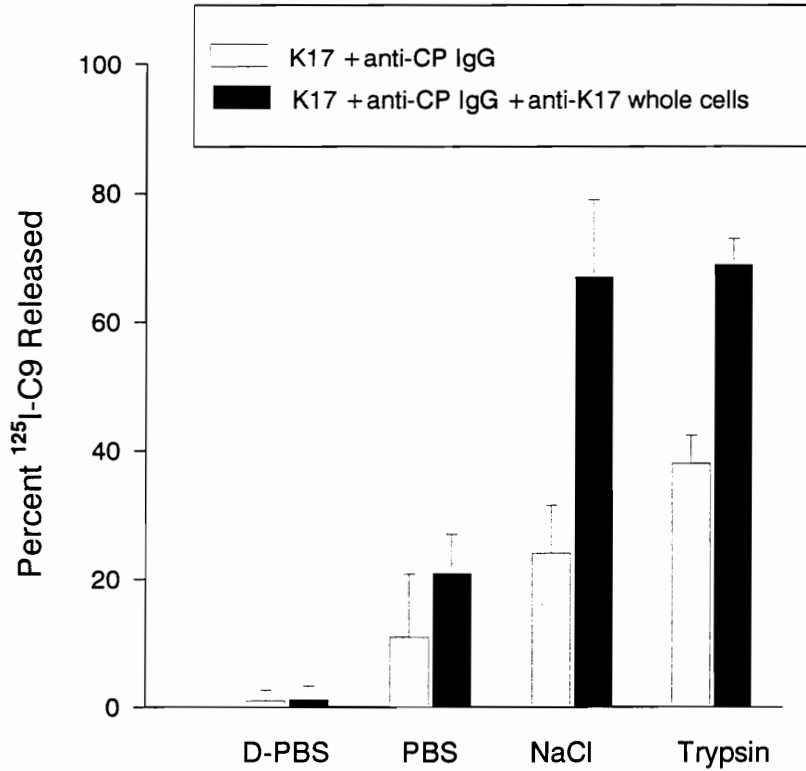


Figure 2.7. Percentage of ^{125}I -C9 bound to *A. pleuropneumoniae* in 40% PCS released after washing and incubation in D-PBS, PBS, 1 M NaCl, or 0.1% trypsin. Anti-capsular polysaccharide IgG and swine anti-K17 were added to final concentrations of 100 $\mu\text{g}/\text{ml}$ and 30%, respectively. Bars represent the mean of three separate experiments. Vertical error bars represent the standard deviation for each group of values. Bacteria were prepared for elution experiments as described in *Materials and Methods*.

DISCUSSION

The complement system is essential for adequate host defense against bacterial infections. Many bacteria that cause infections in man and animals are resistant to complement-mediated killing (Joiner, 1988). For many encapsulated bacteria, such as *H. influenzae* type b, resistance to complement-mediated killing can be overcome by the host's production of antibodies to the capsule, which promote complement-mediated bactericidal activity and opsonization (Robbins, 1978; Tarr et al., 1982). Although complement-mediated opsonization may be more important in overcoming *H. influenzae* type b infection, serum bactericidal activity may also play a role (Noel et al., 1987).

The swine respiratory pathogen, *A. pleuropneumoniae*, is resistant to killing by complement even in the presence of antibody to capsule or somatic antigens (Inzana et al., 1988b; Rycroft and Cullen, 1990). However, a capsule-deficient mutant of *A. pleuropneumoniae* is easily killed by normal serum in the absence of specific antibody (Inzana et al., 1988b). I investigated the mechanism of this resistance in an effort to understand the molecular basis for this virulence property and have identified two distinct mechanisms that contribute to serum resistance in this bacterium.

Encapsulated *A. pleuropneumoniae* was not killed in any of the normal sera tested as a complement source, even in the presence of specific antibody to whole bacteria or affinity-purified antibody to capsular polysaccharide. The capsular polysaccharide appeared to confer serum resistance to *A. pleuropneumoniae* because two capsule-deficient mutants were killed in normal sera. *A. pleuropneumoniae* strains K17 and J45 are phenotypically identical to their respective capsule-deficient mutants, K17-C and J45-C, except for the production of capsular polysaccharide (Inzana and Mathison, 1987; Inzana et al., 1993; Inzana et al., 1990). Therefore, differences in serum sensitivity could not be attributed to differences in LPS structure, which are known to be associated

with serum resistance in other gram-negative bacteria (Joiner et al., 1982a; Joiner et al., 1982b; Joiner et al., 1984; Schiller and Joiner, 1986).

The integrity of the *A. pleuropneumoniae* capsular polysaccharide was examined by electron microscopy to determine whether capsular polysaccharide could prevent specific antibody from reaching the outer membrane of the bacteria. The capsular polysaccharide of *A. pleuropneumoniae* K17 was thick and well adhered to the cell compared to the relatively less adherent capsule of *H. influenzae* type b, a bacterium that is efficiently killed by antibody to capsule and complement (Joiner, 1988; Tarr et al., 1982). Previous electron micrographs demonstrate that *H. influenzae* type b strain Tb-EN produces a relatively thick capsule when homologous antiserum is used for stabilization (Buckmire, 1982). Therefore, the appearance of the *H. influenzae* type b capsule during electron microscopy is dependent upon the method used for stabilization. We determined that the thickness of the K17 capsule after fixation in glutaraldehyde/L-lysine was 50-70 nm, which differed from the thickness of the K17 capsule reported by Jacques et al. (1988) after stabilization with polycationic ferritin (< 10 nm) or homologous antisera (140-150 nm). These differences indicate that capsule thickness observed by electron microscopy is dependent upon the technique used to preserve capsule structure. Therefore, we can only conclude that the *H. influenzae* type b capsular polysaccharide is less adherent to the cell compared to the *A. pleuropneumoniae* capsule under the conditions we used to stabilize the capsule.

Immunogold electron microscopy demonstrated that antibodies to capsular polysaccharide or LPS were bound near or on the outer membrane of *H. influenzae* type b, usually in clumps. Such clumping and proximity of bactericidal antibody to the membrane may be important for bactericidal activity. In contrast, specific antibody to capsular polysaccharide or K17 whole cells bound predominately within the domain of

the capsule and was rarely observed to bind near the outer membrane of *A. pleuropneumoniae* K17. Therefore, capsular polysaccharide may prevent specific antibody from directing the complement MAC onto the outer membrane, or capsular polysaccharide may reduce the efficiency of antibody-mediated complement fixation onto the outer membrane of *A. pleuropneumoniae*. Antibody is known to be important for bactericidal activity because capsule-deficient *A. pleuropneumoniae* is much more efficiently killed in normal rather than precolostral swine serum (Inzana et al., 1988b) and antibody to *H. influenzae* type b capsular polysaccharide is required for killing by the alternative pathway (Tarr et al., 1982). The less adherent capsule of *H. influenzae* type b may allow more specific antibody to reach the membrane, resulting in more efficient killing (Tarr et al., 1982). However, it is possible that the protein A-gold conjugate may have been too large to penetrate the *A. pleuropneumoniae* capsule to react with antibodies at the cell surface.

Bacterial capsules may confer serum resistance in the absence of specific antibody by preventing complement activation (Jarvis and Vedros, 1987; Joiner, 1988; Pluschke et al., 1983) or deposition (Joiner, 1988; Levine et al., 1983) onto bacteria. Therefore, I examined complement activation by *A. pleuropneumoniae* and complement deposition onto the bacterial surface by two independent methods. The serotype 5a capsular polysaccharide did not prevent or reduce complement activation, as determined by the conversion of C3 to split products and consumption of hemolytic complement by K17 and its capsule-deficient mutant. In addition, both K17 and K17-C bound similar levels of C3b, determined by radiolabeling and immunoblotting, indicating that the capsular polysaccharide also did not prevent or reduce C3b deposition. These results were consistent with those of Udeze and Kadis (1992b) who reported that *A. pleuropneumoniae* serotype 1 strain 4074 activated complement and efficiently bound C3. In contrast, I

determined that capsular polysaccharide substantially limited the amount of C9, a component of the MAC, bound to encapsulated *A. pleuropneumoniae* in PCS (in the absence of any antibody). Therefore, capsular polysaccharide appeared to limit deposition of the MAC on *A. pleuropneumoniae* in the absence of added antibody. However, capsular polysaccharide did not prevent the binding of C9 to encapsulated *A. pleuropneumoniae* in NHS, although this binding of C9 was not associated with killing. The decreased binding of C3 and C9 to *A. pleuropneumoniae* in the presence of swine anti-capsular polysaccharide IgG and NHS may be a result of fewer sites available for C3 deposition on the bacterial surface once anti-capsular polysaccharide IgG is bound.

I also present evidence for the existence of a blocking antibody in normal and immune serum that prevented anti-capsular polysaccharide IgG dependent complement-mediated killing of *A. pleuropneumoniae*. This blocking antibody seemed to be an IgG specific for the LPS of *A. pleuropneumoniae*. Swine anti-K17 serum, swine anti-K17 IgG, NSS IgG, and GP anti-K17 LPS IgG were highly effective in blocking the bactericidal activity of anti-capsular polysaccharide IgG for K17 in PCS, an antibody-free complement source. A higher concentration of NSS IgG (25 mg/ml) than swine anti-K17 IgG (5 mg/ml) was required to achieve blocking in PCS and anti-capsular polysaccharide IgG, and 30% NSS and NGPS were unable to block killing. Thus, as would be expected, immune swine serum contained a higher concentration of the blocking antibody than normal serum. The blocking antibodies in normal sera were most likely cross-reacting antibodies generated by exposure to the LPS of other bacteria. The blocking phenomenon I observed did not seem to be the result of a prozone effect since I was able to specifically remove all blocking activity from swine anti-K17 serum by incubating with purified K17 LPS to remove LPS-specific antibodies. This LPS-absorbed serum did not react with K17 LPS on a dot blot, but maintained reactivity to K17 whole cell

proteins as determined by immunoblotting.

My results are similar to those of Corbeil et al. (1988) who reported that an IgG specific for smooth LPS of *Brucella abortus* blocked killing. However, the specificity of this blocking antibody is in contrast to Udeze and Kadis (1992b) who reported that incubation of normal swine serum with column fractions of a saline extract containing either a 27 or 32 kDa *A. pleuropneumoniae* serotype 1 protein removed blocking activity, whereas incubation with LPS did not. It is likely that the 27 and 32 kDa proteins they described are associated with LPS so that incubation of serum with these proteins resulted in the removal of antibodies to LPS as well. These authors attempted to remove only antibodies specific for LPS by incubating normal swine serum with 10-500 μg LPS for 1 hour at 4°C. It is possible that a more extensive incubation was required to sufficiently remove antibodies specific for LPS, or that antibodies specific for *A. pleuropneumoniae* serotype 1 LPS do not block complement-mediated killing of serotype 1 strains as antibodies specific for the serotype 5a LPS block killing of serotype 5a strains.

I could not demonstrate the presence of Fc receptors on *A. pleuropneumoniae*, which supports the concept that blocking antibodies are directed to specific antigens, and are not bound nonspecifically to Fc receptors. The blocking activity I observed could be diluted out of immune serum, confirming that the blocking effect was quantitative: K17 was killed in PCS containing 0.5% hyperimmune swine serum, but killing was blocked at higher concentrations. I also determined that swine anti-K17 IgG and GP anti-K17 LPS, but not NSS IgG, were able to block the killing of capsule-deficient *A. pleuropneumoniae* strain J45-C. Thus, it appeared that a larger amount of anti-LPS antibody was required to block killing of noncapsulated *A. pleuropneumoniae*. It is possible that in the absence of a capsule, which helps to limit the deposition of C9, more

blocking antibody may be needed to overcome the bactericidal effects of complement. Therefore, it is likely that capsular polysaccharide and blocking antibody present in normal and immune serum may act synergistically to inhibit the bactericidal activity of serum for *A. pleuropneumoniae*.

The presence of antibodies in normal or immune sera that block the bactericidal activity of specific antibody has been reported for *Neisseria gonorrhoeae* (Frank, 1992; Joiner et al., 1985), *N. meningitidis* (Jarvis and Griffiss, 1991), and *Brucella abortus* (Corbeil et al., 1988). These blocking antibodies apparently act by directing the deposition of complement components to nonbactericidal locations rather than by decreasing complement activation or deposition (Frank, 1992). In fact, blocking antibodies have been reported to actually increase the binding of C9 to *N. gonorrhoeae* prior to incubation with bactericidal antibody (Joiner et al., 1985). In contrast, we determined that blocking antibody present in immune swine serum decreased the amount of ¹²⁵I-C9 bound to *A. pleuropneumoniae* K17 in PCS in the presence of anti-capsular polysaccharide IgG after 60 minutes incubation. The ¹²⁵I-C9 bound to K17 in PCS in the presence of both blocking antibody and anti-capsular polysaccharide IgG was significantly more easily eluted by 1 M NaCl and 0.1% trypsin than ¹²⁵I-C9 bound to K17 in the absence of blocking antibody. This finding indicated that the ¹²⁵I-C9 and presumably the MAC was not bound hydrophobically to the bacterial surface or in a form that was resistant to protease release. This result is in agreement with Joiner et al. (1982b; 1984) who reported that bound, nonbactericidal C5b-9 (MAC) is easily eluted by salt or trypsin from the surface of serum resistant *E. coli*.

Throughout these studies, complement sources, antibodies, and purified complement components from different species were used, which raises concerns about the compatibility and comparability of these reagents. Swine antibodies and purified human

C9 were clearly able to interact with PCS since we observed killing of *A. pleuropneumoniae* in PCS by swine anti-capsular polysaccharide IgG, and binding of ¹²⁵I-C9 to the bacteria. Therefore, the blocking antibody in swine anti-K17 serum did not appear to be a species-incompatible antibody. Because all complement sources from normal sera we tested displayed similar bactericidal activity against both encapsulated and noncapsulated *A. pleuropneumoniae*, I believe that these sera also displayed similar complement activation and complement binding capabilities and can be compared to one another.

In summary, two mechanisms of resistance to complement-mediated killing may be uniquely combined in *A. pleuropneumoniae*. In normal serum in the absence of specific antibody, capsular polysaccharide limited the amount of antibody and C9 deposited on the bacterial surface but did not prevent complement activation or limit binding of C3 to the bacteria. In addition, blocking antibodies specific for LPS in normal and immune sera contributed to the serum resistance of *A. pleuropneumoniae* by also decreasing the amount of C9 bound to the bacteria and possibly directing the deposition of C9 and therefore the MAC to nonbactericidal sites. It is interesting to note that when a potentially bactericidal antibody, swine anti-capsular polysaccharide IgG, was added to normal serum containing blocking antibody, the amount of C3 and C9 deposited on *A. pleuropneumoniae* was significantly reduced compared to the amount deposited in the presence of serum alone. These two mechanisms of serum resistance may also act at different stages of infection to limit the ability of complement to eliminate *A. pleuropneumoniae in vivo*. The extent of participation of the complement system in defense against *A. pleuropneumoniae* infection has not been established. It is likely that complement-mediated opsonization may have a more significant role in the clearance of this bacterium. However, these results indicate that the potential for the induction of

blocking antibodies should be a concern in the development of vaccines for the control of swine pleuropneumonia.

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CHAPTER 3

Identification and Characterization of a DNA Region Involved in the Export of the Capsular Polysaccharide of *Actinobacillus pleuropneumoniae* Serotype 5a²

ABSTRACT

Actinobacillus pleuropneumoniae synthesizes a serotype-specific capsular polysaccharide that protects this bacterium from phagocytosis and complement-mediated killing. I was interested in identifying the genes involved in *A. pleuropneumoniae* capsular polysaccharide biosynthesis and export. A 5.3 kilobase *Xba*I fragment of *A. pleuropneumoniae* serotype 5a J45 genomic DNA that hybridized with two DNA probes specific for the *Haemophilus influenzae* type b capsulation locus was cloned and partially sequenced. This *A. pleuropneumoniae* DNA fragment encoded four open reading frames, designated *cpxDCBA*. The nucleotide and predicted amino acid sequences of *cpxDCBA* were highly homologous to *H. influenzae* type b *bexDCBA* and *Neisseria meningitidis* group B *ctrABCD*. Homology was also observed between *cpxDCBA* and *Escherichia coli* K1 and K5 *kpsED*, and *kpsMT*. The *bexDCBA*, *ctrABCD*, *kpsED*, and *kpsMT* genes are involved in capsular polysaccharide export. CpxD may be an outer membrane lipoprotein involved in capsular polysaccharide transport across the outer membrane, whereas CpxC, CpxB and CpxA appeared to be components of an ATP-binding cassette transporter required for capsular polysaccharide transport across the inner membrane. The *cpxDCBA* gene cluster was able to partially complement

² This chapter has been submitted for publication to *Journal of Bacteriology*.

kpsM::TnphoA or *kpsT::TnphoA* mutations within a plasmid-encoded *E. coli* K5 *kps* locus and restore sensitivity to a K5-specific bacteriophage, indicating that *cpxDCBA* functioned in capsular polysaccharide export. A *cpxCB* probe hybridized to genomic DNA from all *A. pleuropneumoniae* serotypes tested (1, 2, 5, 7, and 9) indicating that this DNA is conserved among serotypes. These data indicated that we successfully cloned a portion of the *A. pleuropneumoniae* capsulation locus involved in capsular polysaccharide export.

INTRODUCTION

Actinobacillus pleuropneumoniae is an economically important respiratory pathogen of swine. This gram-negative bacterium is surrounded by a serotype-specific, negatively charged capsular polysaccharide that protects it from phagocytosis and complement-mediated killing (Inzana and Mathison, 1987; Inzana et al., 1988b; Ward and Inzana, 1994). Currently, 12 serotypes of *A. pleuropneumoniae* are recognized (Nielsen, 1986). Serotypes vary in virulence (Rosendal et al., 1985) and distribution around the world, making the capsular polysaccharide an important antigen for pathogenic, diagnostic, and epidemiologic studies. The capsular polysaccharide of *A. pleuropneumoniae* is considered to be a major virulence determinant. Noncapsulated mutants isolated after ethyl methanesulfonate mutagenesis are avirulent and do not cause clinical symptoms or the lung lesions characteristic of the disease even when administered at a dose ten times greater than the LD₅₀ of the encapsulated parent strain (Inzana et al., 1993). However, the genomic location, organization, and mechanism of cell-surface expression of this important virulence determinant are unknown.

Capsular polysaccharide biosynthesis and export are complex, multistep processes that

require the participation of many genes within a large gene cluster (Whitfield and Valvano, 1993). The genetic organization of the group II capsule gene loci (Jann and Jann, 1990) of *Haemophilus influenzae* (Kroll et al., 1989; Kroll 1992), *Escherichia coli* (Boulnois et al., 1987; Roberts et al., 1988), and *Neisseria meningitidis* (Frosch et al., 1989) have been studied and are reported to be quite similar (Boulnois and Roberts 1990; Frosch et al., 1991). In each of these species a central, serotype-specific DNA segment necessary for capsular polysaccharide biosynthesis is flanked by DNA required for capsular polysaccharide export. DNA required for capsular polysaccharide export is conserved among serotypes of a given species (Frosch et al., 1989; Kroll et al., 1989; Roberts et al., 1988). In addition, substantial homology is observed among genes required for capsular polysaccharide export to the bacterial surface among different bacterial species expressing group II capsules. Thus, there appears to be a common evolutionary origin for group II capsule export in gram-negative bacteria (Frosch et al., 1991). The *A. pleuropneumoniae* capsular polysaccharides, like the group II capsules of *E. coli*, *H. influenzae* type b, and *N. meningitidis* group B (Jann and Jann, 1990), are co-expressed with many O-antigens, and often contain 3-deoxy-D-manno-2-octulosonic acid (KDO) or phosphate. Therefore, the *A. pleuropneumoniae* capsulation locus may share a similar genetic organization and mechanism of capsular polysaccharide export with *H. influenzae* type b and other bacteria that express group II capsules. The *A. pleuropneumoniae* serotype 5a capsular polysaccharide is an unbranched polymer of a repeating disaccharide unit comprised of α -D-N-acetylglucosamine linked (1 \rightarrow 5) to KDO (Altman et al., 1987). However, extensive physical characterization (e.g. identification of the associated lipid moiety, temperature regulation of expression, and CMP-KDO synthetase activity) beyond the actual carbohydrate structure of the serotype 5a capsular polysaccharide has not been reported. In this chapter, we report using

conserved regions of the *H. influenzae* type b *cap* (*capb*) locus involved in capsular polysaccharide export to identify, clone, and characterize a portion of the *A. pleuropneumoniae* serotype 5a capsulation locus involved in capsular polysaccharide export.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains, plasmids, and bacteriophage used in this study are described in Table 3.1. Plasmids pSKH1 and pSKH2 were provided by S.K. Hoiseth (Praxis Biologics, Rochester, N.Y.). Plasmids pPC6::6, pPC6::17, *E. coli* MS101, and K5-specific bacteriophage were provided by I.S. Roberts (University of Leicester, England). *A. pleuropneumoniae* strains were provided by: B. Fenwick (Kansas State University, Manhattan), serotype 5a strains J45 and K17; L. Hoffman (Iowa State University, Ames), serotype 7 strain 29628; M. Mulks (Michigan State University, East Lansing), serotype 5 strain 178; and J. Nicolet (University of Berne, Switzerland), serotype 9 strain 13261. *A. pleuropneumoniae* serotype 1 strain 4074 and serotype 2 strain 1536 were obtained from the American Type Culture Collection (ATCC; Rockville, Md.). *A. pleuropneumoniae* strains were grown with shaking at 37°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 5 µg/ml nicotinamide adenine dinucleotide. *E. coli* strains were grown in Luria-Bertani (LB) broth (Sambrook et al., 1989) for routine cultivation, or in Terrific broth (Tartof and Hobbes, 1987) for extraction of plasmids. Antibiotics were used in growth media for maintenance of plasmids at the following concentrations: ampicillin (Amp), 100 µg/ml; chloramphenicol (Cm), 30 µg/ml; streptomycin (Sm), 80 µg/ml; tetracycline (Tet), 25 µg/ml.

Table 3.1. Bacterial strains, plasmids, and bacteriophage used in Chapter 3.

Strain, plasmid or bacteriophage	Relevant genotype or characteristics	Source or reference
<i>A. pleuropneumoniae</i> Strains		
4074	serotype 1 strain; (ATCC 27088)	ATCC
1536	serotype 2 strain; (ATCC 27089)	ATCC
J45	serotype 5a strain	Fenwick et al., 1986a
K17	serotype 5a strain	Nielsen, 1986a
178	serotype 5 strain	M. Mulks
29628	serotype 7 strain	L. Hoffman
13261	serotype 9 strain	J. Nicolet
<i>E. coli</i> Strains		
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac (F⁺ proAB lacI^qZΔM15 Tn10)</i> ; Host for recombinant plasmids	Stratagene, La Jolla, Calif.
MS101	<i>serA⁺ rpsL K5⁺; Sm^r</i>	Stevens et al., 1994
Plasmids		
pGEM-3Z	Cloning vector, 2.74 kb; Amp ^r	Promega
pCW-1C	5.3 kb <i>Xba</i> I fragment of J45 cloned into pGEM-3Z	This chapter
pCW-5E	Same insert as pCW-1C, except cloned in opposite orientation	This chapter
pSKH1	4.4 kb <i>Eco</i> RI fragment of <i>H. influenzae capb</i> locus cloned into pBR328; Amp ^r , Tet ^r	Hoiseth et al., 1985
pSKH2	9.0 kb <i>Eco</i> RI fragment of <i>H. influenzae capb</i> locus cloned into pBR328; Amp ^r , Tet ^r	Hoiseth et al., 1985
pPC6::6	Contains the entire <i>E. coli</i> K5 <i>kps</i> locus with a <i>TnphoA</i> insertion in <i>kpsT</i> ; Cm ^r	Pearce and Roberts, 1995
pPC6::17	Contains the entire <i>E. coli</i> K5 <i>kps</i> locus with a <i>TnphoA</i> insertion in <i>kpsM</i> ; Cm ^r	Pearce and Roberts, 1995
Bacteriophage		
K5	Specific for strains expressing the <i>E. coli</i> K5 capsular polysaccharide	I.S. Roberts

Enzymes and reagents. Restriction endonucleases and T4 DNA ligase were purchased from Promega Corporation (Madison, Wis.), 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) and isopropylthio- β -galactopyranoside (IPTG) were purchased from Gold Biotechnology, Inc. (St. Louis, Mo). All other reagents were obtained from Sigma Chemical Company (St. Louis, Mo), except where indicated.

Recombinant DNA methods. Genomic DNA was isolated from broth grown *A. pleuropneumoniae* whole cells by a method described by S. Spinola (personal communication). Briefly, bacteria were resuspended in 10 mM Tris-1 mM EDTA (pH 8.0) and incubated with sodium dodecyl sulfate (0.66%), and RNase (100 μ g/ml) for 1 hour at 37°C. Proteinase K was added to a final concentration of 100 μ g/ml, and the mixture was incubated at 56°C for 1 hour. The mixture was then extracted once with buffered phenol, and four times with buffered phenol-chloroform (Amresco, Inc., Solon, Ohio), and the genomic DNA was ethanol precipitated and resuspended in 10 mM Tris-1 mM EDTA (pH 8.0). Plasmid DNA was isolated by a rapid alkaline lysis method (Ish-Horowicz and Burke, 1981). Restriction fragments required for cloning and probe synthesis were eluted from agarose gels as described (Zhen and Swank, 1993). Restriction digests, agarose gel electrophoresis, and DNA ligations were performed as previously described (Sambrook et al., 1989). Plasmid DNA was transformed into *E. coli* strains by electroporation (Dower et al., 1988) using a BTX ECM 600 electroporator (BTX, Inc., San Diego, Calif.).

DNA hybridization analysis. Restriction endonuclease-digested DNA was transferred by capillary action from 0.7% agarose gels to MagnaGraph nylon membranes (Micron Separations Inc., Westboro, Mass.) using 20X saline sodium citrate (20X SSC

is 3 M NaCl, 300 mM sodium citrate, pH 7) as previously described (Sambrook et al., 1989; Southern, 1975). DNA was covalently linked to nylon membranes by ultraviolet irradiation using a UV Stratalinker (Stratagene, La Jolla, Calif.). Digoxigenin-labeled probes for DNA hybridizations were synthesized by the random primer method using the Genius System nonradioactive labeling and detection kit (Boehringer-Mannheim Corp., Indianapolis, Ind.) according to the manufacturer's directions. *E. coli* colonies containing recombinant plasmids were screened by colony hybridization analysis using digoxigenin-labeled DNA probes according to the Genius System directions. DNA hybridizations were performed at either 55°C or 68°C, as indicated, in solutions containing 5X SSC. The membranes were washed and developed according to the Genius System directions for colorimetric detection.

DNA sequencing and analysis. The nucleotide sequence of both strands of a 4.6 kilobase (kb) *XbaI-ClaI* DNA fragment of pCW-1C was determined by the dideoxy chain-termination method (Sanger et al., 1977) using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio) with α [³⁵S]dATP (DuPont/NEN Research Products, Boston, Mass.). Double stranded DNA templates were sequenced using custom, oligonucleotide primers (DNAgency, Inc., Malverne, Pa.) to continue reading along each strand.

The nucleotide sequence obtained was analyzed using DNASTAR analysis software (DNASTAR, Inc, Madison, Wis.). Sequence similarity searches of the EMBL/GenBank/DDBJ databases were performed using BLAST software (Altschul et al., 1990) at the National Center for Biotechnology Information (Bethesda, Md.).

Sensitivity to capsule-specific bacteriophage. The sensitivity of *E. coli* strains to bacteriophage specific for the *E. coli* K5 capsular polysaccharide was determined by combining 200 μ l of mid-logarithmic phase cultures, grown in LB containing antibiotics (for plasmid maintenance) and 200 μ g/ml IPTG, with 10 μ l of diluted phage stock (approximately 200 PFU total), and 3 ml LB top agar (Sambrook et al., 1989) containing antibiotics and IPTG. This mixture was poured onto prewarmed (37°C) LB agar plates, and allowed to solidify. The plates were incubated overnight at 37°C to allow plaques to form.

Nucleotide sequence accession number. The nucleotide sequence of the 4.6 kb *Xba*I-*Cla*I restriction fragment of pCW-1C was submitted to GenBank and assigned the accession number U36397.

RESULTS

Identification and cloning of a DNA region involved in *A. pleuropneumoniae* encapsulation. To identify the *A. pleuropneumoniae* capsulation locus, Southern blot analyses of *A. pleuropneumoniae* serotype 5a strain J45 genomic DNA with probes specific for contiguous regions of the *H. influenzae* type b capsulation (*capb*) locus were performed. These probes did not hybridize to *A. pleuropneumoniae* genomic DNA under conditions of high stringency (68°C, 5X SSC) (data not shown), but did hybridize under conditions of medium-to-low stringency (55°C, 5X SSC) (Fig. 3.1). A 4.4 kb *Eco*RI fragment of the *H. influenzae capb* locus from pSKH1 (Hoiseth et al., 1985), containing the region 1 *bexD* gene involved in capsular polysaccharide export (Kroll et al., 1990) and two region 2 open reading frames (ORFs) involved in capsular polysaccharide biosynthesis (Van Eldere et al., 1995), hybridized to 1.2 kb *Hind*III and 5.3 kb *Xba*I

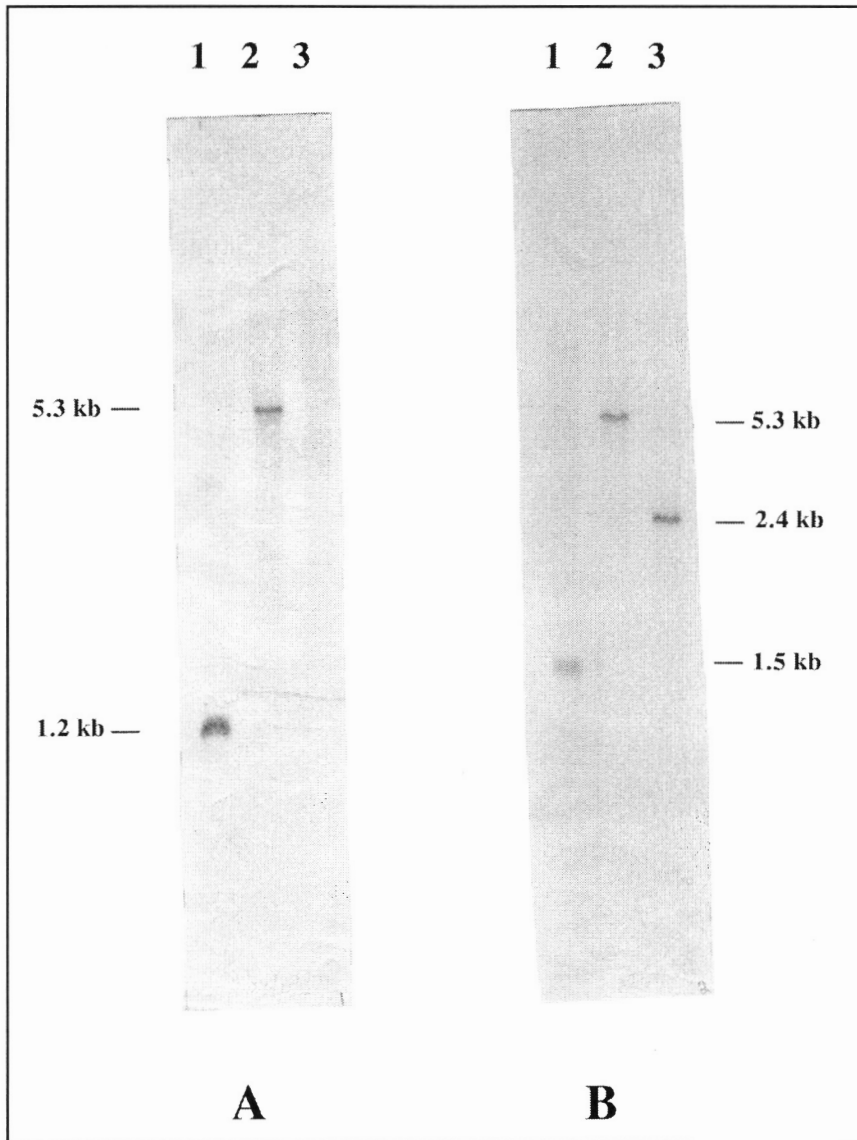


Figure 3.1. Southern blot analysis of *A. pleuropneumoniae* J45 genomic DNA hybridized with digoxigenin-labeled DNA probes specific for the *H. influenzae* type b *cap* locus. Approximately 10 μ g of *A. pleuropneumoniae* J45 total genomic DNA was digested to completion with *Hind*III (lane 1), *Xba*I (lane 2), or *Xho*I (lane 3), electrophoresed through a 0.7% agarose gel, transferred to nylon membranes, and hybridized at 55°C with either the 4.4 kb insert of pSKH1 (panel A), or with the 9.0 kb insert of pSKH2 (panel B). Approximate molecular masses of discrete bands that hybridized with the probes are indicated in kb.

fragments of J45 genomic DNA (Fig. 3.1A). A 9.0 kb *EcoRI* fragment of the *H. influenzae capb* locus from pSKH2 (Hoiseith et al., 1985), containing the region 1 *bexCBA* genes involved in capsular polysaccharide export (Kroll et al., 1990), some uncharacterized region 3 DNA common to several *H. influenzae* serotypes (Kroll et al., 1989), and some region 2 DNA involved in capsular polysaccharide biosynthesis, hybridized to 1.5 kb *HindIII*, 5.3 kb *XbaI*, and 2.4 kb *XhoI* fragments of J45 genomic DNA (Fig. 3.1B). These data indicated that the *H. influenzae* type b and *A. pleuropneumoniae* serotype 5a capsule gene loci shared homologous regions. The *H. influenzae capb* specific probes both contain region 1 DNA involved in capsular polysaccharide export, suggesting that the 5.3 kb *XbaI* genomic DNA fragment from J45 that hybridized to both *H. influenzae capb* probes may contain genes that encode proteins involved in export of the *A. pleuropneumoniae* serotype 5a capsular polysaccharide.

The 5.3 kb *XbaI* genomic DNA fragment from J45 that hybridized with the two *H. influenzae capb* probes was cloned into the *XbaI* site of pGEM-3Z (in both orientations) from *XbaI*-digested J45 genomic DNA fragments in the range of 4.8-6.0 kb that were electroeluted (following electrophoretic separation) from an agarose gel. The resulting plasmids were designated pCW-1C (Fig. 3.2) and pCW-5E. Southern blots were performed to determine if the *H. influenzae* type b *bexD*, *bexC*, *bexB*, and *bexA* genes involved in capsular polysaccharide export hybridized to the *A. pleuropneumoniae* J45 DNA cloned in pCW-1C (data not shown). Probes specific for *bexD*, *bexC*, *bexB*, and *bexA* hybridized to adjacent restriction fragments of pCW-1C in the same order (*bexDCBA*) in which these genes occur in *H. influenzae* (data not shown). These data suggested that I had successfully cloned an *A. pleuropneumoniae* serotype 5a DNA region required for capsular polysaccharide export and that this region was organized in

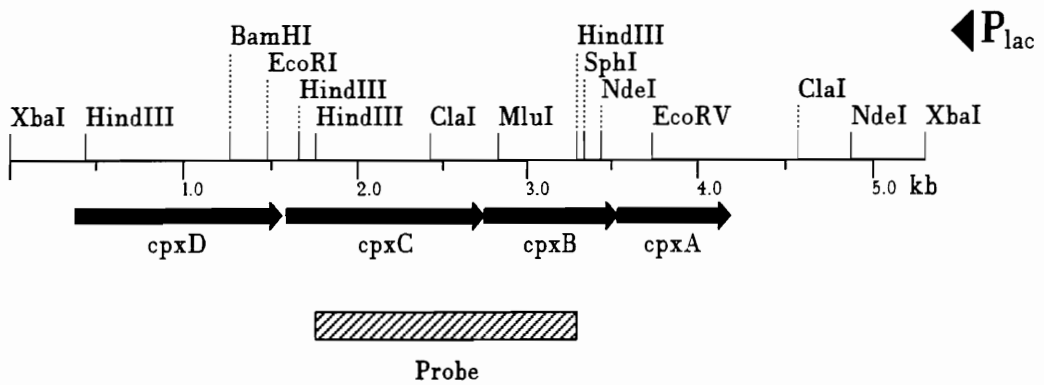


Figure 3.2. Physical map of pCW-1C insert DNA from *A. pleuropneumoniae* J45. The location and direction of transcription of the four major ORFs (*cpxDCBA*) identified by dideoxy sequencing is indicated. The direction of transcription from the *lac* promoter (P_{lac}) located on the plasmid vector is indicated by the arrowhead. The 1.5 kb *HindIII* fragment used as the DNA probe in Fig. 3.6 is shown.

a similar manner to the *H. influenzae* type b *bex* locus.

Nucleotide sequence and analysis of *A. pleuropneumoniae* capsulation DNA. To provide additional evidence for the role of the cloned *A. pleuropneumoniae* J45 DNA in capsular polysaccharide export, the nucleotide sequence of the 4.6 kb *XbaI-ClaI* restriction fragment of pCW-1C was determined (Fig. 3.3). As shown in Fig. 3.2 and Fig. 3.3, four ORFs designated *cpxDCBA* (*cpx* for capsular polysaccharide export) were detected in close proximity on the same DNA strand. The AUG initiation codon of *cpxC* was 26 nucleotides downstream from the UAA termination codon of *cpxD*, whereas the AUG initiation codon of *cpxB* overlapped the UAA termination codon of *cpxC*, and the AUG initiation codon of *cpxA* overlapped the UGA termination codon of *cpxB*. Shine-Dalgarno (Shine and Dalgarno, 1974) ribosome-binding consensus sequences were identified within 17 bases upstream of each AUG initiation codon (Fig. 3.3) and a putative promoter, containing sequences similar to *E. coli* σ^{70} -10 (TATAAT) and -35 (TTGACA) consensus sequences (Hawley and McClure, 1983), was identified upstream of *cpxD* (Fig. 3.3). A palindromic sequence which may function as a rho-independent transcription termination signal (Rosenberg and Court, 1979) was identified downstream from *cpxA* (Fig. 3.3). This genetic organization suggests that *cpxDCBA* are transcribed onto a single, polycistronic mRNA. Unfortunately, Northern blot analyses to verify this transcriptional organization have been unsuccessful (data not shown).

The predicted polypeptides of *cpxDCBA* were comprised of 394 (CpxD), 385 (CpxC), 265 (CpxB), and 216 (CpxA) amino acids, respectively (Fig. 3.3). The predicted molecular masses of CpxD, CpxC, CpxB, and CpxA were 42.1, 43.4, 29.9, and 24.6 kilodaltons (kDa), respectively. The first 20 amino acids of CpxD were characteristic of an N-terminal prokaryotic lipoprotein signal peptide (Pugsley, 1993) (Fig. 3.3).

CTAGACATTACATGATTAATTTATAGGACGAGTCATTATGCTAGACATTAATAATACCTCGAAATACGAATACGTTATGGAGTTACCCTA 90
 GATAGTTGATAAATTTTTTCATCGGAATTTTCATAAATAAATGAGAAATTTATCAACTATCTAGGGGAAACCCGTTATTTATATGGCTT 180
 TTAATTTATAATGTTTATCTGTATAACGCCCTCTTGATTACTTTTTTAAAAAAAATACATTTTTTAATTTGTTGTAATTTCTTA 270
 TATTTAATTTCTCTTGATTAGGTAACCTTATAAATCAACTTTTATGTTGGATTTAAGACATACCTATATAGTTGTTATTCCTTAATATA 360
 TTGAAGAGAAATTAGATGAAACTCATCAAACTTAGATTACTCTTTCTTTGGGGCTGGTTGCTAGTTTGGCTGCCTGCCTCAAGCTTACCC 450
CpxD→ M K L I K L R L L L S L G L V A S L A A C S S L P
 ACTTCAGGGCCTAGCCATAGTGGCATTAGAGGCTAATCCAGAACTCAGATAAACCTTACCAGAACTAATTTAGTGGAGTTAGAT 540
 T S G P S H S A I L E A N S O N S D K P L P E V N L V E L D
 AATGGCTTAGTTCAGCAGTTGTATCAGACTCAGCAAAGTCAGCAATTTCCGGCTTTTAGGCACGGCTGGCGGTGCTGGATATGCCGGT 630
 N G L V Q Q L Y Q T Q Q S Q Q F S G F L G T A G G A G Y A G
 GCGGCAATGTGGGGATGTTCTTGAAATTTCAATTTGGGAAGCGCCACGGCAGTGTGTTGGCGGTACTTTTAGTCTGAAGGGCAA 720
 A V N V G D V L E I S I W E A P P A V L F G G T F S S E G O
 GGTAGCGGGCATTAAACGAATTACGGCGCAAATGGTTAACCAAAACGGTACGGTTACTGTGCCGTTTGGGGTAATATCGTGTGCA 810
 G S G H L T O L P A Q M V N O N G T V T V P F V G N I R V A
 GGTAAAACACCGGAAGCGATTACGTTCTCAAAATGTTGGGGCATTGCAACGTAAAGCGAATCAGCCACAAGTATTAGTAAAAATGCGAAT 900
 G K T P E A I Q S Q I V G A L Q R K A N Q P Q V L V K I A N
 AATAACTCTGCGGATGTTACGGTTATTCGTCAGGGTAACAGTATTCGTATGCCGCTGAGTGCGAATAACGAACGTGTTAGATGCTGTT 990
 N N S A D V T V I R O G N S I R M P L S A N N E R V L D A V
 GCAGCAGTAGCGGTACAACCTGAAAATTTGAAGACGTTACCGTAAAAATTAACGTTGGCTCGCAAGTCAAAACATTAGCGTTTGAACCT 1080
 A A V G G T T E N I E D V T V K L T R G S Q V K T L A F E T
 CTAATTTCCGATCCGGCGCAAATATTATGTTACGTCGCGCGATGTCGTTTCGTTGCTAAACACGCCCTATAGCTTACCGGTTTAGGT 1170
 L I S D P A Q N I M L R A G D V V S L L N T P Y S F T G L G
 GCGTGGGTAACAACCAAGCAAAATGAAATTTCAAGTAAAGGAATTACGTTGCCGAAGCTATCGGTAAGATGGGTGGCCTAATGATACT 1260
 A V G N N O Q M K F S S K G I T L A E A I G K M G G L I D T
 CGTTCCGATCCGAGAGGGGTATTCGCTTCGTCATGTGCCCTTTTCTCAATTAAGTTTAGATCAGCAAAACACAATGGGGAGCGAAAGGC 1350
 R S D P R G V F V F R H V P F S Q L S L D Q O T O W G A K G
 TATGGTATGGGATGAGTATACCACGGTTTATCGTGTGAATTTACTTGAGCCGCAATCACTGTTTTTATTACAACGCTTCCCGATGCAA 1440
 Y G M G M D V P T V Y R V N L L E P Q S L F L L Q R F P M O
 GATAAAGATATTGCTATGTATCAATGCACCGTGTCCGAATCCAAAAATCTTTGAGAATGATTTCTCGATTACTTCCCGGTTACA 1530
 D K D I V Y V S N A P L S E F Q K F L R M I F S I T S P V T

Figure 3.3. Nucleotide sequence of the 4.6 kb *XbaI-ClaI* fragment of pCW-1C, containing *A. pleuropneumoniae* J45 DNA involved in capsular polysaccharide export. The deduced amino acid sequences of the four major ORFs detected in this sequence, *cpxDCBA*, are identified below the nucleotide sequence. Putative ribosome-binding sites (Shine and Dalgarno, 1974) located before each ORF are in boldface, and putative -10 and -35 promoter sequences upstream from *cpxD* are indicated. A potential stem-loop structure downstream from *cpxA* that may function as a rho-independent transcriptional terminator is indicated. The putative cleavage site of the CpxD N-terminal signal peptide is indicated by the arrowhead. The location in CpxA of motif A and motif B consensus sequences of ATP-binding proteins (Walker et al., 1982) is indicated.

AGTACGACTAATGCTATTCGTCCTATTAATATATTGAATTTATAAGGATAAAAATGGAAAACAACATTACGGCAAGTCCGACAGAAAA 1620
 S T T N A I R A Y . CpxC--> M E T T I T A S P T E K
 ACTACAAAAACCGGTTAAACAGAAAAAAGTTGGTTAAAAAAGCTTAATCCGTTATTTGGGTAACGTAGCGATTCTACGGTATTATC 1710
 L Q K P V K Q K K S W L K K L N P L F W V T V A I P T V L S
 AGCCTTTTATTCGGTCTGTTGCTCCGATATTTATATTCGGAATCAAGCTTCGTTGTAAGATCCTCCAAAATCAGACCGCTTTAAC 1800
 A F Y F G S V A S D I Y I S E S S F V V R S P O N Q T A L T
 CGGTGTCGGTGCCTTATTACAAGGTTCCGGATTTCTCGAGCTCAAGATGATACCTATACCGTACAAGAATATATGCATTCTCGTACGGC 1890
 G V G A L L O G S G F S R A Q D D T Y T V Q E Y M H S R T A
 ACTAGAACAGTAAATGAAGAC TTGCCAATACGTGAATACTATGAGAATCAAGGCGATATTATCGCTCGCTTTAATGGATTTGGTTTAAA 1980
 L E Q L M K D L P I R E Y Y E N O G D I I A R F N G F G L N
 TAATAGTAAGAAGCGTTTATAAATATTTCCGAGATCGTTAAGTGTGGACTTGACTCTGTTTCCGGTATCCGACGCTACGTATTCCG 2070
 N S K E A F Y K Y F R D R L S V D F D S V S G I A S L R I R
 AGCATTTAACCGGGAAGAGGGGCAACAATTAATCAAAAATTACTTGCCGAAGGTGAAACGCTTATTAACCGTTTAAACGAACGTGCAAG 2160
 A F N A E E G Q O I N O K L L A E G E T L I N R L N E R A R
 AAAAGATACCTTTTCAATTTGCGGAACAAGCGTTACAGAAGCGAAAAATAATGTAACCGAAACGGCAAAATGCTTTAAGTAATAACCGTAT 2250
 K D T I S F A E Q A V T E A E N N V N E T A N A L S K Y R I
 CAAAAATAAATCTTTGATTTACCGGCACAATCCGCGCTACAACCTTTCATTAATTTCCAGCCTAAAAAGCGAATTGATTCTGTGTAAGAA 2340
 K N K I F D L P A Q S G V O L S L I S S L K S E L I R V E T
 ACAATTTGGCTCAATTGCAATCTATTACACCGGACAACCCACAAGTTGATGCAATTGCTTATGCGCAAAAAAGTTTACGTAAGGAAATCGA 2430
 O L A O L Q S I T P D N P O V D A L L M R O K S L R K E I D
 TGAGCAATCAAAACAGCTTTCCAGTAACAGTAATAGCTCTATTGCTATTTCAAACCTGCCGATTACCAACGCTTAGTACTTGAACAGAGCT 2520
 E Q S K O L S S N S N S S I A I Q T A D Y O R L V L A N E L
 GGCACAGCAACAATTGACCGCAGCATTAACTTCATTACAAAAATACGAAAAATGAAGCGGATCGCCAGCAACTTTATTAGAAGTAATCAG 2610
 A Q Q Q L T A A L T S L Q N T K N E A D R Q O L Y L E V I S
 TCAGCCAAAGCAACCGGACTGGGCGGAAGAGCCTTATCGCTTATAAATATTTTAGCGACATCTTTATCGGCTGTATGCTTTATGGTGT 2700
 Q P S K P D W A E E P Y R L Y N I L A T F F I G L M L Y G V
 ATTAAGTTTATAAATGCAAGCGTAAGAGAGCACAAAACTAATGCAATACGGTGATCAAAACACTTTCCGCAATCTCTCGCCATTCAA 2790
 L S L L I A S V R E H K N . CpxB--> M Q Y G D Q T T F R Q S L A I Q
 GGGAGAGTAATCGGTGCATTACTCATCGGGAAATTTATACGCGTTACGGACGAAAAAATTTGGGTTTTTATGGCTGTTTGTGAGCCG 2880
 G R V I G A L L M R E I I T R Y G R K N L G F L W L F V E P
 CTATTACTCACTTATTTATCGTTTGTGTTGGAATTTATCCGAGCGGATCGCGTTTCCGATTAAATATATTGCTTTTGTGATTACC 2970
 L L L T L F I V L M W K F I R A D R V S D L N I I A F V I T
 GGTTATCCAATGGCCATGATGTGGCGTAATGCGTCAAACCGCACTATCGGTGCAATTTCCGGTAACTTGAGTCTTCTTTATCATCGTAAT 3060
 G Y P M A M M W R N A S N R T I G A I S G N L S L L Y H R N
 GTTCGCGTATTAGATACCTTACTGGCTCGTGCATACCTTGAAGTAGCAGGTGCAACGATTTGCCAAATCATTATTATGGCATTAGTCATT 3150
 V R V L D T L L A R V I L E V A G A T I A Q I I I M A L V I

Figure 3.3. (continued)

TTATTAGGCTGGATAGAAATGCCGAAAGATACGTTTTATATGGTTATGGCGTGGGTATTAATGGCATTTTTGCATTAGGATTAGGCTTG 3240
 L L S W I E M P K D T F Y M V M A W V L M A F F A L G L G L
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 I I C S I A Q K F E A F G K I W G T L S F V L L P L S G A F
 TTCTTTGTCATGCGCTACCAAGCCAAGCTCAACAATATGCAACCTTAATACCGATGATTCACGGCACGGAAATGTTTCGTCACGGTTAT 3420
 F F V H A L P S Q A Q Q Y A T L I P M I H G T E M F R H G Y
 TTTGGCGACAGTGTATCACATATGAAAGTATAAGCTATCTCGTAATATGTGATGTAGCCATGTTACTTTTGGGCTCATTATGGTAAAA 3510
 F G D S V I T Y E S I S Y L V I C D V A M L L F G L I M V K
 AACTTCAGTAAAGGAATCGAACCGCAATGATTAGCGTAAAAAATGTAGTAAAGATTACTATACTCGAAGCGGTAAAAAACCGTATTGC 3600
 N F S K G I E P Q .
CpxA→H I S V K N V S K D Y Y T R S G K K T V L
 AAGATATTAATTTGAGCTGAAAAAAGCGGAGAAAAATCGGTATTTTAGGGCGTAACGGTGCAGGGAAATCAACGCTAATCCGTTTGTAA 3690
 Q D I N F E L K K G E K I G I L **ATP-binding motif A**
 GTGGTGTGAGCGCCCAACATCAGGTAATATGTCGATATCTTGGCCGCTCGCTTTTCAGCGGGCGTTCCAAGGCAGTT 3780
 S G V E P P T S G T I E R N M S I S V P L A F Q R A F Q G S
 TAACCGGTATGGATAATTTACGCTTATTTGCCGTATATATAATGCGGATATTGAATACGTTAAAGCCTTTACCGAAGAATTTCCGAAT 3870
 L T G M D N L R F I C R I Y N A D I E Y V K A F T E E F S E
 TGGGTGATTATTTATATGAGCCGGTAAAGAAATATTCCTCCGGTATGAAAGCCGACTTGCCTTTGCTTTGTCACCTTCGGTAGAGTTG 3960
 L G D Y L Y E P V K K Y S S G M K A R L A F A L S L S V E F
 ATTGCTACTTAATTTGATGAAGTATTGCTGTGGCGATTCCCGTTTTGCGGCAAAATGTAACATGAATATTTGAAAAAGCGCAAAGATC 4050
motif B
 D **C Y L I D** E V I A V G D S R F A A K C K H E L F E K R K D
 GTTCGATTATTTTGGTTTCACATAGCCCGTCTGCAATGAAAGAATATTSCGATAATGCAATGGTATTGGATAAAGGGATTATGTATAAAT 4140
 R S I I L V S H S P S A M K E Y C D N A M V L D K G I M Y K
 TTGAGAATATGGACGAGGCTTACAAGTTCTATAATCAACGCTTTAAAGTCCACATATCCCCCTCTTTAGCAAAGAGGGGCTGGGGGA 4230
 F E N M D E A Y K F Y N S T L
 GATTTGACAGAATAAATAGCGAAGAAATATGCTAATTAACGCTTTTATAAATAAAAAATGAAGTTCATTCAATTTTCATATCTCTAT 4320
stem loop
 TGACAAATCTCCCTAACCCTCTTTACTAAAGAGGGGATTTCCTTTAAAGGTTAACAAATATGATATTGCGAGTAGTTGCAGTTTCC 4410
 GATTAAGTTTTATTAGTTCCCTACGAGATAAGAAAAACCTGAAAAAACAATATTATCCAACATTACGTCAACTGACCGATTCAATTA 4500
 ACTCCGTGCAATCTGTGTTTTCCGTGGTAAATCTCAGGCGTTTTCTATTTGATTCAAAGCCGTCTGTACGATCG 4576

Figure 3.3. (continued)

Cleavage of the CpxD leader peptide by signal peptidase II would leave the processed form of CpxD with an N-terminal cysteine residue available for lipid modification (Pugsley, 1993). The hydropathy plots of CpxD, CpxC, CpxB, and CpxA are shown in Fig. 3.4. CpxC was a relatively hydrophilic protein (Fig. 3.4) with hydrophobic domains near the N- and C-termini that may serve as membrane anchors. CpxB was a very hydrophobic protein (Fig. 3.4) and contained at least 6 potential membrane-spanning α -helical domains, suggesting that CpxB may be an integral membrane protein. CpxA was a relatively small, hydrophilic protein (Fig. 3.4) and contained amino acid sequences matching the A (GRXGXGKST) and B (XXXXD) motif consensus sequences characteristic of ATP-binding proteins (Fath and Kolter, 1993; Higgins, 1992; Walker et al., 1982) (Fig. 3.3).

Homology of *A. pleuropneumoniae* cpxDCBA to capsular polysaccharide transporters. BLAST searches (Altschul et al., 1990) of the combined, nonredundant nucleotide and protein data bases at the National Center for Biotechnology Information (Bethesda, Md.) revealed that *A. pleuropneumoniae* cpxDCBA were highly homologous at both the nucleotide and amino acid levels to *H. influenzae* bexDCBA (region 1) (Kroll et al., 1990) and *N. meningitidis* ctrABCD (region C) (Frosch et al., 1991) (Table 3.2). Homology was also detected between CpxC, CpxB, CpxA, and *E. coli* K1 and K5 KpsE (region 1) (Cieslewicz et al., 1993; Pazzani et al., 1993; Rosenow, et al., 1995), *E. coli* K1 and K5 KpsM (region 3) (Pavelka et al., 1991; Smith et al., 1990), and *E. coli* KpsT (region 3) (Pavelka et al., 1991; Smith et al., 1990), respectively (Table 3.2). A low amount of homology (13.2 %) was observed between CpxD and *E. coli* K1 and K5 KpsD (region 1) (Pazzani et al., 1993; Wunder et al., 1994). The calculated percent similarity of these genes and their predicted protein products are shown in Table 3.2.

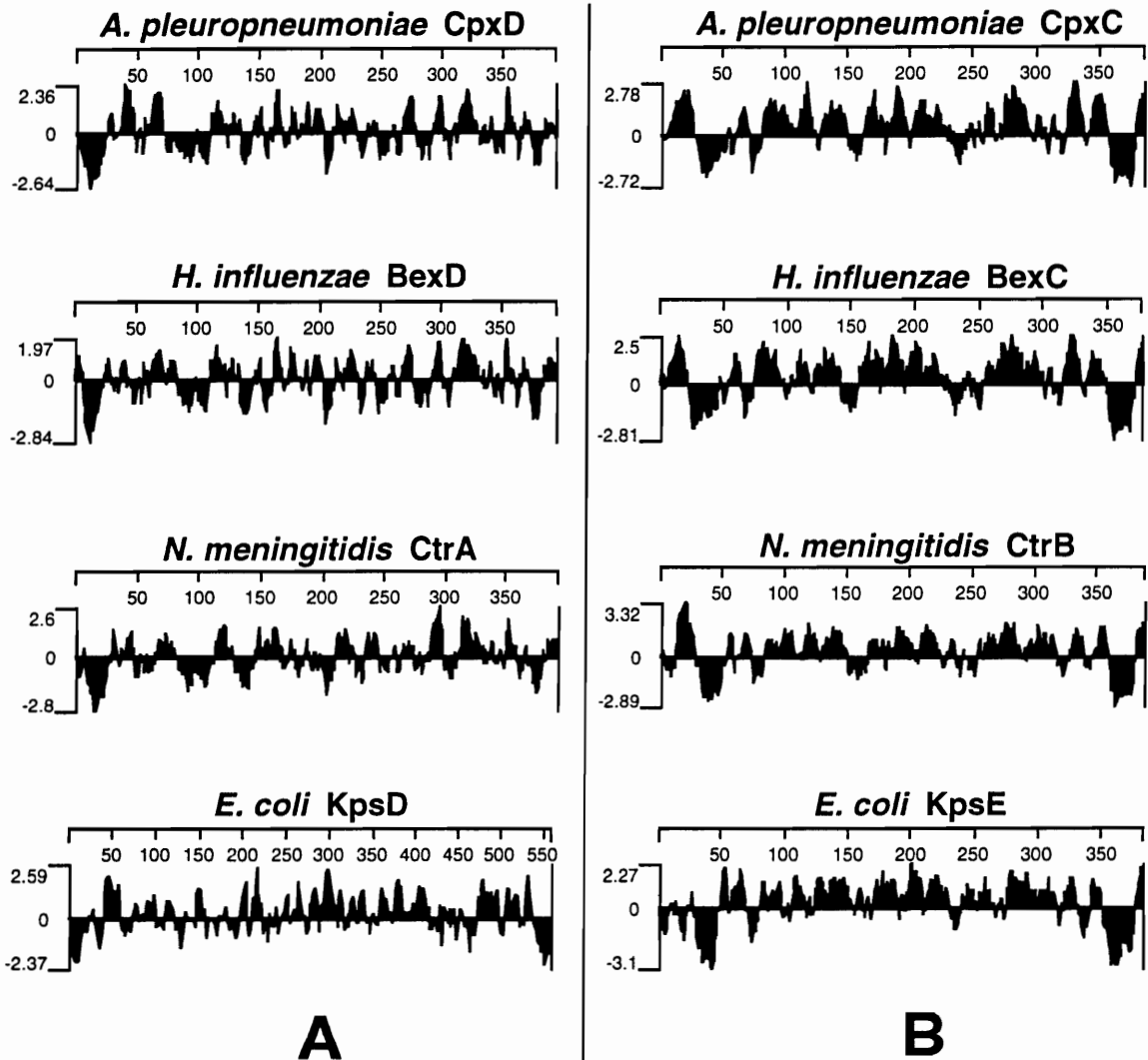
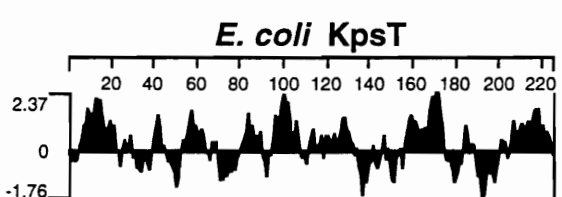
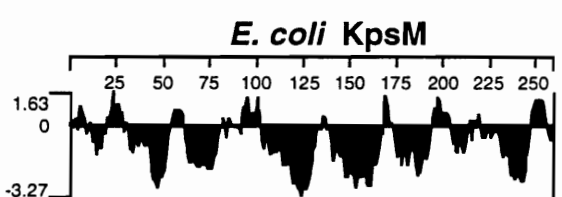
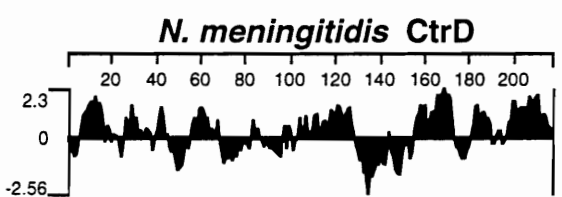
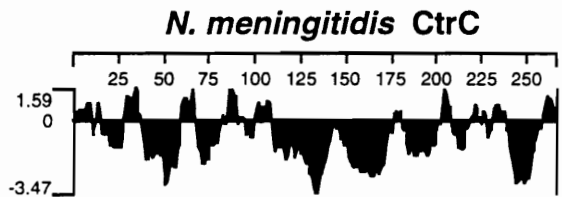
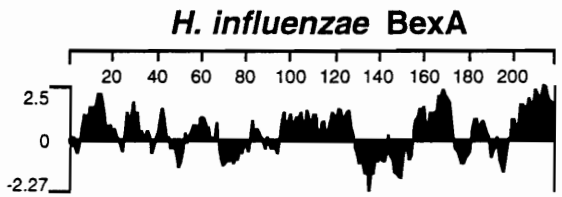
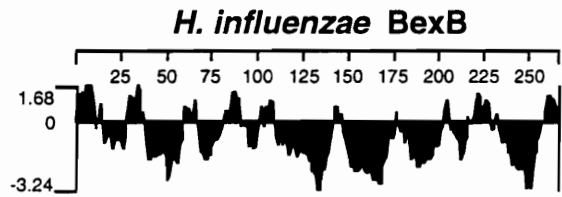
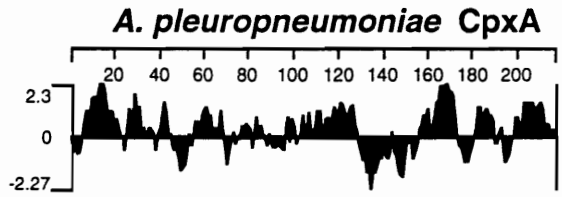
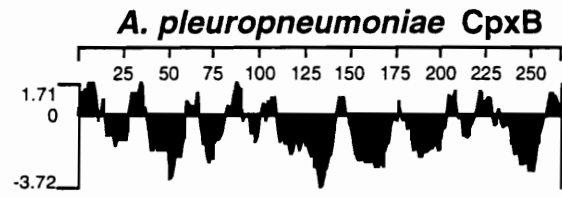


Figure 3.4. Comparison of hydropathy profiles of the predicted amino acid sequences of *A. pleuropneumoniae* J45 *cpxDCBA*, *H. influenzae* type b *bexDCBA*, *N. meningitidis* group B *ctrABCD*, and *E. coli* K5 *kpsED* and *kpsMT* involved in capsular polysaccharide export. Profiles were determined by the algorithm developed by Kyte and Doolittle (1983) using a window size of 9 amino acids. The vertical axis displays relative hydrophilicity with negative scores indicating relative hydrophobicity. The horizontal scale displays a scale of the numbered amino acid residues from the N- to C-terminus of each protein. Panels A, B, C, and D display profiles of CpxD, CpxC, CpxB, and CpxA, and their homologs, respectively.



C

D

Figure 3.4. (continued)

Table 3.2. Pairwise comparison of nucleotide and deduced amino acid (in parentheses) sequences of *A. pleuropneumoniae* J45 *cpxDCBA* to sequences involved in capsular polysaccharide export in *H. influenzae* type b (*bexDCBA*), *N. meningitidis* group B (*ctrABCD*), and *E. coli* K1 and K5 (*kpsED* and *kpsMT*).

	Percent Similarity*			
	<i>cpxD</i>	<i>cpxC</i>	<i>cpxB</i>	<i>cpxA</i>
<i>bexD</i>	66.5 (74.4)			
<i>ctrA</i>	50.6 (50.3)			
<i>kpsD</i>	20.0 (13.2)			
<i>bexC</i>		65.5 (75.1)		
<i>ctrB</i>		52.5 (56.2)		
<i>kpsE</i>		27.0 (23.0)		
<i>bexB</i>			66.9 (75.9)	
<i>ctrC</i>			64.5 (72.9)	
<i>kpsM</i>			26.9 (26.3)	
<i>bexA</i>				75.0 (82.9)
<i>ctrD</i>				70.0 (82.5)
<i>kpsT</i>				46.1 (41.5)

* Percent similarity was determined by analyzing distances of aligned sequences by the Clustal algorithm of DNASTAR sequence analysis software (DNASTAR, Inc., Madison, WI) using the PAM250 residue weight table (protein alignments) and the Weighted residue weight table (nucleotide alignments).

In general, *cpxDCBA* was most similar at both the nucleotide and amino acid levels to the *H. influenzae* region 1 genes *bexDCBA*, and least similar to the *E. coli* K1 and K5 region 1 genes, *kpsED*, and the region 3 genes, *kpsMT*. The predicted protein products of *cpxDCBA* also shared some homology with proteins involved in the export of the *Salmonella typhi* Vi capsular polysaccharide (VexA, VexB, VexC, and VexD) (Hashimoto et al., 1993). CpxD was 21.3 % similar to VexA, CpxC was 20.5% similar to VexD, CpxB was 19.6 % similar to VexB, and CpxA was 26.3 % similar to VexC (data not shown).

The structural homology of the *A. pleuropneumoniae* serotype 5a *cpxDCBA* protein products to their homologs from *H. influenzae* type b, *N. meningitidis* group B and *E. coli* K5 was demonstrated by the similarity of their aligned hydropathy plots (Fig. 3.4). The potential membrane topology of these proteins is also reflected by the hydropathy plots in Fig. 3.4. These similarities suggested that CpxD may be an outer membrane lipoprotein involved in capsular polysaccharide transport across the outer membrane. In addition, these homologies suggested that CpxC, CpxB, and CpxA may be components of a group of ATP-binding cassette (ABC) transporters (Fath and Kolter, 1993; Higgins, 1992) known as the ABC-2 subfamily (Feizer et al., 1992) involved in polysaccharide export across the cytoplasmic membrane: CpxB may be the integral cytoplasmic membrane component, CpxA may be the ATP-binding component, and CpxC may be a cytoplasmic membrane component whose exact function is unknown.

Complementation of *E. coli* K5 *kpsM::TnphoA* and *kpsT::TnphoA*. Attempts to further investigate the role of *cpxDCBA* in capsular polysaccharide export by generating isogenic, noncapsulated *A. pleuropneumoniae cpx* mutants by allelic replacement using a *cpxBA* deletion derivative of pCW-1C were unsuccessful (data not shown). These

mutational studies were probably unsuccessful because of the low DNA transformation efficiency of J45 (my unpublished observations). Therefore, to confirm that *A. pleuropneumoniae* *cpxDCBA* functioned in capsular polysaccharide export, complementation of plasmid-encoded *E. coli* K5 *kps* loci containing *TnphoA* insertions in *kpsM* (pPC6::17) or in *kpsT* (pPC6::6) (Pearce and Roberts, 1995) was performed using pCW-1C and pCW-5E. *E. coli* K5 *kpsMT* are components of a conserved, ATP-driven polysaccharide export apparatus involved in capsular polysaccharide export (Smith et al., 1990). Expression of the *E. coli* K5 capsular polysaccharide was monitored by examining the sensitivity of strains to a K5-specific bacteriophage. This bacteriophage formed plaques on a lawn *E. coli* MS101, a positive control strain that expresses the K5 capsular polysaccharide (Fig. 3.5A) (Stevens et al., 1994), but did not form plaques on *E. coli* XL1-Blue(pPC6::6) (Fig. 3.5B) or XL1-Blue(pPC6::17). Thus, I confirmed previous reports that the *TnphoA* insertions in *kpsM* or *kpsT* eliminate export of the K5 capsular polysaccharide (Pearce and Roberts, 1995). However, K5-specific bacteriophage formed partial, faint plaques on XL1-Blue(pPC6::6/pCW-5E), or XL1-Blue(pPC6::17/pCW-5E) (Fig. 3.5C and D). These results indicated that cloned *A. pleuropneumoniae* *cpxDCBA*, located on the insert of pCW-5E, were able to partially complement *kpsM::TnphoA* and *kpsT::TnphoA* mutations and, therefore functioned in capsular polysaccharide export. It was likely that because *cpxB* was homologous to *kpsM*, and *cpxA* was homologous to *kpsT* (Table 3.2), that *cpxB* and *cpxA* (and not *cpxD* or *cpxC*) were responsible for the respective complementation observed. The plaques observed on XL1-Blue(pPC6::6/pCW-5E) were larger and easier to see than those observed on XL1-Blue(pPC6::17/pCW-5E), indicating that *cpxA* complemented the *kpsT::TnphoA* mutation better than *cpxB* complemented the *kpsT::TnphoA* mutation. The codon usage was more similar between *cpxA* and *kpsT* than between *cpxB* and *kpsM* (data not shown),

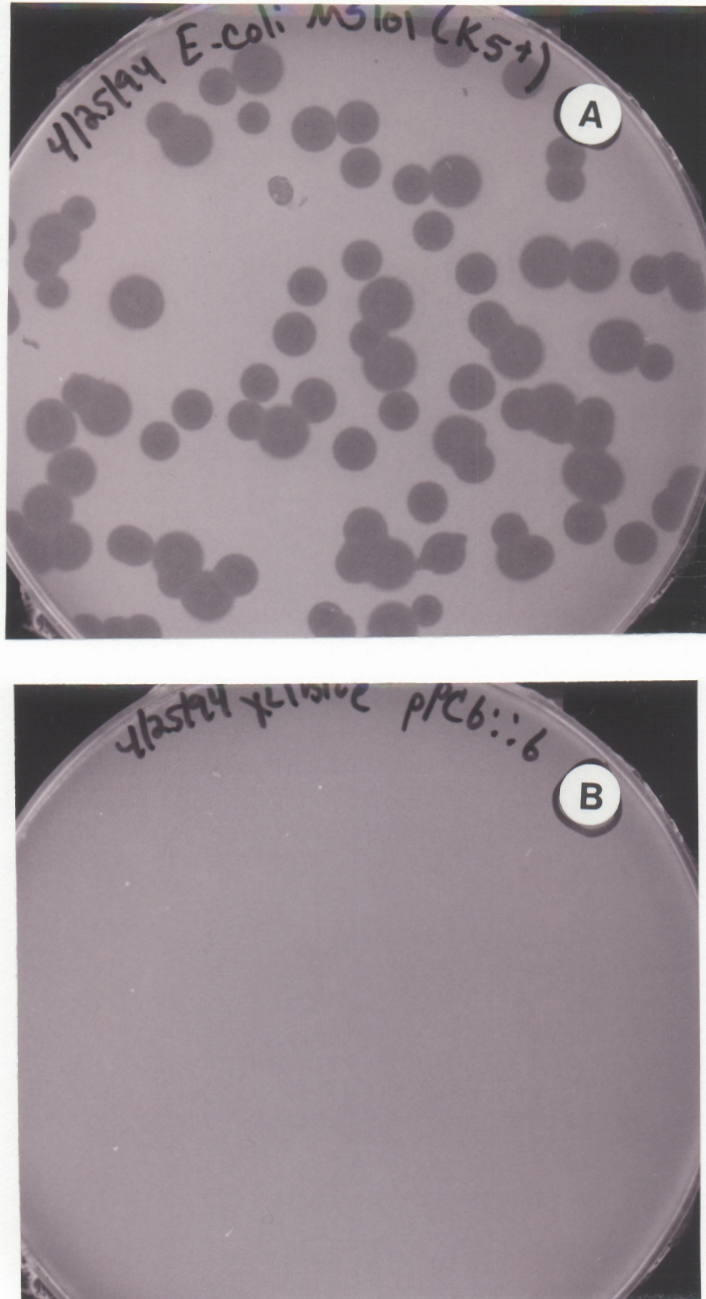


Figure 3.5. Complementation of the *E. coli* K5 *kps* locus, containing *TnphoA* insertions in *kpsM* or *kpsT*, with *A. pleuropneumoniae* J45 *cpxDCBA*. *E. coli* MS101 (panel A), *E. coli* XL1-Blue containing pPC6::6 (panel B), XL1-Blue containing both pCW-5E (*cpxDCBA*) and pPC6::6 (*kpsT*::*TnphoA*) (panel C), or XL1-Blue containing both pCW-5E (*cpxDCBA*) and pPC6::17 (*kpsM*::*TnphoA*) (panel D), were screened for *E. coli* K5 capsular polysaccharide expression using a K5-specific bacteriophage.

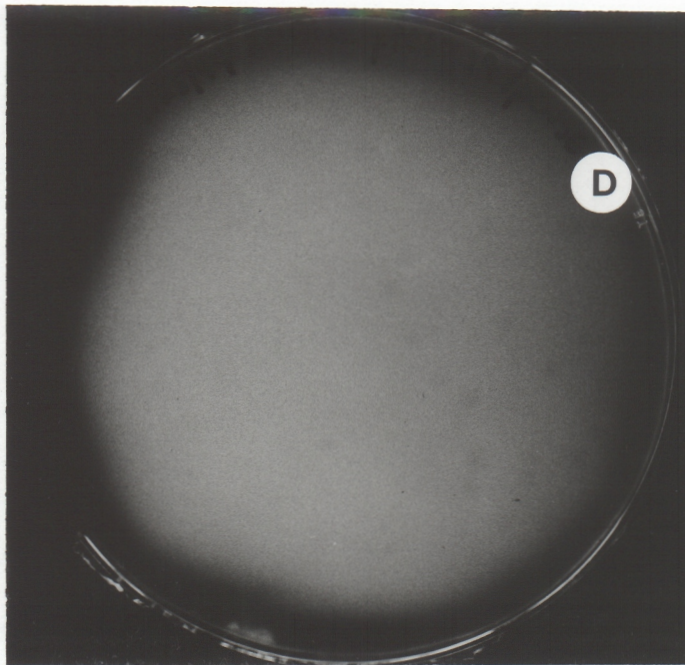
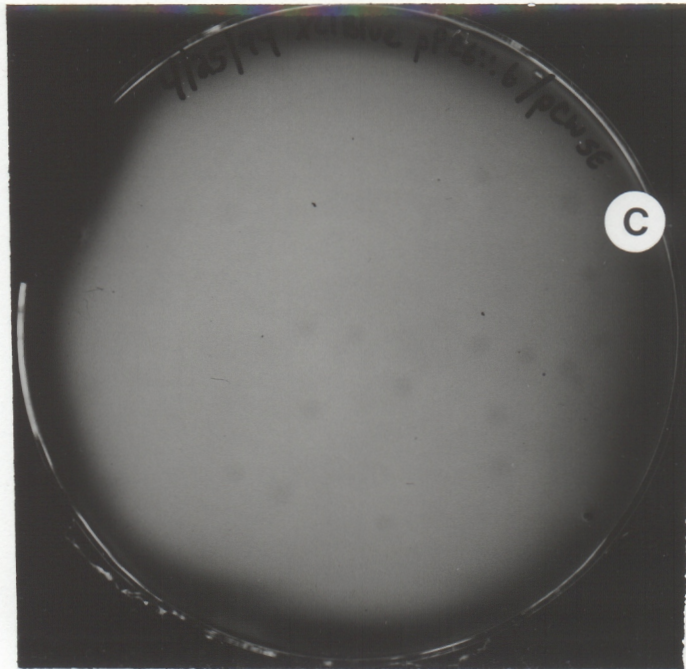


Figure 3.5. (continued)

which may have resulted in better expression of CpxA in *E. coli* and, thus the enhanced complementation of the *kpsT::TnphoA* mutation. The presence of two, distinct plasmids (pPC6::6/pCW-5E) or (pC6::17/pCW-5E) in *E. coli* XL1-Blue was verified by restriction digestion analysis of plasmid DNA isolated from each strain (data not shown).

The ability of *cpxDCBA* to complement *kpsM::TnphoA* and *kpsT::TnphoA* mutations was dependent on the orientation of these genes relative to the *lac* promoter on the pGEM-3Z vector, since K5-specific bacteriophage did not plaque on *E. coli* XL1-Blue(pPC6::6/pCW-1C) or XL1-Blue(pPC6::17/pCW-1C) (data not shown). However, the *cpxDCBA* genes were in the proper orientation to be transcribed from the *lac* promoter in pCW-5E (Fig. 3.2). Therefore, it was probable that either the putative promoter sequence identified upstream from *cpxD* (Fig. 3.3) was not functional in *E. coli*, or that the promoter sequence necessary for transcription of *cpxDCBA* was not located on the 5.3 kb *XbaI* DNA fragment originally cloned in pCW-1C.

Hybridization of *cpxCB* with genomic DNA from several *A. pleuropneumoniae* serotypes. DNA required for capsular polysaccharide export in *H. influenzae* type b, *N. meningitidis* group B, and *E. coli* K1 and K5 is conserved among serotypes that express different capsular polysaccharides in each of these species (Kroll et al., 1989; Roberts et al., 1988). Hybridization of a J45 *cpx*-specific DNA probe to genomic DNA from several *A. pleuropneumoniae* serotypes was examined to determine if *cpxDCBA* was conserved among *A. pleuropneumoniae* serotypes. The 1.5 kb *HindIII* fragment of pCW-1C (indicated in Fig. 3.2) specific for *cpxCB*, was used to probe *BglII*-digested genomic DNA from *A. pleuropneumoniae* serotypes 1, 2, 5, 7, and 9 (Fig. 3.6). The *cpxCB* probe hybridized under conditions of high stringency (68°C, 5X SSC) to an approximate

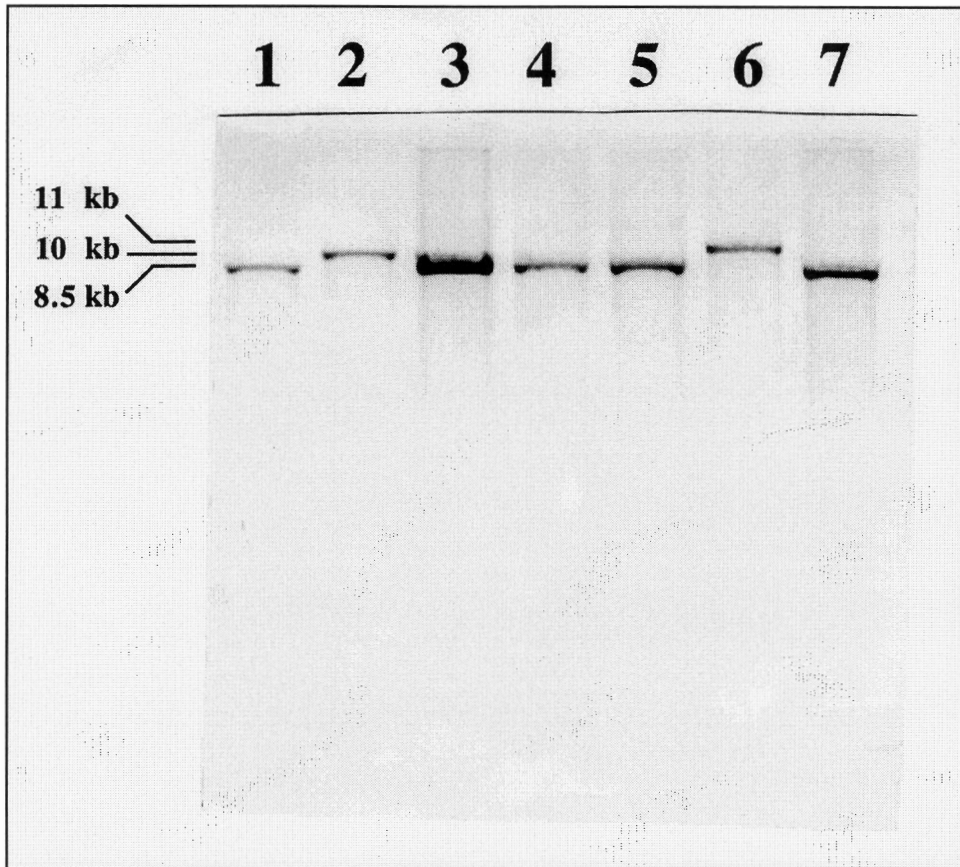


Figure 3.6. Southern blot analysis of *A. pleuropneumoniae* genomic DNA hybridized to a digoxigenin-labeled probe specific for *A. pleuropneumoniae* J45 *cpxCB*. *Bgl*II-digested genomic DNA from serotype 1 strain 4074 (lane 1), serotype 2 strain 1536 (lane 2), serotype 5a strain J45 (lane 3), serotype 5a strain K17 (lane 4), serotype 5 strain 178 (lane 5), serotype 7 strain 29628 (lane 6), and serotype 9 strain 13261 (lane 7) were hybridized with the 1.5 kb *Hind*III fragment of pCW-1C (Fig. 3.2) at 68°C. The approximate molecular mass of each band that hybridized with the probe is indicated in kb.

8.5 kb *Bgl*III fragment from serotypes 1, 5 (three strains), and 9; an approximate 10 kb *Bgl*III fragment from serotype 2; and to an approximate 11 kb *Bgl*III fragment from serotype 7 (Fig. 3.6). Therefore, *cpxCB* was conserved among several *A. pleuropneumoniae* serotypes, although a restriction fragment length polymorphism was observed between some serotypes. These hybridization studies provided further evidence that *cpxDCBA* were involved in *A. pleuropneumoniae* capsular polysaccharide export. I propose to designate the *cpxDCBA* gene cluster as region 1 of the *A. pleuropneumoniae* serotype 5a capsule (*cap*) gene locus, since *cpxDCBA* were most homologous to the region 1 *bexDCBA* gene cluster of the *H. influenzae* type b *cap* locus.

DISCUSSION

In this chapter, I report the identification, cloning, and characterization of four genes involved in the export of the *A. pleuropneumoniae* serotype 5a capsular polysaccharide. This is the first report identifying a DNA region involved in expression of the *A. pleuropneumoniae* capsular polysaccharide. The identification of this region is an important first step toward the complete characterization of the *A. pleuropneumoniae* capsulation locus.

The high degree of homology observed between *A. pleuropneumoniae* J45 (serotype 5a) *cpxDCBA*, *H. influenzae* type b *bexDCBA*, and *N. meningitidis* group B *ctrABCD* at both the nucleotide and amino acid levels suggested that *A. pleuropneumoniae* serotype 5a synthesized and exported a group II capsular polysaccharide. These results were expected because a common molecular origin of capsular polysaccharide export exists in gram-negative bacteria expressing group II capsular polysaccharides (Frosch *et al.*, 1991). However, further phenotypic studies need to be performed to determine if the

capsular polysaccharide of *A. pleuropneumoniae* serotype 5a is truly a group II capsular polysaccharide. The overall organization of the *A. pleuropneumoniae* capsulation locus may resemble the general three-region organization (of a central serotype-specific region flanked on both ends by DNA required for capsular polysaccharide export that is conserved among serotypes) described for the *H. influenzae cap* and *N. meningitidis cps* loci (Frosch et al., 1989; Kroll et al., 1989). The *bexDCBA* and *ctrABCD* genes each comprise an entire region required for capsular polysaccharide export that flanks serotype-specific DNA in *H. influenzae* type b and *N. meningitidis* group B, respectively. The genes involved in *E. coli* K1 and K5 capsular polysaccharide export that are homologous to *cpxDCBA*, *bexDCBA*, and *ctrABCD* are arranged somewhat differently: *kpsD* and *kpsE* are located in region 1 of the *E. coli kps* locus (Cieslewicz et al., 1993; Rosenow et al., 1995; Wunder et al., 1994), whereas *kpsMT* are located in region 3 of the *kps* locus (Pavelka et al., 1991; Smith et al., 1990). Since *cpxDCBA* were contiguous and *cpxCB* hybridized to genomic DNA from several *A. pleuropneumoniae* serotypes, it is likely that *cpxDCBA* comprise an entire region (region 1) required for capsular polysaccharide export that flanks a serotype-specific region required for capsular polysaccharide biosynthesis. I propose to designate *cpxDCBA* as region 1 of the *A. pleuropneumoniae* serotype 5a *cap* locus because these genes were most homologous to the region 1 *bexDCBA* genes of the *H. influenzae* type b *cap* locus.

Although the precise cellular location and function of the protein products of *cpxDCBA* have not been described in this report, analysis of the predicted protein products of *cpxDCBA* and the results of complementation studies indicated that this DNA region was involved in the export of the *A. pleuropneumoniae* serotype 5a capsular polysaccharide. The hydropathic profiles of the predicted protein products of *cpxDCBA*

were strikingly similar to the homologous protein products of *bexDCBA*, *ctrABCD*, *kpsD*, *kpsE*, and *kpsMT*. These data indicated that the membrane location and topology of these proteins were similar. The proteins encoded by *bexDCBA*, *ctrABCD*, *kpsED*, and *kpsMT* are involved with various aspects of capsular polysaccharide export from the cytoplasm to the cell surface (Bronner et al., 1992; Frosch et al., 1991; Kroll et al., 1990; Smith and Roberts, 1990). Analysis of the predicted amino acid sequence of *cpxD*, indicated that the processed form of CpxD may be an outer membrane lipoprotein. The CpxD homologs in *H. influenzae* type b, *N. meningitidis* group B, and *E. coli* K1 (BexD, CtrA, and KpsD, respectively) are located in the outer membrane (Frosch et al., 1991; Frosch et al., 1992; Kroll et al., 1990; Wunder et al., 1994). A *kpsD* deletion mutant accumulates capsular polysaccharide in the periplasmic space, indicating that KpsD is involved in capsular polysaccharide transport across the outer membrane (Wunder et al., 1994). These data suggested that CpxD may be involved in capsular polysaccharide transport across the outer membrane. The nucleotide and predicted protein sequences of *cpxD* were different from those reported for another *A. pleuropneumoniae* lipoprotein, *omlA*. OmlA is a 40 kDa outer membrane lipoprotein of *A. pleuropneumoniae* serotype 5a whose function is unknown (Bunka et al., 1995; Ito et al., 1995).

CpxB and CpxA seemed to be components of an ATP-driven polysaccharide export apparatus known as an ABC-2 transporter (Reizer et al., 1992), where CpxB was the integral membrane component and CpxA was the ATP-binding component. Similar type transporters have been identified which are involved in the export of other bacterial cell surface polysaccharides across the cytoplasmic membrane (Bronner et al., 1994; Fath and Kolter, 1993; Frosch et al., 1991; Kroll et al., 1990; Pavelka et al., 1991; Smith et al., 1990). The predicted function of CpxC in capsular polysaccharide export is less evident. The CpxC homologs in *H. influenzae* type b, *N. meningitidis* group B, and *E. coli* K1

and K5 (BexC, CtrB, and KpsE, respectively) are cytoplasmic membrane proteins that have a substantial periplasmic domain (Cieslewicz et al., 1993; Frosch et al., 1991; Kroll et al., 1990; Rosenow et al., 1995). These proteins are proposed to serve as the second, cytoplasmic membrane component of an ABC-2 type polysaccharide transporter involved in transport across the cytoplasmic membrane (Reizer et al., 1992).

The strongest evidence for the involvement of *cpxDCBA* in *A. pleuropneumoniae* capsular polysaccharide export was demonstrated by the ability of this DNA region to complement *E. coli* K5 *kpsM*::*TnphoA* and *kpsT*::*TnphoA* mutations to restore *E. coli* K5 capsular polysaccharide export to the *E. coli* laboratory strain, XL1-Blue containing a plasmid-encoded K5 *kps* locus. The overall level of complementation was low, since only faint plaques by the K5-specific bacteriophage were observed. The more efficient complementation of the *kpsT*::*TnphoA* mutation compared to the *kpsM*::*TnphoA* mutation by *cpxDCBA* may have been a result of the greater homology and, thus more similar function between CpxA and KpsT than between CpxB and KpsM. It is also possible that CpxA was expressed more efficiently than CpxB in *E. coli* XL1-Blue, resulting in the enhanced complementation of the *kpsT*::*TnphoA* mutation. Several reports have described the complementation of defective capsular polysaccharide export genes with homologous genes from another capsular type of the same species (Roberts et al., 1986; Roberts et al., 1988). However, this is the first report describing the complementation of a defective capsular polysaccharide export gene by a homologous gene from a different bacterial species. These complementation studies indicated that because *cpxDCBA* functioned in the export of the *E. coli* K5 capsular polysaccharide, these genes are likely to function in the export of the *A. pleuropneumoniae* serotype 5a capsular polysaccharide. These studies also indicated that ABC-2 transporters which export capsular polysaccharides may export capsules of different structures, although the

transport of a heterologous capsular polysaccharide may be less efficient. Interestingly, ABC transporters involved in polysaccharide export seem to be specific for a particular type of polysaccharide (capsular polysaccharide versus O-antigen polysaccharide), since Bronner et al. (1994) reported that *kpsMT* could not complement *Klebsiella pneumoniae* serotype O1 LPS O-antigen export defects.

The capsulation locus of *A. pleuropneumoniae* needs further study to characterize the entire genomic DNA region required for capsular polysaccharide expression. Since the entire locus required for capsule expression is linked on the chromosome in *H. influenzae* type b, *N. meningitidis* group B, and *E. coli* K1 and K5, it should be possible to identify the entire capsulation locus of *A. pleuropneumoniae* by genomic walking, using probes specific for *cpxDCBA* to identify and clone contiguous regions. Once the entire locus is identified, mutational and biochemical analyses will be necessary to further characterize the mechanism of capsular polysaccharide expression and determine the role of capsular polysaccharide in virulence and immunoprotection.

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CHAPTER 4

Characterization of a Serotype-Specific DNA Region Involved in the Encapsulation and Virulence of *Actinobacillus pleuropneumoniae* Serotype 5a

ABSTRACT

A DNA region involved in *Actinobacillus pleuropneumoniae* capsular polysaccharide biosynthesis was identified and characterized. A probe specific for the *cpxD* gene involved in the export of the *A. pleuropneumoniae* serotype 5a J45 capsular polysaccharide was used to identify and clone an adjacent 5.8 kilobase *Bam*HI fragment of J45 genomic DNA. Southern blot analyses demonstrated that a portion of this region contained DNA that was serotype-specific. DNA sequence analysis demonstrated that this region contained two complete open reading frames, *cpsA* and *cpsB*, and an incomplete potential third open reading frame, *cpsC*. CpsA and CpsB shared some low homology with glycosyltransferases involved in the biosynthesis of *Escherichia coli* lipopolysaccharide and *Haemophilus influenzae* type b capsular polysaccharide, respectively. A 2.1 kilobase deletion which spanned the cloned *cpsABC* open reading frames was constructed and transferred into the J45 chromosome by homologous recombination to produce the mutant J45-100. This mutant did not produce intracellular or extracellular capsular polysaccharide, indicating that *cpsA*, *cpsB*, and/or *cpsC* were involved in *A. pleuropneumoniae* capsular polysaccharide biosynthesis. The Apx toxin and lipopolysaccharide profiles of J45-100 were identical to the encapsulated parent strain, J45. However, J45-100 grew faster than J45. J45-100 was sensitive to killing

in precolostral calf serum, whereas J45 was not. J45-100 was avirulent when used to challenge pigs intratracheally with 3 times the 50% lethal dose of strain J45. At 6 times the 50% lethal dose of J45, J45-100 caused mild to moderate lung lesions, but not death. These results demonstrated that the capsular polysaccharide is a major determinant of serum-resistance and virulence of *A. pleuropneumoniae*.

INTRODUCTION

Actinobacillus pleuropneumoniae causes swine pleuropneumonia, a severe, frequently fatal, and highly contagious respiratory disease. This disease occurs around the world and results in millions of dollars of losses to the global swine industry. Swine pleuropneumonia is characterized by hemorrhagic and necrotic lung lesions, and pleural adhesions. The vaccines that are currently available to control this disease consist of whole cell bacterins that provide some serotype-specific protection against mortality, but do not protect pigs against the development of the lung lesions characteristic of the disease (Nicolet, 1992). Furthermore, these killed vaccines are ineffective in preventing pigs from becoming subclinical carriers of the disease and transmitting the disease to other pigs (Nicolet, 1992). A considerable amount of research has focused on studying factors involved in *A. pleuropneumoniae* virulence and immunoprotection in order to develop a more effective vaccine to control swine pleuropneumonia.

Convalescent pigs are protected against challenge with homologous and heterologous *A. pleuropneumoniae* serotypes, indicating that an effective vaccine to control this disease may be developed (Crujisen et al., 1995; Nielsen 1979b). *A. pleuropneumoniae* produces several factors including a serotype-specific capsular polysaccharide that enhance its virulence in pigs. Several of these factors, particularly Apx toxins, are

important for the induction of protective immunity in pigs. Several recently developed experimental subunit vaccines consisting of *A. pleuropneumoniae* components such as capsular polysaccharide, lipopolysaccharide (LPS), outer membrane proteins, and Apx toxins have been moderately, but not completely successful in preventing *A. pleuropneumoniae* infection and disease (Byrd and Kadis, 1992; Byrd et al., 1992; Gerlach et al., 1993; Rossi-Campos et al., 1992). Attenuated live vaccines seem to be more efficacious in preventing disease (Inzana et al., 1993a; Inzana et al., 1993b; Rosendal and MacInnes, 1990). In particular, noncapsulated *A. pleuropneumoniae* mutants have potential as live vaccine strains (Inzana et al., 1993a). However, the mutations in these strains have not been characterized and the potential for reversion to the virulent, encapsulated phenotype exists.

Targeted mutagenesis studies in *A. pleuropneumoniae* have been hampered by the difficulty of genetic manipulation in this bacterial species (Jansen et al., 1995b; J. Frey, personal communication). To date, the only genetically-defined recombinant *A. pleuropneumoniae* mutant strains generated are those in which ApxI and/or ApxII expression was eliminated (Jansen et al., 1995b). These mutants, while useful in assisting in the development of a procedure for targeted mutagenesis in *A. pleuropneumoniae*, are likely to be nonprotective when used as immunogens since another Apx toxin-deficient mutant has been reported to be non-protective (Inzana et al., 1991). Thus, no genetically defined, stable, attenuated *A. pleuropneumoniae* strains that are protective have been developed.

In Chapter 3, I reported on the identification and cloning of a conserved DNA region involved in *A. pleuropneumoniae* capsular polysaccharide export. In this chapter, I report the further characterization of the *A. pleuropneumoniae* serotype 5a capsulation locus. *A. pleuropneumoniae* genes involved in capsular polysaccharide export were used

to identify and clone an adjacent DNA region involved in capsular polysaccharide biosynthesis. I also describe the production of an attenuated, recombinant noncapsulated *A. pleuropneumoniae* mutant by allelic exchange.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are as described in Table 4.1. The sources of *A. pleuropneumoniae* strains are as described in Chapter 3. For genomic DNA extraction and for bactericidal assays, *A. pleuropneumoniae* strains were grown with shaking at 37°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 5 µg/ml nicotinamide adenine dinucleotide (NAD) (Sigma Chemical Co., St. Louis, Mo). For electroporation, *A. pleuropneumoniae* strains were grown with shaking at 37°C in tryptic soy broth (Difco Laboratories) containing 0.6% yeast extract (Difco Laboratories) and 5 µg/ml NAD (TSY-N). For pig challenge experiments, *A. pleuropneumoniae* strains were grown with shaking at 37°C in Columbia broth (Difco Laboratories) containing 5 µg/ml NAD. *Escherichia coli* strains were grown in Luria-Bertani broth (Sambrook et al., 1989) for routine cultivation, or in Terrific broth (Tartof and Hobbes, 1987) for extraction of plasmids. Antibiotics were used in growth media for maintenance of plasmids in *E. coli* at the following concentrations: ampicillin (Amp) 100 µg/ml, and kanamycin (Kan) 50 µg/ml. Kanamycin was used at 85 µg/ml for selection of *A. pleuropneumoniae* recombinant mutants.

Calculation of generation time. The generation time of logarithmic phase *A. pleuropneumoniae* strains grown in TSY-N was calculated using the equation:

Table 4.1. Bacterial strains and plasmids used in Chapter 4.

Strain or plasmid	Relevant genotype or characteristics	Source or reference
<i>A. pleuropneumoniae</i> Strains		
4074	serotype 1 strain; (ATCC 27088)	ATCC ^a
1536	serotype 2 strain; (ATCC 27089)	ATCC ^a
J45	serotype 5a strain	Fenwick et al., 1986a
K17	serotype 5a strain	Nielsen, 1986a
178	serotype 5 strain	M. Mulks
29628	serotype 7 strain	L. Hoffman
13261	serotype 9 strain	J. Nicolet
J45-C	noncapsulated mutant isolated after ethyl methanesulfonate mutagenesis of strain J45	Inzana et al., 1993a
J45-100	recombinant noncapsulated mutant derived from strain J45	This chapter
<i>E. coli</i> Strains		
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> (F ⁺ <i>proAB lacI^qZΔM15 Tn10</i>); Host for recombinant plasmids	Stratagene, La Jolla, Calif.
Plasmids		
pGEM-3Z	Cloning vector, 2.74 kb; Amp ^r	Promega
pCW-1C	5.3 kb <i>Xba</i> I fragment of J45 cloned into pGEM-3Z	Chapter 3
pCW-11E	5.8 kb <i>Bam</i> HI fragment of J45 cloned into pGEM-3Z	This chapter
pKS	3.8 kb <i>Bam</i> HI fragment containing the <i>nptI</i> ^b - <i>sacRB</i> cartridge ^c cloned into the <i>Bam</i> HI site of pGEM-3Z; Amp ^r , Kan ^r	S.M. Boyle
pCW11EΔ1KS1	pCW-11E with the 2.1 kb <i>Bg</i> III- <i>Stu</i> I fragment deleted and the 3.8 kb <i>Bam</i> HI <i>nptI-sacRB</i> cartridge from pKS ligated in	This chapter

^a American Type Culture Collection, Rockville, Md.

^b This marker was originally derived from the Tn903 *nptI* gene of pUC4K (Pharmacia Biotech, Piscataway, N.J.).

^c This cartridge has been previously described (Ried and Collmer, 1987).

$R = 1/g$, where R is the average rate of bacterial growth, and g is the generation time of the bacterial population (Pelczar et al., 1993). The average rate of growth, R , was calculated using the following equation: $R = 3.32(\log_{10} N - \log_{10} N_0)/t$, where t is the elapsed time, N is the number of bacteria at time = t , and N_0 is the initial number of bacteria at time = 0 (Pelczar et al., 1993).

DNA hybridization analysis. Restriction endonuclease-digested DNA (approximately 5 μg per lane) was electrophoresed through 0.7% agarose gels and was transferred by capillary action to MagnaGraph nylon membranes (Micron Separations Inc., Westboro, Mass.) using 20X saline sodium citrate (20X SSC is 3 M NaCl, 300 mM sodium citrate, pH 7) as previously described (Sambrook et al., 1989; Southern, 1975). DNA was covalently linked to nylon membranes by ultraviolet irradiation using a UV Stratalinker (Stratagene, La Jolla, Calif.). Digoxigenin-labeled probes for DNA hybridizations were synthesized by the random primer method using the Genius System nonradioactive labeling and detection kit (Boehringer-Mannheim Corp., Indianapolis, Ind.) according to the manufacturer's directions. DNA hybridizations were performed at 68°C in solutions containing 5X SSC. The membranes were washed and developed according to the Genius System directions for colorimetric detection.

Recombinant DNA methods and reagents. Genomic DNA was isolated from broth-grown *A. pleuropneumoniae* cells using a method described by S. Spinola (personal communication). Briefly, bacteria were resuspended in 10 mM Tris-1 mM EDTA (pH 8) and incubated with sodium dodecyl sulfate (0.66%), and RNase (100 $\mu\text{g}/\text{ml}$) for 1 hour at 37°C. Proteinase K was added to a final concentration of 100 $\mu\text{g}/\text{ml}$, and the mixture was incubated at 56°C for 1 hour. The mixture was extracted once with

buffered phenol and four times with buffered phenol-chloroform (Amresco, Inc., Solon, Ohio), and the genomic DNA was ethanol precipitated and resuspended in 10 mM Tris-1 mM EDTA (pH 8). Plasmid DNA was isolated by a rapid alkaline lysis method (Ish-Horowicz and Burke, 1981). Restriction fragments required for cloning and probe synthesis were eluted from agarose gels as described (Zhen and Swank, 1993). Restriction digests, agarose gel electrophoresis, and DNA ligations were performed as previously described (Sambrook et al., 1989). Restriction fragment ends were made blunt-ended by filling in 5' overhangs with nucleotides (dNTPs) using the Klenow fragment of DNA polymerase I, as previously described (Sambrook et al., 1989). Plasmid DNA was transformed into *E. coli* strains by electroporation (Dower et al., 1988) using a BTX ECM 600 electroporator (BTX, Inc., San Diego, Calif.).

Restriction endonucleases and the Klenow fragment of DNA polymerase I were obtained from Promega Corporation (Madison, Wis.). T4 DNA ligase was obtained from Gibco BRL (Gaithersburg, Md.). Nucleotides (dNTPs) for fill-in reactions were obtained from Boehringer-Mannheim Corporation (Indianapolis, Ind.).

DNA sequencing and analysis. The nucleotide sequence of both strands of the 2.7 kilobase (kb) *XbaI-EcoRV* DNA fragment of pCW-11E was determined by the dideoxy chain-termination method (Sanger et al., 1977) using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio) with α [³⁵S]dATP (DuPont/NEN Research Products, Boston, Mass.). Double stranded DNA templates were sequenced using custom, oligonucleotide primers (DNAgency, Inc., Malverne, Pa.) to continue reading along each strand.

The nucleotide sequence obtained was combined with the nucleotide sequence of the 4.6 kb *XbaI-ClaI* DNA fragment of pCW-1C (Chapter 3), and was analyzed using

DNASTAR analysis software (DNASTAR, Inc, Madison, Wis.). Sequence similarity searches of the EMBL/GenBank/DDBJ databases were performed using BLAST software (Altschul et al., 1990) at the National Center for Biotechnology Information (Bethesda, Md.).

Electrotransformation of *A. pleuropneumoniae*. *A. pleuropneumoniae* was grown to midlogarithmic phase in TSY-N, pelleted by centrifugation at 7000 x g at 4°C, and washed four times in a chilled (4°C), filter-sterilized buffer containing 272 mM mannitol, 2.43 mM K₂HPO₄, 0.57 mM KH₂PO₄, 15% glycerol, pH 7.5. This buffer was modified (to contain mannitol in place of sucrose) from a previously described buffer used for washing *A. pleuropneumoniae* cells prior to electroporation (Lalonde et al., 1989b). The cells were then washed one time in chilled, filter-sterilized 15% glycerol, and resuspended to approximately 10¹⁰ CFU/ml in 15% glycerol. Aliquots of this suspension (90 µl) were mixed with 1.5-2.0 µg of plasmid DNA (in 1.5 µl distilled water) that had been purified by cesium chloride density gradient ultracentrifugation (Sambrook et al., 1989), placed in chilled 2 mm gap electroporation cuvettes (BTX, Inc.), and electroporated using a BTX ECM 600 electroporator (BTX, Inc.) set to a charging voltage of 2.5 kV and to a resistance setting of R7 (246 ohms). The actual pulse generated was 2.39 kV delivered over 10.7 milliseconds. After electroporation, the cells were recovered in 1 ml TSY-N containing 5 mM MgCl₂ with gentle shaking for 3.5 hours at 37°C. After recovery, the cells were cultured on TSY-N agar containing 85 µg of kanamycin per ml and were incubated at 37°C.

Immunoblotting. For colony immunoblots, *A. pleuropneumoniae* whole cells grown overnight on TSY-N agar plates were scraped into phosphate-buffered saline (PBS) and

adjusted to 10^9 CFU/ml, as determined spectrophotometrically. Approximately 5×10^4 or 5×10^5 CFU per well was applied to a nitrocellulose membrane (NitroBind; Micron Separations Inc.) using a Bio-Dot apparatus (Bio-Rad Laboratories, Richmond, Calif.). The membrane was placed in chloroform for 15 minutes at room temperature to lyse the bacterial cells on the membrane. The membrane was air dried completely, and incubated for 1 hour at room temperature in Tris-buffered saline, pH 7.5 (TBS) containing 2% skim milk to block nonspecific binding sites on the membrane. The membrane was incubated for 1 hour at room temperature in a 1:200 dilution (in 2% milk-TBS) of an adsorbed swine antiserum that contained antibodies to the serotype 5a capsular polysaccharide, but not other *A. pleuropneumoniae* surface antigens. This capsular polysaccharide-enriched antiserum was prepared by adsorbing hyperimmune swine antiserum to *A. pleuropneumoniae* K17 with a spontaneous noncapsulated mutant, K17-C (Inzana and Mathison, 1987), as described previously (Inzana, 1995). The membrane was washed in TBS containing 0.05% Tween 20, then incubated 1 hour at room temperature in a 1:1000 dilution of rabbit anti-swine IgG conjugated to horseradish peroxidase (heavy and light chains; Cappel, Durham, N.C.). The membrane was washed in TBS, then developed with 4-chloro-1-naphthol (Bio-Rad Laboratories) in TBS containing 0.02% H_2O_2 .

Immunoblotting of *A. pleuropneumoniae* concentrated culture supernatants was performed as described previously (Ma and Inzana, 1990). Briefly, approximately 15 μ g of total culture supernatant protein was separated by discontinuous SDS-PAGE (Laemmli, 1970) through an 8% separating gel. The proteins were transferred to a nitrocellulose membrane (NitroBind; Micron Separations Inc.) by the method of Towbin et al. (1979). The membrane was incubated in TBS containing 2% bovine serum albumin to block nonspecific binding and was cut into strips. The strips were incubated

overnight at 4°C with either a monoclonal antibody specific for the ApxII toxin (Ma and Inzana, 1990) or a monoclonal antibody specific for the ApxI toxin (Devendish et al., 1989; Frey et al., 1992), and washed in TBS. The blot reacted with the ApxII-specific monoclonal antibody was incubated with a 1:2000 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (Cappel), washed in TBS, and developed as described above. The blot reacted with the ApxI-specific monoclonal antibody was incubated with a 1:2000 dilution of goat anti-mouse IgG conjugated to alkaline phosphatase and developed as described previously (Frey et al., 1992).

LPS extraction and electrophoresis. LPS was isolated from *A. pleuropneumoniae* using a micro hot phenol-water extraction method, as previously described (Inzana, 1983). Purified LPS was electrophoresed through a 15% polyacrylamide separating gel containing urea, as described (Inzana et al., 1988). LPS electrophoretic profiles were visualized by staining the gel with ammoniacal silver (Tsai and Frasch, 1982).

Serum bactericidal assay. Sensitivity of *A. pleuropneumoniae* to the bactericidal activity of precolostral calf serum was determined as described in Chapter 2. Percent viability of bacterial strains in 5, 10, 15, 20, 30, 40, and 50% precolostral calf serum was evaluated after 60 minutes incubation at 37°C.

Virulence study. Pigs 7 to 9 weeks of age were obtained from two local herds free from *A. pleuropneumoniae* infection and were distributed randomly into groups. Groups of pigs were housed in separate pens with no direct physical contact permitted between each group. The animal facilities at Virginia Polytechnic Institute and State University are operated and maintained in accordance with the requirements of the American

Association for Accreditation of Laboratory Animal Care. For the challenge experiment, *A. pleuropneumoniae* strains were grown with shaking in Columbia broth (Difco Laboratories) supplemented with 5 $\mu\text{g/ml}$ NAD at 37°C to midlogarithmic phase. The bacteria were pelleted by centrifugation at 7000 x g and resuspended to approximately 10⁹ CFU/ml in PBS. Pigs were challenged intratracheally with 10 ml of a dilution of this suspension following mild sedation with Stresnil (Pittman-Moore, Inc., Washington Crossing, N.J.). Pigs were necropsied as soon as possible after death or immediately after euthanasia with sodium pentobarbital. Lung lesions were scored by a veterinary pathologist according to the following criteria: 0, unremarkable lungs (no gross lesions observed); 1+, 1-10% of lung tissue affected by some combination of congestion, edema, hemorrhage, consolidation, and/or pleuritis; 2+, 11-49% of lung tissue affected; 3+, 50-74% of lung tissue affected; 4+, 75% or greater of lung tissue affected. Lung samples were taken at necropsy from the right cranial-dorsal aspect of the caudal lobe and cultured on brain heart infusion medium containing NAD to detect the presence of *A. pleuropneumoniae*.

RESULTS

Identification and cloning of a serotype-specific *A. pleuropneumoniae* DNA region. To identify and clone *A. pleuropneumoniae* J45 DNA involved in capsular polysaccharide biosynthesis, Southern blot analyses were performed to identify an adjacent DNA region upstream (in the 5' direction) from the *cpxDCBA* gene cluster described in Chapter 3. I expected this upstream DNA region to encode serotype-specific genes involved in capsular polysaccharide biosynthesis because the *A. pleuropneumoniae* capsulation (*cap*) locus seemed to be organized in a manner similar

to the capsulation loci of *Haemophilus influenzae* type b and *Neisseria meningitidis* group B (Chapter 3). *Bam*HI-digested *A. pleuropneumoniae* J45 genomic DNA was probed with the digoxigenin-labeled 1.2 kb *Bam*HI-*Xba*I fragment of pCW-1C that contained a portion of the *cpxD* gene. This *cpxD*-specific probe hybridized to a single, approximate 5.8 kb *Bam*HI J45 genomic DNA fragment (data not shown). This 5.8 kb *Bam*HI fragment was cloned into the *Bam*HI site of pGEM-3Z from *Bam*HI-digested J45 genomic DNA fragments in the range of 5.0 - 6.5 kb that were electroeluted (following electrophoretic separation) from an agarose gel. The resulting plasmid was designated pCW-11E and was restriction mapped (Fig. 4.1). A portion of the pCW-11E insert DNA (the 1.2 kb *Bam*HI-*Xba*I fragment) overlapped the DNA present on the insert of pCW-1C (Chapter 3).

*Bam*HI-digested genomic DNA from several different *A. pleuropneumoniae* serotypes was hybridized with the 2.1 kb *Bgl*III-*Stu*I fragment of pCW-11E (Fig. 4.1) to determine the serotype-specificity of this DNA region (Fig. 4.2). The 2.1 kb *Bgl*III-*Stu*I DNA fragment hybridized to a 5.8 kb *Bam*HI genomic DNA fragment from three *A. pleuropneumoniae* serotype 5 strains tested, but not to genomic DNA from serotypes 1, 2, 7, and 9 (Fig. 4.2). Thus, the *A. pleuropneumoniae* DNA in pCW-11E contained DNA that was specific to serotype 5 strains. Because this DNA was serotype-specific, it was likely to be involved in capsular polysaccharide biosynthesis.

Nucleotide sequence and analysis of a serotype-specific *A. pleuropneumoniae* DNA region. The nucleotide sequence of the 2.7 kb *Xba*I-*Eco*RV DNA fragment of pCW-11E was determined. This nucleotide sequence was combined with the nucleotide sequence of the 4.6 kb *Cla*I-*Xba*I fragment of pCW-1C (described in Chapter 3) and was examined for the presence of open reading frames (ORFs) not previously identified. The

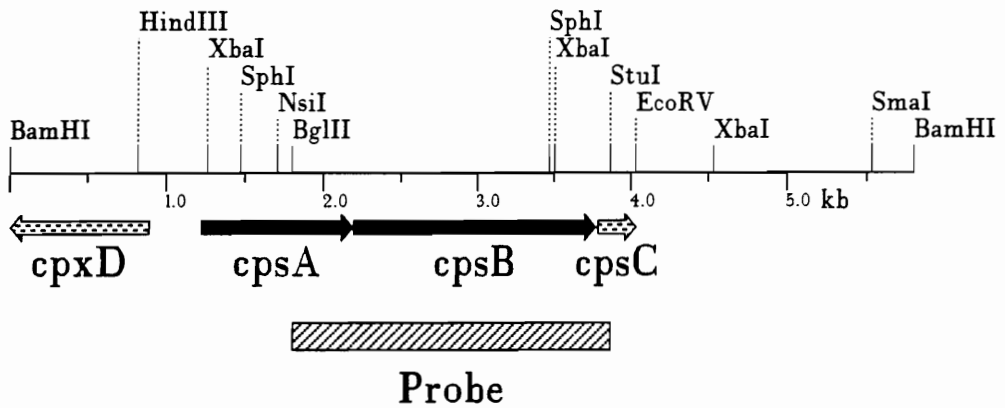


Figure 4.1. Physical map of pCW-11E insert DNA from *A. pleuropneumoniae* J45. The location and direction of transcription of the two complete ORFs (*cpsAB*, solid fill) identified by dideoxy sequencing is indicated. The location of a partial third potential ORF (*cpsC*) is also indicated. The location and direction of transcription of the incomplete *cpxD* gene located on this DNA fragment is also indicated. The 2.1 kb *BglIII*-*StuI* fragment used as the DNA probe in Fig. 4.2 is indicated. Dotted fill indicates incomplete ORFs.

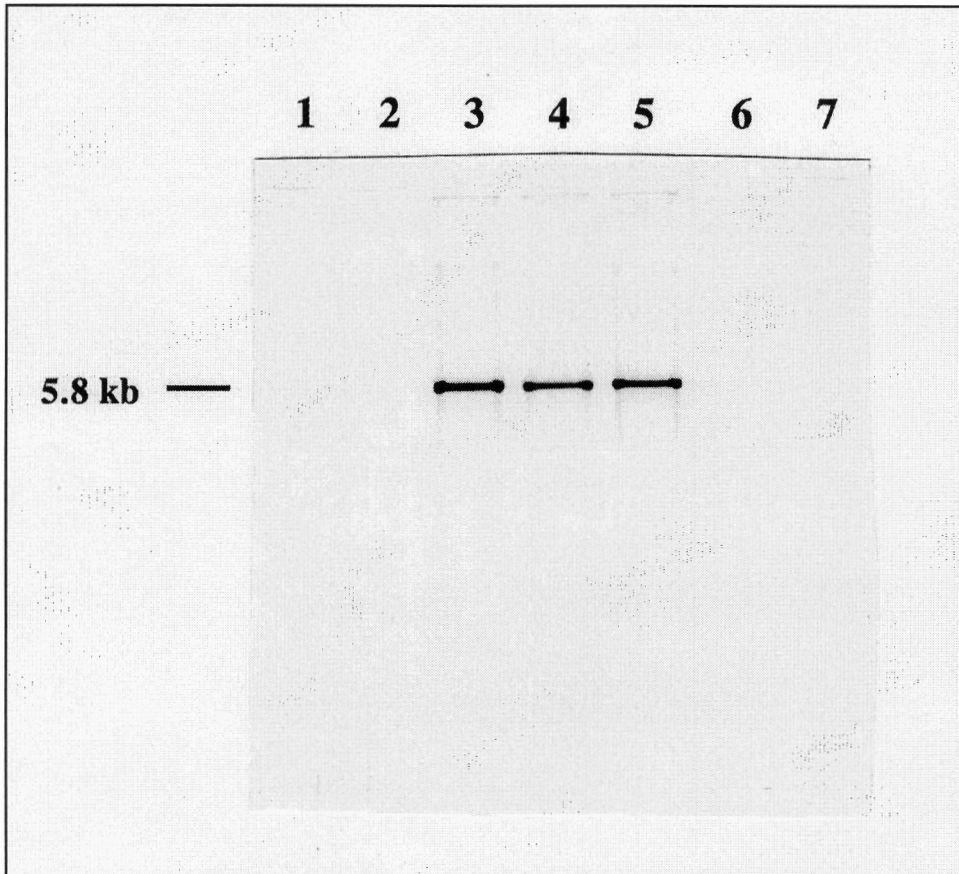


Figure 4.2. Southern blot analysis of *A. pleuropneumoniae* genomic DNA hybridized to the digoxigenin-labeled 2.1 kb *Bgl*III-*Stu*I fragment of pCW-11E. *Bam*HI-digested genomic DNA from serotype 1 strain 4074 (lane 1), serotype 2 strain 1536 (lane 2), serotype 5a strain J45 (lane 3), serotype 5a strain K17 (lane 4), serotype 5 strain 178 (lane 5), serotype 7 strain 29628 (lane 6), and serotype 9 strain 13261 (lane 7) were hybridized with the probe as described in *Materials and Methods*. The molecular mass of the hybridizing bands (in kb) is indicated.

nucleotide sequence of the 3.2 kb *Hind*III-*Eco*RV fragment of pCW-11E containing newly identified ORFs is provided in Fig. 4.3. Two complete ORFs, designated *cpsA* and *cpsB* (*cps* for capsular polysaccharide synthesis), were identified upstream and on the opposite strand from the *cpxD* gene involved in *A. pleuropneumoniae* capsular polysaccharide export (Fig. 4.1 and Fig. 4.3). The AUG initiation codon of *cpsB* was 3 nucleotides downstream from the UAA termination codon of *cpsA*. An AUG initiation codon of a third potential ORF, *cpsC*, was identified 15 bases downstream from the UAA termination codon of *cpsB*. Shine-Dalgarno ribosome-binding consensus sequences (Shine and Dalgarno, 1974) were identified within 13 bases upstream of the AUG initiation codons of *cpsA*, *cpsB*, and *cpsC* (Fig. 4.3). A putative promoter, containing sequences similar to the *E. coli* σ^{70} -10 (TATAAT) and -35 (TTGACA) consensus sequences (Hawley and McClure, 1983) was identified upstream of *cpsA* (Fig. 4.3). The close proximity of *cpsABC* and the identification of a putative promoter upstream suggested that these ORFs may be co-transcribed. The G+C content for the DNA region encoding *cpsABC* was 28%.

The predicted polypeptides of *cpsA* and *cpsB* were comprised of 321 (CpsA) and 526 (CpsB) amino acids (Fig. 4.3). The predicted molecular masses of CpsA and CpsB were 36.9 and 61.7 kilodaltons (kDa), respectively. Hydropathy plots demonstrated that CpsA and CpsB were relatively hydrophilic proteins, suggesting that these proteins may be associated with the *A. pleuropneumoniae* cytoplasmic compartment (data not shown). BLAST searches (Altschul et al., 1990) of the combined, nonredundant nucleotide and protein databases at the National Center for Biotechnology Information did not reveal any substantial homology between *cpsABC* at the nucleotide or amino acid level with other sequences in the databases (data not shown). However, a low level of homology (15% similarity) was observed between CpsA and the *E. coli* Rfb protein, an O-antigen

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AAGCTTGAGCAGGCAGCCAAACTAGCAACCAGCCCCAAGAAAGGAGTAATCTAAGTTTGATGAGTTTCATCTAATTTCTCTTCAATATATTAAAGGAATAACAAC 105
TATATAGGTATGTCCTTAAAAATCCACATAAAGATTGATTTTAAAGTTACCTAATCAAGAGAAATTAATATAAGAAATTTACAACAAATTAATAAATGTTATT 210
TTTTTAAAAAAGTAAATCAAGAGGGCGTTATACAGATAAACATTATAAATTTAAAGCCATATAAAAACGGAGTTTCCCTTAGATAGTTGATAAAATTTCTCA
-35
TTTATATTTATGAAATTCGATGAAAAATTTTCAACTATCTAGGGTAACCCATAACGTATTCGATTTTCAGGAGTATTTTAAATGCTAGCATAATGACTCGT 315
-10
CCTATAATTAATCATGTAATGCTAGAGATATTCAAAGTGGCATATTTAGTTCATTTTAGAATATTTTACTGATTTTGGTCCAAATGAATTTCAACATATTTGTC 525
CpsA -> M S S I M T R
P I I N H V M S R D I Q S G I F S S I L E Y F T D F G S N E F Q H I V
AGTGATATCCCAATACC*GAAGCTAAAGTTTATCACTATCACCGTCCACACCTAGAAAGAAAATATTACCTAATTCGTGTTGACAGTACATCATGACCTCAAT 630
S V S P I P E A K V Y H Y H R P H L E E K L L P N S V C T V H H D L N
GATCCAGATCCTTGGCA*GCTAAGTATAGATTTTCCCTAGATATATGGAAGCTGGGGCTATAAATTTGTTTAAATTACACTCAAAAAGAAAATTTTAAATCTCAG 735
D P D P W H A K Y R F I P R Y M E A G A I I C L N Y T Q K E I L I S Q
GGACTTCCGGAACATAAGTTATTTGTGATTCCTCACGGATATAATCAAAAAGTATTATTTCTCAAGAAAATTAAGAAAATATCAAGTACAGATAAAAATACCTTA 840
G L P E H K L F V I P H G Y N Q K V L F P K K I K E I S S T D K I T L
GGAATGCTTCCAGGAGATATGGTAGAAGAGTAAAAGGAGATGCATATTTATTTGAATTAGCAAAAAGATTAATCCAGACCATTTTAAATTTATTTTGTGGT 945
G I A S R R Y G R R V K G D A Y L F E L A K R L N P D H F K F I F V G
AAAGATAGACAATATAG*GCCTTAGAAATGCAAGATCTAGGATTTGAAGCTCAAGTATATGAAAAGATTGCCATATAGAATTTTCAAGTTTATAATAATATT 1050
K D R Q Y S A L E M Q D L G F E A Q V Y E R L P Y R M F Q S F Y N N I
GATGTACTACTTATGTG*AGTAGTCATGAAGTGGACCTGCAAAATATCCCGAAGCATTAGCTACTGGGACACCTATATTTTCACTAACATAGGTATACCTAAG 1155
D V L L M C S S H E G G P A N I P E A L A T G T P I F S S N I G I P K
GATGTTGTTATTAATATAAAGATGGGTGATTCTAACCTTAGATCCAGATATAGATGCTGAACAGATTAATTTTATTTGCTTGA AAAAACC AAATATATTGAA 1260
D V V I N Y K N G L I L T L D P D I D A E Q I N F I C L E K P N I F E
AATATATTAGATTTTCTACTAAAACAGTCTCCAAGTTTAGCAATTTCTTGGGAGAAATGATTCAACAAAATATTTTAGTTTATAAAAAAATAATTAAGGGTTAA 1365
N I L D F S L K Q S P S L A I S W E K C I Q Q N I L V Y K K I I K G .
TTATGCCATTTCTATTCTAGTACCTGATCTTTACACATTAACAAAAGAACTTTAGTTCATTCTTCAGTTGGATTGAGAAAAATAAAATAATATCCATTTTG 1470
CpsB ->
M S I S I L V P D S L H I N K R N F S S F F S W I E K N K I N I H F
AAAAATAATAAAGATTGGATTTTCAATATATGGTTTTTACGATCAAAAATGGGTATTCTATATGAGAAAATAGATAATCTTACTAAGATTGAAGAAGAGGAAT 1575
E N N N K D W I S L Y G F Y D S K L G I L Y E K I D I L T K I E E E E

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Figure 4.3. Nucleotide sequence of the 3.2 kb *HindIII-EcoRV* fragment of pCW-11E, containing serotype-specific *A. pleuropneumoniae* J45 DNA. The deduced amino acid sequences of the two complete ORFs detected in this sequence, *cpsA* and *cpsB*, and the deduced N-terminal sequence of a third incomplete ORF, *cpsC*, are indicated below the nucleotide sequence. Putative ribosome-binding sites preceding each ORF are in boldface, and putative -10 and -35 promoter sequences upstream from *cpsA* are indicated.

TATTTGCTTTTGTGTTTATGATCTAAATATTTCAATATTTGTAGAGCTGAATTATTATCTTTAGTAGCCACAGACCCGAATGGTATAATGAAGATTATCCTA 1680

L F A F C V Y D L N I F N I C R A E L L S L V A T R P E W Y N E D Y P
ATAACTTAAGAGAAATACAAAAAATCTATACATAATCGAAGTGAATTATTGCAAAACATGGCTGCTTGGTATTGGGTTGATTCTGGAAAAACGCC 1785

N N L R E I Y K K L Y T N N R S E L L Q N M A A A W Y W V D F W K K R
TATCTGAGTAAAACAATCTCTCATTGTTGTATTTTCAGGAGGTTAATTTTACAAAAATCTTTGATTGAGTTATTAATAATACCCAACAAAGTTATGG 1890

L S E L K Q F S H C C V F S G G L I Y Q K S L I E L L K Y T P T K V M
TTATGGAAAGCCTATTACAGGAACGAATATTATTGTGAGGAACGTTATTCATCAATGTCTAATAATAGCGATATTAACATTTAGCTATTTTAACTCTTATA 1995

V M E S L F T G N E Y Y C E E R Y S S I A N N S D I K H L A I F N S Y
AAAAACATTTAGTTCAAAAAGTGAATATGATAAGGAACGAATGAAAGCTATTAATAAGTTCTTATAATGAAAAATAAGAAATGTCCAACAACCTACTGATTCTG 2100

K K T F S S K S E Y D K E R M K A I N K F L L M K N K N V Q Q P T D S
AAATATTAGTATTTAAACAACAAAAACCAATAATTAATCTATTTGGACAAGTGAATAATGATTTTTCAGTCC TAGAATAATAAGGGAGAGGACTATCAACAATTA 2205

E I L V F K Q Q K P I I T I I G O V I N D F S V L E Y K G R G L S T I
AAATCTAAAGAACTATATCTAAACTATCAGAGAATGGATTTAATGTAGTATTA AAAACTCACCTTGGGAAGAGAAAAATAATATCCGTACATCTTTAA 2310

K I Y K E L I S K L S E N G F N V V L K T H P W E E K K N N I R T S L
CTAAAAATATAATAGAAGAAATTTCTAAAAAATCTAACTGAGAATCAACAAGAAATGATAAAAAATAGTTGATCATTCAATAAGAAATTTATTAACAATCTG 2415

T K N I I E E F L K N L T E N Q Q E C I K I V D H Y S I K K L F K Q S
ATTTTATTATAGTTTAAATCTCAAGGGCTCCTGGAAGCTGATTTGATGGTATAAAACCTATACAGTTAGGTAATGCTTTTATGGAAAAAAGGATTACGCT 2520

D F I I S L N S Q G L L E A A F D G I K P I O L G N A F Y G K K G F T
ACGATTATGACTTTTAGATATTGATCAATGGTAAATGACTTAGTAGTAATAAACTTACTCCAACACTATCTTTAGAAGAGTTGATTGTTGCGAAGAGTTCA 2625

Y D Y D F L D I D Q L V N D L V V N K L T P T L S L E E F D L F E E F
TTACTATATTACAAAAAGCATGCTGTTTCTATTCACGGCTCTGGCGTAGTGTTTATCTAGAATATTTAATTTACCTACTATTATACCATTAGTAGAAAAATG 2730

I T I L L Q K H A V S I H A S G V S V L S R I F N L P T I I P L V E N
TCCCTAAGGAGAAGTCTAAAACAACATTACCTACTCAAAAAGATGTGGTAAAAAAGGAAAAATACAACAATGTTAATATGGTTGAGTTACCTAAAGTAGTTCCAC 2835

V P K E K S K T T L P T Q K D V V K K E N T T I V N M V E L P K V V P
AAAGTGATAAGAAATAGGAAATATCAAAAATTTAGAAACAATCCTCGACAATCTTTGCAGATTCTAGGAATCCAGTTATTAGAAGTTAATGATTTTTCCTT 2940

Q S D K N R K Y Q K F R N N P R O F F A D S R N P V I R S L M Y F F P
ATAAATAATATAGGCTAATTTATGTTAAAAAATATCAGCCTTTTGATTTAAGAAAAATAAATGAAGGCCACTCTAGTAATGCTAAGTTAGTTTACATTCTGA 3045

Y K . **CpsC** -> M L K K Y Q P F D L R K I N E G H S S N A K L V L H S E
GGCCTGTAATATAGATGCTAAAATCTCTAAGTTTTCTGTTCCACAAGATGACATTAATTTAGAAAACCTTATTGCAACATTTACTGATAACTATAAAGCACCAGC 3150

A C N I D A K I S K F F C S Q D D I N L E N F I A T F T D N Y K A P E
AGTATATACGGCGATTTTAAAGAATTGTTGATTACACCTAGAGCACC TAAGCTACCAAGAT 3212

V Y T A I L K N C C I T P R A P K L P R

Figure 4.3. (continued)

glycosyltransferase involved in LPS biosynthesis (Cheah and Manning, 1993). A low level of homology (approximately 14% similarity) was detected between CpsB and the region 2 ORF 3 predicted protein product of the *H. influenzae* type b capsulation locus. The ORF 3 predicted protein is involved in the biosynthesis of the polyribosylribitol phosphate capsular polysaccharide of *H. influenzae* type b (Van Eldere et al., 1995). No significant homology was observed between the N-terminal 83 amino acids of CpsC and any proteins in the databases.

Production of kanamycin-resistant, noncapsulated *A. pleuropneumoniae* serotype 5a transformants. Fig. 4.4 schematically outlines the procedures used to produce recombinant, noncapsulated *A. pleuropneumoniae* J45 mutants by homologous recombination and allelic exchange. I first constructed the vector pCW11EΔ1KS1 to use as a nonreplicating, suicide vector to promote the exchange of wild type *A. pleuropneumoniae* capsulation DNA with genetically-altered *A. pleuropneumoniae* capsulation DNA by a double homologous recombination crossover event. The pCW11EΔ1KS1 vector was constructed by first digesting pCW-11E with *Bgl*III and *Stu*I to create a large deletion in serotype-specific *A. pleuropneumoniae* capsulation DNA. The ends of this digested DNA were made blunt-ended, and the large 6.4 kb fragment was ligated to the 3.8 kb *Bam*HI fragment of pKS (also made blunt-ended) containing the *nptI-sacR-sacB* cartridge. This cartridge contains the Tn903 *nptI* gene which is known to confer kanamycin resistance (Kan^r) to *A. pleuropneumoniae* (Tascón et al., 1994), and the *sacRB* sequences that confer sucrose sensitivity (Suc^s) to many gram-negative bacteria (Gay et al., 1983; Ried and Collmer, 1987). The deletion created in pCW11EΔ1KS1 spanned *cpsABC* (Fig. 4.1, Fig 4.4) and was, therefore, likely to affect the protein products of these ORFs.

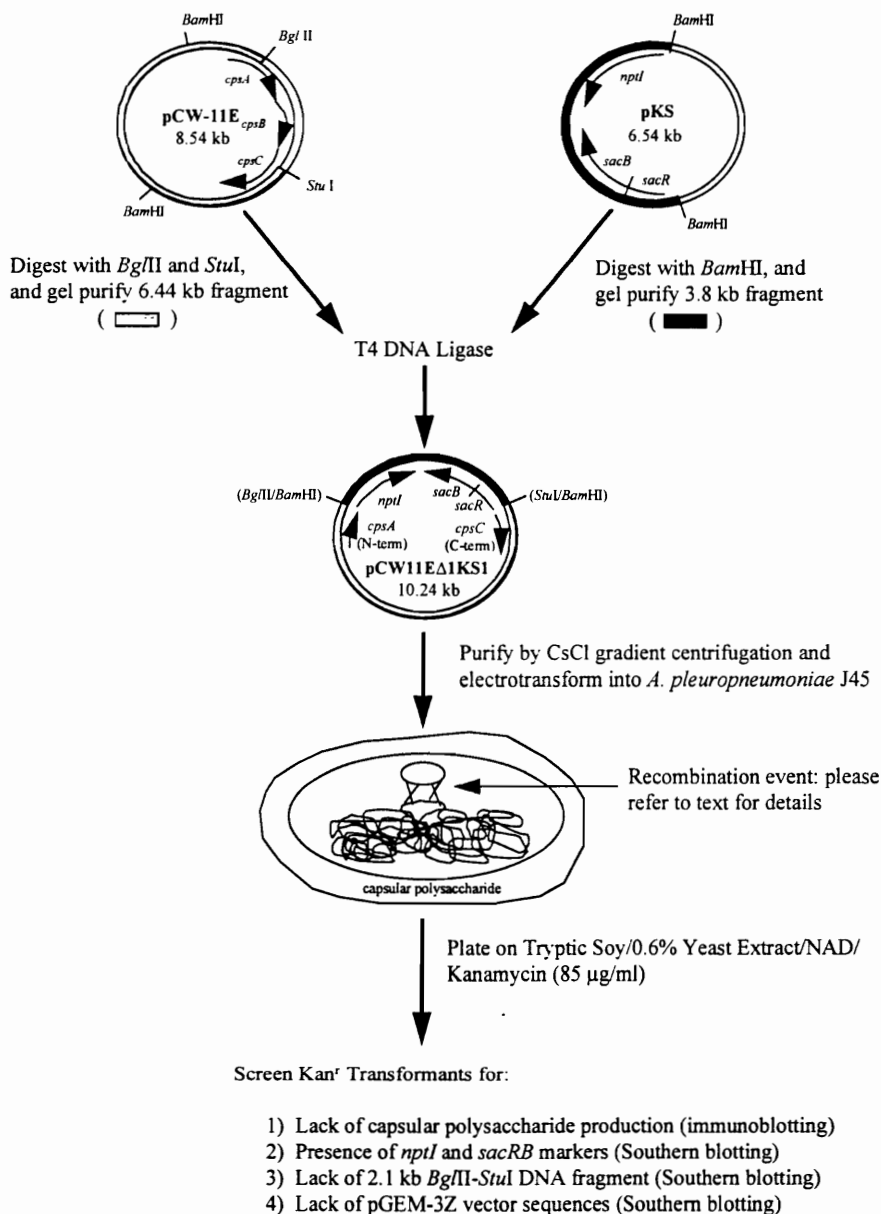


Figure 4.4. Construction of the suicide vector, pCW11EΔ1KS1, and production of noncapsulated mutants of *A. pleuropneumoniae* J45 by allelic exchange. The pCW11EΔ1KS1 plasmid vector was constructed by digesting pCW-11E with *Bgl*II and *Stu*I, making the ends blunt-ended, and ligating the large 6.4 kb fragment to the 3.8 kb *Bam*HI fragment of pKS (also made blunt-ended) containing the *nptI-sacRB* (Kan^r Suc^s) cartridge. Restriction sites in parentheses indicate the original ends of the fragments ligated in pCW11EΔ1KS1. The pCW11EΔ1KS1 vector was electrotransformed into *A. pleuropneumoniae*, and Kan^r transformants were screened as indicated.

The pCW11EΔ1KS1 vector did not replicate in *A. pleuropneumoniae* (my unpublished observations) and, therefore functioned as a suicide vector. After pCW11EΔ1KS1 was electroporated into *A. pleuropneumoniae* J45, seven kanamycin-resistant transformants were obtained after the recovery mixtures were incubated at 37°C for 2 days. Four of these kanamycin-resistant J45 transformants were noniridescent when visualized on plates with an obliquely transmitted light source, suggesting that these transformants were noncapsulated (data not shown). The medium used to grow *A. pleuropneumoniae* prior to electroporation with pCW11EΔ1KS1 was important: noncapsulated kanamycin-resistant transformants were never obtained when *A. pleuropneumoniae* was grown in brain heart infusion supplemented with NAD (data not shown).

Genotypic characterization of the kanamycin-resistant *A. pleuropneumoniae* transformants. Preliminary colony hybridization analyses of the seven kanamycin-resistant transformants revealed that the four transformants which appeared noncapsulated (by visual inspection) hybridized with an *nptI*-specific DNA probe (the 1.24 kb *Pst*I fragment of pKS), but not with probes specific for pGEM-3Z (the 1.1 kb *Bgl*II fragment pGEM-3Z) or the serotype-specific 2.1 kb *Bgl*III-*Stu*I fragment of pCW-11E (data not shown). These results indicated that a double recombination event had occurred in each of these four kanamycin-resistant transformants. In contrast, colonies of the other three kanamycin-resistant transformants hybridized to probes specific for the *nptI* gene, pGEM-3Z, and the 2.1 kb *Bgl*III-*Stu*I fragment of pCW-11E, suggesting that the entire pCW11EΔ1KS1 suicide vector had integrated into the chromosome of these transformants (data not shown). Southern blot analyses of genomic DNA purified from the four kanamycin-resistant, potentially noncapsulated transformants (using the probes

described above) were identical, indicating that the same double recombination event had occurred in each of these transformants (data not shown). One of these transformants was randomly selected for further study and was designated J45-100.

Southern blot analyses of genomic DNA isolated from J45 and J45-100 with DNA probes specific for the *nptI* gene, the 2.1 kb *BglIII-StuI* fragment of pCW-11E, and the 2.1 kb *ClaI* fragment of pCW-1C were performed (Fig. 4.5). The *nptI*-specific DNA probe hybridized to a 5.0 kb fragment of *XbaI*-digested J45-100 DNA, but not to J45 DNA, verifying that the *nptI* marker was in the chromosome of J45-100 (Fig. 4.5A). The hybridization of the *nptI* probe to a 5.0 kb *XbaI* J45-100 genomic DNA fragment was consistent with the size of this *XbaI* fragment in the pCW11EΔ1KS1 suicide vector used to produce J45-100. The 2.1 kb *BglIII-StuI* fragment of pCW-11E hybridized to a 5.8 kb fragment of *BamHI*-digested J45 but not to J45-100 DNA, verifying that this fragment was deleted in J45-100 (Fig. 4.5B). The probe specific for the *cpxCBA* genes (the 2.1 kb *ClaI* fragment of pCW-1C) involved in capsular polysaccharide export hybridized to a 5.3 kb *XbaI* fragment of both J45 and J45-100 (Fig. 4.5C). This result verified that this portion of the *A. pleuropneumoniae* capsulation locus was unaffected by the double recombination event that had occurred within the adjacent DNA region. A probe specific for pGEM-3Z did not hybridize to genomic DNA from either J45 or J45-100, verifying that no vector DNA was contained in the genome of J45-100 (data not shown). Collectively, these DNA hybridization results indicated that the desired double recombination event and allelic exchange had occurred in J45-100.

Phenotypic characterization of the *A. pleuropneumoniae* kanamycin-resistant transformant, J45-100. J45-100 was evaluated for capsular polysaccharide production by colony immunoblotting and latex agglutination. Antiserum containing antibodies

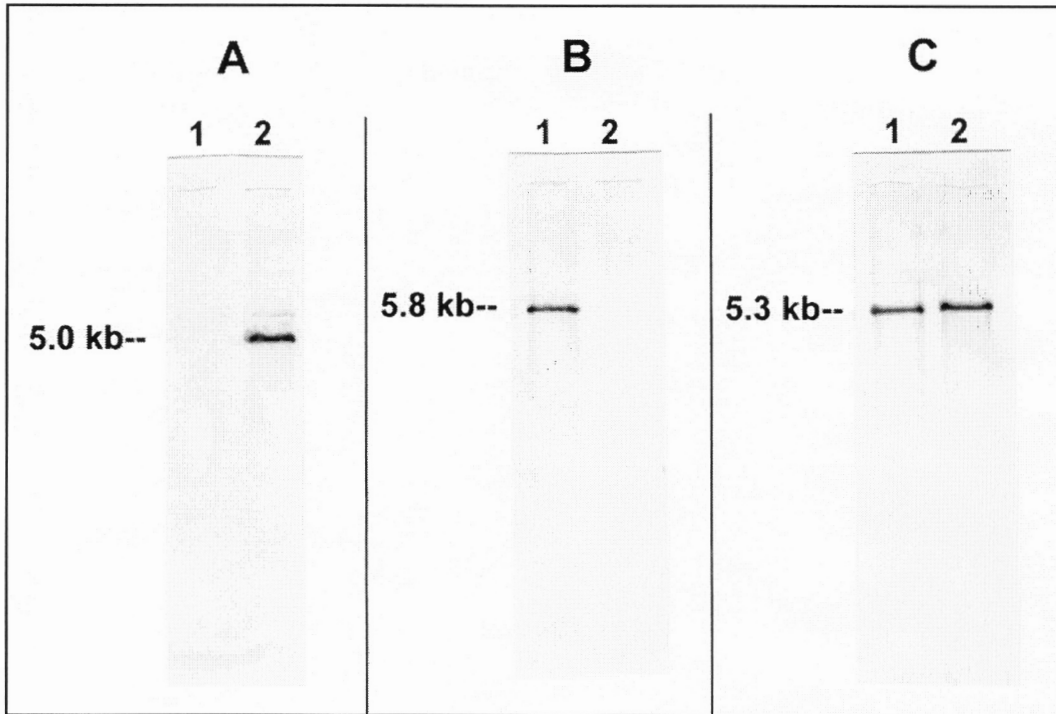


Figure 4.5. Southern blot analysis of genomic DNA isolated from *A. pleuropneumoniae* J45 (lane 1) or J45-100 (lane 2) with digoxigenin-labeled probes specific for *nptI* or portions of the *A. pleuropneumoniae* capsulation locus. *A. pleuropneumoniae* J45 (lane 1) or J45-100 (lane 2) genomic DNA was digested with *Xba*I (panels A and C) or *Bam*HI (panel B), and hybridized with either the 1.24 kb *Pst*I fragment of pKS (*nptI*-specific), panel A; the 2.1 kb *Bgl*III-*Stu*I fragment of pCW-11E (*cpsABC*-specific, see Fig. 4.1), panel B; or the 2.1 kb *Cla*I fragment of pCW-1C (*cpxCBA*-specific, see Fig. 3.2), panel C.

specific for the *A. pleuropneumoniae* serotype 5a capsular polysaccharide, but not other bacterial surface components, reacted with J45 but did not react with J45-100 (Fig. 4.6). Because the bacterial colonies on the membrane had been lysed in chloroform, these results indicated that J45-100 did not produce intracellular or extracellular capsular polysaccharide. Whole or sonicated J45-100 did not agglutinate latex beads that were covalently conjugated to purified antibody to the serotype 5a capsular polysaccharide of *A. pleuropneumoniae* (Inzana, 1995), whereas J45 whole cells and sonicated J45-C cells strongly agglutinated the latex bead reagent (data not shown). These results verified that the deletion engineered into the *cap* locus of *A. pleuropneumoniae* J45-100 resulted in the loss of capsular polysaccharide biosynthesis. Furthermore, these results indicated that a noncapsulated mutant of J45 isolated after ethyl methanesulfonate mutagenesis (Inzana et al., 1993a), J45-C, produced intracellular but not extracellular capsular polysaccharide.

Apx toxin expression and the LPS electrophoretic profiles of J45 and J45-100 were compared to determine if the mutation engineered into the *cap* locus of J45-100 affected these important virulence determinants. No difference in secretion of the 105 kDa ApxI and ApxII toxin proteins into culture supernatant was detected between J45 and J45-100 (Fig. 4.7). In addition, no difference in the LPS electrophoretic profiles of J45 and J45-100 was detected (Fig. 4.8).

The growth of J45 and J45-100 in TSY-N and the sensitivity of J45 and J45-100 to the bactericidal activity of precolostral calf serum were examined to determine the effect of loss of encapsulation on these phenotypic properties. Growth curves of J45 and J45-100 in TSY-N were very similar but not identical (data not shown). However, viable plate counts demonstrated that during the logarithmic phase of growth, J45-100 grew faster (generation time = ca. 23 minutes) than the parent encapsulated strain, J45

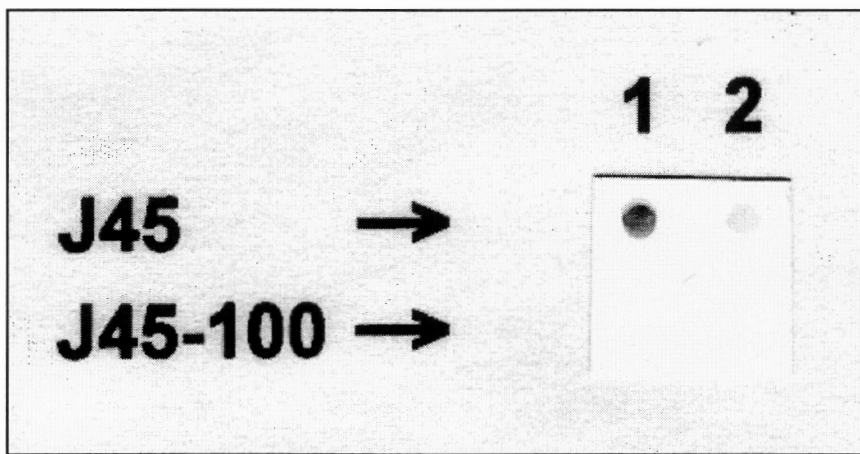


Figure 4.6. Colony immunoblot of *A. pleuropneumoniae* J45 and J45-100 reacted with a capsular polysaccharide enriched swine antiserum. Approximately 5×10^5 (lane 1) or 5×10^4 (lane 2) CFU per well were applied to a nitrocellulose membrane. The membrane was lysed in chloroform and incubated with a swine antiserum that contained antibodies to the serotype 5a capsular polysaccharide but not other *A. pleuropneumoniae* surface antigens.

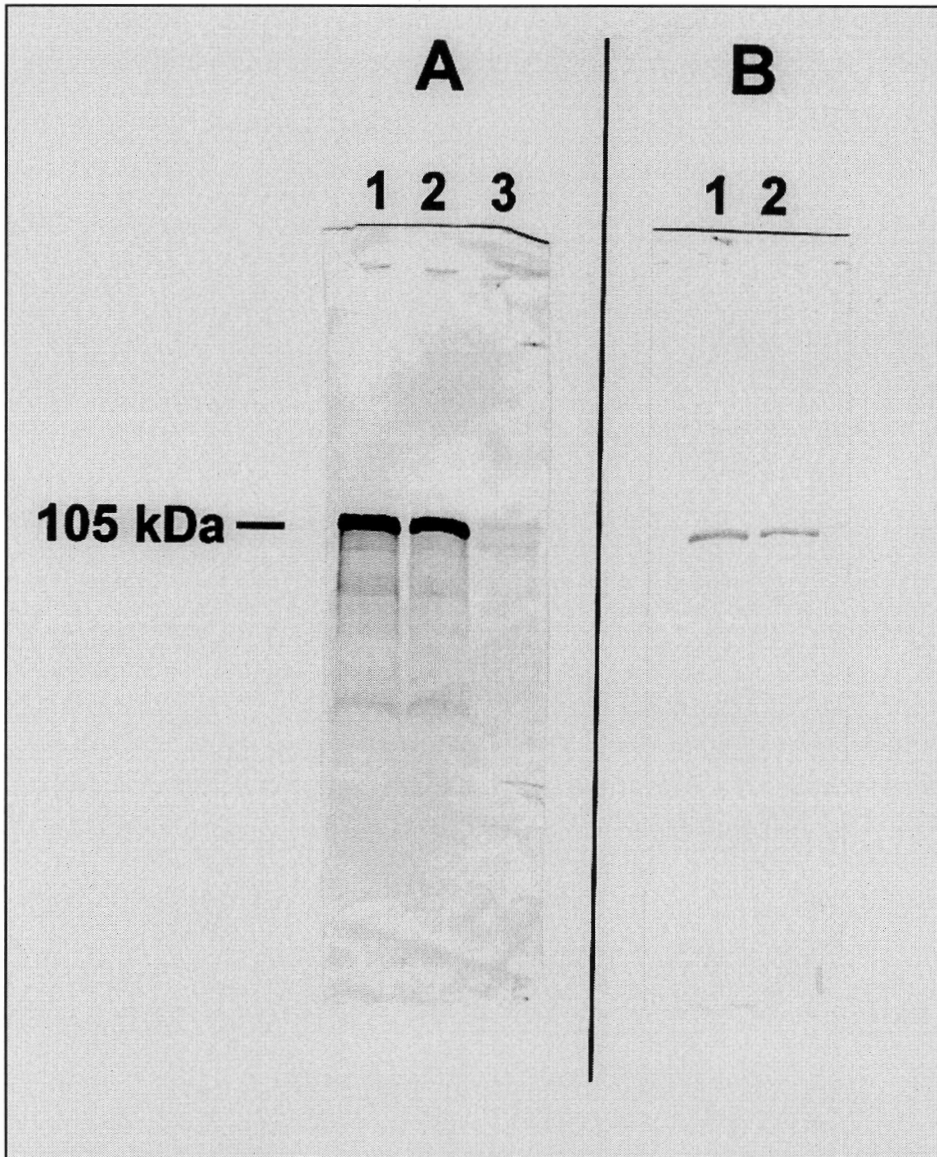


Figure 4.7. Immunoblots of *A. pleuropneumoniae* J45 (lane 1) and J45-100 (lane 2) concentrated culture supernatants. Panel A was reacted with an ApxI-specific monoclonal antibody, and panel B was reacted with an ApxII-specific monoclonal antibody. In panel A, the concentrated culture supernatant of *A. pleuropneumoniae* serotype 2 strain 1536 (lane 3) was included as a negative control because this serotype does not synthesize ApxI. The blot in panel A was reacted with the ApxI-specific monoclonal antibody by J. Frey (University of Berne, Switzerland).

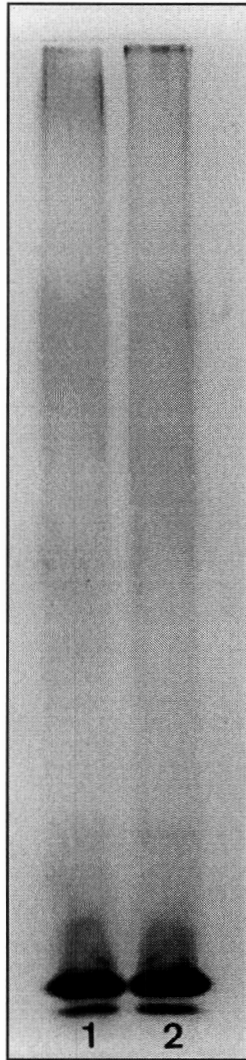


Figure 4.8. Electrophoretic profiles of LPS isolated from *A. pleuropneumoniae* J45 (lane 1) and the recombinant noncapsulated mutant J45-100 (lane 2). LPS was electrophoresed through a 15% separating gel and stained with ammoniacal silver.

(generation time = ca. 28 minutes) (data not shown). The recombinant noncapsulated mutant, J45-100, was efficiently killed within 60 minutes in 10 to 50% precolostral calf serum as a complement source, whereas the encapsulated parent strain, J45, was not killed (Fig. 4.9).

The sucrose sensitivity of J45-100 was examined to determine whether the *sacRB* sequences could function as a counterselectable marker in *A. pleuropneumoniae* and subsequently induce the excision of the *nptI-sacRB* cartridge from the J45-100 chromosome. Broth-grown J45-100 grew very heavily when plated directly or when diluted and then plated on TSY-N or Luria-Bertani (to which 5 μ g/ml NAD was added) medium containing 5% or 8% sucrose (data not shown). The presence of the *sacRB* sequences in the chromosome of J45-100 was verified by Southern blotting (data not shown). These results suggested that either the *sacRB* marker was not expressed in *A. pleuropneumoniae* or possibly that the levan product formed by the *sacRB* levansucrase in the presence of sucrose was not toxic to J45-100.

Intratracheal challenge of pigs with the recombinant *A. pleuropneumoniae* noncapsulated mutant, J45-100. The recombinant noncapsulated mutant, J45-100, did not cause any mortality in pigs when administered at doses 3 and 6 times (1.45×10^7 CFU and 2.95×10^7 CFU, respectively) the 50% lethal dose (LD_{50}) of the encapsulated parent strain, J45 (5×10^6 CFU) (Inzana et al., 1993a) (Table 4.2). In contrast, all three of the pigs challenged with 6.5 times the LD_{50} of J45 developed severe lung lesions and died (Table 4.2).

The five pigs challenged with the lower dose of J45-100 (1.45×10^7 CFU) did not exhibit any clinical symptoms characteristic of swine pleuropneumonia and did not develop any lung lesions. Furthermore, *A. pleuropneumoniae* was not cultured from

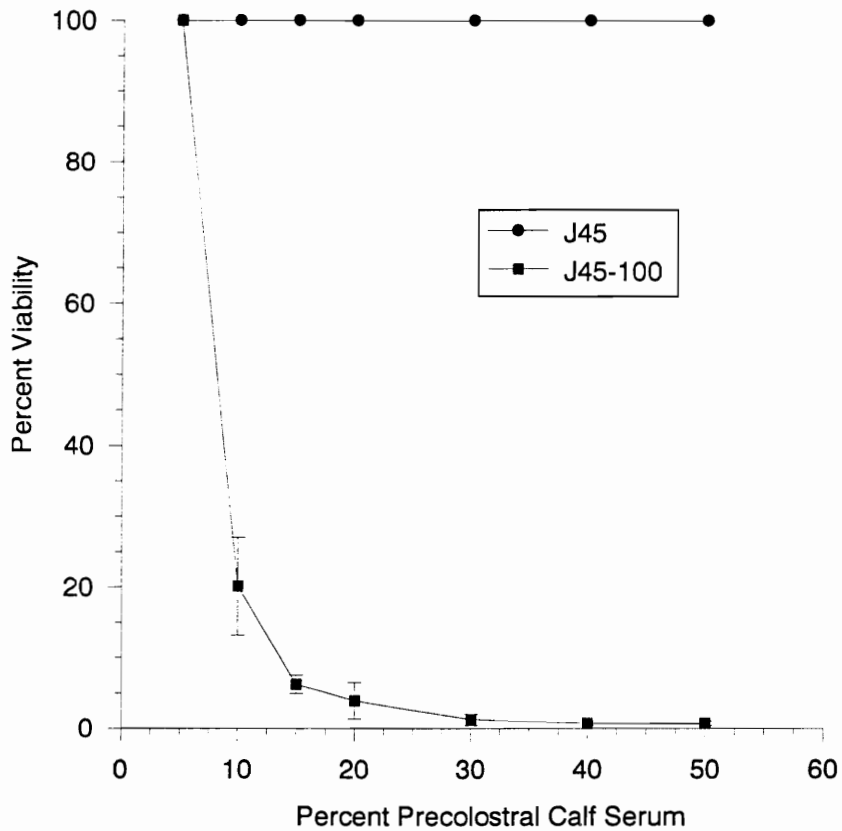


Figure 4.9. Bactericidal activity of precolostral calf serum for *A. pleuropneumoniae* J45 and J45-100. Percent viability of bacterial strains was evaluated after 60 minutes incubation at 37°C. Each data point represents the mean of three separate experiments performed in duplicate. Error bars represent the standard deviation for each mean. The maximum percent viability recorded for J45 was 100%, although these values were typically higher because the bacteria usually grew during the experiment. Values greater than 100% were not recorded because they could not be accurately determined.

Table 4.2. Virulence of *A. pleuropneumoniae* J45 and J45-100 for pigs challenged intratracheally.

Challenge Strain	Challenge Dose	Mean Lung Lesion Score	No. positive/total no. tested	
			Mortality	Recovery ^a
J45	3.30 x 10 ⁷ CFU ^b	3+	3/3 ^c	3/3
J45-100	2.95 x 10 ⁷ CFU	1+	0/5	2/5 ^d
J45-100	1.45 x 10 ⁷ CFU	0	0/5	0/5

^a Recovery of the challenge strain from lung samples taken at necropsy. Pigs challenged with J45-100 were necropsied 4 days post-challenge.

^b This dose is 6.6 times the 50% lethal dose (5 x 10⁶ CFU) reported in a previous study (Inzana et al., 1993a).

^c All of the pigs in this group died within 36 hours post-challenge.

^d Noncapsulated J45-100 was recovered from the lungs of the two pigs with the most severe lung lesions in this group (score = 2+).

lung samples taken four days post-challenge at necropsy. Two of the five pigs challenged with the higher dose of J45-100 (2.95×10^7 CFU) were clinically normal and no lung lesions were observed at necropsy. One pig in this group challenged with the higher J45-100 dose exhibited moderate dyspnea, and at necropsy some lung congestion and slight hemorrhage were observed (lung lesion score = 1+). The remaining two pigs in this group exhibited mild dyspnea, and at necropsy some pleuritis and consolidation were observed (lung lesion score = 2+). *A. pleuropneumoniae* J45-100 was cultured only from these two pigs with the most severe lung lesions. The bacteria recovered from these pigs did not agglutinate the serotype 5a latex bead agglutination reagent. Thus, the recovered bacteria were still noncapsulated, indicating that J45-100 did not revert to the encapsulated phenotype *in vivo*.

DISCUSSION

In this chapter, I report the further characterization of the capsulation (*cap*) locus of *A. pleuropneumoniae* serotype 5a. A portion of the *A. pleuropneumoniae* J45 *cap* locus involved in capsular polysaccharide biosynthesis was cloned and sequenced. This DNA region was located on a 5.8 kb J45 *Bam*HI fragment, that overlapped the *cpxDCBA* gene cluster involved in *A. pleuropneumoniae* capsular polysaccharide export (described in Chapter 3). A portion of this DNA region upstream from *cpxD* hybridized to genomic DNA from only *A. pleuropneumoniae* serotype 5 strains. This result differs from *A. pleuropneumoniae* DNA involved in capsular polysaccharide export that was conserved among serotypes (Chapter 3). The location of this serotype-specific *A. pleuropneumoniae* DNA upstream from *cpxD* is consistent with the location of serotype-specific DNA involved in capsular polysaccharide biosynthesis upstream of the *cpxD*

homologs in *H. influenzae* type b, *bexD*, (Kroll et al., 1990; Van Eldere et al., 1995) and in *N. meningitidis* group B, *ctrA*, (Frosch et al., 1991). These findings provide further evidence that the overall genetic organization of the *A. pleuropneumoniae* *cap* locus may be similar to the organization of the capsulation loci of *H. influenzae* type b and *N. meningitidis* group B. However, the boundaries of the *A. pleuropneumoniae* capsulation locus have not yet been determined.

In general, little homology exists between the genes that encode glycosyltransferases involved in the biosynthesis of structurally distinct capsular polysaccharides (Roberts et al., 1986; Roberts et al., 1988; Edwards et al., 1994; Van Eldere et al., 1995). Therefore, the lack of substantial homology observed at both the nucleotide and amino acid levels between the ORFs (*cpsA*, *cpsB*, and the third potential ORF, *cpsC*) identified in the serotype-specific *A. pleuropneumoniae* DNA region and the sequences in the databases was expected. The low G+C content of the DNA region encoding *cpsABC* (ca. 28%) differs from the overall 42% G+C content reported for NAD-dependent strains of *A. pleuropneumoniae* (Kilian et al., 1978), and from the ca. 40% G+C content of *cpxDCBA* (Chapter 3). These findings suggest a heterologous origin of the genes required for *A. pleuropneumoniae* capsular polysaccharide biosynthesis.

Capsular polysaccharide synthesis occurs on the cytoplasmic side of the gram-negative inner membrane (Whitfield and Valvano, 1993). Since, the CpsA and CpsB proteins were predicted to be relatively hydrophilic, it is likely that these proteins are located within the cytoplasm or associated with the cytoplasmic face of the inner membrane of *A. pleuropneumoniae*.

Genetic manipulations in *A. pleuropneumoniae* have been exceedingly difficult because of the strong restriction barrier expressed by this bacterium (Jansen et al., 1995b; J. Frey, personal communication). Both electroporation and conjugation have

been successfully used to transform plasmid DNA into *A. pleuropneumoniae*, although transformation efficiencies vary among serotypes (Frey, 1992; Lalonde et al., 1989b; Tascón et al. 1993). Tascón et al. (1993) developed a system for random transposon mutagenesis of the *A. pleuropneumoniae* genome and have used this system to construct Apx toxin-deficient mutants (Tascón et al., 1994). However, only Jansen et al. (1995a) have succeeded in producing targeted *A. pleuropneumoniae* knockout mutants (lacking expression of Apx toxins) by allelic exchange. These knockout mutants were produced by electroporating a nonreplicating plasmid vector containing the desired mutation (consisting of a deletion and/or insertion) into *A. pleuropneumoniae* and selecting for transformants resistant to the antibiotic used to inactivate the desired gene. I used a similar type of approach to inactivate *A. pleuropneumoniae* J45 genes involved in capsular polysaccharide biosynthesis by homologous recombination and allelic exchange. The production of these mutants was significant because J45 is one of the more difficult *A. pleuropneumoniae* strains to transform (my unpublished observations). The culture medium used to grow *A. pleuropneumoniae* prior to electroporation seemed to be important for obtaining transformants that had undergone the desired double recombination event: noncapsulated transformants were obtained when *A. pleuropneumoniae* was grown in TSY-N, but not when grown in brain heart infusion. The precise explanation for these differences is unknown. However, brain heart infusion may contain a substance that inhibited the electrotransformation of plasmid DNA into *A. pleuropneumoniae*. Surprisingly, a large proportion of the *A. pleuropneumoniae* kanamycin-resistant transformants obtained (4 out of 7 total in one experiment) had undergone the desired double recombination event. These results were unexpected because single recombination events in which the entire plasmid vector integrates into the genome are more likely to occur (Stibitz, 1994). These results were similar to those

of Jansen et al. (1994a) who also reported a high proportion of *A. pleuropneumoniae* double recombinants after electroporation with a suicide vector. The four *A. pleuropneumoniae* mutants obtained in the present study that had undergone the double recombination event appeared genotypically identical by Southern blot analyses, therefore, only one, J45-100, was selected for further study.

Several important phenotypic characteristics of J45-100 were examined to evaluate the effects of the deletion engineered into the capsulation locus of this strain. J45-100 did not produce intracellular or extracellular capsular polysaccharide. This result was expected since mutations in serotype-specific DNA regions involved in capsular polysaccharide biosynthesis of *N. meningitidis* group B, *E. coli* K1, and *H. influenzae* type b result in strains that do not produce intracellular or extracellular capsular polysaccharide (Frosch et al., 1989; Silver et al., 1984; Van Eldere et al., 1995). These results also indicated that *cpsA*, *cpsB*, and possibly *cpsC* are involved in *A. pleuropneumoniae* serotype 5a capsular polysaccharide biosynthesis. However, the exact contribution of each of these genes to the biosynthesis of the serotype 5a capsular polysaccharide was not determined since all three of these genes were affected by the deletion. It is important to note that J45-100 did not revert to the encapsulated phenotype *in vivo* in the two pigs from which this strain was isolated, indicating the stability of the mutation. Apx toxin secretion and the LPS electrophoretic profiles of J45-100 did not differ from those of the encapsulated parent strain, verifying that the deletion did not affect these important determinants of virulence and immunoprotection. However, J45-100 grew moderately faster (exhibited an ca. 5 minute shorter generation time) than the encapsulated parent strain. In contrast, spontaneous noncapsulated *H. influenzae* type b mutants that produce, but do not export, intracellular capsular polysaccharide grow more slowly and are highly pleomorphic (Hoiseth et al., 1985).

These observations suggested that capsular polysaccharide expression is a metabolically expensive process, and that the accumulation of intracellular capsular polysaccharide is toxic to the bacterial cell.

The sensitivity of J45-100 to the bactericidal activity of precolostral calf serum was examined to ascertain the role of capsular polysaccharide in the serum-resistance of *A. pleuropneumoniae*. J45-100, like the chemically-derived noncapsulated mutants described in Chapter 2, was efficiently killed in precolostral calf serum as a complement source. These results strongly demonstrated that, in the absence of antibody, the capsular polysaccharide was the main determinant of serum-resistance in *A. pleuropneumoniae*.

The recombinant noncapsulated mutant, J45-100, was completely avirulent in nonimmune pigs challenged intratracheally at a dose three times greater than the LD₅₀ of the encapsulated parent strain, J45. When J45-100 was used at a higher dose (six times the LD₅₀ of J45) to challenge pigs, three out of five pigs developed mild to moderate lung lesions, but did not die. It is likely that at this higher dose, J45-100 overwhelmed the natural pulmonary defense mechanisms, such as phagocytosis and killing by pulmonary alveolar macrophages and neutrophils. Once these defense mechanisms were saturated, residual J45-100 was probably able to cause lung lesions since this mutant still produced the highly potent ApxI and ApxII toxins. These challenge studies demonstrated that J45-100 was greatly attenuated in pigs. Thus, the capsular polysaccharide of *A. pleuropneumoniae* can be considered an important virulence determinant. These results validate, but differ slightly, from the results obtained previously with an avirulent *A. pleuropneumoniae* noncapsulated mutant (J45-C) isolated after chemical mutagenesis (Inzana et al., 1993a). The chemically-derived noncapsulated mutant, J45-C, does not cause death or lung lesions at doses up to ten

times greater than the LD₅₀ of the encapsulated parent strain (Inzana et al., 1993a). J45-C produces but does not export capsular polysaccharide. Therefore, J45-C may grow slower than both J45 and J45-100 because of the accumulation of intracellular capsular polysaccharide. It is possible that these small differences in the growth rates of J45-100 and J45-C may account for differences in Apx toxins delivered *in vivo*, and thus in the differences in lesion development observed. However, this hypothesis remains to be proven.

The *sacRB* marker used to construct J45-100 was intended to function as a counterselectable marker to induce the excision of the *nptI-sacRB* cartridge from the genome of J45-100 when this strain was cultured on medium containing sucrose. The *sacB* gene encodes the levansucrase of *Bacillus subtilis* and *sacR* contains regulatory sequences required for the expression of *sacB* (Gay et al., 1983). In the presence of sucrose, levansucrase catalyzes the formation of levan, a fructose polymer that is lethal to many gram-negative bacteria. The *sacB* gene has been used as a counterselectable marker in a wide variety of gram-negative bacteria (Gay et al., 1985; Ried and Collmer, 1987; Schweizer, 1992). However, attempts to cure the *nptI-sacRB* cartridge from the genome of J45-100 by plating the bacteria on medium containing sucrose were unsuccessful. J45-100 grew quite well on medium containing sucrose, indicating that the *sacB* marker was not expressed in *A. pleuropneumoniae* and, thus, did not function as a counterselectable marker. Future work will be needed to optimize the expression of this marker in *A. pleuropneumoniae* so that the cartridge may be successfully excised. If the *sacB* gene is not expressed in *A. pleuropneumoniae*, it may be necessary to produce a new recombinant noncapsulated mutant using a different non-antibiotic resistance marker for positive selection.

In summary, a recombinant noncapsulated *A. pleuropneumoniae* mutant has been

produced that is serum-sensitive and attenuated in pigs. This noncapsulated mutant may be more desirable as a live vaccine candidate than chemically-derived noncapsulated mutants because it is genetically defined, and did not seem to revert. The immunoprotection afforded by this mutant will be evaluated in the future.

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CHAPTER 5

Summary and Future Considerations

The primary focus of the work presented in this dissertation has been the capsular polysaccharide of *A. pleuropneumoniae* serotype 5a. These studies have focused on studying the capsular polysaccharide of serotype 5a because this serotype is commonly isolated from swine pleuropneumonia outbreaks in the United States and Canada (Fedorka-Cray et al., 1993a). At the onset of these studies, the capsular polysaccharide was considered to be a major virulence determinant of *A. pleuropneumoniae*. Previous work demonstrated that a spontaneous noncapsulated mutant was serum-sensitive, indicating that the capsular polysaccharide may protect this bacterium from complement-mediated killing (Inzana et al., 1988b). However, the precise contribution of capsular polysaccharide to *A. pleuropneumoniae* serum-resistance was not known. Previous studies using chemically-induced noncapsulated *A. pleuropneumoniae* mutants also demonstrated the importance of the capsular polysaccharide to virulence and the potential of noncapsulated mutants to be used as live vaccines (Inzana et al., 1993a). However, the role of capsular polysaccharide in the virulence of this bacterium was not known, in part, because genetically-defined noncapsulated *A. pleuropneumoniae* mutants had not been produced.

As the first goal of this project, I sought to determine the mechanism by which capsular polysaccharide contributes to the serum-resistance of *A. pleuropneumoniae*. In Chapter 2, I demonstrated that the capsular polysaccharide prevented complement-mediated killing of *A. pleuropneumoniae* in the absence of specific antibody. The capsular polysaccharide did not prevent complement activation, as has been reported for other bacterial capsular polysaccharides but in the absence of specific antibody, limited

the deposition of C9 onto the bacterial surface. C9 is a component of the membrane attack complex of the complement system required for direct bacterial killing. I also reported the mechanism by which *A. pleuropneumoniae* resists complement-mediated killing in the presence of specific, potentially bactericidal antibody specific for capsular polysaccharide. I identified an antibody present in normal and immune swine serum which bound to *A. pleuropneumoniae* LPS and blocked the complement-mediated killing of this bacterium in the presence of anti-capsular polysaccharide antibody. This LPS-specific blocking antibody reduced the deposition of C9 onto the surface of *A. pleuropneumoniae* and also directed the deposition of C9 to sites on the bacterial surface where it was not stably bound. Since this blocking antibody is present in both normal and immune swine serum, these results suggested that during natural infection with encapsulated *A. pleuropneumoniae* complement-mediated killing probably does not play a role in bacterial clearance. Instead, it is more likely that phagocytosis of *A. pleuropneumoniae* (that has been opsonized by both antibody and complement) by pulmonary alveolar macrophages is the primary host defense mechanism involved in bacterial clearance. Further evidence to support this point is provided by the observations that antibodies specific for *A. pleuropneumoniae* LPS are opsonic (Byrd and Kadis, 1992), and partially protect pigs against swine pleuropneumonia (Byrd et al., 1992; Oishi et al., 1993).

The second major goal of the work presented in this dissertation was to identify and characterize the capsulation locus of *A. pleuropneumoniae*. In Chapter 3, I reported the identification and characterization of a portion of the *A. pleuropneumoniae* capsulation locus involved in capsular polysaccharide export. This region contained four open reading frames, *cpxDCBA*. These genes were highly homologous at both the DNA and deduced amino acid level to genes involved in the export of the capsular polysaccharides

of *H. influenzae* type b and *N. meningitidis* group B, and were moderately homologous to genes involved in capsular polysaccharide export of *E. coli* K1 and K5. Strong evidence for the role of *cpxDCBA* in capsular polysaccharide export was demonstrated by the ability of *cpxDCBA* to complement *E. coli* K5 *kpsM* and *kpsT* mutations to restore capsular polysaccharide export. In chapter 4, I reported the identification and characterization of a DNA region adjacent to the *cpxDCBA* gene cluster that was involved in *A. pleuropneumoniae* capsular polysaccharide biosynthesis. This region contained at least two open reading frames, *cpsA* and *cpsB*, and may contain a third open reading frame, *cpsC*. Collectively, these results suggested that the organization of the *A. pleuropneumoniae* capsulation locus was similar to the general three-region organization of the capsulation loci of *H. influenzae* type b, *N. meningitidis* group B, and *E. coli* K1 and K5. These results provide further evidence that the genes involved in group II capsular polysaccharide expression in different genera of gram-negative bacteria may share a common origin.

The rest of the *A. pleuropneumoniae* capsulation locus may be identified by genomic walking using portions of already cloned capsulation DNA to identify and clone adjacent genomic DNA regions potentially involved in capsular polysaccharide expression. Once these adjacent DNA regions are identified, mutagenesis studies can be performed to delineate the boundaries of the *A. pleuropneumoniae* *cap* locus and to identify the different regions involved in capsular polysaccharide biosynthesis and export. The protein products of the *A. pleuropneumoniae* capsulation locus may also be characterized to assist in studying the mechanisms involved in capsular polysaccharide expression.

Another accomplishment of the work presented in this dissertation was the successful production of a recombinant noncapsulated *A. pleuropneumoniae* mutant, J45-100 (described in Chapter 4). J45-100 contained a deletion in the *cap* locus that spanned

cpsA, *cpsB*, and *cpsC*. This mutant did not produce intracellular or extracellular capsular polysaccharide, verifying that *cpsA*, *cpsB*, and/or *cpsC* were involved in capsular polysaccharide biosynthesis. J45-100 was sensitive to complement-mediated killing in the absence of specific antibody. This finding corroborated the results obtained with spontaneous and chemically-induced noncapsulated mutants, and further demonstrated that the capsular polysaccharide is the main determinant of serum-resistance in the absence of specific antibody (Inzana et al., 1988b; Chapter 2). J45-100 was greatly attenuated in pigs, but was not completely avirulent since some pigs that received an intratracheal dose 6 times the LD₅₀ of the encapsulated parent strain developed mild to moderate lung lesions. This recombinant noncapsulated mutant may potentially prove useful as a live vaccine for the prevention of swine pleuropneumonia. However, immunization studies examining the ability of J45-100 to protect pigs from a potentially lethal challenge with homologous or heterologous serotypes need to be performed. Once the entire *A. pleuropneumoniae* capsulation locus is identified and mutagenized, noncapsulated mutants with defects in different functional regions of the *cap* locus should also be evaluated for their immunoprotective properties. It is possible that noncapsulated mutants which produce intracellular capsular polysaccharide may provide greater protection against disease.

If J45-100 is determined to be immunoprotective, further studies will need to be performed to determine a method for curing the *nptI-sacR-sacB* cartridge from the genome of this strain. These studies may include those to optimize the expression of the *sacB* gene in *A. pleuropneumoniae*, or perhaps may consist of the construction of a new strain using a different marker for selection. Antibiotic resistance markers are undesirable in genetically-engineered microorganisms used as live vaccines because of their potential transfer to other microorganisms. Once the final derivative of the vaccine

strain is developed, the optimal route, dose, adjuvant, and method for commercial preparation need to be evaluated in order to produce an efficient vaccine. While J45-100 may not be the actual strain used as a live vaccine, the results obtained with this strain will be useful in evaluating the potential of recombinant, noncapsulated *A. pleuropneumoniae* mutants as live vaccines.

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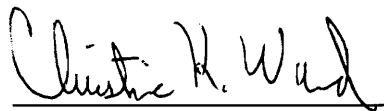
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Vita

Christine Ward was born on March 20, 1967 in Brooklyn, N.Y. At 4 years of age she moved with her parents Robert and Carol Melnik, and sister Joann, to the Long Island town of Huntington where she lived until she graduated from Harborfields High School in Greenlawn, N.Y. in 1985. She then pursued a Bachelor of Science in Biology at Virginia Polytechnic Institute and State University, which she received in May 1989. While studying for her B.S., she met Michael John Ward whom she later married in June 1992. In the fall of 1989, she began a Masters degree program at the Virginia-Maryland Regional College of Veterinary Medicine under the guidance of Dr. Thomas Inzana studying the mechanism of *Actinobacillus pleuropneumoniae* serum-resistance. In the spring of 1993, she transferred into a PhD program, remaining in Dr. Inzana's laboratory to study the genetic basis of *A. pleuropneumoniae* capsular polysaccharide expression.

A handwritten signature in cursive script that reads "Christine K. Ward". The signature is written in black ink and is positioned above a horizontal line.

Christine K. Ward