This is to certify that the

dissertation entitled

Characterization of the genes of Actinobacillus pleuropneumoniae involved in oxidative stress and pathogenesis

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology and
Molecular Genetics

Martha A. Mulks
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Date July 19, 2002

CHARACTERIZATION OF THE GENES OF *ACTINOBACILLUS PLEUROPNEUMONIAE* INVOLVED IN OXIDATIVE STRESS AND PATHOGENESIS

by

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Molecular Genetics

2002

ABSTRACT

CHARACTERIZATION OF THE GENES OF *ACTINOBACILLUS PLEUROPNEUMONIAE* INVOLVED IN OXIDATIVE STRESS AND PATHOGENESIS

By

Robin Jeanne Kastenmayer

Actinobacillus pleuropneumoniae is the causative agent of porcine hemorrhagic pleuropneumonia, a severe and contagious respiratory disease. While many virulence factors in *A. pleuropneumoniae* have been identified, there are still many unanswered questions and unknown proteins which represent potential antibiotic targets or effective vaccines. An *in vivo* technology system (IVET) developed in *A. pleuropneumoniae* was refined so that novel virulence factors could be identified and characterized.

Using this system, twenty five unique *in vivo* induced (*ivi*) clones were identified. These clones contained promoters that were induced during infection of the pig (*in vivo*) but had minimal expression on rich laboratory media (*in vitro*). These genes that were upregulated during infection could be divided into four categories: proteins previously identified as virulence factors, those required for metabolism during rapid growth and colonization, proteins of known function that had not been previously characterized as required for virulence, and unknown proteins.

One of these *ivi* genes encodes Ohr, an organic hydroperoxide reductase. Ohr was shown to be regulated in response to organic peroxides. Ohr functions during the

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oxidative stress that occurs subsequent host immune cell influx and degranulation to release oxygen radicals. Ohr and its regulator are serotype-specific, found only in three of the twelve serotypes. The characterization of *ohr* shows the new information that can be gained from the use of IVET screens to identify virulence genes.

In order to further characterize the identified *ivi* genes, as well as to construct potential vaccines, genetically defined attenuated mutants are required. Mutant construction was attempted for both *ohr* and for *ilvI*, a gene involved in isoleucine-leucine-valine biosynthesis.

The characterization of *ohr* and initial analysis of *ilvI* have shown not only the potential information that can be gained through the use of IVET, but in addition, the many questions that arise. IVET has contributed to the understanding of *A. pleuropneumoniae* pathogenesis through the identification of virulence factors and by providing an initial platform from which more questions can be asked.

ACKNOWLEDGMENTS

I would like to thank my mentor, Martha Mulks, for her encouragement, support and understanding. She has been the primary factor in my growth as an independent scientific researcher and has had a tremendous impact on my career. I wish to thank the members of my committee, Dr. Michael Bagdasarian, Dr. Wendy Champness, Dr. Linda Mansfield, and Dr. Larry Snyder, for their advice and guidance and unwavering support.

I would like to thank the current and former members of the Mulks' laboratory for making it a fun and exciting place to work. Without their help and support, I would not have been able to accomplish this research. I wish to thank Scott Doree, Trevor Wagner, and Traci Eagen for their enjoyable and stimulating discussions of science. I would especially like to thank Dr. Troy Fuller and Dr. Byron Hagewood for their wise advice and assistance in the early stages of my research. In addition, I would like to thank Dr. Fuller and Dr. Rick Martin for two exciting and rewarding collaborations.

I also wish to thank the many people, both within and outside of the College of Veterinary Medicine, who have made the dual degree program possible. Their moral support and belief in myself and the program have been an unwavering strength. I would like to thank Dr. Ron Patterson, Angie Zell, Coreena Spitzley, and Dr. Jerry Dodgson for their aid in establishing this program. I especially wish to thank the current and former Associate Deans for Graduate Studies and Research, in particular, Dr. John Baker. Professional school has been a unique and challenging experience, and one that has shaped my life both personally and professionally. I wish to thank my instructors, classmates and the staff in the college for this wonderful and exciting portion of my life.

I wish to thank the Harold Wetterberg Foundation, the Jaqua Foundation, the College of Veterinary Medicine, and the Graduate School at Michigan State University for their financial support. Without this funding, my program would have been impossible.

Perhaps the most important people to thank are those that are there day after day with unconditional love and support. It is for this that I wish to thank my husband and my family and friends. Without your understanding, I would not have been able to accomplish all that I have done. Without your love, it would not have mattered.

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Introduction

Actinobacillus pleuropneumoniae is a respiratory pathogen of swine that causes a severe contagious hemorrhagic pleuropneumoniae. Several virulence factors have been identified in *A. pleuropneumoniae* and have been shown to be required for infection. While much is known about key virulence factors, a protective vaccine against all *A. pleuropneumoniae* serotypes is not available.

This chapter reviews the epidemiology and pathology of *A. pleuropneumoniae*, in addition to discussing the known *A. pleuropneumoniae* virulence factors, these include capsule, lipopolysaccharide, several exotoxins, pilin, and specific proteins. The discussion of virulence factors includes those involved in survival in response to oxidative stress. The discussion of the virulence factors involved in oxidative stress in *A. pleuropneumoniae* includes those identified in this bacterium, as well as those found in other bacteria and are hypothesized to function as virulence factors in *A. pleuropneumoniae*.

The scope of this dissertation is to expand the knowledge of *A. pleuropneumoniae* pathogenesis by identifying virulence factors required for infection. By identifying these factors, potential subunit vaccines and attenuated mutants for use as vaccines can be developed with the long term plan of creating an effective, heterologous vaccine.

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Epidemiology of Actinobacillus pleuropneumoniae

Since the initial characterization of *Actinobacillus pleuropneumoniae* in 1961, the worldwide significance of this pathogen has grown (26, 49, 83). *A. pleuropneumoniae* is the cause of porcine hemorrhagic pleuropneumonia, which in the acute disease causes high morbidity and mortality. The severity of the clinical signs and the duration of the infection vary with the immunological status of the pig, the degree of exposure, and the environmental conditions (71). In the acute infection, the pigs are febrile and depressed with respiratory signs, such as dypsnea and cough (26, 82). Pigs that recover from these acute infections are often maintain the organism in the nasal passages and respiratory discharges and can propagate the bacteria through aerosal or direct contact with naïve pigs (71). The chronic infection is often missed, since the pigs exhibit no fever, and only occasionally show respiratory distress (71). Often the chronic form is only detected at slaughter by the high prevalence of pleuritis and fibrin deposition in the lungs of the slaughtered swine (81). In either the acute or the chronic infection, the producer is economically affected, either through the loss of individual animals or by a decrease in weight gain in the chronically infected herd (71).

A. pleuropneumoniae is a gram negative pleomorphic member of the *Pasteurellaceae* family, with two biotypes and twelve serotypes in biotype one. The biotypes are based upon a requirement for nicotinamide adenine dinucleotide (NAD), whereas the serotypes are differentiated by capsular and LPS genes (74). In the United States, the predominant serotypes are one, five and seven in the NAD dependent biotype (74).

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Prevention and Treatment of Disease

A. pleuropneumoniae infection can be treated with antimicrobials such as the beta-lactams, chloramphenicol, cotrimoxazole, tetracyclins, and enrofloxacin (71). However, since the lung damage is irreversible, the most effective treatment is prevention. Preventative measures include vaccination with serotype-specific bacterins and improved farm management to prevent exposure of naïve pigs to chronically infected animals. It has been shown that vaccination with a serotype-specific bacterin does not provide heterologous protection against the other serotypes, whereas a natural infection provides protection against subsequent challenge with other serotypes (15, 56, 72). This observation has led to research in the development of vaccines that have the same protective value as a natural infection.

Subunit vaccines composed of outer membrane antigens isolated from one serotype have been proven to provide homologous protection against subsequent challenge by the same serotype and have provided some protection against challenge heterologous serotypes (10, 17, 24, 25). Vaccines consisting of exotoxins and other extracellular proteins have been shown to provide good homologous protection and one vaccine consisting of exotoxins and an outer membrane protein has shown homologous and heterologous protection (22, 93). Attenuated live *A. pleuropneumoniae* vaccines are currently in development with the hope of developing a vaccine that offers complete heterologous protection (39, 40, 51). The use of an attenuated live *A. pleuropneumoniae* that is unable to produce riboflavin as a vaccine provides complete protection against subsequent homologous challenge and reasonable protection against challenge by a

heterologous serotype (39). However, the currently available vaccines have been unable to provide the complete cross protection that occurs following natural infection.

Pathology

A. pleuropneumoniae causes a fulminating bronchopneumonia with an accompanying pleuritis that can resolve to fibrin deposition and regions of necrotic lung tissue in convalescent animals (82). The disease course can range from a subclinical chronic infection to an acute infection in a herd naïve to *A. pleuropneumoniae.* In these immunologically naïve herds, morbidity can be as high as 100 percent (47, 90). The lung necrosis and fibrin deposition that are characteristic of this pneumonia are predominantly seen in the apical and dorso-caudal lung lobes (82).

The diagnosis of porcine pleuropneumonia caused by *A. pleuropneumoniae* can be made initially based upon clinical signs and observation of the lung lesions. The lung damage includes dilation of the alveolar capillaries with erythrocytes, thrombocytes and fibrin. The lung parenchyma shows areas of coagulative necrosis and edema accompanied by an influx of inflammatory cells, such as macrophages, lymphocytes, and neutrophils (5, 63). Bacteria can be routinely cultured from the respiratory surfaces and from the nasal discharge; with occasional isolation from the respiratory associated lymphoid tissues (71).

Virulence Factors

The disease symptoms are due in part to bacterial endotoxin, exotoxins, and capsule, in addition to the activity of specific virulence proteins. Capsule and lipopolysaccharide (LPS) contribute to bacterial survival within the host by protecting against complement-mediated lysis and phagocytosis (16, 27, 52). The role of capsule in preventing phagocytosis can be demonstrated by the addition of anti-capsular antibodies, which when incubated with *A. pleuropneumoniae* allow for effective opsonization of the bacteria by polymorphoneutrophils (52, 79). However, the presence of anticapsular antibodies only provides partial protection during infection, suggesting the presence of other virulence factors required for disease (52, 79). Both LPS endotoxin and exotoxin contribute to the lung damage by causing damage to the lung parenchyma (27, 28).

Lipopolysaccharide (LPS) LPS endotoxin is a common virulence factor in gram negative bacteria and aids in bacterial survival within the host. LPS consists of a lipid portion (lipid A) that is responsible for much of the toxicity associated with endotoxin and a variable oligosaccharide region. The O-antigen oligosaccharides protect the bacteria from complement-mediated lysis by sterically inhibiting insertion of the membrane attack complex (15). In addition, surface-exposed LPS may act as an adhesin by binding respiratory tract mucous and epithelial cells (3, 27). This binding may be a requisite initial event in the disease process (3, 70).

In addition to these roles of LPS, it has been shown that purified endotoxin is toxic to leukocytes, platelets, and vascular endothelium and can cause lung lesions, in a dose-dependent manner (27, 28). These lung lesions are the result of an interstitial

pneumonia but lack the hemorrhage typically seen during infection by *A. pleuropneumoniae*. Perhaps the most important role of LPS in virulence is the effect of LPS on the host immune system. It is believed that the immune cell influx associated with gram-negative endotoxin and the release of proinflammatory mediators, such as IL-1 and TNF-α, is triggered by the presence of the LPS, in particular the lipid portion (18, 68). This results in a classical inflammatory reaction including edema and increased capillary permeability, neutrophil infiltration, and deposition of fibrinogen. The presence of antibodies directed against gram-negative LPS, while not preventing infection from occurring, did cause a decrease in the morbidity and mortality associated with infection by *A. pleuropneumoniae* (29).

This evidence suggests that while LPS may be partially responsible for host immune cell influx and a portion of the disease symptoms, other virulence factors are required to produce the symptoms typically seen during infection.

Exotoxins. A key virulence factor of *A. pleuropneumoniae* was hypothesized to be an excreted toxin, since culture supernatant was toxic for swine monocytes and respiratory epithelium and induced lung lesions when endobronchially inoculated (4). The heat labile protein Apx exotoxins found in *A. pleuropneumoniae* are of the RTX (glycine-rich repeats in structural toxins) family. Four different exotoxins (ApxI-ApxIV), encoded by distinct operons, have been described in *A. pleuropneumoniae*. Each serotype within biotype one contains two or more of these different classes of exotoxins (32, 35, 80). These toxins show both hemolytic and cytotoxic activity and can destroy alveolar macrophages, thymic lymphocytes, erythrocytes, and neutrophils (77).

The mechanism of action of the toxins can be extrapolated from work done with *Escherichia coli* alpha hemolysin, HlyA, which is homologous to ApxI. This toxin inserts into eukaryotic cell membranes, creating pores and leading to osmotic swelling and subsequent cellular lysis (84). This lysis causes the release of oxygen radicals and lysosomal enzymes from the damaged phagocytes, which in turn damage host tissue, resulting in necrosis and hemorrhage (14, 23, 91). Even sublytic doses of toxin cause abnormal phagocytosis and chemotaxis of macrophages and polymorphoneutrophils (23, 88, 91). The presence of the glycine rich repeats allows for efficient binding of calcium and loss of the repeats results in decreases in both calcium binding and hemolytic ability (6). Calcium binding is predicted to occur after export from the bacterial cell (6). The production of the pore occurs via two stages, the reversible adsorption followed by the irreversible insertion of the toxin complex into the eukaryotic cell membrane. This insertion requires proper protein folding to allow for the formation of hydrophobic alpha helices flanked by amphipathic membrane-binding helices (84). A completely formed pore alters the osmotic potential of the cell, in addition to allowing flux of ions across the membrane. Calcium appears to be required for initial insertion, but does not appear to be required for pore formation and may be involved in the proper folding of the protein and exposure of hydrophobic regions (6). The acylation of the protoxin may serve to mediate membrane association and the assembly of the multiple protein pore (84).

The first identified *A. pleuropneumoniae* RTX toxin, ApxI, was shown to be a potent hemolysin and cytotoxin and was strongly immunogenic (35, 69). ApxI is induced by calcium and requires calcium for activity (21, 94). ApxII is a less potent hemolysin, requires a higher concentration of calcium for hemolysis and is not induced by calcium

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(94). ApxI and ApxII share greater than 50% identity at the protein level (35). ApxIII is unable to cause erythrocyte lysis, but is able to cause lysis of alveolar macrophages (57, 64). In addition, ApxIII has a lower sequence homology to either ApxI or ApxII (54). ApxIV toxin, which shows sequence similarity to iron-regulated RTX toxins from *Neiserria meningitidis*, is produced only during infection, with an unknown method of regulation (80).

Each RTX toxin operon comprises four open reading frames encoding the protoxin, an acetylase, and two secretion proteins. The protoxin is activated posttranslationally through protein acylation by the acetylase. The toxin is secreted through a type I secretion apparatus produced by the remaining two proteins within the operon (34, 37). The ApxII protein, which is hemolytic and cytotoxic, is encoded by an operon containing the two structural open reading frames, but without functional secretion genes. ApxII can be secreted by the secretion apparatus of either ApxI or ApxIII (33, 55).

The importance of the RTX exotoxins has been demonstrated by several *A. pleuropneumoniae* mutants that have been characterized. An *A. pleuropneumoniae* serotype 1 mutant, which lacked ApxI and ApxII was shown to have decreased virulence in comparison to the parent strain (53). However, additional factors apart from the exotoxins are required for disease, since piglets with high hemolysin neutralization titers still show morbidity, albeit they have a lesser degree of lung damage than pigs with lower titers (15).

While much has been learned about the structure and the role in virulence of the Apx toxins, the regulation of these genes during infection is still not complete. A gene

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has been identified in *A. pleuropneumoniae* that has been proposed to regulate the expression of these toxins (36). The HlyX protein contains a [4Fe-4S] cluster and is similar to the *Escherichia coli* FNR global regulator, which functions under anoxic conditions (45). Additionally, the HlyX protein may not be the sole regulator of the toxins. ApxIV, which is produced during infection, may be regulated by a complex pathway or by more than one regulator (80).

Fimbriae. Fimbriae and pili are found on the surface of many prokaryotic cells and have been shown to be essential for attachment to host cells and subsequent colonization. *A. pleuropneumoniae* has been shown to express fimbriae during infection, which are lost upon growth on laboratory media (92). Type four fimbriae have recently been described in *A. pleuropneumoniae* serotype one and have been shown to be expressed during growth on a chemically defined media under microaerophilic conditions, suggesting that there exist *A. pleuropneumoniae* genes that are induced during conditions such as the nutrient limitation that occurs during infection of the porcine respiratory tract (96).

Iron scavenging proteins. One of the well characterized host responses to bacterial infection is to limit the free iron concentration, thus limiting an essential element for microbial growth and proliferation. Lactoferrin and transferrin are secreted host factors that are able to bind to free iron and sequester it. In order to obtain the iron necessary for growth, some bacteria have evolved siderophores and iron-regulated proteins that are capable of scavenging the free iron and host sequestered iron for microbial usage. *A. pleuropneumoniae* expresses transferrin-binding proteins (Tbp1 and Tbp2) that are

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specifically induced under iron limitation and are capable of scavenging the iron for use by the bacterium (41, 42, 73). Tbp1 is a transmembrane protein capable of transporting iron across the outer membrane (19), whereas Tbp2 has been shown to be an outer membrane protein that contains an amino-terminal region capable of binding specifically to porcine transferrin and not human or bovine transferrin (41-43, 86). *A. pleuropneumoniae* mutants that lack either or both transferrin-binding proteins are unable to survive within the host or cause disease (2). The Tbp proteins are cotranscribed with two upstream genes, *exbB* and *exbD* (89). ExbBD has been shown to be essential for the utilization of iron from porcine transferrin (89). Three proteins (AfuA, AfuB, and AfuC) have been identified in *A. pleuropneumoniae* and proposed to function in transport of the iron from transferrin and into the bacterium (11). AfuA has homology to a periplasmic iron-binding protein, whereas AfuB and AfuC are most likely membrane transport systems (11). Together the Tbp and the Afu proteins are responsible for acquisition of host iron in order to enable bacterial growth and survival.

Virulence Factors Associated with Oxidative Stress Response

Bacterial Response to Oxygen Radicals

To combat the lethal effect of reactive oxygen species, many bacteria have adopted methods through which to detoxify these oxygen species. The oxygen species are released by host immune cells as well as generated by the aerobic metabolic activities of bacteria and bacterial enzymes (44, 67). Without the proteins necessary to combat these reactive oxygen molecules, bacteria would be unable to survive and hence could not

cause infection. Several genes encoding proteins necessary for survival under oxidative stress have been identified in *A. pleuropneumoniae*. In addition, several proteins necessary for detoxification of oxygen radicals have been identified in other bacteria and could exist in *A. pleuropneumoniae*.

Major reactive oxygen species include the superoxides, peroxides, and hypohalides. Superoxides are detoxified by the superoxide dismutases to form peroxides. These peroxides are in turn degraded by peroxidases and catalases. The cellular damage inflected by these oxygen radicals includes interacting with DNA and proteins to induce DNA mutations and protein denaturation and inactivation, respectively. Additional enzymatic damage results when these reactive oxygen species in turn oxidize the disulfide bonds within proteins and oxidize metals causing protein denaturation and loss of catalytic sites (67). Membrane damage to the cell can also result from autocatalytic oxidation of lipid moities (67). Since the effect of oxygen radicals is so profound and if left unchecked can lead to the destruction of the cell, continual vigilance is required to detect the presence of oxidative damage and activate regulons capable of detoxifying oxygen radicals and repairing the cellular damage. It is by monitoring the oxidative state of the molecules, metals and sulfur moities, that react so vigorously to oxygen radicals, that bacteria can regulate the defense mechanisms involved in oxidative stress response (78). The major global regulators of oxidative defense genes include SoxRS, OxyR and PerR.

Virulence factors of *A. pleuropneumoniae* that respond to oxidative stress. One of the key components in the host reaction to bacterial infection is the respiratory burst and degranulation of host neutrophils and the subsequent release of hypoclorate, superoxides,

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and peroxides. These reactive oxygen species can cause cell membrane lysis, inactivation of metabolic enzymes, oxidation of membrane lipids, and DNA damage (1, 7, 42, 50, 60). *A. pleuropneumoniae* contains superoxide dismutase genes, *sodA* and *sodC,* that produce proteins capable of reducing superoxides. SodC, a copper-zinc containing superoxide dismutase, is constitutively expressed, whereas SodA, a manganese containing superoxide dismutase, has been shown to be produced under anaerobic conditions of iron limitation (61). *A. pleuropneumoniae* has been shown to be catalase positive; i.e., able to reduce hydrogen peroxide to oxygen and water, but the gene responsible for this activity has not been identified (59).

A. pleuropneumoniae also contains Ohr, an organic hydroperoxide reductase that is induced by exposure to organic hydroperoxides and may be responsible for the reduction of organic peroxides that are encountered during exposure to neutrophil contents or those generated during normal cellular metabolism. The identification and characterization of *ohr* is the subject of chapter three of this dissertation.

SoxRS

SoxR, a major sensor of oxidative stress within the cell responds to the oxidative state of a bound metal. SoxR contains two [2Fe-2S] centers that upon oxidation of the iron cause activation of SoxR (20). This activated protein then enhances transcription of *soxS*, which encodes a transcription factor, resulting in increased SoxS production (78). The SoxS protein in turn binds to a consensus site (AnnGCAY) overlapping the –35

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region and interacting with RNA polymerase to activate the SoxS regulon and negatively regulate its own promoter (78).

The *E. coli* genes that are induced by superoxide stress and regulated by the SoxRS system include genes that encode superoxide dismutases, a gene encoding endonucleaseIV, *ahpC*, and *zwf* (46, 58, 62, 95). The superoxide dismutases, which include SodB, SodC, and SodA, are responsible for reducing superoxides to peroxides, which can then be inactivated by catalase or by a different protein, such as alkylhydroperoxide reductase (AhpC). The endonuclease, which is involved in DNA repair, could be responsible for correcting DNA damaged by oxygen radicals (8). The role of Zwf, a glucose-6-phosphate dehydrogenase, could be to supply reducing equivalents in the form of NADH to help reduce oxidized proteins and metals. (46, 95) (75). In addition it has been shown that both OxyR and SoxRS activate Fur, the ferric iron uptake regulator, allowing for modulation of iron concentration (97). This regulation in the concentration of iron is important, since the toxicity of both hydrogen peroxide and superoxides are exacerbated by high concentrations of iron (38, 87, 97).

OxyR

OxyR is activated via the structural change that occurs when an intramolecular disulfide bond between homodimers of OxyR is oxidized. This activated OxyR then binds, with a consensus site of ATAG, to occupy four consecutive DNA major grooves, which in turn results in increased RNA polymerase recruitment and transcription (12). OxyR in turn represses its own transcription, allowing for a constant intracellular

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concentration of protein, which is important for a timely response to an influx of peroxides.

OxyR regulates multiple genes, some of which are regulated by SoxRS as well. The OxyR regulon was initially identified as the proteins that were responsible for adaptation to hydrogen peroxide in *Salmonella*. The genes within the regulon allow *Salmonella* to be grown in the presence of 10 mM hydrogen peroxide, a lethal concentration, after the bacterium has been adapted in 60 μM hydrogen peroxide (13). These genes encode proteins that include: a) those responsible for the reduction of the peroxide to an alcohol, HPI catalase (KatG) and AhpCF; b) those responsible for reducing oxidized enzymes, glutathione reductase (GorA), glutaredoxin I (GrxA), and thioredoxin II (TrxC); and c) those involved in modifying the concentration of intracellular iron, Fur and an iron-sulfur containing protein (FhuF) that is involved in iron uptake (13, 30, 76, 85, 97). In addition, *zwf* and *sodA* are regulated by OxyR to supply reducing equivalents to the cell and protect against oxygen radicals, respectively. Other genes induced by high concentrations of hydrogen peroxide encode proteins responsible for disulfide bond formation (DsbG), formation of heme containing compounds (HemH), and genes in an operon for sulfur mobilization (65, 98, 99).

Per

While OxyR and SoxRS have been found in many bacteria after their initial characterization in *Salmonella* and *E. coli*, there exist additional oxidative stress regulators in other bacteria. Per was initially characterized in *Bacillus subtilis* as a global regulator that responded to iron, manganese, and hydrogen peroxide stress (9). Per has

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been shown to be a metalloregulatory protein that has sequence similarity to Fur and functional analogy to OxyR (9). The genes in the Per regulon include *ahpCF, katA*, and *hemAH*, all of which were shown to contain a consensus Per box in the promoter (CTAtnTTAtAATNATTATAAattA) (9). In addition, Per has been shown to repress itself and *fur* (38). The Per protein found in *Staphyloccocus aureus* has been shown to induce *ahpC, katA*, and *trxB* (48).

Oxidative Regulators in Actinobacillus pleuropneumoniae

The genome of several *Pasteurellaceae* species have been sequenced and can be analyzed for the presence of regulators that respond to the oxidative stress profile of the cell (31, 66). *Haemophilus influenzae* has been shown to contain an *oxyR* homologue and a functional OxyR consensus binding sequence has been identified in the promoter of *katE*, a gene encoding a catalase (31, 78). In *A. pleuropneumoniae* serotype 5, a gene that has homology to *oxyR* has been identified, but a functional analysis of the protein encoded by this gene has not been performed. However, *soxRS* homologues have not been identified in *H. influenzae* or in the other *Pasteurellaceae* members sequenced to date, including *A. pleuropneumoniae* serotype 5 or *A. pleuropneumoniae* serotype 1. Thus, the search for *the A. pleuropneumoniae* regulators of genes that respond to oxidative stress must be found by other mechanisms.

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Scope of Dissertation

The scope of this dissertation is to identify and characterize genes necessary for *A. pleuropneumoniae* pathogenesis. To accomplish this goal, an *in vivo* technology system (IVET) developed in *A. pleuropneumoniae* was refined so that novel virulence factors could be identified and characterized.

Chapter two of this dissertation discusses the twenty five unique *in vivo* induced (*ivi*) clones that were identified. These clones contained promoters that were induced during infection of the pig (*in vivo*) but had minimal expression on rich laboratory media (*in vitro*). These genes that were upregulated during infection could be divided into four categories: proteins previously identified as virulence factors, those required for metabolism during rapid growth and colonization, proteins of known function that had not been previously characterized as required for virulence, and unknown proteins.

Chapter three of this dissertation discusses Ohr, an organic hydroperoxide reductase. Ohr was identified as an *in vivo* induced gene and was shown to be regulated in response to organic peroxides. Ohr functions during the oxidative stress that occurs subsequent to host immune cell influx and degranulation to release oxygen radicals. Ohr and its regulator are serotype specific, found only in three of the twelve serotypes. The characterization of *ohr* shows the new information that can be gained from the use of IVET screens to identify virulence genes.

Chapter four discusses the need to evaluate newly identified virulence genes as potential vaccine candidates. The construction of the genetic tools to make attenuated mutants is described for both *ohr* and for *ilvI*, a gene involved in isoleucine-leucine-

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valine biosynthesis. While the mutants were unable to be constructed, future modifications for successfully obtaining the mutants is discussed.

Chapter five summarizes this dissertation, in order to be able to suggest future experiments to expand upon the current knowledge of *A. pleuropneumoniae* and its virulence factors.

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Chapter 2

Identification of virulence factors of *Actinobacillus pleuropneumoniae* by the use of IVET (*in vivo* expression technology)

A portion of this chapter has been published previously.
Fuller, T. E., R. J. Shea, B. J. Thacker, and M. H. Mulks. 1999. Identification of *in vivo* induced genes of *Actinobacillus pleuropneumoniae*. Microb. Pathog. **27:** 311-327.

Abstract

Actinobacillus pleuropneumoniae promoters that are induced during infection but have minimal expression on rich laboratory media have been identified using an *in vivo* expression technology (IVET) system. This system is composed of an avirulent riboflavin-requiring *A. pleuropneumoniae* mutant (AP233), a promoter-trap vector (pTF86), and an infection model, the natural swine host. The pTF86 vector contains a T_4 terminator, a unique *Bam*HI cloning site, and promoterless copies of the *luxAB* reporter genes and *Bacillus subtilis ribBAH* genes in an *Escherichia coli-A. pleuropneumoniae* shuttle vector. Partially digested *A. pleuropneumoniae* genomic DNA was cloned into pTF86 and electroporated into AP233. Pigs were infected with pools of approximately 600 transformants by endobronchial inoculation. The pigs were euthanized at twelve to sixteen hours after initial infection and surviving bacteria were isolated from the swine lungs. This infection strongly selected for transformants containing cloned promoters that allowed expression of the *ribBAH* genes and complementation and survival of the attenuated mutant. The clones isolated from the pig lungs were assayed for luciferase expression to identify those that had minimal expression *in vitro*. The clones that survived *in vivo*, but which had minimal luciferase expression *in vitro*, are hypothesized to contain cloned promoters that are specifically induced during infection. Twenty-five clones were isolated which contain promoters that are induced during infection. Thirteen clones showed strong homology to known genes. The remaining twelve clones contained either no identifiable open reading frame or not enough sequence information to identify the gene driven by the *in vivo* expressed promoter. Nine of these twelve clones have had

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the in vivo induced gene identified by inverse PCR or by sequence comparison to a database of *A. pleuropneumoniae* serotype 5 genomic DNA sequences. The identification of *in vivo* induced genes has contributed a new understanding to disease caused by *A. pleuropneumoniae*.

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Introduction

Virulence factors of Actinobacillus pleuropneumoniae

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a contagious disease with an acute onset of dyspnea, hyperemia, and pneumonia. This bacterium causes significant lung lesions that are composed of hemorrhage, fibrin deposition, and pleural adhesions. The economic toll of this disease results from both the rapid death of naive swine in peracute infection and from a decreased growth and increased production costs that occur in subclinical chronic infections (23, 48).

Currently identified virulence factors of *A. pleuropneumoniae* include toxins, lipopolysaccharide, and capsule. Capsule and lipopolysaccharide (LPS) have been shown to contribute to bacterial survival in the host by protecting against complement mediated lysis and phagocytosis (6, 10, 24). Purified LPS has been shown to be toxic to leukocytes, platelets, and vascular endothelium. This cellular damage results in non-hemorrhagic pulmonary lesions, often seen during an acute infection (6, 10, 24). The RTX exotoxins (glycine-rich repeats in structural toxins) have been identified in all twelve serotypes of *A. pleuropneumoniae* and have been shown to have both hemolytic and cytotoxic activity (12, 13). The toxins are capable of destroying alveolar macrophages and lymphocytes by creating pores in the eukaryotic cell membranes and causing cell lysis (6, 46, 50). This lysis results in the release of oxygen radicals and lysosomal enzymes from the damaged phagocytes, which in turn damages host tissue by causing necrosis and hemorrhage (4, 8).

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The identification and characterization of the toxins and other known virulence factors have contributed to only a partial understanding of the disease pathogenesis. The RTX toxins and LPS contribute to the disease lesions, but the presence of these virulence factors alone does not explain how the infection spreads through the host and is maintained in the chronically infected herd.

Characterization of virulence genes

In an effort to further understand the pathogenesis of *A. pleuropneumoniae*, an IVET (*in vivo* expression technology) system has been developed to identify genes that are expressed during infection (*in vivo*), but show minimal expression on laboratory media (*in vitro*). The IVET method selects for the promoters of genes that are expressed during an infection. The tools required are an attenuated strain of the pathogen, a promoter trap plasmid that is able to complement this attenuation and restore virulence, and an infection model. The plasmid contains a unique *Bam*HI restriction site into which random genomic DNA fragments can be cloned upstream of a promoterless reporter gene and the promoterless gene that complements the attenuating mutation. If a promoter is contained within the cloned DNA, the promoter-reporter-biosynthetic gene fusion is transcribed during infection, the biosynthetic gene complements the mutation, and the bacteria are able to survive and cause disease *in vivo*. Clones selected *in vivo* by the disease process are expressed during the infection, thus allowing for complementation. The reporter gene is used to quantitate expression to determine the degree of *in vitro* expression. Those that are only induced *in vivo*, but not *in vitro* generally fall into one of four categories of virulence genes. These categories include genes that are known to be

involved in pathogenesis or regulators of these genes, biosynthetic genes that are needed during the rapid growth *in vivo*, genes of known function that have not yet been identified as virulence factors, and unknown genes.

Methods for identification of *in vivo* expressed genes Multiple methods, including IVET, have been developed to allow for the identification of genes that are expressed *in vivo*. One method to identify genes that are induced during infection has been done using in vitro conditions designed to mimic specific environmental conditions encountered *in vivo*, such as nutrient limitation or exposure to oxygen radicals, and then screen for genes that respond to these conditions. This system will miss many of the virulence factors that respond to a more complicated series of environmental stimuli prior to expression (35).

Another method to identify virulence genes is by homology to known virulence factors from other bacteria. This technique has been applied with limited success in identifying possible virulence factors in *A. pleuropneumoniae*. The *sodC* locus (Cu, Zn superoxide dismutase) was identified by constructing PCR primers from regions of high homology to other superoxide dismutases and amplifying *sodC* from *A. pleuropneumoniae* (26). The transferrin binding proteins were isolated by screening for genes that complemented an *E. coli* mutation (2). However, these methods are limited to genes hypothesized to exist in one bacteria based upon the knowledge of another related bacteria. Since little is known about the virulence mechanisms in *A. pleuropneumoniae* or related respiratory pathogens, a different approach is needed for a more thorough screen. The use of genetic tools has replaced the need for screening for individual genes with methods to perform a complete screen for virulence factors.

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These genetic tools include signature-tagged mutagenesis (STM), differential display, and *in vivo* expression technology (IVET). STM is a negative selection process that identifies genes that, when interrupted by a transposon, result in an inability to survive *in vivo*. The process compares an *in vitro* grown pool of transposon-insertion clones to the pool recovered from an infection to identify which transposon mutants were present in the *in vitro* pool but were lost during infection. This *in vivo* loss occurs when the bacteria could not survive without an intact copy of the interrupted gene. This comparison is accomplished by PCR amplification and subsequent hybridization to unique signature sequence tags that are found flanking each transposon. Genes identified by STM have included a secretion apparatus in a *Salmonella typhimurium* pathogenicity island and genes for adhesin maintenance in *Staphylococcus aureus* (34, 42).

Differential display involves comparing the mRNA produced under different conditions. The total mRNA is isolated is isolated from bacteria grown *in vitro* and those recovered after infection. From this mRNA, cDNA is obtained by reverse transcription. The cDNA can then be amplified with polymerase chain reaction (PCR) primers that are specific for a gene of interest, to determine if that gene is transcribed during infection. Another method using differential display involves comparing both pools of cDNA, using gel electrophoresis or Southern blots to determine which transcripts are found under only one condition (9). This comparison can be used to isolate genes which are regulated such that they are only expressed during infection.

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IVET background

The IVET method selects for the promoters of genes that are expressed during an infection. The tools required are an attenuated mutant of the bacteria of interest and a promoter trap plasmid that is able to complement this attenuation and restore virulence. The plasmid consists of a randomly cloned genomic fragment containing a potential promoter fused upstream of a promoterless reporter gene and the promoterless gene that complements the attenuation. The plasmid is transformed into the attenuated bacterial strain. If the promoter fusion is transcribed during infection, the biosynthetic gene complements the mutation and allows the bacteria to survive and cause disease *in vivo*. All clones selected *in vivo* by the disease process are expressed at a high level to allow for complementation. The bacteria are harvested from the infected host and plated *in vitro* on laboratory media. The reporter gene is used to quantitate expression to determine the degree of *in vitro* expression. Those that are also expressed at a high level *in vitro* are likely to be constitutively expressed housekeeping genes, such as those involved in metabolism. Those that are only induced *in vivo* generally fall into one of four categories. These categories include genes that are known to be involved in pathogenesis, biosynthetic genes that are needed during the rapid growth *in vivo*, genes of known function that have not yet been identified as virulence factors, and unknown genes.

IVET was initially developed to identify virulence genes in a *Salmonella typhimurium* BALB/c mouse spleen model. The IVET vector was constructed as a single copy suicide plasmid with the *lacZY* gene as a reporter gene and *purA* to complement the

purine auxotrophy (32, 44). The single copy recombines into the host chromosome to produce two functional promoters, one promoter to drive the expression of the *lacZY* and *purA* genes, while the other promoter remains uninterrupted to continue to regulate the gene of interest. Since this initial work, IVET systems have been continually developed by using different animal models and pathogenic bacteria and by modifying the vector. Some of the modifications have included using different auxotrophic or antibiotic markers to complement the bacterial host, and eliminating or modifying the reporter gene to include a recombinase protein or different colorimetric markers (9, 22, 30, 32, 33, 53, 57).

Materials and methods

Bacterial strains and media. The bacterial strains and plasmids propagated in these strains are listed in Table 1. *A. pleuropneumoniae* were cultured at 35 ℃ in either brain heart infusion (BHI) or heart infusion (HI) (Difco Laboratories, Detroit, Mich.) containing 10 μg/ml β-NAD (V-factor) (Sigma Chemical Company, St. Louis, MO). Riboflavin (Sigma) was added to a final concentration of 200 μg/ml when needed for the propagation of AP233. Ampicillin was added at 50 μg/ml for plasmid maintenance and at 20 μg/ml for selection after electroporation and lung culture. *Escherichia coli* strain XL1-Blue mRF' (Stratagene, LaJolla, Calif.) was cultured in Luria-Bertani media with 100 μg/ml ampicillin added when needed for plasmid selection.

DNA manipulations. DNA modifying enzymes were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, Ind.) and used according to the manufacturer's specifications. PCR was performed using Gibco Taq polymerase (Life Technologies, Rockville, MD). PCR products were separated with a 2% agarose gel containing three parts Gibco (Life Technologies) agarose and one part NuSieve GTG agarose (FMC Bioproducts, Rockland, ME). Plasmid DNA was isolated from bacterial strains using the Qiagen miniprep kit (Qiagen Inc., Valencia, Calif.). *E. coli* transformation was performed by the method of Hanahan (20).

Strain
Strains
AP100
AP233
XL1-B
Plasm
pTF8(
pGZR

a

b

Table 2-1. Strains and plasmids used in this work.

Strain or plasmid	Characteristics	Source
Strains		
AP100	*A. pleuropneumoniae* ATCC 27088, serotype 1A, passaged through pigs	ATCC[a]
AP233	A genetically defined riboflavin auxotroph of AP100	(17)
XL1-Blue mRF'	*E. coli* cloning strain	Promega[b]
Plasmids		
pTF86	*A. pleuropneumoniae* IVET vector containing promoterless *luxAB* and *ribBAH* genes downstream of *Bam*HI cloning site (Figure 1)	(15)
pGZRS18	Ap^r *A. pleuropneumoniae-E. coli* shuttle vector	(55)

a American Type Culture Collection, Rockville, Md.

b Promega Corporation, Madison, Wis

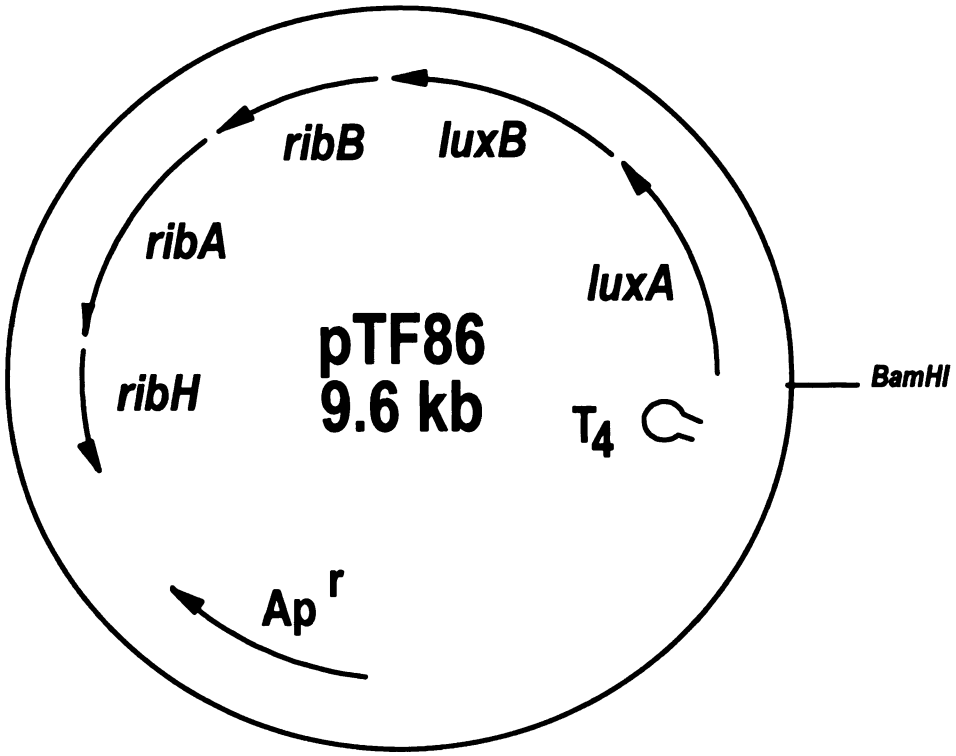

Figure 2-1 Diagram of pTF86, showing the T_4 terminator, the unique *Bam*HI cloning site, and the orientation of the promoterless *luxAB* and *ribBAH* genes.

Copy number determination. The copy number of pTF86 was determined by the method of Xia et al. (56). Briefly, total DNA from AP233 containing piviE was digested with *Sal*I, separated on an agarose gel, and blotted onto a nylon membrane (Schleicher & Schuell, Keene, N.H.). The insert from piviE was labeled with digoxigenin-tagged dUTP (Boehringer-Mannheim Biochemicals). This insert was used as a probe and the blot was developed with the CDP-Star chemiluminescent substrate protocol (Boehringer-Mannheim Biochemicals). The luminescent bands were visualized on X-ray film and the film scanned into an image analysis system (Ambis Inc., San Diego, Calif.). Band densities were quantified using Ambis QuantiProbe version 3.01 software. Copy number

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was calculated as the density of the plasmid band divided by the density of the chromosomal band.

Construction of IVET clones. The protocol for construction and passage of the IVET clones is diagrammed in Figure 2. Genomic DNA from AP233 was prepared using the lysis/proteinase K method of the Gene Fusion Manual (43) and incubated with RNaseA and *Sau*3A to create partially digested fragments. The partially digested DNA was electrophoresed on an 0.8% agarose gel and the region from 400 to 1000 bp was extracted using the Qiaex gel extraction kit (Qiagen). The plasmid, pTF86, was prepared from stationary phase AP100 using Qiagen midi-columns. AP233 competent cells were prepared as previously described and used within three weeks of competency treatment (15). Approximately 15 μg of pTF86 was digested with 30 units of *Bam*HI followed by an ethanol precipitation. This digested DNA was then incubated with 2 units of calf alkaline phosphatase (Boehringer-Mannheim Biochemicals) for forty minutes at 37°C, followed by a phenol-chloroform extraction and ethanol precipitation. A 100 μl ligation consisting of 1 μg of AP233 genomic DNA fragments and 5 μg of cut and phosphatase-treated pTF86 was incubated for 12-16 hours at 4°C with 8 units of T_4 ligase (Boehringer-Mannheim Biochemicals). Two ligation mixtures were combined and extracted with phenol-chloroform, followed by an ethanol precipitation and resuspension in 20 μl of water. AP233 was electroporated using 10 μl of the resuspended ligation mixture (16). Transformants were allowed to recover for four hours in BHIV with 200 μg/ ml riboflavin and then plated on BHIV plates with 200 μg/ml riboflavin and 20 μg/ml ampicillin. Approximately 600 IVET clones were pooled directly from the

Figure 2-2. Diagram of *A. pleuropneumoniae* IVET construction and infection. This flow chart shows the method by which *ivi* clones were constructed using pTF86 and the riboflavin mutant. The clones were then used to infect the swine respiratory model.

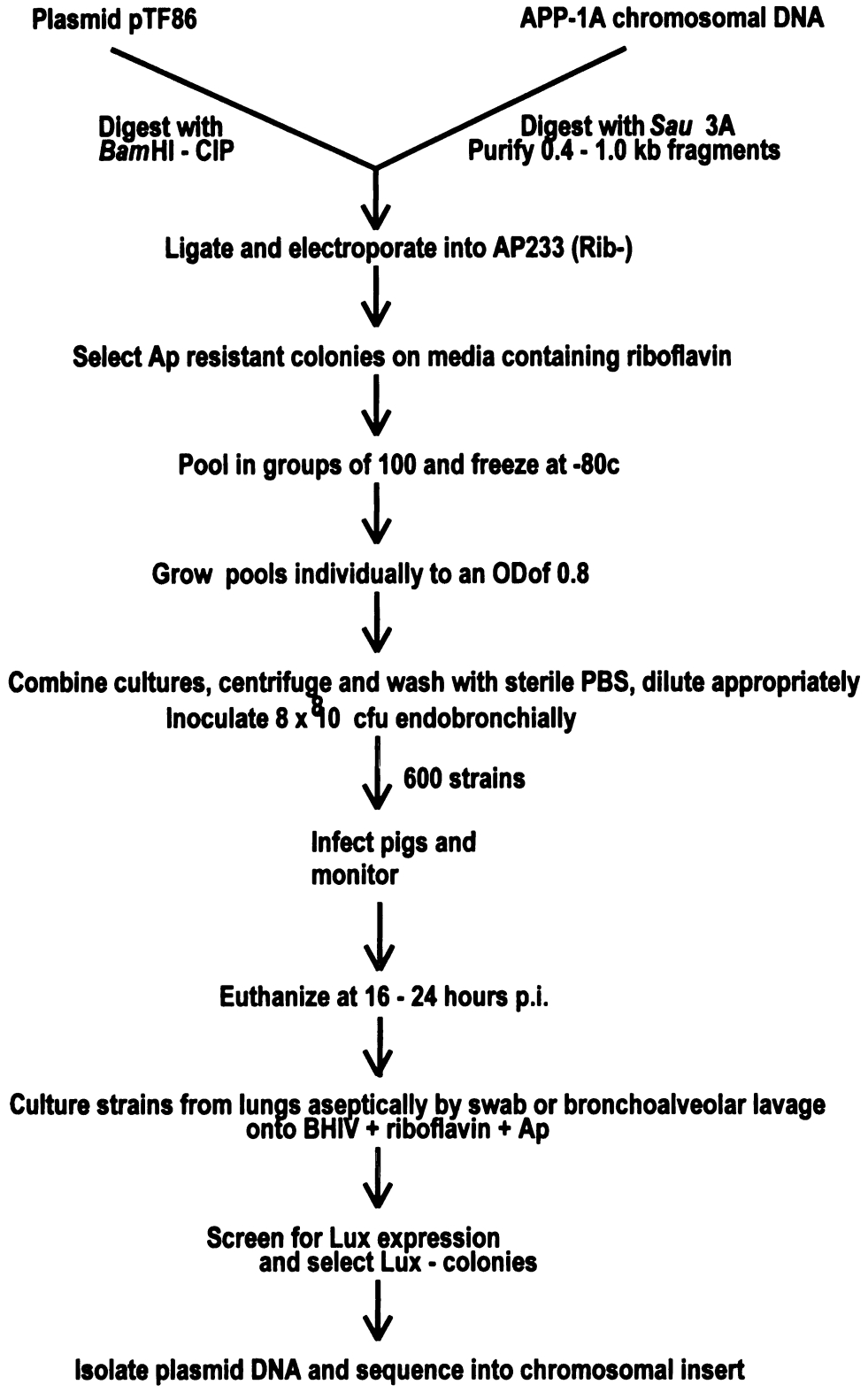
Plasmid pTF86
APP-1A chromosomal DNA
Digest with BamHI - CIP
Digest with Sau 3A
Purify 0.4 - 1.0 kb fragments
Ligate and electroporate into AP233 (Rib-)
Select Ap resistant colonies on media containing riboflavin
Pool in groups of 100 and freeze at -80c
Grow pools individually to an ODof 0.8
Combine cultures, centrifuge and wash with sterile PBS, dilute appropriately
Inoculate 8 x 10^8 cfu endobronchially
600 strains
Infect pigs and monitor
Euthanize at 16 - 24 hours p.i.
Culture strains from lungs aseptically by swab or bronchoalveolar lavage onto BHIV + riboflavin + Ap
Screen for Lux expression and select Lux - colonies
Isolate plasmid DNA and sequence into chromosomal insert

Figure 2-2

electroporated plates without further subculture. Colonies were washed off the plates with HIV broth containing 200 μg/ml riboflavin, 50 μg/ml ampicillin and 5 mM calcium chloride. Filter sterilized glycerol was added to a final concentration of 20%. The mixture was aliquoted into freezer vials and stored at -80°C. In order to estimate the total cell density of the mixture, a portion was diluted ten-fold and an optical density (OD_{520}) was obtained.

Preparation of challenge inocula. Each pool of 600 transformants was thawed and diluted into prewarmed HIV broth containing 5 mM calcium chloride, 200 μg/ml riboflavin, and 50 μg/ml ampicillin, to produce an initial starting volume of 25 ml and an initial OD_{520} of 0.2. Pools were grown in sidearm flasks at 35°C with shaking at 160 rpm to an OD_{520} of 0.8. If needed, cultures were diluted once to prevent overgrowth. An aliquot of the culture was taken for luminometric assays and bacterial counts, and 10 ml was harvested by centrifugation at room temperature and washed once with sterile PBS containing 5 μg/ml riboflavin (PBS-Rib). The bacterial pellet was resuspended in 10 ml of PBS-Rib and diluted if necessary to obtain a desired challenge inoculum of 3×10^8 cfu.

Experimental infections. Eight week old specific pathogen-free castrated male pigs from Whiteshire Hamroc, Inc. (Albion, Ind.) were housed in separate cages in BioSafety Level 2 isolation rooms at the Michigan State University Research Containment Facility. For the challenge procedure, pigs were anesthetized and the challenge inoculum was placed into the lungs by a shallow endobronchial injection. Clinical signs of

pleuropneumonia, including increased respiratory rate, elevated temperature, dypsnea and depression, were monitored and scored as previously described (25). Animals were euthanized at 12 to 16 hours post infection and necropsied. The lungs were examined macroscopically for *A. pleuropneumoniae* lesions, including edema, congestion, hemorrhage, necrosis, fibrosis, and pleuritis.

A. pleuropneumoniae isolates were recovered from aseptic culture of the lung tissue or from bronchoalveolar lavage (BAL). BAL was performed by inserting sterile tubing through the trachea and into the lungs and instilling 200-300 ml of PBS-Rib. The recovered fluid, typically 200-150 ml, was centrifuged to pellet bacteria. The pelleted bacteria were resuspended in PBS-Rib and used for luminometric assay, plate counts, and recovery of isolated colonies. All isolates recovered from the lungs, by either BAL or direct plating, were cultured on BHIV agar plates containing 200 μg/ml riboflavin and 20 μg/ml ampicillin.

The All University Committee on Animal Use and Care at Michigan State University reviewed and approved all protocols for animal experiments. All procedures conformed to university and USDA regulations and guidelines.

Lux analysis. Qualitative screening of *A. pleuropneumoniae* for expression of the *luxAB* genes was performed using a Hamamatsu C1966 Photonic Camera Microscope System. Colonies plated on BHIV plates with 200 μg/ ml riboflavin and 50 μg/ ml ampicillin were exposed to N-decyl aldehyde distributed evenly on a glass petri dish lid. The plate was analyzed with an aperture setting of 11, a digital gain of 2 and photon counting for

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11 seconds. Removal of strongly expressing colonies from the plate was performed as needed to evaluate colonies with lower expression levels.

Quantitative Lux assays were performed using a Turner Model 20e single cell luminometer (Turner Designs, Sunnyvale, Calif.). N-decyl aldehyde substrate was made by sonicating 20 mg/ml Essentially Fatty Acid Free Bovine Serum Albumin (Sigma) dissolved in 1 ml of water with 1 μl/ml N-decyl aldehyde (Sigma) using a sonicating water bath. For analysis, 20 μl of culture was mixed for three seconds with 20 μl of substrate in a polypropylene luminometer cuvette (Turner Designs). The mixture was then read in full integral, autoranging mode without a predelay, a delay of 10 seconds, and an integration time of 30 seconds. Luminometric values were normalized to micro-relative light units per colony forming unit (μRLU/cfu) with the number of bacteria determined by plate counts or by normalizing to the OD_{520} when in pure broth culture.

DNA sequencing. For sequencing of the DNA inserts, a primer complementary to the 5' end of the *luxA* gene (5'-GCTGCCTCCATCCATGGGGTTCCTC-3') and a primer (5'-GGCAACCGTTTCTGCCGGGGAATCC-3') corresponding to the 3' end of the T_4 terminator were used to obtain the initial sequence, after which custom internal primers were designed to obtain additional intervening sequence. Nucleotide sequencing plasmid DNA isolated from *E. coli* was performed using the Sequenase 2.0 kit (U.S. Biochemicals, Cleveland, Ohio) and ^{35}S-dATP (Amersham Corp., Arlington Heights, Ill.).

Inverse PCR. An inverse PCR (iPCR) technique was designed to clone DNA regions flanking the *ivi* sequence (39). 2.5 μg of *A. pleuropneumoniae* serotype 1 genomic DNA was digested to completion with a restriction enzyme, followed by a self-ligation using T4 DNA ligase (Roche) to form closed circular fragments. PCR amplification with AmpliTaq (Roche) was performed with 0.04 μg of ligated DNA using primers that bound within the *ivi* coding region and were oriented to PCR amplify the flanking regions. The resulting PCR fragments were isolated from an agarose gel using the Qiaex II kit (Qiagen) and cloned into the pGEM-T vector (Promega, Madison, Wisc.). The clones were sequenced using an ABI100 Model 377 automated sequencer (Applied Biosystems, Foster City, Calif.). Complete open reading frames (ORF) were identified by comparison of the sequence obtained from the *ivi* clone and the sequence that resulted from the iPCR clones.

Nucleotide and protein sequence analysis. Nucleotide and amino acid sequences were compared with GenBank sequence databases using both the FASTA/TFASTA functions of the GCG Programs and the BLAST suite of programs. All alignments and calculated percent identity and similarity were made using either the GCG Programs or BLAST (19, 47).

Results

AP233/pTF86 lacking an *in vivo* induced promoter is unable to be recovered from infected pigs. Two pigs were each inoculated with AP233/pTF86 at a dose of 2.5 X 10^8 cfu, a dosage identical to that used for the IVET pools. Neither pig showed clinical signs of pleuropneumonia and no visible lesions were present in the lungs upon necropsy, eight to twelve hours post infection. Upon culturing the bronchoalveolar lavage (BAL) fluid and the lungs of the pig that was necropsied after eight hours, less than 10 cfu/ml was recovered from the BAL fluid and a total of 147 colonies were recovered from the culture of the lung sections. In the pig that was euthanized twelve hours post infection, we were unable to isolate bacteria from either the BAL fluid or the lung sections. This is in contrast to the greater than 10^6 cfu/ml BAL fluid and confluent lawns from lung culture that were generally obtained from infection with an IVET pool.

Copy number of IVET vector. The copy number of the IVET plasmid was ascertained in order to determine if the plasmid was being maintained in a high copy number that might permit clones with minimal expression to survive the infection. The copy number of pTF86 was calculated using piviE, since this plasmid contained an insert of 643 bp, which was close in length to the average insert size of 475 bp (16). The copy number, determined to be 8-10 copies per bacterial cell, was calculated by normalizing the intensity of the plasmid DNA to the chromosomal DNA as determined by a Southern blot of AP233/piviE using the piviE insert as the probe (Figure 3).

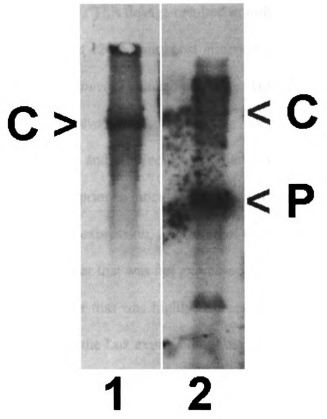
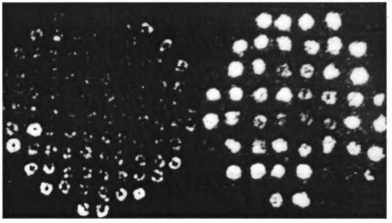


Figure 2-3 Southern blot of AP233 (lane 1) and AP233/piviE (lane 2) when examined with a probe corresponding to piviE. The relative intensities of the chromosomal band (**C**) and the plasmid band (**P**) from AP233/piviE allowed for the calculation of copy number of piviE.

***In vivo* screening of IVET clones.** A total of 11,000 clones were constructed by inserting random genomic DNA fragments in pTF86 and electroporating these plasmids into AP233. A control ligation of alkaline-phosphatase treated vector without insert was performed and indicated that approximately 10% of the total transformants contained no insert. Approximately 5,000 of these clones were used to infect eight pigs, each with 575-750 clones. The remainder of the 11,000 clones was stored at -80°C for future

experiments. The dosage was approximately 3 X 10^8 cfu per pig with a dosage of 5 X 10^5 cfu of each individual clone. This dosage resulted in mild to moderate disease within 8-16 hours, allowing necropsy at 12-16 hours post infection, which minimized the chance of recovering clones lacking an *in vivo* induced promoter (16).

Clones containing *in vivo* induced promoters were able to complement the riboflavin deficiency of AP233 and survive greater than twelve hours after inoculation. Screening of a subset of clones prior to inoculation demonstrated that approximately 85% of the strains had minimal Lux expression, indicative of clones with inserts that lacked a promoter or contained a promoter that was not expressed *in vitro*. The remaining 15% of the clones contained a promoter that was highly expressed prior to passage through the pig. This was in comparison to the Lux expression of the clones isolated after infection, a process that selected for clones with promoters. (Figure 4).



Pre-infection **Post-infection**

Figure 2-4 Photonic camera picture showing a pool of 100 clones before infection and a pool of 50 clones after infection.

***In vitro* screening of IVET clones.** Clones recovered from BAL dilution plates or from lung cultures were plated in groups of 50 and examined using a photonic camera for qualitative identification of clones with low *in vitro* Lux expression. It was often necessary to remove clones that had high Lux expression from the plates during this initial screen in order to identify those with low expression. Clones that had low expression levels were individually frozen and PCR performed from the frozen cultures using primers that annealed to the lux and T_4 terminator regions to determine the size and to group clones that contained inserts of similar sizes. Several clones of each identically sized subgroup were then assayed quantitatively to determine the degree of Lux expression. Clones that had Lux expression levels less than 200 RLU/OD_{520} were partially sequenced using the *lux* primer to further cohort each subgroup. This sequencing was performed using plasmid DNA obtained from *E. coli* transformed with each of the *in vivo* induced plasmids. This strategy led to the identification of 25 unique genetic loci (Table 2, Figure 5).

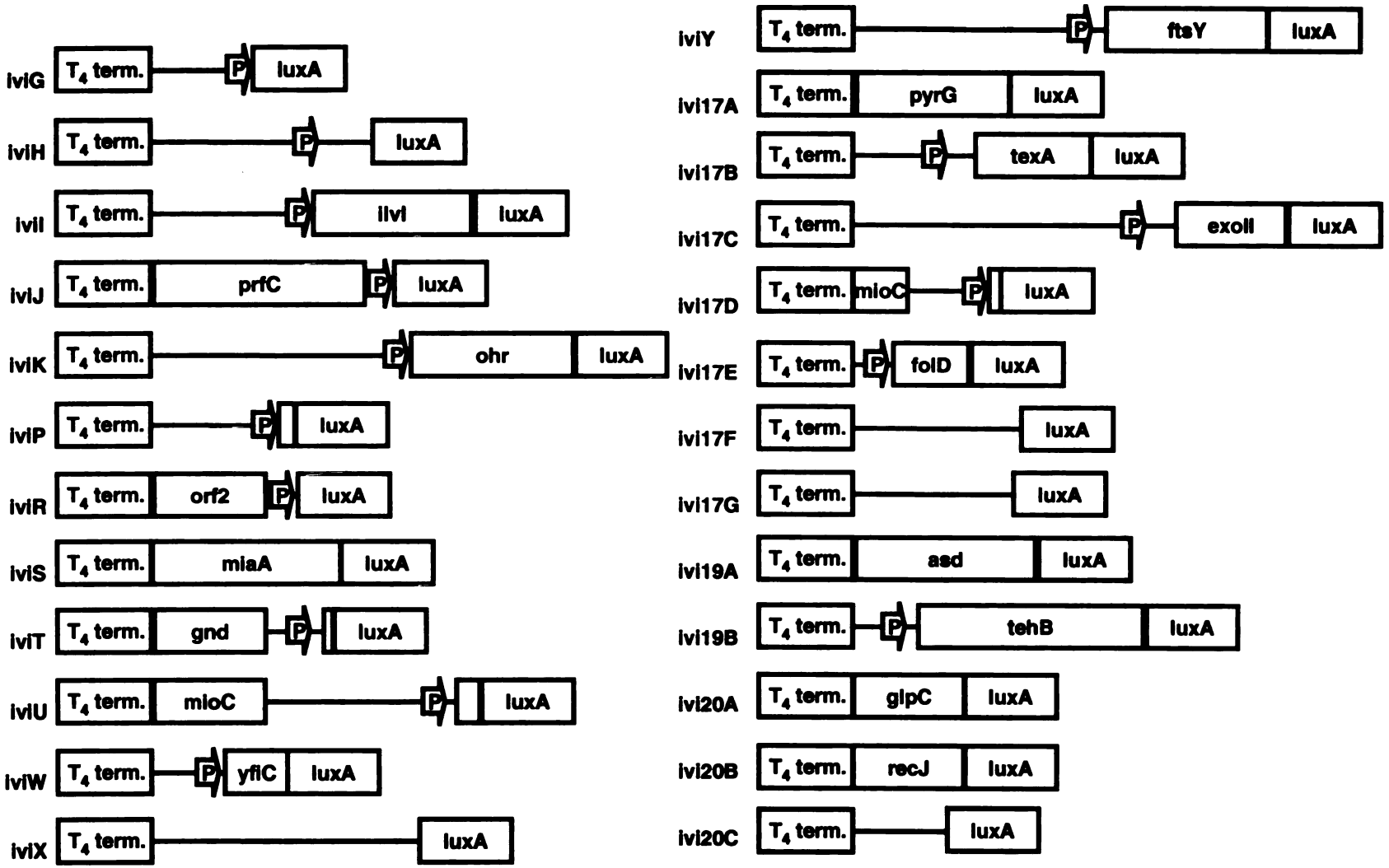
Table 2-2. Identification of ivi insert.

Clone	Database homology (% similarity)	Function of homologue	Span
iviG	*A. pleuropneumoniae* Cps1B (100%)	Capsule biosynthesis	14 aa
iviH	no identifiable ORFs	NA	NA
iviI	*Haemophilus influenzae* IlvI (95%)	Synthesis of branched chain amino acids	81 aa
iviJ	*H. influenzae* PrfC (99%)	Peptide release factor 3	134 aa

Table 2-2 (cont'd).

Clone	Database homology (% similarity)	Function of homologue	Span
iviK	*Vibrio cholerae* Ohr (58%)	Organic hydroperoxidase	97 aa
iviP	*Pasteurella multocida* AmpD (84%)	Amidase	60 aa
iviR	*H. influenzae* ZnuA (90%)	zinc transport	66 aa
iviS	*Shigella flexneri miaA* (68%)	tRNA	352 nt
iviT	*H. influenzae* Gnd(97%)	6-phophogluconate dehydrogenase	58 aa
iviU	*P. multocida* MioC (72%)	Chromosomal replication	67 aa
iviW	*Escherichia coli* YfiC (72%)	Hypothetical orf	41 aa
iviX	no identifiable ORFs	NA	NA
iviY	*E. coli* FtsY (57%)	Cell division/secretion	105 aa
ivi17A	*H. influenzae* PyrG (95%)	CTP synthetase	86 aa
ivi17B	*P. multocida* TexA (92%)	Toxin expression	86 aa
ivi17C	*P. multocida* ExoII (94%)	Acetyl-D-glucosaminadase	60 aa
ivi17D	*P. multocida* MioC (72%)	Chromosomal replication	20 aa
ivi17E	*P.multocida* FolD (78%)	Methylenetetrahydrofolate dehydrogenase/cyclohydrolase	43 aa
ivi17F	no identifiable ORFs	NA	NA
ivi17G	no identifiable ORFs	NA	NA
ivi19A	*A. pleuropneumoniae* Asd (100%)	Aspartate semialdehyde dehydrogenase	69 aa
ivi19B	*H. influenzae* TehB (80%)	Tellurite resistance	148 aa
ivi20A	*H. influenzae* GlpC (91%)	Glycerol-3-Phosphate dehydrogenase	62 aa
ivi20B	*Erwinia chrysanthemi* RecJ (87%)	DNA specific exonuclease	65 aa
ivi20C	no identifiable ORFs	NA	NA

Figure 2-5 Organization of the 25 unique *in vivo* induced inserts (*ivi* clones). Shaded boxes indicate open reading frames. P- indicates a potential promoter. All ORFs fused to *luxA* were preceded by potential ribosome binding site.


ivlG
T4 term.
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T4 term.
P
luxA
ivlI
T4 term.
P
ilvI
luxA
ivlJ
T4 term.
prfC
P
luxA
ivlK
T4 term.
P
ohr
luxA
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luxA
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orf2
P
luxA
ivlS
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miaA
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gnd
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recJ
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ivl20C
T4 term.
luxA

Figure 2-5

Verification of *in vivo* survival. To confirm that the clones identified as *in vivo* induced could be isolated again from an infected pig, groups of five to ten clones were pooled at 5 X 10^5 cfu of each clone and used to infect individual pigs. All pigs developed classical pleuropneumonia symptoms and were euthanized and necropsied 12 to 16 hours post infection. Necropsy and bacterial isolation and characterization was performed as before, with the exception that Lux expression of the recovered clones was not assayed. Sequencing of recovered clones proved that all clones could be recovered from the repeat infection, strengthening the classification of these clones as *in vivo* induced.

Sequence analysis. The complete inserts of the 25 clones were sequenced and both the nucleotide and the predicted amino acid sequences were used to search GenBank databases and a database consisting of sequence information from *A. pleuropneumoniae* serotype 5 (John Nash, personal communication). Based on the initial analysis of the ivi clone, thirteen clones showed strong homology to identified genes, and twelve clones contained either no identifiable open reading frame or not enough sequence information to identify the gene driven by the *in vivo* expressed promoter without determining the downstream sequence through inverse pcr or database analysis (Table 2, Figure 5).

IviI, iviK, iviW, iviY, ivi17b, ivi17c, ivi17e, ivi19a, and ivi19b each contained a region containing a potential promoter, preceding a known partial ORF, oriented upstream of the *luxAB* genes and in the proper orientation to allow expression of the lux and rib genes. While iviJ, iviR, ivi17a, ivi20a, and ivi20b encode a recognizable protein, the orientation or region within the protein are such that the known gene may not be the *in vivo* induced gene. The recognizable gene is described, but with all of these *ivi* clones,

there is the possibility that the promoter is responsible for the expression of a downstream gene of unknown sequence.

The remaining eleven clones did not have enough sequence information to determine the identity of the in vivo induced gene. Both inverse PCR and sequence comparison to the genome of *A. pleuropneumoniae* serotype 5 was used to determine the sequence flanking the promoter within the ivi insert. Through inverse PCR, completed by Trevor Wagner, iviS was identified as Hfq (host integration factor), iviX was identified as VapBC, iviP was confirmed as AmpD, and ivi17g was identified as CoaE.

The remaining seven clones contain inserts having short unidentifiable ORFs, sequences lacking homology to identified proteins, or sequences containing known genes without identifiable promoters to drive expression of the *luxAB* genes. In order to identify the gene expressed by the *ivi* promoter, a database containing *A. pleuropneumoniae* DNA sequence was required to evaluate the flanking DNA. Information for two of the seven clones (iviG and ivi17f) was not able to be found in the *A. pleuropneumoniae* serotype 5 database. IviG was not found in the database and ivi17f was found at the 3' end of a contig and the downstream sequence was unavailable. Thus, the five clones; iviH, iviT, iviU, ivi17D, and ivi20C were used to search the *A. pleuropneumoniae* serotype 5 database.

Thus the information gained from a variety of sources has been assimilated to give the following information about the 25 *ivi* clones that were identified as a result of this IVET study. IviG contains the first 14 amino acids of *cps-1B* (AF518558), one of several proteins involved in capsule biosynthesis. IviH contains no recognizable ORFs in either the *ivi* insert or in the *A. pleuropneumoniae* serotype 5 database.

IviI was identified as IlvI by analysis of the *ivi* clone sequence, which contained 81 aa of the 5' end of IlvI. The full gene was cloned by inverse PCR and is 1689 bp (563 aa) in length. This *A. pleuropneumoniae* protein is 81 % homologous to *P. multocida* IlvI (AAK02954) and 98 % identical to the homologue in *A. pleuropneumoniae* serotype 5. IlvI is acetohydroxy acid synthase III, which is necessary for biosynthesis of isoleucine and valine (45).

IviJ was identified as PrfC by analysis of the *ivi* clone sequence, which contained 134 aa of the 5' end of PrfC. The full gene has not yet been cloned. This *A. pleuropneumoniae* protein is 96 % homologous to *H. influenzae* PrfC (HI1735) and 99 % identical to the homologue in *A. pleuropneumoniae* serotype 5. IviJ contains an antisense ORF with sequence similarity to PrfC, required for protein release from the ribosome (36). PrfC plays a role in protein synthesis and may have increased expression during times of rapid growth and cell division. An antisense RNA to *prfC* might help to regulate the production of new proteins and might transiently limit cell growth during times of nutrient limitation

IviK was identified as Ohr by analysis of the *ivi* clone sequence, which contained 97 aa of the 5' end of Ohr. The full gene was cloned by inverse PCR and is 429 bp (143 aa) in length. This *A. pleuropneumoniae* protein is 62 % homologous to *Pseudomonas aeruginosa* Ohr (AAG06238) and does not have a homologue in *A. pleuropneumoniae* serotype 5. Ohr (organic hydroperoxide reductase), which has been implicated in survival under conditions of oxidative stress (38). The full analysis of Ohr is the subject of Chapter 3 of this dissertation.

IviP was identified as AmpD by analysis of the *ivi* clone sequence, which contained 58 aa of the 5' end of AmpD. The full gene has not been cloned. This *A. pleuropneumoniae* protein is 84 % homologous to P. multocida AmpD (AAK02167) and 98 % identical to the homologue in *A. pleuropneumoniae* serotype 5. AmpD, a N-acetylmuramyl-L-alanine amidase, might function in protection against β- lactam antimicrobials or in intracellular recycling of valuable cell wall components in an energy deficient environment (51).

IviR was identified as ZnuA by analysis of the *ivi* clone sequence, which contained 66 aa of the 5' end of ZnuA. The full gene has not been cloned. This *A. pleuropneumoniae* protein is 92 % homologous to *H. ducreyi* ZnuA (AAF000115.1) and 90 % identical to the homologue in *A. pleuropneumoniae* serotype 5. IviR contains an ORF with sequence similarity to the middle of ZnuA, a periplasmic zinc transport protein (27). This protein is responsible for regulation of zinc levels within the cell and has been shown to be required for virulence in *H. ducreyi* (27). ZnuA has not been fully characterized and the location of the start codon and the active site have not been mapped. It is therefore possible that iviR contains the promoter responsible for expression of a truncated, but functional protein. This hypothesis must be tested by determining if the protein encoded by iviR is able to complement a *H. influenze* mutant that lacks a functional ZnuA (31).

IviS was identified as Hfq cloning the flanking sequence by inverse PCR and finding an ORF with homology to Hfq. This *A. pleuropneumoniae* protein is 84 % homologous to *H. influenzae* Hfq(HI0411) and 99 % identical to the homologue in *A.*

pleuropneumoniae serotype 5. Hfq has been shown to be a RNA binding protein that is required for efficient translation of several key virulence factors, including *oxyS* (58).

IviT and iviU both contain recognizable proteins that are upstream of regions that contain promoters that are required for expression of downstream hypothetical ORFs. The ORFs identified do not have significant homology to hypothetical ORFs from other microbial genomes. IviT is 91% identical to the *A. pleuropneumoniae* serotype 5 genome, and iviU is 98% identical to the *A. pleuropneumoniae* serotype 5 genome.

IviW was identified as YfiC by analysis of the *ivi* clone sequence, which contained 41 aa of the 5' end of YfiC. The full gene has not been cloned. This *A. pleuropneumoniae* protein is 72 % homologous to *E. coli* YfiC (b2575) and 96 % identical to the homologue in *A. pleuropneumoniae* serotype 5. IviW has strong homology to *yfiC*, a gene, encoding a hypothetical protein identified initially during sequencing of the *E. coli* genome that has since been found in the genomes of multiple organisms (1).

IviX was identified as VapB by cloning the flanking sequence using inverse PCR. and is 627 bp (209 aa) in length. This *A. pleuropneumoniae* protein is 89 % homologous to *H. influenzae* VapB (HI0321) and 96% identical to the homologue in *A. pleuropneumoniae* serotype 5. VapBC was shown to be required in *Salmonella dublin* for plasmid stability during nutrient limitation and may play a role in maintaining key virulence genes during rapid growth (35).

IviY was identified as FtsY by analysis of the *ivi* clone sequence, which contained 105 aa of the 5' end of FtsYI. The full gene has not yet been cloned. This *A. pleuropneumoniae* protein is 57 % homologous to *E. coli* FtsY (b3464) and 98 %

identical to the homologue in *A. pleuropneumoniae* serotype 5. IviY was similar to FtsY, which is a required component in bacterial cell division (18, 41).

Ivi17a contains an internal fragment, beginning 140 nucleotides after the start codon, of *pyrG*. The full gene has not yet been cloned. This *A. pleuropneumoniae* protein is 95 % homologous to *H. influenzae* PyrG (HI1077) and 100 % identical to the homologue in *A. pleuropneumoniae* serotype 5. PyrG is a CTP synthetase, that is responsible for the formation of CTP from UTP and ATP (54). CTP is necessary for synthesis of DNA and mRNA, both of which are necessary for cellular growth and replication. The transcriptional start site has been mapped in *E. coli* to a region upstream of the sequence contained in ivi17a, but the intact protein from *A. pleurpopneumoniae* has not been studied (54). It is possible that ivi17a contains the promoter region for a truncated, but functional protein.

Ivi17b was identified as TexA by analysis of the *ivi* clone sequence, which contained 86 aa of the 5' end of TexA. The full gene has not yet been cloned. This *A. pleuropneumoniae* protein is 92 % homologous to *P. multocida* TexA (PM1549) and 98 % identical to the homologue in *A. pleuropneumoniae* serotype 5. Ivi17b encodes an ORF with similarity to TexA, which has been implicated in regulation of toxin expression in *Bordetella pertussis* (14).

Ivi17c was identified as ExoII by analysis of the *ivi* clone sequence, which contained 60 aa of the 5' end of ExoII. The full gene has not been cloned. This *A. pleuropneumoniae* protein is 94 % homologous to *P. multocida* ExoII (PM0081) and 97% identical to the homologue in *A. pleuropneumoniae* serotype 5. Ivi17c encodes an

ORF with sequence homology to ExoII, a β-N-acetylglucosaminidase that may be involved in antigenic variation (3).

Ivi17d was identified as SelA by comparison to the A. pleuropneumoniae serotype 5 database. The insert was 97 % identical to the *A. pleuropneumoniae* serotype 5 genome and the downstream gene was *selA*. SelA is 75% homologous to *H. influenzae* SelA (HI0708). Ivi17d contains a promoter that regulates expression of *selA*, which encodes selenocysteine synthase (11). SelA is responsible for the formation of selenocysteine tRNA which replaces a serine amino acid with a selenocysteine instead, allowing for variation in the protein sequence and quartenary structure. This may be valuable during times of antigenic variation.

Ivi17e was identified as FolD by analysis of the *ivi* clone sequence, which contained 43 aa of the 5' end of FolD. The full gene has not been cloned. This *A. pleuropneumoniae* protein is 78 % homologous to *P. multocida* FolD (PM2085) and 100 % identical to the homologue in *A. pleuropneumoniae* serotype 5. Ivi17e encodes FolD, which is required for synthesis of methyltetrahydrofolate, a precursor of pyrimidines (7).

Ivi17f was unable to be identified. The homologous region in *A. pleuropneumoniae* serotype 5 was at the end of a contig and lacked downstream sequences. There were no ORFs in the insert that had homology to proteins in the microbial genomes.

Ivi17g was identified as CoaE by cloning the flanking regions using inverse PCR and finding an ORF that was 75% homologous to *E. coli* CoaE (ECs0105). This region is 98% identical to the homologue in *A. pleuropneumoniae* serotype 5. CoaE, dephosphocoenzyme A kinase, is a key component, and one of the final steps, in the

biosynthesis of coenzyme A (37). This enzyme may be required for the increased growth and metabolic requirements that occur during infection.

Ivi19a was identified as SodC by analysis of the *ivi* clone sequence, which 69 aa of the 5' end of Asd. This region has been sequenced in *A. pleuropneumoniae* serotype 1 and the downstream gene, divergently transcribed, is *sodC* (26). The *sodC* gene encodes a superoxide dismutase, capable of degrading superoxides to peroxides which can then be further dexotified by other proteins.

Ivi19b was identified as TehB by analysis of the *ivi* clone sequence, which contained 148 aa of the 5' end of TehB. The full gene has not been cloned. This *A. pleuropneumoniae* protein is 80 % homologous to *H. influenzae* TehB (HI1275) and 90% identical to the homologue in *A. pleuropneumoniae* serotype 5. Ivi19b has homology to TehB, which has been implicated in resistance to the heavy metal tellurite and as a potential multi-drug efflux pump (49, 52).

Ivi20a contains an internal fragment, beginning 132 bp from the start codon, of GlpC. The insert contains 62 aa of GlpC. This *A. pleuropneumoniae* protein is 91 % homologous to *H. influenzae* GlpC (HI0683) and 98 % identical to the homologue in *A. pleuropneumoniae* serotype 5. GlpC has been shown to be a membrane bound protein, but its exact function has not been identified and its role in the function of GlpAB as electron transporters has not been established (5). By mapping *glpC* to the *A. pleuropneumoniae* serotype 5 genome, it was discovered that *glpC* is part of a potential operon that encodes an unknown ORF and RibGBAH. This operon structure is not seen downstream of the *E. coli glpC* (5). It is possible that the promoter for this ORF and the *ribGBAH* genes are found internal to *glpC* and that the gene that ivi20a contains the

promoter for is the riboflavin biosynthetic operon and a hypothetical ORF that is a regulator of this operon.

Ivi20b contains an antisense ORF with sequence similarity to RecJ. This insert contains 65 aa of the protein. This *A. pleuropneumoniae* protein is 90 % homologous to *Erwinia chrysanthemi* RecJ (U57963) and 98 % identical to the homologue in *A. pleuropneumoniae* serotype 5. RecJ is a single stranded DNA nuclease that is required for recombination and DNA excision and repair (28, 29). An antisense mRNA might limit recombination during times that DNA repair is not desired, such as when a recombinatorial event might result in antigenic variation.

Ivi20c was identified as TrnS by sequence comparison to the A. pleuropneumoniae serotype 5 genome database. This insert is 97 % identical to the homologous region in *A. pleuropneumoniae* serotype 5. Ivi20c contains sequence upstream of an ORF that has been identified as TrnS, a putative transport protein, which is 77 % homologous to TrnS from *Salmonella enterica* (STY3536). Transport proteins could be involved in uptake of needed nutrients or in export of proteins required for infection such as adhesins or toxins.

Discussion

This study is a continuation of the initial development of an IVET (*in vivo* expression technology) system in *Actinobacillus pleuropneumoniae* (16). Approximately 5000 clones were used to infect swine to select for clones containing promoters that were strongly expressed during infection. Of these 5000, 25 clones contained inserts that were expressed during infection, *in vivo*, and had minimal expression on laboratory media, *in vitro*. While these 5000 clones are an initial beginning of the 30,000 needed for full coverage of the genome, the identification of 25 *in vivo* induced genes has already identified candidate virulence genes. Among these 25 clones are newly identified virulence factors that might be possible targets for vaccine production or metabolic pathways capable of being targeted by antibiotics. This study has also provided new information about the conditions that occur in the pig respiratory tract during infection as select amino acids and metabolic precursors become limiting and the host immune response produces inhospitable conditions.

The *in vivo* induction of *ilvI* and *folD*, suggests that during infection there exists limited nutrients and the invading pathogen must synthesize the precursors needed to produce pyrimidines and branch-chained amino acids by upregulating *folD* and *ilvI*, respectively. This might in turn slow down bacterial growth due to an increased metabolic demand or halt growth entirely if the microbe is unable to synthesize the necessary metabolic components. On rich media, an excess of metabolites potentially exists and thus there is no need to express the genes required for biosynthesis of metabolic precursors. Other clones that contain genes that are responsible for

metabolism include ivi17g, which encodes dephosphokinase coenzymeA, and ivi17a, which encodes CTP synthetase. Dephosphokinase coenzymeA is necessary for the production of coenzymeA, which is a necessary cellular cofactor. CTP synthetase is necessary for production of DNA and mRNA, both of which are required for cellular replication. All of these enzymes are necessary for survival during infection, when the host limits nutrients as part of the immune reaction to bacterial invasion and so could be considered virulence factors.

Another host immune response is the production of oxygen radicals from macrophages. In order to survive this assault, bacteria have acquired enzymes, such as Ohr and SodC, iviK and ivi19a respectively, which might detoxify these oxygen radicals.

Novel *A. pleuropneumoniae* virulence factors have also been identified through the use of this IVET screen. Several proteins have been identified that might alter the antigenic profile of the outer membrane, allowing escape from antibody mediated opsonization. ExoII, a glucosaminidase, could potentially modify bacterial glycoproteins and in doing so, alter the antigenic profile of outer membrane proteins (3). Another potential role for ExoII is to breakdown environmental or host produced glycosides to generate precursor molecules for bacterial metabolism. In addition to ExoII, AmpD and SelA could participate in antigenic variation of membrane proteins. AmpD, a N-acetylmuramyl-L-alanine amidase, could modify the cell wall, making it less antigenically sensitive. AmpD has also been implicated in the resistance to the β-lactam antibiotics, allowing the bacterium to survive in the presence of antimicrobials that are used to treat and prevent infection by *A. pleuropneumoniae* and other bacteria. SelA, by

allowing for the production of a unique amino acid, could alter the quartenary structure of proteins through the formation of a disulfide bond or interaction of the selenium group.

Another category of genes identified through the use of IVET, have been transport proteins. IviR, iviY, ivi19b, and ivi20c have sequence similarity to known or hypothesized transport proteins and efflux pumps. The role of transport proteins is to regulate and maintain the required levels of nutrients in order for cell growth and replication. IviR encodes ZnuA, a zinc transporter that has been implicated in maintaining a nontoxic level of zinc within the cell. In addition, a *H. ducreyi znuA* mutant has been shown to have reduced virulence, perhaps because zinc is required for other virulence factors. IviY encodes FtsY, which has been shown to be a component of SRP (signal recognition particle) a cotranslational protein targeting to the plasma membrane in conjunction with the SecE pathway (18, 41). *SecE* has been previously identified as an *A. pleuropneumoniae in vivo* induced gene, suggesting that protein export to the bacterial membrane is an important feature of the disease, perhaps in the production of required adhesins and recognition particles (16). FtsY has also been shown to be involved in cell division, required during the growth of the bacteria (18). *ftsY* has been identified as an *in vivo* induced gene during a *Pseudomonas aeruginosa* IVET screen, suggesting that FtsY may be a universally important protein and that an antibiotic targeting this enzyme might be useful in multiple bacterial infections (21). Ivi19b encodes TehB. The overexpression of *tehB* increases the resistance to several quaternary ammonium compounds and causes an increase in the efflux of ethidium bromide (49). This efflux pump might play a role in *A. pleuropneumoniae* survival during infection when it is surrounded by toxic molecules generated by macrophage lysosomes.

Another category of genes are regulators of virulence factors. This includes iviS, iviX, and ivi17b. IviS encodes Hfq, a RNA binding protein that has been shown to regulate expression of virulence factors such as *oxyS*. IviX encodes VapBC, a newly identified complex that regulates maintenance of virulence factors on plasmids such that during times of rapid growth, the daughter cells are as virulent as the parent cell. Ivi17b encodes TexA. TexA is a potential regulator of virulence factors, having first been identified as an essential gene in *Bordetella pertussis*, required for the regulation of several toxins. This putative RNA binding factor might be a global regulator of virulence factors, or function in a manner similar to that in *B. pertussis* where it acts in the differential expression of virulence factors (14). In addition, the gene expressed by the promoter in ivi20a might be a regulator of virulence. The ORF found in this clone has homology to GlpC, a protein of unknown function that might help in association of GlpAB to the bacterial membrane. The other role for the potential promoter of ivi20a might be for expression of the downstream ribGBAH operon.

An additional category of ivi clones are those that potentially encode antisense RNA. These clones contain DNA that has homology to the middle of a known gene. IviJ contains *prfC* and ivi20b contains *recJ*, both in an antisense direction. It has yet to be tested to determine if antisense mRNA for these genes are actually present within *A. pleuropneumoniae*. The function of antisense RNA is to limit the expression of proteins that might need to be transiently regulated during times of energy limitation or nutrient stress.

A final category of ivi clones are those that encode hypothetical proteins that are highly conserved through many databases and other unknown genes. Three of the 25 ivi

clones contain promoters of ORFs that have no known function. An additional three clones were unable to be characterized, even in the presence of a database containing DNA sequence from *A. pleuropneumoniae* serotype 5. This is due to the fact that one of the clones was not found in serotype 5, another clone was at the end of a contig and downstream genes could not be characterized, and the final clone was found in the middle of a region of multiple short ORFs that lacked sequence similarity to known proteins.

Thus this IVET study has contributed to the understanding of *A. pleuropneumoniae* pathogenesis through the proposal of several new virulence factors and pathogenically important metabolic pathways. This increase in the understanding of *A. pleuropneumoniae* induced disease, and potentially of similar respiratory infections in other mammalian hosts, might lead to important antibiotic targets or to enhanced vaccine production through the incorporation of important antigens. With the further characterization of the currently identified *in vivo* induced genes from this study and the isolation of more clones from *A. pleuropneumoniae* IVET studies, the understanding of the this disease process will increase dramatically. With the sequencing of the *A. pleuropneumoniae* serotype 1 genome, the flanking regions to these promoters can be determined, easing the characterization steps and allowing further insight into the pathogenesis of *A. pleuropneumoniae* infection.

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Chapter 3

Ohr, Encoding an Organic Hydroperoxide Reductase,

Is an *In Vivo* Induced Gene in *Actinobacillus pleuropneumoniae*

This manuscript has been previously published in Infection and Immunity.
Shea, R. J. and M. H. Mulks. 2002. *ohr*, encoding an organic hydroperoxide reductase, is an *in vivo*-induced gene in *Actinobacillus pleuropneumoniae*. **70:** 794-802.

Abstract

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a disease characterized by pulmonary necrosis and hemorrhage caused in part by neutrophil degranulation. In order to survive within this toxic environment, bacteria have developed enzymes to detoxify the molecules released during degranulation. Many of these enzymes can be considered virulence factors that are only required during infection and are regulated in a manner such that they are only expressed during infection. In an effort to understand the pathogenesis of this disease, we have developed an IVET (in vivo expression technology) system to identify genes that are specifically up-regulated during infection. One of the genes that we have identified as being in vivo induced is *ohr*, encoding organic hydroperoxide reductase, an enzyme that could play a role in detoxification of organic hydroperoxides generated during infection. This identification was based upon protein homology to previously characterized Ohr proteins.

Among the twelve serotypes of *A. pleuropneumoniae*, *ohr* was only found in serotypes 1, 9, and 11. This distribution correlated with increased resistance to cumene hydroperoxide, an organic hydroperoxide, but not to hydrogen peroxide or to paraquat, a superoxide generator. Functional assays of Ohr activity demonstrated that *A. pleuropneumoniae* serotype 1 cultures, but not serotype 5 cultures, were able to degrade cumene hydroperoxide. In *A. pleuropneumoniae* serotype 1, expression of *ohr* was induced by cumene hydroperoxide, but not by either hydrogen peroxide or paraquat. In contrast, an *ohr* gene from serotype 1 cloned into *A. pleuropneumoniae* serotype five was

not induced by cumene hydroperoxide or by other forms of oxidative stress, suggesting the presence of a serotype-specific regulator of *ohr* in *A. pleuropneumoniae* serotype 1. The search for a novel regulator of *ohr* is ongoing.

Introduction

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a disease characterized by massive lung necrosis and pulmonary hemorrhage. This necrosis is due in part to the influx of host immune cells and the release of neutrophil lysosomal contents that include oxygen radicals, which can destroy the invading bacteria as well as the host tissue (28). In an effort to understand the pathogenesis of this respiratory disease and the effect of the host immune response on *A. pleuropneumoniae*, we have developed an in vivo expression technology (IVET) system to identify genes that are expressed during infection but not during growth in vitro on laboratory media (13). One of the genes that we have identified with this selection shows homology to *ohr* (organic hydroperoxide reductase), which has been described in *Xanthomonas campestris* (25), *Pseudomonas aeruginosa* (30), *Enterococcus faecalis* (34), and *Bacillus subtilis* (12). Ohr has been implicated in resistance to and detoxification of the organic peroxides, such as cumene hydroperoxide (25, 30).

Bacteria are frequently exposed to reactive oxygen species during the course of infection. Oxygen radicals in the form of superoxides, hydrogen peroxides, and organic hydroperoxides can result from release of lysosomal contents within inflammatory cells or can be generated by bacterial cellular metabolism (3, 39). During infection of the porcine lung, *A. pleuropneumoniae* is exposed to oxygen radicals in the form of superoxides and peroxides generated by the neutrophil oxidative burst (28). A third class of oxygen radicals, organic hydroperoxides, can be generated either directly within the phagosome or as a consequence of oxygen radicals interacting with the bacterial cell

membrane [reviewed in Miller and Britigan (24)]. To survive and protect its cellular metabolism within this dangerous milieu, *A. pleuropneumoniae* may require enzymes capable of inactivating these oxygen species (21).

A. pleuropneumoniae has been shown previously to contain catalase and two distinct superoxide dismutases, SodA and SodC, which can relieve a portion of the oxidative stress that occurs during infection (22). Enzymes that could detoxify the third category of oxidative stress reagents, the organic hydroperoxides, have not been previously identified in *A. pleuropneumoniae*. In this work, we identify another potentially protective gene, *ohr,* which is specifically induced during infection and produces a protein that is capable of detoxifying organic peroxides encountered by *A. pleuropneumoniae* during infection of the porcine lung.

Materials and methods

Bacterial strains. The *A. pleuropneumoniae* strains that were used in this study and the plasmids propagated in these strains are listed in Table 1. *A. pleuropneumoniae* strains were cultured at 35°C on either brain heart infusion (BHI) (Difco, Detroit, Mich.) or heart infusion (HI) (Difco) media supplemented with V factor (β-NAD) added at 10 μg/ml (BHIV or HIV, respectively). Riboflavin, when needed for maintenance of APP233, was added at 200 μg/ml. Media was supplemented with ampicillin at 50 μg/ml to propagate plasmids or at 20 μg/ml to recover *A. pleuropneumoniae* from porcine lungs and to select for transformants after electroporation. *Escherichia coli* strain XLI-Blue mRF' (Stratagene, LaJolla, Calif.) was used for construction and propagation of plasmids. *E. coli* was cultured on Luria-Bertani (LB) medium (Difco) supplemented with ampicillin at 100 μg/ml.

Molecular manipulations. Genomic DNA from *A. pleuropneumoniae* was prepared according to the lysis/proteinase K method of the Gene Fusion Manual (37). Plasmid DNA was purified using Qiagen spin columns (Qiagen Inc., Valencia, Calif.). DNA modifying enzymes were obtained from Roche (Roche Molecular Biochemicals, Indianapolis, Ind.) and used according to the manufacturer's specifications.

Preparation of electrocompetent *A. pleuropneumoniae* serotype 1 and electroporation of these cells with plasmid DNA prepared from either *E. coli* or *A. pleuropneumoniae* serotype 1 was performed as previously described (15). *A. pleuropneumoniae* serotype 5 (14) was made competent by the method of Ward et al (43)

Table 3-1. Characteristics of bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristics	Source (reference)
APP225	*A. pleuropneumoniae* ATCC 27088, serotype 1A, passaged through pigs	Fuller et al. (15)
APP227	*A. pleuropneumoniae* ISU178, a serotype 5 field isolate, passaged through pigs	Fuller et al. (14)
APP233	A double-crossover riboflavin auxotroph of APP225	Fuller et al. (15)
ATCC 27089	*A. pleuropneumoniae* serotype 2	ATCC [a]
ATCC 27090	*A. pleuropneumoniae* serotype 3	ATCC
ATCC 33378	*A. pleuropneumoniae* serotype 4	ATCC
ATCC 33590	*A. pleuropneumoniae* serotype 6	ATCC
ISU53	*A. pleuropneumoniae* serotype 7	V. Rapp, Iowa State University
405	*A. pleuropneumoniae* serotype 8	R. Nielsen, Denmark
CVJ1326	*A. pleuropneumoniae* serotype 9	R. Nielsen, Denmark
13039	*A. pleuropneumoniae* serotype 10	R. Nielsen, Denmark
56513	*A. pleuropneumoniae* serotype 11	R. Nielsen, Denmark
1096	*A. pleuropneumoniae* serotype 12	R. Nielsen, Denmark

Table 3-1 (cont'd)

Plasmid	Characteristics	Source (reference)
pTF86	*A. pleuropneumoniae* IVET vector containing promoterless *luxAB* and *ribBAH* genes downstream of a unique *Bam*HI cloning site	(13)
piviK	pTF86 containing 801 bp insert with 294 bp of *ohr* in *Bam*HI site	This study
pGEM-T	Ap^r TA cloning vector for PCR fragments	Promega
pinKE	pGEM-T containing 2.5 kb insert with *ohr* inverse PCR fragment	This study
pGZRS18	Ap^r *A. pleuropneumoniae-E. coli* shuttle vector	(44)
pGeohr	pGZRS18 containing full length *ohr* with 360 bp of upstream sequence	This study

a American Type Culture Collection, Manassas, Va.

and electroporated using the same electroporation conditions as for serotype 1, but using plasmid prepared from *E. coli*. *E. coli* transformation was performed by the Hanahan method (17).

Southern blotting. Genomic DNA from all 12 *A. pleuropneumoniae* serotypes (Table 1) was digested to completion using *Eco*RI and separated on a 0.8% agarose gel. DNA fragments were transferred to a Nytran (Schleicher & Schuell, Keene, N.H.) membrane by the method of Southern (36). The membrane was hybridized with a digoxigenin-labeled probe generated by PCR amplification of the complete *ohr* gene using primers (MM150: 5'-GACAAGAATTCAACAAGGACAATATTATG-3' and MM151: 5-CCTAAATCGTCCCAGATCTGGTAGG-3') that flank the open reading frame (Roche PCR DIG synthesis Kit). For high stringency hybridization, blots were incubated for 16 hours at 42°C with the probe diluted in a hybridization buffer that contained 50% formamide, 5 X SSC (sodium chloride, sodium citrate), and 2% blocking reagent (Roche). High stringency washes were performed at 68°C with 0.1 X SSC, 0.1% SDS (sodium dodecyl sulfate). For low stringency hybridization, blots were incubated for 16 hours at 42°C with the probe diluted in an aqueous solution containing 6 X SSC and 1% blocking reagent, with no formamide added. Low stringency blots were washed for 10 minutes at room temperature with 2 X SSC, 0.1% SDS. Hybridizing bands were detected using alkaline-phosphatase tagged anti-digoxigenin and the CDP* Chemiluminescent substrate (Roche).

Infection model. The infection of pigs with either APP233/piviK or APP233/pTF86 was performed as previously described (13). Briefly, each clone was inoculated into HIV broth containing 200 μg/ml riboflavin, 50 μg/ml ampicillin, and 5 mM calcium chloride and grown at 35°C to an OD_{520} of 0.8. The bacteria were washed with saline and resuspended in 10 ml of saline containing 200 μg/ml riboflavin at a final concentration of 7 X 10^8 CFU. The bacterial suspension was inoculated, via an endotracheal catheter, into the lungs of six week old pigs. The pigs were monitored for development of clinical signs of pleuropneumonia as previously described (19) and in conjunction with animal use approval. Sterile lung samples were collected for bacterial isolation and photography following necropsy. Lung samples were examined for *in situ* luciferase activity using a Hamamatsu C1966 Photonic Microscope System (13). All experimental protocols for animal experiments were reviewed by the Michigan State University All University Committee on Animal Use and Care, and all procedures conformed to university and U.S. Department of Agriculture regulations and guidelines.

Cloning of the intact *ohr* gene. An inverse PCR (iPCR) technique was designed to clone the intact *ohr* gene (29). 2.5 μg of *A. pleuropneumoniae* serotype 1 genomic DNA was digested to completion with *Eco*RI, followed by a self-ligation using T4 DNA ligase (Roche) to form closed circular fragments. PCR amplification with AmpliTaq (Roche) was performed with 0.04 μg of ligated DNA using primers (MM139: 5'-AACCAAGTGAACCGTCATCTACTC-3' and MM140: 5'-GTGGCAAAGTCGGCAC AAACC-3') designed from the known sequence of the *iviK* clone, which contained the promoter region and partial open reading frame of the *ohr* gene. These primers bound

within the coding region of *ohr* and were oriented to PCR amplify the flanking regions. The resulting 2.5 kb PCR fragment was isolated from an agarose gel using the Qiaex II kit (Qiagen) and was cloned into the pGEM-T vector (Promega, Madison, Wisc.) to form pinKE. This clone was sequenced using an ABI100 Model 377 automated sequencer (Applied Biosystems, Foster City, Calif.) and a primer internal (MM141: 5'-CTGTAGGCGTGGGAATCGGTC-3') to the known *ohr* sequence. A complete open reading frame (ORF) was identified by comparison of the sequence obtained from the *iviK* clone and the sequence that resulted from the iPCR clone pinKE. The complete *ohr* ORF with the upstream promoter region was amplified from *A. pleuropneumoniae* serotype 1 genomic DNA using *Pfu* polymerase (Promega) and a primer pair (MM138: 5'-GGCTACGAAATATTGGACACG-3' and MM151) that binds 360 bp upstream of the start codon and 50 bp downstream of the stop codon. The PCR product was cloned into *Sma*I cut pGZRS18 to form pGeohr (44). This plasmid was transformed into both *A. pleuropneumoniae* serotype 1 and *A. pleuropneumoniae* serotype 5.

Oxidative stress growth inhibition assay. Hydrogen peroxide (Sigma, St. Louis, Mo.), cumene hydroperoxide (CHP, Sigma) and the superoxide generator paraquat (Sigma) were used as oxidative stress reagents. Disk inhibition assays were used to analyze bacterial sensitivity to these reagents (39). Briefly, 100 µl of an overnight bacterial liquid culture was added to 3 ml of BHIV top agar (0.7%) and poured onto BHIV plates. Filter paper disks (10mm, Whatman #1, Whatman Paper Ltd., Maidstone, England) saturated with 10 µl of 0.88 M hydrogen peroxide, 200 mM CHP, or 0.074 M paraquat were placed onto the hardened top agar (39). Diameters of the zones of growth inhibition were

recorded after 22 hours of incubation at 35° under 5% CO_2. Statistical analysis of zone diameter significance within each treatment group for serotypes containing *ohr* as compared to serotypes lacking the gene was evaluated by a two-tailed Student's t-test. The Analysis ToolPak in Microsoft Excel (2000) was used to perform the t-test under homoscedastic and heteroscedastic conditions.

Oxidative stress and measurement of *lux* expression. Induction of oxidative stress in broth cultures was performed as follows. *A. pleuropneumoniae* strains were grown in 25 ml of BHIV broth containing 50μg/ml ampicillin, at 35° C and shaking at 150 rpm, to an OD_{520} of 0.8, and were then dispensed into a 96 well microtiter plate in 200 μL aliquots. This was followed by a 30 minute incubation period at 35°C under 5% CO_2 prior to addition of the stress reagent to allow for acclimation. For stress induction, stress reagents were added to a final sublethal concentration of 125 μM, 300 μM, or 1 mM CHP; 56 μM hydrogen peroxide; or 50 μM paraquat (39). Aliquots were taken for luminometric assays and primer extension at designated intervals.

Quantitative luciferase assays were performed using a Turner Model 20e luminometer as previously described (13). Briefly, 20 μl of culture was mixed with 20 μl of N-decyl aldehyde in a polypropylene luminometer cuvette. The sample was read in full integral, autoranging mode with a predelay of 0 seconds, a delay of 10 seconds, and an integration time of 30 seconds. The N-decyl aldehyde substrate was made by sonicating 20 mg/ml of Essentially Fatty Acid Free Bovine Serum Albumin (Sigma) with 1 μl/ml of N-decyl aldehyde in a sonicating water bath at room temperature.

Luminometer readings were normalized to relative light units per optical density (RLU/OD_{520}).

Functional analysis of Ohr activity. An assay to evaluate the degradation of CHP was adapted from procedures developed by Dringen et al. (9) and Ochsner et al. (30). *A. pleuropneumoniae* was grown in BHIV broth to early stationary phase (OD_{520} = 0.8-1.0) and diluted with fresh prewarmed media. CHP was added to a final concentration of either 0 μM, 125 μM, 300 μM or 600 μM CHP. Residual CHP concentrations were determined at five minute intervals using a xylenol orange- iron reaction. At each time point, 100 μl of the culture was pelleted and 20 μl of the cell- free supernatant was added to 80 μl of 25 mM sulfuric acid in a 96 well microtiter plate. When all samples had been collected, 100 μl of freshly prepared reaction buffer containing 200 μM xylenol orange (Sigma), 200 μM ammonium ferrous sulfate (Sigma), and 25 mM sulfuric acid was added to each sample. After 10 minutes of incubation at room temperature, absorbance was read at 540 nm using a Bio-Tek ELISA plate reader Model EL310 (Bio-Tek Instruments, Inc., Winooski, VT). Concentrations of CHP in each sample were determined by comparison to a CHP standard curve performed at the time of each assay. Ohr activity was measured as μmoles CHP degraded/ minute.

To evaluate the induction of Ohr activity by CHP, CHP was added to 1 ml of the freshly diluted culture at a final concentration of 0 μM, 125 μM, or 300 μM, and this mixture was held without shaking for 30 minutes at 35° C. A sample was collected to determine the residual CHP concentration. Fresh CHP was added and Ohr activity was assayed as described above.

Primer extension. Primer extension of the *ohr* gene was performed as previously described (8) using a primer (MM220: 5'-CGAGTATGACCATCACGACCGCCACT GC-3') that bound 30 bp into the *ohr* coding region. Bacteria were incubated for 30 minutes in a 96 well microtiter plate under inducing conditions with 1 mM CHP, and without CHP as an non-induced control. The mRNA was isolated using a hot phenol extraction method (45). For reverse transcription, 10 μg of RNA was incubated with 1 pM of ^{32}P-ATP(Amersham Pharmacia Biotech, Piscataway, N.J.)-labeled primer and AMV-Reverse transcriptase (Promega) for 1 hour at 42°C. The samples were separated on an 8% denaturing polyacrylamide gel along with a ^{35}S-dATP (Amersham) sequencing ladder. The sequencing ladder was prepared using the Sequenase v 2.01 kit (Amersham) and the same primer that was used for primer extension (8).

Nucleotide sequence accession number. The sequence reported in this paper has been submitted to GenBank and assigned accession number AF395877.

Protein expression of Ohr. The complete sequence of *ohr* was used to design primers to fuse the gene to a hexa-histidine tag in order to purify Ohr protein. Plasmid pRS97b was constructed by PCR amplifying the majority of the *ohr* ORF using primers MM194 (5'-GCACCATGGAAATTTTTTACAAAACATCAGCAACAGC-3') and MM206 (5'-TACGGATCCATGTAGACGTACGTCCACGTTACC-3') and cloning this 400 bp fragment into pQE60 (Qiagen). The pRS97b plasmid contained the first 134 amino acids of Ohr, including the putative functional region, fused in frame to a C-terminal six

histidine tag. pRS97b was transformed into XL1-Blue mRF' and expression of His-tagged Ohr protein was induced as follows: First, a 50 ml broth culture in LBA^{100} was grown to an OD_{520} of about 0.4 shaking at 150 rpm in 37°C waterbath. IPTG was added to a final concentration of 1mM and the culture was maintained under the same incubation conditions for six hours. After six hours, the 50 ml culture was diluted into 250 ml of fresh LBA^{100} broth containing 1 mM IPTG, and the culture incubated for an additional two hours to allow for a high cell density. Protein could be isolated from this cell culture using the protocol for denaturing conditions. This involved lysing the cells by vortexing with 8M urea. The lysate was then incubated with Ni-NTA resin in a batch format and the mixture was loaded into an empty column. The flow-throughs were collected an analyzed as the resin was washed with increasing elution buffers, of decreasing pH, all of which contained 8M urea. (4^{th} edition QIAexpressionist, Qiagen).

Results

Identification of *ohr* as an in vivo induced gene. We previously developed an IVET (in vivo expression technology) screen to identify genes from *A. pleuropneumoniae* that were induced during infection of the porcine lung but had minimal expression during in vitro growth on laboratory media (13). This IVET system utilized a promoter trap vector (pTF86) with a cloning site for genomic DNA fragments upstream of promoterless copies of the *luxAB* and *ribBAH* genes. A library of random genomic DNA fragments from *A. pleuropneumoniae* serotype 1 was cloned into the pTF86 vector and subsequently electroporated into APP233, a virulent *A. pleuropneumoniae* serotype 1 strain that is unable to produce riboflavin due to a directed mutation within the *ribGBAH* operon (15). When a functional promoter was placed in the cloning site of pTF86, riboflavin was produced and complemented the riboflavin deficiency of the host strain, APP233, restoring full virulence. Without a functional promoter, riboflavin was not produced and APP233 failed to survive and cause disease (15). Thus the initial portion of the IVET procedure utilized infection of the natural host to select clones containing promoters that were expressed during infection. Clones containing functional promoters were isolated from the pig lung and examined for in vitro expression of the *luxAB* genes both quantitatively and qualitatively. Clones that had in vitro expression levels in APP233 that were less than or equal to 200 RLU/OD_{520} were identified as *ivi* (in vivo induced) clones.

Forty-two unique *ivi* clones were identified during this selection. One of these clones, *iviK*, contained an 801 bp insert that included a partial ORF of 98 amino acids

fused to *luxAB*, as well as 507 bp of upstream noncoding sequence. When this ORF, which contained a start codon but lacked a stop codon, was used to search microbial sequence databases, it showed 56% similarity to Ohr from *X. campestris*, an enzyme responsible for protection against organic peroxides (25).

To confirm the in vivo induction of the *iviK* promoter, a pig was infected intratracheally with 7 X 10^8 CFU of APP233/piviK to monitor development and progression of the pulmonary disease. We have previously demonstrated that APP233 alone is avirulent at doses as high as 5 X 10^9 CFU in this animal infection model (13, 15). Within 6 hours, the infected pig developed an increased respiratory rate and fever and showed depression and anorexia. The disease progressed to severe dypsnea by 9 hours post infection. At necropsy, 90-100% of the right lung lobes and the accessory lobe showed edema, hemorrhage, congestion, and regions of necrosis. These symptoms are consistent with peracute pleuropneumonia. A portion of the right caudal lung lobe from this pig was photographed by visible light camera and by photonic camera (Figure 1).

The visible light picture shows regions of severe necrosis and hemorrhage. The photonic camera picture of this same region of lung shows *lux* expression at the edges of this necrotic tissue, which is the region of active infection. In contrast to infection by this *iviK* clone, we have previously shown that infection with a 5 X 10^9 CFU of a clone containing the pTF86 vector only does not result in disease symptoms in the pig (13). When lung tissue isolated from a pig infected with APP233/pTF86 was examined, there were no regions of necrosis or of *lux* expression (Figure 1).

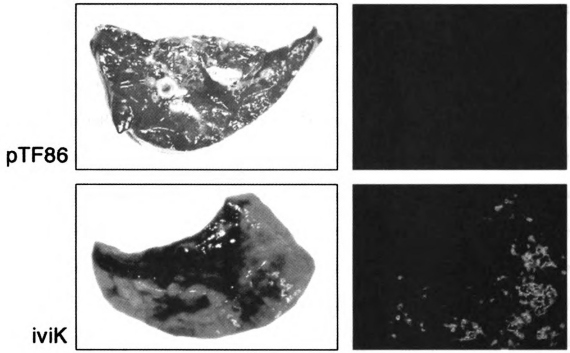


Figure 3-1 Pulmonary damage and in vivo *lux* expression resulting from infection of a swine lung with either APP233/pTF86 (row 1) or APP233/piviK (row 2). For each sample, on the left is an incidental light photograph of the lung specimen. On the right is a corresponding photograph, taken by photonic camera, showing bioluminescence of the same lung specimen. In the absence of a promoter, APP233/pTF86 showed no lung damage and no *lux* expression. In the presence of the piviK plasmid, significant lung damage was apparent and bioluminescence was seen.

Cloning and characterization of full length *ohr*. The nucleotide sequence of *iviK* was used to design inverse PCR primers to clone the full length *ohr*. A 2.5 Kb PCR product was obtained and cloned into pGEM-T to form pinKE. This insert was sequenced, and alignment of this sequence with that of *iviK* demonstrated a contiguous ORF of 432 bp encoding 143 amino acids. This ORF was identified based on sequence homology as *ohr* (Figure 2). Primers were designed to amplify the entire *ohr* gene plus 360 bp of upstream sequence. This PCR product was cloned into the pGZRS18 vector to form pGeohr.

A potential ribosome binding site (AAGGA) at 12 bp upstream of the start codon was identified. Potential transcriptional terminators flanking the ORF were identified

using the GCG Stemloop program (16). The predicted protein sequence was compared to finished and partially finished bacterial genomes deposited in the Microbial Genomes BLAST Databases using the BLAST programs (National Center for Biotechnology, NCBI). The ORF had highest homology to Ohr from *P. aeruginosa* with 62% similarity and 48% identity when examined using BLAST pairwise homology (40) (30). Proteins with strong homology to Ohr were identified from 14 species of bacteria and were aligned to identify regions of identity and similarity (Figure 2). This alignment shows two regions of high conservation that center around conserved cysteines, with the sequence outside of these regions lacking extensive similarity. Analysis of the protein using PSORT to predict cellular localization suggested that Ohr was a cytoplasmic protein (27).

Distribution of *ohr* among serotypes correlates with organic peroxide resistance. To determine if *ohr* was present in all 12 serotypes of *A. pleuropneumoniae*, a Southern blot was performed using *Eco*RI digested genomic DNA from all 12 serotypes as the target and the full length *ohr* from *A. pleuropneumoniae* serotype 1 to construct the probe. Genomic DNA fragments that hybridized to *ohr* were seen only in *A. pleuropneumoniae* serotypes 1, 9, and 11 (Figure 3A), and not found in the other serotypes, under conditions of either high or low stringency.

Sensitivity to oxidative stress reagents for each of the 12 *A. pleuropneumoniae* serotypes was examined using a disk inhibition assay. Overnight cultures were added to top agar and overlaid with disks saturated with hydrogen peroxide, CHP, or the superoxide generator, paraquat. After 22 hours, the zones of growth inhibition were

Figure 3-2. Protein sequence alignment of putative Ohr proteins from 14 bacterial species aligned using Boxshade v3.31 (http://biophysics.med.jhu.edu/prog/boxshade/PC_and_MAC/win16.zip) and ClustalX (41). Black shaded regions indicate residues that are identical in the majority of species. Gray shaded regions indicate residues that are functionally conserved in the majority of species. This alignment highlights the highly conserved regions surrounding the conserved cysteine residues (*). Species abbreviations used are as follows: App: *Actinobacillus pleuropneumoniae*; Pa: *Pseudomonas aeruginosa*; Vc: *Vibrio cholerae*; Sp: *Shewenella putrefaciens*; Lp: *Legionella pneumophila*; Xc: *Xanthomonas campestris*; Cc: *Caulobacter crescentus*; Bs1: *Bacillus subtilis* YklA (OhrA); Ba: *Bacillus anthracis*; Bs2: *B. subtilis* YkzA (OhrB); Dr: *Deinococcus radiodurans*; Sa: *Staphylococcus aureus*; Ef: *Enterococcus faecalis*; Mp: *Mycoplasma pneumoniae.*

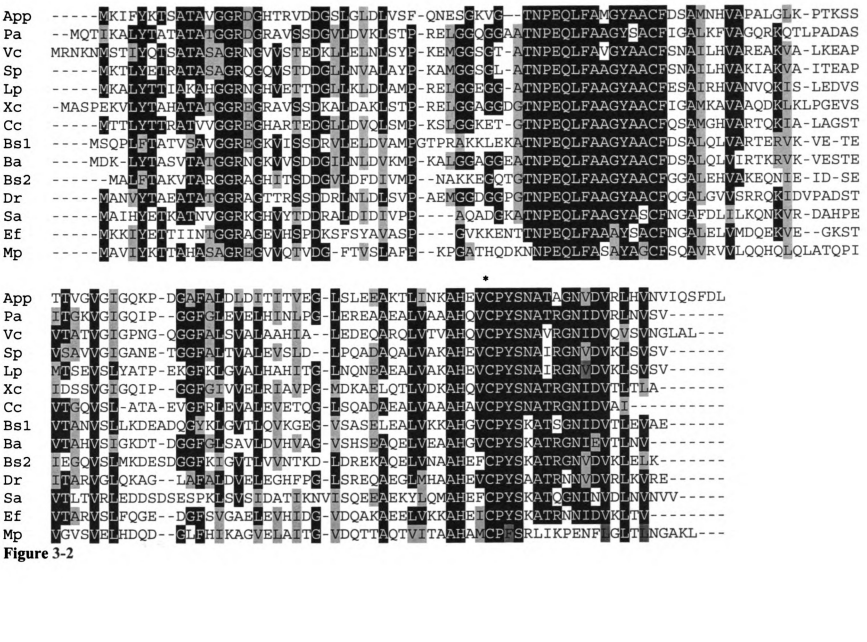
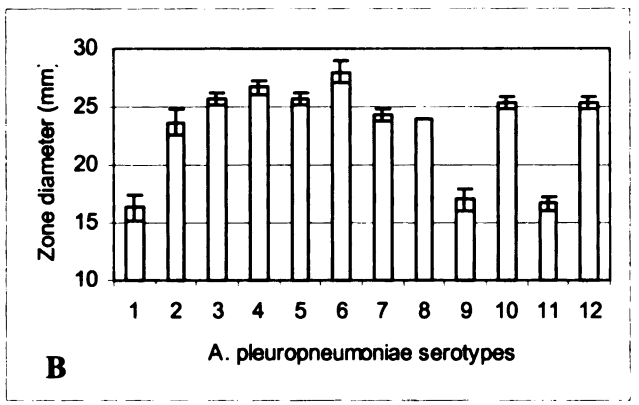
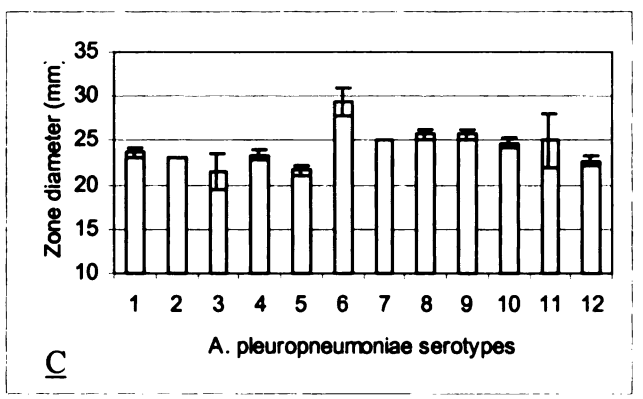


Figure 3-2

Figure 3-3. Correlation of *ohr* presence with resistance to organic hydroperoxides. The presence of the gene was determined by Southern blot of genomic DNA using the intact *ohr* gene as a probe (3A). Molecular weight standards (in kb) are shown on the left. The resistance of each of the 12 *A. pleuropneumoniae* serotypes to oxidative stress agents was determined by measuring the diameter of the zone of growth inhibition due to CHP (3B), hydrogen peroxide (3C) and paraquat (3D). The data presented is the average of three experiments with the standard deviation shown by error bars. The differences in the zone diameters, after incubation with CHP, seen with serotype 1, 9, and 11, in comparison to the remaining serotypes, are statistically significant ($P < 0.0001$).


4.0
3.0
1.6
A
1 2 3 4 5 6 7 8 9 10 11 12


Zone diameter (mm)
30
25
20
15
10
1 2 3 4 5 6 7 8 9 10 11 12
A. pleuropneumoniae serotypes
B


Zone diameter (mm)
35
30
25
20
15
10
1 2 3 4 5 6 7 8 9 10 11 12
A. pleuropneumoniae serotypes
C

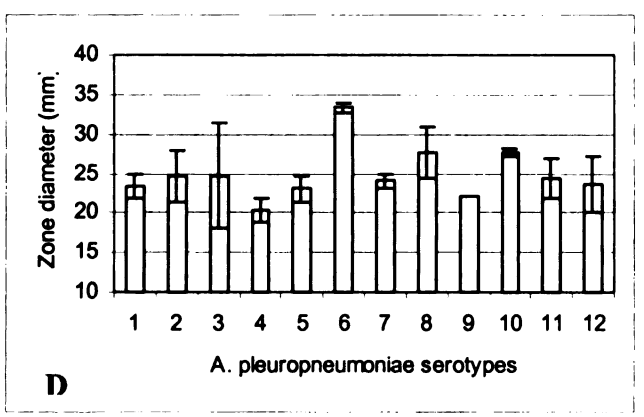

Zone diameter (mm)
40
35
30
25
20
15
10
1 2 3 4 5 6 7 8 9 10 11 12
A. pleuropneumoniae serotypes
D

Figure 3-3

recorded as a measure of the sensitivity of each serotype to the oxidative stress imposed. Eleven of the twelve serotypes showed equivalent sensitivity to hydrogen peroxide and paraquat (Figure 3C, 3D). The exception was serotype 6, which showed a significantly larger zone diameter ($P < 0.01$) with all three forms of oxidative stress, suggesting a decreased resistance to oxidative stress reagents in general for this serotype. Serotypes 2-8, 10, and 12 showed sensitivity to CHP similar to that seen with hydrogen peroxide and paraquat (Figure 3B, 3C, 3D). In contrast, serotypes 1, 9, and 11 showed a significantly reduced zone of growth inhibition upon incubation with CHP, reflecting an increased resistance to oxidative stress caused by organic peroxides (Figure 3B). This increased resistance of serotypes 1, 9, and 11 to CHP was highly statistically significant ($P < 0.0001$), and no statistically significant difference was seen for these serotypes in response to incubation with hydrogen peroxide or paraquat. This increased resistance to CHP, but not to hydrogen peroxide or paraquat, correlated with the presence of the *ohr* gene as shown by Southern blot (Figure 3A).

Induction of *ohr* in response to oxidative stress. To characterize the expression of *ohr* in response to different oxidative stress reagents, induction studies were performed in wild type *A. pleuropneumoniae* serotype 1. The analysis was performed in the wild type strain in order to decrease the oxidative stress imposed on the cultures due to the presence of riboflavin in the media that is necessary for growth of the APP233 strain.

APP225/piviK, which contained the *ohr* promoter-*luxAB* fusion, was induced in microtiter plates with paraquat, CHP, or hydrogen peroxide. Addition of CHP resulted in a rapid increase in *lux* expression in comparison to the noninduced control (Figure 4).

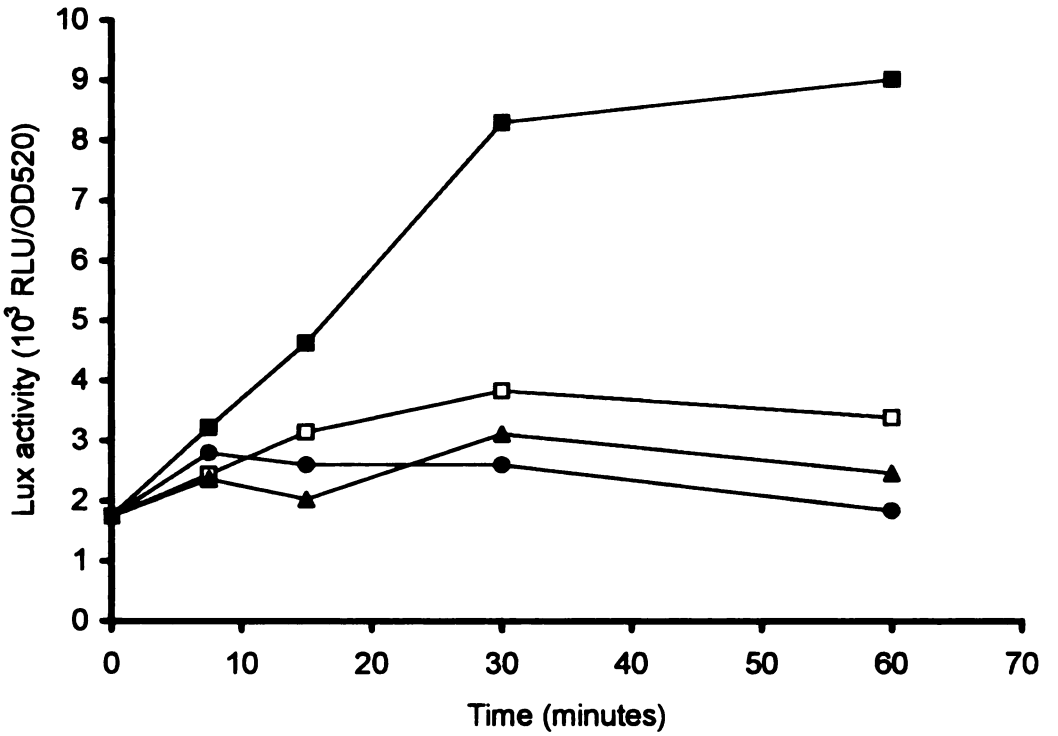


Figure 3-4. Induction of the cloned *ohr* promoter in *A. pleuropneumoniae* serotype 1 (APP225/piviK) by various oxidative stress reagents. 1 mM CHP (■), 50 μM paraquat (●), and 56 μM hydrogen peroxide (▲) were added as oxidative stress reagents and *lux* activity, expressed as RLU/OD_{520}, was measured. *Lux* activity was also measured in control cultures to which no inducing agent was added (□). Data presented are from a representative experiment. Trends were identical in all experiments.

Neither paraquat nor hydrogen peroxide caused any induction, and the level of *lux* expression was equivalent to that seen in the absence of oxidative stress (Figure 4).

Comparison of induction of *ohr* in *A. pleuropneumoniae* serotype 1 and *A. pleuropneumoniae* serotype 5. We compared the expression of the *ohr:lux* fusion using APP225/piviK (serotype 1) and APP227/piviK (serotype 5) during normal growth in culture and under induction by CHP. A growth curve was performed for each serotype to

evaluate the expression level of *ohr* during growth in an aerated broth culture. The expression of *ohr* for both *A. pleuropneumoniae* serotype 1 and *A. pleuropneumoniae* serotype 5, as measured by luciferase activity, remained constant during normal growth, with the increase over time directly correlated to the increase in total cell number (data not shown). The expression level was independent of serotype, with both serotypes showing low but equal expression levels during normal growth in the absence of inducers. This expression level of luciferase in the wild type strains of *A. pleuropneumoniae* serotype 1 and *A. pleuropneumoniae* serotype 5, which averaged 1000 RLU/OD, is greater than that seen in the *A. pleuropneumoniae* serotype 1 riboflavin mutant (APP233), which was never higher than 200 RLU/OD. Expression of luciferase from the pTF86 vector alone, with no insert, showed minimal expression of <50 RLU/OD in both serotypes (data not shown).

Expression of *ohr* under inducing conditions was examined in both *A. pleuropneumoniae* serotype 1 and *A. pleuropneumoniae* serotype 5. Induction of *ohr* in response to incubation with CHP was seen only in *A. pleuropneumoniae* serotype 1 and not in *A. pleuropneumoniae* serotype 5 (Figure 5). APP225/piviK showed rapid induction of expression as measured by Lux assay with a two-fold increase within 10 minutes post exposure to CHP. Lux activity increased over time with maximal levels detected between 30 and 60 minutes after induction. In contrast, APP227/piviK showed no increase in *lux* expression in response to CHP and maintained a level of expression slightly greater than that of the vector only control. This data suggests that incubation with CHP does not cause induction of *ohr* in *A. pleuropneumoniae* serotype 5.

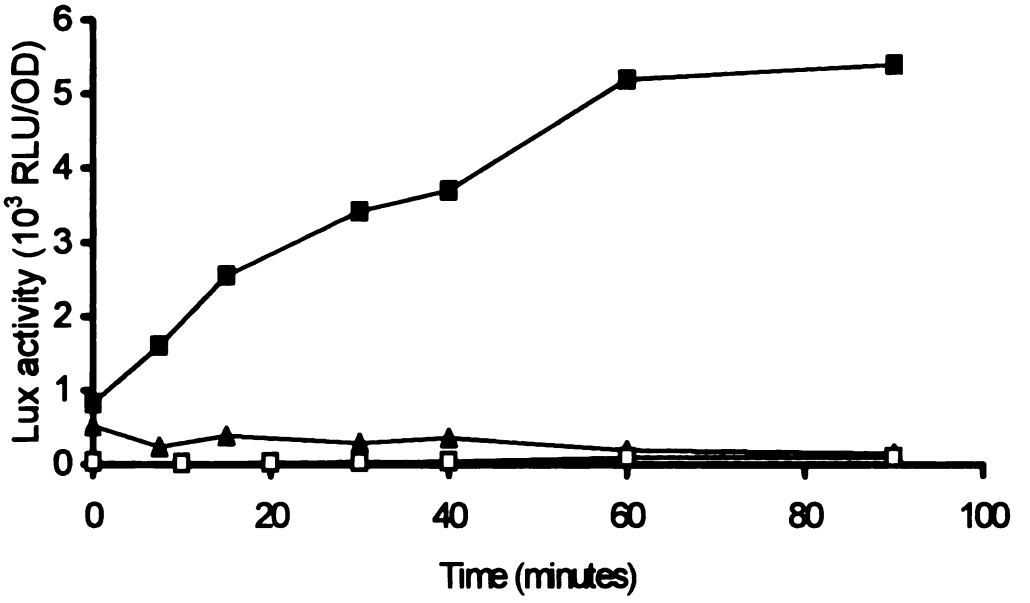


Figure 3-5. Expression of the *ohr* promoter is induced by 125 μM CHP in *A. pleuropneumoniae* serotype 1 and not in serotype 5. *Lux* expression driven by the *ohr* promoter was measured over time in *A. pleuropneumoniae* serotype 1 (APP225/piviK) (■) and in *A. pleuropneumoniae* serotype 5 (APP227/piviK) (▲), in comparison to a vector only control, APP225/pTF86 (□). Data presented are from a representative experiment. Trends were identical in all experiments.

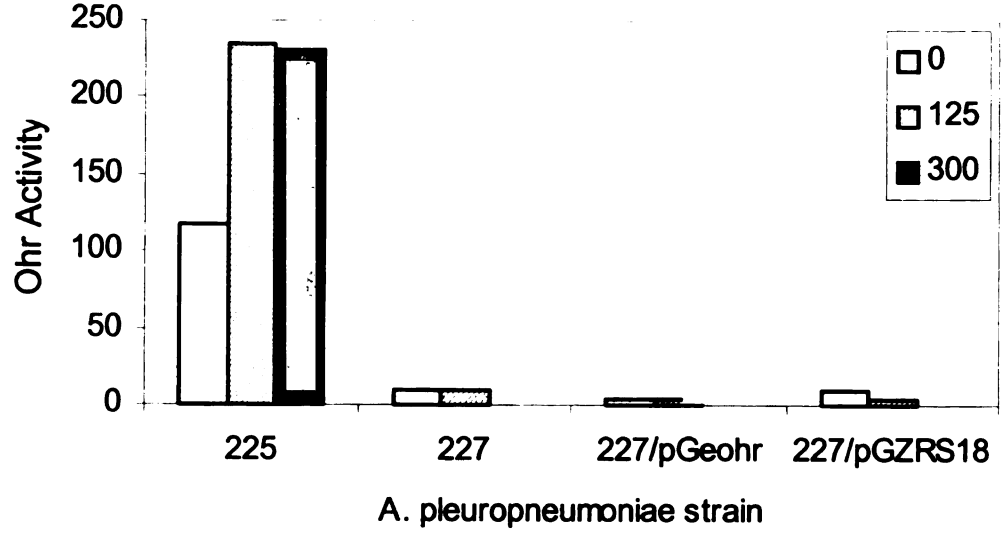
This induction data was confirmed through a functional assay of Ohr enzymatic activity. A xylenol orange colorimetric assay was used to determine the concentration of CHP after incubation of CHP with bacteria. Each serotype was grown in broth and then diluted into fresh media containing 125 μM CHP or 300 μM CHP followed by incubation for 30 minutes to allow for induction. After this induction period, 125 μM CHP, 300 μM CHP, or 600 μM CHP was added and the rate of CHP degradation was measured. APP225, *A. pleuropneumoniae* serotype 1, showed significant Ohr activity, as measured by the rate of CHP degradation, in the absence of induction. Induction of *A. pleuropneumoniae* serotype 1 for 30 minutes with either 125 μM CHP or 300 μM CHP

resulted in an approximately two-fold increase in enzymatic activity, which correlated well with the increase in *ohr* expression as measured by Lux activity (Figure 5, Figure 6).

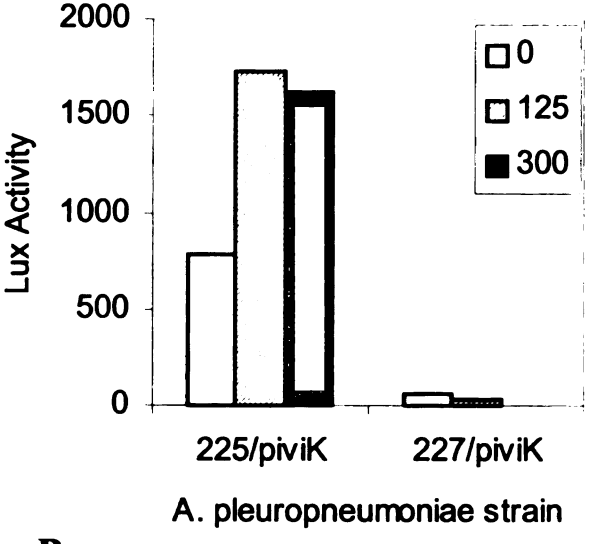
In contrast, neither APP227 (*A. pleuropneumoniae* serotype 5), APP227/pGZRS18, nor APP227/pGeohr showed significant Ohr activity in the absence of induction, and no increase in activity was evident under inducing conditions. These results correlate with and confirm the lack of expression of *ohr* in APP227 as measured by Lux activity (Figure 5, Figure 6). The assays performed with APP227 showed that concentrations of CHP greater than or equal to 300 μM CHP were lethal to the cells and thus induction and assays were performed at 125 μM CHP.

Evaluation of the mRNA start site in serotype 1 under CHP induction. Primer extension was performed using mRNA isolated from APP225/piviK that had been induced with CHP. The major transcriptional start site was located 31 bp upstream of the *ohr* start codon (Figure 7). A –10 region (TAAAAT) was identified 6 bp upstream of the transcription start site. However, no –35 region similar to that found in *A. pleuropneumoniae* housekeeping genes (TTRAA, where R is A or G) could be identified (8). In the region in which a –35 site would be expected to exist, a SoxS binding motif (ACCGCAT) was found (35). Primer extension under non-inducing conditions was also performed using six-fold more RNA. A primer extension product could not be detected under these non-inducing conditions (data not shown).

Figure 3-6. **A)** Ohr activity, expressed as μmoles CHP degraded per minute, is induced by CHP in *A. pleuropneumoniae* serotype 1 (APP225) but not in serotype 5 containing the intact *ohr* gene plus promoter region (APP227/pGeohr). Controls include APP227 with no plasmid and APP227 containing the pGZRS18 shuttle vector only. For induction, CHP was added at final concentrations of 0, 125, or 300 μM to 1 ml broth cultures, which were held for 30 minutes at 35°C. Ohr activity was measured as degradation of CHP. **B)** Lux activity, measured as RLU/OD, is similarly induced in APP225/piviK but not in APP227/piviK. Induction conditions for these assays were identical to those in panel A. Data presented are from a representative experiment. Trends were identical in all experiments.


250
200
150
100
50
0
Ohr Activity
0
125
300
225
227
227/pGeohr
227/pGZRS18
A. pleuropneumoniae strain

A


2000
1500
1000
500
0
Lux Activity
0
125
300
225/piviK
227/piviK
A. pleuropneumoniae strain

B

Figure 3-6

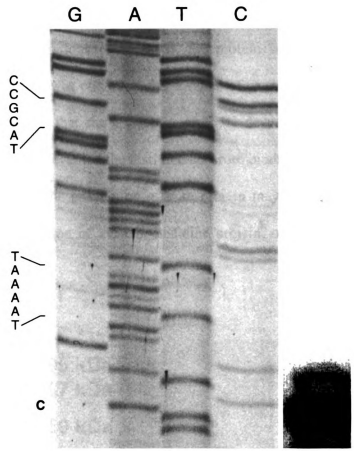


Figure 3-7. Primer extension analysis of APP225/piviK induced with 1 mM CHP. The sequencing reactions and the primer extension reaction were performed with an identical primer. The lane on the right contains the primer extension product. The transcriptional start site is labeled in bold (C). The –10 region (TAAAAT) and the potential SoxS binding motif (CCGCAT) are indicated on the left. An intervening region between the sequencing ladder and the primer extension product was removed, without alteration of alignment.

Evaluation of the Ohr-hexahistidine protein. When XL1-Blue mRF'/pRS97b was induced with 1 mM IPTG, the cell pellet from 50 µl of culture was examined on a 15% SDS-PAGE gel, a heavily stained band of approximately 14 kDa was seen. No similar protein band was seen in 50 µl of uninduced cells grown under the same conditions (Figure 8, lane 5). In initial attempts to purify the protein, minimal binding to the nickel-

NTA column was obtained with non-denaturing, native, conditions (data not shown). Using denaturing conditions (8 M urea), the protein was bound to the column and relatively pure protein was obtained after a series of wash steps (Figure 8, lane 2). This observation suggested that the induction protocol developed led to the formation of inclusion bodies that prevented the histidine tag from binding to the column in the native, nondenatured state. The production of pure protein may prove useful in Ohr-antibody production, crystallization of the Ohr-histidine protein, or in enzymatic assays of pure protein.

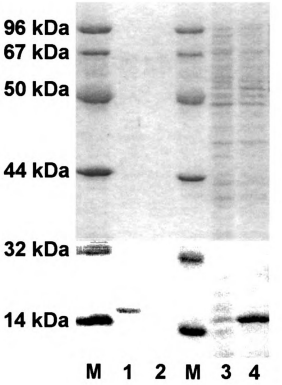


Figure 3-8. Purification of Ohr-hexahistidine protein. A 15% SDS-PAGE denaturing gel was loaded with molecular weight markers (M) and various purification steps of the Ohr protein. Lane 1 contains purified protein, lane 2 contains protein purified from uninduced cells. Lane 3 contains uninduced cell lysate and lane 4 contains whole cell lysate from cells induced with IPTG.

Discussion

Using the IVET system we developed to facilitate identification of *Actinobacillus pleuropneumoniae* genes involved in the pathogenesis of disease, we identified 42 unique promoter clones that were specifically induced during infection of the natural swine host. In this study, we report the identification and characterization of one of these in vivo induced clones, *ohr*, which encodes an organic hydroperoxide reductase that we hypothesize is involved in protection from oxidative stress encountered during infection.

Enzymes responsible for conferring enhanced resistance to oxidative stress encountered during infection of the respiratory tract are potentially important virulence factors for organisms that cause pneumonia, such as *A. pleuropneumoniae* (6, 46). The mechanisms by which *A. pleuropneumoniae* causes disease lead to an environment filled with oxygen radicals (21, 28). Upon infection of the porcine lung by *A. pleuropneumoniae*, the host immune response to bacterial cell components, such as lipopolysaccharide, triggers an influx of inflammatory cells, particularly neutrophils, which limit the bacterial infection. Within this environment, *A. pleuropneumoniae* produces hemolysins and cytotoxins, in the form of RTX toxins (repeats in toxin). These pore-forming RTX toxins secreted by *A. pleuropneumoniae* insert into eukaryotic cell membranes and cause lysis and cell death of neutrophils and macrophages, which in turn releases phagocyte contents such as oxygen radicals in the form of peroxides and superoxides (7, 11, 35). To survive in this environment, *A. pleuropneumoniae* likely requires enzymes that allow the bacteria to evade or detoxify these oxygen radicals. *A. pleuropneumoniae* has previously been shown to produce several enzymes involved in

response to oxidative stress, including catalase and two separate types of superoxide dismutase (20, 22). This is the first report that this pulmonary pathogen produces an additional oxidative stress protectant, an organic hydroperoxide reductase, and the first demonstration that this type of enzyme can be specifically induced in vivo during the course of infection.

Ohr enzymes have been recently described in *Xanthomonas campestris, Pseudomonas aeruginosa*, *Enterococcus faecalis,* and *Bacillus subtilis* and have been shown to be important in the survival of these bacteria when exposed to oxidative stress in vitro, although Ohr has not been previously implicated in virulence, (12, 25, 30, 34). The *ohr* gene from these organisms exhibits a similar pattern of expression to that of *A. pleuropneumoniae ohr.* In each of these organisms, *ohr* is induced specifically in response to organic hydroperoxides, with little or no induction in response to hydrogen peroxide and superoxide (12, 25, 30, 34). This pattern of induction is distinct from that seen with *ahpC*, which encodes Ahp (alkyl hydroperoxide reductase), a second class of organic hydroperoxide reductase found in many bacterial species, including *E. coli, Salmonella typhimurium, B. subtilis,* and *P. aeruginosa* (1, 4, 31, 38). Ahp enzymes are induced by both hydrogen peroxide and organic peroxides but not superoxides (31, 35). We identified putative Ohr sequences, based on homology to these five identified Ohr proteins, from nine additional species (Figure 2), although not from any members of the *Enterobacteriaceae.* The predicted Ohr proteins from these fourteen species share two regions of strong homology that flank conserved cysteines, which may be responsible for the reduction of peroxides to the corresponding alcohol (10). Similar conserved

cysteines are seen in Ahp enzymes, which are functionally similar to Ohr enzymes but not closely related at the DNA level or protein level.

When we examined the type strains of the 12 known serotypes of *A. pleuropneumoniae* for the presence of *ohr* by Southern blot, we were able to detect an *ohr* homologue only in *A. pleuropneumoniae* serotypes 1, 9, and 11 and not in serotypes 2-8, 10, and 12. This distribution correlates with what is known about the relatedness of *A. pleuropneumoniae* serotypes. Serotypes 1, 9, and 11 are closely related to one another, having essentially the same LPS O-antigen chain, the same complement of RTX toxins produced, and an identical genotype for one of these toxins, *apxIA* (18, 32). *A. pleuropneumoniae* serotypes 1 and 9 have also been shown to be closely related to one another and distinct from serotypes 2-8 using multilocus enzyme electrophoresis (26). The differential distribution of *ohr* among the serotypes may reflect both an evolutionary relatedness of these serotypes and a need for detoxification of organic hydroperoxides encountered during the course of infection.

The presence of *ohr* among the 12 serotypes of *A. pleuropneumoniae* correlates with resistance to oxidative stress reagents. Serotypes 2-8, 10, and 12, which do not contain an *ohr* gene, were equally sensitive to all types of oxidative stress agents tested, as judged by the zone of growth inhibition upon exposure to cumene hydroperoxide (CHP), paraquat, and hydrogen peroxide (Figure 3). In contrast, serotypes 1, 9, and 11 were significantly less sensitive to growth inhibition by CHP than the other serotypes, but were similar to the other serotypes in sensitivity to hydrogen peroxide and paraquat. *A. pleuropneumoniae* serotypes 1, 9, and 11 showed an increased resistance to CHP, but not

to hydrogen peroxide or superoxide, that correlates with the presence of the *ohr* gene (Figure 3).

The increased resistance to organic peroxides but not to other forms of oxidative stress seen in *A. pleuropneumoniae* serotypes 1, 9, and 11 correlates well with data on the induction of the *ohr* promoter by various stress reagents in *A. pleuropneumoniae* serotype 1. Induction of *ohr* was measured by luciferase assays using the *ohr* promoter fused to *luxAB* reporter genes and by assay of Ohr enzymatic activity via colorimetric detection of CHP degradation. With both of these methods, *ohr* expression in *A. pleuropneumoniae* serotype 1 was induced by CHP but not by either hydrogen peroxide or paraquat (Figure 4, Figure 6).

We cloned both the intact serotype 1 *ohr* gene plus promoter region and an *ohr:luxAB* gene fusion into *A. pleuropneumoniae* serotype 5, which lacks *ohr*. During growth in broth under non-inducing conditions, serotype 1 and serotype 5 showed low but equivalent expression as assayed by *lux* expression. However, while *A. pleuropneumoniae* serotype 1 is rapidly induced upon exposure to CHP, this induction is not seen in *A. pleuropneumoniae* serotype 5, either as increased expression of luciferase or as increased Ohr enzymatic activity (Figure 5, Figure 6). We conclude that *A. pleuropneumoniae* serotype 5 not only does not contain a WT *ohr* gene but also is unable respond to exposure to CHP by induction of the cloned serotype 1 *ohr* gene. This suggests that *A. pleuropneumoniae* serotype 5 may lack not only the *ohr* gene itself but also additional gene(s) necessary to increase the expression of *ohr* in *A. pleuropneumoniae* serotype 1.

Multiple regulators that respond to oxidative stress have been identified in other prokaryotes, but none have as yet been identified in *A. pleuropneumoniae*. Three of the most well studied regulators of oxidative stress responses in bacteria are OxyR, PerR, and SoxR (2, 35). OxyR, which has been identified in many gram-negative bacteria, is activated by exposure to peroxide, induces expression of *ahpC* (alkyl hydroperoxide reductase) and catalase, and also represses its own expression (30, 42). In both *X. campestris* and *P. aeruginosa*, *ohr* expression was not altered by lack of OxyR (25, 30). In many gram-positive organisms, *ahpC* and catalase are regulated by PerR, a homologue of the ferric uptake regulator Fur, which is functionally analogous to OxyR (2, 5, 35). Both of these regulators have known binding motifs that were not found in the *A. pleuropneumoniae ohr* promoter region (5, 42). SoxR, a transcription factor that is activated by superoxide, induces expression of a second transcription factor, SoxS, which in turn induces expression of superoxide-regulated genes such as *sodA*. SoxRS has also been shown to regulate ahpC in some organisms, but has not been demonstrated to be activated by peroxides (23, 33, 35),. In *P. aeruginosa*, *ohr* induction was not affected by mutations in SoxR (30).

When we examined the promoter region of *A. pleuropneumoniae ohr* for potential regulatory sequences, we identified a potential SoxS box but no PerR or OxyR binding sequences. SoxS in other organisms does not respond to organic peroxides, and the *A. pleuropneumoniae ohr* gene was not induced by superoxide generators. Our results suggest that a novel regulator or regulatory sequence is responsible for induction of *ohr* in *A. pleuropneumoniae*, and that this novel regulator exists in *A. pleuropneumoniae* serotype 1 and not in serotype 5. Further studies are in progress to identify this

regulatory molecule and to evaluate the role of Ohr in pulmonary infection caused by *A. pleuropneumoniae*.

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Chapter 4

Genetic disruption of *ohr* and *ilvI*

Abstract

Actinobacillus pleuropneumoniae is a respiratory pathogen of swine that causes a hemorrhagic pleuropneumonia that results in high morbidity and mortality. A study has been completed to identify *in vivo* induced genes in order to further study the pathogenesis and virulence genes of *A. pleuropneumoniae*. *Ohr* and *ilvI* have been previously identified as two *in vivo* induced genes. These genes were selected in order to further understand how the bacteria adapts to host attempts to limit infection and as a possible antibiotic target, respectively. Ohr is an organic hydroperoxide reductase that could detoxify oxygen radicals that result from the influx of host immune cells (Chapter 3, and reference 15). *IlvI* encodes acetohydroxy acid synthase III, one of several proteins responsible for fulfilling the metabolic requirement for isoleucine and valine (19). In order to assess the role in virulence of these two genes, clones were constructed for gene disruption through allelic replacement and insertion of either a cassette for kanamycin resistance or NAD independence. Multiple matings and electroporations failed to yield a stable double recombinant through homologous recombination. Several methods for improving the selection of double recombinants are discussed.

Introduction

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a disease characterized by massive lung necrosis and pulmonary hemorrhage. This necrosis is due in part to the influx of host immune cells and the release of neutrophil lysosomal contents that include oxygen radicals, which can destroy the invading bacteria as well as the host tissue (10). In addition, the host is able to limit bacterial growth and tissue destruction through prevention of bacterial acquisition essential nutrients.

During infection of the porcine lung, *A. pleuropneumoniae* is exposed to oxygen radicals generated by the neutrophil oxidative burst (10). One class of oxygen radicals, organic hydroperoxides, can be generated either directly within the phagosome or as a consequence of oxygen radicals interacting with the bacterial cell membrane [reviewed in Miller and Britigan (6)]. To survive and protect its cellular metabolism within this dangerous milieu, *A. pleuropneumoniae* contains enzymes capable of inactivating peroxides and superoxides (6). The role of Ohr in the inactivation of organic hydroperoxides has been previously described (Chapter 3 and reference 15) . In order to further the study of this protein and its role in virulence, an *A. pleuropneumoniae ohr-* mutant was required to determine if Ohr was essential for virulence and if this mutant was more susceptible to oxidative stress.

Another gene upregulated during infection was *ilvI*. IlvI, encoded by the ilvIH operon, is homologous to several enzymes involved in the production of the hydrophobic branch chained amino acids, isoleucine, leucine, and valine (13, 18, 19). IlvI encodes

acetohydroxy acid synthase III (AHASIII), one of three isozymes that have been characterized in both *Escherichia coli* and *Salmonella typhimurium*, that can catalyze the condensation of pyruvate with either pyruvate or α-ketobutyrate to form valine or isoleucine, respectively (19). The three isozymes appear to have arisen through gene duplication and can be biochemically differentiated by their inhibition by the branch chained amino acids (17). In *A. pleuropneumoniae* serotype 5, genes encoding homologues of all three isozymes are present, but the function of the proteins encoded by these genes has not yet been analyzed (John Nash, personal communication). AHASIII is inhibited by valine, with the valine inhibition requiring IlvH. It has been proposed that the two proteins encoded by the *ilvIH* operon encode a large catalytic subunit (*ilvI*) and a regulatory small subunit (*ilvH*) (18). *IlvIH* is also regulated by LRP (leucine responsive protein) in response to the concentration of leucine. For *ilvIH*, bound LRP increases the transcription of this operon and high concentrations of leucine reduce expression of the operon by reducing the activation of *ilvIH* by LRP (12, 21).

The role in virulence of *ilvI* is not yet clear. IlvI might be a survival factor directly, since in a hostile, nutrient limiting environment, increased biosynthesis of amino acids would be required. The identification of *ilvI* as an *in vivo* induced gene might also be due to a requirement for this gene in virulence, but rather because LRP and LRP-regulated genes are required for virulence and increased transcription of *ilvI* is a secondary effect due to increased production of LRP.

Materials and Methods

Bacterial strains. *A. pleuropneumoniae* strains were cultured at 35°C on brain heart infusion (BHI) (Difco, Detroit, Mich.) media supplemented with V factor (β-NAD) added at 10 μg/ml (BHIV). Media was supplemented with ampicillin at 50 μg/ml to propagate plasmids or at 20 μg/ml to select for transformants after electroporation. The *A. pleuropneumoniae* strains used were APP225, a virulent nalidixic-acid resistant derivative of *A. pleuropneumoniae* ATCC 27088 (serotype 1A); and APP227, a virulent naladixic-acid resistant derivative of *A. pleuropneumoniae* ISU178 (serotype 5 field isolate) (1, 2). *Escherichia coli* strain XLI-Blue mRF' (Stratagene, LaJolla, Calif.) was used for construction and propagation of pUC and pGZRS-based plasmids. *E. coli* S-17-1 (λ pir) was used for the propagation and mating for all plasmids based on pGP704. *E. coli* was cultured on Luria-Bertani (LB) medium (Difco) supplemented with ampicillin at 100 μg/ml. Isoleucine, leucine, and valine, when required for the construction of *A. pleuropneumoniae ilvI*-., were supplemented at the same concentrations as that found in chemically defined media for the growth of *A. pleuropneumoniae* (5).

Construction of codon usage table. In order to construct degenerate primers to amplify the 3' end of *ilvI*, a codon usage table was required. This was constructed using the codon usage program of GCG (Genetics Computer Group, Madison, WI) with the sequence inputs derived from *A. pleuropneumoniae* gene sequences deposited in GenBank, prior to 1998. The selection criteria for inclusion of a sequence were: the entire protein coding region must be present and must start with a methionine and the

protein must be deemed essential and not required solely for virulence. A total of 28 proteins were included in the codon usage profile. The program generated a table (Table 4-1) for each amino acid and the codon preference determined by percent representation.

Molecular manipulations. A description of the plasmids constructed for this study is listed in Table 4-2. Genomic DNA from *A. pleuropneumoniae* was prepared according to the lysis/proteinase K method of the Gene Fusion Manual (16). Plasmid DNA was purified using Qiagen spin columns (Qiagen Inc., Valencia, Calif.). DNA modifying enzymes were obtained from Roche (Roche Molecular Biochemicals, Indianapolis, Ind.) and used according to the manufacturer's specifications. All PCR amplification was performed with *Pfu* polymerase (Stratagene, LaJolla, CA). All plasmids constructed were sequenced using commercially available pUC18 primers or primers designed based upon known gene sequence. Sequencing was performed using an ABI100 Model 377 automated sequencer (Applied Biosystems, Foster City, Calif.)

Preparation of electrocompetent *A. pleuropneumoniae* serotype 1 and electroporation of these cells with plasmid DNA prepared from either *E. coli* or *A. pleuropneumoniae* serotype 1 was performed as previously described (2, 22). *A. pleuropneumoniae* serotype 5 was made competent by the method of Ward et al (22) and electroporated using the same electroporation conditions as for serotype 1, but using plasmid prepared from *E. coli*. *E. coli* transformation was performed by the Hanahan method (3).

Table 4-1. Codon usage table for *A. pleuropneumoniae*

Amino acid	Codon	Percent
Gly	GGG	9
Gly	GGA	14
Gly	**GGT**	**49**
Gly	GGC	28
Glu	GAG	25
Glu	**GAA**	**75**
Asp	**GAT**	**76**
Asp	GAC	24
Ile	ATA	16
Ile	**ATT**	**54**
Ile	ATC	31
Ala	GCG	29
Ala	GCA	32
Ala	GCT	23
Ala	GCC	15
Arg	AGG	4
Arg	AGA	13
Arg	CGG	12
Arg	CGA	16
Arg	CGT	38
Arg	CGC	17
Met	**ATG**	**100**

Amino acid	Codon	Percent
Ser	AGT	20
Ser	AGC	18
Ser	TCG	16
Ser	TCA	20
Ser	TCT	18
Ser	TCC	8
Lys	AAG	21
Lys	**AAA**	**79**
Asn	**AAT**	**68**
Asn	AAC	32
Tyr	**TAT**	**60**
Tyr	**TAC**	**40**
Thr	ACG	22
Thr	ACA	20
Thr	ACT	26
Thr	ACC	32
Stop	TGA	19
Stop	TAG	19
Stop	**TAA**	**62**
His	**CAT**	**58**
His	CAC	42

Amino acid	Codon	Percent
Trp	**TGG**	**100**
Cys	TGT	42
Cys	**TGC**	**58**
Leu	TTG	19
Leu	**TTA**	**45**
Leu	CTG	8
Leu	CTA	6
Leu	CTT	15
Leu	CTC	7
Phe	**TTT**	**62**
Phe	TTC	38
Gln	CAG	24
Gln	**CAA**	**76**
Pro	**CCG**	**43**
Pro	CCA	33
Pro	CCT	17
Pro	CCC	8
Val	GTG	24
Val	**GTA**	**31**
Val	**GTT**	**31**
Val	GTC	14

Table 4-2. Plasmids constructed in this study

Plasmid (size)	Characteristics	Source
pUC18/pUC19 (2.7 kb)	Ap^r *E. coli* cloning vector	(20)
pUC4K (3.9 kb)	Ap^r, Kan^r *E. coli* vector, from which the kanamycin cassette was derived	Pharmacia
pGP704 (3.7 kb)	Ap^r broad host range suicide vector	(7)
piviK (11.1 kb)	*ohr* (797 nt) insert in pTF86	Chapter 2
pRS91 (3.2 kb)	Entire *ohr* gene, start codon to stop codon, cloned into pUC18	This study
pRS91C (3.2 kb)	*ohr* gene, lacking the first 56 nucleotides, cloned into pUC18	This study
pRS93a (4.4 kb)	*ohr*, from pRS91, with kanamycin, opposite orientation to *ohr*, in middle	This study
pRS93b (4.4 kb)	Identical to pRS93a, kanamycin cassette expressed in same orientation as *ohr*	This study
pRS93KanR (4.4 kb)	*ohr*, from pRS91C, with kanamycin, opposite orientation to *ohr*, in middle	This study
pRS93KanF (4.4 kb)	Identical to pRS93KanR, kanamycin cassette expressed in same orientation as *ohr*	This study
pRS94b (5.5 kb)	Insert from pRS93b (*ohr*-kanamycin) cloned into pGP704	This study
pRS94KanF (5.5 kb)	Insert from pRS93KanF (*ohr*-kanamycin) cloned into pGP704	This study

Table 2. (cont'd)

Plasmid (size)	Characteristics	Source
pivil (10.8 kb)	*ilvI* (622 nt) in pTF86	Chapter 2
pilv5' (3.4 kb)	First 700 nt of *ilvI* cloned into pUC18	This study
pilv5'3' (4.1 kb)	*ilvI*, lacking an internal 300 bp, cloned into pUC18	This study
pilv5'3'Kan (5.7 kb)	pilv5'3' with the kanamycin cassette, in same orientation, in the middle of the gene	This study
pC18ilvKanNad (7.0 kb)	pilv5'3' with the nadV-kanamycin cassette, in same orientation, in the middle of *ilvI*	This study
pC18KnadV (4.3 kb)	*nadV*, with the promoter from the kanamycin cassette, cloned in pUC18	This study
pC18KanNad (5.6 kb)	Kanamycin cassette upstream of *nadV* in pC18KnadV, both genes in same orientation	This study

Construction of mutants by filter mating. Bacterial cultures were grown overnight in BHIV broth, with antibiotics as required, at 35°C. All subsequent steps were performed at room temperature. The total CFUs (colony forming units), as determined by OD_{520}, was calculated. Both donor (*E. coli*) and recipient (*A. pleuropneumoniae*) cells, 100 µl or less of each, were added to 5 ml of 10 mM $MgSO_4$ to obtain set proportions of donor to recipient. Cells were pelleted by centrifugation and resuspended in 0.1 ml of 10 mM $MgSO_4$. The suspension was placed on a sterile filter that was placed on a prewarmed BHIV plate and incubated for four to six hours at 35°C under 5% CO_2. Cells were washed from the filter into sterile phosphate buffered saline (PBS) and centrifuged. The pelleted cells were resuspended in 0.5 ml PBS and plated on BHIV plates containing 125 µg/ml of kanamycin. After incubation for 24-48 hours at 35°C under 5% CO_2, colonies were selected from filter mating plates and screened by PCR and Southern blot for transconjugants.

Southern blotting. Genomic DNA was digested to completion using various restriction enzymes and separated on a 0.8% agarose gel. DNA fragments were transferred to a Nytran (Schleicher & Schuell, Keene, N.H.) membrane by the method of Southern (14). The membrane was hybridized with a digoxigenin-labeled probe generated by PCR amplification of the gene or gene fragment of interest (Roche PCR DIG synthesis Kit). For high stringency hybridization, blots were incubated for 16 hours at 42°C with the probe diluted in a hybridization buffer that contained 50% formamide, 5 X SSC (sodium chloride, sodium citrate), and 2% blocking reagent (Roche). High stringency washes were performed at 68°C with 0.1 X SSC, 0.1% SDS (sodium dodecyl sulfate).

Hybridizing bands were detected using alkaline-phosphatase tagged anti-digoxigenin and the CDP* Chemiluminescent substrate (Roche).

Cloning of the intact *ilvIH* and *ohr* genes. The *ohr* promoter and 291 nt of coding region was encoded in the insert of piviK. The downstream sequence of *ohr* was cloned by using inverse polymerase chain reaction (iPCR) and is described in Chapter 3 of this dissertation. The promoter and 243 nt of coding sequence of ilvI was encoded by pivil. The remainder of *ilvIH*, that which was not contained in ivil, was cloned through the use of degenerate primers based upon IlvIH sequence from other prokaryotes and the *A. pleuropneumoniae* codon usage table. Primer MM219 (5'-CAGHGCRC CRCCNCGWACTTGCATYGG-3') was designed based upon a C-terminus region that was highly conserved among various bacteria, including *Haemophilus influenzae, Pasteurella multocida, Pseudomonas aeruginosa, Escherichia coli*, and *Buchnera* sp. APS. Primer MM219, used in conjunction with primer MM197 (5'-GGACGGTTAAGCTTGCTCAACCGG-3'), was based upon known *A. pleuropneumoniae ilvI* sequence generated from the *in vivo* induced clone containing the 5' end of *ilvI* (pivil). These primers were used in a PCR reaction to generate a 1.5 kb fragment containing the entire *ilvI* gene, lacking the last approximately 30 nucleotides. This fragment was cloned into pUC18 to form pIdeg.

To obtain the sequence of the C-terminus of IlvI, degenerate primers based upon IlvH N-terminus sequence were constructed using IlvH sequence from the same bacteria as were used to design the MM219 *ilvI* primer. Primer MM324 (5'-GGGYTTRTGYA AYTGYTTYTCRATYTGYTCGGG-3') was used to PCR amplify the 3' end of *ilvI* in

conjunction with a primer internal to *ilvI* MM290 (5'-CCCGGATCCGG CTAATATTCCGATTGTGG-3'). The resulting PCR fragment of approximately 800 bp was cloned into pUC18 to generate pILVHdeg. This clone was then sequenced to determine the sequence of the 3' end of the *ilvI* gene.

Construction of ohr-kanamycin suicide vector. The construction of the *ohr*-kanamycin suicide vector is outlined in Figure 4-1. Primers (MM150: 5'-GACAAGAATTCAACAAGGACAATATTATG-3' and MM151: 5'-CCTAAATCG TCCCAGATCTGGTAGG-3') were designed from known *A. pleuropneumoniae ohr* sequence in order to amplify the entire coding region. This fragment, which included the ribosome binding site and the start codon, was cloned into pUC19 to generate pRS91. This plasmid was then digested with *EcoRV* to insert the kanamycin cassette from pUC4K. The resulting plasmid was designated pRS93a, with the kanamycin gene in opposite orientation to *ohr*, or pRS93b, with the kanamycin gene in same orientation as *ohr*. The *ohr*-kanamycin fragment from *Eco*RI and *Xba*I digested pRS93b was then inserted into pGP704 that had been digested with *Eco*RI and *Xba*I to form pRS94b. Plasmids pRS93a, pRS93b, and pRS94b were used during attempts to generate double recombinants using electroporation. Plasmid pRS94b was used for matings.

Additional constructs of *ohr*-kanamycin were made by digesting pRS91 with *Ava*I and *Xba*I to generate an *ohr* clone (pRS91C) that lacked the ribosome binding site and the start codon. This plasmid was then used to insert a blunt-ended kanamycin gene to form pRS93KanF and pRS93KanR, based upon the kanamycin gene in the same

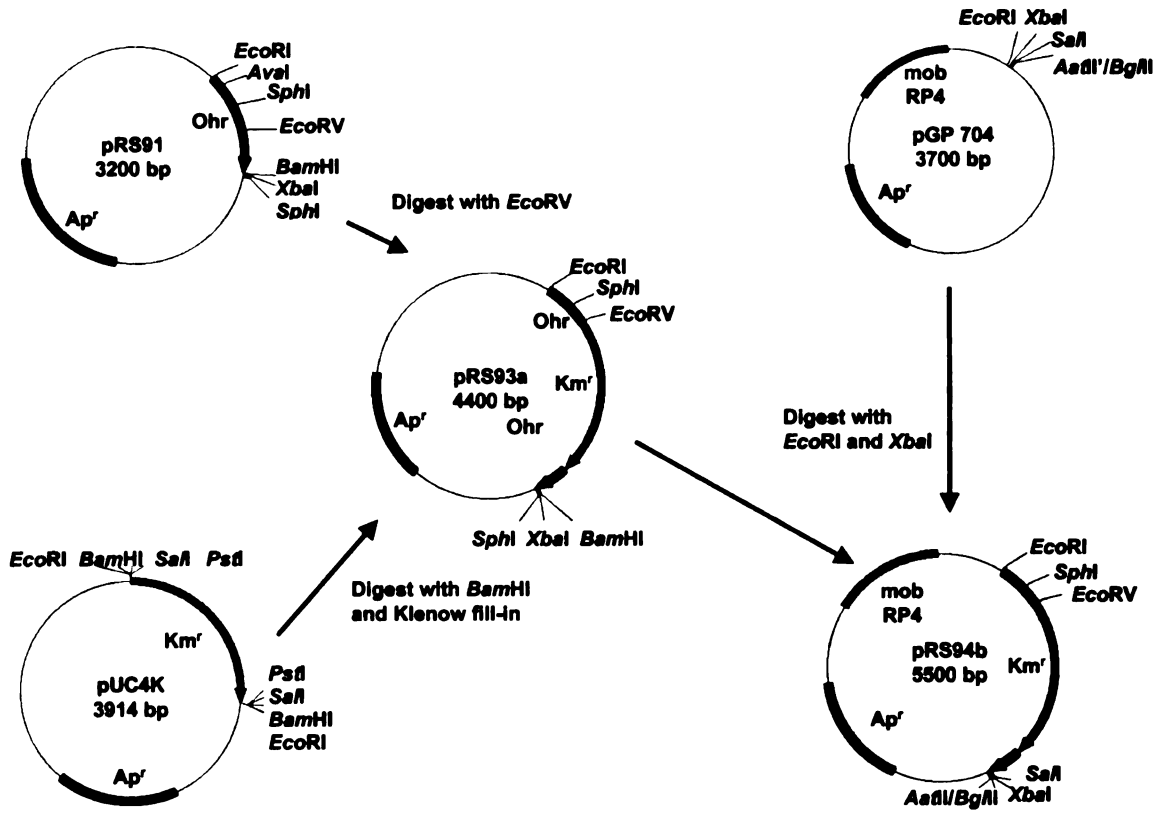

Figure 4-1. Construction of *ohr*-kanamycin suicide vector. The kanamycin resistance gene was cloned into the middle of the *ohr* gene. This was then inserted into the suicide vector, pGP704.

orientation and opposite orientation to ohr, respectively. pRS93KanF was then digested with EcoRI and XbaI to insert into pGP704 to form pRS94KanF.

Construction of ilvI-kanamycin suicide vector. The construction of a plasmid in whichan internal coding region of 0.3 kb of *ilvI* had been replaced with the kanamycin gene is detailed in Figure 4-2. This clone was constructed by PCR amplifying the 3' end of *ilvI* from *A. pleuropneumoniae* genomic DNA using primers MM290 and MM219. This 0.7 kb fragment was inserted into *Sma*I digested pUC18 to form pilvI3'. Next the 5' end of ilvI was PCR amplified using primers MM286 (5'-CGTTGGAGGATTGCATG

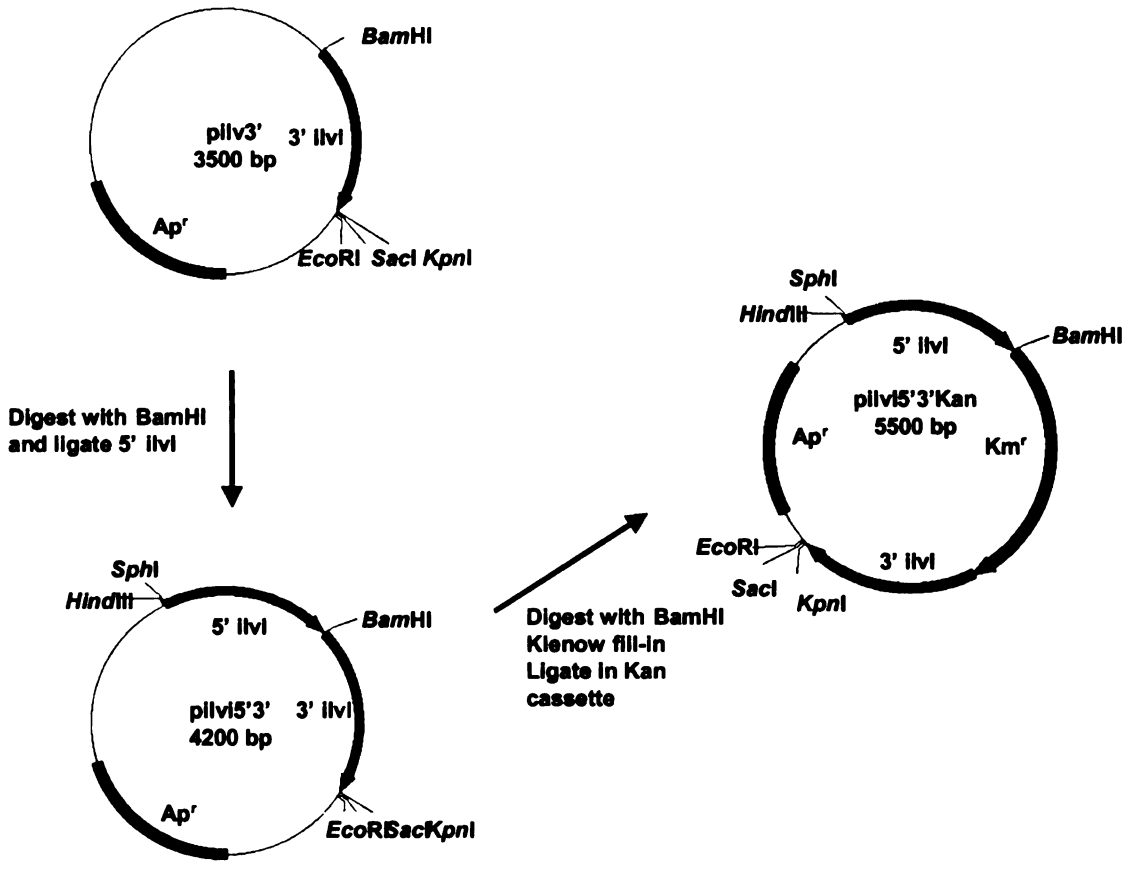

Figure 4-2. Construction of pilvI5'3'Kan. This construct contained the 5' end and the 3' end of ilvI and had the kanamycin resistance gene inserted in place of an internal 300 bp.

CAAAAACTTTCCG-3') and MM288 (5'-GAGAGGATCCCTCCACCAATGTAT AAAACCG -3'). This 0.7 kb fragment was then digested with *Bam*HI and *Sph*I and inserted into *Bam*HI and *Sph*I digested pilvI3' to generate pilvI5'3'. The kanamycin cassette, from *Bam*HI digested pUC4K was then cloned into the *Bam*HI restriction site of pilvI5'3' to form pilvI5'3Kan.

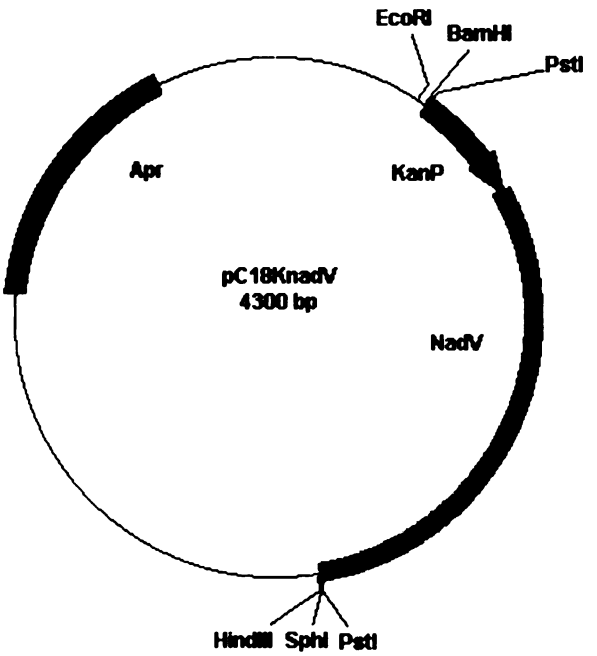

Figure 4-3. Plasmid map of pC18KnadV. This construct contained a promoter upstream of the *nadV* gene that allowed for *nadV* expression independent of orientation within the vector.

Construction of *nadV*-kanamycin cassette. From previous studies, it was shown that the expression of the *nadV* gene was dependent upon the orientation of the gene in the shuttle vector (5). This suggests that the expression of *nadV* was from a promoter within the vector rather than its own promoter. The promoter from the kanamycin cassette from the pUC4K vector was cloned in front of the *nadV* gene to allow for expression of the gene independent of orientation (Figure 4-3).

The promoter region of the kanamycin gene was cloned by PCR amplification using primers MM185 (5'-GTGAGCGGATAACAATTTAAAACAGG-3') and MM207 (5'-CCCGTTGAATATGGCCCATGGCACCCCTTG -3'). Primer MM185 was located

upstream of the nested restriction sites and primer MM207, which contained a *Nco*I restriction site, was centered over the start codon of the open reading frame of this gene. This PCR product was then digested with *Pst*I and *Nco*I and inserted upstream of the *nadV* gene. The *nadV* gene was prepared by PCR amplification with primers MM208 (5'-CGACCATGGATAACCTATTAAATTATAGTAGTCG-3') and MM191 (5'-GCGTATTAACTGCAGAT ATCATAGCGTAGTGCG-3'). MM208 was designed to incorporate a *Nco*I site centered over the start codon and MM191 allowed retention of a *Pst*I site immediately downstream of the stop codon. The *nadV* PCR product was digested with *Nco*I. A three way ligation consisting of the Kan promoter region, the *nadV* gene, and *Pst*I digested pUC18 vector allowed for preparation of the nadV gene downstream of a constitutively expressed promoter. This plasmid was designated pC18KnadV (Figure 4-3). The 1.6 kb insert from pC18KnadV was cloned into both pGZRS18 and pGZRS19 to form GZ18KnadV and pGZ19KnadV, respectively. To provide a double selection cassette, the kanamycin cassette from pUC4K was cloned into the pC18KnadV plasmid. The kanamycin resistance gene was isolated from BamHI digested pUC4K and cloned into BamHI digested pC18KnadV to form pC18KanNad (Figure 4-4).

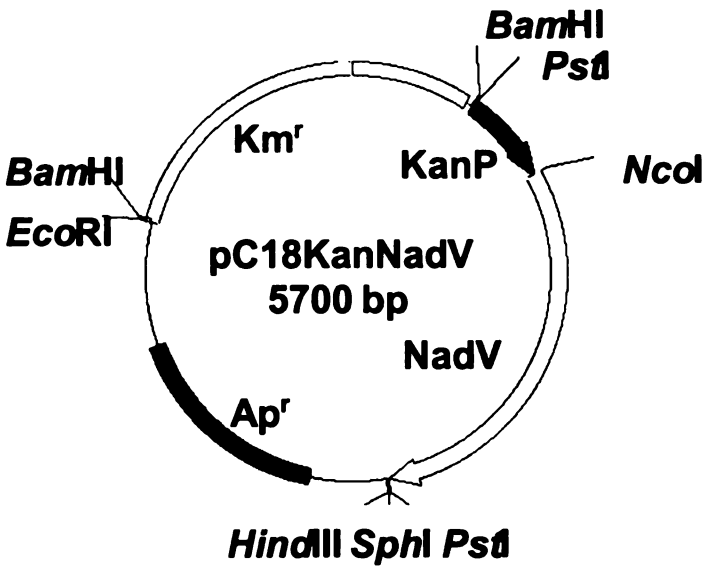

Figure 4-4. Plasmid map of pC18KanNadV. This plasmid contained the double selection cassette of a kanamycin resistance gene and the *nadV* gene allowing for NAD independence. This 2.9 kb insert can be used as a selectable marker in the construction of mutants.

Construction of *ilvI-nadV*-kanamycin suicide vector. The construction of this clone is detailed in Figure 4-5. The pilvI5'3' construct was digested with *Bam*HI and the single stranded ends were filled in using Klenow polymerase to form blunt ends. The *nadV*-Kan^R cassette was PCR amplified from pC18KanNad using the pUC18 forward and reverse primers. The *nadV*-Kan^R cassette was ligated into the center of pilvI5'3', replacing the 0.3 kb internal fragment of ilvI, to form pC18ilvKanNad.

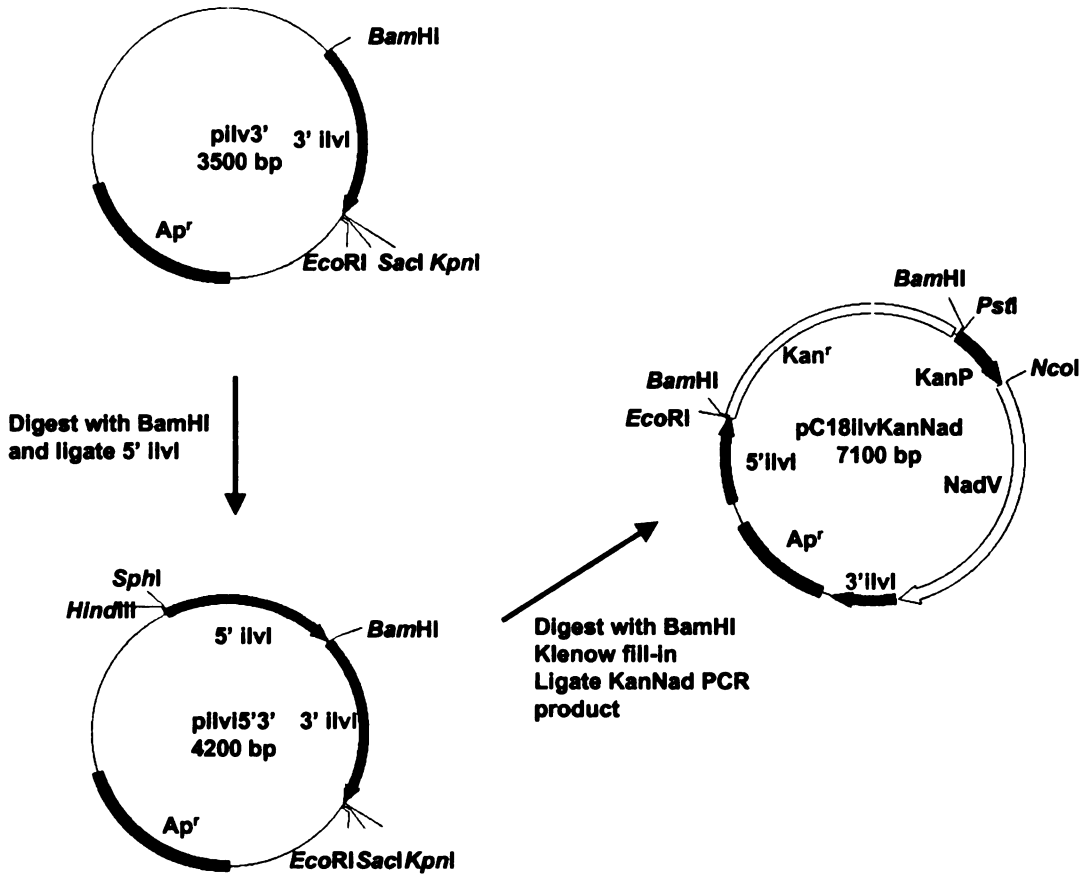

Figure 4-5. Construction of pC18ilvKanNad. This vector was identical to pilv5'3'Kan, but with a double selection (kanamycin resistance and NAD independence) used in place of the selectable kanamycin cassette.

Results and Discussion

Attempts to construct an *ohr-* mutant. Attempts were made to construct a double recombinant *A. pleuropneumoniae ohr-* mutant. These involved mating AP225 with varied concentrations of the donor S17-1 (λ pir)/pRS94b. The mating procedure had already been optimized using *aopA* (9). Fifteen attempts at mating were performed and a total of 250 transconjugates were isolated on BHIV with 125 μg/ml kanamycin. 75% of these were wild type and lacked the kanamycin gene, as determined by lack of a PCR fragment when primers specific to the kanamycin gene were used, and had only one wild type copy of the *ohr* gene, as shown by a 500 bp product when *ohr* specific primers were used for PCR. The remainder of the transconjugates were single crossovers containing two copies of *ohr*, the kanamycin gene, and the pGP704 vector. These single crossovers were confirmed by Southern analysis with a pGP704 specific probe. In addition, many of these single crossovers contained intact pGP704 plasmid that could be isolated with a genomic DNA isolation from the transconjugate and transformed into fresh S17-1 (λpir) to confer kanamycin resistance.

Since the attempts at mating were unsuccessful, methods of DNA uptake by electroporation were tried. Plasmid (pRS93a, pRS93b, and pRS94b) was isolated from *E. coli* and purified using Qiagen columns followed by phenol-chloroform cleanup and ethanol precipitation. A total of 3 electroporations were performed and 80 colonies resulted. Of these 80 colonies, 90% lacked the kanamycin gene, as determined by lack of a PCR fragment when primers specific to the kanamycin gene were used, and had only one wild type copy of the *ohr* gene, as shown by a 500 bp product when *ohr* specific

primers were used for PCR. Six colonies were single crossovers as determined by Southern. No double recombinants were found by Southern analysis.

Since the initial *ohr* constructs contained sequence upstream of the start codon, it was thought that the generation of two functional copies, the wild type and the copy from the suicide vector which contained a ribosome binding site and start codon, might be the cause for failure to obtain double crossovers. Therefore, pRS91C and its derivatives were constructed. These plasmids lacked the first 60 nucleotides, including the ribosome binding site and start codon. Six matings with these constructs resulted in the generation of 14 wild type colonies and 1 single crossover.

At the time these experiments were being performed, a functional assay for the presence of Ohr was not yet in place. The subsequent development of the iron-xylenol orange assay (15) and Chapter 3) might allow for evaluation of the single crossovers for stability of the mutation and attenuation of the Ohr protein. While the disk inhibition assay (Chapter 3) did not allow for differentiation between single crossovers and wildtype cells, the xylenol orange assay might be more consistent and sensitive.

The lack of double crossovers may have resulted from either the essential nature of Ohr or a conditional requirement for Ohr in the presence of oxygen radicals. Pretreating all media by incubating the media with peroxidases might decrease the oxidative stress and allow for propagation of stable double crossovers that were selected against due to the presence of reactive oxygen species and the requirement for enzymes capable of detoxifying oxygen radicals. Another recommendation is that all experiments be conducted anaerobically to decrease the production of oxygen radicals, such that the requirement for detoxifying enzymes are reduced. The final possibility for the lack of

double crossovers is that Ohr is an essential enzyme in *A. pleuropneumoniae*. If Ohr is an essential enzyme, this will be unique among bacteria, since Ohr has been successfully disrupted and mutated via homologous recombination in *Xanthomonas campestris* and *Bacillus subtilis* (8, 11).

Since it was impossible to differentiate between the inability to construct a knockout due to technical difficulties and that due to the essential nature of the gene, an attempt to perfect the mating and electroporation protocols via construction of a knockout of a different gene was performed. For this, an *A. pleuropneumoniae ilvI-* mutant was attempted.

Attempts to construct an *ilvI-* mutant using a kanamycin cassette. Construction of an *ilvI-* mutant was attempted, since it was believed that the conditions for mutant construction, i.e. the requirement for supplementation of isoleucine, leucine, and valine, were known. In addition, it was believed that the larger size and the ability to construct a mutant that had 600-800 bp of sequence flanking the kanamycin cassette would facilitate insertion into the chromosome. Thus, an *ilvI* cassette was prepared for double recombination into the chromosome. This cassette contained *ilvI* sequence in which an internal 300 bp fragment had been removed and the kanamycin resistance gene had been inserted in its place. This construct was electroporated into the *A. pleuropneumoniae* serotype 1 recipient strain. From four electroporations, 104 colonies resulted. Of these, 80 % were wild type and the remainder were single crossovers. Since double crossovers were unable to be generated and all of the transformants were spontaneously kanamycin resistant, the need for a better selectable marker became obvious. It was decided that a

nonantibiotic resistance marker might decrease the percentage of wild type colonies resulting from matings or electroporations. Thus, a gene that would confer NAD independence was a logical choice for replacement of the kanamycin resistance gene.

Construction of a *nadV* expression cassette. From previous studies , it was shown that the *Haemophilus ducreyi nadV* gene apparently lacked a promoter that functioned *in A. pleuropneumoniae* (5). Therefore, to construct a *nadV* expression cassette, a promoter that functions in *A. pleuropneumoniae* was needed upstream to allow expression of the nadV promoter independent of orientation within the vector.

The pUC4K vector contains a kanamycin expression cassette that has previously been shown to be constitutively expressed in *A. pleuropneumoniae* independent of orientation in the vector. The promoter for the kanamycin resistance gene when cloned in front of the nadV gene, allowed for expression independent of orientation within the vector. When either pGZ18KnadV or pGZ19KnadV were electroporated into *A. pleuropneumoniae* serotype 1, both allowed for growth on BHI agar in the absence of exogenous NAD. These results confirm that *nadV* was expressed from the kanamycin promoter, which is functional in *A. pleuropneumoniae.*

Attempts to construct an *ilvI-* mutant using a kanamycin-*nadV* cassette. The plasmid pC18ilvKanNad was purified from *E. coli* and used to electroporate competent *A. pleuropneumoniae* serotype 1. Transformants were plated on BHI without NAD but containing isoleucine, leucine, and valine (BHI+ILV), in addition to BHI+ILV with kanamycin (100 μg/ml). No growth was seen on plates containing kanamycin,

suggesting too rigorous a selection process. After 48 hours, 52 transformants from BHI+ILV were transferred to BHI+ILV containing kanamycin (100 μg/ ml). Over 96% of the colonies that grew on BHI also grew on BHI with kanamycin.

Colonies were subcultured onto BHI plates lacking either NAD or ILV. Four colonies were selected that failed to grow in the absence of exogenous ILV. Southern analysis of these colonies showed that they were single crossovers. Neither double crossovers nor wildtype were identified in this experiment. These results demonstrate that the *nadV* gene can be expressed efficiently in a single copy in the bacterial chromosome and that the NAD independence phenotype conferred by the presence of *nadV* can be used successfully to select mutants in *A. pleuropneumoniae.*

This experiment did not yield any recombinants that were double crossovers. There are several changes that can be made to improve the yield for double recombinants. Additional attempts at repeating the electroporation might yield more transformants and if a double crossover is a rare event, these additional recombinants might be required to be screened prior to discovery of a double crossover. An additional attempt at isolating plasmid DNA for electroporation that was digested such that the DNA contained only the insert without the vector backbone, thereby preventing insertion of the plasmid into the chromosome might yield double crossovers. Additionally, higher concentrations of isoleucine, leucine, or valine, might be required for supplementation of the mutants.

Another tool that is required is a functional screen for lack of AHASIII in the *A. pleuropneumoniae ilvI-* mutant. While assays exist for screening of AHAS isozymes in *E. coli* (4), none have been developed yet for use in *A. pleuropneumoniae.* The perfection of an AHASIII assay in *A. pleuropneumoniae* will permit determination of the

stability of the single crossovers generated. If stable single crossovers exist and inhibit the production of IlvI, then it is possible that these single crossovers can be used for further studies.

The construction of double crossovers of *in vivo* induced genes has proven to be an elusive goal. The inability to construct these double crossovers may be due to the intractability of the system and *A. pleuropneumoniae* genetics, the essential nature of the gene, or to conditions for mutant survivability that have not yet been determined. As more mutants are constructed using the current system (1, 9) (personal communication, S. Doree), the system will become more refined and easier to generate mutants. Additionally, as knowledge is gained about the function of these *in vivo* induced genes in *A. pleuropneumoniae*, the conditions for mutant construction will become more obvious.

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Chapter 5

Summary

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, an economically devastating disease that results in high morbidity and mortality in naïve herds. This bacterium was first identified in 1961 and the agent classified as *Haemophilus pleuropneumoniae* (5, 7). In the thirty-five years following the discovery of this pathogenic bacteria, the study of *H. pleuropneumoniae* has led to its reclassification as *A. pleuropneumoniae* and identification of multiple virulence factors associated with disease (3). These factors include capsule, lipopolysaccharide, exotoxins, iron acquisition proteins, and many metabolic enzymes (riboflavin biosynthesis, two superoxide dismutases, urease genes, amino acid and purine biosynthesis genes).

However, a genetic system for this bacterium was lacking and a genome wide surveillance of virulence factors was not able to be performed. It was only in 1992 that a plasmid shuttle vector was constructed that facilitated horizontal transfer of DNA from *Escherichia coli* to *A. pleuropneumoniae* (1, 8, 9). A protocol for construction of genetically defined mutants of *A. pleuropneumoniae* was developed in 1995 (4). With these tools, it was possible to develop an IVET (*in vivo* expression technology) system that would allow a genome wide study of virulence factors.

This IVET system required an attenuated mutant, an expression vector that contained genes to complement this mutation and genes to evaluate the degree of expression, and an animal infection model. The first experiment using this IVET system occurred in 1996 and the system was perfected within two years (2). Over 20,000 clones have been constructed and used to infect pigs. From these 20,000 clones, 42 unique ivi clones have been identified and are presented in Table 1. This dissertation focused on 25 of these clones that were discussed in Chapter 2. The 25 clones contain *in vivo* induced

Table 5-1. Identification of 42 *ivi* clones

Clone	In vivo induced gene	Protein function	Clone	In vivo induced gene	Protein function
iviA	*mrp*	pyrimidine biosynthesis	ivi17B	*texA*	toxin expression
iviB	hypothetical ORF	NA	ivi17C	*exoII*	glucosaminadase
iviC	hypothetical ORF	NA	ivi17D	*selA*	selenocysteine synthase
iviD	*gufA*	hypothetical protein	ivi17E	*folD*	folate synthesis
iviE	*ybjY*	hypothetical protein	ivi17F	none identified	NA
iviF	*secE-nusG*	secretion-transcription	ivi17G	*coaE*	coenzymeA synthesis
iviG	none identified	NA	ivi19A	*sodC*	superoxide dismutase
iviH	none identified	NA	ivi19B	*tehB*	tellurite resistance
iviI	*ilvIH*	amino acid biosynthesis	ivi20A	*glpC*	electron transport
iviJ	antisense *prfC*	protein translation	ivi20B	antisense *recJ*	exonuclease
iviK	*ohr*	organic hydroperoxidase	ivi20C	*trnS*	transport protein
iviN	hypothetical ORF	NA	ivi21A	hypothetical ORF	NA
iviP	*ampD*	amidase	ivi21B	*trkH*	potassium uptake
iviR	*znuA*	zinc transport	ivi23A	hypothetical ORF	NA
iviS	*hfq*	RNA binding protein	ivi31A	*hemN*	coprogen oxidase
iviT	hypothetical ORF	NA	ivi31D	hypothetical ORF	NA
iviU	hypothetical ORF	NA	ivi31E	hypothetical ORF	NA
iviW	*yfiC*	hypothetical protein	ivi41A	*yihD*	hypothetical protein
iviX	*vapBC*	plasmid maintenance	ivi41B	*lexA*	SOS response repressor
iviY	*ftsY*	cell division/secretion	ivi41C	hypothetical ORF	NA
ivi17A	*pyrG*	CTP synthase	ivi41D	hypothetical ORF	NA

genes identified can be classified into four categories: known virulence genes and regulators of these genes, previously identified genes that have not been previously identified as virulence factors, genes encoding highly conserved hypothetical proteins that are found in the genomes of many pathogens, and previously unidentified genes. Within this group of 25, known virulence factors such as SodC, TehB, FtsY Tex, and VapBC have been identified. These proteins help *A. pleuropneumoniae* escape oxidative stress (SodC), export toxic molecules and proteins (TehB and FtsY), and regulate virulence genes (TexA and VapBC).

Previously identified metabolic genes such as *folD, ilvI, ohr, ampD, exoII, coaE,* and *selA* have taken on a role as virulence factors that are required for growth and pathogenesis during infection. The identification of these genes as *in vivo* induced genes have allowed knowledge about the host response to infection by *A. pleuropneumoniae.* The increased expression of *ilvI, folD,* and *coaE* suggest that nutrient limitation occurs during infection and requires a subsequent increase in enzyme biosynthesis to supply necessary amino acids, nucleotide precursors, and enzymatic cofactors. The expression of *ampD, exoII,* and *selA* suggests that infection of the porcine host requires modification of proteins and cell walls in order to escape immune detection.

In addition to these *ivi* clones that contain proteins known to function as virulence factors or metabolic survival proteins, several clones contain hypothetical ORFs or proteins that have been previously identified, but not yet characterized as virulence factors. The identification of *ohr* as an *in vivo* induced gene and as a prominent virulence factor is a key contribution to the scientific literature (6). This gene has been shown to be induced in response to organic peroxides alone and not other oxygen radicals. In

addition, the gene is found only in three of the twelve serotypes, and it has been shown that *ohr* is regulated by a serotype specific regulator that is not found in *A. pleuropneumoniae* serotype 5, which lacks *ohr*. The identification of *ohr* as an *in vivo* induced gene suggests that in order to escape the destructive potential of the swine immune cells, proteins such as Ohr and SodC are required to detoxify the oxygen radicals. The rapid immune destruction of both bacteria and host tissue by the swine immune system is a key component to porcine pleuropneumonia. In order to survive this dangerous environment, *A. pleuropneumoniae* requires proteins that are unique and have not been previously identified as virulence factors in many of the enteric pathogens. The identification of *in vivo* induced genes in *A. pleuropneumoniae* will aid in the understanding of other respiratory pathogens.

The generation and characterization of these clones has led to new discoveries about *A. pleuropneumoniae*. It has also generated questions that were not able to be asked previously. These questions include why certain genes (iviG and iviK/*ohr*) are only found in *A. pleuropneumoniae* serotype 1 and not in *A. pleuropneumoniae* serotype 5, for which a whole genome sequence has recently become available. Is it possible that one or more pathogenecity islands exist that contain these genes? If so, from whence did these islands arise? Why are they only in certain serotypes and how does the presence of these genes correlate with pathogenesis? Perhaps some of these questions can be answered as more microbial genomes are sequenced and the sequencing of *A. pleuropneumoniae* serotype 1 is completed.

Another set of questions centers around the regulation of these virulence genes. Are sensors present that detect the host immune response and coordinately regulate

groups of genes? What previously unknown regulons will be identified using this system? Can these *in vivo* induced clones be clustered into regulons? If so, what regulator is present? Perhaps these questions can be addressed through the development and use of *A. pleuropneumoniae* genome chips and mimicking of conditions that occur during infection, such as exposure to hydrogen peroxide or superoxides or amino acid limitation.

The final set of questions that arise from these studies concern the significant number of genes that encode proteins of unknown function. What are the functions of these genes? Do they encode virulence factors that are specific to certain pathogens that share unique niches? What is the best way to address the function and classification of these genes? Will classifying by regulator or response to a subset of stressors help to identify the protein's role in virulence? If not, what is the best way to cohort these unknown proteins? Will the characterization of these genes lead to new antibiotic targets or insights into pathways common to multiple pathogens?

This dissertation was begun with the hope that by identifying and characterizing virulence genes, the disease of porcine pleuropneumonia might be abated as new, more effective vaccines were produced and new antibiotic targets were identified. Instead of simplifying this disease, the identification of *in vivo* induced genes, by the use of IVET, has instead presented more questions, which need to be addressed in order to develop more effective vaccines and identify new antibiotic targets. It is the hope that by raising these questions, the devastating disease of porcine pleuropneumonia might be further

studied with the eventual goal of reducing the morbidity and mortality associated with this disease.

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