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IDENTIFICATION AND REGULATION OF *ACTINOBACILLUS PLEUROPNEUMONIAE IN VIVO* INDUCED GENES THAT RESPOND TO BRANCHED-CHAIN AMINO ACID LIMITATION

By

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ABSTRACT

IDENTIFICATION AND REGULATION OF *ACTINOBACILLUS PLEUROPNEUMONIAE IN VIVO* INDUCED GENES THAT RESPOND TO BRANCHED-CHAIN AMINO ACID LIMITATION

By

Trevor Keith Wagner

Actinobacillus pleuropneumoniae is a Gram-negative bacterial pathogen that is the causative agent of a severe hemorrhagic pleuropneumonia in swine. The severe effect of the disease on the swine industry has led to extensive research on development of improved vaccines. To understand the genetic basis of this disease and to identify potential vaccine targets, an *in vivo* expression technology (IVET) system was developed for use in *A. pleuropneumoniae* serotype 1. The IVET system was designed to identify *A. pleuropneumoniae in vivo* induced (*ivi*) genes whose expression is specifically induced during infection of the natural swine host, without the need to identify each individual environmental cue necessary for expression of each gene.

However, to analyze the role of *ivi* genes, it is important to understand the specific cues that regulate expression of each *ivi* gene. It was hypothesized that a limitation of branched-chain amino acids (BCAAs) acts as a signal to induce expression of *ivi* genes in a manner similar to iron limitation. A pool of 32 previously isolated *ivi* promoter clones were analyzed for increased activity of a reporter under BCAA limiting conditions, and 8 *ivi* promoters were identified as up-regulated in this study. These data suggest that the limitation of BCAAs is an important cue in the regulation of *ivi* genes and potentially other virulence genes.

One mechanism known to regulate many *Escherichia coli* genes in response to BCAA limitation is leucine-responsive regulatory protein (Lrp). An *A. pleuropneumoniae* gene similar to Lrp was identified and cloned. Purified *A. pleuropneumoniae* His_6-Lrp bound *in vitro* to 2/8 *ivi* promoters identified to respond to BCAA limitation. A genetically-defined *A. pleuropneumoniae lrp* mutant was constructed and used to show the requirement for Lrp in the regulation of several *A. pleuropneumoniae* genes.

To further understand the role of Lrp in virulence, the *A. pleuropneumoniae lrp* mutant was analyzed in a swine model of respiratory infection. The *lrp* mutant was able to cause disease under the conditions tested, with progression of disease and pathology similar to that seen with wild-type *A. pleuropneumoniae*.

The identification of an environmental stimulus, a regulatory mechanism, and genes regulated by these factors is an important step for understanding the virulence of *A. pleuropneumoniae*. This research offers insight into new avenues of research to further examine the virulence of *A. pleuropneumoniae* and other respiratory pathogens.

Dedicated to:

Ashley Phelps Wagner

Oscar Wagner

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PREFACE

This thesis includes three chapters that are in manuscript format. The second chapter entitled "A subset of *Actinobacillus pleuropneumoniae in vivo* induced promoters respond to branched-chain amino acid limitation" is a full length version of a manuscript that has been submitted to the Federation of European Microbiological Societies (FEMS) Immunology and Medical Microbiology journal, with Dr. Martha H. Mulks as co-author. Chapter three entitled "Identification of *Actinobacillus pleuropneumoniae* leucine-responsive regulatory protein (Lrp) and its involvement in the regulation of *in vivo* induced genes" is a full length version of a manuscript that has been submitted to Infection and Immunity, with Dr. Martha H. Mulks as co-author. Chapter four entitled "A mutation in *Actinobacillus pleuropneumoniae lrp* is not avirulent in a swine respiratory model of infection" will not be submitted at this time, but will provide the basis for a manuscript submitted in the future, with Dr. Martha H. Mulks, Dr. Matti Kiupel, and Dr. Roy Kirkwood as co-authors. As co-authors, Dr. Matti Kiupel provided interpretation of gross and histopathology, while Dr. Roy Kirkwood provided veterinary care and clinical evaluation.

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Actinobacillus pleuropneumoniae: A bacterial swine pathogen causing a deadly hemorrhagic pneumonia and the genetic regulators and host stimuli that mediate virulence gene expression.

Introduction

Actinobacillus pleuropneumoniae is a Gram-negative coccobacillus in the family *Pasteurellaceae* and is the causative agent of porcine contagious pleuropneumonia in swine. *A. pleuropneumoniae* has been identified throughout the world and its efficacy at causing transmittable hemorrhagic pleuropneumonia among swine herds had a significant economic impact to the swine industry.

This chapter reviews the epidemiology, relevance, and pathology of the disease caused by *A. pleuropneumoniae*, while also discussing methods of identification, prevention, treatment, and control of the disease. The major virulence factors of *A. pleuropneumoniae*, as well as the identified environmental stimuli and regulators of virulence gene expression, are discussed. The possibility of the limitation of branched-chain amino acids as a stimulus to induce virulence gene expression and the role of leucine-responsive regulatory protein in regulating that response is hypothesized.

The scope of this thesis is to investigate the limitation of branched-chain amino acids as a cue for *A. pleuropneumoniae* virulence gene expression and to study the role of an *A. pleuropneumoniae* regulator of gene expression to mediate this response. By identifying stimuli and regulators of virulence gene expression, a better genetic understanding of the virulence factors and the conditions under which *A. pleuropneumoniae* expresses them to cause disease will be obtained.

Epidemiology and relevance

The first report of what is now known as an *Actinobacillus pleuropneumoniae* infection occurred in England in 1957 by Pattison et al. (247). In the following 7 years, additional reports by Olander (242) and Shope (288) described a porcine contagious pleuropneumonia from California and Argentina that could result in death in as little as 24 hours from an acute hemorrhagic pleuropneumonia. It has been almost half a century since the first report of *A. pleuropneumoniae* and cases of disease due to this pleomorphic Gram-negative coccobacillus have been identified on all the continents of the world except Antarctica (283, 333). The increased identification of *A. pleuropneumoniae* throughout the world is most likely due to the increasing industrialization of the pork industry.

While *A. pleuropneumoniae* has been identified in many parts of the world, specific serotypes prevail in certain regions (283). The 15 serotypes identified to date are based on differences in the capsular polysaccharide and lipopolysaccharide of each strain (34, 236, 307). The differences in serotype also correlate to differences in the pathogenicity of *A. pleuropneumoniae* with serotypes 1, 5, 7, 9, 10, and 11 being considered to be more virulent than the others (307). Three of these serotypes, 1, 5, and 7, are the most common in the United States (90).

The spread of any given serotype within a geographic region or farm has most likely been limited to direct contact between pigs (288), although recent studies have shown *A. pleuropneumoniae* to be transmitted short distances in the air (171, 312). *A. pleuropneumoniae* does not survive long outside of its only known host, the pig, and

therefore transmission of *A. pleuropneumoniae* by insect, human, or farm vehicle vectors is thought to be minimal to non-existent (90). However, the limited host specificity of *A. pleuropneumoniae* does not decrease its ability to spread. In 1995, almost 1 in 10 farms in the United States were reported to have had an *A. pleuropneumoniae* infection (198). Most of the spread of *A. pleuropneumoniae* has been attributed to the existence of carriers within the swine population, which show no clinical signs of infection but can nonetheless spread virulent *A. pleuropneumoniae* to susceptible pigs (90). These carriers can harbor *A. pleuropneumoniae* in lung lesions, tonsils, and/or nasal cavities (183).

It is not difficult to imagine how *A. pleuropneumoniae* could affect the profit margin of the swine industry. *A. pleuropneumoniae* has been implicated in a 4.6% decrease in pork output (199). Swine farms make money by selling pork meat by weight. The most obvious and most costly effect on a farm is mortality caused by *A. pleuropneumoniae* infection. The rapid transmission of *A. pleuropneumoniae* through a susceptible herd can be devastating. The other method by which *A. pleuropneumoniae* can affect profit is by both slowing down growth through decreased food intake by the pig and by increasing the amounts of food required by the swine to gain a unit of weight (feed conversion efficiency) (302, 303). In one study (265), swine infected subclinically with *A. pleuropneumoniae* gained less weight per day and on average took 5.64 days longer to reach target slaughter weight. However, two other studies by Desrosiers (78) and Hunneman (144) concluded *A. pleuropneumoniae* did not significantly affect weight gain or feed conversion efficiency. Even if weight gain or feed conversion efficiency is not a significant problem, *A. pleuropneumoniae* can result in additional costs for

diagnostic testing, antibiotics, vaccines, veterinarian bills, and the labor to administer medicine to an infected swine herd.

Detection, identification, and pathology of *A. pleuropneumoniae*

Techniques for detection and identification of *A. pleuropneumoniae* have been developed since *A. pleuropneumoniae* was originally identified. There are characteristic signs of infection and disease that can be observed to aid in the diagnosis of *A. pleuropneumoniae* infections. In addition, recent developments in molecular genetics have broadened the ability to detect and identify *A. pleuropneumoniae*. This section will describe the detection, identification, and pathology of *A. pleuropneumoniae* and the resulting disease.

Detection and identification. Analysis of pig sera is one method that has been used to detect *A. pleuropneumoniae* in a swine herd. These serological tests determine whether or not antibodies specific to *A. pleuropneumoniae* are present within the sera of pigs. If specific antibodies are present, pigs testing positive were either previously infected or are currently infected with *A. pleuropneumoniae*. To determine if *A. pleuropneumoniae* is currently within a swine herd, detection by bacteriological culturing can be performed. Samples collected from clean sites in the pig with minimal to no normal flora such as swine lung biopsy samples, can be cultured on a non-selective medium to allow for *A. pleuropneumoniae* growth. In contrast, samples taken from sites colonized with extensive normal flora sites such as the tonsils, the nasal cavity, and the oral cavity are routinely cultured in a medium supplemented with bacitracin to select against the growth of normal flora.

Identification of *A. pleuropneumoniae* can be performed using biochemical tests and serotyping on an isolated bacterium. The biochemical characteristics differentiating *A. pleuropneumoniae* from other bacteria in the upper respiratory tract of swine are that *A. pleuropneumoniae* is urease positive, indole negative, requires nicotinamide adenine dinucleotide (NAD), and ferments mannitol but not melibiose, raffinose, or sorbitol (178, 222). In addition, *A. pleuropneumoniae* is also one of the organisms that shows the characteristic CAMP effect, named after the authors who described it (63), which is a synergistic increase in the hemolytic zone of *A. pleuropneumoniae* colonies when grown next to a β-hemolytic organism such as *Staphylococcus aureus* (178). Furthermore, while the requirement for NAD is diagnostic for most *A. pleuropneumoniae* in North America, there is an NAD-independent biovar (253). Identification of *A. pleuropneumoniae* and its specific serotype can further be achieved by using serotype specific antibodies against an isolated strain with fluorescent antibody assays, coagglutination assays, latex bead agglutination assays, complement fixation assays, or enzyme-linked immunosorbent assays (ELISA).

Recent advances in molecular biology have allowed for the advent of genetic based tests to not only detect if *A. pleuropneumoniae* is present but to also identify the specific serotype. These tests involve polymerase chain reaction (PCR). A plethora of PCR assays have been developed to target *A. pleuropneumoniae* specific genes for purpose of detection or *A. pleuropneumoniae* serotype specific genes for the purpose of identification. More specifically, PCR methods have targeted *A. pleuropneumoniae* genes or combination of genes such as the Apx toxin genes (70, 92, 120, 257, 272, 297), the capsule polysaccharide biosynthetic and export genes (92, 145, 180, 277), the *A.*

pleuropneumoniae outer membrane protein gene, *omlA* (8, 92, 119, 120, 271), the aromatic amino acid biosynthesis gene, *aroA* (92, 133), the disulfide bond formation protein gene, *dsbE* (59, 92), and the transferrin-binding proteins encoded by *tbpA* and *tbpB* (74). Due to the sensitivity of PCR, *A. pleuropneumoniae* can be detected from a variety of sites that were difficult to screen by earlier techniques. Some studies have specifically investigated *A. pleuropneumoniae* detection in the tonsils (59, 92, 120, 121), while others have investigated if *A. pleuropneumoniae* can be detected in the air (271). Detection of *A. pleuropneumoniae* by PCR has become increasingly routine that commercial kits are now available (Adiavet App PCR test; Adiagene, St. Brieuc, France). PCR has truly brought *A. pleuropneumoniae* detection and identification to a new level. However, due to time, cost, and technical issues, PCR methods may not be best suited for the average pig farmer. In this case, aforementioned older techniques or analysis of clinical signs and symptoms may be more useful.

Pathology. *A. pleuropneumoniae* can cause a peracute, acute, subacute, or chronic disease within pigs depending upon the infecting serotype and a variety of other factors such as age, immune status, climate, and the amount or type of exposure the pig had to the pathogen (288, 289, 307). While some infected pigs can be asymptomatic carriers, most infected animals show characteristic clinical signs and symptoms of infection. In general, clinical symptoms can include high fever, labored or rapid breathing, depression, apathy, increased pulse rate, cyanosis, dyspnea, ataxia, coughing, decreased weight gain, and anorexia (283, 288, 289, 307). In the most severe cases, death can occur within 24 to 36 hours from the first sign of infection and extreme hemorrhaging can lead to blood

filled mucous foaming at both the mouth and nostrils (288). In contrast, less severe cases can survive and become healthy with little to no clinical signs.

The pathology of the lung is quite distinct in diseased pigs. Necrosis and hemorrhage are clearly present within the lungs, and focal lung lesions are dark and firm to the touch (288, 307). Both the left and right lungs can be bilaterally infected, although, one lung can show more severe pathology than the other. Unconsolidated areas of the lungs are congested and edematous. Attachment of the lungs to the parietal pleura is characteristic in severe disease and results from fibrinous pleurisy (288, 307). Pathology is mainly limited to the lungs and lymph nodes. The mediastinal and mesenteric lymph nodes are congested and swollen (288). Within the lungs, blood vessels are dilated, bronchial walls are partially detached, fibrinous clots are present, and edema is widespread (288, 307). Neutrophil infiltration is observed and thought to be due to the response of the host immune system to Gram-negative bacterial endotoxin. Histopathology of infected lungs shows dead and streaming neutrophils surrounding local sites of infection with characteristic signs of necrosis in the host tissue.

Prevention, treatment, and control

A. pleuropneumoniae is a pervasive pathogen throughout the world causing morbidity, mortality, and economic hardship for the pork industry. However, there are ways to prevent, treat, and control *A. pleuropneumoniae* disease through use of vaccines, antibiotics, and strict herd management routines.

Vaccines. Vaccines have been used to prevent infection since the late 1800's when Pasteur and Jenner produced vaccines to prevent chicken cholera and smallpox, respectively. After the discovery of *A. pleuropneumoniae* in Argentinean swine (288), it was shown that a subcutaneous injection of *A. pleuropneumoniae* into swine would provide protection against intranasal challenges of *A. pleuropneumoniae* (289). The first *A. pleuropneumoniae* vaccines were bacterins. These vaccines contained whole killed *A. pleuropneumoniae* to illicit a host immune response and build host antibodies against *A. pleuropneumoniae*. While these vaccines were able to decrease mortality rates of swine infected with a homologous serotype, they offered little to no protection against heterologous serotypes (234, 235). The reason for serotype specific protection is probably because killed bacterins do not produce the secreted or surface proteins that one would observe with live virulent bacteria during an infection. To address this problem, bacterial cultures were grown in special conditions, such as iron limitation, to induce expression of immunogenic secreted proteins. One example of this is a study by van Overbeke et al. (330) in which they examined bacterins of *A. pleuropneumoniae* prepared from cultures subjected to NAD restriction and NAD non-restricted conditions. They

showed pigs vaccinated with bacterins prepared with *A. pleuropneumoniae* grown in NAD restricted conditions were better protected. This suggested there were certain *A. pleuropneumoniae* proteins produced in one condition and not in the other. The next generation of vaccines, subunit vaccines, tried to specifically use *A. pleuropneumoniae* products that were either expressed during infection and not specific to one serotype. These subunit vaccines confer better protection against multiple serotypes of *A. pleuropneumoniae* (127).

Many of the initial subunit vaccine studies included the identified *A. pleuropneumoniae* toxins, under the theory that this would allow the vaccinated pig to develop opsonizing antibodies and therefore neutralize the toxins before damage resulted (79). These toxins, named ApxI, ApxII, ApxIII, and ApxIV, are produced by *A. pleuropneumoniae*, although not all serotypes produce the same combination and a given serotype does not produce all of them (5, 95). Subunit vaccines using ApxI, II, and III toxins provided immunity against all serotypes (325). In contrast, final bacterin vaccine preparations most likely do not contain the Apx toxins because they are secreted and are not part of the cells. Furthermore, Apx toxins would likely be degraded in a preparation because they are labile (79, 150). While subunit vaccines offered cross-protection, they only offered partial clinical protection (58). The Apx toxins are not the only proteins expressed by *A. pleuropneumoniae* during infection and are therefore probably not the only characteristic recognized by the immune system of the pig. Vaccines were therefore developed to include the Apx toxins and other proteins expressed during infection such as the transferrin binding proteins (267, 331). These vaccines offered better protection. Subunit vaccines reduce both clinical symptoms and lung lesions and improve growth

and feed conversion efficiency in acute and chronically infected pigs with pleuropneumonia in field trials (196, 197, 211, 254, 324). Currently, Intervet's Porcilis APP vaccine, which contains ApxI, ApxII, ApxIII toxoids and outer membrane proteins of *A. pleuropneumoniae*, is the vaccine that offers the best protection against *A. pleuropneumoniae* infection (315, 316).

Recent advances in molecular genetics have allowed for the construction of live attenuated vaccines. Live attenuated *A. pleuropneumoniae* vaccines use live but less virulent strains of *A. pleuropneumoniae* to elicit the immune response of the host and therefore provide protection against the more virulent wild-type strains. To construct live attenuated vaccines, genes involved in virulence were first mutated by random mutation and more recently through targeted mutation. Unlike bacterins and subunit vaccines, live attenuated vaccines will contain most of the surface components, excreted proteins, and toxins produced during infection thereby allowing the host to fine tune its response to the virulent organism without becoming sick.

Inzana et al. (152) isolated a capsule deficient mutant of *A. pleuropneumoniae* through random chemical mutagenesis and showed a vaccine using it provided protection against both homologous and heterologous strains. Other genes identified to be important to *A. pleuropneumoniae* in causing disease have been targeted for mutation and investigated in the production of live attenuated vaccines. The ApxII toxin gene, *apxIIC*, was mutated to allow for the secretion of an inactive toxin, thereby exposing pigs to a toxin antigen. Vaccinated pigs were protected against a cross-serotype challenge (24, 255). Fuller et al. (108) mutated the riboflavin biosynthetic operon, *ribGBAH*, and showed the *A. pleuropneumoniae* mutant provided protection against challenge with

heterologous serotypes. Other studies showing cross-protection with attenuated *A. pleuropneumoniae* have targeted the urease and ApxII genes, *ureC* and *apxIIA* (310), the essential aromatic amino acid biosynthetic pathway genes, *aroA* (112) and *aroQ* (148), the anaerobic respiration genes, *dmsA* (14) and *aspA* (160), the anaerobic respiration regulator, *hlyX* (16), and the ferric uptake regulator, *fur* (159), have shown cross-protection. While there have been optimistic advances in producing an attenuated strain suitable for a live attenuated vaccine, the targeted mutagenesis strategy may not always target the appropriate genes to cause attenuation (15, 17) or the *A. pleuropneumoniae* mutant may be cleared too rapidly to allow for an immune response (108, 112).

Other types of vaccines are still being investigated. Wilson et al. (346) have tested an abundant *A. pleuropneumoniae* protein as a subunit for a vaccine with success. Seah et al. (282) have investigated what domains of an Apx toxin are suitable for subunit vaccine development, and still others have looked at using an oral administration of bacterin and subunit vaccines (190, 287) as an alternative to subcutaneously injected vaccines which are labor intensive and can produce lesions at the site of injection (131, 300, 301).

Antibiotics. Antibiotics such as tiamulin (7, 49, 172, 278, 280, 291), tilmicosin (224, 270), ceftiofur (53, 87, 217, 270, 295, 319, 347), and enrofloxacin (181, 270) have been used by farmers successfully to treat *A. pleuropneumoniae* infection. *A. pleuropneumoniae* is sensitive to numerous other antibiotics that could be used to treat infections in the field (274). These include penicillin, premafloxacin, cephalothin, lincomycin, streptomycin, neomycin, terramycin, chloramphenicol, sulfonamides,

tetracycline, ampicillin, cephalosporin, colistin, cotrimoxazole, gentamicin, florfenicol, spectinomycin, trimethoprim, and erythromycin (53, 226, 279, 307, 348). While there is a broad choice of antibiotics to treat *A. pleuropneumoniae* infections, recent studies suggest the choice may be becoming smaller with the isolation of *A. pleuropneumoniae* strains resistant to antibiotics commonly used to treat them (54, 153, 154). Depending upon the serotype (103, 104, 146, 147, 226), there have always existed a certain amount of *A. pleuropneumoniae* resistant to penicillin, ampicillin, chloramphenicol, and tetracycline (39, 88, 103, 104, 116, 125, 147, 226, 270, 323, 348). A recent study of strains of *A. pleuropneumoniae* isolated in the Czech Republic between 2001 to 2003 showed a dramatic increase resistance to select antibiotics (228). Strains showing resistance to tetracycline increased in prevalence from 11% to 81.8%, to doxycycline from 9.6% to 61.8%, to nalidixic acid from 2.7% to 45.5%, and to norfloxacin from 0% to 34.6%.

One of the possibilities to explain this dramatic increase in resistance to certain antibiotics is the presence of plasmids encoding antibiotic resistance genes (134). Studies have identified multiple *A. pleuropneumoniae* plasmids that confer resistance to combinations of drugs such as streptomycin, sulfonamide, ampicillin, and nalidixic acid (54, 153, 154). These multiple resistant markers are probably the reason investigators have had to look into treating *A. pleuropneumoniae* infections with not just one antibiotic but combinations of antibiotics (20, 46, 141, 219, 225, 270, 281, 298).

The antibiotic arms race with *A. pleuropneumoniae* is just one negative of using antibiotics to treat *A. pleuropneumoniae* infection. Other drawbacks include the difficulty of administering antibiotics through feed because sick animals decrease feed

intake, adding enough antibiotic into feed to obtain an effective concentration in the serum of the pig, achieving a reliable dose of feed grade antibiotics, and administering direct antibiotic treatment to a large number of pigs (90, 279). Given the limitation of vaccines and antibiotics, managing swine in such a way so *A. pleuropneumoniae* infection does not have the chance to occur is one of the best ways to control this disease.

Management. Herd management is an important additional technique that can be used in conjunction with or even replace, vaccination and antibiotic treatment. Strict management guidelines can greatly reduce the risk of an *A. pleuropneumoniae* outbreak. New pigs should always be obtained from a single source known to be free of *A. pleuropneumoniae* and subsequently quarantined to make sure there are no infectious pigs (314). Pigs should be grouped by their immune status whereby pigs, which are seropositive and seronegative for *A. pleuropneumoniae*, are grouped separately (212). Once grouped, pigs should not be mixed between groups and group densities should be kept low (90, 314). The farm should maintain a group all-in-all-out policy with the pens and barns cleaned when groups are moved in and out (90, 314). The barn should be climate controlled to decrease the stress on the pigs and adequate ventilation should be used to help minimize aerosol transmission of *A. pleuropneumoniae* (90, 144, 314). If an *A. pleuropneumoniae* outbreak does occur, infected pigs should be quarantined and both infected pigs and the group they came from should be treated. If a large scale outbreak occurs, depopulating the farm can be performed as a last resort (90).

Genetic screens for the identification of potential virulence genes

To efficiently engineer vaccines and tailor treatments against pathogens, a solid knowledge of the bacterial genes and mechanisms important in causing disease is paramount. Molecular biology and genetics of bacteria have allowed for the discovery of methods to identify potential virulence genes. This section will examine some of the genetic methods used to identify these genes.

IVET. *In vivo* expression technology (IVET) is a genetic means to identify bacterial genes that are specifically expressed during infection of the host but are not expressed outside the host (207). The rationale of this method is that a bacterium will require certain genes to be transcribed during infection to infect and cause disease. However, a number of these same genes will not be needed, and therefore not transcribed, when the bacterium is growing outside its host. The genes identified by this method, termed *in vivo* induced (*ivi*), could be important in the ability of the pathogen to cause disease and the identification of these genes could lead to targets for vaccine development or drug treatment. IVET was originally developed to identify potential virulence genes in a *Salmonella typhimurium* mouse spleen model (207), and IVET has since been adapted for numerous other pathogens and infection models (reviewed in reference 258).

To identify potential virulence genes and the nascent virulence factors, IVET requires a promoter trap vector with promoterless biosynthetic and reporter genes, a pathogen of interest attenuated in the same biosynthetic gene, and a model of infection. Our laboratory adapted IVET for *A. pleuropneumoniae* and its natural swine host (105).

In brief, genomic DNA from *A. pleuropneumoniae* was digested with *Sau*3AI and restriction fragments were ligated into the promoter trap vector, pTF86 (107). The pTF86 vector contains promoterless riboflavin biosynthetic and luciferase genes. Vector constructs, varying only in the inserted genomic DNA fragment, were transformed into an avirulent *A. pleuropneumoniae* riboflavin auxotroph and resulted in a library of clones. These clones were then used to infect the natural swine host by intraperitoneal injection. Clones with DNA inserts that do not contain promoters are avirulent due to the riboflavin auxotrophy. However, clones with DNA inserts containing promoters that activate during infection will induce the expression of the promoterless luciferase and riboflavin genes within pTF86. Expression of the promoterless riboflavin genes complements the riboflavin auxotrophy, thereby restoring virulence. However, we are interested in promoters that are active during infection but not active when the bacterium is grown on laboratory medium. Therefore, clones isolated after infection can be cultured on laboratory medium and screened for loss of promoter activity via loss of luciferase activity. Only clones able to survive through the infection process and having low luciferase activity *in vitro* were labeled *ivi* clones. Our laboratory has recovered 32 unique *ivi* clones. In general, the genes that the *ivi* promoters control have been identified to be either genes involved in pathogenesis, genes involved in biosynthesis, genes with identified functions not obviously related to pathogenesis, or genes with unknown function (105, 176).

STM. Signature-tagged mutagenesis (STM) is another method, like IVET, to identify genes important in pathogenesis. However, unlike IVET, STM identifies genes critical

for survival during infection. To accomplish this, STM uses transposons with unique DNA sequence tags to mutagenize a pathogen of interest and construct a library of single transposon insertion mutants. The unique sequence tags linked to each transposon insertion allow for identification of an individual mutant within a pool by DNA hybridization. STM pools are used to infect a host in an infection model. An initial pool of mutants before infection can be compared to a pool of mutants recovered after the infection process by surveying for the unique DNA tags and accounting for the mutants lost during the process. Mutants lost during the infection process can be analyzed for the location of the transposon insertion and the mutated gene readily identified. Genes, resulting in inability to survive during infection when mutated, are identified.

STM was first developed in *Salmonella typhimurium* for use in a murine model of typhoid fever (132). Similar to IVET, STM has been adapted to numerous other pathogens and infection models (reviewed in references 10, 269). Also similar to IVET, an STM study has been performed in *A. pleuropneumoniae* and the genes identified to be critical for survival during infection encoded for either potential virulence factors, cellular regulation components, translation components, biosynthetic enzymes, or unknown factors (106).

Virulence genes

For *A. pleuropneumoniae* to cause disease in pigs, it requires a variety of virulence factors to help attach, colonize, replicate, cause damage, and protect itself from the immune system of the host. While factors that directly interact with the host, cause damage, or protect the pathogen from the host have long been considered virulence factors, other factors that allow the bacterium to grow by obtaining essential nutrients in a potentially nutrient limiting environment could also be deemed virulence factors. This section will discuss the current knowledge of the main virulence factors of *A. pleuropneumoniae* and how this pathogen utilizes them to cause the disease known as porcine contagious pleuropneumoniae.

Exotoxins. In 1976, *A. pleuropneumoniae* was observed to have a synergistic hemolytic activity called the CAMP phenomenon in the presence of β-hemolytic organisms such as *Staphylococcus aureus* (63, 178). The hemolytic activity of *A. pleuropneumoniae* differed between serotypes, with more hemolytic acivity correlating to more virulent serotypes. (99). Early studies also showed that bacteria-free culture supernatants or sonicated *A. pleuropneumoniae* would cause lung lesions (266). More specifically, filtered culture supernatants of *A. pleuropneumoniae* were toxic to porcine lung macrophages and peripheral blood monocytes (31), suggesting that at least part of *A. pleuropneumoniae* virulence was due to toxic extracellular *A. pleuropneumoniae* products. However, at the time it was not known whether or not the hemolytic and toxic characteristics of *A. pleuropneumoniae* were due to one or more virulence factors. It is

now known that the hemolytic characteristic of the CAMP phenomenon and the toxicity attributed to *A. pleuropneumoniae*, result from the action of the Apx toxins (164).

A. pleuropneumoniae possesses at least four exotoxins, each with varying hemolytic and/or cytotoxic properties. These groups of toxins have been given a variety of names such as Hly, Cly, App, or Cyt depending on the research group or serotype in which they were identified. A standard has since been decided and the four *A. pleuropneumoniae* toxins are now named ApxI, ApxII, ApxIII, and ApxIV after *A. pleuropneumoniae* RTX toxin (165).

RTX stands for repeat in toxin and these toxins are characterized by their pore forming abilities and glycine-rich nonapeptide repeats Leu/Ile/Phe-Xaa-Gly-Gly-Xaa-Gly-Asn/Asp-Asp-Xaa that bind calcium (339). These type of toxins were first identified in *E. coli*; their hemolytic activity and binding to erythrocytes was shown to be calcium-dependent (36). Genetically, RTX toxins are generally encoded by an operon. In *E. coli*, the HlyA α-hemolysin is encoded by the gene, *hlyA*, which resides in the operon *hlyCABD*. HlyA is initially produced as a prohemolysin, which is activated by the *hlyC* gene product and secreted by the *hlyB* and *hlyD* gene products (89, 340). The same operon structure is found in *A. pleuropneumoniae*.

The Apx toxins not only can cause host cell damage by their variety of hemolytic and cytotoxic properties (173), but they can also protect *A. pleuropneumoniae*. Without Apx toxins, *A. pleuropneumoniae* is readily phagocytosed and killed by host macrophages (68, 317). Furthermore, the Apx toxins in high concentration inhibit the oxidative burst of host immune cells, thereby offering an additional level of protection (317). Apx toxins can target host immune cells such as polymorphonuclear neutrophils

(PMN) and kill them (167). However, at low levels of RTX toxin, the oxidative burst of host immune cells is actually provoked (32, 167, 191, 284, 317) and can result in more damage because of the host cell immune response (208). The hemolytic and cytotoxic activities of RTX toxins are mediated by separate and independent regions of the toxin. The hemolytic activity is mediated by toxin epitopes that allow for non-specific binding to host cells using acyl groups. In contrast, the cytotoxicity is mediated by targeted binding of the RTX repeat region to specific cell types (185). This should emphasize the fact that the main role of Apx toxins is to cause damage to host cells. Without Apx toxins, *A. pleuropneumoniae* is unable to produce lesions and the characteristic disease associated with *A. pleuropneumoniae* (37).

The characteristics of each Apx toxin vary. The ApxI toxin, a 105-kDa protein, was the first of the Apx toxins to be identified (98). Once the *apxIA* gene was cloned and sequenced (96, 126), it was shown to have a 56% DNA similarity to the *E. coli* α-hemolysin gene, *hlyA*. ApxI is a typical RTX toxin with three N-terminal hydrophobic regions, 13 glycine-rich repeats, and a hydrophilic C-terminal region (96). Low levels of calcium are required for both the induction and the activity of ApxI (99). Serotypes 1, 5, 9, 10, 11 and 14 secrete ApxI (34, 94, 173). Serotypes 2, 4, 6, 7, 8, 12, 13, and 15 do not secrete ApxI, even though they have transcriptionally active partial *apxI* operons. In these serotypes, there is a deletion in the *apxICA* genes and therefore no toxin is produced (94, 166). ApxI is able to kill porcine lung macrophages and porcine erythrocytes due to its strong hemolytic and strong cytotoxic properties (173, 329).

ApxII was first identified by Frey and Nicolet (99) and the *apxIICA* genes were subsequently identified (55). Unlike ApxI, the 105-kDa ApxII is weakly hemolytic and

moderately cytotoxic (173). ApxII is not inducible by calcium, but it does requires high levels of calcium for activity (97, 99). Although all serotypes except serotype 10 secrete ApxII (34, 82, 94, 173), none of these serotypes have the *apxIIBD* genes for secretion (294). ApxII is probably secreted by the *apxBD* genes of another toxin.

The third Apx toxin identified, ApxIII, is a 120-kDa protein found to be secreted by serotypes 2, 3, 4, 6, 8, and 15 (34, 94, 173, 268). This toxin has been called pleurotoxin in the past but still has characteristic RTX toxin properties, such as glycine-rich repeats (165) and the ability to bind calcium (329). ApxIII is not hemolytic but is strongly cytotoxic, which explains its ability to kill porcine lung macrophages (173, 329). While ApxIII is strongly cytotoxic, it is not cytotoxic to porcine erythrocytes.

The most recent Apx toxin identified was the 202-kDa ApxIV toxin (273). As with the other Apx toxins, ApxIV has characteristic RTX toxin domains such as a hydrophobic N-terminal end, 24 glycine-rich repeats, and a C-terminal end that binds calcium (273). However, unlike other Apx toxins, ApxIV, encoded by *apxIVA*, seems to be only induced *in vivo* (273). Furthermore, it was shown that the activity of ApxIV, when expressed in *E. coli*, required an upstream open reading frame to allow for hemolytic activity (273). While the *apxIVA* gene has been identified in serotypes 1-15, the expressed ApxIV toxin has only been shown in serotypes 1-12 (34, 61, 62, 272, 273). To date, there have been no investigations into whether or not serotype 13 and 14 produce ApxIV toxin.

While we now know the Apx toxins play a major role in the virulence of *A. pleuropneumoniae*, there is still much that is not known about the Apx toxins. The most recent research into Apx toxins have tried to determine what environmental conditions,

other than the availability of calcium, play a role in regulating Apx toxin expression. Iron represses ApxII expression in later stages of growth (214), but recently Hsu et al. (142) showed both calcium and iron induce ApxI expression through the actions of the ferric uptake regulator (Fur). Further work has shown *apxI* and *apxII* expression is highest at late exponential and early stationary phase (169). However, the regulation of the Apx toxins is unclear under oxygen limitation. One study suggests oxygen limitation does not play a role in the expression of these toxins (168), while another report contradicts this, stating that ApxI is produced aerobically but not anaerobically (341). It remains to be seen what specific environmental factors play a role in the expression of the Apx toxins, especially the *in vivo* induced ApxIV toxin.

Endotoxin. Lipopolysaccharide (LPS), also called bacterial endotoxin, is a major constituent of Gram-negative bacterial cell membranes. LPS is a major virulence factor for all Gram-negative pathogens, including *A. pleuropneumoniae*. It provokes the activation of complement and host immune cells, and leads to a strong immune inflammatory response. In high doses, LPS can elicit an overzealous immune response leading to inflammation, necrosis, and toxicity (215, 318). The structure of LPS consists of 3 regions. Lipid A is the most toxic region of LPS and anchors the whole LPS structure to the bacterial outer membrane. The core is the middle region of LPS and consists of an oligosaccharide containing 3-deoxy-D-manno-2-octulosonic acid (KDO). Finally, the O-antigen consists of a repeating chain of polysaccharides, except for the lipooligosaccharide (LOS) variant in which there is no O-antigen (135). The amount of

O-antigen or lack of O-antigen correlates with the phenotypic appearance of colonies. Smooth colonies have O-antigen while rough colonies do not.

While the role of LPS in virulence has been shown, its role in adhesion of bacteria to host cells is more controversial. Belanger et al. (26) showed smooth strains of *A. pleuropneumoniae* adhere better to porcine tracheal sections. Furthermore, extracted LPS and isolated polysaccharide from LPS were able to inhibit *A. pleuropneumoniae* adhesion to host tissues, suggesting excess LPS and polysaccharide can compete with *A. pleuropneumoniae* for binding to host tissue (245). Antibodies against LPS also inhibit *A. pleuropneumoniae* adhesion to host tissues (246). Recent studies investigating the affinity of *A. pleuropneumoniae* LPS further support the role of LPS in the adherence of *A. pleuropneumoniae* to host tissues by showing phosphatidylethanolamine and certain glycosphingolipids are ligands for *A. pleuropneumoniae* LPS (1, 170).

Spontaneous, undefined LPS mutants have been generated (262), but are unable to shed light on what components of LPS are involved in adherence and virulence. Recently, an *A. pleuropneumoniae* genetically defined LPS mutant in the *galU* gene has been generated using transposon mutagenesis. The *galU* gene, encoding the UTP-α-D-glucose-1-phosphate uridylyltransferase involved in LPS core biosynthesis (263). The mutant still reacted with a monoclonal antibody directed to O-antigen, suggesting O-antigen was still present. However, the mutant was significantly less adherent and less virulent than wild-type *A. pleuropneumoniae*. This specifically showed the LPS core is important in adherence and virulence of *A. pleuropneumoniae*, whereas the O-antigen is not as critical. More LPS mutants are required to determine the exact role of LPS in adherence of *A. pleuropneumoniae* to host tissue, but recent research shows generation of

these mutants may be difficult. A mutation in the gene *rfaE*, was unsuccessful (256) and suggests some genes involved in LPS core biosynthesis are essential.

Fimbriae. To establish infection and disease in the host, a pathogenic bacterium must be able to colonize. The efficient colonization of the host usually requires some type of specific host-pathogen interaction. Fimbriae are one of a variety of factors that can mediate this attachment (136).

Several types of fimbriae, also called pili, have been identified in many Gram-negative pathogens. Type IV fimbriae are one type and are characterized by the fact that the major pilin subunit is first produced as a prepilin and subsequently processed by a prepilin peptidase. In brief, the prepilin peptidase cleaves the target peptide sequence of the prepilin and then methylates the cleaved N-terminus. Once this is performed, the pilus can be assembled (124). Type IV fimbriae have not only been implicated in adherence, but also twitching motility, DNA uptake, and phage infection (305).

While observation of fimbrial structures has been reported for *A. pleuropneumoniae* (81, 322), it was not until recently that researchers identified the amino acid sequence of the major *A. pleuropneumoniae* Type IV fimbrial subunit, ApfA (349), and the *apfABCD* genes encoding for pilus biosynthesis (38, 296).

One of the initial problems in the identification of the Type IV fimbriae was the lack of expression of the fimbriae on complete laboratory medium. Boekema et al. (38) showed Type IV fimbriae could be produced on complete laboratory medium when the *apfABCD* operon was placed under the control of a constitutive promoter. They further showed the expression of *apfABCD* in wild-type *A. pleuropneumoniae* was induced in

chemically defined medium, but environmental factors such as temperature, iron, and NAD concentration had no significant effect. However, host cell contact did induce the expression of the Type IV fimbriae. The specific environmental or host signals which regulate Type IV fimbrial expression in *A. pleuropneumoniae* are currently unknown, but research into environmental cues for virulence gene expression could provide important insight to when and where Type IV fimbriae may be expressed in *A. pleuropneumoniae*.

Capsule. Bacterial capsules, which can be composed of polysaccharides or other compounds, cover the cell surface and protect bacteria from the environment. In pathogenic bacteria, capsules often block antibody attachment to the outer membrane and therefore decrease the chances of being opsonized, phagocytosed, or killed by complement (124). *A. pleuropneumoniae* produces polysaccharide capsule composed of repeating carbohydrates, and the variation in the repeating carbohydrate structure is the basis for the 15 serotypes identified (4, 34, 236, 250, 307). Encapsulated *A. pleuropneumoniae* is resistant to antibody attack (151) and complement mediated attack by specifically blocking the C9 component of the membrane attack complex (151, 337).

The genetic basis for the production of capsule by *A. pleuropneumoniae* is under investigation, but is still not fully understood. Work by Ward and Inzana (336) identified the highly conserved *cpxDCBA* genes for capsule export in *A. pleuropneumoniae* and suggest because of sequence homology that CpxD may be involved in capsular polysaccharide transport across the outer membrane, CpxC may be the second component responsible for transport across the cytoplasmic membrane, CpxB may be an integral membrane protein, and CpxA may be an ATP-binding protein for the transport system.

Using Southern blots and analyzing serotypes 1, 2, 5a, 7, and 9, they showed a *cpxCB* probe hybridized to genomic DNA from all analyzed serotypes. Although no specific expression data has been shown to date, the *cpxDCBA* genes appear to be organized in an operon structure. Investigation of the existence of *cpx* genes in other serotypes has not been reported to date. Attempts to mutate the *cpxBA* capsule transport genes have been unsuccessful in serotype 5a (336). However, given the difficulty of mutating genes in *A. pleuropneumoniae*, this does not indicate with certainty that *cpxBA* mutants or other transport genes are lethal. As an example, a nonencapsulated serotype 1 *cpxC* mutant has been generated (264). No reports on the ability to mutate the *cpxD* gene have been published.

An adjacent set of genes, *cpsABCD*, transcribed in the opposite direction to the capsule transport operon, have been implicated in the biosynthesis of capsule (338). The capsule biosynthesis *cpsABCD* genes do not show a high degree of conservation between *A. pleuropneumoniae* serotypes (338). This is not surprising since the differences in the 15 serotypes of *A. pleuropneumoniae* are based on capsule differences. Similar to the export genes, the biosynthetic genes appear to be arranged in an operon structure, but no evidence beyond gene structure and placement support this. In contrast to the capsule transport genes of *A. pleuropneumoniae*, many single and multiple *cpsABCD* capsule biosynthesis gene deletion mutants have been successfully constructed in serotypes 1 and 5a (18, 338). The roles and regulation of the individual capsule biosynthetic genes are unknown. However, analysis of the predicted amino acid sequences of those that have been sequenced generally shows homology to glycosyltransferases or capsule biosynthetic genes with unknown functions from other organisms (338).

The capsule transport and biosynthesis-deficient mutants reported to date are less virulent in pig experimental infection models and these data support the importance of capsule in the virulence of *A. pleuropneumoniae*. Yet, little is known about when and where *A. pleuropneumoniae* capsule is expressed, what host cues induce the expression, and what factors may regulate the genes. While the amount of available iron does not affect capsule expression significantly (244), there could be other environmental and host cues that could. Host cell contact could be one possible cue that mediates capsule expression. One study showed a nonencapsulated *A. pleuropneumoniae cpxC* mutant was more adherent than wild-type *A. pleuropneumoniae* (264) and suggests there could be a negative correlation between capsule production and host cell attachment. This would make sense since the production of capsule could physically block other important factors, such as adhesins, pili, or toxins, from interacting with the host and a system to control this via host cell contact would be convenient. However, more research is required to address these unknowns.

Iron scavenging. Some bacterial factors are easily characterized as virulence factors because they cause direct damage to the host (Apx toxins and LPS), they allow for adhesion to the host (adhesins and LPS), or they protect bacteria from host defenses (capsule). However, not all bacterial virulence factors are necessarily on the forefront of the battle between the pathogen and the host. When confronted with the environment of the host, the bacterium may be required to obtain essential nutrients that may not be easily obtained. Iron is an important cofactor for bacterial processes and is limiting because host proteins, such as transferrins and lactoferrins, specifically sequester iron

(124). Therefore, iron scavenging is a necessary ability of bacterial pathogens to survive and grow within the iron limiting environment of the host.

Siderophores are small molecules produced and excreted by bacteria into their environment to specifically acquire iron for bacterial use (124). These siderophores bound to iron are then taken up into bacterial cells via specific receptors and uptake systems. However, members of the *Pasteurellaceae* family, of which *A. pleuropneumoniae* is a member, use a slightly different technique. *A. pleuropneumoniae* can acquire iron directly from porcine transferrin via outer membrane proteins that specifically bind transferrin (117, 237). Initial studies identified two outer membrane proteins induced under iron limiting conditions (77). Further studies identified the *A. pleuropneumoniae* genes *tbpA* and *tbpB* encoding for the proteins, TbpA (~100-kDa) and TbpB (~60-kDa), respectively (71, 113, 114, 118, 179). TbpA is highly conserved between serotypes (344) while TbpB is less conserved (114). Gonzalez et al. (117) was able to show these two proteins bind porcine transferrin and are part of a system to obtain sequestered iron from the host. Additional studies by Gerlach et al. (113) and Schryvers et al. (276) showed *A. pleuropneumoniae* TbpB bound specifically to porcine transferrin, but had significantly less affinity to bovine and human transferrin. This suggests these proteins contribute to host cell specificity. The Tbp proteins are absolutely required for the virulence of *A. pleuropneumoniae* and when either is mutated, *A. pleuropneumoniae* is highly attenuated in a swine aerosol model (13). Although *tbpA* and *tbpB* are critical for virulence, they are not the only parts of the transferrin uptake system.

The *tonB*, *exbB*, and *exbD* genes are associated in the same genetic locus with *tbpA* and *tbpB* (311). The *tonB* gene encodes for the TonB protein, which is able to

transduce energy produced at the cytoplasmic membrane of the bacterium and make it available to iron uptake proteins in the outer membrane. The ExbB and ExbD proteins, encoded by *exbB* and *exbD*, respectively, form inner membrane complexes that anchor a TonB dimer in the periplasm. Together, the TonB system provides energy and allows for iron uptake into the cell. An *exbBD* deletion mutant is unable to utilize iron from transferrins (311). In addition to the TonB system, there is a second TonB system identified in *A. pleuropneumoniae* with proteins subsequently named TonB2, ExbB2, and ExbD2 and was present in the 14 serotypes analyzed (23). Although both of these systems are involved in iron uptake in *A. pleuropneumoniae*, they do seem to play different roles. While both *tonB1* and *tonB2* are up-regulated under iron restriction, a *tonB1* mutant is still virulent and a *tonB2* mutant is avirulent (23). This suggests while both of these systems are involved in iron uptake, their roles during an actual infection of the host differ. Insight into their different roles is provided by Beddek et al. (23), who showed TonB2 is important for obtaining iron from hemin, porcine hemoglobin, and ferrichrome while TonB1 is not. They further speculate the affinity of the TonB systems for specific iron sources could in part be due to a TonB1 and TonB2 heterodimer interacting with the transferrin binding proteins in the outer membrane.

These two systems are not the only way for *A. pleuropneumoniae* to acquire iron. One study showed the LPS of *A. pleuropneumoniae* can bind porcine hemoglobin and suggests this could be a way for *A. pleuropneumoniae* to sequestering iron after lysing host cells with Apx toxins (25). More recently, an *A. pleuropneumoniae* system has been identified for uptake of the hydroxamate siderophore, ferrichrome (17, 220). As previously mentioned, siderophores are small molecules excreted by bacteria to acquire

iron. While *A. pleuropneumoniae* can use exogenous siderophores as a source of iron (80), there is no evidence *A. pleuropneumoniae* makes any of its own siderophores (77, 80). The ferric hydroxamate uptake (*fhu*) operon has been extensively studied in *E. coli* and allows the bacterium to utilize iron from ferrichrome. Genetic studies of the *fhuCDBA* operon in *A. pleuropneumoniae* have shown an *fhuA* mutant to be unable to utilize ferrichrome (221), but the mutant is just as virulent as wild-type *A. pleuropneumoniae* in a swine infection model (17). As evident by other factors of iron scavenging systems, some virulence factors allow the pathogen to acquire essential nutrients to survive and grow in a nutrient limiting environment even though they do not cause direct damage to the host or provide direct protection from the host.

Biosynthetic enzymes. Genes encoding for enzymes that allow a bacterium to synthesize essential nutrients, to which the bacterium does not have access in the required concentrations during infection, can be thought of along the same lines as iron scavenging genes. Without these genes, the bacterium is unable to successfully grow and cause disease. Whether these are truly "virulence factors" is a subject for debate, but the fact that mutants in many of these genes are avirulent or attenuated is not.

Mutations in biosynthetic genes involved in the synthesis of aromatic amino acids (3, 21, 40, 51, 52, 91, 137-139, 148, 156, 157, 174, 175, 193, 229, 238, 239, 292, 293, 299, 304, 332), thymine (2, 51, 115, 241), purines (50, 51, 66, 91, 155, 158, 194, 195, 238, 239, 292), and branched-chain amino acids (9, 11, 12, 19, 122, 140, 216, 320, 350) are just some examples of mutations in biosynthetic pathways that lead to attenuation of a variety of different pathogens in their respective hosts. Although some of the

biosynthetic products could be available exogenously to the pathogen, the site of infection may determine whether or not the pathogen has access to enough to grow and cause disease (240). It is clear that certain metabolites and the biosynthetic enzymes needed to produce them are required for a successful infection. While studies investigating biosynthetic genes and their role in virulence have been performed on a variety of bacteria, only a few have been specifically studied in *A. pleuropneumoniae*.

Aromatic amino acids are essential amino acids to mammals and therefore must be obtained through diet. In contrast, most bacteria are able to synthesize their own aromatic amino acids. Hoiseth and Stocker (137) showed a *Salmonella typhimurium* strain auxotrophic for aromatic amino acids was avirulent, and many subsequent studies have yielded similar results with other pathogens. Two *A. pleuropneumoniae* aromatic amino acid biosynthetic genes, *aroA* and *aroQ*, have been mutated, and these mutations decrease the virulence of *A. pleuropneumoniae* in pigs (112, 148). A large number of other pathogens also require aromatic amino acid biosynthesis genes to cause disease. These data indicate the essential aromatic amino acids are an important requirement for *A. pleuropneumoniae* to produce a full infection.

The riboflavin biosynthetic genes are another example of biosynthetic genes critical for some pathogens to cause disease. Riboflavin is an important vitamin for metabolic processes and must be obtained through the diet of the animal. Similar to aromatic amino acids, most bacteria are able to synthesize their own riboflavin. The riboflavin biosynthetic pathway in *A. pleuropneumoniae* was mutated and mutants were unable to cause disease in pigs (109).

The *aroA*, *aroQ*, and *ribBAH* mutants of *A. pleuropneumoniae* support that certain biosynthetic pathways are critical for *A. pleuropneumoniae* to cause disease within its host, similarly to certain iron scavenging genes. These genes do not produce any specific secreted factors like toxin that cause damage or capsular biosynthetic genes that provide protection against the host immune system, but they do offer the pathogen an ability to grow in nutrient-limiting conditions. The ability of the pathogen to survive long enough in the host to cause disease diminishes without these genes.

A. pleuropneumoniae virulence gene stimuli

Bacteria often regulate genes in response to the environment to be able to adapt and survive. Bacteria have developed a wide range of regulatory mechanisms to recognize a range of environmental stimuli and alter gene expression accordingly. Bacterial pathogens often respond to such host environmental conditions as iron concentration, temperature, pH, and oxygen concentration (218). The change in stimuli, between the outside and inside of the host or from one site of infection to another, could allow the pathogen to recognize when it is within the host and when to produce virulence factors. While many stimuli have been identified to regulate virulence gene expression in other organisms, little is known about what stimuli may regulate virulence gene expression in *A. pleuropneumoniae*.

Like many animal pathogens, *A. pleuropneumoniae* respond to iron limiting conditions by expressing genes needed for iron scavenging (23, 71, 77, 113, 114, 118, 179). *A. pleuropneumoniae* is avirulent without some of these gene products (13). Some pathogens, such as *Corynebacterium diptheriae* and *E. coli*, can regulate toxin expression in response to iron concentration (47, 275). Other pathogens can regulate toxin expression in response to calcium concentrations (218). Expression of some *A. pleuropneumoniae* Apx toxins is induced in the presence of calcium (99).

Many pathogens also respond to oxidative stress resulting from the host immune response. Due to the massive host neutrophil response to an *A. pleuropneumoniae* infection (32), and the ability of Apx toxins to kill host immune cells (167), *A. pleuropneumoniae* may need to respond to the oxidative contents of neutrophils. In fact,

the *A. pleuropneumoniae* gene product of *ohr*, which detoxifies the oxidative effect of organic hydrogen peroxides, is induced during infection (286).

A respiratory pathogen, such as *A. pleuropneumoniae*, may encounter a low oxygen environment once the lung becomes necrotic due to infection. This may subsequently act as a signal for virulence gene expression. Baltes et al. (14) showed the gene that encodes part of dimethyl sulfoxide reductase, *dmsA*, is up-regulated during infection and allows the bacteria to produce energy in an oxygen-depleted environment such as a necrotic lung. An *A. pleuropneumoniae dmsA* mutant was attenuated in an *A. pleuropneumoniae* acute infection (14), supporting its critical role in virulence of *A. pleuropneumoniae.*

There may be many unidentified stimuli that induce virulence gene expression for respiratory pathogens such as *A. pleuropneumoniae.* One possible stimulus is the limitation of branched-chain amino acids (BCAA) at the site of infection. BCAAs are essential amino acids that most mammals, including pigs and humans, cannot synthesize on their own and must instead be obtained from their diet. Some evidence suggests the concentrations of free BCAAs within the host can be low in certain areas of the body, such as cerebral spinal fluid (223). Further evidence suggests the lungs of healthy animals are limited in free BCAAs (22, 308). Therefore, it is possible the limitation of free BCAAs, like the limitation of available iron in the host, may be a signal to regulate virulence gene expression. In comparison to iron limitation, the limitation of BCAAs may occur in some parts of the body and not others, thereby affecting only certain pathogens.

A. pleuropneumoniae regulators of gene expression

To respond to a wide range of stimuli, bacteria have developed an equally wide range of ways to regulate genes. Bacteria can either positively or negatively regulate genes and this regulation can be at the transcriptional, translational, or posttranslational level (124). However, there is little known about what *A. pleuropneumoniae* regulators exist and which may be important to change gene expression to suit specific host environments. As an example, *A. pleuropneumoniae* has genes such as *sodC* and *ohr* that encode enzymes that detoxify superoxides and organic peroxides during infection of the host. IVET studies indicate that both of these genes are *in vivo* induced, but regulators for these genes in *A. pleuropneumoniae* have yet to be identified (188, 286). Although two component regulatory systems are prevalent in well studied organisms such as *E. coli*, they have not yet been identified in *A. pleuropneumoniae*. In fact, accurate consensus promoter structures for *A. pleuropneumoniae* have not been identified. The study of *A. pleuropneumoniae* gene regulation is still a novel field and once a complete *A. pleuropneumoniae* genome is assembled and annotated, more of these regulatory systems will likely be identified by sequence similarity.

Two *A. pleuropneumoniae* regulators have been cloned and analyzed to date. HlyX was first identified, albeit incorrectly, as the factor responsible for the CAMP phenomenon of *A. pleuropneumoniae* (63, 100). Its incorrect association with the CAMP phenomenon resulted from the ability of HlyX to activate a cryptic hemolysin activity in *E. coli*, even though HlyX is not itself a hemolysin. It was later correctly identified as a homologue of the *E. coli* fumerate nitrate reductase regulator, FNR (201, 285). FNR is a

global regulator and is responsible for regulating a number of genes under anaerobic conditions.

The gene, *hlyX*, has been cloned, sequenced and shown to complement an *E. coli fnr* mutant (201). However, very little investigation of HlyX in *A. pleuropneumoniae* has been performed. Possible binding sites for HlyX were identified (123) upstream of two genes shown to be induced under anaerobic conditions, the dimethyl sulfoxide reductase gene, *dmsA* (14), and the aspartase gene, *aspA* (160). Using an *A. pleuropneumoniae hlyX* mutant, HlyX was implicated in the regulation of *aspA* and *dmsA* (16). Since hlyX regulates *dmsA* and an *A. pleuropneumoniae dmsA* mutant was shown to be attenuated (14), *hlyX* was identified as a potential target for constructing a live attenuated vaccine. A *hlyX* mutant was tested for attenuation in a swine aerosol infection model and shown to be less virulent and unable to persist in the lungs (16).

The second *A. pleuropneumoniae* regulator studied to date is the ferric uptake regulator (Fur). Fur was first identified in *Salmonella typhimurium* (84), and genes with sequence similarity have been shown to be involved in the regulation of genes associated with iron scavenging in many bacteria. The Fur protein acts as a dimer, can bind iron, and bind to Fur boxes in the promoter regions of genes (75, 124). Classically, in the presence of iron, Fur dimers bind to specific sequences in gene promoters and block transcription. In the absence of iron, Fur dimers release from the DNA and transcription is no longer blocked. In this way, many genes required for iron-scavenging are repressed when sufficient iron is available and derepressed in iron-limiting conditions.

Recently, an *A. pleuropneumoniae* gene similar to *fur* in serotypes 1 and 5 was shown to complement an *E. coli fur* mutant (142). Further studies, using Western blot

analysis of wild-type *A. pleuropneumoniae* and a *fur* mutant, showed *A. pleuropneumoniae* Fur to be involved in the classical type of negative regulation of the transferrin uptake system proteins TbpB and ExbB (159). Furthermore, the *fur* mutant was attenuated in a swine aerosol model of infection (159), suggesting the regulator is important in the virulence of *A. pleuropneumoniae*. However, unlike the classical mechanism, *A. pleuropneumoniae* Fur also acts as an activator. Hsu et al. (142) showed by chloramphenicol acetyl transferase ELISA that calcium and Fur together activate the transcription of the ApxI toxin and this is not traditional Fur reguation.

There may be stimuli to which *A. pleuropneumoniae* respond during infection of the host that have not yet been identified. Along the same lines, there may also be regulators important for the genetic response to the stimuli and for virulence that have also not yet been identified. Investigation into regulators of virulence gene expression in *A. pleuropneumoniae* has only been reported in the last three years. A better understanding of how and under what conditions *A. pleuropneumoniae* causes disease can be obtained by identifying the stimuli and regulatory systems for virulence gene expression.

Branched-chain amino acid biosynthesis and Lrp

Amino acids are required for the synthesis of proteins in organisms ranging from bacteria to humans. Organisms unable to acquire or synthesize amino acids will be unable to support growth and the metabolic processes of life. One group of amino acids are the branched-chain amino acids (BCAA) isoleucine, leucine, and valine, which are characterized by their branched-carbon chain within the R group of their chemical structure. While humans and most mammals lack the ability to synthesize their own BCAAs, most bacteria are able to synthesize their own. When presented with a limitation of BCAAs in the environment, bacteria will most certainly need to synthesize BCAAs to continue to grow. The bacteria will require a group of enzymes to catalyze the formation of intermediary products to eventually synthesize the BCAAs.

The BCAA biosynthetic pathway has been extensively described in *E. coli* and *Salmonella*. The pathway consists of two parallel reactions catalyzed by single enzymes that lead to the synthesis of isoleucine and valine with a branch point at the precursor to valine that leads to the synthesis of leucine (Figure 1). The first committed step in BCAA biosynthesis is performed by an acetohydroxy acid synthase (AHAS) isozyme that catalyzes the decarboxylation of pyruvate and the subsequent acetyl group condensation with α-ketobutyrate or a second pyruvate to form acetohydroxybutyrate or acetolactate in the isoleucine or valine pathways, respectively (321). In *E. coli*, there are three distinct AHAS isozymes, encoded by *ilvIH*, *ilvBN*, and *ilvGM*.

The initial substrates for BCAA biosynthesis can be acquired from other amino acids and other pathways. Threonine deaminase, encoded by *ilvA*, can produce α-

ketobutyrate by acting on threonine, thereby feeding the isoleucine parallel pathway (321). Serine deaminase, encoded by *sdaA*, can produce pyruvate by acting on serine, thereby feeding the valine parallel pathway (313). Pyruvate could also be acquired from other places such as glycolysis via phosphoenolpyruvate or from the degradation of alanine (213) (Figure 1).

The enzymes encoded by two other genes, *ilvC* and *ilvD*, catalyze reactions in the isoleucine and valine parallel pathways to form α-ketomethylvalerate, the precursor to isoleucine, and α-ketoisovalerate, the precursor to valine. These precursors are used in one last parallel reaction catalyzed by the enzyme encoded by *ilvE* to form isoleucine and valine. The synthesis of leucine begins in the valine destined pathway with the precursor to valine and leucine, α-ketoisovalerate. The enzymes encoded by the *leuABCD* operon then catalyze the branched-point reaction to form leucine.

The regulation of BCAA biosynthesis in *E. coli* and *Salmonella* is complex, with controls not only within the BCAA biosynthetic pathway but also in connected pathways, such as the pathways for threonine and serine biosynthesis and catabolism. End product inhibition, enzyme specificity, and the regulation of gene expression are controls responsible for the regulation of BCAA biosynthesis.

End product inhibition occurs when the final product of a reaction pathway can inhibit the start of the reaction. As an example, inhibition of valine biosynthesis occurs if there is already sufficient valine. However, given the fact the parallel pathways of isoleucine and valine use the same enzymes, the end product inhibition of the reaction by excess valine could not only stop valine biosynthesis but also isoleucine biosynthesis. *E.*

coli and *Salmonella* can escape this enzymatic catch-22 because they have isozymes of AHAS that vary in their sensitivity to end product inhibition.

AHAS I and AHAS III are inhibited by valine, while AHAS II is insensitive to valine (321). This permits inhibition of the isozyme(s) that lead to valine biosynthesis without affecting AHAS II, which is more specific to the isoleucine parallel pathway. End product inhibition also affects the *ilvA* encoded threonine deaminase. The major source of the isoleucine precursor, α-ketobutyrate, is from the deamination reaction of threonine by threonine deaminase, and isoleucine can inhibit threonine deaminase (321).

Another point of regulation within the BCAA biosynthetic pathway is AHAS isozyme specificity. Not only can the AHAS isozymes be inhibited differentially, but each has a particular preference for substrate. All AHAS isozymes decarboxylate pyruvate and combine the resulting acetyl group with a secondary substrate, either α-ketobutyrate for the isoleucine parallel pathway or a second pyruvate for the valine parallel pathway. AHAS I prefers pyruvate as the secondary substrate and is therefore tailored to form the valine precursor, acetolactate. AHAS II has a preference for α-ketobutyrate as the secondary substrate and is therefore tailored to form the isoleucine precursor, acetohydroxybutyrate. AHAS III has no strict preference, although it favors isoleucine biosynthesis (321). These preferences allow for the specific regulation of one part of the pathway while limiting the effect on the synthesis of other BCAAs.

Figure 1-1. Branched-chain amino acid biosynthesis pathway. Genes activated by Lrp are shown in bold type. Genes repressed by Lrp are underlined. Gene products: *tdh*, threonine dehydrogenase; *kbl*, glycine acetyltransferase; *ilvA*, L-threonine deaminase; *ilvBN*, acetohydroxy acid synthase isozyme I; *ilvGM*, acetohydroxy acid synthase isozyme II; *ilvIH*, acetohydroxy acid synthase isozyme III; *ilvC*, ketoacid reductoisomerase; *ilvD*, dihydroxy-acid dehydratase; *ilvE*, transaminase B; *leuABCD*, leucine biosynthetic operon; *serA*, 3-phosphoglycerate dehydrogenase; *serB*, phosphoserine phosphatase; *serC*, 3-phosphoserine aminotransferase; *sdaA/sdaB*, serine deaminase; *glyA*, serine hydroxymethyltransferase; *glnALG*, glutamine synthase; *gltBDF*, glutamate synthase. Biosynthetic products: AL, acetolactate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; mTHF, methylenetetrahydrofolate; 3-PHP, 3-phosphohydroxypyruvate; 3-PS, 3-phosphoserine; αKB, α-ketobutyrate; αKG, α-ketoglutarate; AHB, acetohydroxybutyrate; DHMV, dihydroxymethylvalerate; DHIV, dihydroxyisovalerate; αKMV, α-ketomethylvalerate; αKIV, α-ketoisovalerate.

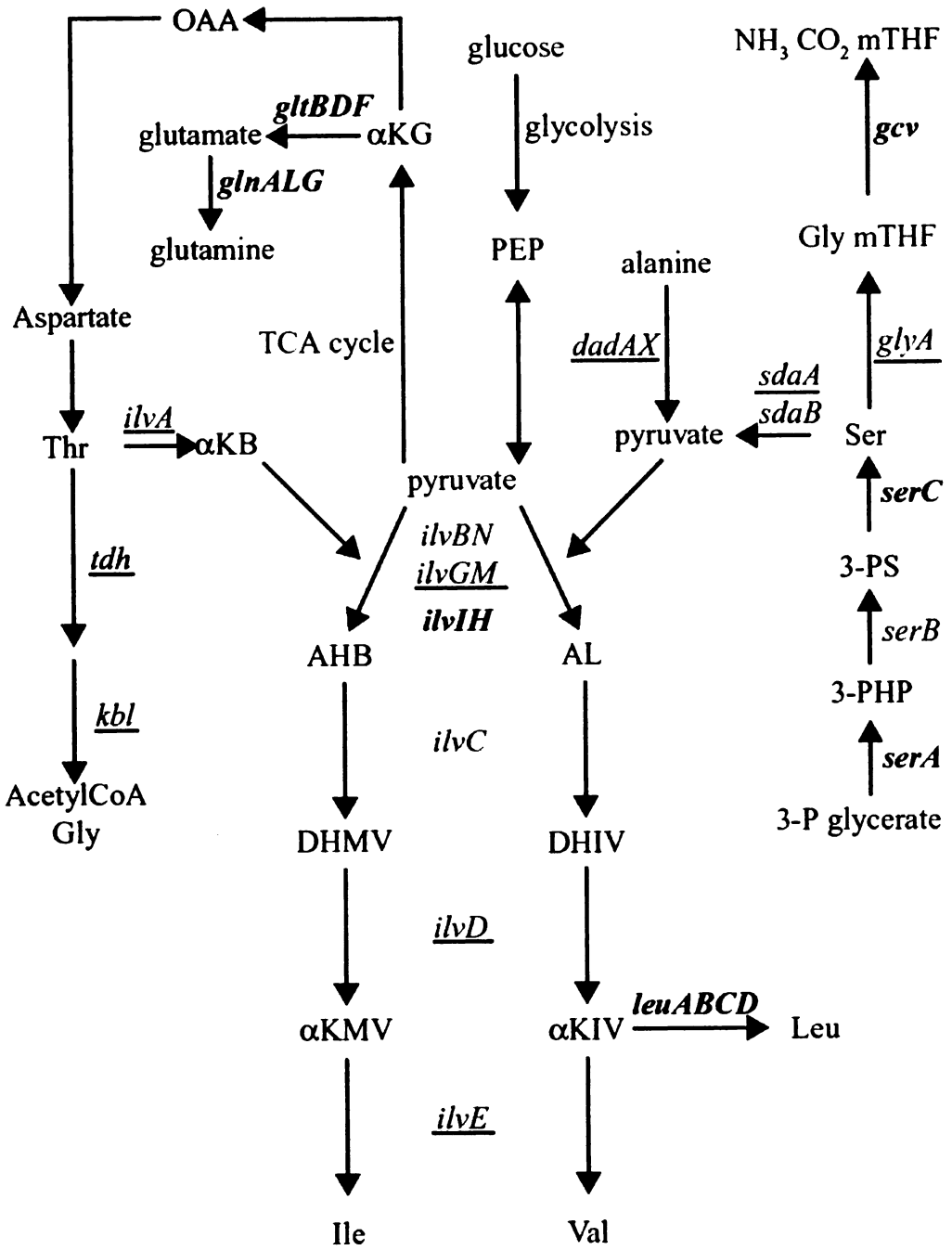
OAA
glucose
NH3 CO2 mTHF
gltBDF
glutamate
αKG
glycolysis
gcv
glnALG
glutamine
PEP
alanine
Gly mTHF
Aspartate
TCA cycle
dadAX
glyA
sdaA
sdaB
ilvA
Thr
αKB
pyruvate
pyruvate
Ser
serC
ilvBN
ilvGM
ilvIH
tdh
3-PS
serB
AHB
AL
3-PHP
kbl
ilvC
serA
AcetylCoA
Gly
DHMV
DHIV
3-P glycerate
ilvD
leuABCD
αKMV
αKIV
Leu
ilvE
Ile
Val

Figure 1-1

The regulation of BCAA biosynthesis can also be achieved at the level of gene expression of the AHAS isozymes. AHAS I is encoded by *ilvBN*, AHAS II is encoded by *ilvGM*, and AHAS III is encoded by *ilvIH* (321). Not all bacteria may have all three functional AHAS isozymes. Due to genetic mutations, *E. coli* K-12 does not make a functional AHAS II and *S. typhimurium* (LT2) does not make a functional AHAS III (321). Analysis of the unfinished *A. pleuropneumoniae* serotype 1 genome indicates it may lack the *ilvB* gene encoding for the large subunit of AHAS I, but does contain genes similar to *ilvGM* and *ilvIH*.

In *E. coli*, the expression of the AHAS isozyme genes can be modulated depending upon what BCAAs are available. The expression of *ilvBN* is derepressed when either leucine or valine are unavailable and the expression of *ilvGM* is derepressed when any of the BCAAs are unavailable (321). The expression of *ilvBN* and *ilvGM* is regulated by an amino acid attenuation mechanism (321). The *leuABCD* operon is also controlled by an attenuation mechanism (321). However, the AHAS isozyme genes of *E. coli* and *Salmonella* are regulated by other means such as protein regulators. Cyclic AMP receptor protein (CRP) positively regulates *ilvBN* expression and integration host factor (IHF) regulates *ilvBN* and *ilvGM* (321).

One of the most extensively studied protein regulators in relation to BCAA biosynthesis in *E. coli* is leucine-responsive regulatory protein (Lrp). Lrp was first identified as a protein that bound to the *E. coli ilvIH* promoter and positively regulated the expression of *ilvIH* in response to the availability of leucine (252, 261). For this reason, Lrp was originally named the *ilvIH*-binding protein (IHB). Historically, Lrp has been independently identified and named OppI (252), *rblA* (313), and LivR (6, 129, 259).

The most extensive characterization of Lrp and its mode of regulation has been in *E. coli* regarding the control of the *ilvIH* operon. Lrp is a monomer but binds as a dimer to sites within the *ilvIH* promoter (334). *E. coli* cells grown on minimal media have ~3000 dimers per cell (345). The Lrp consensus binding site has been identified in *E. coli* to be NNNNN[$^{C}/_{T}$]AG[$^{A}/_{T}$\$^{C}$]A[$^{A}/_{T}$]ATT[$^{A}/_{T}$]T[$^{A}/_{T}$\$^{G}$]CT[$^{A}/_{G}$]NNNNN, where N=G, A, T, or C (67). The Lrp dimers can bind to multiple consensus sites within the *ilvIH* promoter and form a nucleoprotein complex that allows for transcription of *ilvIH* (56, 161, 334).

Structurally, the Lrp monomer has three domains. The N-terminal domain has a helix-turn-helix motif for binding DNA, the middle domain is involved in transcription activation through its interaction with other protein regulators and/or RNA polymerase, and the C-terminal domain is responsible for the leucine response of Lrp and for the ability of Lrp to form dimers and multimers (57, 251). In the case of *ilvIH* regulation, Lrp binds to the *ilvIH* promoter when leucine is not present and activates transcription. The C-terminal domain becomes important when leucine is present; leucine binds to the C-terminal domain of Lrp. When leucine is bound, Lrp does not bind to the *ilvIH* promoter and *ilvIH* expression is not activated. The C-terminal domain is also important for Lrp to form hexadecamers and octamers. When there is a micromolar concentration of Lrp *in vitro*, which is the concentration seen *in vivo* when grown on minimal media (345), Lrp forms hexadecamers and octamers with hexadecamers prevailing when leucine is absent (57). In the presence of millimolar concentrations of leucine, hexadecamers dissociate into octamers (57). In nanomolar Lrp concentrations *in vitro*, Lrp binds DNA as a dimer. Recently, Chen et al. (56) suggested the hexadecameric structure Lrp forms in solution *in vitro* is not important for the activation of *ilvIH*, but rather the dimer-dimer

interactions. Investigations into the dimeric and mulitmeric forms of Lrp and how this affects *ilvIH* expression is the focus of current research. While one type of Lrp regulation may apply to the *ilvIH* operon, Lrp has subsequently been shown to regulate a variety of different genes in a variety of different ways.

Lrp can both positively and negatively regulate gene expression and leucine may antagonize, potentiate, or have no affect on the regulation (reviewed in references 45, 48, 69, 230-232). As examples, *ilvIH* is positively regulated by Lrp and leucine is an antagonist (261); type 1 fimbriae of *E. coli* are are regulated positively by Lrp and leucine potentiates the effect (35, 110); the *sfaA* fimbrial biosynthesis gene is positively regulated by Lrp and leucine has no affect on this regulation (328); the serine deaminase I gene encoded by *sdaA* is repressed by Lrp and leucine acts as an antagonist (192); the *livJ* gene, involved in BCAA transport, is repressed by Lrp and leucine potentiates the effect (192); and Lrp represses its own gene and leucine has no effect on this regulation (192). In general, Lrp promotes the positive regulation of amino acid biosynthetic genes and the negative regulation of amino acid degradation genes. The pathways that best illustrate this generality are the BCAA biosynthetic and related pathways of *E. coli* (Figure 1). Lrp positively regulates, either directly or indirectly, the *ilvIH* operon involved in the biosynthesis of BCAAs (261), the *serA* and *serB* genes involved in serine biosynthesis (209, 259, 313), the *leuABCD* operon involved in leucine biosynthesis (186, 192, 309), and the *gltBDF* and *glnALG* operons involved in glutamate and glutamine biosynthesis, respectively (85, 86). In contrast, Lrp negatively regulates, either directly or indirectly, the *sdaA* and *glyA* genes involved in serine degradation (192, 232, 313), the *dadAX* operon involved in the degradation of alanine (213), the *tdh* and *kbl* genes involved in the

degradation of threonine (192, 259), and the *aidB* gene involved in the breakdown of leucine (187) and possibly isoleucine and valine (http://www.KEGG.com).

However, this type of regulation in *E. coli* may very well be different in other organisms. Within the *E. coli* genome, AHAS II is encoded by the *ilvGMEDA* operon and is negatively regulated by Lrp even though some of the genes do not code for degradative enzymes (260). In contrast, an analysis of the unfinished *A. pleuropneumoniae* serotype 1 genome shows *A. pleuropneumoniae* does not have a complete *ilvGMEDA* operon as *E. coli* does. Instead, *A. pleuropneumoniae* serotype 1 has *ilvGMD* followed by the gene, *gufA*, which encodes for a predicted divalent heavy-metal cation transporter. Given the difference in the order of the genes and the consistency of the operon, it is distinctly possible the genes in *A. pleuropneumoniae* may be regulated differently than they are in *E. coli*. In fact, there is evidence that genes regulated by Lrp differ between organisms. A microarray study showed Lrp affects 10% of the *E. coli* genome (306) and a proteome profile between wild-type and *lrp* mutant *E. coli* showed 30 differences (85). However, there were only 2 differences in the proteome profile of *Haemophilus influenzae* (101), a member of the *Pasteurellaceae* family to which *A. pleuropneumoniae* belongs. Friedberg et al. (101) suggest there may be large differences between the Lrp regulon of *Enterobacteriaceae* to be able to adjust to a wide range of environments they encounter, while organisms that do not encounter a wide range of environments may have a more specific use for Lrp.

Lrp has been identified and studied in many organisms in addition to *E. coli* and *H. influenzae*. These include *Agrobacterium tumefaciens* (60, 162), *Bacillus subtilis* (27, 29, 30, 73, 76), *Citrobacter rodentium* (64), *Enterobacter aerogenes* (102), *Klebsiella*

aerogenes (102, 163), *Methanococcus jannaschi* (243), *Mycobacterium tuberculosis* (290), *Proteus mirabilis* (130, 184), *Pseudomonas putida* (149, 202-206), *Pyrococcus furiosus* (44, 182, 189), *Rhodobacter capsulatus* (177), *Salmonella typhimurium* (102, 210, 233, 335), *Sinorhizobium meliloti* (200), *Sulfolobus acidocaldarius* (83), *Sulfolobus solfataricus* (28, 43, 227), *Zymomonas mobilis* (248, 249). Investigation within these species ranges from simple gene identification, cloning, and sequencing of Lrp homologues to more in depth studies involving the purification, mutation, and biochemical examination of Lrp and its role in regulation. Most studies on Lrp involve the investigation into the regulation of biosynthesis and catabolism genes by Lrp. However, there is some evidence Lrp plays a role in the regulation of genes involved in virulence.

Lrp is involved in the regulation of type I fimbriae in *E. coli*. By binding to a 314-bp element that contains the promoter for the *fimAICDFGH* fimbrial synthesis operon, Lrp can influence whether or not recombination of the element occurs through the action of the FimB and FimE recombinases. The orientation of the element will determine the orientation of the promoter and therefore whether or not fimbriae are expressed for attachment to host tissues (35, 110, 111). Lrp has also been associated with another type of phase variation, that of Pyelonephritis associated pili (pap). Pap pili allow uropathogenic *E. coli* to adhere to the epithelial cells of the urinary tract of the host and aid in attachment and colonization. Regulation of pap pilus expression is partly mediated by Lrp. Lrp can bind to multiple sites within the regulatory region of the pap biosynthesis genes and depending upon the sites to which it binds, allow or sterically inhibit the methylation of one or the other GATC sites found in the promoter (128, 326,

342, 343). Depending upon the methylation pattern at a set of methylation sites in the promoter, pap pili will or will not be synthesized. Lrp regulates additional fimbrial genes in *E. coli* such as *fanABC* (41, 42), *sfaA* (328), *daaABCDE* (33, 327), *foo* (72), and *fae* (143).

Beyond fimbriae, little is known about the role of Lrp in virulence. Recently, Fraser et al. (93) showed the hemolysin toxin of *Proteus mirabilis*, encoded by *hpmBA*, is activated directly by Lrp. They support this by showing that Lrp binds to the *hpmBA* promoter in electrophoretic mobility shift assays (EMSA), and that RNA from an *lrp* mutant does not hybridize with a *hpmA* probe in Northern blots. The expression of another hemolysin, encoded by *xhlA* in *Xenorhabdus nematophila* and required for virulence in insects, decreases in an *lrp* mutant but it is unknown whether this is a result of direct regulation by Lrp (65, 93). This evidence suggests Lrp may also play a role in the regulation of other virulence genes. Given this limited knowledge of the regulation of virulence genes by Lrp, more investigation is warranted to determine the full role of Lrp in virulence gene regulation.

Lrp regulates a variety of genes in a variety of different organisms. While a gene similar to *lrp* can be found in the unfinished *A. pleuropneumoniae* serotype 1 genome, the role of Lrp in *A. pleuropneumoniae* gene regulation is unknown and the role of Lrp in *A. pleuropneumoniae* virulence gene regulation has not been considered.

Scope of Dissertation

The scope of this dissertation is to study the genetic basis of virulence gene regulation in *A. pleuropneumoniae* and to determine an environmental stimulus and regulation mechanism that mediates virulence gene expression and disease. To do this, a library of *A. pleuropneumoniae in vivo* induced (*ivi*) genes was screened and a regulator of a subset of genes was identified.

Chapter two of this dissertation discusses the identification of a subset of *ivi* genes that are up-regulated in response to the limitation of BCAAs. While certain environmental stimuli have been identified to regulate virulence gene expression, little research had been done to investigate the limitation of BCAAs as one of those stimuli. The limitation of BCAAs as an important cue to some pathogens, such as respiratory pathogens, is discussed.

Chapter three of this dissertation discusses the role of Lrp in the regulation of genes involved in the virulence of *A. pleuropneumoniae*. Very little is known about the role of Lrp in virulence gene expression and even less about regulators of virulence gene expression in *A. pleuropneumoniae*. This chapter identifies an *A. pleuropneumoniae* Lrp homologue as a regulator of a subset of genes identified to respond to BCAA limiting conditions in *A. pleuropneumoniae*.

Chapter four discusses the role of Lrp in the ability of *A. pleuropneumoniae* to cause disease. No studies of bacterial pathogens that infect animals have investigated the role of Lrp in pathogenesis. To determine the role of Lrp in virulence, an *A. pleuropneumoniae* Lrp mutant is analyzed in a swine infection model.

Chapter five summarizes the conclusions of this thesis and offers suggestions for future experiments and directions to gain a better understanding of the genetic basis for how *A. pleuropneumoniae* causes disease.

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Chapter 2

A subset of *Actinobacillus pleuropneumoniae in vivo* induced promoters respond to branched-chain amino acid limitation.

Abstract

Actinobacillus pleuropneumoniae is the causative agent of both a chronic and acute form of necrotizing hemorrhagic pleuropneumonia in swine. To understand the genetic basis of this disease, we developed an *in vivo* expression technology system to identify genes whose expression are specifically induced during infection without the need to identify the complete constellation of host environmental cues required for the expression of each gene. In this study, we investigate the possibility that the limitation of branched-chain amino acids is a stimulus that *A. pleuropneumoniae* will encounter during infection and will respond to by up-regulation of genes involved in branched-chain amino acid biosynthesis and virulence. *A. pleuropneumoniae* genetic loci that are specifically induced during infection were screened *in vitro* for expression in response to defined environmental stimuli, including limitation of branched-chain amino acids. Of 32 *in vivo* induced promoter clones screened *in vitro* on chemically defined media with and without isoleucine, leucine, and valine (CDM +/- ILV), eight were induced in CDM –ILV compared to CDM +ILV. We identify the genomic context of each clone and discuss the relevance of each promoter to branched-chain amino acid limitation and virulence. We conclude that limitation of branched-chain amino acids can be a cue for expression of virulence genes in *A. pleuropneumoniae*.

Introduction

Actinobacillus pleuropneumoniae, a Gram-negative coccobacillus in the family *Pasteurellaceae*, is the causative agent of necrotizing hemorrhagic pleuropneumonia in swine (20, 32, 43, 51). This respiratory disease can be found in both acute and chronic forms, where acute forms can cause death in as little as twenty-four hours and chronic forms can result in carrier populations (20). The economic impact of either form can be significant when taking into account reduced weight gain due to infection and the cost of antibiotics and vaccines to treat and control the spread of infection (32).

The severe effect of the disease on the swine industry has led to research on development of improved vaccines and management practices that have ameliorated the economic effects in the United States, but how *A. pleuropneumoniae* infects and causes disease in swine is still not fully understood. It is known that *A. pleuropneumoniae* produces a variety of virulence factors, such as capsular polysaccharides that help the bacteria evade phagocytosis and complement mediated killing (61, 62), toxins and proteases that destroy host neutrophils (16, 42), iron utilization proteins to obtain iron within the host (15, 55), and lipopolysaccharide (LPS) that mediates attachment to host tissues (1, 10, 11, 47). While these key virulence factors have been identified, other as yet unrecognized factors likely also play a role in the virulence of *A. pleuropneumoniae*.

We have previously reported the development of an *in vivo* expression technology (IVET) system for *A. pleuropneumoniae* (24). An IVET system is designed to identify genes whose expression is induced during infection, without the need to identify the full constellation of environmental cues necessary for expression of each gene. However, to

analyze the role of these *in vivo* induced (*ivi*) genes in the disease process and to identify common regulatory pathways that contribute to virulence, it is useful to understand the specific cues that regulate expression of each *ivi* gene.

One of the *A. pleuropneumoniae ivi* genes identified in our studies is *ilvI*, which encodes an enzyme, acetohydroxy acid synthase (AHAS) isozyme III that catalyzes the reaction for the first step in the biosynthesis of the branched-chain amino acids (BCAA), isoleucine, leucine, and valine (24). In addition to the identification of *ilvI* as *in vivo* induced in our *A. pleuropneumoniae* swine lung infection IVET model (24), *ilvI* and *ilvD* mutants were attenuated in a signature-tagged mutagenesis (STM) study in *Neisseria meningitidis* using an infant rat bacteremia model (53) and *ilvA* was identified by STM as required for survival in *Pseudomonas aeruginosa* in a neutropenic mouse septicemia model (60). Another gene implicated in isoleucine biosynthesis, *yjgF*, was identified in an STM screen of *Pasteurella multocida* in a septicemic mouse model (23), and *thrB*, required for the biosynthesis of threonine, which is the precursor for isoleucine, was required for survival of *Staphylococcus aureus* in an STM study using a murine model of bacteremia (38). Recent studies by Ulrich et al. (59) and Atkins et al. (4) showed that *ilvI* mutants of the lung pathogens *Burkholderia mallei* and *B. pseudomallei* were attenuated in mice using an aerosol model. A *leuD* mutation was shown to attenuate the growth of *Mycobacterium. bovis* in macrophages (37) and the virulence of the lung pathogen *M. tuberculosis* (8, 31). Furthermore, inhibitors of BCAA biosynthetic enzymes can prevent the growth of *M. tuberculosis* in the lungs (28, 65). These studies suggest that BCAA biosynthesis is required for survival and virulence in clean sites in the body, such as the lung and blood stream. BCAA biosynthetic genes have not been similarly identified as

critical for virulence in the gastrointestinal tract. Indeed, while leucine auxotrophs were shown to be avirulent in mice in a *Salmonella* systemic model of infection using intraperitoneal injection (5, 6), no genes involved in BCAA biosynthesis have been identified in IVET or STM studies of *Salmonella* using enteric infection models. Furthermore, an STM study of *Vibrio cholerae* in an infant rat gut model showed that four STM mutants that were auxotrophic for isoleucine or isoleucine and valine were not attenuated in virulence (14).

A possible explanation for the requirement for BCAA biosynthesis for bacterial survival in clean sites within the body, but not for survival within the intestinal tract, would be that these amino acids are present in only limited supply within these tissues. Most mammals, including pigs and humans, can not synthesize BCAAs and require these amino acids in their diets. Nutritional studies have shown that the concentration of free amino acids rise in plasma after a protein meal and decrease shortly after. Since the intestinal tract is the entry point of the host's diet and the free amino acids in plasma rise sharply after a protein meal, the free amino acid concentration of all amino acids, including BCAAs, should be high in the gut, although this may vary cyclically with diet. Amino acid concentrations in interstitial fluid are similar to those in plasma (29). In contrast, intracellular concentrations of free amino acids within muscle cells are in general significantly higher (5-10 fold or more) than in the surrounding interstitial fluid, with the exception of several of the essential amino acids, including isoleucine, leucine, and valine, reflecting the cellular biosynthesis of non-essential amino acids (12). Amino acid concentrations in cerebrospinal fluid (CSF), with the exception of glutamine, are significantly lower than in plasma (39). While we are not aware of any studies that have

quantitatively analyzed the concentration of individual free amino acids in the lung, it would be reasonable to predict that these levels are similar to those found in plasma and interstitial fluid.

Qualitative studies suggest that the free amino acid concentration in normal lungs may be low enough to require an infecting pathogen to synthesize their own amino acids rather than survive on exogenous sources. In a recent study, sputum samples from patients with cystic fibrosis (CF) were shown to have on average at least twice the total amino acid content as found in sputum from a control group of non-CF patients (9). When sputum specimens from these CF patients were incorporated into minimal agar medium, 21/22 samples supported the growth of *P. aeruginosa* isolates that were auxotrophic for a variety of amino acids, including isolates requiring leucine, isoleucine, and valine (9). In contrast, 5/6 sputum specimens from control non-CF patients failed to support growth of any of the auxotrophic strains. These results suggest that while *P. aeruginosa* auxotrophs are able to obtain enough amino acids, including BCAAs, exogenously in the lung of a CF patient to survive and multiply, the levels of free amino acids in the sputum of normal individuals are too low to support the growth of bacteria that can not synthesize their own amino acids.

The importance of the expression of the BCAA biosynthetic genes during infection of the host demonstrated by IVET and STM studies suggests the hypothesis that a limitation of BCAAs acts as a signal to induce expression of not only BCAA biosynthetic genes but also other *ivi* genes, which is similar to that which has been well documented for iron limitation.

To test this hypothesis, we investigated whether the limitation of BCAAs is a signal for the induction of *ivi* genes identified using our *A. pleuropneumoniae* IVET system. In this paper, we report the identification of eight *ivi* clones whose expression is modulated by BCAA limitation.

Materials and Methods

Bacterial Strains and growth conditions. The *A. pleuropneumoniae* strains used in this study were AP225 (ATCC 27088), a virulent, nalidixic acid-resistant, serotype 1 strain, and AP233 (25), an avirulent, nalidixic acid-resistant, riboflavin auxotroph derivative of AP225. The plasmids used in this study are listed in Table 1. *A. pleuropneumoniae* strains were cultured in brain heart infusion (BHI; Difco laboratories, Detroit, MI) or chemically defined medium (CDM), incubated at either 35°C with 5% CO_2 for agar media or 35°C and 150 rpm for broth media. The CDM is a modification of the defined medium described by Herriott for *Haemophilus influenzae*(30), with the amino acid stock solution from the *Neisseria* defined medium developed by Morse and Bartenstein (40) substituted for Herriott's amino acid stock solution. The composition of the complete medium and the concentration of its components are listed in Table 2. Solid defined medium was prepared by adding a double-strength solution of the liquid medium to an equal volume of sterile agar (30 g/L). BHI medium was supplemented with nicotinamide adenine dinucleotide (NAD; Sigma Chemical Company, St. Louis, MO) to a final concentration of 10 μg/ml. For growth of AP233, both BHI and CDM media were supplemented with riboflavin (Sigma) to a final concentration of 200 μg/ml. Ampicillin, when required, was added to 20 μg/ml for plasmid selection in *A. pleuropneumoniae*.

When investigating the response of *A. pleuropneumoniae* to the limitation of BCAAs, the amino acids isoleucine, leucine, and valine were excluded from the CDM. For analysis of gene expression on solid media, bacterial strains were picked from 18 h cultures on BHI agar using sterile wooden applicators, spotted onto CDM agar, and

incubated for 48 h. For analysis of gene expression in CDM broth, bacterial strains grown for ~18 h on BHI agar medium were inoculated into 30 ml of CDM broth in 300-ml baffled side arm flasks to an optical density of ~0.1 at 520 nm (OD_{520}) and incubated.

Table 2-1. Characteristics of plasmids used in this study

Plasmid	Characteristic(s)	Reference
pTF86	*A. pleuropneumoniae* IVET vector containing promoterless *luxAB* and *ribBAH* genes downstream of a unique *Bam*HI cloning site.	(24)
pTF7	pTF86 containing a 733-bp insert containing the promoter to the *A. pleuropneumoniae* DNA helicase gene, *dnaB*. The promoter is strongly expressed both *in vivo* during infection and *in vitro* on laboratory medium.	(22)
p*ivi*A	pTF86 containing a 333-bp insert.	(24)
p*ivi*G	pTF86 containing a 211-bp insert.	(24)
p*ivi*I[a]	pTF86 containing a 623-bp insert.	(24)
p*ivi*P[a]	pTF86 containing a 175-bp insert.	(33)
p*ivi*S	pTF86 containing a 352-bp insert.	(33)
p*ivi*U	pTF86 containing a 605-bp insert.	(33)
p*ivi*X	pTF86 containing a 507-bp insert.	(33)
p*ivi*Y	pTF86 containing a 782-bp insert.	(33)
p*ivi*17g	pTF86 containing a 290-bp insert.	(33)

[a] The insert had two genomic fragments inserted into pTF86. The given fragment size is of the one adjacent to the promoterless *luxAB* genes.

Table 2-2. Composition of chemically defined medium for *Actinobacillus pleuropneumoniae*[a]

Component	Concn. (g/L) of stock soln.	Vol. (mL) of stock soln./L of medium	Final conc., mM
Stock solution I		100 ml	
Aspartic acid	5.0		3.76
Glutamic acid	13.0		8.84
NaCl	58.0		100.00
K_2SO_4	10.0		5.75
$MgCl_2$	2.0		2.11
$CaCl_2$	0.222		0.20
EDTA	0.037		0.01
NH_4Cl	2.2		4.07
Stock solution II		80 ml	
Alanine	0.40		0.37
Arginine	0.60		0.24
Asparagine	0.10		0.06
Aspartic acid	2.00		1.25
Cysteine-HCl	0.44		0.70
Cystine[b]	0.28		0.30
Glutamic acid	5.20		2.95
Glutamine	0.20		0.11
Glycine	0.10		0.11
Histidine	0.10		0.04
Isoleucine	0.12		0.08
Leucine	0.36		0.23
Lysine	0.20		0.09
Methionine	0.06		0.03
Phenylalanine	0.10		0.05
Proline	0.20		0.14
Serine	0.20		0.16
Threonine	0.20		0.14
Tryptophan	0.32		0.13
Tyrosine	0.28		0.13
Valine	0.24		0.17
Glutathione (reduced)	0.18		0.05
Stock solution III		20	
Tween 80	1 ml		0.002%
Polyvinyl alcohol	1		0.002%
Glycerol	150 ml		0.30%

Table 2-2 (cont'd).

Component	Concn. (g/L) of stock soln.	Vol. (mL) of stock soln./L of medium	Final conc., mM
Stock solution IV		50	
Uracil[b]	2.0		0.89
Hypoxanthine[b]	1.2		0.44
Solution V		100	
Inosine	10.0		3.73
K_2HPO_4	17.4		10.00
KH_2PO_4	13.6		10.00
Solution VI		0.4	
Thiamine	10.0		0.012
Calcium pantothenate	10.0		0.017
Other stock solutions			
Glucose	180.2	10.0	10
$FeCl_3 \cdot 6H_2O$	0.27	10.0	0.01
NAD	10.0	5.0	0.075

[a] Stock solutions can be prepared individually and then sterilized by autoclaving (solutions I, III, and glucose) or filtration (solutions II, IV, V, and VI) and refrigerated, except for solution VI which should be stored at -20°C. All stock solutions are combined in the proper amounts, adjusted to pH 7.5, and brought to volume with distilled water. The medium is sterilized by filtration and freshly prepared $FeCl_3$ and NAD are added immediately prior to inoculation.

[b] L-cystine was first dissolved in 0.1 N HCl. Hypoxanthine and uracil were dissolved in 0.1 N NaOH.

Luciferase Assays. For qualitative measurement of luciferase activity, the Night Owl molecular light imager LB981 (EG&G Berthold, Oakridge, TN) and WinLight software (EG&G Berthold) were used to analyze the luminescence of *ivi* clones and assign a color from a spectrum relating to the intensity of luciferase expression. Colonies on agar plates were exposed for 2 min to 50 µl N-decyl aldehyde spread evenly across a glass Petri dish lid. The photonic camera was set to a sample format of 120 mm, exposure time of 20 s, time limit of 1 min, single frame accumulation, camera termes of high gain, a 2x2 pixel beginning, and defect correction post processing.

For quantitative measurement of luciferase activity, a Turner model 20e luminometer was utilized as previously described (24). Briefly, 20 µl of broth culture was added to 20 µl of luciferase substrate and mixed for 10 s. The substrate was made by dissolving 20 mg/ml Essentially Fatty acid Free bovine serum albumin (BSA; Sigma) and 1 µl of N-decyl aldehyde in 1 ml of H_2O and sonicating the solution. The luminometer was set to a delay of 10 s, integration of 30 s, and a sensitivity of 39.9%. The luminometer relative light unit (RLU) readings were normalized to optical density of the culture.

Sequence Analysis. The complete DNA sequences of *ivi* clone inserts were previously determined (22, 33), and have been deposited in GenBank under the following accession numbers: clone *ivi*A, DQ370062; *ivi*G, DQ370063; *ivi*I, DQ370055; *ivi*P, DQ370061; *ivi*S, DQ370056; *ivi*U, DQ370060; *ivi*X, DQ370059; *ivi*Y, DQ370058; *ivi*17g, DQ370057; TF7, DQ376028. Computer analysis of the sequences was performed using the Lasergene DNAstar suite of programs (Lasergene, Madison, WI) in addition to the

National Center for Biotechnology Information (NCBI) BLAST algorithms (http://www.ncbi.nlm.nih.gov/BLAST). Identification of putative genes within the *ivi* clone inserts was performed by comparing the translated *ivi* clone insert DNA sequence to GenBank protein submissions using NCBI's BLASTX algorithm. Orientation of putative genes in relation to the *ivi* clone insert was determined by analysis of the unfinished *A. pleuropneumoniae* serotype 1 genome contigs submitted to GenBank.

Results and discussion

***Ivi* Clone Response to BCAA Limitation.** We have previously isolated and identified 32 *A. pleuropneumoniae ivi* clones using an IVET system in a swine animal model (24, 33). Each clone represents an *A. pleuropneumoniae* strain that contains the promoter trap IVET plasmid, pTF86, which consists of promoterless luciferase genes, *luxAB*, promoterless riboflavin genes, *ribBAH*, and a unique *Bam*HI site into which *A. pleuropneumoniae* serotype 1 *Sau*3A1 digested genomic DNA fragments were cloned. When a functional promoter is cloned into pTF86 in the proper orientation, both the luciferase and riboflavin genes are expressed when the promoter is active. Expression of the riboflavin genes complements the deletion in the riboflavin biosynthetic pathway of the attenuated AP233 strain and restores virulence. Expression of the promoterless luciferase genes can be used to measure the promoter activity, both *in vivo* and *in vitro*, by measuring luciferase activity. Each *ivi* clone contains a unique *A. pleuropneumoniae* genomic DNA fragment that allows expression of *ribBAH* and *luxAB in vivo* during infection but not *in vitro* on BHI medium. Each AP233 *ivi* clone was verified as being capable of survival *in vivo* by infection of and reisolation from a second experimentally infected pig.

The identification of BCAA biosynthetic genes as being important during infection, and the possibility of BCAAs being limiting at certain sites of infection, led to the hypothesis that the limitation of BCAAs is a signal to induce the expression of *ivi* genes during infection. To address this hypothesis, we analyzed the *in vitro* activity of the luciferase reporter of the 32 *ivi* clones under BCAA limiting conditions. For

qualitative assays, all 32 *ivi* clones were grown on complete CDM agar (+ILV) or CDM lacking the BCAAs isoleucine, leucine, and valine (-ILV). Eight clones, *ivi*G, *ivi*I, *ivi*P, *ivi*S, *ivi*U, *ivi*X, *ivi*Y, and *ivi*17g, were identified as having putative promoters that were induced on CDM –ILV in comparison to CDM +ILV (Figure 1). Other *ivi* clones, such as *ivi*A, displayed no visible induction on CDM –ILV as compared to CDM +ILV. Several *ivi* clones, including *ivi*P, *ivi*I, *ivi*S, and *ivi*17g also showed an increase in luciferase expression on CDM +ILV compared to growth on BHI (data not shown), suggesting a broad response to CDM in addition to the specific response to BCAA limitation.

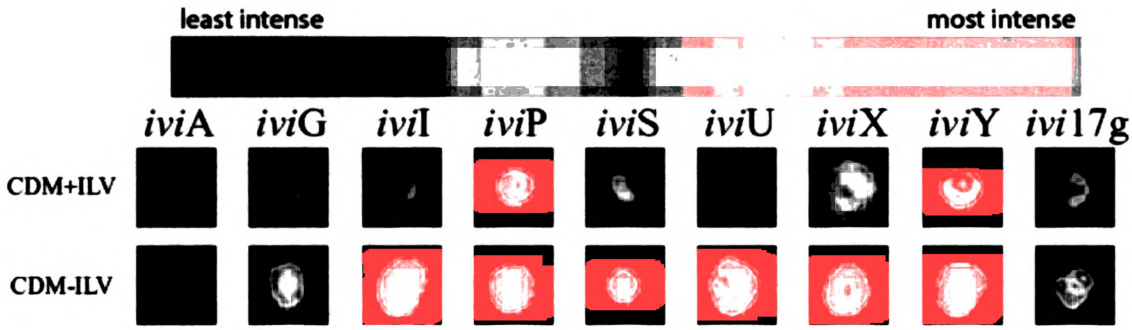

Figure 2-1. Expression of a subset of AP233 *ivi* promoter clones on CDM-ILV agar. *Ivi*G, *ivi*I, *ivi*P, *iviS*, *ivi*U, *ivi*X, *ivi*Y, and *ivi*17g were grown *in vitro* on CDM+ILV and CDM-ILV agar media. A color spectrum legend displays a range of colors relating to the activity of luciferase from the least active (least intense) to most active (more intense). Data presented are from one of four representative experiments. Images in this thesis/dissertation are presented in color.

The luciferase expression of the eight *ivi* clones identified to have increased luciferase activity on CDM –ILV agar medium was analyzed in CDM +/- ILV broth to quantitate luciferase expression (Figure 2). The largest difference in luciferase activity was observed in the *ivi*I clone. A rapid and robust induction of luciferase activity is evident in the first hour of growth of this clone in CDM –ILV when compared to CDM

+ILV. This induction was observed as early as 20 min after inoculation into CDM –ILV (data not shown). The maximum luciferase activity of the *ivi*I clone was observed after 3 h of growth and was 9-fold higher in CDM –ILV when compared to CDM +ILV. The greatest difference occurred at 5 h with an observed over 13-fold difference between CDM –ILV and CDM +ILV. While the induction of luciferase activity in the other seven clones was not as rapid or robust as that displayed by *ivi*I in CDM –ILV, the clones *ivi*G, *ivi*P, and *ivi*X did display significantly higher luciferase activity in CDM –ILV, as compared to CDM +ILV, after 2 h of growth in CDM. In these three clones, luciferase activity in CDM –ILV was generally 3-4 fold higher than that seen in CDM +ILV. Although these four clones differed in the magnitude of luciferase activity, the patterns of luciferase activity over time are similar. In contrast to the *ivi*I, *ivi*G, *ivi*P, and *ivi*X clones, *ivi*U, *ivi*Y, and *ivi*17g are characterized by a slower response to CDM -ILV. In these three clones, it was at least 4-5 h before a significant difference between activity in CDM –ILV and CDM +ILV was observed. The final clone, *ivi*S, did not display a significant difference between luciferase activity in CDM –ILV and CDM +ILV broth media.

Seven of the eight clones tested displayed a significant increase in luciferase activity in CDM –ILV broth compared to CDM +ILV broth at some point during the experiment. These data, together with the luciferase activity of the clones on CDM agar medium, support our hypothesis that a limitation of BCAAs acts as a signal for induction of *ivi* genes.

Figure 2-2. Expression of a subset of AP225 *ivi* promoter clones in CDM-ILV broth. *Ivi*G, *ivi*I, *ivi*P, *iviS*, *ivi*U, *ivi*X, *ivi*Y, and *ivi*17g were grown *in vitro* in CDM +ILV (▲) and CDM -ILV(■) broth. Samples of growing cultures were analyzed for luciferase activity every hour for eight hours. Data points are averages of three experiments with bars indicating standard deviations. There was minimal difference in luciferase activity of the negative control, AP225/pTF7, between the two conditions (data not shown).

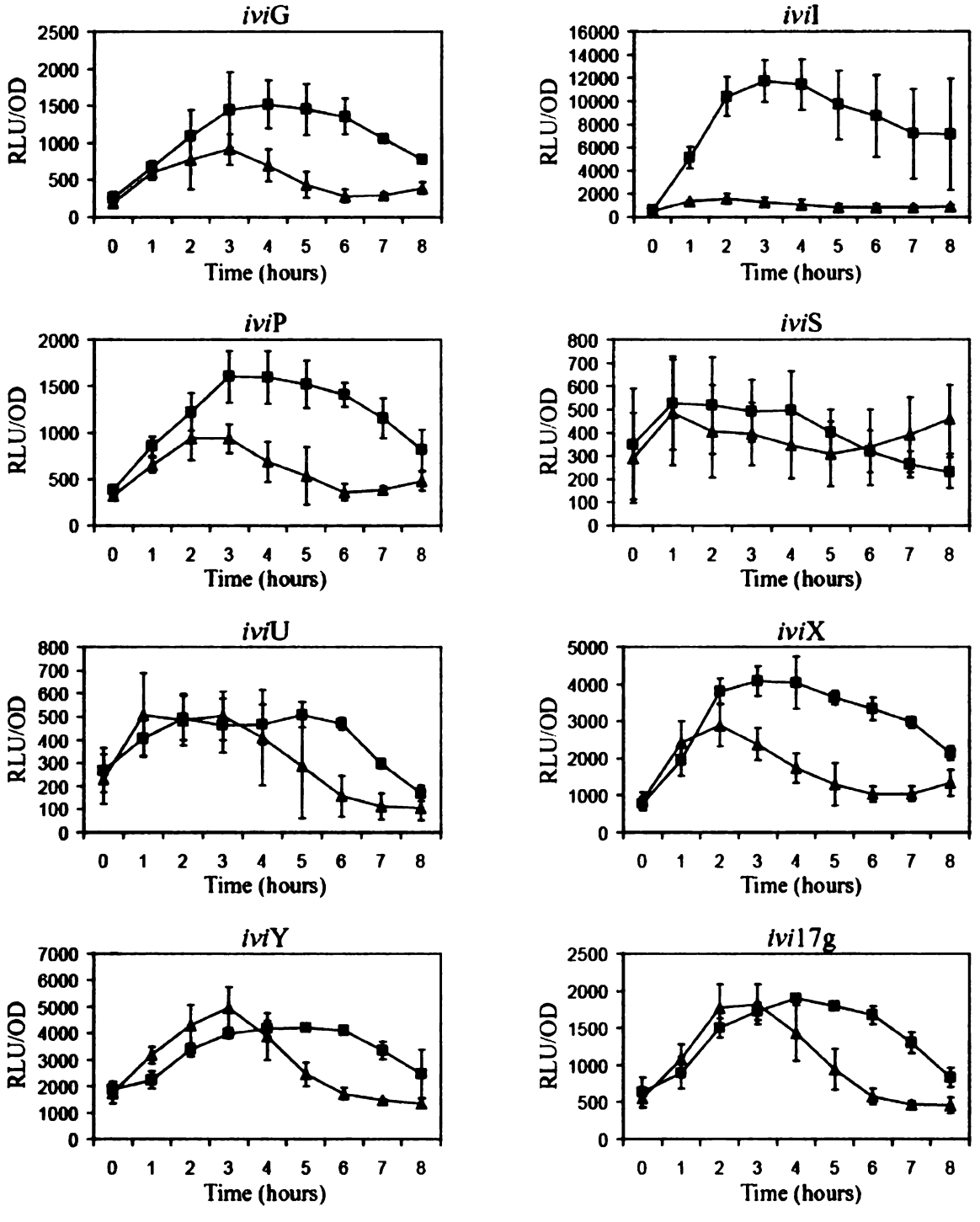

Figure 2-2

The temporal expression of genes in CDM could explain the lack of observed differences in luciferase activity between CDM –ILV and CDM +ILV broth of the *ivi*S clone. As an example, a clone such as *ivi*I in broth responds rapidly, robustly, and stays at a high level of activity throughout the experiment (Figure 2). In contrast, a clone such as *ivi*Y varies in activity over time (Figure 2). It is possible the observed difference of *ivi*S on agar medium could have been at a time when differential luciferase activity was the greatest, for example, in stationary phase, for this clone. In contrast, the time or conditions chosen for analysis of *ivi*S in broth may not have been optimal to observe a difference between CDM +ILV and CDM –ILV.

As with CDM agar media, a broad response to CDM broth medium was observed. All eight *ivi* clones displayed an increase in luciferase activity in the first hour of growth in CDM, irrespective of whether isoleucine, leucine, and valine were present. The *ivi* clones could be initially responding to a shift to a minimal medium such as CDM. However, these clones, with the exception of *ivi*S, are also clearly responding to the limitation of BCAAs, as demonstrated by the observed significant differences in expression between –ILV and +ILV conditions.

Identification of *ivi* genes induced on CDM –ILV media. For each of the *ivi* clones that demonstrated an increased luciferase activity in response to BCAA limitation, the complete nucleotide sequences and predicted amino acid sequences of the inserts were analyzed and compared to GenBank protein sequence submissions using BLASTX (3) to identify sequence similarity to known coding regions. Table 3 shows the bioinformatic results of the GenBank searches while Figure 3 illustrates the approximate size and

orientation of the cloned IVET insert with respect to adjacent coding regions. Homologues of all eight of these loci were found in the unfinished *A. pleuropneumoniae* serotype 1 genome submission and all but *ivi*G were found in the *A. pleuropneumoniae* serotype 5 genome provided by the National Research Council of Canada.

The p*ivi*I insert sequence is 623 bp and has been previously reported to be in the same orientation and have sequence similarity to the 5' end of the *ilvI* gene of *H. influenzae*, a gene involved in the biosynthesis of BCAAs (24). The *ilv*I gene is part of the *ilvIH* operon in *E. coli* and encodes the large subunit of AHAS III involved in BCAA biosynthesis. Three isozymes of AHAS, named AHAS I, II, and III, have been identified in *E. coli* and *Salmonella typhimurium* and are encoded by the operons *ilvBN*, *ilvGM*, and *ilvIH*, respectively. The AHAS isozymes can catalyze the transfer of the active aldehyde group of pyruvate to either ά-ketobutyrate or pyruvate to form acetohydoxybutyrate or acetolactate in the parallel isoleucine or valine biosynthetic pathways, respectively. *A. pleuropneumoniae* contains an intact *ilvIH* operon, as well as a putative *ilvGM* operon for AHAS II, but does not appear to have the *ilvBN* operon for encoding the isozyme AHAS I. The *ilvIH* operon would be predicted to be up-regulated rapidly and robustly in a –ILV environment due to the requirement to synthesize isoleucine, leucine, and valine for survival under these conditions. Other genes may not need to respond as quickly or robustly to these conditions.

Table 2-3. *In vivo* induced genes identified via BCAA limitation

Upstream Gene			*Ivi* clone	Downstream Gene		
Aple locus tag[a]	COG	Sequence similarity[b]		Aple locus tag[a]	COG	Sequence Similarity[b]
N.A.	COG1887: Putative glycosyl/glycerophosphate transferases	*cps1B*, *A. pleuropneumoniae*, E=$1e^{-81}$	*ivi*G	N.A.	No COG listed. Similar to glycosyl transferases	*cps1A*, *A. pleuropneumoniae*, E=0.0
Aple02002220	COG0288, Carbonic anhydrase	*cynT*, *Mannheimia succiniciproducens* MBEL55E, E=e^{-100}	*ivi*I	Aple02001991	COG0028: Thiamine pyrophosphate-requiring enzymes	*ilvI*, *Haemophilus influenzae* Rd KW20, E=$5e^{-38}$
Aple02000242	COG3023, Negative regulator of beta-lactamase expression	*ampD*, *Haemophilus ducreyi* 35000HP, E=$3e^{-79}$	*ivi*P	Aple02000241	COG2961, Protein involved in catabolism of external DNA	*comJ*, *Haemophilus influenzae*, E=e^{-122}
Aple02000645	COG0324, tRNA delta(2)-isopentenylpyrophosphate transferase	*miaA*, *Haemophilus ducreyi* 35000HP, E=e^{-140}	*ivi*S	Aple02000644	COG1923, Uncharacterized host factor I protein	*hfq*, *Haemophilus ducreyi* 35000HP, E=$7e^{-35}$
Aple02000022	COG0716, Flavodoxins	*mioC*, *Haemophilus ducreyi* 35000HP, E=$4e^{-59}$	*ivi*U	Aple02000023	COG1070, Sugar (pentulose and hexulose) kinases	*xylB*, *Mannheimia succiniciproducens* MBEL55E, E=0.0
Aple02001039	COG0086, DNA-directed RNA polymerase, beta' subunit	*rpoC*, *Haemophilus ducreyi* 35000HP, E=0.0	*ivi*X	Aple02001038	COG4456, Virulence-associated protein	*vap*B, *Dichelobacter nodosus*, E=$2e^{-5}$

Table 2-3 (cont'd).

Upstream Gene			*Ivi* clone	Downstream Gene		
Aple locus tag[a]	COG	Sequence similarity[b]		Aple locus tag[a]	COG	Sequence Similarity[b]
Aple02000780	COG0742, N6-adenine-specific methylase	*yhhF*, *Escherichia coli* O157:H7 EDL933, E=$2e^{-42}$	*ivi*Y	Aple02000779	COG0552, Signal recognition particle GTPase receptor	*ftsY*, *Haemophilus ducreyi* 35000HP, E=e^{-161}
Aple02000134	COG3024, Uncharacterized protein conserved in bacteria	*yacG*, *Escherichia coli* CFT073, E=$9e^{-14}$	*ivi*17g	Aple02000135	COG0237, Dephospho-CoA kinase	*coaE*, *Haemophilus ducreyi* 35000HP, E=$7e^{-72}$

[a] The *A. pleuropneumoniae* serovar 1 str. 4074 locus tags were used when available. In the case of *ivi*G, sequence was taken from an unannotated Oklahoma State *A. pleuropneumoniae* contig 269 and therefore had no locus tag.

[b] Genbank sequences were searched using BLASTX using *A. pleuropneumoniae* serotype 1 str. 4074 loci sequences. Results exclude matches to the *A. pleuropneumoniae* 1 str. 4074 database.

Figure 2-3. Graphical representations of *A. pleuropneumoniae* gene orientations in the IVET clone insert region. The black arrows represent the location, orientation, and relative size of the insert within the *ivi* clones (*ivi*G, I, P, S, U, X, Y, and 17g) that triggered the expression of the promoterless luciferase genes within the IVET vector, pTF86, in response to BCAA limitation. The white arrows represent the orientation of the ORF within the region of the *A. pleuropneumoniae* serotype 1 genome. All sizes are approximate.

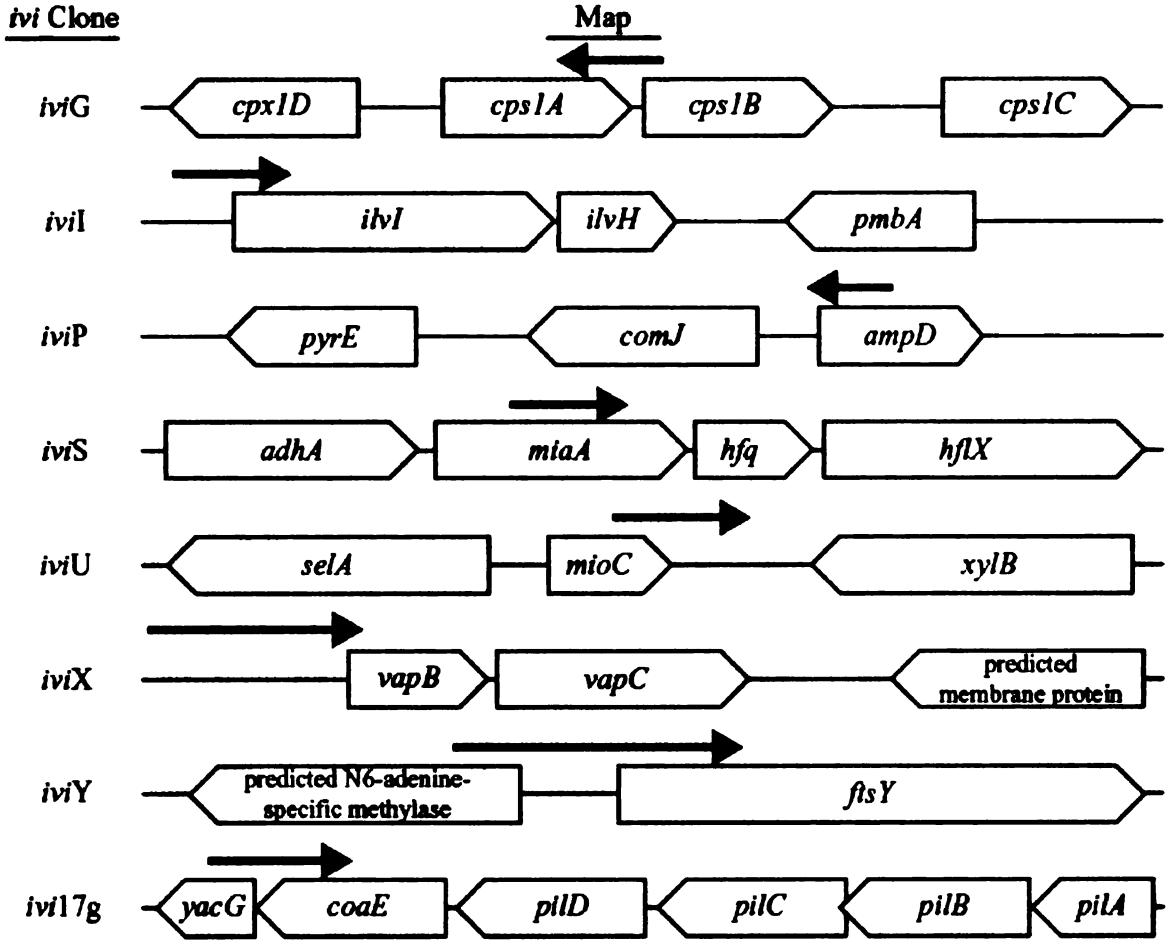
ivi Clone
Map
iviG
cpx1D
cps1A
cps1B
cps1C
iviI
ilvI
ilvH
pmbA
iviP
pyrE
comJ
ampD
iviS
adhA
miaA
hfq
hflX
iviU
selA
mioC
xylB
iviX
vapB
vapC
predicted membrane protein
iviY
predicted N6-adenine-specific methylase
ftsY
ivi17g
yacG
coaE
pilD
pilC
pilB
pilA

Figure 2-3

The p*ivi*X insert of 507 bp does not contain a partial ORF. However, when it is aligned with the *A. pleuropneumoniae* serotype 1 genome sequence, it is located immediately upstream and in the same orientation as a 239 bp ORF with sequence similarity to the *vapB* gene of *Dichelobacter nodosus*. The *vapB* gene has also been annotated as *mvpT* and *vagC* in *Shigella flexneri* and *Idiomarina loihiensis*, respectively (46, 48). This gene has been classified as a virulence-associated protein and has been implicated in the maintenance of the large virulence plasmid of *S. flexneri* through a toxin and antidote post-segregational killing system (50). The two gene toxin/antidote system, *vapBC*, was first described by Pullinger et al. (45) as a virulence plasmid encoded system within *Salmonella dublin* that both conferred the ability to grow in nutrient limiting conditions and was required for plasmid maintenance. A mutant of *vapB* has been shown in *S. dublin* to have reduced virulence (63). Genes similar to this toxin/antidote system have also been identified in the chromosomes of 126 prokaryotes (44). Chromosomally encoded toxin/antidote systems have been shown to target DNA gyrase and mRNA transcripts and proposed to induce a bacteriostatic condition suitable for survival under nutrient stress (reviewed in reference 26). The target of the *vapBC* system is currently unknown, but the system could be involved in induction of a bacteriostatic condition as previously mentioned, allowing for quality control of translational products by reducing the rate of translation, or stabilization of adjacent mobile genetic elements within the chromosome under nutrient limitation (26).

The p*ivi*Y insert is 782 bp in length and includes a partial ORF in the same orientation as the p*ivi*Y insert sequence. The ORF has sequence similarity to the signal recognition particle receptor (SR), *ftsY*, of *H. ducreyi*. The SR has been shown, along

with the signal recognition particle (SRP), to be involved in the targeting of nascent proteins to the bacterial plasma membrane (reviewed in references 17, 35, 52). Upregulation of *ftsY* under BCAA limitation could reflect a need for new proteins such as sensors, receptors, or uptake systems to be targeted to the cell membrane under these conditions.

The p*ivi*S insert has sequence similarity to the middle of the *miaA* gene of *H. ducreyi* and shares the same orientation. The *miaA* gene sequence from *E. coli* encodes a tRNA delta(2)-isopentenylpyrophosphate transferase that is involved in modifying tRNAs which recognize codons beginning with a uridine, such as those for cysteine, leucine, phenylalanine, serine, tryptophan, and tyrosine, and has been shown to be involved the attenuation of the *trp* operon in *E. coli* (19, 34, 64). Downstream of *miaA* gene and the p*ivi*S insert sequence, in the same orientation as the insert sequence, is an ORF with sequence similarity to the *hfq* gene of *H. ducreyi*. The *hfq* gene in *E. coli* encodes the translational regulator, host factor I (HF-I) protein. HF-I has been shown to be a global regulator affecting the expression of more than 30 proteins by altering translation efficiency or transcript stability of mRNA targets (41). HF-I has been shown to negatively regulate the amount of MiaA in both growing and stationary-phase bacteria even though *hfq* expression increases in stationary phase growth (56). While the p*ivi*S insert sequence has sequence similarity to the middle of the *miaA* gene, one report identifies a second promoter for *hfq* expression, $P2_{hfq}$, within this region (57). This would suggest that the *ivi* gene controlled by the promoter in p*ivi*S is *hfq* and not *miaA*. HF-I may be involved in the derepression of attenuated biosynthetic operons that require

tRNAs modified by MiaA, such as for leucine, since it has been shown that the *trp* operon is derepressed through the action of HF-I on MiaA (13).

The p*ivi*P insert has sequence similarity to the *ampD* gene of *H. ducreyi* but has an orientation that is antisense to *ampD*. *AmpD* has been identified as a negative regulator of beta-lactamase expression and as an enzyme involved in cell wall synthesis and intracellular recycling of peptidoglycan fragments. Downstream of the p*ivi*P insert sequence, in the same orientation, is an ORF with sequence similarity to the *comJ* gene. The *comJ* gene has been associated with the competence-induced operon of *H. influenzae* (54) and a mutation consisting of part of the *comJ* gene lead to a decrease in transformation efficiency, DNA binding, and DNA uptake (18). In addition, the *comJ* gene, also known as *yhiR* in *E. coli*, has been implicated in the use of external DNA as a nutrient (21). Either cell wall synthesis is down regulated in response to BCAA limitation through antisense control of *ampD*, or transformation efficiency and potential acquisition of DNA as a nutrient is up-regulated.

The p*ivi*G insert has sequence similarity to 159 bp of the terminal 3' end of the *cps1A* gene and 43 bp of the terminal 5' end of the *cps1B* gene of *A. pleuropneumoniae* serotype 1, but is antisense to both *cps1A* and *cps1B*. *Cps1A* and *cps1B* are involved in the synthesis of *A. pleuropneumoniae* capsular polysaccharide and mutations in these genes affect its virulence in pigs (7), although the specific function of each gene is unknown at this time. The *cps1A* gene of *A. pleuropneumoniae* has sequence similarity to the *fcs1*, *lcbA*, and *xcbA* genes of *H. influenzae* serotype f, *Neisseria meningitidis* serotype L, and *N. meningitidis* serotype X, respectively. All three of these organisms have a similar organization to the three gene *cps1ABC* cluster in *A. pleuropneumoniae*.

Both the *fcs123* and *xcbABC* genes have been shown to be transcribed as operons (49, 58). The reason for a possible promoter within the p*ivi*G insert sequence that is antisense to *cps1AB* is unclear. *A. pleuropneumoniae* serotype 1 capsular polysaccharide is composed of a repeating disaccharide unit containing N-acetyl-2-dioxy-β-D-glucopyranosyl and α-D-galactopyranosyl, with *O*-acetyl groups present in 85% of the repeating units (2). An antisense message could potentially affect the level of *O*-acetylation by decreasing the availability of a transcript from one of the genes potentially involved in the *O*-acetylation reaction, which could alter the antigenic structure of the capsule. Alternatively, an antisense message could modulate expression of capsule biosynthetic genes, reducing the amount of capsule to expose surface adhesins necessary for attachment to respiratory epithelial cells. Clarification of the actual role of each capsule gene is needed to clearly identify the role of an antisense transcript.

The p*ivi*U insert overlaps 207 bp of the 3' end of the gene, *mioC*. Therefore, a promoter within the p*ivi*U insert sequence would most likely not affect the expression of the *mioC* gene, but rather a gene farther downstream. An ORF 441 bp downstream of the p*ivi*U insert sequence and in an antisense orientation has sequence similarity to the *xylB* gene of *Mannheimia succiniciproducens*. The *xylB* gene is annotated as a sugar kinase and is normally part of the *xylAB* operon, which converts xylose to xylulose-5-phosphate, which can then be utilized in the pentose phosphate pathway or the phosphoketolase pathway (27). However, analysis of the *A. pleuropneumoniae* serotype 1 str. 4074 unfinished genome shows that *xylB* is not linked to *xylA*. The potential role of an antisense message to the *xylB* gene that is induced during infection is unclear.

The p*ivi*17g insert has sequence similarity to 102 bp of the terminal 5' end of the 109-bp *yacG* gene of *E. coli* and 207 bp of the terminal 3' end of the *coaE* gene of *Haemophilus decreyi*, but has an orientation that is antisense to both genes. YacG is a hypothetical protein with no known function. The *coaE* gene encodes an enzyme that catalyzes the conversion of dephospho-CoA to coenzyme A (CoA). CoA is an essential cofactor in a range of biochemical reactions. An antisense message could inhibit expression of *coaE* and therefore shut down pathways dependant on coenzyme A, such as BCAA degradation pathways (36).

Summary

We have shown that a subset of previously identified *A. pleuropneumoniae in vivo* induced promoters respond *in vitro* to BCAA limitation. These *in vitro* data suggest that the limitation of BCAA not only induces the expression of BCAA biosynthetic genes such as *ilvI*, but also other genes that are induced during infection of the natural host and therefore potentially play an important role in the infection process. Furthermore, we speculate that BCAA limitation could be responsible for the induction of other *A. pleuropneumoniae* genes not identified in the limited IVET screen. Studies of other host environmental conditions that a pathogen encounters during infection, such as iron limitation have been important in understanding how pathogens cause disease. To fully comprehend the importance of BCAA limitation, further experiments are needed to determine the complete set of genes induced during BCAA limitation, how they are regulated, and whether or not they are important in the virulence of *A. pleuropneumoniae* and other respiratory pathogens. With the *A. pleuropneumoniae* serotype 1 genomic sequence nearing completion, future experiments using genomic chips or microarrays could be performed to address these questions.

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Chapter 3

Identification of *Actinobacillus pleuropneumoniae* leucine-responsive regulatory protein (Lrp) and its involvement in the regulation of *in vivo* induced genes.

Abstract

Actinobacillus pleuropneumoniae is a Gram-negative bacterial pathogen that causes a severe hemorrhagic pneumonia in swine. We have previously shown that the limitation of branched-chain amino acids (BCAAs) is a cue that induces the expression of several *A. pleuropneumoniae* genes associated with virulence. Leucine-responsive regulatory protein (Lrp) is a global regulator and has been shown in *Escherichia coli* to regulate many genes including genes involved in BCAA biosynthesis. We hypothesized that *A. pleuropneumoniae* contains a regulator similar to Lrp and that this protein is involved in the regulation of a subset of genes important during infection and recently shown to have increased expression in the absence of BCAAs. We report the identification of an *A. pleuropneumoniae* serotype 1 gene encoding a protein with similarity to amino acid sequence and functional domains of other reported Lrp proteins. We further show purified *A. pleuropneumoniae* His_6-Lrp binds *in vitro* to the *A. pleuropneumoniae* promoter regions for *ilvI*, antisense *cps1AB*, *lrp*, and *nqr*. A genetically-defined *A. pleuropneumoniae lrp* mutant was constructed using allelic replacement and a sucrose counter-selection method. Analysis of expression from the *ilvI* and antisense *cps1AB* promoters in wild-type, *lrp* mutant, and complemented *lrp* mutant indicated that Lrp is required for induction of expression of *ilvI* under BCAA limitation and suggests that Lrp may also play a role in regulation of the *cps1AB* genes.

Introduction

Actinobacillus pleuropneumoniae is a bacterial pathogen that causes both acute and chronic forms of necrotizing hemorrhagic pleuropneumonia in swine (16, 28, 42, 51). The severe economic effect of this disease on the swine industry has been ameliorated by improvements in detection and prevention of the disease and in management practices. However, the methods by which *A. pleuropneumoniae* infects and causes disease in swine are still not fully understood. While a variety of virulence factors have been reported to contribute to the pathogenesis of *A. pleuropneumoniae* (1, 3, 4, 9, 13, 39, 48, 56, 61, 62), little is known about what signals induce expression of these virulence factors during infection. Certain environmental cues such as iron limitation, heat shock, oxidative stress, and osmotic stress have been shown to play a part in the regulation of virulence genes in other organisms. We have recently shown that the limitation of the branched-chain amino acids (BCAAs), leucine, isoleucine, and valine, is an additional cue that induces the expression of virulence-associated genes *in vitro* (see Chapter 2). However, the mechanism or mechanisms by which these genes are regulated in response to BCAA limitation in *A. pleuropneumoniae* is unknown. To obtain a better understanding of how genes are regulated in response to BCAA limitation, a better understanding of potential regulators in *A. pleuropneumoniae* is needed.

One mechanism known to regulate genes in response to BCAA limitation in prokaryotes is leucine-responsive regulatory protein (Lrp). Lrp was first identified in *Escherichia coli* as the positive regulator of *ilvI* (44, 46), which encodes an enzyme involved in BCAA biosynthesis. Other genes, both activated and repressed by Lrp in *E.*

coli, have been subsequently identified (reviewed in references 6, 7, 14, 40, 41). A DNA microarray study by Tani et al. showed Lrp to be involved in the regulation of up to 10% of all *E. coli* genes either directly or indirectly (54). In general, Lrp positively regulates genes involved in biosynthesis of amino acids and negatively regulates genes involved in catabolism of amino acids in *E. coli*. However, Lrp has been shown to regulate, either directly or indirectly, genes associated with virulence, such as fimbriae in *E. coli* (21, 24, 57, 64, 65) and the *hpmBA* haemolysin operon of *Proteus mirabilis* (17). Recently, Lrp was shown to positively regulate the XhlA haemolysin of *Xenorhabdus nematophila* (10), which is required for virulence in insects.

Genes either directly or indirectly regulated by Lrp may respond to Lrp differently depending upon availability of BCAAs in the environment. Lrp can be a positive or negative regulator with leucine antagonizing the effect of Lrp, potentiating the effect of Lrp, or having no effect on Lrp (34, 54). For example, Lrp positively regulates the *E. coli ilvI* gene in the absence of leucine, but the effect is antagonized in the presence of leucine (46). In contrast, the *livJ* gene, involved in BCAA transport, is repressed by both Lrp and leucine together, but repression is not achieved by either individually (34).

The presence of Lrp and its role in gene expression in *A. pleuropneumoniae* has not been investigated. We hypothesized that *A. pleuropneumoniae* contains an Lrp homologue and that this protein is involved in the regulation of a subset of genes important during infection and recently shown to have increased expression in the absence of BCAAs (see Chapter 2). In this study, we have identified an *A. pleuropneumoniae* serotype 1 gene with similarity to the *lrp* gene of *E. coli*. The *A. pleuropneumoniae* serotype 1 *lrp* gene was cloned, sequenced, expressed in a protein

expression vector, and hexahistidine (His_6)-tagged protein purified. We report *A. pleuropneumoniae* His_6-Lrp binds to the *ilvI* promoter and a potentially antisense promoter to the *A. pleuropneumoniae cps1AB* capsule biosynthetic genes. Furthermore, we report the construction and confirmation of an *A. pleuroepnuemoniae lrp* mutant and show through complementation assays that *A. pleuropneumoniae* Lrp regulates expression of *ilvI* in *A. pleuropneumoniae*.

Materials and Methods

Bacterial Strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *A. pleuropneumoniae* strains were cultured in brain heart infusion (BHI; Difco Laboratories, Detroit, MI) or chemically defined medium (CDM) (see Chapter 2) incubated at either 35°C with 5% CO_2 for agar media or 35°C and 150 rpm for broth media. Media were supplemented with nicotinamide adenine dinucleotide (NAD, also called V factor [V]; Sigma Chemical Company, St. Louis, MO) to a final concentration of 10 μg/ml and riboflavin as needed (Sigma) to a final concentration of 200 μg/ml. Ampicillin and kanamycin, when required, were added to 50 μg/ml for plasmid selection in *A. pleuropneumoniae*. When investigating the response of *A. pleuropneumoniae* to the limitation of BCAAs, the amino acids isoleucine, leucine, and valine were excluded (-ILV) from the complete (+ILV) CDM. For analysis of gene expression in CDM broth, bacterial strains grown for 18 h on BHI agar were inoculated into 5 ml of CDM broth to an optical density of ~0.1 at 520 nm (OD_{520}).

E. coli XL1-Blue mRF' (Stratagene, La Jolla, Calif.) and *E. coli* S17-1 (λ*pir*) (53) were cultured in Luria-Bertani (LB) medium and used for cloning and mating, respectively. Ampicillin and kanamycin, when required, were added to 100 μg/ml and chloramphenicol was added to 10 μg/ml for plasmid selection in *E. coli*.

Table 3-1. Bacterial strains and plasmids used for this study

Strain or plasmid	Characteristic	Reference or origin
Strains		
AP100	*A. pleuropneumoniae* ATCC 27088, serotype 1A, passaged through pigs	ATCC[a]
AP225	A spontaneous nalidixic acid-resistant mutant of AP100	(20)
APTW405	An *lrp* single-crossover mutant of AP225	This work
AP359	An *lrp* double-crossover mutant of AP225	This work
E. coli CV975	F⁻ *ara thi* Δ(*lac-pro*) *ilvIH*::Mu dI1734	(44)
E. coli XL1-Blue mRF'	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacIqZΔM15* Tn*10* (Tetr)]	(Stratagene)
E. coli S17-1 (λ*pir*)	λ*pir recA thi pro hsd* (r_K^- m_K^+) RP4-2-Tc::Mu Km::Tn7 Tmp^r Sm^r	(53)

Table 3-1 (cont'd).

Strain or plasmid	Characteristic	Reference or origin
Plasmids		
pUC19	Ap^r; high-copy-number cloning vector	(58)
pTF86	Ap^r; *A. pleuropneumoniae* IVET vector containing promoterless *luxAB* and *ribBAH* genes downstream of a unique *Bam*HI cloning site	(19)
pQE30	Ap^r; protein expression vector	(QIAGEN)
pCV294	Ap^r; *E. coli* (His_6-Lrp) protein expression vector	(36)
pER187	Ap^r Cm^r; chloramphenicol acetyl-transferase cassette (CAT) containing vector	(49)
pUM24Cm	Cm^r Kan^r; *sacR-sacB-nptI* cassette containing vector	(47)
pGP704	Ap^r; broad-host range suicide vector	(37)
pGZRS39	Kan^r; *A. pleuropneumoniae* shuttle vector	(63)
pTW296	Ap^r; *E. coli ilvIH* promoter (constructed by PCR and cloned into *Bam*HI site of pUC19)	This work

Table 3-1 (cont'd).

Strain or plasmid	Characteristic	Reference or origin
pTW313	Ap^r; *A. pleuropneumoniae lrp* (constructed by PCR and cloned into pQE30)	This work
pTW328	Ap^r; *E. coli ilvIH* promoter (constructed by cloning the ~300-bp *Bam*HI fragment from pTW296 into the *Bam*HI site of pTF86)	This work
pTW338	Ap^r; *rnd'-lrp-ftsK'* (constructed by PCR and cloned into *Sph*I/*Sal*I sites of pUC19)	This work
pTW355	Ap^r; *rnd'-Δlrp-ftsK'* (constructed by inverse PCR with pTW338 as template)	This work
pTW401	Ap^r Cm^r; *rnd'-Δlrp*::CAT-*ftsK'* (constructed by cloning a PCR generated CAT cassette into the *Pst*I site of pTW355)	This work
pTW402	Ap^r Cm^r Kan^r; *rnd'-Δlrp*::CAT-*ftsK'-sacR-sacB-nptI* (constructed by cloning the 3.4-kb *Bam*HI *sacR-sacB-nptI* fragment into a partially *Bam*HI digested pTW401)	This work
pTW404	Ap^r Cm^r Kan^r; *rnd'-Δlrp*::CAT-*ftsK'-sacR-sacB-nptI* (constructed by cloning the *Sph*I/*Sac*I insert of pTW402 into pGP704)	This work
pTW415	Kan^r; *rnd'-lrp-ftsK'* (*Sph*I/*Sac*I insertion of pTW338 cloned into pGZRS39)	This work

Table 3-1 (cont'd).

Strain or plasmid	Characteristic	Reference or origin
p*ivi*A	Ap^r; 333-bp *Sau*3AI AP100 genomic DNA fragment cloned into pTF86	(19)
p*ivi*G	Ap^r; 211-bp *Sau*3AI AP100 genomic DNA fragment cloned into pTF86	(19)
p*ivi*I[b]	Ap^r; 623-bp *Sau*3AI AP100 genomic DNA fragment cloned into pTF86	(19)
p*ivi*P[b]	Ap^r; 175-bp *Sau*3AI AP100 genomic DNA fragment cloned into pTF86	(30)
p*ivi*S	Ap^r; 352-bp *Sau*3AI AP100 genomic DNA fragment cloned into pTF86	(30)
p*ivi*U	Ap^r; 604-bp *Sau*3AI AP100 genomic DNA fragment cloned into pTF86	(30)
p*ivi*X	Ap^r; 490-bp *Sau*3AI AP100 genomic DNA fragment cloned into pTF86	(30)
p*ivi*Y	Ap^r; 782-bp *Sau*3AI AP100 genomic DNA fragment cloned into pTF86	(30)
p*ivi*17g	Ap^r; 290-bp *Sau*3AI AP100 genomic DNA fragment cloned into pTF86	(30)

[a] ATCC, American Type Culture Collection

[b] Two unlinked genomic fragments reside in pTF86. The given fragment size is of the fragment adjacent to the promoterless *luxAB* genes of pTF86.

Molecular Manipulations. Genomic DNA from *A. pleuropneumoniae* was isolated using a QIAGEN-tip 500 according to the QIAGEN Genomic DNA Handbook (QIAGEN Inc., Valencia, CA). Plasmid DNA was purified using QIAprep Spin-columns (QIAGEN). DNA-modifying enzymes were obtained from Roche (Roche Applied Science, Indianapolis, IN) and New England Biolabs (New England Biolabs, Inc., Beverly, MA) and used according to the respective manufacturer's specifications. Electrocompetent AP225 was prepared and electroporated as previously described (20). *E. coli* XL1-Blue mRF' was electroporated using the same conditions as those for *A. pleuropneumoniae*.

Luciferase Assays. For quantitative measurement of luciferase activity, a Turner model 20e luminometer was utilized as previously described (19). Briefly, 20 µl of broth culture was added to 20 µl of luciferase substrate and mixed for 10 s. The substrate was made by dissolving 20 mg/ml Essentially Fatty acid Free bovine serum albumin (BSA; Sigma) and 1 µl of N-decyl aldehyde in 1 ml of H_2O and sonicating the solution. The luminometer was set to a delay of 10 s, integration of 30 s, and a sensitivity of 39.9%. The luminometer relative light unit (RLU) readings were normalized to the optical density units of the culture at 520 nm (OD_{520}).

Induction, purification, and quantification of *A. pleuropneumoniae* and *E. coli* His_6-Lrp. The *A. pleuropneumoniae lrp* gene was amplified by polymerase chain reaction (PCR) using AP100 genomic DNA, *Pfu* turbo DNA polymerase (Stratagene), and *A. pleuropneumoniae lrp* specific primers, MM379-*Sal*I and MM430-*Bam*HI. The *lrp* PCR

product was digested with *Sal*I and *Bam*HI and ligated in frame to similarly digested pQE30 (QIAGEN) to generate pTW313.

The plasmids pTW313 and the *E. coli* His_6-Lrp protein expression vector, pCV294, were separately electroporated into XL1-Blue mRF' and transformants were selected. Two 50 ml cultures were inoculated with an overnight culture of XL1-Blue mRF'/pTW313 and XL1-Blue mRF'/pCV294 and incubated at 35°C and 150 rpm for 4.5 h. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM to each culture and incubated for an additional 3 h to an optical density at 600 nm (OD_{600}) of 0.4. The cultures were centrifuged at 4,000 x g for 20 min. The supernatants were removed, and the cell pellets were frozen at -20°C. Frozen pellets were resuspended in 4 ml ice-cold binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Samples were sonicated for 1 min at a 10% duty cycle and an output setting of 7 until not viscous. Sonicated cultures were centrifuged at 14,000 x g for 20 min at 4°C. Novagen His-Bind Quick 900 Cartridges (EMD Biosciences, Inc., Madison, WI) were used according to the manufacturer's instructions. In brief, the cartridges were equilibrated with 6 ml of binding buffer, and the cell extract supernatants loaded into 60 cc syringes and applied to the cartridges at 2 drops per second. The cartridges were washed with 20 ml of binding buffer, 10 ml of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), and eluted with 4 ml of elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Eluted proteins were dialyzed into 20 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 0.2 mM dithiothreitol, 0.4 M NaCl, using a Centricon 10 (Millipore Co., Bedford, MA) and an equal volume of 100% ultrapure glycerol was added to each sample. Bio-Rad protein micro assays were performed using BSA as a standard to determine final

protein concentrations. The purified *E. coli* His_6-Lrp and *A. pleuropneumoniae* His_6-Lrp protein samples were stored at -80°C until use.

SDS-PAGE. Protein samples were resuspended in an equal volume of 2x SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 25% glycerol, 2.5% SDS, 2.5% β-mercaptoethanol, 1.25% bromophenol blue) and loaded on a 12% polyacrylamide gel, as described by Laemmli (31). Gels were stained with Coomassie blue (22).

Electrophoretic Gel Mobility Shift Assays. *A. pleuropneumoniae* DNA fragments for electrophoretic gel mobility shift assays (EMSA) were isolated by PCR using AP100 genomic DNA and gene specific primers when the promoters for specific genes were analyzed, or plasmid DNA and plasmid specific primers in the case of *ivi* fragments. The 623-bp genomic fragment in p*ivi*I was cloned into pUC19 to generate the *ivi*I PCR template, pTW286. Primers specific to the pUC19 vector, MM531 and MM532, were used in this case to generate the *ivi*I fragment. For all other *ivi* fragments, primers specific to pTF86, MM478-*lux* and MM533-T4, were used.

For an internal control, an *E. coli ilvIH* promoter DNA fragment was PCR-amplified from pTW328 using primers MM478-*lux* and MM533-T4. To generate pTW328, the *ilvIH* promoter was PCR-amplified from a colony of CV975 using primers MM362-*Bam*HI and MM363-*Bam*HI. The PCR product was digested with *Bam*HI and ligated to *Bam*HI digested pUC19 to generate pTW296. The ~300-bp *Bam*HI fragment from pTW296 was ligated to *Bam*HI digested pTF86 to generate pTW328.

PCR products were gel extracted and purified using the QIAEX II system (QIAGEN). For radiolabeling of DNA fragments, 1 pmole of purified DNA fragment was combined with 10 units T4 polynucleotide kinase (Roche), 50 uCi ^{32}P γ-ATP (Amersham Biosciences, Piscataway, N.J.), and 1x polynucleotide kinase buffer in a final volume of 20 µl and incubated at 37°C for 1 h. Completed reactions were inactivated by heating to 68°C for 10 min and cleaned by centrifuging the reaction volume through a quick spin column for radiolabeled DNA purification (Roche). Purified His_6-Lrp was diluted to 5 ng/µl in binding buffer [20 mM Tris-HCl, pH 8.0, 75 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 12.5% glycerol, 0.1 mg/ml BSA, 25 µg/ml poly-(dI-dC)]. Binding reactions containing 0, 5, 30, or 60 ng of His_6-Lrp were incubated with 0.05 pmole of radiolabeled DNA fragment and 0.5 µg poly-(dI-dC) brought to a final volume of 20 µl with binding buffer (8). Binding reactions were incubated at room temperature for 20 min and then stopped by adding 5 µl of STOP solution (USB Co., Cleveland, OH). The entire reaction volume was loaded onto a 5% non-denaturing polyacrylamide gel prepared in 1x TBE, pH 8.0, and electrophoresed at 200 volts for 2-3 h. Gels were dried at 80°C for 40 min and exposed to Amersham Biosciences Hyperfilm MP film.

Filter mating and chloramphenicol selection/sucrose counter-selection. Filter mating between *E. coli* S17-1 (λ*pir*)/pTW404 and AP225 was performed according to the protocol of Mulks and Buysse (38). Transconjugants were isolated on BHIV agar containing 2 µg/ml chloramphenicol and 50 µg/ml nalidixic acid after 48 h and screened by PCR for single or double-crossover events at the *lrp* locus. A single-crossover transconjugant was selected and grown overnight at 35°C and 5% CO_2 on BHIV agar

medium supplemented with 2 μg/ml chloramphenicol. The following day, the single-crossover mutant was inoculated into 1 ml of BHIV supplemented with 5 μg/ml chloramphenicol and grown at 37°C and 220 rpm for 2 h until slightly turbid. At this point, 1 ml of BHIV broth medium supplemented with 20% sucrose and 10 μg/ml chloramphenicol was added to the single-crossover mutant culture to achieve a final concentration of 10% sucrose and 7.5 μg/ml chloramphenicol. This culture was incubated at 37°C and 220 rpm for 5 h to select for chloramphenicol resistance and sucrose insensitivity. Dilutions of the chloramphenicol selection/sucrose counter-selection culture were plated on BHIV agar supplemented with 5 μg/ml chloramphenicol and 10% sucrose and incubated overnight at 35°C and 5% CO_2.

Southern blot Analysis. Chromosomal DNA and plasmid controls were digested with the restriction enzyme *Eco*RI and the DNA fragments were separated on an 0.8% ultrapure agarose gel in Tris-acetate-EDTA (TAE) buffer. Southern blots were performed as described by Sambrook et al. (50). DNA probes were labeled with digoxigenin using either the PCR DIG probe synthesis or the DIG DNA labeling kit (Roche Applied Science, Indianapolis, IN). Probes included an 0.5-kb *lrp* PCR fragment, an 0.8-kb chloramphenicol acetyl-transferase (CAT) cassette fragment, and a 3.7-kb pGP704 (37) fragment. The *lrp* fragment was generated by PCR using the MM430-*Bam*HI and MM379-*Sal*I primers with AP100 genomic DNA as a template. The CAT cassette fragment was generated by PCR using the MM508 and MM509 primers with pER187 (49) as a template. The pGP704 fragment was generated by digesting the plasmid with *Bgl*II. Hybridizations, washes, and developing was performed as by Fuller

et. al (20). Hybridizations were carried out at 42°C for 18 h in 50% formamide, 2% Blocking Solution (Roche), 5X SSC, 0.1% Sarkosyl detergent, and 0.02% SDS. Blots were washed 3 times in 2X SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate (SDS) for 15 min at room temperature and twice in 0.1X SSC-0.1% SDS for 60 min at 68°C. Blots were developed with an alkaline phosphatase-conjugated anti-digoxigenin and CDP-*Star* substrate kit (Roche) according to the manufacturer's instructions.

Complementation Induction Assays. The ~1.9-kb *Sph*I/*Sac*I restriction digest fragment from pTW338 was ligated to similarly digested pGZRS39 to generate the *lrp* complementation plasmid, pTW415. Wild-type (AP225/pGZRS39), *lrp* mutant (AP359/pGZRS39), and complemented mutant (AP359/pTW415) strains containing p*ivi*I or p*ivi*G as a second plasmid were grown overnight on BHIV agar supplemented with 50 μg/ml kanamycin and 50 μg/ml ampicillin for maintenance of both plasmids. A sterile cotton tipped swab was used to resuspend each bacterial strain in 1.2 ml of CDM –ILV broth medium and 100 μl was used to inoculate 5 ml cultures of CDM –ILV and CDM +ILV broth medium for each strain. Cultures were grown at 37°C for 8 h at 220 rpm with samples taken every 1-2 h and analyzed for luciferase expression by quantitative luciferase assays.

Nucleotide sequence accession number. A sequence of the *A. pleuropneumoniae lrp* gene has been deposited in the GenBank database under accession no. DQ370064.

Results

Cloning of APP *lrp*. We previously identified the *A. pleuropneumoniae ilvI* gene as an *in vivo* induced (*ivi*) gene (19), and have shown that expression of the promoterless luciferase genes in pTF86 are induced from the *ilvI* promoter under BCAA limiting conditions (see Chapter 2). *IlvI* has been extensively studied in *E. coli* and shown to be positively regulated by Lrp. To investigate the role of Lrp in the regulation of *ilvI* and other *A. pleuropneumoniae ivi* genes, we identified a gene with similarity to *lrp* in the unfinished *A. pleuropneumoniae* serotype 5 genome by homology to known *lrp* genes. Primers, MM450-*Sph*I and MM451-*Sal*I, were designed from serotype 5 sequence and used to clone a ~1.9-kb region from *A. pleuropneumoniae* serotype 1 into pUC19 (58), which included the 3' end of the upstream *rnd* gene, the complete *lrp* gene, and the 5' end of the downstream *ftsK* gene, to generate pTW338. The insert from pTW338 was sequenced and the translated *lrp* sequence from pTW338 was aligned with Lrp sequences from eight different bacterial species, including four members of the family *Pasteurellaceae* to which *A. pleuropneumoniae* belongs (Figure 1). The translated protein sequence of *A. pleuropneumoniae* is 71% identical to that of *E. coli* Lrp (Figure 1). The alignment showed an overall amino acid sequence conservation, including within the domains for DNA binding, transcriptional activation, and leucine response identified in Lrp from *E. coli* (43) and *Pyrococcus furiosis* (32). The conservation of the domains within *A. pleuropneumoniae* Lrp suggests the domains may have similar functions to those characterized in *E. coli* Lrp.

Figure 3-1. Lrp domain and amino acid alignment. Lrp amino acid sequence from 9 bacterial species aligned with ClustalX (55) and shaded with Boxshade v3.31. Residues that are identical in the majority of species are shown on a black background while residues that are functionally conserved are shown on a gray background. The location of the Lrp functional domains for DNA binding (dashed line), transcriptional activation (solid line), and leucine response (dotted line) are indicated above the sequence (32, 43). The position of the amino acid important in the activation of transcription by Lrp in *E. coli* but unconserved in *A. pleuropneumoniae* is indicated by the symbol *. The following species abbreviations and GenBank numbers were used: Ec, *Escherichia coli* (BAA35614); Ka, *Klebsiella aerogenes* (AAD12584); Aa, *Actinobacillus actinomycetemcomitans*; Pm, *Pasteurella multocida* (AAK02338); Hi, *Haemophilus influenzae* (AAC23241); Hd, *Haemophilus ducreyi* (AAP96279); Ap, *Actinobacillus pleuropneumoniae* serotype 1; Pa, *Pseudomonas aeruginosa* (AAG08693); Lp, *Legionella pneumophila* (AAU27568). The *A. actinomycetemcomitans* sequence was identified by similarity in the University of Oklahoma unfinished *A. actinomycetemcomitans* genome and translated.

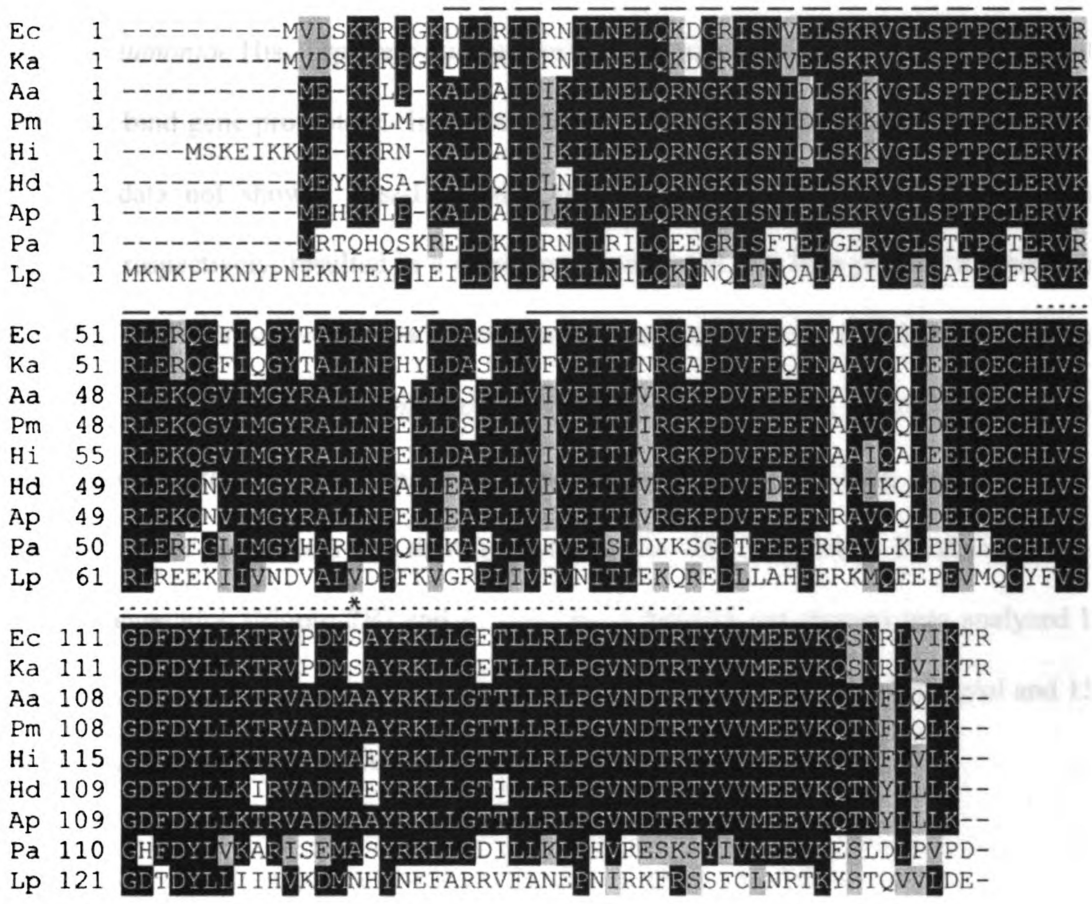
Ec 1 ----------MVDSKKRPGKDLDRIDRNILNELQKDGRISNVELSKRVGLSPTPCLERVR
Ka 1 ----------MVDSKKRPGKDLDRIDRNILNELQKDGRISNVELSKRVGLSPTPCLERVR
Aa 1 ----------ME-KKLP-KALDAIDIKILNELQRNGKISNIDLSKKVGLSPTPCLERVK
Pm 1 ----------ME-KKLM-KALDSIDIKILNELQRNGKISNIDLSKKVGLSPTPCLERVK
Hi 1 ----MSKEIKKME-KKRN-KALDAIDIKILNELQRNGKISNIDLSKKVGLSPTPCLERVK
Hd 1 ----------MEYKKSA-KALDQIDLNILNELQRNGKISNIELSKRVGLSPTPCLERVK
Ap 1 ----------MEHKKLP-KALDAIDLKILNELQRNGKISNIELSKRVGLSPTPCLERVK
Pa 1 ----------MRTQHQSKRELDKIDRNIIRILQEEGRISFTELGERVGLSTTPCTERVR
Lp 1 MKNKPTKNYPNEKNTEYPIEILDKIDRKILNILQKNNQITNQALADIVGISAPPCFRRVK
Ec 51 RLERQGFIQGYTALLNPHYLDASLLVFVEITLNRGAPDVFEQFNTAVQKLEEIQECHLVS
Ka 51 RLERQGFIQGYTALLNPHYLDASLLVFVEITLNRGAPDVFEQFNAAVQKLEEIQECHLVS
Aa 48 RLEKQGVIMGYRALLNPALLDSPLLVIVEITLVRGKPDVFEEFNAAVQQLDEIQECHLVS
Pm 48 RLEKQGVIMGYRALLNPELLDSPLLVIVEITLIRGKPDVFEEFNAAVQQLDEIQECHLVS
Hi 55 RLEKQGVIMGYRALLNPELLDAPLLVIVEITLVRGKPDVFEEFNAAIQALEEIQECHLVS
Hd 49 RLEKQNVIMGYRALLNPALLEAPLLVLVEITLVRGKPDVFDEFNYAIKQLDEIQECHLVS
Ap 49 RLEKQNVIMGYRALLNPELLEAPLLVIVEITLVRGKPDVFEEFNRAVQQLDEIQECHLVS
Pa 50 RLEREGLIMGYHARLNPQHLKASLLVFVEISLDYKSGDTFEEFRRAVLKLPHVLECHLVS
Lp 61 RLREEKIIVNDVALVDEFKVGRPLIVFVNITLEKQREDLLAHEERKMQEEPEVMQGYFVS
Ec 111 GDFDYLLKTRVPDMSAYRKLLGETLLRLPGVNDTRTYVVMEEVKQSNRLVIKTR
Ka 111 GDFDYLLKTRVPDMSAYRKLLGETLLRLPGVNDTRTYVVMEEVKQSNRLVIKTR
Aa 108 GDFDYLLKTRVADMAAYRKLLGTTLLRLPGVNDTRTYVVMEEVKQTNFLQLK--
Pm 108 GDFDYLLKTRVADMAAYRKLLGTTLLRLPGVNDTRTYVVMEEVKQTNFLQLK--
Hi 115 GDFDYLLKTRVADMAEYRKLLGTTLLRLPGVNDTRTYVVMEEVKQTNFLVLK--
Hd 109 GDFDYLLKIRVADMAEYRKLLGTILLRLPGVNDTRTYVVMEEVKQTNYLLLK--
Ap 109 GDFDYLLKTRVADMAAYRKLLGTTLLRLPGVNDTRTYVVMEEVKQTNYLLLK--
Pa 110 GHFDYLVKARISEMASYRKLLGDILLKLPHVRESKSYIVMEEVKESLDLPVPD-
Lp 121 GDTDYLLIIHVKDMNHYNEFARRVFANEPNIRKFRSSFCLNRTKYSTQVVLDE-

Figure 3-1

Purification of *A. pleuropneumoniae* and *E. coli* His_6-*lrp*. To obtain a better understanding of the function of *A. pleuropneumoniae* Lrp, both *E. coli* and *A. pleuropneumoniae* His_6-Lrp were purified for further experiments to determine the ability of Lrp to bind gene promoters. Induction of both *A. pleuropneumoniae* (Figure 2A) and *E. coli* (data not shown) His_6-Lrp using the pQE30 based constructs pTW328 and pCV294, respectively, resulted in a dominantly expressed ~23 kDa protein, as shown by SDS-PAGE. This was ~4.7 kDa larger than the expected size of 18.3 kDa as predicted from the translated *A. pleuropneumoniae lrp* gene and ~4.2 kDa larger than what has been reported for *E. coli* Lrp. The positively charged (His_6)-tag could alter the mobilization of the protein and therefore account for this difference. Purification of *A. pleuropneumoniae* (Figure 2B) and *E. coli* His_6-Lrp (data not shown) was analyzed by SDS-PAGE. The concentration of pure protein was determined to be 1050 ng/µl and 155 ng/µl for *A. pleuropneumoniae* and *E. coli* His_6-Lrp, respectively.

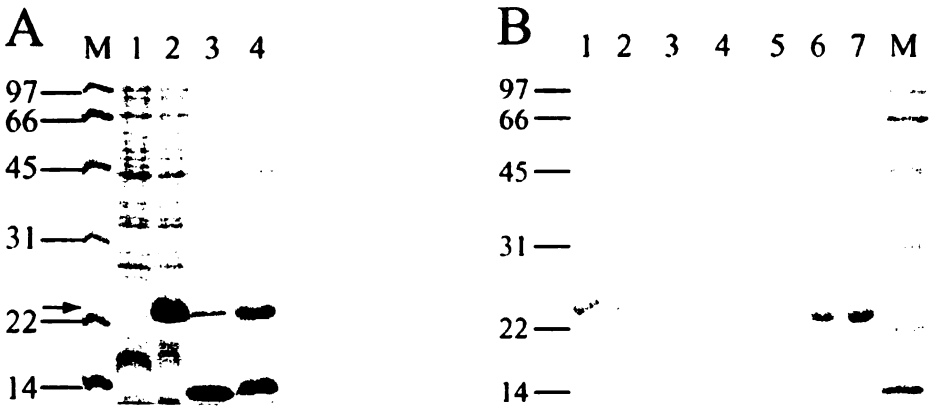

Figure 3-2. Induction and purification of *Actinobacillus pleuropneumoniae* serotype 1 His_6-Lrp. (A) SDS-PAGE of *A. pleuropneumoniae* serotype 1 His_6-Lrp protein induction samples. Lanes in panel A: M, SDS standard; 1, 0.4 ml culture-equivalent of cell extract before induction; 2, 0.1 ml culture-equivalent cell extract after induction; 3, 0.05 ml culture-equivalent induced soluble fraction; 4, 0.05 ml culture-equivalent induced insoluble fraction. Lysozyme, used in the lysis procedure, appears at the 14 kDa size in lanes 3 and 4. (B) SDS-PAGE of *A. pleuropneumoniae* serotype 1 His_6-Lrp purification samples. Lanes in panel A: 1, 35 µl culture-equivalent of cell extract; 2, 35 µl culture-equivalent of cell extract flow through; 3, 17.5 µl culture-equivalent of first wash flow through; 4, 35 µl culture-equivalent of second wash flow through; 5, 35 µl culture-equivalent of protein elution; 6, 140 µl culture-equivalent of concentrated His_6-Lrp; 7, 280 µl culture-equivalent of concentrated His_6-Lrp; M, SDS standard.

His_6-Lrp binding to *A. pleuropneumoniae* promoters. To investigate whether the *A. pleuropneumoniae* Lrp directly regulates expression of *ivi* genes, we analyzed whether purified *A. pleuropneumoniae* His_6-Lrp would bind *in vitro* to the purified DNA inserts of *A. pleuropneumoniae ivi* promoter clones. To first confirm that the electrophoretic gel mobility shift assays (EMSA) were functioning as designed, the *E. coli ilvIH* promoter fragment from pTW328 was analyzed. The mobility of the *E. coli ilvIH* promoter fragment was retarded in an EMSA reaction when either *A. pleuropneumoniae* or *E. coli* His_6-Lrp was present (data not shown). Furthermore, *E. coli* His_6-Lrp bound to the *A. pleuropneumoniae ivi*I insert (data not shown).

We then analyzed eight *ivi* promoter clones that had previously been shown to be induced under BCAA limitation. These included: *ivi*G, *ivi*I, *ivi*P, *ivi*S, *ivi*U, *ivi*X, *ivi*Y, and *ivi*17g (see Chapter 2). *Ivi*A was used as a negative control because the *ivi*A clone did not respond to limitation of BCAAs (see Chapter 2) and the p*ivi*A insert DNA sequence did not have any similarity to published Lrp consensus binding sites (12, 59). Binding of *A. pleuropneumoniae* His_6-Lrp to the *ivi*G and *ivi*I inserts, but not to the *ivi*A control, was demonstrated by EMSA (Figure 3A). The presence of *A. pleuropneumoniae* His_6-Lrp retarded the migration of both the 623-bp *ivi*I and the 211-bp *ivi*G fragments in a dose-dependent manner. The presence of two separate retarded bands with the *ivi*I insert suggests *ivi*I has multiple *A. pleuropneumoniae* His_6-Lrp binding sites. In contrast, *A. pleuropneumoniae* His_6-Lrp did not bind to the *ivi*P, *iviS*, *ivi*U, *ivi*X, ivi*Y*, and *ivi*17g fragments under these assay conditions (data not shown).

Figure 3-3. Analysis of *A. pleuropneumoniae* His_6-Lrp binding to *ivi* clone DNA inserts using electrophoretic mobility shift assays. The *ivi*I, *ivi*G, *ivi*A, *lrp*, and *nqr* labeled DNA fragments were mixed with 0, 5, 30, or 60 ng of His_6-Lrp in a binding reaction.

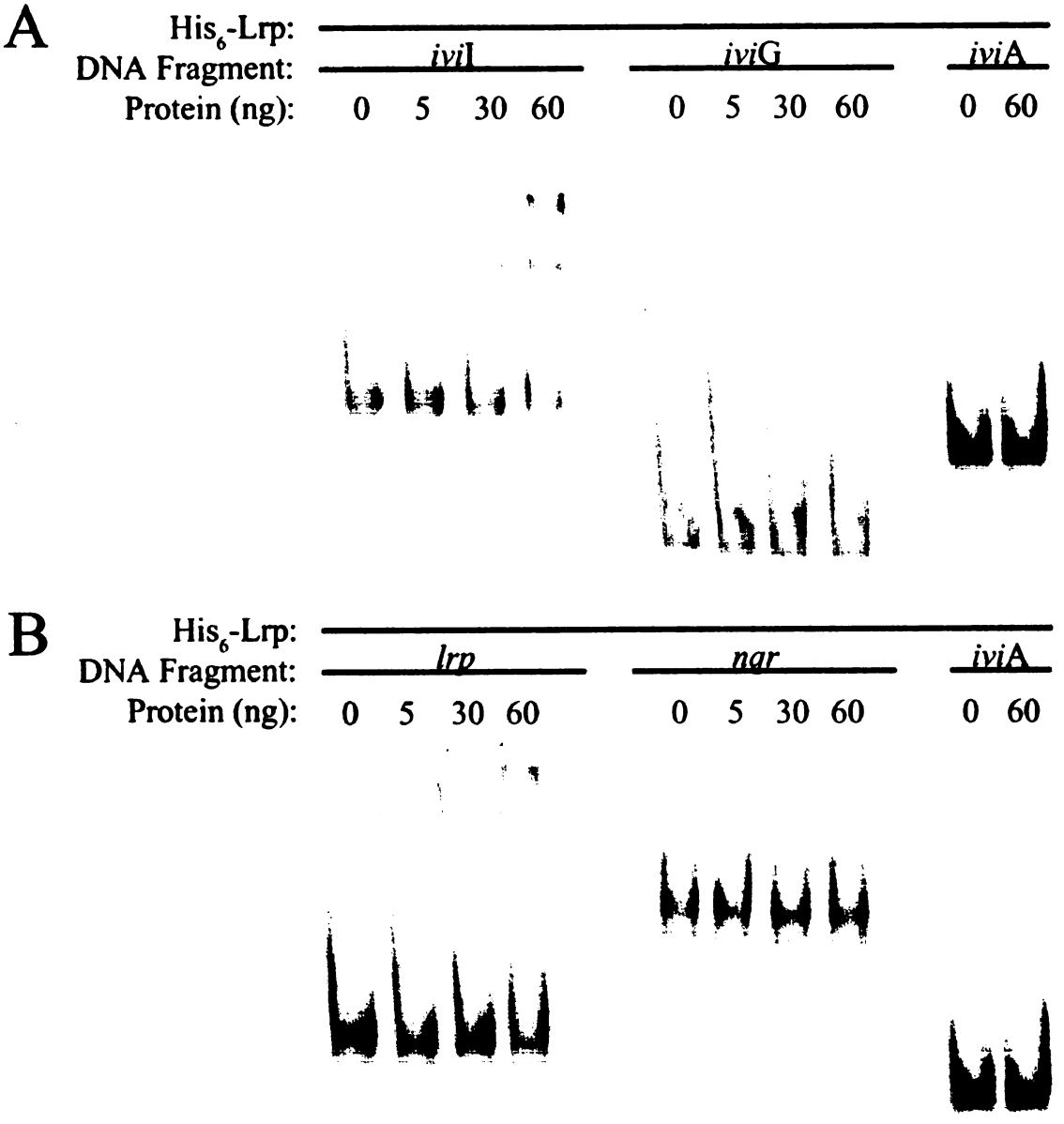
A
His6-Lrp:
DNA Fragment:
ivil
iviG
iviA
Protein (ng):
0 5 30 60
0 5 30 60
0 60
B
His6-Lrp:
DNA Fragment:
lrp
ngr
iviA
Protein (ng):
0 5 30 60
0 5 30 60
0 60

Figure 3-3

The DNA sequences of the inserts to p*ivi*I and p*ivi*G have been identified (see Chapter 2). The p*ivi*I insert sequence has similarity to the 5' end of the *ilvI*, a gene involved in the biosynthesis of BCAAs and likely contains the promoter. The p*ivi*G insert sequence likely also contains a promoter in addition to having sequence similarity to the *cps1AB* genes identified to be involved in the biosynthesis of *A. pleuropneumoniae* serotype 1 capsule.

To obtain a better understanding of the Lrp regulon of *A. pleuropneumoniae*, we extended this analysis to include the promoter regions of other genes either identified in *E. coli* to bind Lrp or identified in *A. pleuropneumoniae* to be involved in virulence. The *A. pleuropneumoniae* putative promoter regions of the Apx toxin genes *apxI*, *apxII*, and *apxIV*, the *apf* type IV fimbriae cluster, the *flp-rcp-tad* locus for type IV fimbriae involved in biofilm formation, the *ilvG* gene involved in BCAA biosynthesis, the *lrp* gene, the *nqr* operon encoding the Na^+-translocating NADH:ubiquinone oxidoreductase, and the *serA* gene involved in serine biosynthesis were isolated by PCR using gene specific primers (Table 2) and analyzed by EMSA. The migration of the 778-bp *nqr* and 751-bp *lrp* promoter fragments were retarded when *A. pleuropneumoniae* His_6-Lrp was added to the reaction (Figure 3B). In contrast, *A. pleuropneumoniae* His_6-Lrp was not observed to bind to the *apxI*, *apxII*, *apxIV*, *apf*, *flp-rcp-tad*, *ilvG*, or *serA* promoter fragments under these assay conditions (data not shown).

Table 3-2. Primers used for this study

Primer	Sequence of primer[a] (5' to 3')	Description
MM362-*Bam*HI	GGCCATCAGTGGATCCAAGAGC	*E. coli ilvIH* promoter forward primer
MM363-*Bam*HI	CTCGGATCCAGACAACATCTCC	*E. coli ilvIH* promoter reverse primer
MM379-*Sal*I	TAATCGTTCAATCGTCGACTAACTCC	*lrp* downstream primer
MM430-*Bam*HI	CTGGGAAGACGGATCCATGGAACATAAAAAAC	*lrp* upstream primer
MM437-*Sph*I	CCCATCGGATCCGTTACATAAGCATGCTCGGC	*nqr* promoter forward primer
MM438-*Bam*HI	GTGTACCTGCGATAGGTGGATCCAAGCC	*nqr* promoter reverse primer
MM450-*Sph*I	GCAGCGGCATGCGTATGGTATTTGTTA	*rnd* internal forward primer
MM451-*Sal*I	AAGCGGTCGACTTTCGTCATTTTGTTGC	*ftsK* internal reverse primer
MM459-*Pst*I	AACTGCAGGAATGTCATTTGGTTTCGGGTGATTTCG	*lrp* inverse PCR forward primer
MM460-*Pst*I	ATCTGCAGGCTTCAAGCAATTCCGGATTCAGTAATG	*lrp* inverse PCR reverse primer
MM478-*lux*	GCTGCCTCCATCCATGGGGTTCCTC	pTF86 *lux* primer
MM480	TGGGATACCGTGCATTACTGAA	*lrp* forward PCR screen primer
MM481	CGGGTAATCGCAGTAAAGTCG	*lrp* reverse PCR screen primer

Table 3-2 (cont'd).

Primer	Sequence of primer[a] (5' to 3')	Description
MM489-*Pst*I	ATGACCCTGCAGACGAATTCGAGCTCGG	pER187::CAT forward primer
MM508	CTAATGAAGAAAGCAGACAAGTA	CAT forward PCR screen primer
MM509	GGGGCAGGTTAGTGACATT	CAT reverse PCR screen primer
MM511-*Pst*I	GTCGACTCTAGACTGCAGCCTCCGTC	pER187::CAT reverse primer
MM525-*Bam*HI	AAAGGGGATCCGCCAGACGAAT	*ilvG* promoter forward primer
MM526-*Xba*I	ACCGACATCTAGACCGACATAAAGTA	*ilvG* promoter reverse primer
MM527-*Bam*HI	GAAATGGATCCGCCTGATTTAGCAC	*lrp* promoter forward primer
MM528-*Xba*I	CGTTCAATTCTAGATTAACTCCTTATTTTA	*lrp* promoter reverse primer
MM529-*Bam*HI	GGAACGGATCCTCAATAACATCGCC	*serA* promoter forward primer
MM530-*Xba*I	CGCATTCTAGAAATTTTTGGTTGAGGC	*serA* promoter reverse primer
MM531	GTTTTCCCAGTCACGACGTTGT	pUC19 forward primer
MM532	CACAGGAAACAGCTATGACCATG	pUC19 reverse primer
MM533-T4	CTGGGGGATGAGTGGCACACC	pTF86 T4 primer

Table 3-2 (cont'd).

Primer	Sequence of primer[a] (5' to 3')	Description
MM534-*Bam*HI	GCAATATCGGATCCGGCTAATGTAATC	*flp-rcp-tad* promoter forward primer
MM535-*Xba*I	TGGCAATGAGTCTAGAAATAAAGAGTGTC	*flp-rcp-tad* promoter reverse primer
MM536-*Bam*HI	AGCGGATCCTAAAACGAGGCACTTCTTG	*apf* promoter forward primer
MM537-*Xba*I	CGATGCCGCCTCTAGATCCGAAAGC	*apf* promoter reverse primer
MM538-*Bam*HI	CCGTAATTGGATCCAAAATACCGTGAAGCAG	*apxI* promoter forward primer
MM539-*Xba*I	AAGTCTAGACATCCGCAACTAGCGAGGCAAC	*apxI* promoter reverse primer
MM540-*Bam*HI	TACGGATCCTTGGTACAAAAAATTTTACAG	*apxII* promoter forward primer
MM541-*Xba*I	CCTTTCTAGAATTGATCATTTAAAGATATTATTGTAG	*apxII* promoter reverse primer
MM544-*Bam*HI	AACCAGGATCCCACAAAGAACACAAGC	*apxIV* promoter forward primer
MM545-*Xba*I	CACAAAACGTCTAGACCCCACCATAAAT	*apxIV* promoter reverse primer

[a] Restriction sites are underlined.

Construction of an *A. pleuropneumoniae lrp* mutant. To further investigate the role of Lrp in the regulation of *ivi* genes responding to limitation of BCAAs, an *A. pleuropneumoniae lrp* mutant was constructed (Figure 4). Primers MM459-*Pst*I and MM460-*Pst*I, both designed with internal *Pst*I restriction sites, were used with an inverse PCR technique to amplify around the pTW338 construct in opposite directions. The 4.5-kb linear product from the inverse PCR, was digested with *Pst*I and ligated to itself to generate the reconstituted plasmid, pTW355. The annealing position of the primers during the inverse PCR resulted in a ~100-bp deletion from the center of *lrp*. An 0.9-kb *Pst*I-digested chloramphenicol acetyl transferase resistance (CAT) cassette, from a PCR reaction using pER187 as template and MM489-*Pst*I and MM511-*Pst*I as primers, was ligated to the newly generated *Pst*I site of pTW355 to generate pTW401. The 3.4-kb *sacR-sacB-nptI Bam*HI fragment from pUM24Cm (47) was ligated to partially *Bam*HI-digested pTW401 to generate pTW402. The *sacR* and *sacB* genes confer sucrose sensitivity in the presence of sucrose and allow for future selection of double-crossover events. The *nptI* gene confers resistance to kanamycin. The *Sph*I/*Sac*I insert from pTW402 was ligated to similarly digested pGP704 (37) to generate the knock-out construct, pTW404. The pTW404 construct was electroporated into *E. coli* S17-1 (λ*pir*) and this strain was mated with AP225, a nalidixic acid resistant derivative of AP100. A single-crossover transconjugant, APTW405, was obtained. A chloramphenicol selection/sucrose counter-selection on APTW405 resulted in chloramphenicol-resistant and sucrose-insensitive bacteria at a density of 6.7×10^7 CFU/ml, suggesting the single-crossover event in APTW405 was forced into a double-crossover event to generate an *lrp* mutant.

Figure 3-4. Construction of the knock-out construct, pTW404. An inverse PCR technique was performed to amplify around the *lrp* containing plasmid, pTW338, in opposite directions and generate a ~100-bp deletion from the center of *lrp* to make pTW355. A chloramphenicol acetyl transferase resistance (CAT) cassette DNA fragment was generated by PCR and cloned into the *Pst*I site of pTW355 to form pTW401. A *Bam*HI fragment, containing the *sacR*, *sacB*, and *nptI*, was excised from pUM24Cm and ligated into a *Bam*HI site in pTW401 to generate pTW402. The insert from pTW402 was cloned into the conjugative suicide vector pGP704 to form pTW404. (see results)

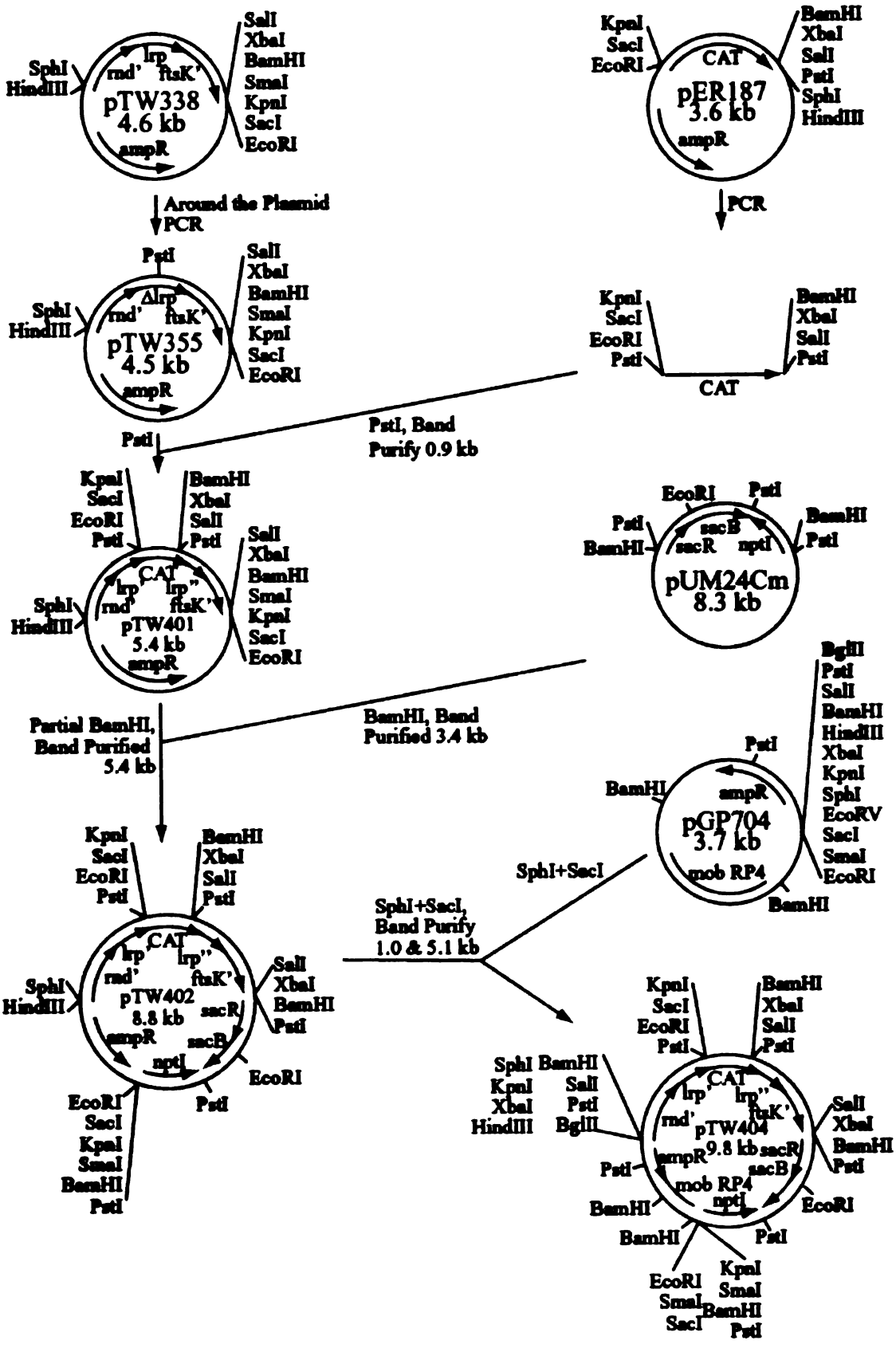
pTW338
4.6 kb
ampR
lrp
rnd'
ftsK'
SphI
HindIII
SalI
XbaI
BamHI
SmaI
KpnI
SacI
EcoRI
Around the Plasmid
PCR
pTW355
4.5 kb
ΔIrp
PstI
pER187
3.6 kb
CAT
PCR
PstI, Band
Purify 0.9 kb
pTW401
5.4 kb
lrp''
pUM24Cm
8.3 kb
sacB
sacR
nptI
Partial BamHI,
Band Purified
5.4 kb
BamHI, Band
Purified 3.4 kb
pGP704
3.7 kb
ampR
mob RP4
BglII
HindIII
XbaI
SphI
EcoRV
SphI+SacI
SphI+SacI,
Band Purify
1.0 & 5.1 kb
pTW402
8.8 kb
sacR
pTW404
9.8 kb

Figure 3-4

***Lrp* Mutant Confirmation.** To confirm that the chloramphenicol selection/sucrose counter selection was successful in producing an *lrp* mutant, twenty potential *lrp* mutants were screened by PCR using *lrp* specific primers, MM480 and MM481. Fifteen of the 20 colonies displayed a single 1-kb product that corresponded to a mutant *lrp* allele. Five colonies displayed both the wild-type 200-bp and mutant 1-kb band predicted for a single-crossover event (data not shown).

Seven of the 15 *lrp* mutants were further characterized and were confirmed as Gram-negative coccobacilli, NAD dependent, chloramphenicol-resistant, sucrose-insensitive, kanamycin-sensitive, and nalidixic acid-resistant. These characteristics were as predicted for a double-crossover *lrp* mutant. A single representative mutant, AP359, was selected for more thorough validation experiments.

Using *lrp* specific primers (MM480 and MM481), a PCR analysis of AP359 resulted in a 1-kb product as predicted for an *lrp* mutant (Figure 5B). In comparison, a predicted 200-bp product was observed for wild-type AP225, and a 1-kb product for *E. coli* S17-1 (λ*pir*)/pTW404 (Figure 5B). The PCR screen supported AP359 as an *lrp* mutant.

Southern blot analyses of genomic DNA prepared from wild-type AP100 and the *lrp* mutant, AP359, are shown in Figure 5C, D, and E. In AP100, the *lrp* probe hybridized to a 5.2-kb *Eco*RI fragment, but there was no reaction with either the CAT or the pGP704 probes. In the *lrp* mutant, the *lrp* probe hybridized to 3.4-kb and 2.6-kb *Eco*RI fragments, while the CAT probe hybridized to a 3.4-kb *Eco*RI fragment; there was no reaction with the pGP704 probe. The hybridization pattern seen with AP359 genomic DNA is the pattern predicted in transconjugants where the wild-type *lrp* locus has been

replaced by the mutated *lrp*::CAT locus by a double-crossover event (Figure 5A). All 3 probes bound to *Eco*RI digested pTW404. These data confirm AP359 as an *A. pleuropneumoniae lrp* deletion disruption mutant.

Figure 3-5. Confirmation of the *A. pleuropneumoniae* lrp mutant. (A) Genetic maps of the *lrp* locus in wild-type, pTW404, and predicted double-crossover mutant strains. The predicted genomic DNA *Eco*RI fragment sizes of wild-type and double-crossover mutant strains are shown along with predicted *Eco*RI fragments for the knock-out construct, pTW404. Abbreviations used: E, *Eco*RI; X_A, site of genetic recombination site A; X_B, site of genetic recombination site B; X_AX_B, resulting double-crossover event at sites X_A and X_B. (B) A 2% agarose gel with PCR reactions using APP *lrp* specific primers, MM480 and MM481. PCR reactions using the following DNA templates were loaded into each lane: AP359 *lrp* mutant genomic DNA (lane 1), AP100 wild-type genomic DNA (lane 2), knock-out construct pTW404 (lane 3), no DNA (lane 4), Invitrogen 1-kb DNA ladder (lane M). Southern blots probed with *lrp* (C), CAT cassette (D), and pGP704 *Bgl*II fragment (E) are shown. Lanes in Southern blot panels: 1, AP100 wild-type genomic DNA; 2, AP359 *lrp* mutant genomic DNA; 3, knock-out construct pTW404.

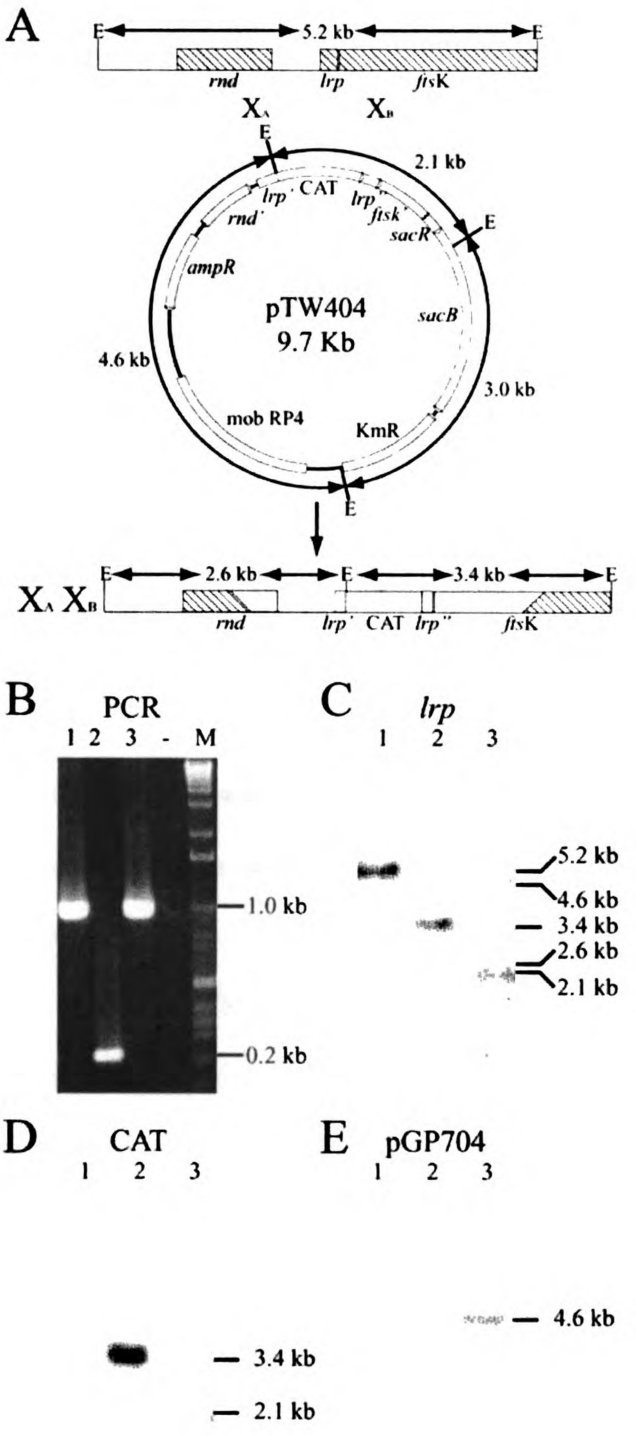
A
E
5.2 kb
E
rnd
lrp
ftsK
X_A
X_B
E
2.1 kb
lrp'
CAT
lrp''
rnd'
ftsk'
sacR
E
ampR
pTW404
9.7 Kb
sacB
4.6 kb
3.0 kb
mob RP4
KmR
E
X_A X_B
E
2.6 kb
E
3.4 kb
E
rnd
lrp'
CAT
lrp''
ftsK
B
PCR
1 2 3 - M
1.0 kb
0.2 kb
C
lrp
1 2 3
5.2 kb
4.6 kb
3.4 kb
2.6 kb
2.1 kb
D
CAT
1 2 3
3.4 kb
2.1 kb
E
pGP704
1 2 3
4.6 kb

Figure 3-5

Luciferase expression from *ivi*I and *ivi*G in wild-type, *lrp* mutant, and complemented *lrp* mutant. To examine the effect of the loss of Lrp on expression from putative Lrp-regulated *ivi* promoters, the luciferase activity of *ivi*I and *ivi*G in each strain was compared in CDM +ILV and CDM –ILV broth media (Figure 6). The luciferase activity of AP225/pGZRS39/p*ivi*I was initially 775 RLU/OD when switched to CDM – ILV, increased to 4479 RLU/OD after one hour, and reached a maximum of 6049 RLU/OD after two hours of incubation (Figure 6A). The luciferase activity was over seven-fold higher in CDM –ILV when compared to CDM +ILV after one hour and over twelve fold higher after two hours, despite minimal change in the growth of the bacteria in CDM –ILV over this time period. No significant change in luciferase activity of AP225/pGZRS39/p*ivi*I was observed in CDM +ILV.

In sharp contrast, the *lrp* mutant (AP359/pGZRS39/p*ivi*I) showed no significant increase in luciferase activity when grown in CDM +ILV or CDM –ILV. When a plasmid containing a wild-type copy of *lrp*, pTW415, was introduced into AP359/p*ivi*I, induction of luciferase expression from p*ivi*I in CDM –ILV was restored to wild-type levels. The complemented mutant showed no significant change in luciferase activity in CDM +ILV. These three strains demonstrated equivalent growth in CDM +ILV but displayed no significant change of luciferase activity from p*ivi*I (Figure 6A). In contrast, while none of the three strains grew well in CDM –ILV, expression from p*ivi*I was strongly up-regulated in the wild-type and complemented mutant strains containing Lrp and unresponsive in the Lrp mutant. These data suggest Lrp is directly involved in the regulation of the *A. pleuropneumoniae ilvIH* promoter within p*ivi*I in response to BCAA limitation.

Figure 3-6. Expression from p*ivi*I and p*ivi*G in wild-type and *lrp* mutant backgrounds. Luciferase assays analyzing the induction of the cloned *A. pleuropneumoniae* serotype 1 *ilvI* promoter (p*ivi*I; panel A) and the putative capsule biosynthesis antisense promoter (p*ivi*G; panel B) in wild-type (AP225/pGZRS39), *lrp* mutant (AP359/pGZRS39), and complemented *lrp* mutant (AP359/pTW415). All strains were grown in CDM +ILV (▲) and CDM -ILV(■). Luciferase activity is expressed as RLU per OD_{520} unit. Data presented are from one of four representative experiments in panel A and one of six representative experiments in panel B. Trends were identical within experiments.

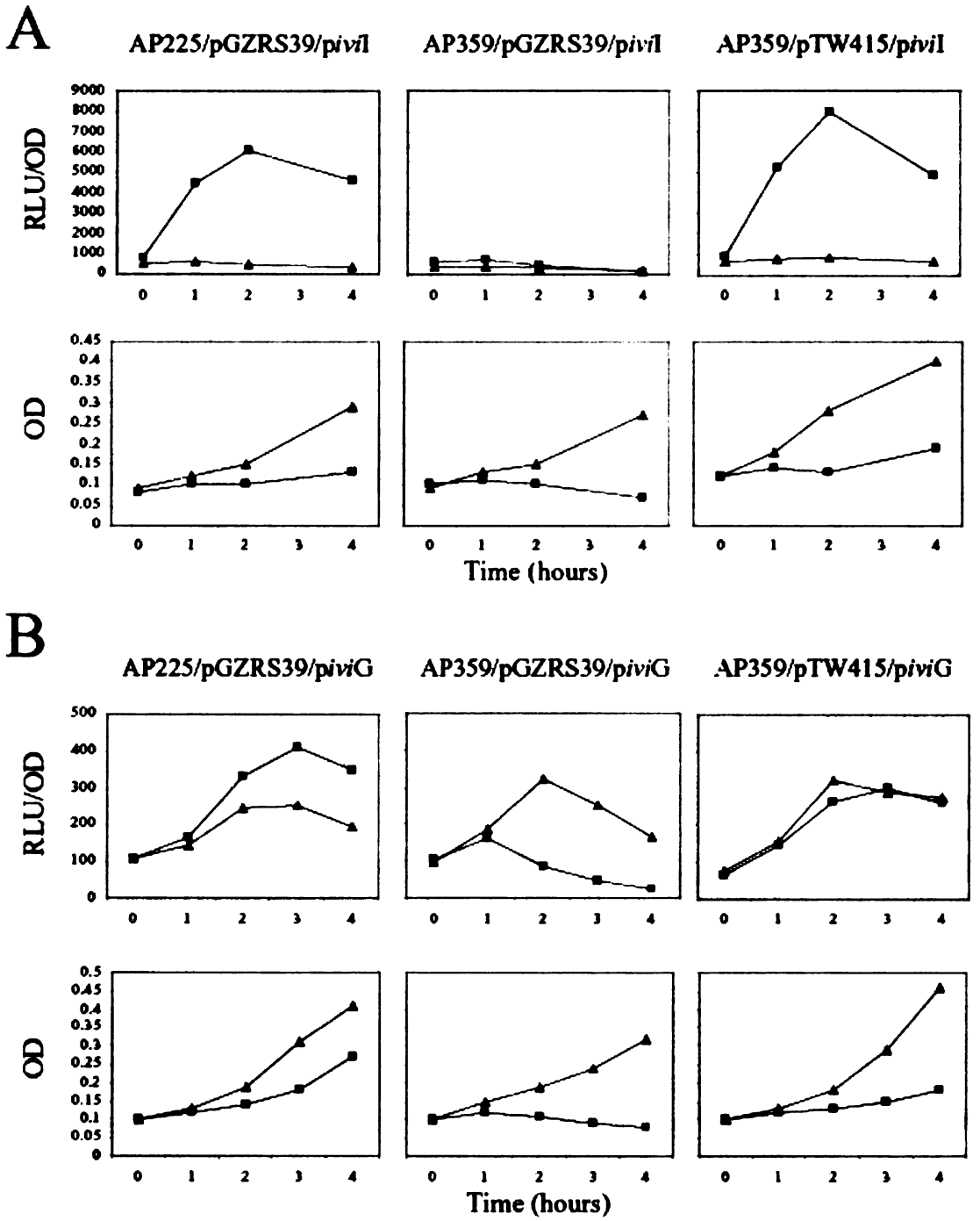
A
AP225/pGZRS39/pivil
AP359/pGZRS39/pivil
AP359/pTW415/pivil
RLU/OD
OD
Time (hours)
B
AP225/pGZRS39/pivG
AP359/pGZRS39/pivG
AP359/pTW415/pivG
RLU/OD
OD
Time (hours)

Figure 3-6

The luciferase activity of strains containing p*ivi*G was also examined. The luciferase activity of AP225/pGZRS39/p*ivi*G was initially 107 RLU/OD when switched to CDM –ILV, increased to 165 RLU/OD after one hour, increased to 389 RLU/OD after two hours, and increased to a maximum of 410 RLU/OD after three hours of incubation (Figure 6B). In comparison, the luciferase activity of AP225/pGZRS39/p*ivi*G in CDM +ILV also increased for the first three hours, although not as much as in CDM –ILV. The greatest difference in luciferase activity in the first three hours was 1.6 fold higher in CDM –ILV than CDM +ILV after 3 hours.

AP359/pGZRS39/p*ivi*G showed an increase in luciferase activity for the first hour when grown in either CDM +ILV or CDM –ILV. However, after two hours the luciferase activity in CDM –ILV decreased to 86 RLU/OD while in CDM +ILV the luciferase activity increased to 325 RLU/OD. When pTW415 was introduced into AP359/p*ivi*G, induction of luciferase expression from p*ivi*G in CDM –ILV was restored to wild-type levels. The complemented mutant showed similar luciferase activity in both CDM –ILV and CDM +ILV. As with strains containing p*ivi*I, the three strains containing p*ivi*G demonstrated equivalent growth in CDM +ILV and displayed low but equal patterns of expression from p*ivi*G (Figure 6B). As with the strains containing p*ivi*I, none of the three strains containing p*ivi*G grew well in CDM –ILV. The expression from p*ivi*G was stronger in the wild-type strain in CDM –ILV as compared to CDM +ILV, greatly reduced in the *lrp* mutant in CDM –ILV, and restored in the complemented *lrp* mutant. While these data suggest that Lrp is also directly involved in the regulation of the *A. pleuropneumoniae* promoter within p*ivi*G, the lack of growth of AP359/pGZRS39/p*ivi*G in CDM –ILV coupled with the much less rapid and robust

response from p*ivi*G as compared to p*ivi*I make this a less convincing conclusion than the regulation of *ilvIH* by Lrp.

Discussion

The study of gene regulators in *A. pleuropneumoniae* has been limited and has yielded the identification of only two to date. These include HlyX (33, 35), a homologue of the *E. coli* global regulator FNR, and the ferric uptake regulator protein, Fur (27). In this study, we report the identification of the *A. pleuropneumoniae lrp* gene and its cloning, sequencing, over-expression and protein purification, and mutation by deletion disruption using a chloramphenicol selection/sucrose counter-selection procedure. The *A. pleuropneumoniae* Lrp is similar in amino acid sequence and function to the extensively studied Lrp from *E. coli*. The functional domains for DNA binding, transcriptional activation, and leucine response identified in *E. coli* Lrp (43) are highly conserved in *A. pleuropneumoniae* and suggest the function of *A. pleuropneumoniae* Lrp may be similar. This is further supported by the *in vitro* binding of purified *A. pleuropneumoniae* His_6-Lrp binding *in vitro* to the *E. coli ilvI* promoter in an EMSA experiment (data not shown).

However, differences may exist between these similar proteins. A study by Platko and Calvo (43) show multiple mutations in Lrp affect DNA-binding, activation, and the leucine response of Lrp. While *A. pleuropneumoniae* has the same amino acids at 21 of the 22 sites identified as critical for these functions, *E. coli* has a serine at position 125 of *E. coli* Lrp whereas *A. pleuropneumoniae* has an alanine at the homologous position (Figure 1). The serine was shown to be important in the activation of transcription by Lrp in *E. coli*. The unconserved alanine in *A. pleuropneumoniae* Lrp suggests a difference in regulation could exist between *E. coli* and *A. pleuropneumoniae*

Lrp. In *Haemophilus influenzae*, a bacterium closely related to *A. pleuropneumoniae*, Lrp was shown to affect the expression of fewer proteins (18) than in *E. coli* (15), which suggests that a difference between the roles of Lrp in *E. coli* and *A. pleuropneumoniae* could be expected.

Our main hypothesis guiding this study was that a subset of *ivi* genes responding similarly to BCAA limitation are also regulated by a similar mechanism. Since Lrp had been identified as a regulator of *ilvI* in *E. coli* (44, 46), we speculated that an *A. pleuropneumoniae* Lrp would not only regulate the *A. pleuropneumoniae ivi* gene, *ilvI* (19), but also other *ivi* genes we previously identified as being up-regulated by BCAA limitation (see Chapter 2).

Since it had been previously shown that the *E. coli* His_6-Lrp behaves as the native protein does (36), we addressed our hypothesis by using purified *A. pleuropneumoniae* His_6-Lrp to determine if *A. pleuropneumoniae* Lrp has a role in the regulation of *ivi* genes shown to induce under BCAA limitation. As shown in Figure 3, *A. pleuropneumoniae* His_6-Lrp bound to DNA fragments from the *ivi*I and *ivi*G clones, but not the negative control, *ivi*A. The observation that two of the eight identified clone inserts bound *A. pleuropneumoniae* His_6-Lrp supports that a subset of clones shown to induce under BCAA limiting conditions are regulated similarly. The remaining six *ivi* clone inserts that did not bind *A. pleuropneumoniae* His_6-Lrp could be regulated by other mechanisms such as other protein regulators or amino acid attenuation, or could also be regulated indirectly by Lrp.

The Lrp regulon in *E. coli* is extensive, including ~10% of all *E. coli* genes (54). We identified *A. pleuropneumoniae* homologues of several genes known to be regulated

by Lrp in *E. coli*, including *ilvG* (45), *serA* (66), and *lrp* itself (60), and tested these by EMSA. Lrp also regulates a variety of virulence-associated genes including fimbriae in *E. coli* (5) and haemolysin in *Xenorhabdus nematophila* (10, 26). Therefore, we also tested binding of Lrp to the putative *A. pleuropneumoniae* promoters of Apx toxin genes, two fimbrial operons, and the *nqr* gene. *A. pleuropneumoniae* Lrp did bind to its own promoter, suggesting that regulation of the Lrp gene may be similar in *A. pleuropneumoniae* and *E. coli*. However, Lrp failed to bind to the *ilvG* or *serA* promoters, which suggests that regulation of BCAA biosynthesis by Lrp in *A. pleuropneumoniae* is different, or at least not as complex, than in *E. coli* (18). *A. pleuropneumoniae* Lrp did not bind to the *apxI*, *apxII*, *apxIV*, *apf*, or *flp-rcp-tad* promoters under the assay conditions used, suggesting that either Lrp does not regulate these genes in *A. pleuropneumoniae*, the effect of Lrp is indirect, or the assay conditions established for binding to the *ilvIH* promoter are not optimal for all DNA fragments. Lrp did bind to the *nqr* operon promoter. *A. pleuropneumoniae nqrA* has been shown to be strongly expressed and antigenic *in vivo* (11) and has been shown to be essential for survival during infection (52). In *Vibrio cholerae*, mutants in Nqr affect virulence gene expression (25). However, there is no *nqr* operon in *E. coli*. This is the first report of potential regulation of *nqr* by Lrp. These results indicate that the *A. pleuropneumoniae* Lrp regulon, while not as extensive as that characterized for *E. coli*, is not limited to genes involved in BCAA biosynthesis and does include both *in vivo* induced and virulence-associated genes.

The ability of *A. pleuropneumoniae* His_6-Lrp to bind to the DNA inserts of *ivi* clones that did not respond to the limitation of BCAAs was not analyzed. Given that

certain promoters can be regulated by *E. coli* Lrp in the presence of leucine and other promoters in the absence of leucine (15, 34, 40), it is distinctly possible Lrp from *A. pleuropneumoniae* may bind to additional *ivi* clone DNA inserts from clones that failed to induce under BCAA limitation. If this is the case, there may be additional *ivi* genes that are regulated by Lrp that have not been identified within the scope of this study.

While the demonstrated binding of recombinant *A. pleuropneumoniae* His_6-Lrp to DNA fragments *in vitro* suggests regulation by Lrp, the expression of these genes may not be regulated by Lrp. To address this question, we constructed an *A. pleuropneumoniae lrp* mutant using a chloramphenicol selection/sucrose counter-selection method and allelic replacement and confirmed this mutant by PCR and Southern blots (Figure 5B) in addition to bacteriological methods.

With an *A. pleuropneumoniae lrp* mutant, it was possible to analyze the luciferase activity of wild-type (AP225/pGZRS39), *lrp* mutant (AP359/pGZRS39), and *lrp* complemented mutant (AP359/pTW415) containing p*ivi*I or p*ivi*G. We showed that an *lrp* mutant containing p*ivi*I is not able to induce the expression of the luciferase reporter in BCAA limiting conditions as was observed in the wild-type strain. Induction was restored in the *lrp* complemented mutant, indicating that Lrp positively regulates the *ilvI* promoter in *A. pleuropneumoniae*. Unfortunately, the *lrp* mutant displays limited to no growth in CDM –ILV. However, despite limited growth of the wild-type strain, AP225/pGZRS39/p*ivi*I, in CDM –ILV, with an increase in optical density of 0.02 in the first hour, we observed RLU/OD to increase almost 500%. Therefore, in the case of *ilvI*, induction can be observed with limited growth due to the rapid and robust response. While the growth of the *lrp* mutant strain, AP359/pGZRS39/p*ivi*I, was also limited and

increased by an optical density of 0.01 in the first hour, the RLU/OD increased only 25%. We are confident the lack of expression from the mutant strain is due to the *lrp* mutation and not the lack of growth. This supports that *A. pleuropneumoniae* Lrp is critical for the regulation of the *ilvI* in *A. pleuropneumoniae*.

In contrast, we were unable to firmly establish the role of *A. pleuropneumoniae* Lrp in the expression of the p*ivi*G promoter. The pattern of the luciferase expression from the p*ivi*G promoter was similar to that seen with p*ivi*I, with the luciferase activity of AP225/pGZRS39/p*ivi*G showing an increase in CDM –ILV as compared to CDM +ILV. However, this increase was dramatically smaller and less rapid than the increase shown in AP225/pGZRS39/p*ivi*I. Due to the smaller induction and the poor growth of the *lrp* mutant in CDM –ILV, we are not as confident in assigning the lack of luciferase induction from strain AP359/pGZRS39/p*ivi*G directly to the *lrp* mutation. The extremely poor growth of the *lrp* mutant in CDM –ILV could influence the assay enough to make the lack of induction appear to be due to the *lrp* mutation when in fact it was due to poor growth. However, since *A. pleuropneumoniae* His_6-Lrp binds to the *ivi*G fragment *in vitro*, the regulation of the *A. pleuropneumoniae* serotype 1 capsule biosynthetic genes by Lrp remains a strong possibility.

In a previous study, we discussed the relevance of the *ivi*I and *ivi*G clones to BCAA limitation and virulence (see Chapter 2). While the discovery of *A. pleuropneumoniae* Lrp binding to and regulating the expression of the *ilvI* promoter in *A. pleuropneumoniae* is novel because few regulators have been examined in the organism, we were not surprised since Lrp had been shown to regulate *ilvI* in *E. coli*. In contrast, the binding of *A. pleuropneumoniae* His_6-Lrp to the putative antisense promoter for

capsule biosynthesis is quite surprising. The antisense nature of the *ivi*G insert to putative capsular biosynthesis genes was discussed previously (see Chapter 2). The fact that *A. pleuropneumoniae* Lrp binds to this region *in vitro* and possibly regulates the expression of the putative promoter raises the possibility that Lrp may play a role in capsule biosynthesis of *A. pleuropneumoniae*. The p*ivi*G insert includes the 5' end of the *cps1B* gene and the 3' end of the *cps1A* gene within the *cps1ABC* operon and therefore could affect the expression of *cps1B* alone, both *cps1AB*, or the entire operon. While the role of each gene in the biosynthesis of capsule has not been established, it is known that serotype 1 capsule is composed of a repeating N-acetyl-2-dioxy-β-D-glucopyranosyl and α-D-galactopyranosyl disaccharide that is partially *O*-acetylated (2). Two possible roles of a promoter antisense to the capsule biosynthesis operon could be to reduce the total amount of capsule or to alter the antigenic structure by reducing the *O*-acetylation. Future experiments comparing the amount or type of capsule produced in wild-type and the *lrp* mutant are needed to address these issues.

In *E. coli*, Lrp has been implicated in the regulation of genes involved in virulence such as fimbriae (5), but to our knowledge, this study is the first time that Lrp has been implicated in the regulation of genes specifically induced during infection of the host. Furthermore, it is interesting to speculate on the affect of an *lrp* mutant on the virulence of *A. pleuropneumoniae* and compare it to what is known about the global regulator, Fur. Like Lrp, Fur has been shown to be both a positive and negative regulator (23) but can be modulated by iron rather than leucine. A Fur mutant in *A. pleuropneumoniae* has recently been shown to have reduced virulence (29). An *A. pleuropneumoniae lrp* mutant may also be attenuated if Lrp is necessary for the correct

regulation of genes important in causing disease. Infection trials with the *lrp* mutant are needed to address this subject.

This is the first report to identify an *A. pleuropneumoniae* Lrp homolog. While the role of Lrp in the regulation of *ilv*I in *E. coli* has been extensively studied, this work addresses the role of *A. pleuropneumoniae* Lrp in the regulation of *A. pleuropneumoniae* virulence-associated genes, *in vivo* induced genes, and BCAA biosynthetic genes. *A. pleuropneumoniae* Lrp was shown to bind to the promoter of *A. pleuropneumoniae ilvI* and regulate the expression under BCAA limitation. Furthermore, Lrp was shown to bind to the putative *nqr* promoter and the *A. pleuropneumoniae* serotype 1 capsule biosynthesis operon, suggesting for the first time that Lrp is involved in the regulation of *A. pleuropneumoniae* serotype 1 capsule biosynthesis and *nqr* expression. We plan to further address the role of an *lrp* mutant in the virulence of *A. pleuropneumoniae* by conducting future infection experiments to address whether or not an *lrp* mutant may lead to an attenuated form of *A. pleuropneumoniae* which could be further used as a live attenuated vaccine to control *A. pleuropneumoniae* disease.

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Chapter 4

A mutation in *Actinobacillus pleuropneumoniae lrp* is not avirulent in a swine respiratory model of infection.

Abstract

Actinobacillus pleuropneumoniae is the causative agent of a severe hemorrhagic pleuropneumoniae in swine and significantly impacts the swine industry. We have previously reported the construction of an *A. pleuropneumoniae* genetically defined leucine-responsive regulatory protein (*lrp*) mutant using allelic exchange and a selection counter-selection method. In this study, we analyze the virulence of the *A. pleuropneumoniae lrp* mutant in a swine model of respiratory infection. We show the *A. pleuropneumoniae lrp* mutant is still able to cause disease in its natural host as demonstrated by mortality, death time, clinical scores, amount of pneumonia, and histopathology. However, the degree of severity of the *lrp* mutant, as compared to the wild-type strain at similar doses, is inconclusive and the role for Lrp in *A. pleuropneumoniae* pathogenesis is unclear.

Introduction

Actinobacillus pleuropneumoniae is the causative agent of a severe hemorrhagic pleuropneumonia in swine. Acute cases of the disease can rapidly lead to death, while chronic cases can lead to carrier populations with poor feed conversion efficiency and decreased growth rate. The effect of the disease on the economics of the swine industry is significant, and improved tools for prevention of *A. pleuropneumoniae* infection have been an important agricultural goal.

Fifteen *A. pleuropneumoniae* serotypes have been identified based on antigenic differences in capsular polysaccharide (6, 30, 34). While vaccines made from killed whole cell bacterins offer only serotype-specific protection at best (27, 28, 32, 35), infection confers protection against multiple serotypes (26, 27, 29). This suggests that live attenuated vaccines could confer cross-serotype protection to pigs, without resulting in typical *A. pleuropneumoniae* disease. Genetic techniques to identify genes critical for *A. pleuropneumoniae* survival and virulence have been developed (1, 11, 12), as well as techniques for the construction of genetically defined *A. pleuropneumoniae* mutants (22, 31). Analysis of such mutants for attenuation in experimental animal models to identify candidates for live attenuated vaccines against *A. pleuropneumoniae* is an ongoing area of research (2, 3, 5, 13, 19, 36, 37).

The regulation of genes important during infection has also been targeted for mutation to construct live attenuated vaccines. DNA methylation, using DNA adenine methylase (Dam), is one way for bacteria to control the expression of a wide range of genes including those encoding for pili in *E. coli* and *Salmonella*. *Salmonella* Dam

mutants were shown to have reduced virulence in mice (15, 17, 18). The cyclic-AMP receptor protein (CRP) is a protein regulator that is involved in the regulation of many genes including toxin and pilus expression in *Vibrio cholerae* (33). *Salmonella* CRP mutants are avirulent in mice (9). Recently, protein regulators of gene expression in *A. pleuropneumoniae* have been targeted. The *A. pleuropneumoniae* anaerobic regulator Fnr homologue, HlyX, and the ferric uptake regulator, Fur, are important in the expression of virulence genes and mutants are attenuated in the natural swine host (2, 4, 16, 19, 20). Mutations in other regulators of virulence gene expression could theoretically result in *A. pleuropneumoniae* mutants deficient in the proper regulation of virulence factors and therefore an attenuated organism.

The leucine-responsive regulatory protein (Lrp) is a global regulator in *Escherichia coli* (reviewed in references 7, 8, 10, 23-25) and regulates genes involved in areas including amino acid biosynthesis, degradation, and transport and pili phase variation. We have previously shown that *A. pleuropneumoniae* serotype 1 Lrp is involved in the regulation of at least two *in vivo* induced genes up-regulated during infection of the natural swine host (see Chapter 3). These include the *ilvI* gene, encoding for the large subunit of acetohydroxy acid synthase III involved in branched-chain amino acid biosynthesis, and the capsule biosynthesis genes of *A. pleuropneumoniae*.

In this paper, we examine the effect of an *lrp* mutation on the virulence of *A. pleuropneumoniae* serotype 1. Under the selected experimental design and conditions, no attenuation in the virulence of *A. pleuropneumoniae lrp* mutant during infection of its natural host was observed.

Materials and methods

Bacterial strains and media. The *A. pleuropneumoniae* strains used in this study were AP225 (14), a virulent, spontaneous nalidixic-acid resistant mutant of the serotype 1 strain ATCC 27088 (American Type Culture Collection, Rockville, MD); AP359 (see Chapter 3), a chloramphenicol and nalidixic acid resistant *lrp* mutant derived from AP225; and AP359/pTW415, the *lrp* mutant strain containing a plasmid with a wild-type allele of *lrp* (see Chapter 3). All strains were cultured at 35°C on either brain heart infusion (BHI), heart infusion (HI), or tryptic soy agar (TSA; Difco Laboratories, Detroit, MI) containing 10 μg/ml nicotinamide adenine dinucleotide (NAD; also called V factor [V]; Sigma Chemical Company, St. Louis, MO). Choramphenicol and nalidixic acid were added to 2 μg/ml and 50 μg/ml, respectively, as needed for selection of strains. Kanamycin was added to 100 μg/ml for selection of the plasmid containing the wild-type *lrp* allele, pTW415 (see Chapter 3).

Experimental infections. Eight-week old specific-pathogen free castrated male pigs (Whiteshire Hamroc, Inc., Albion, IN) were allocated to five challenge groups by a stratified random sampling procedure, balancing each group for body weight. Groups were separated into two biosafety level-2 isolation rooms at the Michigan State University Research Containment Facility, with groups 1 and 2 in one room and 3, 4, and 5 in another. 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.

Bacterial cultures for challenge inocula were grown at 35°C, shaking at 160 rpm, in 30 ml HIV broth containing 5mM $CaCl_2$, in 300-ml baffled side arm flasks, and were harvested at an optical density at 520 nm of 0.9 and a cell density of ~10^9 CFU/ml. Cultures were harvested by centrifugation at room temperature and washed once with sterile phosphate buffered saline (PBS). Cell pellets were resuspended and diluted in PBS to obtain the desired number of CFU/ml. The actual inoculation doses were retrospectively calculated by viable cell counts on agar plates.

Pigs were anesthetized with telazole (6.6 mg/kg of body weight) and xylazine (3.3 mg/kg of body weight) by intramuscular injection. Pigs were subsequently inoculated by percutaneous intratracheal injection posterior to the larynx with the appropriate dose of bacteria resuspended in 10 ml of PBS using an 18 gauge by 3.5 inch disposable spinal tap needle. The anterior of the pig was held in an elevated position for 1-2 min to allow the administered dose to target the lungs. Pigs were monitored for 1 hour post challenge to ensure recovery from anesthesia. Clinical signs of pleuropneumonia, including increased respiration rate and rectal temperature, dyspnea, loss of appetite, and change in activity or attitude (depression), were monitored and scored as previously described (21). Pigs determined by clinical signs to be seriously ill, were euthanized immediately. Survivors were euthanized 39 hr postchallenge. Blood and broncheal alveolar lavage (BAL) fluid were collected for bacteriology. All animals were necropsied shortly after death and brains, joints, heart, liver, kidney, spleen, tonsils, mesenteric and bronchial lymph nodes, stomach, and intestines were examined for any abnormal pathology and infection. Lungs

were examined for signs of *A. pleuropneumoniae* lesions including edema, congestion, hemorrhage, necrosis, abscess, fibrosis, and pleuritis. The percentage of affected lung tissue and pleural surface area was estimated for each of the seven lung lobes and the total percentages for pneumonia was calculated by using a formula that weights the contribution of each lung lobe to the total lung volume (21). Representative samples were collected for histopathology and for bacterial culture.

Histopathology. Samples for histopathology were fixed in 10% formalin and embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin.

Screening for the mutant *lrp* allele. Polymerase chain reaction (PCR) was used to screen for the mutant *lrp* allele. Whole bacterial cells from reisolated strains were used as templates in PCR reactions including 0.025 units *Taq* polymerase, 1X PCR buffer, 1.5 mM $MgCl_2$, 150nM dNTPs, and *lrp* forward and chloramphenicol marker reverse specific primers MM480 (5'-TGGGATACCGTGCATTACTGAA-3') and MM509 (5'-GGGGCAGGTTAGTGACATT-3'), respectively. Reactions were processed in an Applied Biosystems GeneAmp 9700 for 30 cycles consisting of 94°C for 15 sec, 50°C for 30 sec, and 72°C for 1 minute. Reaction products were analyzed by UV transillumination on a 1% agarose gel after electrophoresis and staining with ethidium bromide.

Quantitative bacterial counts from BAL and lung samples. Lung samples each with 10 ml HIV+5 mM $CaCl_2$ were homogenized in a Seward Biomaster 80 stomacher for 150

sec on a normal setting. A series of dilutions in HIV+5 mM $CaCl_2$ were performed on the BAL and homogenized lung samples. Dilutions were cultured in duplicate on selective and nonselective media at 35°C and CFU/ml were measured.

Results

Virulence of the lrp mutant in swine. To determine the effect of an *lrp* mutation on the virulence of *A. pleuropneumoniae*, a swine infection experiment was performed. Six groups of three pigs each, except for two pigs in group 2, were infected with bacteria as follows: group 1, 1 50% lethal dose (LD_{50}) ($5x10^6$ CFU) (21) of wild-type AP225; group 2, $1xLD_{50}$ of the complemented *lrp* mutant AP359/pTW414; group 3 and 4, the *lrp* mutant AP359 at doses equivalent to 1 and 5 times the LD_{50} for the wild-type, respectively; and group 5, PBS.

Mortality, average death time, and clinical score data shown in Tables 1 and 2 indicate that the *lrp* mutant, AP359, was not avirulent in the swine infection model. All pigs infected with wild-type AP225 survived to the end of the experiment and showed less severe symptoms and clinical signs than expected for the challenge dose used. In stark contrast, all pigs infected with the *lrp* mutant required euthanasia within 18 h post infection and showed significant clinical signs of *A. pleuropneumoniae* infection during the time course, including elevated respiration rate, dyspnea, depression, and loss of appetite. Pigs infected with $5xLD_{50}$ of the *lrp* mutant showed the most severe symptoms and on average were euthanized within 13.3 h post infection. Pigs infected with AP359 and AP359 containing the *lrp* gene in *trans* (pTW415) resulted in death times and clinical signs consistent with what would be expected of wild-type infections. Respiration rate, temperature, dyspnea, depression, and loss of appetite of pigs infected with AP359 or AP359/pTW415 were similar at comparable doses. All control pigs challenged with PBS showed no signs of disease during the course of the experiment, as expected.

Clinical scores are normally calculated from experimental data from the entire experiment to accurately compare infections. However, the clinical scores presented in Table 2 are calculated from the first 12 hours post infection because infection with AP359 resulted in death times considerably shorter than the other strains in this experiment. To provide an accurate comparison between AP359 and the other strains, clinical score data was used from time points in which the majority of animals were still alive.

Table 4-1: Dose and mortality data

Group	Strain	LD_{50}[a]	Actual CFU/ Dose[b]	Mortality[c]	Death Time[d]
1	AP225	$1xLD_{50}$	$4x10^6$	0/3	39 h.
2	AP359/ pTW415	$1xLD_{50}$	$3x10^6$	1/2	28.5 h.
3	AP359	$1xLD_{50}$	$4x10^6$	3/3	18 h.
4	AP359	$5xLD_{50}$	$2x10^7$	3/3	13.3 h.
5	PBS	0	0	0/3	39 h.

[a] The established LD_{50} for wild-type *A. pleuropneumoniae* serotype 1 is $5.0x10^6$ CFU for $1xLD_{50}$ and $2.5x10^7$ for $5xLD_{50}$ (21).
[b] Actual CFU/dose is the actual CFU of the prepared dose before challenge.
[c] Mortality reported as number of deceased swine/number of swine in group.
[d] Average time of death of animal within group.

Table 4-2: Clinical score data

Group	Strain	Max RR[a]	Max Temp (°F)[b]	Dyspnea[c]	Depression[d]	Appetite[e]
1	AP225	21.0 +/-2.6	105.2 +/-0.8	0.3	0.7	1.0
2	AP359/pTW415	22.0 +/-2.8	103.9 +/-0.7	1.0	1.5	1.0
3	AP359	21.7 +/-4.9	105.6 +/-1.3	1.0	3.7	2.0
4	AP359	21.3 +/-0.6	101.3 +/-1.9	5.0	5.0	3.0
5	PBS	11.3 +/-1.5	104.8 +/-0.8	0	0	0

[a] Average of the maximum respiration rates for each animal in the group within the first 12 hours post infection (recorded as number of breaths per 15-s observation period).
[b] Average of the maximum rectal temperatures for each animal in the group in degrees Farenheit within the first 12 hours post infection.
[c] Dyspnea measures the degree of respiratory distress. Average of the total scores from each group member recorded at 4, 8, and 12 hours post infection. Range: 0 (normal) – 9 (severe).
[d] Depression evaluates attitude and activity. Average of the total scores from each group member recorded at 4, 8, and 12 hours post infection. Range: 0 (normal) – 9 (severe inactivity).
[e] Appetite measures the willingness of the pig to consume food. Average of the total scores from each group member recorded at 4, 8, and 12 hours post infection. Range: 0 (did eat) - 3 (did not eat).

At necropsy, lungs of challenged pigs were analyzed for differences in gross pathology. PBS-challenged pigs were observed to be normal and extremities and lungs were unremarkable. In contrast, pigs infected with bacteria showed varying signs of cyanosis and hyperemia around the eyes, ears, and snout with more severe cases in pigs challenged with $5xLD_{50}$ of AP359 including cyanosis in the leg, abdomen, and perianal areas. Typical lesions and signs of pleuropneumonia such as hemorrhage, regions of necrosis with fibrin deposits, congestion, edema, and consolidation were observed in

lungs of pigs challenged with AP225, AP359/pTW415, or AP359. Figure 1 shows photographs of representative whole lungs from challenged pigs. The AP359 infected lungs displayed slightly more hemorrhage and necrosis as compared to the lungs of the pigs infected with the wild-type strain at similar doses. Table 3 summarizes the percentage of pneumonia of the lungs from challenged pigs. In general, clinical scores correlated with the severity of lung pathology. The higher dose of AP359 resulted in the most severe pneumonia. Pathology seen in lungs from AP225, AP359, and AP359/pTW415 infected pigs that received $1xLD_{50}$ was less severe and less pneumonia was observed. No significant difference in severity of lung pathology between the $1xLD_{50}$ doses of AP225, AP359, and AP359/pTW415 was observed. These data, including mortality, death time, clinical scores, and lung pathology, are consistent with an acute pleuropneumonia in the lungs of pigs challenged with wild-type, *lrp* mutant, and complemented *lrp* mutant.

Table 4-3: Lung score and bacteriology data

Group	Strain	% Pneumonia[a]	Lung[b]	Blood[b]
1	AP225	8.1 (1.3-15.4)	3/3	0/3
2	AP359/pTW415	14.3 (1.8-26.9)	2/2	0/2
3	AP359	17.3 (4.3-34)	2/3	1/3
4	AP359	36.6 (25.8-48.2)	3/3	0/3
5	PBS	0	0/3	0/3

[a] Average weighted percentage of lung tissue exhibiting *A. pleuropneumoniae* lesions with range within parenthesis.
[b] The data is presented as the number of positive cultures for *A. pleuropneumoniae* per total number examined.

Figure 4-1. Lung and histopathology samples. Gross and microscopic photographs of lungs from challenged pigs are presented in paired panels A and B, respectively. All histopathology slides are at 20X magnification. Each paired sample is labeled (below) with the strain and dose used for challenge: wild-type *A. pleuropneumoniae* at $1xLD_{50}$ (AP225 $1xLD_{50}$), the *A. pleuropneumoniae* lrp mutant at $1xLD_{50}$ (AP359 $1xLD_{50}$), the *A. pleuropneumoniae* lrp mutant at $5xLD_{50}$ (AP359 $5xLD_{50}$), the *A. pleuropneumoniae* complemented lrp mutant at $1xLD_{50}$ (AP359/pTW415 $1xLD_{50}$), and the PBS negative control challenge (PBS). Images in this thesis/dissertation are presented in color.

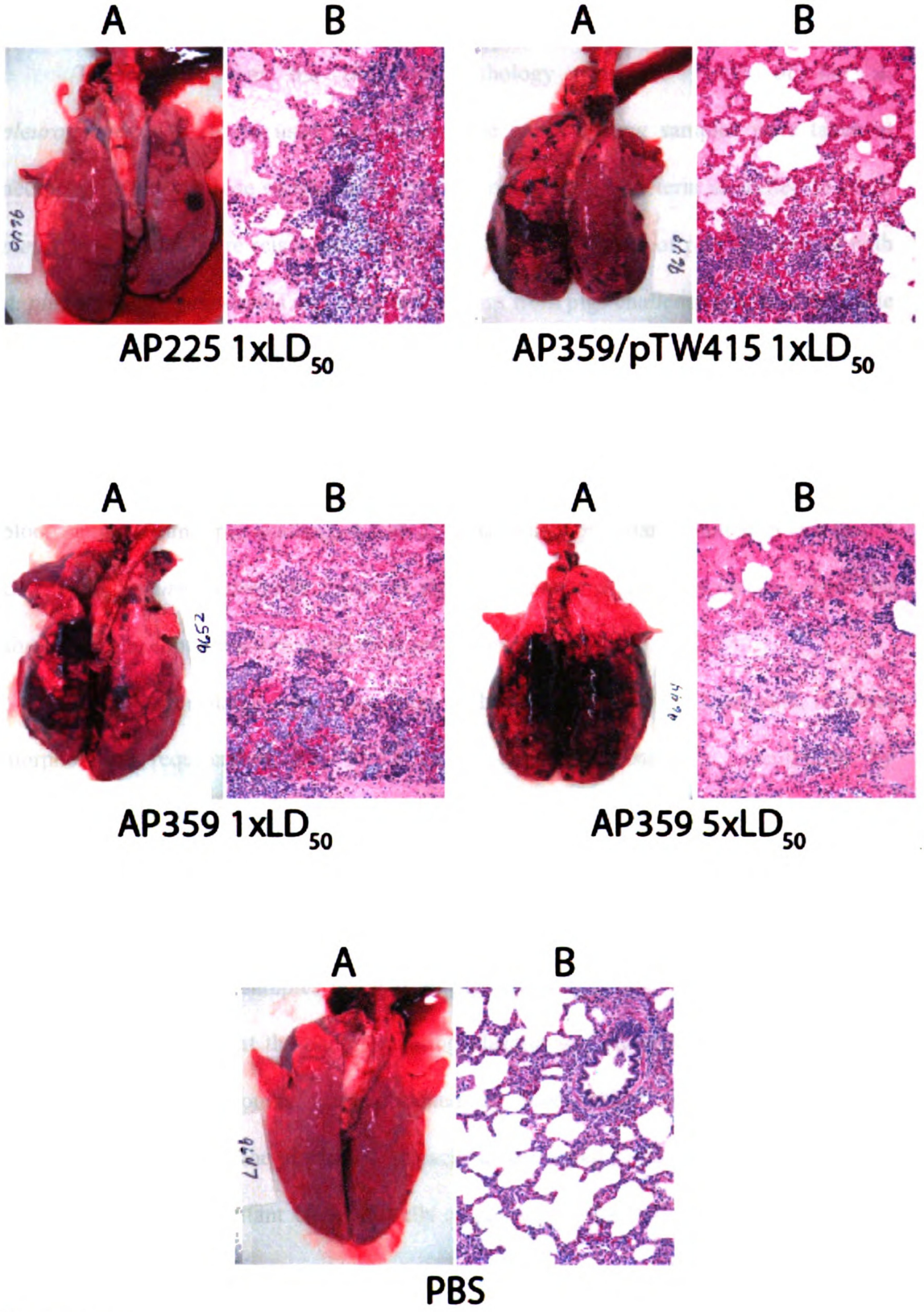
A
B
A
B
AP225 1xLD50
AP359/pTW415 1xLD50
A
B
A
B
AP359 1xLD50
AP359 5xLD50
A
B
PBS

Figure 4-1

To confirm that the observed pathology was the result of the *A. pleuropneumoniae* strains used to challenge the animals, lung samples were taken at necropsy for the purpose of reisolation of bacterial strains. Bacteria identified to be *A. pleuropneumoniae* were reisolated from all but one of the lungs of pigs challenged with *A. pleuropneumoniae* strains and none of the lungs from pigs challenged with PBS (Table 3). Blood from infected animals was also collected and cultured for *A. pleuropneumoniae*. As expected based on results from prior experiments, the majority of blood samples were negative for *A. pleuropneumoniae* (Table 3). Unexpectedly, one blood sample from a pig challenged with $1xLD_{50}$ of the *lrp* mutant resulted in less than 5 CFU of *A. pleuropneumoniae*. Due to the low number of bacteria isolated from the single sample, contamination is the most probable explanation.

Bacteria reisolated from lungs were further characterized by Gram stain, colonial morphology, requirement for V (β-NAD), antibiotic sensitivity, serotyping by coagglutination, and PCR to confirm that the reisolated strains were the same as the inocula used to infect each animal. All reisolated bacteria showed no differences from the strains initially used to challenge the animals (data not shown). Lung and bronchial alveolar lavage (BAL) samples taken from pigs challenged with AP359/pTW415 were analyzed to confirm that the complementing plasmid was maintained during infection. Equivalent bacterial counts were obtained on both BHIV and BHIV containing kanamycin to select for the plasmid from each sample tested (Table 4). This indicates the vast majority of *lrp* mutant bacterial cells containing pTW415 maintained the plasmid

during the course of infection. Furthermore, these data refute any assertion the observed pathology of AP359/pTW415 is due to loss of the complementing plasmid.

Table 4-4: Maintenance of pTW415 during infection

Pig#[a]	Sample[b]	Selection[c]	CFU/ml
9641	Lung	-	$5x10^6$
9641	Lung	+	$5x10^6$
9649	BAL	-	$6x10^8$
9649	BAL	+	$6x10^8$
9649	Lung	-	$9x10^8$
9649	Lung	+	$8x10^8$

[a] Pig 9641 and 9649 were both infected with $1xLD_{50}$ of AP359/pTW415.
[b] Indicates whether the sample was from infected lung or bronchial alveolar lavage (BAL).
[c] Indicates whether the growth medium contained 100 mg/ml kanamycin (+) or not (-).

Histopathology. To investigate the difference in severity of disease between wild-type and *lrp* mutant strains, histopathology was completed on lung tissue samples from challenged animals. The lungs from PBS challenged animals were typical of healthy lungs. The alveolar and bronchiolar epithelia were intact and surround clear air filled spaces, and there was no evidence of microscopic lesions such as edema, hemorrhage, inflammation, and necrosis (Figure 1). In stark contrast, lungs from wild-type infected animals were characterized by severe hemorrhage and necrosis surrounded by streaming neutrophils. There was accumulation of fibrin, blood, and necrotic debris in the affected areas and the alveolar septae were necrotic and no longer delineate between the air spaces of the lung. Edema and hemorrhage were evident resulting in fluid filled alveoli and bronchi and there was loss of nuclear detail within the septae. In comparison, lungs infected with $1xLD_{50}$ of the *lrp* mutant and the complemented *lrp* mutant showed similar microscopic features. The $5xLD_{50}$ dose of the *lrp* mutant shows similar histopathology to

the 1xLD_{50} dose of the *lrp* mutant and complemented *lrp* mutant, but the lungs of higher dose animals were more diffusely affected, had larger numbers of intralesional bacteria, and had more severe inflammation of the vascular walls. When comparing similar challenge doses, there was no significant difference in the histopathology between wild-type, *lrp* mutant, and complemented *lrp* mutant.

Discussion

In this paper we report the results of an animal challenge experiment with an *A. pleuropneumoniae* serotype 1 *lrp* mutant. The *lrp* mutant is a deletion-disruption mutant constructed by allelic exchange with an *lrp* mutant allele containing an inserted chloramphenicol resistance marker (see Chapter 3). We have previously shown that Lrp binds to the promoters of two genes, *ilvI and cps*, that are specifically expressed during infection of the natural swine host and regulates the expression of these genes (see Chapter 3). We hypothesized that the virulence of an *A. pleuropneumoniae lrp* mutant would be attenuated in a swine infection model, in part due to the lack of expression control of these *ivi* genes in an *lrp* mutant.

However, the *lrp* mutant at both doses tested was not avirulent in an experimental infection of the natural swine host when compared to a typical wild-type strain. This result suggests Lrp or the genes identified to be controlled by Lrp in *A. pleuropneumoniae* may not be critical for virulence in the conditions tested. While other *A. pleuropneumoniae* protein regulators, Fur and HlyX, which positively control genes required for *A. pleuropneumoniae* virulence, have been mutated and resulted in attenuated strains of *A. pleuropneumoniae* (4, 19), the *lrp* mutation had no observable effect on virulence. This result is in contrast to our prediction. The *A. pleuropneumoniae lrp* mutant is unable to grow or induce the expression of *ilvI in vitro* under branched-chain amino acid limiting conditions (see Chapter 3). However, this phenotype does not seem to translate to an avirulent organism during infection because the *lrp* mutant was readily reisolated from infected lungs (Table 3), unlike an avirulent *A. pleuropneumoniae*

riboflavin auxotroph (14). The *ilvI* gene product may not be critical during infection because either the other acetohydroxy acid synthase isozymes can compensate for the lack of the *ilvI* product or the amount of branched-chain amino acids available is not limited enough to require *ilvI* expression. In an acute infection with severe hemorrhage and necrosis, the amount of branched-chain amino acids may not be limiting because host cell contents rich in BCAAs are released due to the hemolytic and cytotoxic activity of the Apx toxins. In this case, *A. pleuropneumoniae* may not be required to synthesize its own BCAAs. However, early in infection when little hemorrhage and necrosis are present or when *A. pleuropneumoniae* is phagocytosed, *ilvI* may be more critical. Analysis of an *ilvI* mutant would help to test this hypothesis, however, the role of *ilvI* during infection is unknown because the construction of an *ilvI* mutant was unsuccessful. An *lrp* mutant was proposed to not only improperly regulate *ilvI* but possibly other enzymes involved in BCAA biosynthesis and virulence.

The lack of attenuation of the *lrp* mutant could be explained by reversion of the *lrp* mutant to wild-type by loss of the inserted chloramphenicol resistance marker. This is unlikely for several reasons: 1) The *lrp* mutant is a deletion-disruption mutant and loss of the inserted chloramphenicol marker would still result in a ~100-bp deletion in *lrp*; 2) we observed no reversion from chloramphenicol resistance to susceptibility of the *lrp* mutant when grown on non-selective laboratory media; and 3) the *lrp* allele from bacteria reisolated from *lrp* mutant challenged pigs still contained the chloramphenicol marker in the same location and orientation as the initial inocula.

While the *lrp* mutant and complemented *lrp* mutant infections resulted in an acute pleuropneumonia with high mortality and clinical scores, the experimental wild-type

infection was less severe than expected. In previous studies, the dose of AP225 used has routinely caused death of 50% of the infected animals. Why the degree of infection seen in this experiment is less than usually obtained with this dose is unclear. While the increased mortality, more rapid death times, and more severe clinical scores and gross pathology of the *lrp* mutant in comparison to the wild-type strain suggest the possibility that the *lrp* mutant is more virulent than the wild-type strain, it is important to recognize that only three animals per group were used in this study and that the only groups that are significantly different are the group that received the $5xLD_{50}$ dose of the *lrp* mutant and the uninfected controls.

Since Lrp has been shown to be both a positive and a negative regulator of genes in *E. coli*, it is possible a more virulent strain could result from an *lrp* mutation depending upon the overall change in gene expression. More virulence genes could be induced than repressed and therefore tip the balance of gene expression in *A. pleuropneumoniae* to promote an overall increase in virulence gene expression. Although Lrp was shown not to bind to the promoters of the Apx toxins and therefore is not directly involved in the regulation of these key *A. pleuropneumoniae* virulence genes, it is possible Lrp could indirectly regulate these genes or other virulence genes and drastically alter the virulence of *A. pleuropneumoniae*. To begin to analyze this possibility, a larger study with more animals per group would be necessary to more accurately measure relative virulence of these strains.

Alternatively, the fitness of an *lrp* mutant in comparison to the wild-type strain could be analyzed in a competitive index experiment during infection by measuring survival of each strain by quantitative culturing from lung samples, such as those

presented in Table 4. Furthermore, global gene expression experiments, to determine the Lrp regulon in *A. pleuropneumoniae* and determine if an *lrp* mutation increases the expression of known virulence genes, could be performed to determine the full repertoire of Lrp regulated genes.

This is the first report to specifically address the effects of an *lrp* mutant on the virulence of a pathogen. The lack of avirulence of the *lrp* mutant in the observed animal experiment suggests *lrp* does not have an obvious affect on the virulence of *A. pleuropneumoniae* under the conditions studied. Subtle changes in the virulence of the *lrp* mutant, resulting in slightly less or more virulence, are impossible to determine due to the number of animals infected. Experiments with more animals or competitive indices, and potentially with an aerosol model or lower doses, are needed to characterize the exact change, if any, in virulence of the *lrp* mutant.

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Chapter 5

Summary

Actinobacillus pleuropneumoniae is a bacterial pathogen that causes a severe respiratory disease in swine and is of major economic importance world-wide. The economic impact of *A. pleuropneumoniae* can be significant when taking into account the mortality, morbidity, and veterinary costs associated with the disease. The severe economic impact of *A. pleuropneumoniae* infection has demanded research into ways to prevent, control, and treat pleuropneumonia. Research into *A. pleuropneumoniae* genetics has led to the development of tools and techniques for identifying *A. pleuropneumoniae* genes involved in virulence (1, 7, 8). While many *A. pleuropneumoniae* genes important in the disease process have been identified, very little is known about when and under what conditions the genes are expressed and how the gene expression is controlled to provide for an efficient and successful disease.

The scientific literature, addressing when and under what conditions *A. pleuropneumoniae* genes important for virulence are expressed, is limited. By studying this, a better understanding of how *A. pleuropneumoniae* causes disease can be obtained and therefore improved methods to prevent, treat, and control disease can be developed. Well studied environmental signals for induction of virulence gene expression, such as limitation of iron and an increase in oxidative stress, have been examined in *A. pleuropneumoniae* (4-6, 9-11, 14, 16). However, additional environmental signals could play a role. We have identified that limitation of branched-chain amino acids (BCAAs) induces the expression of genes during infection of the host. This is the first report of BCAA limitation as an environmental cue to which bacterial pathogens respond. It is possible this signal is specific to pathogens infecting certain areas of the host, such as the lungs. The data in this thesis provides the foundation for further research addressing

questions such as: is there truly a concentration difference of free BCAAs between areas of the host; could drugs be engineered to disrupt BCAA biosynthesis and therefore specifically target respiratory pathogens while not affecting the mammalian host or the normal flora of the gastrointestinal tract; what other virulence genes may be expressed in response to a limitation of BCAAs; and how can this knowledge be used to prevent *A. pleuropneumoniae* disease?

Virulence gene regulation in *A. pleuropneumoniae* in response to a stimulus has only recently begun to be investigated. The ferric uptake regulator, Fur, and the anaerobic gene regulator, HlyX, are the only two regulators of gene expression that have been previously identified in *A. pleuropneumoniae* (12, 15). By identifying regulators of virulence gene expression in *A. pleuropneumoniae*, an understanding of how gene expression changes in response to the immediate environment of the pathogen can be obtained. We have shown a third regulator, Lrp, is involved in the regulation of at least two genes induced during infection of the natural host. This is a substantial addition to the *A. pleuropneumoniae* scientific literature. Not only has another regulator of *A. pleuropneumoniae* gene expression been identified and characterized biochemically, but this research shows *A. pleuropneumoniae* Lrp is involved in the regulation of one or more genes shown to be induced during infection. Furthermore, this research suggests, for the first time, that Lrp plays some role in the regulation of capsular polysaccharide biosynthesis, one of the main virulence factors of *A. pleuropneumoniae*. This research creates the ground work for further research into how Lrp may regulate capsule biosynthesis. In addition, this research provides the first insight into the Lrp regulon of *A. pleuropneumoniae* and could lead to further research identifying more of the regulon

through genomic arrays and quantitative reverse transcriptase polymerase chain reaction using bacterial RNA isolated from bronchial alveolar lavage fluid or lung tissue samples from natural infections, or growth under different *in vitro* conditions. The His_6-Lrp protein isolated in during this research could also be used in additional electrophoretic gel mobility shift assays using promoter fragments from other *A. pleuropneumoniae* genes of interest or to the promoters of *in vivo* induced genes that did not respond to BCAA limitation to identify additional genes regulated by Lrp. A more detailed investigation into the *A. pleuropneumoniae* Lrp consensus binding site using footprinting and mutational studies could also be performed.

Disabling a regulator of virulence gene expression through mutation can lead to the lack of control of virulence genes during infection. This can be beneficial in terms of constructing a live attenuated vaccine candidate because mutation of a single gene may not lead to a significant decrease in virulence (2), but mutation of a regulator of virulence genes can lead to more significant attenuation of virulence (3). Only two regulators of virulence gene expression have been targeted for mutation in *A. pleuropneumoniae*. In both cases, mutations in Fur and HlyX resulted in a decrease in virulence of *A. pleuropneumoniae* (3, 13). The research in this thesis expands upon the scientific knowledge of *A. pleuropneumoniae* regulators of gene expression by examining the role of Lrp in the virulence of *A. pleuropneumoniae* in a respiratory model of infection in the natural swine host. We show an *A. pleuropneumoniae* Lrp mutant is not attenuated under the experimental design and conditions tested. While the results were not predicted, the experiment does contribute to the understanding of *A. pleuropneumoniae* disease by showing a mutation in a regulator of genes involved in virulence may not be attenuated,

as it has been shown for Fur and HlyX. The experimental infection results raise many questions. Is an *ilvI* mutant attenuated in pigs even though an *lrp* mutant is not? Could the histopathology lung samples from an *lrp* mutant infected pig show any differences using anti-capsule antibodies when compared to wild-type? Could a quantitative competition infection experiment with wild-type and *lrp* mutant show a more significant difference? What are the differences in gene expression between wild-type and an *lrp* mutant? Does the expression of any of the well studied *A. pleuropneumoniae* virulence factors change in an *lrp* mutant? These questions represent many different paths and projects the research in this thesis could generate.

The scope of this dissertation was to study the genetic basis of virulence gene regulation in *A. pleuropneumoniae* and to determine an environmental stimulus and regulatory mechanism that mediated virulence gene expression and disease. BCAA limitation was identified as an important environmental cue for *A. pleuropneumoniae* gene regulation, and Lrp was shown to be important in this regulation. Both BCAA limitation and Lrp may be important in a broad range of pathogens. The data presented here adds to the understanding of *A. pleuropneumoniae* and possibly other respiratory pathogens while also providing a foundation for further research into *A. pleuropneumoniae* virulence gene environmental stimuli, regulation of virulence gene expression, and how these components interact to cause disease.

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