ROLE OF IN VIVO-INDUCED GENES *ILVI* AND *HFQ* IN THE SURVIVAL AND VIRULENCE OF *ACTINOBACILLUS PLEUROPNEUMONIAE*

By

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ABSTRACT

ROLE OF IN VIVO-INDUCED GENES *ILVI* AND *HFQ* IN THE SURVIVAL AND VIRULENCE OF *ACTINOBACILLUS PLEUROPNEUMONIAE*

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Actinobacillus pleuropneumoniae is the etiological agent of porcine pleuropneumonia, a contagious and often fatal disease of pigs. Infection with this bacterium leads to the development of a fulminant pleuropneumonia resulting in severe damage to the lungs. *A. pleuropneumoniae* genes that are up-regulated during the infection of pig lungs were previously identified in our laboratory and designated as in vivo-induced genes. The role of two such in vivo-induced genes, *ilvI* and *hfq*, in the pathobiology of *A. pleuropneumoniae* is the subject of this dissertation.

The gene *ilvI* encodes an enzyme involved in the biosynthesis of branched-chain amino acids (BCAAs). The leucine-responsive regulatory protein (Lrp) is a transcriptional regulator of the *ilvIH* operon. BCAA biosynthetic genes are associated with virulence in pathogens infecting the respiratory tract and blood stream. Also, twenty five percent of the *A. pleuropneumoniae* in vivo-induced genes were up-regulated under BCAA limitation, suggesting that these BCAAs may be found at limiting concentrations in certain sites of the mammalian body such as the respiratory tract. The concentration of amino acids in the porcine pulmonary epithelial lining fluid was determined and BCAAs were found at limiting concentration in the respiratory tract. Further, the virulence of two BCAA auxotrophs, an *ilvI* mutant and an *lrp* mutant, was tested in a pig infection model and both were found to be attenuated. Finally, inhibitors of BCAA biosynthesis were found to prevent the growth of *A. pleuropneumoniae*.

Hfq, encoding the Host factor Q-beta (Hfq) in *A. pleuropneumoniae*, was identified as an in vivo-induced gene and is also up-regulated under BCAA limitation. Since Hfq is a global regulator which is induced under one of the signals found in the porcine respiratory tract, the role of Hfq in *A. pleuropneumoniae* was analyzed. An *A. pleuropneumoniae hfq* mutant strain failed to form biofilm. Levels of the *pgaC* transcript, encoding the biofilm matrix biosynthetic enzyme, were ~14-fold lower in the *hfq* mutant compared to wild-type strain. The *hfq* mutant displayed enhanced sensitivity to superoxide stress and tellurite. Hfq was found to regulate two virulence-associated phenotypes, biofilm formation and resistance to oxidative stress, in *A. pleuropneumoniae*.

As Hfq was associated with at least two virulence-associated phenotypes, the effect of Hfq on the virulence of *A. pleuropneumoniae* was tested in a pig infection model. Infection with the *hfq* mutant did not result in the development of pleuropneumonia while pigs infected with the wild-type strain exhibited classic signs of pleuropneumonia. Competitive index analysis revealed that the *hfq* mutant is severely attenuated, compared to the wild-type strain.

In summary, studies described in this dissertation have uncovered a new host signal found in the porcine respiratory tract and provide evidence for the potential of inhibitors of BCAA biosynthesis as antibacterial agents. *Hfq*, regulated by BCAA limitation, was found to regulate biofilm formation, resistance to oxidative stress and virulence in *A. pleuropneumoniae*. The role of Hfq in the virulence of a bacterial pathogen during infection of lungs has been examined in the studies described in this dissertation. The *hfq* mutant is highly attenuated and is a potential candidate for the development of a live-attenuated vaccine against porcine pleuropneumonia.

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PREFACE

Three chapters presenting original research, formatted as manuscripts, are included in this dissertation.

Chapter 2, entitled "Branched-chain amino acids are required for the survival and virulence of *Actinobacillus pleuropneumoniae* in swine", has been published in the journal Infection and Immunity with Ms. Rhiannon LeVeque and Drs. Trevor Wagner, Roy Kirkwood, Matti Kiupel and Martha Mulks as co-authors (Volume 77 (11), Pages 4929-4933, 2009). Contribution of co-authors: Ms. Rhiannon LeVeque and Dr. Trevor Wagner constructed the *ilvI* and *lrp* mutants, respectively; Rhiannon LeVeque performed the Q-PCR experiment and helped with animal infection study; Dr. Roy Kirkwood provided veterinary care for the animals; Dr. Matti Kiupel conducted necropsy of pigs and interpreted histopathology results.

Chapters 3 and 4 of this dissertation will be combined into one manuscript and submitted to the journal Infection and Immunity with Drs. Roy Kirkwood, Matti Kiupel and Martha Mulks as co-authors. Contribution of co-authors: Dr. Roy Kirkwood provided veterinary care for the animals; Dr. Matti Kiupel conducted necropsy and interpreted histopathology results.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

AN INTRODUCTION TO *ACTINOBACILLUS PLEUROPNEUMONIAE* AND PORCINE PLEUROPNEUMONIA

INTRODUCTION

Infectious diseases are a major cause of economic loss in swine production. High stocking densities achieved at modern swine rearing facilities lead to an increased risk of rapid spread of infectious diseases. One such important bacterial disease, inflicting significant morbidity and mortality on swine, is porcine pleuropneumonia caused by *Actinobacillus pleuropneumoniae* (101).

A. pleuropneumoniae is an encapsulated Gram-negative pleiomorphic coccobacillus in the family *Pasteurellaceae*. *A. pleuropneumoniae* strains are differentiated into two biotypes based on the requirement for nicotinamide adenine dinucleotide (NAD) for growth and into fifteen serotypes based on the composition of capsular polysaccharides. Serotypes 1 through 12 and 15 belong to biotype 1 and require NAD for growth while serotypes 13 and 14 belong to biotype 2 and do not require NAD for growth (22).

There is a distinct geographical distribution for various serotypes and serotypes 1, 5, and 7 are commonly associated with outbreaks in the USA (22). With no known environmental reservoirs, pigs are the only known natural host for *A. pleuropneumoniae*. In carrier animals, *A. pleuropneumoniae* can be isolated from the tonsillar crypts and serve as a reservoir of infection for naive animals (118). The incidence of *A. pleuropneumoniae* outbreaks has been controlled by the widespread use of multivalent vaccines and maintenance of *A. pleuropneumoniae*-free herds, as well as changes in management practices that limit the mixing of pigs purchased from different breeding herds. However, porcine pleuropneumonia continues to remain as a looming threat to intensive swine farming, both in the US and around the world (49).

Porcine pleuropneumonia. Clinical pleuropneumonia occurs when *A. pleuropneumoniae* spreads either from the tonsils to the lungs in a carrier or is acquired by the naive pigs from infected pigs and colonizes the lungs. Porcine pleuropneumonia is characterized by a fulminating fibrino-hemorrhagic bronchopneumonia marked by severe edema, hemorrhage, necrosis, and fibrinous pleuritis, and is often fatal (22, 49, 101, 102). The clinical presentation and outcome of porcine pleuropneumonia can vary widely with age, immune status and the dose of *A. pleuropneumoniae* acquired by individual pigs. Depending on the severity and progress, this disease can be classified into peracute, acute, or chronic presentation.

Peracute cases of *A. pleuropneumoniae* infection progresses rapidly and pigs are often found dead. If the diseased pigs are identified before they are dead, extreme respiratory distress—dyspnea—with a progressive cyanosis of the whole body is observed. Severe dyspnea is marked by the assumption of a dog-sitting posture and mouth-breathing (49).

Acute infection with *A. pleuropneumoniae* results in severe dyspnea along with cough and mouth-breathing is observed at later stages, shortly before death. Body temperature rises up to 40.5°C and pigs stop feeding. There is reddening of skin over the whole body. Pigs exhibit a marked loss of condition within 24 hours after the onset of clinical signs (49). Some pigs can recover from acute infection and become chronically infected with *A. pleuropneumoniae.* However, distinction between peracute and acute infection is not always evident and depending on a multitude of factors, such as the antibody levels and general health, some animals can recover from peracute and acute infections.

Chronic cases are marked by resolution of pulmonary lesions with formation of fibrous tissue capsule. There can be spontaneous or intermittent cough. Often, these pigs continue to carry *A. pleuropneumoniae* in their tonsils and are a source of infection for piglets and naive

animals (49). Detection and culling of chronically infected animals coupled with replacement of animals from porcine pleuropneumonia-free farms are important preventive measures adopted to minimize the possibility of porcine pleuropneumonia outbreaks.

Pathologic changes in porcine pleuropneumonia. Gross pathologic lesions found in the peracute and acute cases include fibrinous pleurisy, blood tinged fluid in the thoracic cavity, and focal dark demarcated pneumonic lesions on both the right and left lung lobes. Edema is evident in the tracheobronchial lymph nodes, which are distended due to massive polymorphonuclear leukocyte (PMN) infiltration. Trachea and bronchi contain foamy blood-tinged mucus. The cut surface of the lung is friable and small pieces of the lung tissue from pneumonic areas submerge in water while the lung tissue from healthy areas of the lung remain floating in water, indicating the loss of air space due to pneumonia (101). In chronic cases, aging of lesions result in the development of adhesions to the parietal pleura. Pneumonic areas resolve into abscess-like nodules walled off by connective tissue capsule. High prevalence of chronic pleuritis and adhesions in abattoir examinations are suggestive of pleuropneumonia (49).

Histopathology findings in the lung sections include necrosis, hemorrhage, PMN and macrophage infiltration, coccobacilli within and along the alveoli, thrombosis in the alveolar blood vessels, and widespread edema with fibrinous exudate (49). Early stages of infection reveal alveolar septal edema, capillary congestion, platelet aggregation, neutrophil degranulation and robust PMN cell infiltration (18). Microcolonies can be found within the alveoli. Necrotizing vasculitis leads to extensive hemorrhages. In chronic infection, healing by fibrosis around necrotic centers is evident along with macrophage infiltration. Edges of lesions are demarcated by the presence of dead and damaged macrophages, adjoining healthy lung tissue.

Virulence factors. *A. pleuropneumoniae* expresses several established and putative virulence factors to adhere to the porcine respiratory tract, acquire essential nutrients, multiply within porcine lungs, and cause damage to the lung tissue. Studies describing the identification and function of virulence factors in *A. pleuropneumoniae* are reviewed here, in the order of those involved in adherence, nutrient acquisition, colonization, and induction of lesions in the lungs.

Fimbriae. Fimbriae or pili are filamentous surface appendages used by many pathogenic bacteria for attachment to host tissues, which is one of the initial events in colonization. *A. pleuropneumoniae* encodes for proteins involved in the biosynthesis and assembly of fimbrial low-molecular-weight protein (Flp) pili, fine-tangled pili and type IV fimbriae.

Function of the Flp pilus in *A. pleuropneumoniae* is not known, but the Flp pilus is required for tight adherence, biofilm formation, and full virulence in *Aggregatibacter (Actinobacillus) actinomycetamcomitans*, a closely related human periodontal pathogen, in a rat model of periodontitis (111). Since *A. pleuropneumoniae* and *A. actinomycetamcomitans* share homologous genes for biofilm formation and dispersal, Flp pili could also play a role in biofilm formation by *A. pleuropneumoniae* (64). *TadZ,* encoding a protein involved in Flp pilus formation, was identified as one of the transcripts expressed by *A. pleuropneumoniae* during infection of the pig lungs, suggesting that Flp pili could play a role in the virulence (9).

The *ftpA* gene, encoding the major subunit of the fine tangled pilus is found in the *A. pleuropneumoniae* genome sequence (39). Fine tangled pilus was originally identified in *Haemophilus ducreyi*, the etiological agent of chancroid in humans, but the role of this pilus in the pathogenesis of *H. ducreyi* is not fully understood (2). However, neither the presence nor the role of fine tangled pilus in *A. pleuropneumoniae* has been experimentally verified.

Utrera et al. reported the presence of fimbriae in *A. pleuropneumoniae* isolated from infected lung and that the production of fimbriae was lost upon subculture in laboratory medium (114). *A. pleuropneumoniae* produces type IV fimbriae during growth in defined medium under microaerophilic conditions (129). The presence of fimbriae was observed by transmission electron microscopy. The type IV fimbrial protein was detected in Western blots with anti-type IV fimbrial antibodies developed against *Moraxella bovis* type IV fimbriae and was confirmed by N-terminal sequencing (129). Protease treatment reduced the attachment of *A. pleuropneumoniae* to porcine alveolar epithelial cells *in vitro*, showing that proteins are indeed involved in this interaction. Treatment with sodium metaperiodate, a carbohydrate modifying agent, also reduced the attachment of *A. pleuropneumoniae* to porcine alveolar epithelial cells, but the degree of adherence defect was higher with proteinase treatment. Together, these results suggest that both carbohydrates and proteins play a role adherence, but protein-based interactions are more important for adherence than carbohydrate-mediated interactions (88).

Genes involved in the biosynthesis of fimbriae in *A. pleuropneumoniae* are found in the *apfABCD* operon (107). Transcriptional fusion of *luxAB* genes with the *apfABCD* promoter was used to determine the conditions which regulate the expression of the fimbrial operon in *A. pleuropneumoniae*. The *apfABCD* promoter activity was ~8 fold higher in *A. pleuropneumoniae* adherent to porcine lung epithelial cells compared to non-adherent cells. Expression from the *apfABCD* promoter was ~4 fold higher in the porcine lungs, during infection, compared to the negative control plasmid (19). These findings show that the type IV fimbriae are involved in adherence to alveolar epithelial cells and are expressed during infection.

Biofilm. Bacteria are frequently found as sessile communities embedded in a self-produced extracellular polymeric matrix described as a biofilm. Bacterial pathogens growing as a

biofilm have enhanced resistance to host defense mechanisms and antimicrobial agents (52). Many *A. pleuropneumoniae* strains grow as a biofilm with poly-β-1, 6-N-acetyl glucosamine (PGA) as the major biofilm matrix component (58). The biofilm-forming ability of seventy seven field isolates and fifteen reference strains on glass and polystyrene surfaces was tested and 50% of field isolates but only two among the fifteen reference strains formed biofilm (62). Biofilm-positive strains form small, rough colonies on agar, while biofilm-negative strains form large, smooth colonies (62). Kaplan et al. also found that subculture in broth led to the loss of biofilm forming ability, concomitant with a conversion from rough to smooth colony morphology. Since biofilms are commonly formed by field isolates and lost upon subculture in the laboratory, it has been proposed that biofilms could be formed in the pigs and contribute to the persistent colonization of *A. pleuropneumoniae,* such as in the tonsils of carrier pigs (62).

PGA biosynthesis and export is encoded by the *pgaABCD* operon in *A. pleuropneumoniae* (64). The role of each protein in PGA biosynthetic and export machinery has been described in *Escherichia coli* (122). PgaC is an inner membrane-associated glycosyl transferase which catalyzes PGA production; PgaD is also known to co-localize with PgaC, but its exact role in PGA production is unclear; PgaB is a periplasmic deacetylase and partial deacetylation promotes the export of PGA through outer membrane; PgaA is an outer membrane β-barrel protein which forms the channel through which PGA is secreted. *A. pleuropneumoniae* and a closely related human oral pathogen, *A. actinomycetamcomitans* both produce a hexosaminidase, dispersin B encoded by *dspB*, which specifically degrades the PGA matrix (64). Homologues encoding the production of PGA-based biofilms are found in the genomes of several bacterial pathogens, such as *A. actinomycetamcomitans, Bordetella pertussis, B. bronchiseptica, E. coli* O157:H7, uropathogenic *E. coli* CFT073, *Staphylococcus aureus, S.*

epidermidis, Yersinia enterocolitica, and *Y. pestis* (122). However, dispersin B has been identified only in *A. pleuropneumoniae* and *A. actinomycetamcomitans* (63, 64).

Recent studies have unraveled potential regulators of biofilm formation in *A. pleuropneumoniae.* ArcAB is a two-component regulatory system involved in the adaptation to growth under anaerobic conditions. Buettner et al. reported that an *A. pleuropneumoniae arcA* mutant strain was defective in auto-aggregation and biofilm formation on glass surfaces (28). LuxS is the biosynthetic enzyme for autoinducer-2, a bacterial interspecies quorum sensing signaling molecule. A *luxS* mutant strain of *A. pleuropneumoniae* was shown to produce more biofilm than the parent strain (73). The effect of different growth conditions on biofilm formation by *A. pleuropneumoniae* was examined and low levels of zinc in the media were suggested to promote biofilm formation (69). This observation is intriguing because zinc is found at limiting concentrations, similar to iron limitation, within mammalian hosts and could be a host signal for inducing biofilm formation. However, these studies did not attempt to identify a mechanism of regulation of biofilm formation.

Bosse et al. showed that sigma E (extracytoplasmic stress response sigma factor) and histone-like nucleoid structuring protein (H-NS) regulate biofilm formation, at transcriptional level, by modulating the expression from the *pgaABCD* operon in *A. pleuropneumoniae* (23). A mutation in the *rseA* gene, an anti-sigma factor which normally sequesters sigma E in the inner membrane, led to an increased activity of sigma E and induced biofilm formation in a non-biofilm forming strain. Mutation at the *hns* locus also led to the induction of biofilm formation in an otherwise non-biofilm forming strain. Sigma E and H-NS act as positive and negative transcriptional regulators of the *pga* operon expression, respectively. Further studies on the

regulation of biofilm formation in *A. pleuropneumoniae* will expand our knowledge of PGA-based biofilms which are a problem of significant medical importance.

Iron acquisition. Iron is an essential nutrient for most forms of life. Host glycoproteins, such as lactoferrin and transferrin, sequester iron to impede bacterial growth as a part of innate immunity, known as nutritional immunity. However, pathogenic bacteria, including *A. pleuropneumoniae*, are adept at acquiring iron from the host utilizing high-affinity iron acquisition systems. *A. pleuropneumoniae* uses transferrin-binding proteins, hemoglobin-binding proteins, and a ferric hydroxamate uptake system to acquire iron from the porcine respiratory tract (60).

The growth defect of *A. pleuropneumoniae* in iron-restricted media is abrogated by supplementation with porcine transferrin or porcine hemoglobin but not with transferrin or hemoglobin obtained from human and bovine sources indicating that *A. pleuropneumoniae* utilizes iron only from porcine transferrin or hemoglobin (87). Two porcine transferrin-binding membrane proteins (Tbp) were identified by affinity purification with biotinylated porcine transferrin and later designated as TbpA and TbpB (48). Two Ton systems, which transfer energy generated by the proton motive force at the inner membrane to the outer membrane receptors to internalize receptor-bound iron sources, have been identified in *A. pleuropneumoniae*. Tonpitak et al. have shown that ExbB1-ExbD2 proteins of the Ton1 system are required for extracting iron from Tbp-bound transferrin (112). An *A. pleuropneumoniae exbB1D1* mutant was attenuated in porcine infection model and this finding shows that the ability to obtain iron from transferrin is indispensable for virulence (11). The Ton2 system, TonB2-ExbB2-ExbD2, is also involved in the uptake of iron from hemin, porcine hemoglobin and/or ferrichrome and a *tonB2* mutant was attenuated in an acute porcine infection model (14).

Two iron-regulated outer membrane proteins which bind to both hemoglobin and hemin were identified in *A. pleuropneumoniae.* The 75 and 104 kDa proteins were identified by affinity chromatography with hemin-agarose and hemoglobin-agarose, respectively (5). The 75 kDa protein has similarity to iron-regulated outer membrane proteins. The gene encoding the 104 kDa protein has a high degree of homology to bacterial hemoglobin-binding protein genes (*hgbA*) and an *A. pleuropneumoniae hgbA* mutant loses the ability to utilize hemoglobin as the sole source of iron (60).

A. pleuropneumoniae can utilize iron from ferrichrome, a siderophore of the hydroxamate family, and genes encoding a ferric hydroxamate acquisition system are arranged in the *fhuCDBA* operon, which is found in all the serotypes of *A. pleuropneumoniae* (82). FhuA is the outer membrane receptor for ferrichrome; FhuD is the periplasmic protein which translocates FhuA-bound ferrichrome from outer membrane to inner membrane; FhuC and FhuB are inner membrane components of an ABC transporter which internalizes ferrichrome. A *fhuA* mutant strain was unable to utilize ferrichrome as an iron source, showing that FhuA is involved in the uptake or utilization of ferrichrome in *A. pleuropneumoniae.* FhuA was detected in Western blots with serum from *A. pleuropneumoniae* infected pigs, indicating that FhuA is expressed during infection (82). *A. pleuropneumoniae* produces only the ferrichrome uptake system and not the siderophore itself; thus gains iron without spending energy for the production of the siderophore (81). This finding also suggests that *A. pleuropneumoniae* might be part of a community where other bacteria synthesize the ferrichrome siderophore.

The ferric uptake regulator (Fur) is a bacterial transcription factor that regulates the expression of genes involved in iron uptake and metabolism. Fur dimer bound to iron binds to 19-base pair inverted repeats, termed the “Fur boxes”, in the promoter region of Fur-regulated

genes and prevents the expression of genes involved in iron acquisition (71). When the intracellular levels of iron drop, this repression is relieved and iron homeostasis is maintained by bacterial cells. Fur is also known to positively regulate the expression of some genes and this paradox was resolved after the identification of Fur-regulated small RNA—RhyB—first in *E. coli* and later in *Pseudomonas aeruginosa,* where RhyB acts as a post-transcriptional regulator of the genes involved in iron storage (77, 125). An *A. pleuropneumoniae fur* mutant is attenuated in a porcine infection model (59). In summary, the ability to acquire iron from various sources within the pig and the regulation of expression of genes involved in iron metabolism are indispensible for the virulence of *A. pleuropneumoniae.*

Capsule. Variation in the capsular polysaccharide (CPS) is the basis for serotyping *A. pleuropneumoniae* strains (61). CPS is an important virulence determinant used by pathogenic bacteria such as *A. pleuropneumoniae, Haemophilus influenzae, Neisseria meningitidis,* and *Streptococcus pneumoniae* to inhibit killing by host defence mechanisms, including phagocytosis and complement-mediated killing. CPS biosynthesis is encoded by the *cps5A, cps5B*, *cps5C*, and *cps5D* genes in the serotype 5 strains (124). Cps5A, Cps5B and Cps5C are putative glycosyl transferases involved in the biosynthesis of CPS. Cps5D has high degree of homology to KdsA, a protein involved in synthesizing 3-deoxy-D-manno-2-octulosonic acid 8-phosphate, required for the production of both CPS and LPS (124). Export of CPS is enabled by proteins encoded by the *cpxD, cpxC, cpxB,* and *cpxA* genes in *A. pleuropneumoniae* (123). The chemical composition of CPS from various strains of *A. pleuropneumoniae* has been determined. CPS of serotype 5 strains are composed of 2-acetamido-2-deoxy-D-glucose, D-glucose and 3-deoxy-D-manno-2-octulosonic acid (KDO) in the molar ratio of 1:1:1 (4).

Even in the presence of anti-capsule serum, encapsulated *A. pleuropneumoniae* strains are resistant to complement-mediated killing. A noncapsulated mutant of *A. pleuropneumoniae* serotype 5 is sensitive to serum indicating that capsule is involved in protection against killing by the complement system (57). Passive immunization of pigs with anti-capsule serum protected swine from mortality in an experimental *A. pleuropneumoniae* infection but did not prevent the occurrence of hemorrhagic lesions in the lungs. Together, these data show that anti-capsular antibodies aid in the clearance of *A. pleuropneumoniae,* but are not fully protective against the pathological changes induced during infection (57). An uncapsulated mutant generated by Tn-*10* insertion in the *cpxC* gene of *A. pleuropneumoniae* serotype 1 showed 20-fold higher adherence to frozen porcine tracheal ring sections compared to the encapsulated parent strain, indicating that capsule could indeed mask the exposure of surface components required for optimal adhesion. The *cpxC* mutant was attenuated in a pig infection model (93). Genes involved in the export of capsule, *cpxB, cpxC* and *cpxD* were found to be necessary for the survival of *A. pleuropneumoniae* serotype 1 in the pig (99). Thus capsule is an important virulence determinant of *A. pleuropneumoniae.*

Lipopolysaccharide. Lipopolysaccharide (LPS) is a common virulence factor found in Gram-negative bacteria. LPS is composed of the lipid A, the core oligosaccharide, containing 3-deoxy-D-manno-2-octulosonic acid (KDO), and the O-antigen, a variable region of repeating polysaccharide units. Lipid A is responsible for the toxicity of the LPS molecule.

The composition of LPS from various *A. pleuropneumoniae* serotypes has been determined (30). LPS from serotype 1 contains rhamnose and N-acetylglucosamine in the O-antigen while galactose, glucoheptose and mannoheptose are found in the core oligosaccharide (30). LPS of *A. pleuropneumoniae* not only promotes tissue damage by inciting a strong pro-

inflammatory response but is also implicated in the attachment to porcine respiratory tract (90). Pre-incubation of porcine tracheal rings with purified homologous LPS inhibits the adherence of *A. pleuropneumoniae,* indicating that LPS is involved in adhesion, an important early step during infection (17). LPS is also required for the full toxicity of Apx toxins. A mutation in the *galU* gene leads to a truncated outer core of *A. pleuropneumoniae* serotype 1 LPS and the mutant exhibited reduced cytolytic activity, with no change in hemolytic activity, compared to the parent strain (90). Surface plasmon resonance studies revealed that the core oligosaccharide of serotype 1 strains interacts with ApxI and ApxII toxins and this interaction is required for the full cytolytic activity of these toxins (91).

Studies on the interaction of LPS from *A. pleuropneumoniae* serotypes 1 and 2 with porcine respiratory tract mucus led to the identification of two potential LPS receptors (16). Adhesins must be present on the bacterial cell surface, to be able to bind with their cognate receptors. Since *A. pleuropneumoniae* is an encapsulated bacterium, there was uncertainty about the degree of surface exposure of LPS. Paradis et al. used immunoelectron microscopy to test whether LPS is found on the surface of *A. pleuropneumoniae* serotypes 1 and 2, and showed that surface-exposed LPS binds to the porcine lung vascular endothelium, mesenchyme, and tracheal epithelium. They further demonstrated that the high molecular mass polysaccharide fraction of the LPS specifically mediates the adherence of *A. pleuropneumoniae* to porcine lung and tracheal sections and lipid A was not required for this interaction (89).

Binding of whole cells and purified LPS from *A. pleuropneumoniae* to several glycosphingolipids commonly found in mammalian tissue was analyzed by thin-layer chromatography. The core oligosaccharide of *A. pleuropneumoniae* LPS is involved in binding to galactosylceramide, glucosylceramide, lactosylceramide, and sulfatide glycosphingolipids. *A.*

pleuropneumoniae LPS also binds to gangliotriaosylceramide and gangliotetraosylceramide (1). In summary, the LPS of *A. pleuropneumoniae* contributes to virulence by inciting a proinflammatory response, facilitating attachment to respiratory tract and enhancing the cytotoxicity of Apx toxins.

Apx toxins. Apx toxins produced by *A. pleuropneumoniae* belong to the repeats-in-toxin (RTX) family of calcium-dependent pore forming exotoxins. RTX toxins have hydrophobic domains in the N-terminal region and glycine-rich nonapeptide repeats (L/I/F-X-G-G-X-G-N/D-D-X) in the C-terminal region (96). Apx toxins exert their effects on porcine alveolar epithelial cells, erythrocytes, endothelial cells, macrophages and neutrophils and are required for the full virulence of *A. pleuropneumoniae* in pigs (37, 41, 92, 115). Different combinations of four Apx toxins are secreted by various strains (41). ApxI is a 105 kDa protein with strong hemolytic and cytolytic activity secreted by serotypes 1, 5, 9, 10, and 11. ApxII is a 103-105 kDa protein with weak hemolytic and moderate cytolytic activity secreted by all serotypes except serotype 10. ApxIII is a 120 kDa protein secreted by serotypes 2, 3, 4, 6, and 8 that has strong cytolytic function but lacks hemolytic activity. Production of ApxIV, a 202 kDa protein with a weak hemolytic activity, is induced only during porcine infection in all serotypes (96).

Genes encoding the structural toxins and export machinery for ApxI are found in the *apxICABD* operon. ApxIA is the protoxin; ApxIC is an acyl transferase required for post-transcriptional activation of the protoxin; ApxIB is an inner membrane-associated ATPase; ApxID is an adaptor protein required for membrane fusion. ApxB and ApxD form the inner membrane translocon that interacts with TolC in the outer membrane to form a functional type I secretion system to export the toxin (110). ApxII is encoded by genes in the *apxIICA* operon and ApxII is secreted through the ApxI secretion machinery (104). Production and export of ApxIII

is encoded by the *apxIIICABD* operon (41). ApxIVA requires the product of an ORF found immediately upstream of *apxIVA* for hemolytic activity (96). Liu et al. have shown that ApxIV is required for the full virulence of *A. pleuropneumoniae* in pigs (74).

Apx toxins are involved in the production of hemorrhagic necrosis, a pathognomonic lesion for porcine pleuropneumonia, observed in the lungs (33, 92). Apx toxins have a concentration-dependent effect on porcine neutrophils. At low concentrations, Apx toxins induce a robust oxidative burst in neutrophils while higher concentrations lead to the death of neutrophils, resulting in the release of reactive molecules, including reactive oxygen species, which then cause extensive damage to the lung tissue (37). Due to the production of Apx toxins during infection, *A. pleuropneumoniae* has greater access to intracellular iron reserves of the porcine host than other bacterial pathogens which do not produce such toxins.

Resistance to oxidative stress. *A. pleuropneumoniae* encounters reactive oxygen species, such as hydrogen peroxide, organic hydroperoxides, and superoxide, in porcine lungs, both within the phagocytes and in the extracellular milieu due to cytolytic activity of Apx toxins on neutrophils and macrophages (98). The respiratory burst is a rapid increase in oxygen consumption by professional phagocytes, such as neutrophils and monocytes, leading to the production of superoxide, which is then converted into hydrogen peroxide by dismutation (95). Superoxide and hydrogen peroxide are precursors for toxic reactive oxygen species, including hydroxyl radicals, singlet oxygen and ozone, intended to destroy pathogens. Neutrophil myeloperoxidase uses hydrogen peroxide to generate hypochlorous acid, a potent cytotoxin (95). These reactive molecules are produced primarily to kill invading pathogens, but there can be collateral damage to the host tissues. Some of the pathological changes associated with porcine

pleuropneumonia have been attributed to the damage caused by excessive accumulation of reactive oxygen species released from dying neutrophils (49).

An in vivo expression technology screen of *A. pleuropneumoniae* in the porcine host led to identification of several genes involved in oxidative stress resistance, including *ohr*, encoding an organic hydroperoxide reductase; *tehB*, encoding a putative S-adenosyl methyltransferase involved in resistance to tellurite; and *sodC*, encoding a periplasmic copper-zinc superoxide dismutase, as in vivo induced genes (66, 98). Together, these observations indicate that *A. pleuropneumoniae* encounters oxidative stress within pig lungs and responds by up-regulating the expression of genes involved in protection against oxidative stress.

Shea et al. identified that an organic hydroperoxide reductase, encoded by *ohr*, is involved in protection against organic hydroperoxides in *A. pleuropneumoniae* (98). The *ohr* gene is found only in serotypes 1, 9 and 11 and is up-regulated in vitro during growth in the presence of cumene hydroperoxide, an organic hydroperoxide, but not with hydrogen peroxide or paraquat, a superoxide generator. The presence of *ohr* correlates with an increased resistance of *A. pleuropneumoniae* strains to cumene hydroperoxide, as determined by a top-agar growth inhibition assay. A recent report showed that the *ohr* gene is found on a 9.3 kilo base pair genomic island and is co-transcribed with a *gst* gene, encoding a glutathione-S-transferase (127). Glutathione-S-transferase activity is required for the function of *ohr*, probably by acting as a reducing partner. The genomic island hosting *ohr* and gst was proposed as a horizontally transferred locus (127).

The bacterial arsenal against oxidative stress includes superoxide dismutases (Sod), which convert superoxide into hydrogen peroxide which is subsequently converted into water by peroxidases and catalases (56). *A. pleuropneumoniae* encodes two Sods, SodA and SodC, which

are found in the cytoplasm and periplasm, respectively (70). Manganese is the co-factor for SodA while copper and zinc act as co-factors for SodC. SodB, an iron containing cytoplasmic enzyme regulated by Fur, is absent in *A. pleuropneumoniae* but found in other bacteria such as *E. coli* (56). Sheehan et al. constructed an *A. pleuropneumoniae sodC* mutant strain which was severely impaired in survival when exposed to superoxide in vitro (100). Since SodC is found in the periplasm and superoxide does not cross the inner membrane, it was hypothesized that SodC is involved in protection against superoxide generated by phagocytic cells and a *sodC* mutant strain would be attenuated during porcine infection. Contrary to the hypothesis, the *sodC* mutant and wild-type strain produced similar gross and microscopic lesions in the pig lungs.

Functional genomics studies. To identify the genes expressed by *A. pleuropneumoniae* during infection of pig lungs, functional genomics studies such as transcriptional profiling during infection, in vivo expression technology (IVET), signature-tagged mutagenesis (STM), and selective capture of transcribed sequences (SCOTS) have been performed (8, 9, 35, 42, 45, 99, 120). These studies have unraveled a number of genes critical for the virulence of *A. pleuropneumoniae* in pigs.

In vivo expression technology. IVET was developed as a genetic screen using the host as a selective medium to identify genes that are up-regulated during infection (76). An IVET study was conducted previously in our laboratory to identify genes which are up-regulated during *A. pleuropneumoniae* (serotype 1) infection of the porcine lungs. A highly attenuated *A. pleuropneumoniae* riboflavin auxotrophic mutant was used as the host strain for the *A. pleuropneumoniae*-IVET vector (44). A promoter-trap IVET vector containing a T4 terminator, a unique BamHI recognition site, a promoterless copy of *luxAB* reporter genes from *Vibrio*

harveyi, and a promoterless copy of *ribBAH* genes from *Bacillus subtilis*, in tandem, was generated in a low-copy number plasmid.

Random genomic DNA fragments from *A. pleuropneumoniae* were cloned into the IVET vector and transformed into the riboflavin mutant strain. Approximately 20,000 transformants, in pools of 600-800 transformants per pig, were used for infecting pigs and the lungs were collected at 12 to 16 hours post-infection. The riboflavin mutant does not survive longer than 8 hours in the pig lungs. If the random DNA fragment in the IVET vector contains a promoter that is up-regulated during infection, riboflavin is synthesized and the riboflavin mutant survives in the pig lungs. If there is no promoter activity in the random DNA fragment, riboflavin is not synthesized and the transformant cannot survive in the porcine lungs. Production of luciferase from the *luxAB* genes was used to screen for promoter activity during in vitro growth and after pig infection. *A. pleuropneumoniae* promoters which were not expressed on laboratory media but up-regulated during infection of the pig lungs were designated as in vivo-induced genes (45).

A total of thirty two promoters were found to be expressed only in vivo-and nucleotide sequencing was used to identify them (Table 1-1) (66, 120). In vivo induced genes include the *vapBC*, a toxin-antitoxin system; *comJ*, involved in competence; genes involved in response to oxidative stress *ohr*, *sodC*, and *tehB*; and many clones antisense orientation to known genes. These antisense transcripts could regulate the expression of capsule or antigenic variation of capsular polysaccharides. Genes involved in important biosynthetic pathways, such as *ilvI*, *folD*, *pyrG*, *ribG*, and *selA* were also identified as in vivo induced genes. Proteins encoded by *gufA*, *acrD*, and *znuA* genes are involved in transport or efflux and could be important for the acquisition of nutrients. Regulatory proteins, including *texA*, *nusG*, and *hfq* were also identified to be up-regulated during infection of the pig lungs.

Table 1-1. *A. pleuropneumoniae* in vivo-induced genes

IVET Clone	Closest Homologue	Function
iviA	*mrp*	ATPase involved in chromosome partitioning
iviB		Antisense to *cps1B*
iviC		Sugar transporter of the major permease superfamily
iviD	*gufA*	Predicted zinc transporter
iviE	*acrA/macA*	Membrane fusion protein involved in macrolide efflux
iviF	*nusG*	Transcriptional antiterminator
iviG		**Antisense to *cps1A cps1B***
iviI	***ilvI***	**Branched-chain amino acid biosynthesis, acetohydroxy acid synthase isozyme III – large subunit**
iviJ		Predicted integral cytoplasmic membrane protein
iviK	*ohr*	Organohydroperoxide reductase
iviN	*ung*	Uracil-DNA glycosylase
iviP	***comJ***	**Competence-related protein**
iviQ	*znuA*	Zinc uptake
iviR	*ubiD*	Polyprenyl p-hydroxybenzoate Decarboxylase
iviS	***hfq***	**Host factor 1 RNA-binding regulatory protein**
iviT		Conserved hypothetical protein
iviU		**Antisense to *xylB* sugar kinase**
iviW	*smtA*	Predicted SAM-methyltransferase
iviX	***vapBC***	**Toxin-antitoxin system**
iviY	***yhhF***	**N6-adenine specific methylase**
ivi17a	*pyrG*	Pyrimidine biosynthesis – CTP synthase
ivi17b	*texA*	Transcriptional regulator
ivi17c	*exoII*	Beta-hexosaminidase
ivi17d	*selA*	Selenocysteine synthase
ivi17e	*folD*	Folate biosynthesis
ivi17f		Antisense to *fhaB*, hemagglutinin or hemolysin-like protein
ivi17g		**Antisense to *coaE*, dephosphocoenzyme A kinase**
ivi19a	*sodC*	Cu-Zn superoxide dismutase
ivi19b	*tehB*	Tellurite resistance, SAM dependent methyltransferase
ivi20a	*ribG*	Riboflavin biosynthesis
ivi20b		Antisense to *recJ*, single-stranded DNA specific exonuclease
ivi20c		Antisense to putative sodium/sulfate transporter

Bold typeface indicates BCAA-responsive IVET clones (66, 120)

Signature-tagged mutagenesis. STM is a modification of transposon mutagenesis, where multiple mutants with unique sequence-tags can be screened for attenuation in a single animal (54). Sequence-tags from the input pool used for infection are compared to the output pool recovered after infection to determine the mutants which fail to survive during infection. Therefore, STM studies can be used to identify genes required for survival and virulence during infection.

Fuller et al. have screened a total of 800 *A. pleuropneumoniae* serotype 1 transposon insertion mutants for virulence defect in a pig infection model and identified that insertional mutation in 20 different loci led to a loss of virulence (42). The *exbB* gene, involved in iron acquisition, was identified in both the STM studies and this highlights the importance of iron uptake during the growth of *A. pleuropneumoniae* in the pig lungs.

Sheehan et al. screened a total of 2064 mini-Tn*10* transposon mutants in a serotype 1 strain for survival in the porcine lungs and 105 mutants, with transposon insertions in 55 different genes, were found to have a survival defect during growth in the pig lungs (99). Previously described *A. pleuropneumoniae* virulence genes, such as the genes involved in the biosynthesis of capsule and LPS were identified in this study (99). Regulatory genes, including *fur, luxS, rpoE* and *rseA,* were also identified as necessary for the virulence of *A. pleuropneumoniae*. The *rseA* gene encodes an anti-sigma factor, which sequesters sigma E at the inner membrane. Sigma E acts as a positive transcriptional regulator of the *pgaABCD* operon, involved in biofilm formation. It is interesting to note that the *rseA* mutant, which over-expresses PGA-based biofilm, was defective in survival in the pig lungs (23, 99). Further evaluation of the role of PGA-based biofilm in the virulence of *A. pleuropneumoniae* is required to differentiate the effects of PGA on virulence versus pleiotropic effects of sigma E over-expression in an *rseA*

mutant. The number of mutants tested in both STM studies is not enough to obtain a complete coverage of the *A. pleuropneumoniae* genome. Nevertheless, these STM studies have identified genes that are required for the full virulence of *A. pleuropneumoniae* in pigs.

Selective capture of transcribed sequences. SCOTS technology can be used to detect transcripts which are expressed under specific conditions and was developed to identify the transcripts expressed by *Mycobacterium tuberculosis* during infection of the human macrophages (50). Transcripts extracted from *A. pleuropneumoniae* infected lung tissue were hybridized to biotinylated bacterial genomic DNA and purified by streptavidin-based affinity purification. A PCR-based subtractive hybridization was employed to remove cDNA from transcripts which were expressed during growth in laboratory media, thereby enriching for transcripts expressed in vivo. Resulting cDNA libraries were sequenced to identify genes which were expressed only during infection. While IVET and STM approaches use pools of transformants for infection and defined time points to collect surviving transformants, SCOTS offers more plasticity with regards to analysis of gene expression during different phases in the progression of infection.

SCOTS technology has been utilized to study gene expression during acute and chronic *A. pleuropneumoniae* infection. After seven days of growth of *A. pleuropneumoniae* serotype 9 strain within the porcine lungs, a total of 46 transcripts including *apxIVA*, encoding the ApxIV protoxin, *hgbA*, encoding the hemoglobin-binding protein A, *tbpA*, encoding the transferring-binding protein B, *tadZ*, encoding the fimbriae-like protein involved in Flp pilus biogenesis, and *pomA*, encoding an immunogenic outer membrane protein, were identified by SCOTS (9).

Gene expression in *A. pleuropneumoniae* serotype 7 following twenty one days of growth in the porcine lungs was analyzed by the same group (8). As predicted, genes involved in anaerobic metabolism were identified as highly expressed genes during chronic infection. A

total of thirty six genes, including *aasP*, a serine protease auto-transporter protein and *hlyX*, a global regulator of anaerobic metabolism, were found to be expressed by *A. pleuropneumoniae* in necrotic lung tissue. HlyX is the homologue of fumarate nitrate reductase (FNR) regulator which governs anaerobic metabolism in *E. coli* (10). The expression of *aasP* was dependent on HlyX in *A. pleuropneumoniae* (8). A *hlyX* mutant strain was highly attenuated in a porcine infection model (10).

The results from IVET and SCOTS studies reflect the course of progression of porcine pleuropneumonia. During acute infection, *A. pleuropneumoniae* encounters oxidative stress and responds by up-regulating the expression of genes such as *sodC* and *ohr*. As the disease progresses, *A. pleuropneumoniae* starts to encounter oxygen deprivation, eventually leading to near anaerobic conditions and reacts by expressing the genes required for survival under anaerobic conditions, such as *hlyX*.

Transcriptional profile during infection. Recently, a study on the transcriptional profile of *A. pleuropneumoniae* serotype 5b during natural infection was published (35). A total of 150 genes were found to be differentially expressed during infection of the pig lung, compared to growth in laboratory medium. Genes up-regulated during infection include *hbpA*, encoding the hemoglobin-binding protein A; *irp*, encoding a Ton-dependent receptor potentially involved in hemin transport; *apfB* and *apfC*, encoding the type IV fimbriae; and *APL_0959*, encoding a filamentous hemagglutinin/adhesin.

In contrast to the IVET study, where *hfq* was identified as an in vivo induced gene, Deslandes et al. found that the *hfq* transcript was 2.1-fold down-regulated during infection (35). This discrepancy could be due to difference in strains, serotype 1 in IVET versus serotype 5b in transcriptional profiling, used in these studies or due to different degree of progression of porcine

pleuropneumonia. In the IVET study, pigs were euthanized 12 to 16 hours post infection while in the transcriptional profiling experiment tissue was collected from a pig naturally infected with *A. pleuropneumoniae* and exhibiting signs of acute disease for 24 hours; thus the exact duration of infection is not clear. Alternatively, Hfq could have different roles during acute and chronic infection as well as when colonizing the tonsils and the lungs.

These functional genomics studies have not only contributed to enhancing our knowledge on *A. pleuropneumoniae* gene expression during infection but information gleaned from these studies will also help develop strategies to combat bacterial pathogens of the mammalian respiratory tract.

Branched-chain amino acid limitation as a mammalian host signal. The *A. pleuropneumoniae* IVET study revealed that the promoter region of the *ilvIH* operon is up-regulated during infection of the pig lungs (45). The genes, *ilvI* and *ilvH* encode the large and small subunits of acetohydroxyacid synthase (AHAS) isozyme III, respectively. AHAS is a thiamin and flavin adenine dinucleotide-dependent enzyme which forms hetero-tetramers with two large and small subunits and catalyzes the first step in the biosynthesis of branched chain amino acids (BCAAs), isoleucine, leucine and valine, in bacteria (130). A survey of IVET, STM and transcriptional profiling studies conducted in bacterial pathogens revealed that BCAA biosynthetic genes were critical for the virulence of pathogens causing pneumonia and septicemia but were not identified to play a role in the virulence of gastrointestinal tract pathogens (32, 120).

Genes involved in BCAA biosynthesis, including *ilvC, ilvD, ilvI, leuA,* and *leuC,* are required for the survival and multiplication of *Brucella suis* in the human monocyte/macrophage cell line THP-1, indicating that the *Brucella*-containing phagosome has low levels of BCAAs

(67). The gene *ilvA*, encoding threonine dehydratase, was identified as an in vivo induced gene in an IVET screen with *Pseudomonas aeruginosa* in a neutropenic mouse model model of infection (121). A mini-Tn *10*-based STM study uncovered that *yjgF*, involved in regulating the biosynthesis of isoleucine, is required for the virulence of *Pastuerella multocida* in a mouse model of septicemia (43). The gene *thrB*, encoding homoserine kinase, whose product feeds into BCAA biosynthetic pathway, was found to be essential for the survival of *S. aureus* in a murine model of bacteremia (79).Transcriptional profiling of *P. multocida* in a fowl cholera infection model revealed that the *ilvH* gene was expressed at higher levels during growth in chicken blood compared to growth in rich media (25). A DNA microarray analysis of gene expression in *P. multocida* during infection identified *ilvD*, encoding dihydroxy-acid dehydratase, and *ilvM*, encoding the small subunit of AHAS isozyme I as genes upregulated during infection of the chicken liver (24). An STM study of *Neisseria meningitidis* in an infant rat model revealed that the *ilvI* gene was critical for survival in the blood, an essential step to establish septicemia (109).

BCAA biosynthetic mutants of bacterial pathogens causing pneumonia and septicemia were found to be attenuated in animal infection models. Atkins et al. demonstrated that an *ilvI* mutant strain of *Burkholderia pseudomallei* was highly attenuated and induced protective immunity in a murine model of melioidosis (6). An *ilvI* mutant strain of *B. mallei* was also attenuated in a murine model of glanders, a disease of zoonotic importance, and induced protective immunity against *B. mallei* infection in mice (113). A *leuD* mutant of *Mycobacterium bovis* BCG, generated by transposon mutagenesis, lost its ability to survive in mice compared to its parent strain (78). The *leuD* mutant was also defective in intracellular replication in the human monocyte/macrophage cell line THP-1, suggesting that the availability of leucine is limited in the phagolysosomal compartment (12). These studies led to the analysis of role of

leuD in the virulence of *M. tuberculosis* (55). A leucine auxotroph of *M. tuberculosis* was defective in intracellular survival in THP-1 cells and was attenuated in virulence in a severe combined immunodeficient mouse model of infection. The *leuD* mutant tested for use as a live attenuated vaccine and compared to the standard vaccine strain, BCG. The *leuD* mutant did induce a protective immune response but it was less effective than BCG. This difference was attributed to the failure of the *leuD* mutant to replicate within macrophages and survive in the host long enough to produce antigens critical for the development of protective immunity (55).

Twenty five percent (eight genes from a total of thirty two) of *A. pleuropneumoniae* in vivo induced genes, including the genes *ilvI* and *hfq*, were up-regulated when grown in chemically defined medium lacking BCAAs, indicating that limitation of BCAA could be a host signal (120). *A. pleuropneumoniae* may respond to this signal by not only regulating the expression of BCAA biosynthetic genes but also other potential virulence-associated genes. Such a genetic program could be mediated by regulatory proteins, such as Lrp, a transcriptional regulator of genes involved in BCAA metabolism, including *ilvI*, and/or Hfq, a pleiotropic post-transcriptional regulator.

Non-competitive inhibitors of plant AHAS have been used as effective herbicides for several decades (130). BCAAs are essential amino acids for mammals, thereby reducing the potential toxicity concerns of AHAS inhibitors. Extensive toxicological testing has shown that they are a relatively safe group of compounds (53). The effect of AHAS inhibitors on the survival and multiplication of *B. suis* was tested, as a previous study had demonstrated that BCAA biosynthetic genes were required for multiplication within human macrophages. AHAS inhibitors abolished the multiplication of *B. suis* in THP-1 cells and prevented the growth of *B. suis* in vitro, in minimal medium (21). Since a *leuD* mutant of *M. tuberculosis* was attenuated,

the potential of AHAS inhibitors as antituberculosis agents was tested (51). Sulphometuron methyl, a member of the sulfonylurea class of AHAS inhibitors, had an in vitro minimum inhibitory concentration (MIC) of 20 μM against *M. tuberculosis.* When this compound was administered to mice with established tuberculosis, a reduction in bacterial load of the lungs was observed but there was no difference in bacterial load of the spleen (51). However, a mechanism for the differential activity of sulphometuron methyl against *M. tuberculosis* in the lungs versus the spleen was not identified in that study. Zohar et al. cloned the genes encoding AHAS from *M. avium* and showed that members of sulfonylurea class of AHAS inhibitors, such as metsulfuron methyl, inhibit the catalytic activity of mycobacterial AHAS (130).

These observations suggest that the availability of BCAAs could be a hitherto unrecognized signal in the mammalian host and the ability to synthesize BCAAs may be important for the survival and virulence of pathogens colonizing certain niches, such as the lungs and blood, within the host.

Host factor Q-beta (Hfq). Hfq was originally identified as a factor required for the replication of RNA bacteriophage Q-β in *Escherichia coli* (40). Hfq is closely related to Sm and Sm-like proteins associated with spliceosomes and RNA degradosomes found in eukaryotes and archaea, respectively (97). As a pleiotropic post-transcriptional regulator, Hfq modulates translation and transcript stability in bacteria (26). Hfq is a key mediator in the small non-coding regulatory RNA (sRNA) mediated post-transcriptional regulation. Bacterial sRNAs are usually not translated into proteins and vary in length from 50 to 500 base pairs (15). However, there are several sRNAs which do not require Hfq for binding with target transcripts (15).

Hfq contains the Sm1 and Sm2 motifs required for the RNA binding activity. Hfq proteins reveal an α helix in the N-terminal region followed by five β strands. The first three β

strands form the Sm1 motif while the Sm2 motif is formed by the fourth and fifth β strands. Hexamerization of Hfq monomers occur by interactions between residues from β4 and β5 strands of the participating subunits (27). There are at least two distinct RNA binding sites, a proximal side and a distal side, on an Hfq hexamer. The proximal side is involved in binding to sRNAs and A/U rich tracts adjacent to stem-loop structures while the distal side interacts with the poly-A tails (27). Hfq also interacts with the C-terminal scaffold region of RNase E, an endoribonuclease and is proposed to form specialized RNA degradation machinery for destabilizing transcripts targeted by specific Hfq-binding sRNAs, such as SgrS and RhyB (84).

Doughnut shaped homo-hexamers of Hfq bind to the A/U rich regions in the 5' untranslated regions (5' UTR) of transcripts and sRNA, facilitating the formation of mRNA:sRNA duplexes by incomplete base-pairing. This interaction either enhances or blocks the access of ribosomes to the translation initiation region and the mRNA:sRNA duplex may be targeted to degradation, although inhibition of translation alone is sufficient for gene silencing (85, 86). For example, DsrA sRNA binds to Hfq and the 5'UTR of RpoS mRNA to relieve an intramolecular secondary structure that prevents the access of the ribosome to the translation initiation region on the transcript, facilitating the translation of sigma S (116). On the contrary, OxyS sRNA expressed in cells exposed to oxidative stress binds to the RpoS mRNA in the presence of Hfq and prevents its translation (128). Caution should be exercised while interpreting the phenotypes observed in *hfq* mutants, as there could be several levels of indirect regulation perhaps mediated by global regulators such as sigma S. In *E. coli*, Hfq has been shown to bind to the 5' UTR of its own transcript and acts as an autorepressor of translation (117). sRNAs play a number of regulatory roles in the physiology as well as the virulence of bacterial pathogens by acting as ultra sensitive switches in adaptation to changing environmental conditions and Hfq is

required for many of the sRNA regulated phenotypes by virtue of its role in mRNA:sRNA interaction (72).

Hfq is implicated in biofilm formation by uropathogenic *E. coli*, although a mechanism for how this effect is mediated is not known (68). The role of Hfq in biofilm formation is well understood in *V. cholerae* (72). Hfq is integral to the quorum sensing-regulated expression of genes involved in virulence and biofilm formation. Under low cell density conditions, encountered by *V. cholerae* in the human intestine, transcription factor LuxO is phosphorylated. Sigma 54 and phosphorylated LuxO together activate the transcription of four quorum sensing-regulated-sRNAs (Qrr 1-4). Qrrs, in the presence of Hfq, bind to the HapR mRNA, the master regulator of virulence gene expression in *V. cholerae,* and target the complex for degradation. HapR is a repressor of genes involved in virulence and biofilm formation. By negatively regulating the expression of a negative regulator, Hfq and Qrrs facilitate the expression of genes involved in virulence and biofilm formation. However, the role of Hfq in regulating PGA-based biofilm is not known.

Hfq has been shown to play an important role in the stress resistance and virulence of bacterial pathogens. Detailed studies on the role of Hfq in the physiology and virulence have been reported for *Salmonella enterica* (29, 31, 103, 126). In *S. enterica* serovar Typhimurium, *hfq* mutants are characterized by: attenuation in a mouse model of infection; multiplication defect within macrophages; reduced expression of genes in the *Salmonella* pathogenicity island 1; reduced motility; defective adherence; and reduced invasion (103). Further, the role of the *S. enterica* serovar Typhimurium *hfq* mutant strain as a live attenuated vaccine was tested in a murine model of typhoid and was found to induce protective immunity (3). In laboratory media, *hfq* mutants exhibit minor growth defects marked by a longer lag phase and a lower viable count

during stationary phase, compared to wild-type (31). In Gram-positive pathogens, *Listeria monocytogenes* and *S. aureus,* the *hfq* mutants do not exhibit a growth defect (20, 34).

The *hfq* mutants of several bacterial pathogens, such as *B. abortus,* uropathogenic *E. coli, Francisella tularensis, N. meningitidis*, *P. aeruginosa, S. enterica* serovars Typhimurium and Enteritidis, *V. cholerae, Y. pestis* and *L. monocytogenes,* were attenuated in murine infection models (34, 36, 38, 47, 65, 68, 80, 83, 94, 103, 105, 106). *B. cepacia* and *Shigella sonnei hfq* mutants were attenuated in *Caenorhabditis elegans* and guinea pig infection models, respectively (83, 106). However, *hfq* mutants of respiratory tract pathogens, including *P. aeruginosa* and *Y. pestis,* were not tested for virulence in a pneumonia model.

Hfq has been implicated in resistance to oxidative stress in bacteria. *B. abortus hfq* mutants are more sensitive to killing by hydrogen peroxide than the parental strain (94). A later study identified that there is also reduced expression of SodC, a periplasmic Cu-Zn superoxide dismutase, in the *B. abortus hfq* mutant (46). *Hfq* mutants of *B. cepacia, M. catarrhalis, N. meningitidis, Sinorhizobium meliloti* and uropathogenic *E. coli* reveal high sensitivity to methyl viologen, a redox-cycling agent which generates intracellular superoxide radicals (7, 13, 38, 68, 106). *P. aeruginosa hfq* mutants produced less catalase, which catalyzes the conversion of hydrogen peroxide into water, compared to the wild-type strain (105). In *Y. pestis, hfq* mutants were more sensitive to killing by hydrogen peroxide (47). However, *hfq* mutant strains of *S. aureus* and *V. parahaemolyticus* exhibit higher resistance to oxidative stress induced by hydrogen peroxide and superoxide compared to parent strain (75, 108). These data show that Hfq could either positively or negatively regulate resistance to oxidative stress. Enhanced sensitivity to oxidative stress may be at least in part responsible for the virulence defect observed in the *hfq* mutants in a number of bacterial pathogens.

Hfq is implicated in the virulence of several bacterial pathogens. Further research on Hfq activity may help in identification of inhibitors of Hfq function, which could be developed as potential broad spectrum anti-virulence agents.

Scope of the dissertation. The scope of this dissertation is to unravel the role of two *A. pleuropneumoniae in vivo* induced genes, *ilvI* encoding the BCAA biosynthetic enzyme acetohydroxy acid synthase and *hfq* encoding the RNA chaperone Hfq, in the pathogenesis of porcine pleuropneumonia.

Chapter two describes the identification of a novel host signal, limitation of BCAA in the porcine pulmonary epithelial lining fluid (ELF). BCAA limitation was previously shown to up-regulate expression from *ilvI* and *hfq* promoters in vitro (120). Growth rates of the wild-type, *ilvI* mutant, and *lrp* mutant strains in chemically defined medium containing physiological amounts of BCAA were determined. Lrp is a positive transcriptional regulator of *ilvI* under BCAA-limiting conditions in *A. pleuropneumoniae* (119). Transcription from the *ilvI* and *lrp* promoters in varying levels of BCAA was analyzed by quantitative PCR and luciferase reporter gene assays. The role of *ilvI* and *lrp* in the virulence of *A. pleuropneumoniae* was tested in a porcine infection model. Small-molecule inhibitors of BCAA biosynthesis were shown to inhibit the growth of *A. pleuropneumoniae* in media lacking BCAA. This study identified that limitation of BCAA is a novel host signal in the porcine respiratory tract and identified that small molecule inhibitors of BCAA biosynthesis could be developed as niche-specific antibacterial agents against bacterial pathogens of respiratory tract.

Chapter three discusses the regulation of two virulence-associated phenotypes, PGA-based biofilm formation and superoxide stress resistance, by the *in vivo* induced gene *hfq*. The *hfq* mutant does not form biofilm and this effect is due to the reduced

levels of *pgaC* transcript in the mutant strain. PgaC is the glycosyl transferase which catalyzes the formation of PGA polymers. Cell-associated PGA content was ten-fold lower in the mutant compared to wild-type strain. Since PGA biosynthetic genes and Hfq are widely distributed among bacteria, this study has broad relevance. Hfq was also identified as a regulator of the expression of cysteine synthase, CysK. The *hfq* mutant has enhanced sensitivity to superoxide stress, induced by methyl viologen and potassium tellurite, compared to wild-type strain. Both these phenotypes suggest that an *hfq* mutant might be attenuated in a porcine infection model.

Chapter four describes the contribution of Hfq to the virulence of *A. pleuropneumoniae* in a porcine infection model using a competitive index experimental design. Equal numbers of wild-type and *hfq* mutant bacteria were mixed and used for inoculating pigs. Bronchoalveolar lavage fluid and samples from various lung lobes were collected at specific times post-inoculation or when pigs exhibited moderate distress during clinical evaluation. Numbers of the wild-type and *hfq* mutant bacteria were enumerated by plate counts. Competitive indices were calculated as a measure of virulence and the *hfq* mutant was found to be highly attenuated in pigs. Pigs infected with wild-type alone revealed classic clinical signs of porcine pleuropneumonia and severe lung damage. On the contrary, pigs infected with the *hfq* mutant did not develop signs of porcine pleuropneumonia and lungs were found to resemble lungs from uninfected pigs. Together, our data demonstrates that Hfq is indispensable for the virulence of *A. pleuropneumoniae* in pigs and is a potential candidate for the development of a live attenuated vaccine.

Chapter five summarizes the key findings reported in this dissertation and raises new questions about Hfq-mediated regulation. Briefly, research described in this dissertation has identified a novel host signal and expanded our knowledge on the regulation of virulence genes by Hfq in bacterial pathogens. Suggestions for conducting further experiments to understand the regulation of virulence gene expression in *A. pleuropneumoniae* are included.

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CHAPTER II

BRANCHED-CHAIN AMINO ACIDS ARE REQUIRED FOR THE SURVIVAL AND VIRULENCE OF *ACTINOBACILLUS PLEUROPNEUMONIAE* IN SWINE

ABSTRACT

In *Actinobacillus pleuropneumoniae*, which causes porcine pleuropneumonia, *ilvI* was identified as an in vivo-induced (*ivi*) gene and encodes the enzyme acetohydroxyacid synthase (AHAS) required for branched-chain amino acid (BCAA) biosynthesis. *ilvI* and 7 of 32 additional *ivi* promoters were upregulated in vitro when grown in chemically defined medium (CDM) lacking BCAA. Based on these observations, we hypothesized that BCAA would be found at limiting concentrations in pulmonary secretions and that *A. pleuropneumoniae* mutants unable to synthesize BCAA would be attenuated in a porcine infection model. Quantitation of free amino acids in porcine pulmonary epithelial lining fluid showed concentrations of BCAA ranging from 8 to 30 μmol/liter, which is 10 to 17% of the concentration in plasma. The expression of both *ilvI* and *lrp*, a global regulator that is required for *ilvI* expression, was strongly upregulated in CDM containing concentrations of BCAA similar to those found in pulmonary secretions. Deletion-disruption mutants of *ilvI* and *lrp* were both auxotrophic for BCAA in CDM and attenuated compared to wild-type *A. pleuropneumoniae* in competitive index experiments in a pig infection model. Wild-type *A. pleuropneumoniae* grew in CDM+BCAA but not in CDM−BCAA in the presence of sulfonylurea AHAS inhibitors. These results clearly demonstrate that BCAA availability is limited in the lungs and support the hypothesis that *A. pleuropneumoniae*, and potentially other pulmonary pathogens, uses limitation of BCAA as a cue to regulate the expression of genes required for survival and virulence. These results further suggest a potential role for AHAS inhibitors as antimicrobial agents against pulmonary pathogens.

INTRODUCTION

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a disease of significant economic importance throughout the swine-raising areas of the world (6, 48). This pathogen possesses several well-studied virulence factors, including Apx toxins (20), capsular polysaccharides (57, 58), lipopolysaccharide (1, 17, 41), fimbriae (63), and iron scavenging proteins (13, 50), which aid in the pathogenesis of acute pleuropneumonia marked by edema, hemorrhage, and necrosis (6, 26). In a search for additional virulence factors of this pathogen, we developed an in vivo expression technology (IVET) system and used this genetic tool to identify *A. pleuropneumoniae* gene promoters that are upregulated in vivo in the swine lung during infection compared to growth on laboratory media (22, 55).

One of the *A. pleuropneumoniae* in vivo-induced (*ivi*) promoters that we identified drives the *ilvIH* operon, which encodes both large and small subunits of acetohydroxy acid synthase isozyme III (AHAS) (55). AHAS enzymes catalyze pivotal steps in the biosynthesis of the branched-chain amino acids (BCAA) isoleucine, leucine, and valine (31). In a survey of IVET, signature-tagged mutagenesis, and microarray studies of other pathogens, we observed that genes involved in BCAA biosynthesis were frequently identified in studies of pathogens that cause pneumonia, meningitis, or septicemia but not in pathogens of the gastrointestinal tract (55). This observation suggests that the ability to synthesize BCAA is critical for pathogens of the respiratory tract but not for gastrointestinal pathogens. BCAA are essential amino acids that must be acquired from ingested food for most mammals, including humans and pigs, and it is possible that fluids in “clean” body sites such as the lungs have only limited supplies of BCAA compared to the digestive tract.

To test whether limitation of BCAA affects the expression of *A. pleuropneumoniae* genes that are induced in vivo, we compared expression from the *A. pleuropneumoniae ivi* promoters in a chemically defined medium (CDM) containing or lacking BCAA (55). We found that 25% (8 of 32) of the *ivi* promoters were upregulated during growth in CDM lacking BCAA compared to complete CDM. These included the *ilvI* promoter, as well as promoters for other genes potentially involved in survival within the host and virulence, such as *hfq*, a global regulator that binds sRNAs and mRNA and affects expression of virulence-associated genes in many pathogens (9, 49). These results strongly suggest that the environmental conditions encountered by *A. pleuropneumoniae* during infection of the swine lung include limitation of BCAA.

The goals of the present study were to quantify free BCAA in porcine pulmonary secretions, to evaluate the effect of these concentrations of BCAA on expression of genes required for BCAA biosynthesis, and to test whether *A. pleuropneumoniae*mutants that cannot synthesize BCAA were attenuated. *A. pleuropneumoniae* deletion-disruption mutants of the *ilvI* biosynthetic gene and the *lrp* gene, which encodes a global regulator required for expression of several genes involved in BCAA biosynthesis, were constructed and shown to be attenuated in a porcine infection model. The low levels of available BCAA in pulmonary secretions and the attenuation of these mutants led us to examine the effect of small molecule inhibitors of AHAS on growth of *A. pleuropneumoniae* in vitro. Several AHAS inhibitors were shown to prevent growth in CDM lacking BCAA but not in complete CDM. These results demonstrate that *A. pleuropneumoniae*, and likely other bacterial pathogens of the respiratory tracts of other mammals, encounter conditions where BCAA are available only in limited supply during infection, that these low levels of BCAA can affect bacterial gene expression, and that these pathogens must be able to synthesize BCAA to survive and cause disease in the lung.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and growth conditions. The bacterial strains and plasmids used in the present study are listed in table 2-1. *A. pleuropneumoniae* strains were routinely grown on Bacto brain heart infusion (BHI) (Becton Dickinson, Sparks, MD) or CDM (55) supplemented with 10 mg of NAD (V factor; Sigma Chemical, St. Louis, MO)/ml and incubated either at 35°C with 5% CO_2 for agar media or at 35°C shaking at 160 rpm for broth media.

To make CDM containing various concentrations of BCAA, a BCAA stock was added separately to CDM lacking BCAA to final concentrations equivalent to 10, 20, 50, or 100% of the BCAA concentration in complete CDM. For growth rate, in vitro competitive index, and experimental infection experiments, Bacto heart infusion broth (Becton Dickinson) supplemented with 10 mg of NAD/ml was also used. For plasmid selection in *A. pleuropneumoniae*, ampicillin and kanamycin were added at 50 mg/ml. For mating experiments, nalidixic acid was added at 50 mg/ml, and chloramphenicol was added at 2 mg/ml.

Escherichia coli XL1-Blue (Stratagene, La Jolla, CA) and *E. coli* S17-1 (λ*pir*), used for cloning and mating, respectively, were grown on Luria-Bertani (LB) medium at 37°C on agar medium and at 37°C with rapid shaking in broth medium. For plasmid selection in *E. coli*, ampicillin was added at 100 mg/ml, kanamycin was added at 100 mg/ml, and chloramphenicol was added at 10 mg/ml.

Primers used for construction of plasmids or for quantitative reverse transcription-PCR (RT-PCR) are listed in table 2-2.

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

Strain or plasmid	Characteristics[a]	Source or reference
Strains		
A. pleuropneumoniae		
AP100	ATCC 27088, serotype 1A, passaged through pigs	ATCC[b]
AP225	*A. pleuropneumoniae* ATCC 27088, serotype 1A, nalidixic acid resistant, passaged through pigs	23
AP359	*lrp* double-crossover mutant of AP225	54
AP364	*ilvI* single-crossover mutant of AP225	This study
AP365	*ilvI* double-crossover mutant of AP225	This study
E. coli		
XL1-Blue mRF'	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* (F'*proABlacI*^{q}ZΔM15 Tn*10* (Tetr))	Stratagene
S17-1(λ*pir*)	Δ*pir recA thi pro hsd* ($rK^{-}mK^{+}$) RP4-2-Tc::Mu Km::Tn*7*; Tmp^{r} Sm^{r}	43
Plasmids		
pUC18	Ap^{r}; high-copy-number cloning vector	62
pGZRS18/19	Ap^{r}; *A. pleuropneumoniae-E. coli* shuttle vectors	59
pGZRS39	Kan^{r}; *A. pleuropneumoniae-E. coli* shuttle vector	59
pER187	Ap^{r}, Cm^{r}; CAT cassette-containing vector	42
pUC4K	Ap^{r}, Kan^{r}; Kan-cassette containing vector; source of the Kan promoter	53
pUM24Cm	Cm^{r}, Kan^{r}; *sacR-sacB-nptI* cassette-containing vector	40
pGP704	Ap^{r}; broad-host-range suicide vector	34
pGP704SacKan	Cm^{r}, Kan^{r}; *sacR-sacB-nptI* from pUM24Cm cloned into pGP704	This study
pTW415	Kan^{r}; *rnd'-lrp-ftsK'* from AP100 cloned into pGZRS39	54

Table. 2-1. (Cont'd)

Strain or plasmid	Characteristics[a]	Source or reference
pTW429	Ap^r; 632-bp of the 5' end of *ilvI* and ~800-bp of the 3' end of *ilvI* amplified by PCR from AP100 and cloned into *Sph*I/*Sal*I digested pUC18	This study
pRL100	Ap^r, Cm^r; 300-bp containing Kan promoter from pUC4K cloned upstream of the CAT cassette of pER187	This study
pRL101	Ap^r, Cm^r; KanP-CAT cassette from pRL100 inserted into *Nsi*I site in center of *ilvI* in pTW429	This study
pRL102	Ap^r, Cm^r, Kan^r; 2.5 kb fragment from pRL101 containing Δ*ilvI*::KanP-CAT cloned into pGP704SacKan	This study
pRL103	Ap^r; 2.8-kb fragment containing full *ilvIH* genes amplified from AP100 genomic DNA cloned into *Sal*I/*Xba*I digested pGZRS19	This study
pIviI	Ap^r; pTF86 *A. pleuropneumoniae* IVET vector containing a 623-bp insert with the *ilvI* promoter upstream of promoterless *luxAB* and *ribBAH* genes	22
pTW429	Ap^r; 632-bp of the 5' end of *ilvI* and ~800-bp of the 3' end of *ilvI* amplified by PCR from AP100 and cloned into *Sph*I/*Sal*I digested pUC18	This study

[a] Cm^r, chloramphenicol resistance; Tet^r, tetracycline resistance; Tmp^r, trimethoprim resistance; Ap^r, ampicillin resistance; Sm^r, streptomycin resistance; Kan^r, kanamycin resistance. [b] ATCC, American Type Culture Collection

Collection and analysis of BALF. Bronchoalveolar lavage fluid (BALF) was collected from five healthy 12- to 14-week-old Yorkshire-Landrace pigs by using standard veterinary procedures (52). All animal use protocols were approved by the Michigan State University Institutional Animal Care and Use Committee. Pigs were kept off feed for 4 h prior to the collection of BALF. Pigs were anesthetized, placed in ventral recumbency, and intubated to ensure respiratory function, and a flexible catheter was inserted through the tracheal tube. A total

of 25 ml of phosphate-buffered saline (PBS) containing 0.0005% methylene blue, kept at body temperature, was slowly introduced into the lungs. After 1 min, the fluid was aspirated with a

Table. 2-2. Oligonucleotide primers used in this study

Primer	Sequence (5' to 3')[a]	Description
MM727	CGTTGGAGGATTGCATGCAAAAACTTTCCG	Forward primer for construction of pTW429 (first *ilvI* fragment)
MM512	GAGAATGCATCTCCACCAATGTATAAAACCG	Reverse primer for construction of pTW429 (first *ilvI* fragment)
MM513	AACCGTTATGCATTATATTCCGATTGTGGG	Forward primer for construction of pTW429 (second *ilvI* fragment)
MM734	CTGATGTCGACCTACGCATCTGTTCTC	Reverse primer for construction of pTW429 (second *ilvI* fragment)
MM588	CCATGCCGCGTGAATGA	16S rRNA forward Q-PCR primer
MM589	TTCCTCGCTACCGAAAGAACTT	16S rRNA reverse Q-PCR primer
MM590	TGTCGGTCAGCACCAAATGT	*ilvI* forward Q-PCR primer
MM591	GCGACGAGGTTTTTCAAACG	*ilvI* reverse Q-PCR primer
MM592	AATTGCTTGAAGCACCGCTATT	*lrp* forward Q-PCR primer
MM593	CGTCCGGCTTACCTCTGACT	*lrp* reverse Q-PCR primer

[a]Restriction sites inserted by PCR are underlined

syringe. This procedure was repeated with a second 25 ml of lavage fluid. The two samples were combined, and the total volume of the aspirated BALF was measured. BALF was centrifuged at 1,300 × *g* for 20 min to remove cells and filtered through a Millipore Centrifree cartridge (Millipore, Billerica, MA).

The volume of pulmonary epithelial lining fluid (ELF) in the recovered BALF was calculated both by measurement of the concentration of methylene blue in the BALF (3, 52) and by using urea as a marker of dilution (39). Concentration of free amino acids and of urea in the BALF were measured by physiological amino acid analysis on a Hitachi I-8800 amino acid analyzer (Hitachi High Technologies America, Pleasanton, CA) (45) at the Michigan State University Macromolecular Structure Facility and compared to the concentration of urea in a plasma sample collected immediately prior to the lavage procedure. Using urea as a marker for dilution, which was found to be more reproducible than the methylene blue method, the volume of ELF in each sample was calculated as follows: (the concentration of urea in the BALF × the volume of BALF)/the concentration of urea in plasma (39). The dilution factor was calculated as the volume of BALF divided by the volume of ELF. The concentration of each amino acid in ELF was calculated as the concentration in BALF times the dilution factor.

Luciferase reporter assays. Expression from the *ilvI* promoter was quantified by using a luciferase expression plasmid (pIviI) in which the *ilvI* promoter drives the expression of promoterless *luxAB* genes (54). Wild-type *A. pleuropneumoniae* strain AP225 containing pIviI grown overnight on BHI agar supplemented with 10 mg of V factor/ml (BHIV) containing 50 mg of ampicillin/ml was suspended in CDM−BCAA and then diluted to an optical density at 520 nm (OD_{520}) of 0.2 in 30 ml of prewarmed CDM containing 50 mg of ampicillin/ml and supplemented with 0, 10, 20, 50, or 100% of the concentration of BCAA found in complete CDM. Growth, measured as OD_{520}, and luciferase activity were determined at 0, 1, 2, and 3 h time points. Luciferase activity was measured as relative light units (RLU) using *N*-decyl aldehyde substrate (Sigma) and a Turner model 20e luminometer (Turner Designs, Sunnyvale, CA) as previously described (22, 55). Each sample was measured in triplicate, and the average

RLU were normalized to the optical density of the culture. Three biological replicates of the complete experiment were performed.

Quantitative RT-PCR analysis of gene expression. Wild-type *A. pleuropneumoniae* AP100 was grown to mid-exponential phase (OD_{520} 0.5 to 0.6) in complete CDM+BCAA and cells pelleted by centrifugation and resuspended to an OD_{520} of ~0.2 in 30 ml of prewarmed CDM supplemented with 0, 10, 20, 50,or 100% of the concentration of BCAA found in complete CDM. One hour after the shift to fresh medium, 30 ml of ice-cold methanol was added to each culture to stop growth, the resulting samples were chilled on ice for at least 5 min, and the cells were pelleted by centrifugation. RNA was isolated by using an RNeasy Midi kit protocol (Qiagen, Valencia, CA), with the modification that 5 mg of lysozyme/ml was used instead of the recommended 400 μg/ml. Residual genomic DNA was removed with Turbo DNase (Ambion, Austin, TX), and the RNA was concentrated. The RNA concentration was measured by using a spectrophotometer (Nanodrop, Wilmington, DE), and the RNA quality was verified by gel electrophoresis. The RNA was used as a template for RT, using random hexamers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR (Q-PCR) was performed using gene-specific primers (Table 2) and SYBR green PCR Core reagents (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. The results were analyzed by using the SDS 2.1 software (Applied Biosystems) and the relative standard curve method, with normalization to 16S rRNA. Each sample was measured in triplicate, and three biological replicates of the complete experiment were performed.

Construction and verification of an *A. pleuropneumoniae ilvI* mutant. To construct an *A. pleuropneumoniae ilvI* mutant, we used a similar technique to that previously reported by our laboratory for the construction of an *lrp* mutant (54). The 5' and 3' ends of the *ilvI* gene from *A.*

pleuropneumoniae AP100 were amplified from genomic DNA by PCR and cloned into pUC18. Primers MM727 and MM512 (Table 2) were used to amplify a 653-bp fragment from the 5' end of the *ilvI* gene, which was digested with SphI and NsiI. Primers MM513 and MM734 were used to amplify a 789-bp fragment from the 3' end of the gene, which was digested with NsiI and SalI. These amplicons were ligated into pUC18 which had been digested with SphI and SalI, resulting in pTW429, which contains the *ilvI* gene with 259 bp deleted from the center of the gene. Next, the Kanamycin promoter-chloramphenicol resistance (Cmr) cassette was digested from pRL100 with PstI and ligated into the newly generated NsiI site of pTW429 to generate pRL101. The 2.5-kb insert containing *ilvI-5'–KanP-cat–ilvI-3'* was digested from pRL101 with SphI and SacI and ligated into SphI/SacI-digested pGP704SacKan to generate pRL102. This conjugal suicide plasmid was electroporated into *E. coli* S17-1(λ*pir*) and conjugated into nalidixic acid-resistant *A. pleuropneumoniae* AP225 as previously described (35). Transconjugants were isolated on BHIV agar supplemented with 2 μg of chloramphenicol/ml and 50 μg of nalidixic acid/ml. Screening for single- or double-crossover mutants was performed by PCR analysis of the *ilvI* locus. A single-crossover transconjugant (AP364) was exposed to chloramphenicol selection and sucrose counterselection, as previously described (35), to generate a double-crossover *ilvI* mutant. This mutant, designated AP365, was confirmed by PCR and Southern blot to contain the deleted-disrupted *ilvI* gene and the Cm^r cassette in the appropriate location in the AP225 chromosome but not the pGP704 vector, the kanamycin resistance gene, or the *sacR-sacB* cassette.

Preparation of challenge inocula. Bacterial cultures were grown at 35°C, shaking at 160 rpm, in heart infusion broth containing 10 μg of V factor/ml and 5 mM calcium chloride ($CaCl_2$) to an OD_{520} of 0.8. Cells were harvested by centrifugation for 10 min at 5,000 × *g*,

washed once with sterile PBS, diluted in PBS to the appropriate cell density, and administered within 60 min of preparation. The actual CFU/ml in the inocula were calculated by viable cell count on BHIV agar.

Experimental infection of pigs. Three separate experimental infection experiments were performed. In the first experiment, 15 10-week-old specific-pathogen-free (SPF) Yorkshire-Landrace crossbred pigs (Whiteshire Hamroc, Albion, IN) were divided into five groups of three pigs by a random-stratified sampling procedure, balancing each group for body weight. Group 1 was challenged by percutaneous intratracheal inoculation with 4×10^6 CFU of wild-type *A. pleuropneumoniae* AP225 in 10 ml of PBS, as previously described (23, 28). Group 2 received 4×10^6 CFU of AP359, an *lrp* mutant of AP225 (54). Group 3 received 2×10^7 CFU of AP359. Group 4 received 4×10^6 CFU of AP359 complemented with plasmid pTW415, which contains the *lrp* gene in a pGZRS18 vector (54). Group 5 received 10 ml of PBS.

In the second experiment, eight 10-week-old SPF pigs were divided into four groups of two pigs. Group 1 was challenged by intratracheal inoculation with 10^6 CFU of wild-type *A. pleuropneumoniae* AP225 in 10 ml of PBS. Group 2 received 10^6 CFU of AP359, the *lrp* mutant, by intratracheal inoculation. Group 3 received a mixture of 5×10^5 CFU each of the wild type and the mutant intratracheally. Group 4 received with 2.5×10^6 each of the wild type and the mutant in 2 ml of PBS, inoculated intranasally.

In the third experiment, 10 10-week-old SPF pigs were divided into four groups. Group 1 (three pigs) received 10^6 CFU of AP225. Group 2 (three pigs) received 5×10^5 CFU each of the wild type and the *lrp* mutant. Group 3 (two pigs) received 5×10^5 CFU of the wild type and the

ilvI mutant. Group 4 (two pigs) received PBS only. All pigs in this experiment were inoculated intratracheally.

After experimental infection, pigs were monitored for the development of clinical signs of pleuropneumonia, including elevated rectal temperature, increased respiratory rate, dyspnea, decreased appetite, and decreased activity (depression), as previously described (23, 28). Pigs were euthanized by lethal injection either when mild-to-moderate clinical signs, particularly dyspnea and/or depression, were seen or at the end of the experiment. All animals were necropsied, and lungs were examined macroscopically for pleuropneumonia lesions. The percentage of lung tissue and pleural surface area affected was estimated for each of the seven lung lobes, and the total percent pneumonia and pleuritis was calculated by using a formula that weights the contribution of each lung lobe to the total lung volume (28). BALF and lung tissue samples from six areas of the lungs were collected and processed for culture and histopathology in all experiments and for quantitative plate counts in experiments 2 and 3. All animal use protocols were approved by the Michigan State University Institutional Animal Care and Use Committee.

Determination of competitive indices. For in vivo competitive index determination, serial 10-fold dilutions of BALF samples and of lung samples homogenized in a Stomacher-80 (Seward Laboratory Systems, Bohemia, NY) were plated on BHIV agar containing 25 μg of nalidixic acid/ml for a total viable count of wild-type and mutant bacteria and on BHIV containing 1 μg of chloramphenicol/ml to select for the mutants. The numbers of wild-type bacteria were calculated as the CFU on BHIV containing nalidixic acid minus the CFU on BHIV containing chloramphenicol. The competitive index for each sample was calculated by using the following formula: the competitive index at time X = the ratio of mutant to the wild type at time

X/the ratio of the mutant to the wild type at time zero. The average competitive index for each animal was calculated as the average of all seven specimens (BALF plus lung), with the exception that data for any specimen with no growth were excluded. Similar methods were used to calculate competitive indices in broth media.

Determination of MICs of AHAS inhibitors. Four sulfonylurea herbicide compounds—metsulfuron methyl, primisulfuron methyl, and chlorsulfuron (all purchased from Riedel-de-Haen Laborchemikalien, Seelze, Germany) and chlorimuron ethyl (Chem Service, West Chester, PA)—and the two imidazolinone herbicide compounds imazapyr and imazaquin (Riedel-de-Haen) were tested for their ability to inhibit the growth of *A. pleuropneumoniae* in CDM + BCAA and CDM − BCAA. Serial two-fold dilutions of each inhibitor were made in CDM + BCAA and CDM − BCAA in either test tubes or microtiter plates. *A. pleuropneumoniae* AP100 was grown overnight on BHIV agar and suspended in either CDM + BCAA or CDM − BCAA, and this suspension was used to inoculate both tubes and microtiter plate wells at a concentration of ~5×10^7 CFU/ml. Tubes were incubated overnight at 35°C shaking at 160 rpm; microtiter plates were incubated overnight at 35°C under 5% CO_2. MICs were determined as the lowest concentration of chemical compound that inhibited growth. The full experiment was repeated four times.

RESULTS

Analysis of free amino acid concentration in porcine pulmonary ELF. To determine whether BCAA are present in only limited amounts in the porcine lung, we measured the concentrations of free amino acids in porcine plasma and BALF from healthy Yorkshire-Landrace feeder pigs and calculated the levels of free amino acids in pulmonary ELF using urea as a marker of dilution. Free amino acid concentrations in porcine plasma and ELF are shown in table 2-3. The amino acid concentrations found in plasma were similar to those previously reported for porcine plasma (11). Many amino acids, including the essential amino acids lysine and threonine, were present in ELF at ~50% of the concentration in plasma, and aspartic acid and glutamic acid were present in higher concentrations in ELF than in plasma. In contrast, for the BCAA leucine, isoleucine, and valine, the available levels in pulmonary ELF ranged from 8.4 to 30 μmol/liter, which was 9.8 to 16.8% of the levels found in porcine plasma. The concentrations of BCAA in porcine ELF were 10 to 20% of the amounts of these amino acids in the CDM used for *A. pleuropneumoniae* (55).

Expression of genes involved in BCAA biosynthesis in CDM. We have previously shown that *A. pleuropneumoniae ilvI*, which is required for BCAA biosynthesis, is upregulated both in vivo during infection (22) and in CDM lacking BCAA (CDM − BCAA) compared to complete CDM (CDM + BCAA) (55) and that leucine-responsive regulatory protein, encoded by the gene *lrp*, is required for the response of *ilvI* to BCAA limitation (54). To determine whether expression of *ilvI* and *lrp* correlates with the concentrations of BCAA available in the porcine lung, we used luciferase reporter assays and quantitative RT-PCR to measure the expression of these genes in CDM samples containing various concentrations of BCAA.

Table. 2-3. Concentration of free amino acids in porcine plasma and pulmonary ELF

Amino acid [a]	Amino acid concentration in µmol/liter (avg ± SD)		Avg ELF/plasma ratio(%) [b]	Amino acid concentration in CDM in µmol/liter
	Plasma	ELF		
Alanine	509 ± 204	92.9 ± 45.2	20.5%	370
Arginine	29.6 ± 39.1	2.6 ± 5.7	24.4%	240
Aspartic acid	5.9 ± 3.9	47.9 ± 25.8	640%	1250
Cysteine	71.7 ± 15.8	8.7 ± 10.9	10.5%	700
Glutamic acid	132 ± 41.7	172 ± 80.4	114%	2950
Glutamine	926 ± 612	76.3 ± 61.4	12.8%	110
Glycine	816 ± 166	291 ± 177	38.7%	110
Histidine	15.7 ± 20.5	0.1 ± 0.1	59.9%	43
Isoleucine *	80.6 ± 9.8	8.4 ± 9.5	9.8%	80
Leucine *	146 ± 25.7	25.5 ± 21.8	16.6%	230
Lysine *	57.1 ± 82.3	11.9 ± 26.7	54.2%	90
Methionine *	27.7 ± 7.2	1.6 ± 1.5	5.2%	30
Phenylalanine *	68.3 ± 18.1	18.1 ± 11.8	26.1%	50
Proline	820 ± 206	345 ± 202	46.2 %	140
Serine	125 ± 26.4	58.2 ± 39.2	42.9%	160
Threonine *	63.6 ± 11.9	28.2 ± 15.9	46.8%	140
Tyrosine	62.2 ± 13.1	5.5 ± 4.1	9.2%	130
Valine *	171 ± 30	30.4 ± 23.4	16.8%	170

[a] Values for asparagine, cystine, and tryptophan were below detectable limits on most or all samples. * Essential amino acid.

[b] ELF/plasma amino acid concentration ratios were calculated for each pig, and the averages of these ratios are presented. Avg = average, SD = standard deviation, ELF = epithelial lining fluid, CDM = chemically defined medium

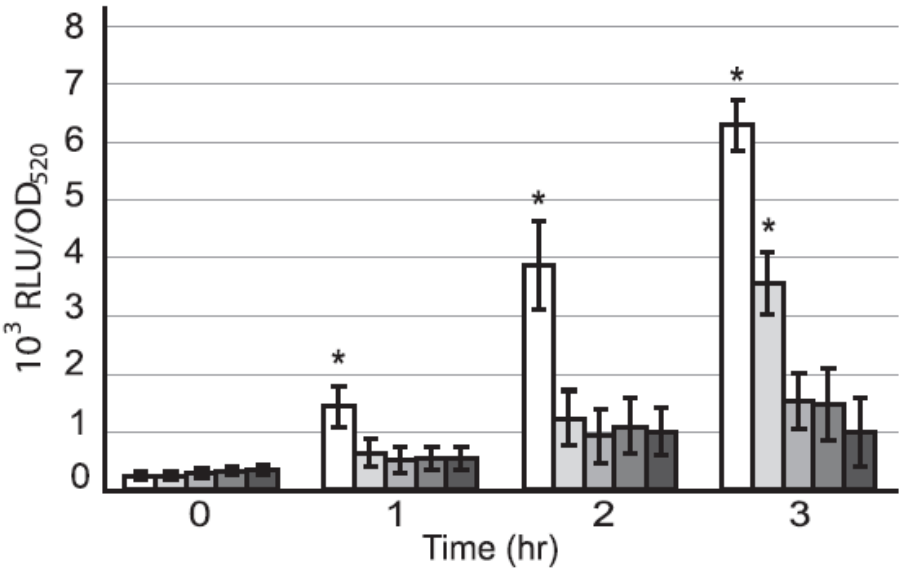

Figure. 2-1. Expression from the *ilvI* promoter in CDM containing various concentrations of BCAA. *A. pleuropneumoniae* AP225/pIviI was grown in CDM containing 0, 10, 20, 50, and 100% of the amount of BCAA in complete CDM + BCAA. Growth was measured as the OD_{520}, and the luciferase activity expressed from the *ilvI* promoter-*luxAB* fusions was measured as RLU. Luciferase activity was normalized to RLU per OD_{520} for each sample. The data are presented as the means ± the standard deviations from three separate experiments. Asterisks indicate values that are significantly different ($P \leq 0.02$) from all other values at the same time point, as determined by using Student's *t*-test.

Expression of *ilvI*, as measured using a luciferase reporter assay (Fig. 2-1), increased rapidly in CDM − BCAA (0% BCAA), with a 6-fold increase at 1 h and a 26-fold increase at 3 h, compared to the 0-h time point. *ilvI* expression was significantly higher ($P \leq 0.02$) in CDM − BCAA at 1, 2, and 3 h than in all other concentrations of BCAA tested. Expression of *ilvI* also increased significantly, albeit more slowly, in CDM plus 10% BCAA, reaching 14-fold times the baseline level by 3 h, which was significantly increased compared to expression in 20, 50, and 100% BCAA ($P \leq 0.01$).

We also measured changes in expression of both *ilvI* and *lrp* in CDM containing different concentrations of BCAA by quantitative RT-PCR (Table 2-4). At 1 h after a shift to fresh

medium, the expression of *ilvI* was strongly upregulated in CDM − BCAA compared to CDM plus 100% BCAA and moderately upregulated in CDM plus 10% and plus 20% BCAA, which paralleled the results seen with the reporter assays. Expression of *lrp* was also upregulated in CDM − BCAA and CDM plus 10% BCAA, although to a much smaller degree than *ilvI*. The results using reporter assays and Q-PCR indicated that expression of *ilvI* and *lrp* was highest in CDM containing no BCAA but also increased in CDM containing the low concentrations of BCAA found in porcine ELF.

Table. 2-4. Q-PCR analysis of *ilvI* and *lrp* expression

Medium	Avg ± SD[a]	
	ilvI	***lrp***
CDM+0% BCAA	27.1 ± 0.32	2.82 ± 0.48
CDM+10% BCAA	6.93 ± 1.32	2.35 ± 0.34
CDM+20% BCAA	6.21 ± 1.99	1.89 ± 0.72
CDM+50% BCAA	3.91 ± 1.91	1.70 ± 0.93

[a] Data are presented as the ratio of the concentration of specific RNA from cultures grown in the medium indicated divided by the concentration of RNA from cultures grown in CDM + 100% BCAA. Transcript levels for each gene were normalized to the level of 16S rRNA. The data represent the average of triplicate samples from two replicate experiments.

Growth of wild-type, *ilvI* mutant, and *lrp* mutant *A. pleuropneumoniae* in CDM. To investigate whether the levels of BCAA in porcine ELF are sufficient for the growth and virulence of *A. pleuropneumoniae* that is unable to synthesize these amino acids, we constructed *A. pleuropneumoniae* serotype 1 strains with mutations in the *ilvI* gene (AP365) (see Materials

and Methods) and in the *lrp* gene (AP359) (54). The construction of the *ilvI* mutant was confirmed by both PCR and Southern blot analyses. PCR analysis showed the predicted 2.5-kb

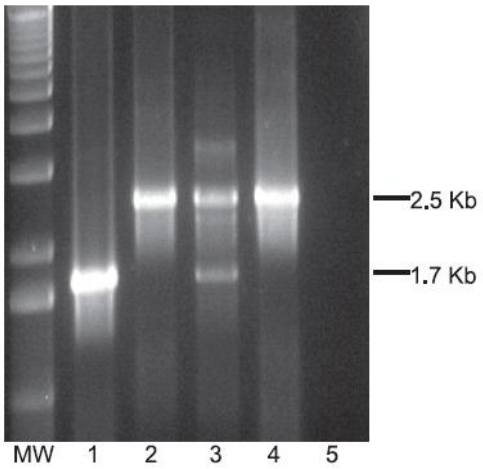

Figure. 2-2. Confirmation of the *ilvI* mutant. PCR was performed using *A. pleuropneumoniae ilvI*-specific primers MM727 and MM734 and the following templates: AP225 wild-type genomic DNA (lane 1), AP365 double-crossover *ilvI* mutant (lane 2), AP364 single-crossover *ilvI* mutant (lane 3), knockout plasmid pRL102 (lane 4), and no DNA (lane 5).

product with *ilvI*-specific primers in AP365, the double-crossover mutant, compared to a 1.7-kb product in wild-type AP225; both products were seen with AP364, a single-crossover mutant (Fig.2-2). In addition, AP365 was shown to contain the Kan-promoter-Cmr cassette in a chromosomal location immediately upstream of the *ilvH* gene and to lack the pGP704 vector (data not shown).

Growth of wild-type *A. pleuropneumoniae* AP100, the *ilvI* mutant, a complemented *ilvI* mutant containing plasmid pRL103, the *lrp* mutant, and a complemented *lrp* mutant containing plasmid pTW415 were compared on CDM agar plates containing various concentrations of BCAA. Both mutants grew well on 100 and 50% BCAA but showed reduced growth on 20% BCAA, a faint haze of growth on 10% BCAA, and no growth on 0% BCAA (data not shown). In

contrast, wild-type *A. pleuropneumoniae* and both complemented mutants grew well on all concentrations of BCAA.

Exponential growth rates in CDM broth containing various concentrations of BCAA were determined for wild-type *A. pleuropneumoniae* and both *ilvI* and *lrp* mutants (Table 2-5). Specific growth rates were highest for all three strains in CDM plus 100% BCAA and dropped for all three strains as the concentration of BCAA in the medium dropped. The growth rates for the wild type were higher than for the *lrp* mutant, although both strains grew in all of the media, despite very low growth rates in CDM – BCAA. The growth rates were lowest in all media for the *ilvI* mutant, which failed to grow at all in CDM – BCAA and grew very poorly in CDM plus 10% BCAA.

Table 2-5. Specific growth rates of wild-type, *ilvI* mutant, and *lrp* mutants in chemically defined medium containing various concentrations of branched chain amino acids

Medium	**Avg specific growth rate μ (h^{-1}) ± SD**[a]		
	AP100 (wild-type)	**AP365 (*ilvI* mutant)**	**AP359 (*lrp* mutant)**
CDM + 100%BCAA	0.91 ± 0.08	0.67 ± 0.08	0.75 ± 0.09
CDM + 20% BCAA	0.60 ± 0.09	0.47 ± 0.06	0.53 ± 0.05
CDM + 10% BCAA	0.41 ± 0.01	0.34 ± 0.04	0.42 ± 0.04
CDM + 0% BCAA	0.33 ± 0.03	NG	0.26 ± 0.04

[a] Data are presented as the averages from at least three separate growth curves. The specific growth rate μ was calculated as $\ln 2/T_d$, where T_d is the doubling time during exponential growth. Avg = average, SD = standard deviation, NG = no growth.

Evaluation of virulence of *A. pleuropneumoniae ilvI* and *lrp* mutants in pigs. To measure the relative virulence of an *A. pleuropneumoniae lrp* mutant, we first compared wild-type *A. pleuropneumoniae* AP225, the *lrp* mutant (AP359), and a complemented mutant (AP359/pTW415) for virulence in pigs at a relatively high infective dose of 4×10^6 CFU/pig. At this dose, all pigs except the uninfected control group developed clinical signs of pneumonia, including elevated rectal temperature, dyspnea, depression, and loss of appetite, within 4 to 8 h postinfection, and most of the infected animals were euthanized due to moderate-to-severe dyspnea and/or depression within 16 to 20 h postinfection. At this dose, there were no significant differences between the groups receiving wild-type AP225, the *lrp* mutant, and the complemented mutant in either percent pneumonia (average = 8.1, 17.3, and 14.3%, respectively), maximum temperature (average = 106.1, 106.2, and 104.0°F, respectively), maximum respiratory rate (85, 85, and 86 breaths/min, respectively), or other parameters measured. The group of pigs that received the *lrp* mutant at a higher dose of 2×10^7 CFU developed disease more rapidly, with all three animals showing clinical signs at 4 h postinfection and requiring euthanasia between 10 and 14 h postinfection. This group had a higher average percent pneumonia of 36.6%. *A. pleuropneumoniae* was cultured from all infected animals but not from uninfected controls. Gross pathology showed typical lesions of severe pleuropneumonia in all infected animals, including hemorrhage, regions of necrosis with fibrin deposits, congestion, edema, and consolidation. Histopathology showed large areas of severe hemorrhage and necrosis surrounded by streaming neutrophils, with bacteria visible within these lesions. There was accumulation of fibrin, blood, and necrotic debris in the affected areas. The alveolar septae were necrotic, with loss of nuclear detail and loss of delineation between air spaces, and vascular walls were inflamed. Edema and hemorrhage were evident, resulting in fluid-filled

alveoli and bronchi. The animals receiving the higher dose of the *lrp* mutant were most severely affected, but there was no visible difference between the wild type, the *lrp* mutant, and the complemented mutant receiving the same dose. PBS-challenged control animals did not develop any clinical signs of pneumonia and showed no lung lesions at necropsy. In this experiment, where all animals were infected with a high infective dose and rapidly developed disease, the *A. pleuropneumoniae lrp* mutant showed no loss in virulence compared to wild-type *A. pleuropneumoniae*.

Although the methods used in this first experiment were similar to those we have previously used successfully to measure the attenuation of a riboflavin-requiring mutant of *A. pleuropneumoniae* (23), we were concerned that the high infective dose and rapid severe hemorrhagic lung damage might have masked small differences in virulence between the *lrp* mutant and wild-type *A. pleuropneumoniae*. Therefore, we used a lower infective dose and a more sensitive technique, competitive index analysis, to measure the relative virulence of both *lrp* and *ilvI* mutants in pigs in two sets of experiments. In these experiments, most pigs were infected intratracheally with 10^6 CFU of bacteria, either of the wild-type or mutant alone or as a mixture of 5×10^5 CFU of the wild type and 5×10^5 CFU of the mutant. To test whether *lrp* might be critical for initial adherence and survival in the upper respiratory tract rather than at entry into the lung, two animals were infected with a combination of the wild type and the *lrp* mutant by the intranasal route rather than the intratracheal route. Animals were monitored for clinical signs of disease as in the previous experiment. At this lower infective dose, all animals developed elevated rectal temperatures but otherwise showed only mild clinical signs of respiratory disease, with the exception of pigs 2199 and 2202, which developed moderate-to-severe disease. The two pigs infected with the *lrp* mutant alone showed less severe clinical signs,

lower percent pneumonia, and less severe pathology than that seen in pigs infected with either the wild-type alone or with a mixture of wild-type and *lrp* mutant (data not shown).

Pigs were euthanized either as disease signs became moderate to severe or at 18, 42, and 66 h postinfection, and BALF and lung samples from six different lung locations were collected.

Table 2-6. Competitive index analysis of virulence of *A. pleuropneumoniae* mutants[a]

Mutant	Pig #	Dose (cfu)	Route	Time PI (hr)	Ave CI
ilvI	33	1×10^6	Intratracheal	42	0.22
	38	1×10^6	Intratracheal	66	0.05
lrp	35	1×10^6	Intratracheal	18	0.24
	2199	1×10^6	Intratracheal	32	0.14
	29	1×10^6	Intratracheal	42	0.06
	41	1×10^6	Intratracheal	66	<0.06
	2203	1×10^6	Intratracheal	66	0.04
	2202	5×10^6	Intranasal	18	0.44
	2205	5×10^6	Intranasal	42	0.19

[a] The table includes data on all pigs from experiments 2 and 3 that received both mutant and wild-type strains. Pigs 2199, 2202, 2203, and 2205 were from experiment 2. Pigs 29, 33, 35, 38, and 41 were from experiment 3.
cfu = colony formning units, PI = post-infection, hr = hour, Ave = average, CI, competitive index.

The CFU/ml of BALF or per g of lung tissue were measured for both the wild type and the mutant, and competitive indices were calculated as a measure of how well the mutant competed with virulent wild-type *A. pleuropneumoniae* (Table 2-6). In these competitive index experiments, we found that neither the *lrp* mutant nor the *ilvI* mutant competed well with wild-type *A. pleuropneumoniae*. This was true for the *lrp* mutant in infections by both the intratracheal and the intranasal routes. For both mutants, the competitive index tended to drop

over time, with average CIs of 0.2 to 0.4 for animals euthanized 18 h postinfection, 0.06 to 0.22 at 32 to 42 h, and 0.04 to 0.06 at 66 h. The competitive index was always lower than 1 and declined as the disease progressed. These results indicate that both the *ilvI* mutant and the *lrp* mutant are attenuated in our swine experimental infection model.

Effect of AHAS inhibitors on growth of *A. pleuropneumoniae*. Six compounds that are inhibitors of plant AHAS enzymes routinely used in herbicides, namely, the sulfonylureas metsulfuron methyl, primisulfuron methyl, chlorimuron ethyl, and chlorsulfuron and the imidazolinones imazapyr and imazaquin, were tested for their ability to inhibit the growth of wild-type *A. pleuropneumoniae*. All four of the sulfonylureas that were tested inhibited the

Table 2-7. Minimum inhibitory concentrations of AHAS inhibitors against *A. pleuropneumoniae* AP100 in CDM + BCAA and CDM − BCAA

AHAS Inhibitor	Avg MIC (µg/ml) [a]	
	CDM + BCAA	**CDM − BCAA**
Sulfonylureas		
Chlorimuron ethyl	> 2000	1
Chlorsulfuron	> 2000	2
Metsulfuron methyl	2000	0.5
Primisulfuron	> 2000	16
Imidazolinones		
Imazapyr	> 2000	≥ 1000
Imazaquin	> 2000	≥ 2000

[a] Average (Avg) of at least four independent replicate experiments. AHAS = acetohydroxyacid synthase

growth of *A. pleuropneumoniae* in CDM − BCAA but not CDM + BCAA (Table 2-7). Metsulfuron methyl was the most effective, with an MIC of 0.5 nmol/ml (0.2 µg/ml), whereas primisulfuron methyl was the least effective, with an MIC of 16 nmol/ml (7.5 µg/ml). In contrast, neither of the imidazolinones tested inhibited growth of *A. pleuropneumoniae* in either CDM + BCAA or CDM − BCAA.

DISCUSSION

Our IVET studies on *A. pleuropneumoniae* (22, 55) and a review of other similar studies (7, 8, 21, 33, 46, 56) identified genes involved in BCAA biosynthesis as in vivo induced or required for survival and virulence in pathogens of relatively clean body sites such as the lungs, cerebrospinal fluid, and bloodstream, but not in pathogens of the gastrointestinal tract. Further, we found that 25% of the *A. pleuropneumoniae* promoters that we had identified as specifically induced in vivo were upregulated in vitro in CDM lacking BCAA (55). These studies suggested that a previously unrecognized environmental condition—limitation of the BCAA leucine, isoleucine, and valine—exists in the healthy mammalian lung, that bacteria can sense this environmental condition and respond to it, and that pulmonary pathogens unable to synthesize BCAA will be attenuated. Since no data were available on the actual concentrations of BCAA in mammalian lung fluids, we first measured the free amino concentrations in porcine pulmonary ELF and serum. We found that most amino acids, with the exception of aspartic acid and glutamic acid, were present in lower concentrations in ELF than in serum, although many were present at roughly 40 to 50% of the serum level, including the essential amino acids lysine and threonine. In contrast, the essential BCAA were present in pulmonary ELF at ~10 to 17% of the concentration in serum. This is the first report of actual free amino acid concentrations in porcine ELF. When tested in vitro in a CDM, the low concentrations of BCAA found in pulmonary ELF led to reduced growth rates of wild-type *A. pleuropneumoniae*, as well as to increased expression of genes required for BCAA biosynthesis. During infection, even a slight reduction in growth rate of a pathogen can allow the host natural defenses to clear the pathogen before disease develops.

To test whether the ability to synthesize BCAA is critical for survival and virulence of *A. pleuropneumoniae* in its natural swine host, we constructed two mutants that require exogenous BCAA for growth. The first mutant contained a mutation in the *ilvI* gene, which encodes the large subunit of acetohydroxy acid synthase, an enzyme required for biosynthesis of all three BCAAs. The *A. pleuropneumoniae* genome contains two sets of genes that encode putative AHAS enzymes, *ilvIH* and *ilvGM* (18, 61), and there was concern that the *ilvGM*-encoded enzyme might substitute for that encoded by *ilvIH*. However, the *ilvI* mutant failed to grow in vitro in the absence of exogenous BCAA, indicating that the *ilvGM*-encoded AHAS enzyme did not substitute for the *ilvIH*-encoded AHAS under these conditions.

The second mutant was constructed to knock out *lrp*, which encodes a global regulator of BCAA synthesis and degradation in *E. coli* (10, 12, 38). We have previously shown that an *lrp* homologue is required for *ilvI* expression in *A. pleuropneumoniae* (54). Lrp is frequently a pleiotropic regulator of a wide variety of genes in addition to those involved in amino acid biosynthesis and catabolism, although the Lrp regulon varies between even closely related species (14, 30). Lrp has also been shown to regulate a variety of virulence-associated genes in many other pathogens (2, 19, 24, 29, 32, 60). Although Lrp is essential for virulence in *Xenorhabdus nematophila* (15, 29), it appears to act as a virulence repressor in *Salmonella enterica* serovar Typhimurium (2).

In initial attenuation experiments comparing wild-type and Lrp *A. pleuropneumoniae* in experimental infections in pigs using the high inoculating dose of 4×10^6 CFU, the pigs rapidly developed severe hemorrhagic pneumonia, and there was no obvious difference in severity or pathology between the wild-type and mutant infections. However, when a lower inoculating dose of 10^6 was used, the pigs infected with the *lrp* mutant showed less severe clinical signs and less

severe pathology than those infected with the wild type. We chose to use a competitive index design for further studies. Competitive index experiments can be a more sensitive measure of virulence attenuation than standard infection experiments to calculate differential LD_{50} values for wild-type and mutant strains (4). In competitive index experiments using a combined dose of 1×10^6 CFU for intratracheal inoculation and 5×10^6 CFU for intranasal inoculation, we found that neither the *lrp* mutant nor the *ilvI* mutant competed well with wild-type *A. pleuropneumoniae* and that the *lrp* mutant was attenuated in animals infected by both methods.

The observation that the *lrp* mutant is virulent at high doses but attenuated at lower doses might be explained by the disease process. At high infecting doses, lung damage occurs very rapidly in this disease. This is most likely due to the extracellular Apx toxins, since growth of the infecting inoculum under conditions that enhance Apx toxin production leads to increased virulence and increased rapidity of disease progression. The Apx toxins, which can be hemolytic or cytotoxic or both, are the key virulence factors leading to severe lung damage (47). These toxins kill neutrophils that are attracted to the site of infection, releasing toxic neutrophil contents into tissues, which causes tissue damage; the Apx toxins are also cytotoxic for porcine alveolar epithelial cells (27, 51). The severe damage to cells and tissues that results can lead to release of cytoplasmic contents such as hemoglobin and free amino acids from host cells. In addition, *A. pleuropneumoniae* produces extracellular proteases that may degrade proteins within the lung tissues (36, 37). Together, Apx toxins plus secreted proteases could release sufficient BCAA to allow the *lrp* mutant to survive and grow. In contrast, at lower infecting doses, there is generally a lag of several hours before the development of clinical signs, and the reduction in tissue damage at the early stages of infection may result in decreased release of free amino

acids from host cells and therefore insufficient levels of BCAA to allow multiplication of the infecting *lrp* or *ilvI* mutants. It is likely that BCAA are present in limiting amounts at the initial stages of *A. pleuropneumoniae* infection and that the damage caused by the disease leads to increased availability of BCAA.

We observed that limitation of BCAA stimulated the expression of both *ilvI* and *lrp* in the present study, with levels of gene expression increased with decreasing concentrations of BCAA. Expression measured both by Q-PCR to quantitate RNA transcripts for *ilvI* and *lrp* and by luciferase reporter assay for *ilvI* showed elevated RNA and reporter enzyme at 1 h after a shift to conditions of BCAA limitation. We had previously reported that BCAA limitation induced expression in vitro of 25% of the *A. pleuropneumoniae* gene promoters identified as specifically expressed in vivo in our IVET studies (55). These results suggest that low levels of BCAA may be an important environmental cue regulating gene expression in *A. pleuropneumoniae* and other pulmonary pathogens. Recent studies in our laboratory to identify the *A. pleuropneumoniae* transcriptome in response to limitation of BCAA indicate that expression of many genes is modulated by BCAA limitation, including increased expression of several adhesins (M. H. Mulks, unpublished data). These results suggest a model in which BCAA limitation acts as an early signal regulating expression of genes required for survival and virulence, such as adhesins, whose expression may later be altered by increased levels of BCAA.

The results reported here demonstrate that the ability to synthesize BCAA is critical for the survival and virulence of *A. pleuropneumoniae* in the swine lung. These results suggested that BCAA biosynthesis is a potential target for the development of antimicrobials against *A. pleuropneumoniae* and similar pathogens. Many AHAS inhibitors have been developed as potent herbicides that show little toxicity for mammals (16). We tested two classes of inhibitors of

AHAS enzymes, sulfonylureas and imidazolinones, for their ability to inhibit growth of *A. pleuropneumoniae*. We predicted that these inhibitors would have minimal effect on growth in CDM + BCAA, where the ability to synthesize BCAA is not required because there are sufficient levels of exogenous BCAA, and would have significant effect on growth in CDM − BCAA, where the bacteria must be able to synthesize BCAA. Our results show that all four sulfonylureas that were tested inhibited growth of wild-type *A. pleuropneumoniae* in CDM − BCAA but not in CDM + BCAA. However, neither imidazolinone had any effect on growth in either medium. Since both classes of compounds inhibit AHAS enzymes, the difference is likely to be in uptake of the compound into bacterial cells. Sulfonylureas have also been shown to inhibit growth of *Mycobacterium tuberculosis* both in vitro and in a mouse model (25, 44) and of *Brucella suis* in macrophages (5). These results indicate that AHAS inhibitors have excellent potential for development as antimicrobials against infections of the respiratory tract or other "clean" body sites, although their utility against fulminant *A. pleuropneumoniae* infection may be limited in vivo due to the effect of the Apx toxins. However, only a few pulmonary pathogens, such as *A. pleuropneumoniae* and *Mannheimia haemolytica*, produce large amounts of repeat-in-toxin toxins, and AHAS inhibitors may function well in vivo against pathogens that cause less destruction of tissues and blood cells, or as prophylactic measures against spread of diseases such as porcine pleuropneumonia within a herd.

In summary, our IVET studies on *A. pleuropneumoniae* led us to hypothesize that BCAA limitation is an environmental condition encountered by this pathogen in the healthy pig lung and that the ability to synthesize BCAA would be critical for full virulence of this pathogen. We have shown that BCAA are indeed present in only limited amounts in porcine pulmonary ELF, that the growth of wild-type *A. pleuropneumoniae* was reduced in vitro when such low levels of

BCAA were available and was inhibited by sulfonylureas, and that two mutants unable to synthesize BCAA were attenuated in an experimental infection model in swine. We have also shown that the concentration of available BCAA affects expression of *ilvI* and *lrp*, with increased expression correlating with decreased BCAA concentration. Further studies to identify the full *A. pleuropneumoniae* transcriptome that responds to BCAA limitation and regulatory molecules that control this response are in progress. These results demonstrate how data from IVET and signature-tagged mutagenesis studies can be used to define the environment encountered by pathogens in vivo and to suggest promising avenues for the development of new tools to control infectious diseases.

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CHAPTER III

THE RNA CHAPERONE HFQ REGULATES BIOFILM FORMATION AND IS REQUIRED FOR OXIDATIVE STRESS RESISTANCE IN *ACTINOBACILLUS PLEUROPNEUMONIAE*

ABSTRACT

Actinobacillus pleuropneumoniae is the etiological agent of porcine pleuropneumonia, an economically important disease of pigs. The *hfq* gene in *A. pleuropneumoniae*, encoding the RNA chaperone and post-transcriptional regulator Hfq, is up-regulated both during infection of porcine lungs and during growth in media lacking branched-chain amino acids. Since biofilm genes are up-regulated in *A. pleuropneumoniae* grown under branched-chain amino acid limitation and Hfq regulates biofilm formation in *Vibrio cholerae*, we investigated the role of *hfq* in biofilm formation by *A. pleuropneumoniae*. An *hfq* mutant strain was constructed in a biofilm-positive *A. pleuropneumoniae* serotype 1 strain. The *hfq* mutant was defective in biofilm formation on abiotic surfaces. The *pgaC* transcript, encoding the biosynthesis of poly-β-1, 6-N-acetylglucosamine (PGA), a major biofilm matrix component, was found to be fourteen-fold lower in the *hfq* mutant compared to wild-type. Cysteine synthase, implicated in resistance to oxidative stress and tellurite in bacteria, was not found at detectable levels by SDS-PAGE analyses in the outer membranes isolated from the *hfq* mutant. Therefore, the effect of Hfq on oxidative stress and tellurite resistance was tested. The *hfq* mutant displayed enhanced sensitivity to superoxide produced by methyl viologen and tellurite, but not to inorganic or organic peroxides. These phenotypes were readily reversed by the provision of *hfq in trans.* These findings clearly demonstrate that the in vivo-induced gene *hfq* is involved in the regulation of PGA-based biofilm formation and resistance to superoxide stress and tellurite in *A. pleuropneumoniae*. This is the first report of the regulation of PGA-based biofilm by Hfq.

INTRODUCTION

A. pleuropneumoniae is an encapsulated Gram-negative pleiomorphic coccobacillus in the family *Pasteurellaceae* and is the etiological agent of porcine pleuropneumonia (3). Porcine pleuropneumonia is characterized by a fulminating fibrino-hemorrhagic bronchopneumonia, which is often fatal. Although the incidence of outbreaks has decreased in the developed world, porcine pleuropneumonia remains as a major cause of economic loss in intensive swine production around the world (20). *A. pleuropneumoniae* uses several virulence factors—including the Apx toxins, capsular polysaccharides and lipopolysaccharides—to evade clearance by phagocytes and induce tissue damage resulting in edema, hemorrhage and necrosis of various lung lobes in addition to fibrinous pleuritis (3, 20). Pigs are the only known host for *A. pleuropneumoniae* and there are no environmental reservoirs for this pathogen.

An in vivo expression technology study was performed previously in our laboratory to identify *A. pleuropneumoniae* genes which are up-regulated during infection of the porcine lungs (17). Thirty-two promoters—including the promoter region for *hfq*, which encodes host factor Q-β (Hfq)—were identified to be induced during infection (18, 29, 51). Twenty five percent of the in vivo-induced genes, including *hfq*, were up-regulated under limitation of the branched-chain amino acids (BCAA) isoleucine, leucine and valine in laboratory media (51). We have recently identified that limitation of BCAAs is a host signal encountered by *A. pleuropneumoniae* in the pig lungs and the ability to synthesize BCAAs is essential for the survival and virulence of *A. pleuropneumoniae* in swine lungs (46). A transcriptomics study revealed that the *pgaABCD* operon, encoding the production and export of the principal biofilm matrix polysaccharide—poly-β-1, 6-N-acetylglucosamine (PGA)—is ~14-fold up-regulated under BCAA limitation in *A. pleuropneumoniae* (R. M. LeVeque and M. H. Mulks, unpublished data). Since the *A.*

pleuropneumoniae strain used in the transcriptome analyses does not form biofilm, a biofilm-positive strain was used in further studies on biofilm formation.

Hfq was identified as a factor required for the replication of RNA bacteriophage Q-β in *Escherichia coli* (15). Hfq is a pleiotropic post-transcriptional regulator which modulates translation and transcript stability by acting as an RNA chaperone in bacteria (6). Doughnut-shaped homo-hexamers of Hfq bind to the A/U rich regions in the 5' untranslated regions (UTR) of transcripts, and small regulatory RNAs (sRNA), facilitating the formation of mRNA:sRNA duplexes by incomplete base-pairing. This interaction either enhances or blocks the access of ribosomes to the translation initiation region and the mRNA:sRNA duplex may be targeted to degradation, although inhibition of translation alone is sufficient for silencing the expression of a target transcript (36). Small RNAs play a number of regulatory roles in the physiology as well as the virulence of bacterial pathogens by acting as ultra sensitive switches in adaptation to changing environmental conditions (38). However, Hfq can also act as a regulator independent of sRNAs. For instance, in *E. coli* Hfq binds to the 5' UTR of its own transcript and autoregulates translation (49).

Bacteria are frequently found as sessile communities embedded in a self-produced extracellular polymeric matrix described as a biofilm. Bacterial pathogens growing as a biofilm have enhanced resistance to host defense mechanisms and antimicrobial agents (24). Biofilm formation is common among field isolates of *A. pleuropneumoniae* but laboratory strains rarely form biofilm (26). The biofilm lifestyle has been proposed to play a role in colonization of the porcine respiratory tract (25, 26). However, the role of PGA in the colonization and virulence of *A. pleuropneumoniae* remains to be experimentally verified. PGA, whose biosynthesis and export is encoded by the *pgaABCD* operon, is the major biofilm matrix component facilitating

auto-aggregation and biofilm formation in *A. pleuropneumoniae* (25, 28). *A. pleuropneumoniae* also produces dispersin B, a hexosaminidase, which degrades PGA (28). PGA-based biofilms are important virulence factors in several pathogens, including *Acinetobacter baumannii, A. actinomycetamcomitans, Bordetella pertusis, Staphylococcus aureus* and *S. epidermidis* (10, 11, 21, 50, 52). Hfq is implicated in biofilm formation by uropathogenic *E. coli* and *Vibrio cholerae* (30, 33). However, the role of Hfq in the regulation of PGA-based biofilm is not known.

In this report, we provide evidence for the regulation of PGA-based biofilm formation by Hfq. Studies to identify additional Hfq-regulated factors involved in biofilm formation led to the finding that cysteine synthase, CysK, is not found at detectable levels in the membrane fraction obtained from the *hfq* mutant strain. Since CysK is involved in resistance to oxidative stress, we tested the ability of the *hfq* mutants to survive under oxidative stress and found that Hfq is required for resistance against superoxide stress and tellurite in *A. pleuropneumoniae*. In summary, Hfq, encoded by the in vivo-induced gene *hfq*, regulates PGA-based biofilm formation and superoxide stress resistance in *A. pleuropneumoniae.*

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used for this work are listed in table 3-1. *A. pleuropneumoniae* strains were grown in brain heart infusion (BHI) (Becton Dickinson and Company, Sparks, MD) supplemented with 10 µg/ml of NAD (V factor), BHIV, unless stated otherwise. The *hfq* mutant and complemented mutant were grown in BHIV containing 1.5 µg/ml of chloramphenicol, and 50 µg/ml of ampicillin, respectively. Plate cultures were incubated at 35°C under 5% CO_2, and broth cultures were incubated in a water bath set at 35°C and shaking at 160 rpm.

For determination of growth pattern, *A. pleuropneumoniae* strains were grown in BHIV containing 50 µg/ml of ampicillin and 0.25 µg/ml of dispersin B (Kane Biotech Inc, Winnipeg, Canada), and optical density at 520 nm (OD_{520} nm) was determined at 30 minute intervals. The wild-type and *hfq* mutant strains were transformed with pGZRS19 to confer ampicillin resistance. Dispersin B was added to prevent cellular aggregation to facilitate determination of accurate optical density values (27). For determination of biofilm production over time and RNA extraction, *A. pleuropneumoniae* strains were grown in chemically defined medium (CDM) (51).

E. coli XL1-Blue was used for cloning and grown in Luria Bertani medium at 37°C. Broth cultures were incubated in a water bath set at 37°C and shaking at 200 rpm. For antibiotic selection, 30 µg/ml of chloramphenicol or 100 µg/ml of ampicillin was used as needed.

Oligonucleotide primers used in this study are listed in table 3-2.

Table. 3-1. Strains and plasmids used in this study

Strains or plasmids	Description[a]	Source or reference
Strains		
A. pleuropneumoniae		
AP 100	ATCC 27088, serotype 1A, passaged through pigs	ATCC[b]
AP 93-9	Serotype 1 field isolate (Wild-type)	12
AP 371	Double cross over mutant at the *hfq* locus in AP 93-9 (Δ*hfq*)	This study
AP 372	Complemented mutant (Δ*hfq*+*hfq*)	This study
AP 373	AP 371 with pGZRS19	This study
AP 382	AP 93-9 with pGZRS19	This study
E.coli XL1-Blue mRF'	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*[F' *proAB lacI*^{q} *Z*ΔM15 Tn *10* (Tet^{R})]	Stratagene
Plasmids		
pUC18	Ap^{R}; high-copy-number cloning vector	24
pGZRS18/ 19	Ap^{R}; *A. pleuropneumoniae-E. coli* shuttle vector	23
pSS102	Ap^{R}; 5' end and 3' end of *hfq* were cloned into pUC18 with an internal NsiI site	This study
pSS103	Ap^{R}, Cm^{R}; *cat* cassette expressed from *nptI* promoter inserted into NsiI site of pSS102	This study, 21
pSS104	Complementation plasmid with *hfq* gene and its promoter region cloned into pGZRS19	This Study

[a] Ap^{R} – ampicillin resistance, Cm^{R} – chloramphenicol resistance, [b] American Type Culture Collection

Table. 3-2. Oligonucleotide primers used in this study

Primer ID	Sequence[a]	Feature
MM 496	AAGGTTGATATGTCCGCACC	*omlA* forward primer
MM 497	CACCGATTACGCCTTGCCA	*omlA* reverse primer
MM 721	GGTTTGCATGCCGAATTATTG	Forward primer for cloning 5' end of *hfq* into pSS102, and construction of pSS104
MM 722	GACGAAATGCATTCAAATATGG	Reverse primer for cloning 5' end of *hfq* into pSS102
MM 724	GTACATGCATGTTCCGTTTCTC	Forward primer for cloning 3' end of *hfq* into pSS102
MM 725	GCCCAGTCGACGTACCAAAC	Reverse primer for cloning 3' end of *hfq* into pSS102
MM 728	CGGATCCATGGCAAAAGGTCAATC	Forward primer binding to the *hfq* start codon
MM 729	CAAGTCGACTTATTCCGCTTTATCCGC	Reverse primer for construction of pSS104
MM 588	CCATGCCGCGTGAATGA	16S rRNA forward primer, Q-PCR
MM 589	TTCCTCGCTACCGAAAGAACTT	16S rRNA reverse primer, Q-PCR
MM 845	CGATAAGTGGGCGAAAAGAGA	*pgaC* forward primer, Q-PCR
MM 846	CCGGAGTTCGCTTGGTGTA	*pgaC* reverse primer, Q-PCR
MM 847	GATCAAAAACAACTTAAGCGAACTCA	*dspB* forward primer, Q-PCR
MM 848	CGGGCAAATCGGCTCTT	*dspB* reverse primer, Q-PCR
MM 863	CTGCTCGTTCCGTTTCTCATAA	*hfq* forward primer, Q-PCR
MM 864	ACCGCAGGAGCTTGTTGAGT	*hfq* reverse primer, Q-PCR

[a]Underlined sequences indicate restriction sites

Effect of BCAA limitation on *pgaC* expression. To determine the effect of BCAA limitation on wild-type *pgaC* expression, biofilm-negative (AP 100) and biofilm-positive (AP 93-9) strains grown on BHIV plates were used to inoculate 32 ml cultures in complete CDM for acclimatization. Cultures were grown to mid-log phase (OD_{520} of 0.5 - 0.6) at 35°C, shaking at 160 rpm in 300 ml baffled-flasks. Two 15 ml samples of each strain were centrifuged to pellet the cells. Pellets were resuspended in 30 ml of either CDM containing the branched-chain amino acids leucine, isoleucine, and valine (CDM+BCAA) or lacking these amino acids (CDM-BCAA)

and grown for 1 hour at the same conditions used for acclimatization. At the end of 1 hour incubation, 30 ml of ice-cold methanol was added to the cultures, mixed and stored at -20°C for 1 hour. 1.5 ml aliquots were centrifuged at 16,000 × *g* for 2.5 minutes at 4°C and pellets were stored at -80°C.

RNA isolation. For RNA extraction, cell pellets were resuspended in 90 µl of Tris EDTA (TE) (10 mM Tris, 1mM EDTA, pH8). 10 µl of a 50 mg/ml lysozyme solution was added and the samples were incubated for 10 minutes at room temperature. RNA was extracted using Qiagen RNeasy mini columns (Qiagen, Valencia CA), with an on-column DNase treatment, following the manufacturer's instructions. The concentration, purity and integrity of RNA samples were determined using a Nanodrop spectrophotometer (Nanodrop Products, Wilmington, DE) and agarose gel electrophoresis. Any additional contaminating DNA was removed by DNase treatment with the Turbo DNAfree kit according to manufacturer's instructions (Ambion, Austin, TX). RNA was reverse transcribed using a Superscript II reverse transcription kit (Invitrogen, Carlsbad, CA). Three 10-fold dilutions of cDNA were used in Q-PCR with SYBR green PCR core reagents in a StepOnePlus real time PCR system (Applied Biosystems, Foster City, CA). Data was normalized with 16S rRNA gene as the endogenous control and the results were analyzed by a ΔΔCt method described by Pfaffl to calculate fold change in gene expression (39). Each sample was measured in duplicate and the experiment in its entirety was repeated three times.

Construction and verification of an *A. pleuropneumoniae hfq* mutant. To construct an *A. pleuropneumoniae hfq* mutant strain, we first constructed a plasmid (pSS103) containing the 3' end of *miaA*, 5' end of *hfq*, promoter of *nptI*, chloramphenicol acetyl transferase (*cat*) cassette, 3' end of *hfq*, and 5' end of *hflX* in tandem, with an internal deletion of 153bp from the 279 bp

hfq gene, in the pUC18 vector (Fig. 3-1). 644 base pairs from the 5' end of *hfq* and the upstream flanking region and 617 base pairs from the 3' end of *hfq* and the downstream flanking region were amplified with primer pairs MM 721-MM 722 and MM 724-MM 725 from AP 100 genomic DNA and cloned into pUC18 to generate the plasmid pSS102. This construct has a unique NsiI site between the 5' and 3' ends of *hfq*. A *cat* cassette expressed from an *nptI* promoter, obtained from the plasmid pRL100 (46), was cloned into the NsiI site of pSS102 to engineer pSS103. A deletion-disruption mutation at the *hfq* locus in AP 93-9 (wild-type serotype 1 field isolate) was introduced by allelic exchange using natural transformation (4). Dispersin B (0.5 µg/ml) was added to the medium during preparation of naturally competent AP 93-9, to prevent PGA-mediated autoaggregation. The transformants were selected by plating on BHIV containing 2 µg/ml of chloramphenicol. Genomic DNA isolated from the transformants was screened by PCR analysis of the *hfq* locus to confirm the mutant construct (Fig. 3-1). The *hfq* mutant was designated as AP 371.

The entire *hfq* ORF and 595 bp of upstream region including the promoter was amplified with primers MM 721 and MM 729, and cloned into the *A. pleuropneumoniae*:*E. coli* shuttle vector pGZRS19 for *trans* complementation (53). The complementation construct, pSS104, was verified by PCR, restriction mapping, and sequencing. AP 371 was transformed with pSS104 to generate the complemented mutant AP 372 (AP 371/pSS104). The shuttle vector pGZRS19 was transformed into AP 93-9 and AP 371 to generate AP 382 (AP 93-9/pGZRS19) and AP 373 (AP 371/pGZRS19), respectively, as empty vector controls.

Determination of biofilm biomass. Wild-type *A. pleuropneumoniae* grown overnight on BHIV plates was used to inoculate either BHIV or CDM broth to an OD_{520} nm of ~0.05. Three

ml of these suspensions were transferred to each well in a 6 well tissue culture treated polystyrene plate (Corning, Corning, NY). Six identical plates were set up to quantify biofilms at 0, 3, 6, 9, 12, and 24 hours. Absorbance (OD_{520} nm) was recorded at the beginning and the end of incubation period to monitor growth. Polystyrene plates were incubated at 35°C under 5% CO_2. A crystal violet binding assay was used to determine the biomass of biofilm (37). At the end of the incubation period, contents of the wells were aspirated and wells were rinsed three times with water and dried. 500 µl of Gram's crystal violet solution was added to each well (Becton Dickinson and Company, Sparks, MD) and incubated for 2 minutes at room temperature. The crystal violet solution was aspirated, the wells were rinsed three times with water to remove unbound stain, and the plates were dried at 37°C for an hour. 3 ml of 33% acetic acid solution (v/v in water) was added to each well to dissolve biofilm-bound crystal violet. Plates were placed on an orbital shaker shaking at 100 rpm for 10 minutes and the optical density was measured as absorbance at 590 nm. All optical density measurements for this experiment were made using a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA). The zero hour time point was used to detect the baseline non-specific binding to polystyrene. Results reported are the mean and standard deviation of triplicate samples from four independent experiments.

To measure biofilm production in the wild-type (AP 93-9/pGZRS19), *hfq* mutant (AP 371/pGZRS19), and complemented mutant (AP 371/pSS104), inocula were prepared in BHIV supplemented with ampicillin (50 µg/ml) as described above. Ampicillin was added to the medium to ensure the maintenance of the complementation plasmid and vector alone controls. To analyze multiple technical replicates, the assay was modified to a 96 well tissue culture plate format using 200 µl of inoculum per well. Plates were processed essentially as described above

except that 200 µL of water or Gram's crystal violet solution or 33% acetic acid was used. Results reported are the mean and standard deviation from four independent experiments with eight technical replicates per experiment.

RNA extraction from biofilm. Wild-type (AP 93-9) and *hfq* mutant (AP 371) strains, were grown in CDM as described for the biofilm assay in 6 well plates. From wells containing wild-type *A. pleuropneumoniae,* planktonic and biofilm cells were collected separately. Since the *hfq* mutant did not form biofilm, only the planktonic cells were available for harvesting. 500 µl of planktonic cells, from wells with the wild-type or *hfq* mutant, were mixed with 500 µl of 100% ice cold methanol and centrifuged at 4°C, 16,000 × *g* for 2.5 minutes. To collect biofilm, the supernatant was aspirated from the wells and 3 ml of 50% ice cold methanol was added per well. A cell scraper was used to detach the sessile cells. Aliquots of 1 ml were centrifuged at 4°C, 16,000 × *g* for 2.5 minutes. The supernatant was removed and cell pellets were stored at -80°C.

RNA extraction and Q-PCR were performed as described above with one modification in lysozyme treatment. To cell pellets resuspended in 400 µl of TE 8, 100 µl of 50 mg/ml lysozyme solution was added and incubated for 10 minutes at room temperature. Each sample was measured in triplicate and the experiment in its entirety was repeated two times.

Detection of PGA by Congo red staining. Strains were grown for 6 hours in 3 ml of BHIV supplemented with ampicillin (50 µg/ml), with or without dispersin B at 1.5 µg/ml, in polystyrene 6 well plates. Crude polysaccharide extracts were prepared as described previously, with changes as follows (28). For the wild-type and complemented mutant strains, planktonic and biofilm cells were harvested and processed separately. Since *A. pleuropneumoniae* does not form biofilm in the presence of dispersin B, only planktonic cells were available from wells

treated with dispersin B. Biofilms were collected from the culture wells with a cell scraper and resuspended in 500 µl of a resuspension buffer (50 mM sodium acetate, pH 5.8, with 100 mM sodium chloride). Cells were harvested by centrifugation and pellets resuspended in 500 µl resuspension buffer were transferred to 1.5 ml tubes and centrifuged at 16,000 × g for 3 minutes. Supernatants were discarded and wet weight of pellets (mg) was recorded. Pellets were stored at -20°C until further processing. Pellets were resuspended in 40 µl per 10 mg of wet weight in resuspension buffer and an equal volume of Tris buffer (10 mM Tris-HCl, pH 8, 5 mM EDTA and 0.5% SDS) was added. Tubes were vortexed briefly and placed in boiling water bath for 10 minutes before centrifuging at 16,000 × g for 1 minute. Supernatants were discarded and crude polysaccharide pellets were resuspended in 20 µl per 10 mg wet weight of cell pellet. 10 µl of crude polysaccharide suspension was applied to a well in a polystyrene 96 well tissue culture treated plate, 2 wells per sample and dried at 55°C for 1 hour. Wells were stained with 200 µl of 1% Congo red for 5 minutes at room temperature, rinsed three times with 200 µl of water and dried. PGA-bound Congo red was solubilized in 200 µl of DMSO (50). The plates were placed on an orbital shaker for 10 minutes and OD_{500} nm was measured in a SpectraMax M5 spectrophotometer.

Preparation of outer membrane fractions. Strains were grown in 100 ml of BHIV for 9 hours (stationary phase) and cells were harvested by centrifugation at 4°C. Cell pellets were processed according to the method described by Cruz et al. to obtain membrane fractions (12). Briefly, pellets were treated with lysozyme to generate spheroplasts which were subsequently ruptured with ten bursts of sonication, each burst was fifteen seconds long, at 30% duty cycle and 40% power, respectively, in a Branson sonifier (Branson, Danbury, CT). The total membrane fraction was isolated by ultracentrifugation of whole cell sonicate at 150,000 × g for

1 hour. Cytoplasmic membrane and outer membrane (OM) were separated from the total membrane fraction by sucrose density gradient centrifugation. Aliquots were stored at -20°C for further analysis.

SDS-PAGE. Protein concentration in the OM fractions was determined by Bradford's method (Bio-Rad, Hercules, CA). 2.5 µg of OM samples from the wild-type and *hfq* mutant were separated on 12% SDS-PAGE gels using the discontinuous buffer system of Laemmli (12, 32). Gels were stained with Coomassie blue-silver to compare the protein profile of the *hfq* mutant to the parent strain as described by Cruz et al. (12). The experiment was repeated three times. A differentially expressed protein, found only in the wild-type, was excised from the gel and submitted for MALDI-TOF mass spectrometry analysis at the Michigan State University Research Technology Support Facility.

Oxidative stress protection assays. Cumene hydroperoxide, hydrogen peroxide, methyl viologen (paraquat), and potassium tellurite (Sigma, St. Louis, MO), were used to induce oxidative stress. Growth inhibition assays on top agar plates were performed as described previously (42). Briefly, *A. pleuropneumoniae* strains grown in BHIV broth for 16 hours were diluted 10 fold in 0.7% BHIV agar and poured on top of BHIV agar in plates. After solidification of top agar, filter paper discs, 7 mm in diameter (Whatman Paper Ltd, Maidstone, England), soaked in 10 µl of 200 mM cumene hydroperoxide, 880 mM hydrogen peroxide, 74 mM methyl viologen, or 390 mM potassium tellurite, were placed on the plates. After 22 hours of incubation, the diameter of zones of growth inhibition around the discs was measured. Three independent experiments were performed.

To determine the effect of Hfq on oxidative stress in the absence of PGA, experiments were done as described above, except for the addition of dispersin B (1.5 µg/ml) to media used for overnight growth. The entire experiment was repeated four times, independently.

Statistical analyses. Experiments—effects of Hfq on biofilm formation, PGA production and oxidative stress—were repeated at least three times and data were analyzed by Student's *t*-test for unpaired samples with equal variance. In all cases, a *P* value of less than 0.05 was considered as a statistically significant difference.

RESULTS

The *pgaC* transcript is up-regulated under BCAA limitation. To test the effect of BCAA limitation on biofilm formation, the expression of *pgaC* and *hfq* genes in biofilm-positive (AP 93-9) and biofilm-negative (AP 100) wild-type *A. pleuropneumoniae* strains was compared in BCAA-replete and BCAA-limited media. There was no detectable difference in the expression of the *hfq* gene, by qPCR, in either biofilm-positive or biofilm-negative strains due to BCAA limitation. The *pgaC* transcript in the biofilm-negative strain was ~eight-fold up-regulated under BCAA limitation, indicating that the expression of the *pgaC* gene is modulated by BCAA availability in *A. pleuropneumoniae* AP100. In contrast, in the biofilm-positive strain, the expression of the *pgaC* gene was not altered due to the availability of BCAAs in the growth media. However, expression of *pgaC* during growth in complete CDM was ~six-fold higher in the biofilm-positive strain than in the biofilm-negative strain. It is not clear why there is a higher basal level of expression of *pgaC* in the biofilm-positive strain, but this may have masked any minor difference in the expression of *pgaC* in response to BCAA limitation. The role of Hfq in biofilm formation in *A. pleuropneumoniae* was analyzed in the biofilm-positive strain.

Construction of an *hfq* mutant. The predicted *A. pleuropneumoniae* Hfq protein has high homology to Hfq proteins from several bacteria, including *E. coli,* and contains the Sm1 and Sm2 motifs which are conserved among bacterial Hfqs (41). A BLASTP search revealed that the *A. pleuropneumoniae* Hfq protein has 88% identity to *E. coli* Hfq. To analyze the function of Hfq in *A. pleuropneumoniae*, an *hfq* mutant strain was constructed. A deletion-disruption mutation at the *hfq* locus was introduced by allelic exchange using natural transformation with a linearized *hfq* mutation plasmid construct (Fig. 3-1 A and B). *A. pleuropneumoniae* was transformed with the *hfq* mutation construct and chloramphenicol-resistant transformants were

selected. Transformants were verified as *A. pleuropneumoniae* by PCR using oligonucleotide primers specific for the *omlA* gene (data not shown) (22). The transformants were further analyzed by PCR to verify the presence, location, size, and orientation of the insert in the chromosome (Fig. 3-1C). The full length *hfq* gene, including the promoter region, was cloned to generate a complemented mutant strain. PCR with primers to amplify the full length *hfq* gene revealed 279 bp amplicons from the wild-type and the complementation plasmid templates, while 1300 bp amplicons were amplified from the *hfq* mutant and the knock-out construct templates (Fig. 3-1C), indicating that the *cat* cassette and the *nptI* promoter were inserted by a double cross-over event at the chromosomal *hfq* locus.

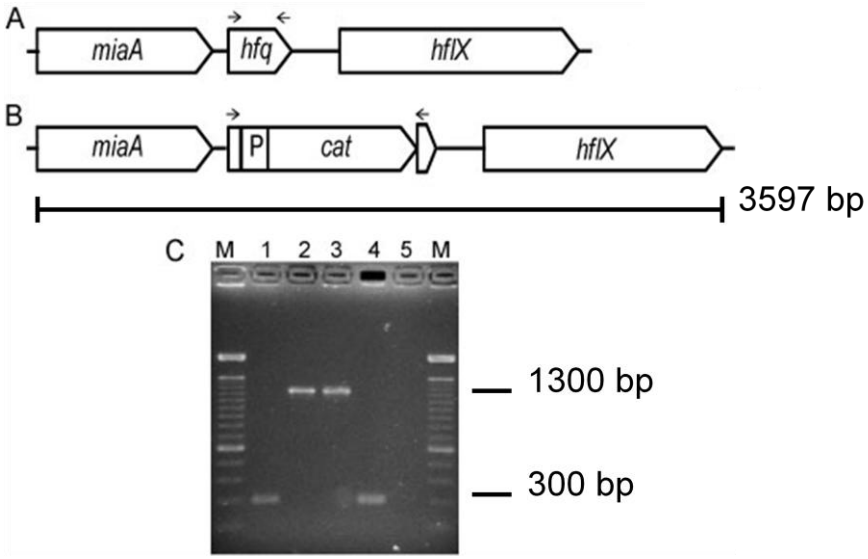

Figure. 3-1. Construction of an *A. pleuropneumoniae hfq* mutant strain. A- Schematic representation of the *hfq* locus in the wild-type strain. B- Schematic representation of the *hfq* locus in the *hfq* mutant strain. C. PCR verification of the *hfq* mutant. Primers MM 728 and MM 729 binding to the *hfq* start and stop codon regions, respectively, indicated as arrows in panels A and B, were used with the following templates: wild-type (lane 1), Δ*hfq* (lane 2), knockout plasmid (lane 3), complementation plasmid (lane 4) and no template control (lane 5). M = molecular size marker.

Effect of Hfq on growth. Growth patterns of the wild-type, *hfq* mutant and complemented mutant strains were determined in BHIV, a rich medium used for cultivating *A. pleuropneumoniae* (Fig. 3-2). The *hfq* mutant exhibited a reduced growth rate, in the log phase compared to the wild-type and complemented mutant but all the strains used in this experiment reached similar optical density values in the stationary phase.

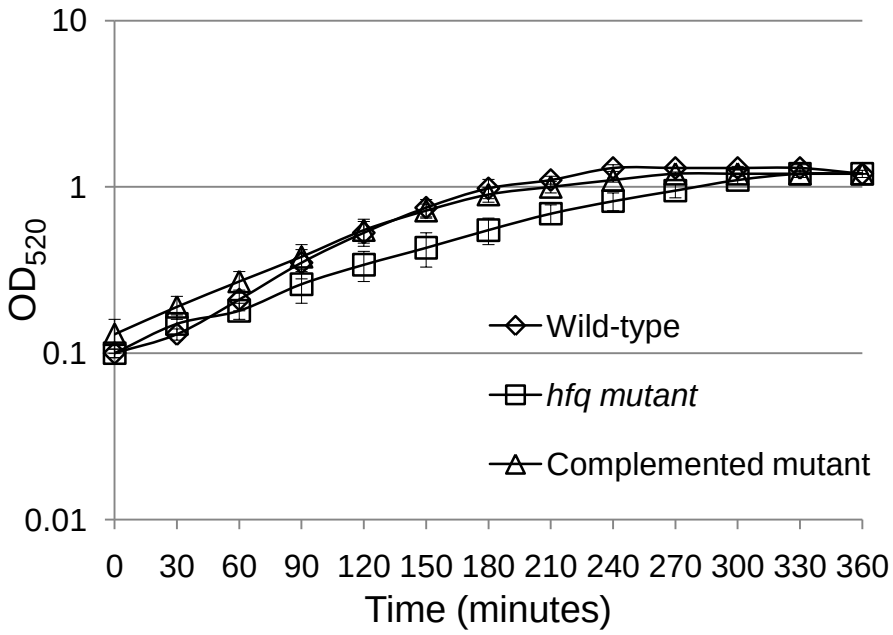

Figure. 3-2. Effect of mutation of *hfq* on growth. Results presented here are mean and standard deviation (error bars) from four independent experiments.

Provision of *hfq* on a low copy number plasmid reversed the growth defect, showing that the reduced growth rate observed is due to loss of Hfq and not due to potential polar effects of the mutation (Fig. 3-2).

Hfq is required for biofilm formation. Changes in *A. pleuropneumoniae* biofilm biomass over time were measured to understand the kinetics of biofilm formation and determine a suitable time point to conduct further studies on biofilm formation. An *A. pleuropneumoniae*

serotype 1 field isolate (AP 93-9) was grown under static conditions in six well plates. Kinetics of growth of AP 93-9 was similar in both BHIV and CDM media under the culture conditions used in this experiment, although the maximum growth as determined by turbidity was lower in CDM (Fig. 3-3A). Biofilm formed at the bottom of the wells was indirectly quantified using a crystal violet binding assay at 0, 3, 6, 9, 12 and 24 hours of incubation (Fig. 3-3B) (37). Biofilm biomass peaked at 6 hours of growth and remained high until 12 hours in BHIV (Fig. 3-3B). In CDM, maximum biofilm biomass was reached at 3 hours and was retained up to 12 hours.

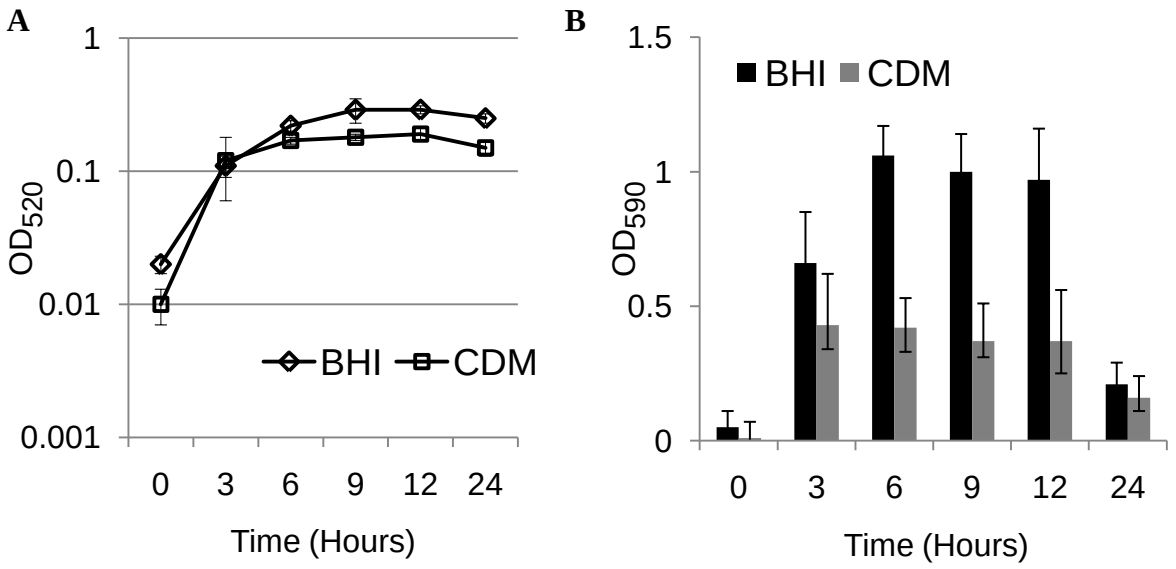

Figure. 3-3. Growth and biofilm formation by wild-type *A. pleuropneumoniae*. A. Growth of AP 93-9 in six well plates. B. Biofilm formed on polystyrene was measured using a crystal violet.binding assay. Mean and standard deviation (error bars) of data from four independent experiments are shown.

After 24 hours of growth, the amount of biofilm was lower than biofilm at 12 hours but remained higher than the baseline value in both BHIV and CDM. Over all, higher biofilm biomass was observed in BHIV compared to CDM (Fig. 3-3B). Similar results were obtained when this experiment was repeated in 96 well plates.

Biofilm formation by the wild-type, *hfq* mutant, and complemented mutant strains grown in 96 well polystyrene plates in BHIV was assessed by a crystal violet binding assay. The wild-type and complemented mutant strains produced 54-fold and 49-fold more biofilm, respectively, than the *hfq* mutant strain (Fig. 3-4), clearly indicating that Hfq is required for biofilm formation in *A. pleuropneumoniae*. Similar results were observed when this experiment was repeated in CDM.

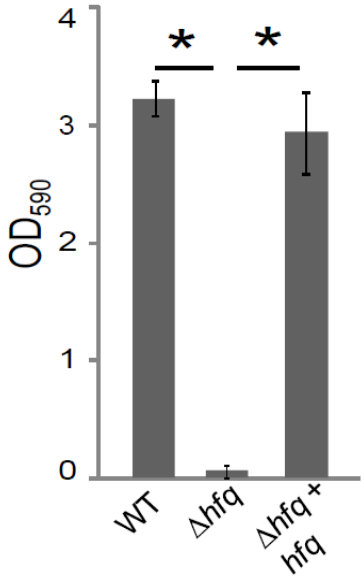

Figure. 3-4. Effect of Hfq on biofilm formation in *A. pleuropneumoniae*. Biofilm produced by *A. pleuropneumoniae* strains, after six hours of growth in BHIV, was stained with crystal violet and absorbance at 590 nm was measured. The data presented here are the mean and standard deviation (error bars) from four independent experiments. **P*<0.01.

Hfq regulates the levels of *pgaC* and *dspB* mRNA. PgaC is an inner membrane-associated glycosyl transferase which synthesizes the biofilm matrix polysaccharide, PGA (28). DspB is a hexosaminidase which degrades PGA and promotes the dispersal of cells from a PGA-based biofilm (28). The levels of *pgaC* and *dspB* transcripts in the wild-type biofilm, wild-type supernatant and the *hfq* mutant were measured to test if there are Hfq-dependent changes in the

level of these transcripts. Quantitative PCR revealed a 14-fold reduction in the level of *pgaC* mRNA and a 6-fold reduction in the level of *dspB* mRNA in the *hfq* mutant compared to the wild-type biofilm (Table 3-3). A 7-fold reduction in the level of *pgaC* mRNA and 2-fold reduction in the level of *dspB* mRNA in the *hfq* mutant compared to the wild-type planktonic cells was observed (Table. 3-3). There was ~2-fold difference in the level of *pgaC* and *dspB* transcripts between the wild-type biofilm and the wild-type planktonic cells. These results demonstrate that expression of both *pgaC* and *dspB* was reduced in the *hfq* mutant compared to wild-type, and that these differences were due to the effects of Hfq and not to differences in biofilm versus planktonic growth (Table 3-3).

Table. 3-3. Q-PCR analysis of *pgaC* and *dspB* transcripts

Comparison	***pgaC***[a]	***dspB***[a]
hfq mutant/WT Biofilm	-14.8 ± 2.6	-6.6 ± 2.9
hfq mutant/WT Planktonic	-7.8 ± 3.1	-2 ± 0.7
WT Planktonic/WT Biofilm	-1.7 ± 0.3	-2.4 ± 0.7

[a]Fold change in transcript levels were calculated using the 16S rRNA gene as an endogenous control for normalization. Down-regulation is indicated by negative sign. Samples were analyzed in triplicate. Data presented are mean and standard deviation from two independent experiments.

Hfq is required for PGA production in *A. pleuropneumoniae*. To test whether the Hfq-mediated difference in the *pgaC* transcript level was reflected in the amount of cell-associated PGA content, crude polysaccharides from the wild-type, *hfq* mutant and complemented mutant cells were extracted and the PGA content was determined using a Congo red binding assay (50). The Congo red binding assay was validated by comparing crude polysaccharide extracts from cells grown in the absence or presence of dispersin B. Crude polysaccharide extracts from dispersin B-treated cells revealed very low levels of Congo red

binding compared to untreated cells, indicating that Congo red primarily bound to PGA under these conditions (Fig. 3-5). The wild-type and complemented mutant strains produced ~10-fold more PGA than the *hfq* mutant (Fig. 3-5). PGA content of biofilm and planktonic cells from the wild-type and complemented mutant strains were similar (Fig. 3-5). Data presented here demonstrates that Hfq is required for the production of normal levels of PGA in *A. pleuropneumoniae*.

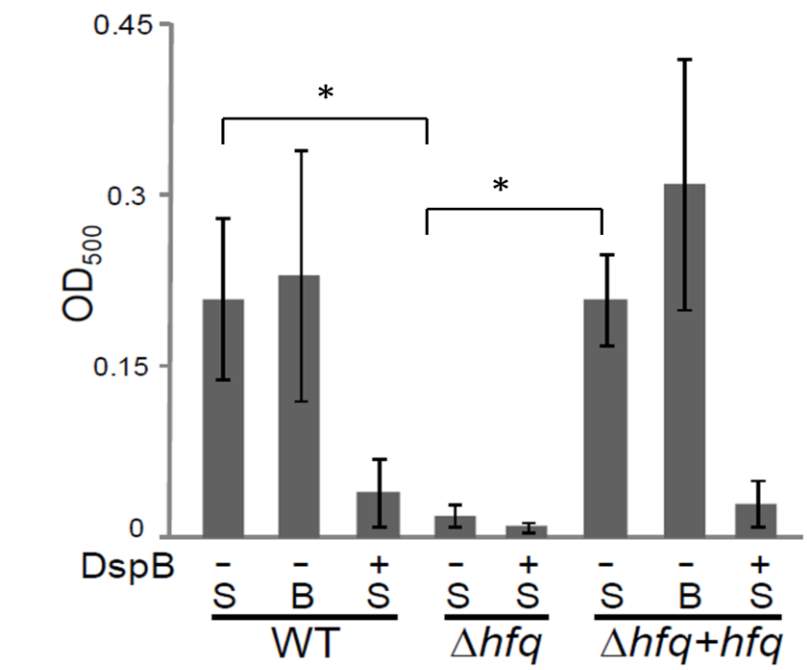

Figure. 3-5. Determination of PGA production in *A. pleuropneumoniae.* PGA content in the crude polysaccharide extracts were quantified using a Congo red binding assay. The data presented here are the mean and standard deviation (error bars) from three independent experiments. * $P<0.01$. DspB = dispersin B, S = supernatant, and B = biofilm.

Hfq is required for the production of cysteine synthase. To identify Hfq-regulated surface proteins which could affect biofilm formation, membrane fractions were isolated from the wild-type and *hfq* mutant strains grown to stationary phase in BHIV. Outer membrane fractions were

analyzed by SDS-PAGE and a 34 kD protein was found only in the wild-type. Based on peptide mass fingerprint obtained from MALDI-TOF mass spectrometry, the differentially expressed protein was identified as a cysteine synthase, CysK (Fig. 3-6). Cysteine synthases are known to be involved in protection against oxidative stress and tellurite in bacteria, and this finding led us to probe the role of Hfq in response to oxidative stress and tellurite resistance in *A. pleuropneumoniae* (2, 16).

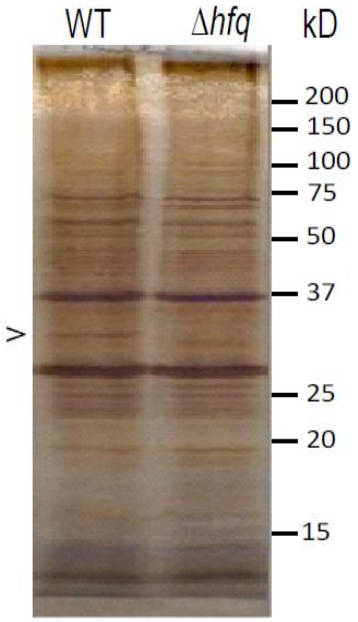

Figure. 3-6. SDS-PAGE analyses of outer membrane proteins. A 34 kD-protein (arrow head) was found only in the outer membrane fraction from wild-type and was identified as a cysteine synthase, CysK, by mass spectrometry. Locations of molecular mass markers are indicated. The experiment was repeated twice and an image of a representative gel is shown here. WT = wild-type, Δ*hfq* = *hfq* mutant. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Hfq is involved in protection against superoxide stress and tellurite. Since the *hfq* mutant did not produce CysK at wild-type levels, we hypothesized that an *A. pleuropneumoniae hfq* mutant would be hypersensitive to oxidative stress compared to the wild-type strain. *A.*

pleuropneumoniae is exposed to several forms of oxidative stress induced by organic hydroperoxides, hydrogen peroxide and superoxide anion during infection of the pig lungs (42). The following agents, which replicate various types of oxidative stress encountered by this pathogen in porcine lungs, were tested in a top agar growth inhibition assay system: cumene hydroperoxide, an organic hydroperoxide; hydrogen peroxide; methyl viologen and potassium tellurite. Both methyl viologen and potassium tellurite are known to generate superoxide radicals within bacterial cells (2, 9). Although complete details of toxicity exerted by tellurite are not fully understood, tellurite is known to induce superoxide stress (9) (see Discussion). The *hfq* mutant displayed increased sensitivity to methyl viologen and potassium tellurite compared to wild-type. This phenotype could be readily reversed by providing *hfq* in *trans* (Fig. 3-7). The wild-type and *hfq* mutant strains displayed similar sensitivity to cumene hydroperoxide and hydrogen peroxide (data not shown). These results indicate that Hfq plays a role in protection against superoxide stress but not against organic hydroperoxide (cumene hydroperoxide) or hydrogen peroxide stress in *A. pleuropneumoniae.*

Cells in a bacterial biofilm are exposed to conditions such as high cell density, nutrient deprivation and accumulation of waste products. Therefore, cells in a biofilm can respond differently to various stressors, including oxidative stress, compared to their planktonic counterparts (44). Since the *hfq* mutant was defective in biofilm formation, we tested whether the role of Hfq in superoxide stress resistance is independent of its role in PGA-based biofilm

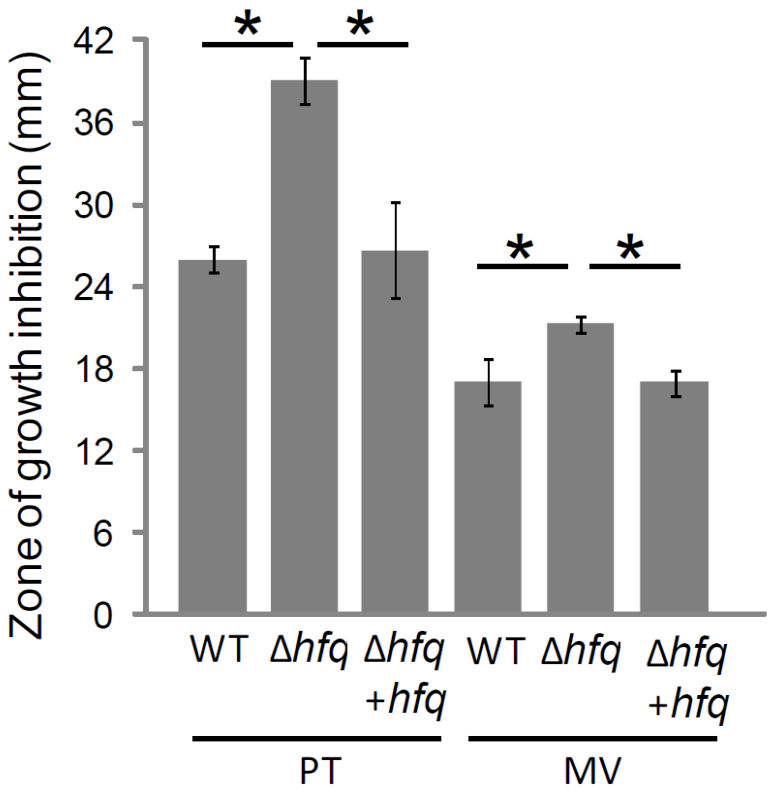

Figure. 3.7. Role of Hfq in resistance to oxidative stress. Sensitivity of *A. pleuropneumoniae* strains to potassium tellurite (PT) and methyl viologen (MV) were determined in a growth inhibition assay. Mean and standard deviation (error bars) of zones of growth inhibition from three independent experiments are presented here. WT = wild-type, Δ*hfq* = *hfq* mutant and Δ*hfq*+ *hfq* = complemented mutant. **P*<0.05.

formation. All strains were grown in the presence of dispersin B to eliminate PGA-based biofilm and top agar growth inhibition assays were performed. The *hfq* mutant remained hypersensitive to methyl viologen and potassium tellurite compared to the wild-type and complemented mutant strains, indicating that the effect of Hfq on superoxide resistance in *A. pleuropneumoniae* is independent of its role in the regulation of PGA biosynthesis (Fig. 3-8).

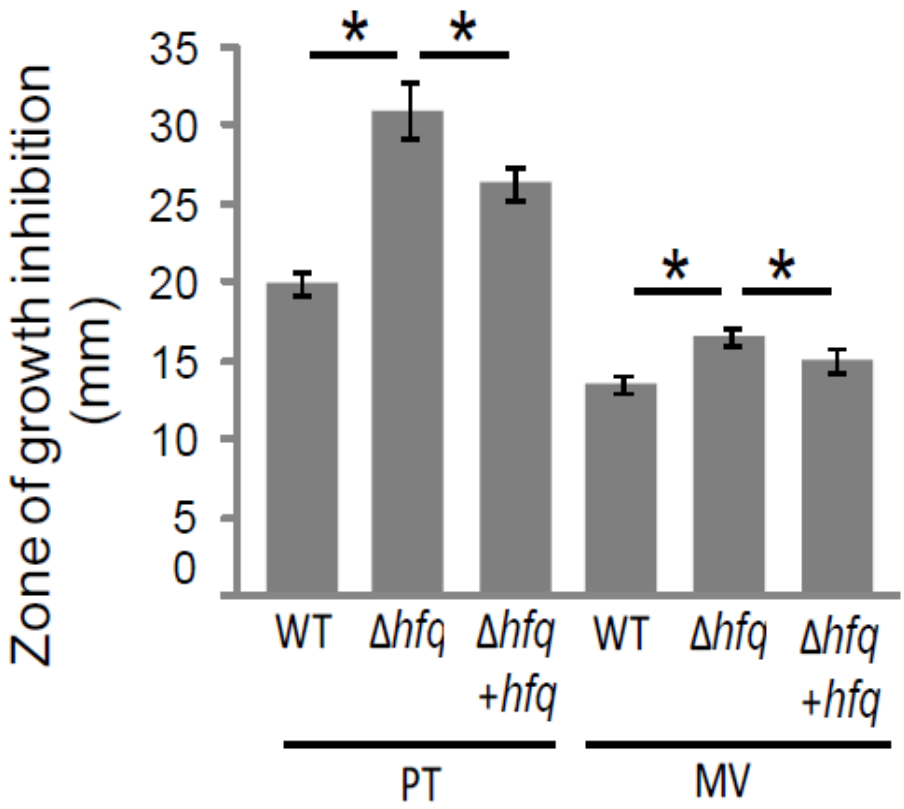

Figure. 3-8. Effect of Hfq on resistence to superoxide stress and tellurite in the presence of dispersin B. *A. pleuropneumoniae* strains grown in the presence of dispersin B were used to determine sensitivity to potassium tellurite (PT) and methyl viologen (MV) in a growth inhibition assay. Mean and standard deviation (error bars) of zones of growth inhibition from four independent experiments are presented here. WT = wild-type, Δ*hfq* = *hfq* mutant and Δ*hfq*+ *hfq* = complemented mutant. **P*<0.05.

DISCUSSION

Previous work on *A. pleuropneumoniae* in our laboratory led to the identification of *hfq* as an in vivo-induced gene that is up-regulated in vitro during growth in CDM lacking BCAAs (51). Genes in the *pgaABCD* operon were also found to be up-regulated under BCAA limitation (R. M. LeVeque and M. H. Mulks, unpublished data). Since *hfq* is an in vivo-induced gene in *A. pleuropneumoniae* and Hfq regulates biofilm formation in another bacterium, *V. cholerae*, we investigated the potential role of Hfq in the regulation of biofilm formation in *A. pleuropneumoniae*.

First, the effect of BCAA limitation on the expression of *pgaC* and *hfq* genes in *A. pleuropneumoniae* was studied. The *pgaC* gene was up-regulated under BCAA limitation in ATCC 27088, the type strain for *A. pleuropneumoniae,* a strain that does not form biofilm under the conditions we have tested. However, there was no difference in the expression of the *pgaC* gene under BCAA limitation in a biofilm-positive field isolate (AP 93-9). Furthermore, our data shows that expression of the *pgaC* gene in the biofilm-positive strain was higher than the biofilm-negative strain when both strains were grown in complete CDM. It is possible that a gene or multiple genes encoding either structural components or regulatory proteins involved in biofilm formation might be missing or altered in the *A. pleuropneumoniae* type strain, ATCC 27088, resulting in lower levels of the *pgaC* transcript compared to field isolate AP 93-9. Field isolates of *A. pleuropneumoniae* readily form biofilm but this phenotype is lost upon subculture in broth (26). The molecular mechanism(s) involved in the loss of biofilm-forming ability during culture in laboratory media remains to be determined.

Previously, BCAA limitation was shown to up-regulate the expression of *hfq* in the biofilm-negative strain (51). However, expression of *hfq* was not induced in either the biofilm-positive or biofilm-negative strains under BCAA limitation in the current study. This difference might be reconciled by differences in the assay conditions used in these two studies. In the current experiment, cells were grown in CDM ± BCAA for 1 hour before harvesting for RNA extraction. In the previous study described by Wagner and Mulks, expression of the luciferase reporter gene fusion to the *hfq* promoter was measured in bacteria grown overnight on CDM ± BCAA plates (51).

Homologues of *hfq* are found in all members of the *Pasteurellaceae* family whose genomes have been sequenced, but their function has not been demonstrated. To investigate the role of a predicted *hfq* homologue in *A. pleuropneumoniae,* we constructed an *hfq* mutant strain. Loss of Hfq has been shown to cause variable effects on the growth of bacteria in laboratory media, ranging from extended doubling time to no effect (8). We observed a slightly reduced growth rate in the *A. pleuropneumoniae hfq* mutant strain.

Potential regulators of biofilm formation have been identified in *A. pleuropneumoniae.* Buettner et al. reported that an *A. pleuropneumoniae arcA* mutant strain was defective in auto-aggregation and biofilm formation on glass surfaces (7), suggesting that ArcA positively regulates biofilm formation. ArcAB is a two-component global regulatory system involved in the adaptation to growth under anaerobic conditions, and is required for full virulence of *A. pleuropneumoniae* and for persistence in chronic infection (7). A *luxS* mutant strain of *A. pleuropneumoniae* was shown to produce more biofilm than the parent strain (34). LuxS is the biosynthetic enzyme for autoinducer-2, a bacterial interspecies quorum sensing signaling molecule (40). This result suggests that biofilm formation is down-regulated under high cell

density conditions in *A. pleuropneumoniae*. The effect of different growth conditions on biofilm formation by *A. pleuropneumoniae* was examined and low levels of zinc in the media were shown to promote biofilm formation (31). This finding is intriguing because zinc limitation is a condition encountered by bacterial pathogens within mammalian hosts and could be a host signal for inducing biofilm formation. While these studies identified potential regulators and environmental conditions that affect biofilm formation, they did not identify molecular mechanisms for these effects.

In *A. pleuropneumoniae* serotype 1 strain 4074 (ATCC 27088), sigma E (σ^E - extracytoplasmic stress response sigma factor) and histone-like nucleoid structuring protein (H-NS) regulate biofilm formation at the transcriptional level by modulating the expression of the *pgaABCD* operon (5). A mutation in *rseA*, which encodes an anti-sigma factor which normally sequesters σ^E in the inner membrane, led to increased activity of σ^E and induced biofilm formation in a strain that did not normally form biofilm. A mutation at the *hns* locus also led to the induction of biofilm formation in an otherwise non-biofilm forming strain. σ^E and H-NS act as positive and negative transcriptional regulators of the *pgaABCD* operon expression, respectively (5). Here, we report the identification of Hfq as yet another regulator of PGA-based biofilm formation in *A. pleuropneumoniae*.

E. coli and *Salmonella enterica* serovar Typhimurium *hfq* mutants reveal an extracytoplasmic stress phenotype including the activation of the σ^E regulon, due to dysregulation of membrane protein expression (14, 23). Conversely, we have found that in an *A. pleuropneumoniae hfq* mutant the expression of the *pgaABCD* operon and biofilm formation,

which are induced by σ^E, are down-regulated. These observations suggest that the extracytoplasmic stress response might not be activated in an *A. pleuropneumoniae hfq* mutant. Comparative analyses of the transcriptome and proteome of the *hfq* mutant, the σ^E mutant and the wild-type strain might shed light on the extent of involvement of the extracytoplasmic stress response in the phenotypes displayed by an *A. pleuropneumoniae hfq* mutant strain. Hfq could regulate the expression of the *pgaABCD* operon at post-transcriptional level either directly by binding to stabilize the transcript with or without sRNA partners or indirectly by modulating the activity of other negative regulators, including H-NS.

Homologues encoding the enzymes involved in the production of PGA are found in the genomes of *Acinetobacter baumannii, A. pleuropneumoniae , A. actinomycetamcomitans, B. pertusis, B. bronchiseptica, E. coli* O157:H7, uropathogenic *E. coli* CFT073, *S. aureus, S. epidermidis, Yersinia enterocolitica,* and *Y. pestis* (10, 52). Studies on the role of Hfq in PGA-based biofilms formed by these pathogens will expand our knowledge on the regulatory molecules governing the development of medically-important biofilms and inhibitors of such regulators can be developed for controlling biofilm infections.

Bacterial pathogens such as *A. pleuropneumoniae* encounter oxidative stress due to oxygen radicals generated by phagocytic cells during infection. Hfq has been implicated in resistance to oxidative stress in bacteria. For example, in a *Brucella abortus hfq* mutant there is reduced expression of the *sodC* gene, which encodes a periplasmic Cu-Zn superoxide dismutase (19). *Hfq* mutants of *Burkholderia cepacia, Moraxella catarrhalis, Neisseria meningitidis, Sinorhizobium meliloti* and uropathogenic *E. coli* have enhanced sensitivity to methyl viologen, a redox-cycling agent which generates superoxide within bacterial cells (1, 2, 13, 30, 43).

However, *hfq* mutants of *S. aureus* and *V. parahaemolyticus* exhibit higher resistance to oxidative stress induced by hydrogen peroxide and superoxide compared to the parent strain (35, 45). These observations show that Hfq is involved in the regulation of resistance to oxidative stress in bacteria.

Our effort to identify Hfq-regulated membrane proteins that might be involved in biofilm formation led us to the finding that the cysteine synthase (CysK) protein is found only in the wild-type compared to the *hfq* mutant. Cysteine synthases are known to be involved in resistance to oxidative stress and tellurite in bacteria (2, 16). Cysteine is a precursor for glutathione, whose primary role is to maintain the redox status of the bacterial cytoplasm. Furthermore, cysteine residues are critical for the repair of iron-sulfur containing enzymes that are damaged by oxidative stress. *A. pleuropneumoniae* is exposed to oxidative stress induced by organic hydroperoxides, hydrogen peroxide and superoxide anion during infection of porcine lungs. Several genes involved in oxidative stress resistance, including *ohr*, encoding an organic hydroperoxide reductase; *tehB*, encoding a putative S-adenosyl methyltransferase involved in resistance to tellurite; and *sodC*, encoding a periplasmic copper-zinc superoxide dismutase, were identified as in vivo-induced genes in *A. pleuropneumoniae* (29).

We have shown that Hfq is required for optimal resistance against oxidative stress induced by methyl viologen in *A. pleuropneumoniae.* Sequenced *A. pleuropneumoniae* genomes encode two superoxide dismutases, SodA and SodC . Hfq may alter the expression of these key enzymes involved in detoxifying superoxide. In *E. coli*, Hfq is known to regulate the expression of SodB at the post-transcriptional level, but *A. pleuropneumoniae* lacks *sodB* (48). Enhanced sensitivity to oxidative stress might be, at least in part, responsible for the virulence defect of *hfq* mutants in a number of bacterial pathogens (8).

While genes identified as conferring resistance to tellurite have been found in many bacterial pathogens, tellurite is not found in significant amounts within mammalian hosts (9). Therefore, the true role of these "tellurite-resistance" genes in mammalian pathogens is not understood. The mechanisms involved in the toxicity of tellurite are also not fully understood, but tellurite is known to trigger production of superoxide (9). The *Haemophilus influenzae tehB* gene is essential for resistance against tellurite and oxidative stress and for virulence in a mouse model of invasive disease (54). *TehB* was also identified as an in vivo-induced gene in *A. pleuropneumoniae* (29). Together, these studies show that tellurite resistance genes might not only play a role resistance against tellurite but might also be involved in oxidative stress resistance and virulence of bacterial pathogens. Studies have shown that Hfq is required for resistance against superoxide stress in bacteria (8) but the role of Hfq in resistance to tellurite is not known. Cysteine synthases are known to be involved in bacterial resistance against tellurite, although exact role of these enzymes in the protection against tellurite toxicity is not clear (16). One possible mechanism could be due to the requirement of cysteine for production of molecules involved in protection against oxidative stress, including glutathione. Expression of the *cysK* gene cloned from *Bacillus stearothermophilus* in *E. coli* confers ~ten-fold higher resistance, indicated by changes in minimum inhibitory concentration, against tellurite (47). Furthermore, the *cysK* gene is up-regulated when grown in media containing tellurite suggesting that cysteine biosynthesis is important for alleviating the toxic effects of tellurite (16). Since an *A. pleuropneumoniae hfq* mutant produced low levels CysK, the effect of Hfq on tellurite resistance was tested and we found that Hfq is involved in resistance to tellurite in *A. pleuropneumoniae.*

In summary, evidence for the biological functions of an in vivo-induced gene, *hfq*, in *A. pleuropneumoniae* is presented here. This is the first report on the role of Hfq in any member of

the *Pasteurellaceae* family. We found that Hfq regulates two virulence-associated phenotypes, PGA-based biofilm formation and resistance against oxidative stress and tellurite in *A. pleuropneumoniae* (26, 29). Evaluation of the virulence of an *A. pleuropneumoniae hfq* mutant in a porcine pleuropneumonia model will shed light on the role of Hfq in the virulence of this important pathogen.

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CHAPTER IV

THE IN VIVO-INDUCED GENE HFQ IS ESSENTIAL FOR THE SURVIVAL AND VIRULENCE OF ACTINOBACILLUS PLEUROPNEUMONIAE IN SWINE

ABSTRACT

Porcine pleuropneumonia, caused by *Actinobacillus pleuropneumoniae*, imposes a significant economic burden on swine production. The predicted *A. pleuropneumoniae hfq* homologue, encoding the RNA chaperone and post-transcriptional regulator Hfq, was previously identified as an in vivo-induced gene and was shown to regulate biofilm formation in *A. pleuropneumoniae*. Furthermore, an *hfq* mutant was hypersensitive to superoxide stress and tellurite. Here, we assessed the fitness of the *hfq* mutant in the porcine respiratory tract. Pigs infected with the wild-type strain displayed classic pleuropneumonia lesions in the lungs whereas the pigs infected with the *hfq* mutant did not reveal tissue damage in the lungs. During mixed-infection, the *hfq* mutant was out-competed by the wild-type strain, with competitive indices ranging from 10^{-2} to 10^{-9}, indicating that Hfq is required for survival and virulence in pigs. Our data demonstrates that the in vivo-induced gene *hfq* is essential for the survival and virulence of *A. pleuropneumoniae* in pigs and is a potential candidate for the development of a live attenuated vaccine.

INTRODUCTION

Porcine pleuropneumonia is a disease of significant economic importance for swine producers around the world. Porcine pleuropneumonia is caused by *A. pleuropneumoniae,* an encapsulated Gram-negative pleiomorphic coccobacillus in the family *Pasteurellaceae* (3). Pigs infected with *A. pleuropneumoniae* develop a fibrino-hemorrhagic bronchopneumonia (8). Due to better management practices, such as the all-in all-out system, and improved vaccines, the economic loss imposed by porcine pleuropneumonia has declined during the last decade in industrialized countries (18). However, porcine pleuropneumonia remains an important disease in the developing countries such as China (35), and still causes significant morbidity of swine in the US, Canada and Europe.

Several virulence factors produced by *A. pleuropneumoniae*—including the pore-forming Apx toxins, capsular polysaccharides and lipopolysaccharides—provide protection from phagocytes and induce damage in the lung tissue (3). This disease is characterized by sudden death with blood-tinged nasal discharge in peracute cases. Pigs developing an acute infection exhibit severe respiratory distress and often die within twenty-four hours after the onset of clinical signs. Typical gross pathologic lesions associated with acute *A. pleuropneumoniae* infection are edema, hemorrhage and necrosis of lung lobes in addition to fibrinous pleuritis and engorgement of tracheobronchial lymph nodes (3, 18). Lesions evident upon histopathological examination include necrosis, hemorrhage, influx of neutrophils and macrophages, widespread edema with extremely diminished air spaces and presence of coccobacilli (18).

To identify genes involved in the virulence of *A. pleuropneumoniae,* an in vivo expression technology study was performed previously in our laboratory (15). The promoter

region of the *hfq* gene, which encodes host factor Q-β (Hfq), was identified to be induced during the infection of pig lungs. Hfq was originally identified because of its role in the replication of RNA bacteriophage Q-β in *Escherichia coli* (13).

Studies performed in the last two decades have revealed that Hfq acts as an RNA chaperone and pleiotropic post-transcriptional regulator in bacteria. Furthermore, Hfq plays an important role in the small regulatory RNA-mediated post-transcriptional regulation of gene expression (5). Due to dysregulation of the expression of multiple genes in the absence of Hfq, the *hfq* mutants of several bacterial pathogens have been found to be attenuated in murine infection models (6). However, *hfq* mutants of pathogens which could cause severe pneumonia, such as *Pseudomonas aeruginosa* and *Yersinia pestis*, have not been tested for virulence in the respiratory tract (17, 30). In this paper, the ability of an *A. pleuropneumoniae hfq* mutant to survive and cause disease in the lungs was tested using a pleuropneumonia infection model in the natural swine host. To our knowledge, this is the first detailed report of the role of Hfq in the virulence of a respiratory tract pathogen in the lungs.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this work are listed in table 4-1.

Table. 4-1. Strains used in this study

Strains	Description	Source or reference
AP 93-9	*A. pleuropneumoniae* serotype 1 field isolate (wild-type)	12
AP 371	Double cross over mutant at the *hfq* locus in AP 93-9 (Δ*hfq*), Cm^r	Chapter 3
AP 93-9-1	AP 93-9 re-isolated from pig lungs (wild-type)	This study
AP 371-1	AP 371 re-isolated from pig lungs (Δ*hfq*)	This study

Cm^r – chloramphenicol resistance

For infection experiments, *A. pleuropneumoniae* strains were grown in 25 ml of heart infusion broth (Becton Dickinson and Company, Sparks, MD) supplemented with 10 µg/ml of NAD (V factor) (Sigma, St. Louis, MO), 5 mM calcium chloride, and dispersin B (0.5 µg/ml) (Kane Biotech Inc, Winnipeg, Canada) and optical density (absorbance) at 520 nm (OD_{520}) was measured over time. Dispersin B is a hexosaminidase which degrades poly-N-acetylglucosamine—a polysaccharide adhesin—and was added to broth cultures to prevent autoaggregation. Broth cultures were incubated at 35°C with shaking at 160 rpm. Ten ml of culture per sample was collected when broth cultures reached an OD_{520} of 0.8 and centrifuged to pellet the cells. Cell pellets were washed once in phosphate-buffered saline (PBS) pH 7.4 before diluting in PBS to appropriate colony forming units/ml (CFU/ml) for animal infection.

Viable counts in the inocula were determined by plating the doses used for infection both at the time the doses were prepared and at the same time when the pigs were infected. Serial dilutions were plated on brain heart infusion (BHIV) (Becton Dickinson and Company, Sparks, MD) plates for the wild-type strain and BHIV supplemented with 1.5 µg/ml of chloramphenicol for the *hfq* mutant strain. Plates were incubated at 35°C under 5% CO_2 for 24 hours.

Infection experiments. The protocol and procedures used in pig infection experiments were reviewed and approved by the Michigan State University Institutional Animal Care and Use Committee. Eight-week-old *A. pleuropneumoniae*-free pigs were purchased from the Michigan State University Swine Research Facility. Animal infection experiments were conducted in two phases.

Phase I. The objective of the first phase was to re-isolate wild-type and *hfq* mutant strains from pig lungs. This step was performed to ensure that virulence lost during laboratory culture, if any, could be revived. Pigs, sedated with a telazol, ketamine and xylazine cocktail (100 mg of each compound per ml) at a dosage of 2.5 mg/kg of body weight, were placed in dorsal recumbency on an inclined V-shaped board so that the head was at an elevated position compared to the chest. Pigs were inoculated with bacteria in 10 ml of PBS by percutaneous intratracheal injection using 18 gauge × 3.5" spinal tap needles. One pig was infected with 5×10^6 CFU of the wild-type strain. Two pigs were infected with the *hfq* mutant strain: one pig received 3×10^6 CFU and the second pig received 6×10^7 CFU. Two pigs served as uninfected controls. One pig received 10 ml of PBS intratracheally and the second pig received 2 ml of PBS intranasally (1 ml/nostril).

Pigs were monitored for the development of clinical signs of porcine pleuropneumonia at two hour intervals as previously described (21). Respiratory rate was measured as the number of breaths in a 15 second period. Degree of respiratory distress, dyspnea, was scored on a scale of 0 to 3 with 0 being normal and 3 being extreme dyspnea. Physical inactivity (depression) was scored on a scale of 0 to 3 with 0 being normal and actively maintaining flight distance and 3 being moribund. Rectal temperature was measured, although we have noted that elevated rectal temperatures do not always correspond to severe lung pathology. Appetite, indicated by feeding readily upon provision of feed, was also recorded.

Pigs were euthanized at 17 hours post-infection (HPI) and parts of the lung exhibiting pleuropneumonia-like lesions were collected for culture. Samples were plated on BHIV and BHIV supplemented with 1.5 µg/ml of chloramphenicol. The wild-type and *hfq* mutant strains isolated from the pigs were confirmed as *A. pleuropneumoniae* by Gram's stain, requirement for V factor and PCR with primers (AAGGTTGATATGTCCGCACC and CACCGATTACGCCTTGCCA) for the *A. pleuropneumoniae omlA* gene (20). Co-agglutination was used to verify that the wild-type and *hfq* mutant re-isolated from pigs were serotype 1 strains (25). PCR was performed using primers (CGGATCCATGGCAAAAGGTCAATC and CAAGTCGACTTATTCCGCTTTATCCGC) that bind to the start and stop codon regions of the *hfq* gene to confirm that the strains re-isolated from pig lungs were identical to the strains used for infection. Since Hfq affects biofilm formation, a crystal violet binding assay for biofilm production by the wild-type and *hfq* mutant re-isolated from pigs was conducted and compared to the strains used for infection.

Phase II. In the second phase, wild-type (AP 93-9-1) and *hfq* mutant (AP 371-1) strains re-isolated from pigs were used for competitive index analyses. Fourteen pigs were divided into

four groups with 3-4 pigs per group using a random-stratified sampling method, balancing each group for body weight and sex. Littermates were allotted to different groups. Groups one, two and three were infected intratracheally with inocula in 10 ml PBS. Group one, containing three pigs, was infected with 4×10^6 CFU of the wild-type *A. pleuropneumoniae* strain AP93-9-1. Group two, containing three pigs, received 4×10^6 CFU of the *hfq* mutant strain AP371-1. Group three, with four pigs, received a 1:1 mixture of wild-type and the *hfq* mutant, at a total of 4×10^6 CFU. Group four, containing four pigs, was infected with a 1:1 mixture of wild-type and the *hfq* mutant, at a total of 4×10^6 CFU, in 2 ml of PBS by the intranasal route.

Clinical evaluation was performed as described above at 2 hour intervals for the first 12 hours and at 4 to 6 hour intervals afterwards until the end of the experiment. Pigs were euthanized either at specific times (16, 40 and 64 HPI) or when they reached either a dyspnea score of 3, a physical inactivity score of 3, or a score of 2 in both dyspnea and physical inactivity. Pigs exhibiting a blood-tinged nasal discharge were euthanized immediately. Time of euthanasia was recorded to calculate the duration of infection as hours post-infection (HPI). Tracheotomy was performed on all pigs, except PBS controls, immediately after opening the carcass to collect bronchoalveolar lavage fluid (BALF) by infusing 200 ml of PBS into the lungs. During necropsy, lungs from all pigs were scored for gross lesions. Percent pleuritis and pneumonia were estimated using a formula that accounts the contribution of each lung lobe to the total volume of the lung (21). Representative samples from all six lung lobes from each pig were collected for culture and histopathological examination.

Preparation of samples for histopathology. Samples were fixed in 10% formalin and processed at the Histopathology Laboratory in the Diganostic Center for Population and Animal Health at Michigan State University.

Determination of competitive indices. Lung samples were homogenized in heart infusion broth using a Stomacher-80 (Seward Laboratory Systems, Bohemia, NY). Serial 10-fold dilutions of lung homogenates and BALF were plated on both BHIV and BHIV supplemented with chloramphenicol (1.5 µg/ml). Total number of viable bacteria was determined by counting colonies on BHIV agar plates. The number of *hfq* mutants was enumerated by counting colonies on BHIV with chloramphenicol. The number of wild-type bacteria was calculated by subtracting the number of *hfq* mutants from the total number of viable bacteria. Select colonies were verified as *A. pleuropneumoniae* by assessing NAD-dependence and by PCR with primers for the *omlA* gene. The structure of the *hfq* locus was also verified with PCR using primers which bind to the start and stop codon regions of the *hfq*. Competitive index for each sample was calculated as the ratio of the number of *hfq* mutant bacteria to the number of wild-type bacteria at any time divided by the ratio of the number of *hfq* mutant to the number of wild-type in the inoculum. Analysis of the doses used for competitive index infections showed that the *hfq* mutant and wild-type were present in equal numbers in the inocula. Therefore, the ratio of the *hfq* mutant divided by the wild-type strain is 1. The competitive indices from each of six lung lobes and BALF were used to calculate an average competitive index at a given time for each pig.

In vitro competitive indices were determined by growing the wild-type and *hfq* mutant in a mixed culture in BHIV and CDM. Cultures were serially diluted and plated to enumerate the number of wild-type and *hfq* mutant bacteria. Competitive indices were calculated as described above.

RESULTS

Passage of wild-type and the *hfq* mutant through pigs. The wild-type and *hfq* mutant strains were used for infecting pigs and re-isolated from pig lungs. Growth was observed in plates inoculated with swabs obtained from the wild-type infected pig and the pig which received the higher dose of the *hfq* mutant (6×10^7 CFU). No growth was observed on plates from the pig which received the lower dose of the *hfq* mutant (3×10^6 CFU), or from the no inoculum controls. Both strains were verified as *A. pleuropneumoniae* by PCR and as serotype 1 by co-agglutination. The wild-type strain formed biofilm on polystyrene while the *hfq* mutant was defective in biofilm formation. These strains were used in the competitive index experiments.

Experimental infections to determine the competitive fitness of the *hfq* mutant. Pigs infected with the wild-type and mixed inoculum (wild-type and *hfq* mutant) by the intratracheal route began to manifest increased respiratory rate and developed signs of depression by 15-18 HPI. One pig infected with the wild-type strain had a blood-tinged nasal exudate at 16 HPI and was euthanized immediately. Pigs infected with either wild-type or mixed inocula, intratracheally, developed progressively severe dyspnea (Table 4-2). All of the pigs infected with either wild-type alone or with wild-type and *hfq* mutant developed dypnea and/or depression scores, measured according to the clinical scoring guidelines described in Materials and Methods, requiring euthanasia prior to the planned duration of the experiment (64 HPI). Pigs infected with the *hfq* mutant developed mild, transient depression by 18 HPI. Neither pigs infected intratracheally with the *hfq* mutant alone nor those infected with the wild type:*hfq* mutant mixture by the intranasal route exhibited any sign of respiratory distress throughout the course of the experiment (64 HPI) (Table 4-2).

Table 4-2. Clinical evaluation of pigs

Group	RR[a]	Dyspnea[b]	% Pneumonia[c]	% Pleuritis[d]
wild-type	20 ± 1	2 ± 1	28.1 ± 12.2	13.9 ± 9.9
hfq mutant	14 ± 1.2	0	2 ± 3.2	1.1 ± 1.8
CI IT	19 ± 3.3	1.75 ± 0.6	34 ± 9.5	15.8 ± 13.2
CI IN	12 ± 0.5	0	0	0
Control	11.5 ± 1	0	0	0
Normal	8-10	0	0	0

[a]Average maximum respiratory rate ± standard deviation, breaths per 15 second period, observed after infection. [b]Average maximum dyspnea score ± standard deviation, measured as degree of respiratory distress with 0 = normal, 1 = slight, 2 = moderate, 3 = severe, observed after infection. [c]Average ± standard deviation of percent of lung tissue displaying pleuropneumonia-like lesions. [d]Average ± standard deviation of percent of pleural surface displaying inflammation. CI = competitive index, IT = intratracheal, IN = intranasal. Number of pigs per treatment: wild-type = 3, *hfq* mutant = 3, CI IT = 4, CI IN = 4, PBS control = 2

Gross lesions. Lungs from pigs infected either with wild-type or a mixture of wild-type and *hfq* mutant revealed large areas of necrosis and hemorrhage with edema (Fig. 4-1). Fibrin deposits were found on the pleural surface indicating fibrinous pleuritis. Cut surfaces of the lungs were friable and sequestration of the necrotic areas was evident. The lesions were primarily distributed on the dorso-caudal areas of the right caudal and left caudal lobes. There were no remarkable changes in the appearance of lungs from the *hfq* mutant infected pigs, except for a small pneumonic area found in the left caudal lobe of one pig. Lungs from pigs infected with the wild-type strain revealed pneumonic changes over, on average, 28% of their surface area. In the *hfq* mutant infected group, pneumonic changes covered, on average, 2% of the lung area. Pleuritis, a hallmark lesion of *A. pleuropneumoniae* infection, was found over 13% and 1% of the lung surface in the wild-type and *hfq* mutant infected groups, respectively. These findings clearly indicate that the *hfq* mutant is highly attenuated compared to wild-type strain (Table 4-2).

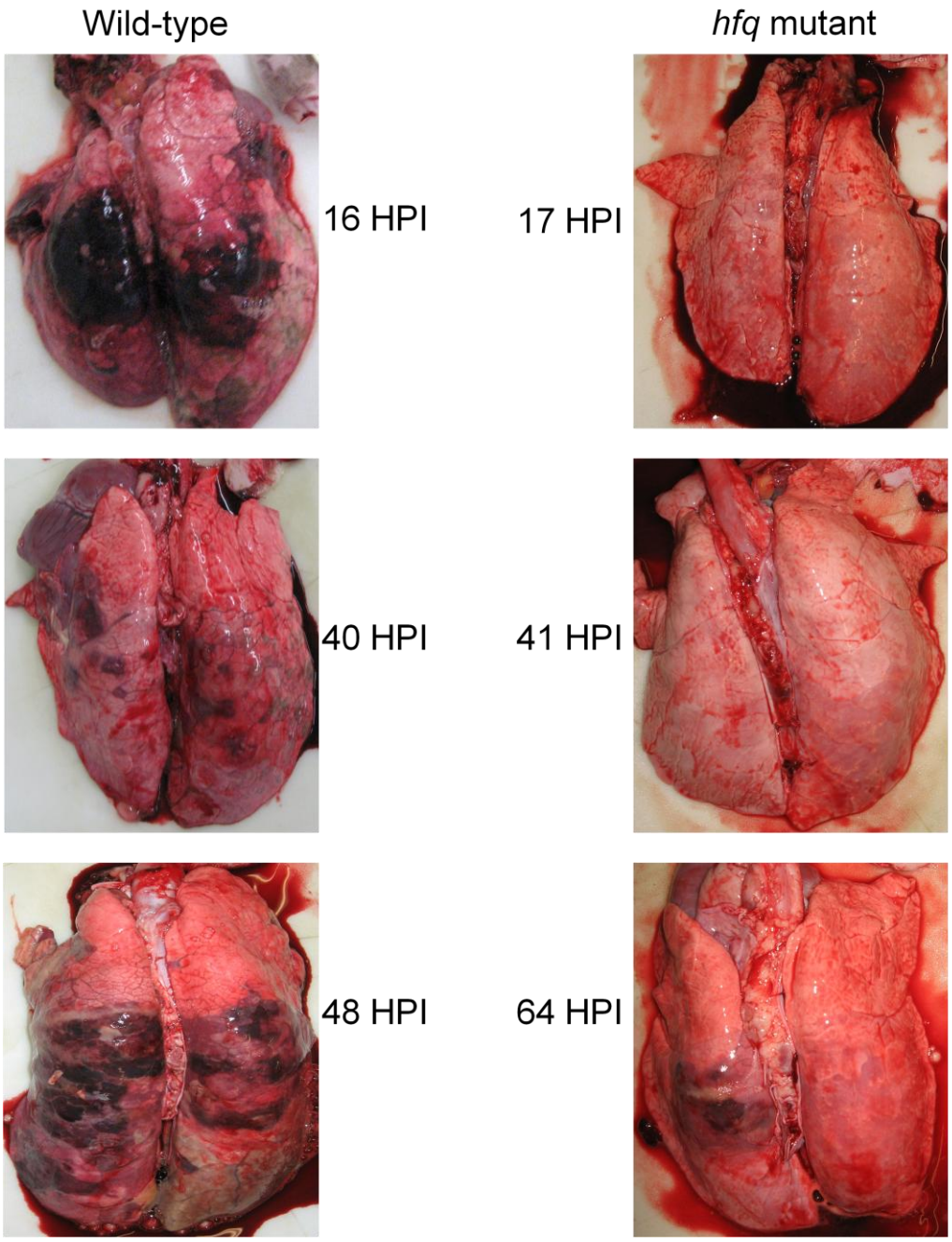

Figure. 4-1. Photographs of lungs from the pigs infected with wild-type and the *hfq* mutant. Infection with the wild-type resulted in the development of fibrino-hemorrhagic bronchopneumonia whereas lungs from *hfq* mutant-infected pigs did not reveal pneumonic changes except for the focal lesion observed in the pig euthanized at 64 HPI. HPI = hours post-infection. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation

The pigs inoculated with PBS and with a mixed inoculum by intranasal route did not reveal lung damage during necropsy.

Microscopic lesions. Formalin-fixed paraffin-embedded lung sections stained with eosin and hematoxylin were used to determine histopathological changes (Fig. 4-2). Lung sections from pigs infected with the wild-type strain revealed extensive areas of diffuse lobular pneumonia, with severe hemorrhage and necrosis sequestered by streaming neutrophils, as well as fibrinous pleuritis. There was accumulation of bacteria, fibrin, blood, and necrotic debris including lysed neutrophils in the affected areas. The alveolar septae were necrotic, with loss of nuclear detail and loss of delineation between air spaces. There was vascular thrombosis, and vascular walls were inflamed. Edema and hemorrhage were evident, resulting in fluid-filled alveoli and bronchi. These lesions were characterized as acute pleuropneumonia upon histopathological examination. In contrast, lung sections from the pigs infected with the *hfq* mutant and the PBS controls showed no evidence of pleuropneumonia. These pigs showed normal lung architecture and normal pleura, with well-defined alveolar septae with occasional neutrophils and alveolar macrophages, with the exception of a small pneumonic focus in the left caudal lobe of one of the *hfq* infected pigs which was histopathologically indistinguishable from the wild-type infected pig lungs.

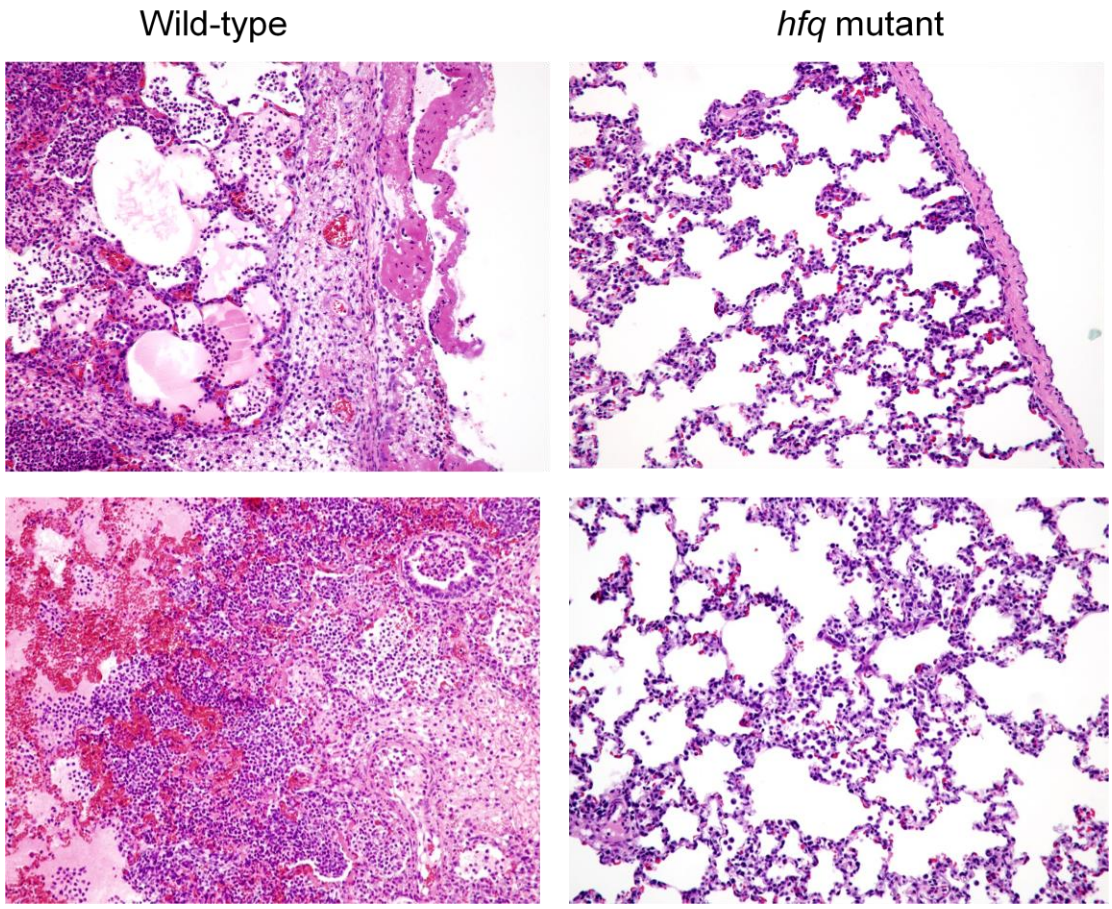

Figure. 4-2. Images of lung sections. Lung sections from the wild-type infected pigs revealed extensive necrosis and hemorrhage with heavy influx of neutrophils. Lung sections from the *hfq* mutant infected pigs did not display significant changes in the histological appearance. Representative images (20X magnification) are shown here.

***An A. pleuropneumoniae hfq* mutant is attenuated in pigs.** Competitive index (CI) is a sensitive technique to compare the fitness of two strains under the same conditions (2). When a mutant and corresponding wild-type parent strain are compared in an animal infection model, a CI value less than 1 indicates that the mutant has reduced fitness in vivo and suggests attenuation of virulence compared to wild-type. In vitro CI calculated during co-culture of the wild-type and *hfq* mutant strain was close to 1, indicating that the *hfq* mutant is not defective in survival in laboratory media. Average competitive indices determined from data for BALF and all six lung

lobes were as follows: 3.7×10^{-5} at 16 HPI, 2.1×10^{-5} and 5.6×10^{-6} at 40 HPI and 5×10^{-3} at 49 HPI (Table 4-3). These data demonstrate that the *hfq* mutant has a severe defect in ability to survive during infection of pigs, compared to wild-type *A. pleuropneumoniae*

The group with four pigs inoculated with a 1:1 mixture of wild-type and the *hfq* mutant, adding to a total of 5×10^{6} CFU by intranasal route did not exhibit any gross lesions at necropsy. Data from these pigs were not used for the determination of competitive indices.

Table. 4-3. Competitive index analysis of the *hfq* mutant in pigs

Pig Number	Time (HPI)	Sample	CI
14	16	BAL	6×10^{-5}
		RCR	2×10^{-5}
		RM	5.5×10^{-6}
		RC	1×10^{-4}
		LC	2.4×10^{-5}
		LCR	1.6×10^{-6}
		ACC	4×10^{-5}
		Average	3.7×10^{-5}
21	40	BAL	2.4×10^{-6}
		RCR	2.9×10^{-6}
		RM	3.3×10^{-5}
		RC	2×10^{-9}
		LC	2×10^{-9}
		LCR	3.3×10^{-9}
		ACC	3.3×10^{-7}
		Average	5.6×10^{-6}
18	40	BAL	5.5×10^{-5}
		RM	1.4×10^{-5}
		RC	1.5×10^{-6}
		LC	1.3×10^{-6}
		LCR	9×10^{-7}
		ACC	5.4×10^{-5}
		Average	2.1×10^{-5}
11	49	BAL	1×10^{-4}
		RCR	3×10^{-3}
		RM	1×10^{-2}
		RC	1.4×10^{-4}
		LC	1×10^{-6}
		LCR	1.6×10^{-3}
		ACC	1.7×10^{-2}
		Average	5×10^{-3}

Table. 4-3. (Cont'd)

HPI = hours post-infection, CI = competitive index, BAL = bronchoalveolar lavage, RCR = right cranial lobe, RM = right medial lobe, RC = right caudal lobe, LC = left caudal lobe, LCR = left cranial lobe, ACC = accessory lobe

DISCUSSION

The *hfq* gene, encoding Hfq, was identified as an in vivo-induced gene in *A. pleuropneumoniae* (22). Further studies revealed that the *hfq* gene is up-regulated during growth in media lacking BCAAs (33). Hfq has been implicated in the virulence of uropathogenic *Escherichia coli, Francisella tularensis, Neisseria meningitidis, Pseudomonas aeruginosa, Salmonella enterica* serovar Typhimurium, *Vibrio cholerae, Yersinia pestis* and *Listeria monocytogenes* in murine infection models (9, 11, 12, 17, 23, 24, 29, 30). Zhou et al. reported that an *hfq* mutant strain of *A. pleuropneumoniae* is attenuated in virulence at a meeting but a detailed report has not been published (36).

The microenvironments at various niches within a mammalian host present a different set of cues to the invading pathogens. We have previously reported that BCAAs are found at limiting concentrations in the respiratory tract of healthy pigs (31). Since the lungs exhibit a unique microenvironment and the role of Hfq in the virulence of a respiratory tract pathogen during lung infection is not known, the survival and virulence of *an A. pleuropneumoniae hfq* mutant in a porcine pleuropneumonia infection model was tested.

Pigs were infected with the wild-type, *hfq* mutant, or a mixture of wild-type and *hfq* mutant strains. Pigs infected with the *hfq* mutant alone failed to develop clinical pleuropneumonia, which was evident in the pigs infected with the wild-type. Pigs infected with a mixture of wild-type and *hfq* mutant by the intratracheal route displayed clinical signs of pleuropneumonia. Determination of bacterial load in the lung tissue and BALF revealed that the wild-type clearly out-competed the *hfq* mutant, indicating that Hfq is essential for the survival of *A. pleuropneumoniae* in the swine lungs.

The average CI in the pig which survived for 49 HPI was 5×10^{-3} and higher than the average CI from pigs euthanized earlier. Extensive damage to the lung tissue over time due to the presence of the wild-type strain in a CI experiment might provide a conducive environment for the growth of the *hfq* mutant and might be responsible for the higher CI observed in the pig euthanized at 49 HPI compared to 16 and 40 HPI. Together, the data from infection with individual strains and mixed infection reveal that the *A. pleuropneumoniae hfq* mutant strain is defective in survival in the pig lungs and is attenuated in virulence.

The virulence defect observed in the *A. pleuropneumoniae hfq* mutant strain could be due to failure to produce PGA and/or cysteine synthase and/or as yet unidentified factors. Polysaccharide intercellular adhesin (PGA produced by *Staphyloccus* species) has been shown as an important virulence factor in *S. aureus* and *S. epidermidis,* where it is required for the production of stable biofilm on catheters and implants (19). In *A. actinomycetamcomitans*, a periodontal pathogen closely related to *A. pleuropneumoniae,* PGA is required for survival within human and mouse macrophages (32). While it has been shown that fresh field isolates of *A. pleuropneumoniae* generally produce PGA-based biofilms, it has not been established that biofilms contribute to virulence in *A. pleuropneumoniae.* It is clear that production of biofilms is not essential for experimental infection models via the intratracheal route, since *A. pleuropneumoniae* ATCC 27088, which is a biofilm-negative strain, is highly virulent (16). PGA was shown to promote colonization of *B. pertussis* in the upper respiratory tract of a rat model of *Bordetella* colonization (10). PGA-based biofilm formation might play a significant role in the persistent colonization of the upper respiratory tract of pigs by *A. pleuropneumoniae*. However, further studies using an aerosol infection model and a *pgaABCD* mutant are required to determine the role of PGA-based biofilms in the colonization of *A. pleuropneumoniae* in pigs.

Cysteine synthase, CysK, catalyzes the production of L-cysteine in bacteria and cysteine is a precursor for glutathione production (14). Glutathione is required for the maintenance of normal redox status in bacterial cells (14). CysK has been shown to be involved in resistance to oxidative stress and tellurite in other bacteria (1, 14). *A. pleuropneumoniae* is exposed to oxidative stress during infection of the porcine lungs (28). Since the *hfq* mutant is defective in resistance to superoxide, it is possible that the impaired virulence of the *hfq* mutant could be due to the failure in the production of CysK. Analysis of a *cysK* mutant strain will be necessary to test the relationship between CysK and superoxide resistance in *A. pleuropneumoniae.* While a tellurite resistance gene, *tehB*, has been identified as an in vivo-induced gene in *A. pleuropneumoniae*, a signal for the induction of this gene within porcine lungs is unclear (22). Tellurite is extremely toxic for many bacteria even at levels as low as one µg/ml (7). Recently, the *tehB* gene in *Haemophilus influenzae* was demonstrated to be required for resistance to oxidative stress and tellurite (34). Whitby et al. demonstrated that *tehB* is required for the virulence of *H. influenzae* in a rat model (34). Together, these observations suggest that the tellurite resistance gene *tehB* might also be involved in resistance to oxidative stress in bacteria.

Infection with live *A. pleuropneumoniae* leads to better cross-protection against infection with heterologous serotypes whereas bacterin preparations offer limited cross protection (26). However, a live attenuated vaccine is not commercially available yet for the prevention of *A. pleuropneumoniae* infections (27). Given the morbidity and mortality caused by *A. pleuropneumoniae*, at least in the developing world, there is a need for better vaccines to protect against this disease. Since the *A. pleuropneumoniae hfq* mutant is defective in survival and virulence during infection the vaccine potential of this mutant warrants further consideration.

In summary, we have previously reported that Hfq regulates PGA-based biofilm formation and is required for oxidative stress resistance in *A. pleuropneumoniae*. Since biofilm formation and oxidative stress resistance have been proposed to be important for the virulence of *A. pleuropneumoniae*, we tested the effect of Hfq on survival and virulence during infection and found that the *hfq* mutant is defective in survival and virulence in a natural host infection model. Data presented in this paper demonstrates that Hfq is critical for the pathogenesis of pneumonia

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CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

SUMMARY

The goal of the work described in this dissertation was to analyze the role of two in vivo-induced genes, *ilvI* and *hfq*, in the pathobiology of *Actinobacillus pleuropneumoniae.* In vivo-induced genes are the genes that are up-regulated during infection. Understanding the roles of the in vivo-induced genes in *A. pleuropneumoniae* not only advances our knowledge of the virulence of this pathogen but also sheds light on gray areas in the role of bacterial genes involved in the pathogenesis of bacterial pneumonia. Furthermore, the use of a natural host infection model of *A. pleuropneumoniae* in pigs provides better insight into the host-pathogen interaction compared to studying pathogens in heterologous host systems. Important contributions of the research described in this dissertation to scientific literature include the identification of a novel host signal in the lungs, demonstration that the ability to synthesize branched-chain amino acids is necessary for the survival of a pulmonary pathogen, and unraveling the role of Hfq in poly-N-acetylglucosamine-based biofilm formation and in vivo fitness in a pleuropneumonia infection model.

Previous studies suggested that the limitation of branched-chain amino acids (BCAA) could be a host signal encountered by *A. pleuropneumoniae* and other pulmonary pathogens in the mammalian lung (20). Evidence for BCAA limitation in the lungs is presented in the second chapter of this dissertation. The actual concentration of free amino acids in the porcine pulmonary epithelial lining fluid was determined and BCAA were found at low concentrations, ~10 to 17% of plasma levels (19). Growth of *A. pleuropneumoniae* in the laboratory in physiological concentrations of BCAA revealed a reduced growth rate; any reduction in growth rate can have a detrimental effect on virulence during infection. Furthermore, mutations in *ilvI*—leading to BCAA auxotrophy—and *lrp,* which encodes a global regulator involved in BCAA

biosynthesis, were found to be attenuating in a pig model of pleuropneumonia, demonstrating that the ability to synthesize BCAAs is critical for the virulence of *A. pleuropneumoniae* in porcine lungs (19).

As BCAA levels were found to be low in porcine pulmonary epithelial lining fluid, the effect of inhibitors of BCAA biosynthesis on the survival of *A. pleuropneumoniae* in media containing and lacking BCAA was tested. Inhibitors of BCAA biosynthesis prevented the growth of *A. pleuropneumoniae* only in media lacking BCAA (19). This finding highlights the potential of inhibitors of BCAA biosynthesis as antibacterial agents against pathogens colonizing sites with limited availability of BCAAs, such as the respiratory tract. BCAA biosynthetic inhibitors belong to a relatively safe group of compounds, the sulfonylureas, that have been used as herbicides for several decades (12). Considering the rapid rise in antibiotic resistance and the small number of compounds in the antibiotic development pipeline, this study has opened up a potential new use for a group of existing compounds.

Although the idea of using inhibitors of BCAA biosynthesis as potential lung-specific antibacterial agents emerged after studies with *A. pleuropneumoniae* in a pig model of pleuropneumonia, the efficacy of these compounds to prevent bacterial growth cannot be tested in this model system. *A. pleuropneumoniae* elaborates pore-forming hemolysins and cytolysins, which damage the host cells to release their contents, such as amino acids and iron (8). Therefore, BCAA limitation is a condition found in the healthy lungs and the milieu at the site of infection changes rapidly due to the action of toxins elaborated by *A. pleuropneumoniae.* A pathogen causing pneumonia without extensive necrosis and hemorrhage, such as *Haemophilus influenzae,* is a suitable candidate for evaluation of the capacity of inhibitors of BCAA biosynthesis to limit the growth of bacteria in animal models of pneumonia.

Studies on the transcriptome of *A. pleuropneumoniae* in response to BCAA limitation revealed that many genes in addition to the genes involved in BCAA biosynthesis are modulated under those conditions. The *pgaABCD* operon, involved in biofilm matrix synthesis, is ~14-fold up-regulated under BCAA limitation but this effect is not modulated by Lrp (R. M. LeVeque and M. H. Mulks, unpublished data). In *A. pleuropneumoniae,* the *hfq* gene, which encodes the RNA chaperone and post-transcriptional regulator Hfq, was identified as one of the in vivo-induced genes which was up-regulated under BCAA limitation (16, 20). In *Vibrio cholerae* and *Escherichia coli*, Hfq has been shown to affect biofilm formation (17, 18). This led us to examine the role of Hfq in *A. pleuropneumoniae* and Hfq was found to regulate biofilm formation and resistance to oxidative stress.

The strain of *A. pleuropneumoniae* used in the transcriptome analyses does not form biofilm under the conditions we have tested. Therefore, further studies on the role of Hfq in the regulation of biofilm formation were conducted in a biofilm-positive strain. Basal level of expression of the *pgaC* gene, encoding an enzyme involved in biofilm matrix production, was higher (six-fold) in the biofilm-positive strain compared to the biofilm-negative strain. Failure of biofilm formation by the laboratory strains might also be due to mutation in other genes (structural or regulatory) which could be essential for biofilm formation in *A. pleuropneumoniae.*

Biofilm-based infections are recalcitrant to antibiotic treatment and are a major concern, especially with catheters and implants, in health care settings (11). Poly-β-1, 6-N-acetylglucosamine (PGA)-based biofilms are formed by pathogens such as *Acinetobacter baumannii, Aggregatibacter actinomycetamcomitans, Bordetella pertusis, Staphylococcus aureus* and *S. epidermidis* (5, 7, 10, 15). *A. pleuropneumoniae* forms a PGA-based biofilm on abiotic surfaces (13). The biofilm-positive phenotype is common among field isolates while

laboratory strains rarely form biofilm (14). However, the presence of *A. pleuropneumoniae* biofilm within the porcine respiratory tract and its role in the disease triggered by *A. pleuropneumoniae* remain to be experimentally verified.

Hfq regulates biofilm formation in *V. cholerae* by acting in the quorum sensing-mediated pathway leading to the degradation of the *hapR* transcript, which is a major negative regulator of biofilm formation (18). In uropathogenic *E. coli*, an *hfq* mutant was found to be defective in biofilm formation but a mechanism for the Hfq-mediated regulation of biofilm formation was not identified (17). Research described in the third chapter of this dissertation provides several lines of evidence for the regulation of PGA-based biofilm production by Hfq in *A. pleuropneumoniae.* This is the first report on the role of Hfq in regulation of PGA-based biofilm formation in bacteria and is an important contribution to knowledge on both biofilm formation and the role of Hfq in bacteria. Homologues of both the *hfq* gene and genes involved in PGA biosynthesis are found at least in *A. baumannii, A. pleuropneumoniae , A. actinomycetamcomitans, B. pertusis, Escherichia coli, S. aureus,* and *Yersinia pestis* (3, 21). This study has uncovered a link between Hfq and PGA-based biofilms. Other groups working with pathogens capable of forming PGA-based biofilms will eventually enrich our knowledge on the role of Hfq in PGA production in multiple systems.

It was hypothesized that Hfq regulates surface components, such as outer membrane proteins, which could be important in biofilm formation by *A. pleuropneumoniae.* Studies on Hfq-mediated changes in the outer membrane fractions revealed that a cysteine synthase, CysK, which co-purified with the outer membrane fraction, was found at detectable levels only in the wild-type strain. Although CysK does not display characteristics of membrane proteins, it has been found to co-purify with membrane fractions from *Brucella abortus* (6). It is also possible

that CysK could be a contaminant from cytoplasmic contents during isolation of bacterial membrane fractions. However, CysK was consistently found only in the outer membrane fractions obtained from the wild-type strain. CysK catalyzes the final step in the biosynthesis of L-cysteine, which is a precursor for coenzymeA, cystine, glutathione and thiamine and is known to be involved in resistance to oxidative stress and tellurite in bacteria (9). Glutathione, in particular, is a vital player in maintaining a reducing environment within bacterial cells (9). Since the *hfq* mutant was defective in the production of CysK, the role of Hfq in resistance to oxidative stress and tellurite was tested in *A. pleuropneumoniae.* Genes involved in oxidative stress resistance, *ohr* and *sodC,* and tellurite resistance, *tehB,* were identified as in vivo-induced genes in *A. pleuropneumoniae,* suggesting that the ability to withstand oxidative stress is critical for the survival and/or virulence of this pathogen (16). The reason for up-regulation of the tellurite resistance gene during infection is not apparent, although recent studies have demonstrated that the *tehB* gene in *H. influenzae* is not only involved in resistance to tellurite but also in resistance to oxidative stress (22). An *A. pleuropneumoniae hfq* mutant exhibited increased sensitivity to methyl viologen, a redox cycling agent which produces superoxide within bacterial cells, as well as to tellurite (1). Current understanding of the mechanisms of toxicity induced by tellurite is incomplete, but tellurite is known to produce superoxide within bacterial cells and could be an important factor in inducing damage (4).

Finally, the effect of Hfq on the virulence of *A. pleuropneumoniae* was tested in an experimental infection model of porcine pleuropneumonia in the natural swine host. While Hfq has been implicated in the virulence of several bacterial pathogens, the role of Hfq in the virulence of pathogens causing pneumonia in the lungs has not been established (3). The ability of the *hfq* mutant to survive within pig lungs during co-infection with the wild-type strain was

evaluated using a competitive index experimental design. The *hfq* mutant strain was found to be highly attenuated, with competitive indices ranging from 10^{-2} to 10^{-9}. Since the *hfq* mutant is severely attenuated, it is a potential candidate for the development of a live attenuated vaccine. Further studies to determine the 50% lethal dose and ability to induce a protective immune response in pigs are required to evaluate the vaccine potential of the *A. pleuropneumoniae hfq* mutant. If the *hfq* mutant is deemed fit for development as a live attenuated vaccine, a markerless *hfq* mutant strain should be generated. The *hfq* mutant strain used in the experiments described in this dissertation contains a chloramphenicol resistance marker. If this strain is used for vaccination, there is a possibility of disseminating the gene involved in chloramphenicol resistance in the environment.

The virulence defect observed in the *hfq* mutant could be at least in part due to defect in production of PGA and/or cysteine synthase. However, Hfq is a pleiotropic regulator and it is possible that in addition to its role in PGA production, superoxide resistance and tellurite resistance, Hfq could affect the expression of other known or novel factors involved in the virulence of *A. pleuropneumoniae* (see future directions).

In summary, a model for the role of in vivo-induced genes *ilvI* and *hfq* and the regulatory protein, Lrp, during infection is presented here. *A. pleuropneumoniae* is transmitted between pigs by contact with infectious nasal secretions and aerosol. Upon entry into the lungs, *A. pleuropneumoniae* is exposed to various signals unique to mammalian hosts such as a shift in temperature, iron limitation and BCAA limitation. Cues perceived within the lungs result in the expression of the in vivo-induced genes in addition to the constitutively expressed genes. Two such in vivo-induced genes, *ilvI* and *hfq,* are induced due to the low levels of BCAAs present in

the pulmonary epithelial lining fluid. Genes encoding regulatory proteins governing the expression of in vivo-induced genes, such as *lrp*, are likely also to be up-regulated during infection. Regulators such as Hfq and Lrp could then mediate global changes in gene expression. Increased expression of *ilvI*, which is regulated by Lrp, is required for the production of sufficient BCAA, endogenously, to survive within the lungs. This initial phase of survival and multiplication within the lungs is important for the production of a sufficient amount of the Apx toxins to induce tissue damage thereby triggering a strong inflammatory response. However, due to the action of Apx toxins, the microenvironment at the site of infection rapidly transforms from a BCAA limiting environment to a nutrient rich environment. Thus the limitation of BCAAs is likely an important signal only during the early stages of the infection.

Hfq, also induced during BCAA limitation, could play multiple roles during early stages of infection. Hfq regulates the production of PGA and PGA mediates autoaggregation and biofilm formation in *A. pleuropneumoniae.* PGA could also act as an adhesin to mediate binding to the porcine respiratory tract. Due to shear size, *A. pleuropneumoniae* aggregates might present a special challenge for the clearance by the phagocytic cells. Additionally, high cell density conditions encountered within such aggregates could initiate global changes in gene expression that are favorable for this bacterium during infection. The host immune system recognizes LPS and other pathogen-associated molecular patterns in *A. pleuropneumoniae,* leading to the dispatch of phagocytic cells (neutrophils and macrophages) to the infection site within the lungs. *A. pleuropneumoniae* is exposed to oxidative stress both within and outside the phagocytes. The respiratory burst in neutrophils and monocytes results in the production of reactive oxygen molecules including superoxide anion. Apx toxins cause extensive damage to host cell membranes and the site of infection becomes replete with contents of lysed cells including

phagocytes. Hfq could enhance survival under oxidative stress by promoting the production of CysK, which is in turn required for biosynthesis of glutathione, a key regulator of redox status within bacterial cells. Hfq could also act directly on superoxide dismutases to promote their expression at a post-transcriptional level. Superoxide dismutases catalyze the conversion of superoxide anions into hydrogen peroxide which is rapidly converted into water by the action of catalases. Additionally, Hfq could regulate the expression of *tehB,* involved in tellurite resistance, although the exact role of *tehB* in the virulence of *A. pleuropneumoniae* remains to be determined. Taken together, *ilvI*, which encodes a BCAA biosynthetic enzyme; *lrp*, which encodes a global transcriptional regulator that also regulates *ilvI*; and *hfq,* which encodes a global post-transcriptional regulator are all involved in promoting the survival and fitness of *A. pleuropneumoniae* during infection of the pig lungs.

Future directions. The research presented in this dissertation identifies a host signal, BCAA limitation that could be targeted for the development of site-specific antibacterial agents. Elucidation of the role of a BCAA biosynthetic enzyme in *A. pleuropneumoniae* shows the importance of bacterial biosynthetic genes during infection. In light of our findings, biosynthetic genes identified to be involved in the virulence of other bacterial pathogens by functional genomic studies should be re-examined to find if any of those pathways can be targeted for developing antibacterial agents. Furthermore, a global regulatory protein, Hfq, which is implicated in the virulence of many important pathogens such as *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium, *S. aureus*, and *Y. pestis* , was found to be essential for the virulence of *A. pleuropneumoniae* (3). Further studies directed at the identification and development of specific inhibitors of Hfq might lead to the development of a novel group of antibacterial agents. These are examples of how information obtained from research on genes

playing an important role during infection, such as the in vivo-induced genes, could be used to detect potential targets for the development of control strategies against bacterial pathogens. The work presented in this dissertation takes us one step closer towards realizing that goal.

While answering many questions regarding the role of *ilvI* and *hfq* in the pathobiology of *A. pleuropneumoniae,* the work presented in this dissertation also raises new questions. One of the key questions is whether Hfq binds to any of the transcripts known to be regulated by Hfq in *A. pleuropneumoniae*. In *E. coli*, Hfq binds to its mRNA and/or sRNA targets to mediate changes in stability and/or translation (2). Identification of Hfq-binding RNAs (both mRNA and sRNA) would take us one step closer towards understanding Hfq-mediated post-transcriptional regulation in *A. pleuropneumoniae.* If Hfq binds to the *pgaABCD* mRNA and/or the *cysK* mRNA, it is possible that Hfq could directly regulate these transcripts. Additional studies would then be required to demonstrate the specific regions on the transcripts required for binding to Hfq. To achieve this goal, the *hfq* gene—from start to stop codon—was cloned in frame into an overexpression vector to isolate hexa-histidine-tagged-Hfq (6H-Hfq). Purified 6H-Hfq was immobilized on magnetic beads by metal affinity chromatography and incubated with RNA extracted from *A. pleuropneumoniae.* At the time of writing this dissertation, Hfq-bound RNA of sufficient quantity and quality for reverse transcription (RT) had not been obtained. If sufficient Hfq-bound RNA could be obtained, then an RT-PCR to look at potential Hfq-binding transcripts, including 5' untranslated regions of *hfq*, *pgaABCD* and *cysK*, could be performed. However, if Hfq does not bind to these mRNAs in *A. pleuropneumoniae,* then it may be difficult to validate this assay. Alternatively, cDNA synthesized from Hfq-bound RNA could be used for the construction of clone libraries and sequencing to identify the Hfq-binding transcripts. Size exclusion methods could be applied to enrich the RNA samples for small RNAs (< 500 bp) to

facilitate the detection of small RNAs in *A. pleuropneumoniae.* Further optimization of this system will provide a relatively simple tool for the identification of Hfq-binding transcripts in *A. pleuropneumoniae*.

Since Hfq is a pleiotropic regulator, understanding Hfq-mediated changes in the transcriptome will help to identify both direct and indirect targets of Hfq in *A. pleuropneumoniae* and enrich our knowledge on Hfq-mediated regulation. A direct comparison design with two-color microarrays could be used to compare the transcriptome of the *hfq* mutant to wild-type. A whole genome tiling array for *A. pleuropneumoniae* was developed previously in our laboratory and could be used for this study. Gene expression data obtained from a tiling array could be used to identify signals in the intergenic regions to identify potential small RNAs. Differentially regulated transcripts will provide targets for further evaluation of their role in the virulence of *A. pleuropneumoniae*. Since Hfq is a post-transcriptional regulator, it is possible that altered transcript levels may not match changes in protein levels. Therefore protein levels of at least some of the Hfq-regulated transcripts would have to be verified.

Construction of defined mutation in *pgaA* and *cysK* in *A. pleuropneumoniae* was attempted during the course of the studies described in this dissertation; however, those mutants could not be generated. Given the difficulty of genetic manipulation in *A. pleuropneumoniae*, this was not completely unexpected. Only the natural transformation approach was tried to generate both those mutant strains, so an alternative method of using a conjugal suicide vector combined with a counter-selectable marker, described in the second chapter of this dissertation, under construction of the *ilvI* mutant, could be tried.

Biofilm-forming ability of the *pgaA* mutant strain could be compared to the *hfq* mutant and wild-type strain to test if the defect in biofilm formation observed in the *hfq* mutant is due to low levels of PGA production. Currently, there is no evidence for the role of PGA produced by *A. pleuropneumoniae* in the colonization or virulence within the natural host, swine. Comparison of the virulence and colonization potential, especially in the tonsils and upper respiratory tract, of the *pgaA* mutant to the wild-type strain using an aerosol route of infection might explain the role of PGA in the pathogenesis of porcine pleuropneumonia.

Comparing the ability of a *cysK* mutant to withstand superoxide stress and tellurite with the wild-type and *hfq* mutant strain would clarify whether the superoxide and tellurite sensitivity phenotypes observed in the *hfq* mutant are because of low levels of CysK production. Competitive index experiments with the *pgaA* and *cysK* mutants compared to the wild-type and *hfq* mutant might help to explain the role of PGA and CysK in the virulence defect observed in the *hfq* mutant.

Additional studies proposed here will help advance our understanding of the role of Hfq specifically in *A. pleuropneumoniae* and are broadly applicable to the role of Hfq in bacterial pathogenesis. In the long term, better understanding of the physiological conditions encountered by pathogens at the site of infection coupled with the knowledge of regulatory systems governing the expression of genes involved in virulence will help develop novel therapeutic and prophylactic strategies to control infectious diseases.

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