This is to certify that the

dissertation entitled

Identification of In Vivo Induced Genes
in Actinobacillus pleuropneumoniae

presented by

Troy Eugene Fuller

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology

Martha H. Mulks
Major professor

Date 2-21-97

# IDENTIFICATION OF *IN VIVO* INDUCED GENES IN *ACTINOBACILLUS PLEUROPNEUMONIAE*

By

Troy Eugene Fuller

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Microbiology

1997

# ABSTRACT

# IDENTIFICATION OF *IN VIVO* INDUCED GENES IN *ACTINOBACILLUS PLEUROPNEUMONIAE*

By

Troy Eugene Fuller

*Actinobacillus pleuropneumoniae* (APP) is the causative agent of porcine pleuropneumonia, a severe and often fatal respiratory disease in swine. In order to develop effective vaccines and antibiotics, there is a need for a better understanding of the pathogenesis of APP. The goal of this research was to identify APP genes that are specifically induced *in vivo* during infection.

The 4300 bp riboflavin operon (*ribGBAH*) of APP serotype 5 was cloned and sequenced. The biosynthetic functions of these genes were confirmed by sequence homology with known riboflavin biosynthesis genes, by complementation of known *Escherichia coli* riboflavin mutants, by analysis of the plasmid encoded proteins and by analysis of the recombinant riboflavin product including spectral analysis, mass spectroscopy and fluorescence. A deletion disruption riboflavin-requiring mutant of a serotype 1 strain was made by targeted mutagenesis of the riboflavin operon. This mutant (AP233) is avirulent at a dosage which is 500 times the established wild type $LD_{50}$. AP233, as a live vaccine, provides a degree of protection against both serotype 1 and serotype 5 strains when administered intramuscularly and supplemented with enough riboflavin to permit two to three generations of growth *in vivo*.

An IVET (*in vivo* expression technology) system was constructed for APP that is able to identify genes that are induced *in vivo*, i.e., during infection, and not *in vitro*. An IVET vector was assembled using the plasmid pGZRS-19 containing in sequence the $T_4$ terminator, a unique *Bam*HI cloning site, promoterless *luxAB* genes from *Vibrio* *harveyi* and a promoterless *Bacillus subtilis ribBAH* operon. It was demonstrated that wild type APP contains no native bioluminescence, that the *B. subtilis ribBAH* genes can complement the attenuating riboflavin mutation in AP233 thus restoring virulence and that the $T_4$ terminator effectively eliminates background expression from the pGZRS-19 vector. This IVET system was used to identify five *in vivo* induced genes in APP, two of which were putatively identified as the *mrp* gene and the *secE-nusG* operon. The further identification of *in vivo* induced genes will help us better understand and control the pathogenesis of APP.

Dedicated to:

Michelle Fuller

James Fuller and Karen Hanes

I

I

D

Th

Th

Dr

Jar

a

c

Mich

# ACKNOWLEDGMENTS

My years in graduate school could not have been accomplished without the support of many. I wish to thank the following:

Dr. Martha H. Mulks, for all of your guidance, mentoring both in and out of the laboratory and for dedicating yourself to my professional development as a scientist.

Dr. Brad Thacker, for all of your expertise and help with the animal experiments.

Drs. Pat Oriel, Michael Bagdasarian, Paul Coussens, Linda Mansfield and Ron Patterson, members of my graduate committee for your advice, assistance and excellent questions.

Drs. Sadhana (Chauhan) Motin, Vladimir Motin and Wilma Cruz, for patiently helping me get started during my first year and for teaching me many valuable techniques.

The United States Department of Agriculture for 3 years of support through a National Needs Graduate Fellowship in Animal Biotechnology

The DuVall family for their gracious financial support during my last year

Dr. David Kreps, my first mentor and inspiration for pursuing microbiology as a profession

James and Sandra Fuller and Karen and Robert Hanes, my parents, for all of your love and support and for allowing me to pursue my own interests and to make my own decisions.

Michelle Fuller, my wife, without whom my accomplishments would not matter.

# PREFACE

This thesis includes four chapters that are in manuscript format. The second chapter entitled "Characterization of *Actinobacillus pleuropneumoniae* Riboflavin Biosynthesis Genes" is a full length version of a manuscript that was published in note format in the Journal of Bacteriology Volume 177 (1995) pages 7265-7270, with Dr. Martha H. Mulks as co-author. Chapter three entitled "A Riboflavin Auxotroph of *Actinobacillus pleuropneumoniae* is Attenuated in Swine" was published in Infection and Immunity Volume 64 (1996) pages 4659-4664, with Dr. Martha H. Mulks and Dr. Brad J. Thacker as co-authors. Chapters four and five will be submitted to Infection and Immunity with Dr. Martha H. Mulks and Dr. Brad J. Thacker as co-authors. As a co-author, Dr. Brad Thacker provided veterinary care and expertise, clinical evaluation and pathological interpretation for the animal experiments.

# TABLE OF CONTENTS

LIST OF TABLES ........ ix

LIST OF FIGURES ........ x

CHAPTER 1
GENERAL INTRODUCTION ........ 1
- *Actinobacillus pleuropneumoniae* ........ 2
  - Epidemiology and Pathology ........ 2
  - Virulence Factors ........ 3
  - Prevention and Control ........ 9
  - Genetics ........ 13
- Genetic Technology for the Study of Pathogenesis ........ 15
  - *In Vivo* Expression Technology ........ 15
  - Signature-Tagged Mutagenesis ........ 18
  - Resolvase-Based IVET ........ 18
  - Subtractive Hybridization ........ 19
- References ........ 21

CHAPTER 2
CHARACTERIZATION OF *ACTINOBACILLUS PLEUROPNEUMONIAE* RIBOFLAVIN BIOSYNTHESIS GENES ........ 36
- Abstract ........ 37
- Introduction ........ 38
- Materials and Methods ........ 43
- Results ........ 46
- Discussion ........ 59
- Acknowledgments ........ 61
- References ........ 62

CHAPTER 3
A RIBOFLAVIN AUXOTROPH OF *ACTINOBACILLUS PLEUROPNEUMONIAE* IS ATTENUATED IN SWINE ........ 66
- Abstract ........ 67
- Introduction ........ 68
- Materials and Methods ........ 71
- Results ........ 76
- Discussion ........ 84
- Acknowledgments ........ 86
- References ........ 87

CHAPTER 4
A RIBOFLAVIN AUXOTROPH AS A LIVE-ATTENUATED VACCINE AGAINST *ACTINOBACILLUS PLEUROPNEUMONIAE* ........ 91
Abstract ........ 92
Introduction ........ 93
Materials and Methods ........ 96
Results ........ 100
Discussion ........ 106
Acknowledgments ........ 110
References ........ 111

CHAPTER 5
IDENTIFICATION OF *IN VIVO* INDUCED GENES IN *ACTINOBACILLUS PLEUROPNEUMONIAE* ........ 115
Abstract ........ 116
Introduction ........ 117
Materials and Methods ........ 123
Results ........ 127
Discussion ........ 138
Acknowledgments ........ 141
References ........ 142

CHAPTER 6
SUMMARY ........ 145

# LIST OF TABLES

Table 2.1 Comparison of Amino Acid Sequence Similarity Between Riboflavin Synthesis Proteins .......... 54

Table 3.1 Characteristics of Bacterial Strains and Plasmids .......... 72

Table 3.2 Mortality and Lung Score Data .......... 83

Table 3.3 Clinical Score Data .......... 83

Table 4.1 Serologic Analysis of Samples Collected at Challenge for Trial 1.... 103

Table 4.2 Mortality and Lung Score Data for Trial 1 .......... 104

Table 4.3 Clinical Sign Data for Trial 1 .......... 104

Table 4.4 Mortality and Lung Score Data for Trial 2 .......... 105

Table 4.5 Clinical Sign Data for Trial 2 .......... 105

F

F

F

F

F

F

Fi

Fi

# LIST OF FIGURES

Figure 2.1 Proposed Bacterial Riboflavin Biosynthesis Pathway .................. 39-40

Figure 2.2 Physical Map of the Construct pTF10 .................. 47

Figure 2.3 Spectral Analysis of Riboflavin and Recombinant Riboflavin .................. 48

Figure 2.4 Complete Nucleotide Sequence of APP *ribGBAH* Genes and Flanking Regions .................. 49-53

Figure 2.5 Complementation of *E. coli* Mutants by Cloned APP *rib* Genes .................. 57

Figure 2.6 Minicell Analysis of pTF10 and Deletions .................. 58

Figure 3.1 Construction of pTF67a .................. 77

Figure 3.2 Southern Blots of *Hin*dIII or *Eco*RI Digested DNA from Mutants and Controls .................. 78

Figure 3.3 Schematic Structure of the *rib* Locus of Parent and Mutant Strains in Double and Single Cross-Over Events .................. 79

Figure 3.4 Phenotypic Analysis of AP100, AP225 and AP233 .................. 81

Figure 5.1 Construction of the IVET Vector pTF86 .................. 129

Figure 5.2 Identification of *In Vivo* Induced Genes .................. 130

Figure 5.3 Primary Screening for Minimally Expressed IVET Clones in AP233 .................. 132

Figure 5.4 *In Vitro* Quantitative Lux Analysis of Selected IVET Clones .................. 133

Figure 5.5 Amino Acid Alignment of IVA and Mrp .................. 134

Figure 5.6 Amino Acid Alignment of IVF and SecE/NusG .................. 136

Figure 5.7 *In Vivo* Induction of IVET Clones .................. 137

# Chapter 1

# GENERAL INTRODUCTION

v

l

t

w

c

wh

Th

are

neu

vuln

stag

neut

With

inflan

fibrin,

## *ACTINOBACILLUS PLEUROPNEUMONIAE*

### 1. Epidemiology and Pathology:

*Actinobacillus pleuropneumoniae* (APP) is a gram negative, encapsulated, hemolytic, pleiomorphic rod belonging to the family *Pasteurellaceae*. APP strains can be divided into two biotypes based on their nicotinamide adenine dicnucleotide dependence (biotype 1) or independence (biotype 2). APP is the etiologic agent of porcine contagious pleuropneumonia, a severe and often fatal respiratory disease of swine (38,112,141). The disease has become a major economic problem in swine-raising areas throughout the world since its discovery and identification over 30 years ago (38,67,102,112,119,123,141,142). Pleuropneumonia is typically characterized as an acute, necrotizing, exudative bronchopneumonia with accompanying hemorrhage, vascular injury and fibrinous inflammation of the pleural cavity (8,30,38,94,112,141,168). Increased industrialization of swine farms has led to situations which are conducive to transmission and infection. Disease outbreaks are frequently associated with stresses which might depress normal respiratory defenses such as transportation, abrupt climatic change, overcrowding or poor ventilation (38,112,139,141).

The disease may occur in a number of forms ranging from a peracute phase which normally results in high morbidity and mortality, to a chronic carrier phase (112). The disease most commonly affects 3 to 5 month-old pigs even though pigs of all ages are susceptible. It has been proposed that the gradual loss of maternal toxin-neutralizing antibodies is the reason that animals at the age of 12 weeks are particularly vulnerable in an endemically infected herd (20,21). The pathology during the initial stages of pleuropneumonia includes congestion and platelet aggregation, edema, and neutrophil infiltration, as well as development of multifocal petechial lesions (8,94,168). With progression, the alveoli, bronchioles, and bronchi become filled with an inflammatory exudate that contains macrophages, dead and degenerate neutrophils, fibrin, hemorrhage, and bacteria (112). It has also been suggested that the severity of r

r

s

re

m

mu

clo

neg

mut

Anticlinical signs may be enhanced by a synergistic co-infection with viral agents, such as pseudorabies virus (138). This synergism may be due to viral mediated hindrance of normal physical respiratory defense mechanisms such as mucociliary clearance (111).

## 2. Virulence Factors:

Several proven and suspected virulence factors of APP include capsular polysaccharide (CPS), lipopolysaccharide (LPS), extracellular toxins, and surface proteins (OMPs). These factors may contribute to the ability to survive *in vivo* by escaping from or neutralizing host defenses.

There are currently 12 recognized serotypes based on CPS, the serotype-specific antigen of APP. Serotypes 1 and 5 can both be divided further into subtypes A and B. The composition and structure of the CPS of all 12 serotypes has been determined (124). CPS does not cause pulmonary lesions when administered endobronchially (41,68), does not demonstrate toxic activity (39,41,68) and does not activate the complement cascade (68). The role of CPS is believed to be protection against specific (bactericidal killing) and nonspecific (phagocytosis) respiratory defense mechanisms because encapsulated APP strains are resistant to antibody and complement mediated killing and to phagocytosis by PMNs (68,69,134). The mechanism for this resistance appears to be inhibition of the binding of C9 and membrane attack complex to the cells (163). Non-encapsulated mutants of APP serotypes 1 and 5 have been shown to be at least 10 fold less virulent than their respective wild type strains (71,130) although this work was done with chemically mutagenized bacteria leading to the possibility of additional unknown attenuating mutations. The gene cluster involved in the biosynthesis of CPS in APP-5 has been cloned, and two genes homologous to polysaccharide export genes from other gram negative bacteria have been identified (164). Further work with genetically defined mutants has recently been reported to confirm the previous attenuation findings (163). Anti-CPS antibodies provide serotype-specific protection against severe disease c

i

a

fa

ac

lik

LP

pn

infl

res

(4,5

LPS,

expe

symptoms, but they do not prevent colonization or chronic infection (68,69,113,131,132) suggesting the importance of other virulence factors.

LPS, or endotoxin, is produced by all gram-negative bacteria. As with CPS, the composition and structure of the O-antigens (the outermost oligosaccharide chain of LPS) has been determined for all 12 serotypes of APP (124). Structural similarities between LPS O-side chains of some serotypes may explain many cross reactions in serotyping. Although serotyping by antigenic differences in CPS is standard, it has been proposed that APP strains be classified using both LPS (O) and capsule (K) designations, as is done with other gram-negative bacteria (9,110,124). However, the creation of additional subtypes using multiple antigens would likely lead to a decreased reliability in the typing due to antisera variation.

Except for the typical hemorrhagic necrosis, LPS may cause many of the pathologic lesions in the lungs of swine infected with APP, including inflammatory edema, neutrophil infiltration, increased capillary permeability, hemorrhage into alveoli, intravascular platelet aggregation and fibrinous thrombosis (41,154). Death during acute infection may even be due to septic shock rather than to the pulmonary ventilation failure associated with extensive lung damage (94). LPS is known to specifically induce activated complement, production of fibrin and production of pro-inflammatory cytokines like interleukin 1 and tumor necrosis factor (10,24,105). Administered intratracheally, LPS can induce lesions ranging from mild interstitial pneumonia to consolidating lobular pneumonia with inflammatory cell infiltration (41,154). In addition to its role in inflammatory reactions, smooth LPS plays a role in adherence of APP to porcine respiratory tract cells, to mucus secretions and to porcine tracheal rings in organ culture (4,5,122). The lipid A-core region also has been shown to bind porcine hemoglobin (3).

Immunization of swine with a J5 strain of *E. coli,* which produces a non-toxic LPS, provided a significant reduction in clinical signs and protection from mortality in experimental infections and field trials; however, it did not prevent APP infection (37,40).

t
h
to
th

9,
wh
cel
reg
(46,
sero
bind
The
hemo

Possible reasons for this lack of protection from infection might be that antibodies against LPS may be blocking the ability of complement components to bind to APP cells (165), the LPS may be too dissimilar, or perhaps it is even partially hidden due to CPS.

Three extracellular RTX (Repeat-In-Toxin) toxins have been described in APP and are termed ApxI, II and III. An additional cryptic hemolysin, the source of some confusion, has also been identified. All field isolates of APP produce one or more of these toxins depending on the serotype (2,85). The genes encoding all three Apx toxins have been sequenced by multiple laboratories (14-6,43,46,49,79-81,83). ApxI and ApxIII are encoded by typical RTX toxin operons, consisting of four contiguous genes, *apxCABD* (14,43,46,49,79,80,83), encoding respectively the toxin activator, the pro-toxin and the two products necessary for a single step secretion pathway. The ApxII operon is truncated, containing only the *apxC* and *apxA* genes (15,81,96); therefore, secretion of ApxII is apparently mediated through ApxI or ApxIII B and D (81,96). RTX toxins kill eukaryotic cells by forming pores in the cell membrane. Biophysical studies have recently determined the channel size and channel-forming activity of all three Apx toxins (101), and the pore forming activity appears to directly correlate with the activity of the toxins.

ApxI is a strongly hemolytic and cytolytic toxin produced by serotypes 1, 5a, 5b, 9, 10, and 11 (44,50-2,85). Like other RTX toxins, the ApxI toxin is a pore-forming toxin which binds calcium and requires calcium for activity, specifically for binding to target cells such as erythrocytes and neutrophils (28,49,90,160). Expression of ApxI is regulated at the transcriptional level by calcium through an unknown mechanism (46,52,63). ApxII is a weakly hemolytic and weakly cytolytic toxin produced by all APP serotypes except serotype 10 (45,52,54,85,86,144). The ApxII toxin requires calcium for binding to target cells, but unlike ApxI its production is not induced by calcium (160). The cytotoxic and hemolytic domains of this toxin are distinct and separable with the hemolytic activity being more dependent on activation and secretion than cytotoxic activity (103,152). ApxIII requires calcium for binding to porcine neutrophils and is strongly cytotoxic for porcine alveolar macrophages and neutrophils; however, unlike ApxI and ApxII it is not able to bind to erythrocytes and is therefore not hemolytic (85,95,136,160). It is produced by serotypes 2, 3, 4, 6 and 8 (85,95).

In addition to the three Apx toxins, several laboratories have identified an APP gene encoding a cryptic hemolysin, HlyX, which is expressed in *E. coli* but not in APP (53,76,93). HlyX is homologous to the FNR protein of *Escherichia coli*, which regulates several genes involved in anaerobic respiration (60,97,145). It was originally thought that the HlyX was responsible for the CAMP reaction hemolysis seen with APP, but now this phenomena has been attributed to the Apx toxins (47,78). There is currently no evidence that the *hly*X gene is involved in virulence in *A. pleuropneumoniae,* and the reason for its hemolytic activity in *E. coli* remains to be determined. Perhaps it regulates the expression of yet another cryptic hemolysin in *E. coli.*

The APX toxins are perhaps the most studied virulence factors in the pathogenesis of APP. APP culture supernatants are toxic *in vitro* for a wide variety of porcine cells including erythrocytes, pulmonary macrophages and neutrophils (7,85,129,160,161). The ApxI and ApxII toxins from serotype 1 have been shown to stimulate the oxidative metabolism of porcine PMNs at low doses (153), kill PMNs at higher doses (153), and damage porcine alveolar macrophages (18,147). Likewise, the ApxII and ApxIII toxins from serotype 2 stimulate neutrophils and porcine pulmonary alveolar macrophages at low doses and kill at higher doses (31,33). The activities of these toxins *in vivo* probably interfere with pulmonary macrophage and neutrophil function, thereby decreasing effective phagocytosis and killing. This lack of clearance could then allow the rapid multiplication of bacterial cells leading to acute pleuropneumonia. In addition, damage to phagocytic cells will result in the release of lysosomal contents and oxygen radicals contributing to massive inflammation and necrosis.

a

t

c

A

c

(9

fu

gr

na

se

*infl*

sug

Toxin negative mutants of APP have been shown to be avirulent, with the degree dependent on which toxins are knocked out. *In vivo* studies using isogenic toxin-negative mutants conclusively prove that the RTX toxins are critical to the pathogenesis of APP infection. Even though toxin is critical to the virulence of the organism, toxin neutralizing antibodies are not sufficient to completely protect against pleuropneumonia. Mutants of APP-1 lacking ApxI and ApxII do not activate or kill porcine neutrophils (82) and do not cause significant clinical signs or lesions in pigs at a dose that is normally 100% lethal with a wild type APP-1 strain (148). In contrast, APP-1 mutants that can still produce ApxII but which lack ApxI are still pathogenic, although they are less virulent than wild type APP-1 (149). An ApxI-, ApxII- mutant of APP-5 caused neither mortality nor lung lesions in either mice or pigs, even when tested at 10 times the $LD_{50}$ for the wild type strain (70). An ApxII- ApxIII- mutant of APP-2 was also attenuated compared to the wild type strain (23). However, an ApxII- ApxIII+ mutant of APP-2 was still able to cause pleuropneumonia and typical acute lesions in pigs, although the disease in animals infected with this mutant was less severe than in those infected with the wild type strain (137). An ApxII- mutant of APP-7 was also attenuated in pigs and could not colonize the host as well as the wild type strain (58). These results suggest that the ApxII toxin is critical when it is the only toxin produced.

Outer membrane protein (OMP) profiles of APP strains show three major common proteins of 38-42 kDa, 29 kDa, and 16-17 kDa, as well as 10-20 minor proteins (98,108,126,135). The 29 kDa OMP is a heat-modifiable protein which has not been further characterized (126). The 16-17 kDa OMP was reported initially as a common gram negative antigen (98), and the gene has recently been cloned, characterized and named *palA* (48). It is predicted to be a 16.2 kDa protein which is homologous in sequence to the peptidoglycan-associated lipoprotein of *E. coli* and the *Haemophilus influenzae* OMP P6 (48). Amino terminal sequencing of a 40 kDa major OMP from APP suggests that it is related to a porin protein from *Pasteurella multocida* (64). Our lab has tested purified OMPs as subunit vaccines; however, vaccination of pigs with a purified 38-42 kDa OMP or with a 48 kDa OMP (AopA) (22) did not provide protection against APP challenge (106). A 42 kDa OMP expressed by all APP serotypes, which is homologous to the P2 OMP of *H. influenzae,* does provide good protection against APP challenge when used in combination with Apx toxins in a subunit vaccine (158-9).

A common host defense mechanism is the sequestration of iron from the pathogen. APP responds to conditions of iron-limitation by inserting new OMPs into its outer membrane including OMPs of 60-65, 76-79, and 99-105 kDa rather than by production of iron scavenging siderophores. These iron regulated OMPs are expressed *in vivo* as detected by convalescent sera from infected pigs (26,57,117,128). The sarkosyl-soluble 60-65 kDa OMP (TfbA) is a transferrin binding outer membrane lipoprotein homologous to other TbpII type proteins (57,59). Similar Tfb proteins are made by all 12 APP serotypes, although there are three antigenically distinct families (59). The TfbA protein from APP-7 has been tested in combination with ApxII from APP-7 as a vaccine, providing significant protection against challenge with APP-7 but not with APP-1 (133). The gene for the type one transferrin binding protein (*tbpI*) is located downstream of *tbpII* (*tfbA*) and has recently been cloned and sequenced from serotype 1 (25).

Another sarkosyl-soluble outer membrane lipoprotein (OmlA) which is recognized by convalescent serum has been cloned from serotypes 1 and 5 (11,56,75). Southern blot analysis demonstrated that all APP serotypes contain DNA homologous to the *omlA* gene, and immunoblots detected homologous proteins to the serotype 1 OmlA produced by APP 2, 8, 9, 11, and 12, and to the serotype 5 OmlA in serotype 10. Vaccination with the recombinant protein provided protection against mortality when challenged with the homologous serotype but still showed limited protection against clinical signs and lung lesions. No data on protection against heterologous challenge has been reported with this lipoprotein.

There are various other environmental conditions besides iron restriction that stimulate the production of additional OMPs. For instance, the presence of maltose in the medium (27) and variation of NAD concentration (120) can induce the production of unique OMPs in APP. Several of these OMPs are expressed *in vivo* as detected by convalescent pig sera. It is unknown whether they have any significant role in virulence.

Adhesins are important for attaching and localizing the pathogen to invade or to prevent physical clearance by the mucosal ciliary system. When the normal mucosal ciliary system is inhibited by atropine or xylocaine, infection rapidly progresses seeming to implicate adhesion as an important process. APP adheres to porcine tracheal rings, tracheal epithelial cells, lung tissue, respiratory mucus, and hemoglobin (3,4,6,77,122) and has been shown to closely associate with alveolar and bronchial epithelial cells *in vivo* during the initial stages of infection (32). The high molecular weight O antigens of lipopolysaccharide have been identified as potential adhesins (4,122), while capsule seems to inhibit adherence (6,77). APP cells freshly isolated from infected pigs have also been shown to possess fimbriae that were rapidly lost upon subculture (155), but their role in attachment has yet to be proven.

### 3. Prevention and Control:

Rapid progress has been made in the basic science of *Actinobacillus pleuropneumoniae* (APP) in the past several years, yet APP continues to be a serious worldwide economic problem in the swine industry. Current methods of treatment are costly as well as fairly ineffective for preventing lung damage. Antibiotics such as penicillin, ampicillin, chloramphenicol and cotrimoxazole are fairly effective against APP (112) in the initial phase of the disease before significant tissue damage occurs. Antibiotic therapy, however, will not eliminate infection in a herd as chronic infections may persist in abscesses and tonsils of the swine or the onset of pleuropneumonia may be too rapid to treat effectively (112). Increasing consumer concerns for food safety

a
tr
r
h
c
v
c
c
t
v

tr
v
v
ir
p
a
p
ir
p
(1
m

(9
(1
of
ho

also discourage the use of high levels of antibiotics for prophylactic feeding or even treatment. In addition, the use of antibiotics encourages the development of antibiotic resistant strains as seen with many different pathogens. Naturally occurring plasmids have been identified in APP which encode resistance to many of the major antibiotics currently in use. Although herd management techniques such as early segregated weaning are gaining in popularity and effectiveness, the risk of a herd to potential disaster is great because the animals' immune systems may not be primed specifically or nonspecifically to prevent disease. There is also no difference in the number of days to market or in the final weights between early segregated weaned animals and those vaccinated and raised in the conventional manner (34).

It is more cost-effective and practical to focus on prevention rather than treatment post-infection; however, APP prevention is still limited by the lack of effective vaccines. Current commercial vaccines are killed whole cell vaccines, or bacterins, which have been shown to induce mainly capsular polysaccharide (CPS) specific immune responses (113). A serious limitation of these APP bacterins is their inability to provide protection against heterologous serotypes. Unfortunately, there are no other available types of vaccines, subunit or live, which are both economical and cross-protective. Another limitation of the bacterin is its ability to induce mainly systemic immunity rather than specific secretory mucosal immunity. There are many other problems that result in vaccine failure, including production of ineffective bacterins (150), blockage of immune response by passive immunity (151), and the existence of many serotypes and subtypes of APP (84,116).

Many of the OMPs previously described appear to be common to all serotypes (98,108,126,135), and several outer membrane vaccines have been tested against APP (17,35,109,127,150). Several outer membrane vaccines prepared by sarkosyl extraction of total APP cell membranes have provided significant if not complete protection against homologous challenge (17,35,127). However, a vaccine which used APP-5 outer

a

a

p

d

m

att

vir

viru

the

membranes prepared by sucrose density centrifugation as the antigen provided complete protection against APP-5 challenge and also reasonable protection against a heterologous APP-1 challenge (109). This suggests that sarkosyl soluble OMPs, such as TfbA (57), OmlA (11,56,75) and our AopA (22), that are present in a sucrose density gradient preparation, may contribute to cross-protective immunity.

Extracellular toxins should be included in a true cross-protective vaccine. Several labs have demonstrated that a concentrated cell culture supernatant containing hemolysin is an effective vaccine (1,35,36,87,118,150). The addition of this hemolysin-containing supernatant to whole cell bacterins can also improve vaccine efficacy (151). A purified cytotoxin preparation from APP-1, which likely contained both ApxI and ApxII, conferred good protection against APP-1 challenge (29). A trivalent vaccine containing ApxI, ApxIII, and a 42 kDa OMP conferred significant protection against challenge with both APP-1 and APP-2 (89,158,159). In contrast, a recombinant ApxII from APP-7 provided protection against death following challenge with APP-7, but no cross-protection against APP-1 (133), likely due to the absence of ApxI in the vaccine preparation. A vaccine containing the ApxI protein conjugated to CPS and detoxified LPS from APP-1 provided only minimal protection against APP-1 challenge (12).

Finally, several groups have begun testing avirulent or attenuated strains of APP as live vaccines (71,121,130,156). While several of these have elicited good protection and cross-protection (71,121,156), the use of live vaccines in the field is problematic, particularly when the attenuating lesions in the vaccine strains have not been genetically defined. Many live-attenuated vaccines have been selected by passage on laboratory media or by chemical mutagenesis without fully understanding what causes the attenuation. With undefined attenuating mutations the possibility of reversion to virulence may be strong especially if the mutations are merely point mutations. The virulent strain would also likely have a selective advantage over the attenuated strain in the host. The protection data from these live vaccines do suggest, however, that

a

l

u

in

in

a

a

in

im

W

an

sh

me

app

env

virul

have

rational development of attenuated strains with carefully defined deletion mutations that limit growth *in vivo* and prevent reversion to wild type is a promising approach to improved APP vaccines.

In contrast to bacterin vaccinated animals, which are protected against infection with homologous serotypes but not against heterologous serotypes (39, 113, 115, 146), animals infected either naturally or experimentally with a more virulent form of APP are also strongly resistant to both homologous and heterologous serotype infection (19,113,114,116). These reports suggest that there are common antigens expressed during an infection that result in good cross-protective immunity but that these antigens are not expressed, masked or are altered in killed whole cell vaccines. Work in our lab has demonstrated that APP produces antigens which are expressed *in vivo* but not *in vitro* under standard culture conditions. It is possible that the absence of these antigens in whole cell bacterins prepared *in vitro* results in reduced protection and that their inclusion in a subunit vaccine would elicit cross-protective immunity against APP. It may also be possible that a live strain produces immunomodulatory compounds which lead to a different immune response and better protection.

Two methods currently exist for identifying *in vivo* expressed antigens in APP: immunological identification and mimicry of host conditions in laboratory media. The immunological approach involves screening bacteria grown *in vivo* and *in vitro* by Western blot with convalescent pig sera and then comparing the results to identify antigens that are produced only *in vivo*. Using immunological techniques, our lab has shown that on SDS-PAGE gels of *in vivo* grown APP whole cell lysates and outer membranes there are several bands between 106 kDa and 13 kDa which do not appear *in vitro* (106). The other approach has been directed at duplicating host environmental conditions on laboratory media to identify environmentally regulated virulence factors that may be important in host-pathogen interaction. Both approaches have limitations in that immunological screening mainly identifies surface-associated

a
b
s
d
4

m
ce
int
Se
inc
APP
Lalo
APP

antigens and that media mimicry will perhaps never duplicate all of the possible host conditions. Historically, research has focused on the studies of antigens expressed *in vitro*; however, this at best only gives a fractional picture of virulence-associated characteristics. Increasing attention is being given to the environmentally-stimulated regulatory mechanisms that modulate bacterial attributes in the host (61,62,104). Known environmental regulatory signals for bacterial virulence genes include temperature, osmotic strength, pH, oxygen levels, availability of iron, calcium levels, carbon sources, urea, growth phase, stress, starvation and availability of other nutrients such as amino acids (61,62, 104).

Modern molecular genetic tools are rapidly becoming available to identify APP genes that are specifically expressed *in vivo* during infection of the natural host. Some of these genes will likely encode infection-associated antigens, which can then be cloned, purified, and tested as components of subunit vaccines against APP. In addition, other *in vivo* expressed genes are likely to encode enzymes for critical biochemical pathways that could be used for the development of genetically defined, stable deletion mutants for use as modified live vaccines, and potentially for the development of antibiotics that target these specific biochemical pathways.

## 4. Genetics:

Significant advances in genetic studies on *A. pleuropneumoniae* have been made within the last few years. While it does not seem to be possible to transform certain serotypes of APP at high frequency (42,92,106), plasmids can be electroporated into many serotypes of APP, although generally not into serotype 5 (42,91,92,167). Several types of native plasmids of APP have been described and assigned to incompatibility groups (73,74,140,169). Several shuttle vectors that replicate in both APP and *E. coli* have been constructed from these naturally occurring plasmids. Lalonde et al (92) developed several based on an APP Inc H3 plasmid, using the native APP chloramphenicol resistance ($Cm^R$) gene. One of the plasmids can be conjugally v

t

f

t

p

s

(

pr

bi

us

pro

sys

her

transferred into APP. Frey (42) constructed a set of vectors based on the RSF1010 replicon, with a chloramphenicol resistance ($Cm^R$) gene derived from *Staphylococcus aureus*. West et al (167) have constructed several vectors based on an APP Inc H2 plasmid that contain either the β-lactamase gene from Tn3, under the control of a putative APP promoter, or the kanamycin resistance gene from Tn903, as well as the *lacZ* alpha-complementation region containing a multiple cloning site from pUC18/19. Hodgson et al have developed vectors which are also able to function in *E. coli*, *Pasteurella haemolytica* and APP (66). In addition, West has constructed a cosmid vector that works well in APP (166).

Three different systems for construction of chromosomal mutants of APP have been described. A system for targeted mutagenesis of APP, based on a conditionally replicating plasmid vector and insertional mutagenesis by homologous recombination, has been described and used to produce hemolysin-negative mutants of APP (82). While the initial premise of using a temperature sensitive (Ts) replicon in this system was promising, the Ts-vector does not appear to replicate in APP, and the efficiency of this system is quite low. Because this system is based on electroporation, it will not function well with APP-5 (82). Mulks and Buysee have recently developed a system for targeted mutagenesis of APP that utilizes a broad-host range conjugative suicide vector, pGP704, that functions in both APP-1 and APP-5 (107). This system has been used successfully to construct knock-out mutants of the *aopA* gene in APP-1 and APP-5 (107), which fail to express the 48 kDa outer membrane protein. We have also previously reported its use to construct riboflavin mutants of the *ribBAH* riboflavin biosynthetic genes in serotypes 1 and 5 (55). A transposon mutagenesis procedure, using a mini-Tn10 derivative delivered via a conjugative suicide plasmid, can be used to produce random mutations in the chromosome of most serotypes of APP (148). This system has been successfully used to produce both tryptophan auxotrophs and hemolysin-deficient mutants of APP serotype 1 (82,148).

I

(

c

(S

th

g

su

R

1.

wh

infe

## GENETIC TECHNOLOGIES FOR THE STUDY OF BACTERIAL PATHOGENESIS

In the search for a more complete understanding of bacterial pathogenesis, new genetic techniques are emerging which can be used to identify genes that are expressed specifically during infection and not during growth on standard laboratory media. These techniques are able to identify a wide variety of gene products that are infection-associated. These genes may produce important antigens, enzymes that are necessary for production of critical growth factors not available *in vivo* (e.g., purines), factors which allow survival within host cells (e.g., enzymes that inactivate phagocytic killing mechanisms), or toxins that are not produced under standard *in vitro* conditions (104). Identification of such *in vivo* expressed genes should lead to further insights into the pathogenesis of APP disease, to the identification of important antigens for inclusion in subunit vaccines and to the development of defined avirulent mutants for use as live vaccines.

Four genetic methods for the analysis of genes involved in the pathogenesis of bacteria have been described. The first, designated *in vivo* expression technology (IVET) uses survival in the natural host to identify genes specifically turned on *in vivo* during infection (99,143). The second, designated signature-tagged mutagenesis (STM), uses negative selection with unique transposon mutants to identify mutations that adversely affect survival in the natural host (65). The third relies on a heritable genetic change promoted by the induction of the enzyme resolvase, and the fourth is subtractive hybridization (SH) which identifies differentially expressed genes based on RNA profiles.

### 1. *In vivo* expression technology:

An IVET (*in vivo* expression technology) genetic system has been developed which allows positive selection of bacterial genes that are specifically expressed during infection and has been used to identify *in vivo* induced genes in the gram negative

r
r
e

v
f
a
g
w
th
th
tr
ch
ge
ch
clo
inte
no

pathogen *Salmonella typhimurium* (99,143). The purpose of the system is to use reporter genes to identify promoters that are turned on specifically in the host. Once identified, the genes regulated by these promoters can be cloned, sequenced and studied further. There are five basic requirements for constructing an IVET system. First, it is necessary to have a biochemical mutant that attenuates growth of the organism *in vivo* resulting in lack of survival. The second requirement is to have a promoterless copy of a wild type gene from a different organism that will complement the attenuating biochemical lesion to restore growth capability when expressed *in vivo*. Third, it is necessary to have an appropriate vector allowing introduction of the DNA into the organism. Fourth, a quantifiable promoterless lacZY or similar reporter gene is needed to monitor *in vitro* expression, and finally one needs an animal model of disease.

In Mahan's system, a *purA* auxotroph of *S. typhimurium* was used. A delivery vector was constructed that contained a promoterless copy of a wild type *purA* gene fused to a promoterless *lacZY* gene, which encodes the enzyme β-galactosidase, using a broad host range suicide vector. Next, random fragments of the *S. typhimurium* genome were inserted upstream of the *purA* gene, creating transcriptional fusions that would allow any properly positioned *S. typhimurium* promoters to drive expression of the *purA:lacZY* fusion. The entire library of transcriptional fusions was introduced into the *S. typhimurium purA* auxotroph, and recombinants which contained the transcriptional fusion integrated by a single crossover event into the bacterial chromosome at the site homologous to the genomic fragment upstream of the *purA* gene were selected. Thus, the integration event generated strains where the native chromosomal promoter would drive expression of the *purA-lacZY* genes, while the cloned promoter would drive expression of the chromosomal gene at the site of integration. This assures that there would be no disruption of chromosomal genes and no loss of expression of any potential virulence factors. The pool of *S. typhimurium* strains containing integrated *purA-lacZY* fusions was injected into mice, and surviving bacteria recovered 3 days later from the mouse spleens. The environmental conditions in the host induced transcription from infection-induced promoters, thus expressing the wild type biochemical gene and relieving the attenuation. Only strains which contained a transcriptional fusion that expressed the *purA* gene *in vivo* should have survived. All recovered strains isolated from the infected mice were screened *in vitro* for expression of the *lacZY* gene. Strains which survived in mice, but which minimally expressed β-galactosidase activity *in vitro*, contained promoters that were turned on *in vivo* and off *in vitro*. From the strains identified, the *in vivo* expressed (*ivi*) genes were cloned and knock-out mutants of three different *S. typhimurium ivi* genes (*carAB*, *pheST-himA*, *rfb*) have been shown to be attenuated in mice (99).

A similar system to identify *in vivo* expressed genes has been developed for use in the intracellular pathogen *Legionella pneumophila*, using an avirulent *thyA* mutant, an independently replicating plasmid, and infection of a macrophage cell culture rather than an intact host (72,125). Other IVET selection methods for *in vivo* expressed promoters, in addition to complementation of defined biochemical mutants, have been developed for use in other bacteria. One form of selection involves the use of a promoterless antibiotic-resistance cassettes, such as chloramphenicol acetyltransferase (100). Cloned promoters are selected for expression during infection by administering the antibiotic and isolating surviving bacteria. These antibiotic selections appear to function best in tissue culture or small animals rather than in whole animal models because of the difficult pharmacokinetics. Finally, a modified IVET system has been developed for use with *Pseudomonas aeruginosa* consisting of an avirulent *purEK* mutant, plasmid pBR322, a promoterless *purEK* operon and a mouse model of infection (162). One major limitation to IVET type selections is the necessity for a high enough level of expression during the entire infection process to reisolate the organism containing an *in vivo* expressed promoter.

I

t

c

A

r

la

la

b

ic

w

3.

res

her

for

### 2. Signature-tagged mutagenesis (STM):

This system overcomes the problem of an auxotrophic or antibiotic based IVET system because genes do not have to be expressed during the entire infection process to be reisolated. In this system, transposons carrying unique DNA sequence tags are used to create a collection of mutants by insertional mutagenesis (65). The pool of mutants is used to infect a host animal, and surviving bacteria are recovered from the infected host after several days. Using the unique DNA sequence tags and appropriate PCR primers, probes can be created for colony blot hybridization. It is then possible to identify which specific members of the original mutant pool are absent upon recovery of bacteria from the host. These mutants should carry mutations in genes necessary for survival in the host. When this system was tested with *Salmonella typhimurium*, almost half of the mutations identified were in genes known to be involved in the virulence of this organism. The STM system has the power to identify genes that may be turned on only transiently. However, the STM system is at this time not applicable to *A. pleuropneumoniae*, since the transposon (Tn5) used as the basis for the system does not function in APP. Additional problems may also exist with the use of this system in larger animals. If the entire population of surviving clones is not reisolated due to the large size of the lungs or other organs, then mutants will be falsely identified as not being able to survive. A certain subset of virulence genes (e.g. toxins) will likely not be identified because other clones in the pool will express them and the transposon mutant will be able to replicate normally even though it is not able to express these genes.

### 3. Resolvase based IVET:

An extremely promising selection system uses *in vivo* induction of *tnpR* resolvase, a site-specific recombinase of the transposable element $\gamma\delta$ to induce a heritable, detectable genetic change. In this construct, expression of resolvase, even for a very brief period, results in excision of a tetracycline resistance cassette flanked by

direct repeats of the DNA sequence at which the resolvase functions (13). Any bacterial cell in which the resolvase is expressed will give rise to progeny that are tetracycline-sensitive. This selection system has the power to identify promoters that are only transiently expressed *in vivo* because all strains survive whether the cloned promoter is not turned on, turned on transiently, or turned on constitutively during infection. It has not yet been demonstrated whether this system would be functional in APP, but if so it would potentially be the most sensitive and least technical method to identify *in vivo* expressed genes. One limitation, as the system is currently designed, is the reliance on screening for the loss of antibiotic resistance after *in vivo* passage. A second limitation is the sensitivity of the system; it can be too sensitive yielding a high level of background with the false identification of genes being turned on. Finally, all promoters turned on *in vivo* will be identified, not just those that are *in vivo* specific; thus, pre-screening of the pools is required to eliminate *in vitro*-expressed clones. As a result, this system will likely not identify genes that are minimally to moderately expressed *in vitro* and induced *in vivo*.

### 4. Subtractive hybridization:

Subtractive hybridization is based on identifying differences in RNA transcripts (157) between bacteria isolated from an infected animal and bacteria grown in the laboratory. One limitation on this type of procedure is the technical difficulty in isolation and manipulation of bacterial RNA to obtain a cDNA clone. Another limitation is that subtractive hybridization results may change dramatically depending on the stage of infection at which bacteria are isolated therefore an impractical number of animals may be needed in order to truly identify all genes involved in pathogenesis. Also, this technique will tend to identify "all or none" expressed genes rather than *in vivo* induced genes which have some level of constitutive expression.

The choice of these systems for applications in different organisms will vary with the genetic tools that are functional in the organism and perhaps with the type of animal used. Each system has advantages and disadvantages, but as a whole they provide tools that will revolutionize the study of pathogenesis. In previous IVET studies in *Salmonella typhimurium* (100,143), approximately 5% of the bacteria recovered from infected animals had cloned promoters that were turned on *in vivo* and turned off *in vitro*, indicating that they were expressed specifically during infection. Many of the genes identified as having promoters specifically turned on *in vivo* or as being required for survival *in vivo* (13,65,99,162) have been genes involved in the biosynthesis of purines, pyrimidines, and essential amino acids, such as *purD, pyrE,* and *carAB*, while others have been genes known to encode virulence factors, such as *invA, invG, spvA*, *fadB*, and *virB* or even transcriptional regulators such as Fur. These infection associated genes can be divided into four different classes of *in vivo* expressed genes: 1) enzymes in critical biochemical pathways; 2) known virulence factors, or genes involved in the synthesis of such factors, such as genes for LPS biosynthesis/modification; 3) new genes, not identifiable by comparison with known sequence data; and 4) genes involved in transcriptional regulation or signal transduction. *In vivo*-expressed genes that can be identified as steps in critical biochemical pathways, such as purine biosynthesis, may be candidates for production of knock-out mutants and testing as live avirulent vaccines. Any of the genes identified by homology with virulence genes of other pathogens are likely to be of extreme interest for antibiotic and vaccine development, and further work with the unidentifiable genes will open up new horizons and lead to a better understanding of pathogenesis.

1

2.

3.

4.

5.

6.

7.

8.

9.

10.

11.

## REFERENCES

1. **Beaudet, R., G. McSween, G. Boulay, P. Rousseau, J.G. Bisaillon, J.P. Descoteaux, and R. Ruppanner.** 1994. Protection of mice and swine against infection with *Actinobacillus pleuropneumoniae* by vaccination. Vet. Microbiol. 39:71-81.

2. **Beck, M., J. F. van den Bosch, I. M. Jongenelen, P. L. Loeffen, R. Nielsen, J. Nicolet, and J. Frey.** 1994. RTX toxin genotypes and phenotypes in *Actinobacillus pleuropneumoniae* field strains. J. Clin. Microbiol. 32:2749-2754.

3. **Belanger, M., C. Begin, and M. Jacques.** 1995. Lipopolysaccharides of *Actinobacillus pleuropneumoniae* bind pig hemoglobin. Infect. Immun. 63:656-662.

4. **Belanger, M., D. Dubreuil, J. Harel, C. Girard, and M.Jacques.** 1990. Role of lipopolysaccharides in adherence of *Actinobacillus pleuropneumoniae* to porcine tracheal rings. Infect. Immun. 58:3523-3530.

5. **Belanger, M., D. Debreuil, and M. Jacques.** 1994. Proteins found within porcine respiratory tract secretions bind lipopolysaccharides of *Actinobacillus pleuropneumoniae*. Infect. Immun. 62:868-873.

6. **Belanger, M., S. Rioux, B. Foiry, and M. Jacques.** 1992. Affinity for porcine respiratory tract mucus is found in some isolates of *Actinobacillus pleuropneumoniae*. FEMS Microbiol. Lett. 97:119-126.

7. **Bendixen, P.H., P.E. Shewen, and S. Rosendal.** 1981. Toxicity of *Haemophilus pleuropneumoniae* for porcine lung macrophages, peripheral blood monocytes, and testicular cells. Infect. Immun. 33:673-676.

8. **Bertram, T.A.** 1985. Quantitative morphology of peracute pulmonary lesions in swine induced by *Haemophilus pleuropneumoniae*. Vet. Pathol. 22:598-609.

9. **Beynon, L. M., D. W. Griffith, J. C. Richards, and M. B. Perry.** 1992. Characterization of the lipopolysaccharide O-antigens of *Actinobacillus pleuropneumoniae* serotypes 9 and 11: Antigenic relationships among serotypes 9, 11, and 1. J. Bacteriol. 174:5324-5331.

10. **Bradley, S. G.** 1979. Cellular and molecular mechanisms of action of bacterial endotoxins. Ann. Rev. Microbiol. 33: 67-94.

11. **Bunka, S., C. Christensen, A. A. Potter, P. J. Willson, and G. F. Gerlach.** 1995. Cloning and characterization of a protective outer membrane lipoprotein of *Actinobacillus pleuropneumoniae* serotype 5. Infect. Immun. 63:2797-2800.

12. **Byrd, W., B.G. Harmon and S. Kadis.** 1992. Protective efficacy of conjugate vaccines against experimental challenge with porcine *Actinobacillus pleuropneumoniae*. Vet. Immunol. Immunopathol. **34**:307-324.

13. **Camilli, A. and J. J. Mekalanos.** 1995. Use of recombinase gene fusions to identify *Vibrio cholerae* genes induced during infection. Mol. Microbiol. **18**:671-683.

14. **Chang, Y.F., J. Shi, D.P. Ma, S.J. Shin, and D.H. Lein.** 1993. Molecular analysis of the *Actinobacillus pleuropneumoniae* RTX toxin-III gene cluster. DNA Cell. Biol. **12**:351-362.

15. **Chang, Y.F., R.Y. Young, and D.K. Struck.** 1991. The *Actinobacillus pleuropneumoniae* hemolysin determinant: unlinked *appCA* and *appBD* loci flanked by pseudogenes. J. Bacteriol. **173**:5151-5158.

16. **Chang, Y.F., R. Young, and D.K. Struck.** 1989. Cloning and characterization of a hemolysin gene from *Actinobacillus (Haemophilus) pleuropneumoniae*. DNA **8**:635-647.

17. **Chiang, Y.W., T.F. Young, V.J. Rapp-Gabrielson, and R.F.Ross.** 1991. Improved protection of swine from pleuropneumonia by vaccination with proteinase K-treated outer membrane of *Actinobacillus (Haemophilus) pleuropneumoniae*. Vet. Microbiol. **27**:49-62.

18. **Chung, W.B., L. Backstrom, J. McDonald, and M.T. Collins.** 1993. *Actinobacillus pleuropneumoniae* culture supernatants interfere with killing of *Pasteurella multocida* by swine pulmonary alveolar macrophages. Can. J. Vet. Res. **57**:190-197.

19. **Cruijsen, T., L.A. van Leengoed, M. Ham-Hoffies, and J.H. Verheijden.** 1995. Convalescent pigs are protected completely against infection with a homologous *Actinobacillus pleuropneumoniae* strain but incompletely against a heterologou-serotype strain. Infect. Immun. **63**:2341-2343.

20. **Cruijsen, T., L.A.M.G. vanLeengoed, E.M. Kamp, A. Bartelse, A. Korevaar and J.H.M. Verheijden.** 1995. Susceptibility to *Actinobacillus pleuropneumoniae* infection in pigs from an endemically infected herd is related to the presence of toxin neutralizing antibodies. Vet. Microbiol. **47**:219-228.

21. **Cruijsen, T., L.A.M.G. van Leengoed, E.M. Kamp, W.A. Hunneman, K. Riepema, A. Bartelse and J.H.M. Verheijden.** 1995. Prevalence and development of antibodies neutralizing the haemolysin and cytotoxin of *Actinobacillus pleuropneumoniae* in three infected pig herds. Vet. Quarterly. **17**:96-100.


2
2
2
3
31
32

22. **Cruz, W.T., Y. A. Nedialkov, B.J. Thacker, and M.H. Mulks**. 1996. Molecular characterization of a common 48 kDa outer membrane protein of *Actinobacillus pleuropneumoniae*. Infect. Immun. **64**: 83-90.

23. **Cullen, J. M., and A. N. Rycroft**. 1994. Phagocytosis by pig alveolar macrophages of *Actinobacillus pleuropneumoniae* serotype 2 mutant strains defective in haemolysin II (ApxII) and pleurotoxin (ApxIII). Microbiology **140**:237-244.

24. **Cybulsky, M. I., M. K. W. Chan, and H. Z. Movat**. 1988. Acute inflammation and microthrombosis induced by endotoxin, interleukin-1, and tumor necrosis factor and their implication in gram negative infections. Lab. Invest. **58**:365-378.

25. **Daban, M., A. Medrano, and E. Querol**. 1996. Cloning, sequencing and expression of the transferrin-binding protein 1 gene from Actinobacillus pleuropneumoniae. Biochem. J. **315**:257-264.

26. **Deneer, H.G., and A.A. Potter**. 1989. Effect of iron restriction on the outer membrane proteins of *Actinobacillus (Haemophilus) pleuropneumoniae*. Infect. Immun. **57**:798-804.

27. **Deneer, H.G., and A.A. Potter**. 1989. Identification of a maltose-inducible major outer membrane protein in *Actinobacillus (Haemophilus) pleuropneumoniae*. Microb. Pathog. **6**:425-432.

28. **Devenish, J., and S. Rosendal**. 1991. Calcium binds to and is required for biological activity of the 104-kilodalton hemolysin produced by *Actinobacillus pleuropneumoniae* serotype 1. Can. J. Microbiol. **37**:317-321.

29. **Devenish, J., S. Rosendal, and J.T. Bosse**. 1990. Humoral antibody response and protective immunity in swine following immunization with the 104-kilodalton hemolysin of *Actinobacillus pleuropneumoniae*. Infect. Immun. **58**:3829-3832.

30. **Didier, P. J., L. Perino, and J. Urbance**. 1984. Porcine *Haemophilus* pleuropneumonia: Microbiologic and pathologic findings. J. Am. Vet. Med. Assoc. **184**:716-719.

31. **Dom, P., F. Haesebrouck, and P. DeBaetselier**. 1992. Stimulation and suppression of oxygenation activity of porcine pulmonary alveolar macrophages by *Actinobacillus pleuropneumoniae* and its metabolites. Am. J. Vet. Res. **53**:1113-1118.

32. **Dom, P., F. Haesebrouck, R. Ducatelle, and G. Charlier**. 1994. *In vivo* association of *Actinobacillus pleuropneumoniae* serotype 2 with the respiratory epithelium of pigs. Infect. Immun. **62**:1262-1267.

3

3

3

3

3

3

3

4

4

42

43

33. **Dom, P., F. Haesebrouck, E.M. Kamp, and M.A. Smits**. 1992. Influence of *Actinobacillus pleuropneumoniae* serotype 2 and its cytolysins on porcine neutrophil chemiluminescence. Infect. Immun. **60**:4328-4334.

34. **Drum, S., W.E. Marsh, M.A. Mellencamp and R.D. Walker**. 1996. Effects of segregated early weaning on growth performance of pigs from nursery to slaughter. Abstracts, 77th Conference of Research Workers in Animal Diseases, abstract #64.

35. **Fedorka-Cray, P.J., M.J. Huether, D.L. Stine, and G.A. Anderson**. 1990. Efficacy of a cell extract from *Actinobacillus (Haemophilus) pleuropneumoniae* serotype 1 against disease in swine. Infect. Immun. **58**:358-365.

36. **Fedorka-Cray, P.J., D.L.Stine, J.M. Greenwald, J.T. Gray, M.J. Heuther, and G. A. Anderson**. 1993. The importance of secreted virulence factors in *Actinobacillus pleuropneumoniae* bacterin preparation: a comparison. Vet. Microbiol. **37**:85-100.

37. **Fenwick, B. W., J. S. Cullor, B. I. Osburn, and H. J. Olander**. 1986. Mechanisms involved in protection provided by immunization against core lipopolysaccharides of *Escherichia coli* J5 from lethal *Haemophilus pleuropneumoniae* infections in swine. Infect. Immun. **53**:298-304.

38. **Fenwick, B., and S. Henry**. 1994. Porcine pleuropneumonia. J. Am. Vet. Med. Assoc. **204**:1334-1340.

39. **Fenwick, B. W., and B. I. Osburn**. 1986. Immune responses to the lipopolysaccharides and capsular polysaccharides of *Haemophilus pleuropneumoniae* in convalescent and immunized pigs. Infect. Immun. **54**:575-582.

40. **Fenwick, B. W., B. I. Osburn, J. S. Cullor, S. C. Henry, and H. J. Olander**. 1986. Mortality in swine herds endemically infected with *Haemophilus pleuropneumoniae*: Effect of immunization with cross-reacting lipopolysaccharide core antigens of *Escherichia coli*. Am. J. Vet. Res. **47**:1888-1891.

41. **Fenwick, B. W., B. I. Osburn, and H. J. Olander**. 1986. Isolation and biological characterization of two lipopolysaccharides and a capsular-enriched polysaccharide preparation from *Haemophilus pleuropneumoniae*. Am. J. Vet. Res. **47**:1433-1441.

42. **Frey, J.** 1992. Construction of a broad host range shuttle vector for gene cloning and expression in *Actinobacillus pleuropneumoniae* and other *Pasteurellaceae*. Res. Microbiol. **143**: 263-269.

43. **Frey, J., M. Beck, U. Stucki, and J. Nicolet**. 1993. Analysis of hemolysin operons in *Actinobacillus pleuropneumoniae*. Gene **123**:51-58.

44. **Frey, J., J.T. Bosse, Y.F. Chang, J.M. Cullen, B. Fenwick, G.F. Gerlach, D. Gygi, F. Haesebrouck, T.J. Inzana, R. Jansen, et al.** 1993. *Actinobacillus pleuropneumoniae* RTX-toxins: uniform designation of haemolysins, cytolysins, pleurtoxin, and their genes. J. Gen. Microbiol. **139**:1723-1728.

45. **Frey, J., J.B. Deillon, D. Gygi, and J. Nicolet.** 1991. Identification and partial characterization of the hemolysin (HlyII) of *Actinobacillus pleuropneumoniae* serotype 2. Vet. Microbiol. **28**:303-312.

46. **Frey, J., A. Haldimann, J. Nicolet, A. Boffini, and P. Prentki.** 1994. Sequence analysis and transcription of the *apxI* operon (hemolysin I) from *Actinobacillus pleuropneumoniae*. Gene **142**:97-102.

47. **Frey, J., R. Kuhn, and J. Nicolet.** 1994. Association of the CAMP phenomenon in *Actinobacillus pleuropneumoniae* with the RTX toxins ApxI, ApxII and ApxIII. FEMS Microbiol. Lett. **124**:245-251.

48. **Frey, J., P. Kuhnert, L. Villiger and J. Nicolet.** 1996. Cloning and characterization of an Actinobacillus pleuropneumoniae outer membrane protein beloning to the family of PAL lipoproteins. Res. Microbiol. **147**:351-361.

49. **Frey, J., R. Meier, D. Gygi, and J. Nicolet.** 1991. Nucleotide sequence of the hemolysin I gene from *Actinobacillus pleuropneumoniae*. Infect. Immun. **59**:3026-3032.

50. **Frey, J., and J. Nicolet.** 1990. Hemolysin patterns of *Actinobacillus pleuropneumoniae*. J. Clin. Microbiol. **28**:232-236.

51. **Frey, J., and J. Nicolet.** 1988. Purification and partial characterization of a hemolysin produced by *Actinobacillus pleuropneumoniae* type strain 4074. FEMS Microbiol. Lett. **55**:41-46.

52. **Frey, J., and J. Nicolet.** 1988. Regulation of hemolysin expression in *Actinobacillus pleuropneumoniae* serotype 1 by $Ca^{2+}$. Infect. Immun. **56**: 2570-2575.

53. **Frey, J., J. Perrin, and J. Nicolet.** 1989. Cloning and expression of a cohemolysin, the CAMP factor of *Actinobacillus pleuropneumoniae*. Infect. Immun. **57**:2050-2056.

54. **Frey, J., H. van den Bosch, R. Segers, and J. Nicolet.** 1992. Identification of a second hemolysin (HlyII) in *Actinobacillus pleuropneumoniae* serotype 1 and expression of the gene in *Escherichia coli*. Infect. Immun. **60**:1671-1676.

55. **Fuller, T.E., B.J. Thacker and M.H. Mulks.** 1996. A riboflavin auxotroph of *Actinobacillus pleuropneumoniae* is attenuated in swine. Infect. Immun. **64**:4659-4664

56. **Gerlach, G.F., C. Anderson, S. Klashinsky, A. Rossi-Campos, A.A. Potter, and P.J. Willson**. 1993. Molecular characterization of a protective outer membrane lipoprotein (OmlA) from *Actinobacillus pleuropneumoniae* serotype 1. Infect. Immun. **61**:565-572.

57. **Gerlach, G.F., C. Anderson, A.A. Potter, S. Klashinsky, and P.J. Willson**. 1992. Cloning and expression of a transferrin-binding protein from *Actinobacillus pleuropneumoniae*. Infect. Immun. **60**:892-898.

58. **Gerlach, G.-F., C. Anderson, A. Rossi-Campos, and A.A.Potter**. 1992. The role of the 103 Kd *Actinobacillus pleuropneumoniae* cytolysin in virulence and the association of the encoding gene with insertion sequence-like elements. Proc. Int. Pig Vet. Soc. **12**:199.

59. **Gerlach, G.F., S. Klashinsky, C. Anderson, A.A. Potter, and P.J. Willson**. 1992. Characterization of two genes encoding distinct transferrin-binding proteins in different *Actinobacillus pleuropneumoniae* isolates. Infect. Immun. **60**:3253-3261.

60. **Green, J., A.D. Sharrocks, J.I. MacInnes, and J.R. Guest**. 1992. Purification of HlyX, a potential regulator of haemolysin synthesis, and properties of HlyX:FNR hybrids. Proc. R. Soc. Lond. B. Biol. Sci. **248**:79-84.

61. **Griffiths, E**. 1991. Environmental Regulation of Bacterial Virulence-Implications for Vaccine Design and Production. Trends in Biotechnology **9**:309-315.

62. **Gross, R**. 1993. Signal Transduction and Virulence Regulation in Human and Animal Pathogens. FEMS Micro. Rev. **104**:301-326

63. **Gygi, D., J. Nicolet, C. Hughes, and J. Frey**. 1992. Functional analysis of the Ca(2+)-regulated hemolysin I operon of *Actinobacillus pleuropneumoniae* serotype 1. Infect. Immun. **60**:3059-3064.

64. **Hartmann, L., W. Schroder, and A. Lubke**. 1995. Isolation of the major outer-membrane protein of *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis*. J. Vet. Med. B. **42**:59-63.

65. **Hensel, M. J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden**. 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science **269**:400-403.

66. **Hodgson, A.L.M., C. L. Wright, C.A. Podgson, R.A. Strugnell and C.T. Prideaux**. 1996. Genetic manipulation of Haemophilus, Actinobacillus and Pasteurella (HAP). Proc, HAP-96, abstract #21.

67. **Hunneman, W.A**. 1986. Incidence, economic effects, and control of *Haemophilus pleuropneumoniae* infections in pigs. Vet. Q. **8**:83-87.

6

6

7

7

7

7

7

7

76

77

78

68. **Inzana, T. J.** 1991. Virulence properties of *Actinobacillus pleuropneumoniae*. Microb. Path. **11**: 305-316.

69. **Inzana, T. J., J. Ma, T. Workman, R. P. Gogolewski, and P. Anderson.** 1988. Virulence properties and protective efficacy of the capsular polymer of *Haemophilus (Actinobacillus) pleuropneumoniae* serotype 5. Infect. Immun. **56**: 1880-1889.

70. **Inzana, T. J., J. Todd, J. Ma, and H. Veit.** 1991. Characterization of a non-hemolytic mutant of *Actinobacillus pleuropneumoniae* serotype 5: role of the 110 kilodalton hemolysin in virulence and immunoprotection. Microb. Path. **10**:281-296.

71. **Inzana, T. J., J. Todd, and H. P. Veit.** 1993. Safety, stability, and efficacy of nonencapsulated mutants of *Actinobacillus pleuropneumoniae* for use in live vaccines. Infect. Immun. **61**:1682-1686.

72. **Isberg, R.R.** 1994. Intracellular trafficking of *Legionella pneumophila* within phagocytic cells, pp.263-278. *In* V.L. Miller et al (ed). Molecular Genetics of Bacterial Pathogenesis. ASM Press, Washington, DC.

73. **Ishii, H., T. Fukuyasu, S. Iyobe, and H. Hashimoto.** 1993. Characterization of newly isolated plasmids from *Actinobacillus pleuropneumoniae*. Am. J. Vet. Res. **54**:701-708.

74. **Ishii, H., F. Hayashi, S. Iyobe, and H. Hashimoto.** 1991. Characterization and classification of *Actinobacillus (Haemophilus) pleuropneumoniae* plasmids. Am. J. Vet. Res. **52**:1816-1820.

75. **Ito, H., I. Uchida, T. Sekizaki, E. Ooishi, T. Kawai, T. Okabe, A. Taneno, and N. Terakado.** 1995. Molecular cloning of an *Actinobacillus pleuropneumoniae* outer membrane lipoprotein (OmlA) from serotype 5a. Microb. Pathog. **18**:29-36.

76. **Ito, H., I. Uchida, T. Sekizaki, and N. Terakado.** 1993. A cryptic DNA sequence, isolated from *Actinobacillus pleuropneumoniae*, confers a hemolytic activity upon *Escherichia coli* K12 strains. J. Vet. Med. Sci. **55**:173-175.

77. **Jacques, M., M. Belanger, G. Roy, and B. Foiry.** 1991. Adherence of *Actinobacillus pleuropneumoniae* to porcine tracheal epithelial cells and frozen lung sections. Vet. Microbiol. **27**:133-143.

78. **Jansen, R., J. Briaire, E. M. Kamp, A. L. Gielkins, and M. A. Smits.** 1995. The CAMP effect of *Actinobacillus pleuropneumoniae* is caused by Apx toxins. FEMS Microbiol. Lett. **126**:139-143.

7

8

8

8

8

8

8

8

8

88

89.

90.

79. **Jansen, R., J. Briaire, E.M. Kamp, A.L. Gielkens, and M.A. Smits**. 1993. Cloning and characterization of the *Actinobacillus pleuropneumoniae*-RTX-toxin III (ApxIII) gene. Infect. Immun. **61**:947-54.

80. **Jansen, R., J. Briaire, E. M. Kamp, A.L. Gielkens, and M.A. Smits**. 1993. Structural analysis of the *Actinobacillus pleuropneumoniae*-RTX-toxin I (ApxI) operon. Infect. Immun. **61**:3688-3695.

81. **Jansen, R., J. Briaire, E.M. Kamp, and M.A. Smits**. 1992. Comparison of the cytolysin II genetic determinants of *Actinobacillus pleuropneumoniae* serotypes. Infect. Immun. **60**:630-636.

82. **Jansen, R., J. Briaire, H.E. Smith, P. Dom, F. Haesebrouck, E.M. Kamp, A.L.J. Gielkens, and M.A. Smits**. 1995. Knockout mutants of *Actinobacillus pleuropneumoniae* serotype 1 that are devoid of RTX toxins do not activate or kill porcine neutrophils. Infect. Immun. **63**:27-37.

83. **Jansen, R., J. Briaire, A. B. van Geel, E. M. Kamp, A. L. Gielkins, and M. A. Smits**. 1994. Genetic map of the *Actinobacillus pleuropneumoniae* RTX-toxin (Apx) operons: characterization of the ApxIII operons. Infect. Immun. **62**:4411-4418.

84. **Jolie, R.A.V., M. H. Mulks, and B. J. Thacker**. 1994. Antigenic differences within *Actinobacillus pleuropneumoniae* serotype 1. Vet. Microbiol. **38**:329-349.

85. **Kamp, E.M., J. K. Popma, J. Anakotta, and M. A. Smits**. 1991. Identification of hemolytic and cytotoxic proteins of *Actinobacillus pleuropneumoniae* by use of monoclonal antibodies. Infect. Immun. **59**:3079-3085.

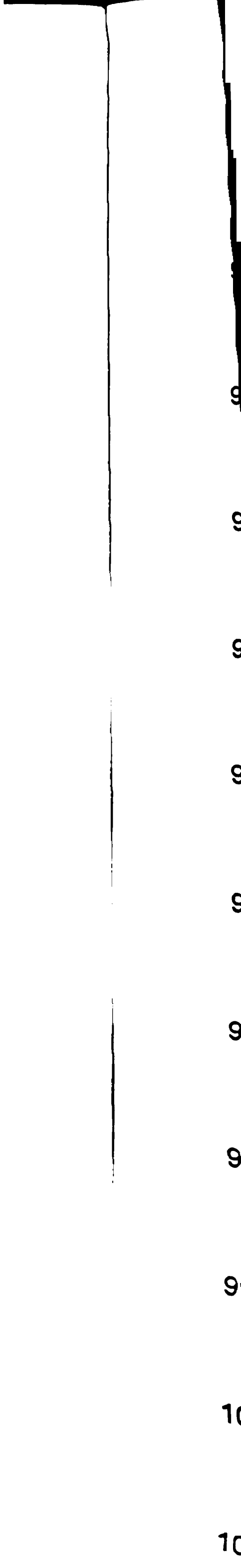
86. **Kamp, E.M., and L. A. van Leengoed**. 1989. Serotype-related differences in production and type of heat-labile hemolysin and heat-labile cytotoxin of *Actinobacillus (Haemophilus) pleuropneumoniae*. J. Clin. Microbiol. **27**:1187-1191.

87. **Kamp, E.M., T.M.M. Vermeulen, T. Cruysen, J.E. van Dijk, K. Riepema, M.A. Smits, and J.M. van Leeuwen**. 1992. *Actinobacillus pleuropneumoniae* cytotoxins: Cross protective effect of a crude cytolysin vaccine. Proc. Int. Pig Vet. Soc. 12:209.

88. **Kamp, E.M., T.M.M.Vermeulen, M.A.Smits, and J. Haagsma**. 1994. Production of Apx toxins by field strains of *Actinobacillus pleuropneumoniae* and *Actinobacillus suis*. Infect. Immun. **62**: 4063-4065.

89. **Kobisch, M., and J. F. van den Bosch**. 1992. Efficacy of an *Actinobacillus pleuropneumoniae* subunit vaccine. Proc. Int. Pig. Vet. Soc. 12:216.

90. **Lalonde, G., T.V. McDonald, P. Gardner, and P.D. O'Hanley**. 1989. Identification of a hemolysin from *Actinobacillus pleuropneumoniae* and



characterization of its channel properties in planar phospholipid bilayers. J. Biol. Chem. **264**:13559-13564.

91. **Lalonde, G., J. F. Miller, L. S. Tompkins, and P. D. O'Hanley.** 1989. Transformation of *Actinobacillus pleuropneumoniae* and analysis of R factors by electroporation. Am. J. Vet. Res. **50**:1957-1960.

92. **Lalonde, G., and P. D. O'Hanley.** 1989. Development of a shuttle vector and a conjugative transfer system for *Actinobacillus pleuropneumoniae*. Gene **85**:243-246.

93. **Lian, C.J., S. Rosendal, and J. I. MacInnes.** 1989. Molecular cloning and characterization of a hemolysin gene from *Actinobacillus (Haemophilus) pleuropneumoniae*. Infect. Immun. **57**:3377-3382.

94. **Liggett, A. D., L. R. Harrison, and R. L. Farrell.** 1987. Sequential study of lesion development in experimental haemophilus pleuropneumonia. Res. Vet. Sci. **42**:204-221.

95. **MacDonald, J., and A. N. Rycroft.** 1993. *Actinobacillus pleuropneumoniae* haemolysin II is secreted from *Escherichia coli* by *A. pleuropneumoniae* pleurotoxin secretion gene products. FEMS Microbiol. Lett. **109**:317-322.

96. **MacDonald, J., and A.N. Rycroft.** 1992. Molecular cloning and expression of *ptxA*, the gene encoding the 120-kilodalton cytotoxin of *Actinobacillus pleuropneumoniae* serotype 2. Infect. Immun. **60**:2726-2732.

97. **MacInnes, J.I., J. E. Kim, C.-J. Lian, and G.A. Soltes.** 1990. *Actinobacillus pleuropneumoniae hlyX* gene homology with the *fnr* gene of *Escherichia coli*. J. Bacteriol. **172**:4587-4592.

98. **MacInnes, J.I., and S. Rosendal.** 1987. Analysis of major antigens of *Haemophilus (Actinobacillus) pleuropneumoniae* and related organisms. Infect. Immun. **55**:1626-1634.

99. **Mahan, M.J., J.M. Slauch, and J.J.Mekalanos.** 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259**:686-688.

100. **Mahan, M.J., J.W. Tobias, J.M. Slauch, P. C. Hanna, R. J. Collier, and J. J. Mekalanos.** 1995. Antibiotic-based selection for bacterial genes that are specifically induced during infection of a host. PNAS **92**:669-673.

101. **Maier, E., N. Reinhard, R. Benz and J. Frey.** 1996. Channel-forming activity and channel size of the RTX toxins ApxI, ApxII, and ApxIII of *Actinobacillus pleuropneumoniae*. Infect. Immun. **64**:4415-4423.

1

1

1

1

10

10

11

11

112

113

114

115

102. **Matthews, P. R. J., and I. H Pattison**. 1961. The identification of a *Haemophilus*-like organism associated with pneumonia and pleurisy in the pig. J. Comp. Pathol. **71**:44-52.

103. **McWhinney, D.R., Y-F. Chang, R. Young, and D.K.Struck**. 1992. Separable domains define target cell specificities of an RTX hemolysin from *Actinobacillus pleuropneumoniae*. J. Bacteriol. **174**:291-297.

104. **Mekalanos, J.J.** 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. **174**:1-7.

105. **Morrison, D. C., and J. L. Ryan**. 1987. Endotoxins and disease mechanisms. Ann. Rev. Med. **38**:417-432.

106. **Mulks, M.H.** Unpublished data.

107. **Mulks, M.H., and J. M. Buysse**. 1995. A targeted mutagenesis system for *Actinobacillus pleuropneumoniae*. Gene **165**:61-66.

108. **Mulks, M.H., D.Y. Hunter-Simon, J.W.Sprague, & B.J.Thacker**. 1986. Identification of immunogenic outer membrane proteins of *Haemophilus pleuropneumoniae* in infected swine. Proc. Int. Pig Vet. Soc. **9**:266.

109. **Mulks, M.H., and B.J.Thacker**. 1988. Efficacy of an *Haemophilus pleuropneumoniae* outer membrane subunit vaccine in swine. Proc. Int. Pig Vet Soc. **10**:81.

110. **Nakai, T., E. Ono, K. Ike, and K. Kume**. 1992. Serotyping of *Actinobacillus pleuropneumoniae* strains by use of purified capsular polysaccharide or lipopolysaccharide. Proc. Int. Pig Vet. Soc. **12**:186.

111. **Narita, M., K. Kawashima, T. Morozumi and H. Takashima**. 1995. Effect of physical defenses of the respiratory tract on the development of pneumonia in pigs inoculated endobronchially with *Actinobacillus pleuropneumoniae*. J. Vet. Med. Sci. **57**:839-844.

112. **Nicolet, J.** 1992. Actinobacillus pleuropneumoniae. pp. 401-408. In A. D. Leman et al, eds. Diseases of swine. Iowa State University Press, Ames, Iowa.

113. **Nielsen, R.** 1984. *Haemophilus pleuropneumoniae* serotypes - Cross protection experiments. Nord. Vet. Med. **36**:221-234.

114. **Nielsen, R.** 1979. *Haemophilus parahaemolyticus* serotypes: pathogenicity and cross immunity. Nord. Vet. Med. **31**:407-413.

115. **Nielsen, R.** 1976. Pleuropneumonia of swine caused by *Haemophilus parahaemolyticus*. Studies on the protection obtained by vaccination. Nord. Vet. Med. **28**:337-338.

116. **Nielsen, R.** 1974. Serological and immunological studies of pleuropneumonia of swine caused by *Haemophilus parahaemolyticus*. Acta Vet. Scand. **15**:80-89.

117. **Niven, D.F., J. Donga, and F.S. Archibald.** 1989. Responses of *Haemophilus pleuropneumoniae* to iron restriction: changes in the outer membrane protein profile and the removal of iron from porcine transferrin. Mol. Microbiol. **3**:1083-1089.

118. **Oishi, E., T. Kitajima, Y. Koyama, T. Ohgitani, S. Katayama, and T. Okabe.** 1995. Protective effect of the combined vaccine prepared from cell-free-antigen of Actinobacillus pleuropneumoniae serotypes 1, 2 and 5 in pigs. J. Vet. Med. Sci. **57**:1125-1128.

119. **Olander, H.J.** 1963. A septicaemic disease of swine and its causative agent *Haemophilus parahaemolyticus*. Ph.D. thesis, U. California.

120. **O'Reilly, T., D.F. Niven, and M.R. Brown.** 1991. Phenotypic variation in the outer membrane protein composition of *Actinobacillus (Haemophilus) pleuropneumoniae*: non-specific effect of exogenous pyridine nucleotide supply. Vet. Microbiol. **29**:159-172.

121. **Paltineanu, D., R. Pambucol, E. Tirziu, and I. Scobercea.** 1992. Swine infectious pleuropneumonia: Aerosol vaccination with a live attenuated vaccine. Proc. Int. Pig. Vet. Soc. **12**:214.

122. **Paradis, S.E., D. Debreuil, S. Rioux, M. Gottschalk, and M. Jacques.** 1994. High-molecular-mass lipopolysaccharides are involved in *Actinobacillus pleuropneumoniae* adherence to porcine respiratory tract cells. Infect. Immun. **62**:3311-3319.

123. **Pattison, I. H., D. G. Howell, and J. Elliot.** 1957. A *Haemophilus*-like organism isolated from pig lung and the associated pneumonic lesions. J. Comp. Path. **67**:320-329.

124. **Perry, M.B., E. Altman, J.-R. Brisson, L. M. Beynon, and J. C. Richards.** 1990. Structural characteristics of the antigenic capsular polysaccharides and lipopolysaccharides involved in the serological classification of *Actinobacillus pleuropneumoniae* strains. Serodiag. Immunother. Infect. Dis. **4**:299-308.

125. **Rankin, S., and R. R. Isberg.** 1993. Identification of *Legionella pneumophila* loci expressed in response to an intracellular environment. Infect. Agents Dis. **2**:269-271.

126. **Rapp, V.J., R.S.Munson, & R.F.Ross.** 1986. Outer membrane profiles of *Haemophilus pleuropneumoniae*. Infect. Immun. **52**:414-420.

127. **Rapp, V.J., and R.F. Ross.** 1988. Immunogenicity of outer membrane components of *Haemophilus (Actinobacillus) pleuropneumoniae*. Can. Vet. J. **29**:585-587.

128. **Ricard, M.A., F.S. Archibald, and D.F. Niven.** 1991. Isolation and identification of a putative porcine transferrin receptor from *Actinobacillus pleuropneumoniae* biotype 1. J. Gen. Microbiol. **137**:2733-2740.

129. **Rosendal, S., D.S. Carpenter, W.R. Mitchell, and M.R. Wilson.** 1981. Vaccination against pleuropneumonia in pigs caused by *Haemophilus pleuropneumoniae*. Can. Vet. J. **22**:34-35.

130. **Rosendal, S., J. Devenish, J.I. MacInnes, J.H. Lumsden, S. Watson, and H. Xun.** 1988. Evaluation of heat-sensitive, neutrophil-toxic, and hemolytic activity of *Haemophilus (Actinobacillus) pleuropneumoniae*. Am. J. Vet. Res. **49**:1053-1058.

131. **Rosendal, S., and J. I. MacInnes.** 1990. Characterization of an attenuated strain of *Actinobacillus pleuropneumoniae*, serotype 1. Am. J. Vet. Res. **51**:711-717.

132. **Rosendal, S., O. P. Miniats, and P. Sinclair.** 1986. Protective efficacy of capsular extracts of *Haemophilus pleuropneumoniae* in pigs and mice. Vet. Microbiol. **12**:229-240.

133. **Rossi-Campos, A., C. Anderson, G.F. Gerlach, S. Klashinsky, A.A. Potter, and P.J. Willson.** 1992. Immunization of pigs against *Actinobacillus pleuropneumoniae* with two recombinant protein preparations. Vaccine. **10**:512-518.

134. **Rycroft, A. N., and J. M. Cullen.** 1990. Complement resistance in *Actinobacillus (Haemophilus) pleuropneumoniae* infection of swine. Am. J. Vet. Res. **51**:1449-1453.

135. **Rycroft, A.N., and D.J. Taylor.** 1987. Preparation and characterization of envelope proteins from *Haemophilus pleuropneumoniae*. Vet. Microbiol. **15**:303-314.

136. **Rycroft, A.N., D. Williams, J.M. Cullen, and J. Macdonald.** 1991. The cytotoxin of *Actinobacillus pleuropneumoniae* (pleurotoxin) is distinct from the haemolysin and is associated with a 120 kDa polypeptide. J. Gen. Microbiol. **137**:561-568.

137. **Rycroft, A.N., D. Williams, I.A. McCandlish, and D.J. Taylor.** 1991. Experimental reproduction of acute lesions of porcine pleuropneumonia with a haemolysin-deficient mutant of *Actinobacillus pleuropneumoniae*. Vet. Rec. **129**:441-443.

138. **Sakano, T., I. Shibata, Y. Samegai, A. Taneda, M. Okada, T. Irisawa, and S. Sato**. 1993. Experimental pneumonia of pigs infected with Aujesky's disease virus and *Actinobacillus pleuropneumoniae*. J. Vet. Med. Sci. **55**:575-579.

139. **Sanford, S. E., and G. K. A. Josephson**. 1981. Porcine *Haemophilus pleuropneumoniae* epizootic in Southwestern Ontario: Clinical, microbiological, pathological, and some epidemiologic findings. Can. J. Comp. Med. **45**:2-7.

140. **Scholz, P., V. Haring, B. Wittmann-Liebold, K. Ashman, M. Bagdasarian, and E. Scherzinger**. 1989. Complete nucleotide sequence and gene organization of the broad host range plasmid RSF1010. Gene **75**:271-288.

141. **Sebunya, T.N.K. and Saunders, J.R.** 1983. *Haemophilus pleuropneumoniae* Infection in Swine: A Review. J. Am. Vet. Med. Assoc. **182**:1331-1337.

142. **Shope, R.E.** 1964. Porcine contagious pleuropneumonia. I. Experimental transmission, etiology, and pathology. J. Exp. Med. **119**:357-368.

143. **Slauch, J.M., M.J. Mahan, and J.J. Mekalanos**. 1994. *In vivo* expression technology for selection of bacterial genes specifically induced in host tissues. Meth. Enzymol. **235**:481-492.

144. **Smits, M.A., J. Briaire, R. Jansen, H.E. Smith, E. M. Kamp. and A.L. Gielkens**. 1991. Cytolysins of *Actinobacillus pleuropneumoniae* serotype 9. Infect. Immun. **59**:4497-4504.

145. **Soltes, G., and J.I. MacInnes**. 1994. Regulation of gene expression by the HlyX protein of *Actinobacillus pleuropneumoniae*. Microbiology **140**:839-845.

146. **Stine, D. L., P. J. Fedorka-Cray, M. J. Huether, M. J. Gentry, and G. A. Anderson**. 1994. Comparison of serum responses in swine after vaccination and challenge exposure with *Actinobacillus pleuropneumoniae*. Am. J. Vet. Res. **55**:1238-1243.

147. **Tarigan, S., R. F. Slocombe, G. F. Browning, and W. Kimpton**. 1994. Functional and structural changes of porcine alveolar macrophages induced by sublytic doses of a heat-labile, hemolytic, cytotoxic substance produced by *Actinobacillus pleuropneumoniae*. Am. J. Vet. Res. **55**:1548-1557.

148. **Tascon, R. I., E. F. Rodriguez-Ferri, C. B. Gutierrez-Martin, I. Rodriguez-Barbosa, P. Berche, and J. A. Vazquez-Boland**. 1993. Transposon mutagenesis in *Actinobacillus pleuropneumoniae* with a Tn*10* derivative. J. Bacteriol. **175**:5717-5722.

149. **Tascon, R.I., J.A. Vazquez-Boland, C.B. Gutierrez-Martin, I. Rodriguez-Barbosa, and E.F. Rodriguez-Ferri**. 1994. The RTX haemolysins ApxI and ApxII are major virulence factors of the swine pathogen *Actinobacillus*

*pleuropneumoniae*: evidence from mutational analysis. Mol. Microbiol. 14:207-216.

150. **Thacker, B.J., M.H. Mulks, and R.A.V Jolie.** 1992. Development of improved *Actinobacillus pleuropneumoniae* vaccines. Proc. Int. Pig Vet Soc. 12:193.

151. **Thacker, B.J., M.H. Mulks, F. Udeze, and R.R. Simonson.** 1994. Development of disrupted whole-cell *Actinobacillus pleuropneumonaie* (APP) vaccines. HAP-94 Proc., abstract # P35.

152. **Tu, A.H.T., C. Hausler, R. Young, and D.K. Struck.** 1994. Differential expression of the cytotoxic and hemolytic activities of the ApxIIA toxin from *Actinobacillus pleuropneumoniae*. Infect. Immun. 62:2119-2121.

153. **Udeze, F.A., and S. Kadis.** 1992. Effects of *Actinobacillus pleuropneumoniae* hemolysin on porcine neutrophil function. Infect. Immun. 60:1558-1567.

154. **Udeze, F.A., K. S. Latimer, and S. Kadis.** 1987. Role of *Haemophilus pleuropneumoniae* lipopolysaccharide endotoxin in the pathogenesis of porcine *Haemophilus* pleuropneumonia. Am. J. Vet. Res. 48:768-773.

155. **Utrera, V., and C. Pijoan.** 1991. Fimbriae in *A. pleuropneumoniae* strains isolated from pig respiratory tracts. Vet. Rec. 128:357-358.

156. **Utrera, V., C. Pijoan, and T. Molitor.** 1992. Evaluation of the immunity induced in pigs after infection with a low virulence strain of *A. pleuropneumoniae* serotype 1. Proc. Int. Pig. Vet. Soc. 12:213.

157. **Utt, E.A., J.P. Brousal, L.C. Kikuta-Oshima and F.D. Quinn.** 1995. The identification of bacterial gene expression differences using mRNA-based isothermal subtractive hybridisation. Can. J. Microbiol. 41:152-156.

158. **van den Bosch, J.F., I.M.C.A. Jongenelen, A.N.B. Pubben, F.G.A. van Vugt, and R.P.A.M. Segers.** 1992. Protection induced by a trivalent *A. pleuropneumoniae* subunit vaccine. Proc. Int. Pig. Vet. Soc. 12:194.

159. **van den Bosch, J.F., A.N.B. Pubben, F.G.A. van Vugt, and R.P.A.M. Segers.** 1994. Synergistic effect of a P2-homologous outer membrane protein and ApxI toxin in protection against *Actinobacillus pleuropneumoniae*. HAP-94 Proceedings, abstract # T12.

160. **van Leengoed, L.A., and H.W. Dickerson.** 1992. Influence of calcium on secretion and activity of the cytolysins of *Actinobacillus pleuropneumoniae*. Infect. Immun. 60:353-359.

161. **van Leengoed, L.A., E.M. Kamp, and J.M. Pol.** 1989. Toxicity of *Haemophilus pleuropneumoniae* to porcine lung macrophages. Vet. Microbiol. 19:337-349.

162. **Wang, J., A. Mushegian, S. Lory and S. Jin**. 1996. Large-scale isolation of candidate virulence genes of Pseudomonas aeruginosa by in vivo selection. PNAS **93**:10434-10439.

163. **Ward, C.K., and T.J. Inzana**. 1996. Mutations in capsular polysaccharide biosynthesis render Actinobacillus pleuropneumoniae avirulent. HAP-96 Proc., abstract #25.

164. **Ward, C.K., and T.J.Inzana**. 1994. Cloning of a DNA region putatively involved in the encapsulation of *Actinobacillus pleuropneumoniae*. HAP-94 Proc., abstract # T25.

165. **Ward, C.K., and T.J. Inzana**. 1994. Resistance of *Actinobacillus pleuropneumoniae* to bactericidal antibody and complement is mediated by capsular polysaccharide and blocking antibody specific for lipopolysaccharide. J. Immunol. **153**:2110-2121.

166. **West, S.E.H., and M.J.M. Romero**. 1995. Construction of an *Actinobacillus pleuropneumoniae-Escherichia coli* cosmid cloning vector. Gene **160**:87-88.

167. **West, S.E.H., M.J.M. Romero, L.B. Regassa, N.A. Zielinski, and R.A. Welch**. 1995. Construction of *Actinobacillus pleuropneumoniae-Escherichia coli* shuttle vectors: Expression of antibiotic resistance genes. Gene **160**:81-86.

168. **Westfall, J. A., A. Chattergee, and N. V. Anderson**. 1994. *Actinobacillus pleuropneumoniae* in gnotobiotic piglets: ultrastructural changes in the pulmonary alveoli with dose and time. J. Submicrosc. Cytol. Pathol. **26**:41-50.

169. **Willson, P.J., H.G. Deneer, A. Potter, and W. Albritton**. 1989. Characterization of a streptomycin- sulfonamide resistance plasmid from *Actinobacillus pleuropneumoniae*. Antimicr. Agents Chemother. **33**:235-238.

# Chapter 2

# CHARACTERIZATION OF *ACTINOBACILLUS PLEUROPNEUMONIAE* RIBOFLAVIN BIOSYNTHESIS GENES

## ABSTRACT

Four genes encoding enzymes involved in riboflavin biosynthesis have been cloned from *Actinobacillus pleuropneumoniae* (APP) serotype 5. These genes, which are encoded on a single 5.0 kb *Hind*III DNA fragment and are expressed from the endogenous APP promoter in *E. coli* DH5α, specify the production of large amounts of a bright yellow, water-soluble, fluorescent compound identified as riboflavin by mass spectroscopy and characteristic absorbance spectra. Nucleotide sequence analysis of the cloned DNA demonstrated four open reading frames of 1232, 647, 1205, and 461 bps. Based on homology with the riboflavin biosynthetic operon of *Bacillus subtilis*, these ORFs were designated *ribG*, *ribB*, *ribA*, and *ribH*. Predicted amino acid sequences showed strong homology with related enzymes from *B. subtilis* and *E. coli*, and suggested that the APP *ribGBAH* genes encode, respectively, a *rib*-specific deaminase, the α-subunit of riboflavin synthase, a bifunctional enzyme containing GTP cyclohydrase and 3,4-dihydroxy 2-butanone 4-phosphate synthase (DHBP) activities, and the β-subunit of riboflavin synthase. Minicell analysis and SDS-PAGE demonstrated bands of comparable molecular weights to those predicted from the sequence. The cloned APP genes were able to complement mutants of *E. coli* deficient in GTP cyclohydrase II (RibA), 3,4-DHBP synthase (RibB) and riboflavin synthase (RibC) activities. The APP genes were expressed constitutively in *E. coli,* presumably due to the lack of a cloned trans-acting repressor such as a homologue to the *B. subtilis ribC* gene product. Further analysis of the APP *rib* operon and its regulation should yield interesting information on APP genetics and on the role of riboflavin biosynthesis in the pathogenesis of infections caused by this organism.

## INTRODUCTION

*Actinobacillus pleuropneumoniae* (APP) is the causative agent of porcine pleuropneumonia (9,23,39). The disease is characteristically an acute necrotizing hemorrhagic bronchopneumonia, with accompanying fibrinous pleuritis (9,39). Pleuropneumonia is an economically devastating, severe and often fatal disease with clinical courses ranging from peracute to chronic infection (9,14). The existence of at least twelve antigenically distinct capsular serotypes (31) has made development of a cross-protective vaccine difficult. Killed whole cell bacterins provide at best serotype-specific protection (25,26,35,43). In contrast, natural or experimental infection with virulent APP frequently elicits protection against reinfection with any serotype (24,25,27). Avirulent strains of APP have been tested as live vaccines and have elicited cross-protective immunity against subsequent challenge (15,28,44). However, the use of live vaccines in the field is problematic, particularly when the attenuating lesions in the vaccine strain have not been genetically defined. Development of attenuated strains with defined biochemical mutations that limit growth *in vivo* and prevent reversion to wild type is a promising approach to improved vaccines against APP infection.

Riboflavin (vitamin B2), a precursor of the coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), is essential for basic metabolism. It is synthesized by plants and by most microorganisms but not by higher animals (1). Many pathogenic bacteria are apparently unable to utilize flavins from their environment and are entirely dependent on endogenous production of riboflavin (38). Therefore, riboflavin biosynthesis may be essential for survival of pathogens *in vivo*, and mutations in the riboflavin biosynthetic pathway may be attenuating.

The proposed metabolic pathway for bacterial riboflavin synthesis shown in Figure 2.1 begins with guanosine triphosphate (GTP ) as the precursor (for a review

**Figure 2.1 - Proposed Bacterial Riboflavin Biosynthesis Pathway.** Proposed gene functions are as indicated although the functions of *ribG* and *ribT* have not been determined conclusively. Structures correspond to the following: I, GTP; II, 2,5-diamino-6-(ribosylamino)-4(3H)-pyrimidinone 5'-phosphate; III, 5-amino-6-(ribosylamino)-2,4(1H,3H)-pyrimidinedione 5'-phosphate; IV, 5-amino-6-(ribitylamino)-2,4(1H,3H)-pyrimidinedione 5'-phosphate; V, 5-amino-6-(ribitylamino)-2,4(1H,3H)-pyrimidinedione; VI, ribulose 5'-phosphate; VII, 3,4-dihydroxy-2-butanone 4-phosphate; VIII, 6,7-dimethyl-8-ribityllumazine; IX, riboflavin. Structures are adapted from Bacher (1).

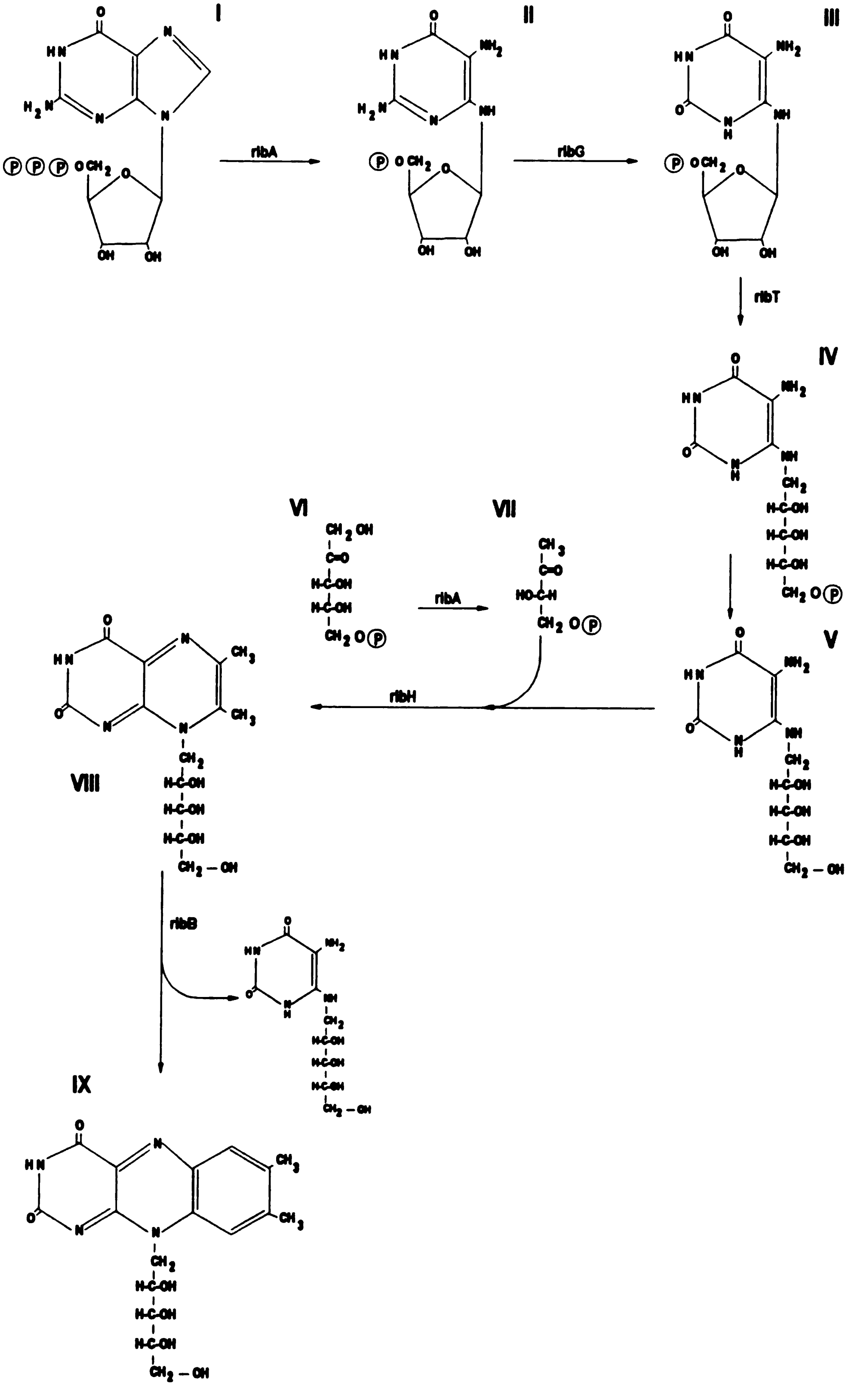


Figure 2.1

see reference 1). The most extensively studied system for bacterial riboflavin synthesis is *Bacillus subtilis* (for a review see reference 29). The *B. subtilis* riboflavin synthesis genes are located and co-regulated in an operon structure (12) that consists of five open reading frames designated as *ribG*, *rib B*, *rib A*, *ribH* and *ribT* (19,29). The *ribGBAHT* genes encode, respectively, a *rib*-specific deaminase; the α-subunit of riboflavin synthase (lumazine synthase); a bifunctional enzyme containing GTP cyclohydrase and 3,4-dihydroxy 2-butanone 4-phosphate synthase (DHBP) activities; the β-subunit of riboflavin synthase; and a *rib*-specific reductase (29). The complete sequence of the *B. subtilis* riboflavin operon has been determined in two individual laboratories (19,30). The *B. subtilis* structural *ribGBAHT* genes code for predicted proteins of 361 (MW 39,700), 215 (MW 23,600), 398 (MW 43,800), 154 (MW 16,900), and 124 (MW 13,600) amino acids in length (19, 29). Two functional promoters have been identified in the *B. subtilis rib* operon. The main promoter, P1, for which a transcriptional start site has been determined 294 bps upstream of *ribG* (12,30), is responsible for transcription of all five structural genes (12). Another promoter, P2, produces a secondary transcript encoding the last three genes of the operon, *ribAHT* (12). A possible third promoter has been postulated that would express *ribH* (7). In addition, the operon has been shown to be transcriptionally coregulated (30) by a trans-acting repressor, RibC (3,6), which acts at a regulatory site, *ribO* (3,20), upstream of *ribG*, apparently by a transcription termination-antitermination mechanism (29). The RibC repressor appears to respond to FMN and FAD, as well as to riboflavin and several of its biosynthetic intermediates, and regulates expression from both *rib*P1 and *rib*P2 (4,20,29).

*E. coli* is the second most chemically characterized system for riboflavin synthesis. In contrast to *B. subtilis*, the *rib* genes of *E. coli* are scattered around the chromosome and are expressed constitutively (2,46). Rather than having a bifunctional *ribA*, *E. coli* has two separate genes, *ribB* and *ribA*, that encode the functions of 3,4-

DHBP synthase (34) and GTP cyclohydrase II (33), respectively. *ribB* is homologous to the 5' end of *B. subtilis ribA* while *ribA* is homologous to the 3' end (33,34). *E. coli* genes with sequence homology to the *B. subtilis ribG* (42), *ribH* (42), and *ribB* genes have also been identified.

In this study, we have identified a fragment of APP serotype 5 chromosomal DNA that triggers overproduction of riboflavin when cloned in *E. coli*. Nucleotide sequence analysis demonstrated four open reading frames with significant identity and a similar operon arrangement to the *ribGBAH* genes from *Bacillus subtilis*.

## MATERIALS AND METHODS

**Bacterial strains and media.** *A. pleuropneumoniae* ISU178, a serotype 5 strain, was cultured at 37°C in brain heart infusion broth or agar (Difco Laboratories, Detroit, MI) containing 10 μg/ml β-NAD (Sigma Chemical Company, St. Louis, MO). *E. coli* DH5-α (*supE44*, *ΔlacU169*, (*ϕ80lacZ*ΔM15), *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*) was used for construction of the APP genomic library. *E. coli* strain DS410 (*azi-8, tonA2, minA1, minB2, rpsL135, xyl-7, mtl-2, thi-1,* λ-) was used for minicell isolation and protein labeling experiments. *E. coli ribA*:Tn5 (BSV18), *ribB*:Tn5 (BSV11) and *ribC*:Tn5 (BSV13) mutants used for complementation studies were described by Bandrin et al (2) and were kindly provided by Barbara Bachmann (*E. coli* Genetic Stock Center, Yale University). *E. coli* strains were cultured in Luria-Bertani medium or in M9 (36) supplemented with 15 g/L NZ amine (Sigma) and with riboflavin at 200 μg/mL when necessary. Ampicillin was added to 100 μg/ml for plasmid selection.

**DNA manipulations.** DNA modifying enzymes were supplied by Boehringer-Mannheim Biochemicals (Indianapolis, IN) and used according to manufacturer's specifications. Genomic and plasmid DNA preparations, gel electrophoresis, and *E. coli* transformation were all performed by conventional methods (36).

**Cloning and sequencing.** APP serotype 5 genomic DNA was digested with *Hin*dIII and fragments ranging in size from 4 to 7 kb were ligated into the *Hin*dIII site in the polylinker of the plasmid vector pUC19 (45). A recombinant plasmid, designated pTF10, which overproduced riboflavin was isolated from this library. Unidirectional nested deletions were constructed with exonuclease III and S1 nuclease digestion, using the Erase-a base system (Promega Corp., Madison, WI). Nucleotide sequencing was performed on alkali-denatured double-stranded DNA by the dideoxy chain-termination method of Sanger *et al.* (37) using the Sequenase 2.0 kit (U.S. Biochemical, Cleveland, OH) and [$^{35}$S]dATP (Amersham Corp., Arlington Heights, IL). Sequencing primers used included universal forward and reverse primers for pUC sequencing (U.S. Biochemicals), as well as several oligonucleotide primers designed from previously obtained sequence data. These internal primers were synthesized by the Michigan State University Macromolecular Structure Facility and included MM4 (5'-AAT-CCG-GCA-AAA-ATT-GAA-GGC-3'), MM5 (5'-GCA-CCG-TGA-CGC-ACT-AAC-G-3'), MM6 (5'-GCG-CCA-ATA-CTT-GCT-CAC-CG-3'), MM9 (5'-GGT-TTC-TTT-ATT-CGT-ATG-CGG-3'), MM10 (5'-TGA-AGA-AGT-CGG-CAA-ATT-GCT-C-3'), MM11 (5'-CGG-ATT-GGG-ATT-CGT-CCA-GCC-3'), MM13 (5'-GGC-GAC-ACG-ATT-GCG-GTG-3'), MM14 (5'-GCC-AGT-TAG-TGC-AGA-CAG-CG-3'), and MM38 (5'-CTC-ACC-GGT-TCC-TGC-CAA-ACC-3').

DNA sequences were analyzed using the GCG sequence analysis programs (11).

**Mass spectroscopy.** Positive and Negative Ion Fast Atom Bombardment (FAB) mass spectroscopy was performed at the Michigan State University Mass Spectroscopy Facility.

**Quantification of riboflavin.** Bacterial cells were pelleted in a microfuge, and the absorbance at 445 nm of the culture supernatant was measured using a Beckman DU-7 spectrophotometer (Beckman Instruments, Fullerton, CA). The absorbance at 445 nm was multiplied by a factor of 31.3 to yield the riboflavin concentration in mg/liter (10).

**Minicell Analysis.** The minicell-producing *E. coli* strain DS410 (32) was transformed by calcium chloride/heat shock treatment with pUC19 or pTF *rib* clones. Transformant colonies which produced a large number of minicells were selected by microscopy. Cultures were grown overnight at 37°C in 500 ml LB broth, and minicells were isolated by differential centrifugation followed by glass fiber filtration as described by Christen et al (8). Minicells were resuspended to an $OD_{590}$ of 0.5-1.0 in 200 µl labeling mix (22.0 ml M9 media, 20.0 ml 50 mM HEPES pH 7.5, 2.5 ml of 20% glucose, 0.05 ml of 10 mg/ml adenine, 0.05 ml of 10 mg/ml pyridoxine, 5.0 ml of NEDA amino acid stock (21) lacking methionine and cysteine, and 0.2 ml of 10 mg/ml cycloserine-D) and incubated at 37°C for 30 minutes. Trans-label ([$^{35}$S]methionine plus [$^{35}$S]cysteine, ICN Biomedicals, Irvine, CA) was added to a final concentration of 22 μ Ci per reaction and cells were incubated at 37°C for 1 hour. Total and TCA precipitable counts were measured by liquid scintillation counting to determine amount of incorporation. Cells were pelleted in a microfuge and washed with cold HEPES (50 mM, pH7.5) plus 10 mM methionine plus 10 mM cysteine. Labeled proteins (50,000 cpm/lane) were separated by discontinuous SDS-PAGE on a 12% polyacrylamide gel and were visualized by autoradiography on Kodak XAR-5 film.

**Nucleotide sequence accession number.** The nucleotide sequence of the *A. pleuropneumoniae ribGBAH* genes has been submitted to GenBank and assigned an accession number of: U27202.

## RESULTS

**Identification of a riboflavin producing clone.** A genomic library of *A. pleuropneumoniae* serotype 5 DNA was constructed in pUC19 and transformed into *E. coli* DH5-α. A single clone, designated pTF10 (Figure 2.2), containing a 5.0 kb insert, was identified that produced a bright yellow extracellular, water-soluble compound that fluoresced under ultraviolet light. The compound was crudely purified by filtration through a 3000 Da cut off membrane filter (Amicon Corporation, Bedford, MA). Absorbance spectra of this compound in aqueous solution under neutral conditions showed absorbance peaks at 373 and 443 nm, which coalesced to a single peak at 388 nm under acidic conditions; these results compared well to a riboflavin standard (Figure 2.3). Positive and negative ion fast atom bombardment mass spectroscopy indicated that the compound was a flavin (data not shown). Culture of *E. coli* DH5-*α*/ pTF10 in M9 medium plus NZ-amine plus 0.6% glucose yielded 10 mg riboflavin per liter in 24 hours.

**Sequence of APP rib genes.** The nucleotide sequence and corresponding predicted amino acid sequence of a 4312 bp region of the insert in pTF10 is shown in Figure 2.4. Four open reading frames of 1232, 647, 1205, and 461 bp were observed that encoded proteins with predicted molecular masses of 45,438 Da, 23,403 Da, 44,739 Da and 16,042 Da, respectively. Based on homology with the riboflavin biosynthetic genes of *B. subtilis* (Table 2.1), these ORFs were designated *ribG*, *ribB*, *ribA*, and *ribH*, respectively. All four ORFs were preceded by potential ribosome binding sites (RBS), although the RBS upstream of *ribG* is not as strong as the other three. Production of riboflavin by this clone is not dependent on its orientation in pUC19 or on induction by IPTG, indicating that it is produced under the control of a native promoter included in the cloned DNA fragment. A consensus promoter sequence of the -35/-10 type (12) was identified within the sequenced region 224 bp upstream from the *ribG* start codon. A second potential consensus promoter was

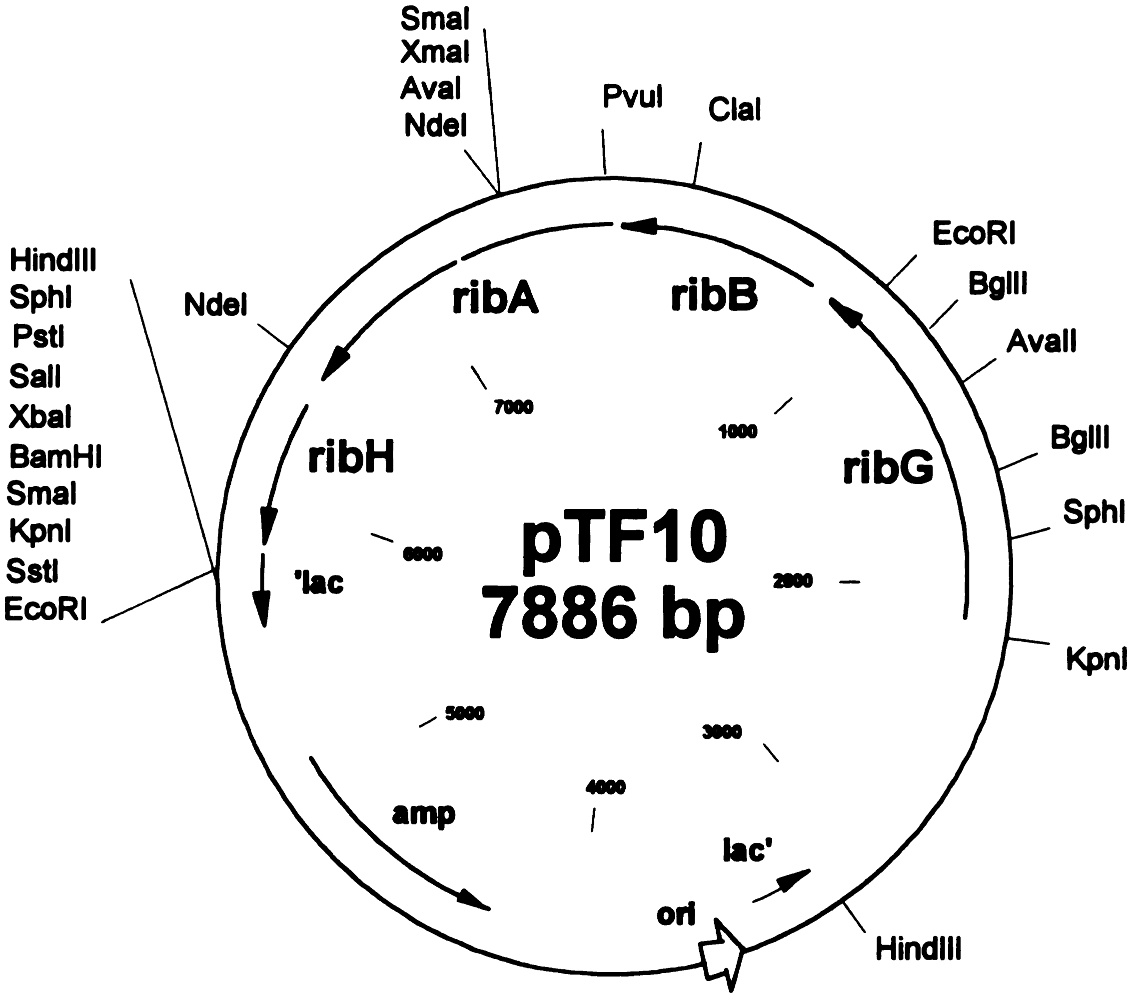


**Figure 2.2 - Physical Map of the Construct pTF10.**

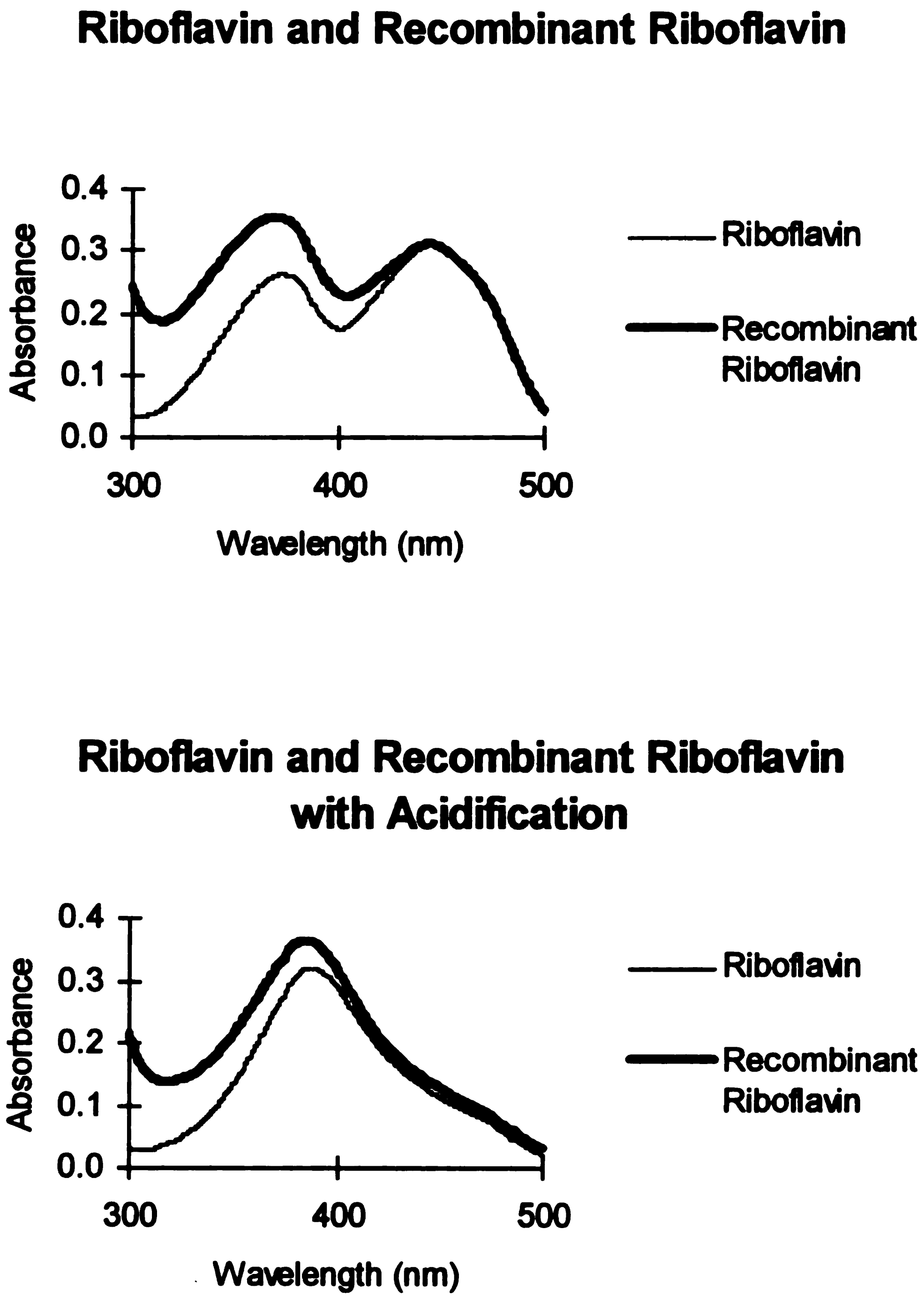


**Figure 2.3 - Spectral Analysis of Riboflavin and Recombinant Riboflavin.**

**Figure 2.4 - Complete Nucleotide Sequence of APP *ribGBAH* Genes and Flanking Regions.** The amino acid translations are shown for *ribG*, *ribB*, *ribA*, and *ribH* and correspond to base pairs 330-1560, 1685-2330, 2393-3596 and 3709-4168. Putative ribosome binding sites are underlined. Potential promoters for the operon and for *ribH* are double-underlined. An inverted repeat that may function as a transcription terminator is overlined.

```
   1   AATTCGGTCGGACGTACTTT ATTTGAGCATATCAATGAAG GAGGTTTTGATTATGTGATT

                                                        -35
  61   TCAGAGTGTGAAACCTGTAA ATGGCAGATTGATATGTCGA GCAATGTGACTTGTTTACAT

                -10
 121   CCGATTACTTTATTATCAAT GGCATTGGATAAACGCTAAT TCTTGCTTGACTTTGACAAT

 181   CAAAAGTCGCAAATTTGCAA CAATTTTTTAATAATCTTCA GGGCAGGGTGAAATTCCCGA

 241   TCGGCGGTAAAGTCCGCGAG CCGAACGAAAAAGGTTTGGC AGGAACCGGTGAGATTCCGG

                                     M  K  L  P   C  K  R  W  F  F  L
 301   TACCGACAGTATAGTCTGGA TGGAAGAAGATGAAATTACC GTGTAAGCGGTGGTTTTTCC

         S  F  L  Q  A  L   R  S  K  D  F  K  A   F  F  I  I  R  V  N
 361   TATCTTTTTTACAAGCCTTG AGATCGAAAGATTTCAAGGC TTTTTTCATCATTAGGGTAA

         M  P  V  M  C  F   P  L  P  S  N  S  F   K  T  M  T  D  L  D
 421   ACATGCCTGTAATGTGTTTT CCTCTGCCCTCAAATAGTTT CAAAACAATGACGGATTTAG

         Y  M  R  R  A  I   A  L  A  K  Q  G  L   G  W  T  N  P  N  P
 481   ACTATATGCGCCGTGCCATT GCACTGGCAAAACAAGGTTT AGGCTGGACGAATCCCAATC

         L  V  G  C  V  I   V  K  N  G  E  I  V   A  E  G  Y  H  E  K
 541   CGCTTGTCGGTTGTGTAATT GTCAAAAACGGTGAAATCGT TGCCGAAGGTTACCATGAAA

         I  G  G  W  H  A   E  R  N  A  V  L  H   C  K  E  D  L  S  G
 601   AGATTGGTGGATGGCATGCG GAACGTAATGCCGTTTTACA TTGTAAGGAAGATCTTTCCG

         A  T  A  Y  V  T   L  E  P  C  C  H  H   G  R  T  P  P  C  S
 661   GGGCGACTGCTTATGTAACG CTTGAGCCTTGTTGTCATCA CGGCCGCACGCCGCCTTGTT

         D  L  L  I  E  R   G  I  K  K  V  F  I   G  S  S  D  P  N  P
 721   CGGATTTATTAATTGAACGA GGCATTAAAAAAGTATTTAT CGGTTCGAGCGATCCGAATC

         L  V  A  G  R  G   A  N  Q  L  R  Q  A   G  V  E  V  V  E  G
 781   CTTTAGTAGCAGGGCGGGGA GCAAATCAGCTACGCCAAGC CGGCGTGGAAGTGGTGGAAG

         L  L  K  E  E  C   D  A  L  N  P  I  F   F  H  Y  I  Q  T  K
 841   GTTTACTCAAAGAAGAATGT GATGCGTTAAACCCGATTTT TTTCCACTATATTCAAACTA

         R  P  Y  V  L  M   K  Y  A  M  T  A  D   G  K  I  A  T  G  S
 901   AACGTCCGTATGTGCTAATG AAATATGCCATGACGGCAGA CGGCAAAATTGCAACCGGTA

         G  E  S  K  W  I   T  G  E  S  A  R  A   R  V  Q  Q  T  R  H
 961   GCGGCGAATCCAAATGGATT ACCGGTGAATCGGCAAGAGC AAGAGTGCAGCAAACACGTC

         Q  Y  S  A  I  M   V  G  V  D  T  V  L   A  D  N  P  M  L  N
1021   ATCAATATAGTGCGATTATG GTCGGTGTAGATACGGTACT TGCCGATAACCCGATGTTAA

         S  R  M  P  N  A   K  Q  P  V  R  I  V   C  D  S  Q  L  R  T
1081   ATAGCCGAATGCCGAATGCG AAACAACCGGTCCGGATTGT CTGCGATAGCCAATTACGTA

         P  L  D  C  Q  L   V  Q  T  A  K  E  Y   R  T  V  I  A  T  V
1141   CACCGTTAGATTGCCAGTTA GTGCAGACAGCGAAAGAATA TCGCACCGTAATTGCAACCG
```

```
         S  D  D  L  Q  K  I  E  Q  F  R  P  L   G  V  D  V  L  V  C
1201  TTAGTGACGATTTGCAAAAA ATTGAACAATTTAGACCGCT TGGCGTAGATGTATTAGTGT

         K  A  R  N  K  R  V  D  L  Q  D  L  L   Q  K  L  G  E  M  Q
1261  GTAAAGCACGAAACAAGCGG GTAGATTTGCAAGATCTTTT GCAAAAGCTCGGTGAAATGC

         I  D  S  L  L  L  E  G  G  S  S  L  N   F  S  A  L  E  S  G
1321  AGATCGACAGCCTCTTATTG GAAGGCGGTTCAAGTTTGAA TTTCAGTGCGTTAGAAAGCG

         I  V  N  R  V  H  C  Y  I  A  P  K  L   V  G  G  K  Q  A  K
1381  GTATCGTGAATCGAGTACAT TGTTATATTGCGCCTAAATT AGTCGGTGGTAAACAAGCGA

         T  P  I  G  G  E  G  I  Q  Q  I  D  Q   A  V  K  L  K  L  K
1441  AAACCCCAATCGGCGGTGAG GGAATTCAACAAATCGACCA AGCGGTTAAATTAAAATTGA

         S  T  E  L  I  G  E  D  I  L  L  D  Y   V  V  I  S  P  L  *
1501  AATCGACCGAACTCATCGGC GAAGATATTTTGTTGGATTA TGTAGTCATCTCCCCTCTTT


1561  AGCAAAGAGGGGTCGGGGGA GATTTGAGATAATGTTGAAA TTTACACCGCCTTTCACTTT


1621  GGCGTTGTTAAATCTCCCCT AACCCCTCTTTACAAAAGAG AGGGATCAATAATGAGGAAA

           M  F  T  G  I  I  E  E  V  G  K  I    A  Q  I  H  K  Q  G
1681  TTATATGTTCACAGGTATTA TTGAAGAAGTCGGCAAAATT GCTCAAATTCATAAGCAAGG

       E  F  A  V  V  T  I   N  A  T  K  V  L    Q  D  V  H  L  G  D
1741  CGAATTTGCGGTAGTCACAA TTAATGCGACCAAAGTATTA CAAGACGTTCATTTAGGCGA

       T  I  A  V  N  G  V   C  L  T  V  T  S    F  S  S  N  Q  F  T
1801  CACGATTGCGGTGAACGGCG TATGTTTAACCGTAACTTCT TTTTCGAGTAATCAGTTTAC

       A  D  V  M  S  E  T   L  K  R  T  S  L    G  E  L  K  S  N  S
1861  CGCCGATGTAATGTCGGAAA CGTTAAAACGTACTTCATTA GGCGAATTAAAGTCGAATAG

       P  V  N  L  E  R  A   M  A  A  N  G  R    F  G  G  H  I  V  S
1921  TCCGGTTAATTTAGAACGCG CGATGGCGGCAAACGGACGT TTCGGCGGACACATCGTTTC

       G  H  I  D  G  T  G   E  I  A  E  I  T    P  A  H  N  S  T  W
1981  GGGGCATATTGACGGCACCG GCGAAATTGCGGAAATCACA CCGGCACATAATTCGACATG

       Y  R  I  K  T  S  P   K  L  M  R  Y  I    I  E  K  G  S  I  T
2041  GTATCGCATTAAAACCTCTC CAAAATTAATGCGTTATATT ATTGAGAAAGGTTCGATCAC

       I  D  G  I  S  L  T   V  V  D  T  D  D    E  S  F  R  V  S  I
2101  CATTGACGGTATTAGCCTGA CCGTAGTCGATACCGATGAT GAAAGTTTCCGTGTATCGAT

       I  P  H  T  I  K  E   T  N  L  G  S  K    K  I  G  S  I  V  N
2161  TATTCCGCATACGATTAAAG AAACCAATTTAGGTTCGAAA AAAATCGGCAGTATTGTCAA

       L  E  N  D  I  V  G   K  Y  I  E  Q  F    L  L  K  K  P  A  D
2221  TTTAGAAAATGATATTGTCG GTAAATATATCGAACAGTTT TTACTGAAAAAGCCGGCGGA

       E  P  K  S  N  L  S   L  D  F  L  K  Q    A  G  F  *
2281  TGAGCCGAAAAGTAATCTTA GTTTAGACTTTTTAAAGCAG GCGGGATTTTAAGATTTGTA

                                                             M  T  D
2341  GGACACACTGAGTGTATCCT ACCGACAAAAATATATATTT TAGGAAAAGAAGATGACAGA
```

```
       F  Q  F  S  K  V  E   D  A  I  E  A  I   R  Q  G  K  I  I  L
2401  TTTCCAATTTTCAAAAGTAG AAGATGCGATCGAAGCGATT CGACAAGGCAAAATCATTTT

       V  T  D  D  E  D  R   E  N  E  G  D  F   I  C  A  A  E  F  A
2461  AGTGACTGACGATGAAGATC GCGAAAACGAAGGCGATTTT ATCTGTGCGGCGGAATTTGC

       T  P  E  N  I  N  F   M  A  T  Y  G  K   G  L  I  C  T  P  I
2521  CACACCGGAAAATATCAATT TTATGGCAACTTACGGCAAA GGTTTGATTTGTACGCCGAT

       S  T  E  I  A  K  K   L  N  F  H  P  M   V  A  V  N  Q  D  N
2581  TTCAACCGAAATCGCTAAAA AATTAAATTTCCATCCGATG GTTGCGGTCAATCAAGATAA

       H  E  T  A  F  T  V   S  V  D  H  I  D   T  G  T  G  I  S  A
2641  TCATGAAACGGCGTTTACCG TATCGGTGGATCATATTGAT ACGGGAACGGGTATCTCAGC

       F  E  R  S  I  T  A   M  K  I  V  D  D   N  A  K  A  T  D  F
2701  TTTTGAGCGTTCGATTACCG CAATGAAAATTGTCGATGAT AATGCTAAAGCAACGGATTT

       R  R  P  G  H  M  F   P  L  I  A  K  E   G  G  V  L  V  R  N
2761  CCGCCGCCCGGGGCATATGT TTCCGTTAATCGCTAAAGAA GGTGGAGTGTTAGTGCGTAA

       G  H  T  E  A  T  V   D  L  A  R  L  A   G  L  K  H  A  G  L
2821  CGGTCATACCGAAGCAACAG TGGATTTAGCTCGTTTAGCC GGTTTAAAACACGCCGGTTT

       C  C  E  I  M  A  D   D  G  T  M  M  T   M  P  D  L  Q  K  F
2881  ATGTTGTGAAATTATGGCGG ATGACGGCACGATGATGACT ATGCCGGATCTACAAAAATT

       A  V  E  H  N  M  P   F  I  T  I  Q  Q   L  Q  E  Y  R  R  K
2941  TGCGGTAGAACACAATATGC CGTTTATCACGATTCAACAA TTACAAGAATATCGCCGTAA

       H  D  S  L  V  K  Q   I  S  V  V  K  M   P  T  K  Y  G  E  F
3001  GCATGACAGCTTGGTGAAAC AAATTTCTGTGGTAAAAATG CCGACAAAATACGGTGAGTT

       M  A  H  S  F  V  E   V  I  S  G  K  E   H  V  A  L  V  K  G
3061  TATGGCACATAGCTTTGTTG AAGTGATTTCAGGTAAAGAA CACGTTGCGTTAGTCAAAGG

       D  L  T  D  G  E  Q   V  L  A  R  I  H   S  E  C  L  T  G  D
3121  CGATTTAACCGACGGTGAGC AAGTATTGGCGCGTATCCAT TCGGAATGTTTAACCGGTGA

       A  F  G  S  Q  R  C   D  C  G  Q  Q  F   A  A  A  M  T  Q  I
3181  CGCTTTCGGTTCTCAACGTT GTGATTGCGGTCAGCAATTT GCCGCAGCAATGACCCAAAT

       E  Q  E  G  R  G  V   I  L  Y  L  R  Q   E  G  R  G  I  G  L
3241  TGAGCAAGAGGGCAGAGGTG TGATTCTGTATTTACGCCAA GAAGGTCGTGGTATCGGTTT

       I  N  K  L  R  A  Y   E  L  Q  D  K  G   M  D  T  V  E  A  N
3301  AATCAATAAGCTACGTGCTT ACGAACTACAAGATAAAGGG ATGGATACCGTTGAAGCGAA

       V  A  L  G  F  K  E   D  E  R  E  Y  Y   I  G  A  Q  M  F  Q
3361  CGTCGCTTTAGGATTTAAAG AAGACGAACGTGAGTACTAT ATCGGTGCACAAATGTTCCA

       Q  L  G  V  K  S  I   R  L  L  T  N  N   P  A  K  I  E  G  L
3421  GCAGTTAGGCGTAAAATCGA TCCGTTTATTAACCAATAAT CCGGCAAAAATTGAAGGCTT

       K  E  Q  G  L  N  I   V  A  R  E  P  I   I  V  E  P  N  K  N
3481  AAAAGAGCAAGGATTAAATA TCGTTGCACGTGAGCCGATT ATTGTAGAACCGAACAAAAA
```

```
        D  I  D  Y  L  K  V   K  Q  I  K  M  G   H  M  F  N  F  *
3541  TGACATTGATTACCTAAAAG TCAAACAGATAAAAATGGGG CATATGTTTAACTTCTAACT

                                                             -35
3601  TTAACAACCGTATGTAGTAT TAGGGAAGCAAGCGTTGCGT CCCTACTATAGAATGATACA

                      -10                                M  A  K  I
3661  AGCGGTCACTTTTTTATAAA ATTTTGCATATTTCGAGAGG ACAAAAAAATGGCAAAGATT

       T  G  N  L  V  A  T   G  L  K  F  G  I  V   T  A  R  F  N  D
3721  ACAGGTAACTTAGTTGCGAC AGGTTTAAAATTCGGTATTG TAACCGCACGTTTCAACGAT

       F  I  N  D  K  L  L   S  G  A  I  D  T  L   V  R  H  G  A  Y
3781  TTTATCAACGATAAATTATT AAGCGGTGCAATTGATACGT TAGTGCGTCACGGTGCGTAT

       E  N  D  I  D  T  A   W  V  P  G  A  F  E   I  P  L  V  A  K
3841  GAAAACGATATTGATACGGC ATGGGTTCCGGGTGCATTTG AGATTCCATTAGTTGCGAAA

       K  M  A  N  S  G  K   Y  D  A  V  I  C  L   G  T  V  I  R  G
3901  AAAATGGCAAACAGCGGTAA ATATGATGCGGTAATCTGTT TAGGTACGGTAATTCGCGGT

       S  T  T  H  Y  D  Y   V  C  N  E  A  A  K   G  I  G  A  V  A
3961  TCGACAACTCACTATGATTA CGTATGTAATGAAGCGGCAA AAGGTATCGGTGCGGTAGCA

       L  E  T  G  V  P  V   I  F  G  V  L  T  T   E  N  I  E  Q  A
4021  TTAGAAACCGGCGTACCGGT AATTTTCGGTGTATTAACCA CAGAAAATATTGAACAGGCG

       I  E  R  A  G  T  K   A  G  N  K  G  S  E   C  A  L  G  A  I
4081  ATTGAACGCGCGGGTACTAA AGCAGGTAATAAAGGTTCAG AATGTGCATTAGGCGCAATC

       E  I  V  N  V  L  K   A  I  *
4141  GAAATAGTAAACGTATTAAA AGCGATCTAATTTTCGTTTG ACGTGCTAAAAACAAGCGGT

4201  CGTTTTTGACTGGAATTTTG CAAATTTCCCGTTAAAAACG ACCGCTTATATTTTATGTCT

4261  AGTAAAGACCTTCTTTCTCG TACCAGATTTTGTTGATATA TAGCAAGCTTGG  4312
```

## Table 2.1 - Comparison of Amino Acid Sequence Similarity Between Riboflavin Synthesis Proteins.[a]

| | % Similarity with *A. pleuropneumoniae* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | RibG | | RibB | | RibA | | RibH | |
| **Bacterium** | **Compared with** | **%** | **Compared with:** | **%** | **Compared with:** | **%** | **Compared with:** | **%** |
| *Bacillus subtilis* | RibG | 63 | RibB | 69 | RibA | 73 | RibH | 83 |
| *Escherichia coli*[b] | RibG | 62 | RibC | 58 | RibB<br>RibA | 63<br>73 | RibH | 74 |
| *Haemophilus influenzae*[c] | RibG | 58 | RibC | 60 | RibB<br>RibA | 65<br>71 | RibE | 75 |
| *Photobacterium leiognathi* | | NA | RibI | 64 | RibII | 61 | RibIII | 69 |
| *Photobacterium phosphoreum*[d] | | NA | RibI | 63 | RibII<br>RibIV | 59<br>63 | RibIII | 73 |
| *Vibrio harveyi* | | NA | | NA | LuxH | 59 | | NA |

[a] Identity is expressed in percent similarity as calculated by the GCG Needleman-Wunsch algorithm (22). Proteins with partial identity were compared to the entire appropriate APP Rib protein.

[b] *E.coli* RibB is homologous to the 5' end of APP RibA. *E.coli* RibA is homologous to the 3' end of APP RibA.

[c] *H.influenzae* RibB is homologous to the 5' end of APP RibA. *H.influenzae* RibA is homologous to the 3' end of APP RibA.

[d] *P.phosphoreum* RibIV is homologous to the 3' end of APP RibA.

identified between the genes *ribA* and *ribH*. However, no consensus promoter was identified between *ribB* and *ribA*, as is found in *B. subtilis*. The ORF encoding *ribH* is followed by an inverted repeat stem-loop structure with a $\Delta G = -56.0$ that may function as a rho-dependent transcriptional terminator (13).

**Homology of APP rib genes.** Predicted amino acid sequences of the APP RibGBAH proteins were compared with *B. subtilis* RibGBAH (19); *E. coli* RibA, RibB, RibC, RibG, and RibH (33,34,42); *Photobacterium leiognathi* RibI-III (17), *Photobacterium phosphoreum* RibI-IV (16), and *Vibrio harveyi* LuxH (41) proteins, using the GCG Gap program (Table 2.1). APP RibG showed 62-63% similarity to the RibG proteins from *B. subtilis* and *E. coli*. APP RibB showed 58-69% and APP RibH showed 69-83% similarity to homologous genes from *B. subtilis*, *E. coli*, and *Photobacterium* species. APP RibA showed 73% similarity to the entire RibA protein of *B. subtilis* and 61% to the RibII protein of *P. leiognathi*, both of which encode a bifunctional enzyme catalyzing two distinct steps in the riboflavin pathway. In addition, the carboxy terminal half of APP RibA, encompassing ~200 amino acids, shows 59-63% similarity to *E. coli* RibB, and *V. harveyi* LuxH, which encode 3,4-DHBP synthase. The N-terminal region of APP RibA, encompassing the remaining ~200 amino acids, shows 63-73% similarity to *E. coli* RibA and *P. phosphoreum* RibIV, which encode GTP cyclohydrase II.

**Complementation of *E. coli* mutants.** The original pTF10 clone and several deletion derivatives were tested for their abilities to complement *ribA* (GTP cyclohydrase II), *ribB* (3,4-DHBP synthase), and *ribC* (β- subunit of riboflavin synthase) mutations in *E. coli* (2) (Figure 2.5). Complementation of the *E. coli* mutation was determined by restoration of the ability to grow on M9 minimal medium in the absence of riboflavin. Plasmids containing a complete copy of the APP *ribB* gene complemented the *E. coli ribC* mutation. Plasmids containing the 5' end of APP *ribA* complemented the *E. coli ribB* mutation. Plasmids containing a complete copy of APP *ribA* complemented both *E. coli ribB* and *ribA* mutations.

**Minicell analysis.** Plasmid pTF10 and its deletion derivatives were transformed into the minicell-producing *E. coli* strain DS410, and the proteins encoded by these plasmids were radioactively labeled, separated by SDS-PAGE, and visualized by autoradiography. Compared with the pUC19 vector, plasmid pTF10 shows four unique proteins with apparent molecular masses of 45 kDa, 27.7 kDa, 43.7 kDa, and 14.8 kDa (Figure 2.6), which correspond well with the sizes predicted for the RibG, RibB, RibA, and RibH proteins by amino acid sequence data. The RibG protein did not appear to be as strongly expressed as RibB, RibA, and RibH. Analysis of proteins encoded by plasmid pTF19 (Figure 2.5), which contains no *ribH* and a slightly truncated *ribA* gene, eliminates the 14.8 kDa protein (RibH) and truncates the 43.7 kDa protein (RibA) to 42.5 kDa (Figure 2.6). Plasmid pTF12 (Figure 2.5), which does not contain *ribB, ribA*, or *ribH* genes, does not express the 27.7, 43.7, or 14.8 kDa proteins (data not shown).

**Complementation of E.coli Mutants**

| ribA - | ribB - | ribC - | |
|---|---|---|---|
| nd | - | - | pTF12 |
| - | - | + | pTF26 |
| - | - | nd | pTF23 |
| - | + | nd | pTF22 |
| - | + | nd | pTF19 |
| + | + | nd | pTF18 |
| + | + | + | pTF10 |

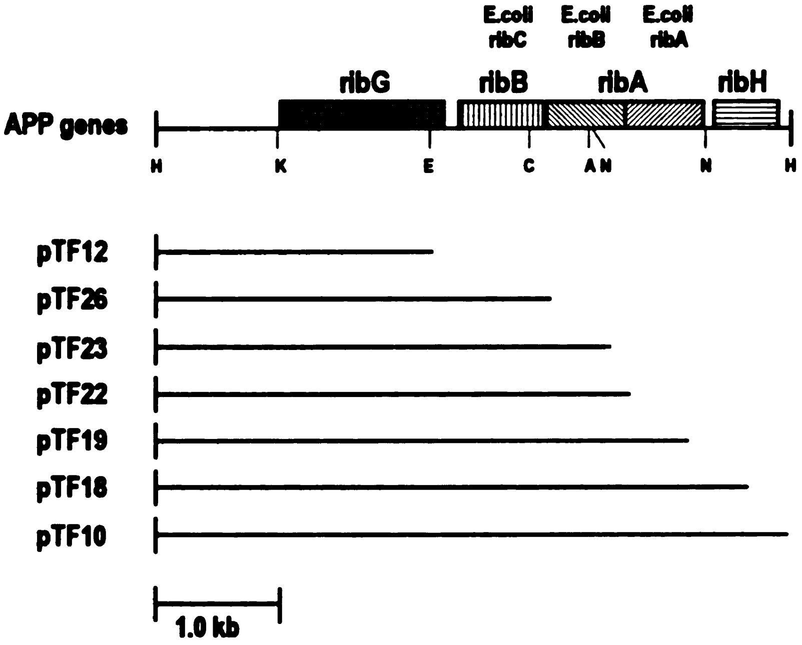


**Figure 2.5 - Complementation of *E. coli* Mutants by Cloned APP *rib* Genes.** A physical map for the APP *ribGBAH* genes is shown as well as several deletions that were made from the 3' end of the APP *rib* clone. The *E. coli* gene designations are indicated above their APP homologues. A "+" indicates complementation of the indicated *E. coli* mutation by the recombinant plasmid. nd = not done. Enzyme abbreviations: A, *Ava*I; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nde*I

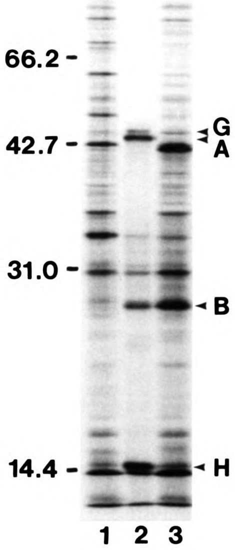


**Figure 2.6 - Minicell Analysis of pTF10 and Deletions.** Minicells contained: Lane 1, pUC19; Lane 2, pTF10; Lane 3, pTF19. Molecular weight standards are indicated on the left. Proteins encoded by the APP genes are indicated by the arrows on the right. Apparent molecular weights for the APP Rib enzymes are: RibG, 45 kDa; RibA, 43.7 kDa; RibB, 27.7 kDa, and RibH, 14.8 kDa.

## DISCUSSION

In this paper, we report the identification, cloning and complete nucleotide sequence of four genes from *Actinobacillus pleuropneumoniae* that are involved in riboflavin biosynthesis. The cloned genes can specify production of large amounts of riboflavin in *E. coli*, can complement several defined genetic mutations in riboflavin biosynthesis in *E. coli*, and are homologous to riboflavin biosynthetic genes from both *E. coli* and *Bacillus subtilis*. The genes have been designated APP *ribGBAH* due to their similarity in both sequence and arrangement to the *B. subtilis ribGBAH* operon.

The DNA sequence data, complementation, and minicell analysis strongly suggest that the four *rib* genes are transcribed from a single APP promoter upstream of the *ribG* gene. This promoter, among the first described for housekeeping genes in APP, is a good match for an *E. coli* consensus -35/-10 promoter. There is a 4 of 6 bp match at the -35 region, a 17 bp interval, a 4 of 6 bp match at the -10 region, an 8 bp interval, and a CAT box at the -1/+1 site. There is also a second potential promoter located between *ribA* and *ribH*, although it is not clear whether this promoter is functional.

Biosynthesis of riboflavin by APP appears to be more similar to that in the gram-positive bacterium *B. subtilis* than in the gram-negative bacterium *E. coli*. First, APP *rib* genes are arranged in an operon similar to that seen in *B. subtilis*, rather than scattered throughout the chromosome as is found in *E. coli*. However, the *B. subtilis rib* operon contains a fifth gene, *ribT*, that is proposed to mediate the third step in riboflavin biosynthesis; it is unlikely that a *ribT* homologue is present as part of the operon in APP because of the presence of a strong inverted repeat following *ribH* and the lack of a likely reading frame downstream. Second, APP contains a *ribA* gene that encodes a bifunctional enzyme with both GTP cyclohydrase II and DHPB synthase activities, as is found in *B. subtilis*; *E. coli* has two genes, *ribA* and *ribB*, that encode these two enzymes separately. Finally, the APP riboflavin biosynthetic enzymes are more similar at the amino acid level to the enzymes of *B. subtilis* than to those of *E. coli*, although alignment of the proteins from all three sources shows highly conserved sequences (data not shown).

It should be noted that in three bioluminescent species from the family *Vibrionaceae*, *Vibrio harveyi*, *Photobacterium leiognathi*, and *P. phosphoreum*, riboflavin biosynthesis genes have been shown to be closely linked to the *lux* operon (10,11,41). $FMNH_2$ is the substrate for the light-emitting reaction, and therefore an increase in bioluminescence requires an increased supply of the cofactor. Since riboflavin is the precursor for FMN, linkage and possibly coordinate regulation of *lux* and *rib* genes may facilitate the expression of bioluminescence in these bacteria.

The recombinant *E. coli* DH5-α containing plasmid pTF10 showed a marked increase in extracellular riboflavin production, most likely due to the lack of regulation (40) and high copy number of the cloned synthetic genes (45). Although the APP *rib* operon is similar in structure to that of *B. subtilis*, it is not yet known whether the genes are tightly regulated in APP by a repressor similar to *B. subtilis* RibC, or whether they are constitutively expressed as appears to be true in *E. coli* (33). We hypothesize that APP must synthesize riboflavin to meet its own metabolic demands during infection, since riboflavin is not synthesized by mammals and therefore is not likely to be freely available to APP within its porcine host. Further study of this model operon should reveal interesting information on regulation and promoter/operon structure in APP, as well as information on the role of riboflavin biosynthesis in APP infection.

## ACKNOWLEDGMENTS

Mass spectral data were obtained at the Michigan State University Mass Spectrometry Facility which is supported, in part, by a grant (DRR-00480) from the Biotechnology Research Technology Program, National Center for Research Resources, National Institutes of Health.

We also thank Barbara Bachmann for supplying the *E.coli* mutant strains, Vladimir Motin for interpretation of Russian papers, and Robert Hausinger, Patrick Oriel and Douglas Gage for helpful discussion.

## REFERENCES

1. **Bacher, A.** 1991. Biosynthesis of flavins, p. 215-259. *In* F. Muller (ed.), Chemistry and Biochemistry of Flavins, vol. 1. Chemical Rubber Co., Boca Raton, FL.

2. **Bandrin, S.V., P.M. Rabinovich, and A.I. Stepanov.** 1983. Three linkage groups of genes involved in riboflavin biosynthesis in *Escherichia coli.* Sov. Genet. **19:**1103-1109.

3. **Bresler, S.E., E.I. Cherepenko, T.P. Chernik, V.L. Kalinin, and D.A. Perumov.** 1970. Investigation of the operon of riboflavin synthesis in *Bacillus subtilis*. I. Genetic mapping of the linkage group. Genetika **6:**116-124.

4. **Bresler, S.E., E.A. Glazunov, G.I. Chernik, T.N. Shevchenko, and D.A. Perumov.** 1973. Investigation of the operon of riboflavin synthesis in *Bacillus subtilis*. V. Flavin mononucleotide and flavin adenine dinucleotide as effectors in the operon of riboflavin biosynthesis. Genetika **9:**84-91.

5. **Bresler, S.E., V.L. Kalinin, A.S. Kriviskii. D.A. Perumov. and T.P. Chernik.** 1969. Mutant of *Bacillus subtilis* synthesizing notable amounts of riboflavin. Genetika **5:**133-138.

6. **Bresler, S.E., D.A. Perumov, G.I. Chernik, A.P. Skvortsova.** 1976. Investigation of the operon of riboflavin synthesis in *Bacillus subtilis.* XI. Determination of the type of regulation by a test for dominance of operator-constitutive and regulator-constitutive mutations. Genetika **12:**124-130.

7. **Chikindas, M.L., G.I. Morozov, V.N. Mironov, E.V. Luk'yanov, V.V. Emel'yanov, and A.I. Stepanov.** 1988. Regulatory regions of the riboflavin operon in *Bacillus subtilis*. Dokl. Akad. Nauk SSSR **298:**997-1000.

8. **Christen, A.A., M.L. Pall, T. Manzara and P.F. Lurquin.** 1983. Rapid isolation of *Escherichia coli* minicells by glass-fiber filtration: study of plasmid-coded polypeptides. Gene **23:**195-198.

9. **Fenwick, B.** 1994. Porcine pleuropneumonia. J. Am. Vet. Med. Assoc. **204:**1334-1340.

10. **Foster, E.W., D.C. Gyure, D.L. Heefner, C.A. Weaver, M.J. Yarus, L.A. Burdzinski.** June 1992. U.S. Patent 5,120,655.

11. **Genetics Computer Group.** Sept. 1994. Program Manual for the Wisconsin Package, Ver. 8. Genetics Computer Group. Madison, Wisconsin.

12. **Hawley, D.K. and W.R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids. Res. **11:**2237-2255.

13. **Holmes, W.M., T. Platt, and M. Rosenber.** 1983. Termination of transcription in *E. coli*. Cell **32**:1029-1032.

14. **Hunneman, W.A.** 1986. Incidence, economic effects, and control of *Haemophilus pleuropneumoniae* infections in pigs. Vet. Quarterly **8**:83-87.

15. **Inzana, T.J., J. Todd, and H.P. Veit.** 1993. Safety, stability and efficacy of nonencapsulated mutants of *Actinobacillus pleuropneumoniae* for use in live vaccines. Infect. Immun. **61**:1682-1686.

16. **Lee, C.Y., D.J. O'Kane, and E.A. Meighen.** 1994. Riboflavin synthesis genes are linked with the *lux* operon of *Photobacterium phosphoreum*. J. Bacteriol. **176**:2100-2104.

17. **Lee, C.Y. and E.A. Meighen.** 1992. The lux genes in *Photobacterium leiognathi* are closely linked with genes corresponding in sequence to riboflavin synthesis genes. Biochem. Biophys. Res. Commun. **186**:690-697.

18. **Mironov, V.N., M.L. Chikindas. A.S. Kraev, A.I. Stepanov. and K.G. Skryabin.** 1990. Operon organization of genes of riboflavin biosynthesis in *Bacillus subtilis*. Dok. Akad. Nauk SSSR **312**:237-240.

19. **Mironov, V.N., A.S. Kraev, B.K. Chernov, A.V. Ul'yanov. Y.B. Golva, G.E. Pozmogova, M.L. Simonova, V.K. Gordeev, A.I. Stepanov, and K.G. Skryabin.** 1989. Genes of riboflavin biosynthesis of *Bacillus subtilis*-complete primary structure and model of organization. Dokl. Akad. Nauk SSSR **305**:482-487.

20. **Mironov, V.N., D.A. Perumov, A.S. Kraev, A.I. Stepanov, K.G. Skryabin.** 1990. Unusual structure in the regulation region of the *Bacillus subtilis* riboflavin biosynthesis operon. Molekulyamaya Biologiya **24**:256-261.

21. **Morse, S.A. and L. Bartenstein.** 1980. Purine metabolism in *Neisseria gonorrhoeae*: the requirement for hypoxanthine. Can. J. Microbiol. 26:13-20.

22. **Needleman, S.B. and C.D. Wunsch.** 1970. A general method applicable to the search for similarities in the amino acid sequence of two proteins. J. Molec. Biol. **48**:443-453.

23. **Nicolet, J.** 1992. *Actinobacillus pleuropneumoniae*, p. 401-408. *In* A.D. Leman et al (eds), Diseases of Swine, 7th edition. Iowa State University Press, Ames, Iowa.

24 **Nielsen, R.** 1979. *Haemophilus parahaemolyticus* serotypes: pathogenicity and cross immunity. Nord. Vet. Med. **31**:407-413.

25. **Nielsen, R.** 1984. *Haemophilus pleuropneumoniae* serotypes - Cross protection experiments. Nord. Vet. Med. **36**:221-234.

26. **Nielsen, R.** 1976. Pleuropneumonia of swine caused by *Haemophilus pleuropneumoniae*. Studies on the protection obtained by vaccination. Nord. Vet. Med. **28**:337-338.

27. **Nielsen, R.** 1974. Serological and immunological studies of pleuropneumonia of swine caused by *Haemophilus parahaemolyticus*. Acta Vet. Scand. **15**:80-89.

28. **Paltineanu, D., R. Pambucol, E. Tirziu, and I. Scobercea.** 1992. Swine infectious pleuropneumonia: Aerosol vaccination with a live attenuated vaccine. Proc. Int. Pig. Vet. Soc. **12**:214

29. **Perkins, J.B., and J.G. Pero.** 1993. Biosynthesis of riboflavin, biotin, folic acid, and cobalamin, p. 319-334. *In* A. Sonenshein (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology. Washington, D.C.

30. **Perkins, J.B., J.G. Pero, and A. Sloma.** January 1991. Riboflavin overproducing strains of bacteria. European patent application 0405370.

31. **Perry, M.B., E. Altman, J.-R. Brisson, L.M. Beynon, and J.C. Richards.** 1990. Structural characteristics of the antigenic capsular polysaccharides and lipopolysaccharides involved in the serological classification of *Actinobacillus pleuropneumoniae* strains. Serodiag. Immunother. Infect. Dis. **4**:299-308.

32. **Reeve, J.** 1977. Bacteriophage infection of minicells: a general method for identification of in vivo bacteriophage directed polypeptide biosynthesis. Molec. Gen. Genet. **158**:73-79.

33. **Richter, G., H. Ritz, G. Katzenmeier, R. Volk, A. Kohnle, F. Lottspeich, D. Allendorf, and A. Bacher.** 1993. Biosynthesis of riboflavin: Cloning, sequencing, mapping and expression of the gene coding for GTP cyclohydrolase II in *Escherichia coli.* J. Bacteriol. **175**:4045-4051.

34. **Richter, G., R. Volk, C. Krieger, H.W. Lahm, U. Rothlisberger, and A. Bacher.** 1992. Biosynthesis of riboflavin: cloning, sequencing, and expression of the gene coding 3,4-dihydroxy-2-butanone 4-phosphate synthase of *Escherichia coli.* J. Bacteriol. **174**:4050-4056.

35. **Rosendal, S., D.S. Carpenter, W.R. Mitchell, and M.R. Wilson.** 1981. Vaccination against pleuropneumonia in pigs caused by *Haemophilus pleuropneumoniae*. Can. Vet. J. **22**:34-35.

36. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual, 2nd. ed. Cold Spring Harbor Press, Cold Spring Harbor, New York.

37. **Sanger, F., S. Milken, and A.R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. **74**:5463-5467.

38. **Schott, K., J. Kellermann, F. Lottspeich, and A. Bacher.** 1990. Riboflavin synthases of *Bacillus subtilis*: purification and amino acid sequence of the $\alpha$-subunit. J. Biol. Chem. **265**:4204-4209.

39. **Sebunya, T.N.K., and J.R. Saunders.** 1983. *Haemophilus pleuropneumoniae* infection in swine: A review. J. Amer. Vet. Med. Asscoc. **182**:1331-1337.

40. **Shavlovskii, G.M., and E.M. Logvinenko.** 1988. Flavin oversynthesis in microorganisms and its molecular mechanisms (review). Prikladnaya Biokhimiya i Mikrobiologiya **24**:435-447.

41. **Swartzman, E., C. Miyamoto, A. Graham, and E.A. Meighen.** 1990. Delineation of the transcriptional boundaries of the *lux* operon of *Vibrio harveyi* demonstrates the presence of two new *lux* genes. J. Biol. Chem. **265**:3513-3517.

42. **Tetsuya, T., C. Ueguchi, K. Shiba., and K. Ito.** 1992. Insertional disruption of the *nusB* (*ssyB*) gene leads to cold-sensitive growth of *Escherichia coli* and suppression of the *secY24* mutation. Mol. Gen. Genet. **234**:429-432.

43. **Thacker, B.J., and M.H. Mulks.** 1988. Evaluation of commercial *Haemophilus pleuropneumoniae* vaccines. Proc. Int. Pig Vet. Soc. **10**:87.

44. **Utrera, V., C. Pijoan, and T. Molitor.** 1992. Evaluation of the immunity induced in pigs after infection with a low virulence strain of *A. pleuropneumoniae* serotype 1. Proc. Int. Pig Vet. Soc. **12**:213.

45. **Vieira, J. and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259-268.

46. **Wilson, A.C. and A.B. Pardee.** 1962. Regulation of flavin synthesis by *Escherichia coli*. J. Gen. Microbiol. **28**:283-303.

# Chapter 3

# A RIBOFLAVIN AUXOTROPH OF *ACTINOBACILLUS PLEUROPNEUMONIAE* IS ATTENUATED IN SWINE

# ABSTRACT

*Actinobacillus pleuropneumoniae* is the etiological agent of a highly contagious and often fatal pleuropneumonia in swine. A riboflavin-requiring mutant of *A. pleuropneumoniae* serotype 1, designated AP233, was constructed by deleting a portion of the riboflavin biosynthetic operon (*ribGBAH*) and replacing it with a gene cassette encoding kanamycin resistance. The genes affected included both the α and β-subunits of riboflavin synthase as well as a bifunctional enzyme containing GTP cyclohydrase and 3,4-dihydroxy-2-butanone-4-phosphate synthase (DHBP) activities. AP233 was unable to grow in the absence of exogenous riboflavin, but otherwise was phenotypically identical to the parent wild type strain. Experimental infection studies in pigs demonstrated that the riboflavin-requiring mutant was unable to cause disease, based on mortality, lung pathology and clinical signs, at dosages as high as 500 times the normal $LD_{50}$ of the wild type parent. This is the first demonstration of the attenuation of *A. pleuropneumoniae* by introduction of a defined mutation in a metabolic gene and the first demonstration that mutations in the genes required for riboflavin biosynthesis can lead to attenuation in a bacterial pathogen.

# INTRODUCTION

*Actinobacillus pleuropneumoniae*, a gram negative coccobacillus belonging to the family *Pasteurellaceae*, is the causative agent of porcine pleuropneumonia (4,20,32). This disease is characteristically an acute necrotizing hemorrhagic bronchopneumonia, with accompanying fibrinous pleuritis (4,32). Pleuropneumonia is an economically devastating, severe and often fatal disease with clinical courses ranging from hyperacute to chronic infection (4,9). The existence of at least twelve antigenically distinct capsular serotypes (28) has made development of a cross-protective vaccine difficult. Killed whole cell bacterins provide at best serotype-specific protection (22,23,29,37). In contrast, natural or experimental infection with a highly virulent serotype of *A. pleuropneumoniae* elicits protection against reinfection with any serotype (21,22,24). In several recent studies, attenuated strains of *A. pleuropneumoniae* produced by chemical mutagenesis, serial passage, or other undefined spontaneous mutation have been tested as live vaccines, with promising results (10,27,38). However, the use of live vaccines in the field is problematic, particularly when the attenuating lesion in the vaccine strain has not been genetically defined. A well-defined mutation that prevents reversion to wild-type would be extremely desirable for the development of a live attenuated vaccine against pleuropneumonia. We hypothesize that a mutation in a critical biosynthetic pathway which limits growth *in vivo* but does not otherwise alter expression of important antigens such as capsular polysaccharide, lipopolysaccharide and extracellular toxins, could produce an attenuated vaccine strain capable of inducing cross-protective immunity against *A. pleuropneumoniae*.

A variety of mutations in biosynthetic pathways are known to be attenuating in other organisms. Lesions in *aro* (6-8, 13, 17, 26, 39), *pur* (26,33), and *thy* (1) loci, which affect the biosynthesis of aromatic amino acids, purines and thymine, respectively, are attenuating because they eliminate the ability of the bacterium to synthesize critical compounds that are not readily available within mammalian hosts. For example, *aro* mutants of *Salmonella* and *Shigella* species have been shown to be attenuated in their natural hosts (6-8, 13, 17, 26). Lesions that affect the biosynthesis of LPS (3,25) and of cyclic AMP (14, 36) have also been shown to be attenuating in *Salmonella* species. It is important to note that not all attenuating mutations are good vaccine candidates in different organisms because some attenuating mutations result in poor persistence and immunogenicity (26,33).

Riboflavin (vitamin B2), a precursor of the coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), is essential for basic metabolism. It is synthesized by plants and by most microorganisms but not by higher animals (2). Many pathogenic bacteria are apparently unable to utilize flavins from their environment and are entirely dependent on endogenous production of riboflavin (31). Even with the ability to utilize exogenous riboflavin, there may not be enough of the vitamin present in mammalian host tissues to permit growth, particularly not in sites devoid of normal bacterial flora. Therefore, we hypothesized that riboflavin biosynthesis would be essential for survival of *A. pleuropneumoniae in vivo*, and that mutations in the riboflavin biosynthetic pathway would be attenuating due to the scarcity of riboflavin present on the mucosal surfaces of the respiratory tract.

Previously we reported the identification of a fragment of *A. pleuropneumoniae* serotype 5 chromosomal DNA that triggers overproduction of riboflavin when cloned in *E. coli*. Nucleotide sequence analysis demonstrated four open reading frames with significant identity and a similar operon arrangement to the *ribGBAH* genes from *Bacillus subtilis* (5). The *ribGBAH* genes encode, respectively, a *rib*-specific deaminase, the $\alpha$-subunit of riboflavin synthase, a bifunctional enzyme containing GTP cyclohydrase and 3,4-dihydroxy-2-butanone-4-phosphate synthase activities, and the $\beta$-subunit of riboflavin synthase (5). In this study we report the construction of a deletion-disruption riboflavin mutant of *A. pleuropneumoniae* serotype 1 and show that this mutation attenuates *A. pleuropneumoniae in vivo*. This is the first published report demonstrating that riboflavin auxotrophy can lead to attenuation of a bacterial pathogen in its natural host.

## MATERIALS AND METHODS

**Bacterial strains and media.** The bacterial strains and plasmids used in this study are listed in Table 3.1. *A. pleuropneumoniae* strains were cultured at 37 °C in either brain heart infusion (BHI), heart infusion (HI), or tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI) containing 10 μg/ml β-NAD (V factor) (Sigma Chemical Company, St. Louis, MO). Riboflavin (Sigma) was added to a final concentration of 200 μg/ml when needed. *E. coli* strains were cultured in Luria-Bertani medium. Ampicillin was added to 100 μg/ml and kanamycin to 50 μg/ml for plasmid selection in *E. coli* strains. For *A. pleuropneumoniae* strains, 50 μg/ml kanamycin sulfate and 25 μg/ml nalidixic acid were added as required, except for selection after matings which were performed with 100 μg/ml kanamycin sulfate and 50 μg/ml nalidixic acid.

**DNA manipulations.** DNA modifying enzymes were supplied by Boehringer-Mannheim Biochemicals (Indianapolis, IN) and used according to the manufacturer's specifications. Genomic DNA was prepared according to the lysis/proteinase K method of the Gene Fusion Manual (34). Plasmid DNA preparations, agarose gel electrophoresis, and *E. coli* transformation were all performed by conventional methods (30).

**Filter mating targeted mutagenesis.** Filter mating between *E. coli* S17-1 (λ*pir*) /pTF67A and AP225 was performed according to the protocol of Mulks and Buysse (19). Briefly, bacterial cultures were grown overnight at 37°C. Equal cell numbers of donor and recipient cultures, as determined by optical density at 520 nm, were added to 5 ml 10 mM $MgSO_4$ and the bacteria pelleted by centrifugation. The pellet containing the cell mating mixture, resuspended in 100 μl of 10 mM $MgSO_4$, was plated on a sterile filter on BHIV + riboflavin agar and incubated for 3 h at 37°C. Cells were washed from the filter in sterile phosphate buffered saline (pH 7.4), centrifuged, resuspended in 400 μl BHIV broth and plated in 100 μl aliquots on BHIV containing riboflavin, kanamycin, and nalidixic acid. Kanamycin and nalidixic acid resistant

## Table 3.1 - Characteristics of Bacterial Strains and Plasmids.

| Strain / Plasmid | Characteristics | Source/Reference |
|---|---|---|
| **Strain** | | |
| *E. coli* DH5-α | *supE44*, Δ*lacU169*, (φ80*lacZ*ΔM15), *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1* | BRL (USA) |
| *E. coli* DH5-α (λpir) | λpir, *supE44*, Δ*lacU169*, (φ80*lacZ*ΔM15), *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1* | Mulks & Buysse |
| *E. coli* S17-1 ( λpir) | λ pir, *recA*, *thi*, *pro*, *hsd*, (r-m+), RP4-2, (Tc::Mu), (Km::Tn7), [TmpR], [SmR] | Simon et al. |
| AP100 | *A. pleuropneumoniae* ATCC 27088, serotype 1, passaged through pigs | ATCC |
| AP106 | *A. pleuropneumoniae* ISU178, a serotype 5 field isolate, passaged through pigs | Iowa State University |
| AP225 | A spontaneous nalidixic acid resistant mutant of AP100 | This work |
| AP233 | A double cross-over riboflavin auxotroph of AP225 | This work |
| AP234 | A single cross-over riboflavin auxotroph of AP225 | This work |
| **Plasmid** | | |
| pUC19 | Ap$^{R}$ cloning vector | Vieira & Messing |
| pUC4K | Ap$^{R}$, Km$^{R}$ vector, source of the *kan* cassette | Pharmacia (USA) |
| pGP704 | Ap$^{R}$ broad host range suicide vector | Miller & Mekalanos |
| pGZRS19 | Ap$^{R}$ APP-E.coli shuttle vector | West et al. |
| pTF10 | AP106 *ribGBAH* genes cloned into pUC19 | Fuller & Mulks |
| pTF66 | A 2.9 kb fragment containing AP106 *ribBAH* in pGP704 | This work |
| pTF67a | pTF66 with all of *ribA* and part of *ribB* deleted and replaced with the *kan* cassette from pUC4K | This work |
| pTF76 | 5.2 Kb insert from pTF10 cloned into pGZRS19 | This work |

colonies were selected from filter mating plates and screened for riboflavin auxotrophy by replica plating onto TSAV, observing for inability to grow in the absence of added riboflavin.

**Southern Analysis of Transconjugants.** Chromosomal DNA and plasmid controls were digested with the appropriate restriction enzymes and the DNA fragments were separated on an 0.7% ultrapure agarose gel in TAE buffer. Southern blots were performed as described by Sambrook et al (30). DNA probes were labeled with digoxygenin by random priming using the Genius V. 3.0 kit from Boehringer Mannheim. Probes included the 5.2 Kb insert from pTF10 containing the intact riboflavin operon from AP106 (Rib), the 1.4 Kb *ClaI/NdeI* fragment deleted from the riboflavin operon in the construction of pTF67a (R.Del.), the 1.2 Kb kanamycin cassette from pUC4K (Km) and the intact plasmid pGP704 (pGP704). Hybridization was carried out in 50% formamide at 42°C for 16h. Blots were washed twice in 2 X SSC/0.1% SDS for 15 min at room temperature, then twice in 0.1X SSC/0.1% SDS for 30 min at 65°C. Blots were developed with alkaline phosphatase-conjugated anti-digoxygenin and colorimetric substrate (Boehringer Mannheim) according to the manufacturer's instructions.

**Phenotypic analysis of mutant strains.** Whole cell lysates and supernatants of AP100, AP225 ($Nal^R$), and AP233 ($Km^R$, $Nal^R$, Rib-) were prepared from overnight cultures grown in HIV + 5 mM $CaCl_2$ + appropriate antibiotics. Cells were separated by microcentrifugation and resuspended in SDS-PAGE sample buffer (16). The culture supernatant was precipitated with an equal volume of 20% trichloroacetic acid (TCA) and resuspended in SDS-PAGE sample buffer. Cellular polysaccharides, including lipopolysaccharide (LPS) and capsular polysaccharide, were prepared according to the cell lysis/proteinase K method of Kimura et al (15). All samples were analyzed on an 0.125% SDS-12% acrylamide gel using a discontinuous buffer system (16). Samples were transferred to nitrocellulose according to standard protocols (30) and probed with convalescent serum from a pig infected with *A. pleuropneumoniae* serotype 1.

Antigen-antibody complexes were detected with horseradish peroxidase-conjugated protein A (Boehringer Mannheim) and the colorimetric substrate 4-chloro-naphthol (BioRad, Hercules, CA).

Production of serotype-specific capsular polysaccharide was measured by coagglutination assay using hyperimmune rabbit anti-sera complexed to *Staphylococcus aureus* whole cells (11).

**Electroporation of *A. pleuropneumoniae*.** AP233 was grown in 100 ml BHIV with riboflavin at 37°C, with shaking at 150 RPM, to an $OD_{520}$ of 0.7. Cells were chilled on ice and centrifuged at 5,000 X *g* at 4°C for 10 min. Cells were washed twice in ice cold sterile 15% glycerol. Cells were resuspended in 2 ml 15% glycerol and frozen in 50 μl aliquots using a dry ice-ethanol bath. Plasmid DNA was added to an aliquot of competent cells thawed on ice and then transferred to a 0.1 cm gap electroporation cuvette (BioRad). Cells were electroporated using a Gene Pulser II (BioRad) with the following settings: voltage, 1.8 kV; resistance, 200 Ω; capacitance, 25 μFd.

**Experimental infections.** Eight-week-old specific-pathogen-free castrated male pigs (Whiteshire Hamroc, Inc., Albion, IN) were allotted to six challenge groups by a stratified random sampling procedure, balancing each group for body weight. Each challenge group was housed in a separate BSL-2 isolation room at the Michigan State University Research Containment Facility. All experimental protocols for animal experiments were reviewed by the Michigan State University All University Committee on Animal Use and Care, and all procedures conformed to university and USDA regulations and guidelines.

For preparation of challenge inocula, bacteria were grown in 30 ml HIV + 5 mM $CaCl_2$ + riboflavin and antibiotics as needed, in 300 ml baffled side-arm flasks, at 37°C with shaking at 160 RPM, to an $OD_{520}$ of 0.8. Ten ml of each culture was harvested by centrifugation at room temperature and washed once with sterile 0.9 % saline. The cell pellet was resuspended in 10 ml of saline and diluted in saline to obtain the desired cfu/ml. The actual inoculating doses were retrospectively calculated by viable cell counts on agar plates.

For the challenge procedure, pigs were anesthetized by intravenous injection with ketamine (4.4 mg/kg) and xylazine (1.65 mg/kg) and inoculated by percutaneous intratracheal injection with the appropriate dose of bacteria suspended in 10 ml saline. Clinical signs of pleuropneumonia, including respiration rate, temperature, dyspnea, appetite and activity/attitude (depression), were monitored and scored as previously described (12). Seriously ill animals, as determined by severe dyspnea and/or depression, were euthanized immediately. Survivors were euthanized three days post-challenge. All animals were necropsied, and lungs were examined macroscopically for *A. pleuropneumoniae* lesions, including edema, congestion, hemorrhage, necrosis, abscessation, fibrosis, and pleuritis. The percentage of lung tissue and pleural surface area affected was estimated for each of the seven lung lobes, and the total % pneumonia and % pleuritis calculated using a formula that weights the contribution of each lung lobe to the total lung volume (12). Representative lung samples were collected for histopathology and for bacterial culture.

## RESULTS

**Construction of *A. pleuropneumoniae rib* mutants.** To construct riboflavin-requiring auxotrophic mutants of *A. pleuropneumoniae*, a suicide plasmid with part of the riboflavin operon deleted and replaced with a kanamycin-resistance ($Km^R$) cassette was designed (Figure 3.1). A 2.9 kb *Eco*RI fragment from pTF10 (5) containing the *A. pleuropneumoniae ribBAH* genes was cloned into the *Eco*RI site of the conjugative suicide vector pGP704 (18) to create plasmid pTF66. pTF66 was digested with *Cla*I and *Nde*I to excise the 3' end of *ribB* and the entire *ribA* gene. After Klenow treatment of the DNA, the 1.2 kb $Km^R$ cassette, excised with *Eco*RI from pUC4K , was blunt-end ligated into the *rib* deletion site to create pTF67a.

pTF67a was transformed into *E. coli* S17-1 (λ*pir*) and mobilized into AP225 ($Nal^R$ ) to produce >100 transconjugant colonies demonstrating resistance to both nalidixic acid and kanamycin. Transconjugants were replica plated onto TSAV and TSAV + riboflavin to assess the requirement for riboflavin and the stability of the riboflavin auxotrophy. Two classes of transconjugants were found. The majority of the transconjugants, e.g. AP234, were unstable and produced revertants capable of growth without supplemental riboflavin in the absence of kanamycin selection. One transconjugant, AP233, was very stable, maintaining kanamycin resistance as well as the inability to grow without exogenous riboflavin. All transconjugants were confirmed as *A. pleuropneumoniae* by gram stain, colonial morphology, and requirement for V factor (β-NAD).

**Southern blot analysis of transconjugants.** Two transconjugants were selected for further analysis based on their phenotypes as potential single (AP234) and double cross-over mutants (AP233). Southern blot analysis of transconjugant genomic DNA from the two mutants indicated that AP233 and AP234 were indeed double and single cross-over insertion mutants respectively (Figure 3.2). Predicted band sizes for single and double cross-over events are shown in Figure 3.3.

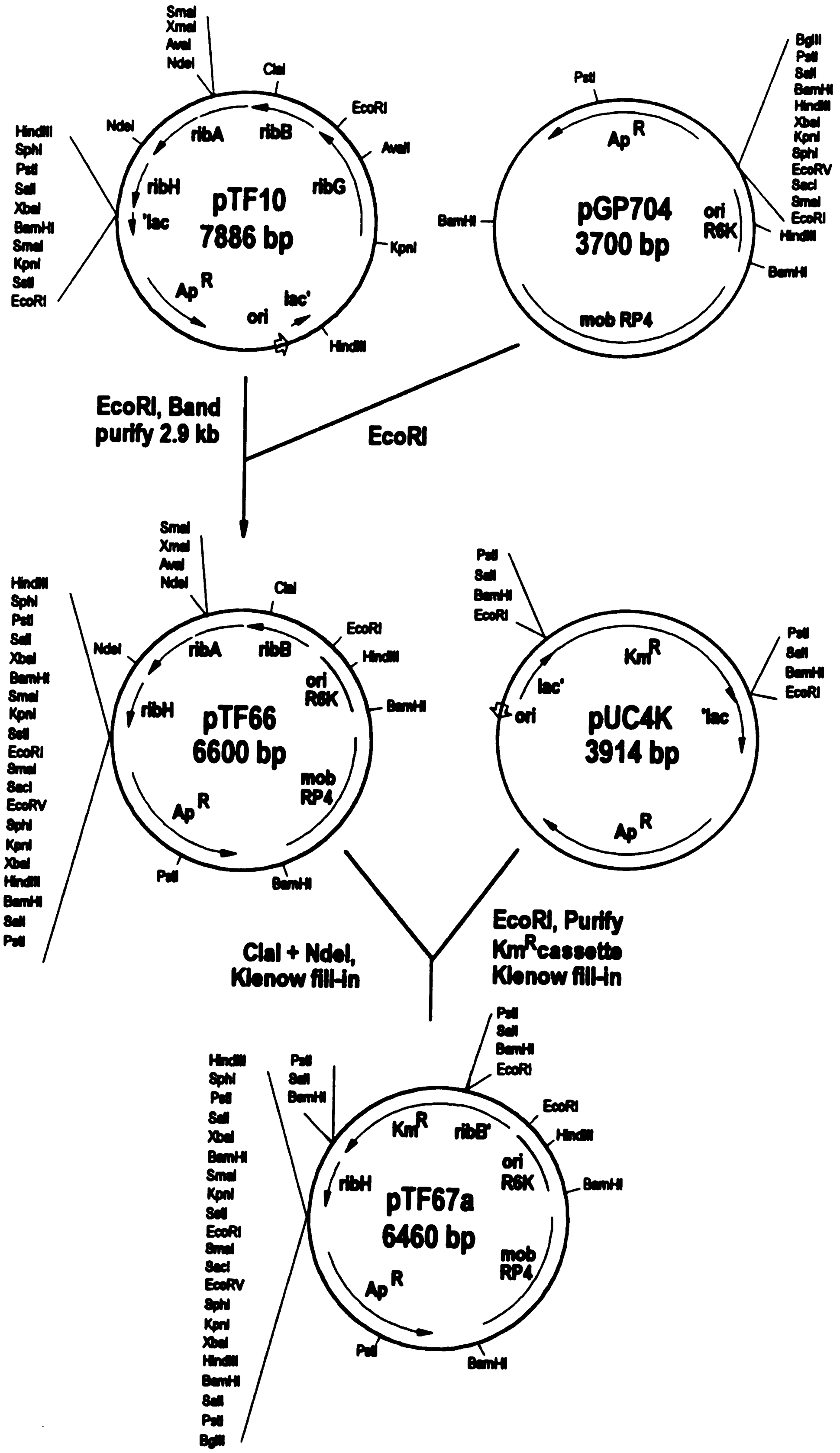


Figure 3.1 - Construction of pTF67a.

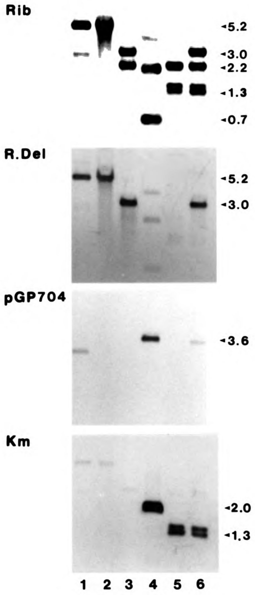


**Figure 3.2 - Southern blots of *Hin*dIII or *Eco*RI Digested DNA from Mutants and Controls.** Blots were prepared in quadruplicate and hybridized at high stringency with one of four probes: Rib, the entire *ribGBAH* operon from pTF10; R. Del., the deleted portion (*Cla*I/*Nde*I fragments) of the *ribGBAH* operon; pGP704, the entire plasmid; Km, the kanamycin cassette from pUC4K. Lanes: 1, pTF10 digested with *Hin*dIII; 2, AP106 + *Hin*dIII; 3, AP100 + *Hin*dIII; 4, pTF67a + *Eco*RI; 5, AP233 + *Hin*dIII; and 6, AP234 + *Hin*dIII.

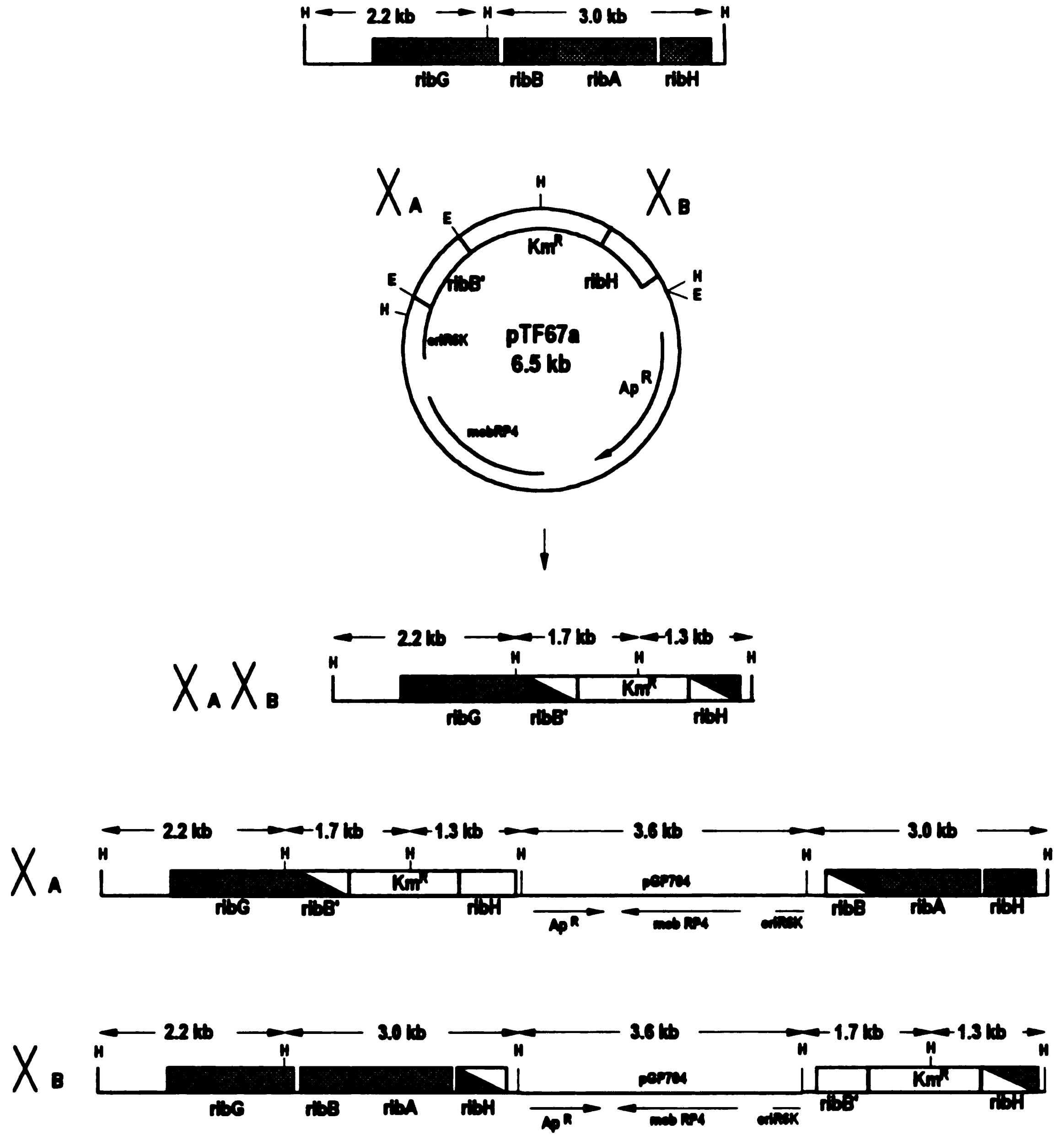


**Figure 3.3 - Schematic Structure of the *rib* Locus of Parent and Mutant Strains in Double and Single Cross-Over Events.** The predicted sizes of *Hin*dIII genomic fragments are shown for two possible single cross-over events and for a double cross-over event. The results show that for AP233 the chromosomal *rib* operon has been replaced with the cloned riboflavin operon containing the Km$^R$ cassette by a double cross-over event, while AP234 is the result of a single cross-over event either upstream or downstream of the kanamycin cassette. Restriction enzymes used: E = *Eco*RI; H = *Hin*dIII.

Genomic DNA from AP233 contained a 2.2 Kb *Hin*dIII fragment that hybridized with the riboflavin operon (Rib) probe, as well as 1.7 and 1.3 Kb fragments that hybridized with both the Rib and Km probes; however, there was no reaction with either pGP704 nor the deleted portion of the riboflavin operon (Figure 3.2). This is the pattern of hybridization predicted in transconjugants that replaced the wild type riboflavin operon with the mutated *rib*::Km$^R$ locus by a double-crossover event (Figure 3.3). In contrast, genomic DNA from AP234 shows the presence of DNA homologous to the fragment deleted from the riboflavin operon (R. del), pGP704, and the kanamycin cassette (Figure 3.3). This is the pattern of hybridization predicted in transconjugants that inserted the entire pTF67a plasmid into the wild type *rib* operon by a single crossover event (Figure 3.2).

**Phenotypic analysis of the *A. pleuropneumoniae rib* mutant.** Whole cell lysates, TCA-precipitated culture supernatants, and polysaccharide preparations were analyzed on silver stained SDS-PAGE and on immunoblots developed with convalescent swine sera. No differences in protein, LPS, extracellular toxin, or capsular polysaccharide profiles were detected between wild type AP100, its Nal$^R$ derivative AP225, and the riboflavin mutant AP233 (Figure 3.4). There was no difference in reactivity with serotype-specific antisera as determined by coagglutination assay (data not shown).

**Complementation of the *rib* mutation with a cloned wild type *rib* operon.** The 5.2 Kb insert from pTF10, containing the wild-type *A. pleuropneumoniae* riboflavin operon, was cloned into pGZRS19, an *E. coli-A. pleuropneumoniae* shuttle vector (41), to form pTF76. pTF76 was transformed into AP233 by electroporation, restoring the ability of AP233 to grow in the absence of exogenous riboflavin and restoring the virulence of the mutant (Table 3.2 and Table 3.3).

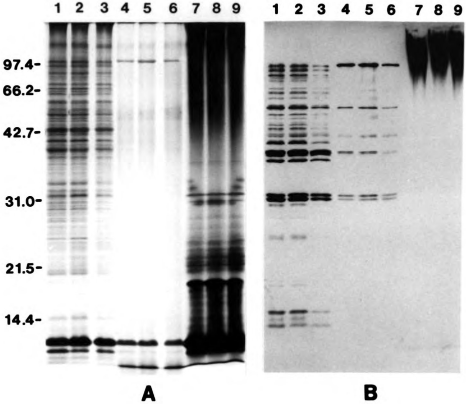


**Figure 3.4 - Phenotypic Analysis of AP100, AP225 and AP233.**

Panel A: All samples were boiled for 5 minutes before SDS-PAGE on a 12% gel.
Panel B: Western blot analysis was performed using convalescent $\alpha$-APP-1 swine serum and detected colorimetrically with a protein A-HRP system.

Lanes 1-3 are whole cell lysates of AP100, AP225 and AP233.
Lanes 4-6 are TCA precipitated supernatants of AP100, AP225 and AP233
Lanes 7-9 are LPS preparations of AP100, AP225 and AP233.

**Attenuation of virulence of the *rib* mutant in swine.** Six groups of three pigs each were infected with: group 1, 1 $LD_{50}$ ($5 \times 10^6$ cfu) of AP225; groups 2-5, AP233 at doses equivalent to 4, 20, 100, and 500 times the wild-type $LD_{50}$; and group 6, AP233/pTF76 at a dose equivalent to 1 $LD_{50}$ for the wild-type. Mortality, lung score, and clinical score data, shown in Tables 3.2 and 3.3, all indicate that the riboflavin auxotroph is avirulent in pigs at doses as high as 500 times the wild-type $LD_{50}$. The pigs infected with the *rib* mutant AP233 displayed no dyspnea, elevated respiration rate, depression, or loss of appetite, and had no typical pleuropneumonic pathology at necropsy at even the highest dose tested. In contrast, 1 of 3 pigs infected with the wild-type AP225 strain died, and all three exhibited significant clinical signs of infection, including elevated respiration rates, dyspnea, depression, loss of appetite, and fever, and severe pneumonia and pleuritis was evident at necropsy. Pigs infected with AP233 containing the riboflavin genes in *trans* (pTF76) also exhibited obvious clinical signs and significant pneumonia and pleuritis, although somewhat less severe than the wild-type strain. These results indicate that restoration of the ability to synthesize riboflavin does restore virulence.

Bacteria were readily reisolated at necropsy from the lungs of pigs receiving AP225 and AP233 /pTF76. All reisolated organisms were characterized by gram stain, colonial morphology, requirement for V factor (β-NAD), antibiotic sensitivity, and serotyping by coagglutination. Reisolated organisms showed no differences from the initial inocula, including the presence of plasmid pTF76 in bacteria reisolated from pigs infected with AP233/pTF76. In contrast, we were unable to recover organisms from the lungs of animals infected with AP233 and euthanized 48 hours post infection.

## Table 3.2 - Mortality and Lung Score Data.

| Group | Strain | Dose ($LD_{50}$)[a] | Mortality | % Pneumonia[b] | % Pleuritis[c] |
|---|---|---|---|---|---|
| 1 | AP225 (WT) | 1 | 1/3 | 66.7 | 71.7 |
| 2 | AP233 (Rib-) | 4 | 0/3 | 0 | 0 |
| 3 | AP233 (Rib-) | 20 | 0/3 | 0 | 0 |
| 4 | AP233 (Rib-) | 100 | 0/3 | 0 | 0 |
| 5 | AP233 (Rib-) | 500 | 0/3 | 0 | 0 |
| 6 | AP233+ pTF76 | 1 | 0/3 | 27.6 | 20.2 |

[a] Doses are multiples of the established wild-type $LD_{50}$ of 5.0 x $10^6$ cfu (12).

[b] % lung tissue exhibiting *A. pleuropneumoniae* lesions

[c] % pleural surface area exhibiting pleuritis

## Table 3.3 - Clinical Score Data

| Group | Strain | Dose ($LD_{50}$)[a] | RR Max[b] | Temp Max[c] | Dyspnea[d] | Depression[e] | Appetite[f] |
|---|---|---|---|---|---|---|---|
| 1 | AP225 | 1 | 20 | 105.7 | 5.5 | 6.7 | 4.2 |
| 2 | AP233 | 4 | 8 | 102.5 | 0 | 0 | 0 |
| 3 | AP233 | 20 | 8 | 103.3 | 0 | 0 | 0 |
| 4 | AP233 | 100 | 8 | 103.5 | 0 | 0 | 0 |
| 5 | AP233 | 500 | 8 | 102.8 | 0 | 0 | 0 |
| 6 | AP233 + pTF76 | 1 | 19.3 | 105.4 | 4.5 | 4.7 | 3.7 |
|  | Normal |  | 8.0 | <103.0 | 0 | 0 | 0 |
|  | Maximum |  | 25 |  | 15 | 15 | 5 |

[a] Doses are multiples of the established wild-type $LD_{50}$ of 5.0 x $10^6$ cfu (12).

[b] Maximum respiratory rate observed after challenge. Respiratory rate recorded as number of breaths per 15 sec observation period.

[c] Maximum rectal temperature after challenge, in degrees Fahrenheit.

[d] Dyspnea score measures degree of respiratory distress and labored breathing. Scored as 0 = normal; 1 = slight; 2 = moderate; 3 = severe. Total score = sum of scores taken at 12 hour intervals after challenge.

[e] Depression score evaluates attitude and activity. Scored as 0 = normal; 1= slight inactivity; 2 = moderate; 3 = severe. Total score = sum of scores taken at 12 hour intervals after challenge.

[f] Appetite was scored as 0 = did eat; 1 = did not eat. Total score = number of 12 hour periods not eating over 60 hour observation period.

## DISCUSSION

In this paper, we report the construction of a serotype 1 *Actinobacillus pleuropneumoniae* deletion-disruption riboflavin mutant that is attenuated *in vivo*. The *A. pleuropneumoniae ribGBAH* operon was disrupted by deleting an internal segment of the operon (*ribBA*) and replacing it with a $Km^R$ cassette using a targeted mutagenesis technique (19). A stable riboflavin-requiring, $Km^R$ mutant, AP233, was phenotypically identical to its wild-type parent based on analysis of proteins, extracellular toxin, LPS, and capsular polysaccharide by SDS-PAGE, immunoblot, and coagglutination.

A riboflavin mutant of *A. pleuropneumoniae* serotype 5 was also constructed and was also found to be attenuated in a preliminary animal challenge experiment. However, further studies were conducted in serotype 1 because serotype 5 seemed to be very resistant to transformation by standard heat shock or electroporation procedures. In order to complement the *rib* mutation *in trans,* and for ease of future genetic manipulations, it was necessary to use a serotype 1 strain for these studies.

Experimental infection of pigs, the only natural host for *A. pleuropneumoniae,* demonstrated that the riboflavin-requiring mutant was unable to cause disease at dosages as high as 500 times the $LD_{50}$ for the wild-type parent. In the four groups of pigs infected with AP233 by intratracheal inoculation, there was no mortality, no significant clinical signs were observed, and no typical pleuropneumonic lesions were discovered upon necropsy. Complementation of AP233 in *trans* with the wild-type *A. pleuropneumoniae* riboflavin operon restored both the ability to grow without exogenous riboflavin and virulence, demonstrating that the riboflavin mutation itself is responsible for the attenuation *in vivo*.

It is important to note that the riboflavin-requiring mutant used in these studies is a deletion mutant, with ~1.4 Kb of the riboflavin operon removed from the chromosome and replaced with an antibiotic resistance marker. We observed neither reversion to prototrophy nor loss of kanamycin resistance in this mutant in the laboratory. In the preliminary experiment with a serotype 5 riboflavin mutant, we were able to reisolate the mutant from the pig lungs at 16 hours post-infection. All colonies isolated in this experiment were kanamycin-resistant, nalidixic acid-resistant, and riboflavin requiring, suggesting that reversion to prototrophy and thus virulence will not occur *in vivo*.

In the dosage trial experiments, AP233 was not recovered from the lungs of infected swine at 48 hours post-infection. These results may indicate poor persistence of the organism *in vivo*, a potential problem for its use as a live-attenuated vaccine. If necessary, sufficient exogenous riboflavin could be added to the vaccine to allow the organism to replicate minimally and therefore persist long enough to induce a protective immune response.

This is the first report that a mutation in riboflavin biosynthesis in a pathogenic bacterium is attenuating. This finding represents a new addition to the group of biosynthetic mutations which can be used to construct attenuated strains of bacteria. This is also the first report of a genetically defined attenuated mutant of *A. pleuropneumoniae* that is still capable of production of all of the major known virulence factors of this organism, including extracellular toxins and capsular polysaccharide. We intend to continue studies on the use of riboflavin mutants of *A. pleuropneumoniae* as candidates for a new generation of live attenuated vaccines against this disease. In addition, the fact that riboflavin biosynthesis is essential for this pathogen and is not synthesized by higher eukaryotes could potentially lead to discovery of a new generation of antibiotics which inhibit riboflavin biosynthesis.

## ACKNOWLEDGMENTS

This research was supported by grants from the State of Michigan Research Excellence Fund Center for Animal Production Enhancement and from the Michigan State University Office of Intellectual Property. T.E. Fuller is supported by a U.S.D.A. National Needs Graduate Fellowship in Animal Biotechnology.

## REFERENCES

1. **Ahmed, Z.U., M.R. Sarker, and D.A. Sack.** 1990. Protection of adult rabbits and monkeys from lethal shigellosis by oral immunization with a thymine-requiring and temperature-sensitive mutant of *Shigella flexneri* Y. Vaccine. **8**:153-158.

2. **Bacher, A.** 1991. Biosynthesis of flavins. p. 215-59. *In* F. Muller (ed.), Chemistry and Biochemistry of Flavins, Vol. 1. Chemical Rubber Company, Boca Raton, Fla.

3. **Collins, L.V., S. Attridge, and J. Hackett.** 1991. Mutations at *rfc* or *pmi* attenuate *Salmonella typhimurium* virulence for mice. Infect. Immun. **59**:1079-1085.

4. **Fenwick, B. and S. Henry.** 1994. Porcine pleuropneumonia. J. Am. Vet. Med. Assoc. **204**:1334-1340.

5. **Fuller, T.E. and M.H. Mulks.** 1995. Characterization of *Actinobacillus pleuropneumoniae* riboflavin biosynthesis genes. J. Bacteriol. **177**:7265-7270.

6. **Hoiseth S. K. and B.A.D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature (london). **291**:238-239.

7. **Homchampa, P., R. A. Strugnell and B. Adler.** 1992. Molecular analysis of the *aroA* gene of *Pasteurella multocida* and vaccine potential of a constructed *aroA* mutant. Mol. Microbiol. **6**:3585-3593.

8. **Homchampa, P., R. A. Strugnell and B. Adler.** 1994. Construction and vaccine potential of an *aroA* mutant of *Pasteurella haemolytica*. Vet. Microbiol. **42**:35-44.

9. **Hunneman, W.A.** 1986. Incidence, economic effects, and control of *Haemophilus pleuropneumoniae* infections in pigs. Vet. Quarterly **8**:83-87.

10. **Inzana, T.J., J. Todd, and H.P. Veit.** 1993. Safety, stability and efficacy of nonencapsulated mutants of *Actinobacillus pleuropneumoniae* for use in live vaccines. Infect. Immun. **61**:1682-1686.

11. **Jolie, R. A. V., M. H. Mulks, and B. J. Thacker.** 1994. Antigenic differences within *Actinobacillus pleuropneumoniae* serotype 1. Vet. Microbiol. **38**:329-349.

12. **Jolie, R. A. V., M. H. Mulks, and B. J. Thacker.** 1995. Cross-protection experiments in pigs vaccinated with *Actinobacillus pleuropneumoniae* subtypes 1A and 1B. Vet. Microbiol. **45**:383-391.

13. **Karnell, A., P.D. Cam, N. Verma and A.A. Lindberg**. 1993. *AroD* deleteion attenuates *Shigella flexneri* strain 2457T and makes it a safe and efficacious oral vaccine in monkeys. Vaccine 8:830-836.

14. **Kelly, S.M., B.A. Bosecker and R. Curtiss III**. 1992. Characterization and protective properties of attenuated mutants of *Salmonella cholerasuis*. Infect. Immun. 60:4881-4890.

15. **Kimura, A. and E.J. Hansen**. 1986. Antigenic and phenotypic variations of *Haemophilus influenzae* type B lipopolysaccharide and their relationship to virulence. Infect. Immun. 51:69-79.

16. **Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

17. **Lindberg, A.A., A. Karnell, B.A.D. Stocker, S. Katakura, H. Sweiha and F.P. Reinholt**. 1988. Development of an auxotrophic oral live *Shigella flexneri* vaccine. Vaccine 6:146-150.

18. **Miller, V.L. and J.J. Mekalanos**. 1988. A novel suicide vector and its use in construction of insertion mutants: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. 170:2575-2583.

19. **Mulks, M.H. and J.M. Buysse**. 1995. A targeted mutagenesis system for *Actinobacillus pleuropneumoniae*. Gene 165:61-66.

20. **Nicolet, J.** 1992. *Actinobacillus pleuropneumoniae*, p. 401-408. *In* A.D. Leman et al (eds), Diseases of Swine, 7th edition Iowa State University Press, Ames, Iowa.

21. **Nielsen, R.** 1979. *Haemophilus parahaemolyticus* serotypes: pathogenicity and cross immunity. Nord. Vet. Med. 31:407-413.

22. **Nielsen, R.** 1984. *Haemophilus pleuropneumoniae* serotypes - Cross protection experiments. Nord. Vet. Med. 36:221-234.

23. **Nielsen, R.** 1976. Pleuropneumonia of swine caused by *Haemophilus pleuropneumoniae*. Studies on the protection obtained by vaccination. Nord. Vet. Med. 28:337-338.

24. **Nielsen, R.** 1974. Serological and immunological studies of pleuropneumonia of swine caused by *Haemophilus parahaemolyticus*. Acta Vet. Scand. 15:80-89.

25. **Nnalue, N.A., and B.A.D. Stocker**. 1987. Tests of the virulence and live-vaccine efficacy of auxotrophic and *galE* derivatives of *Salmonella cholerasuis*. Infect. Immun. 55:955-962.

26. **O'Callaghan, D. D. Maskell, F.Y. Lieu, C.S.F. Easmon and G. Dougan**. 1988. Characterization of aromatic and purine dependent *Salmonella typhimurium*: attenuation, persistence and ability to induce protective immunity in BALB/c mice. Infect. Immun. **56**:419-423.

27. **Paltineanu, D., R. Pambucol, E. Tirziu, and I. Scobercea**. 1992. Swine infectious pleuropneumonia: Aerosol vaccination with a live attenuated vaccine. Proc. Int. Pig. Vet. Soc. **12**:214.

28. **Perry, M.B., E. Altman, J.-R. Brisson, L.M. Beynon, and J.C. Richards**. 1990. Structural characteristics of the antigenic capsular polysaccharides and lipopolysaccharides involved in the serological classification of *Actinobacillus pleuropneumoniae* strains. Serodiag. Immunother. Infect. Dis. **4**:299-308.

29. **Rosendal, S., D.S. Carpenter, W.R. Mitchell, and M.R. Wilson**. 1981. Vaccination against pleuropneumonia in pigs caused by *Haemophilus pleuropneumoniae*. Can. Vet. J. **22**:34-35.

30. **Sambrook, J., E.F. Fritsch and T. Maniatis**. 1989. Molecular Cloning: A Laboratory Manual, 2nd. ed. Cold Spring Harbor Press, Cold Spring Harbor, New York.

31. **Schott, K., J. Kellerman, F. Lottspeich and A. Bacher**. 1990. Riboflavin synthases of *Bacillus subtilis*: purification and amino acid sequence of the α-subunit. J. Biol.Chem. **265**:4204-4209.

32. **Sebunya, T.N.K. and J.R. Saunders**. 1983. *Haemophilus pleuropneumoniae* infection in swine: a review. J. Am. Vet. Med. Assoc. **182**:1331-1337.

33. **Sigwart, D.F., B.A.D. Stocker, and J.D. Clements**. 1989. Effect of a *purA* mutation on the efficacy of *Salmonella* live vaccine vectors. Infect. Immun. **57**:1858-1861.

34. **Silhavy, T.J.** 1984. DNA extraction from bacterial cells. p. 137-139. *In* Experiments with Gene Fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

35. **Simon, R., U. Priefer, and A. Puhler**. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technol. **1**:784-791.

36. **Tacket, C.I., D.M. Hone, R. Curtiss III, S.M. Kelly, G. Losonsky, L. Guers. A.M. Harris, R. Edelman. M.M. Levine**. 1992. Comparison of the safety and immunogenicity of Δ*aroC* Δ*aroD* and Δ*cya*Δ*crp* *Salmonella typhi* strains in adult volunteers. **60**:536-541.

37. **Thacker, B.J., and M.H. Mulks**. 1988. Evaluation of commercial *Haemophilus pleuropneumoniae* vaccines. Proc. Int. Pig Vet. Soc. **10**:87.

38. **Utrera, V., C. Pijoan, and T. Molitor**. 1992. Evaluation of the immunity induced in pigs after infection with a low virulence strain of *A. pleuropneumoniae* serotype 1. Proc. Int. Pig Vet. Soc. **12**:213.

39. **Vaughan, L.M., P.R. Smith, and T.J. Foster**. 1993. An aromatic-dependent mutant of the fish pathogen *Aeromonas salmonicida* is attenuated in fish and is effective as a live vaccine against the Salmonid disease furunculosis. Infect. Immun. **61**:2172-2181.

40. **Vieira, J. and J. Messing**. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259-268.

41. **West, S.E.H., M.J.M. Romero, L.B. Regassa, N.A. Zielinski, and R.A. Welch**. 1995. Construction of *Actinobacillus pleuropneumoniae-Escherichia coli* shuttle vectors: expression of antibiotic resistance genes. Gene **160**: 81-86.

# Chapter 4

# A RIBOFLAVIN AUXOTROPH OF *ACTINOBACILLUS PLEUROPNEUMONIAE* AS A LIVE-ATTENUATED VACCINE

## ABSTRACT

*Actinobacillus pleuropneumoniae* (APP) is a gram negative pleiomorphic rod that is the causative agent of a severe, highly infectious and often fatal pleuropneumonia in swine. We have previously reported the construction of a genetically defined stable riboflavin auxotroph (AP233) by replacing a portion of the APP serotype 1 strain (ATCC 27088) riboflavin biosynthetic operon (*ribGBAH*) with an antibiotic cassette encoding resistance to kanamycin. The riboflavin mutant was previously shown to be avirulent at a dosage equivalent to 500 times the established $LD_{50}$ for the wild type parent. We have tested AP233 in two studies for its ability to stimulate protective immunity against pleuropneumonia. In the first study, four groups of six pigs each were inoculated with 5 x $10^8$ cfu of AP233 either by intratracheal or intramuscular injection, with or without the addition of 5 μg/ml of riboflavin to the live vaccine inoculum. Two additional groups of six pigs each were either vaccinated with a serotype 1A bacterin or kept as unvaccinated controls. The pigs received a second vaccination at three weeks followed by experimental challenge with 1 $LD_{50}$ of serotype 1A two weeks later. Results of the challenge experiment indicate that intramuscular vaccination with the live attenuated *rib* mutant, in a vaccine formulation that included a limiting amount of exogenous riboflavin, provided significant protection against homologous challenge with virulent *A. pleuropneumoniae*. In a second study, two groups of six pigs each were vaccinated twice with 5 x $10^9$ cfu of AP233 in PBS + 5 μ g/ml riboflavin by intramuscular injection. Two additional groups of six pigs each were sham vaccinated with PBS + 5 μg/ml riboflavin. Challenge with both a homologous (serotype 1) strain and a heterologous (serotype 5) strain demonstrated limited protection against pleuropneumonia. It is inconclusive at this time as to the degree of protection obtained by vaccination with AP233.

## INTRODUCTION

*Actinobacillus pleuropneumoniae* (APP) is the causative agent of porcine pleuropneumonia, a severe and often fatal respiratory disease of swine (5,24,37) first described over 30 years ago (38). While our understanding of the pathogenesis of APP infections and the virulence factors involved has increased dramatically over the past decade, there is still no safe effective vaccine against APP that provides protection against all the major serotypes (26,27,33,40). This is in part due to the fact that there are twelve antigenically distinct capsular serotypes (31) as well as antigenic subtypes of some serotypes (17, 25), and vaccination with a killed whole cell vaccine prepared from one serotype does not generally confer protection against other serotypes (26,27,33,40), or even subtypes of the same serotype (16). Current commercial vaccines are still primarily killed whole cell bacterins, which generally reduce mortality from APP infection but frequently fail to prevent severe morbidity and economic loss due to chronic effects of the disease on growth rate and feed efficiency (5,13).

In contrast to bacterin vaccines, natural or experimental infection with a virulent strain of APP generally elicits protection against reinfection with any serotype (26,27,33,40), implying that there are protective antigens or immunomodulatory compounds produced by the organism *in vivo* which are not produced *in vitro*. This suggests that a live avirulent vaccine, with limited replication ability, would have the potential to produce these factors and elicit broad cross-protective immunity against all serotypes of APP. Avirulent strains of APP, including capsule-deficient strains and other strains for which the attenuating lesion is completely undefined, have been tested as live vaccines and have elicited cross-protective immunity against subsequent challenge (14,30,34,42). However, the use of live vaccines in the field is problematic, particularly when the attenuating lesions in the vaccine strain are not genetically defined. The development of attenuated strains of APP with genetically defined bi

us

ge

ta

co

1

c

p

r

t

s

biochemical mutations that limit growth *in vivo* and prevent reversion to wild type for use as live avirulent vaccines has, until recently, been impossible due to the lack of genetic tools for manipulation of APP chromosomal genes. We have developed a targeted mutagenesis system for APP (23) and are now using this methodology to construct genetically defined deletion mutants of APP.

Many live-attenuated strains of organisms such as *Shigella flexneri* (1, 18,20,28,35), *Salmonella typhi* (8), *Salmonella typhimurium* (4,10), *Salmonella choleraesuis* (19), *Aeromonas salmonicida* (44), *Bacillus anthracis* (15), *Bordetella pertussis* (32), *Yersinia enterocolitica* (3), *Pasteurella haemolytica* (11) and *Pasteurella multocida* (12) provide moderate-to-excellent protection against infection, varying with the attenuating lesion and the dosage regimen. Many of these live-attenuated vaccine strains have been constructed by disrupting biochemical pathways or critical virulence genes. For example, live attenuated vaccines have been constructed using mutations in *aro* (3,10,11,12,15,18,20,32,44), *icsA* (35), *iuc* (35), *guaB-A-virG* (28), *cya-crp* (19), *thy* (1), *rfc, pmi* (4) and *gal* (8), which respectively encode aromatic amino acid biosynthetic enzymes, intracellular spreading factors, siderophores, guanine nucleotide biosynthetic enzymes, adenylate cyclase and cAMP, thymine biosynthetic enzymes, and enzymes involved in lipopolysaccharide biosynthesis. Live vaccines appear to hold much promise for the future of vaccination, due to the ability to vaccinate with a small amount of organism, the production and more natural presentation of protective antigens, and the ability to stimulate the production of cytokines influencing the immune system (21).

The goal of this research was to test a genetically defined attenuated biochemical mutant of APP as a live-attenuated vaccine (LAV). Previously we reported the cloning and characterization of the operon encoding riboflavin biosynthesis from APP. Riboflavin (vitamin B2), a precursor of the coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), is essential for basic metabolism. It is synthesized by plants and by most microorganisms but not by higher animals (2). Therefore, exogenous riboflavin is not likely to be freely available on the mucosal surfaces of the respiratory tract. In addition, many pathogenic bacteria are apparently unable to utilize flavins from their environment and are entirely dependent on endogenous production of riboflavin (36). We have constructed genetically defined mutants of APP that lack part of the riboflavin biosynthetic operon (6) and require exogenous riboflavin for growth. We have confirmed that riboflavin biosynthesis is essential for survival of APP *in vivo* and that mutations in the riboflavin biosynthetic pathway are highly attenuating (7).

In this study we have conducted two vaccine trials to test the potential efficacy of this riboflavin-requiring mutant of APP as a live-attenuated vaccine. The results presented here demonstrate that intramuscular vaccination with the live riboflavin auxotroph can elicit some protective immunity against challenge with two different serotypes of virulent wild type *A. pleuropneumoniae*.

## MATERIALS AND METHODS

**Animals.** Eight-week-old crossbred (Yorkshire/Landrace) castrated male pigs from a herd known to be free of *A. pleuropneumoniae* and related respiratory pathogens (Whiteshire Hamroc, Inc., Albion, IN) were allotted to challenge groups by a stratified random sampling procedure, balancing each group for body weight. Each challenge group was housed in a separate BSL-2 isolation room at the Michigan State University Research Containment Facility and fed a standard antibiotic-free diet provided by the Michigan State University Swine Research and Teaching Center. All experimental protocols for animal experiments were reviewed by the Michigan State University All University Committee on Animal Use and Care, and all procedures conformed to university and USDA regulations and guidelines.

**Bacterial Strains.** *A. pleuropneumoniae* ATCC 27088 (APP-1A), reisolated from experimentally infected pigs, was used as the serotype 1 challenge strain. *A. pleuropneumoniae* ISU178 (APP-5), also reisolated from infected pigs, was used as the serotype 5 challenge strain. AP233 (7), a Km$^r$, Nal$^r$, riboflavin-requiring mutant of APP-1A, was used as the live attenuated vaccine strain.

**Media/Culture Conditions.** All bacteria were grown in heart infusion broth (HI) (Difco, Detroit, MI) with 10 μg/ml β-nicotinamide adenine dinucleotide (V factor) (Sigma, St. Louis, MO). Riboflavin (Sigma) was added at 200 μg/ml for growth of strain AP233. Kanamycin was used at 100 μg/ml and nalidixic acid was used at 50 μg/ml in agar plates.

**Preparation of Vaccines.** AP233 for the live vaccine was grown in 30 ml HIV broth + 5 mM $CaCl_2$ + 200 μg/ml riboflavin, in 300 ml baffled side-arm flasks at 37°C with shaking at 160 RPM, to an optical density at 520 nm of 0.8. Bacteria were harvested by centrifugation, washed once in phosphate buffered saline (PBS), pH 7.0, diluted in PBS to the appropriate cell density, and used immediately as vaccine. For vaccine groups containing riboflavin, 5 μg/ml riboflavin was added to the PBS (PBS-

Rib) used for washing and resuspension. The actual inoculating doses were retrospectively calculated by viable cell counts on agar plates.

To prepare the formalinized whole cell bacterin, virulent APP-1A bacteria were similarly grown in HIV broth + 5 mM $CaCl_2$ to an optical density at 520 nm of 0.8. Bacteria were harvested by centrifugation and washed once with Tris-acetate-EDTA-DTT buffer. Bacteria were resuspended in buffer containing 0.2% formalin to a concentration of $5 \times 10^9$ cfu/ml, and kept at room temperature for 1 hour, then stored at 4°C. Each vaccine dose contained 1 ml formalinized cells, 0.5 ml saline, and 0.5 ml Emulsigen adjuvant (MVP Laboratories, Ralston, Nebraska).

**Vaccination.** In trial one, six groups (six pigs per group) of six- to eight-week-old APP-free pigs were vaccinated twice with a 3 week interval. Group 1 was vaccinated intratracheally (IT) with $5 \times 10^8$ cfu (100 X the established $LD_{50}$ for wild type APP-1A) of live AP233 in 10 ml of sterile PBS by percutaneous transtracheal inoculation, after anesthesia with xylazine (1.65 mg/kg) and telazol (4.4 mg/kg) administered intramuscularly. Group 3 received $5 \times 10^8$ cfu of live AP233 intramuscularly (IM) in 2 ml PBS. Groups 2 and 4 received the same treatment as Groups 1 and 3, respectively, except that the bacteria were suspended in PBS containing 5 μg/ml riboflavin. Group 5 received a formalinized whole cell bacterin prepared from APP-1A containing the equivalent of $5 \times 10^9$ cfu per dose in 2 ml of 25% Emulsigen adjuvant (MVP Laboratories, Ralston, Nebraska). Group 6 was unvaccinated controls.

In trial two, four groups (six pigs per group) of six- to eight- week- old APP-free pigs were vaccinated twice with a 3 week interval. Groups 1 and 2 were vaccinated with $2 \times 10^9$ cfu (400 X the wild type $LD_{50}$) of live AP233 intramuscularly in 2 ml PBS-Rib. Groups 3 and 4 were sham vaccinated with 2 ml PBS-Rib.

Pigs were monitored for 24 hours post-vaccination for any clinical signs of APP infection, as described below.

**Experimental Challenge.** Two weeks after the second vaccination, all pigs were challenged with either 1 $LD_{50}$ (5 X $10^6$ cfu) of virulent wild type APP-1A or 1 $LD_{50}$ (2 X $10^7$ cfu) of virulent wild type APP-5A, by percutaneous intratracheal inoculation (41). For preparation of the challenge inoculum, bacteria were grown to an $OD_{520}$ of 0.8 in HIV broth containing 5 mM $CaCl_2$, washed once in sterile saline, and diluted in saline to the appropriate cell density. Pigs were anesthetized by intravenous injection with ketamine (4.4 mg/kg) and xylazine (1.65 mg/kg) and inoculated by percutaneous intratracheal injection with the appropriate dose of bacteria suspended in 10 ml saline. Clinical signs of pleuropneumonia, including increased respiration rate, elevated rectal temperature, dyspnea, decreased appetite and activity/attitude (depression), were monitored and scored as previously described (16). Seriously ill animals, as determined by severe dyspnea and/or depression, were euthanized immediately. Survivors were euthanized three days post-challenge in trial 1 and four days post-challenge in trial 2. All animals were necropsied, and lungs were examined macroscopically for *A. pleuropneumoniae* lesions, including edema, congestion, hemorrhage, necrosis, abscess, fibrosis, and pleuritis. The percentage of lung tissue and pleural surface area affected was estimated for each of the seven lung lobes, and the total % pneumonia and % pleuritis calculated using a formula that weights the contribution of each lung lobe to the total lung volume (16). Representative lung samples were collected for histopathology and for bacterial culture. The neck muscles of all animals were also examined for any macroscopic signs of injection site reactions such as granuloma formation. Protection against challenge was measured as a reduction in mortality, in the severity of lung lesions, and in the severity and duration of clinical signs as compared to the unvaccinated control animals.

**Serologic analysis of immune responses.** Sera were collected from all pigs on days 0, 21, and 35, i.e., prior to the first vaccination, at the second vaccination, and at challenge. Antibody titers against APP were quantitated by indirect ELISA against APP outer membranes (16), hemolysin neutralization (22), and complement fixation (9).

**Statistical analysis.** Statistical analysis of the data was conducted using the Statistix microcomputer program (Analytical Software, St. Paul, MN) for analysis of variance (ANOVA) and Epistat (T.L. Gustafson, Round Rock, TX) for nonparametric analyses.

## RESULTS

**Addition of riboflavin to the vaccine inoculum.** In preliminary studies, it was found that riboflavin-requiring strains of APP failed to persist in the porcine respiratory tract for more than 16-24 hours. To permit expression of infection-associated antigens by the live attenuated vaccine strain of bacteria after immunization of pigs, we needed to ensure that the bacteria had sufficient available riboflavin to permit 2-3 generations of growth *in vivo*. We determined that addition of 5-10 μg of riboflavin per ml of the vaccine inoculum was sufficient to permit this amount of growth, and no more, *in vitro*. Therefore, as part of the first vaccine trial, we compared intratracheal (IT) and intramuscular (IM) administration of the live attenuated vaccine, with and without the addition of 5 μg/ml exogenous riboflavin.

**Safety**. Pigs were monitored post-vaccination for any clinical signs of APP disease, such as fever, dyspnea, and increased respiratory rate, and for injection site reactions in animals that received IM vaccines. The intramuscular administration of AP233 as a live vaccine caused no significant clinical signs other than slight depression and decreased appetite for less than eight hours post vaccination and caused no injection site reaction. In contrast, bacterin-vaccinated animals showed mild fever, depression and decrease in appetite for eight to sixteen hours post vaccination, and injection site granulomas in several cases. Intratracheal immunization with the LAV did cause slight clinical signs, including increased respiratory rates, fever, decreased appetite, and mild depression for 8-16 hours post-immunization. These results demonstrate that the live intramuscular vaccine is at least as safe as, if not safer than, a formalinized whole cell bacterin of the type currently used commercially.

**Immunogenicity.** The immune responses of the pigs to vaccination in the first vaccine trial, were evaluated by ELISA against APP outer membranes (16), hemolysin neutralization titer (22), and complement fixation (9)(Table 4.1). At challenge, the bacterin-vaccinated animals showed significant ELISA and complement fixation titers, but low or negative hemolysin neutralization titers. The four groups receiving live vaccines showed low or negative ELISA and CF titers. However, the animals that were vaccinated intramuscularly did show significant hemolysin neutralization titers, indicating that hemolysin was expressed *in vivo* by the LAV bacteria.

**Protection against experimental challenge.** In the first vaccine trial, all vaccine groups were challenged with virulent APP-1A, the homologous serotype to the vaccine strain. The mortality and clinical data from this homologous challenge are reported in Table 4.2 and Table 4.3. APP was cultured from the lungs of all the pigs except for 1 animal in Group 4. All cultures were confirmed as APP-1A by gram stain, requirement for NAD, and coagglutination. In this experiment, the LAV prepared with exogenous riboflavin and delivered intramuscularly (Group 4) provided complete protection against mortality (0/5 animals died) and significant reductions in lung damage and some clinical signs of pleuropneumonia. In contrast, 6/6 unvaccinated control animals died from overwhelming pleuropneumonia as a result of this experimental challenge. Other LAV formulations, as well as the formalinized bacterin, did not afford significant protection.

Note that the experimental challenge in this case was overwhelming. Normally, in our hands, the killed whole cell bacterin provides 100% protection against mortality and some reduction in morbidity; whereas in this experiment, half (3/6) of the bacterin-vaccinated animals died. In addition, all (6/6) unvaccinated control animals died, indicating that the dose administered was much higher than the intended wild type $LD_{50}$. However, despite the enormous challenge, the group that received the LAV IM with riboflavin added had significant reductions in mortality and in % pneumonia as determined by Least Significant Difference (LSD) analysis ($p<0.05$). No other groups were significantly different.

The second vaccine trial was conducted to determine whether intramuscular administration of the LAV, with limiting amounts of riboflavin added to the inoculum, could elicit cross-protective immunity against challenge with a heterologous serotype of APP (APP-5). In this experiment, the concentration of bacteria in the vaccine dosage was increased four-fold from the dosage previously used; however, the amount of riboflavin in the dosage was kept constant at 5 μg/ml. Mortality, lung lesion scores, and clinical scores from trial 2 are shown in Table 4.4 and Table 4.5. It is clear that, as in trial 1, the challenge doses were still overwhelming, even though the challenge dose of APP-1A was decreased to half that administered in trial 1. All sham vaccinated controls, whether challenged with APP-1A or APP-5, died from severe pleuropneumonia in less than 48 hours. The vaccinated group challenged with the homologous serotype (Group 1) did show a reduction in mortality and a significant reduction in clinical signs of APP infection, but no statistically significant reduction in lung pathology. The vaccinated group challenged with the heterologous serotype (Group 3) showed slightly, but not significantly, reduced mortality, death time, and clinical scores. APP of the appropriate challenge serotype was cultured from the lungs of all experimentally infected pigs.

# Table 4.1 - Serologic Analysis of Samples Collected at Challenge for Trial 1.

| Group | Vaccine | HNT[1] | ELISA-APP1[2] | CF (Log2)[3] |
|---|---|---|---|---|
| 1 | Live, IT, PBS | $3129 \pm 1478^{b}$ | $227 \pm 90^{b}$ | $1.7 \pm 2.8^{b}$ |
| 2 | Live, IT, PBS + riboflavin | $2520 \pm 741^{b}$ | $164 \pm 73^{b,c}$ | $1.6 \pm 3.1^{b}$ |
| 3 | Live, IM, PBS | $10760 \pm 6245^{a}$ | $120 \pm 32^{b,c}$ | $0.0 \pm 0.0^{b}$ |
| 4 | Live, IM, PBS + riboflavin | $6293 \pm 2662^{a,b}$ | $236 \pm 173^{b}$ | $2.0 \pm 4.0^{b}$ |
| 5 | APP-1A bacterin | $3035 \pm 285^{b}$ | $1119 \pm 170^{a}$ | $24.3 \pm 7.4^{a}$ |
| 6 | Unvaccinated control | $2240 \pm 243^{b}$ | $67 \pm 21^{c}$ | $0.0 \pm 0.0^{b}$ |

[1] Hemolysin neutralization titer; <3000 = negative; 3000-6000 = suspect; >6000 = positive. Assays were performed in the laboratory of Dr. Brad Fenwick, Kansas State University.

[2] ELISA vs APP-1 outer membranes; <200 = negative, 200-300 = suspect, >300 = positive. Assays were performed in the laboratory of Dr. Martha H. Mulks, Michigan State University.

[3] Complement fixation test; 0 = negative; >0 = positive; data presented as geometric mean titer. Assays were performed at the Veterinary Diagnostic Laboratory, Iowa State University.

### Table 4.2 - Mortality and Lung Score Data for Trial 1.

| Group | Vaccine[1] | Mortality | % Pneumonia[2] | % Pleuritis[3] |
|---|---|---|---|---|
| 1 | Live, IT, PBS | 3/5 | $58.6 \pm 23.5^{a,b}$ | $73.3 \pm 39.3^{a}$ |
| 2 | Live, IT, PBS + riboflavin | 6/6 | $63.2 \pm 8.2^{a,b}$ | $66.7 \pm 51.6^{a}$ |
| 3 | Live, IM, PBS | 4/6 | $57.7 \pm 23.2^{b}$ | $73.3 \pm 42.5^{a}$ |
| 4 | Live, IM, PBS + riboflavin | 0/5 | $24.5 \pm 15.0^{c}$ | $21.5 \pm 20.7^{a}$ |
| 5 | APP-1A bacterin | 3/6 | $54.1 \pm 24.8^{b}$ | $73.9 \pm 41.2^{a}$ |
| 6 | Unvaccinated control | 6/6 | $80.9 \pm 13.2^{a}$ | $83.3 \pm 40.8^{a}$ |

[1] IT: live vaccine administered by intratracheal inoculation; IM: live vaccine administered by intramuscular injection. Inoculating dose is $5x10^{8}$ cfu.

[2] Percentage of lung tissue exhibiting *A. pleuropneumoniae* lesions; results presented as mean ± standard deviation.

[3] Percentage of pleural surface area exhibiting pleuritis; results presented as mean ± standard deviation.

[a..c] Values with different superscripts among the six vaccine groups were significantly different ($p<0.05$) by Least Significant Difference (LSD) analysis.

### Table 4.3 - Clinical Score Data for Trial 1.

| Group[1] | RR Max[2] | Dyspnea[3] | Depression[4] | Appetite[5] |
|---|---|---|---|---|
| 1 | $22.0 \pm 5.2^{a}$ | $1.80 \pm .45^{a,b}$ | $1.40 \pm .55^{a}$ | $2.00 \pm .71^{a}$ |
| 2 | $19.7 \pm 5.7^{a}$ | $2.17 \pm .41^{a}$ | $1.67 \pm .82^{a}$ | $0.75 \pm .96^{b,c}$ |
| 3 | $19.2 \pm 1.2^{a}$ | $1.83 \pm .41^{a,b}$ | $1.27 \pm .75^{a}$ | $2.33 \pm 1.21^{a}$ |
| 4 | $18.2 \pm 3.4^{a}$ | $1.20 \pm .75^{b}$ | $0.40 \pm .89^{a}$ | $0.20 \pm .45^{c}$ |
| 5 | $23.3 \pm 1.6^{a}$ | $1.83 \pm .52^{a,b}$ | $1.83 \pm .75^{a}$ | $1.67 \pm 1.03^{a,b}$ |
| 6 | $23.0 \pm 5.8^{a}$ | $2.33 \pm .52^{a}$ | $1.83 \pm .75^{a}$ | $1.83 \pm .90^{a,b}$ |
| Norm. | 8.0 | 0 | 0 | 0 |
| Max. | 25 | 3 | 3 | 3 |

[1] See Table 4.2 for vaccination types.

[2] Maximum respiratory rate observed after challenge. Respiratory rate recorded as number of breaths per 15 sec observation period.

[3] Maximum dyspnea score observed after challenge. Dyspnea score measures degree of respiratory distress and labored breathing. Scored as 0 = normal; 1 = slight; 2 = moderate; 3 = severe.

[4] Maximum depression score observed after challenge. Depression score evaluates attitude and activity. Scored as 0 = normal; 1= slight inactivity; 2 = moderate; 3 = severe.

[5] Appetite was scored as 0 = did eat; 1 = did not eat. Total score = number of 12 hour periods not eating over 36 hour observation period.

[a..c] Values with different superscripts among the six vaccine groups were significantly different ($p<0.05$) by Least Significant Difference (LSD) analysis.

## Table 4.4 - Mortality and Lung Score Data for Trial 2.

| Group | Vaccine[1] | Challenge | Mortality | Death Time (hrs)[2] | % Pneumonia[3] | % Pleuritis[4] |
|---|---|---|---|---|---|---|
| 1 | APP-1A, PBS-Rib | APP-1A | 2/6 | 74.0 ± 16[a] | 53.4 ± 26.5[a] | 51.9 ± 34.4[a] |
| 2 | PBS-Rib | APP-1A | 6/6 | 26.0 ± 13.1[b,c] | 60.3 ± 4.6[a] | 66.7 ± 51.6[a] |
| 3 | APP1-A, PBS-Rib | APP-5 | 4/5 | 40.0 ± 28.1[b] | 54.6 ± 10.2[a] | 49.0 ± 50.1[a] |
| 4 | PBS-Rib | APP-5 | 6/6 | 18.7 ± 3.3[c] | 61.0 ± 5.6[a] | 65.8 ± 51.0[a] |

[1] Live vaccine administered by intramuscular injection. Inoculating dose is $2x10^{9}$ cfu.

[2] Results presented as mean ± standard deviation.

[3] Percentage of lung tissue exhibiting *A. pleuropneumoniae* lesions; results presented as mean ± standard deviation.

[4] Percentage of pleural surface area exhibiting pleuritis; results presented as mean ± standard deviation.

[a..c] Values with different superscripts among the six vaccine groups were significantly different ($p<0.05$) by Least Significant Difference (LSD) analysis.

## Table 4.5 Clinical Score Data for Trial 2.

| Group[1] | RR Max[2] | Dyspnea[3] | Depression[4] | Appetite[5] |
|---|---|---|---|---|
| 1 | 13.7 ± 3.0[b] | 1.17 ± 0.98[a] | 1.17 ± 0.98[a] | 0.5 ± 0.55[a] |
| 2 | 19.0 ± 3.8[a] | 2.33 ± 0.52[b] | 2.17 ± 0.75[b] | 2.0 ± 0[b] |
| 3 | 15.6 ± 1.5[a,b] | 2.20 ± 0.45[b] | 1.80 ± 0.45[a,b] | 1.6 ± 0.89[b] |
| 4 | 14.0 ± 2.8[b] | 2.80 ± 0.45[b] | 2.20 ± 0.45[b] | 2.0 ± 0[b] |
| Norm. | 8.0 | 0 | 0 | 0 |
| Max. | 25 | 3 | 3 | 3 |

[1] See Table 4.4 for vaccination types.

[2] Maximum respiratory rate observed after challenge. Respiratory rate recorded as number of breaths per 15 sec observation period.

[3] Maximum dyspnea score observed after challenge. Dyspnea score measures degree of respiratory distress and labored breathing. Scored as 0 = normal; 1 = slight; 2 = moderate; 3 = severe.

[4] Maximum depression score observed after challenge. Depression score evaluates attitude and activity. Scored as 0 = normal; 1= slight inactivity; 2 = moderate; 3 = severe.

[5] Appetite was scored as 0 = did eat; 1 = did not eat. Total score = number of 4 hour periods not eating over 12 hour observation period

[a..c] Values with different superscripts among the six vaccine groups were significantly different ($p<0.05$) by Least Significant Difference (LSD) analysis.

## DISCUSSION

Current commercial bacterin vaccines do not provide effective cross-protective immunity against all strains of APP. However, infection with a virulent strain of APP does elicit cross-protection against other serotypes. This suggests that there are common antigens expressed during infection that elicit cross-protective immunity and that these antigens may not be present in killed vaccines prepared from bacteria grown in the laboratory. This was certainly true in the past when APP bacterins were routinely prepared in media that did not elicit expression of Apx toxins. It remains to be determined what other *in vivo* specific antigens and virulence factors of APP are produced as well as the specific environmental stimuli, such as temperature, lack of available iron, pH, or osmotic conditions, that trigger their production. One way to produce an APP vaccine that contains these cross-protective antigens, without identifying them or the growth conditions which induce their production, is to use a biochemically attenuated strain of APP that is still capable of synthesis of these antigens and virulence factors as a live vaccine.

The genetically defined riboflavin auxotroph of APP serotype 1 used in these studies as a live attenuated vaccine is phenotypically identical to its wild type parent and is fully capable of synthesis of known virulence factors such as extracellular Apx toxins, outer membrane proteins, lipopolysaccharide, and capsular polysaccharide (7).

In previous attenuation studies (7), we found that AP233, the riboflavin-requiring vaccine strain, failed to persist in the porcine respiratory tract for more than 16 hours. Poor persistence of live vaccine strains *in vivo* can lead to a failure to elicit a protective immune response (29,39). In order to assure that infection-associated antigens would be expressed by this live attenuated vaccine strain after immunization of pigs, we needed to ensure that the bacteria had sufficient available riboflavin to permit 2-3 generations of growth *in vivo*. We determined *in vitro* that addition of 5 μg of riboflavin per ml to our vaccine inoculum containing 5 X $10^8$ cfu was sufficient to permit this amount of growth.

In trial 1, we compared intratracheal (IT) and intramuscular (IM) administration of the live attenuated vaccine, with and without the addition of 5 µg/ml exogenous riboflavin. Intramuscular vaccination with the live attenuated riboflavin-requiring APP mutant, with the addition of a limited amount of exogenous riboflavin, led to complete protection against mortality and to significant reductions in lung damage and clinical signs of pleuropneumonia. Intratracheal immunization with the same dosage of live vaccine did not elicit the same degree of protective immunity. As predicted, the addition of a limited amount of riboflavin to the live vaccine inoculum did improve the efficacy of the vaccine, at least with IM immunization, most probably due to improved persistence of the vaccine strain *in vivo*. We did not predict that IM immunization would be more effective than the intratracheal route. This could be due to dispersal of the IT dosage throughout the lung, and subsequent lack of containment of enough riboflavin in close proximity to the bacterial cells to permit sufficient *in vivo* replication and persistence. It is also possible that intratracheal inoculation circumvented important antigen sampling sites in the nasal passages that are implicated in the generation of a protective immune response (43).

The live vaccine administered intramuscularly did not provide complete protection against clinical signs of pleuropneumonia in this experiment. We suggest two possible reasons for this result. First, the challenge administered, although designed to be an $LD_{50}$ dose, was overwhelming, with 6/6 control animals killed in less than 24 hours and 3/6 animals vaccinated with a bacterin that usually affords significant protection also killed. Second, the dose of live vaccine used was determined based on the dosage that could be safely administered intratracheally. It should be possible to use a higher dose for intramuscular immunization without adverse effects.

Serologic analysis indicates that both forms of IM vaccination elicited high toxin neutralization titers, but not significant ELISA or complement fixation (CF) titers. However, only the IM vaccination with riboflavin elicited good protection. In contrast, the whole cell bacterin elicited strong ELISA and CF titers, but elicited no hemolysin neutralizing antibody and did not confer adequate protection against severe challenge. This strongly suggests that the current serologic tests are not truly indicative of protective immune responses. Certainly the antigen preparation used for this ELISA, which is comprised of APP outer membranes prepared from bacteria grown in brain heart infusion, does not contain APP antigens that are only expressed *in vivo*. Therefore, if antibodies against *in vivo* expressed antigens are critical to protection, an accurate measure of the protective immune response would not possible with this ELISA.

In the second vaccine trial, we attempted to duplicate, and perhaps improve, the homologous protection seen in trial 1 and to extend these results to include heterologous protection. However, there was a significant loss in homologous protection in trial 2 as compared to trial 1, and we failed to achieve significant heterologous protection. We believe this is the result of increasing the dosage of AP233 fourfold without correspondingly increasing the amount of riboflavin in the dosage. In the second trial, there was one-quarter the amount of riboflavin available to each organism and thus not enough to allow sufficient growth *in vivo*. Therefore this vaccination was analogous to a bacterin vaccine, which we know is not entirely protective against pleuropneumonia in our challenge model. Because of this lack of consistency in our two trials, we are unable to prove conclusively the degree to which AP233 is protective against both homologous and heterologous challenge.

It is also interesting to note that we recently began acquiring our specific pathogen free pigs from a high health status herd and using the new Biosafety Level 2 Michigan State University Research Containment Facility. We have determined that the $LD_{50}$ for these animals kept under these very clean conditions is at least two fold and likely a full log lower than had been previously established for a typical herd in a more typical environment. It is evident that we will need to re-establish an $LD_{50}$ dosage for future experiments in order to obtain accurate protection data. This may also have implications for many of the high health status herds which are being managed to prevent diseases like pleuropneumonia as these herds may be extremely sensitive to potential outbreaks of disease.

In summary, a deletion-disruption riboflavin mutant (AP233) of APP serotype 1 (ATCC 27088) which requires riboflavin for growth is highly attenuated in the porcine host, causing no clinical signs, no typical pleuropneumonic lung pathology, and no mortality. AP233 was tested as a live attenuated vaccine, prepared either with or without the addition of riboflavin to the vaccine inoculum and delivered either intratracheally or intramuscularly. Intramuscular vaccination with 5 X $10^8$ cfu of live AP233 with 5 μg/ml of exogenous riboflavin provided complete protection against mortality and significant reductions in lung damage and clinical signs of pleuropneumonia. However, intramuscular vaccination with 2 x $10^9$ cfu of live AP233 and 5 μg/ml of exogenous riboflavin provided only moderate protection against homologous challenge and minimal protection against heterologous challenge. We will need to repeat these vaccination experiments, altering the concentration of bacteria and/or the amount of riboflavin in the vaccination inoculum and lowering the challenge dose before conclusively evaluating the protective effects of vaccination with AP233.

## ACKNOWLEDGMENTS

This research was supported by grants from Pfizer Animal Health and the National Pork Producers' Council.

T. E. Fuller is supported by a U.S.D.A. National Needs Graduate Fellowship in Animal Biotechnology.

# REFERENCES

1. **Ahmed, Z.U., M.R. Sarker, and D.A. Sack**. 1990. Protection of adult rabbits and monkeys from lethal shigellosis by oral immunization with a thymine-requiring and temperature-sensitive mutant of *Shigella flexneri* Y. Vaccine **8**:153-158.

2. **Bacher, A**. 1991. Biosynthesis of flavins. p. 215-59. *In* F. Muller (ed.), Chemistry and Biochemistry of Flavins, Vol. 1. Chemical Rubber Company, Boca Raton, Fla.

3. **Bowe, F., P. O'Gaora, D. Maskell, M. Cafferkey and G. Dougan**. 1989. Virulence, persistence and immunogenicity of *Yersinia enterocolitica* O:8 aroA mutants. Infect. Immun. **57**:3234-3236.

4. **Collins, L.V., S. Attridge, and J. Hackett**. 1991. Mutations at *rfc* or *pmi* attenuate *Salmonella typhimurium* virulence for mice. Infect. Immun. **59**:1079-1085.

5. **Fenwick, B. and S. Henry**. 1994. Porcine pleuropneumonia. J. Am. Vet. Med. Assoc. **204**:1334-1340.

6. **Fuller, T.E. and M.H. Mulks**. 1995. Characterization of *Actinobacillus pleuropneumoniae* riboflavin biosynthesis genes. J. Bacteriol. **177**:7265-7270.

7. **Fuller, T.E., B.J. Thacker, and M.H. Mulks**. 1996. A riboflavin auxotroph of *Actinobacillus pleuropneumoniae* is attenuated in swine. Infect. Immun. **64**:4659-4664.

8. **Germanier, R. and E. Furer**. 1975. Isolation and characterisation of *galE* mutant Ty21a of *Salmonella typhi*: a candidate strain for a live oral typhoid vaccine. J. Infect. Dis. **131**:553-558.

9. **Hoffman, L.J.** 1989. *Actinobacillus (Haemophilus) pleuropneumoniae*: Use of coagglutination and complement fixation to determine the relationship between presence of organisms and antibody titer in slaughterhouse pigs. J. Vet. Diagn. Invest. **1**:12-15.

10. **Hoiseth S. K. and B.A.D. Stocker**. 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature (london). **291**:238-239.

11. **Homchampa, P., R. A. Strugnell and B. Adler**. 1994. Construction and vaccine potential of an *aroA* mutant of *Pasteurella haemolytica*. Vet. Microbiol. **42**:35-44.

12. **Homchampa, P., R. A. Strugnell and B. Adler.** 1992. Molecular analysis of the *aroA* gene of *Pasteurella multocida* and vaccine potential of a constructed *aroA* mutant. Mol. Microbiol. **6**:3585-3593.

13. **Hunneman, W.A.** 1986. Incidence, economic effects, and control of *Haemophilus pleuropneumoniae* infections in pigs. Vet. Quarterly **8**:83-87.

14. **Inzana, T.J., J. Todd, and H.P. Veit.** 1993. Safety, stability and efficacy of nonencapsulated mutants of *Actinobacillus pleuropneumoniae* for use in live vaccines. Infect. Immun. **61**:1682-1686.

15. **Ivins, B.E., S.L. Welkos, G.B. Knudson and S.F. Little.** 1990. Immunization against anthrax with aromatic compound-dependent (Aro-) mutants of *Bacillus anthracis* and with recombinant strains of *Bacillus subtilis* that produce anthrax protective antigen. Infect. Immun. **58**:303-308.

16. **Jolie, R.A.V., M.H. Mulks, and B.J. Thacker.** 1995. Cross-protection experiments in pigs vaccinated with *Actinobacillus pleuropneumoniae* subtypes 1A and 1B. Vet. Microbiol. **45**:383-391.

17. **Jolie, R.A.V., M. H. Mulks, and B. J. Thacker.** 1994. Antigenic differences within *Actinobacillus pleuropneumoniae* serotype 1. Vet. Microbiol. **38**:329-349.

18. **Karnell, A., P.D. Cam, N. Verma and A.A. Lindberg.** 1993. *AroD* deleteion attenuates *Shigella flexneri* strain 2457T and makes it a safe and efficacious oral vaccine in monkeys. Vaccine **8**:830-836.

19. **Kelly, S.M., B.A. Bosecker and R. Curtiss III.** 1992. Characterization and protective properties of attenuated mutants of *Salmonella cholerasuis*. Infect. Immun. **60**:4881-4890.

20. **Lindberg, A.A., A. Karnell, B.A.D. Stocker, S. Katakura, H. Sweiha and F.P. Reinholt.** 1988. Development of an auxotrophic oral live *Shigella flexneri* vaccine. Vaccine **6**:146-150.

21. **Mekalanos, J.J.** 1994. Live attenuted vaccine vectors. Intl. J. Tech. Assess. Hlth Care **10**:131-142.

22. **Montaraz, J.A., B. Fenwick, H. Hill and M. Rider.** 1996. Evaluating antibody isotype-specific ELISA, complement fixation, and ApxI hemolysin neutralization tests to detect serum antibodies in pigs infected with Actinobacillus pleuropneumoniae serotype 1. Swine Health and Production **4**:79-83.

23. **Mulks, M.H. and J.M. Buysse.** 1995. A targeted mutagenesis system for *Actinobacillus pleuropneumoniae*. Gene **165**:61-66.

24. **Nicolet, J.** 1992. *Actinobacillus pleuropneumoniae*, p. 401-408. *In* A.D. Leman et al (eds), Diseases of Swine, 7th edition Iowa State University Press, Ames, Iowa.

25. **Nielsen, R.** 1986. Serology of *Haemophilus (Actinobacillus) pleuropneumoniae* serotype 5 strains: establishment of subtypes A and B. Acta Vet. Scand. **27**: 49-58.

26. **Nielsen, R.** 1984. *Haemophilus pleuropneumoniae* serotypes - Cross protection experiments. Nord. Vet. Med. **36**:221-234.

27. **Nielsen, R.** 1974. Serological and immunological studies of pleuropneumonia of swine caused by *Haemophilus parahaemolyticus*. Acta Vet. Scand. **15**:80-89.

28. **Noriega, F.R., G. Losonsky, C. Lauderbaugh, F.M. Liao, J.Y. Wang and M.M. Levine**. 1996. Engineered Δ*guaB-A* Δ*virG Shigella flexneri* 2a strain CVD 1205: construction, safety, immunogenicity and potential efficacy as a mucosal vaccine. Infect. Immun. **64**:3055-3061.

29. **O'Callaghan, D. D. Maskell, F.Y. Lieu, C.S.F. Easmon and G. Dougan**. 1988. Characterization of aromatic and purine dependent *Salmonella typhimurium*: attenuation, persistence and ability to induce protective immunity in BALB/c mice. Infect. Immun. **56**:419-423.

30. **Paltineanu, D., R. Pambucol, E. Tirziu, and I. Scobercea.** 1992. Swine infectious pleuropneumonia: Aerosol vaccination with a live attenuated vaccine. Proc. Int. Pig. Vet. Soc. **12**:214

31. **Perry, M.B., E. Altman, J.-R. Brisson, L.M. Beynon, and J.C. Richards.** 1990. Structural characteristics of the antigenic capsular polysaccharides and lipopolysaccharides involved in the serological classification of *Actinobacillus pleuropneumoniae* strains. Serodiag. Immunother. Infect. Dis. **4**:299-308.

32. **Roberts, M., D. Maskell, P. Novotny and G. Dougan**. 1990. Construction and characterization in vivo of Bordetella pertussis aroA mutants. Infect. Immun. **58**:732-739.

33. **Rosendal, S., D.S. Carpenter, W.R. Mitchell, and M.R. Wilson.** 1981. Vaccination against pleuropneumonia in pigs caused by *Haemophilus pleuropneumoniae*. Can. Vet. J. **22**:34-35.

34. **Rosendal, S., and J. I. MacInnes**. 1990. Characterization of an attenuated strain of *Actinobacillus pleuropneumoniae*, serotype 1. Am. J. Vet. Res. **51**: 711-717.

35. **Sansonetti P.J. and J. Arondel.** 1989. Construction and evaluation of a double mutant of Shigella flexneri as a candidate for oral vaccination against shigellosis. Vaccine **7**:443-450.

36. **Schott, K., J. Kellerman, F. Lottspeich and A. Bacher.** 1990. Riboflavin synthases of *Bacillus subtilis*: purification and amino acid sequence of the α-subunit. J. Biol. Chem. **265**:4204-4209.

37. **Sebunya, T.N.K. and J.R. Saunders.** 1983. *Haemophilus pleuropneumoniae* infection in swine: a review. J. Am. Vet. Med. Assoc. **182**:1331-1337.

38. **Shope, R. E.** 1964. Porcine contagious pleuropneumonia. 1. Experimental transmission, etiology, and pathology. J. Exp. Med. **119**:357-368.

39. **Sigwart, D.F., B.A.D. Stocker, and J.D. Clements.** 1989. Effect of a *purA* mutation on the efficacy of *Salmonella* live vaccine vectors. Infect. Immun. **57**:1858-1861.

40. **Thacker, B.J., and M.H. Mulks.** 1988. Evaluation of commercial *Haemophilus pleuropneumoniae* vaccines. Proc. Int. Pig Vet. Soc. **10**:87.

41. **Thacker, B. J., M. H. Mulks, B. Yamini, & J. Krehbiel.** 1988. Clinical, immunological, hematological, microbiological, and pathological evaluation of a percutaneous intratracheal injection *Haemophilus pleuropneumoniae* challenge model. Proc. Int. Pig Vet. Soc. **10**:69.

42. **Utrera, V., C. Pijoan, and T. Molitor.** 1992. Evaluation of the immunity induced in pigs after infection with a low virulence strain of *A. pleuropneumoniae* serotype 1. Proc. Int. Pig Vet. Soc. **12**:213.

43. **van Ginkel, F.D., J.L. VanCott, S. Yamamoto, M. Marinaro, K. Fujihashi, M. Yamamoto, M.N. Kweon, H. Kiyono and J.R. McGhee.** 1996. Current vaccination approaches with emphasis on mucosal pathogens and immunity in the respiratory tract. Abstracts, HAP 96. #27.

44. **Vaughan, L.M., P.R. Smith, and T.J. Foster.** 1993. An aromatic-dependent mutant of the fish pathogen *Aeromonas salmonicida* is attenuated in fish and is effective as a live vaccine against the Salmonid disease furunculosis. Infect. Immun. **61**:2172-2181.

## Chapter 5

# DEVELOPMENT OF AN IVET (*IN VIVO* EXPRESSION TECHNOLOGY) SYSTEM FOR *ACTINOBACILLUS PLEUROPNEUMONIAE*

## ABSTRACT

To aid in the identification of genes involved in the pathogenesis of APP infection, we have developed an *in vivo* expression technology (IVET) system to identify APP gene promoters that are specifically induced *in vivo* during infection. A functional APP IVET system requires a defined biochemically attenuated APP mutant, the wild type biochemical gene(s) from a different source that can complement the mutation, a readily quantifiable reporter gene with no background in APP and a delivery vector. We have constructed an APP mutant that lacks most of the operon required for synthesis of riboflavin and that fails to survive *in vivo*. When the *ribBAH* genes from *Bacillus subtilis* were cloned into this Rib- mutant under the control of a functional APP promoter, both ability to grow without riboflavin and virulence were restored. We have shown that the *luxAB* genes from *Vibrio harveyi* can be used to monitor gene expression in APP and that APP is not natively bioluminescent. Our initial APP-IVET promoter-trap vector (pTF86) contains, in sequence, the T4 terminator, a unique *Bam*HI site, a promoterless copy of the *B. subtilis ribBAH* genes, and a promoterless copy of the *V. harveyi luxAB* genes in the *E. coli*-APP shuttle vector pGZRS-19. Plasmid pools were constructed by cloning *Sau*3A fragments from APP into the *Bam*HI site in pTF86 and transforming into the APP Rib- mutant. Pigs were infected with pools of 400 transformants by endobronchial inoculation and surviving bacteria isolated from the pigs' lungs at 12 to 24 hours post-infection. Only those transformants containing cloned promoters active *in vivo* should have survived due to expression of the cloned *ribBAH* genes. Strains that survived *in vivo,* but which minimally expressed Lux *in vitro*, should contain cloned promoters that are specifically induced *in vivo*. We report the identification of 5 clones containing promoters that are induced *in vivo* (during infection). Two of these clones were putatively identified by amino acid sequence similarity as the *secE-nusG* operon and the *mrp* gene. This is the first report of an IVET system for use in the family *Pasteurellaceae*.

## INTRODUCTION

*Actinobacillus pleuropneumoniae* (APP) is the causative agent of an economically devastating, acute necrotizing hemorrhagic bronchopneumonia with accompanying fibrinous pleuritis (4,12,19,29). We have chosen to use APP as a model system to study the pathogenesis of respiratory disease in order to identify potential targets for the development of new live vaccines and antibiotics. There are many characteristics which APP shares with other members of the family *Pasteurellaceae*. For instance, RTX type toxins are also produced by *Actinobacillus suis* and *Pasteurella haemolytica* (30). Also, as with *Pasteurella multocida* and *Pasteurella haemolytica*, there are multiple antigenic serotypes (24,30), and killed whole cell bacterins provide at best serotype-specific protection or no signifcant protection (20,22,26,30,33) while natural or experimental infection elicits good cross-protective immunity (20,21,23,30). This suggests that there are cross-protective antigens expressed *in vivo* which are not expressed during *in vitro* growth for bacterin production.

Historically, research on this and other pathogens has focused on the studies of antigens expressed *in vitro*; however, this at best only gives a fractional picture of virulence-associated characteristics. Increasing attention is being given to the environmentally-stimulated regulatory mechanisms that modulate bacterial attributes in the host (9,10,16). Known environmental regulatory signals for bacterial virulence genes include temperature, osmotic strength, pH, oxygen levels, availability of iron, calcium levels, carbon sources, urea, growth phase, stress, starvation and availability of other nutrients such as amino acids (9,10,16). Attempts to mimic host environmental conditions during *in vitro* growth have demonstrated that there are many differences in gene expression in response to various environmental signals. A serious limitation of this technique is the multitude of environmental factors that can induce gene expression. One or more of these factors, individually or in combination, could produce a wide variety of responses in the pathogen. Which combination of known environmental stimuli are important at any given stage of infection is virtually impossible to determine *in vitro*. In addition, there may be many environmental conditions currently undiscovered in the host which have a significant effect on gene expression.

Another method for studying these antigens is to use convalescent antisera to directly compare the antigenic profiles of bacteria isolated directly from the site of infection with bacteria that have been cultured in the laboratory. We have used bronchoalveolar lavage (BAL) to recover bacteria directly from the lungs of infected pigs and have demonstrated that there are indeed differing immunogenic bands on Western blot with convalescent pig sera as compared to bacteria cultured in the lab (18). This technique is also restrictive because it will only identify antigenic differences and will not identify regulatory or non-immunogenic factors. Also, further analysis of these factors is limited by the ability to identify, clone and express these antigens *in vitro*.

In view of these limitations, genetic technology has emerged to the forefront as a means to study bacterial pathogenesis. Genetic techniques have been recently described that can be used to identify genes that are specifically induced only during infection and not during growth on standard laboratory media. The power in these techniques is that they can identify genes required for survival in the host such as enzymes necessary for production of critical growth factors not available *in vivo* (e.g., purines and aromatic amino acids), factors which allow survival within host cells (e.g., enzymes that inactivate phagocytic killing mechanisms), or even toxins that have not yet been identified because they are not produced under standard *in vitro* culture conditions (16). Identification of such *in vivo* expressed genes should lead to further insights into the pathogenesis of APP disease as well as to identification of important antigens for inclusion in subunit vaccines and to development of defined avirulent mutants for use as live vaccines.

Four genetic methods for the study of bacterial pathogenesis have been described to date: subtractive hybridization, *in vivo* expression technology (IVET), resolvase based-IVET and signature-tagged mutagenesis (STM). Subtractive hybridization is based on identifying differences in RNA transcripts (34) between bacteria isolated from an infected animal and bacteria grown in the laboratory. Limitations may be the technical difficulty in isolation and manipulation of bacterial RNA to obtain a cDNA clone, and the results may change dramatically depending on the stage of infection at which bacteria are isolated necessitating a large number of animals in order to truly perform a saturation study of all the genes involved in pathogenesis. Also, this technique will tend to identify "all or none" expressed genes rather than *in vivo* induced genes which have some level of constitutive expression.

A second method termed IVET has been developed which allows positive selection of bacterial genes that are specifically expressed during infection and has been used to identify *in vivo* induced genes in the pathogen *Salmonella typhimurium* (15,32). The basis of this system is to use a tandem set of *in vivo* and *in vitro* promoterless "reporter" genes to identify promoters and genes in random genomic fragments which are turned on specifically in the host. The host organism is a biochemically attenuated mutant which is unable to survive unless complemented by expression of the wild type biochemical genes driven by a cloned promoter (*in vivo* "reporter" gene). Bacterial strains that survive *in vivo* are isolated and expression of the *in vitro* reporter gene is measured. The reisolated strains which do not express the quantifiable reporter gene contain *ivi* (*in vivo* induced) promoters that are turned on *in vivo* and off *in vitro*. Knock-out mutants of three different *S. typhimurium ivi* genes (*carAB*, *pheST-himA*, *rfb*) have been shown to be attenuated in mice (15).

Modifications of this system using different promoterless genes for the selection process, such as chloramphenicol acetyltransferase (16), have been developed. Cloned promoters are selected for expression during infection by treating with the antibiotic and isolating surviving bacteria. These antibiotic selections would appear to function best in tissue culture or in small animal models rather than in large animal models because of pharmacokinetics. Another system to identify *in vivo* expressed genes has been developed for use in the intracellular pathogen *Legionella pneumophila*, using an avirulent *thyA* mutant, an independently replicating plasmid, and infection of a macrophage cell culture rather than an intact host (13,25). Finally, a modified IVET system has been developed for use with *Pseudomonas aeruginosa* consisting of an avirulent *purEK* mutant, plasmid pBR322, a promoterless *purEK* operon and a mouse model of infection (35). The major limitation with the IVET technology is its ability to recover only promoters which are expressed throughout the infection process. Promoters which are expressed only transiently or during latter stages of infection will not be recovered because of the lack of complementation and thus the lack of survival of the biochemical mutant.

The remaining two technologies are designed to overcome the limitations of IVET and subtractive hybridization and can identify genes that are expressed only transiently or at low levels. Signature-tagged mutagenesis (STM) overcomes the problem of an IVET type system in that genes do not have to be expressed during the entire infection process to be reisolated. In this system, transposons carrying unique DNA sequence tags are used to construct a collection of mutants by insertional mutagenesis (11). The pool of mutants is used to infect a host animal, and surviving bacteria are recovered from the infected host after several days. Using the unique DNA sequence tags to make probes, it is possible to identify which specific members of the original mutant pool are absent upon recovery of bacteria from the host. These mutants are predicted to carry mutations in genes necessary for survival in the host. The STM system is not yet applicable to *A. pleuropneumoniae*, since the transposon's (Tn5) antibiotic marker does not appear to function in APP. The system also requires the generation of individually tagged transposon mutants of each new organism to be studied, and in large animal models the entire surviving pool will be difficult to reisolate, possibly leading to the false identification of mutants as unable to survive. A certain subset of virulence genes (e.g. toxins) will likely not be identified because other clones in the pool will express them and the transposon mutant will be able to replicate normally even though it is not able to express these genes.

The fourth and perhaps most promising selection system based on resolvase activity uses *in vivo* induction of *tnpR* resolvase, a site-specific recombinase of the transposable element $\gamma\delta$, to induce a heritable, detectable genetic change. In this system, even brief expression of the promoterless resolvase by a cloned promoter results in excision of a tetracycline resistance cassette flanked by direct repeats of the DNA sequence at which the resolvase functions (1). Any bacterial cell in which the resolvase is expressed will give rise to progeny that are tetracycline-sensitive. This selection system has the power to identify promoters that are only transiently expressed *in vivo* because all strains survive whether or not the promoter is inactive, turned on transiently, or turned on constitutively during infection. A second limitation as it currently exists is the reliance on a negative selection requiring replica plating of colonies to determine whether they have lost antibiotic resistance. Another limitation is the sensitivity of the system; it can be too sensitive yielding a high level of background with the false identification of genes being turned on. Another limitation is that all promoters turned on *in vivo* will be identified, not just those that are *in vivo* specific; thus, pre-screening of the pools is required to eliminate *in vitro*-expressed clones. As a result, this system will likely not identify genes that are minimally to moderately expressed *in vitro* and induced *in vivo*. It is not yet clear whether this system could be made to function in APP.

Many of the genes identified as having promoters specifically turned on *in vivo* or as being required for survival *in vivo* (1,11,15,35) have been genes involved in the biosynthesis of purines, pyrimidines, and essential amino acids, such as *purD, pyrE,* and *carAB*, while others have been genes known to encode virulence factors, such as *invA, invG, spvA*, *fadB*, and *virB* or even transcriptional regulators such as Fur. Previously identified genes have been divided into four different classes of *ivi* expressed genes: 1) enzymes in critical biochemical pathways; 2) known virulence factors, or genes involved in the synthesis of such factors, such as genes for LPS biosynthesis/modification; 3) new genes, not identifiable by comparison with known sequence data; and 4) genes involved in transcriptional regulation or signal transduction. *In vivo* expressed genes that can be identified as steps in critical biochemical pathways, such as purine biosynthesis, may be targets for the production of knock-out mutants to be used as live avirulent vaccines or may be targets for a new generation of antibiotics.

In this study, we report the construction and utilization of a modified IVET system for APP based on an attenuated riboflavin mutant of APP, the shuttle vector pGZRS-19 (36), the $T_4$ terminator, the *Bacillus subtilis ribBAH* genes and the *luxAB* genes from *Vibrio harveyi.* Using this system initially with a limited number of clones, representing approximately 10% of the number required to be truly representative of the entire genome, we have identified five clones that are specifically induced *in vivo*. Two of these clones have been putatively identified as clones of the *secE-nusG* operon and the *mrp* gene. The identity of the other clones remains undetermined, but they will perhaps be the most interesting clones as no homologous genes have previously been sequenced.

## MATERIALS AND METHODS

**Bacterial strains and media.** *A. pleuropneumoniae* strains were cultured at 37 °C in either brain heart infusion (BHI) or heart infusion (HI) (Difco Laboratories, Detroit, MI). containing 10 μg/ml β-NAD (V factor) (Sigma Chemical Company, St. Louis, MO). Riboflavin (Sigma) was added to a final concentration of 200 μg/ml as necessary. *E. coli* strains were cultured in Luria-Bertani medium. Ampicillin was added to 100 μg/ml for plasmid selection in *E. coli* strains. For *A. pleuropneumoniae* strains, 50 μg/ml ampicillin was added except for selection after electroporation which was done with 20 μg/ml ampicillin.

**DNA manipulations.** DNA modifying enzymes were supplied by Boehringer-Mannheim Biochemicals (Indianapolis, IN) and used according to the manufacturer's specifications. Genomic DNA was prepared according to the lysis/proteinase K method of the Gene Fusion Manual (31). Plasmid DNA preparations, agarose gel electrophoresis, and *E. coli* transformation were all performed by conventional methods (27).

**PCR.** PCR was performed to eliminate *Xba*I and *Bam*HI restriction sites and to introduce *Sph*I sites in the 5' and 3' ends of the *luxAB* genes. Primers were MM43 (5'-GCA-GCA-TGC-ACT-AGA-GGA-ACC-CCA-TG-3')for the forward and MM44 (5'-GCA-GCA-TGC-GTT-AAA-CGT-TAC-GAG-TG-3') for the reverse direction. The PCR reaction was carried out using Vent polymerase (New England Biolabs, Beverly, MA), 450 pmol of each primer, 100 ng of the pRL1062a template, 2 mM $MgSO_4$ with 2mM dNTPs in a reaction volume of 100 ul. Reaction conditions were repeated for 30 cycles with 1 minute at 95°C for denaturing, 1 minute at 48°C for annealing and 2-1/2 minutes at 72°C for polymerization. The desired 2.2 kb PCR product was purified by phenol-chloroform extraction, restriction digested with *Sph*I and isolated by purification from an agarose gel with the QIAEX gel extraction kit (QIAGEN, Chatsworth, CA).

**Electroporation of *A. pleuropneumoniae*.** AP233 was grown in 100 ml BHIV + riboflavin at 37°C, with shaking at 150 RPM, to an $OD_{520}$ of 0.7. Cells were chilled on ice and centrifuged at 5,000 x g at 4°C for 10 min. Cells were washed twice in 10.0 ml ice cold sterile 15% glycerol. Cells were resuspended in 2.0 ml 15% glycerol and frozen in 50 μl aliquots using a dry ice-ethanol bath. Plasmid DNA was added to an aliquot of competent cells thawed on ice and then transferred to a 0.1 cm gap electroporation cuvette (BioRad, Hercules, CA). Cells were electroporated using a Gene Pulser II (BioRad) with the following settings: voltage, 1.8 kV; resistance, 200 Ω; capacitance, 25 μFd. Cells were allowed to recover in BHIV + riboflavin at 37°C with slow shaking for approximately 3 to 4 hours before plating on selective media.

**Experimental infections.** Eight-week-old specific-pathogen-free castrated male pigs (Whiteshire Hamroc, Inc., Albion, IN) were housed in separate cage units in BSL-2 isolation rooms at the Michigan State University Research Containment Facility. All experimental protocols for animal experiments were reviewed by the Michigan State University All University Committee on Animal Use and Care, and all procedures conformed to university and USDA regulations and guidelines.

For preparation of challenge inocula, each pool of approximately 100 transformants was added to 3 ml HIV + 5 mM $CaCl_2$ + riboflavin + ampicillin to produce a starting $OD_{520}$ of 0.2. Pools were grown in 20x100 mm culture tubes, at 37°C with shaking at 160 RPM, to an $OD_{520}$ of 0.8. Appropriate amounts of each pool were combined to produce 10.0 ml culture containing ~ $2 \times 10^6$ cfu each of 300-400 individual strains. The combined culture was harvested by centrifugation at 2100 x g at room temperature and washed once with sterile PBS pH 7.0 with 5 μg/ml riboflavin (PBS-Rib). The cell pellet was resuspended in 10 ml of PBS-Rib and diluted to obtain the desired $8 \times 10^8$ cfu dose.

For the challenge procedure, pigs were anesthetized by intravenous injection with xylazine (1.65 mg/kg) and telazol (4.4 mg/kg) and inoculated by shallow endobronchial injection with the 8 x $10^8$ cfu suspended in 10 ml saline. Clinical signs of pleuropneumonia, including increased respiration rate, elevated temperature, dyspnea, decreased appetite and depression were monitored and scored as previously described (14). Animals were euthanized at approximately 12 hours post infection. All animals were necropsied, and lungs were examined macroscopically for *A. pleuropneumoniae* lesions, edema, congestion, hemorrhage, necrosis, abscessation, fibrosis and pleuritis. APP strains were recultured by one of two methods. Bronchoalveolar lavage (BAL) (18) was performed by insertion of a tube into the lungs followed by the instillation and recovery of PBS-Rib. The recovered BAL fluid was first centrifuged at 450 x g to pellet eukaryotic cells or extraneous tissue and then the supernatant was centrifuged at 2100 x g to pellet any bacterial cells. The pellet was resuspended in 1 ml of PBS-Rib and either used directly for Lux quantitation or diluted appropriately for plate counts and recovery of single colonies. The second method consisted of excising the lungs and using sterile scalpel blades to make incisions every 1-2 cm which were swabbed with sterile cotton-tipped applicators and cultured onto BHIV$R^{200}A^{50}$ agar plates.

**Lux Analysis**: Qualitative screening of strains for expression of the *luxAB* genes *in vitro* was performed on a Hamamatsu C1966 Photonic Microscope System. Briefly, colonies on agar plates were exposed to 50 µl of N-decyl aldehyde distributed evenly on a glass petri dish lid. The plate was analyzed using appropriate sensitivity settings, which varied with the amount of expression by the colonies on each plate. Final screening to select clones for quantitation after eliminating strong Lux+ colonies was done with an aperture setting of 11, digital gain of 2 and photon counting for 59 seconds. Quantitative analysis of Lux expression was performed using a Turner Model 20e Luminometer. N-decyl aldehyde (Sigma) substrate was made by dissolving 20 mg/ml Essentially Fatty Acid Free BSA (Sigma) in 1.0 ml of $H_2O$ with 1 µl/ml N-decyl aldehyde. This mixture was sonicated in a glass screw cap test tube for approximately 1 hour to disperse the N-decyl aldehyde into micelles. For analysis, 20 µl of the culture was mixed with 20 μl of substrate in polypropylene luminometer cuvettes (Turner Designs, Sunnyvale, CA) and mixed for 10 seconds. The mixture was then read in full integral, autoranging mode with a pre-delay of 0 seconds, delay of 10 seconds, and integration time of 30 seconds. Luminometer readings were normalized to micro-relative light units per colony forming unit (μRLU/CFU) with the number of bacteria in the sample as determined by plate counts on selective media, or by normalizing to the $OD_{520}$ when in pure culture.

**DNA/Protein sequence analysis.** Nucleotide and amino acid sequences were searched against the GenBank database using the FASTA and TFASTA functions of the GCG Programs (8). All alignments, calculated percent identities, and similarities reported were made using the GAP function of the GCG Programs (8).

## RESULTS

In order to construct an IVET system we need an attenuated biochemical mutant and a vector which contains the following: random chromosomal DNA fragments from the organism, promoterless wild type biochemical genes from a different organism to complement the attenuating mutation, and a quantitative/qualitative reporter gene. We have previously reported the cloning of the riboflavin operon from APP (6) and the construction of a defined deletion-disruption riboflavin mutant of APP that is attenuated in swine (7). In this paper, we report the construction of an APP IVET vector and its use in our riboflavin auxotroph to identify *in vivo* induced genes in *Actinobacillus pleuropneumoniae*.

**Verification of complementation by *Bacillus subtilis ribBAH* genes.** A 2.7 kb *Eco*RV fragment containing a promoterless copy of the *ribBAH* genes from *Bacillus subtilis* strain 168 was excised from plasmid pRF2 (provided by Dr. John Perkins, OmniGene Bioproducts Inc., Cambridge, MA) and ligated into the *Sma*I site of pGZRS-19 (36) to construct plasmid pTF74. This plasmid was electroporated into AP233, a riboflavin-requiring, nalidixic acid and kanamycin resistant attenuated mutant of APP serotype 1. The restoration of virulence was determined as described previously (7). A dosage of AP233 + pTF74 equivalent to the wild type $LD_{50}$ was administered to 3 six- to eight-week-old pigs, resulting in significant signs of pleuropneumonia including increased respiration rate, dyspnea, depression, loss of appetite and pneumonia and pleuritis as determined by necropsy. These results indicate that the *B. subtilis ribBAH* genes are able to complement the AP233 riboflavin mutation *in trans* as AP233 is normally unable to cause disease at a dosage which is 500 times the established wild type $LD_{50}$.

**Verification of Lux Activity in *Actinobacillus pleuropneumoniae*.** A promoterless *luxAB* operon from *Vibrio harveyi* was cloned in both orientations into the APP-*E. coli* shuttle vectors pGZRS-18 and pGZRS-19. When the resulting four plasmids were transformed into AP233 and screened for Lux activity, all four conferred detectable Lux activity, indicating background expression from unidentified promoters in both vectors. In contrast, neither AP233 alone nor AP233 containing either pGZRS-18 or pGZRS-19 showed detectable Lux activity, indicating that there is no native bioluminescence in APP.

**Construction of the IVET Vector: pTF86.** Figure 5.1 outlines the construction of the IVET vector pTF86. A 2.3 kb fragment containing the promoterless *luxAB* genes was cloned from pRL1062a (provided by Dr. Peter Wolk, MSU-PRL, East Lansing, MI) by PCR, replacing the *XbaI/BamHI* sites with *SphI* sites for insertion into pGZRS-19 to make pTF77b. The 2.7 kb *Eco*RV fragment containing the promoterless *B. subtilis ribBAH* genes from pRF2 was blunt-end ligated into the Klenow-filled *Hin*dIII site of pTF77b to make pTF85. The $T_4$ terminator from pJFF224-NX (5) was inserted into pTF85 on a 0.3 kb *KpnI-XbaI* fragment to make pTF86. There is a unique *Bam*HI site upstream of the promoterless *luxAB*:*ribBAH* fusion into which *Sau*3A fragments can be cloned. AP233 containing pTF85, which does not contain the $T_4$ terminator, is able to grow without riboflavin and is weakly Lux positive. pTF86 is unable to grow without riboflavin and is Lux negative, indicating that the $T_4$ terminator does indeed prevent background expression from the pGZRS-19 vector.

**Construction of an APP promoter library in pTF86.** Chromosomal DNA fragments generated by digestion with *Sau*3A, 0.4 to 1.0 kb in size, were purified from an agarose gel and ligated into the *Bam*HI site in pTF86. The ligation mix was electroporated into AP233. Single colonies were selected, and sub-cultured onto BHIV$R^{200}A^{50}$ plates in groups of 100. Colonies were washed off each plate and frozen at -70°C in HIVR broth + 5 mM $CaCl_2$ + 20% glycerol.

***In vivo* screening of IVET clones.** An initial library of 1800 clones was screened for the identification of chromosomal fragments containing *in vivo* induced promoters (Figure 5.2). Groups of 300 to 400 clones were used to infect pigs by

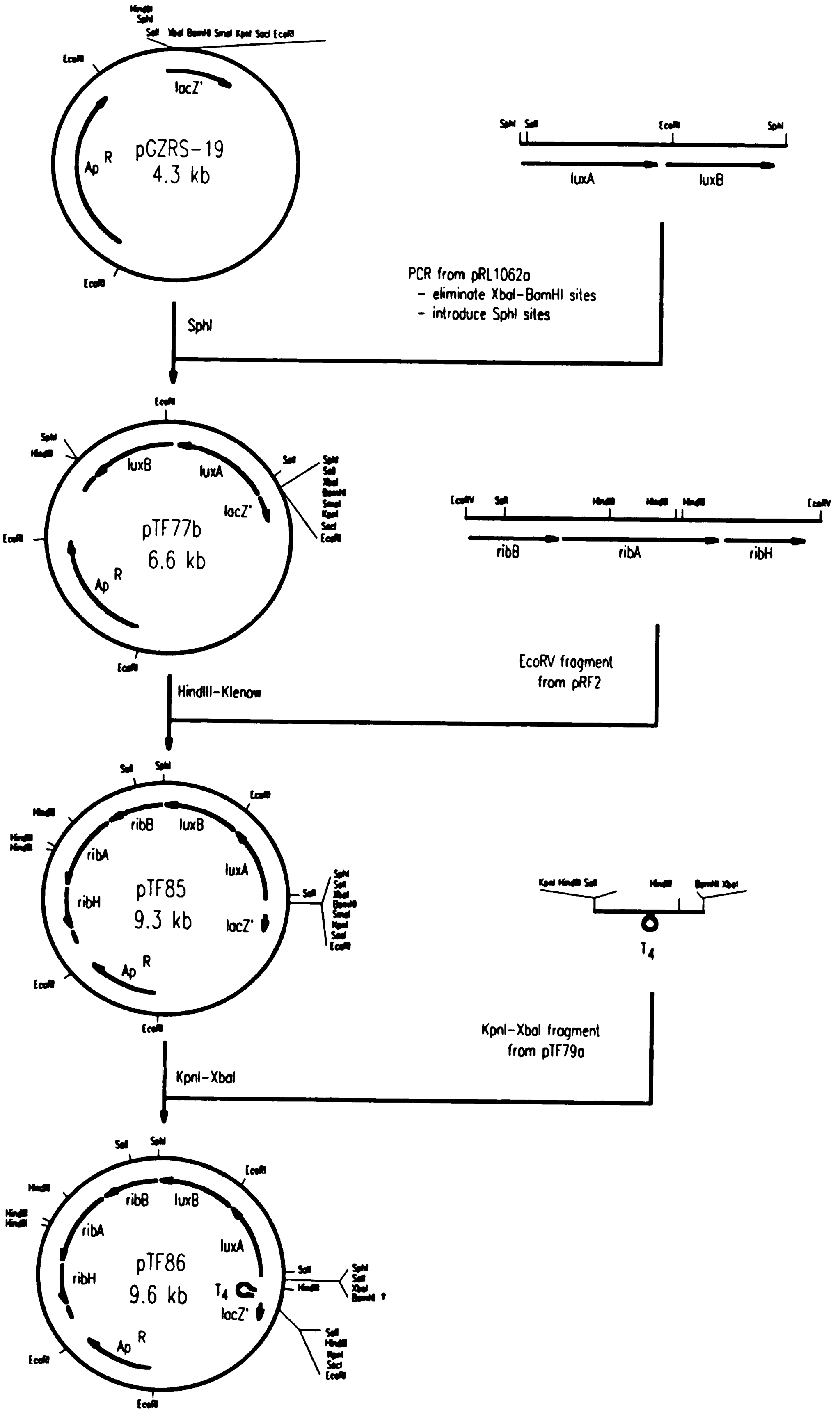


**Figure 5.1 - Construction of the IVET Vector pTF86.**

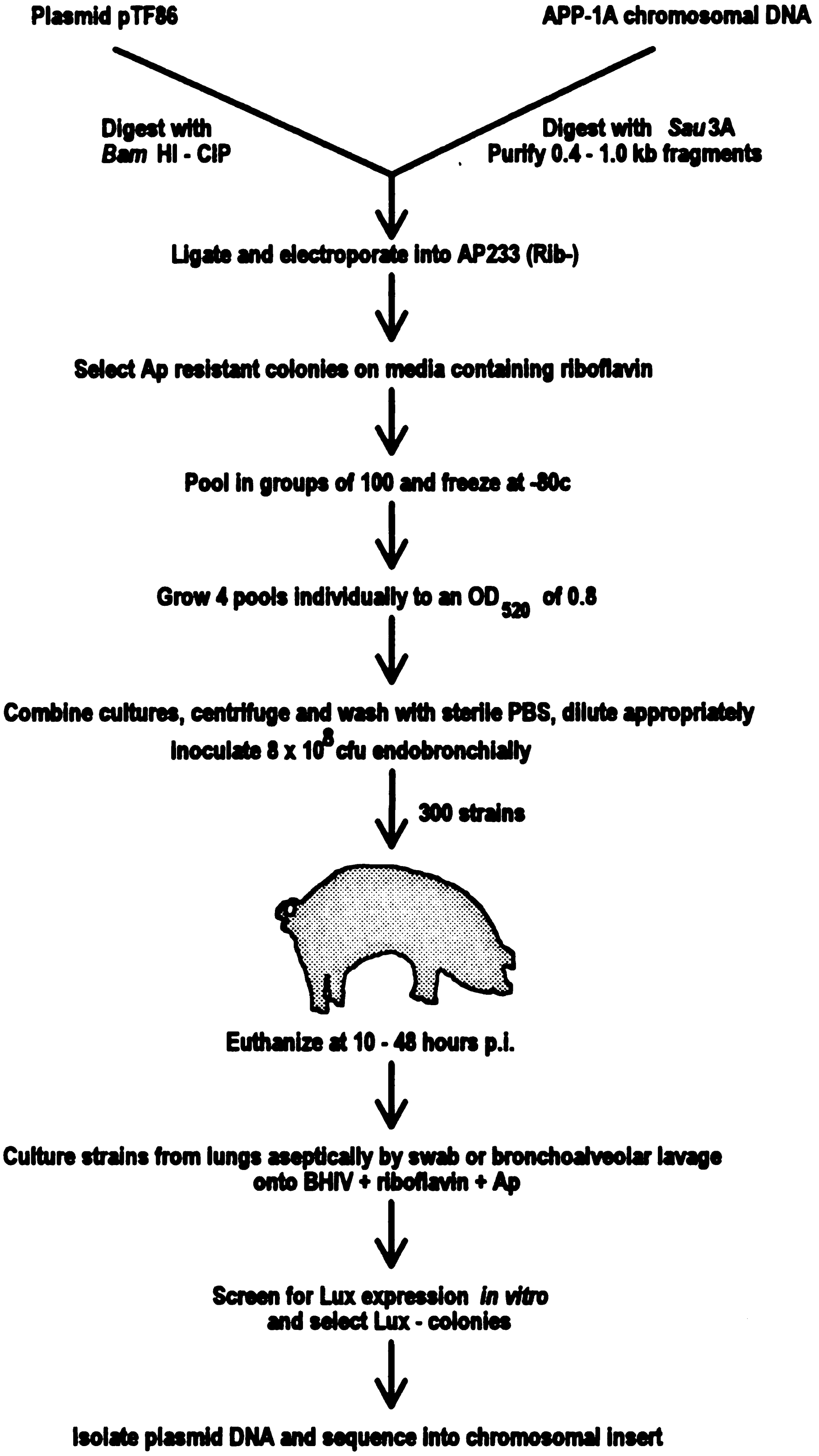


**Figure 5.2 - Identification of *In Vivo* Induced Genes.**

endobronchial inoculation. At either 10, 24 or 48 hours post infection, the pigs were euthanized and necropsied, and surviving bacteria were isolated from the lungs. Screening of this pool of clones *in vitro* before *in vivo* passage demonstrated that approximately 90% to 95% of the pool was composed of strains which had minimal or no Lux expression, presumably containing no functional promoters or promoters which were not expressed *in vitro*, and that 5% to 10% were Lux+. After reisolation from infected pigs, the entire pool was approximately 1% Lux- and 99% Lux+, indicating a strong selection *in vivo* for those clones which had promoters expressed *in vivo*.

**Quantitation of *In vivo* induced (*ivi*) clones.** A total of 750 individual colonies were isolated from four pigs infected with the IVET pools. An example of the range of promoter activity observed during primary qualitative screening is shown in Figure 5.3. Clones with expression between the negative controls (pTF86 and pGZRS19) and a minimally expressed clone (IVF) are selected for further analysis. Clones containing strong *in vitro* expression (e.g. IVH) are likely to not be induced *in vivo*. Forty-six reisolated clones were selected during a primary screen on agar plates as being Lux negative or as having relatively minimal Lux expression. Sixteen additional clones were selected that contained strong *in vitro* promoters for future promoter studies as little is known about APP promoter structure. All 62 clones were assayed for Lux activity *in vitro* (Figure 5.4) and those with an activity of less than 200 RLU/$OD_{520}$ were partially sequenced with a primer reading upstream of the *luxA* gene in plasmid pTF86. The 20 selected clones that were sequenced represented 5 unique clones.

**Recovery of pTF85.** As a positive control in a preliminary experiment, pTF85 (Figure 5.1) was included in an equimolar amount with 300 other clones. This clone was reisolated after 24 hours and was confirmed by sequencing to demonstrate the lack of a *Sau*3A insert and $T_4$ terminator. The reisolation of this clone, with Lux activity of only 75.9 RLUs per $OD_{520}$ indicates that this system is extremely sensitive to expression by a cloned promoter.

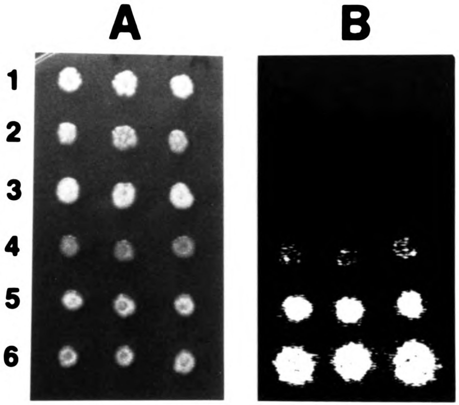


**Figure 5.3 - Primary Screening for Minimally Expressed IVET Clones in AP233.**

Panel A is a incidental light photograph of triplicate colonies on an agarose plate. Panel B is the corresponding photograph of the bioluminescence

| | |
|---|---|
| Row 1 | pGZRS-19 |
| Row 2 | pTF86 |
| Row 3 | IVA (*mrp* promoter) |
| Row 4 | IVF (*secE-nusG* promoter) |
| Row 5 | IV42 (moderately expressed promoter) |
| Row 6 | IVH (strong promoter) |

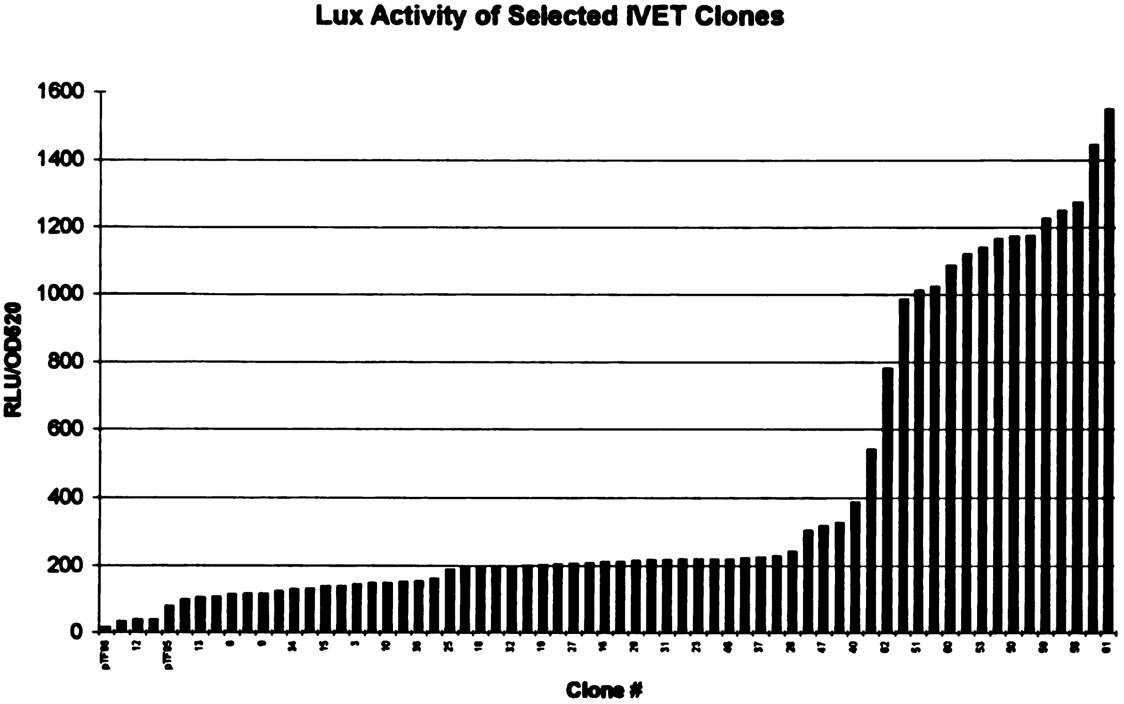


Figure 5.4 - *In Vitro* Quantitative Lux Analysis of Selected IVET Clones.

**Sequence comparison.** TFASTA and FASTA searches were performed against the GenBank database with either the nucleotide sequence or predicted amino acid sequences of the IVET clones, and two of the clones were putatively identified. The predicted amino acid sequence from IVA had 54.8% similarity and 45.2% identity with the Mrp (methionyl t-RNA synthetase related protein) from *Haemophilus influenzae* and 61.3% similarity and 45.2% identity with *E. coli* Mrp protein (Figure 5.5). The 31 amino acid overlap shown in Figure 5.5 occurs 50 amino acids from the amino terminus of *E.coli* Mrp (a 369 amino acid polypeptide) .

**Amino Acid Alignment of APP IVA with *E. coli* Mrp**

```
APP              MPFAWNSGFEALKADTEVKLKQVTGANEVKW
                 |||.|:|:||.||.:....| .:|||..:.|
E.c.   ...TLHVELVMPFVWHSAFEELKEQCSAELLRITGAKAIDWKLSHNI...
```

**Amino Acid Alignment of APP IVA with H. *Influenzae* Mrp**

```
APP              MPFAWNSGFEALKADTEVKLKQVTGANEVKW
                 :||||||| |.||....  | ..|:.. :||
H.i.   ...TLRIELQLPFAWNSGAEQLKQAVSDALLKATDCKLIKWAVAYQI...
```

**Figure 5.5 - Amino Acid Alignment of IVA and Mrp.** Identical residues are indicated with " | " and conserved residues are indicated with " : " or " . "

The two predicted ORFs within the sequence for IVF were homologous to the carboxy terminus of SecE (a 127 amino acid polypeptide) and the amino terminus of NusG (a 181 amino acid polypeptide) which are separated by two nucleotides in *E. coli* and co-transcribed. ORF1 (25 amino acids) was 88% similar and 60% identical with the carboxy terminus of *Haemophilus influenzae* SecE and 88% similar and 64% identical with the carboxy terminus of *Escherichia coli* SecE. ORF2 (40 amino acids) was 63.3% similar and 55.3% identical with NusG from *Haemophilus influenzae* and 60% identical and 54% similar with NusG from *Escherichia coli*. (Figure 5.6).

The remaining three *ivi* clones could not be assigned a putative identity based on their sequence.

**Measurement of *in vivo* induction.** To confirm that the reisolated clones had indeed been induced *in vivo*, each clone was quantitated for Lux expression *in vivo*. One clone which contained a good constitutive promoter (IVH) was used as a control for the regeneration of virulence of AP233. Another previously identified clone containing a weak *in vitro* and *in vivo* promoter and partial ORF putatively identified as a thioredoxin (TrxA) homologue was also included in these experiments. A dosage of 5 x $10^8$ cfu of each IVET clone was administered to six- to eight-week-old pigs by endobronchial instillation. After eight hours, animals were euthanized and bronchoalveolar lavage (BAL) was performed to reisolate surviving bacteria. Animals infected with all IVET clones except the pig containing the TrxA clone showed significant clinical signs and evidence of acute pleuropneumonia at necropsy. Bacteria obtained by BAL after differential centrifugation to remove eukaryotic contaminants were immediately assayed for Lux activity and plate counts of viable bacteria were determined to standardize the readings. Each clone was induced *in vivo* ranging from 8 to 88 fold induction (Figure 5.7) except the *trxA* clone which was unable to be accurately quantitated due to the lack of survival within the host.

### Amino Acid Alignment of APP IVF with *H. influenzae* SecE

```
APP           VVSLVLWGIDSIIVTLVTFLTNLRF
              :.||.:|::||||||::.|||:|||
H.i.     ...TMIASLFFWAVDSIIVTVINFLTDLRF
```

### Amino Acid Alignment of APP IVF with *H. influenzae* NusG

```
APP      MSEVENTEATKMRWYVLQAFSGFENRVAVTLRDISNYTKW
            .|:|...|.||||||||||||.|||:|||:. .  .
H.i.       MTEETTVSKKRWYVLQAFSGFESRVALTLREYIKQQQMEDQFG...
```

### Amino Acid Alignment of APP IVF with *E. coli* SecE

```
APP           VVSLVLWGIDSIIVTLVTFLTNLRF
              |:||:|||:|:|:| ||.|:|.|||
E.c.     ...TAVMSLILWGLDGILVRLVSFITGLRF
```

### Amino Acid Alignment of APP IVF with *E. coli* NusG

```
APP      MSEVENTEATKMRWYVLQAFSGFENRVAVTLRDISNYTKW
               .||.|.||||:|||||||.|||..||:  .. .
E.c.          MSEAPKKRWYVVQAFSGFEGRVATSLREHIKLHNMEDLFG...
```

**Figure 5.6 - Amino Acid Alignment of IVF and SecE/NusG.** Identical residues are indicated with " | " and conserved residues are indicated with " : " or " . "

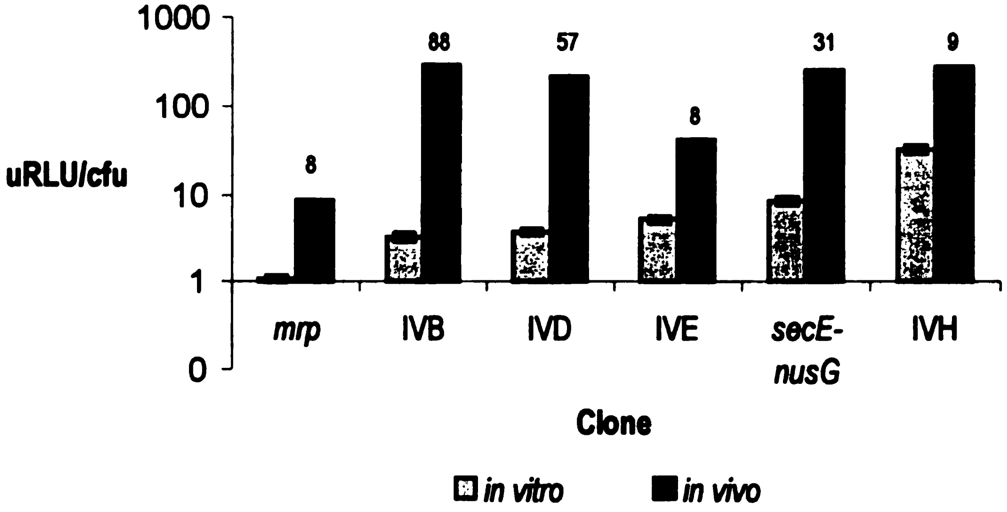


**Figure 5.7 - *In Vivo* Induction of IVET Clones.** Fold induction is indicated above each *in vivo* bar.

# DISCUSSION

In this study we report the construction of an IVET system for use in *Actinobacillus pleuropneumoniae*. To our knowledge, this is the only IVET system which has been made specifically for use in an infection model of the pathogen's natural host rather than a laboratory animal or tissue culture model. We have demonstrated that a functional IVET vector can be constructed with the *E. coli*-APP shuttle vector pGZRS-19 (36), the T4 terminator, the promoterless wild type riboflavin biosynthesis genes (*ribBAH*) from *Bacillus subtilis* and the promoterless *luxAB* reporter genes from *Vibrio harveyi*. We have shown that the *Bacillus subtilis* genes can complement the riboflavin mutation in AP233 restoring the ability to grow without riboflavin and restoring virulence. We have shown that there is no background bioluminescence in APP and that the *luxAB* genes can be used as a simple and quantifiable reporter of gene expression in APP. Finally, our IVET system has been used to identify 5 specifically *in vivo* induced genes which are likely to be important for survival *in vivo* or virulence.

Two of the *in vivo* induced clones have been putatively identified based upon their sequence homology as clones of the *secE-nusG* (3, 28) operon and the *mrp* gene (2). SecE is an essential integral cytoplasmic membrane protein necessary for the signal sequence dependent export of proteins (3,28). It is likely that the increase in *in vivo* expressed proteins (e.g. transferrin binding protein) which contain a signal sequence for export may require a concomitant increase in the secretion machinery necessary for its export. It is unknown what role the NusG protein, known to be involved in lambda antitermination, plays as no bacteriophage have been previously identified in APP; however, in *E. coli* NusG has been shown to be essential for viability and perhaps this protein also may play a role in the antitermination regulation of virulence genes. The Mrp protein has been putatively identified as a potential ATPase (2) which may be involved in the salvage of nucleosides *in vivo* for production of recycled nucleotides or for obtaining carbon, nitrogen or energy. One other possibility is that *mrp* gene expression by virtue of its overlap in the antisense orientation with a *metG* promoter, can regulate the expression or activity of methionyl t-RNA synthetase (2). It remains to be determined what the exact role for these clones and the unidentified clones is in the pathogenesis of APP.

We have had some problems identifying significant *in vivo* induction and obtaining exact quantitative numbers for our IVET clones *in vivo*. Part of this problem is that the riboflavin mutant AP233 is unable to survive well when diluted and plated for colony counts. Another potential problem is that a clone containing the IVET vector with a strong constitutive promoter (IVH) also appeared to be induced *in vivo*. It is unknown whether this induction is due to experimental artifact or whether it is indeed induced 9 fold *in vivo*. If this clone is not induced *in vivo* and represents the error in our measurement, then perhaps only clones IVB, IVE and IVF (*secE-nusG)* are truly induced *in vivo*. However, the virulence of AP233 containing these IVET plasmids can also yield important information. For example, the weak *trxA* promoter in the IVET plasmid in AP233 did not restore virulence even at 100 times the normal wild type $LD_{50}$ dosage, indicating there was not enough expression of the wild type *rib* genes to overcome the attenuating mutation. We also recovered approximately 1000 fold fewer organisms from the animal containing this clone. In contrast, the other IVET clones restored the virulence of AP233 to a level equal or surpassing that of IVH which we know contains a strong promoter. This would suggest that even though all 5 clones have similar minimal expression to the *trxA* clone *in vitro*, they are induced to a level at least equal to the expression of IVH, and thus all 5 clones could qualitatively be said to be induced *in vivo*.

It will likely be necessary in the future to alter our IVET procedure to provide a more effective screen for *in vivo* induced promoters. It is important that the *in vivo* selection process is allowed to continue for an appropriate amount of time such that weak promoters are not carried through and reisolated. Also, it may be necessary to pre-screen the IVET pools and eliminate strong constitutive promoter clones. This would allow the use of a greater number of colonies in each animal as well as perhaps extending the time available for selection because the animal would not become seriously ill as fast. To overcome the problem of quantitation of Lux activity from IVET clones *in vivo*, it may be necessary to re-transform any IVET plasmids containing potential *in vivo* induced promoters into the wild type strain of APP serotype 1, which appears to be less fragile and able to provide more accurate plate counts. This will provide a more accurate quantitative measurement but will not reconfirm the ability of the promoter to restore virulence by expression of the wild type riboflavin genes. The further identification of genes specifically induced *in vivo* will likely lead to new insights about the pathogenesis of this organism and may identify targets for the production of new antibiotics or live attenuated vaccines.

## ACKNOWLEDGMENTS

This research was supported by a grant from the U.S.D.A. T. E. Fuller is supported by a U.S.D.A. National Needs Graduate Fellowship in Animal Biotechnology.

## REFERENCES

1. **Camilli, A., and J. J. Mekalanos.** 1995. Use of recombinase gene fusions to identify *Vibrio cholerae* genes induced during infection. Mol. Microbiol. **18**:671-683.

2. **Dardel, F., M. Panvert and G. Fayat.** 1990. Transcription and regulation of expression of the *Escherichia coli* methionyl t-RNA synthetase gene. Mol. Gen. Genet. **223**:121-133.

3. **Downing, W.L., S.L. Sullivan, M.E. Gottesman and P.P. Dennis.** 1990. Sequence and transcriptional pattern of the essential *Escherichia coli secE-nusG* operon. J. Bacteriol. **172**:1621-1627.

4. **Fenwick, B. and S. Henry.** 1994. Porcine pleuropneumonia. J. Am. Vet. Med. Assoc. **204**:1334-1340.

5. **Frey, J.** 1992. Construction of a broad host range shuttle vector for gene cloning and expression in *Actinobacillus pleuropneumoniae* and other *Pasteurellaceae*. Res. Microbiol. **143**:263-269.

6. **Fuller, T.E. and M.H. Mulks.** 1995. Characterization of *Actinobacillus pleuropneumoniae* Riboflavin Biosynthesis Genes. J. Bacteriol. **177**:7265-7270.

7. **Fuller, T.E., B.J. Thacker and M.H. Mulks.** 1996. A riboflavin auxotroph of Actinobacillus pleuropneumoniae is attenuated in swine. Infect. Immun. **64**:4659-4664.

8. **Genetics Computer Group.** Sept. 1994. Program Manual for the Wisconsin Package, Ver. 8. Genetics Computer Group. Madison, Wisconsin.

9. **Griffiths, E.** 1991. Environmental Regulation of Bacterial Virulence- Implications for Vaccine Design and Production. Trends in Biotechnology **9**:309-315.

10. **Gross, R.** 1993. Signal Transduction and Virulence Regulation in Human and Animal Pathogens. FEMS Micro. Rev. **104**:301-326.

11. **Hensel, M. J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden.** 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science **269**:400-403.

12. **Hunneman, W.A.** 1986. Incidence, economic effects, and control of *Haemophilus pleuropneumoniae* infections in pigs. Vet. Quarterly **8**:83-87.

13. **Isberg, R.R.** 1994. Intracellular trafficking of *Legionella pneumophila* within phagocytic cells, pp.263-278. *In* V.L. Miller et al (ed). Molecular Genetics of Bacterial Pathogenesis. ASM Press, Washington, DC.

14. **Jolie, R. A. V., M. H. Mulks, and B. J. Thacker.** 1995. Cross-protection experiments in pigs vaccinated with *Actinobacillus pleuropneumoniae* subtypes 1A and 1B. Vet. Microbiol. **45**:383-391.

15. **Mahan, M.J., J.M. Slauch, and J.J.Mekalanos.** 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259**:686-688.

16. **Mahan, M.J., J.W. Tobias, J.M. Slauch, P. C. Hanna, R. J. Collier, and J. J. Mekalanos.** 1995. Antibiotic-based selection for bacterial genes that are specifically induced during infection of a host. PNAS **92**: 669-673.

17. **Mekalanos, J.J.** 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. **174**:1-7.

18. **Mulks, M.H.** Unpublished data.

19. **Nicolet, J.** 1992. *Actinobacillus pleuropneumoniae*, p. 401-408. *In* A.D. Leman et al (eds), Diseases of Swine, 7th edition Iowa State University Press, Ames, Iowa.

20. **Nielsen, R.** 1984. *Haemophilus pleuropneumoniae* serotypes - Cross protection experiments. Nord. Vet. Med. **36**:221-234.

21. **Nielsen, R.** 1979. *Haemophilus parahaemolyticus* serotypes: pathogenicity and cross immunity. Nord. Vet. Med. **31**:407-413.

22. **Nielsen, R.** 1976. Pleuropneumonia of swine caused by *Haemophilus pleuropneumoniae*. Studies on the protection obtained by vaccination. Nord. Vet. Med. **28**:337-338.

23. **Nielsen, R.** 1974. Serological and immunological studies of pleuropneumonia of swine caused by *Haemophilus parahaemolyticus*. Acta Vet. Scand. **15**:80-89.

24. **Perry, M.B., E. Altman, J.-R. Brisson, L.M. Beynon, and J.C. Richards.** 1990. Structural characteristics of the antigenic capsular polysaccharides and lipopolysaccharides involved in the serological classification of *Actinobacillus pleuropneumoniae* strains. Serodiag. Immunother. Infect. Dis. **4**:299-308.

25. **Rankin, S., and R. R. Isberg.** 1993. Identification of *Legionella pneumophila* loci expressed in response to an intracellular environment. Infect. Agents Dis. **2**:269-271.

26. **Rosendal, S., D.S. Carpenter, W.R. Mitchell, and M.R. Wilson.** 1981. Vaccination against pleuropneumonia in pigs caused by *Haemophilus pleuropneumoniae*. Can. Vet. J. **22**:34-35.

27. **Sambrook, J., E.F. Fritsch and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual, 2nd. ed. Cold Spring Harbor Press, Cold Spring Harbor, New York.

28. **Schatz, P.J., P.D. Riggs, A. Jacq, M.J. Fath and J. Beckwith.** 1989. The *secE* gene encodes an integral membrane protein required for protein export in *Escherichia coli*. Genes Dev. **3**:1035-1044.

29. **Sebunya, T.N.K. and J.R. Saunders.** 1983. *Haemophilus pleuropneumoniae* infection in swine: a review. J. Am. Vet. Med. Assoc. **182**:1331-1337.

30. **Shewen, P.E. and J.A. Rice Conlon.** 1993. 18/*Pasteurella*, p. 216-225. *In* C.L. Gyles and C.O. Thoen (eds), Pathogenesis of Bacterial Infections in Animals, 2nd edition Iowa State University Press, Ames, Iowa.

31. **Silhavy, T.J.** 1984. DNA extraction from bacterial cells. p. 137-139. *In* Experiments with Gene Fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

32. **Slauch, J.M., M.J. Mahan, and J.J. Mekalanos.** 1994. *In vivo* expression technology for selection of bacterial genes specifically induced in host tissues. Meth. Enzymol. **235**:481-492.

33. **Thacker, B.J., and M.H. Mulks.** 1988. Evaluation of commercial *Haemophilus pleuropneumoniae* vaccines. Proc. Int. Pig Vet. Soc. **10**:87.

34. **Utt, E.A., J.P. Brousal, L.C. Kikuta-Oshima and F.D. Quinn.** 1995. The identification of bacterial gene expression differences using mRNA-based isothermal subtractive hybridisation. Can. J. Microbiol. **41**:152-156.

35. **Wang, J., A. Mushegian, S. Lory and S. Jin.** 1996. Large-scale isolation of candidate virulence genes of Pseudomonas aeruginosa by in vivo selection. PNAS **93**:10434-10439.

36. **West, S.E.H., Romero, M.J.M., Regassa, L.B., Zielinski, N.A. and R.A. Welch.** 1995. Construction of *Actinobacillus pleuropneumoniae-Escherichia coli* shuttle vectors: expression of antibiotic resistance genes. Gene **160**:81-86.

# Chapter 6

# SUMMARY

Our long term objectives were to better understand the pathogenesis of *Actinobacillus pleuropneumoniae* infections as a model of respiratory disease and to use this information to generate better vaccines and new antibiotics. To contribute to these objectives, the goal of this thesis research was to design and utilize a genetic system to identify specifically *in vivo* induced (e.g., infection associated) genes in this organism. At the time this project was started little was known about the genetics of the swine pathogen *Actinobacillus pleuropneumoniae*. Only one unstable shuttle vector and one transposon mutagenesis system were available to us, and there were no defined biochemical mutants.

We have identified, cloned and completely sequenced four genes from *Actinobacillus pleuropneumoniae* that are involved in riboflavin biosynthesis. The cloned genes can specify production of large amounts of riboflavin in *E. coli*, can complement several defined genetic mutations in riboflavin biosynthesis in *E. coli*, and are homologous to riboflavin biosynthetic genes from both *E. coli* and *Bacillus subtilis*. The genes have been designated APP *ribGBAH* due to their similarity in both sequence and arrangement to the *B. subtilis ribGBAH* operon. The DNA sequence data, complementation, and minicell analysis strongly suggest that the four *rib* genes are transcribed from a single APP promoter upstream of the *ribG* gene. Biosynthesis of riboflavin by APP appears to be more similar to that in the gram-positive bacterium *B. subtilis* than in the gram-negatives *E. coli* or *Haemophilus influenzae*. Further study of this model operon should reveal interesting information on regulation and promoter/operon structure in APP, as well as information on the role of riboflavin biosynthesis in APP infection.

We hypothesized that APP must synthesize riboflavin to meet its own metabolic demands during infection, since riboflavin is not synthesized by mammals and therefore is not likely to be freely available to APP within its porcine host, especially within the relatively sterile lungs. We constructed a serotype 1 *Actinobacillus pleuropneumoniae* deletion-disruption riboflavin mutant that is attenuated *in vivo*. The *A. pleuropneumoniae ribGBAH* operon was disrupted by deleting an internal segment of the operon (*ribBA*) and replacing it with a $Km^R$ cassette using a targeted mutagenesis technique. A stable riboflavin-requiring, genetically-defined, $Km^R$ mutant, AP233, was phenotypically identical to its wild type parent based on analysis of proteins, extracellular toxin, LPS, and capsular polysaccharide by SDS-PAGE, immunoblot, and coagglutination.

Experimental infection of pigs, the only natural host for *A. pleuropneumoniae,* demonstrated that the riboflavin-requiring mutant was unable to cause disease including lung lesions or clinical signs at dosages as high as 500 times the $LD_{50}$ for the wild-type parent. It is important to note that the riboflavin-requiring mutant used in these studies is a deletion mutant, with ~1.4 kb of the riboflavin operon removed from the chromosome and replaced with an antibiotic resistance marker. We observed neither reversion to prototrophy nor loss of kanamycin resistance in this mutant in the laboratory suggesting that reversion to prototrophy and thus virulence will not occur *in vivo*.

This is the first report that a mutation in riboflavin biosynthesis in a pathogenic bacterium is attenuating. This finding represents a novel addition to the group of biosynthetic mutations which can be used to construct attenuated strains of bacteria. This is also the first report of a genetically-defined attenuated mutant of *A. pleuropneumoniae* that is still capable of production of all of the major known virulence factors of this organism, including extracellular toxins and capsular polysaccharide. The fact that riboflavin biosynthesis is essential for this pathogen and that riboflavin is not synthesized by higher eukaryotes could potentially lead to discovery of a new generation of antibiotics which inhibit riboflavin biosynthesis.

Live-attenuated vaccine strains of numerous pathogenic organisms are being developed and appear to hold the greatest promise for the future of vaccination. Many of these strains contain mutations in biochemical pathways which are necessary for survival inside the host. We have tested our riboflavin-requiring mutant of APP as a live-attenuated vaccine. Poor persistence of live vaccine strains *in vivo* can lead to a failure to elicit a protective immune response. In order to assure that infection-associated antigens would be expressed by the live-attenuated vaccine strain of bacteria after immunization of pigs, we needed to ensure that the bacteria had sufficient available riboflavin to permit 2-3 generations of growth. An IM immunization with a dose containing sufficient riboflavin gave very good protection against APP in the face of an overwhelming challenge. Further experimentation with varying dosages and riboflavin concentrations is required in order to determine the optimal vaccine regimen to elicit good protection.

We have constructed an IVET system for use in our riboflavin-requiring strain of *Actinobacillus pleuropneumoniae,* the first of its kind for use in the family *Pasteurellaceae*. We have demonstrated that a functional IVET vector can be constructed with the *E. coli*-APP shuttle vector pGZRS-19, the T4 terminator, the promoterless wild type riboflavin biosynthesis genes (*ribBAH*) from *Bacillus subtilis* and the promoterless *luxAB* reporter genes from *Vibrio harveyi*. We have shown that the *Bacillus subtilis* genes can complement the riboflavin mutation in AP233, restoring the ability to grow without riboflavin and restoring virulence. We have shown that there is no background bioluminescence in APP and that the *luxAB* genes can be used as a simple and quantifiable reporter of gene expression in APP. Finally, our IVET system has been used to identify 5 specifically *in vivo* induced genes which are likely to be important for growth or virulence of APP in its natural host. Based upon their sequence similarity, two of the identified clones have been predicted to encode polypeptides homologous to *E. coli* SecE/NusG and *E. coli* Mrp. It remains to be determined what the exact role for these clones and the unidentified clones is in the pathogenesis of APP.

With a rapid mutation rate and the potential for horizontal gene transfer, pathogens seem to have the advantage in the continual struggle against their hosts. We now realize that antibiotics and vaccines are not the magical answers they were originally thought to be. Therefore, it is imperative that we continue to use modern genetic technology to study the pathogenesis of organisms which are presently causing disease and anticipate how to overcome the next resurgence of diseases that seem to be irrelevant at this time. The further identification of genes specifically induced *in vivo* will likely lead to new universal insights about the pathogenesis of these organisms and may identify targets for the production of a new generation of antibiotics or new live attenuated vaccines.