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Vaccines of pasteurellaceae mutants and vaccination method

Abstract
A live vaccine of recombinant mutants of a member of the family Pasteurellaceae lacking a rib gene necessary for production of riboflavin as well as a method of vaccination therewith is described. The vaccine is effective against members of the family Pasteurellaceae.

Keywords
Veterinary Diagnostic & Production Animal Medicine, Microbiology

Disciplines
Comparative and Laboratory Animal Medicine | Veterinary Medicine | Veterinary Microbiology and Immunobiology

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VACCINES OF PASTEURELLACEAE MUTANTS AND VACCINATION METHOD

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Provisional application No. 60/007,764, filed on Nov. 30, 1995.

Int. Cl. 6 A61K 39/00; A61K 39/102; A61K 45/00; A61K 39/12; C12N 1/36
U.S. Cl. 424/184.1; 424/256.1; 424/282.1; 424/256.1; 424/255.1; 424/282.1; 435/245; 935/65; 935/66; 935/72
Field of Search 424/184.1; 256.1; 424/282.1; 200.1; 235.1; 255.1; 282.1; 435/245; 935/65; 66, 72

References Cited
U.S. PATENT DOCUMENTS
4,888,170 A 12/1989 Curtiss, III
5,429,818 A * 7/1995 Inzana
5,456,914 A 10/1995 Stine et al.
5,468,485 A 11/1995 Curtiss, III
5,925,354 A * 7/1999 Fuller et al.
6,022,728 A * 2/2000 Mulks et al.

FOREIGN PATENT DOCUMENTS
CA 2045950 12/1992
WO 9049921 5/1994

OTHER PUBLICATIONS
Fuller, T.E. et al., Infection and Immunology Nov. 1996 p. 4659–4664.

Primary Examiner—Nita Minniefield

ABSTRACT
A live vaccine of recombinant mutants of a member of the family Pasteurellaceae lacking a rib gene necessary for production of riboflavin as well as a method of vaccination therewith is described. The vaccine is effective against members of the family Pasteurellaceae.

11 Claims, 12 Drawing Sheets
FIG. 1
FIG. 8A
FIG. 8B
1 VACCINES OF PASTEURELLACEAE MUTANTS AND VACCINATION METHOD

CROSS REFERENCE TO RELATED CASES

This application is a divisional of application Ser. No. 08/741,327, filed on Oct. 28, 1996 now U.S. Pat. No. 5,925,354, which includes the disclosure in provisional patent application serial No. 60/007,764 filed Nov. 30, 1995, for Characterization of Actinobacillus pleuropneumoniae Riboflavin Biosynthesis Genes, hereby incorporated by refer-
ence.

TECHNICAL FIELD

The invention relates to vaccines and in particular, live vaccines against Actinobacillus pleuropneumoniae (APP) and related bacterial pathogens. The invention is also concerned with recombinant techniques for preparing such a vaccine.

BACKGROUND OF THE INVENTION

An organism known as Actinobacillus pleuropneumoniae (APP) is a gram negative coccobacillus organism that is found in the pig and causes pneumonia in the pig. This disease is characterized by an acute necrotizing hemorrhagic bronchopneumonia, with accompanying fibrin-


12:214) (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). 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 rever-


22:150–158) loci, which affect the biosynthesis of aromatic amino acids, purines, and thymine, respectively, are attenu-


Cam, N. Verma and A. A. Lindberg. 1985. AroD deletion attenuates Stigmella flexneri strain 25477 and makes it a safe
and efficacious oral vaccine in monkeys. Vaccine. 8:830–836) (Lindberg, A. A., A. Karnell, B. A. D. Stocker, S.
of aromatic and purine dependent Salmonella typhimurium: attenuation, persistence and ability to induce protective
immunity in BALB/c mice. Infect. Immun. 56:419–423) Lesions that affect the biosynthesis of LPS (Collinge, V. S.
of autoreactive and gale derivatives of Salmonella cholerasuis. Infect. Immun. 55:955–962) and of cyclic AMP
(Kelly, S. M., B. A. Bosceker and R. Curtiss III. 1992. Characterization and protective properties of attenuated
nogenicity of DeltaC DeltaD Salmonella typhii strains in adult volunteers. Infect. Immun. 60:536–541) 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 (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)

Riboflavin (vitamin B2), a precursor of the coenzymes flavin adenine dinucleotide (FAD) and flavin mononucle-
otide (FMN), is essential for basic metabolism. It is synthesized by plants and by most microorganisms but not
of Flavins, Vol. I. Chemical Rubber Company, Boca Raton, Fla.). Many pathogenic bacteria are apparently unable
to utilize flavins from their environment and are entirely dependent on endogenous production of riboflavin (Schott,
acid sequence of the α-subunit. J. Biol.Chem. 265:4204–4209). 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.

Vaccines are preparations used to prevent specific dis-
eases in animals by inducing immunity. This is accom-
plished by exposing a patient to an antigen from an agent
capable of causing a particular disease which, in turn, causes
the immune system of the patient to produce large Quantities of antibody. The presence of the antibody in the patient’s blood protects the patient from a later attack by the disease-
causing agent. Vaccines may either be composed of subunits
of the agent, or the live or killed agent itself. If a live vaccine
is to be used, its virulence must be attenuated in some way;
otherwise, the vaccine will cause the disease it is intended to
protect against. See U.S. Pat. No. 5,429,818, Col. 1

Most current vaccines against APP are killed whole cell
bacteria, that is, whole bacterial cells killed by heat treat-
ment or formalinization, suspended in an adjuvant solution.
Some alternative ways of attempting to develop vaccines
against APP are the use of subunit vaccines and the use of
non-encapsulated mutants.

The use of a protease lysate of the outer membrane of A.
pleuropneumoniae cells as a vaccine against APP infection is
described in U.S. Pat. No. 5,332,572.

The use of extracellular proteins and/or hemolysins from
APP as vaccines against APP infection is described in U.S.
595,188, CA 2045950, and EP No. 453,024.

The use of non-encapsulated mutants of APP is described
in U.S. Pat. No. 5,429,818. It disclosed that the capsule of
such bacteria is required for virulence. Therefore, the prepa-
ration of a mutant of APP that was a non-encapsulated
mutant was described as a vaccine.

A method of administering vaccines to pigs by a tran-
stracheal intrapulmonary immunization is described in U.S.
Pat. No. 5,456,914.

A vaccine for the immunization of an individual against
Salmonella cholerasuis utilizing derivatives that are inac-
capable of producing functional adenylate cyclase and/or
cyclic AMP receptor protein is described in U.S. Pat.
5,468,485. The avirulent S. cholerasuis was made avirulent
by an inactivating mutation in a cya gene and an inactivating
mutation in a crp gene. Similar techniques are described in
other bacteria in U.S. Pat. Nos. 5,424,065; 5,389,386; 5,387,
744 and 4,888,170.

To protect animals from lung disease, it is needed to
achieve a sufficiently high level of antibodies, particularly
IgA antibodies, in the lungs to prevent adherence of invading
microorganisms to mucosal surfaces and neutralize poten-
tially damaging virulence factors. Antibodies in the patient’s
serum or at the mucosal surfaces can be important to
protection. One of the reasons for using a live vaccine
instead of a killed whole cell bacterin is that a live vaccine,
given intranasally or orally, can induce specific local secre-
tory antibody in the secretions that cover mucosal surfaces.
This local antibody is often quite helpful for protection
against diseases that infect at or through mucosal surfaces.

None of the patents pertain to a recombinant technique for
a relatively convenient method for obtaining genetically
defined mutants for use in a vaccine against APP.

It is believed 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 induc-
ing cross-protective immunity against A. pleuropneumoniae.

It is believed that riboflavin biosynthesis would be essen-
tial 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.

It is an object of the present invention to describe the use
of mutations in the riboflavin biosynthetic pathway to con-
struct attenuated strains of pathogenic bacteria for use as live vaccines, with a riboflavin-requiring mutant of APP used as a specific example.

It is an object of the present invention to describe a live vaccine against APP utilizing a riboflavin mutation in the APP genome.

SUMMARY OF THE INVENTION

Described is a live vaccine against bacterial pathogens comprising a recombinant riboflavin-requiring mutant having a mutation that inactivates riboflavin biosynthesis therein. In particular, this includes bacterial pathogens in the family Pasteurellaceae, which include animal pathogens as Actinobacillus pleuropneumoniae, Actinobacillus suis, Haemophilus parasuis, Pasteurella haemolytica and Pasteurella multocida, as well as human pathogens Haemophilus influenzae and Haemophilus ducryi.

Also described is a live vaccine against Actinobacillus pleuropneumoniae (APP) comprising a recombinant APP having an inactivating mutation therein.

BRIEF DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

FIG. 1 describes a proposed metabolic pathway for bacterial riboflavin synthesis.

FIG. 2 is a physical map of a plasmid construct pTF-10, which comprises a 5.2 Kb fragment of A. pleuropneumoniae chromosomal DNA, including the APP riboflavin biosynthetic operon, cloned into the vector pUC19.

FIGS. 3A and 3B show the absorbance spectra of aqueous solutions at neutral pH (Panel A) and acidified aqueous solutions (Panel B) of the product excreted into the growth medium by E. coli DH5α/pTF10 (solid line) and a standard riboflavin preparation (dotted line).

FIGS. 4A to 4D show the complete nucleotide sequence of APP ribGBAH operon and flanking regions and the predicted amino acid sequences of the encoded proteins.

FIG. 5 shows the complementation of E. coli riboflavin-requiring mutants by cloned APP rib genes. A physical map for the APP ribGBAH genes is shown as well as several deletions.

FIG. 6 shows a minicell analysis of the proteins encoded by pTF10 and its deletions.

FIG. 7 shows the construction of pTF67a, a suicide delivery vector containing a portion of the APP rib operon with a part of ribB and all of ribA deleted and replaced with a gene cassette encoding resistance to the antibiotic kanamycin.

FIG. 8A is an analysis of rib-transconjugants of A. pleuropneumoniae serotype 1.

FIG. 8B is a southern blot analysis of chromosomal DNA from A. pleuropneumoniae serotype 1 rib mutants.

DESCRIPTION OF PREFERRED EMBODIMENTS

The present application pertains to the development of attenuated mutants of the pathogenic bacterium A. pleuropneumoniae which contain mutations in the genome, specifically in the genes encoding the enzymes involved in the biosynthesis of riboflavin. By “mutation” is meant not just a random selection of variations of the genome of APP but utilization of well known recombinant techniques for specifically modifying the genome of APP. Accordingly, therefore, it is desirable to ascertain the riboflavin biosynthesis genes of APP.

By “attenuated” is meant a reduction in the severity, virulence or vitality of the disease causing agent.

After determining the sequence and organization of the riboflavin genes, one is then able to modify APP by removing some or all of such genes, thereby attenuating the pathogen, i.e., making the pathogen avirulent.

After a strain of avirulent APP is obtained, it could then be utilized as a live vaccine. Described below are the detailed steps broadly outlined above.

Identifying, Cloning, and Sequencing of the Riboflavin Biosynthesis Genes from APP

Cloning of riboflavin genes from APP is described in the paper entitled “Characterization of APP Riboflavin Biosynthesis Genes”, Journal of Bacteriology, December, 1995, pages 7265–7270 by Fuller and Mulkis. This is incorporated herein by reference.

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 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 FIG. 1 begins with guanosine triphosphate (GTP) as the precursor (for a review 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 coregulated 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 cyclohydrolase and 3,4-dihydroxy
2-butanoate 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 (19,20). The B. subtilis structural ribGBAH1T 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 base pairs (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 transacting 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 P1 and 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 cyclohydrolase 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. Identified herein is a fragment of APP serotype 5 chromosomal DNA that triggers overproduction of riboflavin when cloned in E. coli. Nuclease sequence analysis demonstrated four open reading frames with significant identity and a similar operon arrangement to the ribGBAH1T genes from Bacillus subtilis. Materials and Methods

Bacterial strains and media. B. pleurophagum nae ISU178, a serotype 5 strain, was cultured at 37°C in brain heart infusion broth or agar (Difco Laboratories, Detroit, Mich.) containing 10 µg/ml novobiocin and amikacin (NAD) (Sigma Chemical Company, St. Louis, Mo.). E. coli DH5α (supE44), AcacU169, (80lacZAM15), 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, rpsL35, xyl-7, nth-2, thi-1, h-) was used for miniincell isolation and protein labeling experiments. E. coli ribA-Tn5 (BSV18), ribB-Tn5 (BSV11) and ribC:Tn5 (BSV3) mutants used for complementation studies were described by Brandin et al (2) and are available from 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) 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, Ind.) and used according to manufacturer’s specifications. Genomic and plasmid DNA preparations, gel electrophoresis, and E. coli transformation were performed by conventional methods (36).

Cloning and sequencing. APP serotype 5 genomic DNA was digested with HindIII and fragments ranging in size from 4 to 7 kb were ligated into the HindIII site in the polylinker of the plasmid vector pUC19 (45). A recombinant plasmid, designated pFT10, 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, Wis.). 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, Ohio) and [³²P]dATP (adenosine triphosphate) (Amersham Corp., Arlington Heights, Ill.). 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'-AAE-CCG-CCA-AAA-ATT-GAA-GGC-3') (Sequence ID No: 1), MM5 (5'-GCA-CCG-TGA-GCC-3') (Sequence ID No: 2), MM6 (5'-GCG-CCA-ATA-CTT-GCT-CAC-3') (Sequence ID No: 3), MM9 (5'-GGT-CTT-TTT-ATT-GGT-CCG-3') (Sequence ID No: 4), MM10 (5'-TCA-AGA-AGT-GGG-GAA-3') (Sequence ID No: 5), MM11 (5'-GCT-GTT-AGG-ATG-CCA-GCC-3') (Sequence ID No: 6), MM13 (5'-GCG-C-AC-AGT-CAG-ATG-3') (Sequence ID No: 7), MM14 (5'-GCC-AGT-AGC-AGA-3') (Sequence ID No: 8), and MM38 (5'-CTC-ACC-GCT-TTC-TGC-CAA-ACC-3') (Sequence ID No: 9).

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 microcentrifuge, and the absorbance at 445 nm of the culture supernatant was measured using a Beckman DU-7 spectrophotometer (Beckman Instruments, Fullerton, Calif.). The absorbance at 445 nm was multiplied by a factor of 31.3 to yield the riboflavin concentration in mg/liter (10).

Mycelium Analysis. The mycelial-producing E. coli strain DS410 (32) was transformed by calcium chloride/heat shock treatment with pUC19 or pTb rib clones. Transformant colonies which produced a large number of mycellines were selected by microscopy. Cultures were grown overnight at 37°C in 500 mL LB broth, and mycellines were isolated by differential centrifugation followed by glass fiber filtration as described by Christen et al (8). Mycellines were resuspended to an OD₅₉₀ of 0.5-1.0 in 200 µl labeling mix (22.0 mL M9 media, 20.0 mL 50 mM HEPES (N-2-hydroxyethyl)piperazine-N'-2 ethanesulfonic acid) pH 7.5, 2.5 mL of 20% glucose, 0.05 mL of 10 mg/ml adenosine, 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 cyclsiner- D) and incubated at 37°C for 30 minutes. Trans-labeled (³¹P) methionine plus (³¹S) cysteine, ICN Biomedicals, Irvine, Calif.) was added to a final concentration of 22 µCi per reaction and cells were incubated at 37°C for 1 hour. Total and TCA (trichloroacetic acid) precipitable counts were measured by liquid scintillation counting to determine amount of incorporation. Cells were pelleted in a microcentrifuge and washed with cold HEPES (50 mM, pHi7.5) plus 10 mM methionine plus 10 mM cysteine. Labeled proteins (50,000 cpm/lane) were separated by dis-
continuous 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 was 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 (Fig. 2), containing a 5.2 kbp 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, Mass.). 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 (Figs. 3A and 3B). Positive and negative ion fast atom bombardment mass spectrometry 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. pTF10 was deposited under the terms of the Budapest Treaty at the American Type Culture Collection, 10801 University Boulevard, Manassas, Va. 20110-2209 on Aug. 31, 2000 as ATCC PTA-2438. All restrictions on availability to the public of the deposited material will be irrevocably removed upon granting the patent.

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 Figs. 4A to 4D. 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 (see below), 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 ribB 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 to 10 type (12) was identified within the sequenced region 224 bp upstream from the ribB start codon. A second potential consensus promoter was identified between the genes ribA and ribB. 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 ΔG of ~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 Rib-III (17), Photobacterium phosphoreum RibIV (16), and Vibrio harveyi LuxH (43) proteins, using the GCG Gap program (Table 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 Photobacrerium 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 59–65% similarity to E. coli RibB, and V. harveyi LuxH, but encode 3,4-DHP synthase. For 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 cyclohydrolase II.

![Table 1](image)

**Table 1**

<table>
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<tr>
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<th>%</th>
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</table>

*Identity is expressed in percent similarity as calculated by the Genetics Computer Group Needleman-Wunsch algorithm (22). Proteins with partial identity were compared with the entire appropriate A. pleuropneumoniae Rib protein.

Complementation of E. coli mutants. The original pTF10 clone and several deletion derivatives were tested for their abilities to complement ribA (GTP cyclohydrolase II), ribB (3,4-DHB synthase), and ribC (β-subunit of riboflavin synthase) mutations in E. coli (2) (FIG. 5).
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* ribBC 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 ribB 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 (Fig. 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 (Fig. 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 (Fig. 6). Plasmid pTF12 (Fig. 8), which does not contain ribH, ribA, or ribH genes, does not express the 27.7, 43.7, or 14.8 kDa proteins (data not shown).

Described above is 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 (55–10 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 ~1/4 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, ribE, that is proposed to mediate the third step in riboflavin biosynthesis; it is unlikely that a ribE 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 cyclohydrolase II and DHPP 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). FMNH₂ 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). It is believed 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.

### Attenuation of Rib-Mutants of *A. pleuropneumoniae*

Applicants have constructed deletion-disruption riboflavin-requiring mutants of *A. pleuropneumoniae* serotypes 1 and 5.

Applicants have conducted experiments to confirm that the Rib-APP mutants constructed are attenuated in swine.

In a preliminary experiment, seven 8- to 10-week old pigs were used. Three pigs were infected endobronchially with NaI (resistant to the antibiotic malidixic acid) derivatives of wild type virulent APP-1 or APP-5; three were infected with APP-5 Rib-mutants; and one was used as an uninfected control. The APP strains, dosages used for infection, and results are summarized below in Table 2. Animals were euthanized when clinical signs became severe or at 12 hours post-infection. The animals were necropsied and the lungs examined for gross pathology and histopathology, and lungs were cultured to recover APP.

### Table 2

<table>
<thead>
<tr>
<th>Strain and Description</th>
<th>Dosage</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>APP22: APP-1, NaI&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2 × 10⁶ cfu</td>
<td>Died, 4+ hrs; 4+ penciclate hemorhagic pneumonia lesions</td>
</tr>
<tr>
<td>APP27: APP-5, NaI&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 × 10⁶ cfu</td>
<td>Died, 6+ hrs; 4+ penciclate hemorhagic pneumonia lesions</td>
</tr>
<tr>
<td>APP28: APP-5, NaI&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 × 10⁶ cfu</td>
<td>Died, 4+ hrs; 4+ penciclate hemorhagic pneumonia lesions</td>
</tr>
<tr>
<td>APP29: APP-5, NaI&lt;sup&gt;b&lt;/sup&gt;, Km&lt;sup&gt;b&lt;/sup&gt;, Rib&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 × 10⁶ cfu</td>
<td>Mild clinical signs; 1+ mild pneumonia lesions</td>
</tr>
<tr>
<td>APP30: APP-5, NaI&lt;sup&gt;b&lt;/sup&gt;, Km&lt;sup&gt;b&lt;/sup&gt;, Rib&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 × 10⁶ cfu</td>
<td>Mild clinical signs; 1+ mild pneumonia lesions</td>
</tr>
<tr>
<td>APP31: APP-5, NaI&lt;sup&gt;b&lt;/sup&gt;, Km&lt;sup&gt;b&lt;/sup&gt;, Rib&lt;sup&gt;b&lt;/sup&gt;</td>
<td>None</td>
<td>— 1+ mild pneumonia lesions (empyplasm)</td>
</tr>
</tbody>
</table>

Note that the dosage used in all of these animals was about 200 times the LD₅₀ (50% lethal dose, or the dose that will kill 50% of the animals exposed) for the wt (wild type)
APP parent strains. The Na⁺ derivatives of the wild type) parent strains retained virulence, triggering severe fibrino-
 supplicative hemorrhagic pneumonia and death within 4 
 hours. The Rib- mutants caused minimal clinical signs 
 (increased respiration rate and slight fever) and at most mild 
 signs of pneumonia, including some consolidation but no 
 hemorrhagic necrosis, as compared to the uninfected con-
 trol. These were not SPF (specific pathogen free) pigs, and 
 there were histologic lesions suggestive of mild mycoplasma 
 infection, in all of the pigs, including the uninfected control 
 (Table 2).

Described below is the construction of a deletion-
 disruption riboflavin mutant of A. pleuropneumoniae sere-
 type 1 (APP-1) and detailed analysis of the attenuation of 
 this APP-1 Rib-mutant in vivo in swine.

Materials and Methods

Bacterial strains and media. The bacterial strains and 
 plasmids used in this study are listed in Table 1. A. pleu-
 ro pneumoniae strains were cultured at 37°C in either brain 
 heart infusion (BHI), heart infusion (HI), or tryptic soy agar 
 (TSA) (Difco Laboratories, Detroit, Mich.) containing 10 
 μg/ml NAD (V factor) (Sigma Chemical Company, St. 
 Louis, Mo.). Riboflavin (Sigma) was added to a final con-
 centration 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 naldixic acid were 
 added as required, except for selection after matings which 
 were performed with 100 μg/ml kanamycin sulfate and 50 μg/ml 
 naldixic acid.

DNA manipulations. DNA modifying enzymes were sup-
 plied by Boehringer-Mannheim Biochemicals (Indianapolis, 
 Ind.) and used according to the manufacturer’s specifi-
 cations. Genomic DNA was prepared according to the lysis/ 
 proteinase K method of the Gene Fusion Manual (Silihavy, 
 T. J. 1984. DNA extraction from bacterial cells. p. 137–139. 
 In Experiments with Gene Fusions. Cold Spring Harbor 
 Laboratory, Cold Spring Harbor, N.Y.). Plasmid DNA 
 preparations, agarose gel electrophoresis, and E. coli trans-
 formation were all performed by conventional methods 
 (Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. Molecu-
 lar Cloning: A Laboratory Manual, 2nd. ed. Cold Spring 
 Harbor Press, Cold Spring Harbor, N.Y.)

Filter mating targeted mutagenesis. Filter mating between 
 E. coli S17-1 (λpir)/pTF67A and AP225 was performed 
 according to the protocol of Mulsks and Buysses (Mulsks, 
 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 
 of 10 mM MgSO₄, and the bacteria pelleted by centrifugation.

The pellet containing the cell mating mixture, resuspended 
 in 100 μl of 10 mM MgSO₄, was plated on a sterile filter on 
 BHI+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 BHI 
 broth and plated in 100 μl aliquots on BHI containing 
 riboflavin, kanamycin, and nalidixic acid. Kanamycin and 
 nalidixic acid resistant colonies were selected from filter 
 mating plates and screened for riboflavin auxotrophy by 
 replica plating onto TSA, observing for inactivity to grow in 
 the absence of added riboflavin.

Southern Analysis of Transconjugants. Chromosomal 
 DNA and plasmid controls were digested with the appro-
 priate 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 
 Molecular Cloning: A Laboratory Manual, 2nd. ed. Cold 
 probes were labeled with digoxigenin by random priming 
 using the Genius V. 3.0 kit from Boehringer Mannheim.

Probes included the 5.2 Kb insert from pTI10 containing the 
 intact riboflavin operon from A. pleuropneumoniae (RbbH), the 
 Cla/NdeI fragment deleted from the riboflavin operon in the 
 construction of pTF67a (R.De.), and 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 16 h. Blots were washed twice in 2×SSC/0.1% 
 SDS for 15 min at room temperature, then twice in 0.1× 
 SSC/0.1% SDS for 30 min at 65°C. Blots were developed 
 with alkaline phosphatase-conjugated anti-digoxigenin and 
 colorimetric substrate (Boehringer Mannheim) according to 
 the manufacturer’s instructions.

Phenotypic analysis of mutant strains. Whole cell lysates 
 and supernatants of AP100, AP225 (Na⁺), and AP233 (Km⁺, 
 Na⁺, Rib-) were prepared from overnight cultures 
 grown in HIV+5 mM CaCl₂+-appropriate antibiotics.

APP225 and AP233 were deposited under the terms of 
 the Budapest Treaty at the American Type Culture Collection 
 on Aug. 31, 2000 as ATCC PTA-2429 and ATCC PTA-2430, 
 respectively. All restrictions on availability to the public 
 of the deposited material will be irrevocably removed upon 
 granting the patent. Cells were separated by microcentrifu-
 gation and resuspended in SDB-PAGE loading buffer 
 the assembly of the head of bacteriophage T4. Nature 
 227:680–685). The culture supernatant was precipitated 
 with an equal volume of 20% trichloroacetic acid (TCA) and 
 resuspended in SDB-PAGE (sodium dodecyl sulfate-
 polyacrylamide gel electrophoresis) sample buffer. Cellular 
 polysaccharides, including lipopolysaccharide (LPS) and 
 capsular polysaccharide, were prepared according to the cell 
 lysis/proteinase K method of Kimura et al (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) . All 
 samples were analyzed on a 0.125% SDS-12% acrylamide 
 Cleavage of structural proteins during the assembly of 
 were transferred to nitrocellulose according to standard 
 Molecular Cloning: A Laboratory Manual, 2nd. ed. Cold 
 Spring Harbor Press, Cold Spring Harbor, N.Y.) and probed with convalescent serum from a pig 
 infected with A. pleuropneumoniae serotype 1. Antibody-
 antibody complexes were detected with horseradish 
 peroxidase-conjugated protein A (Boehringer Mannheim) 
 and the colorimetric substrate 4-chloro-naphthol (BioRad, 
 Hercules, Calif.).

Production of serotype-specifc capsular polysaccharide was 
 measured by coagglutination assay using hyperimmune 
 rabbit anti-sera complexed to Staphyloccoccus aureus whole 
 Antigenic differences within Actinobacillus pleuropneumo-

Electroporation of A. pleuropneumoniae. AP233 was 
 grown in 100 ml BHI with riboflavin at 37°C, with 
 shaking at 150 RPM, to an OD₅₆₀ of 0.7. Cells were chilled 
 on ice and centrifuged at 5,000 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, 200Ω; capacitance, 25 μF.

Experimental infections. Eight-week-old, specific-pathogen-free, castrated, male pigs (Whiteshire Hamroc, Inc., Albion, Ind.) 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 (biosafety level) 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 CaCl2+riboflavin and antibiotics as needed, in 300 ml baffled side-arm flasks, at 37°C with shaking at 160 RPM, to an OD520 (optical density) 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 pneumonopneumonia, including increased respiratory rate, fever, dyspnea, decreased appetite and activity/attitude (depression), were monitored and scored as previously described (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). 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 (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). 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 (KmR) cassette was designed (FIG. 7). A 2.9 kb EcoRI fragment from pTF10 (Fuller, T. E. and M. H. Mulks. 1995. Characterization of Actinobacillus pleuropneumoniae ribollavin biosynthesis genes. J. Bacteriol. 177:7265–7270) containing the A. pleuropneumoniae ribBAH genes was cloned into the EcoRI site of the conjugal suicide vector pGPF704 (18) to create plasmid pTF66. pTF66 was deposited under the terms of the Budapest Treaty at the American Type Culture Collection on Aug. 31, 2000 as ATCC PTA-24357. All restrictions on availability to the public of the deposited material will be irrevocably removed upon granting the patent. pTF66 was digested with ClaI and NdeI to excise the 3’ end of ribB and the entire ribA gene. After Klenow treatment of the DNA, the 1.2 kb KmR cassette, excised with EcoRI from pUC4K, was blunt-end ligated into the rib deletion site to create pTF67a.

pTF67a was transformed into E. coli S17-1 (λpir) and mobilized into AP225 (NaR) 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 (FIG. 8A). Predicted band sizes for single and double cross-over events are shown in FIG. 8A. Genomic DNA from AP233 contained a 2.2 Kb HindIII 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 pGPF704 nor the deleted portion of the riboflavin operon (FIG. 8B). This is the pattern of hybridization predicted in transconjugants that replaced the wild type riboflavin operon with the mutated rib::KmrR locus by a double-crossover event (FIG. 8A). In contrast, genomic DNA from AP234 shows the presence of DNA homologous to the fragment deleted from the riboflavin operon (R. del), pGPF704, and the kanamycin cassette (FIG. 8B). 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 (FIG. 8A).

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 sera. No differences in protein, LPS, extracellular toxin, or capsular polysaccharide profiles were detected between wild type AP100, its NaR derivative AP225, and the riboflavin mutant AP233 (data not shown). 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 pGZRSv19, an *E. coli*-A. pleuropneumoniae shuttle vector (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), to form pTF76. pTF76 was deposited under the terms of the Budapest Treaty at the American Type Culture Collection on Aug. 31, 2000 as ATCC PTA-2436. All restrictions of availability to the public of the deposited material will be irrevocably removed upon granting the patent. 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 (see below).

Attenuation of virulence of the rib mutant in swine. Six groups of three pigs each were infected with: group 1, 1 LD<sub>50</sub> (5×10<sup>6</sup> cfu) of AP225; groups 2–5, AP233 at doses equivalent to 4, 20, 100, and 500 times the wild-type LD<sub>50</sub>; and group 6, 1 wild-type LD<sub>50</sub> of AP233/pTF76. Mortality, lung score, and clinical score data, shown in Tables 3, 4 and 5, all indicate that the riboflavin auxotroph is avirulent in pigs at doses as high as 500 times the wild-type LD<sub>50</sub>. The pigs infected with the rib mutant AP233 displayed no dyspnea, elevated respiratory 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 APP disease, including elevated respiratory rates, dyspnea, depression, loss of appetite, and fever, and severe pneumonia and pleuritis was evident at necropsy. Pigs infected with AP225 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

<table>
<thead>
<tr>
<th>Strain/ Plasmid</th>
<th>Characteristics</th>
<th>Source/ Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>apr</em>, supF44, AbcU169, (Δ601acZAM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1</td>
<td>BRL, USA</td>
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<tr>
<td>E. coli DH5α</td>
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<tr>
<td><em>apri</em>, supF44, AbcU169, (Δ601acZAM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1</td>
<td>Mulks &amp; Buysse</td>
<td></td>
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<tr>
<td>E. coli S171-1</td>
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<td></td>
</tr>
<tr>
<td><em>apri</em>, recA, thi, pro, hsd, (r-m+), RP4-2, (Tc:Mo), Km::Tn7, [TnpR], [SniR]</td>
<td>Simon et al.</td>
<td></td>
</tr>
<tr>
<td>AP100</td>
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<tr>
<td><em>A. pleuropneumoniae</em> ATCC 27088,</td>
<td>ATCC</td>
<td></td>
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</table>

### TABLE 4

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain</th>
<th>Dose (LD&lt;sub&gt;50&lt;/sub&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mortality</th>
<th>% Pneumonia&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% Pleuritis&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AP225 (WT)</td>
<td>1</td>
<td>1/3</td>
<td>66.7</td>
<td>71.7</td>
</tr>
<tr>
<td>2</td>
<td>AP233 (Rib+)</td>
<td>4</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>AP233 (Rib+)</td>
<td>20</td>
<td>0.5</td>
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<td>0</td>
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<tr>
<td>4</td>
<td>AP233 (Rib+)</td>
<td>100</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
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<tr>
<td>5</td>
<td>AP233 (Rib+)</td>
<td>500</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
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<tr>
<td>6</td>
<td>AP233 + pTF76</td>
<td>1</td>
<td>0.3</td>
<td>27.6</td>
<td>20.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Doses are multiples of the established wild-type APP 225-corescrete type 1 LD<sub>50</sub> of 5.0 × 10<sup>6</sup> cfu (12)

<sup>b</sup>Percentage of lung tissue exhibiting *A. pleuropneumoniae* lesions

<sup>c</sup>Percentage of pleural surface area exhibiting pleuritis
### Table 5

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain</th>
<th>Dose (LD&lt;sub&gt;50&lt;/sub&gt;)</th>
<th>RR Max&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Temp Max&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Dyspno&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Depression&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Appetite&lt;sup&gt;e&lt;/sup&gt;</th>
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<td>105.7</td>
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<td>2</td>
<td>AP233</td>
<td>5</td>
<td>8</td>
<td>102.5</td>
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<sup>a</sup> Doses are multiples of the established wild-type APP225-serotype 1 LD<sub>50</sub> of 5.0 x 10<sup>5</sup> cfu (12).
<sup>b</sup> Maximum respiratory rate observed after challenge. Respiratory rate recorded as number of breaths per 15 sec observation period.
<sup>c</sup> Maximum rectal temperature after challenge, in degrees Fahrenheit.
<sup>d</sup> Depression 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.
<sup>e</sup> Appetite was scored as: 0 = did not eat; 1 = did not eat. Total score = number of 12 hour periods not eating over 60 hour observation period.

### Discussion

Above is shown 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 (ribBAH) with replacing it with a Km<sup>b</sup> cassette using a targeted mutagenesis technique (Mullus, M. H. and J. M. Buyssse. 1995. A targeted mutagenesis system for *Actinobacillus pleuropneumoniae*. Gene 165:61–66). A stable riboflavin-requiring, Km<sup>b</sup> 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 desirable 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<sub>50</sub> 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 observed at 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. Neither reversion to prototrophy nor loss of kanamycin resistance in this mutant in the laboratory was observed. In the preliminary experiment with a serotype 5 riboflavin mutant, it was possible to reisolate the mutant from the 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 experiment, 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. 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. The above represents a new addition to the group of bio-synthetic mutations that can be used to construct attenuated strains of bacteria. It also shows a genetically modified attenuated mutant of APP that is capable of production of all of the major virulence factors of this organism, including extracellular toxins and capsular polysaccharide.

### Evaluation of a Riboflavin-Requiring Auxotrophic Mutant of *Actinobacillus pleuropneumoniae* as a Genetically Defined Live Attenuated Vaccine Against Porcine Pleuropneumonia

The applicants have evaluated a genetically defined riboflavin-requiring attenuated mutant of *Actinobacillus pleuropneumoniae* as a live avirulent vaccine that provides immunity against experimental challenge with a virulent strain of *A. pleuropneumoniae*.

The specific aims of this study were: 1) to evaluate whether respiratory exposure to a live attenuated vaccine APP strain elicits protection against subsequent experimental challenge with virulent *A. pleuropneumoniae*; and 2) to determine whether addition of exogenous riboflavin to the vaccine dosage improves persistence, and therefore immunogenicity and protection; and 3) to compare the protection afforded by respiratory exposure to that elicited by intramuscular (IM) immunization with the live vaccine, which is a more commercially feasible vaccination route.

### Materials and Methods

**Animals.** In this study, 6-to-8 week old crossbred (Yorkshire/Landrace) barrows from a herd known to be free
of *A. pleuropneumoniae* and related respiratory pathogens were used. Pigs were housed in the Michigan State University Research Containment Facility and fed a standard antibiotic-free diet provided by the MSU Swine Research and Teaching Center.

Preparation of Vaccines

1. Live vaccine: The bacterial strain used to prepare the live attenuated vaccine was AP233, a derivative of the species type strain, ATCC27088 (here designated APP-1A) that is resistant to nalidixic acid (NAI), resistant to kanamycin (Kan), and that requires riboflavin (Rib) because it contains a riboflavin-biosynthetic operon that has been mutated by deletion-disruption with a kanamycin resistance cassette. Bacteria for the live vaccine were grown in heart infusion broth containing 10 µg/ml NAD (nicotinic adenine dinucleotide)+5 mM CaCl₂+200 µg/ml riboflavin, at 37 C, to an optical density at 520 nm of 0.8. Bacteria were harvested, washed once in phosphate buffered saline (PBS), pH 7.0, diluted in phosphate buffered saline (PBS) or PBS containing 5 µg/ml riboflavin to the appropriate cell density, and used immediately as vaccine.

2. Bacterin: Virulent APP-1A bacteria were grown in heart infusion broth containing 10 µg/ml NAD (nicotinic adenine dinucleotide)+5 mM CaCl₂ at 37 C, shaken at 160 rpm, 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 5×10⁴ 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 Eumuligen adjuvant (MVP Laboratories, Ralston, Nebr.).

These were six treatment groups (six pigs/group) in this study. Pigs were blocked by starting weight and randomly assigned to treatment groups. The animals were vaccinated twice at a 3 week interval, and challenged with virulent APP serotype 1A (APP-1) two weeks after the second vaccination. Group 1 received 5×10⁹ cfu (100x the 50% lethal dose previously established for the wild type parent strain [WT LD₅₀]) of live AP233, our APP-1 riboflavin-requiring mutant, in 10 ml of sterile PBS, by percutaneous tracheal inoculation, as in our challenge model (described below). Group 2 received the same treatment as Group 1, except the bacteria were suspended in 10 ml of PBS containing 5 µg/ml riboflavin, a concentration of exogenous riboflavin sufficient to permit 2–3 generations of growth. Group 3 received 5×10⁹ cfu of live AP233, intramuscularly in 2 ml PBS. Group 4 received the same treatment as Group 3, except the bacteria were suspended in PBS plus 5 µg/ml riboflavin. Group 5 received a formalized whole cell bacterin prepared from APP-1, which contained the equivalent of 5×10⁹ cfu per dose, in 2 ml of 25% Eumuligen adjuvant (MVP Laboratories, Ralston, Nebr.). Group 6 were unvaccinated controls.

Experimental challenge. Two weeks after the second vaccination, all groups of pigs were challenged with virulent wild type APP-1A, using an experimental challenge model (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; Thacker, B. J., M. H. Mulks, B. Yamini, & J. Krehb. 1988. Clinical, immunological, hemato logical, microbiological, and pathological evaluation of a percutaneous intratracheal injection *Haemophilus pleuropneumoniae* challenge model. Proc. Int. Pig Vet. Soc. 10: 69). For the challenge inoculum, bacteria were grown to late exponential phase in heart infusion broth containing 10 µg/ml NAD 5 mM CaCl₂, washed once in sterile saline, and diluted in saline to the appropriate cell density. Pigs were anesthetized by intravenous injection with a mixture of ketamine (6.6 mg/kg) and xylazine (1.65 mg/kg) and inoculated transtracheally with 1 L.D₅₀ (5×10⁹ cfu) of APP-1 suspended in 10 ml saline. Clinical signs, including increased rectal temperature, increased respiration rate, dyspnea, decreased appetite, and depression, were monitored at 4 hour intervals for the first 24 hours post infection, and at 12 hour intervals thereafter. Severely ill animals, as determined by the severity of clinical signs, were euthanized by overdose with a pentobarbital solution (Brevital) delivered intravenously and necropsied immediately. Three days post-infection, all surviving pigs were euthanized and necropsied, and gross pathology of the lungs examined and compared. Lungs were examined macroscopically for APP lesions, including edema, congestion, hemorrhage, infarction, necrosis, abscess, fibrosis, and pleuritis. The percentage of lung tissue and surface area affected was estimated for each of the seven lung lobes, and the data inserted into a formula that weights the contribution of each lung lobe to give a total percentage of lung involvement and affected pleural surface (Thacker, B. J., M. H. Mulks, B. Yamini, & J. Krehb. 1988. Clinical, immunological, hemato logical, microbiological, and pathological evaluation of a percutaneous intratracheal injection *Haemophilus pleuropneumoniae* challenge model. Proc. Int. Pig Vet. Soc. 10: 69). Tissue samples were collected and processed for histopathology, and for culture of APP to confirm infection. Protection of pigs 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. Statistical analysis of the data was conducted using the Statistix microcomputer program (Analytical Software, Tallahassee, Fla.) for analysis of variance (ANOVA) and Epistat (T. L. Gustafson, Round Rock, Tex.) for nonparametric analyses.

Results

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 Group 3, 4, and 5 animals. The bacterin vaccinated animals (Group 5) showed mild fever, depression, and decrease in appetite for 8–16 hours post-vaccination, which is a common reaction to bacterin vaccines. Several of the Group 5 animals had granulomatous reactions at the injection site in the neck muscle, which were detected at necropsy. The Group 1 and 2 animals, which received intratracheal immunizations, showed increased respiratory rates, fever, decreased appetite, and mild depression for 8–16 hours post-immunization. The Group 3 and 4 animals, which received the intramuscular vaccine, showed only slight depression and decreased appetite for <8 hours, and no significant fever or increase in respiratory rate. No injection site reactions were detected in the Group 3 or 4 animals at necropsy. These results demonstrate that the live intramuscular vaccine is at least as safe as, if not safer than, a formalized bacterin of the type routinely used commercially at this time.


At challenge, the bacterin-vaccinated animals showed significant ELISA and complement fixation titers, but low or negative hemolysis neutralization titers. The four groups receiving live vaccines showed low or negative ELISA and CF titers. However, the Group 3 and 4 animals did show significant hemolysis neutralization titers.

| Table 6 | Serologic analysis of serum samples collected at challenge. |
|-----------------|-----------------|-----------------|-----------------|
| Group # Vaccine | ELISA-APP1 | CF2 |           |
| 1 Live, I, PBS   | 3129 ± 1478a   | 227 ± 90a      | 1.7 ± 2.6b     |
| 2 Live, I, PBS + riboflavin | 2520 ± 741b | 164 ± 72b      | 1.3 ± 3.1c     |
| 3 Live, IM PBS   | 10760 ± 6245b | 120 ± 20bd     | 0.0 ± 0.0d     |
| 4 Live, IM PBS + riboflavin | 6293 ± 2626bc | 230 ± 173b     | 2.0 ± 4.0bc    |
| 5 APP-IA bacterin | 3035 ± 285b   | 1119 ± 170b    | 24.3 ± 7.4b    |
| 6 Unvaccinated control | 2240 ± 243b | 67 ± 21b      | 0.7 ± 0.7b     |

1 Hemolysis neutralization titer; <3000 = negative; 3000–6000 = suspect; >6000 = positive. Assays performed in the laboratory of Dr. Brad Pennik, Kansas State University.
2 ELISA vs APP-1 outer membranes; <200 = negative; 200–500 = suspect; >500 = positive. Assays performed in the laboratory of Dr. Martha H. McNeil, Michigan State University.
3 Complement fixation test; reported as geometric mean titer 0 = negative; >0 = positive. Assays performed at the Veterinary Diagnostic Laboratory, Iowa State University.

Addition of riboflavin to the 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. Poor persistence of live vaccine strains in vivo can lead to a failure to elicit a protective immune response. *A. pleuropneumoniae* and other related pathogens can produce infection-associated antigens when grown in an appropriate host. These are antigens that are only produced by the bacterium when it is grown within a host animal, presumably due to specific environmental stimuli such as temperature, lack of available iron, pH, or osmotic conditions (Mekalanos, J. J. 1992. Environmental signals controlling expression of virulence determinants in bacteria. Infect. Immun. 174:1–7). Such infection-associated antigens are not produced when the bacterium is grown in vitro in standard laboratory media. In order to assure that such infection-associated antigens would be expressed by the live attenuated vaccine strain of bacteria after immunization of pigs, it was necessary to ensure that the bacteria had sufficient available riboflavin to permit 2–3 generations of growth. It was determined that addition of 5–10 μg of riboflavin per ml of the vaccine inoculum was sufficient to permit this amount of growth. Therefore, as part of this vaccine trial, intratracheal (IT) and intramuscular (IM) administration of the live attenuated vaccine, with and without the addition of 5 μg/ml exogenous riboflavin, were compared.

Riboflavin may be added to permit two generations of growth such that the amount may vary from about 1 to about 10 μg/ml.

Protection against challenge. In this experiment, the live attenuated vaccine prepared with exogenous riboflavin and delivered intramuscularly (Group 4) provided complete protection against mortality (0/5 animals died) and a significant reduction in lung damage and in some clinical signs of pleuropneumonia (Tables 2 and 3). In contrast, 6/6 unvaccinated control animals died from overwhelming pleuropneumonia as a result of this experimental challenge. Other live vaccine formulations, as well as the formalinized bacterin, afforded less protection than the intramuscular immunization containing riboflavin. It is concluded that 1) intramuscular immunization with this live vaccine does elicit significant protection against APP infection; 2) that intratracheal immunization does not elicit the same degree of protection; and 3) that the addition of exogenous riboflavin improves the efficacy of the live vaccine.

Lung cultures. APP was cultured from the lungs of all the challenged pigs except for 1 animal in Group 4. All cultures were confirmed as APP-IA by gram stain, requirement for NAD, and coagglutination.

| Table 7 | Mortality and Lung Score Data |
|-----------------|-----------------|-----------------|-----------------|
| Group # Vaccine | Mortality | % Pneumonia | % Pleuritis |
| 1 Live, I, PBS   | 3/5 | 58.6 ± 23.5% | 73.3 ± 36.3% |
| 2 Live, I, PBS + riboflavin | 6/6 | 63.2 ± 8.2% | 66.7 ± 51.6% |
| 3 Live, IM PBS   | 4/6 | 57.7 ± 23.2% | 73.3 ± 42.5% |
| 4 Live, IM PBS + riboflavin | 8/8 | 24.5 ± 18.0% | 21.5 ± 20.7% |
| 5 APP-IA bacterin | 3/5 | 54.3 ± 24.0% | 73.9 ± 41.2% |
| 6 Unvaccinated control | 6/6 | 80.9 ± 13.2% | 83.3 ± 40.8% |
### TABLE 7-continued

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<th>Group</th>
<th>Vaccine</th>
<th>Mortality</th>
<th>% Pneumonia</th>
<th>% Pleuritis</th>
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<td>1.40 ± 5.5</td>
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<td>2 Live, IT, PBS + riboflavin</td>
<td>19.7 ± 5.7</td>
<td>2.17 ± 4.1</td>
<td>1.67 ± 3.2</td>
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<td>3 Live, IM, PBS</td>
<td>19.2 ± 1.2</td>
<td>1.83 ± 4.1</td>
<td>1.27 ± 3.5</td>
<td>2.35 ± 1.2</td>
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<tr>
<td>4 Live, IM, PBS + riboflavin</td>
<td>18.5 ± 3.4</td>
<td>1.50 ± 7.5</td>
<td>0.40 ± 8.9</td>
<td>0.20 ± 4.8</td>
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<tr>
<td>5 APP-1A bacterin</td>
<td>23.3 ± 6.8</td>
<td>1.83 ± 5.2</td>
<td>1.83 ± 7.5</td>
<td>1.67 ± 1.0</td>
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<td>23.0 ± 5.8</td>
<td>2.33 ± 5.2</td>
<td>1.83 ± 7.5</td>
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**Note:** Values with different superscripts among the six vaccine groups were significantly different (p < 0.05) by Least Significant Difference (LSD) analysis.

### TABLE 8

<table>
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<tr>
<th>Group</th>
<th>Vaccine</th>
<th>RR Max</th>
<th>Temp Max</th>
<th>Dypnea</th>
<th>Depression</th>
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<td>104.7</td>
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<td>2.00 ± 7.1</td>
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<td>2 Live, IT, PBS + riboflavin</td>
<td>19.7 ± 5.7</td>
<td>104.0</td>
<td>104.0</td>
<td>2.17 ± 4.1</td>
<td>1.67 ± 3.2</td>
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<td>3 Live, IM, PBS</td>
<td>19.2 ± 1.2</td>
<td>104.7</td>
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<td>1.83 ± 4.1</td>
<td>1.27 ± 3.5</td>
<td>2.35 ± 1.2</td>
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<tr>
<td>4 Live, IM, PBS + riboflavin</td>
<td>18.5 ± 3.4</td>
<td>104.2</td>
<td>104.2</td>
<td>1.50 ± 7.5</td>
<td>0.40 ± 8.9</td>
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<td>5 APP-1A bacterin</td>
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<td>6 Unvaccinated control</td>
<td>23.0 ± 5.8</td>
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<td>1.83 ± 7.5</td>
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</table>

**Note:** Values with different superscripts among the six vaccine groups were significantly different (p < 0.05) by Least Significant Difference (LSD) analysis.

It is concluded that intramuscular vaccination with the live attenuated riboflavin-requiring *A. pleuropneumoniae* mutant, with the addition of a limited amount of exogenous riboflavin, led to complete protection against mortality and to significant reduction in lung damage and clinical signs of pleuropneumonia.

The applicants have determined that other related species of pathogenic bacteria in the Family Pasteurellaceae contain homologous genes encoding riboflavin biosynthetic enzymes. Southern blot analysis of genomic DNA from *Actinobacillus suis* and *Pasteurella haemolytica* demonstrated that these species contain DNA fragments that are highly homologous to the rib genes from *A. pleuropneumoniae*. Genomic DNA from these species was digested with the restriction endonucleases EcoRI and HindIII, fragments separated on an agarose gel, and the fragments transferred to nitrocellulose. The nitrocellulose blot was probed with a digoxigenin-labelled probe prepared from the ribGBAH operon from APP serotype 5, at 42°C, in a hybridization cocktail that included 50% formamide, 5xSSC (20xSSC contains 3 M NaCl and 0.3 M sodium citrate, pH 7.0), 0.1% N-laurylsarcosine, and 0.02% SDS. The blot was washed under high stringency conditions, including two 15 minute washes at room temperature in 2xSSC, 0.1% SDS, followed by two 30 minute washes at 68°C in 0.1xSSC, 0.1% SDS. Phosphorylase a contains an ~12 kb DNA HindIII fragment that hybridized with the rib probe, while A. suis contains three EcoRI fragments of ~4.4, 2.5, and 1.0 kb that are highly homologous to the APP-5 rib probe. These data suggest that these species of bacteria contain riboflavin operons that are similar to that analyzed from APP serotype 5.

A live avirulent vaccine against APP is desirable. There are a variety of different kinds of vaccines produced to elicit protection against bacterial diseases. Some of the most effective are purified toxins converted to toxoids. These toxoid vaccines are often very safe, and can be very effective against diseases where a toxin is the major virulence factor. Examples would be current vaccines against tetanus and diphtheria. These vaccines do not prevent acquisition and carriage of the causative organism, e.g., *Clostridium tetani*, the causative agent of tetanus, or *Corynebacterium diphtheriae*, the agent of diphtheria. Rather, they prevent the deleterious effects of the toxin by eliciting antibodies that neutralize the toxin in other cases where a key virulence factor has been identified, purified protein or polysaccharide vaccines have been produced. Examples here would be the *E. coli* pilin vaccine against porcine colibacillosis and the capsular polysaccharide vaccines now available against *Haemophilus influenzae* B, *Streptococcus pneumoniae*, and some serotypes of *Neisseria meningitidis*. These vaccines either prevent initial adherence of the pathogen, as in the case of the pilin vaccine, or enhance phagocytosis and clearance of the pathogen, as in the case of the pilin vaccine, or enhance phagocytosis and clearance of the pathogen, as in the case of the pilin vaccine, or enhance phagocytosis and clearance of the pathogen, as in the case of the pilin vaccine.
problem with all of these types of vaccines is that they generally induce systemic humoral immunity, i.e., serum antibodies. It is difficult to induce local secretory immunity with these types of vaccines. Live avirulent vaccines, where the recipient of the vaccine receives a dose of infectious but non-virulent bacteria, can be an improvement over purified subunit or killed whole cell vaccines, for several reasons. First, the vaccine dose can often be administered to the same region of the body that is normally infected by the pathogen, e.g., orally for a gastrointestinal pathogen or as a nasal spray for a respiratory pathogen. This can elicit local secretory immunity as well as systemic humoral immunity. Second, live avirulent vaccines can often be administered as a single dose rather than multiple doses, because the organism can continue to grow and replicate within the host, providing a longer term exposure to important antigens that a single dose of killed vaccine. Finally, live avirulent vaccines may provide exposure to important bacterial antigens not contained in killed vaccines grown in the laboratory. For example, if a bacteria produces important antigens or virulence factors whose expression is induced by in vivo environmental signals, these antigens may not be contained in a vaccine prepared from bacteria grown in vitro in laboratory media. It is desirable for a vaccine to elicit cross-protective immunity against the different serotypes of APP. It is known that vaccination with a killed whole cell vaccine prepared from a single serotype of APP will usually not elicit cross-protective immunity against other serotypes. However, infection with a virulent strain of APP will generally elicit at least some degree of cross-protection against other serotypes. One explanation for this phenomenon is that antigens may be expressed by APP during growth in vivo that elicit a cross-protective immune response, and that these antigens are not contained in most bacteria vaccines.

Specifically, it has been shown that extracellular toxins, referred to as hemolysins/cytolysins, are produced by APP in vivo but are not produced under the culture conditions typically used for producing killed whole cell vaccines. The applicants have shown that riboflavin-requiring mutants of APP can be effective as a live avirulent vaccine. There are two basic methods or producing live avirulent vaccine strains. One is to knock out a critical virulence factor necessary for survival in vivo and perhaps also for disease/damage to the host. An example would be Inzana’s non-capsulated APP mutants. These mutants are unable to synthesize capsular polysaccharide, which acts in vivo to protect the bacterium from phagocytosis and clearance by alveolar macrophages. Non-capsulated mutants simply can not survive long enough in vivo to cause disease. They do, however, presumably express all the other important virulence factors and therefore should elicit an immune response against antigens other than capsular polysaccharide.

A second method to produce live avirulent vaccines is to knock out genes in biosynthetic pathways known to be critical for survival in vivo. For example, the availability of compounds such as purines and aromatic amino acids is limited in mammalian hosts. Bacterial pathogens must be able to synthesize these compound themselves, or scavenge them from host tissues. Mutations in the biosynthetic pathways for purines and aromatic amino acids have been used to construct bacterial mutants that can not survive long in vivo, and thus have potential for use as attenuated vaccines. Much of the current research on genetically engineered live avirulent vaccines has been done with members of the genus Salmonella. These studies show that parA mutants are avirulent but poorly immunogenic (O’Callaghan et al., 1988), while mutations in the chorismate pathway, including aroA, aroC, and aroD, are attenuated and can be effective as live oral vaccines (Doggett & Curtiss, 1992; Tacket et al., 1992). In addition, Salmonella strains carrying cya and crp mutations, which produce mutants that lack the enzyme adenylate cyclase and the cyclic AMP receptor protein, which are required for the expression of numerous critical genes in bacteria, have been shown to be both avirulent and immunogenic (Doggett & Curtiss, 1992; Tacket et al., 1992; Kelly et al., 1992).

Riboflavin is an essential vitamin and biosynthetic precursor for the coenzymes FMN and FAD. It is synthesized by most bacteria, but not by mammals. Therefore, it is expected that riboflavin would be in limited supply in a mammalian host and that a bacterium incapable of synthesizing its own riboflavin would be attenuated. This has been shown above. It has also been shown above that Rib-mutants can survive long enough in the host to be immunogenic and effective as a live avirulent vaccine.

The rib-APP mutant may be combined with a sterile, buffered, isotonic, pharmaceutically-acceptable and compatible aqueous carrier such as saline, or saline derivative such as citrate-buffered saline, tris-buffered saline, Ringer’s Solution or tissue culture medium, and the like, preferably having a physiologic pH. An antigen composition may also include a suitable compatible adjuvant such as aluminum hydroxide, parafin-based oils, averdine, muramyl dipeptide, and the like, to stabilize the antigen in solution, and/or an immunomodulator such as a recombinant cytokine or interleukin such as IL-1, IL-5, IL-6, TGF-beta, or gamma interferon, and the like, to enhance the IgA antibody response. However, the adjuvant chosen should not contain any preservative, such as formalin, that would be deleterious to a live vaccine. In the experiments described above, no adjuvant was used.

The vaccine composition may be formulated for administration as a single injection of about 0.5 to 10 ml. The composition may also be in the form for administration in a series of biweekly or monthly injections of about 0.5 to 10 ml each, until the desired level of immunity is achieved. Preferably, the composition is formulated for a single administration to the animal.

The vaccine composition as described herein may be formulated with a pharmaceutically-acceptable biodegradable encapsult material for administration by transcutaneous subcutaneous, intramuscular injection. The vaccine may also be supplied orally or intranasally. These vehicles comprise substances that are essentially nontoxic and nontherapeutic such as saline and derivatives of saline such as citrate-buffered saline, tris-buffered saline and Ringer’s Solution, dextrose solution, Hank’s Solution, tissue culture medium, and the like. The antigen composition may also include minor but effective amounts of pharmaceutically-accepted adjuvants, buffers and preservatives to maintain isotonicity, physiological pH, and stability. Adjuvants useful in the composition include, but are not limited to, for example, parafin based oils, averdine, muramyl dipeptide, and oil-in-water-based adjuvants, and the like. Examples of suitable buffers include but not limited to, phosphate buffers, citrate buffers, carbonate buffers, TRIS buffers, and the like. It is also envisioned that the antigen may be combined with a biocompatible, and optimally synergistic, immunomodulator that cooperatively stimulates IgA Antibody production, as for example, but not limited to, recombinant cytokines such as TGF-beta, interferons, activating factors, chemotactants, interleukins such as IL-1, IL-2, IL-4, IL-5, IL-6 and the like, and other like substances.
While the forms of the invention herein disclosed constitute presently preferred embodiments, many others are possible. It is not intended here to mention all the possible equivalent forms or ramifications of the invention. It is understood that the terms used herein are merely descriptive, and that various changes may be made without departing from the spirit or scope of the invention.

REFERENCES


SEQUENCE LISTING

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<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide primer for DNA sequencing rib

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Ala His Ser Phe Val Glu Val Ile Ser Gly Lys His Val Ala Leu
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475 480 485

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Phe
550

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Glu Gly Asp Phe Ile Cys Ala Ala Glu Phe Ala Thr Pro Glu Asn Ile
35 40 45

Asn Phe Met Ala Thr Tyr Gly Lys Gly Leu Ile Cys Thr Pro Ile Ser
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Thr Glu Ile Ala Lys Leu Asn Phe His Pro Met Val Ala Val Asn
65 70 75 80

Gln Asp Asn His Glu Thr Ala Phe Thr Val Ser Val Asp His Ile Asp
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100
Thr Gly Thr Gly Ile Ser Ala Phe Glu Arg Ser Ile Thr Ala Met Lys
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Ile Val Asp Asp Asn Ala Lys Ala Thr Asp Phe Arg Arg Pro Gly His
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His Thr Glu Ala Thr Val Asp Leu Ala Arg Ala Gly Leu Lys His
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Ala Gly Leu Cys Cys Glu Ile Met Ala Asp Asp Gly Thr Met Met Thr
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Met Pro Asp Leu Gln Phe Ala Val Glu His Asn Met Pro Phe Ile
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Lys Gin Gly Phe Ala Val Val Thr Ala Thr Lys Val Leu
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cag gcc gtt cat tta ggc gac acc att gcg gtc aac gta gta ttt tta gln asp val his leu gly asp thr ile ala val asp val gly val cys leu
35 40 45
acc gta act tct ttt tcc aat cag ctt acc ggc gat gta aag tgg thr val thr ser phe ser ser asn phe thr ala asp val met ser
50 55 60
qaa acq cta aat cgt act tca tta ggc gaa tta aag tcc aat cag glu thr leu lys arg thr ser leu gly glu leu lys ser asn ser pro
65 70 75 80
gtt aat tta gaa cgc gac atg cgc gaa aac gaa ctt ggc gaa cac val asp leu glu arg ala met ala ala asn gly phe gly gly his
85 90 95
atc gtt tcc ggg cat att gac ggc acc ggc gaa att ggc gaa asc aca ile val ser gly his ile asp gly thr gly ile ala glu ile thr
100 105 110
cog gca cat atg cca tgg tca cgc att aas acc tct cca aas tta pro ala his asn ser thr trp tyr arg ile lys thr ser pro lys leu
115 120 125
atg ctt cat att att gag aas gtt tcc atc acc att gag ggt att aac arg tyr ile ile lys gly ser ile ile asp gly ile ile ser
130 135 140
ctg acc gta gtc gat acc gat gaa aat tgg tca cta gta cta cta gta leu thr val val asp thr asp gly ser phe arg val ser ile ile
145 150 155 160
cog cat acc aat aas gag acc gct tta gat cga aas aas aas aas asc gac pro his thr ile lys thr asp leu glu ser lys ile gly ser
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thr val thr ser phe ser ser asn glu phe thr ala asp val met ser
50 55 60
val glu thr leu lys arg thr ser leu gly glu leu lys ser asn ser pro
65 70 75 80
val asp leu glu arg ala met ala alan gly arg phe gly gly his
85 90 95
ile val ser gly his ile asp gly thr gly glu ile ala glu ile thr
100 105 110
pro ala his asn ser thr trp tyr arg ile lys thr ser pro lys leu
**US 6,410,021 B1**

---continued---

115  120  125

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Leu Thr Val Val Asp Thr Asp Glu Ser Phe Arg Val Ser Ile Ile

Pro His Thr Ile Lys Glu Thr Asn Leu Gly Ser Lys Lys Ile Gly Ser

Ile Val Asn Leu Glu Asn Ile Val Gly Lys Tyr Ile Glu Gin Phe

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Phe Leu Lys Gin Ala Gly Phe

210  215

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Leu Arg Ser Lys Asp Phe Pro Leu Val Asn Leu Met

20  25  30

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Pro Val Met Cys Phe Pro Leu Pro Ser Asn Ser Phe Lys Thr Met Thr

35  40  45

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Asp Leu Asp Tyr Met Arg Arg Ala Ile Ala Leu Lys Gin Gly Leu

50  55  60

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Gly Trp Thr Asn Pro Asn Leu Val Gly Cys Val Ile Val Lys Asn

65  70  75  80

gtt gaa ctc gtt gcc gaa gtt tac cag aag att gtt gga tgg cag

Gly Ile Val Ala Glu Gly Tyr His Glu Lys Ile Gly Gly Trp His

85  90  95

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Ala Glu Asp Arg Asn Val Leu His Cys Lys Gly Asp Leu Ser Gly Ala

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act gct tat gta aag ctt gac cgt tgt gcc ctc gac gcc cgg cag cgc

Thr Ala Tyr Val Thr Leu Glu Pro Cys His His Gly Arg Thr Pro

115 120 125

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Pro Cys Asp Ser Leu Val Glu Arg Gly Ile Lys Lys Val Phe Ile

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Gly Ser Ser Asp Pro Asn Pro Leu Val Ala Gly Arg Gly Asn Gin

145 150 155 160

cga ccc gac cgc ggt gaa gtg gaa gat gtt ctc ctc aca gaa

Leu Arg Gin Ala Gly Val Val Gly Leu Val Lys Gly Leu

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Cys Asp Ala Leu Aas Pro Ile Phe Phe His Tyr Ile Gin Thr Lys Arg

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576
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acc ggt agc ggc gaa tcc aaa tgg att acc ggt gas tgg cga aga gca 210 215 220

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Arg Val Gin Gin Thr Arg His Gin Tyr Ser Ala Ile Met Val Gin Val 225 230 235 240

qat acq gta ctt gcc gat acc ccg atg tta aat acc cga atg ccg aat 760

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Ala Lys Gin Pro Val Arg Ile Val Cys Asp Ser Gin Leu Arg Thr Pro 915 920 925

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Leu Asp Cys Gin Leu Val Gin Thr Ala Lys Gin Thr Arg Thr Val Ile 965 970 975

gas acc gat ggt gag gat tgg csa aas att gag csa tgg tta aat aca ccg 990

Thr Val Ser Ser Leu Ser Gin Aan Gin Leu Pro Gin Phe Arg Pro Leu 995 1000 1005

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Gly Val Asp Val Val Cys Lys Ala Arg Aan Lys Arg Val Asp Leu 1065 1070 1075

csa gat ctt tgg csa aag ctc ggt gag aag cag atc gcc gcc ctc tta 1100

Gln Asp Leu Leu Gin Lys Leu Gly Met Gin Ile Asp Ser Gin Leu Leu 1105 1110 1115

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Pro Val Met Cys Phe Pro Leu Pro Ser Aan Ser Phe Lys Thr Met Thr 35 40 45

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What is claimed is:

1. A live vaccine against members of the family of Pasteurellaceae comprising a recombinant mutant of a member of the family of Pasteurellaceae lacking a rib gene necessary for the production of riboflavin in a pharmaceutically acceptable carrier.

2. The vaccine of claim 1 wherein said members of the family of Pasteurellaceae are selected from the group consisting of Pasteurella multocida, Pasteurella haemolytica, Actinobacillus suis, Haemophilus parasuis, Haemophilus influenzae, and Haemophilus ducreyi.

3. The vaccine of claim 1 wherein said rib gene is selected from the group consisting of rib G, rib A, rib B, and rib H.

4. A method of vaccinating a mammal in need thereof comprising administering to the mammal an effective vaccinating amount of a live vaccine comprising a recombinant mutant of a member of the family of Pasteurellaceae lacking a rib gene necessary for the production of riboflavin in a pharmaceutically acceptable carrier.

5. A method of stimulating the immune system of a mammal in need thereof comprising the steps of:

(a) providing a recombinant Pasteurellaceae mutant having an inactivating mutation in one or more rib genes necessary for the production of riboflavin; and

(b) administering an effective immunogenic amount of the recombinant Pasteurellaceae mutant in a pharmaceutically acceptable carrier to a mammal in need thereof, thereby causing an antigenic response thereto, which stimulates the immune system in the mammal.

6. A method of inducing protective immunity in a mammal in need thereof against disease caused by Family Pasteurellaceae comprising the step of administering to the mammal an effective amount of a recombinant Pasteurellaceae mutant having an inactivating mutation in one or more rib genes necessary for the production of riboflavin in a pharmaceutically acceptable carrier such that the mutant causes protective immunity in the mammal against Pasteurellaceae.

7. The vaccine of claim 1 wherein the pharmaceutically acceptable carrier further comprises riboflavin.

8. The method of claim 4 wherein the pharmaceutically acceptable carrier further comprises riboflavin.

9. The method of claim 5 wherein the pharmaceutically acceptable carrier further comprises riboflavin.

10. The method of claim 5 or 6 wherein the rib genes are selected from the group consisting of rib G, rib A, rib B, and rib H.

11. The method of claim 4 wherein said rib gene is selected from the group consisting of rib G, rib A, rib B, and rib H.

* * * * *
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

**Title page.**
Insert Item [73] as follows: -- [73] Assignees: Michigan State University, East Lansing, Michigan; and Iowa State University Research Foundation, Inc., Ames Iowa --

**Column 2.**
Line 2, “mutagenesis” should be -- mutagenesis --.

**Column 3.**
Line 25, “gale derivatives” should be -- gale derivatives --.

**Column 4.**
Line 1, “Quantities” should be -- quantities --.

**Column 5.**
Line 41, “shows” after “show” and before “the” should be deleted.

**Column 6.**
Line 55, “may be” should be -- may be --.

**Column 7.**
Line 55, “(BSV3) mutants” should be -- (BSV13) mutants --.
Line 64, “according Lo” should be -- according to --.

**Column 8.**
Line 28, “(5’GGC-C-AC-AGC)” should be -- (5’-GGC-GAC-ACG) --.

**Column 12.**
Table 2, line 47, (in the heading), “either wide” should be -- either wild --.

**Column 14.**
Line 25, “APP225 and APP33” should be -- AP225 and AP233 --.
Line 32, “Laemmli” should be -- Laemmli --.
Line 56, “colorimetric” should be -- colorimetric --.

**Column 17.**
Table 3, line 62, “(801acZ)” should be -- (Φ801acZ) --.
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 18,
Table 3, line 33, “AP106 ribGBAH” should be -- AP106 ribBAH --.
Table 4, line 63, “wild-type APP 225-serotype” should be -- wild type AP 225-serotype --.

Column 19,
Table 5, line 13, “APP225-serotype” should be -- AP225-serotype --.
Line 30, “ribGBAH operon” should be -- ribBAH operon --.

Column 21,
Line 22, “APP-LA bacteria” should be -- APP-1A bacteria --.

Column 24,
Line 3, “addition or” should be -- addition of --.
Line 37, “From overwhelming” should be -- from overwhelming --.
Line 49, “except: for 1 animal” should be -- except for 1 animal --.
Line 51, “APP-LA” should be -- APP-1A --.

Column 26,
Line 54, “toxin, n other” should be -- toxin, In other --.
Lines 63-64, “case of the pilin vaccine, or enhance In the” should be -- case of the capsular polysaccharide vaccines. In the vet --.

Column 27,
Line 16, “that a” should be -- than a --.
Line 19, “virulence actors” should be -- virulence factors --.
Line 47, “phagocytosis” should be -- phagocytosis --.

Signed and Sealed this
Twenty-fifth Day of March, 2003

JAMES E. ROGAN
Director of the United States Patent and Trademark Office