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A Riboflavin Auxotroph of *Actinobacillus pleuropneumoniae* Is Attenuated in Swine

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

Actinobacillus pleuropneumoniae, a gram-negative coccobacillus belonging to the family *Pasteurellaceae*, is the causative agent of porcine pleuropneumonia (4, 20, 32). This disease is characteristically an acute necrotizing hemorrhagic bronchopneumonia, with accompanying fibrinous pleuritis (4, 32). Pleuropneumonia is an economically devastating, severe, and often fatal disease with clinical courses ranging from hyperacute to chronic infection (4, 9). The existence of at least 12 antigenically distinct capsular serotypes (28) has made development of a cross-protective vaccine difficult. Killed whole-cell bacterins provide at best serotype-specific protection (22, 24, 29, 37). In contrast, natural or experimental infection with a highly virulent serotype of *A. pleuropneumoniae* elicits protection against reinfection with any serotype (21, 23, 24). In several recent studies, attenuated strains of *A. pleuropneumoniae* produced by chemical mutagenesis, serial passage, or other undefined spontaneous mutation have been tested as live vaccines, with promising results (10, 27, 38). However, the use of live vaccines in the field is problematic, particularly when the attenuating lesion in the vaccine strain has not been genetically defined. A well-defined mutation that prevents reversion to wild-type would be extremely desirable for the development of a live attenuated vaccine against pleuropneumonia. We hypothesize that a mutation in a critical biosynthetic pathway which limits growth in vivo but does not otherwise alter the expression of important antigens such as capsular polysaccharide, lipopolysaccharide (LPS), and extracellular toxins could produce an attenuated vaccine strain capable of inducing cross-protective immunity against *A. pleuropneumoniae.*

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

Riboflavin (vitamin B_2), a precursor of the coenzymes flavin adenine dinucleotide and flavin mononucleotide, is essential for basic metabolism. It is synthesized by plants and by most microorganisms but not by higher animals (2). Many pathogenic bacteria are apparently unable to utilize flavins from their environment and are entirely dependent on endogenous production of riboflavin (31). Even with the ability to utilize exogenous riboflavin, there may not be enough of the vitamin present in mammalian host tissues to permit growth, particularly not in sites devoid of normal bacterial flora. Therefore, we hypothesized that riboflavin biosynthesis would be essential for survival of *A. pleuropneumoniae* in vivo and that mutations in the riboflavin biosynthetic pathway would be attenuating because of the scarcity of riboflavin present on the mucosal surfaces of the respiratory tract.

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

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TABLE 1. Characteristics of bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source (reference)
Strain		
E. coli DH5-α	*supE44 ΔlacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	BRL[a]
E. coli DH5-α (λ*pir*)	λ*pir supE44 ΔlacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Mulks and Buysse (19)
E. coli S17-1 (λ*pir*)	λ*pir recA thi pro hsd* (r_K^- m_K^+) RP4-2-Tc::Mu Km::Tn*7* Tmp^r Sm^r	Simon et al. (35)
AP100	*A. pleuropneumoniae* ATCC 27088 serotype 1, passaged through pigs	ATCC[b]
AP106	*A. pleuropneumoniae* ISU178, a serotype 5 field isolate, passaged through pigs	Iowa State University
AP225	A spontaneous nalidixic acid-resistant mutant of AP100	This work
AP233	A double-crossover riboflavin auxotroph of AP225	This work
AP234	A single-crossover riboflavin auxotroph of AP225	This work
Plasmid		
pUC19	Ap^r cloning vector	Vieira and Messing (40)
pUC4K	Ap^r Km^r vector, source of the *kan* cassette	Pharmacia[c]
pGP704	Ap^r broad-host-range suicide vector	Miller and Mekalanos (18)
pGZRS19	Ap^r *A. pleuropneumoniae-E. coli* shuttle vector	West et al. (41)
pTF10	AP106 *ribGBAH* genes cloned into pUC19	Fuller and Mulks (5)
pTF66	2.9-kb fragment containing AP106 *ribBAH* in pGP704	This work
pTF67a	pTF66 with all of *ribA* and part of *ribB* deleted and replaced with the *kan* cassette from pUC4K	This work
pTF76	5.2-kb insert from pTF10 cloned into pGZRS19	This work

[a] BRL, Gibco-BRL, Grand Island, N.Y.
[b] ATCC, American Type Culture Collection.
[c] Pharmacia Biotech, Inc., Piscataway, N.J.

demonstrating that riboflavin auxotrophy can lead to attenuation of a bacterial pathogen in its natural host.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used in this study are listed in Table 1. *A. pleuropneumoniae* strains were cultured at 37°C in either brain heart infusion (BHI), heart infusion (HI), or tryptic soy agar (TSA) (Difco Laboratories, Detroit, Mich.) containing 10 μg of NAD (also called V factor [V]) (Sigma Chemical Company, St. Louis, Mo.) per ml. Riboflavin (Sigma) was added to a final concentration of 200 μg/ml when needed. *E. coli* strains were cultured in Luria-Bertani medium. Ampicillin and kanamycin were added to 100 and 50 μg/ml, respectively, for plasmid selection in *E. coli* strains. For *A. pleuropneumoniae* strains, kanamycin sulfate (50 μg/ml) and nalidixic acid (25 μg/ml) were added as required, except for selections after matings, which were performed with 100 and 50 μg of kanamycin sulfate and nalidixic acid, respectively, per ml.

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

Filter mating targeted mutagenesis. Filter mating between *E. coli* S17-1 (λ*pir*)/pTF67A and AP225 was performed according to the protocol of Mulks and Buysse (19). Briefly, bacterial cultures were grown overnight at 37°C. Equal cell numbers of donor and recipient cultures, as determined by optical density at 520 nm, were added to 5 ml of 10 mM $MgSO_4$, and the bacteria were pelleted by centrifugation. The pellet containing the cell mating mixture, resuspended in 100 μl of 10 mM $MgSO_4$, was plated on a sterile filter on BHI-V-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 of BHI-V broth and plated in 100-μl aliquots on BHI-V 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-V, with observation for inability to grow in the absence of added riboflavin.

Southern analysis of transconjugants. Chromosomal DNA and plasmid controls were digested with the appropriate restriction enzymes, and the DNA fragments were separated on a 0.7% ultrapure agarose gel in Tris-acetate-EDTA (TAE) buffer. Southern blots were performed as described by Sambrook et al. (30). DNA probes were labeled with digoxigenin by random priming with the Genius V (version 3.0) kit from Boehringer Mannheim. Probes included the 5.2-kb insert from pTF10 containing the intact riboflavin operon from AP106 (Rib), the 1.4-kb *Cla*I-*Nde*I fragment deleted from the riboflavin operon in the construction of pTF67a (R.Del.), the 1.2-kb kanamycin cassette from pUC4K (Km), and the intact plasmid pGP704 (pGP704). Hybridization was carried out in 50% formamide at 42°C for 16 h. The blots were washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 15 min at room temperature and then twice in 0.1× SSC–0.1% SDS for 30 min at 65°C. The blots were developed with alkaline phosphatase-conjugated antidigoxigenin and colorimetric substrate (Boehringer Mannheim) according to the manufacturer's instructions.

Phenotypic analysis of mutant strains. Whole-cell lysates and supernatants of AP100, AP225 (Nal^r), and AP233 (Km^r Nal^r Rib^-) were prepared from overnight cultures grown in HI-V–5 mM $CaCl_2$ and appropriate antibiotics. Cells were separated by microcentrifugation and resuspended in SDS polyacrylamide gel electrophoresis (PAGE) sample buffer (16). The culture supernatant was precipitated with an equal volume of 20% trichloroacetic acid and resuspended in SDS-PAGE sample buffer. Cellular polysaccharides, including LPS and capsular polysaccharide, were prepared according to the cell lysis-proteinase K method of Kimura and Hansen (15). All samples were analyzed on a 0.125% SDS–12% acrylamide gel by using a discontinuous buffer system (16). Samples were transferred to nitrocellulose according to standard protocols (30) and probed with serum from a convalescent pig infected with *A. pleuropneumoniae* serotype 1. Antigen-antibody complexes were detected with horseradish peroxidase-conjugated protein A (Boehringer Mannheim) and the colorimetric substrate 4-chloro-naphthol (Bio-Rad, Hercules, Calif.).

The production of serotype-specific capsular polysaccharide was measured by a coagglutination assay using hyperimmune rabbit antisera complexed to *Staphylococcus aureus* whole cells (11).

Electroporation of *A. pleuropneumoniae*. AP233 was grown in 100 ml of BHI-V with riboflavin at 37°C, with shaking at 150 rpm, to an optical density at 520 nm 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 of 15% glycerol and frozen in 50-μl aliquots in 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 (Bio-Rad). Cells were electroporated with a Gene Pulser II (Bio-Rad) with the following settings: voltage, 1.8 kV; resistance, 200 Ω; 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 biosafety level-2 isolation room at the Michigan State University Research Containment Facility. All experimental protocols for animal experiments were reviewed by the Michigan State University All University Committee on Animal Use and Care, and all procedures conformed to university and U.S. Department of Agriculture regulations and guidelines.

For preparation of challenge inocula, bacteria were grown in 30 ml of HI-V–5 mM $CaCl_2$–riboflavin and antibiotics as needed, in 300-ml baffled side arm flasks, at 37°C with shaking at 160 rpm, to an optical density at 520 nm of 0.8. Ten milliliters 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 number of CFU per milliliter. The actual inoculation 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 of body weight) and xylazine (1.65 mg/kg) and inocu-

lated by percutaneous intratracheal injection with the appropriate dose of bacteria suspended in 10 ml of saline. Clinical signs of pleuropneumonia, including increased respiration rate and temperature, dyspnea, loss of appetite, and change in activity or attitude (depression), were monitored and scored as previously described (12). Seriously ill animals, as determined by severe dyspnea and/or depression, were euthanized immediately. Survivors were euthanized 3 days postchallenge. All animals were necropsied, and their lungs were examined macroscopically for signs of *A. pleuropneumoniae* lesions, including edema, congestion, hemorrhage, necrosis, abscess, fibrosis, and pleuritis. The percentage of lung tissue and pleural surface area affected was estimated for each of the seven lung lobes, and the total percentages for pneumonia and pleuritis were calculated by using a formula that weights the contribution of each lung lobe to the total lung volume (12). Representative lung samples were collected for histopathology and for bacterial culture.

RESULTS

Construction of *A. pleuropneumoniae rib* mutants. To construct riboflavin-requiring auxotrophic mutants of *A. pleuropneumoniae*, a suicide plasmid with part of the riboflavin operon deleted and replaced with a kanamycin resistance (Km^r) cassette was designed (Fig. 1). A 2.9-kb *Eco*RI fragment from pTF10 (5) containing the *A. pleuropneumoniae ribBAH* genes was cloned into the *Eco*RI site of the conjugative suicide vector pGP704 (18) to create plasmid pTF66. pTF66 was digested with *Cla*I and *Nde*I to excise the 3′ end of *ribB* and the entire *ribA* gene. After Klenow treatment of the DNA, the 1.2-kb Km^r cassette, excised with *Eco*RI from pUC4K, was blunt-end ligated into the *rib* deletion site to create pTF67a.

pTF67a was transformed into *E. coli* S17-1 (λ*pir*) and mobilized into AP225 (Nal^r) to produce >100 transconjugant colonies demonstrating resistance to both nalidixic acid and kanamycin. Transconjugants were replica plated onto TSA-V and TSA-V-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 (β-NAD).

Southern blot analysis of transconjugants. Two transconjugants were selected for further analysis on the basis of their phenotypes as potential single (AP234)- and double (AP233)-crossover mutants. Southern blot analysis of transconjugant genomic DNA from the two mutants indicated that AP233 and AP234 were indeed double- and single-crossover insertion mutants, respectively (Fig. 2). Predicted band sizes for single- and double-crossover events are shown in Fig. 2A. Genomic DNA from AP233 contained a 2.2-kb *Hin*dIII fragment that hybridized with the riboflavin operon (Rib) probe as well as 1.7- and 1.3-kb fragments that hybridized with both the Rib and Km probes; however, there was no reaction with either pGP704 or the deleted portion of the riboflavin operon (Fig. 2B). This is the pattern of hybridization predicted in transconjugants that replaced the wild-type riboflavin operon with the mutated *rib*::Km^r locus by a double-crossover event (Fig. 2A). In contrast, genomic DNA from AP234 shows the presence of DNA homologous to the fragment deleted from the riboflavin operon (R.Del.), pGP704, and the kanamycin cassette (Fig. 2B). 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. 2A).

Phenotypic analysis of the *A. pleuropneumoniae rib* mutant. Whole-cell lysates, trichloroacetic acid-precipitated culture supernatants, and polysaccharide preparations were analyzed on silver-stained SDS-polyacrylamide gels and on immunoblots

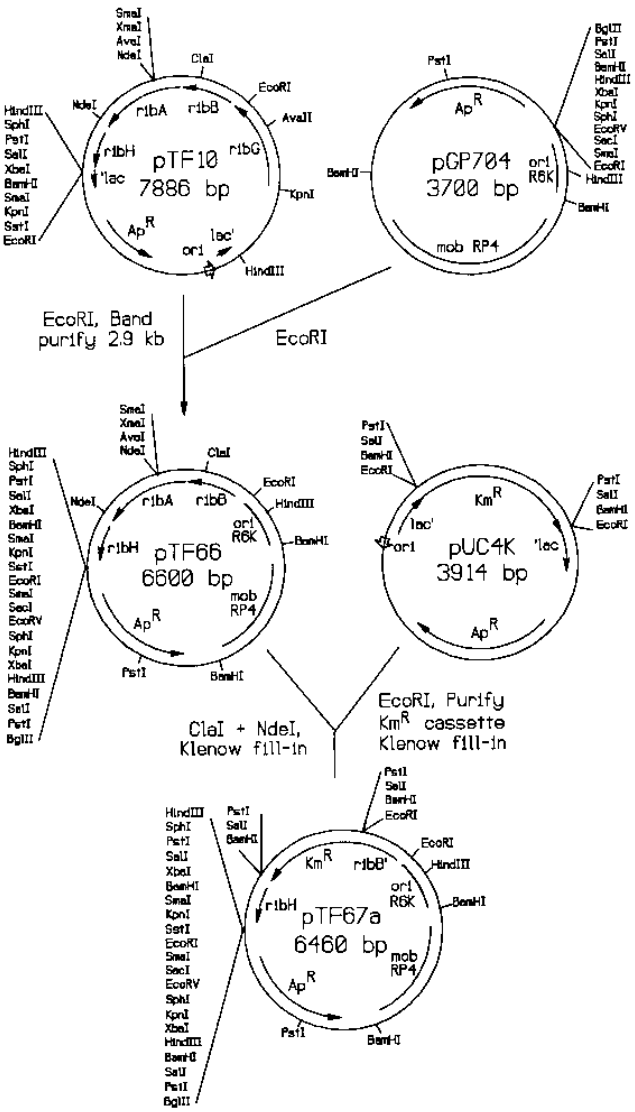

FIG. 1. Construction of pTF67A. The entire riboflavin operon, containing the *ribGBAH* genes from AP106, was cloned into pUC19 to make pTF10 (5). A 2.9-kb fragment containing the *ribBAH* portion of the riboflavin operon was excised from pTF10 with *Eco*RI and ligated into the *Eco*RI site of the conjugative suicide vector pGP704 to form pTF66. A 1.4-kb *Cla*I-*Nde*I fragment, which contains all of *ribA* and part of *ribB*, was deleted and replaced with the Km^r cassette from pUC4K to create pTF67a.

developed with sera from convalescent swine. No differences in protein, LPS, extracellular toxin, or capsular polysaccharide profiles of the wild-type AP100, its Nal^r derivative AP225, and the riboflavin mutant AP233 (data not shown) were detected. There was no difference in reactivity with serotype-specific antisera as determined by coagglutination assay (data not shown).

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

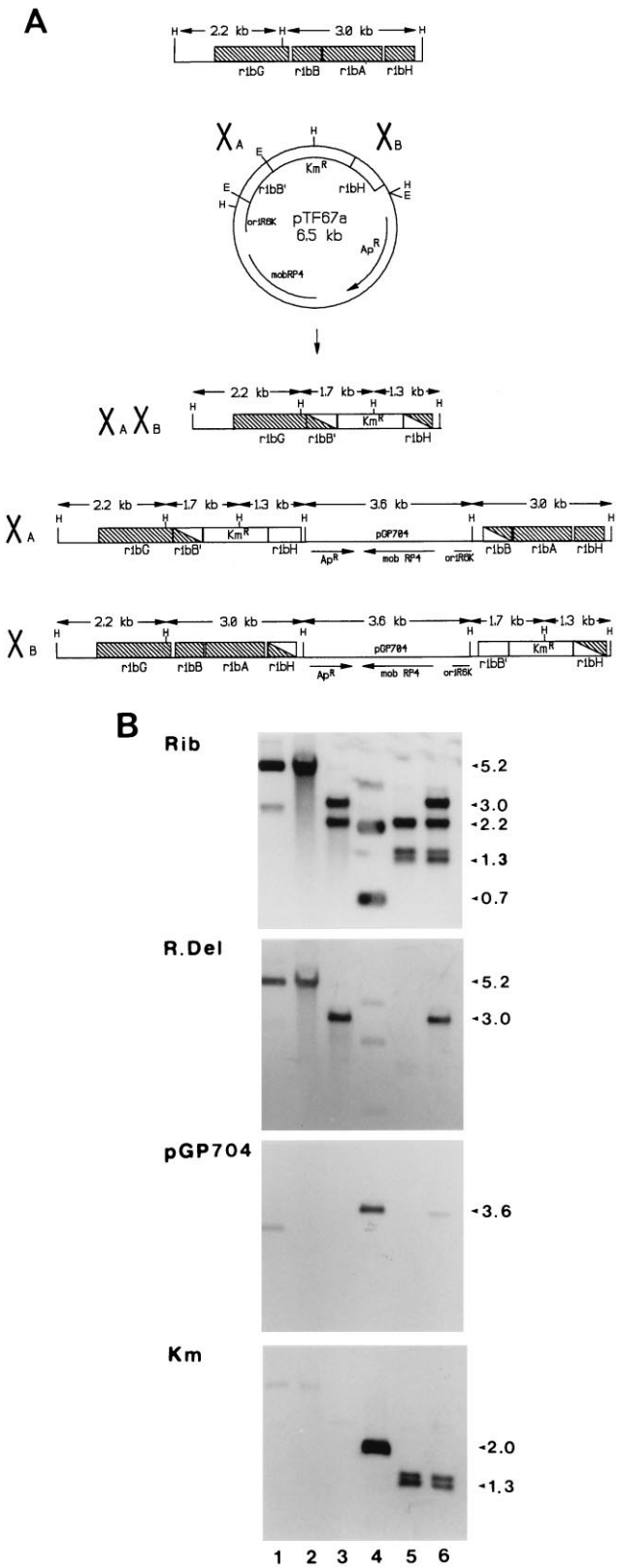

FIG. 2. Southern blot analysis of chromosomal DNA from AP100 *rib* mutants. (A) Schematic of the structure of the *rib* locus of parent and mutant strains in double- and single-crossover events. The predicted sizes of *Hin*dIII genomic fragments for two possible single-crossover events and for a double-crossover event are shown. The results show that for AP233 the chromosomal *rib* operon has been replaced with the cloned riboflavin operon containing the Km^r cassette by a double-crossover event, while AP234 is the result of a single-crossover event either upstream or downstream of the kanamycin cassette. Abbreviations for restriction enzymes used: E, *Eco*RI; H, *Hin*dIII. (B) Southern blots of *Hin*dIII- or *Eco*RI-digested DNA from mutants and controls. Blots were prepared in quadruplicate and hybridized at high stringency with one of four probes: Rib, the entire *ribGBAH* operon from pTF10; R.Del., the deleted portion (*Cla*I-*Nde*I fragments) of the *ribGBAH* operon; pGP704, the entire plasmid; Km, the kanamycin cassette from pUC4K. Lanes: 1, pTF10 digested with *Hin*dIII; 2, AP106 digested with *Hin*dIII; 3, AP100 digested with *Hin*dIII; 4, pTF67a digested with *Eco*RI; 5, AP233 digested with *Hin*dIII; and 6, AP234 digested with *Hin*dIII. Numbers to the right of the gels are fragment sizes (in kilobases).

TABLE 2. Mortality and lung score data

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

[a] Doses are multiples of 5.0×10^6 CFU, the established LD_{50} for the wild type (12).
[b] Mortality reported as number of deceased swine/number of swine in group.
[c] Percentage of lung tissue exhibiting *A. pleuropneumoniae* lesions.
[d] Percentage of pleural surface area exhibiting pleuritis.
[e] WT, wild type.

Attenuation of virulence of the *rib* mutant in swine. Six groups of three pigs each were infected with bacteria as follows: group 1, 1 50% lethal dose (LD_{50}) (5×10^6 CFU) of AP225; groups 2 to 5, AP233 at doses equivalent to 4, 20, 100, and 500 times the LD_{50} for the wild type; and group 6, AP233/pTF76 at a dose equivalent to 1 LD_{50} for the wild type. Mortality, lung score, and clinical score data, shown in Tables 2 and 3, all indicate that the riboflavin auxotroph at doses as high as 500 times the LD_{50} for the wild type is avirulent in pigs. The pigs infected with the *rib* mutant AP233 displayed no dyspnea, elevated respiration rate, depression, or loss of appetite and had no typical pleuropneumonic pathology at necropsy even if they had received the highest dose tested. In contrast, one of three pigs infected with the wild-type AP225 strain died and all three exhibited significant clinical signs of infection, including elevated respiration rate, dyspnea, depression, loss of appetite, and fever and severe pneumonia and pleuritis were evident at necropsy. Pigs infected with AP233 containing the riboflavin genes in *trans* (pTF76) also exhibited obvious clinical signs and significant pneumonia and pleuritis, although such signs were somewhat less severe than those exhibited by pigs infected with 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 that received AP225 and AP233/pTF76. All reisolated organisms were characterized by Gram stain, colonial morphology, requirement for V (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 that were infected with AP233 and euthanized 48 h postinfection.

DISCUSSION

In this paper, we report the construction of a serotype 1 *A. pleuropneumoniae* deletion-disruption riboflavin mutant that is

TABLE 3. Clinical score data

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

[a] Doses are multiples of 5.0×10^6 CFU, the established LD_{50} for the wild type (12).
[b] Maximum respiratory rate (recorded as number of breaths per 15-s observation period) observed after challenge.
[c] Maximum rectal temperature, in degrees Fahrenheit, after challenge.
[d] The dyspnea score measures the degree of respiratory distress and labored breathing: 0, normal; 1, slight; 2, moderate; 3, severe. The reported score is the sum of the scores taken at 12-h intervals after challenge.
[e] The depression score evaluates attitude and activity: 0, normal; 1, slight inactivity; 2, moderate inactivity; 3, severe inactivity. The reported score is the sum of the scores taken at 12-h intervals after challenge.
[f] Appetite was scored as follows: 0, did eat; 1, did not eat. The reported score is the number of 12-h periods during which animals did not eat over the 60-h observation period.

attenuated in vivo. The *A. pleuropneumoniae ribGBAH* operon was disrupted by deleting an internal segment of the operon (*ribBA*) and replacing it with a Km^r cassette by a targeted-mutagenesis technique (19). A stable riboflavin-requiring, Km^r mutant, AP233, was phenotypically identical to its wild-type parent on the basis of analyses 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 with serotype 1 because serotype 5 seemed to be very resistant to transformation by standard heat shock or electroporation procedures. In order to complement the *rib* mutation in *trans*, and for ease of future genetic manipulations, it was necessary to use a serotype 1 strain for these studies.

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

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

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

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

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