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Molecular Gene Cloning and Nucleotide Sequencing and Construction of an *aroA* Mutant of *Pasteurella haemolytica* Serotype A1

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The *aroA* gene of *Pasteurella haemolytica* serotype A1 was cloned by complementation of the *aroA* mutation in *Escherichia coli* K-12 strain AB2829. The nucleotide sequence of a 2.2-kb fragment encoding *aroA* predicted an open reading frame product 434 amino acids long that shows homology to other bacterial AroA proteins. Several strategies to inactivate *aroA* were unsuccessful. Gene replacement was finally achieved by constructing a replacement plasmid with *aroA* inactivated by insertion of a *P. haemolytica* ampicillin resistance fragment into a unique *NdeI* site in *aroA*. A hybrid plasmid was constructed by joining the *aroA* replacement plasmid with a 4.2-kb *P. haemolytica* plasmid which encodes streptomycin resistance. Following *PhaI* methylation, the replacement plasmid was introduced by electroporation into *P. haemolytica* NADC-D60, a plasmidless strain of serotype 1A. Allelic exchange between the replacement plasmid and the chromosome of *P. haemolytica* gave rise to an ampicillin-resistant mutant which grew on chemically defined *P. haemolytica* medium supplemented with aromatic amino acids but failed to grow on the same medium lacking tryptophan. Southern blot analysis confirmed that *aroA* of the mutant was inactivated and that the mutant was without a plasmid.

The microorganism *Pasteurella haemolytica* biotype A, serotype 1, is the principal causative agent of pneumonic pasteurellosis in cattle. With the development of techniques for introducing exogenous DNA into *P. haemolytica* (5), it now may be possible to produce site-specific mutations in this bacterium. Such mutants would prove extremely useful in investigating the molecular mechanisms of pathogenesis of *P. haemolytica*. Also, genetic manipulation of *P. haemolytica* may permit construction of rationally attenuated strains to test as live vaccine candidates.

Attenuated auxotrophic mutants were first described by Bacon et al. in 1950 (3). In that study, it was reported that attenuated auxotrophs of *Salmonella typhi* defective in the aromatic amino acid biosynthetic pathway were avirulent in mice. Subsequently, it has been demonstrated in widely diverse bacteria that disrupting the aromatic amino acid biosynthetic pathway produces attenuated organisms. For example, attenuated strains of the invasive bacteria *S. typhi* (3), *Salmonella typhimurium* (11), *Shigella flexneri* (22), and *Yersinia enterocolitica* (4) were generated by introducing mutations in their respective *aroA* genes. Also, attenuation was produced in the noninvasive bacteria *Bordetella pertussis* (19) and *Pasteurella multocida* (13) through *aroA* inactivation. These *aroA* mutant strains are unable to synthesize chorismic acid from which *p*-aminobenzoic acid, dihydrobenzoate, and aromatic amino acids are produced. It is likely that the absence of one or more of these compounds in vivo is responsible for the poor growth of *aroA* mutants in the hosts.

The interest in constructing attenuated bacterial strains as vaccine candidates can be attributed to the superior protection afforded by live vaccines. In general, live vaccines elicit a stronger cell-mediated response in the host than do bacterins (7). Also, the superior immunity provided by attenuated organisms compared with that provided by killed bacteria may be explained by induced expression of stress proteins and, possibly, of certain toxins within the host. The immune response generated by live organisms would be directed against these abundant proteins and thereby provide better protection.

Because most vaccines to prevent pneumonic pasteurellosis are killed formulations, we decided to construct an *aroA* mutant of *P. haemolytica* to test for efficacy as a live vaccine. To accomplish this goal, we cloned and determined the nucleotide sequence of *P. haemolytica aroA*. A number of replacement plasmids and hybrid plasmids consisting of a replacement plasmid joined to plasmids of *P. haemolytica* were constructed. Following methylation by *PhaI* methyltransferase, the plasmids were introduced into *P. haemolytica*. One of these constructs, a hybrid plasmid which joined the replacement plasmid harboring insertionally inactivated *aroA* to the *P. haemolytica* plasmid encoding streptomycin resistance, was used to produce an *aroA* mutant by gene replacement.

MATERIALS AND METHODS

Molecular cloning and Southern blot analysis of *P. haemolytica aroA*. Genomic DNA was prepared from *P. haemolytica* NADC-D60 by previously described methods (5). Southern blotting of *P. haemolytica* restriction fragments fractionated by electrophoresis on 0.75% agarose was performed as described previously (5). Blots were hybridized with a radioactively labeled 1.3-kb *Escherichia coli aroA* fragment. The *aroA* probe was amplified (Gene-AMP; Perkin-Elmer Inc., Branchburg, N.J.) from *E. coli* X-L1 Blue (Stratagene, Inc., La Jolla, Calif.) genomic DNA by PCR. The primers (5′-AGGCTGCCTG GCTAATCCGCGCCAG-3′ and 5′-TTCATGGAATCCCTT GACGTTACAACCCATC-3′) used in the PCR hybridize with *E. coli aroA* nucleotides 1308 through 1332 and to the complementary strand of nucleotides −3 through 28, respectively. The primers were synthesized with an oligonucleotide synthesizer (Applied Biosystems Inc.) by the Nucleic Acids Facility, Iowa State University, Ames. DNA was radiolabeled with [α-^{32}P]dCTP with a random-priming kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Nylon membranes (Hybond-N; Amersham Corp., Arlington Heights, Ill.) were

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source or reference
Strains		
E. coli		
AB2829	K-12 strain with mutation in *aroA*	18
DH10B	Cloning strain used in this work	BRL
*Pha*IMtase	Recombinant DH108B carrying *Pha*I methyltransferase gene on cosmid vector	This work
S17-1	Donor strain containing chromosomally integrated RP4 transfer functions	20
XL1-Blue	Strain used for DNA sequencing	Stratagene
P. haemolytica		
NADC-D60	Serotype 1, plasmidless	NADC[a] (R. Briggs)
NADC-D70	Serotype 1 containing pD70	NADC (R. Briggs)
NADC-D80	Serotype 1 containing pD80	NADC (R. Briggs)
Plasmids		
pSK	Cloning vector (Amp^r)	Stratagene
pBC SK	Cloning vector (Cm^r)	Stratagene
pD70	4.2-kb plasmid encoding Sm^r	NADC (R. Briggs)
pD80	4.2-kb plasmid encoding Amp^r	NADC (R. Briggs)
pPh*aroA*1	3.2-kb *Hin*dIII fragment containing *P. haemolytica aroA* (pSK)	This work
pPh*aroA*2	*Hin*dIII-*Cla*I digest of pPh*aroA*1 resulting in 2.2-kb *aroA* fragment (pSK)	This work
pPh*aroA*3	Same insert as pPh*aroA*2 on pBC SK	This work
pPhΔ*aroA*Cm^r	*Sty*I-*Nde*I digest of pPh*aroA*2 Cm^r fragment inserted into deletion site	This work
pPhΔ*aroA*Cm^rpD80	*Sma*I-digested pPhΔ*aroA*Cm^r joined to *Sca*I-digested pD80	This work
pPhAmp^r	2.2-kb *Sau*3A fragment of pD80 cloned into pBC SK	This work
pPh*aroA*$^-$$Amp^r$	Amp^r fragment of pD80 inserted into unique *Nde*I site of pPh*aroA*3	This work
pPh*aroA*$^-$$Amp^r$pD70	*Hin*dIII-digested pPh*aroA*$^-$$Amp^r$ joined to *Hin*dIII-digested pD70	This work

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incubated with a prehybridization solution of 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 5× Denhardt's solution (15), 0.1% sodium dodecyl sulfate (SDS), 10 μg of sonicated salmon sperm DNA per ml, and 50% formamide at 42°C for 2 h. After removal of the prehybridization solution, the filters were incubated with hybridization solution (same as the prehybridization solution but lacking Denhardt's solution) containing 10^7 cpm of ^{32}P-labeled probe and 50% formamide at 42°C. After hybridization for 18 h, membranes were washed twice with 1× SSC–0.1% SDS for 10 min each time at room temperature and two times with 1× SSC–0.1% SDS buffer at 42°C for 15 min each time. Membranes were exposed to X-AR (Eastman Kodak Co., Rochester, N.Y.) at −80°C for 24 h. A positive signal corresponding to a 3.2-kb *Hin*dIII fragment of *P. haemolytica* chromosomal DNA was identified.

*Hin*dIII-digested *P. haemolytica* DNA fragments ranging from 3.0 to 3.4 kb in length were electroeluted from a 1% agarose gel. The *Hin*dIII genomic fragments were added to *Hin*dIII-digested alkaline phosphatase-treated pBluescript SK^- vector (Stratagene, Inc.) and ligated overnight at 10°C with T4 ligase (Bethesda Research Laboratories [BRL], Gaithersburg, Md.). The ligation mix was diluted 1:10 with distilled water and electroporated with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) into *E. coli aroA* mutant AB2829 (18). A recombinant plasmid, pPh*aroA*1, complemented AB2829 growth on M9 minimal medium containing phosphate buffer, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.2% glucose, thiamine (10 μg/ml), 1.5% Noble agar (Difco), and 50 μg of ampicillin per ml (1). *Cla*I-*Hin*dIII double digestion of pPH*aroA*1 produced a 2.2-kb fragment which when cloned into the *Acc*I and *Hin*dIII sites of pBluescript SK^- gave rise to pPh*aroA*2. The recombinant plasmid, pPh*aroA*2, which also complemented growth of AB2829 on minimal plates, was used to sequence *P. haemolytica aroA*. Plasmid pPh*aroA*3 was generated by subcloning the 2.2-kb fragment of pPh*aroA*2 into pBC SK^+ (Stratagene Inc.).

DNA sequencing and analysis. DNA sequencing was done by the dideoxy nucleotide termination method with single- or double-stranded templates by using the Sequenase 2.0 kit (United States Biochemicals, Cleveland, Ohio). A series of ordered deletions were made in *P. haemolytica aroA* on pPH*aroA*2 by using an Erase-a-Base kit (Promega Corp., Madison, Wis.). Gaps in the sequence were completed by using DNA primers synthesized by the DNA Core Facility at Iowa State University. DNA sequence analysis was done with MacDNASIS Pro (Hitachi Software Ltd., San Bruno, Calif.) and MacVecvtor (Kodak Co., New Haven, Conn.) software.

Construction of replacement plasmids and the production and characterization of a *P. haemolytica aroA* mutant. Construction of the replacement plasmid pPhΔ*aroA*Cm^r (Table 1) and the hybrid plasmid pPhΔ*aroA*Cm^rpD80 is described in the preceding study (5). The steps involved in the construction of the other plasmids used in this study (Table 1) are presented below.

Plasmid pD80, a 4.2-kb ampicillin resistance (Amp^r) encoding plasmid of *P. haemolytica* (2, 14), was partially digested with *Sau*3A and ligated into the *Bam*HI site of pBC SK^+ (Stratagene Inc.). The ligation mix was diluted 1:10 in distilled water and electroporated into *E. coli* DH-10B (BRL). After recovery in 1 ml of SOC medium containing 2% Bacto Tryptone, 0.5% Bacto Yeast Extract, 8 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 20 mM glucose (15), at 37°C the cells were spread onto B-broth plates, containing 1% Bacto Tryptone, 0.8% NaCl, 2% Difco agar, thiamine (10 μg/ml), 0.002% 5-chloro-4-bromo-3-indolyl-β-D-galactopyranoside (X-Gal), 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG), and 50 μg of ampicillin per ml. Plasmid pPhAmp^r contained a 2.2-kb *P. haemolytica* fragment which imparted ampicillin resistance to *E. coli* to up to 100 μg/ml. Plasmid pPhAmp^r was digested with *Hin*dIII and *Xba*I, and the fragment ends were made blunt by incubation with deoxynucleoside triphosphates and the large Klenow fragment of *E. coli* polymerase I. The fragment encoding ampicillin resistance was electroeluted and used to inactivate *aroA* of pPh*aroA*3. To do so, pPh*aroA*3 was digested

at a unique *Nde*I site contained in *aroA* (see Fig. 2) and the fragment ends were made blunt as described above. The ampicillin resistance fragment was blunt end ligated with pPh*aroA*3 to generate pPh*aroA*$^-$Amp^r. To produce a hybrid plasmid, pPh*aroA*$^-$Amp^r was digested with *Hin*dIII and dephosphorylated with calf alkaline phosphatase. A 4.2-kb plasmid, designated pD70, which encodes streptomycin resistance (Sm^r) (6), was isolated from *P. haemolytica* NADC-D70 by previously described methods. Plasmid pD70 was digested at a unique *Hin*dIII recognition site and ligated with *Hin*dIII-digested pPh*aroA*$^-$Amp^r to generate the hybrid plasmid pPh*aroA*$^-$Amp^{r}pD70. Plasmid pPh*aroA*$^-$Amp^{r}pD70 was electroporated into *E. coli Pha*IMtase (Table 1). Strain *Pha*IMtase is *E. coli* DH-10B (BRL) which contains the *Pha*I methyltransferase gene on cosmid vector pLAFRX, which was derived from pLAFR2 (9). Then the *Pha*I methyl-modified hybrid plasmid was isolated by the alkaline lysis method (15) and CsCl purified. One microgram of plasmid and 100 μl of *P. haemolytica* NADC-D60 (a plasmidless isolate of serotype A1) were transferred to a 0.1-cm cuvette and electroporated at 15,000 V/cm with 800 Ω. The resultant time constant was approximately 9 mc. Cells were transferred to 1 ml of Bacto Columbia broth (Difco Labs, Detroit, Mich.) and incubated at 37°C for 1 h. One milliliter of Columbia broth containing 20 μg of ampicillin was added, and the cells were incubated for an additional hour and then spread on Difco Columbia blood agar plates containing 5% bovine blood and 10 μg of ampicillin per ml. Eight ampicillin-resistant *P. haemolytica* colonies were isolated after incubation at 37°C for 18 h. The colonies were then transferred to Bacto Columbia broth containing 1 μg of ampicillin per ml and incubated at 37°C. Daily passage into fresh medium containing 1 μg of ampicillin per ml was carried out for 3 days, at which time the cultures were transferred onto Columbia broth blood agar plates containing 10 μg of ampicillin per ml and incubated at 37°C overnight. The next day the colonies were replica plated onto Columbia broth blood agar plates containing 10 μg of chloramphenicol per ml or 50 μg of streptomycin per ml. The colonies were also replica plated onto a chemically defined medium for *P. haemolytica* cultivation (23). The defined medium contained 15 amino acids and included the aromatic amino acids phenylalanine and tyrosine but not tryptophan. Clones unable to grow on the chemically defined medium for *P. haemolytica* cultivation were presumed to be *aroA* mutants. Genomic DNAs isolated from clones with Amp^r Cm^s Sm^s AroA$^-$ phenotypes were analyzed by Southern blotting. Southern blotting was performed as described previously with the exception that after hybridization the membranes were washed twice for 10 min each in 1× SSC–0.1% SDS at 42°C and twice more for 15 min each in 0.1× SSC–0.1% SDS at 65°C.

Nucleotide sequence accession number. The *P. haemolytica aroA* sequence data reported here have been deposited in GenBank under accession number U03068.

RESULTS AND DISCUSSION

Cloning of *P. haemolytica aroA*. Restriction fragments of *P. haemolytica* genomic DNA were fractionated by agarose gel electrophoresis. The fragments were probed for homology to *E. coli aroA* by Southern analysis. Under conditions of low stringency, a 3.2-kb *Hin*dIII fragment of *P. haemolytica* genomic DNA hybridized with radiolabeled *E. coli aroA* (Fig. 1). The *Hin*dIII fragment was isolated from an agarose gel by electroelution and was cloned into *Hin*dIII-digested pBluescript SK$^-$. The recombinant plasmid, pPH*aroA*1, bearing *P. haemolytica aroA* was identified by complementation of *E. coli aroA* mutant AB2829 on M9 minimal medium containing ampicillin. A *Cla*I-*Hin*dIII double digest of pPh*aroA*1 generated a 2.2-kb fragment which was cloned into the *Acc*I and *Hin*dIII sites of pBluescript SK$^-$, giving rise to pPh*aroA*2. Plasmid pPh*aroA*2 also complemented growth of *E. coli* AB2829 on M9 minimal medium. Plasmid pPh*aroA*2 was used to determine the nucleotide sequence of *P. haemolytica aroA* presented here.

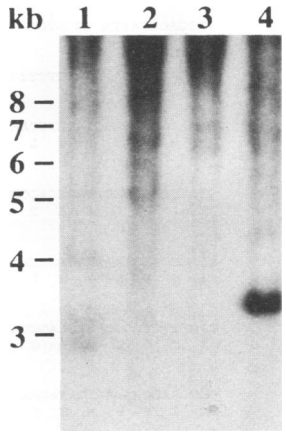

FIG. 1. Southern blot analysis of *P. haemolytica* NADC-D60 DNA digested with *Eco*RI (lane 1), *Cla*I (lane 2), *Pst*I (lane 3), or *Hin*dIII (lane 4). The membrane was hybridized with an *E. coli aroA* probe, and washing was performed under low-stringency conditions.

Nucleotide sequence and analysis of *P. haemolytica aroA*. The nucleotide sequence and the deduced amino acid sequence of *P. haemolytica aroA* and its product are shown in Fig. 2. Sequence analysis on *P. haemolytica aroA* revealed an open reading frame of 1,302 bases with a coding capacity of 434 amino acid residues. The deduced molecular weight is 47,296, and the G+C content of the *aroA* coding region product is 43%. The predicted amino acid sequence of *P. haemolytica* AroA showed 75, 70, 69, and 68% identity with the AroA proteins of *P. multocida* (13), *Klebsiella pneumoniae* (21), *Y. entercolitica* (17), and *E. coli* (8), respectively.

P. haemolytica aroA, like *P. multocida aroA* (13), appears to be transcribed from its own promoter. This differs from the usual genetic arrangement in gram-negative bacteria, in which *aroA* and *serC* constitute an operon with *aroA* distal to the promoter (8, 12, 17). Evidence to support this claim includes the findings that (i) the nucleotide sequence upstream of *aroA* on clone pPh*aroA*2 shows no homology with *serC* genes and (ii) complementation of *E. coli* AB2829 by *P. haemolytica aroA* contained on the 2.2-kb fragment is independent of the fragment's orientation on the cloning vector (data not shown).

Construction of a *P. haemolytica aroA* mutant. The purpose of this investigation was to develop methods in order to genetically engineer a rationally attenuated strain of *P. haemolytica*. To achieve this goal, a number of replacement plasmids were constructed, each considered an improvement on the previous one which failed to produce an *aroA* mutant. First the deletion plasmid pPhΔ*aroA*Cm^r (Table 1) was constructed from pPh*aroA*2 as described previously (5) and amplified in *E. coli Pha*IMtase. Although resistant to *Pha*I digestion (data not shown), introduction of pPhΔ*aroA*Cm^r into *P. haemolytica* NADC-D60 by electroporation failed to generate Cm^r colonies. The inability to transform *P. haemolytica* with pPhΔ*aroA*Cm^r indicated that plasmids containing a ColE1 origin do not replicate in this bacterium.

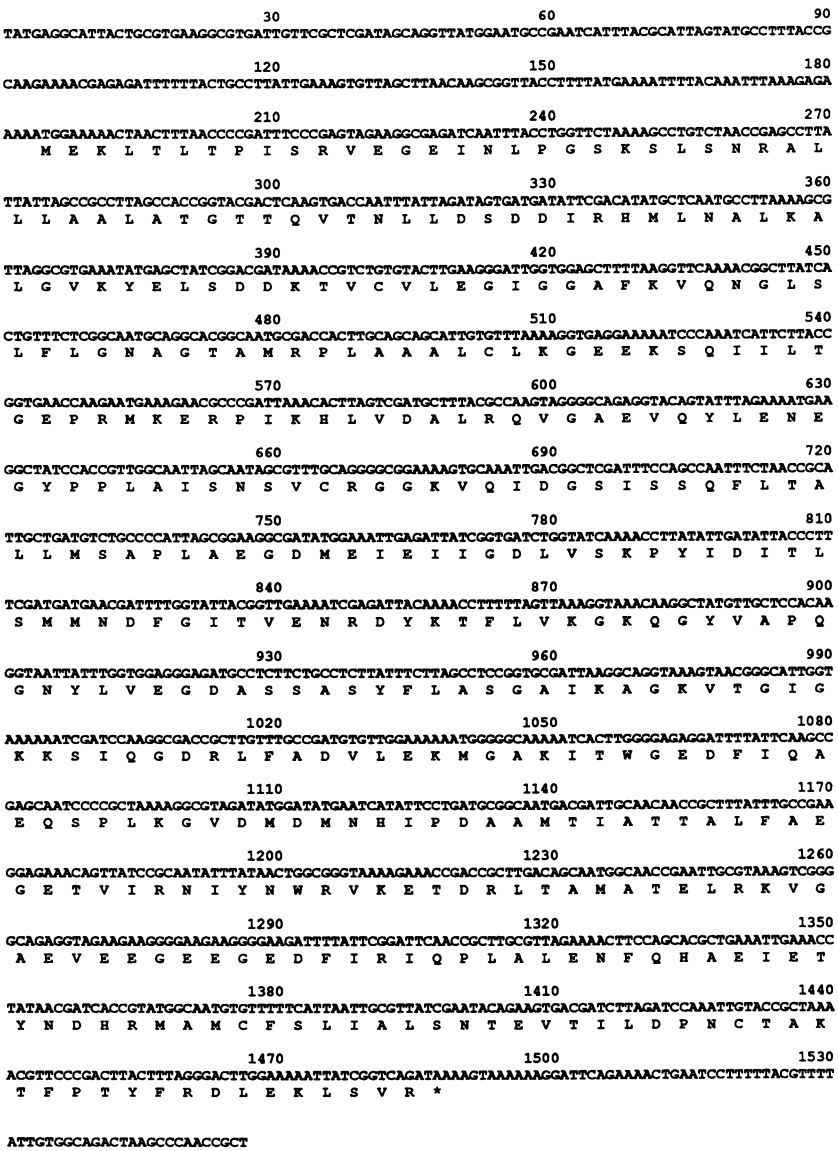

FIG. 2. Nucleotide sequence of and deduced amino acid sequence encoded by *P. haemolytica aroA*.

Since we have shown that the *Pha*I-methylated hybrid plasmid consisting of plasmid pPhΔ*aroA*Cm^r joined with pD80 (Amp^r) from *P. haemolytica* could be used to transform *P. haemolytica* NADC-D60 (5), we investigated whether *aroA* mutants might arise after transformation with the hybrid plasmid. The hybrid plasmid was found, however, to be stable after multiple passages without selection, so no recombinant *P. haemolytica* could be detected. Since previous investigation demonstrated that the hybrid plasmid more efficiently transforms *P. haemolytica* when isolated from the homologous bacterium than when isolated from *E. coli* (5), an attempt was made to generate a suicide plasmid from the hybrid plasmid isolated from *P. haemolytica*. Linear and T4 ligase-treated relaxed circular pPhΔ*aroA*Cm^r plasmids were generated from the hybrid plasmid after restriction enzyme digestion. Neither version resulted in Cm^r colonies upon reintroduction into *P. haemolytica*. Although pPhΔ*aroA*Cm^r was treated with the Klenow fragment and deoxynucleoside triphosphates prior to treatment with T4 ligase, examination showed that most of the sample remained linear rather than circular. The results, then, are not totally unexpected, since there have been few reports of the successful establishment of linear DNA into bacteria (10, 16, 24).

Because initial selection on chloramphenicol has been inferior to selection on ampicillin in our laboratory (5), we decided to change the antibiotic resistance cassette in our replacement plasmid from Cm^r to Amp^r (5). A *Sau*3A-generated 2.2-kb fragment from pD80 from *P. haemolytica* which conferred ampicillin resistance was cloned into the *Bam*HI site of pBC SK, resulting in pPhAmp^r. To demonstrate that pPhAmp^r did not possess the pD80 origin of replication, pPhAmp^r was isolated from *E. coli* containing *Pha*I methyltransferase by methods described previously, CsCl purified, and introduced into *P. haemolytica* by electroporation. Since the *Pha*I-methylated plasmid did not confer ampicillin resistance to *P. haemolytica* NADC-D60, we concluded that the fragment encoding

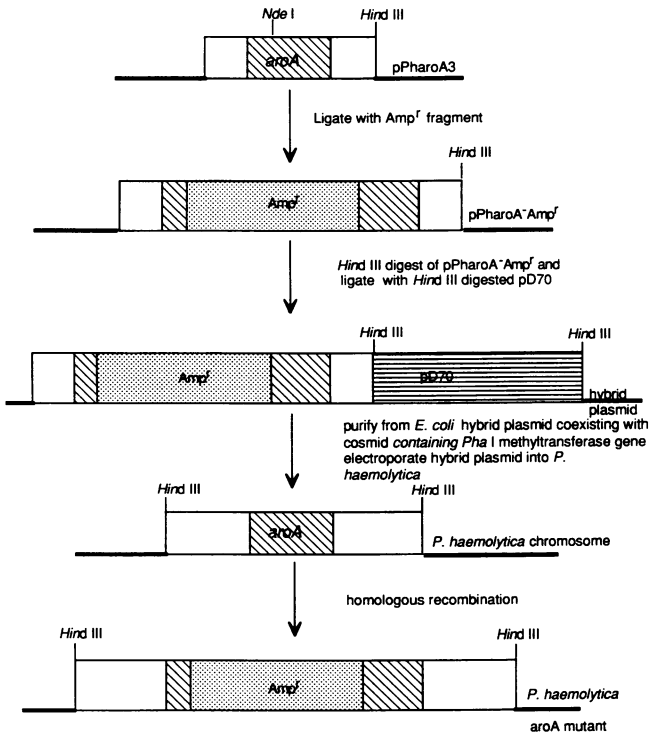

FIG. 3. Construction of a *P. haemolytica aroA* mutant. The hybrid plasmid pPh*aroA*⁻Amp^r pD70 shown above was successfully used to produce an *aroA* mutant.

β-lactamase did not contain the pD80 origin of replication. The 2.2-kb Amp^r fragment, made blunt, was used to insertionally inactivate *aroA* upon introduction into the unique *Nde*I site of *aroA*. The resulting plasmid, pPh*aroA*⁻Amp^r (Fig. 3), after passage through *E. coli Pha*^r MTase failed to generate ampicillin-resistant colonies of *P. haemolytica*.

To increase the likelihood of selecting an *aroA* mutant arising by homologous recombination between inactivated *aroA* on the deletion plasmids and the *P. haemolytica* chromosome, we constructed a second hybrid plasmid. This hybrid construct joined a 4.2-kb *P. haemolytica* plasmid encoding streptomycin resistance (6), designated pD70, with the replacement plasmid pPh*aroA*⁻Amp^r. Because pD70 may be unstable in *P. haemolytica* (4a), we hypothesized that the hybrid plasmid pPh*aroA*⁻Amp^r pD70 also would be unstable in *P. haemolytica*. Thus, after repeated passages in medium containing ampicillin, a *P. haemolytica* clone transformed with pPh*aroA*⁻Amp^r pD70 should eventually give rise to mutants via homologous recombination which express β-lactamase by means of chromosomally encoded Amp^r genes. And some of these mutants would be expected to have inactivated *aroA* genes.

The hybrid plasmid was constructed by joining *Hin*dIII-digested pD70 with *Hin*dIII-digested pPh*aroA*⁻Amp^r. The resultant hybrid plasmid, pPh*aroA*⁻Amp^r pD70 (Fig. 3), was isolated from *E. coli Pha*IMtase, purified by standard methods, and introduced into *P. haemolytica* NADC-D60 by electroporation. Transformation efficiency of the hybrid plasmid yielded approximately 10^1 ampicillin-resistant colonies per μg of DNA. Eight Amp^r clones were grown overnight in Columbia broth containing 1 μg of ampicillin per ml. Chromosomal DNAs from the parental strain and from the Amp^r clones were digested with *Hin*dIII and analyzed by Southern blotting with *P. haemolytica aroA*, pBC SK, and pD70 radiolabeled probes. The results indicated that intact pPh*aroA*⁻Amp^r pD70 was present in the Amp^r clones (data not shown). The eight Amp^r cultures were transferred to Columbia broth containing 1 μg of ampicillin per ml and cultured at 37°C. The bacteria were transferred to fresh media daily, and this process was continued for approximately 100 generations. The eight cultures were streaked for isolation without antibiotic selection, and a single colony of each was passed into Columbia broth containing either 1 μg of ampicillin per ml or 1 μg of chloramphenicol per ml. Two of the eight survived on the broth containing ampicillin; none surviving on chloramphenicol-containing broth. Passage from ampicillin-containing broth onto blood agar plates containing either ampicillin or chloramphenicol or streptomycin confirmed that the two clones were Amp^r, Cm^s Sm^s. Also, the two Amp^r clones were spread onto plates of chemically defined medium for *P. haemolytica* cultivation (23). This medium lacks the aromatic amino acid tryptophan. The parent strain grew on the defined medium, but the Amp^r clones did not. Upon addition of tryptophan to the defined medium, growth of the Amp^r clones was comparable to that of

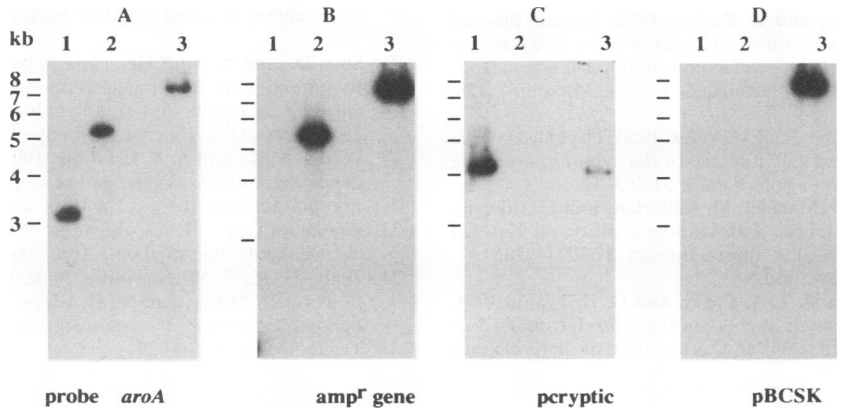

FIG. 4. Southern hybridization of genomic DNAs from the parental strain *P. haemolytica* NADC-D60 (lanes 1), the *aroA* mutant (lanes 2), and the hybrid plasmid pPh*aroA*⁻Amp^r pD70 (lanes 3). All the DNAs used in the blots shown here were digested with *Hin*dIII. (A) Probed with *P. haemolytica aroA*; (B) probed with *P. haemolytica* Amp^r fragment; (C) probed with *P. haemolytica* pD70 plasmid; (D) probed with pBCSK. DNA was isolated from *P. haemolytica* NADC-D70 and run in lane 1 of blot C to demonstrate that if plasmid DNA was present in the bacteria it would also be present in our DNA preparations.

the parent strain. The *E. coli aroA* mutant AB2829 also required tryptophan to grow on the chemically defined medium for *P. haemolytica* cultivation. DNAs from the two colonies with Amp^r Cm^s Sm^s *aroA* mutant phenotypes were analyzed by Southern blotting. The results indicated that both had insertionally inactivated *aroA* genes. Moreover, Southern blotting also confirmed that both pD70 and pBC SK sequences were no longer present in the *aroA* mutants (Fig. 4).

The experimental approach that successfully produced an *aroA* mutant required repeated passage of *P. haemolytica* transformed with the hybrid plasmid pPh*aroA*$^-$Amp^{r}pD70. Although this hybrid plasmid was able to replicate in *P. haemolytica*, improving the chance for recombination, its instability allowed recovery of plasmidless *aroA* mutants. The failure of the replacement plasmid alone to generate antibiotic-resistant colonies of *P. haemolytica* may be explained by the low transformation frequencies of *Pha*I-methylated plasmids which were insufficient to routinely produce mutants by the rare event of homologous recombination. In other bacteria, conjugative suicide vectors have been shown to be highly efficient for gene replacement constructions. For this reason, we constructed a derivative of pPh*aroA*$^-$Amp^{r}pD70 which contained *mob* (20), the P-type-specific recognition site for mobilization, inserted in the unique *Bam*HI site of the polylinker. The hybrid plasmid was mobilized from *E. coli* S17-1 (20) with and without *Pha*I methyltransferase on a compatible vector. No *P. haemolytica* transconjugates were detected from donors lacking *Pha*I methyltransferase; approximately 10^{-9} arose following matings with donors which contained *Pha*I methyltransferase. Plasmid recovered from the transconjugates apparently had undergone rearrangement, because their apparent molecular weights differed from that of the donor plasmid (data not shown).

To our knowledge, the work described here is the first report of site-directed gene replacement in *P. haemolytica*. Whether the *P. haemolytica aroA* mutant is attenuated is yet to be determined. Future work aimed at assessing the suitability of the *aroA* mutant as a live vaccine against pneumonic pasteurellosis in cattle and sheep is planned.

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