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Methylation of DNA can be a critical step in the introduction of DNA into P. haemolytica. A methyltransferase has been isolated and molecularly cloned for this purpose. Use of the methyltransferase has allowed construction of defined, attenuated aroA mutants for use as vaccines to protect cattle.

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(54) Title: CONSTRUCTION OF *PASTEURELLA HEAMOLYTICA* VACCINES

(57) Abstract

Methylation of DNA can be a critical step in the introduction of DNA into *P. haemolytica*. A methyltransferase has been isolated and molecularly cloned for this purpose. Use of the methyltransferase has allowed construction of defined, attenuated aroA mutants for use as vaccines to protect cattle.

CONSTRUCTION OF *PASTEURELLA HAEMOLYTICA* VACCINES

TECHNICAL AREA OF THE INVENTION

The invention relates to the area of bacterial genetic engineering. In particular, it relates to the bacteria *Pasteurella haemolytica*.

BACKGROUND OF THE INVENTION

The microorganism *P. haemolytica* biotype A, serotype 1, is the principal causative agent of pneumonic pasteurellosis in cattle. If techniques could be developed for introducing exogenous DNA into *P. haemolytica*, it would be possible to produce site-specific mutations in this bacterium. Such mutants could provide "rationally" attenuated strains for use as live vaccines.

Attenuated auxotrophic mutants were first described by Bacon and Burrows in the early 1950's. They 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 *Salmonella typhi*, *Salmonella typhimurium*, *Shigella flexneri*, and *Yersina enterocolitica*, were generated by introducing mutations in their respective *aro*A genes. Also attenuation was produced in the non-invasive bacteria *Bordetella pertussis* and *Pasteurella multocida* through *aro*A inactivation. Strains which carry *aro*A mutations 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 *aro*A mutants in the hosts.

Live attenuated bacterial strains generally provide superior protection as compared to killed bacterial vaccines (bacterins). In general, live vaccines elicit a stronger cell mediated response in the host than do bacterins. The superior immunity provided by attenuated live organisms may be explained by their ability to induce 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.

There is a long-felt and continuing need in the art for veterinary vaccines to protect cattle from *P. haemolytica* infection. There also is a need for techniques for introducing DNA into *P. haemolytica.*

SUMMARY OF THE INVENTION

It is an object of an aspect of the invention to provide methods for mutagenizing *P. haemolytica.*

It is another object of an aspect of the invention to provide a *P. haemolytica* gene for production of an enzyme for use in preparing genetic material for introduction into *P. haemolytica.*

It is yet another object of an aspect of the invention to provide an enzyme for use in preparing genetic material for introduction into *P. haemolytica.*

It is still another object of an aspect of the invention to provide a plasmid for unstable introduction of genetic material into *P. haemolytica.*

It is an object of an aspect of the invention to provide *P. haemolytica* mutant strains.

It is another object of an aspect of the invention to provide live, attenuated vaccines against *P. haemolytica* infection.

It is another object of the invention to provide genetically engineered *P. haemolytica.*

These and other objects of an aspect of the invention are provided by one or more of the embodiments described below. In one embodiment of the invention a method for site-directed mutagenesis of *P. haemolytica* is provided. The method comprises the steps of: isolating a DNA region from *P. haemolytica* in which region a mutation is desired; introducing a mutation into said DNA region to form a mutated DNA region; methylating said mutated DNA region with a methylating enzyme, to form methylated DNA, which methylated DNA is refractory to endonuclease cleavage at GATGC and GCATC sequences; introducing said methylated DNA into *P. haemolytica* to form transformants; and screening said transformants for those which have said mutation in said region on chromosomal DNA of said *P. haemolytica* cell.

In an alternative embodiment of the invention site-directed mutagenesis of *P. haemolytica* is accomplished by the steps of: isolating a DNA region from *P. haemolytica* in which region a mutation is desired; introducing a mutation into said DNA region to form a mutated DNA region; introducing said methylated DNA into a *P. haemolytica* cell which does not express a *Pha*I restriction endonuclease, to form transformants; and screening said transformants for those which have said mutation in said region on chromosomal DNA of said *P. haemolytica* cell.

In another embodiment of the invention an isolated and purified gene is provided. The gene encodes *Pha*I methyltransferase.

In still another embodiment of the invention another isolated and purified gene is provided. The gene encodes *Pha*I restriction endonuclease.

In yet another embodiment of the invention a preparation of *Pha*I methyltransferase is provided. The preparation is free from *Pha*I restriction endonuclease.

In still another embodiment of the invention a preparation of *Pha*I restriction endonuclease is provided. The preparation is free from *Pha*I methyltransferase.

In another embodiment of the invention a chimeric plasmid is provided which is suitable for unstable introduction of genetic material into *P. haemolytica*. The plasmid comprises a 4.2 kb *P. haemolytica* plasmid encoding a streptomycin resistance determinant deposited at the American Type Culture Collection as Accession No. ATCC 69499; and a plasmid which cannot replicate in *P. haemolytica*.

In an additional embodiment of the invention a *P. haemolytica* mutant is provided. The mutant is made by the process of the invention described in more detail below.

In another embodiment of the invention a *P. haemolytica* mutant is provided which does not express the *Pha*I restriction endonuclease.

In another embodiment of the invention a *P. haemolytica aro*A mutant is provided.

In still another embodiment of the invention a vaccine is provided. The vaccine comprises an attenuated, live, mutant of *P. haemolytica* which has an *aro*A mutation.

In yet another embodiment of the invention an isolated and purified *P. haemolytica* strain is provided. The strain has been genetically modified by the introduction of DNA.

According to one aspect of the invention, there is provided a method for producing a mutation in a particular region of DNA of a *Pasteurella haemolytica* genome:

isolating the region of the genome from *P. haemolytica;*

introducing a mutation into the region to form a mutated DNA region;

methylating the mutated DNA region with a methylating enzyme which inhibits endonuclease cleavage at a recognition sequence selected from the group consisting of 5'-GATGC-3' and 5'-GCATC-3', to form methylated DNA;

introducing the methylated DNA into a *P. haemolytica* cell to form transformants; and

screening the transformants for those which have the mutation in the region on chromosomal DNA of the *P. haemolytica* cell.

According to another aspect of the invention, there is provided an isolated and purified gene encoding *Pha*I methyltransferase which comprises the *Pha*I methyltransferase encoding nucleotide sequence of a plasmid deposited under ATCC Accession No. 69500.

According to a further aspect of the invention, there is provided an isolated and purified gene encoding *Pha*I restriction endonuclease which comprises the *Pha*I restriction endonuclease encoding nucleotide sequence of a plasmid deposited under ATCC Accession No. 69500.

According to another aspect of the invention, thes is provided a preparation of *Pha*I methyltransferase free from *Pha*I restriction endonuclease.

According to a further aspect of the invention, there is provided a preparation of *Pha*I endonuclease free from *Pha*I methyltransferase.

According to another aspect of the invention, there is provided a chimeric plasmid for unstable introduction of genetic material into *Pasteurella haemolytica* comprising two plasmids covalently linked to each other, wherein the first plasmid is a 4.2 kb Str^R plasmid of *P. haemolytica* deposited at the American Type Culture Collection as Accession No. ATCC 69499; and the second plasmid is a plasmid which cannot replicate in *P. haemolytica.*

According to a further aspect of the invention, there is provided a *Pasteurella haemolytica* strain NADC-D60aroA, deposited under ATCC Accession No.55518.

According to another aspect of the invention, there is provided a *Pasteurella haemolytica* strain which harbors a mutation which abolishes expression of *Pha*I restriction endonuclease.

According to a further aspect of the invention, there is provided a vaccine comprising an attenuated, live, mutant *of Pasteurella haemolytica,* which comprises an *aro*A mutation.

According to another aspect of the invention, there is provided a method for producing a mutation in a particular region of DNA of a *Pasteurella haemolytica* genome:

isolating the region of the genome from *P. haemolytica;*

introducing a mutation into the region to form a mutated DNA region;

introducing the mutated DNA region into a *P. haemolytica* cell which does not express a *Pha*I restriction endonuclease, to form transformants; and

screening the transformants for those which have the mutation in the region on chromosomal DNA of the *P. haemolytica* cell.

According to a further aspect of the invention, there is provided an isolated and purified non-naturally occurring *haemolytica* strain which has been genetically modified by the stable introduction of DNA wherein the introduced DNA has recombined with genomic DNA of *P. haemolytica*.

These and other embodiments of the invention provide the art with the means to construct desirable mutants of the economically important and previously intractable pathogen *P. haemolytica.*

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Determination of *Pha*I cleavage positions alongside that of *Sfa*NI Lanes 1 and 3 cut with *Pha*I*;* lanes 2 and 4 cut with *Sfa*NI. The cleavage products of

*Pha*I and *Sfa*NI migrated 0.5 bp faster than the corresponding sequence bands because the labeled primer for extension had a *5'* phosphate, whereas the primer for sequencing did not (Juarin et al., *Gene 39*:191-201 (1985)).

Figure 2. Protection against *Pha*I digestion by cloned *Pha*I methyltransferase. Lanes 1 and 2 plasmid pPhΔAroACm^R-pD80 from *E. coli* DH10B incubated without and with *Pha*I. Lanes 3 and 4 plasmid pPhΔAroACm^R-pD80 from *E. coli Pha*IMtase incubated without and with *Pha*I

Figure 3. Southern blot analysis of *P. haemolytica* strain NADC-D60 DNA digested with *Eco*RI lane 1, *Cla*I lane 2, *Pst*I lane 3, or *Hind*III lane 4. The membrane was hybridized with an *E. coli aro*A probe and washing was performed under low-stringency conditions.

Figure 4. Nucleotide sequence and deduced amino acid sequence of *P. haemolytica aro*A.

Figure 5. Construction of a *P. haemolytica aro*A mutant. The hybrid plasmid pPharoA-Amp^{R}pD70 was successfully used to produce an *aro*A mutant.

Figure 6. Southern hybridization of genomic DNAs from the parental strain, *P. haemolytica* strain NADC-D60, the *aro*A mutant, and *P. haemolytica* strain NADC-D70 and the hybrid plasmid pPharoA⁻Amp^{R}pD70. All the DNAs used in the blots shown here were digested with *Hin*dIII. **Figure 6A.** Lanes: 1, *P. haemolytica* strain NADC-D60; 2, *aro*A mutant; 3, pPharoA⁻Amp^{R}pD70 probed with *P. haemolytica aro*A. **Figure 6B.** Lanes: 1, *P. haemolytica* strain NADC-D60; 2, *aro*A mutant; 3, pPharoA⁻Amp^{R}pD70 probed with *P. haemolytica* Amp^R fragment. **Figure 6C.** Lanes: 1, *P. haemolytica* strain NADC-D70; 2, *aro*A mutant; 3, pPharoA⁻Amp^{R}pD70 probed with *P. haemolytica* Amp^R plasmid. **Figure 6D.** 1, *P. haemolytica* strain NADC-D60; 2, *aro*A mutant; 3, pPharoA⁻Amp^{R}pD70 probed with pBCSK. DNA was isolated from *P. haemolytica* strain NADC-D70 and run in Lane 1 of blot B to demonstrate that if plasmid DNA was present in the bacteria it would also be present in our DNA preparations.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

It is a discovery of the present invention that *P. haemolytica* contains at least one restriction-modification system, called herein the *Pha*I system. Both the restriction endonuclease and the methyltransferase have been molecularly cloned. One such molecular clone (*E. coli Pha*IMtase) has been deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, 20852, USA, on December 2, 1993, under the terms of the Budapest Treaty as Accession No. ATCC 69500. A preliminary sequence of the methyltransferase gene has been determined. The predicted amino acid sequence of the methyltransferase contains sequence motifs which are consistent with an adenine-methylating specificity.

Provided with the molecular clone of *PhaI*Mtase (Accession No. ATCC 69500) one of ordinary skill in the art can readily obtain a preparation of either or both enzymes free of other *P. haemolytica* proteins. A lysate of a non-*P. haemolytica* bacterium carrying one of the cloned enzymes would provide such a preparation. If one desires a preparation of each of the enzymes free of the other enzyme, one of skill in the art can readily subclone to separate the two genes. The methyltransferase gene has been cloned into a plasmid which when introduced into a cell produces *Pha*I methyltransferase but is free of the *Pha*I restriction endonuclease. The *Pha*I restriction endonuclease gene can be cloned on a plasmid free of the methyltransferase gene by introduction of the cloned gene into host cells which express either the *Pha*I or the *Sfa*NI methyltransferase.

Provided with *Pha*IMtase (ATCC Accession No. ATCC 69500) one of skill in the art can also readily obtain an isolated and purified gene encoding either or both the *Pha*I restriction and methyltransferase enzymes. Standard techniques, such as cesium chloride gradients, phenol and chloroform extractions, etc., can be used for purifying plasmid DNA from the deposited *E. coli* bacteria. The genes can be isolated together from the deposited bacteria, or they can be subcloned, as discussed above, to isolate the two genes from each other.

It has also been discovered by the present inventors, that a barrier to transformation of *P. haemolytica* can be overcome by treating DNA with a methylating enzyme, such as the *Pha*I methyltransferase. Such enzymes modify DNA substrates such that endonucleases which recognize 5′-GATGC-3′ or 5′-GCATC-3′ sequences are inhibited in their ability to digest such modified substrates. Examples of such endonucleases are *Pha*I endonuclease and *Sfa*NI endonuclease. While applicants do not wish to be bound by any particular hypothesis on the mechanism of action of such methyltransferase enzymes, it appears that the *Pha*I methyltransferase methylates specific adenine residues in DNA.

Methylation of DNA substrates for transformation (electroporation, or other means of introduction of DNA into cells) can be accomplished *in vitro* or *in vivo*. For *in vitro* methylation, DNA is incubated with a preparation of methyltransferase in the presence of a methyl donor, such as S-adenosylmethionine (SAM). *In vivo* methylation can be accomplished by passaging the DNA substrate through a bacterium which contains an appropriate methyltransferase, such as *Pha*I or *Sfa*NI methyltransferase. A mutant or natural variant of *P. haemolytica* which lacks the *Pha*I endonuclease could also be used to prepare DNA for subsequent introduction into *P. haemolytica*. Such a mutant can be made *inter alia* according to the method for site-directed mutagenesis disclosed herein.

Site-directed mutagenesis of *P. haemolytica* can be accomplished according to the present invention by first isolating a wild-type DNA region from *P. haemolytica*. As described below in the examples, an *aro*A gene can be isolated using *aro*A DNA from other bacteria as hybridization probes. The sequence of the *P. haemolytica aro*A gene is shown in SEQ ID NO. 3. Similarly other genes can be isolated from *P. haemolytica*. Another desirable gene for mutations is the *Pha*I endonuclease gene, which is provided in *Pha*IMtase (ATCC Accession No. ATCC 69500). Other genes in which mutations may be desirable are genes in the leukotoxin operon (C, A, B, D) and neuraminidase. A mutation is created in the isolated, wild-type DNA region according to any method known in the art. For example, the isolated DNA can be chemically mutagenized, either in a bacterium or *in vitro*. Alternatively, restriction endonucleases can be used to create precise deletions or insertions *in vitro*. Other methods as are known in the art can be used as is desirable for a particular application.

After *P. haemolytica* DNA has been isolated and mutagenized, it is methylated as described above. Then it can be introduced into *P. haemolytica* according to any technique known in the art, including but not limited to transfection, transformation, electroporation, and conjugation. Alternatively, rather than methylating the mutagenized DNA and introducing it into a *P. haemolytica* which expresses *Pha*I restriction endonuclease, one can omit the methylation of the mutagenized DNA and introduce the mutagenized DNA into a *P. haemolytica* cell which does not express the *Pha*I restriction endonuclease. Such cells can be isolated from nature by extensive screening, isolated following chemical mutagenesis of a cell which does express the *Pha*I restriction endonuclease, or made by the site-directed mutagenesis method disclosed herein.

According to one aspect of the invention, the mutagenized and methylated *P. haemolytica* DNA region is introduced into a *P. haemolytica* cell on a plasmid which includes a *P. haemolytica* approximately 4.2 kb streptomycin resistance determining plasmid (pD70). This plasmid has also been deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, 20852, USA, on December 2, 1993, under the terms of the Budapest Treaty as Accession

No. ATCC 69499. While applicants do not wish to be bound by any particular theory, it appears that the pD70 streptomycin resistance determining plasmid allows the introduced DNA to be replicated and maintained, albeit unstably, for a period of time sufficient to allow gene conversion (replacement of the chromosomal copy of the gene with the introduced mutant copy of the gene) to occur. Gene conversion can be monitored *inter alia* by Southern hybridization with probes to the gene of interest, by screening for genetic markers on the introduced DNA construct (such as ampicillin^R or streptomycin^R), and by screening for the presence/absence of plasmid in the transformed cells' progeny.

A chimeric plasmid, as described above, is provided which is suitable for the unstable introduction of DNA into *P. haemolytica*. The chimeric plasmid comprises the approximately 4.2 kb streptomycin resistance determining plasmid, pD70, as well as a plasmid which cannot replicate in *P. haemolytica* but can replicate in another cell type. To use such a chimeric plasmid, typically a region of the chromosome of *P. haemolytica* which has been mutagenized is ligated into the plasmid. Maintenance of the chimeric plasmid in *P. haemolytica* can be selected, for example by using an appropriate antibiotic to which the plasmid confers resistance. After a selected number of generations, antibiotic selection can be removed, and the cells tested to determine whether the introduced region of *P. haemolytica* has replaced the genomic copy.

Also provided by the present invention are mutant strains made by the disclosed method of site-directed mutagenesis. One such mutant (NADC-D60 *aro*A$^-$) has been deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, 20852, USA, on December 2, 1993, under the terms of the Budapest Treaty as Accession No. ATCC 55518. Such mutants can provide the veterinary arts with attenuated, live strains of *P. haemolytica* which are suitable for vaccines to induce protective immunity against *P. haemolytica* infection. For vaccine production, it is desirable that the mutation which attenuates the *P. haemolytica* be an essentially non-reverting mutation. Typically these are deletion or insertion mutations, the latter not being caused by a transposable element. Strains which contain multiple attenuating mutations may also be used, so that the risk of reversion to a wild-type, virulent *P. haemolytica* is vanishingly small.

Another mutant strain which can be made by the site-directed mutagenesis method disclosed is one which is *Pha*I restriction endonuclease negative. Such a strain is useful for genetic engineering in *P. haemolytica*. Such a strain can be a recipient of DNA which is not *Pha*I methyltransferase methylated, yet would yield DNA which is *Pha*I methyltransferase methylated.

The present invention thus allows those of ordinary skill in the art to stably introduce DNA into *P. haemolytica*. The DNA can be from other strains or species. The DNA can be artificially modified or in its native state. If recombination into the genome is desired two regions of flanking homology are preferred. Such techniques are generally known for other bacteria, but have been hitherto unsuccessful in *P. haemolytica* due to its restriction system.

Vaccines are typically formulated using a sterile buffered salt solution. Sucrose and/or gelatin may be used as stabilizers, as is known in the art. It is desirable that the *P. haemolytica* vaccines of the invention be administered by the intranasal or intratracheal route, but subcutaneous, intramuscular, intravenous injections also may be used. Suitable formulations and techniques are taught by Kucera U.S. 4,335,106, Gilmour U.S. 4,346,074, and Berget U.S. 4,957,739. Typically, between 10^7 and 10^{11} CFU are administered per dose, although from 10^5 to 10^3 CFU can be used. Adjuvants also may be added.

EXAMPLES

Example 1

This example demonstrates the isolation and characterization of the type IIs restriction endonuclease *Pha*I.

Bacterium, growth, and crude extract

Pasteurella haemolytica serotype 1, strain NADC-D60, was grown 16 hours on 4 Columbia blood agar base plates (100ml total volume, Difco, Detroit, MI) without supplemental blood. The cells were harvested in TE (10mM Tris, 1mM EDTA, pH 8.0), pelleted by centrifugation at 16,000 G for 5 minutes at

4°C, and washed once in TE. The washed pellet was resuspended in 1.5 ml chromatography running buffer (20mM $NaPO_4$, 10mM 2-mercaptoethanol, pH 7.5, 4 C) and placed on ice. The bacterial cells were disrupted by sonication for 2 minutes in 15 second bursts. Debris and unbroken cells were removed by centrifugation at 16,000 G for 10 minutes and then filtration of supernatant through a 0.45 um HA membrane. No further treatment of the crude extract was performed prior to chromatography.

Chromatographic separation of proteins

All chromatographic procedures were performed at room temperature. Prepacked heparin-sepharose columns [Econopac heparin columns, Bio-Rad, Richmond, CA] were equilibrated as recommended by the manufacturer. A flow rate of 0.5 ml/minute was used for separation, controlled by 2 HPLC pumps and a controller [Beckman Instruments, Inc, Fullerton, CA]. One ml of crude extract was injected and 10 ml of running buffer was used to wash the column. A linear gradient from 0 to 0.5 M NaCl in 60 ml of running buffer was used to elute proteins. The column was washed with 2M NaCl in running buffer at 2.0 ml/minute as recommended by the manufacturer, then re-equilibrated to initial conditions of 0 M NaCl in running buffer prior to additional runs. Fractions (1.0 ml) were stored on ice prior to activity assay, then frozen at -20°C.

Assay for restriction endonuclease activity

Aliquots, 5 μl, of the chromatographic fractions were incubated with 1 μl 12 mM MgCl and 0.25 μg unmethylated bacteriophage lambda DNA (New England Biolabs) at 37°C for 2 hours. After addition of tracking dye, and electrophoresis on a 1% agarose gel in TBE buffer, the banding patterns were visualized by ethidium bromide staining and UV illumination. The active fractions (6ml) were pooled, concentrated 10-fold on 30,000 MW cutoff ultrafilters, and brought to final concentrations of 150 mM NaCl, 10 mM $NaPO_4$, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 0.25 μg/ml BSA, and 50:50 vol:vol glycerol [pH 7.5] for storage at -20°C.

<u>Determination of the recognition sites for *Pha* I</u>

The recognition sequence was identified using digestion of pBluescript™ (Stratagene, LaJolla, CA), which resulted in 4 fragments of approximate size 1476, 1057, 252, and 184 base pairs. Double digestion with *Pha*I and either *Xho*I or *Sac*I, which cut at opposite ends of the polylinker, showed that one *Pha*I site mapped at approximately nucleotide 1245, and another at 2735. Additional double digestions with *Ava*II, *Bgl*II, *Dra*II, *Pvu*I and *Sca*I were used to map the remaining 2 *Pha*I sites at approximately nucleotides 2300 and 2490, consistent with the sequences 5'-GATGC-3' and 5'GCATC-3'. Further confirmation was made with *Pha*I digests of ΦX174 and pUC19 DNA, and by sequencing pBluescript *Pha*I fragments filled in and cloned into pBluescript.™ Single-stranded ΦX174 DNA was digested to determine if *Pha*I has activity on this substrate.

<u>Determination of the cleavage sites for *Pha* I</u>

The cleavage site was identified by digestion of a primed-synthesis reaction on pBluescript derivatives (Brown et al. (1980) J. Mol. Biol. 140:143-148). An oligonucleotide containing the *Pha*I site was annealed and ligated with *Sma* I-cleaved pBluescript SK+ and SK-DNA. Single-stranded DNA containing each orientation was selected and used for the template. Four standard dideoxy DNA sequencing reactions were performed for each template with an appropriate primer. Additional reactions containing no dideoxy terminator were extended through the *Pha*I site with the Klenow fragment of DNA polymerase I using ^{32}P-endlabelled primer with both templates. The extension reaction was stopped by chloroform extraction followed by ethanol precipitation. *Pha*I or *Sfa* NI endonuclease was added to the additional reactions and allowed to digest the DNA for 2 minutes. The reaction was stopped by addition of gel loading buffer and heating to 80°C for 3 minutes.

A new restriction endonuclease, *Pha*I, an isochizomer of *Sfa*NI (Roberts (1990) Nucl. Acids Res. 18 (Suppl.), 2331-2365), was isolated from *Pasteurella haemolytica* serotype 1, strain NADC-D60, obtained from pneumonic bovine lung. *Pha*I recognizes the 5 base non-palindromic sequence 5'-GCATC-3' and

5′-GATGC-3′. Cleavage occurs five bases 3′ from the former recognition site and nine bases 5′ from the latter recognition site.

Under our experimental conditions, endonuclease activity was eluted from heparin-sepharose columns by 275 to 325 mM NaCl. A single pass through these columns was sufficient to allow identification of both the DNA recognition specificity and cleavage site. Approximately 5000 units of *Pha*I per gram of wet cells were recovered. In contrast to *Sfa*NI, optimal conditions for *Pha*I digestion required NaCl or KCl concentrations below 50 mM; >50% reduction in activity was observed at the 100 mM NaCl optimum of *SfaI*NI.

Digests of pBluescript resulted in 4 fragments of approximate size 1476, 1057, 252 and 184 bp. Double digestion with *Pha*I and either *Xho*I or *Sac*I mapped 2 *Pha*I sites, one at approximately nucleotide 1245, and another at 2735 of pBluescript. Additional double digestions with *Pha*I and each of *Ava*II, *Bgl*I, *Dra*I, *Pvu*I, or *Sca*I mapped the remaining 2 *Pha*I sites at approximately nucleotides 2300 and 2490, consistent with the sequences 5′-GATGC-3′ and 5′-GCATC-3′. Digests of pUC19, and ΦX174 confirmed the recognition specificity of 5′-GCATC-3′, which is the same as that of *Sfa*NI. Double digests of pBluescript with *Pha*I and *Sfa*NI resulted in patterns identical to those using either enzyme alone. DNA containing the recognition sequence 5′-GATGC-3′ cut 9 nucleotides 5′ to the end of the recognition site with both *Pha*I and *Sfa*NI. (Figure 1, lanes 1 and 2) DNA containing the recognition sequence 5′-GCATC-3′ cut 5 nucleotides 3′ to the end of the recognition site with both *Pha*I and *Sfa*NI. (Figure 1, lanes 3 and 4)

5′...GCATCNNNNN↓NNNNN...3′

3′...CGTAGNNNNN NNNNN↑...5′

These data confirm that *Pha*I is a true isoschizomer of *Sfa*NI. *Pha*I like *Sfa*NI is a type IIs enzyme (Roberts, *Nucleic Acids Res. 18*:2331-2365 (1990)). The type IIs restriction enzymes, like the more common type II restriction enzymes, recognize specific sequences and cleave at predetermined sites. Type IIs enzymes, however, neither recognize palindromic sequences nor cleave internally to the recognition sequence (Szybalski, *Gene 100*:13-26 (1991)).

Example 2

This example demonstrates the molecular cloning of *Pha*I endonuclease and methyltransferase.

Cosmid Library Construction

High-molecular weight DNA for cosmid cloning was prepared by the large scale DNA isolation method described for gram-negative bacteria in Ausabel et al. (*Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley Interscience, NY, NY (1987)). Approximately 100 μg of *P. haemolytica* strain NADC-D60 genomic DNA was digested with 100U of *Apo*I in NEB buffer #3 at 50°C for 10 minutes. Following digestion, the DNA was phenol-chloroform extracted and ethanol precipitated. The DNA was resuspended in 100 μl TE and layered onto a linear gradient of 10-40% sucrose (Schwartz-Mann Ultrapure) in 10 mM Tris HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0. After centrifugation in a SW40 (Beckman Inst.) at 20,000 RPM for 20 hr, gradient fractions were collected and restriction fragments of approximately 30 kb in length were ligated into *Eco* RI-digested calf alkaline phosphatase-treated cosmid vector pLAFRX (Ausabel, *supra*). A standard ligation mixture contained 1 μg vector, 3 μg *P. haemolytica* DNA and 5 Weiss U of T4 ligase in a volume of 10 μl. The ligation mixture was incubated at 10°C for 16 hr. The DNA was packaged using Promega packaging extract (Promega, Madison, WI) according to the manufacturers' recommendations. *E. coli* HB101 transduced with the recombinant cosmid library were plated on 2XYT plates containing 10 μg/ml tetracycline. Cloning efficiencies were approximately 10^4 recombinant colonies per μg of genomic DNA.

Cloning of *Pha*I endonuclease and methyltransferase gene

Approximately 1 μg of the recombinant *P. haemolytica* cosmid library was digested with *Pha*I restriction enzyme. The digested DNA was phenol-chloroform-isoamyl alcohol-extracted, ethanol precipitated, and resuspended in TE buffer. The DNA was electroporated into *E. coli* AP1-200-9 (Piekarowicz et al., *Nucl. Acids Res. 19*:1831-1835 (1991)) and the cells were plated on LB-broth plates containing 20 μg/ml tetracycline and 35 μg/ml Xgal. The transformed cells were incubated at 42°C for 18 hours and transferred to 30°C for 4 hours. The cells were moved again to 42°C and blue colonies, indicating the presence of a cloned methyltransferase gene, were isolated and analyzed. The colonies were screened for restriction endonuclease activity by the technique of Schleif (*Method in Enzymology,* vol. 65, part I, pp. 19-23 (1980)). Double-stranded DNA mini-preps isolated from restriction endonuclease-positive colonies were analyzed for resistance to digestion by *Pha*I. Recombinant colonies resistant to *Pha*I digestion were presumed to contain a *Pha*I methyltransferase gene. Cosmid DNA from these cells was electroporated into *E. coli* DH10B (BRL, Gaithersburg, Maryland) and the cells were plated on LB-broth plates containing 20 μg/ml tetracycline. The transformants containing the *Pha*I methyltransferase gene were designated *E. coli* strain *Pha*IMtase.

After digestion with *Pha*I and transformation of AP1-200-9 strain of *E. coli*, fifteen cosmid clones of *P. haemolytica* genomic DNA were tested for endonuclease activity. The nine clones which were endonuclease-positive were tested for *Pha*I methyltransferase activity. All nine expressed methyltransferase activity in addition to endonuclease activity, as evidenced by resistance to digestion by *Pha*I of genomic DNA recovered from transformed *E. coli*. The selective recovery of clones containing functional methyltransferase was due to previous digestion of the cosmid library with *Pha*I prior to transformation of *E. coli*. Recovery of clones containing both *Pha*I endonuclease and methyltransferase activity is not surprising since restriction and modification enzymes have previously been shown to be closely linked (the proximity of such genes has obvious implications to gene inheritance and the survival of the organism). The AP1-200-9 strain of *E. coli* (used to screen the cosmid library in this experiment) was designed by Piekarowicz et al., to give color selection for DNA-modifying enzymes (genes). The *mrr* and *mcr* systems, with a temperature-sensitive phenotype, induce inducible locus of the SOS response allows for color selection. All the transformants were blue after incubation at the permissive temperature for the *mcr/mrr* systems. Recovery of clones containing both *Pha*I endonuclease and methyltransferase activity is not surprising since restriction and modification enzymes have previously been shown to be closely linked (the proximity of such genes has obvious implications to gene inheritance and to the survival of the organism). (Wilson et al., *Annu. Rev. Genet.* *25*:585-627 (1991).)

Example 3

This example demonstrates the construction and methylation of a hybrid shuttle vector for introduction of DNA to *P. haemolytica*.

The following hybrid DNA construct was generated during attempts to introduce site-directed mutations into *P. haemolytica*. The *aro*A gene of *P. haemolytica*, contained on a *Hin*dIII-*Acc*I fragment of genomic DNA from strain NADC-D60, was ligated into the *Hin*dIII-*Acc*I site of pBluescript. A 700 bp fragment was excised from the coding region of the *aro*A gene by double digestion with *Nde*I and *Sty*I. Following digestion, the fragment ends were made blunt by treatment with the Klenow fragment of *E. coli* polymeraseI and deoxynucleoside triphosphates. The deleted plasmid was excised from a 1% agarose gel and electroeluted. The eluted DNA, designated pPhΔaroA2, was phenol-chloroform extracted and ethanol precipitated. The fragment was resuspended in TE buffer and ligated with the Cm^R gene isolated from pBR325. The Cm^R gene was excised from pBR325 by double digestion with *Aat* II and *Cla*I and the fragment was made blunt and purified by the above methods. The Cm^R fragment ligated with pPhΔaroA2 was given the designation pPhΔaroACm^R. Transformation of *E. coli* DH10B with pPhΔaroACm^R conferred Cm^R to the bacterium.

The hybrid plasmid pPhΔaroACm^RpD80 was constructed by ligating *SmaI* digested pPhΔaroACm^R with *Sca*I digested pD80 (4.2 kb amp^R plasmid from *P. haemolytica* serotype 1 strain NADC-D80). The resultant hybrid plasmid, approximately 11 kb in size, contained a ColE1 and *P. haemolytica* ori, amp^R, and Cm^R.

For methylation, the hybrid plasmid was electroporated into *E. coli* strain DH10B with or without a cosmid containing cloned *Pha*I methyltransferase gene. Plasmid DNA was isolated and purified by CsCl gradient centrifugation. *Pha*I methyltransferase-treated hybrid plasmid was electroporated into *P. haemolytica* strain NADC-D60 and then was reisolated by the above procedures. Plasmid DNA was reisolated from an ampicillin-resistant *P. haemolytica* transformant by the above procedures. The isolated plasmid DNA was tested for resistance to PhaI digestion as shown in Figure 2.

Example 4

This example demonstrates that methylated DNA, but not unmethylated DNA, is able to transform *P. haemolytica.*

Pasteurella haemolytica strain NADC-D60 was grown in 250 ml Columbia broth (Difco) 3 hours at 37°C with shaking to late logarithmic phase. The bacteria were centrifuged at 5000 G 15 minutes and the pellet resuspended in 272 mM sucrose at 0°C. The bacteria were washed 4 times in 272 mM sucrose with 5 minute centrifugation at 16,000 G and finally suspended at 50:50 vol:vol packed bacteria:272 mM sucrose on ice. Competent bacteria (100 μl) were mixed with 1 μg hybrid plasmid DNA (harvested from three sources: *E. coli* DH10B with methyltransferase (*Pha*IMtase); *E. coli* DH10B without methyltransferase; *P. haemolytica* NADC-D60) in 3 separate 1mm electroporation cuvettes (Bio-Rad), plus a fourth no-DNA control. The cells were quickly electroporated after addition of DNA (Bio-Rad Gene pulser) at 1500 V, 800 ohm, 25 uFd with resultant time constants ranging from 7.8 to 8.9 msec. Columbia broth (1ml, 0°C) was immediately added to the electroporated cells and the suspension was kept on ice approximately 10 minutes. The electroporated cells were allowed to recover at 37°C with gentle shaking for 1 hour, then broth containing 20 μg/ml ampicillin was added to bring the final ampicillin concentration to 10 μg/ml and the cells were incubated an additional hour at 37°C with shaking. Ten-fold dilutions were plated in duplicate onto blood agar plates containing 5% bovine blood and 10 μg/ml ampicillin. Undiluted cells electroporated with hybrid plasmid obtained from *E. coli* containing *Pha*I methyltransferase were plated on 2 μg/ml chloramphenicol after the first hour of recovery. Colonies were enumerated after overnight incubation at 37°C and representative colonies were checked for plasmid content.

Hybrid plasmid (pPhΔaroACm^{R}pD80) passed through *E. coli* containing *Pha*I methyltransferase in a cosmid was able to transform *P. haemolytica* serotype 1. The hybrid plasmid was stably maintained through multiple passages under selective pressure. Whereas DNA not exposed to *Pha*I methyltransferase was unable to transform *P. haemolytica*, DNA methylated by *Pha*I methyltransferase in *E. coli* yielded 10^3 transformants per μg plasmid (Table 1). Plasmid DNA passed through *P. haemolytica* yielded 10^5 transformants per μg plasmid. This experiment demonstrates that the restriction-modification system of *Pha*I is important for introduction of exogenous DNA into *P. haemolytica* serotype 1.

The plating efficiency of transformants was 2 logs lower on chloramphenicol than on ampicillin. All transformants recovered, however, were resistant to both ampicillin, and chloramphenicol upon passage.

The possibility that a system similar to *E. coli mcr, mrr*, is active in *P. haemolytica* was investigated by passage of pPhΔroACm^{R}pD80 through *E. coli* strain GM2163 previously transformed with the recombinant cosmid containing *Pha*I methyltransferase (Raleigh et al., *Proc. Natl. Acad. Sci. 83*:9070-9074 (1986)). Since strain GM2163 is dam-, the resultant DNA would only be modified at *Pha*I sites (Marinus et al., *Mol. Gen. Genet. 192*:288-289 (1983)). Efficiency of transformation with this DNA, however, was not substantially different than that using DNA obtained from *Pha*I Mtase which is dam-methylated (Table 1). It is possible a second restriction system, not readily detectable in cell extracts, is active in *P. haemolytica* A1. Genes have been described in *Neisseria gonnorhea* MS11 which encode for restriction enzymes which are expressed at levels too low to detect biochemically (Stein et al., *J. Bact. 74*:4899-4906 (1992).

Table 1. Transformation efficiency of *P. haemolytica* NADC-D60 with hybrid plasmid pPhΔaroACm[r]pD80 purified from various sources[a].

Source of DNA[b]	Amp[R] transformants[c] CFU/μg DNA	Cm[R] transformants[d] CFU/μg DNA
E. coli DH10B	0	nd[e]
*E. coli Pha*IMtase	$1x10^3$	5
E. coli GM2163	$5x10^2$	nd
P. haemolytica NADC-D60	$1x10^5$	nd

[a] One μg DNA introduced by electroporation using same competent cell preparation.

[b] Purified by CsCl-EtBr gradient centrifugation.

[c] Colonies on plates containing 10 μg/ml ampicillin, cells recovered 2 hours prior to plating.

[d] Colonies on plates containing 2 μg/ml chloramphenicol, cells recovered 1 hours prior to plating.

[e] Not done.

This experiment demonstrates that the restriction-modification system of *Pha*I plays an important role in the difficulties researchers have encountered in their attempts to introduce exogenous DNA into *P. haemolytica* serotype 1. Protection against *Pha*I activity may allow genetic manipulation of this organism, which could lead to dramatic improvements in our understanding of pathogenesis and control of pneumonic pasteurellosis in cattle.

Example 5

This example demonstrates the molecular cloning and sequencing of *P. haemolytica aro*A.

Cloning of *P. haemolytica aro*A. Restriction fragments of *P. haemolytica* genomic DNA were fractionated by agarose gel electrophoresis. The fragments were probed for homology to a 1.3 kb *E. coli aro*A fragment 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 aro*A (Fig. 3). The *Hin*dIII fragment was isolated from an agarose gel by electroelution and it was cloned into *Hin*dIII digested pSK. The recombinant plasmid, pPharoA1, bearing *P. haemolytica aro*A was identified by complementation of *E. coli aro*A mutant AB2829 on M9 minimal media containing ampicillin. A *Cla* I, *Hin*dIII double digest of pPharoA1 generated a 2.2-kb fragment which was cloned into the *Acc*I and *Hin*dIII sites of pSK- giving rise to pPharoA2. Plasmid pPharoA2 also complemented growth of *E. coli* AB2829 on M9 minimal media. This plasmid was used to determine the sequence of *P. haemolytica aro*A.

Southern Blotting and Molecular Cloning of *P. haemolytica aro*A Methods. *P. haemolytica* genomic DNA was prepared by the method for isolating DNA from gram-negative bacteria. Southern blotting of *P. haemolytica* restriction fragments fractionated by electrophoresis on a 0.75% agarose was performed as described previously. Blots were hybridized with a radioactively labeled 1.3-kb *E. coli aro*A fragment. The *aro*A probe was amplified (Gene-AMP by Perkin Elmer Inc., Branchburg, N.J.) from *E. coli* X-L1 Blue (Stratagene, Inc. S.D. CA) genomic DNA using PCR. The primers: 5'-TTCATGGAATCCCTTGACGTTACAACCCATC-3' and 5'-AGGCTGCCTGGCTAATCCGCGCCAG-3' used in the PCR reaction hybridize with *E. coli aro*A nucleotides -3 through 28 and 1308 through 1323 respectively. The primers were synthesized using an Applied Biosystems oligonucleotide synthesizer (Applied Biosystems Inc.) by the Nucleic Acids Facility, Iowa State University (Ames, IA). DNA was radiolabeled with [α-^{32}P] dCTP using a random priming kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Nylon membranes (Hybond-N, Amersham Corp., Arlington Heights, IL) were incubated with hybridization solution 5X SSC (1X SSC is 0.15M NaCl and 0.015M sodium citrate), 5X Denhardts solution (Maniatis, *Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982)), 0.1% SDS, 10 μg/ml sonicated salmon sperm DNA, containing 1x10^7 CPM of ^{32}P-labeled probe and 50% formamide at 42°C. After hybridization for 18 hours, membranes were washed twice with 1X SSC and 0.1% SDS for 10 minutes at RT and two times with 1X SSC and 0.1% SDS buffer at 42°C for 15 minutes. Membranes were exposed to X-AR (Eastman Kodak Co., Rochester, NY) at -80°C for 24 hours. A positive signal corresponding to a 3.2-kb *Hind*III fragment of *P. haemolytica* chromosomal DNA was identified.

*Hind*III digested *P. haemolytica* DNA fragments ranging from 3.0-3.4 kb in length were electroeluted from a 1% agarose gel. The *Hind*III genomic fragments were added to *Hind*III digested alkaline phosphatase treated pSK-vector (Stratagene, Inc. S.D. CA) and ligated overnight at 10°C with T4 ligase (BRL, Gaithersburg, MD). The ligation mix was diluted 1:10 with distilled water and electroporated using a Gene Pulser (Bio-Rad Laboratories, Richmond, CA) into *E. coli aro*A mutant AB2829 (Pittard et al., *J. Bact. 91*:1494-1508 (1966)). A recombinant plasmid, pPharoA1, complemented AB2829 grown on M9 minimal media 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/ml ampicillin (Ausubel et al., *supra*). A *Cla*I, *Hind*III double digest of pPharoA1 produced a 2.2 kb fragment which when cloned into the *Acc*I, *Hind*III sites of pSK- gave rise to pPharoA2. The recombinant plasmid, pPharoA2, which also complemented growth of AB2829 on minimal plates, was used to sequence *P. haemolytica aro*A.

Nucleotide sequence of *P. haemolytic aro*A. The nucleotide sequence and the deduced amino acid sequence of *P. haemolytica aro*A are shown in Fig. 4 and SEQ ID NOS: 3 and 4. An open reading frame of 1302 bases with a coding capacity of 434 amino acid residues was discerned. The deduced molecular weight is 47,296 and the G+C content of the *aro*A coding region is 43%. The predicted amino acid sequence of *P. haemolytica aro*A showed 75, 70, 69, and 68% identity with *Pasteurella multocida, Klebsiella pneumoniae, Yersenia entercolitica,* and *Escherichia coli*, respectively.

*P. haemolytica aro*A, like *P. multocida aro*A (Homchampa et al. *Molec. Microbiol. 23*:3585-3593 (1992)), appears to be transcribed from its own promoter. This differs from the usual genetic arrangement in gram-negative bacteria where *aro*A and *ser*C constitute an operon with *aro*A distal to the promoter. Evidence to support this claim are the findings that: (1) the nucleotide sequence upstream of *aro*A on clone pPharoA2 shows no homology with *ser*C genes and (2) complementation of *E. coli* AB2829 by *P. haemolytica aro*A contained on the 2.2 kb fragment is independent of the fragment's orientation on the cloning vector.

DNA sequencing and Analysis. DNA sequencing was done by the dideoxy nucleotide termination method with single or double stranded templates using the Sequenase™ 2.0 kit (United States Biochemicals, Cleveland, OH). A series of ordered deletions were made in *P. haemolytica aro*A contained on pPharoA2 using an Erase-a-base kit (Promega Corp. Madison, WI). Gaps in the sequence were completed using DNA primers synthesized by the DNA core facility at Iowa State University (Ames, IA). DNA sequence analysis was done with MacDNASIS Pro (Hitachi Software Ltd., San Bruno, CA) and MacVector (Kodak Co., New Haven, CT) software.

Example 6

This example demonstrates the construction of a defined *P. haemolytica aro*A mutant.

Construction of a *P. haemolytica aro*A mutant. The deletion plasmid, pPhΔaroACm^R (Table 2), was constructed from pPharoA2 as described above and amplified in *E. coli* containing a cosmid clone carrying the *Pha*I methyltransferase gene on a 20-kb *P. haemolytica* DNA fragment. Although resistant to *Pha*I endonuclease digestion, introduction of pPhΔaroACm^R into *P. haemolytica* strain NADC-D60 by electroporation failed to generate Cm resistant colonies. The inability to transform *P. haemolytica* with pPhΔaroACm^R suggested that plasmids containing a ColE1 origin do not replicate in this bacterium.

Table 2. Bacterial strains and plasmids used

Strains	Characteristics	Source/Reference
E. coli		
AB2829	K-12 strain with mutation in *aroA*	Pittard and Wallace (1966)
DH10B	Cloning strain used in this work	BRL
XL 1-Blue	Strain used for DNA sequencing	Stratagene
P. haemolytica		
NADC-D60	Serotype 1 plasmidless	NADC/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
pBCSK	cloning vector (Cm^R)	Stratagene
pD70	4.2 kb plasmid encoding $streptomycin^R$	NADC/R. Briggs
pD80	4.2 kb plasmid encoding Amp^R	NADC/R. Briggs
pPharoA1	3.2 kb *Hin*dIII fragment containing *P. haemolytica* aroA (pSK)	This work
pPharoA2	*Hin*dIII *Cla*I digest of pPharoA1 resulted in 2.2 kb *aroA* fragment (pSK)	This work
pPharoA3	same insert as pPharoA2 on pBCSK	This work
pPhΔaroACm^R	*Sty*I *Nde*I digest of pPharoA2 Cm^R fragment inserted into deletion site	This work
pPhΔaroACm^{R}pD80	*Sma*I digested pPhΔaroACm^R joined to *Sca*I digested pD80	This work
pPhAmp^R	2.2 kb *Sau* 3A fragment of pD80 cloned into pBCSK	This work
pPharoA·Amp^R	Amp^R fragment of pD80 inserted into unique *Nde*I site of pPharoA3	This work
pPharoA·Amp^{R}pD70	*Hin*dIII digested pPharoA·Amp^R joined to *Hin*dIII digested pD70	This work

Since we have shown that the *Pha*I methylated hybrid plasmid consisting of plasmids pPhΔaroACm^R joined with *P. haemolytica* pD80 (Amp^R) could be used to transform *P. haemolytica* strain NADC-D60 (see above), we investigated the possibility that *aro*A mutants might arise after transformation with the hybrid plasmid by recombination with the genomic copy of the *aro*A gene, i.e., "replacement" of the gene. *P. haemolytica* harboring the hybrid plasmid pPhΔaroACm^{R}pD80 were passed for >100 generations in Columbia broth without antibiotics and plated onto blood-agar plates. The colonies were then replica plated onto blood-agar plates containing 5 μg/ml ampicillin. All colonies had an Amp^R phenotype, suggesting that the plasmid was stable in *P. haemolytica*. This was confirmed by Southern blot analysis which showed that intact plasmid was present in all the Amp^R colonies that were analyzed.

Because the number of *P. haemolytica* transformants generated with hybrid plasmid pPhΔaroACm^{R}pD80 (Amp^{R}Cm^R) was 100-fold greater with plasmid isolated from *P. haemolytica* (10^5 CFU/μg DNA) than from *E. coli* containing the *Pha*I methyltransferase gene (see above), we reasoned that a replacement plasmid isolated from *P. haemolytica* would be resistant to enzymatic digestion upon reintroduction into *P. haemolytica*, and thus more likely to give rise to mutants via homologous recombination. The improved efficiency is presumed to be the result of DNA modifications in *P. haemolytica* in addition to that of *Pha*I methylation.

With this in mind, hybrid plasmid pPhΔaroACm^R pD80 was isolated from *P. haemolytica* strain NADC-D60 and CsCl purified by the methods described previously. The hybrid plasmid was digested with *Hin*dIII and *Xba*I to produce two fragments consisting of pD80 and pPhΔaroACm^R . Linear deletion plasmid, pPhΔaroACm^R, was isolated by electroelution and purified using "GlassMAX™" beads (BRL, Gaithersburg, MD). Approximately 5 μg of the linear plasmid was electroporated into *P. haemolytica* NADC-D60. The cells were recovered in 1 ml Columbia broth and shaken at 37°C for 1 hour prior to plating on Blood-agar plates containing 10 μg/ml chloramphenicol. No Cm^R colonies were detected after incubation at 37°C for 48 hours. However, this result was not totally unexpected since there have been few reports of the successful establishment of linear DNA into bacteria.

Five μg of linearized pPhΔaroACm^R, isolated from *P. haemolytica*, was treated with Klenow and deoxynucleoside triphosphates to produce blunt ends. The DNA was then ligated with T4 ligase overnight to form a circular replacement plasmid. The plasmid was phenol chloroform extracted, ethanol precipitated, resuspended in distilled water, and reintroduced into *P. haemolytica* by electroporation. The cells were transferred to Columbia broth and allowed to recover for 1 hour. The cells were spread on blood-agar plates containing antibiotic and incubated at 37°C for 48 hours. This experiment also failed to generate Cm^R *P. haemolytica* colonies.

Additional efforts to produce an *aro*A mutant resulted in construction of a new replacement plasmid in which *aro*A was insertionally inactivated by the *P. haemolytica* β-lactamase gene. This antibiotic resistance cassette was chosen to select gene replacement candidates because we had found that survival of *P. haemolytica* transformed with pPhΔaroACm^{R}pD80 was approximately 100-fold greater (10^3 CFU/μgDNA) on blood-agar plates containing ampicillin than on blood-agar plates containing chloramphenicol.

Molecular cloning of *P. haemolytica* β-lactamase gene was done as follows. Purified pD80 was partially digested with *Sau3A*, phenol-chloroform extracted, and ethanol precipitated. The fragments were resuspended in T.E. and ligated overnight into *BamHI*-digested pBCSK (Stratagene Inc., La Jolla, CA). The ligated mixture was diluted 1:10 with water and electroporated into *E. coli* DH10B. The cells were recovered in 1 ml SOC for 1 hour and spread on LB-plates containing 50 μg/ml ampicillin and 20 μg/ml chloramphenicol. Restriction enzyme analysis on plasmid isolated from an ampicillin, chloramphenicol resistant *E. coli* clone revealed a 2.2 kb *P. haemolytica* insert in pBCSK. This plasmid was designated pPhAmp^R. To demonstrate that pPhAmp^R did not possess the pD80 origin of replication, the plasmid was amplified in *E. coli* DH10B which also contained the *Pha*I methyltransferase clone. Plasmid pPhAmp^R was isolated from *E. coli* as described previously, CsCl purified and introduced into *P. haemolytica* by electroporation. Since this plasmid did not confer ampicillin resistance to *P. haemolytica* strain NADC-D60, we concluded that the antibiotic resistant fragment did not contain the pD80 origin of replication and that the fragment encoding the β-lactamase gene could be used to construct a deletion plasmid.

Construction of the deletion plasmid involved the following. The β-lactamase gene was excised from pPhAmp^R by *Hind*III, *Xba*I digestion and treated with Klenow and deoxy-ribonucleotides to generate blunt ends. The β-lactamase gene was ligated into the Klenow treated unique *Nde*I site of pPharoA3 (Fig. 5) to produce pPharoA$^-$Amp^R. Insertional inactivation of *aro*A on pPharoA$^-$amp^R was demonstrated by failure of the plasmid to complement AB2829. Plasmid pPharoA$^-$Amp^R was amplified in *E. coli* DH10B (BRL) which also contained the recombinant cosmid carrying *Pha*I methylase recombinant cosmid. Although *Pha*I methylated pPharoA$^-$Amp^R was resistant to digestion by *Pha*I, introduction of this plasmid into *P. haemolytica* failed to generate ampicillin resistant colonies.

To increase the likelihood of allelic replacement between the deletion plasmid's inactivated *aro*A and *P. haemolytica* chromosome, we constructed an *aro*A$^-$ mutant-hybrid plasmid consisting of pPharoA$^-$Amp^R and a 4.2-kb *P. haemolytica* plasmid (pD70, which confers streptomycin resistance (Sm^R)) (Fig. 5). The Sm^R plasmid was isolated from *P. haemolytica* using methods described previously. The str^R plasmid was digested at a unique *Hind*III site and ligated with *Hind*III digested pPharoA$^-$Amp^R. The resultant hybrid plasmid, pPharoA$^-$Amp^{R}pD70 (Fig. 5), was *Pha*I methyltransferase modified in *E. coli* DH10B containing the cosmid clone of the *Pha*I methylase gene. The hybrid plasmid was isolated from *E. coli*, CsCl purified and introduced into *P. haemolytica* strain NADC-D60 by electroporation. The cells were resuspended in Columbia broth for 2 hours at 37°C and spread on blood-agar plates containing 10 μg/ml ampicillin. Transformation efficiency of the hybrid plasmid yielded approximately 10^1 ampicillin resistant colonies/μg DNA. Eight Amp^R colonies were grown overnight in Columbia broth containing 1 μg/ml ampicillin. Chromosomal DNAs from the parental strain and from the Amp^R colonies were digested with *Hind*III and probed by Southern blotting with *P. haemolytica aro*A, pBCSK, and pD70. The results indicated that intact pPharoA$^-$Amp^{R}pD70 was present in the Amp^R colonies.

Eight Amp^R clones were grown overnight in Columbia broth containing 1 μg/ml ampicillin. Chromosomal DNAs from the parental strain and from the Amp^R clones were digested with *Hind*III and analyzed by Southern blotting with *P. haemolytica aro*A, pBCSK, and pD70 radio-labeled probes. The results indicated that intact pPharoA$^-$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/ml ampicillin 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/ml ampicillin or 1 μg/ml chloramphenicol. Two of the eight survived on the broth containing ampicillin, none on chloramphenicol. Passage from ampicillin broth onto blood-agar plates containing either ampicillin or chloramphenicol or streptomycin confirmed 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 (Wessman, *Applied Microbiol. 14*:597-602 (1966)). 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 the parent strain. The *E. coli aro*A mutant AB2829 also required tryptophan to grow on the chemically-defined medium for *P. haemolytica* cultivation. DNAs from the two colonies possessing Amp^R, Cm^S, Sm^S,*aro*A$^-$ phenotypes were analyzed by Southern blotting. The results indicated that both had insertionally inactivated *aro*As. Moreover, Southern blotting also confirmed that both pD70 and pBCSK sequences were no longer present in the *aro*A mutants (Figure 6).

Construction methods for *P. haemolytica* mutants. The 4.2 kb ampicillin resistance encoding plasmid of *P. haemolytica* (pD80) was partially digested with *Sau3A* and ligated into the *BamHI* site of pBCSK$^+$ (Cm^R) (Stratagene Inc., La Jolla, CA). The ligation mix was diluted 1:10 in distilled water and electroporated into *E. coli* DH-10B (BRL, Gaithersburg, MD). After recovery in 1 ml SOC at 37°C, the cells were spread onto B-agar plates containing 50 μg/ml ampicillin. 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 digestion and the fragment ends were made blunt by incubation with deoxynucleotide triphosphates and the large Klenow fragment of *E. coli* polymerase I. The fragment encoding ampicillin resistance was electroeluted. *P. haemolytica aro*A contained on pPharoA3 was digested at an unique restriction site within the coding region of *aro*A with *Nde*I and the fragment ends were made bunt as described previously. The fragment encoding ampicillin resistance was blunt-end ligated with T4 ligase into pPharo2 thus generating pPharoA$^-$Amp^R. Plasmid pPharoA$^-$Amp^R was digested with *Hin*dIII and dephosphorylated with calf alkaline phosphatase. A 4.2 kb plasmid encoding Sm^R isolated from *P. haemolytica* strain NADC-D70 (Chang et al., *J. DNA Sequencing and Mapping 3*:89-97 (1992)) was also digested with *Hin*dIII and the two plasmids were ligated with T4 ligase to generate the hybrid plasmid pPharoA$^-$Amp^{R}pD70. The hybrid plasmid was electroporated into *E. coli Pha* IMtase which contained the *Pha*I methyltransferase gene on cosmid pLAFRX (Ausubel, *supra*).

P. haemolytica strain NADC-D60 is a plasmidless strain which was isolated from a cow with pneumonic pasteurellosis. The *Pha*I methylated hybrid plasmid was CsCl purified and 1 μg plasmid and 30 μl of *P. haemolytica* strain NADC-D60 were transferred to an 0.2 cm. cuvette and electroporated at 15,000 volts/cm with 800 ohms. The resultant time constant was approximately 9 milliseconds. Cells were transferred to 2 ml Bacto Columbia broth (Difco Labs, Detroit, MI) and incubated at 37°C for two hours and spread on Difco Columbia blood-agar plates containing 10 μg/ml ampicillin. Eight ampicillin resistant *P. haemolytica* colonies were isolated after incubation at 37°C for 18 hours. The colonies were then transferred to Bacto-Columbia broth containing 1 μg/ml ampicillin and incubated at 37°C. Daily passage into fresh medium containing 1 μg/ml ampicillin was carried out for three days at which time the cultures were transferred onto Columbia broth blood-agar plates containing 10 μg/ml ampicillin and incubated at

37°C overnight. The next day, colonies were replica-plated onto Columbia broth blood-agar plates containing 10 μg/ml or 50 μg/ml streptomycin and a chemically-defined medium for *P. haemolytica* cultivation (Wessman, *supra*). The defined medium contains 15 amino acids and includes the aromatic amino acids phenylalanine and tyrosine but not tryptophan. The clones unable to grow on the chemically-defined medium for *P. haemolytica* cultivation were presumed to be *aroA*$^-$. Genomic DNA isolated from colonies with Amp^R, Cm^S, Sm^S, *aro*A- 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 minutes each in 1x SSC and 0.1% SDS at 42° C and twice more for 15 minutes each in 0.1x SSC and 0.1% SDS at 65° C.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: Biotechnology Research and Development Corporation and The United States of America

(ii) TITLE OF INVENTION: CONSTRUCTION OF PASTEURELLA HAEMOLYTICA VACCINES

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Banner, Birch, McKie and Beckett
(B) STREET: 1001 G Street, N.W.
(C) CITY: Washington
(D) STATE: D.C.
(E) COUNTRY: U.S.A.
(F) ZIP: 20001

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 06-DEC-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kagan, Sarah A.
(B) REGISTRATION NUMBER: 32,141
(C) REFERENCE/DOCKET NUMBER: 48294

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 202-508-9100
(B) TELEFAX: 202-508-9299
(C) TELEX: 197430 BBMB UT

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGGCTGCCTG GCTAATCCGC GCCAG 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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TTCATGGAAT CCCTTGACGT TACAACCCAT C                                    31
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1556 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Pasteurella haemolytica

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 184..1486

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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TATGAGGCAT TACTGCGTGA AGGCGTGATT GTTCGCTCGA TAGCAGGTTA TGGAATGCCG      60

AATCATTTAC GCATTAGTAT GCCTTTACCG CAAGAAAACG AGAGATTTTT TACTGCCTTA     120

TTGAAAGTGT TAGCTTAACA AGCGGTTACC TTTTATGAAA ATTTTACAAA TTTAAAGAGA     180

AAA ATG GAA AAA CTA ACT TTA ACC CCG ATT TCC CGA GTA GAA GGC GAG       228
    Met Glu Lys Leu Thr Leu Thr Pro Ile Ser Arg Val Glu Gly Glu
      1               5                  10                  15

ATC AAT TTA CCT GGT TCT AAA AGC CTG TCT AAC CGA GCC TTA TTA TTA       276
Ile Asn Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ala Leu Leu Leu
                 20                  25                  30

GCC GCC TTA GCC ACC GGT ACG ACT CAA GTG ACC AAT TTA TTA GAT AGT       324
Ala Ala Leu Ala Thr Gly Thr Thr Gln Val Thr Asn Leu Leu Asp Ser
             35                  40                  45

GAT GAT ATT CGA CAT ATG CTC AAT GCC TTA AAA GCG TTA GGC GTG AAA       372
Asp Asp Ile Arg His Met Leu Asn Ala Leu Lys Ala Leu Gly Val Lys
         50                  55                  60
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TAT GAG CTA TCG GAC GAT AAA ACC GTC TGT GTA CTT GAA GGG ATT GGT     420
Tyr Glu Leu Ser Asp Asp Lys Thr Val Cys Val Leu Glu Gly Ile Gly
     65                  70                  75

GGA GCT TTT AAG GTT CAA AAC GGC TTA TCA CTG TTT CTC GGC AAT GCA     468
Gly Ala Phe Lys Val Gln Asn Gly Leu Ser Leu Phe Leu Gly Asn Ala
 80                  85                  90                  95

GGC ACG GCA ATG CGA CCA CTT GCA GCA GCA TTG TGT TTA AAA GGT GAG     516
Gly Thr Ala Met Arg Pro Leu Ala Ala Ala Leu Cys Leu Lys Gly Glu
                100                 105                 110

GAA AAA TCC CAA ATC ATT CTT ACC GGT GAA CCA AGA ATG AAA GAA CGC     564
Glu Lys Ser Gln Ile Ile Leu Thr Gly Glu Pro Arg Met Lys Glu Arg
            115                 120                 125

CCG ATT AAA CAC TTA GTC GAT GCT TTA CGC CAA GTA GGG GCA GAG GTA     612
Pro Ile Lys His Leu Val Asp Ala Leu Arg Gln Val Gly Ala Glu Val
        130                 135                 140

CAG TAT TTA GAA AAT GAA GGC TAT CCA CCG TTG GCA ATT AGC AAT AGC     660
Gln Tyr Leu Glu Asn Glu Gly Tyr Pro Pro Leu Ala Ile Ser Asn Ser
    145                 150                 155

GTT TGC AGG GGC GGA AAA GTG CAA ATT GAC GGC TCG ATT TCC AGC CAA     708
Val Cys Arg Gly Gly Lys Val Gln Ile Asp Gly Ser Ile Ser Ser Gln
160                 165                 170                 175

TTT CTA ACC GCA TTG CTG ATG TCT GCC CCA TTA GCG GAA GGC GAT ATG     756
Phe Leu Thr Ala Leu Leu Met Ser Ala Pro Leu Ala Glu Gly Asp Met
                180                 185                 190

GAA ATT GAG ATT ATC GGT GAT CTG GTA TCA AAA CCT TAT ATT GAT ATT     804
Glu Ile Glu Ile Ile Gly Asp Leu Val Ser Lys Pro Tyr Ile Asp Ile
            195                 200                 205

ACC CTT TCG ATG ATG AAC GAT TTT GGT ATT ACG GTT GAA AAT CGA GAT     852
Thr Leu Ser Met Met Asn Asp Phe Gly Ile Thr Val Glu Asn Arg Asp
        210                 215                 220

TAC AAA ACC TTT TTA GTT AAA GGT AAA CAA GGC TAT GTT GCT CCA CAA     900
Tyr Lys Thr Phe Leu Val Lys Gly Lys Gln Gly Tyr Val Ala Pro Gln
    225                 230                 235

GGT AAT TAT TTG GTG GAG GGA GAT GCC TCT TCT GCC TCT TAT TTC TTA     948
Gly Asn Tyr Leu Val Glu Gly Asp Ala Ser Ser Ala Ser Tyr Phe Leu
240                 245                 250                 255

GCC TCC GGT GCG ATT AAG GCA GGT AAA GTA ACG GGC ATT GGT AAA AAA     996
Ala Ser Gly Ala Ile Lys Ala Gly Lys Val Thr Gly Ile Gly Lys Lys
                260                 265                 270

TCG ATC CAA GGC GAC CGC TTG TTT GCC GAT GTG TTG GAA AAA ATG GGG    1044
Ser Ile Gln Gly Asp Arg Leu Phe Ala Asp Val Leu Glu Lys Met Gly
            275                 280                 285

GCA AAA ATC ACT TGG GGA GAG GAT TTT ATT CAA GCC GAG CAA TCC CCG    1092
Ala Lys Ile Thr Trp Gly Glu Asp Phe Ile Gln Ala Glu Gln Ser Pro
        290                 295                 300

CTA AAA GGC GTA GAT ATG GAT ATG AAT CAT ATT CCT GAT GCG GCA ATG    1140
Leu Lys Gly Val Asp Met Asp Met Asn His Ile Pro Asp Ala Ala Met
    305                 310                 315
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ACG ATT GCA ACA ACC GCT TTA TTT GCC GAA GGA GAA ACA GTT ATC CGC      1188
Thr Ile Ala Thr Thr Ala Leu Phe Ala Glu Gly Glu Thr Val Ile Arg
320                 325                 330                 335

AAT ATT TAT AAC TGG CGG GTA AAA GAA ACC GAC CGC TTG ACA GCA ATG      1236
Asn Ile Tyr Asn Trp Arg Val Lys Glu Thr Asp Arg Leu Thr Ala Met
            340                 345                 350

GCA ACC GAA TTG CGT AAA GTC GGG GCA GAG GTA GAA GAA GGG GAA GAA      1284
Ala Thr Glu Leu Arg Lys Val Gly Ala Glu Val Glu Glu Gly Glu Glu
        355                 360                 365

GGG GAA GAT TTT ATT CGG ATT CAA CCG CTT GCG TTA GAA AAC TTC CAG      1332
Gly Glu Asp Phe Ile Arg Ile Gln Pro Leu Ala Leu Glu Asn Phe Gln
    370                 375                 380

CAC GCT GAA ATT GAA ACC TAT AAC GAT CAC CGT ATG GCA ATG TGT TTT      1380
His Ala Glu Ile Glu Thr Tyr Asn Asp His Arg Met Ala Met Cys Phe
385                 390                 395

TCA TTA ATT GCG TTA TCG AAT ACA GAA GTG ACG ATC TTA GAT CCA AAT      1428
Ser Leu Ile Ala Leu Ser Asn Thr Glu Val Thr Ile Leu Asp Pro Asn
400                 405                 410                 415

TGT ACC GCT AAA ACG TTC CCG ACT TAC TTT AGG GAC TTG GAA AAA TTA      1476
Cys Thr Ala Lys Thr Phe Pro Thr Tyr Phe Arg Asp Leu Glu Lys Leu
            420                 425                 430

TCG GTC AGA T AAAAGTAAAA AAGGATTCAG AAAACTGAAT CCTTTTTACG            1526
Ser Val Arg

TTTTATTGTG GCAGACTAAG CCCAACCGCT                                     1556
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 434 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Met Glu Lys Leu Thr Leu Thr Pro Ile Ser Arg Val Glu Gly Glu Ile
  1               5                  10                  15

Asn Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ala Leu Leu Leu Ala
            20                  25                  30

Ala Leu Ala Thr Gly Thr Thr Gln Val Thr Asn Leu Leu Asp Ser Asp
        35                  40                  45

Asp Ile Arg His Met Leu Asn Ala Leu Lys Ala Leu Gly Val Lys Tyr
    50                  55                  60

Glu Leu Ser Asp Asp Lys Thr Val Cys Val Leu Glu Gly Ile Gly Gly
65                  70                  75                  80

Ala Phe Lys Val Gln Asn Gly Leu Ser Leu Phe Leu Gly Asn Ala Gly
                85                  90                  95
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Thr Ala Met Arg Pro Leu Ala Ala Ala Leu Cys Leu Lys Gly Glu Glu
100 105 110

Lys Ser Gln Ile Ile Leu Thr Gly Glu Pro Arg Met Lys Glu Arg Pro
115 120 125

Ile Lys His Leu Val Asp Ala Leu Arg Gln Val Gly Ala Glu Val Gln
130 135 140

Tyr Leu Glu Asn Glu Gly Tyr Pro Pro Leu Ala Ile Ser Asn Ser Val
145 150 155 160

Cys Arg Gly Gly Lys Val Gln Ile Asp Gly Ser Ile Ser Ser Gln Phe
165 170 175

Leu Thr Ala Leu Leu Met Ser Ala Pro Leu Ala Glu Gly Asp Met Glu
180 185 190

Ile Glu Ile Ile Gly Asp Leu Val Ser Lys Pro Tyr Ile Asp Ile Thr
195 200 205

Leu Ser Met Met Asn Asp Phe Gly Ile Thr Val Glu Asn Arg Asp Tyr
210 215 220

Lys Thr Phe Leu Val Lys Gly Lys Gln Gly Tyr Val Ala Pro Gln Gly
225 230 235 240

Asn Tyr Leu Val Glu Gly Asp Ala Ser Ser Ala Ser Tyr Phe Leu Ala
245 250 255

Ser Gly Ala Ile Lys Ala Gly Lys Val Thr Gly Ile Gly Lys Lys Ser
260 265 270

Ile Gln Gly Asp Arg Leu Phe Ala Asp Val Leu Glu Lys Met Gly Ala
275 280 285

Lys Ile Thr Trp Gly Glu Asp Phe Ile Gln Ala Glu Gln Ser Pro Leu
290 295 300

Lys Gly Val Asp Met Asp Met Asn His Ile Pro Asp Ala Ala Met Thr
305 310 315 320

Ile Ala Thr Thr Ala Leu Phe Ala Glu Gly Glu Thr Val Ile Arg Asn
325 330 335

Ile Tyr Asn Trp Arg Val Lys Glu Thr Asp Arg Leu Thr Ala Met Ala
340 345 350

Thr Glu Leu Arg Lys Val Gly Ala Glu Val Glu Glu Gly Glu Glu Gly
355 360 365

Glu Asp Phe Ile Arg Ile Gln Pro Leu Ala Leu Glu Asn Phe Gln His
370 375 380

Ala Glu Ile Glu Thr Tyr Asn Asp His Arg Met Ala Met Cys Phe Ser
385 390 395 400

Leu Ile Ala Leu Ser Asn Thr Glu Val Thr Ile Leu Asp Pro Asn Cys
405 410 415

Thr Ala Lys Thr Phe Pro Thr Tyr Phe Arg Asp Leu Glu Lys Leu Ser
420 425 430

Val Arg

ANNEX M3

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 3, line 27-28 of the description [1]

A. IDENTIFICATION OF DEPOSIT [1]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country) [4]

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit [4]	Accession Number [4]
2 December 1993 (02.12.93)	69499

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

P. haemolytica containing a 4.2kb plasmid

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [6] (If the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [8] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., " Accession Number of Deposit ")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

Elnora Y. Rivera
PCT International Division
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

ANNEX M3

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 5, line 18 of the description [1]

A. IDENTIFICATION OF DEPOSIT [1]

Further deposits are identified on an additional sheet ☐ [1]

Name of depositary institution [4]

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country) [4]

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit [4]: 2 December 1993 (02.12.93)

Accession Number [4]: 69500

B. ADDITIONAL INDICATIONS [1] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

E. coli PhaIMtase

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [1] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [1] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [1] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

Elnora Y. Rivera
PCT International Division

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [1]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 8, line 26 of the description [1]

A. IDENTIFICATION OF DEPOSIT [1]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country) [4]

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit [5]	Accession Number [6]
2 December 1993 02.12.93	55518

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

P. haemolytica

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

Elnora Y. Rivera
PCT International Division

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

CLAIMS:

1. A method for producing a mutation in a particular region of DNA of a *Pasteurella haemolytica* genome:

isolating said region of the genome from *P. haemolytica;*

introducing a mutation into said region to form a mutated DNA region;

methylating said mutated DNA region with a methylating enzyme which inhibits endonuclease cleavage at a recognition sequence selected from the group consisting of 5′-GATGC-3′ and 5′-GCATC-3′, to form methylated DNA;

introducing said methylated DNA into a *P. haemolytica* cell to form transformants; and

screening said transformants for those which have said mutation in said region on chromosomal DNA of said *P. haemolytica* cell.

2. The method of claim 1 wherein said step of methylating is performed by passage of said DNA region through a methylating cell containing *Pha*I methylase.

3. The method of claim 1 wherein said step of methylating is performed by passage of said DNA region through a methylating cell containing *Sfa*NI methylase.

4. The method of claim 1 wherein the step of methylating is performed *in vitro.*

5. The method of claim 1 wherein the methylating enzyme is *Pha*I methyltransferase.

6. The method of claim 1 wherein the methylating enzyme is *Sfa*NI methyltransferase.

7. The method of claim 2 wherein said methylating cell is a *P. haemolytica* strain which contains no *Pha*I restriction endonuclease activity.

8. The method of claim 2 wherein said methylating cell is a bacterium other than *P. haemolytica* which contains a gene encoding *Pha*I methylase.

9. The method of claim 2 wherein said methylating cell is a bacterium other than *Streptococcus faecalis* which contains a gene encoding *Sfa*NI methylase.

10. The method of claim 1 wherein said methylated DNA is introduced into *P. haemolytica* on a plasmid containing a *P. haemolytica* 4.2 kb Str^R plasmid deposited under ATCC Accession No. 69499.

11. The method of claim 10 further comprising:

screening said transformants for loss of said 4.2 kb Str^R plasmid.

12. A chimeric plasmid for unstable introduction of genetic material into *Pasteurella haemolytica* comprising two plasmids covalently linked to each other, wherein the first plasmid is a 4.2 kb Str^R plasmid of *P. haemolytica* deposited at the American Type Culture Collection as Accession No. ATCC 69499; and the second plasmid is a plasmid which cannot replicate in *P. haemolytica*.

13. The chimeric plasmid of claim 12 further comprising:

a region of the chromosome of *P. haemolytica* wherein said region harbors a mutation.

14. A *Pasteurella haemolytica* strain NADC-D60aroA, deposited under ATCC Accession No.55518.

15. A *Pasteurella haemolytica* strain which harbors a mutation which abolishes expression of *Pha*I restriction endonuclease.

16. A vaccine comprising an attenuated, live, mutant *of Pasteurella haemolytica,* which comprises an *aro*A mutation.

17. The vaccine of claim 16 wherein said mutation is a non-reverting mutation.

18. The vaccine of claim 16 wherein said mutation is an insertion mutation.

19. The vaccine of claim 16 wherein said mutation is genetically linked to a selectable marker.

20. A method for producing a mutation in a particular region of DNA of a *Pasteurella haemolytica* genome:

isolating said region of the genome from *P. haemolytica;*

introducing a mutation into said region to form a mutated DNA region;

introducing said mutated DNA region into a *P.*

haemolytica cell which does not express a *Pha*I restriction endonuclease, to form transformants; and

screening said transformants for those which have said mutation in said region on chromosomal DNA of said *P. haemolytica* cell.

21. The method of claim 20 wherein said *P. haemolytica* cell which does not express a *Pha*I restriction endonuclease is a natural isolate.

22. The method of claim 20 wherein said *P. haemolytica* cell which does not express a *Pha*I restriction endonuclease is a mutant made by chemical mutagenesis.

23. The method of claim 20 wherein said *P. haemolytica* cell which does not express a *Pha*I restriction endonuclease is a mutant made by the method of claim 1.

FIG. 1

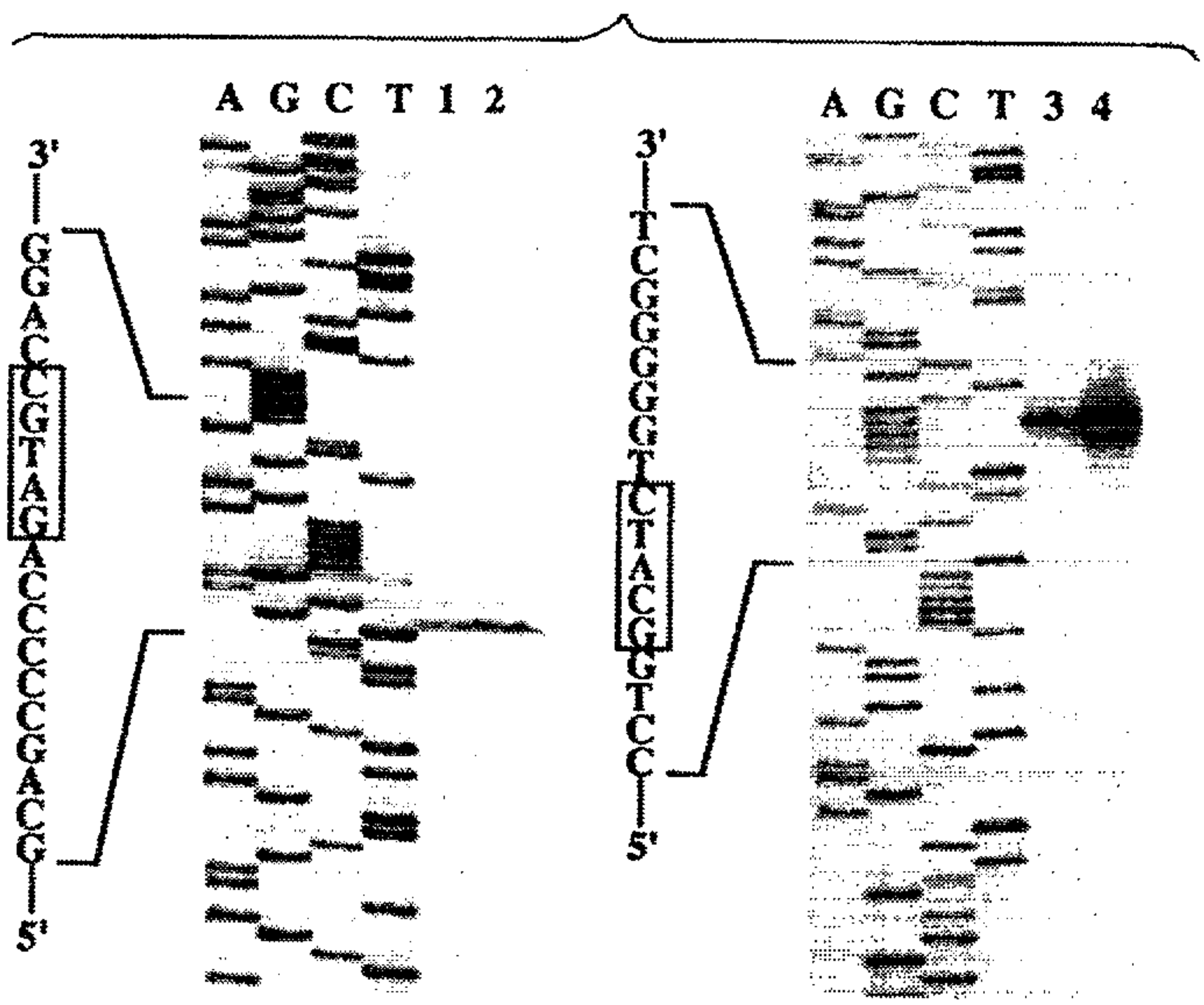
A G C T 1 2
3'
G
G
A
C
C
G
T
A
G
A
C
C
C
C
C
G
A
C
G
5'
A G C T 3 4
3'
T
C
G
G
G
G
G
T
C
T
A
C
G
G
T
C
C
5'

FIG. 2

FIG. 3

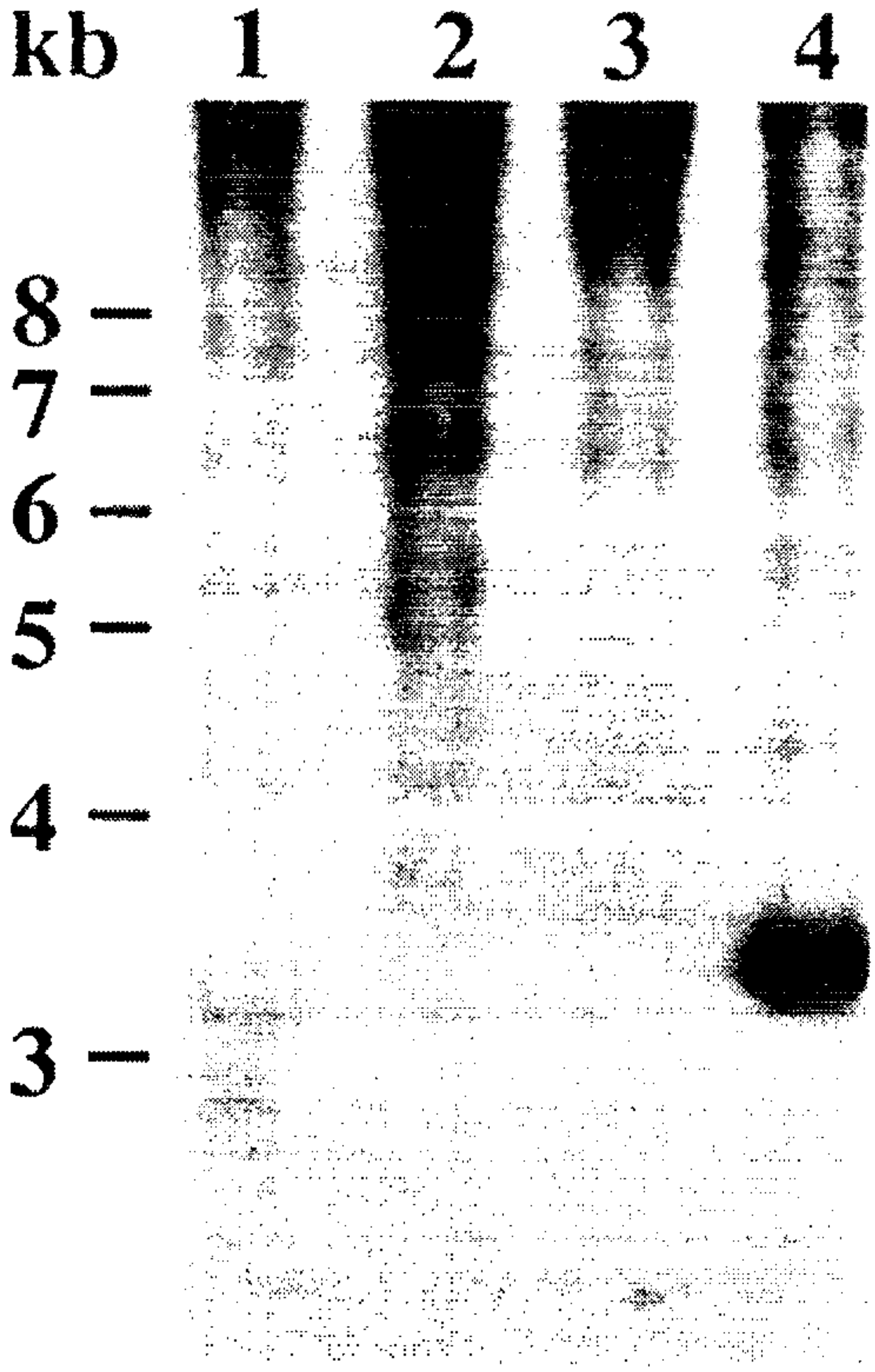

FIG. 4A

```
                              30                            60                            90
TATGAGGCATTACTGCGTGAAGGCGTGATTGTTCGCTCGATAGCAGGTTATGGAATGCCGAATCATTTACGCATTAGTATGCCTTTACCG

                             120                           150                           180
CAAGAAAACGAGAGATTTTTTACTGCCTTATTGAAAGTGTTAGCTTAACAAGCGGTTACCTTTTATGAAAATTTTACAAATTTAAAGAGA

                             210                           240                           270
AAAATGGAAAAACTAACTTTAACCCCGATTTCCCGAGTAGAAGGCGAGATCAATTTACCTGGTTCTAAAAGCCTGTCTAACCGAGCCTTA
   M  E  K  L  T  L  T  P  I  S  R  V  E  G  E  I  N  L  P  G  S  K  S  L  S  N  R  A  L

                             300                           330                           360
TTATTAGCCGCCTTAGCCACCGGTACGACTCAAGTGACCAATTTATTAGATAGTGATGATATTCGACATATGCTCAATGCCTTAAAAGCG
 L  L  A  A  L  A  T  G  T  T  Q  V  T  N  L  L  D  S  D  D  I  R  H  M  L  N  A  L  K  A

                             390                           420                           450
TTAGGCGTGAAATATGAGCTATCGGACGATAAAACCGTCTGTGTACTTGAAGGGATTGGTGGAGCTTTTAAGGTTCAAAACGGCTTATCA
 L  G  V  K  Y  E  L  S  D  D  K  T  V  C  V  L  E  G  I  G  G  A  F  K  V  Q  N  G  L  S

                             480                           510                           540
CTGTTTCTCGGCAATGCAGGCACGGCAATGCGACCACTTGCAGCAGCATTGTGTTTAAAAGGTGAGGAAAAATCCCAAATCATTCTTACC
 L  F  L  G  N  A  G  T  A  M  R  P  L  A  A  A  L  C  L  K  G  E  E  K  S  Q  I  I  L  T

                             570                           600                           630
GGTGAACCAAGAATGAAAGAACGCCCGATTAAACACTTAGTCGATGCTTTACGCCAAGTAGGGGCAGAGGTACAGTATTTAGAAAATGAA
 G  E  P  R  M  K  E  R  P  I  K  H  L  V  D  A  L  R  Q  V  G  A  E  V  Q  Y  L  E  N  E

                             660                           690                           720
GGCTATCCACCGTTGGCAATTAGCAATAGCGTTTGCAGGGGCGGAAAAGTGCAAATTGACGGCTCGATTTCCAGCCAATTTCTAACCGCA
 G  Y  P  P  L  A  I  S  N  S  V  C  R  G  G  K  V  Q  I  D  G  S  I  S  S  Q  F  L  T  A

                             750                           780                           810
TTGCTGATGTCTGCCCCATTAGCGGAAGGCGATATGGAAATTGAGATTATCGGTGATCTGGTATCAAAACCTTATATTGATATTACCCTT
 L  L  M  S  A  P  L  A  E  G  D  M  E  I  E  I  I  G  D  L  V  S  K  P  Y  I  D  I  T  L
```

FIG. 4B

```
                840                                     870                                     900
TCGATGATGAACGATTTTGGTATTACGGTTGAAAATCGAGATTACAAAACCTTTTTAGTTAAAGGTAAACAAGGCTATGTTGCTCCACAA
 S  M  M  N  D  F  G  I  T  V  E  N  R  D  Y  K  T  F  L  V  K  G  K  Q  G  Y  V  A  P  Q

                930                                     960                                     990
GGTAATTATTTGGTGGAGGGAGATGCCTCTTCTGCCTCTTATTTCTTAGCCTCCGGTGCGATTAAGGCAGGTAAAGTAACGGGCATTGGT
 G  N  Y  L  V  E  G  D  A  S  S  A  S  Y  F  L  A  S  G  A  I  K  A  G  K  V  T  G  I  G

               1020                                    1050                                    1080
AAAAAATCGATCCAAGGCGACCGCTTGTTTGCCGATGTGTTGGAAAAAATGGGGGCAAAAATCACTTGGGGAGAGGATTTTATTCAAGCC
 K  K  S  I  Q  G  D  R  L  F  A  D  V  L  E  K  M  G  A  K  I  T  W  G  E  D  F  I  Q  A

               1110                                    1140                                    1170
GAGCAATCCCCGCTAAAAGGCGTAGATATGGATATGAATCATATTCCTGATGCGGCAATGACGATTGCAACAACCGCTTTATTTGCCGAA
 E  Q  S  P  L  K  G  V  D  M  D  M  N  H  I  P  D  A  A  M  T  I  A  T  T  A  L  F  A  E

               1200                                    1230                                    1260
GGAGAAACAGTTATCCGCAATATTTATAACTGGCGGGTAAAAGAAACCGACCGCTTGACAGCAATGGCAACCGAATTGCGTAAAGTCGGG
 G  E  T  V  I  R  N  I  Y  N  W  R  V  K  E  T  D  R  L  T  A  M  A  T  E  L  R  K  V  G

               1290                                    1320                                    1350
GCAGAGGTAGAAGAAGGGGAAGAAGGGGAAGATTTTATTCGGATTCAACCGCTTGCGTTAGAAAACTTCCAGCACGCTGAAATTGAAACC
 A  E  V  E  E  G  E  E  G  E  D  F  I  R  I  Q  P  L  A  L  E  N  F  Q  H  A  E  I  E  T

               1380                                    1410                                    1440
TATAACGATCACCGTATGGCAATGTGTTTTTCATTAATTGCGTTATCGAATACAGAAGTGACGATCTTAGATCCAAATTGTACCGCTAAA
 Y  N  D  H  R  M  A  M  C  F  S  L  I  A  L  S  N  T  E  V  T  I  L  D  P  N  C  T  A  K

               1470                                    1500                                    1530
ACGTTCCCGACTTACTTTAGGGACTTGGAAAAATTATCGGTCAGATAAAAGTAAAAAAGGATTCAGAAAACTGAATCCTTTTTACGTTTT
 T  F  P  T  Y  F  R  D  L  E  K  L  S  V  R  *

ATTGTGGCAGACTAAGCCCAACCGCT
```

FIG. 5

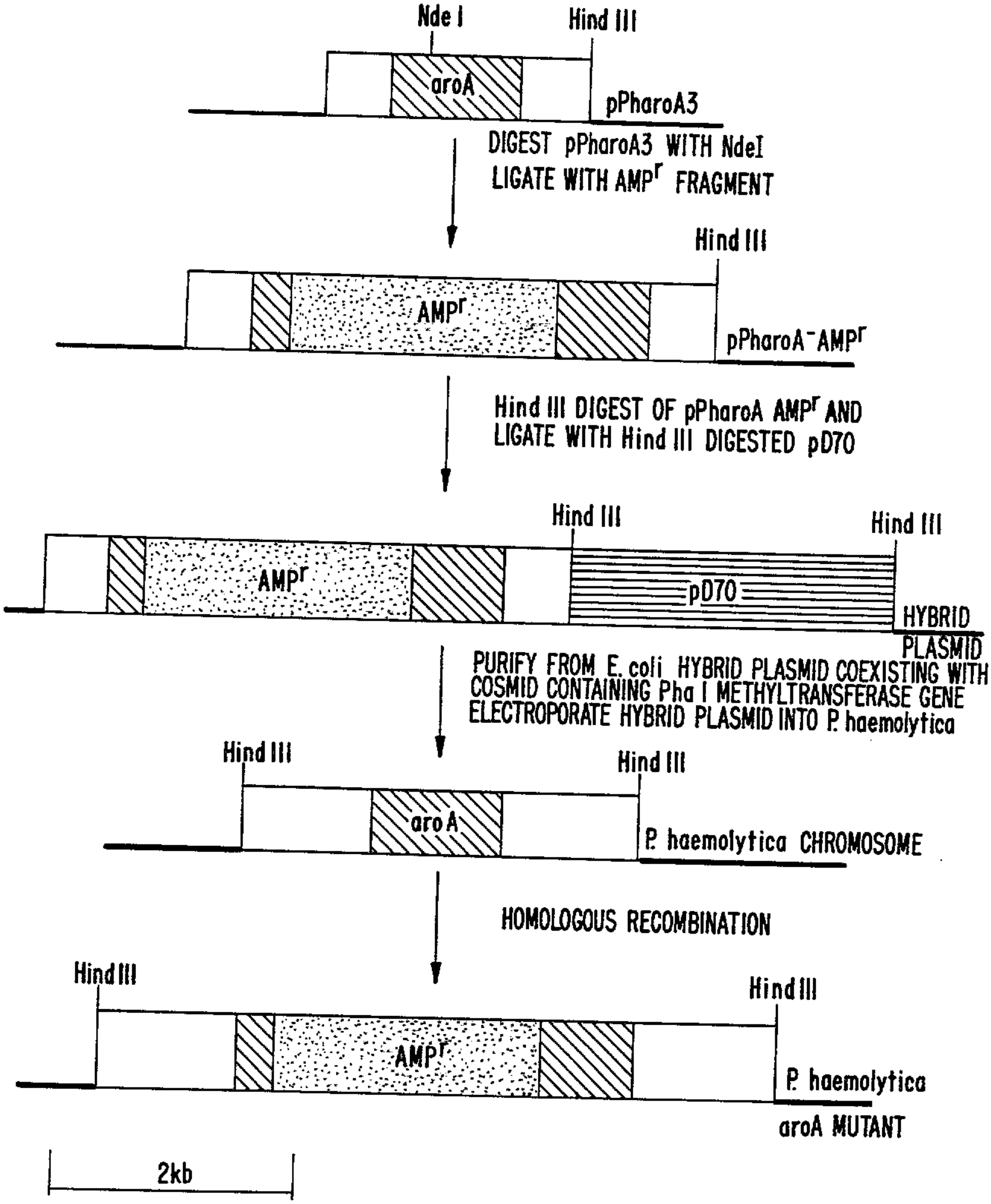
Nde I
Hind III
aroA
pPharoA3
DIGEST pPharoA3 WITH NdeI
LIGATE WITH AMPr FRAGMENT
Hind III
AMPr
pPharoA-AMPr
Hind III DIGEST OF pPharoA AMPr AND
LIGATE WITH Hind III DIGESTED pD70
Hind III
Hind III
AMPr
pD70
HYBRID PLASMID
PURIFY FROM E. coli HYBRID PLASMID COEXISTING WITH
COSMID CONTAINING Pha I METHYLTRANSFERASE GENE
ELECTROPORATE HYBRID PLASMID INTO P. haemolytica
Hind III
Hind III
aroA
P. haemolytica CHROMOSOME
HOMOLOGOUS RECOMBINATION
Hind III
Hind III
AMPr
P. haemolytica
aroA MUTANT
2kb

FIG. 6