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(54) Title: MOLECULAR GENETIC CONSTRUCTION OF VACCINE STRAINS OF PASTEURELLACEAE			
(57) Abstract Tools for genetically engineering <i>Pasteurellaceae</i> are provided. Replication-conditional plasmids which are useful for the <i>Pasteurellaceae</i> have been isolated and characterized. The plasmids can be utilized for delivery of DNA segments into the <i>Pasteurellaceae</i> in situations where control of extrachromosomal replication desired, such as in achieving allelic exchange or site-directed mutagenesis. A restriction endonuclease, <i>Hsol</i>, was isolated from a bovine lung isolate of <i>Haemophilus somnus</i>. The enzyme was found to be a true isoschizomer of <i>HinPI</i>, a commercially available enzyme originally isolated from <i>Haemophilus influenzae</i> PI. Commercially available <i>HhaI</i> methyl transferase was found to protect against cleavage by both enzymes. Methylation of foreign plasmid DNA was found to enhance transformation of <i>Haemophilus somnus</i> in excess of four orders of magnitude.			

MOLECULAR GENETIC CONSTRUCTION OF VACCINE STRAINS OF *PASTEURELLACEAE*

BACKGROUND OF THE INVENTION

Pasteurella haemolytica, *P. multocida*, and *Haemophilus somnus* are members of the Family *Pasteurellaceae*. Each is involved in respiratory disease syndromes in domestic cattle. These organisms have proven difficult to genetically manipulate, and therefore the construction of live, attenuated vaccines has been hampered.

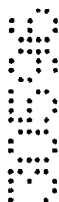
Live attenuated bacterial strains generally provide superior protection as compared to killed bacterial vaccines (bacterins). In general, live vaccines elicit a stronger cell mediated response in the host than do bacterins. The superior immunity provided by attenuated live organisms may be explained by their ability to induce expression of stress-proteins and, possibly, of certain toxins within the host. The immune response generated by live organisms can be directed against these abundant proteins and thereby provide better protection.

There is a need in the art for live, attenuated vaccines against respiratory disease syndrome in domestic cattle caused by the *Pasteurellaceae*. There is also a need for techniques and tools to facilitate the construction of such vaccines.

SUMMARY OF THE INVENTION

The invention provides a replication-conditional plasmid.

The invention also provides a cell-free preparation of a plasmid which is purified from genomic DNA and which is replication-conditional.



The invention further provides *Pasteurellaceae* host cells which comprise a plasmid which is replication-conditional.

The invention still further provides methods for introducing DNA into *H. somnus*.

The invention provides methods for mutagenizing *H. somnus*.

The invention also provides *H. somnus* transformant strains.

The invention further provides *H. somnus* mutant strains.

The invention still further provides genetically engineered *H. somnus*.

In another aspect the invention provides a method of introducing a DNA segment into a *Pasteurellaceae* genome.

In a further aspect the invention provides genetically modified *Pasteurellaceae*.

These and other aspects of the invention are provided by one or more embodiments described below. In one embodiment a plasmid which is conditional for replication in *H. somnus*, *P. multocida*, and *P. haemolytica* is provided.

In another embodiment of the invention a cell-free preparation of plasmid DNA is provided. The plasmid DNA is purified free of genomic DNA. The plasmid is temperature-conditional for replication in *H. somnus*, *P. multocida*, and *P. haemolytica*.

In another embodiment of the invention a host cell of the family *Pasteurellaceae* is provided. The host cell comprises a plasmid which is replication-conditional in *H. somnus*, *P. multocida*, and *P. haemolytica*.

In one embodiment of the invention, a method of introducing DNA to *H. somnus* is provided. The method comprises the steps of: providing a DNA molecule; methylating the DNA molecule with a methyl transferase enzyme having a recognition site of 5'-GCGC-3', to form methylated DNA and transforming *H. somnus* cells with the methylated DNA.

In another embodiment of the invention a method for producing a mutation in a particular region of DNA of an *H. somnus* genome is provided. The method comprises the steps of: isolating a region of the genome from *H. somnus*; introducing



5 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 5'-GCGC-3' to form methylated DNA; introducing the methylated DNA into an *H. somnus* cell to form transformants; and screening the transformants for those which have the mutation in the region on chromosomal DNA
10 of the *H. somnus* cell.

In yet another embodiment of the invention a preparation is provided of an isolated *HsoI* restriction endonuclease.

In still other embodiments *H. somnus* mutants and transformants made by the process of the invention are provided.

15 In another embodiment of the invention a method is provided of introducing a DNA segment to a *Pasteurellaceae* genome comprising: administering to a *Pasteurellaceae* cell a recombinant construct comprising the DNA segment and a plasmid which is temperature-conditional for replication in the *Pasteurellaceae* cell to form transformants; subjecting the transformants to a non-permissive temperature;
20 screening the transformants for the presence of the DNA segment; and screening the transformants for the absence of the plasmid.

In yet another embodiment a genetically modified *Pasteurellaceae* is provided which is made by the method of administering to a *Pasteurellaceae* cell a recombinant construct comprising the DNA segment and a plasmid which is temperature-
25 conditional for replication in the *Pasteurellaceae* cell to form transformants; subjecting the transformants to a non-permissive temperature; screening the transformants for the presence of the DNA segment; and screening the transformants for the absence of the plasmid.

30 These and other embodiments of the invention provide the art with the means to construct desirable mutants and transformants of the economically important and previously intractable *Pasteurellaceae* family of pathogens.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

35 In the generation of defined mutations in the *Pasteurellaceae*, it is desirable to introduce a segment of the genome carrying a defined mutation which has been made

5 either *in vitro* or in another bacterium. Typically, the segment of the genome is introduced on a plasmid. In order to "transfer" the defined mutation from the incoming DNA to the genome, homologous recombination is required. A single recombination event will result in integration of the entire plasmid, which results in one wild-type and one mutant copy of the gene. A second recombination event is desirable, to delete the
10 wild-type copy of the gene and the vector sequences. Since the occurrence of the desired double recombination is a rare event, occurring in only a fraction of the cells which receive the introduced DNA, increasing the number of cells which contain the introduced DNA will increase the recovery of cells which are double recombinants. The use of a plasmid which can replicate in the *Pasteurellaceae* increases the number
15 of cells which have the introduced DNA. However, the presence of a plasmid in cells which may ultimately be used for vaccines is undesirable, as such plasmids often contain ecologically and medically undesirable drug- and toxin-resistance determinants.

 The inventors have solved this problem by creating a plasmid which does not
20 replicate under defined conditions, *i.e.*, which is conditional for replication. Thus genomic DNA carrying a defined mutation introduced to cells via the plasmid can be present in many copies in many directly transformed and progeny cells, by growing the cells at the permissive temperature. This feature increases the absolute number of desired double recombinants obtained by increasing the starting population of cells
25 carrying the DNA-segment. In addition, by switching to the non-permissive conditions (*e.g.*, high temperature), one can eliminate plasmids which are episomal. It is a discovery of the present invention that placing such plasmid-bearing bacteria at a higher temperature which does not permit efficient plasmid replication, results in quick loss of the plasmid.

30 The conditionality of the replication of the plasmids of the present invention can be based on any selectable phenotype. For example, the plasmids might be unable to replicate in the presence of a particular agent, such as a drug or toxin. The plasmids might not be able to replicate in the presence or absence of certain metabolites or salts. Temperature-conditionality has been demonstrated for a mutant of pD70, but other
35 conditionalities can be used, as well as other plasmids which replicate in the

5 *Pasteurellaceae*. Particularly preferred are those plasmids of the same incompatibility group as pD70.

The plasmids of the present invention can be purified according to any art recognized method from genomic DNA. Typical separations of plasmid from genomic DNA in a cell-free preparation are electrophoretic, chromatographic, density gradient
10 sedimentation, alkaline lysis, etc. The plasmids can be introduced into bacterial host cells of the *Pasteurellaceae* by any means known in the art, including transformation, conjugation, liposome mediated gene transfer, particle bombardment, etc. Any *Pasteurellaceae* host can be used.

Plasmid mutations may be induced by any means known in the art. These
15 include *in vitro* or *in vivo* chemical mutagenesis, passage through a mutator strain, etc. Even spontaneous mutations can be used if one is willing to screen more extensively. Particularly preferred are deletions and insertions which are non-reverting. Such mutations are easily generated *in vitro* using restriction enzymes, for example. Conditional mutations are most likely missense mutations, but nonsense mutations can
20 also be used in the presence of a temperature-sensitive suppressor tRNA.

The temperature-conditional plasmids of the present invention can be administered to a *Pasteurellaceae* cell according to standard methods known in the art, including, but not limited to electroporation, transformation, transfection, transduction. One can screen by genetic or physical methods to detect those cells which have
25 received the plasmid DNA. Subsequently, one can screen among the plasmid recipients for those which have lost the plasmid and retained the DNA of interest carried on the plasmid. The screening methods can be genetic or physical, such as screening for a phenotype or screening for the presence of a particular DNA sequence in the cell by hybridization.

30 It is an additional discovery of the present invention that *H. sommus* contains a restriction-modification system, called herein the *HsoI* system. The *HsoI* restriction endonuclease has been isolated and its cleavage sequence determined to be 5'-GCGC-3'. It has also been discovered by the present inventors, that a barrier to transformation of *H. sommus* can be overcome by treating DNA with a methylating enzyme, such as
35 the *HsoI* methyl transferase (M. *HsoI*). Such enzymes modify DNA substrates such

5 that endonucleases which recognize 5'-GCGC-3' sequences are inhibited in their ability to digest such modified substrates. The methyl transferases produce a site which is 5'-GmCGC-3', i.e., the 5' cytosine is methylated. Examples of such methyl transferases are *HsoI* methyl transferase, *HinPI* methyl transferase, and *HhaI* methyl transferase, which is commercially available from New England Biolabs, Beverly, MA, 01915. Cells containing such methyl transferase enzymes can also be used. Preferably, 10 these are recombinant cells with the methyl transferase enzymes introduced, so that they lack the cognate restriction enzyme. Alternatively, they are mutant or natural variants which lack the cognate restriction enzyme. In some instances, it may be possible to passage DNA through cells which have both the restriction and methyl transferase enzymes, if the former is less active (slower) or less prevalent than the latter. 15

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 methyl transferase in the presence of a methyl donor, such as S-adenosylmethionine (SAM). *In vivo* methylation 20 can be accomplished by passing the DNA substrate through a bacterium which contains an appropriate methyl transferase, such as *HsoI*, *HinPI*, or *HhaI* methyl transferase. A mutant or natural variant of *H. somnus* which lacks the *HsoI* endonuclease could also be used to prepare DNA for subsequent introduction into *H. somnus*. Such a mutant can be made *inter alia* according to the method for site-directed mutagenesis disclosed herein. 25

Site-directed mutagenesis of *H. somnus* can be accomplished according to the present invention by first isolating a wild-type DNA region from *H. somnus*. A mutation is created in the isolated, wild-type DNA region according to any method 30 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 *H. somnus* DNA has been isolated and mutagenized, it is methylated as 35 described above. Then it can be introduced into *H. somnus* 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 *H. somnus* which expresses *HsaI* restriction endonuclease, one can omit the methylation of the mutagenized DNA and introduce the mutagenized DNA into an *H. somnus*, *H. haemolyticus*, or *H. influenza* cell which does not efficiently express the *HsaI* restriction endonuclease or an isoschizomer of it. Such cells can be isolated from nature by extensive screening, isolated following chemical mutagenesis of a cell which does express the *HsaI* restriction endonuclease, or made by the site-directed mutagenesis method disclosed herein. According to one aspect of the invention, the mutagenized and methylated *H. somnus* DNA region is introduced into a *P. multocida* 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. Derivatives of this plasmid or other pD70-incompatible plasmids (ie. plasmids of the same incompatibility group) can be used similarly. An incompatibility group is a group of plasmids which are so similar that a second member of the group cannot be added to a cell containing a first member of the group. The origin of replication of pD70 can be isolated on a 1.2 kb *Sau3AI* fragment immediately downstream of the streptomycin resistance determinant. 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.

Also provided by the present invention are mutant and transformant strains made by the disclosed methods of transformation and/or site-directed mutagenesis. Such mutants can provide the veterinary arts with attenuated, live strains of Pasteurellaceae, which are suitable for vaccines to induce protective immunity against Pasteurellaceae infection. For vaccine production, it is desirable that the mutation which attenuates the bacterium 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 bacterium is vanishingly small.



5 Suitable attenuating mutants may be, for example, auxotrophic. Mutants with altered virulence factors may also be used.

 One mutant strain which can be made by the site-directed mutagenesis method disclosed is one which is *HsoI* restriction endonuclease negative. Such a strain is useful for genetic engineering in *H. sommus*. Such a strain can be a recipient of DNA
10 which is not *HsoI* methyl transferase methylated, yet would yield DNA which is *HsoI* methyl transferase methylated.

 A preparation of isolated *HsoI* endonuclease can be prepared *inter alia* by passing a cell-free lysate of *H. sommus* over a column of heparin-sepharose. Other known techniques for isolating restriction endonucleases can be used as is appropriate.
15 Typically the specific activity of such a preparation will be enriched as compared to the cell-free lysate.

 The present invention thus allows those of ordinary skill in the art to stably introduce DNA into *H. sommus*. The DNA can be from other strains or species. The DNA can be artificially modified or in its native state. If recombination into the
20 genome is desired two regions of flanking homology are preferred. Such techniques are generally known for other bacteria, but have been hitherto unsuccessful in *H. sommus* 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
25 *Pasteurellaceae* 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
30 also may be added.

EXAMPLES

Example 1: Plasmid construction and mutagenesis. The 4.2 kb plasmid (pD70) which encodes streptomycin resistance in *Pasteurella haemolytica* serotype 1 has been
35 previously sequenced and described (Chang, Tatum). The plasmid was linearized by

*Hind*III digestion and made blunt by treatment with dNTPs and Klenow fragment. A kanamycin cassette (Genblock) previously digested with *Bam*HI and made blunt as above was ligated into pD70 to produce pD70kan. This plasmid was amplified in *E. coli* DH10B containing *Pha*I methyltransferase on cosmid *Pha*Imtase. The plasmid was introduced into *P. haemolytica* strain NADC-D153. Plasmid DNA (1 µg) recovered from a kanamycin-resistant transformant was mutagenized with hydroxylamine for 90 minutes at 70° C as previously described. The treated DNA was dialyzed against TE, ethanol-precipitated, and resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8.0).

Example 2: Recovery of temperature-sensitive plasmids. *Pasteurella haemolytica* serotype 1, strain NADC D153, was grown to late logarithmic phase in 100 ml columbia broth at 37° C with gentle shaking. The bacteria were pelleted by centrifugation at 5000 x G and washed in 100 ml 272 mM sucrose at 0° C. The pellet was resuspended in an equal volume of 272 mM sucrose at 0° C. Competent cells (100µl) were placed into six 0.1 cm electroporation cuvettes and mixed with 100 ng of the treated DNA. The cells were electroporated (GenePulser, Bio-Rad) at 18,000 V/cm and 800 ohms yielding time constants ranging from 11-12 msec. Immediately after each electroporation the cells were resuspended in 1.0 ml columbia broth at 0° C.

Recovery was for 2 hours at 30° C. The suspension was spread (100 µl / plate) onto columbia agar (Difco) plates containing 50 µg/ ml kanamycin. Plates were incubated 28 hours at 30°C then transferred to 42° C for 6 hours. Colonies were picked which were smaller than typical colonies and dotted onto kanamycin plates. After overnight incubation at 30° C, growth from each selected colony was duplicated onto columbia agar plates with and without kanamycin and then incubated overnight at 42°C. Growth from non-selective plates was transferred to kanamycin plates which were incubated overnight at 30° C. Clones which failed to grow with selection at 42° C but which grew well on selective media at 30° C after passage without selection at 42° C were presumed to be temperature-sensitive for kanamycin expression. Similar clones which exhibited reduced growth on selective plates at 30° C after passage without



5 selection at 42° C were presumed to be temperature-sensitive for plasmid maintenance. Four of the latter clones were selected for further study.

10 **Example 3: Testing of temperature-sensitive plasmids.** Plasmid DNA was recovered by an alkaline lysis procedure from four *P. haemolytica* clones presumed to contain temperature-sensitive plasmid origins of replication. The DNA was electroporated into *P. multocida* strain NADC TT94, a bovine lung isolate which is Carter-Heddlestone type A:3. After specific methylation with *HhaI* (as previously described) the DNA was electroporated into *H. somnus* strain HS91, a bovine lung isolate. Transformants were grown overnight on columbia agar plates supplemented with 5% bovine blood and 50 ug / ml kanamycin at 30° C in 10% CO₂. Duplicate 15 broth cultures were inoculated with transformants of each organism and plasmid. One culture was grown overnight at 40° C, the other at 30° C. Plasmid was recovered from each broth by an alkaline lysis procedure and resolved on 1% agarose gels. Selective and non-selective plates were struck for subjective assessment of percentage CFU resistant to kanamycin after passage. 20

Example 4: Properties of the replication-conditional plasmid. *Pasteurella haemolytica* was transformed to kanamycin resistance by the mutagenized plasmid at an efficiency of about 6x10⁴ CFU/ug DNA. Of the transformants, about 1% (360) were 25 found to form atypically small colonies after the incubation at 42° C. Passage of these colonies at either 30° C or 42° C on plates with or without kanamycin revealed that about 90% of the transformants were temperature sensitive for expression of kanamycin-resistance (about 10% failed to grow on the first passage and were not tested further). These organisms formed colonies on selective or non-selective plates at 30° C but failed to grow on selective plates at 42° C. Passage of growth from non-selective plates at 42° C to selective plates at 30° C resulted in heavy growth, 30 indicating plasmid was still present. A 5.5 kb plasmid was detected in plasmid preparations out of representatives of these cultures.

Ten of the 360 colonies behaved on passage similar to the colonies containing 35 temperature-sensitive kanamycin genes except growth was reduced on selective media

5 at 30° C after passage without selection at 42° C. These colonies appeared to vary in percentage resistant to kanamycin after passage without selection at 42° C, indicating possible differences in their degree of instability at non-permissive temperature (42° C). Four were selected based on their low yield of kanamycin-resistant colonies after passage without selection at the non-permissive temperature.

10 The four temperature-sensitive plasmids were readily introduced into *P. multocida* strain NADC-TT94 and into *H. somnus* strain NADC-HS91 (after appropriate methylation). The plasmids behaved as they did in *P. haemolytica*. No growth was observed on selective plates incubated at 40° C and no plasmid was detected in broth cultures grown without selection at 40° C. Cultures transformed with wild-type plasmid grew well under selection at 40° C and yielded plasmid DNA
15 from non-selective broth cultures at that temperature. All cultures grew and yielded plasmid when grown with or without selection at 30° C. The results indicate that plasmid replication is temperature-conditional in each of the three species bacteria.

One of the temperature-conditional plasmids called pBB192 has been deposited
20 in *P. haemolytica* NADC D153 at the American Type Culture Collection, Rockville, MD, 20852 under Accession No. ATCC 55893 on December 2, 1996.

Example 5: Isolation and characterization of restriction endonuclease, *HsoI*, from *Haemophilus somnus* and protection of heterologous DNA by *HhaI* methyl transferase.
25

Chromatographic fractions exhibiting endonuclease activity eluted from the heparin-sepharose columns by 680 and 760 mM NaCl (960 - 1060 μ S). A single pass through these columns was sufficient to identify both the recognition specificity and cleavage site. Digestion of lambda DNA with the concentrated *HsoI* preparation
30 resulted in a distinctive restriction fragment pattern identical to that produced by *HhaI*, a commercially available restriction endonuclease isolated from *Haemophilus haemolytica*. The cleavage site (5'...G↓CGC...3') was found to differ from that of *HhaI*, producing a 5' overhang identical to that produced by *HinPI*.

Methods Used

Bacterium, growth, and crude extract. *Haemophilus somnus*, strain 2336

(kindly supplied by Lynette Corbeil, San Diego, CA), was grown for 16 h on eight chocolate agar plates (Columbia blood agar base; Difco, Detroit, Mich, supplemented with 5% defibrinated bovine blood at 90°C, 200-ml total volume). The cells were harvested in TE (10 mM Tris, 1 mM EDTA, pH 8.0), pelleted by centrifugation at 16,000 x g for 5 min at 4°C, and washed once in TE. The washed pellet was resuspended in 12 ml chromatography running buffer (20 mM sodium phosphate, 10 mM 2-mercaptoethanol, pH 8.0, 0° C) and placed on ice. The bacterial cells were disrupted by sonication for 2 min in 15-s bursts. Debris and unbroken cells were removed by centrifugation at 16,000 x g for 10 min, and the supernatant was filtered through a 0.45-µm-pore-size membrane (Millex-HA; Millipore Corp., Bedford, Mass.). 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, Calif) were equilibrated as recommended by the manufacturer. A flow rate of 1.0 ml/min was used for separation, using a gradient low pressure automated chromatography system (Automated Econo-System; Bio-Rad, Richmond, Calif). Five ml of crude extract was injected and a linear gradient from 0 to 1.0 M NaCl in 60 ml of running buffer was used to elute proteins. Fractions (1 ml) were stored on ice prior to activity assay. A second identical chromatographic separation was performed with a new column from which active fractions were collected and pooled for storage.

Assay for restriction endonuclease activity. Aliquots (5 µl) of the chromatographic fractions were incubated with 1 µl of React 1 (BRL, Gaithersburg, MD) and 0.5 µl of unmethylated bacteriophage lambda DNA (0.5 µg/µl; New England Biolabs, Beverly, Mass.) at 37° C for 2 h. After addition of tracking dye and electrophoresis on a 1% agarose gel in Tris-borate-EDTA buffer, the banding patterns were visualized by ethidium bromide staining and UV illumination. The fractions corresponding with DNA cleavage activity were pooled from the second chromatographic separation, concentrated 20-fold on 30,000-molecular-weight-cutoff



ultrafilters, and brought to final concentrations of 150 mM NaCl, 10 mM sodium phosphate, 0.1 mM EDTA, 5mM 2-mercaptoethanol, 0.25 µg of bovine serum albumin per ml, and 50:50 (vol/vol) glycerol, pH 8.0, for storage at -20°C. The concentrated preparation was designated *HsoI*.

Determination of the recognition and cleavage sites for *HsoI*. The recognition sequence was identified by digestion of pBluescript (Stratagene, La Jolla, Calif.) and of lambda DNA. The cleavage site was identified by digestion of a primed-synthesis reaction on pBluescript. An oligonucleotide primer was synthesized which is complementary with sequences 3' from an *HsoI* site of pBluescript. Single-stranded DNA was used for the template. Standard dideoxy DNA sequencing reactions were performed and an additional reaction containing no dideoxy terminator was extended through the *HsoI* site with the Klenow fragment of DNA polymerase I by using ³²P-end-labeled primer. The extension reaction was stopped by phenol-chloroform extraction followed by ethanol precipitation. *HsoI* or *HhaI* (New England Biolabs) was added to the additional reactions and allowed to digest the DNA for 2 min. The reaction was stopped by addition of gel loading buffer and heating to 80° C for 3 min.

Example 6: Transformation of *H. somnus* with methylated DNA

DNA obtained from *Haemophilus somnus* or from *E. coli* and *in vitro* methylated with *HhaI* methyl transferase was resistant to cleavage by both *HsoI* and *HhaI*. Protection by *in vitro* methylation was found to often be partial, based on electrophoretic mobility of DNA after digestion with and without prior *in vitro* methylation, even when the substrate DNA had been phenol-chloroform-isoamyl alcohol extracted and then purified by CsCl gradient centrifugation.

Introduction of plasmid DNA into *Haemophilus somnus* was enhanced about 4 orders of magnitude by previous *in vitro* methylation of the plasmid. Each of the pD70-based plasmids transformed *H. somnus*, but efficiency dropped as the size increased. It is possible a second restriction-modification system may be responsible for the marked reduction in efficiency as plasmid size is increased. The possibility of systems analogous to *mcr* or *mrr* in *E. coli* was not investigated. Partial rather than complete protection conferred by *in vitro* methylation could also account for the reduction.



5 No ampicillin-resistant transformants were recovered, indicating either that the
ampicillin-resistance cassette of pD80 does not express in *H. somnus* or that the origin
of replication does not function. A pD70-based replicon containing the pD80
ampicillin-resistance cassette in the *Hind*III site transformed *H. somnus* to yield
streptomycin-resistant colonies. Those colonies failed to replicate on ampicillin-
10 containing media, indicating the ampicillin cassette does not function in *H. somnus*.
The pD80 origin of replication was not tested further.

 The kanamycin-resistance cassette derived from Tn903 was found to be
excellent for selection of transformants. Streptomycin provided only fair selection.
Transformants containing both streptomycin- and kanamycin-resistance cassettes were
15 more robust on kanamycin selection than on streptomycin. Conversely, untransformed
colonies were common on streptomycin selection but were not encountered on
kanamycin selection.

 A second strain of *H. somnus*, 649 (kindly supplied by Dr. Lynette Corbeil),
was not transformed by derivatives of pD70. This strain was found to harbor a small
20 plasmid which we presume to be incompatible with pD70. This plasmid, like pD70,
might serve as a useful vector for the introduction of DNA into the bacterium.

 The restriction-modification system carried by *Haemophilus somnus* are useful
to genetically manipulate this pathogen. Specific methylation against the restriction
endonuclease allows introduction of foreign DNA. Two replicons, both based on
25 similar origins of replication, were discovered which may be of use as vectors for the
introduction of foreign genes.

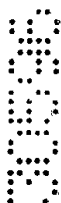
Methods Used

Construction and methylation of shuttle vector. A derivative of pD70, the
4.2 kb streptomycin-resistance plasmid of *Pasteurella haemolytica* serotype 1, was
30 previously constructed during experiments involving that bacterium. Briefly, the 2.2
kb *Pst*I fragment of pD70 containing streptomycin-resistance was excised from a 1%
agarose gel, electroeluted, and ligated with a *Pst*I kanamycin cassette derived from
Tn903.(Genblock, Pharmacia) The resulting plasmid conferred kanamycin-resistance
in *E. coli* and in *P. haemolytica*. The plasmid was methylated with commercially
35 available *Hha*I methyl transferase according to instructions. Other plasmids based on

the pD70 origin of replication were tested, including intact pD70, pD70kan (pD70 with the kanamycin cassette blunt-ligated into the unique *Hind*III site), and pD80 (the 4.2 kb *P. haemolytica* plasmid encoding for ampicillin-resistance).

Electroporation of methylated DNA into *Haemophilus somnus*.

Haemophilus somnus strain NADC Hs91 (pneumonic bovine lung isolate) was grown in 100 ml Levinthal's broth at 37°C in 10% CO₂ to late logarithmic phase, approximately four hours. The growth was pelleted by centrifugation at 5000 x G for fifteen minutes and washed once in 100 ml 272 mM sucrose at 0° C. The pellet was resuspended 1:3 packed bacteria : 272 mM sucrose on ice. Competent bacteria (100 ml) were mixed with 100 ng plasmid DNA either unmethylated or *in vitro* methylated in 0.1 cm electroporation cuvettes (Bio-Rad). The cells were quickly electroporated after addition of DNA (Gene pulser, Bio-Rad) at 18,000 V/cm, 800 ohm, 25 mF with resultant time constants ranging from 11 to 15 msec. Levinthal's broth (1 ml, 0°C) was immediately added to the electroporated cells and the suspension was incubated at 25° C approximately 10 minutes. The cells were then recovered at 37° C with 10% CO₂ for 2 hours. Ten-fold dilutions were plated onto chocolate agar plates (Columbia blood agar base with 5% defibrinated bovine blood) containing 50 mg / ml kanamycin, 100 mg / ml streptomycin, or 20 mg/ ml ampicillin. Colonies were enumerated after 36 hours incubation at 37°C with 10% CO₂. Representative colonies were examined for plasmid content using a rapid alkaline lysis procedure.



Example 7: Use of a temperature conditional replicon to generate an *aroA* deletion mutant of *Pasteurella multocida*

Previous attempts to produce gene-replacement mutants of *P. multocida* in our lab were hindered by poor electroporation efficiencies and by replication of ColEI- based replicons in *P. multocida* (unpublished results). In addition, products of gene-replacement typically contain foreign antibiotic resistance genes which may preclude or delay particular uses of otherwise desirable mutants. The shuttle plasmid constructed here was used to overcome those problems.

The origin of replication of the *P. haemolytica* plasmid pD70 was found to be within a 1.2 kb *Sau*3AI fragment downstream from the streptomycin coding region. Together with a kanamycin cassette derived from Tn903, the vector (pBB192) proved



to replicate in members of the Family *Pasteurellaceae* but not particularly well in *E. coli*, requiring cloning of sequences relying on the pD70 origin to be performed in a *Pasteurellaceae* host. While unsuitable for use in *P. multocida*, a derivative of the shuttle vector was constructed which contains a ColE1 origin of replication in the BamHI site (pBB192C) to facilitate construction in *E. coli* of temperature-conditional constructs for use in *P. haemolytica* or *H. somnus* target organisms (unpublished results). Plasmid pBB192C replicated efficiently in *E. coli*.

Approximately 100 *P. multocida* transformants were recovered on kanamycin at 30°C from electroporation with 25 ng of replacement plasmid. Passage of broth cultures from 6 representative colonies to kanamycin plates at 40°C resulted in about 20 well isolated colonies from each 10 µl inoculum, but the number of colonies produced varied among the 6 cultures. The colony size varied significantly on each plate, yielding a number of small colonies and a few large colonies. The relative proportion of these sizes varied among the 6 cultures. Southern blot analysis of genomic DNA from the colonies (probing with *aroA*) revealed that the small colonies were products of single crossover events. The large colonies contained sequences homologous to *aroA* which were not similar in size to the replacement plasmid in addition to a fragment consistent with wild-type chromosomal *aroA*. The large colonies were not examined further. Our interpretation of the data is that the integrated replacement plasmid destabilizes the chromosome, resulting in a substantial reduction in replication rate and therefore conferring small colony size. The replacement plasmid, however, replicates so inefficiently by itself at the non-permissive temperature that colonies do not form at all under kanamycin selection. This situation put strong selective pressure to rearrange the plasmid for improved replication or to integrate into chromosome plasmid sequences containing the kanamycin gene, resulting in some potential unlikely products.

Passage of growth from products of single-crossover events without kanamycin selection resulted in >99% loss of kanamycin resistance in a single passage. These results indicate substantial instability of the single-crossover product. Among 500 isolated colonies from such a passage, 5 failed to grow on both defined medium and under kanamycin selection. Southern blot analysis confirmed the loss of DNA



5 sequences homologous to the deleted *ClaI* - *EcoRV* fragment, failed to show
homology with plasmid vector, and showed a reduction of about 300 bp in size of
chromosomal *aroA*. Results of PCR analysis indicated a 300 bp reduction in product
size. Sequencing of the PCR product confirmed a deletion extending from the *EcoRV*
10 site to slightly beyond the *ClaI* site, 5'-ATTGATAT-GAACCAT-3', which does not
alter the reading frame of downstream DNA sequences.

The temperature-sensitive shuttle vector separated the operations of bacterial
transformation from that of selection of crossover products in gene-replacement. It
also simplified the generation of products without foreign selectable markers. The
instability of single-crossover products appeared to facilitate resolution of the plasmid
15 from the chromosome to generate deletion mutants without use of negative selection
afforded by such genes as *SacB*. Since the vector replicates temperature-conditionally
in *P. haemolytica* and in *H. somnus*, it is likely that it should be equally useful in these
or other *Pasteurellaceae* as well.

The *P. multocida aroA* mutant constructed here which was deposited on
20 December 2, 1996, at the ATCC, and given the accession number ATCC 55892,
differs from that described by Homchampa et al because the present strain is of bovine
origin, a deletion was introduced in *aroA*, and no foreign DNA sequences are present
in the product. This mutant can be used as an attenuated live vaccine.

Methods Employed

25 **Construction temperature-sensitive shuttle vector.** A shuttle-vector was
constructed based on the previously described temperature-sensitive origin of
replication of pD70, the streptomycin-resistance plasmid isolated originally from *P.*
haemolytica serotype 1 (Tatum et al, Chang). A PCR product approximately 1450 bp
was produced from temperature-sensitive pD70kan #192 using forward primer 5'-
30 GCCTGTTTTTCCTGCTC-3' and reverse primer 5'-
CCTGCGGTGTAAGTGTATT-3'. The product was digested with *Sau3A1* to
completion to produce an approximately 1.2 kb fragment. A kanamycin resistance
cassette from Tn903 (GenBlock, Pharmacia) was digested with *EcoRI*, ligated into the
EcoRI site of pBC SK (Stratagene), and electroporated into *E. coli* strain 30-9G to
35 produce *PhaI*-methylated plasmid DNA (Briggs et al). The approximately 1.3 kb

kanamycin-resistance cassette was excised from the methylated plasmid with BamHI and ligated overnight to the 1.2 kb *Sau3A*I. The ligation mixture was electroporated into *P. haemolytica* strain NADC-D153 and plasmid was recovered from kanamycin resistant colonies. It was found that a portion of the pBC SK multiple cloning site from the *Eco*R1 site to the BamHI site had been transferred along with the kanamycin cassette, resulting in a 2.5 kb plasmid with a unique *Eco*R1 site and an effectively unique BamHI site which replicates in *P. haemolytica*, *P. multocida*, and *H. somnus* at 30°C but very poorly in *E. coli*. The plasmid was named pBB 192.

Cloning and Deletion of *P. multocida aroA*. A 1.2kb PCR product containing the *aroA* gene was produced using forward primer 5'-TTACTCTCAATCCCATCAGCTATA-3' and reverse primer 5'-CTATCTGTAGGCTACTTCGCGTG-3'. The product was cloned into a vector which contains *Eco*R1 sites flanking the PCR product insert (TA vector, Invitrogen). The insert was excised with *Eco*R1 and ligated into the *Eco*R1 site of pUC9. The product was double digested with *Cla*I and *Eco*RV to remove an approximately 300bp fragment. The ends of the remaining plasmid were made blunt using Klenow fragment of DNA polymerase I and dNTPs then ligated upon themselves to generate a deletion of *aroA*. The deleted plasmid was amplified in *E. coli* strain 30-9G to *Pha*I methylate the DNA then digested with *Eco*R1 and electrophoresed on an agarose gel to confirm the deletion. The *Pha*I-methylated *Eco*R1 *aroA* fragment containing the deletion was ligated into the *Eco*R1 site of pBB192 to create pBB 192PmΔ*aroA*. The ligation mixture was electroporated into *P. haemolytica* strain NADC-D153 and plasmid was recovered from kanamycin-resistant colonies.

Production of *P. multocida* single-crossover products. *Pasteurella multocida* strain NADC-TT94, a bovine lung isolate of Carter-Heddleston type A:3, was grown 4 hours in 100 ml columbia broth containing 2,500 U hyaluronidase. Growth was centrifuged at 5000 × G for 15 minutes and washed twice in 272 mM sucrose at 0° C then resuspended in 1 ml 272 mM sucrose. The cells (100 μl) were electroporated (Gene Pulser, Bio-Rad) with 25ng pBB192PmΔ*aroA* in a 0.1cm cuvette at 1.8kv, 800Ω,



5 and 25 μ F with a resultant time constant of 14.7 ms. The cells were immediately resuspended in 1 ml columbia broth at 0° C then incubated for 2 hours at 30° C. The recovered cells were spread 100 μ l/plate on columbia agar plates containing 50 μ g/ml kanamycin then incubated 24 h at 30° C. Six colonies were passed separately to 5 ml columbia broth containing 50 μ g / ml kanamycin which were incubated 18 hours at 30
10 C. Growth was spread (10 μ l / plate) onto columbia agar plates with kanamycin which were incubated 24 hours at 40 C. Representative colonies were passed to columbia broth (25 ml) with kanamycin (to confirm single crossover products by Southern blot analysis) and to columbia broth (5 ml) without kanamycin (to screen for deletion mutants) and incubated overnight at 40 C.

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Screening for *P. multocida* deletion mutants. From the non-selective broth culture above, columbia agar plates were struck for isolated colonies and incubated overnight. Five-hundred isolated colonies were passed into microtiter plates containing 100 μ l columbia broth/well and incubated 6 hours. Growth (1 μ l) was passed into each of two microtiter plates containing either 100 μ l/well columbia broth with kanamycin or 100 μ l/well chemically defined medium lacking tryptophane based on that of Wessman et al and that of Watko et al. Wells which grew only on the original non-selective microtiter plate but not on either kanamycin or defined medium were suspected deletion mutants. These were passed for Southern blot analysis and for PCR analysis using forward primer 5'-CTACCCACCTATCGCCATTG-3' and reverse primer 5'-TCCGCCCCACCTTA-3'. The PCR product from one of the deletion mutants was cloned for sequencing of the deletion.

Where the terms "comprise", "comprises", "comprised" or "comprising" are used in this specification, they are to be interpreted as specifying the presence of the stated features, integers, steps or components referred to, but not to preclude the presence or addition of one or more other feature, integer, step, component or group thereof.



The claims defining the invention are as follows:

1. A method of introducing DNA to *H. somnus*, comprising the steps of:
 providing a DNA molecule;
 methylating the DNA molecule with a methyl transferase having a recognition site of 5'-GCGC-3', to form methylated DNA;
 transforming *H. somnus* cells with the methylated DNA.
2. The method of claim 1, wherein the methyl transferase enzyme produces a site 5'-GmCGC-3'.
3. The method of claim 1, wherein the methyl transferase enzyme is selected from the group consisting of: *M. HsaI*, *M. HhaI*, *M. HinPI*.
4. A method for producing a mutation in a particular region of DNA of a *H. somnus* genome comprising:
 isolating a region of the genome from *H. somnus*;
 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 5'-GCGC-3' to form methylated DNA;
 introducing the methylated DNA into an *H. somnus* cell to form transformants; and
 screening said transformants for those which have said mutation in said region on chromosomal DNA of said *H. somnus* cell.
5. The method of claim 4, wherein said step of methylating is performed by passage of said DNA region through a methylating cell containing *M. HhaI* or *M. HinPI* methyl transferase.
6. The method of claim 4, wherein the step of methylating is performed *in vitro*.
7. The method of claim 4, wherein the methylating enzyme is *M. HhaI* or *M. HinPI* methyl transferase.
8. The method of claim 4, wherein said methylated DNA is introduced into *H. somnus* on a plasmid comprising pD70, a *P. haemolytica* 4.2 kb Str^R plasmid deposited at the ATCC as Accession No. ATCC 69499.
9. The method of claim 4, wherein said methylated DNA is introduced in *H. somnus* on a plasmid comprising an origin of replication of pD70, a *P. haemolytica* 4.2 kb Str^R plasmid deposited at the ATCC as Accession No. ATCC 69499.
10. The method of claim 8 further comprising:
 screening said transformants for loss of said 4.2 kb Str^R plasmid.



11. A method for producing a mutation in a particular region of DNA of a *H. somnus* genome comprising:
 - isolating a region of the genome from *H. somnus*;
 - introducing a mutation into the region to form a mutated DNA region;
 - introducing the mutated DNA into an *H. somnus* cell which lacks an *HsoI* restriction endonuclease to form transformants; and
 - screening said transformants for those which have said mutation in said region on chromosomal DNA of said *H. somnus* cell.
12. A preparation of an isolated *HsoI* endonuclease.
13. An *H. somnus* mutant made by the method of claim 4.
14. An *H. somnus* transformant made by the method of claim 1.
15. An isolated and purified *H. somnus* transformant which has been genetically modified by the stable introduction of DNA.
16. The *H. somnus* strain of claim 15, wherein the introduced DNA has recombined with genomic DNA of *H. somnus*.
17. A method of claim 1 of introducing DNA to *H. somnus*, comprising the steps of:
 - providing a DNA molecule;
 - methylating the DNA molecule with a methyl transferase having a recognition site of 5'-GCGC-3', to form methylated DNA;
 - transforming *H. somnus* cells with the methylated DNA,
 which method is substantially as herein described with reference to the accompanying Examples.
18. A method of claim 4 for producing a mutation in a particular region of DNA of a *H. somnus* genome comprising:
 - isolating a region of the genome from *H. somnus*;
 - 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 5'-GCGC-3' to form methylated DNA;
 - introducing the methylated DNA into an *H. somnus* cell to form transformants; and
 - screening said transformants for those which have said mutation in said region on chromosomal DNA of said *H. somnus* cell,



which method is substantially as herein described with reference to the accompanying Examples.

19. A method of claim 11 for producing a mutation in a particular region of DNA of a *H.somnus* genome comprising:
- isolating a region of the genome from *H. somnus*;
 - introducing a mutation into the region to form a mutated DNA region;
 - introducing the mutated DNA into an *H.somnus* cell which lacks an *HsoI* restriction endonuclease to form transformants; and
 - screening said transformants for those which have said mutation in said region on chromosomal DNA of said *H. somnus* cell,
- which method is substantially as herein described with reference to the accompanying Examples.
20. An isolated and purified *H. somnus* transformant which has been genetically modified by the stable introduction of DNA, substantially as herein described with reference to the accompanying Examples.
21. The *H. somnus* strain of claim 15 wherein the introduced DNA has recombined with genomic DNA of *H. somnus*, substantially as herein described with reference to the accompanying Examples.

Dated this 8th day of February 2001

BIOTECHNOLOGY RESEARCH AND DEVELOPMENT CORPORATION OF PEORIA,
ILLINOIS AND U.S. DEPARTMENT OF AGRICULTURE

By their patent attorneys
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