



US006086894A

# United States Patent [19]

[11] **Patent Number:** **6,086,894**

**Inzana et al.**

[45] **Date of Patent:** **Jul. 11, 2000**

[54] **RECOMBINANT VACCINE FOR DISEASES CAUSED BY ENCAPSULATED ORGANISMS**

### FOREIGN PATENT DOCUMENTS

9310815 10/1993 WIPO .

[75] Inventors: **Thomas J. Inzana**, Blacksburg, Va.;  
**Christine Ward**, Irving, Tex.

### OTHER PUBLICATIONS

Frosch et al. (1991) *Mol. Microbiol.* vol. 5(5), 1251–1263.  
Ley et al (1995) *Vaccine* vol. 13(4), 401–107.  
Kozel et al (1992) *Infect. & Immunity* vol. 60(8), 3122–3127.

[73] Assignee: **Virginia Tech Intellectual Properties, Inc.**, Blacksburg, Va.

*Primary Examiner*—Anthony C. Caputa  
*Assistant Examiner*—Khalid Masood  
*Attorney, Agent, or Firm*—Whitham, Curtis, & Whitham

[21] Appl. No.: **08/673,814**

[22] Filed: **Jun. 27, 1996**

### [57] ABSTRACT

[51] **Int. Cl.**<sup>7</sup> ..... **A61K 39/102**; C12N 1/36

[52] **U.S. Cl.** ..... **424/235.1**; 424/93.2; 424/256.1;  
424/825; 424/932; 435/243; 435/245; 435/252.3;  
435/172.3; 56/23.1; 56/24.32; 56/24.33

Vaccines for diseases caused by normally encapsulated organisms are produced by genetically modifying those organisms by deleting the genes encoding for capsule synthesis or a portion thereof sufficient to produce non-encapsulated mutants of the organisms. As an example, a live, attenuated strain of *Actinobacillus pleuropneumoniae* genetically modified with a large deletion in a chromosomal regions of the DNA which encodes for capsule synthesis is a safe and effective vaccine against swine pleuropneumonia.

[58] **Field of Search** ..... 424/93.2, 256.1,  
424/825, 235.1, 932; 435/243, 245, 252.3,  
172.3; 536/23.1, 24.32, 24.33

### [56] References Cited

#### U.S. PATENT DOCUMENTS

5,429,818 7/1995 Inzana .

**4 Claims, 10 Drawing Sheets**

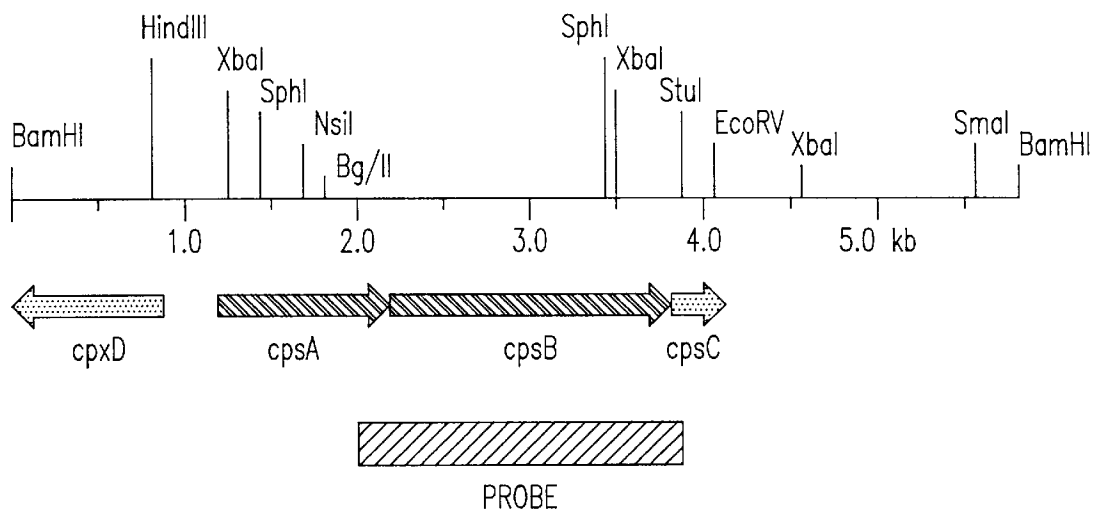


FIG.1

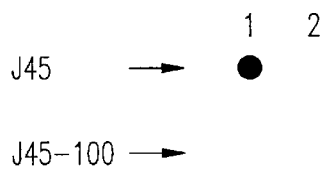


FIG.6

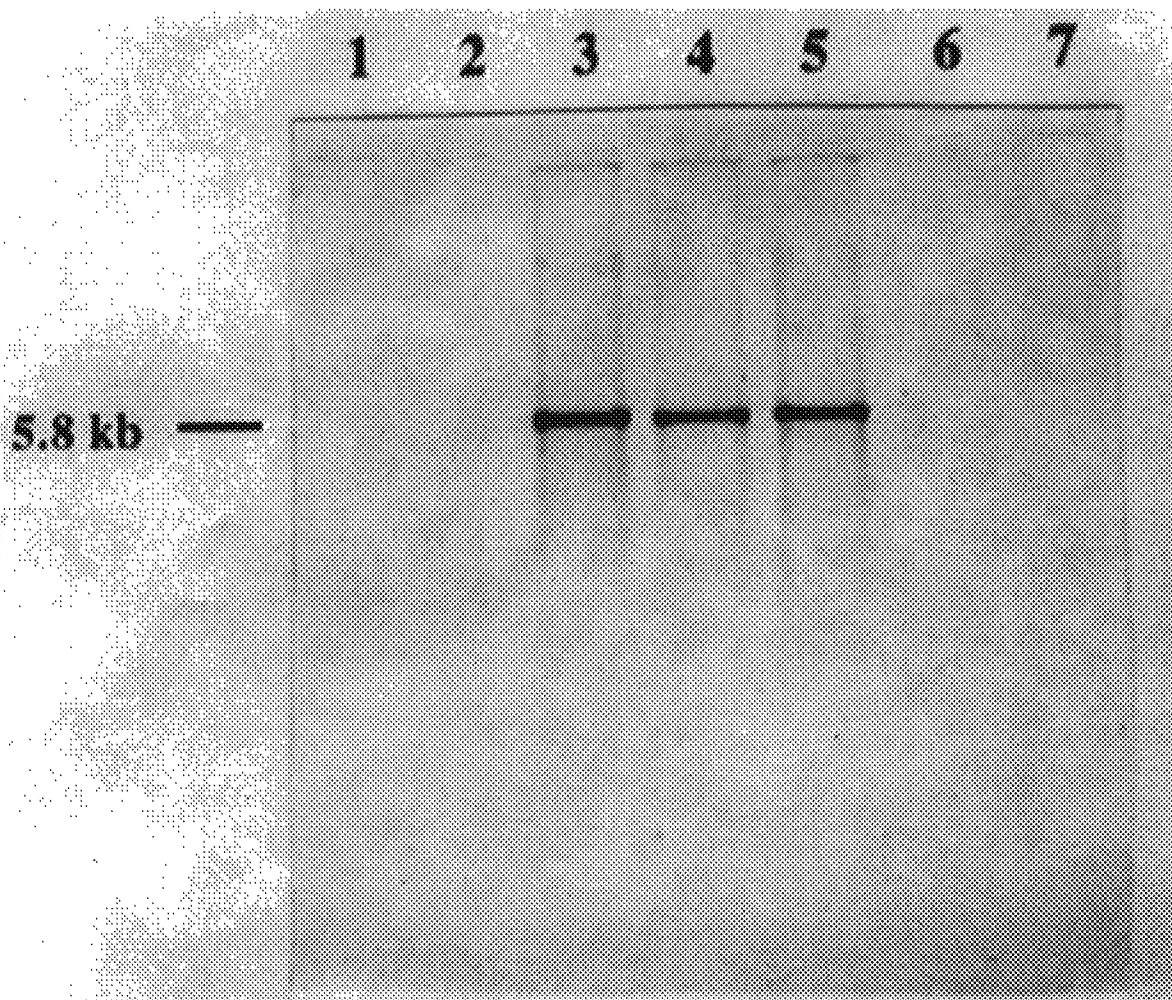


FIG.2

AAGCTTGAGCAGGCAGCCAACTAGCAACCAGCCCCAAAGAAAGGAGTAATCTAAGTTTGATGAGTTTCATCTAATTTCTCTTCAATATATTAAGGAATAACAAC 105

TATATAGGTATGCTTAAAATCCACATAAAGATTGATTTAATAAGTTACCTAATCAAGAGAATTAATATAAGAAATTTACAAAACAATTAATAATGTTATT 210

TTTTAAAAAAGTAATCAAGAGGGCGTTATACAGATAAACATTATAATTTAAAGCCATATAAAATACGGAGTTTCCCCTAGATAGTTGATAAATTTCTCA 315

TTATATTTATGAAATCCGATGAAAAATTTCAACTATCTAGGGTAACTCCATAACGTATTCGTATTTCCAGGAGTATTTTAAATGTCTAGCATAATGACTCGT 420

CCTATAATTAATCATGTAATGTCTAGAGATATCAAAGTGGCATATTAGTTCTATTTTAGAATATTTACTGATTTTGGTCCAATGAATTTCAACATATTGC 525

P I I N H V M S R D I Q S G I F S S I L E Y F T D F G S N E F Q H I V  
 AGTGTATCTCCAATACCTGAAGCTAAAGTTTATCACTATCACCGTCCACACCTAGAAGAAAAATTATTACCTAATCTGTTTGACAGTACATCATGACCTCAAT 630

S V S P I P E A K V Y H Y H R P H L E E K L L P N S V C T V H H D L N  
 GATCCAGATCCTTGGCATGCTAAGTATAGATTTATTCCTAGATATATGGAAGCTGGGCTATAATTTGTTAAATTACACTCAAAAAGAAATTTAATATCTCAG 735

D P D P W H A K Y R F I P R Y M E A G A I I C L N Y T Q K E I L I S Q  
 GGACTCCGGAACATAAGTATTTGTGATTCCTCAGGATATAATCAAAAAGTATTATTCCTAAGAAAAATTAAGAAATATCAAGTACAGATAAAATACCTTA 840

G L P E H K L F V I P H G Y N Q K V L F P K K I K E I S S T D K I T L  
 GGAATGCTTCACGGAGATATGGTAGAAGAGTAAAAGGAGATGCATATTTATTTGAATTAGCAAAAAGATTAATCCAGACCATTTAAATTTATTTTGTGGT 945

G I A S R R Y G R R V K G D A Y L F E L A K R L N P D H F K F I F V G  
 AAAGATAGACAATATAGTGCCTTAGAAATGCAAGATCTAGGATTTGAAGCTCAAGTATATGAAAGATTGCCATATAGAATGTTTCAAAGTTTTATAATAATTT 1050

K D R Q Y S A L E M Q D L G F E A Q V Y E R L P Y R M F Q S F Y N N I  
 GATGACTACTTATGTAGTAGTCATGAAGTGGACCTGCAATATCCCCAAGCATTAGCTACTGGGACACCTATATTTTCATCTAACATAGGTATACCTAAG 1155

D V L L M C S S H E G G P A N I P E A L A T G T P I F S S N I G I P K  
 GATGTTGTTATTATAAGAATGGTTGATTCTAACCTTAGATCCAGATATAGATGCTGAACAGATTAATTTTATTTGCCCTGAAAAACCAATATATTTGAA 1260

D V V I N Y K N G L I L T L D P D I D A E Q I N F I C L E K P N I F E  
 AATATATAGATTTTCACTAAAACAGTCTCCAAGTTAGCAATTTCTTGGGAGAAATGATTCAACAAAATATTTAGTTTATAAAAAATAATTAAGGGTTAA 1365

N I L D F S L K Q S P S L A I S W E K C I Q Q N I L V Y K K I I K G  
 TTATGTCCATTTCTATTCTAGTACCTGATTCCTTACACATTAACAAAAGAACTTTAGTTCATCTTCAGTTGAGAAAAATAAAATAATATCCATTTTG 1470

CpsB →  
 M S I S I L V P D S L H I N K R N F S S F F S W I E K N K I N I H F  
 AAAATAATAAAGATTGGATTCATTATATGTTTTACGATTCAAAATGGGTTTCTATATGAGAAAATAGATATTTACTAAGATTGAAGAGAGGAAT 1575

E N N N K D W I S L Y G F Y D S K L G I L Y E K I D I L T I E E E E

FIG.3A

TATTGGCTTTTGTGTTTATGATCTAAATATTTTCAATATTTGTAGAGCTGAATTATTATCTTTAGTAGCCACAAGACCCGAATGGTATAATGAAGATTATCCTA 1680

L F A F C V Y D L N I F N I C R A E L L S L V A T R P E W Y N E D Y P  
ATAACTTAAGAGAAATATACAAAACTCTATACTAATAATCGAAGTGAATTATTGCAAAACATGGCTGCTGCTTGGTATTGGGTGATTCTCGAAAAACGCC 1785

N N L R E I Y K K L Y T N N R S E L L Q N M A A A W Y W V D F W K K R  
TATCTGAGTAAACAATTCTCTCATTGTTGTATTTTCAGGAGGTTAATTTATCAAAAATCTTTGATTGAGTATTAAAATATACTCCAACATAAAGTATGG 1890

L S E L K Q F S H C C V F S G G L I Y Q K S L I E L L K Y T P T K V W  
TTATGGAAGCCATTATACAGGAACGAATATTATTGTGAGGAACGTATTTCATCAATTGCTAATAATAGCGATATTAACATTTAGCTATTTTAACTCTTATA 1995

V W E S L F T G N E Y Y C E E R Y S S I A N N S D I K H L A I F N S Y  
AAAAACATTAAGTCAAAAAGTGAATATGATAAGGAACGAATGAAAGCTATTAAAGTCTCTATTAAAGAAAAATAAGAATGCAACACACTACTGATTCTG 2100

K K T F S S K S E Y D K E R M K A I N K F L L M K N K N V Q Q P T D S  
AAATATTAGTATTTAAACAACAAAAACCAATAATACTATTATTGGACAAGTGATAAATGATTTTCAGTCTAGAATATAAAGGGAGAGACTATCAACAATTA 2205

E I L V F K Q Q K P I I T I I G Q V I N D F S V L E Y K G R G L S T I  
AAATCTAAAGAACCTTATATCTAAACTATCAGAGAATGGATTAATGTAGTATTAATAACTCACCCCTGGGAAGAGAAAAATAATATCCGTACATCTTAA 2310

K I Y K E L I S K L S E N G F N V V L K T H P W E E K K N N I R T S L  
CTAAAAATAATAGAAGATTTCTAAAAATCTAACTGAGAAATCAACAAGATGTATAAAAATAGTTGATCACTATTCAATAAAGAAATTTTAAACAATCTG 2415

T K N I I E E F L K N L T E N Q Q E C I K I V D H Y S I K K L F K Q S  
ATTTTATTAGTTTAAATCTCAAGGCCCTCTTCAAGCTGCATTGATGGTATAAAACCTATACAGTTAGGTAATGCTTTTATGAAAAAAGGATTCACGT 2520

D F I I S L N S Q G L L E A A F D G I K P I Q L G N A F Y G K K G F T  
ACGATTAGACTTTTATAGATTGATCAATTGGTAATGACTTAGTAGTAATAAATACTACTCAACACTATCTTTAGAAGAGTTTATTGTTGCGAAGAGTCA 2625

Y D Y D F L D I D Q L V N D L V V N K L T P T L S L E E F D L F E E F  
TACTATATTATACAAAACGATGCTGTTTCTATTACGCCCTCTGGCGTAAGTGTTTTATCTAGAATATTTAATTTACCTACTATTATACCATTAGTAGAAAATG 2730

I T I L L Q K H A V S I H A S G V S V L S R I F N L P T I I P L V E N  
TCCCTAAGGAGAAGTCTAAAAACAACATTACCTACTCAAAAAGATGTGTAAAAAGGAAAATACAACAATTGTAATATGGTTGAGTTACCTAAAGTAGTCCAC 2835

V P K E K S K T T L P T Q K D V V K K E N T T I V N W V E L P K V V P  
AAAGTGATAAGAATAGGAATATCAAAAATTAGAAAATCCTCGACAATCTTTGCAGATTCTAGGAATCCAGTTATTAGAAGTTAATGTATTTTTCCCTT 2940

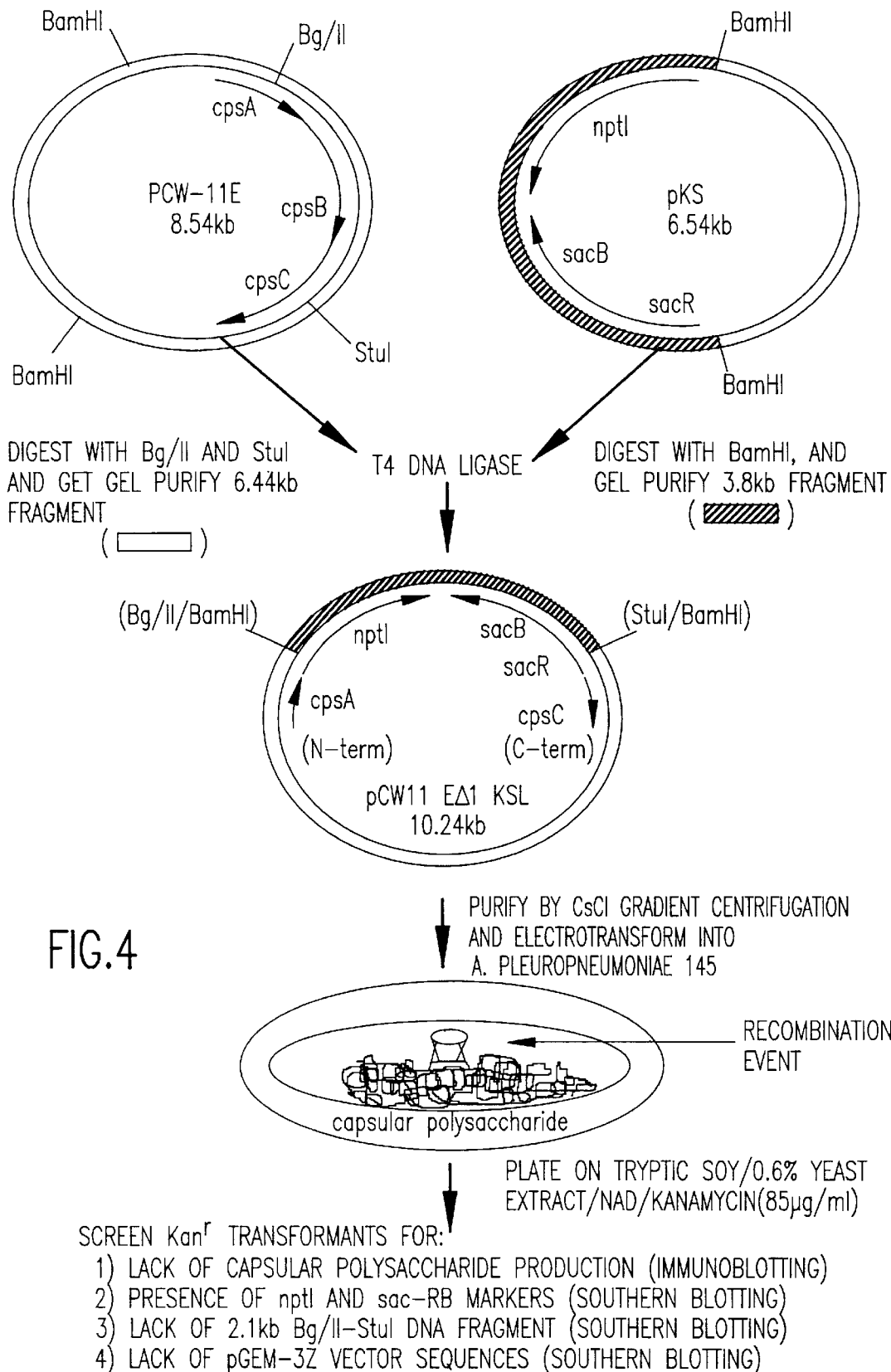
Q S D K N R K Y Q K F R N N P R Q F F A D S R N P V I R S L W Y F F P  
ATAAATAATATAGTCTAATTTATGTTAAAAATATCAGCCTTTTGATTAAGAAAAATAAATGAAGCCACTCTAGTAATGCTAAGTTAGTTTACATTCTGA 3045

Y K . CpsC → M L K K Y Q P F D L R K I N E G H S S N A K L V L H S E  
GGCCTGTAATATAGATGCTAAAATCTCTAAGTTTTCTGTTTACAAGATGACATTAATTTAGAAAATTTTATGCAACATTTACTGATACTATAAAGCACCAGA 3150

A C N I D A K I S K F F C S Q D D I N L E N F I A T F T D N Y K A P E  
AGTATATACGCCATTTTAAAGAATTGTTGATTACACCTAGAGCACCTAAGCTACCAAGAT 3212

V Y T A I L K N C C I T P R A P K L P R

FIG.3B



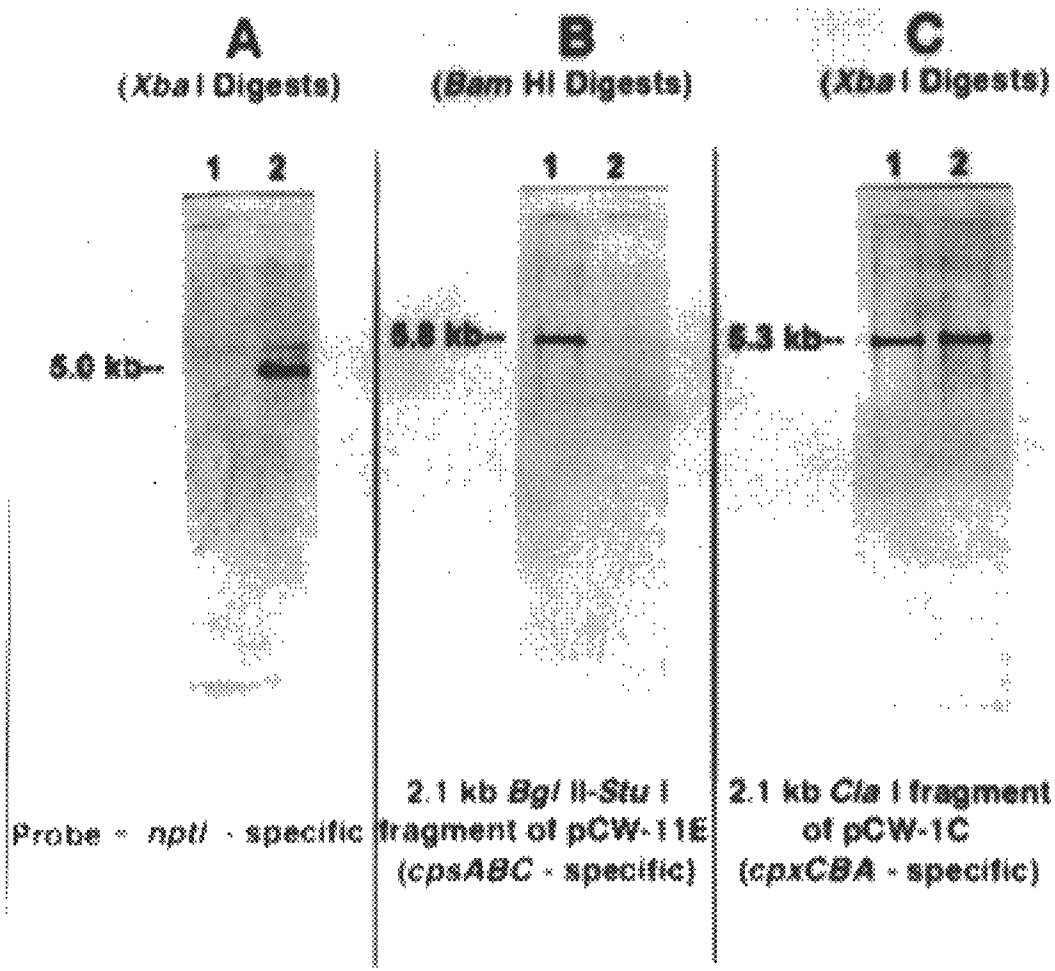


FIG.5

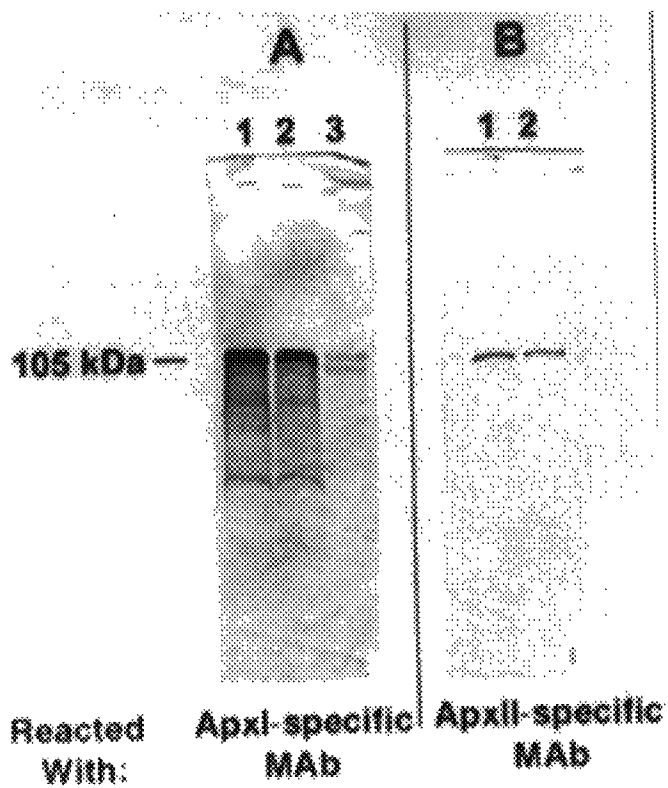


FIG.7

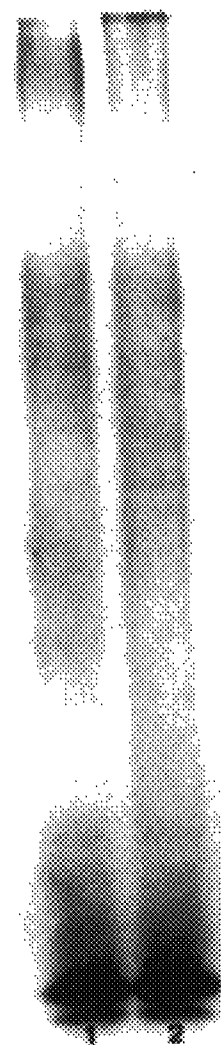


FIG.8



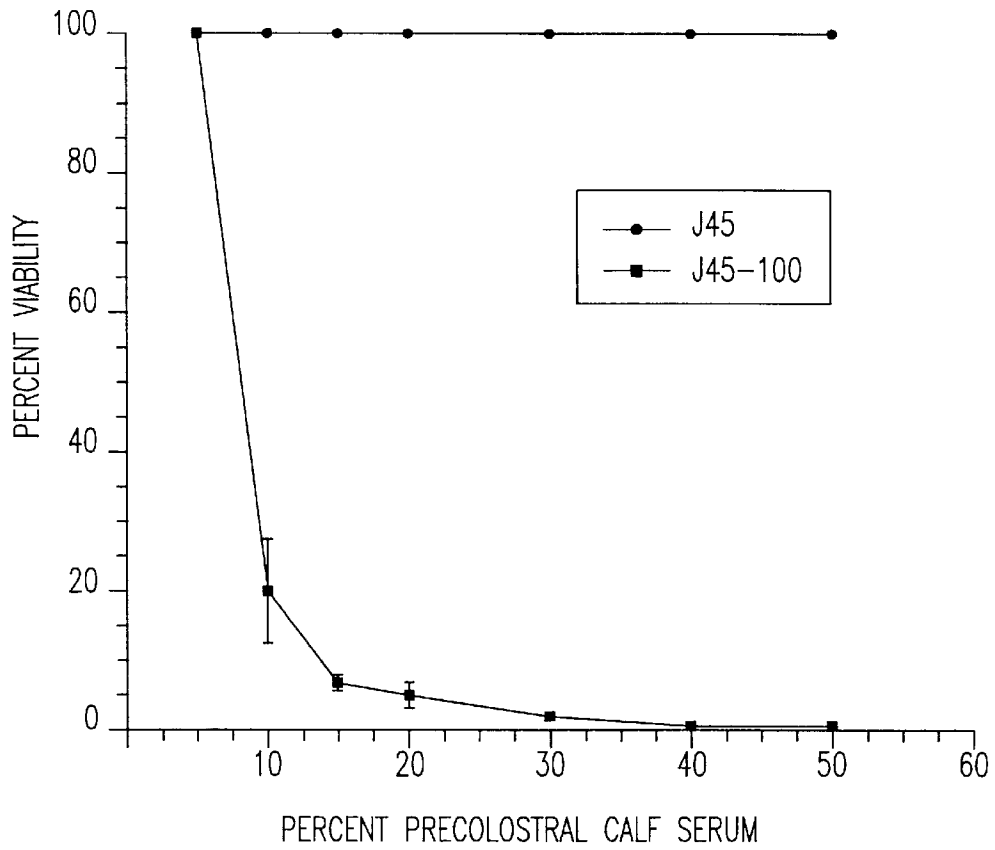


FIG.9

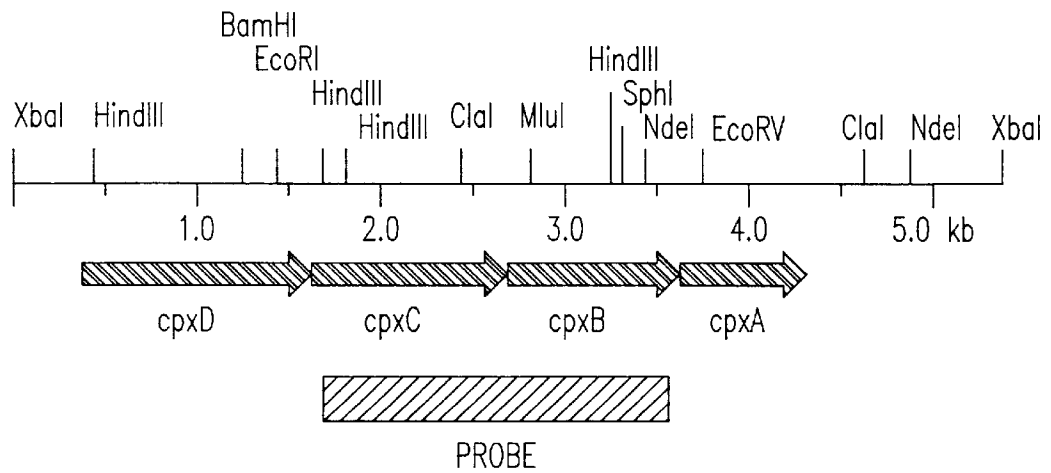


FIG.11

CTAGACATTACATGATTAATTATAGGACGAGTCATTATGCTAGACATTAATAACTCCTGAAATACGAATACGTTATGGAGTTACCCTA 90  
 GATAGTTGATAAATTTTTCATCGGAATTCATAAATATAAATGAGAAATTTATCAACTATCTAGGGGAAACTCCGTATTTTATATGGCT 180  
 TAAATTATAATGTTTATCTGTATAACGCCCTCTTGATTTACTTTTTTTAAAAAATACATTTTTTAATTTGTTGTAAATTTCTTA 270  
 TATTTAATTTCTCTTGATTAGGTAACCTTATAAAATCAATCTTTATGTGGATTTAAGACATACCTATATAGTTGTTATTCCTTAATATA 360  
 TTGAGAGAAATTAGATGAAACTCATCAAACCTAGATTACTCCTTTCTTTGGGCTGGTGTAGTTGGCTGCCTGCTCAAGCTTACCC 450  
 CpxD → M K L I K L R L L L S L G L V A S L A A C S S L P  
 ACTTCAGGCCCTAGCCATAGTCCGATTTAGAGGCTAATCCAGAACTCAGATAAACCTTTACCGGAAGTTAATTTAGTGGAGTTAGAT 540  
 T S G P S H S A I L E A N S Q N S D K P L P E V N L V E L D  
 AATGGCTTAGTTCAGCAGTTGTATCAGACTCAGCAAAGTCAGCAATTTCCGGCTTTTATAGGCACGGCTGGCGGTGCTGGATATGCCGT 630  
 N G L V Q Q L Y Q T Q Q S Q Q F S G F L G T A G G A G Y A G  
 CGCGTCAATGTGGGGATGTTCTTGAAATTTCAATTTGGGAAGCGCCACCGGCAGTGTGTTGGCGGTACTTTAGTTCTGAAGGGCAA 720  
 A V N V G D V L E I S I W E A P P A V L F G G T F S S E G Q  
 GGTAGCGGCATTTAACGCAATTACCGCGCAATGGTTAACCAAAACGGTACGGTACTGTCCGTTTGTGGGTAATATTCGTGTGCA 810  
 G S G H L T Q L P A Q M V N Q N G T V T V P F V G N I R V A  
 CGTAAACACCGGAAGCGATTCACTCAAAATGTTGGGCATTGCAACGTAAGCGAATCAGCCACAAGTATTAGTAAAAATTCGCAAT 900  
 G K T P E A I Q S Q I V G A L Q R K A N Q P Q V L V K I A N  
 AATAACTCTCGGATGTTACGGTATTCTGTCAGGTAACAGTATTCTGATGCCGCTGAGTCCGAATAACGAACGTGTGTAGATGCTGTT 990  
 N N S A D V T V I R Q G N S I R M P L S A N N E R V L D A V  
 GCAGCAGTAGCCGTACAACCTGAAATATTGAAGACGTACCGTAAATTAACCTGCTGGCTCGCAAGTCAAAACATTAGCGTTTGAACCT 1080  
 A A V G T T E N I E D V T V K L T R G S Q V K T L A F E T  
 CTAATTTCCGATCCGGCGCAAAATATTATGACGTGCCGGCGATGTCGTTCCGTTGCTAAACACCGCTTATAGCTTTACCGGTTTAGTT 1170  
 L I S D P A Q N I M L R A G D V V S L L N T P Y S F T G L G  
 CGCGTGGTAAACACAGCAATGAAATTTCTCAAGTAAAGGAATTACGCTGCGGAAGCTATCGGTAAGATGGGTGGCCTAATTGATACT 1260  
 A V G N N Q Q M K F S S K G I T L A E A I G K M G G L I D T  
 CGTTCGGATCCGAGAGGGGTAATTCGTTCCGTCATGTGCCTTTTTCTCAATTAAGTTTAGATCAGCAAACACAATGGGAGCGAAAGGC 1350  
 R S D P R G V F V F R H V P F S Q L S L D Q Q T Q W G A K G  
 TATGGTATGGGTATGGATGTACCCAGGTTTATCGTGTGAATTTACTTGAGCCGAATCACTGTTTTTATTACAACGCTTCCCGATGCAA 1440  
 Y G M G M D V P T V Y R V N L L E P Q S L F L L Q R F P M Q  
 GATAAAGATATTGCTATGTATCAATGCACCGTTGTCGGAATTCAAAAATCTTGAGAATGATTTTCTCGATTACTTCGCCGGTTACA 1530  
 D K D I V Y V S N A P L S E F Q K F L R M I F S I T S P V T

FIG.10A

AGTACCGACTAATGCTATTCGTGCCTATTAATATATTGAATTTATAAGGATAAAAATATGAAACAACCTATTACGGCAAGTCCGACAGAAAA  
 S T T N A I R A Y . CpxC → M E T T I T A S P T E K 1620  
 ACTACAAAAACCGGTTAAACAGAAAAAGTTGGTTAAAAAGCTTAATCCGTTATTTGGGTAAGTGTAGCGATTCTACGGTATTATC  
 L Q K P V K Q K K S W L K K L N P L F W V T V A I P T V L S 1710  
 AGCCTTTTATTCGGTCTGTTGCTCCGATATTATATTCGGAATCAAGCTTCGTTGTAAGATCTCCTCAAATCAGACCGCTTAAAC  
 A F Y F G S V A S D I Y I S E S S F V V R S P Q N Q T A L T 1800  
 CGGTGTCCGGTGCCTTATTACAAGGTTCCGGATTTCTCGAGCTCAAGATGATACTTATACCGTACAAGAATATATGCATTCTCGTACGGC  
 G V G A L L Q G S G F S R A Q D D T Y T V Q E Y M H S R T A 1890  
 ACTAGAACAGTTAATGAAAGACTTGCCAATACGTGAATACTATGAGAATCAAGGCGATATTATCGCTCGCTTAAATGGATTGGTTTAAA  
 L E Q L M K D L P I R E Y Y E N Q G D I I A R F N G F G L N 1980  
 TAATAGTAAAGAAGCGTTTTATAAATATTTCCGAGATCGCTTAAGTGTGGACTTTGACTCTGTTCCGGTATCGCCAGCTTACCTATTCCG  
 N S K E A F Y K Y F R D R L S V D F D S V S G I A S L R I R 2070  
 AGCATTTAACCGGAAGAGCGGCAACAATAATCAAAAATACTTCCCGAAGGTGAAACGCTTATTAACCGTTAAACGAACGTGCAAG  
 A F N A E E G Q Q I N Q K L L A E G E T L I N R L N E R A R 2160  
 AAAAGATACCATTTCAATTCGCGAACAAGCGGTACAGAAGCGGAAAATAATGTAACGAAACGGAAATGCTTTAAGTAAATACCGTAT  
 K D T I S F A E Q A V T E A E N N V N E T A N A L S K Y R I 2250  
 CAAAAATAAAATCTTTGATTTACCGGCACAATCCGGCGTACAACCTTCATTAATTTCCAGCCTAAAAACGGAATTGATTGCGTGTAGAAC  
 K N K I F D L P A Q S G V Q L S L I S S L K S E L I R V E T 2340  
 ACAATGGCTCAATGCAATCTATTACCGGACAACCCACAAGTGTATGCAATGCTTATGCCCAAAAAAGTTTACGTAAGGAAATCGA  
 Q L A Q L Q S I T P D N P Q V D A L L M R Q K S L R K E I D 2430  
 TGAGCAATCAAACAGCTTCCCACTAACACTAATAGCTCTATTGCTATTCAAACCTGCCATTACCACCGCTTAGTACTTGCAAACGAGCT  
 E Q S K Q L S S N S N S S I A I Q T A D Y Q R L V L A N E L 2520  
 GGCACAGCAACAATTGACCGCAGCATTAACTCATTACAAAAACGAAAAATGAAGCGGATCGCCAGCAACTTTATTTAGAAGTAAATCAG  
 A Q Q Q L T A A L T S L Q N T K N E A D R Q Q L Y L E V I 2610  
 TCAGCCAAGCAAACCGGACTGGCGGAAGAGCCTTATCGCTATATAATATTTAGCCGACATTCTTTATCGGTCTGATGCTTTATGGTGT  
 Q P S K P D W A E E P Y R L Y N I L A T F F I G L M L Y G V 2700  
 ATTAAGTTTATTAATGCAAGCGTAAGAGAGCACAAAACTAATGCAATACGGTGATCAAACAACCTTTCCGCCAATCTCTCGCCATTCAA  
 L S L L I A S V R E H K N . CpxB → M Q Y G D Q T T F R Q S L A I Q 2790  
 GGGAGAGTAATCGTGCATTACTCATCGGGAAATTTATTACGCGTTACGGACGAAAAAATTTGGGTTTTTATGGCTGTTGTTGAGCCG  
 G R V I G A L L M R E I I T R Y G R K N L G F L W L F V E P 2880  
 CTATTACTCACTTATTTATCGTTTTGATGTGGAATTTATCCGAGCGGATCGCGTTTTCCGATTTAAATATTATGCTTTTGTGATTACC  
 L L L T L F I V L M W K F I R A D R V S D L N I A F V I T 2970  
 GGTATCCAATGCCCATGATGTGGCGTAATCGGTCAAACCGCACTATCGGTGCAATTTCCGGTAACCTGAGTCTTCTTATCATCGTAAT  
 G Y P M A M M W R N A S N R T I G A I S G N L S L L Y H R N 3060  
 GTTCCCGTATTAGATACCTTACTGGCTCGTGTCACTTGAAGTAGCAGGTGCAACGATTGCCAAATCATTATTATGGCATTAGTCATT  
 V R V L D T L L A R V I L E V A G A T I A Q I I M A L V I 3150

FIG. 10B

## RECOMBINANT VACCINE FOR DISEASES CAUSED BY ENCAPSULATED ORGANISMS

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

The invention relates generally to vaccines used in veterinary applications and, more particularly, to a live, recombinant, attenuated vaccine for disease states that are caused by organisms that include capsule where the presence of the capsule is required for virulence but not immunoprotection. The invention has specific application to a recombinantly produced vaccine that has been engineered such that it lacks capsule.

#### 2. Description of the Prior Art

Vaccines are preparations used to prevent specific diseases in animals and humans by inducing immunity. This is accomplished by exposing a patient to an antigen for a particular disease which, in turn, causes the immune system of the patient to produce large quantities of antibody. The presence of the antibody in the patient's blood protects the patient from a later attack by the disease causing agent. Vaccines may either be composed of subunits of the agent, or the live or killed agent itself. For example, poliomyelitis, commonly referred to as "polio", is typically prevented by either administering a live, attenuated oral poliovirus vaccine, which is common practice for treating children, or by administering a killed or inactivated poliovirus vaccine, which is the usual practice for treating adults since they are generally at higher risk for contracting polio from the live vaccine. If a live vaccine is to be used, its virulence must be attenuated in some way; otherwise the virus in the vaccine will cause the disease it is intended to protect against.

A number of diseases are caused by encapsulated bacteria wherein the capsule, which is the gum-like layer of polysaccharide or polypeptide exterior to the cell wall of these bacteria, is required for pathogenesis. Swine pleuropneumonia is one example, and virulence factors for *Actinobacillus pleuropneumoniae*, the bacterium which causes the disease, include capsular polysaccharide, endotoxin, and protein exotoxins. Swine pleuropneumonia is one of the major respiratory diseases affecting swine production throughout the world, and accounts for millions of dollars in annual losses to the industry in the United States alone.

U.S. Pat. No. 5, 429,818 to Inzana, which is herein incorporated by reference, discloses that non-encapsulated mutants of *Actinobacillus pleuropneumoniae* are avirulent and capable of providing excellent protection against subsequent exposure to the virulent bacteria. The non-encapsulated mutants described in Inzana were prepared by ethylmethanesulfonate mutagenesis. However, such procedures have the disadvantages that some spontaneous or chemically induced mutants may not be stable, and the nature of the mutation(s) is (are) unknown.

### SUMMARY OF THE INVENTION

It is an object of this invention to provide a safe and effective, live, attenuated, recombinant vaccine for diseases caused by bacteria and fungi which are normally encapsulated and where the capsule is required for virulence but not immunoprotection.

It is another object of this invention to genetically engineer certain bacteria or fungi to lack capsule such that they are rendered avirulent and the genetic nature of the mutation is known.

It is yet another object of this invention to provide a safe and effective, live, attenuated, recombinant vaccine for pleuropneumonia.

According to the invention, a recombinant, live, attenuated strain of *Actinobacillus pleuropneumoniae* which has been genetically engineered to lack capsule has been produced. Since the capsule is required for virulence, but not immunoprotection, the strain will be useful as a vaccine against swine pleuropneumonia. The vaccine was produced by cloned plasmid vector that cannot replicate in *A. pleuropneumoniae*. The capsule export and synthesis genes of *A. pleuropneumoniae* serotype 5 were sequenced. A large deletion was made in the cloned synthesis genes for the capsule, and genes encoding for kanamycin resistance and sucrose sensitivity were then cloned into the deleted site to serve as marker genes. This suicide vector was inserted into a virulent *A. pleuropneumoniae* serotype 5 strain using electroporation in order to obtain a homologous recombination event by double cross over between homologous regions of the chromosome and plasmid. Four isolates were obtained, and each lacked iridescence suggesting a lack of capsule. The lack of capsule and the deleted region of the capsule genes was confirmed in one strain by dot blotting and Southern blotting, respectively. The presence of the marker genes in the recombinant strain was also confirmed. No other change in any other phenotypic properties could be identified, and the marker genes were not found in other regions of the chromosome. The recombinant strain, referred to as J45-100, was very serum sensitive, had reduced virulence in pigs at ten times the 50% lethal dose for the parent strain, and should provide protection for swine against pleuropneumonia.

This invention will be useful for producing vaccines against any encapsulated organism that produces toxins or other virulence factors where the capsule is required for virulence but not immunoprotection. All that will be required will be to clone the genes encoding for capsule synthesis for the organism, and then delete and replace the section of the cloned gene with a marker gene on a suicide vector, and then introduce the vector into the desired organism and screen for a genetically modified organism that lacks capsule. The invention should be useful in producing vaccines for additional bacteria infectants including, but not limited to, *Pasteurella multocida*, *Pasteurella haemolytica*, and *Pseudomonas aeruginosa*, as well as fungi such as *Cryptococcus neoformans* which is a pathogen associated with acquired immune deficiency syndrome (AIDS) in cats and humans.

### BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, aspects and advantages will be better understood from the following detailed description of the preferred embodiments of the invention with reference to the drawings, in which:

FIG. 1 is a physical map of pCW-11 E cloned DNA from the capsule synthesis region of *A. pleuropneumoniae* J45. The location and direction of transcription of the two complete ORFs (cpsA and cpsB, solid fill) identified by dideoxy sequencing is indicated. The location of a partial third potential ORF (cpsC) is also indicated. The location and direction of transcription of the incomplete capsule export gene cpxD located on this DNA fragment is also indicated. The 2.1 kb BglIII-StuI fragment used as the DNA probe in FIG. 2 is indicated. Dotted fill indicates incomplete ORFs.

FIG. 2 is a southern blot analysis of *A. pleuropneumoniae* genomic DNA hybridized to the digoxigenin-labeled 2.1 kb BglIII-StuI fragment of pCW11E. BamHI-digested genomic DNA from serotype 1 strain 4074 (lane 1), serotype 2 strain

1536 (lane 2), serotype 5a strain J45 (lane 3), serotype 5a strain K17 (lane 4), serotype 5 strain 178 (lane 5), serotype 7 strain 29628 (lane 6), and serotype 9 strain 13261 (lane 7) were hybridized with the probe as described below. The molecular mass of the hybridizing bands (in kb) is indicated.

FIGS. 3a and 3b present the nucleotide sequence of the 3.2 kb HindIII-EcoRV fragment of pCW-11E, containing the serotype-specific *A. pleuropneumoniae* J45 DNA (SEQ ID NO. 1). The deduced amino acid sequences of the two complete ORFs detected in this sequence, *cpsA* (SEQ ID NO.2) and *cpsB* (SEQ ID NO.3), and the deduced N-terminal sequence of a third incomplete ORF, *cpsC* (SEQ ID NO. 4), are indicated below the nucleotide sequence. Putative ribosome-binding sites preceding each ORF are in boldface, and putative -10 and -35 promoter sequences upstream from *cpsA* are indicated.

FIG. 4 describes construction of the suicide vector containing the deleted capsule synthesis DNA, pCW11 EΔ1 KS1, and production of noncapsulated mutants of *A. pleuropneumoniae* J45 by allelic exchange. The pCW11EΔ1KS1 plasmid vector was constructed by digesting pCW-11E with BglIII and StuI, making the ends blunt-ended, and ligating the large 6.4 kb fragment to the 3.8 kb BamHI fragment of pKS (also made bluntended) containing the nptI-sacRB (Kan<sup>r</sup> Suc<sup>r</sup>) cartridge. Restriction sites in parentheses indicate the original ends of the fragments ligated in pCW11EΔ1KS1. The pCW11EΔ1KS1 vector was electrotransformed into *A. pleuropneumoniae*, and non-capsulated Kan<sup>r</sup> transformants were screened by lack of iridescence on media containing 85 μg/ml of kanamycin.

FIG. 5 is a southern blot analysis of genomic DNA isolated from *A. pleuropneumoniae* J45 (lane 1) or J45-100 (lane 2) with digoxigenin-labeled probes specific for nptI or portions of the *A. pleuropneumoniae* capsulation locus. *A. pleuropneumoniae* J45 (lane 1) or J45-100 (lane 2) genomic DNA was digested with XbaI (panels A and C) or BamHI (panel B), and hybridized with either the 1.24 kb PstI fragment of pKS (nptI-specific), panel A; the 2.1 kb BglIII-StuI fragment of pCW-11E (*cpsABC*-specific, see FIG. 1), panel B; or the 2.1 kb C/al fragment of pCW-1C (*cpxCBA*-specific, see FIG. 3.2), panel C.

FIG. 6 is a colony immunoblot of *A. pleuropneumoniae* J45 and J45-100 reacted with a capsular polysaccharide specific swine antiserum. Approximately  $5 \times 10^5$  (lane 1) or  $5 \times 10^4$  (lane 2) CFU per well were applied to a nitrocellulose membrane. The membrane was lysed in chloroform and incubated with a swine antiserum that contained antibodies to the serotype 5a capsular polysaccharide but not other *A. pleuropneumoniae* surface antigens.

FIG. 7 shows immunoblots of *A. pleuropneumoniae* J45 (lane 1) and J45-100 (lane 2) concentrated culture supernatants containing predominately the exotoxins Ap<sub>xI</sub> and Ap<sub>xII</sub>. Panel A was reacted with an Ap<sub>xI</sub>-specific monoclonal antibody, and panel B was reacted with an Ap<sub>xII</sub>-specific monoclonal antibody. In panel A, the concentrated culture supernatant of *A. pleuropneumoniae* serotype 2 strain 1536 (lane 3) was included as a negative control because this serotype does not synthesize Ap<sub>xI</sub>. The blot in panel A was reacted with the Ap<sub>xI</sub>-specific monoclonal antibody.

FIG. 8 shows the electrophoretic profiles of LPS isolated from *A. pleuropneumoniae* J45 (lane 1) and the recombinant noncapsulated mutant J45-100 (lane 2). LPS was electrophoresed through a 15% separating gel and stained with ammoniacal silver.

FIG. 9 shows the bactericidal activity of precolostral calf serum for *A. pleuropneumoniae* J45 and J45-100. Percent

viability of bacterial strains was evaluated after 60 minutes incubation at 37° C. Each data point represents the mean of three separate experiments performed in duplicate. Error bars represent the standard deviation for each mean. The maximum percent viability recorded for J45 was 100%, although these values were typically higher because the bacteria usually grew during the experiment. Values greater than 100% were not recorded because they could not be accurately determined.

FIGS. 10a and 10b present the nucleotide sequence of the 3.2 kb XbaI-ClaI fragment of pCW-1C encoding for the capsule export genes of *A. pleuropneumoniae* J45 DNA (SEQ ID NO. 5). The deduced amino acid sequences (SEQ ID Nos. 6-9) of proteins involved in the export of the *A. pleuropneumoniae* serotype 5a capsular polysaccharide are presented.

FIG. 11 is a physical map of pCW-1C DNA from *A. pleuropneumoniae* J45

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

The invention contemplates using a live, recombinantly produced, avirulent, strain of a microorganism (i.e., bacteria or fungus) which has been genetically engineered to be non-capsulated as a vaccine against diseases caused by the microorganism. The invention will have utility in preventing diseases wherein the capsule of the microorganism is required for virulence but not immunoprotection, and where the disease is caused by toxins or other virulence factors. As a particular example of the invention, a non-capsulated strain of *Actinobacillus pleuropneumoniae* has been produced and should be useful as a vaccine against pleuropneumonia in swine. The chief feature of the invention is the genetic modification of the microorganism, which, in a specific embodiment is *Actinobacillus pleuropneumoniae*, to include a deletion in its deoxyribonucleic acid (DNA) in the region encoding for capsule synthesis. For exemplary purposes only, the synthesis of a transformed *Actinobacillus pleuropneumoniae* serotype 5 mutant is disclosed; however, it should be understood that other serotypes could be prepared in a manner similar to that which is described below, and would be useful in a vaccine alone or in combination with one or more recombinant mutants of different serotypes.

The strain described below, along with other strains of noncapsulated, toxigenic bacteria or other microorganisms generated according to the procedures described below, will make excellent vaccines because they are avirulent, but produce all the antigens necessary for the host to make a protective immune response. The vaccines can be administered by a variety of methods; however, intramuscular or subcutaneous injection is preferred. The advantage of these live vaccines is that the toxins that are primarily responsible for the disease and other components only made by live organisms or in vivo, will be made at the immunization site and the host will make an immune response which protects itself from the lesions caused by the toxins. Hence, the disease (acute or chronic) does not occur. The organisms cannot disseminate, however, because without capsule, they are extremely serum sensitive, and are cleared immediately in the bloodstream or respiratory tract. In addition, as a live vaccine, the cell-mediated immune response will be greater and the protection will last longer than with killed vaccines.

#### EXAMPLE

A DNA region involved in *Actinobacillus pleuropneumoniae* capsular polysaccharide biosynthesis was identified and

characterized. A probe specific for the *cpxD* gene involved in the export of the *A. pleuropneumoniae* serotype 5a J45 capsular polysaccharide was used to identify and clone an adjacent 5.8 kilobase BamHI fragment of J45 genomic DNA. Southern blot analyses demonstrated that a portion of this region contained DNA that was serotype-specific. DNA sequence analysis demonstrated that this region contained two complete open reading frames, *cpsA* and *cpsB*, and an incomplete potential third open reading frame, *cpsC*. *cpsA* and *cpsB* shared some low homology with glycosyltransferases involved in the biosynthesis of *Escherichia coli* lipopolysaccharide and *Haemophilus influenzae* type b capsular polysaccharide, respectively. A 2.1 kilobase deletion which spanned the cloned *cpsABC* open reading frames was constructed and recombined into the J45 chromosome by allelic exchange to produce the mutant J45-100. This mutant did not produce intracellular or extracellular capsular polysaccharide, indicating that *cpsA*, *cpsB*, and/or *cpsC* were involved in *A. pleuropneumoniae* capsular polysaccharide biosynthesis. The Apx toxin and lipopolysaccharide profiles of J45-100 were identical to the encapsulated parent strain, J45. However, J45-100 grew faster in vitro than J45. J45-100 was sensitive to killing in precolostral calf serum, whereas J45 was not. J45-100 was avirulent when used to challenge pigs intratracheally with 3 times the 50% lethal dose of strain J45. At 6 times the 50% lethal dose of J45,

## MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are as described in Table 1. For genomic DNA extraction and for bactericidal assays, *A. pleuropneumoniae* strains were grown with shaking at 37° C. in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 5 µg/ml nicotinamide adenine dinucleotide (NAD) (Sigma Chemical Co., St. Louis, Mo.). For electroporation, *A. pleuropneumoniae* strains were grown with shaking at 37° C. in tryptic soy broth (Difco Laboratories) containing 0.6% yeast extract (Difco Laboratories) and 5 µg/ml NAD (TSY-N). For pig challenge experiments, *A. pleuropneumoniae* strains were grown with shaking at 37° C. in Columbia broth (Difco Laboratories) containing 5 µg/ml NAD. *Escherichia coli* strains were grown in Luria-Bertani broth (Sambrook et al., 1989) for routine cultivation, or in Terrific broth (Tartof and Hobbes, 1987) for extraction of plasmids. Antibiotics were used in growth media for maintenance of plasmids in *E. coli* at the following concentrations: ampicillin (Amp) 100 µg/ml, and kanamycin (Kan) 50 µg/ml. Kanamycin was used at 85 µg/ml for selection of *A. pleuropneumoniae* recombinant mutants.

TABLE 1

Bacterial strains and plasmids used		
Strain or plasmid	Relevant genotype or characteristics	Source or reference
<i>A. pleuropneumoniae</i> Strains		
4074	serotype 1; (ATCC 27088)	ATCC <sup>a</sup>
1536	serotype 2; (ATCC 27089)	ATCC <sup>a</sup>
J45	serotype 5a	Fenwick et al., 1986a
K17	serotype 5a	Nielsen, 1986a
178	serotype 5	M. Mulks
29628	serotype 7	L. Hoffman
13261	serotype 9	J. Nicolet
J45-C	noncapsulated mutant isolated after ethyl methanesulfonate mutagenesis of strain J45	Inzana et al., 1993a
J45-100	recombinant noncapsulated mutant derived from strain J45	This chapter
<i>E. coli</i> Strains		
XL1-Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac (F <sup>+</sup> proAB lacI <sup>q</sup> ZAM15 Tn10); Host for recombinant plasmids	Stratagene, LaJolla, Calif.
Plasmids		
pGEM-3Z	Cloning vector, 2.74 kb; Amp <sup>r</sup>	Promega
pCW-1C	5.3 kb XbaI fragment of J45 cloned into pGEM-3Z	Chapter 3
pCW-11E	5.8 kb BamHI fragment of J45 cloned into pGEM-3Z	This work
pKS	3.8 kb BamHI fragment containing the nptI <sup>b</sup> -sacRB cartridge <sup>c</sup> cloned into the BamHI site of pGEM-3Z; Amp <sup>r</sup> , Kan <sup>r</sup>	S. M. Boyle
pCW11EA1KS1	pCW-11E with the 2.1 kb BglIII-StuI fragment deleted and the 3.8 kb BamHI nptI-sacRB cartridge from pKS ligated in	This chapter

<sup>a</sup>American Type Culture Collection, Rockville, Md.

<sup>b</sup>This marker was originally derived from the Tn903 nptI gene of pUC4K (Pharmacia Biotech, Piscataway, N.J.).

<sup>c</sup>This cartridge has been previously described (Ried and Collmer, 1987).

J345-100 caused mild to moderate lung lesions, but not death. These results demonstrated that the capsular polysaccharide is a major determinant of serum-resistance and virulence of *A. pleuropneumoniae*.

Calculation of generation time. The generation time of logarithmic phase *A. pleuropneumoniae* strains grown in TSY-N was calculated using the equation:  $R=1/g$ , where R is the average rate of bacterial growth, and g is the genera-

tion time of the bacterial population (Pelczar et al., 1993). The average rate of growth,  $R$ , was calculated using the following equation:  $R=3.32(\log_{10} N-\log_{10} N_0)/t$ , where  $t$  is the elapsed time,  $N$  is the number of bacteria at time= $t$ , and  $N_0$  is the initial number of bacteria at time= $0$  (Pelczar et al., 1993).

DNA hybridization analysis. Restriction endonuclease-digested DNA (approximately 5  $\mu$ g per lane) was electrophoresed through 0.7% agarose gels and was transferred by capillary action to MagnaGraph nylon membranes (Micron Separations Inc., Westboro, Mass.) using 20 $\times$  saline sodium citrate (20 $\times$  SSC is 3 M NaCl, 300 mM sodium citrate, pH 7) as previously described (Sambrook et al., 1989; Southern, 1975). DNA was covalently linked to nylon membranes by ultraviolet irradiation using a UV Stratalinker (Stratagene, La Jolla, Calif.). Digoxigenin-labeled probes for DNA hybridizations were synthesized by the random primer method using the Genius System nonradioactive labeling and detection kit (Boehringer-Mannheim Corp., Indianapolis, Ind.) according to the manufacturer's directions. DNA hybridizations were performed at 68 $^{\circ}$  C. in solutions containing 5 $\times$ SSC. The membranes were washed and developed according to the Genius System directions for calorimetric detection.

Recombinant DNA methods and reagents. Genomic DNA was isolated from broth-grown *A. pleuropneumoniae* cells using a method described by S. Spinola. Briefly, bacteria were resuspended in 10 mM Tris-1 mM EDTA (pH 8) and incubated with sodium dodecyl sulfate (0.66%), and RNase (100  $\mu$ g/ml) for 1 hour at 37 $^{\circ}$  C. Proteinase K was added to a final concentration of 100  $\mu$ g/ml, and the mixture was incubated at 56 $^{\circ}$  C. for 1 hour. The mixture was extracted once with buffered phenol and four times with buffered phenol-chloroform (Amresco, Inc., Solon, Ohio), and the genomic DNA was ethanol precipitated and resuspended in 10 mM Tris-1 mM EDTA (pH 8). Plasmid DNA was isolated by a rapid alkaline lysis method (Ish-Horowicz and Burke, 1981). Restriction fragments required for cloning and probe synthesis were eluted from agarose gels as described (Zhen and Swank, 1993). Restriction digests, agarose gel electrophoresis, and DNA ligations were performed as previously described (Sambrook et al., 1989). Restriction fragment ends were made blunt-ended by filling in 5' overhangs with nucleotides (dNTPs) using the Klenow fragment of DNA polymerase 1, as previously described (Sambrook et al., 1989). Plasmid DNA was transformed into *E. coli* strains by electroporation (Dower et al., 1988) using a BTX ECM 600 electroporator (BTX, Inc., San Diego, Calif.).

Restriction endonucleases and the Klenow fragment of DNA polymerase I were obtained from Promega Corporation (Madison, Wis.). T4 DNA ligase was obtained from Gibco BRL (Gaithersburg, Md.). Nucleotides (dNTPs) for fill-in reactions were obtained from Boehringer-Mannheim Corporation (Indianapolis, Ind.).

DNA sequencing and analysis. The nucleotide sequence of both strands of the 2.7 kilobase (kb) XbaI-EcoRV DNA fragment of pCW-11E was determined by the dideoxy chain-termination method (Sanger et al., 1977) using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio) with  $\alpha^{35}$ [S]dATP (DuPont/NEN Research Products, Boston, Mass.). Double stranded DNA templates were sequenced using custom, oligonucleotide primers (DNAgency, Inc., Malverne, Pa.) to continue reading along each strand.

The nucleotide sequence obtained was combined with the nucleotide sequence of the 4.6 kb XbaI-ClaI DNA fragment

of pCW-1C encoding for the upstream capsule structural genes (FIG. 10), and was analyzed using DNASTAR analysis software (DNASTAR, Inc, Madison, Wis.). Sequence similarity searches of the EMBUGenBank/DBJ databases were performed using BLAST software (Altschul et al., 1990) at the National Center for Biotechnology Information (Bethesda, Md.).

Conserved regions of the *H. influenzae* type b cap (capb) locus involved in capsular polysaccharide export was used to identify, clone, and characterize a portion of the *A. pleuropneumoniae* serotype 5a capsulation locus involved in capsular polysaccharide export. Southern blot analyses of *A. pleuropneumoniae* serotype 5a strain J45 genomic DNA with probes specific for contiguous regions of the *H. influenzae* type b capsulation (capb) locus were performed. These probes did not hybridize to *A. pleuropneumoniae* genomic DNA under conditions of high stringency (68 $^{\circ}$  C., 5 $\times$  SSC), but did hybridize under conditions of medium-to-low stringency (55 $^{\circ}$  C., 5 $\times$  SSC). A 4.4 kb EcoRI fragment of the *H. influenzae* capb locus from the plasmid pSKH1 containing the region 1 bexD gene involved in capsular polysaccharide export and two region 2 open reading frames (ORFs) involved in capsular polysaccharide biosynthesis, hybridized to 1.2 kb HindIII and 5.3 kb XbaI fragments of J45 genomic DNA. A 9.0 kb EcoRI fragment of the *H. influenzae* capb locus from the plasmid pSKH2, containing the region 1 bexCBA genes involved in capsular polysaccharide export, some uncharacterized region 3 DNA common to several *H. influenzae* serotypes, and some region 2 DNA involved in capsular polysaccharide biosynthesis, hybridized to 1.5 kb HindIII, 5.3 kb XbaI, and 2.4 kb XhoI fragments of J45 genomic DNA. These data indicated that the *H. influenzae* type b and *A. pleuropneumoniae* serotype 5a capsule gene loci shared homologous regions. The *H. influenzae* capb specific probes both contain region 1 DNA involved in capsular polysaccharide export, suggesting that the 5.3 kb XbaI genomic DNA fragment from J45 that hybridized to both *H. influenzae* capb probes may contain genes that encode proteins involved in export of the *A. pleuropneumoniae* serotype 5a capsular polysaccharide. The 5.3 kb XbaI genomic DNA fragment from J45 that hybridized to the two *H. influenzae* capb probes was cloned into the XbaI site of the plasmid pGEM-3Z (in both orientations) from XbaI-digested J45 genomic DNA fragments in the range of 4.8 to 6.0 kb that were electroeluted (following electrophoretic separation) from an agarose gel. One of the resulting plasmids was designated pCW-1C. Southern blots were performed to determine if the *H. influenzae* type b bexD, bexC, bexB, and bexA hybridized to adjacent fragments of pCW-1C in the same order (bexDCBA) in which these genes occur in *H. influenzae*. The results suggested that the *A. pleuropneumoniae* serotype 5a DNA region required for capsular polysaccharide export had been successfully cloned, and that this region was organized in a similar manner to the *H. influenzae* type b bex locus.

The nucleotide sequence of the 4.6 kb XbaI-ClaI restriction fragment of pCW-1C was determined and a 3.2 kb XbaI-ClaI restriction fragment is presented in FIGS. 10a-b. Four ORFs (shown in FIGS. 10a-b (SEQ ID NO.5) and FIG. 11) designated cpxDCBA (cpx is used to designate capsular polysaccharide export) were detected in close proximity on the same DNA strand. The AUG initiation codon of cpxC (SEQ ID NO. 7) was 26 nucleotides downstream from the UAA termination codon of cpxD (SEQ ID NO. 6), whereas the AUG initiation codon of cpxB (SEQ ID NO.8) overlapped the UAA termination codon of cpxC (SEQ ID NO.7), and the AUG initiation codon of cpxA (not shown) over-

lapped the UGA termination codon of *cpxB* partially present (SEQ ID NO. 8). Shine-Dalgarno ribosome binding consensus sequences were identified within 17 bases upstream of each AUG initiation codon and a putative promoter containing sequences similar to *E. coli*  $\sigma^{70}$ -10 (TATAAT) and -35 (TTGACA) consensus sequences was identified upstream of *cpxD* (SEQ ID NO. 6). A palindromic sequence which may function as a rho-independent transcription termination signal was identified downstream of *cpxA* (SEQ ID NO. 9). The genetic organization suggests that *cpxDCBA* are transcribed onto a single, polycistronic mRNA.

Electrotransformation of *A. pleuropneumoniae*. *A. pleuropneumoniae* was grown to midlogarithmic phase in TSY-N, pelleted by centrifugation at 7000×g at 4° C., and washed four times in a chilled (4° C.), filter-sterilized buffer containing 272 mM mannitol, 2.43 mM K<sub>2</sub>HPO<sub>4</sub>, 0.57 mM KH<sub>2</sub>PO<sub>4</sub>, 15% glycerol, pH 7.5. This buffer was modified (to contain mannitol in place of sucrose) from a previously described buffer used for washing *A. pleuropneumoniae* cells prior to electroporation (Lalonde et al., 1989b). The cells were then washed one time in chilled, filter-sterilized 15% glycerol, and resuspended to approximately 10<sup>10</sup> CFU/ml in 15% glycerol. Aliquots of this suspension (90  $\mu$ l) were mixed with 1.5–2.0  $\mu$ g of plasmid DNA (in 1.5  $\mu$ l distilled water) that had been purified by cesium chloride density gradient ultracentrifugation (Sambrook et al., 1989), placed in chilled 2 mm gap electroporation cuvettes (BTX, Inc.), and electroporated using a BTX ECM 600 electroporator (BTX, Inc.) set to a charging voltage of 2.5 kV and to a resistance setting of R7 (246 ohms). The actual pulse generated was 2.39 kV delivered over 10.7 milliseconds. After electroporation, the cells were recovered in 1 ml TSY-N containing 5 mM MgCl<sub>2</sub> with gentle shaking for 3.5 hours at 37° C. After recovery, the cells were cultured on TSY-N agar containing 85  $\mu$ g of kanamycin per ml and were incubated at 37° C.

Immunoblotting. For colony immunoblots, *A. pleuropneumoniae* whole cells grown overnight on TSY-N agar plates were scraped into phosphatebuffered saline (PBS) and adjusted to 10<sup>9</sup> CFU/ml, as determined spectrophotometrically. Approximately 5×10<sup>4</sup> or 5×10<sup>5</sup> CFU per well was applied to a nitrocellulose membrane (NitroBind; Micron Separations Inc.) using a Bio-Dot apparatus (Bio-Rad Laboratories, Richmond, Calif.). The membrane was placed in chloroform for 15 minutes at room temperature to lyse the bacterial cells on the membrane. The membrane was air dried completely, and incubated for 1 hour at room temperature in Tris-buffered saline, pH 7.5 (TBS) containing 2% skim milk to block nonspecific binding sites on the membrane. The membrane was incubated for 1 hour at room temperature in a 1:200 dilution (in 2% milk-TBS) of an adsorbed swine antiserum that contained antibodies to the serotype 5a capsular polysaccharide, but not other *A. pleuropneumoniae* surface antigens. This capsular polysaccharide-enriched antiserum was prepared by adsorbing hyperimmune swine antiserum to *A. pleuropneumoniae* K17 with a spontaneous noncapsulated mutant, K17-C (Inzana and Mathison, 1987), as described previously (Inzana, 1995). The membrane was washed in TBS containing 0.05% Tween 20, then incubated 1 hour at room temperature in a 1:1000 dilution of rabbit anti-swine IgG conjugated to horseradish peroxidase (heavy and light chains; Cappel, Durham, N.C.). The membrane was washed in TBS, then developed with 4-chloro-1-naphthol (Bio-Rad Laboratories) in TBS containing 0.02% H<sub>2</sub>O<sub>2</sub>.

Immunoblotting of *A. pleuropneumoniae* concentrated culture supernatants was performed as described previously

(Ma and Inzana, 1990). Briefly, approximately 15  $\mu$ g of total culture supernatant protein was separated by discontinuous SDS-PAGE (Laemmli, 1970) through an 8% separating gel. The proteins were transferred to a nitrocellulose membrane (NitroBind; Micron Separations Inc.) by the method of Towbin et al. (1979). The membrane was incubated in TBS containing 2% bovine serum albumin to block nonspecific binding and was cut into strips. The strips were incubated overnight at 4° C. with either a monoclonal antibody specific for the *Apxl* toxin (Ma and Inzana, 1990) or a monoclonal antibody specific for the *Apxl* toxin (Devendish et al., 1989; Frey et al., 1992), and washed in TBS. The blot reacting with the *Apxl*-specific monoclonal antibody was incubated with a 1:2000 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (Cappel), washed in TBS, and developed as described above. The blot reacting with the *Apxl*-specific monoclonal antibody was incubated with a 1:2000 dilution of goat anti-mouse IgG conjugated to alkaline phosphatase and developed as described previously (Frey et al., 1992).

LPS extraction and electrophoresis. LPS was isolated from *A. pleuropneumoniae* using a micro hot phenol-water extraction method, as previously described (Inzana, 1983). Purified LPS was electrophoresed through a 15% polyacrylamide separating gel containing urea, as described (Inzana et al., 1988). LPS electrophoretic profiles were visualized by staining the gel with ammoniacal silver (Tsai and Frasch, 1982).

Serum bactericidal assay. Sensitivity of *A. pleuropneumoniae* to the bactericidal activity of precolostral calf serum was determined. Percent viability of bacterial strains in 5, 10, 15, 20, 30, 40, and 50% precolostral calf serum was evaluated after 60 minutes incubation at 37° C.

Virulence study. Pigs 7 to 9 weeks of age were obtained from two local herds free from *A. pleuropneumoniae* infection and were distributed randomly into groups. Groups of pigs were housed in separate pens with no direct physical contact permitted between each group. The animal facilities at Virginia Polytechnic Institute and State University are operated and maintained in accordance with the requirements of the American Association for Accreditation of Laboratory Animal Care. For the challenge experiment, *A. pleuropneumoniae* strains were grown with shaking in Columbia broth (Difco Laboratories) supplemented with 5  $\mu$ g/ml NAD at 7000×g and resuspended to approximately 10<sup>9</sup> CFU/ml in PBS. Pigs were challenged intratracheally with 10 ml of a dilution of this suspension following mild sedation with Stresnil (Pittman-Moore, Inc., Washington Crossing, N.J.). Pigs were necropsied as soon as possible after death or immediately after euthanasia with sodium pentobarbital. Lung lesions were scored by a veterinary pathologist according to the following criteria: 0, unremarkable lungs (no gross lesions observed); 1+, 1–10% of lung tissue affected by some combination of congestion, edema, hemorrhage, consolidation, and/or pleuritis; 2+, 11–49% of lung tissue affected; 3+, 50–74% of lung tissue affected; 4+, 75% or greater of lung tissue affected. Lung samples were taken at necropsy from the right cranial-dorsal aspect of the caudal lobe and cultured on brain heart infusion medium containing NAD to detect the presence of *A. pleuropneumoniae*.

## RESULTS

Identification and cloning of a serotype-specific *A. pleuropneumoniae* DNA region. To identify and clone *A. pleuropneumoniae* J45 DNA involved in capsular polysaccharide biosynthesis, Southern blot analyses were performed to identify an adjacent DNA region upstream (in the 5'



direction) from the cpxDCBA gene cluster involved in the export of capsule polysaccharide described above (FIGS. 10a–b and 11). It was expected this upstream DNA region would encode serotype-specific genes involved in capsular polysaccharide biosynthesis because the *A. pleuropneumoniae* capsulation (cap) locus seemed to be organized in a manner similar to the capsulation loci of *Haemophilus influenzae* type b and *Neisseria meningitidis* group B. BamHI-digested *A. pleuropneumoniae* J45 genomic DNA was probed with the digoxigenin-labeled 1.2 kb BamHI-XbaI fragment of pCW-1C that contained a portion of the cpxD gene. This cpxD-specific probe hybridized to a single, approximate 5.8 kb BamHI J45 genomic DNA fragment (data not shown). This 5.8 kb BamHI fragment was cloned into the BamHI site of pGEM-3Z from BamHI-digested J45 genomic DNA fragments in the range of 5.0–6.5 kb that were electroeluted (following electrophoretic separation) from an agarose gel. The resulting plasmid was designated pCW-11E and was restriction mapped (FIG. 1). A portion of the pCW-11 E insert DNA (the 1.2 kb BamHI-XbaI fragment) overlapped the DNA present on the insert of pCW-1C.

BamHI-digested genomic DNA from several different *A. pleuropneumoniae* serotypes was hybridized with the 2.1 kb BglIII-StuI fragment of pCW-11 E (FIG. 1) to determine the serotype-specificity of this DNA region (FIG. 2). The 2.1 kb BglIII-StuI DNA fragment hybridized to a 5.8 kb BamHI genomic DNA fragment from three *A. pleuropneumoniae* serotype 5 strains tested, but not to genomic DNA from serotypes 1, 2, 7, and 9 (FIG. 2). Thus, the *A. pleuropneumoniae* DNA in pCW-11 E contained DNA that was specific to serotype 5 strains. Because this DNA was serotype-specific, it was likely to be involved in capsular polysaccharide biosynthesis.

Nucleotide sequence and analysis of a serotype-specific *A. pleuropneumoniae* DNA region. The nucleotide sequence of the 2.7 kb XbaI-EcoRV DNA fragment of pCW-11E was determined. This nucleotide sequence was combined with the nucleotide sequence of the 4.6 kb ClaI-XbaI fragment of pCW-1C and was examined for the presence of open reading frames (ORFs) not previously identified. The nucleotide sequence of the 3.2 kb HindIII-EcoRV fragment of pCW-11E containing newly identified ORFs is provided in FIG. 3. Two complete ORFs, designated cpsA and cpsB (cps for capsular polysaccharide synthesis), were identified upstream and on the opposite strand from the cpxD gene involved in *A. pleuropneumoniae* capsular polysaccharide export (FIG. 1 and FIG. 3). The AUG initiation codon of cpsB was 3 nucleotides downstream from the UAA termination codon of cpsA. An AUG initiation codon of a third potential ORF, cpsC, was identified 15 bases downstream from the UAA termination codon of cpsB. Shine-Dalgarno ribosome-binding consensus sequences (Shine and Dalgarno, 1974) were identified within 13 bases upstream of the AUG initiation codons of cpsA, cpsB, and cpsC (FIG. 3). A putative promoter, containing sequences similar to the *E. coli* <sup>-70</sup>-10 (TATAAT) and <sup>-35</sup> (TTGACA) consensus sequences (Hawley and McClure, 1983) was identified upstream of cpsA (FIG. 3). The close proximity of cpsABC and the identification of a putative promoter upstream suggested that these ORFs may be co-transcribed. The G+C content for the DNA region encoding cpsABC was 28%.

The predicted polypeptides of cpsA and cpsB were comprised of 321 (CpsA) and 526 (CpsB) amino acids (FIG. 3). The predicted molecular masses of CpsA and CpsB were 36.9 and 61.7 kilodaltons (kDa), respectively. Hydropathy plots demonstrated that CpsA and CpsB were relatively

hydrophilic proteins, suggesting that these proteins may be associated with the *A. pleuropneumoniae* cytoplasmic compartment (data not shown). BLAST searches (Altschul et al., 1990) of the combined, nonredundant nucleotide and protein databases at the National Center for Biotechnology Information did not reveal any substantial homology between cpsABC at the nucleotide or amino acid level with other sequences in the databases (data not shown). However, a low level of homology (15% similarity) was observed between CpsA and the *E. coli* Rfb protein, an O-antigen glycosyltransferase involved in LPS biosynthesis (Cheah and Manning, 1993). A low level of homology (approximately 14% similarity) was detected between CpsB and the region 2 ORF 3 predicted protein product of the *H. influenzae* type b capsulation locus. The ORF 3 predicted protein is involved in the biosynthesis of the polyribosylribitol phosphate capsular polysaccharide of *H. influenzae* type b (Van Eldere et al., 1995). No significant homology was observed between the N-terminal 83 amino acids of CpsC and any proteins in the databases.

Production of kanamycin-resistant, noncapsulated *A. pleuropneumoniae* serotype 5a transformants. FIG. 4 schematically outlines the procedures used to produce recombinant, noncapsulated *A. pleuropneumoniae* J45 mutants by homologous recombination and allelic exchange. The vector pCW11EΔ1 KS1 was first constructed to use as a nonreplicating, suicide vector to promote the exchange of wild type *A. pleuropneumoniae* capsulation DNA with genetically-altered *A. pleuropneumoniae* capsulation DNA by a double homologous recombination crossover event. The pCW11 EΔ1 KS1 vector was constructed by first digesting pCW-11 E with BglIII and StuI to create a large deletion in serotype-specific *A. pleuropneumoniae* capsulation DNA. The ends of this digested DNA were made blunt ended, and the large 6.4 kb fragment was ligated to the 3.8 kb BamHI fragment of pKS (also made blunt ended) containing the nptI-sacR-sacB cartridge. This cartridge contains the Tn903 nptI gene before which is known to confer kanamycin resistance (Kan<sup>r</sup>) to *A. pleuropneumoniae* (Tascon et al., 1994), and the sacRB sequences that confer sucrose sensitivity (Suc<sup>s</sup>) to many gram-negative bacteria (Gay et al., 1983; Ried and Collmer, 1987). The deletion created in pCW11EΔ1KS1 spanned cpsABC (FIG. 1, FIG. 4) and was, therefore, likely to affect the protein products of these ORFs.

The pCW11EΔ1KS1 vector did not replicate in *A. pleuropneumoniae* and, therefore, functioned as a suicide vector. After pCW11 EΔ1 KS1 was electroporated into *A. pleuropneumoniae* J45, seven kanamycin-resistant transformants were obtained after the recovery mixtures were incubated at 37° C. for 2 days. Four of these kanamycin resistant J45 transformants were noniridescent when visualized on plates with an obliquely transmitted light source, suggesting that these transformants were noncapsulated (data not shown). The medium used to grow *A. pleuropneumoniae* prior to electroporation with pCW11EΔ1KS1 was a factor since noncapsulated kanamycin-resistant transformants were never obtained when *A. pleuropneumoniae* was grown in brain heart infusion supplemented with NAD.

Genotypic characterization of the kanamycin-resistant *A. pleuropneumoniae* transformants. Preliminary colony hybridization analyses of the seven kanamycin-resistant transformants revealed that the four transformants which appeared noncapsulated (by visual inspection) hybridized with an nptI-specific DNA probe (the 1.24 kb PstI fragment of pKS), but not with probes specific for pGEM-3Z (the 1.1 kb BglIII fragment pGEM-3Z) or the serotype-specific 2.1 kb

BglIII-Stul fragment of pCW-11E (data not shown). These results indicated that a double recombination event had occurred in each of these four kanamycin-resistant transformants. In contrast, colonies of the other three kanamycin-resistant transformants hybridized to probes specific for the *nptI* gene, pGEM-3Z, and the 2.1 kb BglIII-Stul fragment of pCW-11E, suggesting that a single cross over had occurred and the entire pCW11EA1KS1 suicide vector had integrated into the chromosome of these transformants (data not shown). Southern blot analyses of genomic DNA purified from the four kanamycin-resistant, potentially noncapsulated transformants (using the probes described above) were identical, indicating that the same double recombination event had occurred in each of these transformants. One of these transformants was randomly selected for further study and was designated J45-100.

Southern blot analyses of genomic DNA isolated from J45 and J45-100 with DNA probes specific for the *nptI* gene, the 2.1 kb BglIII-Stul fragment of pCW-11E, and the 2.1 kb Clal fragment of pCW-1C were performed (FIG. 5). The *nptI*-specific DNA probe hybridized to a 5.0 kb fragment of Xbal-digested J45-100 DNA, but not to J45 DNA, verifying that the *nptI* marker was in the chromosome of J45-100 (FIG. 5A). The hybridization of the *nptI* probe to a 5.0 kb Xbal J45-100 genomic DNA fragment was consistent with the size of this Xbal fragment in the pCW11E-1KS1 suicide vector used to produce J45-100. The 2.1 kb BglIII-Stul fragment of pCW-11E hybridized to a 5.8 kb fragment of BamHI-digested J45 but not to J45-100 DNA, verifying that this fragment was deleted in J45-100 (FIG. 5B). The probe specific for the *cpxCBA* genes (the 2.1 kb Clal fragment of pCW-1C) involved in capsular polysaccharide export hybridized to a 5.3 kb Xbal fragment of both J45 and J45-100 (FIG. 5C). This result verified that this portion of the *A. pleuropneumoniae* capsulation locus was unaffected by the double recombination event that had occurred within the adjacent DNA region. A probe specific for pGEM-3Z did not hybridize to genomic DNA from either J45 or J45-100, verifying that no vector DNA was contained in the genome of J45-100. Collectively, these DNA hybridization results indicated that the desired double recombination event and allelic exchange had occurred in J45-100.

Phenotypic characterization of the *A. pleuropneumoniae* kanamycin-resistant transformant, J45-100. J45-100 was evaluated for capsular polysaccharide production by colony immunoblotting and latex agglutination. Antiserum containing antibodies specific for the *A. pleuropneumoniae* serotype 5a capsular polysaccharide, but not other bacterial surface components, reacted with J45 but did not react with J45-100 (FIG. 6). Because the bacterial colonies on the membrane had been lysed in chloroform, these results indicated that J45-100 did not produce intracellular or extracellular capsular polysaccharide. Whole or sonicated J45-100 did not agglutinate latex beads that were covalently conjugated to purified antibody to the serotype 5a capsular polysaccharide of *A. pleuropneumoniae* (Inzana, 1995), whereas J45 whole cells and sonicated J45-C cells strongly agglutinated the latex bead reagent (data not shown). These results verified that the deletion engineered into the cap locus of *A. pleuropneumoniae* J45-100 resulted in the loss of capsular polysaccharide biosynthesis. Furthermore, these results indicated that a noncapsulated mutant of J45 isolated after ethyl methanesulfonate mutagenesis (Inzana et al., 1993a), J45-C, produced intracellular but not extracellular capsular polysaccharide.

Apx toxin expression and the LPS electrophoretic profiles of J45 and J45-100 were compared to determine if the

mutation engineered into the cap locus of J45-100 affected these important virulence determinants. No difference in secretion of the 105 kDa ApxI and ApxII toxin proteins into culture supernatant was detected between J45 and J45-100 (FIG. 7). In addition, no difference in the LPS electrophoretic profiles of J45 and J45-100 was detected (FIG. 8).

The growth of J45 and J45-100 in TSY-N and the sensitivity of J45 and J45-100 to the bactericidal activity of precolostral calf serum were examined to determine the effect of loss of encapsulation on these phenotypic properties. Growth curves of J45 and J45-100 in TSY-N were similar but not identical (data not shown). However, viable plate counts demonstrated that during the logarithmic phase of growth, J45-100 grew faster (generation time=ca. 23 minutes) than the parent encapsulated strain, J45 (generation time=ca. 28 minutes) (data not shown). The recombinant noncapsulated mutant, J45-100, was efficiently killed within 60 minutes in 10 to 50% precolostral calf serum as a complement source, whereas the encapsulated parent strain, J45, was not killed (FIG. 9).

The sucrose sensitivity of J45-100 was examined to determine whether the *sacRB* sequences could function as a counterselectable marker in *A. pleuropneumoniae* and subsequently induce the excision of the *nptI*-*sacRB* cartridge from the J45-100 chromosome. Broth-grown J45-100 grew very heavily when plated directly or when diluted and then plated on TSY-N or Luria-Bertani (to which 5 llg/ml NAD was added) medium containing 5% or 8% sucrose. The presence of the *sacRB* sequences in the chromosome of J45-100 was verified by Southern blotting. These results suggested that either the *sacRB* marker was not expressed in *A. pleuropneumoniae* or possibly that the levan product formed by the *sacRB* levansucrase in the presence of sucrose was not toxic to J45-100.

Intratracheal challenge of pigs with the recombinant *A. pleuropneumoniae* noncapsulated mutant, J45-100. The recombinant noncapsulated mutant, J45-100, did not cause any mortality in pigs when administered at doses 3 and 6 times (1.45×10<sup>7</sup> CFU and 2.95×10<sup>7</sup> CFU, respectively) the 50% lethal dose (LD<sub>50</sub>) of the encapsulated parent strain, J45 (5×10<sup>6</sup> CFU) (Inzana et al., 1993a) (Table 2). In contrast, all three of the pigs challenged with 6.5 times the LD<sub>50</sub> of J45 developed severe lung lesions and died (Table 2).

TABLE 2

Virulence of *A. pleuropneumoniae* J45 and J45-100 for pigs

Challenge Strain	Challenge Dose	Mean Lung Lesion Score	Number positive/ total number tested	
			Mortality	Recovery <sup>a</sup>
J45	1.6–3.30 × 10 <sup>7</sup> CFU <sup>b</sup>	4+	3/4 <sup>c</sup>	4/4
J45-100	1.5 × 10 <sup>7</sup> CFU	0	0/5	0/5
J45-100	3.0 × 10 <sup>7</sup> CFU	1+	0/5	2/5 <sup>d</sup>
J45-100	8.4 × 10 <sup>7</sup> CFU	1+	1/4 <sup>e</sup>	4/4 <sup>b</sup>
J45-100	1.8 × 10 <sup>8</sup> CFU	2+	0/4	4/4 <sup>d</sup>
J45-C	1.7 × 10 <sup>8</sup> CFU	1+	0/2	2/2 <sup>d</sup>

<sup>a</sup>Recovery of the challenge strain from lung samples taken at necropsy. Pigs challenged with J45-100 were necropsied 4 days post-challenge.

<sup>b</sup>This dose is 6.6 times the 50% lethal dose (5 × 10<sup>6</sup> CFU) reported in a previous study (Inzana et al., 1993a).

<sup>c</sup>All of the pigs in this group died within 36 hours post-challenge.

<sup>d</sup>*A. pleuropneumoniae* was recovered from the lungs, and was confirmed to be noncapsulated by lack of iridescence and failure to agglutinate serotype 5-specific sensitized latex particles.

<sup>e</sup>Necropsy of the one pig that died indicated that death was due to misadministration of challenge dose.

The five pigs challenged with the lower dose of J45-100 (1.45×10<sup>7</sup> CFU) did not exhibit any clinical symptoms

characteristic of swine pleuropneumonia and did not develop any lung lesions. Furthermore, *A. pleuropneumoniae* was not cultured from lung samples taken four days post-challenge at necropsy. Two of the five pigs challenged with the higher dose of J45-100 ( $2.95 \times 10^7$  CFU) were clinically normal and no lung lesions were observed at necropsy. One pig in this group challenged with the higher J45-100 dose exhibited moderate dyspnea, and at necropsy some lung congestion and slight hemorrhage were observed (lung lesion score=1+). The remaining two pigs in this group exhibited mild dyspnea, and at necropsy some pleuritis and consolidation were observed (lung lesion score=2+). *A. pleuropneumoniae* J45-100 was cultured only from these two pigs with the most severe lung lesions. The bacteria recovered from these pigs did not agglutinate the serotype 5a latex bead agglutination reagent. Thus, the recovered bacteria were still noncapsulated, indicating that J45-100 did not revert to the encapsulated phenotype in vivo.

While nptI (confers resistance to kanamycin) and SacB/SacR (confers sensitivity to sucrose) genes were cloned into the deletion site, these genes were only intended to be used as marker genes. Alternative marker genes may also be employed. It may be preferable to avoid using an antibiotic resistant marker such as nptI for health and safety related reasons, or to provide a mechanism for curing or inactivating the antibiotic marker. Suitable non-antibiotic markers might include mercury resistance.

The non-capsulated strain of *Actinobacillus pleuropneumoniae* serotype 5 produced according to the above procedures only produces two of the three toxins made by *Actinobacillus pleuropneumoniae*. While the modified *Actinobacillus pleuropneumoniae* is protective and

immunogenic, it may also be useful to clone the third RTX toxin gene into the deletion site. This may be done by cloning the RTX toxin gene into the kanamycin gene cassette of strain J45-100, thus inactivating the kanamycin gene.

The vaccine should preferably be provided in a form similar to other vaccines well known in the art. It is preferable that the vaccine will be bottled as a lyophilized mixture, and can include one or more serotypes of mutant strains. To preserve viability, a substance such as Columbia broth, trehalose, or albumin, glycerol, or some other agent would be included. The contents of the lyophilized mixture would only need to be rehydrated with sterile water or saline and injected (intramuscular, intravenous, intraperitoneal, subcutaneous, etc.). The vaccine may also be formulated for other modes of administration as well (e.g., oral, transdermal, sublingual, etc.) using appropriate carrier matrixes (e.g., starch, polysaccharides, oils, liposomes, gums, etc.).

The dose of the vaccine provided to an animal will depend on such factors as the age or sex of the animal, and the mode of delivery. In all cases, a sufficient quantity of the live, avirulent, non-capsulated *Actinobacillus pleuropneumoniae* should be provided to give rise to an immunogenic response in the vaccinated animal. Successful results have been obtained with 2 immunizations 2 to 3 weeks apart of  $10^9$  colony forming units.

While the invention has been described in terms of its preferred embodiments, those skilled in the art will recognize that the invention can be practiced with modification within the spirit and scope of the appended claims.

---

SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 8

## (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 3212 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 400..1362

(ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 1368..2945

(ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 2963..3211

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

```

AAGCTTGAGC AGGCAGCCAA ACTAGCAACC AGCCCCAAAG AAAGGAGTAA TCTAAGTTTG      60
ATGAGTTTCA TCTAATTTCT CTTCATATA TTAAGGAATA ACAACTATAT AGGTATGTCT      120
TAAAATCCAC ATAAGATTG ATTTAATAA GTTACCTAAT CAAGAGAAAT TAAATATAAG      180
AAATTTACAA ACAATTTAAA AAATGTATTT TTTTAAAAA AAAGTAAATC AAGAGGGGCG      240

```

-continued

TTATACAGAT AACATTATA ATTTAAAAGC CATATAAAAT ACGGAGTTTC CCCTAGATAG	300
TTGATAAATT TCTCATTTAT ATTTATGAAA TTCCGATGAA AAATTTATCA ACTATCTAGG	360
GTAACTCCAT AACGTATTCG TATTTTCAGGA GTATTTTTA ATG TCT AGC ATA ATG Met Ser Ser Ile Met 1 5	414
ACT CGT CCT ATA ATT AAT CAT GTA ATG TCT AGA GAT ATT CAA AGT GGC Thr Arg Pro Ile Ile Asn His Val Met Ser Arg Asp Ile Gln Ser Gly 10 15 20	462
ATA TTT AGT TCT ATT TTA GAA TAT TTT ACT GAT TTT GGT TCC AAT GAA Ile Phe Ser Ser Ile Leu Glu Tyr Phe Thr Asp Phe Gly Ser Asn Glu 25 30 35	510
TTT CAA CAT ATT GTC AGT GTA TCT CCA ATA CCT GAA GCT AAA GTT TAT Phe Gln His Ile Val Ser Val Ser Pro Ile Pro Glu Ala Lys Val Tyr 40 45 50	558
CAC TAT CAC CGT CCA CAC CTA GAA GAA AAA TTA TTA CCT AAT TCT GTT His Tyr His Arg Pro His Leu Glu Glu Lys Leu Leu Pro Asn Ser Val 55 60 65	606
TGT ACA GTA CAT CAT GAC CTC AAT GAT CCA GAT CCT TGG CAT GCT AAG Cys Thr Val His His Asp Leu Asn Asp Pro Asp Pro Trp His Ala Lys 70 75 80 85	654
TAT AGA TTT ATT CCT AGA TAT ATG GAA GCT GGG GCT ATA ATT TGT TTA Tyr Arg Phe Ile Pro Arg Tyr Met Glu Ala Gly Ala Ile Ile Cys Leu 90 95 100	702
AAT TAC ACT CAA AAA GAA ATT TTA ATA TCT CAG GGA CTT CCG GAA CAT Asn Tyr Thr Gln Lys Glu Ile Leu Ile Ser Gln Gly Leu Pro Glu His 105 110 115	750
AAG TTA TTT GTG ATT CCT CAC GGA TAT AAT CAA AAA GTA TTA TTT CCT Lys Leu Phe Val Ile Pro His Gly Tyr Asn Gln Lys Val Leu Phe Pro 120 125 130	798
AAG AAA ATT AAA GAA ATA TCA AGT ACA GAT AAA ATT ACC TTA GGA ATT Lys Lys Ile Lys Glu Ile Ser Ser Thr Asp Lys Ile Thr Leu Gly Ile 135 140 145	846
GCT TCA CGG AGA TAT GGT AGA AGA GTA AAA GGA GAT GCA TAT TTA TTT Ala Ser Arg Arg Tyr Gly Arg Arg Val Lys Gly Asp Ala Tyr Leu Phe 150 155 160 165	894
GAA TTA GCA AAA AGA TTA AAT CCA GAC CAT TTT AAA TTT ATT TTT GTT Glu Leu Ala Lys Arg Leu Asn Pro Asp His Phe Lys Phe Ile Phe Val 170 175 180	942
GGT AAA GAT AGA CAA TAT AGT GCC TTA GAA ATG CAA GAT CTA GGA TTT Gly Lys Asp Arg Gln Tyr Ser Ala Leu Glu Met Gln Asp Leu Gly Phe 185 190 195	990
GAA GCT CAA GTA TAT GAA AGA TTG CCA TAT AGA ATG TTT CAA AGT TTT Glu Ala Gln Val Tyr Glu Arg Leu Pro Tyr Arg Met Phe Gln Ser Phe 200 205 210	1038
TAT AAT AAT ATT GAT GTA CTA CTT ATG TGT AGT AGT CAT GAA GGT GGA Tyr Asn Asn Ile Asp Val Leu Leu Met Cys Ser Ser His Glu Gly Gly 215 220 225	1086
CCT GCA AAT ATC CCC GAA GCA TTA GCT ACT GGG ACA CCT ATA TTT TCA Pro Ala Asn Ile Pro Glu Ala Leu Ala Thr Gly Thr Pro Ile Phe Ser 230 235 240 245	1134
TCT AAC ATA GGT ATA CCT AAG GAT GTT GTT ATT AAT TAT AAG AAT GGG Ser Asn Ile Gly Ile Pro Lys Asp Val Val Ile Asn Tyr Lys Asn Gly 250 255 260	1182
TTG ATT CTA ACC TTA GAT CCA GAT ATA GAT GCT GAA CAG ATT AAT TTT Leu Ile Leu Thr Leu Asp Pro Asp Ile Asp Ala Glu Gln Ile Asn Phe 265 270 275	1230
ATT TGC CTT GAA AAA CCA AAT ATA TTT GAA AAT ATA TTA GAT TTT TCA Ile Cys Leu Glu Lys Pro Asn Ile Phe Glu Asn Ile Leu Asp Phe Ser 280 285 290	1278

-continued

CTA AAA CAG TCT CCA AGT TTA GCA ATT TCT TGG GAG AAA TGT ATT CAA Leu Lys Gln Ser Pro Ser Leu Ala Ile Ser Trp Glu Lys Cys Ile Gln 295 300 305	1326
CAA AAT ATT TTA GTT TAT AAA AAA ATA ATT AAG GGT TAATT ATG TCC Gln Asn Ile Leu Val Tyr Lys Lys Ile Ile Lys Gly Met Ser 310 315 320 1	1373
ATT TCT ATT CTA GTA CCT GAT TCT TTA CAC ATT AAC AAA AGA AAC TTT Ile Ser Ile Leu Val Pro Asp Ser Leu His Ile Asn Lys Arg Asn Phe 5 10 15	1421
AGT TCA TTC TTC AGT TGG ATT GAG AAA AAT AAA ATA AAT ATC CAT TTT Ser Ser Phe Phe Ser Trp Ile Glu Lys Asn Lys Ile Asn Ile His Phe 20 25 30	1469
GAA AAT AAT AAT AAA GAT TGG ATT TCA TTA TAT GGT TTT TAC GAT TCA Glu Asn Asn Asn Lys Asp Trp Ile Ser Leu Tyr Gly Phe Tyr Asp Ser 35 40 45 50	1517
AAA TTG GGT ATT CTA TAT GAG AAA ATA GAT ATT CTT ACT AAG ATT GAA Lys Leu Gly Ile Leu Tyr Glu Lys Ile Asp Ile Leu Thr Lys Ile Glu 55 60 65	1565
GAA GAG GAA TTA TTT GCT TTT TGT GTT TAT GAT CTA AAT ATT TTC AAT Glu Glu Glu Leu Phe Ala Phe Cys Val Tyr Asp Leu Asn Ile Phe Asn 70 75 80	1613
ATT TGT AGA GCT GAA TTA TTA TCT TTA GTA GCC ACA AGA CCC GAA TGG Ile Cys Arg Ala Glu Leu Leu Ser Leu Val Ala Thr Arg Pro Glu Trp 85 90 95	1661
TAT AAT GAA GAT TAT CCT AAT AAC TTA AGA GAA ATA TAC AAA AAA CTC Tyr Asn Glu Asp Tyr Pro Asn Asn Leu Arg Glu Ile Tyr Lys Lys Leu 100 105 110	1709
TAT ACT AAT AAT CGA AGT GAA TTA TTG CAA AAC ATG GCT GCT GCT TGG Tyr Thr Asn Asn Arg Ser Glu Leu Leu Gln Asn Met Ala Ala Ala Trp 115 120 125 130	1757
TAT TGG GTT GAT TTC TGG AAA AAA CGC CTA TCT GAG TTA AAA CAA TTC Tyr Trp Val Asp Phe Trp Lys Lys Arg Leu Ser Glu Leu Lys Gln Phe 135 140 145	1805
TCT CAT TGT TGT GTA TTT TCA GGA GGT TTA ATT TAT CAA AAA TCT TTG Ser His Cys Cys Val Phe Ser Gly Leu Ile Tyr Gln Lys Ser Leu 150 155 160	1853
ATT GAG TTA TTA AAA TAT ACT CCA ACT AAA GTT ATG GTT ATG GAA AGC Ile Glu Leu Leu Lys Tyr Thr Pro Thr Lys Val Met Val Met Glu Ser 165 170 175	1901
CTA TTT ACA GGA AAC GAA TAT TAT TGT GAG GAA CGT TAT TCA TCA ATT Leu Phe Thr Gly Asn Glu Tyr Tyr Cys Glu Glu Arg Tyr Ser Ser Ile 180 185 190	1949
GCT AAT AAT AGC GAT ATT AAA CAT TTA GCT ATT TTT AAC TCT TAT AAA Ala Asn Asn Ser Asp Ile Lys His Leu Ala Ile Phe Asn Ser Tyr Lys 195 200 205 210	1997
AAA ACA TTT AGT TCA AAA AGT GAA TAT GAT AAG GAA CGA ATG AAA GCT Lys Thr Phe Ser Ser Lys Ser Glu Tyr Asp Lys Glu Arg Met Lys Ala 215 220 225	2045
ATT AAT AAG TTC CTA TTA ATG AAA AAT AAG AAT GTC CAA CAA CCT ACT Ile Asn Lys Phe Leu Leu Met Lys Asn Lys Asn Val Gln Gln Pro Thr 230 235 240	2093
GAT TCT GAA ATA TTA GTA TTT AAA CAA CAA AAA CCA ATA ATT ACT ATT Asp Ser Glu Ile Leu Val Phe Lys Gln Gln Lys Pro Ile Ile Thr Ile 245 250 255	2141
ATT GGA CAA GTG ATA AAT GAT TTT TCA GTC CTA GAA TAT AAA GGG AGA Ile Gly Gln Val Ile Asn Asp Phe Ser Val Leu Glu Tyr Lys Gly Arg 260 265 270	2189
GGA CTA TCA ACA ATT AAA ATC TAT AAA GAA CTT ATA TCT AAA CTA TCA Gly Leu Ser Thr Ile Lys Ile Tyr Lys Glu Leu Ile Ser Lys Leu Ser 2237	

-continued

275	280	285	290	
GAG AAT GGA TTT AAT GTA GTA TTA AAA ACT CAC CCT TGG GAA GAG AAA Glu Asn Gly Phe Asn Val Val Leu Lys Thr His Pro Trp Glu Glu Lys 295 300 305				2285
AAA AAT AAT ATC CGT ACA TCT TTA ACT AAA AAT ATA ATA GAA GAA TTT Lys Asn Asn Ile Arg Thr Ser Leu Thr Lys Asn Ile Ile Glu Glu Phe 310 315 320				2333
CTA AAA AAT CTA ACT GAG AAT CAA CAA GAA TGT ATA AAA ATA GTT GAT Leu Lys Asn Leu Thr Glu Asn Gln Gln Glu Cys Ile Lys Ile Val Asp 325 330 335				2381
CAC TAT TCA ATA AAG AAA TTA TTT AAA CAA TCT GAT TTT ATT ATT AGT His Tyr Ser Ile Lys Lys Leu Phe Lys Gln Ser Asp Phe Ile Ile Ser 340 345 350				2429
TTA AAT TCT CAA GGG CTC CTT GAA GCT GCA TTT GAT GGT ATA AAA CCT Leu Asn Ser Gln Gly Leu Leu Glu Ala Ala Phe Asp Gly Ile Lys Pro 355 360 365 370				2477
ATA CAG TTA GGT AAT GCT TTT TAT GGA AAA AAA GGA TTC ACG TAC GAT Ile Gln Leu Gly Asn Ala Phe Tyr Gly Lys Lys Gly Phe Thr Tyr Asp 375 380 385				2525
TAT GAC TTT TTA GAT ATT GAT CAA TTG GTA AAT GAC TTA GTA GTA AAT Tyr Asp Phe Leu Asp Ile Asp Gln Leu Val Asn Asp Leu Val Val Asn 390 395 400				2573
AAA CTT ACT CCA ACA CTA TCT TTA GAA GAG TTT GAT TTG TTC GAA GAG Lys Leu Thr Pro Thr Leu Ser Leu Glu Glu Phe Asp Leu Phe Glu Glu 405 410 415				2621
TTC ATT ACT ATA TTA TTA CAA AAG CAT GCT GTT TCT ATT CAC GCC TCT Phe Ile Thr Ile Leu Leu Gln Lys His Ala Val Ser Ile His Ala Ser 420 425 430				2669
GGC GTA AGT GTT TTA TCT AGA ATA TTT AAT TTA CCT ACT ATT ATA CCA Gly Val Ser Val Leu Ser Arg Ile Phe Asn Leu Pro Thr Ile Ile Pro 435 440 445 450				2717
TTA GTA GAA AAT GTC CCT AAG GAG AAG TCT AAA ACA ACA TTA CCT ACT Leu Val Glu Asn Val Pro Lys Glu Lys Ser Lys Thr Thr Leu Pro Thr 455 460 465				2765
CAA AAA GAT GTG GTA AAA AAG GAA AAT ACA ACA ATT GTT AAT ATG GTT Gln Lys Asp Val Val Lys Lys Glu Asn Thr Thr Ile Val Asn Met Val 470 475 480				2813
GAG TTA CCT AAA GTA GTT CCA CAA AGT GAT AAG AAT AGG AAA TAT CAA Glu Leu Pro Lys Val Val Pro Gln Ser Asp Lys Asn Arg Lys Tyr Gln 485 490 495				2861
AAA TTT AGA AAC AAT CCT CGA CAA TTC TTT GCA GAT TCT AGG AAT CCA Lys Phe Arg Asn Asn Pro Arg Gln Phe Phe Ala Asp Ser Arg Asn Pro 500 505 510				2909
GTT ATT AGA AGT TTA ATG TAT TTT TTC CCT TAT AAA TAATATAGGT Val Ile Arg Ser Leu Met Tyr Phe Phe Pro Tyr Lys 515 520 525				2955
CTAATTT ATG TTA AAA AAA TAT CAG CCT TTT GAT TTA AGA AAA ATA AAT Met Leu Lys Lys Tyr Gln Pro Phe Asp Leu Arg Lys Ile Asn 1 5 10				3004
GAA GGC CAC TCT AGT AAT GCT AAG TTA GTT TTA CAT TCT GAG GCC TGT Glu Gly His Ser Ser Asn Ala Lys Leu Val Leu His Ser Glu Ala Cys 15 20 25 30				3052
AAT ATA GAT GCT AAA ATC TCT AAG TTT TTC TGT TCA CAA GAT GAC ATT Asn Ile Asp Ala Lys Ile Ser Lys Phe Phe Cys Ser Gln Asp Asp Ile 35 40 45				3100
AAT TTA GAA AAC TTT ATT GCA ACA TTT ACT GAT AAC TAT AAA GCA CCA Asn Leu Glu Asn Phe Ile Ala Thr Phe Thr Asp Asn Tyr Lys Ala Pro 50 55 60				3148

-continued

---

GAA GTA TAT ACG GCG ATT TTA AAG AAT TGT TGT ATT ACA CCT AGA GCA	3196
Glu Val Tyr Thr Ala Ile Leu Lys Asn Cys Cys Ile Thr Pro Arg Ala	
65 70 75	

CCT AAG CTA CCA AGA T	3212
Pro Lys Leu Pro Arg	
80	

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 321 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

Met Ser Ser Ile Met Thr Arg Pro Ile Ile Asn His Val Met Ser Arg	
1 5 10 15	
Asp Ile Gln Ser Gly Ile Phe Ser Ser Ile Leu Glu Tyr Phe Thr Asp	
20 25 30	
Phe Gly Ser Asn Glu Phe Gln His Ile Val Ser Val Ser Pro Ile Pro	
35 40 45	
Glu Ala Lys Val Tyr His Tyr His Arg Pro His Leu Glu Glu Lys Leu	
50 55 60	
Leu Pro Asn Ser Val Cys Thr Val His His Asp Leu Asn Asp Pro Asp	
65 70 75 80	
Pro Trp His Ala Lys Tyr Arg Phe Ile Pro Arg Tyr Met Glu Ala Gly	
85 90 95	
Ala Ile Ile Cys Leu Asn Tyr Thr Gln Lys Glu Ile Leu Ile Ser Gln	
100 105 110	
Gly Leu Pro Glu His Lys Leu Phe Val Ile Pro His Gly Tyr Asn Gln	
115 120 125	
Lys Val Leu Phe Pro Lys Lys Ile Lys Glu Ile Ser Ser Thr Asp Lys	
130 135 140	
Ile Thr Leu Gly Ile Ala Ser Arg Arg Tyr Gly Arg Arg Val Lys Gly	
145 150 155 160	
Asp Ala Tyr Leu Phe Glu Leu Ala Lys Arg Leu Asn Pro Asp His Phe	
165 170 175	
Lys Phe Ile Phe Val Gly Lys Asp Arg Gln Tyr Ser Ala Leu Glu Met	
180 185 190	
Gln Asp Leu Gly Phe Glu Ala Gln Val Tyr Glu Arg Leu Pro Tyr Arg	
195 200 205	
Met Phe Gln Ser Phe Tyr Asn Asn Ile Asp Val Leu Leu Met Cys Ser	
210 215 220	
Ser His Glu Gly Gly Pro Ala Asn Ile Pro Glu Ala Leu Ala Thr Gly	
225 230 235 240	
Thr Pro Ile Phe Ser Ser Asn Ile Gly Ile Pro Lys Asp Val Val Ile	
245 250 255	
Asn Tyr Lys Asn Gly Leu Ile Leu Thr Leu Asp Pro Asp Ile Asp Ala	
260 265 270	
Glu Gln Ile Asn Phe Ile Cys Leu Glu Lys Pro Asn Ile Phe Glu Asn	
275 280 285	
Ile Leu Asp Phe Ser Leu Lys Gln Ser Pro Ser Leu Ala Ile Ser Trp	
290 295 300	
Glu Lys Cys Ile Gln Gln Asn Ile Leu Val Tyr Lys Lys Ile Ile Lys	
305 310 315 320	

-continued

Gly

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 526 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

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

```

Met Ser Ile Ser Ile Leu Val Pro Asp Ser Leu His Ile Asn Lys Arg
 1           5           10           15
Asn Phe Ser Ser Phe Phe Ser Trp Ile Glu Lys Asn Lys Ile Asn Ile
          20           25           30
His Phe Glu Asn Asn Asn Lys Asp Trp Ile Ser Leu Tyr Gly Phe Tyr
          35           40           45
Asp Ser Lys Leu Gly Ile Leu Tyr Glu Lys Ile Asp Ile Leu Thr Lys
          50           55           60
Ile Glu Glu Glu Glu Leu Phe Ala Phe Cys Val Tyr Asp Leu Asn Ile
          65           70           75           80
Phe Asn Ile Cys Arg Ala Glu Leu Leu Ser Leu Val Ala Thr Arg Pro
          85           90           95
Glu Trp Tyr Asn Glu Asp Tyr Pro Asn Asn Leu Arg Glu Ile Tyr Lys
          100          105          110
Lys Leu Tyr Thr Asn Asn Arg Ser Glu Leu Leu Gln Asn Met Ala Ala
          115          120          125
Ala Trp Tyr Trp Val Asp Phe Trp Lys Lys Arg Leu Ser Glu Leu Lys
          130          135          140
Gln Phe Ser His Cys Cys Val Phe Ser Gly Gly Leu Ile Tyr Gln Lys
          145          150          155          160
Ser Leu Ile Glu Leu Leu Lys Tyr Thr Pro Thr Lys Val Met Val Met
          165          170          175
Glu Ser Leu Phe Thr Gly Asn Glu Tyr Tyr Cys Glu Glu Arg Tyr Ser
          180          185          190
Ser Ile Ala Asn Asn Ser Asp Ile Lys His Leu Ala Ile Phe Asn Ser
          195          200          205
Tyr Lys Lys Thr Phe Ser Ser Lys Ser Glu Tyr Asp Lys Glu Arg Met
          210          215          220
Lys Ala Ile Asn Lys Phe Leu Leu Met Lys Asn Lys Asn Val Gln Gln
          225          230          235          240
Pro Thr Asp Ser Glu Ile Leu Val Phe Lys Gln Gln Lys Pro Ile Ile
          245          250          255
Thr Ile Ile Gly Gln Val Ile Asn Asp Phe Ser Val Leu Glu Tyr Lys
          260          265          270
Gly Arg Gly Leu Ser Thr Ile Lys Ile Tyr Lys Glu Leu Ile Ser Lys
          275          280          285
Leu Ser Glu Asn Gly Phe Asn Val Val Leu Lys Thr His Pro Trp Glu
          290          295          300
Glu Lys Lys Asn Asn Ile Arg Thr Ser Leu Thr Lys Asn Ile Ile Glu
          305          310          315          320
Glu Phe Leu Lys Asn Leu Thr Glu Asn Gln Gln Glu Cys Ile Lys Ile
          325          330          335
Val Asp His Tyr Ser Ile Lys Lys Leu Phe Lys Gln Ser Asp Phe Ile

```



-continued

340				345				350							
Ile	Ser	Leu	Asn	Ser	Gln	Gly	Leu	Leu	Glu	Ala	Ala	Phe	Asp	Gly	Ile
		355					360							365	
Lys	Pro	Ile	Gln	Leu	Gly	Asn	Ala	Phe	Tyr	Gly	Lys	Lys	Gly	Phe	Thr
		370				375					380				
Tyr	Asp	Tyr	Asp	Phe	Leu	Asp	Ile	Asp	Gln	Leu	Val	Asn	Asp	Leu	Val
		385				390					395				400
Val	Asn	Lys	Leu	Thr	Pro	Thr	Leu	Ser	Leu	Glu	Glu	Phe	Asp	Leu	Phe
						405					410				415
Glu	Glu	Phe	Ile	Thr	Ile	Leu	Leu	Gln	Lys	His	Ala	Val	Ser	Ile	His
						420					425				430
Ala	Ser	Gly	Val	Ser	Val	Leu	Ser	Arg	Ile	Phe	Asn	Leu	Pro	Thr	Ile
						435					440				445
Ile	Pro	Leu	Val	Glu	Asn	Val	Pro	Lys	Glu	Lys	Ser	Lys	Thr	Thr	Leu
						450					455				460
Pro	Thr	Gln	Lys	Asp	Val	Val	Lys	Lys	Glu	Asn	Thr	Thr	Ile	Val	Asn
						465					470				475
Met	Val	Glu	Leu	Pro	Lys	Val	Val	Pro	Gln	Ser	Asp	Lys	Asn	Arg	Lys
						485					490				495
Tyr	Gln	Lys	Phe	Arg	Asn	Asn	Pro	Arg	Gln	Phe	Phe	Ala	Asp	Ser	Arg
						500					505				510
Asn	Pro	Val	Ile	Arg	Ser	Leu	Met	Tyr	Phe	Phe	Pro	Tyr	Lys		
						515					520				525

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 83 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

Met	Leu	Lys	Lys	Tyr	Gln	Pro	Phe	Asp	Leu	Arg	Lys	Ile	Asn	Glu	Gly
1				5					10					15	
His	Ser	Ser	Asn	Ala	Lys	Leu	Val	Leu	His	Ser	Glu	Ala	Cys	Asn	Ile
				20					25					30	
Asp	Ala	Lys	Ile	Ser	Lys	Phe	Phe	Cys	Ser	Gln	Asp	Asp	Ile	Asn	Leu
				35					40					45	
Glu	Asn	Phe	Ile	Ala	Thr	Phe	Thr	Asp	Asn	Tyr	Lys	Ala	Pro	Glu	Val
				50					55					60	
Tyr	Thr	Ala	Ile	Leu	Lys	Asn	Cys	Cys	Ile	Thr	Pro	Arg	Ala	Pro	Lys
				65					70					75	80
Leu	Pro	Arg													

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 3150 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 376..1557

(ix) FEATURE:  
 (A) NAME/KEY: CDS

-continued

(B) LOCATION: 1586..2740

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 2743..3150

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTAGACATTA CATGATTAAT TATAGGACGA GTCATTATGC TAGACATTAA AAATACTCCT	60
GAAATACGAA TACGTTATGG AGTTACCCTA GATAGTTGAT AAATTTTCA TCGGAATTC	120
ATAAATATAA ATGAGAAATT TATCAACTAT CTAGGGGAAA CTCGCTATTT TATATGGCTT	180
TTAAATTATA ATGTTTATCT GTATAACGCC CCTCTTGATT TACTTTTTTT TAAAAAAAAT	240
ACATTTTTTA ATTTGTTTGT AAATTTCTTA TATTTAATTT CTCTTGATTA GGTAACCTAT	300
TAAATCAAT CTTTATGTGG ATTTTAAGAC ATACCTATAT AGTTGTTATT CCTTAATATA	360
TTGAAGAGAA ATTAG ATG AAA CTC ATC AAA CTT AGA TTA CTC CTT TCT TTG	411
Met Lys Leu Ile Lys Leu Arg Leu Leu Leu Ser Leu	
1 5 10	
GGG CTG GTT GCT AGT TTG GCT GCC TGC TCA AGC TTA CCC ACT TCA GGC	459
Gly Leu Val Ala Ser Leu Ala Ala Cys Ser Ser Leu Pro Thr Ser Gly	
15 20 25	
CCT AGC CAT AGT GCG ATT TTA GAG GCT AAT TCC CAG AAC TCA GAT AAA	507
Pro Ser His Ser Ala Ile Leu Glu Ala Asn Ser Gln Asn Ser Asp Lys	
30 35 40	
CCT TTA CCG GAA GTT AAT TTA GTG GAG TTA GAT AAT GGC TTA GTT CAG	555
Pro Leu Pro Glu Val Asn Leu Val Glu Leu Asp Asn Gly Leu Val Gln	
45 50 55 60	
CAG TTG TAT CAG ACT CAG CAA AGT CAG CAA TTT TCC GGC TTT TTA GGC	603
Gln Leu Tyr Gln Thr Gln Gln Ser Gln Gln Phe Ser Gly Phe Leu Gly	
65 70 75	
ACG GCT GGC GGT GCT GGA TAT GCC GGT GCG GTC AAT GTG GGG GAT GTT	651
Thr Ala Gly Gly Ala Gly Tyr Ala Gly Ala Val Asn Val Gly Asp Val	
80 85 90	
CTT GAA ATT TCA ATT TGG GAA GCG CCA CCG GCA GTG TTG TTT GGC GGT	699
Leu Glu Ile Ser Ile Trp Glu Ala Pro Pro Ala Val Leu Phe Gly Gly	
95 100 105	
ACT TTT AGT TCT GAA GGG CAA GGT AGC GGG CAT TTA ACG CAA TTA CCG	747
Thr Phe Ser Ser Glu Gly Gln Gly Ser Gly His Leu Thr Gln Leu Pro	
110 115 120	
GCG CAA ATG GTT AAC CAA AAC GGT ACG GTT ACT GTG CCG TTT GTG GGT	795
Ala Gln Met Val Asn Gln Asn Gly Thr Val Thr Val Pro Phe Val Gly	
125 130 135 140	
AAT ATT CGT GTT GCA GGT AAA ACA CCG GAA GCG ATT CAG TCT CAA ATT	843
Asn Ile Arg Val Ala Gly Lys Thr Pro Glu Ala Ile Gln Ser Gln Ile	
145 150 155	
GTT GGG GCA TTG CAA CGT AAA GCG AAT CAG CCA CAA GTA TTA GTA AAA	891
Val Gly Ala Leu Gln Arg Lys Ala Asn Gln Pro Gln Val Leu Val Lys	
160 165 170	
ATT GCG AAT AAT AAC TCT GCG GAT GTT ACG GTT ATT CGT CAG GGT AAC	939
Ile Ala Asn Asn Asn Ser Ala Asp Val Thr Val Ile Arg Gln Gly Asn	
175 180 185	
AGT ATT CGT ATG CCG CTG AGT GCG AAT AAC GAA CGT GTG TTA GAT GCT	987
Ser Ile Arg Met Pro Leu Ser Ala Asn Asn Glu Arg Val Leu Asp Ala	
190 195 200	
GTT GCA GCA GTA GGC GGT ACA ACT GAA AAT ATT GAA GAC GTT ACC GTA	1035
Val Ala Ala Val Gly Thr Thr Glu Asn Ile Glu Asp Val Thr Val	
205 210 215 220	
AAA TTA ACT CGT GGC TCG CAA GTC AAA ACA TTA GCG TTT GAA ACT CTA	1083
Lys Leu Thr Arg Gly Ser Gln Val Lys Thr Leu Ala Phe Glu Thr Leu	
225 230 235	

-continued

ATT TCC GAT CCG GCG CAA AAT ATT ATG TTA CGT GCC GGC GAT GTC GTT	1131
Ile Ser Asp Pro Ala Gln Asn Ile Met Leu Arg Ala Gly Asp Val Val	
240 245 250	
TCG TTG CTA AAC ACG CCT TAT AGC TTT ACC GGT TTA GGT GCG GTG GGT	1179
Ser Leu Leu Asn Thr Pro Tyr Ser Phe Thr Gly Leu Gly Ala Val Gly	
255 260 265	
AAC AAC CAG CAA ATG AAA TTC TCA AGT AAA GGA ATT ACG CTT GCC GAA	1227
Asn Asn Gln Gln Met Lys Phe Ser Ser Lys Gly Ile Thr Leu Ala Glu	
270 275 280	
GCT ATC GGT AAG ATG GGT GGC CTA ATT GAT ACT CGT TCG GAT CCG AGA	1275
Ala Ile Gly Lys Met Gly Gly Leu Ile Asp Thr Arg Ser Asp Pro Arg	
285 290 295 300	
GGG GTA TTC GTC TTC CGT CAT GTG CCT TTT TCT CAA TTA AGT TTA GAT	1323
Gly Val Phe Val Phe Arg His Val Pro Phe Ser Gln Leu Ser Leu Asp	
305 310 315	
CAG CAA ACA CAA TGG GGA GCG AAA GGC TAT GGT ATG GGT ATG GAT GTA	1371
Gln Gln Thr Gln Trp Gly Ala Lys Gly Tyr Gly Met Gly Met Asp Val	
320 325 330	
CCG ACG GTT TAT CGT GTG AAT TTA CTT GAG CCG CAA TCA CTG TTT TTA	1419
Pro Thr Val Tyr Arg Val Asn Leu Leu Glu Pro Gln Ser Leu Phe Leu	
335 340 345	
TTA CAA CGC TTC CCG ATG CAA GAT AAA GAT ATT GTC TAT GTA TCA AAT	1467
Leu Gln Arg Phe Pro Met Gln Asp Lys Asp Ile Val Tyr Val Ser Asn	
350 355 360	
GCA CCG TTG TCC GAA TTC CAA AAA TTC TTG AGA ATG ATT TTC TCG ATT	1515
Ala Pro Leu Ser Glu Phe Gln Lys Phe Leu Arg Met Ile Phe Ser Ile	
365 370 375 380	
ACT TCG CCG GTT ACA AGT ACG ACT AAT GCT ATT CGT GCC TAT	1557
Thr Ser Pro Val Thr Ser Thr Thr Asn Ala Ile Arg Ala Tyr	
385 390	
TAATATATTG AATTTATAAG GATAAAAT ATG GAA ACA ACT ATT ACG GCA AGT	1609
Met Glu Thr Thr Ile Thr Ala Ser	
1 5	
CCG ACA GAA AAA CTA CAA AAA CCG GTT AAA CAG AAA AAA AGT TGG TTA	1657
Pro Thr Glu Lys Leu Gln Lys Pro Val Lys Gln Lys Lys Ser Trp Leu	
10 15 20	
AAA AAG CTT AAT CCG TTA TTT TGG GTA ACT GTA GCG ATT CCT ACG GTA	1705
Lys Lys Leu Asn Pro Leu Phe Trp Val Thr Val Ala Ile Pro Thr Val	
25 30 35 40	
TTA TCA GCC TTT TAT TTC GGT TCT GTT GCT TCC GAT ATT TAT ATT TCG	1753
Leu Ser Ala Phe Tyr Phe Gly Ser Val Ala Ser Asp Ile Tyr Ile Ser	
45 50 55	
GAA TCA AGC TTC GTT GTA AGA TCT CCT CAA AAT CAG ACC GCT TTA ACC	1801
Glu Ser Ser Phe Val Val Arg Ser Pro Gln Asn Gln Thr Ala Leu Thr	
60 65 70	
GGT GTC GGT GCC TTA TTA CAA GGT TCC GGA TTT TCT CGA GCT CAA GAT	1849
Gly Val Gly Ala Leu Leu Gln Gly Ser Gly Phe Ser Arg Ala Gln Asp	
75 80 85	
GAT ACT TAT ACC GTA CAA GAA TAT ATG CAT TCT CGT ACG GCA CTA GAA	1897
Asp Thr Tyr Thr Val Gln Glu Tyr Met His Ser Arg Thr Ala Leu Glu	
90 95 100	
CAG TTA ATG AAA GAC TTG CCA ATA CGT GAA TAC TAT GAG AAT CAA GGC	1945
Gln Leu Met Lys Asp Leu Pro Ile Arg Glu Tyr Tyr Glu Asn Gln Gly	
105 110 115 120	
GAT ATT ATC GCT CGC TTT AAT GGA TTT GGT TTA AAT AAT AGT AAA GAA	1993
Asp Ile Ile Ala Arg Phe Asn Gly Phe Gly Leu Asn Asn Ser Lys Glu	
125 130 135	
GCG TTT TAT AAA TAT TTC CGA GAT CGC TTA AGT GTG GAC TTT GAC TCT	2041
Ala Phe Tyr Lys Tyr Phe Arg Asp Arg Leu Ser Val Asp Phe Asp Ser	



-continued

---

Ile	Ala	Phe	Val	Ile	Thr	Gly	Tyr	Pro	Met	Ala	Met	Met	Trp	Arg	Asn				
				75					80					85					
GCG	TCA	AAC	CGC	ACT	ATC	GGT	GCA	ATT	TCC	GGT	AAC	TTG	AGT	CTT	CTT				3048
Ala	Ser	Asn	Arg	Thr	Ile	Gly	Ala	Ile	Ser	Gly	Asn	Leu	Ser	Leu	Leu				
			90					95					100						
TAT	CAT	CGT	AAT	GTT	CGC	GTA	TTA	GAT	ACC	TTA	CTG	GCT	CGT	GTC	ATA				3096
Tyr	His	Arg	Asn	Val	Arg	Val	Leu	Asp	Thr	Leu	Leu	Ala	Arg	Val	Ile				
		105					110					115							
CTT	GAA	GTA	GCA	GGT	GCA	ACG	ATT	GCC	CAA	ATC	ATT	ATT	ATG	GCA	TTA				3144
Leu	Glu	Val	Ala	Gly	Ala	Thr	Ile	Ala	Gln	Ile	Ile	Ile	Met	Ala	Leu				
	120					125					130								
GTC	ATT																		3150
Val	Ile																		
135																			

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 394 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met	Lys	Leu	Ile	Lys	Leu	Arg	Leu	Leu	Leu	Ser	Leu	Gly	Leu	Val	Ala				
1				5					10					15					
Ser	Leu	Ala	Ala	Cys	Ser	Ser	Leu	Pro	Thr	Ser	Gly	Pro	Ser	His	Ser				
		20						25					30						
Ala	Ile	Leu	Glu	Ala	Asn	Ser	Gln	Asn	Ser	Asp	Lys	Pro	Leu	Pro	Glu				
		35					40					45							
Val	Asn	Leu	Val	Glu	Leu	Asp	Asn	Gly	Leu	Val	Gln	Gln	Leu	Tyr	Gln				
	50					55					60								
Thr	Gln	Gln	Ser	Gln	Gln	Phe	Ser	Gly	Phe	Leu	Gly	Thr	Ala	Gly	Gly				
	65				70				75						80				
Ala	Gly	Tyr	Ala	Gly	Ala	Val	Asn	Val	Gly	Asp	Val	Leu	Glu	Ile	Ser				
				85					90					95					
Ile	Trp	Glu	Ala	Pro	Pro	Ala	Val	Leu	Phe	Gly	Gly	Thr	Phe	Ser	Ser				
		100						105					110						
Glu	Gly	Gln	Gly	Ser	Gly	His	Leu	Thr	Gln	Leu	Pro	Ala	Gln	Met	Val				
		115					120					125							
Asn	Gln	Asn	Gly	Thr	Val	Thr	Val	Pro	Phe	Val	Gly	Asn	Ile	Arg	Val				
	130					135					140								
Ala	Gly	Lys	Thr	Pro	Glu	Ala	Ile	Gln	Ser	Gln	Ile	Val	Gly	Ala	Leu				
	145				150					155					160				
Gln	Arg	Lys	Ala	Asn	Gln	Pro	Gln	Val	Leu	Val	Lys	Ile	Ala	Asn	Asn				
			165						170					175					
Asn	Ser	Ala	Asp	Val	Thr	Val	Ile	Arg	Gln	Gly	Asn	Ser	Ile	Arg	Met				
		180						185					190						
Pro	Leu	Ser	Ala	Asn	Asn	Glu	Arg	Val	Leu	Asp	Ala	Val	Ala	Ala	Val				
		195					200					205							
Gly	Gly	Thr	Thr	Glu	Asn	Ile	Glu	Asp	Val	Thr	Val	Lys	Leu	Thr	Arg				
	210					215						220							
Gly	Ser	Gln	Val	Lys	Thr	Leu	Ala	Phe	Glu	Thr	Leu	Ile	Ser	Asp	Pro				
	225				230					235					240				
Ala	Gln	Asn	Ile	Met	Leu	Arg	Ala	Gly	Asp	Val	Val	Ser	Leu	Leu	Asn				
			245						250						255				

-continued

---

Thr Pro Tyr Ser Phe Thr Gly Leu Gly Ala Val Gly Asn Asn Gln Gln  
                   260                                  265                                  270  
 Met Lys Phe Ser Ser Lys Gly Ile Thr Leu Ala Glu Ala Ile Gly Lys  
                   275                                  280                                  285  
 Met Gly Gly Leu Ile Asp Thr Arg Ser Asp Pro Arg Gly Val Phe Val  
                   290                                  295                                  300  
 Phe Arg His Val Pro Phe Ser Gln Leu Ser Leu Asp Gln Gln Thr Gln  
                   305                                  310                                  315                                  320  
 Trp Gly Ala Lys Gly Tyr Gly Met Gly Met Asp Val Pro Thr Val Tyr  
                   325                                  330                                  335  
 Arg Val Asn Leu Leu Glu Pro Gln Ser Leu Phe Leu Leu Gln Arg Phe  
                   340                                  345                                  350  
 Pro Met Gln Asp Lys Asp Ile Val Tyr Val Ser Asn Ala Pro Leu Ser  
                   355                                  360                                  365  
 Glu Phe Gln Lys Phe Leu Arg Met Ile Phe Ser Ile Thr Ser Pro Val  
                   370                                  375                                  380  
 Thr Ser Thr Thr Asn Ala Ile Arg Ala Tyr  
                   385                                  390

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 385 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Glu Thr Thr Ile Thr Ala Ser Pro Thr Glu Lys Leu Gln Lys Pro  
   1                  5                                  10                                  15  
 Val Lys Gln Lys Lys Ser Trp Leu Lys Lys Leu Asn Pro Leu Phe Trp  
                   20                                  25                                  30  
 Val Thr Val Ala Ile Pro Thr Val Leu Ser Ala Phe Tyr Phe Gly Ser  
                   35                                  40                                  45  
 Val Ala Ser Asp Ile Tyr Ile Ser Glu Ser Ser Phe Val Val Arg Ser  
                   50                                  55                                  60  
 Pro Gln Asn Gln Thr Ala Leu Thr Gly Val Gly Ala Leu Leu Gln Gly  
                   65                                  70                                  75                                  80  
 Ser Gly Phe Ser Arg Ala Gln Asp Asp Thr Tyr Thr Val Gln Glu Tyr  
                   85                                  90                                  95  
 Met His Ser Arg Thr Ala Leu Glu Gln Leu Met Lys Asp Leu Pro Ile  
                   100                                  105                                  110  
 Arg Glu Tyr Tyr Glu Asn Gln Gly Asp Ile Ile Ala Arg Phe Asn Gly  
                   115                                  120                                  125  
 Phe Gly Leu Asn Asn Ser Lys Glu Ala Phe Tyr Lys Tyr Phe Arg Asp  
                   130                                  135                                  140  
 Arg Leu Ser Val Asp Phe Asp Ser Val Ser Gly Ile Ala Ser Leu Arg  
                   145                                  150                                  155                                  160  
 Ile Arg Ala Phe Asn Ala Glu Glu Gly Gln Gln Ile Asn Gln Lys Leu  
                   165                                  170                                  175  
 Leu Ala Glu Gly Glu Thr Leu Ile Asn Arg Leu Asn Glu Arg Ala Arg  
                   180                                  185                                  190  
 Lys Asp Thr Ile Ser Phe Ala Glu Gln Ala Val Thr Glu Ala Glu Asn  
                   195                                  200                                  205  
 Asn Val Asn Glu Thr Ala Asn Ala Leu Ser Lys Tyr Arg Ile Lys Asn

-continued

210	215	220
Lys Ile Phe Asp Leu Pro Ala Gln Ser Gly Val Gln Leu Ser Leu Ile 225 230 235 240		
Ser Ser Leu Lys Ser Glu Leu Ile Arg Val Glu Thr Gln Leu Ala Gln 245 250 255		
Leu Gln Ser Ile Thr Pro Asp Asn Pro Gln Val Asp Ala Leu Leu Met 260 265 270		
Arg Gln Lys Ser Leu Arg Lys Glu Ile Asp Glu Gln Ser Lys Gln Leu 275 280 285		
Ser Ser Asn Ser Asn Ser Ser Ile Ala Ile Gln Thr Ala Asp Tyr Gln 290 295 300		
Arg Leu Val Leu Ala Asn Glu Leu Ala Gln Gln Gln Leu Thr Ala Ala 305 310 315 320		
Leu Thr Ser Leu Gln Asn Thr Lys Asn Glu Ala Asp Arg Gln Gln Leu 325 330 335		
Tyr Leu Glu Val Ile Ser Gln Pro Ser Lys Pro Asp Trp Ala Glu Glu 340 345 350		
Pro Tyr Arg Leu Tyr Asn Ile Leu Ala Thr Phe Phe Ile Gly Leu Met 355 360 365		
Leu Tyr Gly Val Leu Ser Leu Leu Ile Ala Ser Val Arg Glu His Lys 370 375 380		
Asn 385		

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 136 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Gln Tyr Gly Asp Gln Thr Thr Phe Arg Gln Ser Leu Ala Ile Gln 1 5 10 15
Gly Arg Val Ile Gly Ala Leu Leu Met Arg Glu Ile Ile Thr Arg Tyr 20 25 30
Gly Arg Lys Asn Leu Gly Phe Leu Trp Leu Phe Val Glu Pro Leu Leu 35 40 45
Leu Thr Leu Phe Ile Val Leu Met Trp Lys Phe Ile Arg Ala Asp Arg 50 55 60
Val Ser Asp Leu Asn Ile Ile Ala Phe Val Ile Thr Gly Tyr Pro Met 65 70 75 80
Ala Met Met Trp Arg Asn Ala Ser Asn Arg Thr Ile Gly Ala Ile Ser 85 90 95
Gly Asn Leu Ser Leu Leu Tyr His Arg Asn Val Arg Val Leu Asp Thr 100 105 110
Leu Leu Ala Arg Val Ile Leu Glu Val Ala Gly Ala Thr Ile Ala Gln 115 120 125
Ile Ile Ile Met Ala Leu Val Ile 130 135

## 41

We claim:

1. A vaccine comprised of an avirulent, non-capsulated serotype 5 *Actinobacillus pleuropneumoniae* bacterium, said bacterium lacking DNA sequences coding for capsule synthesis.
2. A method of immunizing swine against pleuropneumonia, comprising the step of administering to said swine an immunogenic dose of a vaccine comprising an avirulent, non-capsulated serotype 5 *Actinobacillus pleuropneumoniae* bacterium which lacks DNA sequences coding for capsule synthesis.

## 42

3. The method of claim 2 wherein said step of administering is achieved by injecting the vaccine intramuscularly or subcutaneously.

4. A method of preparing a vaccine to prevent diseases caused by *Actinobacillus pleuropneumoniae* serotype 5 bacteria, comprising the steps of:

- identifying genes encoding for capsule synthesis in said bacteria; and
- deleting said genes in said bacteria encoding for capsule synthesis to produce non-capsulated mutants of said bacteria.

\* \* \* \* \*



UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : **6,086,894**

DATED : **July 11, 2000**

INVENTOR(S) : **Inzana, et al**

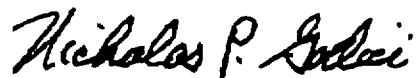
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

**Claim 1, line 3, "bacteiium" should read ---- bacterium ----.**

Signed and Sealed this

Twenty-ninth Day of May, 2001

Attest:



NICHOLAS P. GODICI

Attesting Officer

Acting Director of the United States Patent and Trademark Office