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Pasteurella multocida Produces a Protein with Homology to the P6 Outer Membrane Protein of *Haemophilus influenzae*

RICKIE W. KASTEN,[1] LORI M. HANSEN,[1] JULIAN HINOJOZA,[2]† DAVID BIEBER,[2]‡ WILLIAM W. RUEHL,[2]§ AND DWIGHT C. HIRSH[1]*

Department of Pathology, Microbiology, and Immunology, School of Veterinary Medicine, University of California, Davis, California 95616,[1] and Division of Laboratory Animal Medicine, Stanford University Medical Center, Stanford, California 94305[2]

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An antibody specific for a 16-kDa outer membrane protein of a rabbit strain of *Pasteurella multocida* was used to probe representatives of all 16 somatic serotypes of *P. multocida*, as well as the vaccine strains CU and M9, and all were shown to express the protein. The gene encoding this protein was cloned and sequenced and found to have extensive sequence homology with the gene encoding the P6 protein of *Haemophilus influenzae*. The protein in *P. multocida* has been designated P6-like. The gene encoding the P6-like protein was used to probe members of the family *Pasteurellaceae* and other gram-negative bacteria. Representatives of all 16 somatic serotypes (as well as the vaccine strains CU and M9) of *P. multocida* hybridized with the P6-like gene under conditions of high stringency. The DNA from *H. influenzae* hybridized weakly with the P6-like gene under these conditions, but *Pasteurella haemolytica* (representatives of A and T biotypes), *Bordetella bronchiseptica*, *B. avium*, *Actinobacillus suis*, *A. suis*-like, *A. lignieresii*, *A. ureae*, *A. rossii*, *A. pleuropneumoniae*, *A. equuli*, and various members of the family *Enterobacteriaceae* (*Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella typhimurium*) did not hybridize detectably. Under conditions of lower stringency, the P6-like gene also hybridized strongly with DNA from *P. multocida*, *H. influenzae*, and *A. rossii* but weakly with DNA from *P. haemolytica* and members of the genus *Actinobacillus*. These results suggest that the P6-like protein of *P. multocida* might be useful as an immunizing product to protect poultry from avian cholera. This suggestion stems from (i) our finding that the P6-like protein in *P. multocida* is widely distributed among all the somatic serotypes and (ii) the previous work of others demonstrating that the P6 protein of *H. influenzae* elicits a protective immune response in animal models of human disease.

Avian cholera, caused by *Pasteurella multocida*, is a major infectious disease of turkeys raised for food (18, 38). In California, a state that grows approximately 20 million turkeys annually, there are 30 to 40 outbreaks of avian cholera per year affecting 3 million to 4 million turkeys (17). In 1987, the cost of avian cholera was $0.40 per turkey or $18,750 per flock (based on an average flock size of 40,000 turkeys) (3). Given the current gross margin associated with turkey production, this amount is large enough to turn a profit into a potentially disastrous loss.

Current measures taken by the poultry industry to prevent avian cholera include immunization procedures using whole-cell bacterins or live vaccines composed of attenuated strains of *P. multocida*. Neither is satisfactory. Bacterins, though effective, must be given parenterally and are efficacious only for the somatic serotypes (currently 16 types) comprising the bacterin preparation (10, 11). Parenteral vaccination necessitates the handling of each bird at least two times. Since average flock sizes are large, over 40,000 birds per flock in California, this is an extremely labor intensive practice. Attenuated, live vaccines are usually administered in the drinking water for vaccination of turkeys, doing away with the problem of handling individual birds (2, 23). In addition, live vaccines protect against disease that might follow infection by any of the 16 somatic serotypes (2, 13, 15, 23). A major risk intrinsic to the use of live products is the production of avian cholera by the vaccine strain (40).

A virus-vectored vaccine would circumvent the problems described above. Vaccinia virus, for example, has been adapted to vector a variety of proteins to various host species (19, 24), most notably rinderpest virus-encoded proteins in cattle (47). Likewise, the fowlpox virus has been used to vector proteins that express protective epitopes (41, 46). Unfortunately, a gene(s) encoding conserved determinants containing protective epitopes has not been identified for avian strains of *P. multocida.*

We have isolated and sequenced a gene from *P. multocida* that encodes a 16-kDa protein with extensive sequence homology with the gene encoding the P6 protein of *Haemophilus influenzae*. Since there is compelling evidence that the P6 protein induces a protective immune response in human beings, we explored further the distribution of the gene encoding the P6-like protein. We show that the gene is present and expressed in all 16 somatic serotypes of *P. multocida*. These findings, as well as those of others pertaining to *H. influenzae*, strongly suggest that the P6-like protein may be a useful inclusion in a vaccine for avian cholera.

* Corresponding author. Mailing address: Department of Pathology, Microbiology, and Immunology, School of Veterinary Medicine, University of California, Davis, CA 95616. Phone: (916) 752-3144. Fax: (916) 752-3349. Electronic mail address: dchirsh@ucdavis.edu.

† Present address: Department of Pathology, Stanford University Medical Center, Stanford, CA 94305.

‡ Present address: Department of Medicine, Division of Infectious Diseases, Stanford University Medical Center, Stanford, CA 94305.

§ Present address: Deprenyl Animal Health, Inc., Overland Park, KS 66210.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains of *P. multocida* used in this study were X-73 (somatic serotype [ss] 1), M-1404 (ss 2), P-1059 (ss 3), P-1662 (ss 4), P-1702 (ss 5), P-2192 (ss 6), P-1997 (ss 7), P-1581 (ss 8), P-2095 (ss 9), P-2100 (ss

10), P-903 (ss 11), P-1573 (ss 12), P-1591 (ss 13), P-2225 (ss 14), P-2237 (ss 15), P-2723 (ss 16), M9 (ss 3,4), and CU (ss 3,4). *P. multocida* T16 (ss 3,4) was isolated from the oropharynx of a live, apparently normal turkey that was part of a California flock affected with avian cholera, and *P. multocida* 2 was isolated from the respiratory tract of a rabbit. *Bordetella avium* ATCC 35086, *B. bronchiseptica* C15 (Veterinary Medical Teaching Hospital, University of California, Davis, culture collection), *Pasteurella haemolytica* biotype A, serotype 1 (kindly provided by E. L. Biberstein, University of California, Davis), *P. haemolytica* biotype T, serotype 10 (kindly provided by E. L. Biberstein), *H. influenzae* isolates R15670, R15672, R15704, R15712, R15758, and R15791 (kindly provided by J. R. Carlson, University of California, Davis), *Actinobacillus lignieresii* ATCC 19393, *A. suis* ATCC 15560, *A. pleuropneumoniae* 545, serotype 5 (kindly provided by E. L. Biberstein), *A. suis*-like 3042A (Veterinary Medical Teaching Hospital, University of California, Davis, culture collection), *A. equuli* ATCC 19392, *A. rossii* ATCC 27072, *A. ureae* ATCC 25976, *Escherichia coli* 1206 (Veterinary Medical Teaching Hospital, University of California, Davis, culture collection), *Klebsiella pneumoniae* C3 (Veterinary Medical Teaching Hospital, University of California, Davis, culture collection), and *Salmonella typhimurium* 91-4428 (Veterinary Medical Teaching Hospital, University of California, Davis, culture collection) were also used. Other microorganisms and plasmids used in this study were *E. coli* DH5α and *E. coli* SK8201 (containing plasmid pWSK29; kindly provided by S. R. Kushner, University of Georgia, Athens) (45). The cosmid vector pLorsal is a derivative of pLorist2 but differs by the addition of a *Sal*I site inserted between the *Hin*dIII and *Bam*HI sites (kindly provided by S. Muir, Scripps Institute of Medical Research, La Jolla, Calif.) (6).

Gene cloning. DNA was purified from *P. multocida* T16 and digested with limiting dilutions of *Sau*3A (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) to yield a random partial digest of the chromosome (20, 36). Random-sized fragments were fractionated on a 10 to 50% sucrose gradient, and fractions of 30- to 50-kb fragments were pooled and dialyzed against TE (10 mM Tris, 1 mM EDTA [pH 8]) (36). The fragments were ligated into the dephosphorylated *Bam*HI site of pLorsa1, and the cosmid was packaged into phage heads by using Gigapak II (Stratagene, La Jolla, Calif.). *E. coli* DH5α cells were infected with phage and plated onto selective medium (LB agar containing 50 μg of kanamycin per ml). After 2 days of incubation at 37°C in air, colony lifts were made by using nitrocellulose filters (Schleicher & Schuell, Keene, N.H.). The lifts were digested overnight with colony lysis buffer (100 mM Tris [pH 8.0], 150 mM NaCl, 5 mM $MgCl_2$, 1.5% bovine serum albumin, 40 μg of lysozyme per ml, 1 μg of DNase I per ml) (36) and then probed with rabbit antiserum to *P. multocida* outer membrane proteins. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Zymed Laboratories, South San Francisco, Calif.) was used as the secondary antibody. The substrate, 4-chloro-1-naphthol (0.5 mg/ml; Bio-Rad, Richmond, Calif.), was diluted in substrate buffer (8.4 mM Tris–17% methanol [pH 7.4] with 15 μl of 30% H_2O_2).

Cosmid hybrids expressing proteins reacting with the anti-*P. multocida* serum were subcloned into pWSK29. Briefly, plasmid DNA was extracted, purified on a CsCl gradient, and partially digested with *Sau*3A. DNA, 2 to 6 kb in size, was ligated into the dephosphorylated *Bam*HI site of pWSK29. After transformation of *E. coli* DH5α and plating onto selective medium (LB agar containing 100 μg of ampicillin per ml, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal]), ampicillin-resistant white colonies were picked and analyzed on Western blots (immunoblots) (1, 43).

Antibody production. Rabbit antiserum used to probe the *Pasteurella* library was raised against *P. multocida* protein which was obtained by using the procedure employed for the purification of pili from *Moraxella bovis* (35). Briefly, *P. multocida* 2 was grown to confluence on 5% sheep blood agar plates at 37°C overnight. Membrane-bound proteins were sedimented and then fractionated by repeated sedimentation in ethanolamine buffers of various pHs as described previously (35). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of the final product demonstrated a single band of 16 kDa after staining with Coomassie brilliant blue.

Pasteurella-free New Zealand White rabbits were inoculated subcutaneously with 100 μg of the purified protein emulsified with incomplete Freund's adjuvant. The animals were boosted after 6 weeks and bled 2 weeks later.

Sequence determination and analysis. The nucleic acid sequence was determined by the dideoxy-chain termination method, using a Sequenase version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, Ohio) (37). Initial primers were standard M13/reverse M13, and additional synthetic primers were constructed as needed. DNA was labeled with [α-^{35}S]dATP by the protocol provided with the DNA sequencing kit. The sequence data provided herein were the result of sequencing twice in one direction and once in the reverse direction. Sequence analyses were conducted with Genepro software version 2.0 (Hoefer Scientific Instruments, San Francisco, Calif.) and the Genetics Computer Group software package (Genetics Computer Group, Madison, Wis.). Amino acid comparisons were made using GenBank accession number M19391 for *H. influenzae* (28).

SDS-PAGE and Western blotting. Whole-cell proteins were prepared from bacterial cultures as previously described (31). For PAGE, 75 μg of protein was used per well. Samples were heated to 100°C for 5 min in SDS sample buffer (35). The preparations were subjected to SDS-PAGE with 15% separating gels (22). The separated proteins were transferred to nitrocellulose paper by using a Trans-blot tank transfer cell (Bio-Rad Laboratories, Hercules, Calif.) overnight at room temperature and at 55 V in blotting buffer (20 mM Tris, 150 mM glycine, 20% methanol) (43).

Blots were blocked in NET buffer (0.2% gelatin, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.05% Triton X-100 [pH 7.4]) and stained as previously described (1). Horseradish peroxidase-conjugated protein A (Zymed Laboratories) was used to determine the location of antibody bound to proteins on Western blots.

Dot blots. Bacteria were cultured in 5 ml of brain heart infusion broth at 37°C in air except for *H. influenzae*, which was grown on chocolate agar plates at 37°C in a candle jar. All media were incubated in air except the chocolate agar plates, which were incubated in a candle jar. Whole-cell DNA was isolated by selective precipitation with hexadecyltrimethylammonium bromide (Sigma Chemical Company, St. Louis, Mo.) (1). The DNA concentrations were determined by fluorometry using the manufacturer's protocol (TKO 100 fluorometer; Hoefer Scientific Instruments, San Francisco, Calif.).

The DNA was diluted to 40 μg/ml in TE, heated to 100°C for 5 min, and then placed in ice. One volume of 20× SSC (3 M sodium chloride, 0.3 M sodium citrate [pH 7.0]) was added, and 50 μl containing 1 μg of DNA was spotted onto a nylon membrane (Hybond-N+ [Amersham]) by using a Bio-Dot apparatus (Bio-Rad) (1). The membrane was air dried, and the DNA was fixed by UV light (UV Crosslinker; Fisher Scientific, Pittsburgh, Pa.).

The gene encoding the P6-like protein, within pWSK29, was amplified by PCR using appropriately constructed primers and conditions (1). The following primers, based on the sequence of the gene encoding the P6-like protein, were used:

5′TGTAGATCTTTAGTATGCTAACACAGCACG3′
5′TCTGGATCCATGAAAAAACTAACTAAAGTA3′

The amplified product was used at a final concentration of 10 ng/ml in hybridization fluid. Labeling of DNA, hybridization, and detection were performed as prescribed by the manufacturer (ECL Labelling Kit; Amersham). Stringency was determined by the primary wash buffer at 40°C containing either 1× SSC (lower stringency) or 0.1× SSC (high stringency). Film (Hyperfilm-MP; Amersham) was exposed for 10 min.

Nucleotide sequence accession number. The DNA sequence of the gene encoding the P6-like protein has been deposited in GenBank under accession number U16849.

RESULTS

Antiserum RC-C, which recognizes a 16-kDa protein from a rabbit *P. multocida* isolate, reacted to a protein of similar size (16 kDa) in all 16 somatic serotypes of *P. multocida*, as well as the vaccine strains CU and M9 (Fig. 1).

A *P. multocida* genomic library was constructed in the expression vector pLorsal. This library was probed with antiserum RC-C, and a colony which reacted with the antibody was selected for study. Attempts to subclone the insert DNA into the high-copy-number plasmid pUC18 were unsuccessful. Subcloning into the low-copy-number plasmid pWSK29 resulted in the isolation of stable clones. One clone (designated F168N) contained a 2,189-bp insert. F168N expressed a new 16-kDa protein which reacted with antiserum RC-C (Fig. 2).

The DNA sequence was determined for the insert, and an open reading frame was found approximately 1,270 bases downstream from the M13 primer binding site. The open reading frame of 450 bases encoded a polypeptide of 150 amino acids (predicted protein size of 16,213 Da) (Fig. 3). The first 20 amino acids comprised a potential leader peptide, rich in hydrophobic amino acids, and ended in a possible lipoprotein signal peptide, Leu-Ala-Ala-Cys. Analysis of the open reading frame for homology to sequences stored in GenBank revealed extensive homology with the P6 protein of *H. influenzae*. Bestfit analysis between the P6 gene of *H. influenzae* and the cloned *P. multocida* gene resulted in a value of 81 for percent identity. A comparison of amino acid sequences deduced from the nucleotide sequence produced a Bestfit analysis of 91% similarity and 83% identity (Fig. 4). The protein encoded by the cloned gene from *P. multocida* will hereafter be referred to as the P6-like protein.

Several gram-negative microorganisms were analyzed to determine the distribution of the gene encoding the P6-like protein. The P6-like gene hybridized, under high stringency, with DNA from representatives of all somatic serotypes of *P. mul-*

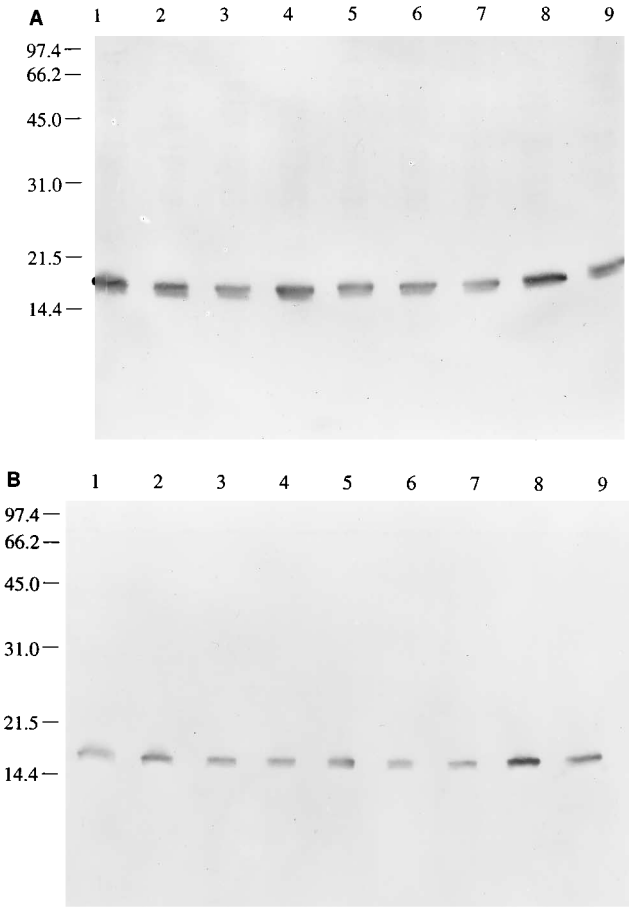

FIG. 1. Immunoblot analysis of whole-cell lysates of *P. multocida* probed with anti-*P. multocida* 16-kDa protein. (A) Lane 1, CU; lane 2, M9; lane 3, ss 1; lane 4, ss 2; lane 5, ss 3; lane 6, ss 4; lane 7, ss 5; lane 8, ss 6; lane 9, ss 7. (B) Lane 1, ss 8; lane 2, ss 9; lane 3, ss 10; lane 4, ss 11; lane 5, ss 12, lane 6, ss 13; lane 7, ss 14; lane 8, ss 15, lane 9, ss 16. Molecular mass standards (in kilodaltons) are noted on the left.

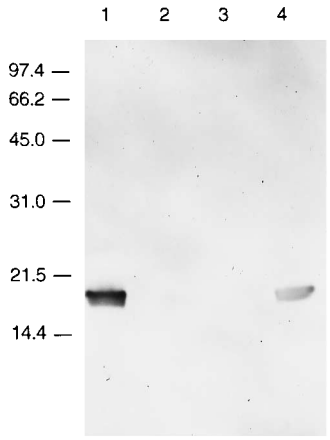

FIG. 2. Immunoblot analysis of whole-cell lysates of *P. multocida* T16 and *E. coli* recombinants. Lane 1, *P. multocida* T16; lane 2, *E. coli* DH5α; lane 3, *E. coli* DH5α containing pWSK29; lane 4, *E. coli* DH5α recombinant clone F168N. Molecular mass standards (in kilodaltons) are noted on the left.

tocida, as well as the vaccine strains CU and M9. The DNA from isolates of *H. influenzae* hybridized minimally to the gene, while isolates of the genera *Actinobacillus* and *Bordetella* and selected members of the family *Enterobacteriaceae* did not hybridize detectably (Fig. 5). If, however, the blots were washed under conditions of lower stringency, the P6-like gene also hybridized strongly with DNA obtained from *H. influenzae* and *A. rossii* but weakly with DNA from *P. haemolytica* and other members of the genus *Actinobacillus* (Fig. 6).

DISCUSSION

The purpose of this study was to identify and clone a gene encoding a protein from *P. multocida* with potential for use as part of a vaccine for avian cholera. In this report, we describe the cloning and sequencing of a gene encoding a conserved 16-kDa protein.

We had originally hypothesized that an adhesin protein on the surface of *P. multocida* would be highly conserved and thus suitable for inclusion in an immunizing preparation. By using the method described for the isolation of pilin from *M. bovis*, we isolated a protein of the appropriate size (16 kDa). Antiserum produced to this 16-kDa outer membrane protein reacted to all somatic serotypes of *P. multocida* tested, to the strain from which the gene encoding the P6-like protein (ss 3,4) was obtained, and to the vaccine strains CU and M9 (both ss 3,4). These data indicate that the protein is conserved and expressed, traits that are important if the protein is under consideration for inclusion in a vaccine.

Initial attempts to subclone this gene into pUC18 were unsuccessful. Our hypothesis that the gene product was toxic (pUC18 may have up to 300 copies per cell) appeared correct since successful subcloning was readily accomplished with the low-copy-number plasmid pWSK29 (6 to 8 copies per cell) (45).

The nucleotide sequence of the cloned *Pasteurella* gene showed extensive homology to the gene encoding the P6 protein of *H. influenzae* (81% identity) but not to described pilin proteins (41.5% identity; results not shown). Up to now, the P6 protein was thought to be unique to *H. influenzae*, though some homology with other low-molecular-mass proteins has been reported (5). However, *Pasteurella* and *Haemophilus* species share strong taxonomic associations, and so the presence of a P6-like protein in members of the genus *Pasteurella* is not surprising.

The narrow distribution of the gene encoding the P6-like protein, as evidenced by lack of strong hybridization under low stringency, was surprising, especially with respect to some of

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M  K  K  L  T  K  V  L  L  V  A  G  S  V  A  V  L
ATGAAAAAACTAACTAAAGTATTATTAGTTGCTGGTTCTGTTGCTGTATTA      51

A  A  C  G  S  S  K  K  D  E  S  A  G  Q  M  F  G
GCTGCTTGTGGTTCATCTAAAAAAGATGAAAGCGCTGGTCAAATGTTTGGC     102

G  Y  S  V  Q  D  L  Q  Q  R  Y  N  T  V  Y  F  G
GGTTATTCAGTACAAGATTTACAACAACGTTATAATACCGTGTATTTCGGC     153

F  D  K  Y  N  I  E  G  E  Y  V  Q  I  L  D  A  H
TTCGATAAATACAATATCGAAGGTGAATATGTACAAATTTTAGATGCACAT     204

A  A  F  L  N  A  T  P  A  T  K  V  V  V  E  G  N
GCGGCGTTCTTAAATGCAACACCAGCAACGAAAGTCGTTGTTGAAGGTAAC     255

T  D  E  R  G  T  P  E  Y  N  I  A  L  G  Q  R  R
ACCGATGAACGCGGTACACCAGAATATAACATCGCATTAGGTCAACGTCGT     306

A  D  A  V  K  H  Y  L  S  A  K  G  V  Q  A  G  Q
GCAGATGCAGTAAAACATTATTTATCAGCGAAAGGTGTACAAGCTGGTCAA     357

V  S  T  V  S  Y  G  E  E  K  P  A  V  L  G  H  D
GTATCAACAGTTTCTTACGGTGAAGAGAAACCTGCTGTGTTAGGTCACGAT     408

E  A  A  Y  S  K  N  R  R  A  V  L  A  Y  *
GAAGCAGCTTACTCTAAAAACCGTCGTGCTGTGTTAGCATACTAA           453
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FIG. 3. Nucleotide and deduced amino acid sequences of the *P. multocida* gene encoding the 16-kDa protein. The asterisk denotes the termination codon.

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A  MKKLTKVLLVAGSVAVLAACGSSKKD...ESAGQMFGGYSVQDLQQRYNT 47
B  MNKFVKSLLVAGSVAALAACSSSNNDAAGNGAAQTFGGYSVADLQQRYNT 50

A  VYFGFDKYNIEGEYVQILDAHAAFLNATPATKVVVEGNTDERGTPEYNIA 97
B  VYFGFDKYDITGEYVQILDAHAAYLNATPAAKVLVEGNTDERGTPEYNIA 100

A  LGQRRADAVKHYLSAKGVQAGQVSTVSYGEEKPAVLGHDEAAYSKNRRAV 147
B  LGQRRADAVKGYLAGKGVDAGKLGTVSYGEEKPAVLGHDEAAYSKNRRAV 150

A  LAY                                               150
B  LAY                                               153
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FIG. 4. Comparison of the amino acid sequences of *P. multocida* 16-kDa protein (A) and *H. influenzae* P6 protein (B). Identity (solid line) and similarity (≥0.5, double dot; ≥0.1, single dot) are indicated.

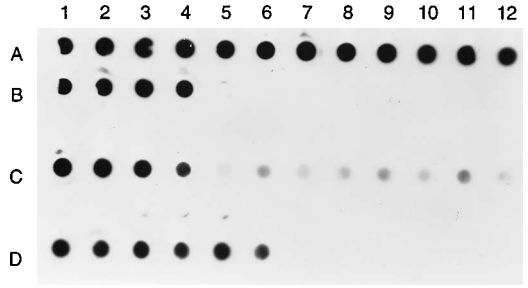

FIG. 6. Dot blot hybridization of chromosomal DNA from various microorganisms probed with the *P. multocida* P6-like gene. The blot was processed under low-stringency conditions. See the legend to Fig. 5 for row and lane contents.

the members of the family *Pasteurellaceae*. The weak degree of hybridization between the P6-like gene and DNA from *P. haemolytica* and some of the members of the genus *Actinobacillus* (excluding *A. rossii*) indicates some minor degree of homology with a related gene, weak homology with domains of unrelated genes, or some totally nonspecific interaction. We are uncertain why *A. rossii* would possess a DNA sequence that hybridized strongly, albeit under conditions of lower stringency, with the gene encoding the P6-like protein. Under conditions of high stringency, only the *P. multocida* isolates hybridized strongly with the P6-like gene, whereas the *H. influenzae* isolates did so weakly. These findings probably reflect the degree of similarity between the P6-like gene of *P. multocida* and the P6 gene of *H. influenzae*.

The importance of the P6 protein has been studied over the last several years (8, 9, 27–30). An extensive variety of typeable and nontypeable strains of *H. influenzae* possess the P6 protein. Some strains of *H. influenzae* possess a P6 protein that varies slightly from the consensus sequence with the loss of a specific epitope (27). These strains are unusually virulent and tend to be more invasive. The role, if any, of the P6-like protein in virulence is unknown.

The P6 protein has been postulated to have a structural function within the bacterial cell (29). Since it is a lipoprotein and associated with the peptidoglycan, it may act as an anchor to bind the outer membrane to the peptidoglycan layer. The surface exposure of the protein has been documented by immunoelectron microscopy of intact cells (28), and specific surface epitopes have been identified by monoclonal antibodies (30). Our data show that a potential leader sequence consistent with a signal peptide (Leu-Ala-Ala-Cys) described for lipoproteins was also found with the P6-like protein (32).

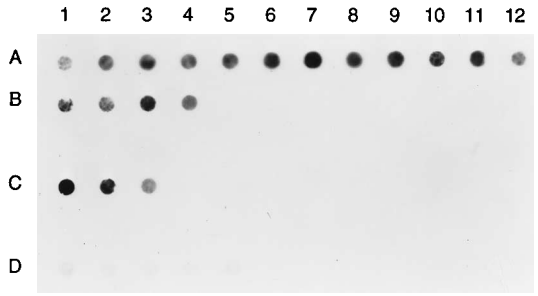

FIG. 5. Dot blot hybridization of chromosomal DNA from various microorganisms probed with the *P. multocida* P6-like gene. The blot was processed under high-stringency conditions. Row A: lane 1, *P. multocida* ss 1; lane 2, ss 2; lane 3, ss 3; lane 4, ss 4; lane 5, ss 5; lane 6, ss 6; lane 7, ss 7; lane 8, ss 8; lane 9, ss 9; lane 10, ss 10; lane 11, ss 11; lane 12, ss 12. Row B: lane 1, *P. multocida* ss 13; lane 2, ss 14; lane 3, ss 15; lane 4, ss 16. Row C: lane 1, *P. multocida* T16; lane 2, *P. multocida* CU; lane 3, *P. multocida* M9; lane 4, *A. rossii* ATCC 27072; lane 5, *A. ureae* ATCC 25976; lane 6, *A. lignieresii* ATCC 19393; lane 7, *A. equuli* ATCC 19392; lane 8, *A. suis* ATCC 15560; lane 9, *A. suis*-like 3052A; lane 10, *A. pleuropneumoniae* 545; lane 11, *P. haemolytica* A1; lane 12, *P. haemolytica* T10. Row D: lane 1, *H. influenzae* R15670; lane 2, *H. influenzae* R15672; lane 3, *H. influenzae* R15704; lane 4, *H. influenzae* R15712; lane 5, *H. influenzae* R15758; lane 6, *H. influenzae* R15791; lane 7, *E. coli* DH5α; lane 8, *E. coli* 1206; lane 9, *B. avium* ATCC 35086; lane 10, *B. bronchiseptica* C15; lane 11, *S. typhimurium* 91-4428; lane 12, *K. pneumoniae* C3.

The P6-like protein has potential value as part of an immunizing product since it is conserved, and the gene encoding it is expressed by all somatic serotypes of *P. multocida* affecting poultry. Whether an immune response to epitopes expressed on this protein is protective remains to be demonstrated. It is conceivable that proteins on the surface of *P. multocida* would not be accessible to antibody because of the capsule produced by this microorganism. However, antibodies specific to outer membrane proteins of avian strains of *P. multocida* have been shown to bestow protection in passive protection trials (42, 44). In addition, capsule production by this microorganism is decreased under conditions of iron limitation, conditions that would prevail in vivo (21).

There are, however, some other points to discuss with respect to the P6-like protein as a potential vaccine candidate for poultry. It has been recognized for some time that bacterins made from *P. multocida* grown on artificial media induce a protective immune response only against the somatic serotype comprising the bacterin (12). On the other hand, if poultry are vaccinated with live bacteria, then protection is achieved regardless of the infecting serotype (12, 16). That there are protective epitopes expressed in vivo is strongly suggested by the finding that killed *P. multocida* previously grown in vivo (in embryonated eggs, tissue, or in the bloodstream of turkeys) will elicit protection regardless of the somatic serotype of the infecting strain (13, 14). Therefore, there appears to be a determinate(s) that is responsible for cross-protection that is made in vivo (33, 34, 44). Some have suggested that the cross-protection factor is an iron-regulated outer membrane protein, though this notion has not been convincingly supported experimentally (4, 7, 39). Whether the P6-like protein will elicit

protection, let alone cross-protection, remains to be determined.

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