INFECTION AND IMMUNITY, Feb. 2004, p. 1195–1198 Vol. 72, No. 2
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.2.1195–1198.2004

Identification of Five Outer Membrane-Associated Proteins among Cross-Protective Factor Proteins of *Pasteurella multocida*

Louisa B. Tabatabai* and Emilie S. Zehr

National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa

Received 30 June 2003/Returned for modification 4 September 2003/Accepted 23 October 2003

Fowl cholera is caused by *Pasteurella multocida* serovars A:1, A:3, and A:4. The 39-kDa cross-protective factor protein and four other membrane proteins of the membrane proteome of *P. multocida* were identified. We determined that the 39-kDa cross-protective protein was *Pasteurella* lipoprotein B, or PlpB.

Fowl cholera continues to be of major concern to the poultry industry, especially for turkey growers. Fowl cholera costs the turkey industry millions of dollars annually. The disease is caused primarily by three serotypes of *Pasteurella multocida*, A:1, A:3, and A:4. A live attenuated vaccine strain, strain M-9 (serotype 3,4), has been implicated in outbreaks associated with vaccination (3, 4, 6, 8, 14). Commercial bacterins prepared from serotypes A:1, A:3, and A:4 confer cross protection and are currently used to control outbreaks of disease in poultry flocks. Recently, Rimler (11) identified an outer membrane-associated protein with a molecular mass of approximately 39 kDa that was believed to be one of the cross-protective antigens. However, the identity of the cross-protective antigen expressed by the *P. multocida* serovars was not known (11). The objectives of this study were to (i) identify the 39-kDa protein by using trypsin digestion followed by peptide mass fingerprinting with matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) and (ii) identify the remaining proteins of the outer membrane proteome.

The outer membrane protein extract was prepared by Rimler (11) from in vitro-grown *P. multocida* serotype A:3 strain P-1059. Briefly, the bacterial pellet was suspended in a solution containing lysozyme, EDTA, and Triton X-100. The viscosity was reduced by incubating the suspension with DNase and hyaluronidase (12). The suspension was centrifuged at 100,000 $\times$ *g* for 1 h. The supernatant was removed, and the pellet was extracted with 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS). The extract was stored frozen at −80°C.

The CHAPS-solubilized proteins were denatured under reducing conditions (7) and applied to either a 10-well (10 μg of protein per well) or a two-dimensional-well (250 μg of protein) 4 to 12% bis-Tris gradient gel (Invitrogen, Carlsbad, Calif.) by using the 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (MOPS) buffer system (Invitrogen). Gels were stained with Coomassie brilliant blue R-250 and destained as described previously (7).

Gel plugs were removed from the stained bands of the gel by using a blunt-cut 16-gauge needle. The gel plugs were deposited into a 96-well microtiter plate and digested with trypsin by using the automated digester ProGest (Genomic Solutions, Ann Arbor, Mich.). The extracted peptides were desalted using a C_{18} ZipTip (Millipore, Bedford, Mass.), mixed with an equal volume of saturated α-cyano-hydroxy-cinnamic acid (Sigma Chemical Co., St. Louis, Mo.) in 30% acetonitrile–0.2% tri-

* Corresponding author. Mailing address: National Animal Disease Center, ARS, USDA, 2300 Dayton Rd., Ames, IA 50010. Phone: (515) 294-6284. Fax: (515) 294-0453. E-mail: lbt@iastate.edu.

TABLE 1. MS-Fit analysis of five *P. multocida* membrane proteins

Band	Gel molecular weight	Molecular weight/pI	% Match of peptides	Identity[a] (accession no.)
1	117	117/5.6	4	Put Vir Fact (15603684)
2	79	79.7/9.2	11	LipA/PhyA (15602638)
3	68	47.8/5.9	1	HemY (15603680)
4	52	37.4/7.7	7	Unkn lipoprot (15602915)
5	39	30/5.2	7	PlpB (15603595)

[a] Put Vir Fact, putative virulence factor by homology to *Pseudomonas syringae* and *Yersinia pestis* proteins (10); LipA/PhyA, lipase A, replaces phospholipid in hyaluronic acid (2, 10, 15); HemY, protoporphyrinogen oxidase by homology to *Haemophilus influenzae* protein (10); Unkn lipoprot, unknown lipoprotein of membrane biogenesis by homology to *H. influenzae* protein (10).

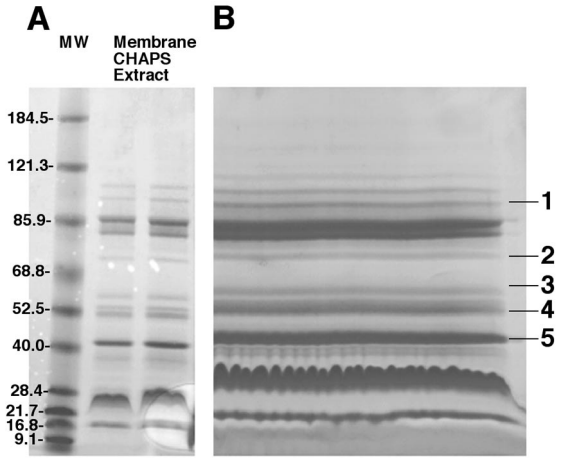

FIG. 1. SDS-PAGE of CHAPS-solubilized *P. multocida* outer membrane fraction. An overnight culture of *P. multocida* A:3 strain P-1059 in a peptone-based medium was harvested by centrifugation, lysed with EDTA, lysozyme, and Triton X-100, treated with DNase and hyaluronidase, and centrifuged at 100,000 $\times$ *g*. The pellet was extracted with CHAPS, centrifuged at 100,000 $\times$ *g*, and analyzed by SDS-PAGE. (A) Ten micrograms of protein per lane. (B) Two hundred fifty micrograms of protein. MW, molecular weight.

A Peptide 4- NVSELQDGATVAVPNNPSNLG
Peptide 11- LAIVGNTFVFPIAAYSK

B gi|15603595|ref|NP_246669.1| PlpB [Pasteurella multocida] –276 amino acids

1 MKLTKLFGLA TLVSAVALAG CKDDKPAAAA APQEPAARKL
41 TVGVMTGAEA QVTEVAAKIA KEKYNIDVKL VEFTEYTQPN
81 DALTKGDLDA NAFQHKPYMD KEVEQRGYKL AIVGNTFVFP
121 IAAYSKKIKN VSELQDGATV AVPNNPSNLG RALLLLEKQG
161 LIKLKDPSNL FSTSIDVIEN PKNLQIKEVE GSLLPRMLDD
201 VDFAIINNNY AVQGLTAEKD GIFVEDKDSP YVNLVVSRED
241 NKDNEAIKDF VKAFQTEEV YQEALKHFQG GVVKGW

FIG. 2. N-terminal sequence of the isolated tryptic peptides and protein sequence of the lipoprotein B. (A) Amino acid sequences of peptides 4 and 11. (B) Protein sequence of lipoprotein B, PlpB. Sequences corresponding to those of peptides 4 and 11 are underlined. The notation at the top of panel B indicates the database search result and accession numbers.

fluoroacetic acid, and spotted onto the target plate. MALDI mass spectra were obtained by using a Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, Calif.). We used the MS-Fit search engine of the Prospector website of the University of California at San Francisco (http://prospector.ucsf.edu/) to search the nonredundant database of the National Center for Biotechnology Information. The identity of the 39-kDa protein was confirmed by automated Edman degradation. Briefly, 15 gel plugs were treated with trypsin as described above and the extracted peptides (20 μl) in 1% formic acid were injected onto the C_{18} column (Vydac; 2.1 by 250 mm) equilibrated in 0.1% (vol/vol) trifluoroacetic acid–MilliQ water. The peptide fragments were eluted with a linear gradient of 0.08% (vol/vol) trifluoroacetic acid–80% (vol/vol) acetonitrile–MilliQ water for 100 min at a flow rate of 200 μl/min and monitored at 214 nm. Each peak was collected into

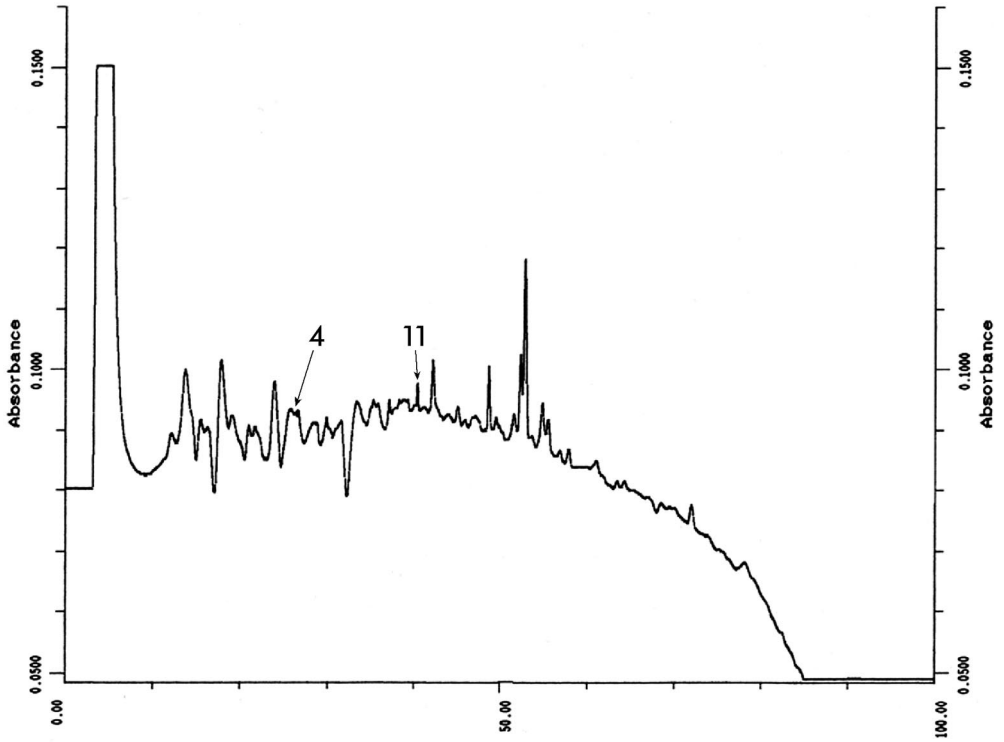

FIG. 3. Results of C_{18} high-performance liquid chromatography of a trypsin digest of band 5 protein. Multiple gel plugs were digested with trypsin using the automated digester. Twenty microliters of extracted and concentrated peptide was loaded onto a C_{18} column and eluted with a linear gradient of acetonitrile in aqueous trifluoroacetic acid. The absorbance was monitored at 214 nm. Each peak was collected into a methanol-washed microfuge tube and analyzed by MALDI-TOF mass spectrometry. Peptide peaks 4 and 11 were analyzed by N-terminal sequencing.

TABLE 2. Peptide masses and peptide sequences predicted for the tryptic digest of the 39-kDa protein by using MS-Fit[a]

Peptide	Predicted mi-*m*/*z*[b]	Predicted av-*m*/*z*[c]	Observed mi-*m*/*z*[b]	Start position	End position	No. of missed cleavages	Database sequence
11	1811.0107	1812.1761	1811.0319	110	126	0	(K)LAIVGNTFVPIAAYSK(R)
4	2252.1271	2253.4490	2252.1667	130	151	0	(K)NVSELQDGATVAVNNPSNLG(R)

[a] Predicted peptide masses and predicted peptide sequences were determined from the mass spectrometry data obtained by MALI-TOF mass spectrometry using the MS-Fit algorithm of the University of California at San Francisco Prospector database (http://prospector.ucsf.edu) as described in the text. Only the peptide masses for the two sequenced peptides are indicated.
[b] mi-*m*/*z*, monoisotopic mass/charge ratio.
[c] av-*m*/*z*, average mass/charge ratio.

a methanol-washed microfuge tube. A 0.5-μl sample was prepared for MALDI-TOF as described above and subjected to MALDI-TOF mass spectrometry using the Dynamo (Thermo-Bioanalysis, Hemel Hempstead, United Kingdom) mass spectrometer. Based on the MALDI spectra obtained (data not shown), two fractions containing one major peptide (peak 4 and peak 11) were selected for Edman degradation. Fractions were dried down, redissolved in 30% (vol/vol) acetonitrile–0.2% (vol/vol) trifluoroacetic acid, and analyzed by automated Edman degradation using an Applied Biosystems model 494 Procise sequencer (Iowa State University Protein Facility).

The protein profile of 10 μg of cross-protective CHAPS-solubilized fraction is shown in analytical format in Fig. 1A. The same fraction at a 25-fold-higher concentration is shown in preparative format in Fig. 1B. The latter gel shows 11 major proteins of which the 39-kDa protein and four other proteins (marked as bands 1 through 5) gave mass spectra with sufficient information for database searching. The lack of data from the remaining bands may indicate a lack of trypsin cleavage sites in these proteins. The identity of the 39-kDa protein (Fig. 1B, band 5) was consistent with the molecular masses of tryptic peptides of the *Pasteurella* lipoprotein B, PlpB, listed in the National Center for Biotechnology Information database (1) for *P. multocida* (Table 1 and Table 2). N-terminal sequencing of peptide peaks 4 and 11 (Fig. 2) showed amino acid sequences identical to those obtained by peptide mass fingerprinting and database searching using MS-Fit. Similarly, the molecular masses of the purified peptide fragments obtained

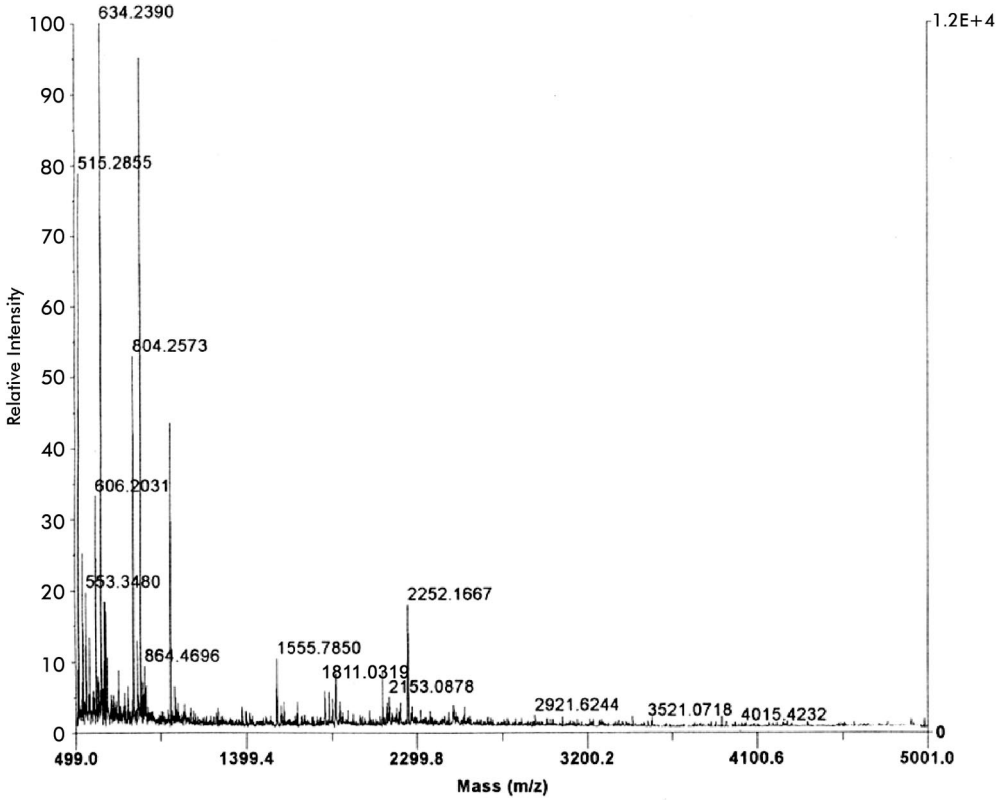

FIG. 4. MALDI-TOF mass spectrum of a trypsin digest of band 5 protein. Protein plugs from the gel were treated with trypsin by using an automated digester. The extracted peptides were desalted, mixed with an equal volume of saturated α-cyano-hydroxy-cinnamic acid in 30% acetonitrile–0.2% trifluoroacetic acid, and analyzed by MALDI-TOF mass spectrometry. The peak with an *m*/*z* of 2252.1667 is clearly visible. Relative intensities are given as percentages.

by high-performance liquid chromatography (Fig. 3) and analyzed by MALDI-TOF (Fig. 4) were similar to the molecular masses observed for the unfractionated trypsin digest.

Table 1 shows the MS-Fit results with the mass spectra of tryptic digests prepared from proteins of bands 1, 2, 3, and 4 of the preparative sodium dodecyl sulfate (SDS) gel (Fig. 1B). These proteins include two membrane biogenesis proteins and two virulence-related proteins. The reason for analyzing these proteins is that often improved protection was observed with the cruder fraction compared to that with the purified 39-kDa protein (11) and it is likely that more than one protein is necessary to afford complete protection.

The gel molecular weights of proteins in bands 1 and 2 correspond to the molecular weights of the proteins determined based on amino acid composition as identified in the database, whereas those of the proteins in bands 3, 4, and 5 do not. For these three proteins (HemY, the unknown lipoprotein, and PlpB), the gel molecular weights do not correspond to the actual molecular weights based on amino acid composition. This is not uncommon for membrane proteins, which may migrate anomalously in SDS-polyacrylamide gel electrophoresis (PAGE) (5, 9, 13). The percentages of matched peptides for the identified proteins varied from 11 to 1% (Table 1), and this is not uncommon for membrane proteins.

Identification of the 39-kDa outer membrane protein as the *Pasteurella* lipoprotein B, or PlpB, a member of a family of virulence-related proteins, should now permit cloning and expression of PlpB for vaccine experiments.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Boyce, J. D., J. Y. Chung, and B. Adler.** 2000. Genetic organisation of the capsule biosynthetic locus of *Pasteurella multocida* M1404 (B:2). Vet. Microbiol. **72:**121–134.
3. **Christensen, J. P., and M. Bisgaard.** 2000. Fowl cholera. Rev. Sci. Tech. **19:**626–637.
4. **Christiansen, K. H., K. P. Snipes, and D. W. Hird.** 1991. Transmission of *Pasteurella multocida* on California turkey premises in 1988–1989. Avian Dis. **36:**262–271.
5. **Diedrich, D. L., M. A. Stein, and C. A. Schnaitman.** 1990. Association of *Escherichia coli* K-12 OmpF trimers with rough and smooth lipopolysaccharides. J. Bacteriol. **172:**5307–5311.
6. **Hird, D. W., T. E. Carpenter, K. P. Snipes, D. C. Hirsch, and R. H. McCapes.** 1991. Case control study of fowl cholera outbreaks in meat turkeys in California August 1985-July 1986. Am. J. Vet. Res. **52:**212–216.
7. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
8. **Lee, M. D., R. E. Wooley, J. R. Glissen, and J. Brown.** 1988. Comparison of *Pasteurella multocida* serotype 3,4 isolates from turkeys with fowl cholera. Avian Dis. **32:**501–508.
9. **Lobos, S. R., and G. C. Mora.** 1991. Alteration in the electrophoretic mobility of OmpC to variations in the ammonium persulfate concentration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Electrophoresis **12:**448–450.
10. **May, B. J., Q Zhang, L. L. Li, M. L. Paustian, T. S. Whittam, and V. Kapur.** 2001. Complete genomic sequence of *Pasteurella multocida*, Pm70. Proc. Natl. Acad. Sci. USA **98:**3460–3465.
11. **Rimler, R. B.** 2001. Purification of a cross-protective antigen from *Pasteurella multocida* grown *in vitro* and *in vivo*. Avian Dis. **45:**572–580.
12. **Rimler, R. B., and K. R. Rhoades.** 1981. Lysates of turkey-grown *Pasteurella multocida*: protection against homologous and heterologous serotype challenge exposures. Am. J. Vet. Res. **42:**2117–2121.
13. **Rosenbusch, J. P.** 1976. Characterization of the major envelope protein from *Escherichia coli*. J. Biol. Chem. **249:**8019–8029.
14. **Snipes, K. P., D. C. Hirsch, R. W. Kasten, T. E. Carpenter, D. W. Hird, and R. H. McCapes.** 1990. Differentiation of field isolates of *Pasteurella multocida* serotype 3,4 from live vaccine strain by genotypic characterization. Avian Dis. **34:**419–424.
15. **Townsend, K. M., J. D. Boyce, J. Y. Chung, A. J. Frost, and B. Adler.** 2001. Genetic organization of *Pasteurella multocida cap* loci and development of a multiplex capsular PCR typing system. J. Clin. Microbiol. **39:**924–929.

Editor: J. T. Barbieri