INFECTION AND IMMUNITY, Apr. 1986, p. 175–182
0019-9567/86/040175-08$02.00/0

Vol. 52, No. 4

Biochemical and Immunological Characterization of Cell Surface Proteins of *Pasteurella multocida* Strains Causing Atrophic Rhinitis in Swine

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Received 10 June 1985/Accepted 23 December 1985

In a previous paper (B. Lugtenberg, R. van Boxtel, and M. de Jong, Infect. Immun., 46:48–54, 1984) we showed that among 34 isolates from swine the membrane protein and lipopolysaccharide (LPS) patterns, as analyzed by sodium dodecyl sulfate-gel electrophoresis, could be classified into three and six patterns, respectively. In all cases a certain LPS pattern was correlated with a certain protein pattern. Certain combinations of types of cell surface proteins and LPSs were correlated with pathogenicity, the latter property being judged by the guinea pig skin test. In the present paper the immunological and biochemical properties of cell surface constituents were analyzed. The reaction between electrophoretically separated cell surface constituents with guinea pig and sow antisera showed that LPS as well as several proteins were immunogenic. Among these is protein H, whose electrophoretic mobility is the main criterium for typing of cell envelope protein patterns. Protein H was the most heavily labeled component when whole cells were iodinated by the Iodo-Gen procedure, showing its accessibility at the cell surface. These properties of protein H make it an attractive vaccine candidate. Further biochemical analyses revealed that protein H shares many properties with pore proteins of members of the family *Enterobacteriaceae*. One of these properties, association between pore proteins and peptidoglycan, was used as the basis for a simple procedure developed to partially purify protein H.

Pasteurella multocida is the causative agent of disease in a variety of animals and birds. Strong indications for an interaction between *P. multocida* and *Bordetella bronchiseptica* in causing atrophic rhinitis have recently been obtained (4, 15). The guinea pig skin test is a good indicator of pathogenicity (11). Pathogenicity of *P. multocida* is correlated with exotoxin activity (M. F. de Jong, H. L. Oei, and G. J. Tetenburg, Proc. Int. Pig Vet. Soc., Copenhagen, Denmark, p. 211, 1980).

A biochemical analysis of cell surface proteins and lipopolysaccharides (LPSs) from 34 pathogenic and nonpathogenic *P. multocida* strains by sodium dodecyl sufate (SDS)-polyacrylamide gel electrophoresis revealed that with respect to the protein and LPS patterns these strains can be divided into three (I, II, and III) and six (a through f) classes, respectively. All combinations of protein and LPS types could be correlated with the presence or absence of virulence. Since properties of cell surface proteins or LPSs or both may be important in diagnosis and vaccination (11), we extended these studies. We now describe experiments designed to characterize the biochemical and immunological properties of the cell surface proteins in more detail. Since differences in protein patterns are mainly due to differences in the electrophoretic mobility of protein H (11), particular attention was paid to the properties of this major protein. The results show that protein H is surface exposed and immunogenic.

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MATERIALS AND METHODS

Strains and growth conditions. Relevant properties of the *P. multocida* strains used are listed in Table 1. P (problem) and C (certificate of health) herds are herds in which atrophic rhinitis has been diagnosed and is absent, respectively. The pathogenic character of the strains has been judged by the guinea pig skin test (for rationale, see reference 11). Positive and negative results of this test are indicated with + and −. The strains have also been classified with respect to the patterns of their cell envelope proteins (I, II, and III) and LPSs (classes a through f). Unless otherwise indicated, cells were grown in fresh meat broth at 37°C under vigorous aeration. In a few cases L-broth (13) was used as the growth medium.

Antisera. Results for antisera obtained after immunization of guinea pigs and sows are shown in Table 2. Cpb-GpHi 65, male, *P. multocida*-free, guinea pigs were used. The vaccine for animal 1 was prepared by adding 0.5 ml of a solution of penicillin G (50,000 IU/ml to 10 ml of a 24-h culture in meat broth of the pathogenic *P. multocida* isolate. The suspension was incubated for 6 h at 37°C, a procedure which kills the bacteria as judged by incubating 0.1 ml of the suspension on blood agar plates. For the first vaccination, a volume of 0.2 ml was injected intracutanously at each of two spots. This resulted in weak skin positive reaction. Revaccinations were carried out at days 14 and 35 by injection of 0.5 ml of the suspension in each of the hind legs. Blood samples were obtained by heart-puncture under anesthesia 2 weeks after the last vaccination. Guinea pig 2, used to raise antiserum against whole cells of the toxin-negative *P. multocida* strain of the same herd, was treated in exactly the same way except that the blood sample was gathered at day 35, before the last

TABLE 1. Strains and their relevant properties[a]

Strain	AR pathogenicity[b]	Farm type[c]	Electrophoretic pattern[d]	
			Protein	LPS
S1-2	+	P	I	a
Da9	+	P	I	a
M2	+	P	I	c
M7-5	+	P	I	f
4B8	−	C	II	b
H202	−	P	II	b
Ba4-6	−	P	II	b
Gritt 4-6	+	P	III	a
JH1	+	P	III	c
JH4	+	P	III	c
H4-4	(+)/−	P	III	c
L8-2	−	C	III	d
Mark 1	−	P	III	a
P1	+	P	III	c
P7	−	P	III	a

[a] See reference 13 for more detailed information.
[b] AR, Atrophic rhinitis; +, positive; (+)/−, doubtful; −, negative.
[c] P, Disease present; C, no disease detected.
[d] The results refer to patterns of cell envelope proteins and LPS obtained after SDS-polyacrylamide gel electrophoresis (11).

vaccination. Guinea pig 3 was used to raise antiserum against the atrophic rhinitis toxin-containing supernatant of the pathogenic strain described above. The culture filtrate of a 48-h culture was freed from bacteria by sequential filtration through filters (Millipore Corp., Bedford, Mass.) with pore sizes of 0.45 and 0.2 μm. A 10-fold dilution of the filtrate in a solution of 135 mM NaCl was injected according to the same schedule used to obtain the other guinea pig antisera. Guinea pig 4 was used as a control.

Sow antisera V734 and V737 were raised using an atrophic rhinitis commerical vaccine based on formalin-killed, washed, whole cell bacterins. As strong indications have been obtained that both *P. multocida* and *B. bronchiseptica* interact in causing atrophic rhinitis (15) the vaccine contains, in addition to two *P. multocida* isolates with different somatic antigens, a pathogenic *B. bronchiseptica* isolate. Because we never observed positive reactions when sera raised against *B. bronchiseptica* alone were tested against *P. multocida* cell envelope preparations, it can be assumed that the reactions described in this paper are due to the *P. multocida* components of the vaccine. Sows were vaccinated intramuscularly four times. The first two vaccinations were given with an interval of 6 weeks. Revaccinations were given 6 and 12 months later. The sera were gathered a few days before farrowing, which was approximately 2 months after the last vaccination. Sow 64 was vaccinated four times with another *P. multocida* atrophic rhinitis vaccine based on the filter-sterilized culture supernatant of *P. multocida* CVI40456, containing approximately 20 mouse 50% lethal dose units per ml. The supernatant fluid was emulsified with an equal volume of Freund incomplete mineral oil adjuvant. The sow was vaccinated intramuscularly with 2 ml of this emulsion at 8 and 4 weeks before farrowing, during the first pregnancy, after starting vaccination of the herd. During the following two pregnancies a 2-ml revaccination was given 1 month before the expected date of farrowing. The blood sample was gathered a few days before farrowing, which was approximately 4 weeks after the last vaccination.

Surface labeling of whole cells. Overnight cultures were centrifuged, and the cells were washed twice with phosphate-buffered saline (10 mM phosphate buffer [pH 7.5], 140 mM NaCl). Surface labeling was carried out by using the Iodo-Gen procedure (21). The cells were incubated with [^{125}I]iodide in a glass tube coated with the catalyzer 1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril. Efficient labeling of a cell surface protein with radioactive iodide can only occur upon contact with the catalyzer. The procedure was carried out as described (21) for 5 min at room temperature. Labeled cell envelope polypeptides were identified after SDS-polyacrylamide gel electrophoresis (5×10^3 to 10×10^3 cpm per slot) and subsequent autoradiography for 24 h at −80°C.

Toxin. A preparation of partly purified atrophic rhinitis toxin was kindly supplied by Ph. van der Heyden, Lelystad, The Netherlands. The toxin was partly purified (4a) from the filter-sterilized supernatant fluid of *P. multocida* CDI 40456 after 90% ammonium sulfate precipitation and by column chromatography, using Sephacryl S300 and DEAE-Sephacel.

Isolation and analysis of cell envelope fractions. Cell envelopes were isolated by differential centrifugation after disruption of stationary-phase cells by sonication (11). The procedure used for treatment of cell envelopes with trypsin has been described previously (14). Extraction of cell envelopes with Triton X-100 in the presence of 10 mM $MgCl_2$ was carried out as described by Schnaitman (20) with minor modifications (9). To isolate complexes of certain proteins with peptidoglycan or with peptidoglycan-lipoprotein, we followed the SDS-heat treatment of cell envelopes as described by Rosenbusch (18) with some modification (7). To optimize the isolation of such complexes, temperatures below 60°C were also used.

For the analysis of protein by SDS-polyacrylamide gel electrophoresis, cell envelopes were usually completely solubilized by boiling in sample buffer (8). In a few cases boiling was replaced by incubation for 20 min at 37°C, conditions which leave some complexes intact. Protein bands were indicated by their apparent molecular weights. For the analysis of the LPS patterns, cell envelopes solubilized by boiling in SDS were treated with proteinase K before

TABLE 2. Antisera obtained after immunization of guinea pigs and sows[a]

Animal species and no.	Antigen used for immunization[b]	Electrophoretic patterns of cell envelope	
		Proteins	LPS
Guinea pig			
1	*P. multocida* Mark (+)	ND[c]	ND
2	*P. multocida* Mark (−)	III	c
3	Bacteria-free supernatant fluid of Mark (+)	ND	ND
4	None	—	—
Sow			
V734 and	*P. multocida* P1 (+), *P. multocida*	III	a
V737	P7 (−), and *B. bronchiseptica*[d]	III	c
64	Cell-free supernatant fluid of *P. multocida* CVI 40456 (+)[e]	ND	ND

[a] Guinea pigs were from a population free from *B. bronchiseptica* and *P. multocida*.
[b] + and − refer to the atrophic rhinitis pathogenic (+) and nonpathogenic (−) character as judged from the guinea pig skin test (13).
[c] ND, Not determined.
[d] First Ducht atrophic rhinitis commercial vaccine available. In addition to a pathogenic and a nonpathogenic *P. multocida* strain, it contained a *B. bronchiseptica* strain.
[e] First experimental vaccine containing cell-free supernatant fluid.

electrophoresis to degrade proteins (11). Electrophoresis of proteins was usually carried out in gel system A (8), but sometimes modifications of this system, designated as gel systems B and C, were also used to obtain a slightly different resolution. The modified gel system B was used for the separation of LPSs (11). Proteins were stained with fast green FCF (Sigma Chemical Co., St. Louis, Mo.) (8), and LPSs were stained by using a slightly modified (11) silver staining procedure of Tsai and Frasch (23).

Gel immunoradioassay. The original procedure for the immunological detection of antigens in thin longitudinal sections of SDS-polyacrylamide gels has been described by Van Raamsdonk et al. (27). Poolman et al. (16) introduced the radioassay. Briefly, after electrophoretic separation of the antigens in SDS-polyacrylamide gels, the gel was cut into four parts ca. 5 by 5 cm. These quarters were frozen and up to 20 identical thin longitudinal sections were cut. After removal of SDS, these sections were incubated sequentially with antiserum and with iodinated *Staphylococcus aureus* protein A, which binds to immunoglobulin G of both swine and guinea pigs. The reacting antigens were detected by autoradiography. We have introduced several modifications to reduce the background (14) or to shorten the time necessary for the procedure (2).

RESULTS

Biochemical properties of *P. multocida* cell envelope proteins. Previous characterization of the cell envelope protein patterns of 34 *P. multocida* strains revealed three distinct protein patterns designated as I, II, and III (Fig. 1 and reference 11). The electrophoretic mobility of protein H, one band of the doublet bands H (heavy) and W (weak) in the middle of the gel, is the major criterion for determining the type of cell envelope protein pattern. The electrophoretic mobility of the W proteins is indistinguishable for the three types of strains (11). Our recent attempts to characterize the properties of the cell envelope proteins further revealed the following. (i) Growth of strains Me2, Ba4-6, and Gritt 4-6, representing types I, II, and III cell envelope protein patterns, respectively, in L-broth instead of in meat broth did not significantly change the cell envelope protein pattern (data not shown). (ii) Incubation of cell envelopes with trypsin (50 μg/ml), a treatment which degrades all cytoplasmic membrane proteins and many outer membrane proteins of *Escherichia coli* (5), solubilized the 65,000 (65K) and 50K proteins but did not solubilize the proteins H and W of strains Da-9 (type I), 4B8 (type II) and L8-2 (type III) (data not shown). (iii) With the same strains, we observed that none of the proteins 65K, 50K, H, and W were solubilized by extraction of cell envelopes with Triton X-100 in the presence of 10 mM Mg^{2+} (data not shown), a treatment that solubilizes cytoplasmic membrane proteins of *E. coli* (8, 20). (iv) When solubilization of the sample by boiling for 5 min in sample buffer was replaced by incubation for 20 min at 37°C, subsequent analysis of the solubilized proteins of the representative strains S1-2 (type I), 4B8 (type II), and Gritt 4-6 (type III) showed almost exactly the same gel pattern except that band H was absent (Fig. 2). Application of the gel immunoradioassay technique on such gels (see below), using an antibody preparation that reacted strongly with the H band of boiled preparations, showed no reaction at the electrophoretic position of the H band but a new antigen was detected in a position 0.5 to 1.0 cm from the top of the running gel, strongly suggesting that protein H is relatively resistant to solubilization by incubation at 37°C. Pore proteins of *E. coli* K-12 show the same response to the solubilization temperature as H proteins (unpublished results).

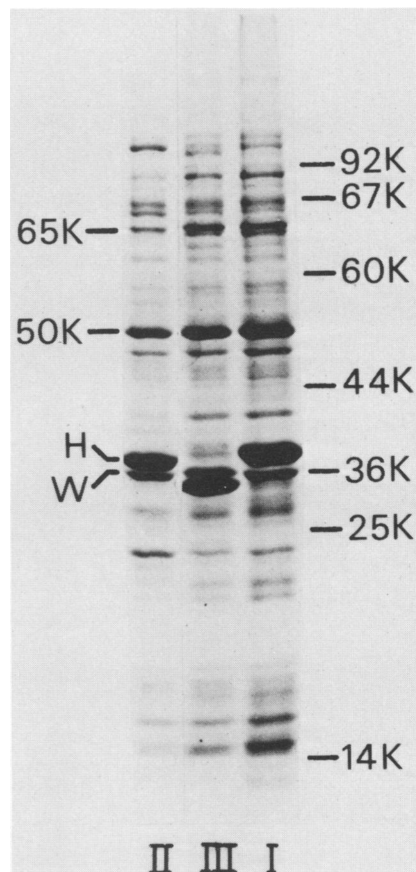

FIG. 1. Different cell envelope protein patterns of *P. multocida* strains obtained when boiled cell envelope samples were resolved by SDS-polyacrylamide gel electrophoresis in gel system C. The lanes represent the following strains. II, the P^- strain Ba4-6; III, the P^+ strain JH-1; I, the P^+ strain M2. The positions of molecular weight standard proteins are indicated at the right. A number of typical *P. multocida* proteins are indicated at the left. For further details see reference 9.

Immunogenicity of cell envelope constituents. As protective antigens should be immunogenic, electrophoretically separated cell surface constituents of *P. multocida* were tested for their ability to react with available sera from guinea pigs and sows immunized with vaccines containing whole cells or supernatant fluids or both of *P. multocida*.

Guinea pig antisera had been raised against strains from farm Mark which had been characterized with respect to their pathogenic properties but not with respect to their biochemical properties. The antigens consisted of cell envelopes of well-characterized strains which had been solubilized in SDS either mildly, i.e., 20 min at 37°C to conserve as many antigenic determinants as possible, or completely, i.e., 5 min at 100°C. The 37°C treatment solubilizes most proteins into monomers, protein H being the major exception (Fig. 2). Reactions of antigens with antibodies were tested with the gel immunoradioassay.

Using completely solubilized antigens of cells of strains S1-2 (P^+, I, a), 4 B-8 (C^-, II, b), Gritt 4-6 (P^+, III, a), and M 7-5 (P^+, I, f), guinea pig sera raised against whole cells of either a toxin-producing strain (Fig. 3, lane b) or against a toxin-negative strain (Fig. 3, lane d) showed heavy reac-

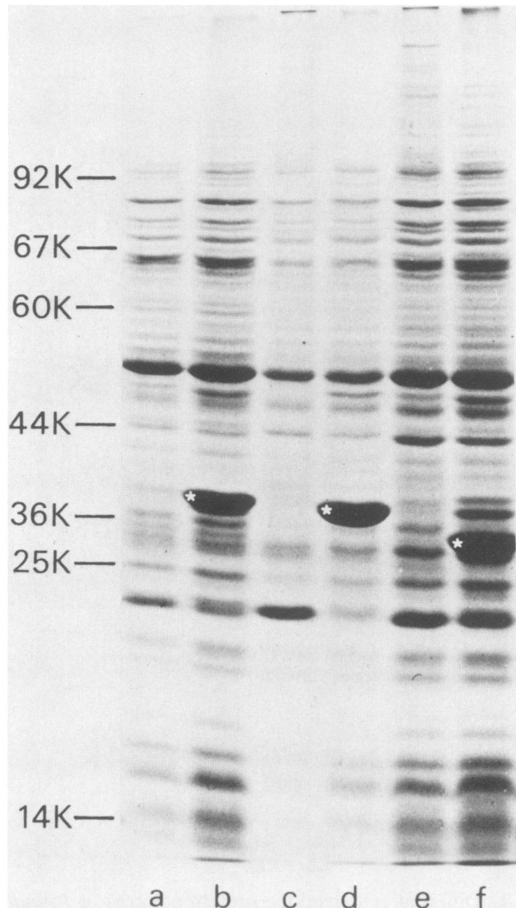

FIG. 2. Effect of solubilization temperature on cell envelopes protein patterns. Cell envelopes of strains S1-2 (P^+, I, a), (lanes a and b), 4B8 (C^-, II, b) (lanes c and d), and Gritt 4-6 (P^+, III, a) (lanes e and f) were either incubated in sample buffer for 20 min at 37°C (lanes a, c, and e) or boiled for 5 min (lanes b, d, and f), followed by electrophoresis in gel system A. The heavy band H (indicated with asterisks) is only present in boiled preparations. The positions of molecular weight markers are as indicated.

tions, although to a different extent, with the H band of cell envelopes of type III protein pattern (Fig. 3, lanes b and d), whereas the reactions with the H bands of cell envelopes of protein pattern types I and II were positive but considerably weaker (data not shown). In contrast to the antiserum against the toxin-positive strain (Fig. 3, lane b), that against the toxin-negative strain consistently showed a clearly positive reaction in the position of LPS (Fig. 3, lane d) and sometimes showed a weaker reaction in the position of a protein band with an apparent molecular weight of 25,000. These results clearly show that H protein and LPS of whole cells of *P. multocida* can be immunogenic in guinea pigs.

To conserve as many antigenic determinants as possible, the cell envelopes were also solubilized at 37°C before electrophoresis (Fig. 3, lanes a and c). The reaction in the position of the H band was absent, and that in the position of LPS monomers was weaker or absent. Moreover, a long smear appeared in the upper 20% of the gel which seems to be caused by a large number of discrete bands. Evidence that the latter antigens can tentatively be identified as protein H-LPS complexes will be presented below.

Serum raised against the culture supernatant of the same toxin-producing strain in guinea pig 3 was also allowed to react with cell envelope antigens. This antiserum showed heavy reactions with the H band, to a lesser extent with LPS and some proteins in boiled preparations (Fig. 3, lanes f and h), and with the pore protein-LPS complexes in the case of samples incubated at 37°C (Fig. 3, lanes e and g). In all cases the reactions were stronger with cell envelopes of types III and II than with those of type I. Surprisingly, no reaction was observed with 37°C treated or boiled preparations of the partly purified atrophic rhinitis toxin.

Sera of four vaccinated sows immunized with *B. bronchiseptica* and *P. multocida* were allowed to react with the electrophoretically separated constituents of boiled cell envelopes of a variety of strains mentioned in the legend to Fig. 4. In all four cases positive reactions were found. Examples of the reactions found for eight strains with two of the sera are given in Fig. 4. Usually the reactions were very similar for the various strains. Specifically, no consistent differences were found between toxin-positive and toxin-negative strains. For most strains positive reactions were detected with antigens in the following electrophoretic positions (the number of positive sera of the four sera tested is given in parenthesis). Top of running gel (twice), 100K (three times), about four bands ranging from 70K to 100K (once), 65K (four times), 50K (four times), H (three times), L (twice), 30K (four times), and LPS (four times).

A surprising observation was that, although the two *P. multocida* strains present in the vaccine are both of cell envelope protein type III, serum V734 showed positive reactions with the H protein of cells with cell envelope protein types II (Fig. 4B, lanes a and b) and I (Fig. 4B, lanes g and h), but not with those of cell envelope protein type III (Fig. 4B, lanes c to f). However, a later serum from the same animal tested against preparations solubilized at 37°C instead of at 100°C showed a positive reaction with preparations of all protein types in the region where protein H-LPS complexes are found. Since in this case no reaction was found in the position of LPS monomers, the most likely explanation is that the antibodies recognize the native form but not the denatured form of protein H of strains of cell envelope protein type III.

Finally, when the serum of a sow (no. 64) which had been immunized with the supernatant fluid of the toxin-producing strain CVI40456 was tested against boiled cell envelope preparations from strains representing all three types of cell envelope proteins, the only positive reaction was observed in the position of protein H. With 37°C-treated cell envelopes, the only reacting antigens were multiple bands in the position of the putative protein-LPS complexes. As this antiserum does not contain antibodies against LPS monomers but is active against protein H monomers, these results provide evidence for the presence of protein H in the smear putatively suggested to contain protein-LPS complexes.

Cell surface localization of cell envelope proteins. Since cell surface components which are immunogenic may be useful for protection of animals by vaccination as well as for many diagnostic purposes, we labeled the cell surface of whole cells by using the Iodo-Gen procedure. Whole cells of strains M2 and P7-5/05097-2, representing the two cell envelope protein types among which pathogenic strains have been found (11), were iodinated. After isolation of cell envelopes and separation of the boiled constituents by electrophoresis, subsequent autoradiographic analysis revealed a relatively low number of labeled bands (Fig. 5). Comparison with the stained gel showed for both strains that the heaviest iodinated band corresponds with protein H, whereas the radioac-

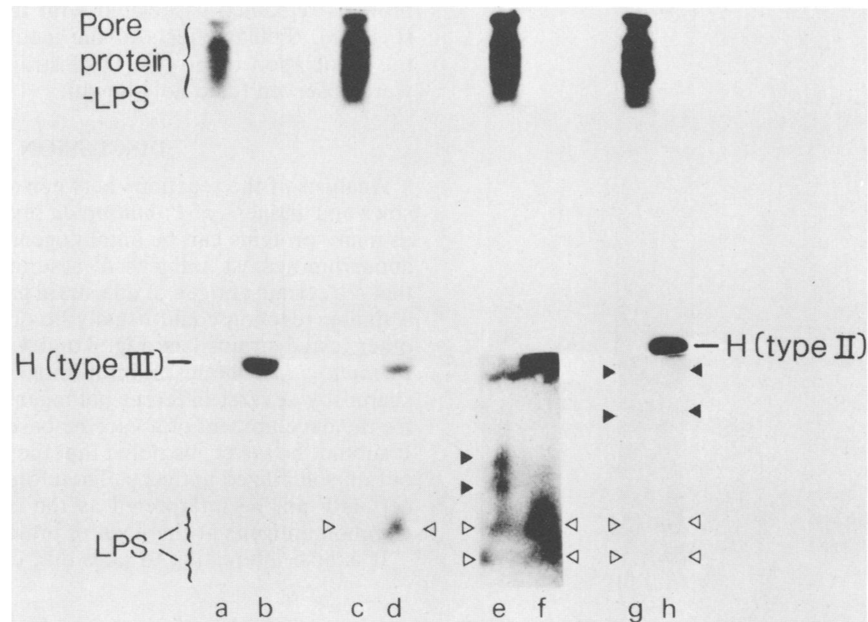

FIG. 3. Gel immunoradioassay of guinea pig antisera with electrophoretically separated antigens of *P. multocida*. Cell envelopes of strains Gritt 4-6 (P^+, III, a) (lanes a through f) and 4B8 (C^-, II, b) (lanes g and h) were solubilized at 37°C (lanes a, c, e, and g) or at 100°C (lanes b, d, f, and h). After electrophoresis in gel system A, thin longitudinal sections were incubated with antiserum raised in guinea pig 1 against whole cells of a toxin-producing strain (lanes a and b), raised in guinea pig 2 against whole cells of a toxin-negative strain (lanes c and d), or raised in guinea pig 3 against the extracellular fluid of the same toxin-producing strain (lanes e, f, g, and h). After allowing the bound immunoglobulin G to react with ^{125}I-protein A, the radioactivity was detected by autoradiography. The positions of the relevant constituents are indicated by closed and open triangles, representing protein and LPS antigens, respectively.

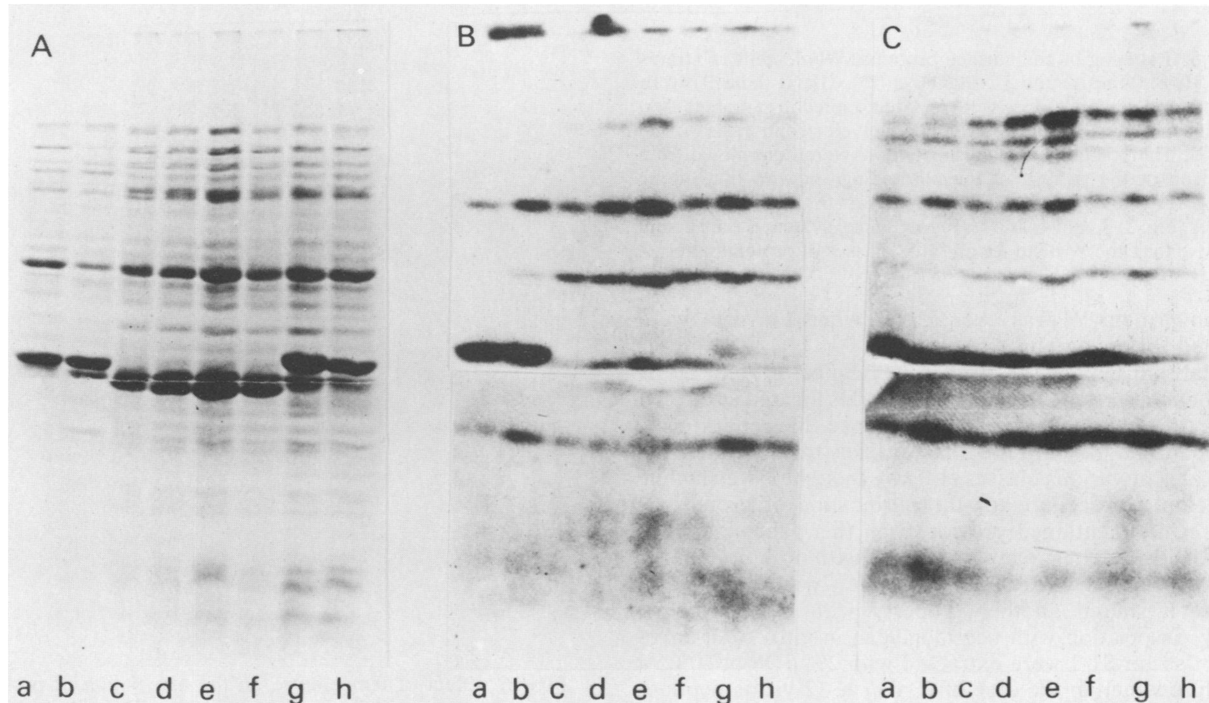

FIG. 4. Reactions of sera of vaccinated sows with electrophoretically separated antigens of *P. multocida* strains from which strain designation and, in parenthesis, pathogenicity, cell envelope protein type, and LPS type, respectively, are indicated below. Cell envelopes of strains (lanes): a, H202 (P^-, II, b); b, Ba4-6 (P^-, II, b); c, L8-2 (C^-, III, a); d, Mark 1 (P^-, III, a); e, H4-4 ($P^{(+)/-}$, III, c); f, JH-4 (P^+, III, c); g, M2 (P^+, I, e); and h, S1-2 (P^+, I, a) were dissolved in sample buffer by boiling, and the constituent molecules were separated by SDS-polyacrylamide gel electrophoresis using gel system A. Subsequently, the gel was treated as explained in the legend to Fig. 3. (A) stained gel, (B and C) autoradiograms of gel copies treated with indiluted antisera V734 and V737, respectively. Autoradiography was carried out for 4 days with a reflection screen.

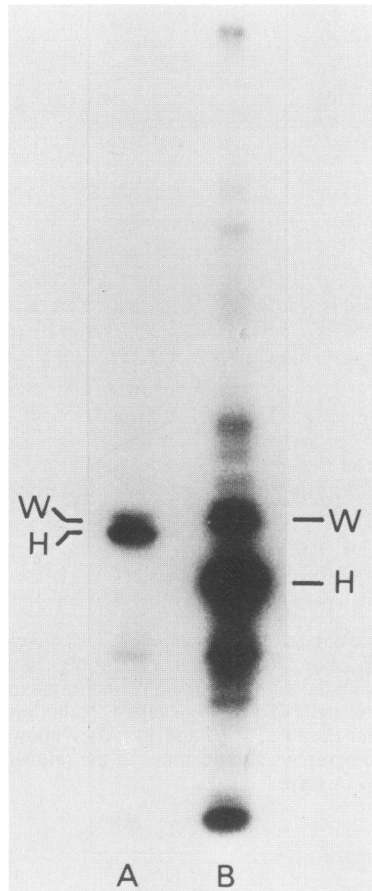

FIG. 5. Labeling of cell surface proteins. Whole cells of strains M2 (P^+, I, e) (lane A) and P7-5/05097-2 (P^-, III, a) (lane B) were iodinated, and cell envelopes were isolated, boiled in sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis in gel system B. The positions of bands H and W were determined from comparison of the pattern of the autoradiogram with that of the stained gel. It should be noted that, in contrast to the situation in gel system A (Fig. 2, 3, and 4) and C (Fig. 1), in gel system B the H band runs faster than the W band for all three classes of protein patterns.

tivity in protein W was even larger when the data were corrected for the amount of protein.

Partial purification of protein H. Since protein H is strongly immunogenic (Fig. 3 and 4) and is located at the cell surface of whole cells (Fig. 5), and since differences in its electrophoretic mobility are a major basis for distinguishing the isolates of various classes (13), we thought it worthwhile to develop a procedure for the purification of protein H. Since results mentioned previously in this paper indicated that protein H shares many properties with pore proteins of members of the *Enterobacteriaceae*, we investigated whether it shared another property with pore proteins, namely, association with peptidoglycan in vitro. Cell envelopes of strain S1-2 were extracted with 2% SDS at 60°C, a procedure which in the case of *E. coli* K-12 yields peptidoglycan with practically pure protein noncovalently attached to it (7, 18). Indeed protein H was the only protein detected in the material that was not solubilized by this treatment, but the yield was only 5 to 10% of the total amount (compare lanes a and c in Fig. 6). By decreasing the temperature during the extraction to 37°C, the yield of protein H increased to about 50%, whereas only traces of a few other proteins remained associated with the peptidoglycan layer (Fig. 6b). Similar effects of the incubation temperature on the association of protein H of strains of types II and III were observed (data not shown).

DISCUSSION

Analysis of the reactions between sera of guinea pigs and sows and antigens of *P. multocida* revealed that LPS as well as many proteins can be immunogenic. By testing a certain antiserum against antigens of a series of strains we found that if a certain antigen of one strain gave a positive reaction, a similar reaction could usually be observed for most or all other tested strains (see Fig. 3 and 4). The observation that *P. multocida* contains several antigens that are apparently shared by several different pathogenic strains is promising for the development of a vaccine based on protein antigens. It should, however, be noted that the reactions were carried out on solubilized antigens. Therefore, this observation can certainly not be interpreted as the frequent occurrence of common antigens at the level of intact cells.

It is also interesting to note that certain antigenic deter-

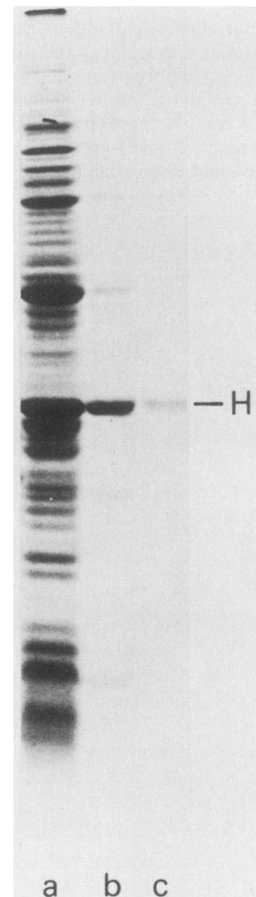

FIG. 6. Effect of temperature on the association of protein H with peptidoglycan. Cell envelopes of strain S1-2 (P^+, I, a) were incubated in 2% SDS at 37 and at 60°C. Peptidoglycan-protein complexes were isolated by centrifugation as described in the text. The resulting pellet was boiled in sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis in gel system A. Lane a, cell envelopes; lanes b and c, peptidoglycan-protein complexes isolated after incubation of cell envelopes at 37 and 60°C, respectively. The position of protein H is indicated.

minants seem to be more or less specific for strains belonging to a certain protein type, e.g., it appears that antibodies against the 50K protein react with the 50K protein of the tested strains of protein types I and III but not with those of protein type II (Fig. 4). Such proteins could be used as target for the immunological diagnosis of pathogenic strains.

Because a protective antigen should be located at the surface of the cell, iodination experiments were performed to identify these antigens. These showed that proteins H and W are among the major surface-exposed proteins (Fig. 5). Special attention was paid to the properties of protein H since its electrophoretic mobility in SDS-polyacrylamide gels has been used to classify strains of *P. multocida* (11). This protein shares many properties with pore proteins of members of the *Enterobacteriaceae* (10), i.e., insolubility in Triton X-100 in the presence of Mg^{2+}, resistance to degradation by trypsin, resistance to solubilization to free monomers in SDS at 37°C (Fig. 2) (the latter property presumably being the result of a strong affinity for LPS), the formation of tight complexes with peptidoglycan (Fig. 6), and the localization at the cell surface (Fig. 5).

Antibodies were allowed to react with two types of antigens, namely, monomeric molecules, which were obtained by boiling cell envelope samples, as well as with 37°C-treated cell envelopes. The latter samples can contain completely or partly unfolded monomeric proteins, LPS, and complexes of proteins or LPS or both. Among the antigens present after 37°C treatment but absent after boiling are a series of bands which often appears as a smear with a relatively low electrophoretic mobility (e.g., see Fig. 3 lanes a, c, e, and g). The following lines of evidence indicate that this smear contains, or even consists of, complexes of protein H and LPS. (i) Complexes of pore proteins and LPS have been reported to run in these positions as multiple bands (4, 26). (ii) Appearance of these antigens in 37°C-treated preparations coincided with the disappearance of the H band (compare lanes b and a and lanes d and c in Fig. 3) as well as with the virtual disappearance of the LPS band (lanes d and c in Fig. 3). (iii) Antiserum from sow 64 contains antibodies against H protein monomers but not against LPS monomers. The serum reacts with the multiple bands (see above).

The immunogenic complexes of protein H and LPS described in this paper have probably been described earlier. For example, Prince and Smith (17) described that the α-complex, one of the three types of *P. multocida* antigens which is immunogenic and closely bound to the cell wall, probably consist of a polysaccharide-protein complex. Moreover, a protective antigen extracted from turkey-pathogenic *P. multocida* P-1059 contains three protein subuntis of 44K, 31K, and 25K, as well as one carbohydrate band in the electrophoretic position of proteins with an apparent molecular weight below 20,000 (22). The only carbohydrate-containing cell envelope molecules found in our experiments in this electrophoretic position were LPSs (11). The strong immunogenicity and protective activity of outer membrane protein-(lipo)polysaccharide complexes has been shown earlier for members of the *Enterobacteriaceae* (J. Dankert, H. Hofstra and T. S. Veninga, FEMS Symp. on Microbial Envelopes, 1980, abstr. no. 51; N. Kuusi, M. Nurminen, H. Saxén and P. H. Mäkelä, FEMS Symp. on Microbial Envelopes, 1980, abstr. no. 50; 6, 12) and *Neisseria meningitidis* (3). The observed reactions of antiserum against culture supernatants with the outer membrane constituents, H protein and LPS support our assumption (11) that extracellular material is rich in outer membrane vesicles. Moreover, it has even been reported that pore proteins are enriched in outer membrane vesicles of *E. coli* (28).

Based on its affinity for peptidoglycan, protein H can be largely purified by a very simple procedure (see Fig. 6). It is likely that procedures that have been applied successfully for the further purification of the pore proteins of members of the *Enterobacteriaceae*, discussed in reference 10, can also be used for the final purification steps of protein H. Purified preparations of protein H can be used for raising polyclonal or monoclonal antibodies against the protein. Our previous results showed that all tested strains with cell envelope type I are pathogenic and those with cell envelope type II are nonpathogenic (11). Therefore, it is likely that antibodies that discriminate strains with cell envelope protein types I, II, and III can be used to diagnose the pathogenic character of approximately half of the strains, thereby limiting the number of painful and elaborate guinea pig skin tests that must be performed to strains of envelope protein type III. Antibodies that do not clearly discriminate between the H proteins of the various *P. multocida* strains could, if they can be raised by vaccination, provide protection of animals against *P. multocida*. Antibodies of both types of specificity can indeed by obtained in the case of *E. coli* PhoE pore proteins. Among monoclonal antibodies raised against PhoE protein-peptidoglycan complexes, one class can be found that discriminates in whole cells between the three *E. coli* K-12 pore proteins in that only PhoE protein is recognized. By using another monoclonal antibody PhoE proteins of a large number of different members of the *Enterobacteriaceae* can be recognized (25).

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