INFECTION AND IMMUNITY, Oct. 1986, p. 109–117
0019-9567/86/100109-09$02.00/0

Vol. 54, No. 1

Heat-Modifiable Envelope Proteins of *Bordetella pertussis*

SANDRA K. ARMSTRONG AND CHARLOTTE D. PARKER*

Department of Microbiology, School of Medicine, University of Missouri-Columbia, Columbia, Missouri 65212

Received 18 February 1986/Accepted 27 June 1986

Several envelope proteins of *Bordetella pertussis* demonstrated differences in electrophoretic mobility, depending upon solubilization temperature before sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These proteins were exposed on the cell surface as judged by their accessibility to radiolabeling with ^{125}I. Monoclonal antibodies to two of the heat-modifiable proteins (M_rs of 18,000 and 91,000) reacted with intact cells in immunofluorescence microscopy experiments, also indicating surface exposure of these two proteins. Two-dimensional gel electrophoresis revealed that two heat-modifiable proteins (a major protein with an M_r of 38,000 and one with an M_r of 18,000) migrated as higher-M_r moieties when solubilized at low temperatures (25°C). Three proteins (M_rs of 91,000, 32,000, and 30,000) and possibly a fourth (31,000) migrated as lower-M_r species when solubilized at 25°C, as revealed in the two-dimensional gel system; these three proteins were found only in virulent *B. pertussis* and were not detected in a phase IV avirulent strain nor in a strain modulated to phenotypic avirulence by growth in nicotinic acid. The 38,000 molecular-weight protein (38K protein) and a 25K protein were found to be noncovalently associated with the underlying peptidoglycan. Small amounts of the 91K and 18K proteins were also found associated with peptidoglycan.

Certain outer membrane proteins (OMPs) of gram-negative bacteria have been termed heat modifiable with regard to their migration behavior upon sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (2, 11, 18, 27, 28). This behavior is determined by the solubilization conditions before electrophoresis. Solubilization of these proteins at certain temperatures results in altered electrophoretic mobility, presumably due to heat-induced changes in protein-SDS interactions. Other conditions known to alter protein migration in SDS-PAGE gels include the presence of 2-mercaptoethanol (2-ME) in the solubilizing buffer and the percentage of acrylamide in the gels (11, 28). Alterations in protein migration due to variations in electrophoresis technique can make identification of specific OMPs difficult; this has been especially true in the study of *Escherichia coli* OMPs (2, 4, 28).

The gel protein profiles of cell envelopes of *Bordetella pertussis* have been shown to be altered by solubilization temperature before analysis by tube gel SDS-PAGE (20). *B. pertussis* exhibits a phase change in which virulent phase I organisms display a specific OMP profile while avirulent phase IV cells demonstrate altered OMP profiles and lack virulence-associated factors such as pertussis toxin, dermonecrotic toxin, and filamentous hemagglutinin (5, 7, 20, 26). Growth of phase I organisms in medium containing high magnesium or nicotinic acid results in a phenomenon termed modulation which mimics the phenotypic effects of phase degradation (29, 31). In a study by Parton and Wardlaw, some of the temperature-induced migration changes involved envelope proteins found only in virulent phase I cells but not in avirulent phase IV or magnesium ion-modulated *B. pertussis* (20).

In the present study we sought to further characterize the identities of the heat-modifiable envelope proteins of *B. pertussis* and to examine their distribution in a virulent strain and its avirulent derivative. We used a combination of techniques such as surface radioiodination, two-dimensional SDS-PAGE, and Western immunoblotting with cell surface-specific monoclonal antibodies (MAbs) to demonstrate that virulent *B. pertussis* cells possess at least five heat-modifiable proteins which are exposed on the cell surface. Three (or four) of these proteins were not observed in avirulent cells. Migration of the cell envelope proteins was unaltered by the presence or absence of 2-ME in the SDS-PAGE solubilization buffer, and several proteins were found to be noncovalently associated with the underlying cell peptidoglycan.

(Portions of this research have been presented [S. K. Armstrong and C. D. Parker, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, D108, p. 72].)

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. pertussis* UT25, a virulent clinical isolate (7), was maintained in its virulent phase, lyophilized or stored in blood or 15% glycerol at −70°C. Strain UT25D, an avirulent phase IV derivative of UT25, was obtained by serial passage on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) containing decreasing amounts of defibrinated sheep blood (7). All strains were cultivated in modified Stainer-Scholte medium as described previously (29) or on Bordet-Gengou plates containing 20% defibrinated sheep blood. Modulated cells (UT25NA) were obtained by passaging UT25 twice in modified Stainer-Scholte medium containing 500 μg of nicotinic acid (Sigma Chemical Co., St. Louis, Mo.) per ml (29).

Radioiodination of intact bacteria. Whole cells of *B. pertussis* were radioiodinated by the technique of Richardson and Parker (24) adapted from Markwell (17). Cells were harvested from broth or plates and washed twice in phosphate-buffered saline (PBS), and approximately 0.1 g (wet weight) of cells was resuspended in 0.5 ml of PBS. The bacterial suspension was transferred to a 1-dram (ca. 3.7 ml) glass vial, and 0.5 mCi of $Na^{125}I$ (Amersham Corp., Arlington Heights, Ill.) was added. The reaction was initiated by the addition of four or five Iodo-beads (Pierce Chemical Co., Rockford, Ill.) which had been previously rinsed with PBS. After a 7-min incubation at room temperature with gentle agitation, the reaction was terminated by removal of the bacterial suspension from the Iodo-beads. After addition of

* Corresponding author.

0.2 ml of a 6-mg/ml solution of NaI in PBS, the total number of radioactive counts in the suspension was measured with a gamma counter (model 8000; Beckman Instruments, Inc., Fullerton, Calif.). The radiolabeled bacteria were pelleted in a microcentrifuge, washed twice with the NaI-PBS solution and once with PBS, and then suspended in PBS. Pellet-associated counts were then measured with the gamma counter. Typical experiments yielded from 7 to 14% incorporation of ^{125}I into whole cells.

Crude OMP preparation. The technique of Schneider and Parker was modified to obtain Triton X-100-insoluble envelope fractions enriched in OMPs (29). Cells which had been grown in modified Stainer-Scholte medium were pelleted by centrifugation at 6,000 × *g* for 15 min. Approximately 2.0 g (wet weight) of cells was suspended in 10 ml of a cold solution containing 0.75 M sucrose and 10 mM Tris (pH 7.8), after which 20 ml of cold 1.5 mM EDTA was slowly added. RNase and DNase (1 mg each) were added, and the cells were disrupted by sonication under cooling conditions; total cell envelopes were prepared and then extracted with Triton X-100 as has been described (29).

Peptidoglycan-associated proteins. Peptidoglycan-associated proteins were identified by two methods. The first was based on the technique of Hindahl and Iglewski (12), which was modified from that of Lugtenberg et al. (16). A total cell envelope pellet was suspended in 15 mM Tris (pH 8.0) with 2% SDS, and samples were incubated for 15 min at 37, 50, 60, 80, and 100°C. The peptidoglycan and any associated proteins were pelleted by centrifugation at 100,000 × *g* for 1 h, suspended in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4), washed and repelleted two additional times with the same buffer, and examined by SDS-PAGE.

The second method was adapted from Darveau et al. (3). Total cell envelopes were suspended in 15 mM Tris (pH 8.0) containing 2% SDS and incubated at 37°C for 30 min. After this material was centrifuged at 100,000 × *g* for 1 h the pellet was washed twice with the SDS buffer. The washed pellet was resuspended in the same buffer containing 0.1 M NaCl and centrifuged at 100,000 × *g* as before. The supernatant and peptidoglycan pellet (suspended in 10 mM HEPES) were analyzed by SDS-PAGE.

Analysis by SDS-PAGE. A modified Laemmli (14) procedure was used to examine proteins on 7.5 to 20% gradient gels (29). Gel dimensions were 0.75 mm by 12 cm by 14 cm, except those gels used for resolving the second dimension in the two-dimensional gel experiments, which were 1.5 mm thick. Urea (0.5 M; Bio-Rad Laboratories, Richmond, Calif.) was used in both the stacking and separation gels. Protein determinations were performed by the method of Lowry et al. (15) with bovine serum albumin as the standard. Samples were solubilized in digestion buffer containing final concentrations of 0.625% SDS, 0.625% 2-ME, 1.5% urea, 6.25% glycerol, and 0.125 M Tris (pH 6.8) at 100°C for 5 min unless otherwise stated. Each gel lane was loaded with 20 μg of protein or 50,000 cpm. Gels were stained with Coomassie blue by the technique of Fairbanks et al. (6).

Heat-modifiable proteins were identified by using a two-dimensional SDS-PAGE technique adapted from Hindahl and Iglewski (12) and Russell (27). Radioiodinated UT25 or UT25D whole cells were solubilized in sample digestion buffer at 25 or 100°C for 5 min and subjected to SDS-PAGE in the gel system described above. Each gel lane was excised after electrophoresis, boiled in digestion buffer without 2-ME for 5 min, placed horizontally atop a 1.5-mm-thick 7.5 to 20% gradient gel, and electrophoresed at 1.0 W for 18 h.

TABLE 1. Characteristics of monoclonal antibodies used in this study[a]

Organism, strain	Reactivity as determined by using the following tests and antibodies:			
	Western immunoblots		Fluorescent antibody microscopy	
	1B6	P8E7	1B6	P8E7
B. pertussis				
UT 25	+	+	+	+
UT25D	+	−	+	−
UT25NA	+	−		
B. bronchiseptica SC89-22-2	+	−		
B. parapertussis ONT1-2	+	−		

[a] MAbs 1B6 (19) and P8E7 (8, 19) are both of the immunoglobulin G isotype and have been described previously. The sizes of the antigens detected by 1B6 and P8E7 in the Western immunoblots were 18 and 91 kilodaltons, respectively. Symbols: +, positive reaction; −, negative reaction.

The proteins were transferred to nitrocellulose for Western immunoblot analysis (30) and subsequent autoradiography.

MAbs. MAb-secreting hybridomas were produced, screened, and subcloned as described by Frank and Parker (9), using modifications of the techniques of Kohler and Milstein (13). The properties of the MAbs used in this study are described in Table 1.

MAb 1B6 was generated by subcutaneous immunization of BALB/c mice with 2 μg of UT25 Triton X-100-insoluble envelope proteins (denatured by boiling for 5 min in a solution of 0.625% SDS, 0.625% 2-ME, 0.125M Tris [pH 6.8], and 1.5% urea) in incomplete Freund adjuvant. The mice received intraperitoneal booster injections at 3-, 4-, and 5-week intervals with the same denatured antigen preparation. Hybrid colonies were screened for MAb production by using an enzyme-linked immunosorbent assay with UT25 Triton-insoluble envelope proteins as the antigen.

Western immunoblot analysis. Proteins subjected to SDS-PAGE were transferred to nitrocellulose by the method of Towbin et al. (29), incubated with undiluted MAb culture supernatants, reacted with a 1:2,000 dilution of goat anti-mouse immunoglobulin G peroxidase conjugate (Cooper Biomedical, Inc., West Chester, Pa.) and developed with 4-chloro-1-naphthol as described previously (8). Dried blots containing radioiodinated proteins were incubated against Kodak X-Omat XAR-2 film (Eastman Kodak Co., Rochester, N.Y.) for 24 h at room temperature to produce an autoradiogram. If desired, blots were reprobed with antibody by soaking dried, previously probed, and photographed nitrocellulose blots in 0.9% NaCl–10 mM Tris buffer (pH 7.4) for 5 min, draining the buffer, incubating in the desired antibody, and processing in the usual fashion.

RESULTS

One-dimensional SDS-PAGE and immunoblot analyses. Two types of preparations were examined: (i) an outer membrane fraction, and (ii) surface-radioiodinated whole cells.

(i) Triton X-100-insoluble envelope fractions. A Triton X-100-insoluble OMP preparation from virulent UT25 solubilized at various temperatures and analyzed by SDS-PAGE demonstrated the presence of heat-modifiable proteins. The presence or absence of 2-ME in the solubilization

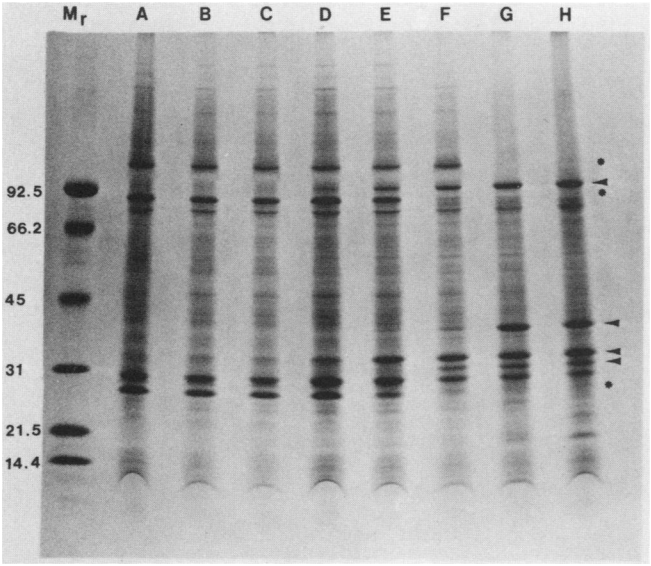

FIG. 1. SDS-PAGE analysis of virulent UT25 crude OMPs solubilized at different temperatures. Crude OMPs were prepared as described in the text and solubilized in the presence or absence of 2-ME before electrophoresis. Solubilization conditions: lane A, 25°C, without 2-ME; lane b, 25°C, with 2-ME; lane C, 35°C, with 2-ME; lane D, 50°C, with 2-ME; lane E, 60°C, with 2-ME; lane F, 80°C, with 2-ME; lane G, 100°C, with 2-ME; lane H, 100°C, without 2-ME. Asterisks indicate the location of protein bands which are not visible at higher solubilization temperatures; arrows show bands which become evident only at higher solubilization temperatures. Molecular size standards (lane M_r, shown in kilodaltons): phosphorylase B, 92.5; bovine serum albumin, 66.2; ovalbumin, 45; carbonic anhydrase, 31; soybean trypsin inhibitor, 21.5; lysozyme, 14.4.

buffer had no apparent effect on the gel migration characteristics of the crude OMPs (Fig. 1). Proteins of apparent molecular sizes of 91 and 32 kilodaltons (kDa) were obtained by using solubilization temperatures in excess of 35°C, while two proteins of apparent molecular weights of 38,000 and 30,000 were observed when the sample was solubilized at a temperature greater than 60°C. Certain protein bands apparent at lower solubilization temperatures were not seen in samples solubilized at higher temperatures (indicated by asterisks in Fig. 1). The uppermost asterisk indicates a high-M_r zone of the protein profile which showed a protein band within an area of gel distortion. Because this band and the distortion disappeared at a solubilization temperature greater than 80°C, this area could represent one or more proteins or oligomers which are unable to migrate further into the matrix at the acrylamide concentration found in that zone of the gradient gel.

(ii) Surface-radioiodinated cells. When surface-radioiodinated cells were examined, the heat-modifiable 38K protein was easily detected in virulent UT25, avirulent UT25D, and modulated UT25NA samples solubilized at 100°C (Fig. 2a, lanes B, D, and F). When the radioiodinated samples were solubilized at 56°C, little or no 38K protein was observed in the cell samples (Fig. 2a, lanes A, C, and E). Virulent UT25 showed a 91K protein in the sample solubilized at 100°C which was not detected in the sample solubilized at 56°C, although a protein with an M_r of about 86,000 was shown to be unique to the UT25 56°C sample. Avirulent UT25D and UT25NA cells did not possess the 91K protein and did not demonstrate an 86K protein when solubilized at 56°C. All three strains examined showed a 76K surface protein when solubilized at 100°C but appeared to lack this protein when solubilized at 56°C. A protein with an M_r of approximately 76,000 was observed in the crude OMP analysis (Fig. 1) of UT25 but did not appear to be heat modifiable. The heat-modifiable 32K and 30K proteins seen in Fig. 1 were not readily identified in surface-radioiodinated cells. The high-molecular-weight white bands appearing in lanes A, C, and E of Fig. 2a of the blot autoradiogram corresponded with the high-molecular-weight distorted zones of the Coomassie blue-stained gel in Fig. 1 and possibly represented aggregates or oligomers of proteins which were not transferred to the nitrocellulose during electrophoretic blotting.

(iii) Immunoblot analysis of surface-radioiodinated cells. Probing the nitrocellulose blot of ^{125}I-labeled whole-cell proteins used to prepare the autoradiogram with cell-surface-reactive MAbs 1B6 and P8E7 revealed that the protein antigens were heat modifiable in distinct ways (Fig. 2b). MAb 1B6 reacted with an 18K antigen of UT25, UT25D, and UT25NA when the samples were solubilized at 100°C for 5 min; when these samples were solubilized at 56°C, the only reactivity observed was a diffuse area in the high-molecular-weight (ca. 95,000) region. When MAb P8E7 was reacted against this nitrocellulose blot, the reactive antigen was present only in virulent UT25. This antigen had an apparent molecular size of 91 kDa when the sample was solubilized at 100°C and a molecular size of 86 kDa when the sample was solubilized at 56°C. By aligning the autoradiogram over the probed immunoblot, it was apparent that these bands were identical to the 91K and 86K surface proteins observed in the autoradiogram of this nitrocellulose blot (Fig. 2a).

Two-dimensional SDS-PAGE and immunoblot analyses. (i) Virulent UT25. A two-dimensional SDS-PAGE system was used to characterize further the radioiodinated cell surface proteins (12, 27). In this system, if the solubilization temperature before the second-dimensional PAGE is identical to the solubilization temperature before the first-dimensional PAGE, the proteins will all migrate so as to form a diagonal pattern in the second dimensional gel, and this serves as a control. Varying the solubilization temperature before the second-dimensional PAGE will affect the heat-modifiable proteins only, causing them to migrate in areas other than the diagonal pattern, with proteins which decrease in apparent molecular weight with increasing temperatures lying to one side of the diagonal pattern and those which increase in apparent size with increasing temperatures lying to the other side. Boiling radioiodinated UT25 cells in the presence of 2-ME before SDS-PAGE in the first dimension resulted in a diagonal line of proteins after resolubilization at 100°C and electrophoresis in the second dimension (Fig. 3a). When the labeled-cell preparation was solubilized at 25°C before the first dimension of SDS-PAGE, several proteins failed to migrate on the diagonal line (Fig. 3b) after solubilization at 100°C and electrophoresis in the second dimension. Two proteins migrated with apparent molecular weight values of 38,000 and 18,000 when first solubilized at 100°C, while these same proteins migrated as high-M_r species when first solubilized at 25°C. The 18K protein was only faintly visible on the autoradiogram shown in Fig. 3 but became evident after longer exposure (see Fig. 5 for immunoblot). Three proteins migrated with apparent molecular weights of 91,000, 32,000, and 30,000 (and possibly a fourth at about 31,000) when solubilized by boiling before first-dimensional SDS-PAGE but migrated as lower-M_r moieties when solubilized at 25°C before first-dimensional electrophoresis.

Similar experiments with UT25 Triton-insoluble crude OMPs showed the presence of the heat-modifiable 91K,

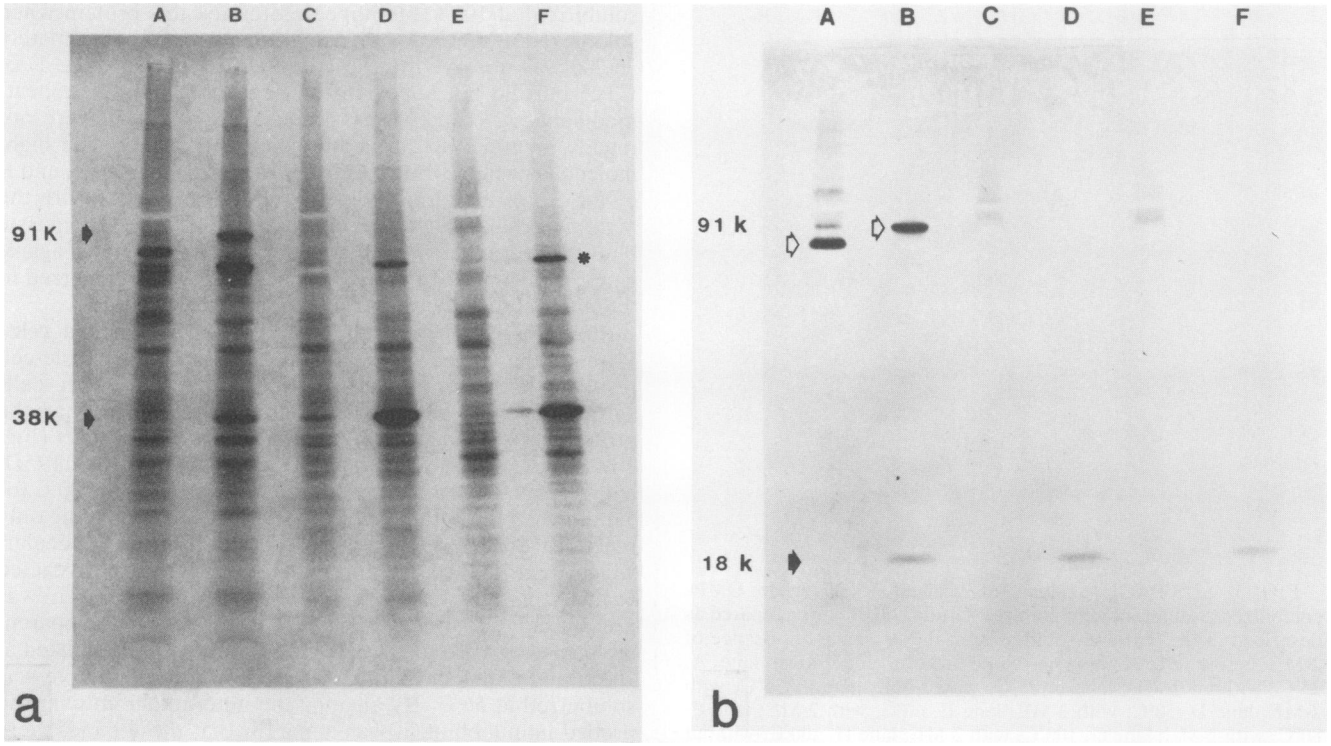

FIG. 2. Western blot analysis of surface-radioiodinated proteins of UT25. Whole cells were radioiodinated and subjected to electrophoresis after solubilization at either 56 or 100°C in the presence of 2-ME. Lane A, UT25 solubilized at 56°C; lane B, UT25 solubilized at 100°C; lane C, UT25D solubilized at 56°C; lane D, UT25D solubilized at 100°C; lane E, UT25NA solubilized at 56°C; lane F, UT25NA solubilized at 100°C. (a) Autoradiogram made from the nitrocellulose blot of the samples. The arrow at 91K indicates a virulence-associated protein seen in UT25 solubilized at 100°C. The asterisk indicates a protein with an apparent M_r of 76,000, and the arrow at 38K indicates the major protein observed in all samples solubilized at 100°C. (b) Immunoblot of the same nitrocellulose sheet incubated with MAbs P8E7 and 1B6. Open arrows show the bands of MAb P8E7 reactivity; the solid arrow at 18K shows MAb 1B6 activity in the lanes in which the samples were solubilized at 100°C. The molecular sizes indicated were estimated by reference to the protein standards described in the legend to Fig. 1.

38K, 32K, 30K, and 18K proteins (data not shown). The heat-modifiable 31K protein was not observed in these experiments, a finding which could indicate that it is a surface protein which is soluble in Triton X-100.

(ii) Avirulent UT25D and UT25NA. The heat-modifiable 91K, 32K, 31K, and 30K proteins were not demonstrable in radioiodinated avirulent UT25D when examined by two-dimensional SDS-PAGE (Fig. 4b). These missing proteins are those which migrated in gels as lower-M_r species if solubilized at 25°C before electrophoresis in the first dimension. The 38K and 18K surface proteins were present in UT25D and migrated in the two-dimensional gels just as did those proteins of the virulent strain. Two-dimensional SDS-PAGE analysis of UT25NA OMPs showed protein patterns similar to those of UT25D, including the absence of the 91K, 32K, 31K, and 30K proteins (data not shown). The virulence-associated 30K doublet described in this paper refers to the 32K and 30K proteins seen in the Triton-insoluble preparation as well as in ^{125}I-labeled cells.

(iii) Monoclonal antibody analyses of two-dimensional Western blots. The nitrocellulose sheets used to prepare the autoradiograms in Fig. 3 and 4 were subjected to immunoblotting analyses sequentially with MAbs 1B6 and P8E7. The immunoblots of UT25 and UT25D cells solubilized at 100°C before first-dimensional SDS-PAGE showed immunological reactivity of MAb 1B6 in areas of the blots that corresponded exactly with the 18K regions of the diagonal protein patterns of the autoradiograms in Fig. 3a and 4a. The two-dimensional immunoblot of these same samples solubilized at 25°C before first-dimensional PAGE demonstrated MAb 1B6 reactivity in an area to the left of the diagonal pattern, indicating that at 25°C, this protein behaved as a high-molecular-weight aggregate. Probing the same nitrocellulose sheets with MAb P8E7 demonstrated a lack of reactivity with UT25D, whereas the UT25 91K antigen was observed to migrate on the diagonal when solubilized at 100°C before first-dimensional SDS-PAGE. Solubilization at 25°C before first-dimensional electrophoresis resulted in the migration of the antigen in the second dimensional gel slightly to the right of the protein diagonal, indicating a form with a lower apparent molecular weight existing at 25°C.

UT25 total envelope proteins analyzed by two-dimensional SDS-PAGE and immunoblotting with MAbs 1B6 and P8E7 demonstrated patterns of immunological reactivity similar to those of radioiodinated whole cells (Fig. 5). MAbs 1B6 and P8E7 reacted with their corresponding 18K and 91K antigens located on a diagonal line when the proteins were first solubilized at 100°C (Fig. 5A). When the two antigens were first solubilized at 25°C before first-dimensional PAGE, their migration in the second-dimensional gel departed from the diagonal protein pattern as expected (Fig. 5B). The additional minor areas of reactivity on the diagonal pattern of Fig. 5B are probably the result of smaller breakdown products of the 91K protein and disaggregation of the 18K complex due to preparation of the envelopes or storage at −20°C. The properties of the heat-modifiable proteins of *B. pertussis* are summarized in Table 2.

Peptidoglycan-associated proteins. Because some heat-

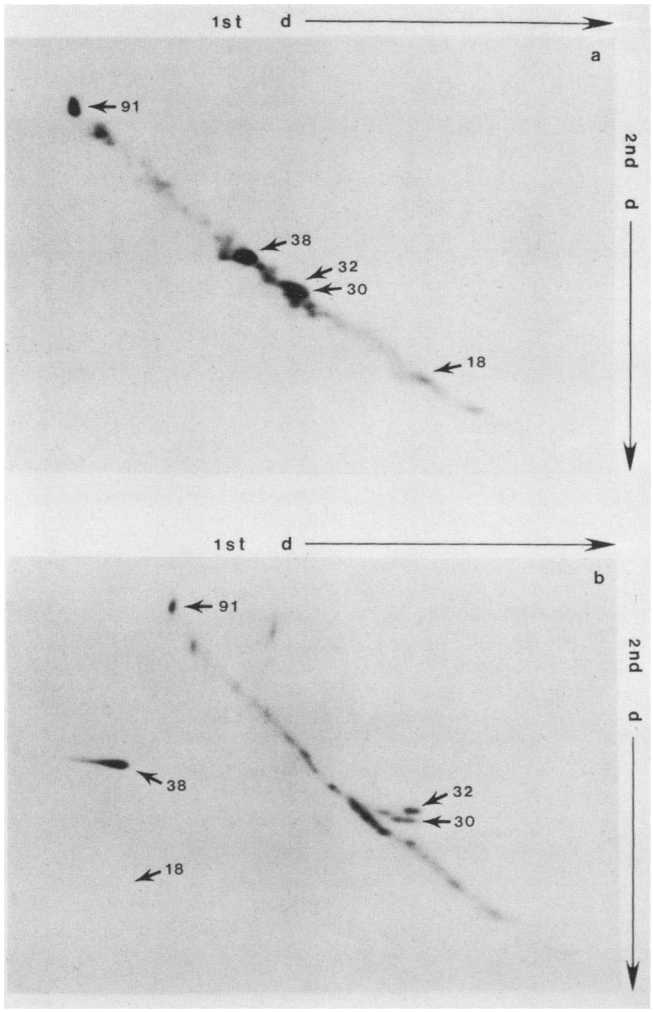

FIG. 3. Autoradiogram of surface-radioiodinated proteins of virulent *B. pertussis* analyzed by two-dimensional SDS-PAGE. For electrophoresis in the first dimension, radioiodinated samples of UT25 were solubilized at either 25 or 100°C in sample buffer containing 2-ME. Gel lanes excised from the first-dimensional gel were treated in sample buffer at 100°C for 5 min before second-dimensional PAGE. Numbers refer to the molecular sizes (in kilodaltons) of the proteins. When solubilized at 100°C before being run in the first dimension, all proteins migrated in a diagonal pattern (a). The sample solubilized at 25°C before first-dimensional PAGE is also shown (b). The 38K and 18K proteins migrated as large aggregates at 25°C, while the 91K, 32K, and 30K proteins behaved as smaller-sized molecules. As explained in the text, the 18K protein was not well labeled but was apparent in two-dimensional autoradiograms exposed for longer periods of time. Crude OMPs similarly analyzed also showed the 18K protein to migrate to the left of the diagonal pattern.

modifiable OMPs of gram-negative organisms may also be noncovalently associated with the cell murein layer, the envelope proteins of UT25 were examined for this characteristic. When cell envelopes were incubated at different temperatures in a buffer containing 2% SDS, major bands of 38,000 and 25,000 as well as other minor bands were found to remain with the cell peptidoglycan at temperatures up to 80°C (Fig. 6, lanes C through F). Incubation of the peptidoglycan sample at 100°C caused the release of the murein-associated proteins, leaving a pellet of crude peptidoglycan after ultracentrifugation (lane G). The 38K peptidoglycan-associated protein corresponded with the 38K major envelope protein seen in virulent and avirulent UT25 cells. A second technique was used to identify peptidoglycan-associated proteins. Treatment of the pellet resulting from the incubation of envelopes with 2% SDS at 37°C with the same SDS buffer containing 0.1 M NaCl resulted in the release of the 38K protein and several minor proteins (Fig. 6, lane H). The 25K protein, a small amount of the 38K protein, and two lower-M_r minor proteins remained noncovalently associated with the murein (Fig. 6, lane I).

Immunoblot analyses of cell envelopes treated at 50°C in SDS and envelopes treated at 37°C with SDS and then SDS-NaCl showed that the 18K protein reactive with MAb 1B6 was associated with the peptidoglycan at 50°C (Fig. 7, lane A) and was released from the peptidoglycan with 0.1 M NaCl (lane B). The 91K antigen recognized by MAb P8E7 was present in the soluble proteins released from envelopes treated with SDS-NaCl but was not observed in the crude peptidoglycan fraction of the envelope sample treated in SDS at 50°C. It is likely that this protein is associated with peptidoglycan at temperatures less than 50°C. The relative

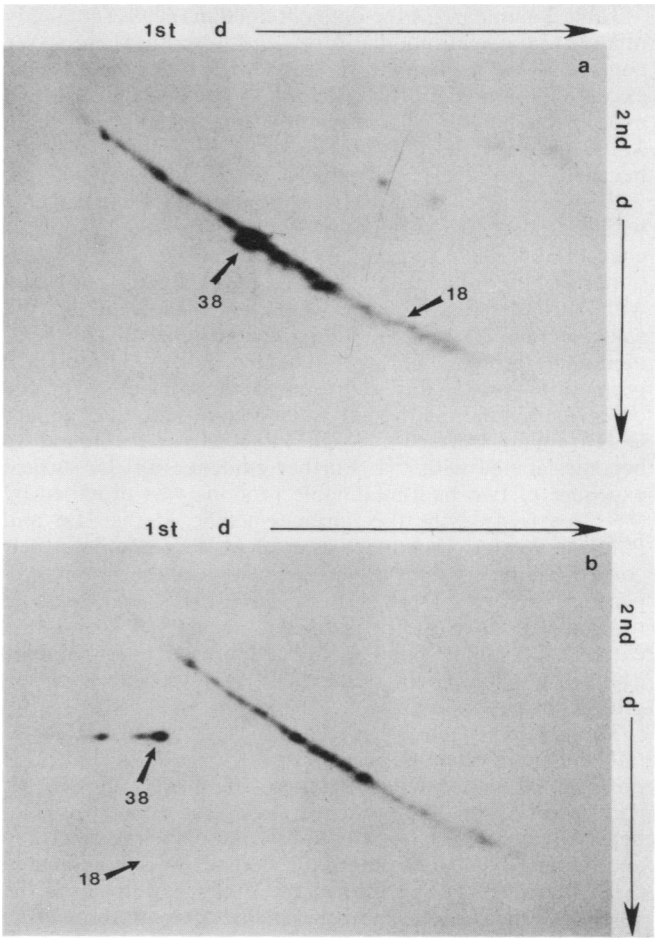

FIG. 4. Autoradiogram of surface-radioiodinated proteins of avirulent UT25D analyzed by two-dimensional SDS-PAGE. The conditions for electrophoresis were identical to those described in the legend to Fig. 3. Samples were solubilized at 100°C (a) or 25°C (b) before running in the first dimension. Arrows show the locations of the 38K and 18K proteins in each experiment. The location of the 18K protein was revealed by using longer exposure times.

abundance of the 18K and 91K proteins in these preparations was minor compared with that of the 38K and 25K proteins. The characteristics of the heat-modifiable proteins, including their association with peptidoglycan, are summarized in Table 2.

DISCUSSION

Heat-modifiable proteins have been described in a variety of gram-negative bacteria including *E. coli*, *Salmonella typhimurium*, *Neisseria* species, and *Pseudomonas aeruginosa* (11, 18, 27). It has been proposed that the β-sheet structure of these proteins determines the extent of SDS binding before examination by SDS-PAGE (2, 18). Proteins which increase in apparent molecular weight upon heating have been determined to have a high content of β-sheet structure. Proteins of the other class, those which decrease in apparent molecular weight upon heating, are often porin proteins (2). Although some porins also possess significant β-sheet structure, they are usually trimeric and noncovalently associated with the underlying peptidoglycan. Heating the higher-molecular-weight trimers in SDS causes them to dissociate and migrate in electrophoretic gels as lower-molecular-weight monomers.

Table 2 summarizes the data obtained in the present study relative to the heat-modifiable proteins described herein. We consider these proteins to be true OMPs (with the possible exception of the 31K protein), not only because they are heat modifiable, but also because they are insoluble in Triton X-100 and are vectorially labeled with ^{125}I in intact cells, and because some are peptidoglycan associated and reactive with cell surface-specific MAbs. In our initial experiments with a crude OMP preparation, we demonstrated the presence of several heat-modifiable proteins by using conventional and two-dimensional SDS-PAGE. Results obtained with surface-radioiodinated whole cells analyzed by the same techniques corroborated the existence of the heat-modifiable proteins and also indicated that the crude OMP preparation reasonably represented the molecules of the outer membrane. Although it is not certain that all are OMPs, many surface-exposed molecules were found to become labeled with ^{125}I. Further evidence for the surface exposure of two heat-modifiable proteins was provided by their reactivity with the surface-specific MAbs 1B6 and P8E7. In Western immunoblots, both MAbs recognized their corresponding epitopes regardless of the solubilization temperature before SDS-PAGE. Crude OMPs and surface-radiolabeled envelope proteins did not appear to possess extensive disulfide bonding, as the presence of reducing agent in the solubilizing buffer had no detectable effect on protein migration in gels.

Several investigators have used the method of ^{125}I vectorial labeling of intact *B. pertussis* cells to study the envelope proteins associated with virulence (10, 21, 23). Gow et al. (10) observed the 30K virulence-associated doublet in unlabeled envelopes but reported a low radiolabeling efficiency and, hence, could not detect the doublet in radioiodinated cells. Peppler (21) and Redhead (23) successfully used the whole-cell radioiodination technique to compare the surface exposed envelope proteins of avirulent and virulent *B. pertussis*. One study (21) showed a doublet (25,500 and 30,500) from domed hemolytic colony types which was absent in colonies which were neither domed nor hemolytic. A 200K protein and an 80K protein seemed to be virulence associated, but this association was not observed in all of the strains examined (21). In another study, virulent, avirulent, and magnesium ion-modulated cells were surface radioiodinated and examined (23). It was demonstrated that only certain strains could be modulated to cause a decrease or disappearance of the 28K and 30K proteins. The absence of the virulence-associated doublet in labeled avirulent-phase strains was not discussed in the report, and the autoradiograms were difficult to interpret. In all three of the studies involving surface radioiodination of cells, a major radiola-

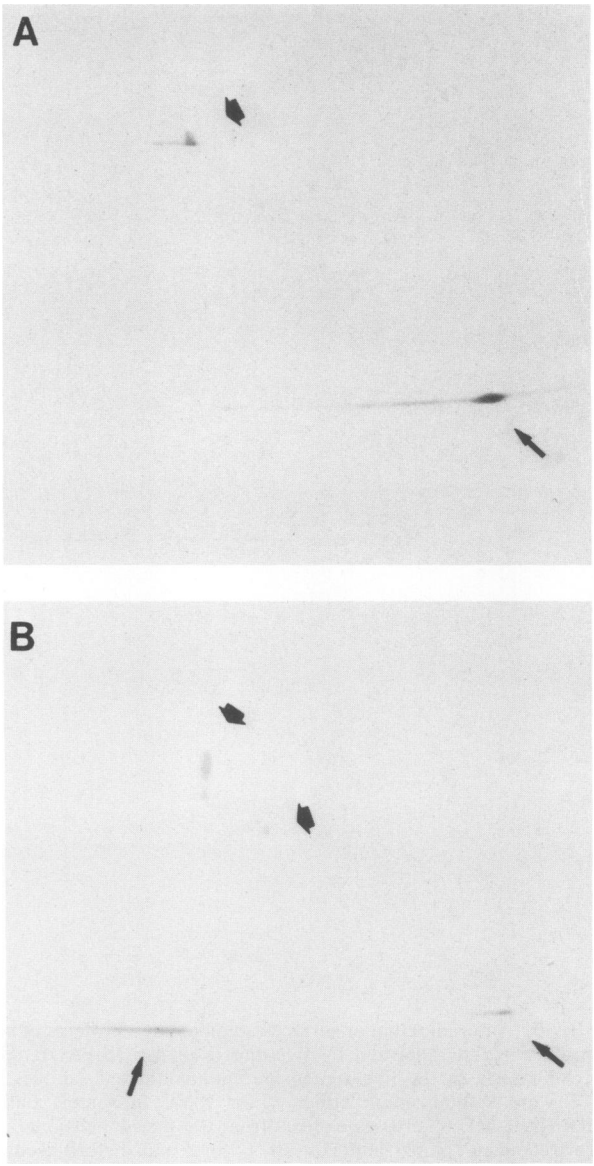

FIG. 5. Immunoblots of virulent UT25 total envelope proteins analyzed by two-dimensional SDS-PAGE. The nitrocellulose sheets containing blotted two-dimensional gel proteins were probed first with MAb P8E7 and then MAb 1B6. (A) Reactivity of MAb P8E7 (short arrow) and MAb 1B6 reactivity (long arrow), with both antigens falling on the diagonal protein pattern which results when the sample is solubilized at 100°C before running in the first dimension. (B) The sample was solubilized at 25°C before electrophoresis in the first dimension; MAb P8E7 (short arrows) reacted with the 91K antigen migrating slightly to the right of the diagonal pattern, while MAb 1B6 (long arrows) reacted with the 18K protein, which behaved as a large aggregate, with some 18K protein reactivity on the diagonal region.

TABLE 2. Summary of the properties of heat-modifiable *B. pertussis* envelope proteins

Envelope protein size (kDa)	Property[a]						
	Availability for ^{125}I surface labeling	Presence in Triton-insoluble envelope fraction	Heat modifiability[b]		Noncovalent association with peptidoglycan	Association only with virulent cells	Surface-specific MAb reactivity
			High to low M_r	Low to high M_r			
91	+	+	NA	+	+[c]	+	+[d]
38	+	+	+	NA	+	−	ND
32	+	+	NA	+	NK	+	ND
31	+	−	NA	+	NK	+	ND
30	+	+	NA	+	NK	+	ND
18	+	+	+	NA	+	−	+[e]

[a] +, Positive for indicated property; −, negative for indicated property; NA, not applicable; ND, not determined; NK, not known.
[b] Change shown is that observed upon heating.
[c] Associated with peptidoglycan under mild ionic conditions and temperatures of less than 50°C.
[d] Reactive with MAb P8E7.
[e] Reactive with MAb 1B6.

beled 35K to 40K protein was observed, as well as minor lower-molecular-weight (17K to 20K) proteins. These proteins were observed in all phenotypes studied, and it is likely that they are the same as the 38K and 18K heat-modifiable surface proteins that we have demonstrated in this report. The 18K protein was radioiodinated poorly, suggesting that it may have few tyrosine residues. This protein reacted with a cell surface-specific MAb, strongly suggesting cell surface-exposed domains.

Parton and Wardlaw studied the cell envelopes of virulent phase, avirulent phase, and magnesium ion-modulated cells by using tube gel SDS-PAGE (20). They reported the presence of two virulence-associated (30K and 33.5K) proteins which were not seen in avirulent or modulated cells. When the solubilization temperature was 37°C, it was observed that the 30K and 33.5K doublet as well as a 46K band failed to migrate to their expected R_f values. By eluting the proteins from two lower-molecular-weight (ca. 20,000) bands seen in the sample solubilized at 37°C and solubilizing them at 100°C before electrophoresis, it was demonstrated that the 30K doublet migrated as a 20K doublet at 37°C. The form of the 46K protein solubilized at 37°C was not detected. Although other investigators have examined the envelope proteins of virulent, avirulent, and modulated strains (5, 21, 23, 26, 29) and have observed the virulence-associated doublet, the study of Parton and Wardlaw (20) is the only

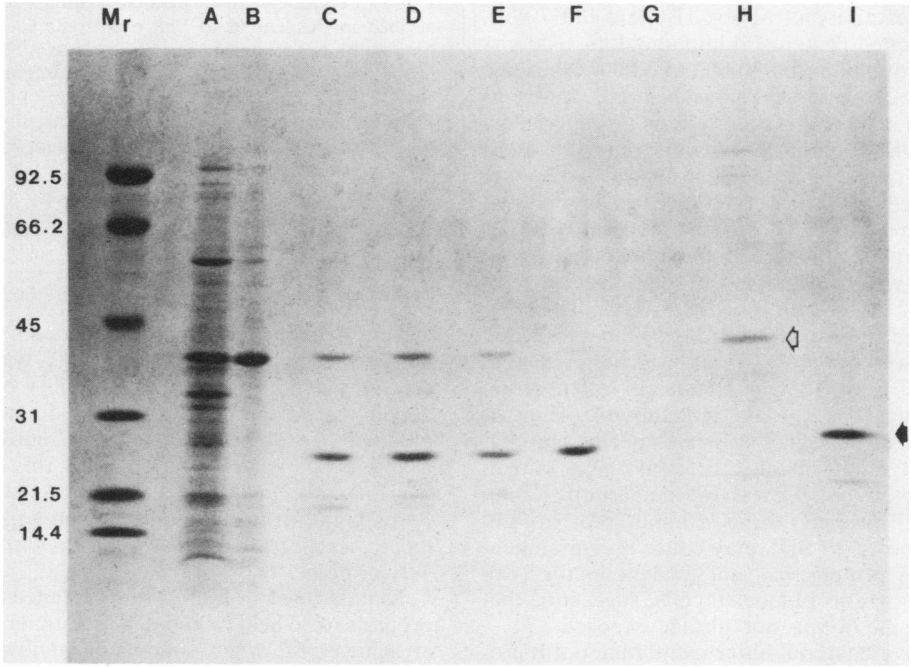

FIG. 6. SDS-PAGE of peptidoglycan-associated proteins of UT25. Lane A, virulent UT25 envelopes without SDS treatment; lane B, avirulent UT25D envelopes without SDS treatment. Cell envelopes of virulent UT25 were treated with SDS at the following temperatures, and the insoluble material was analyzed by SDS-PAGE: lane C, 37°C; lane D, 50°C; lane E, 60°C; lane F, 80°C; lane G, 100°C. Cell envelopes treated with SDS at 37°C were then treated with SDS-NaCl as described in the text. This material was centrifuged, and the supernatant (lane H) and pellet material (lane I) were analyzed. The open arrow shows the 38K protein, and the closed arrow indicates an approximately 25K protein. Lane M_r shows the molecular size standards described in the legend to Fig. 1.

one in which the solubilization temperature of the samples was altered to show heat-modifiable proteins. Because these molecules exist in their natural membrane-associated state not at 100°C but at 35 to 37°C, boiling antigen preparations for SDS-PAGE and immunoblotting may destroy or otherwise alter important epitopes. The split-gel technique used in the Parton and Wardlaw study (20) allowed comparison between two samples in one gel, but accurate estimations of molecular weights and comparisons with other samples were not possible. By using conventional and two-dimensional SDS-PAGE, we were able to confirm the presence of a surface-exposed, virulence-associated, heat-modifiable doublet at an M_r of approximately 30,000 and showed that these proteins behave as lower-M_r moieties at a decreased solubilization temperature.

Virulence-associated envelope proteins other than the 30K doublet have been found in *B. pertussis*. Their molecular weights, when they were solubilized at 100°C, were reported to be 98,000 and 88,000 (5); 90,000, 86,000, 81,000, and 33,000 (26); 200,000, and 80,000 (21); or in the 60,000 to 90,000 range (29). Our demonstration of a virulence-associated 91K heat-modifiable surface protein correlates well with these previous findings. MAb P8E7, in addition to reacting specifically with this protein in an enzyme-linked immunosorbent assay and an immunofluorescence assay, recognized the 91K antigen in immunoblots, regardless of solubilization temperature. This antibody will be useful as a probe to study phase variation and modulation of *B. pertussis*.

Further examination of the cell envelope of virulent *B. pertussis* revealed several proteins which were noncovalently associated with the underlying peptidoglycan layer. Three of the major proteins (the 91K, 38K, and 18K proteins) were identical to three of the heat-modifiable molecules described in this report. The 18K and 91K proteins were identified by reactivity of MAbs 1B6 and P8E7 with peptidoglycan-associated proteins in immunoblots. The virulence-associated proteins in the 30,000 to 32,000 M_r range did not appear to be peptidoglycan-associated. MAbs to these proteins would be useful in determining this characteristic. The 25K protein shown in Fig. 6 appeared to be more tightly associated with the peptidoglycan than was the 38K protein as it remained with the peptidoglycan after incubation above 80°C or in 0.1 M NaCl. In other experiments (not shown), this 25K protein was heat modifiable and appeared as a high-molecular-weight aggregate if solubilized at temperatures less than 100°C before SDS-PAGE. The 25K protein was observed only in the peptidoglycan-associated protein preparations of virulent UT25 and was not evident in similar preparations from UT25D or BP338, a virulent strain derived from Tohama I (32) (data not shown). Thus, it appears that the 25K protein may be strain specific and that it was lost during selection for the UT25D avirulent phenotype. The 25K protein may exist in the Triton X-100-insoluble crude OMP preparation as a minor protein, and treatment of envelopes with SDS may cause an enrichment of this protein. This protein was not evident in the two-dimensional autoradiograms of labeled cells, suggesting that it either lacked tyrosine or was not surface exposed.

Many gram-negative bacterial outer membrane porin proteins are associated with peptidoglycan in a manner similar to that observed in UT25 (2, 4, 16). This characteristic prompted us to purify the 38K protein in its high-molecular-weight oligomeric form and show that it functions as an anion-selective porin in lipid bilayers (1). The molecular size of the purified protein monomer was calculated to be 40 kDa by linear regression analysis with a 12% acrylamide gel system, whereas its apparent molecular weight in the gradient gels used in the present study was 38,000. This discrepancy demonstrates the variability of protein migration, depending on the gel system implemented. The 18K heat-modifiable protein consistently copurified with the porin despite repeated gel filtration steps; this led us to believe that the 18K protein is closely associated with the 38K porin. Perhaps the heat modifiability seen with the 18K protein is merely a reflection of its association with the heat-modifiable 38K protein.

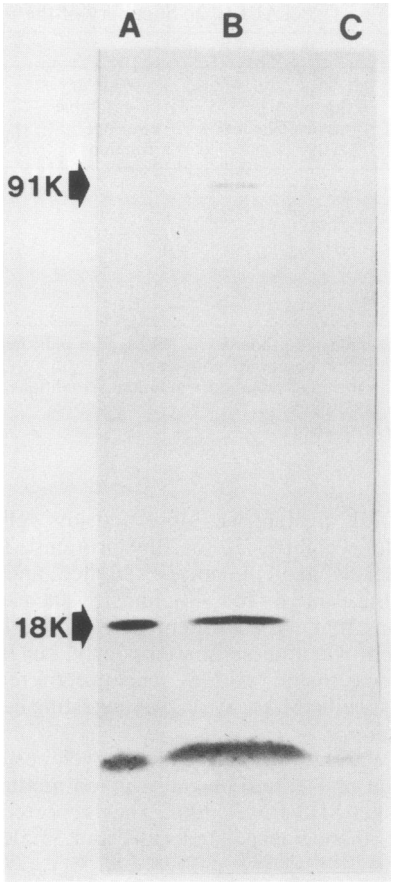

FIG. 7. Immunoblot of peptidoglycan-associated proteins of UT25. Peptidoglycan-associated proteins were obtained as described in the legend to Fig. 6 and subjected to SDS-PAGE and immunoblotting with MAbs P8E7 and 1B6. Shown are immunoblots of the pellet material resulting from treatment of cell envelopes with SDS at 50°C (lane a), and the supernatant material (lane b) and the pellet material (lane c) resulting from treatment of cell envelopes with SDS at 37°C and subsequent treatment with SDS-NaCl. Arrows show areas of reactivity for MAb P8E7 (91K) and MAb 1B6 (18K). Reactive bands at the bottom of the blot resulted from probing the nitrocellulose blot with a monoclonal antibody specific for *Bordetella* lipopolysaccharide (8).

That all three or four of the virulence-associated envelope proteins were heat modifiable in a similar way (they migrated at a lower M_r when solubilized at lower temperatures) is interesting. Because this modifiability is probably a result of a unique molecular configuration causing alterations in the binding of SDS, this molecular characteristic may impart a virulence-associated trait to the bacterial cell. An example of this would be the cell hydrophobicity seen in virulent *B. pertussis*, which is markedly decreased in magnesium ion-

and nicotinic acid-modulated cells (25). Differences in the lipopolysaccharide of strains varying in virulence characteristics have been demonstrated (22); these differences may relate to cell surface hydrophobicity or the presence of the virulence-associated proteins or both. The contribution of individual virulence-associated proteins to the pathogenesis of *B. pertussis* will be difficult to study, as the expression of the three or four proteins we and others have studied appears to be coordinately regulated with the other virulence factors such as pertussis toxin, hemolysin, dermonecrotic toxin, and filamentous hemagglutinin (31).

ACKNOWLEDGMENTS

This research was supported by Department of Health and Human Services grant AI 17682 to C.D.P. from the National Institutes of Health.

LITERATURE CITED

1. **Armstrong, S. K., T. R. Parr, Jr., C. D. Parker, and R. E. W. Hancock.** 1986. *Bordetella pertussis* major outer membrane porin protein forms small, anion-selective channels in lipid bilayer membranes. J. Bacteriol. **166**:212–216.
2. **Benz, R.** 1985. Porin from bacterial and mitochondrial outer membranes. CRC Crit. Rev. Biochem. **19**:145–190.
3. **Darveau, R. P., S. MacIntyre, J. T. Buckley, and R. E. W. Hancock.** 1983. Purification and reconstitution in lipid bilayer membranes of an outer membrane, pore-forming protein of *Aeromonas salmonicida*. J. Bacteriol. **156**:1006–1011.
4. **DiRienzo, J. M., K. Nakamura, and M. Inouye.** 1978. The outer membrane proteins of gram-negative bacteria: biosynthesis, assembly, and functions. Annu. Rev. Biochem. **47**:481–532.
5. **Ezzell, J. W., W. J. Dobrogosz, W. E. Kloos, and C. R. Manclark.** 1981. Phase-shift markers in *Bordetella*: alterations in envelope proteins. J. Infect. Dis. **143**:562–569.
6. **Fairbanks, G., T. L. Steck, and D. F. H. Wallack.** 1971. Electrophoretic analysis on the major polypeptide of the human erythrocyte membrane. Biochemistry **10**:2606–2617.
7. **Field, L. H., and C. D. Parker.** 1979. Differences observed between fresh isolates of *Bordetella pertussis* and their laboratory passaged derivatives, p. 124–132. *In* C. R. Manclark and J. C. Hill (ed.), International Symposium on Pertussis. U.S. Department of Health, Education and Welfare, Washington, D.C.
8. **Frank, D. W., and C. D. Parker.** 1984. Isolation and characterization of monoclonal antibodies to *Bordetella pertussis*. J. Biol. Stand. **12**:353–365.
9. **Frank, D. W., and C. D. Parker.** 1984. Interaction of monoclonal antibodies with pertussis toxin and its subunits. Infect. Immun. **46**:195–201.
10. **Gow, J. A., R. Parton, and A. C. Wardlaw.** 1976. Radiolabelling of *Bordetella pertussis* envelope proteins by the ^{125}I-lactoperoxidase method. Microbios **15**:209–219.
11. **Hancock, R. E. W., and A. M. Carey.** 1979. Outer membrane of *Pseudomonas aeruginosa*: heat- and 2-mercaptoethanol-modifiable proteins. J. Bacteriol. **140**:902–910.
12. **Hindahl, M. S., and B. H. Iglewski.** 1984. Isolation and characterization of the *Legionella pneumophila* outer membrane. J. Bacteriol. **159**:107–113.
13. **Kohler, G., and C. Milstein.** 1975. Continuous culture of fused cells secreting antibody of predefined specificity. Nature (London) **256**:495–497.
14. **Laemmli, U. K.** 1970. Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
15. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
16. **Lugtenberg, B., H. Bronstein, N. Van Selm, and R. Peters.** 1977. Peptidoglycan-associated outer membrane proteins in gram-negative bacteria. Biochim. Biophys. Acta **465**:571–578.
17. **Markwell, M. A. K.** 1982. A new solid-state reagent to iodinate proteins. I. Conditions for the efficient labeling of antiserum. Anal. Biochem. **125**:427–432.
18. **Nakamura, K., and S. Mizushima.** 1976. Effects of heating in dodecyl sulfate solution on the conformation and electrophoretic mobility of isolated major outer membrane proteins from *Escherichia coli* K-12. J. Biochem. **80**:1411–1422.
19. **Parker, C. D., S. K. Armstrong, and D. W. Frank.** 1985. Surface antigens of *Bordetella pertussis*, p. 117–127. *In* M. Z. Atassi, and H. L. Bachrach (ed.), Immunobiology of proteins and peptides-III: viral and bacterial antigens. Plenum Publishing Corp., New York.
20. **Parton, R., and A. C. Wardlaw.** 1975. Cell-envelope proteins of *Bordetella pertussis*. J. Med. Microbiol. **8**:47–57.
21. **Peppler, M. S.** 1982. Isolation and characterization of isogenic pairs of domed hemolytic and flat nonhemolytic colony types of *Bordetella pertussis*. Infect. Immun. **35**:840–851.
22. **Peppler, M. S.** 1984. Two physically and serologically distinct lipopolysaccharide profiles in strains of *Bordetella pertussis* and their phenotype variants. Infect. Immun. **43**:224–232.
23. **Redhead, K.** 1983. Variability in the surface exposure of the outer membrane proteins of *Bordetella pertussis*. FEMS Microbiol. Lett. **17**:35–39.
24. **Richardson, K., and C. D. Parker.** 1985. Identification and characterization of *Vibrio cholerae* surface proteins by radioiodination. Infect. Immun. **48**:87–93.
25. **Robinson, A., A. R. Gorringe, L. I. Irons, and C. W. Keevil.** 1983. Antigenic modulation of *Bordetella pertussis* in continuous culture. FEMS Microbiol. Lett. **19**:105–109.
26. **Robinson, A., and D. C. Hawkins.** 1983. Structure and biological properties of solubilized envelope proteins of *Bordetella pertussis*. Infect. Immun. **39**:590–598.
27. **Russell, R. R. B.** 1976. Two-dimensional SDS-polyacrylamide gel electrophoresis of heat-modifiable outer-membrane proteins. Can. J. Microbiol. **22**:83–91.
28. **Schnaitman, C. A.** 1974. Outer membrane proteins of *Escherichia coli*. III. Evidence that the major protein of *Escherichia coli* O111 outer membrane consists of four distinct polypeptide species. J. Bacteriol. **118**:442–453.
29. **Schneider, D. R., and C. D. Parker.** 1982. Effect of pyridines on phenotypic properties of *Bordetella pertussis*. Infect. Immun. **38**:548–553.
30. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
31. **Wardlaw, A. C., R. Parton, and M. J. Hooker.** 1976. Loss of protective antigen, histamine-sensitizing factor and envelope polypeptides in cultural variants of *Bordetella pertussis*. J. Med. Microbiol. **9**:89–100.
32. **Weiss, A. A., E. L. Hewlett, G. A. Myers, and S. Falkow.** 1983. Tn*5*-induced mutations affecting virulence factors of *Bordetella pertussis*. Infect. Immun. **42**:33–41.