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# Characterization of the Outer Membrane Proteins of *Bordetella avium*

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**The outer membrane proteins of *Bordetella avium* were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sarkosyl-insoluble outer membrane protein-enriched profiles from 50 virulent *B. avium* isolates, containing major 21,000- and 37,000-molecular-weight proteins (21K and 37K proteins, respectively) and at least 13 less intensely stained proteins with molecular weights ranging from 13,500 to 143,000, were very similar. The 21K, 27K, 31K, and 37K outer membrane proteins were shown to be associated noncovalently with the underlying peptidoglycan layer. It was necessary to treat cell envelopes with 2% sodium dodecyl sulfate and at temperatures in excess of 60°C for 15 min to release these proteins. Exposure of proteins on the cell surface of *B. avium* was assessed by labeling with $^{125}I$ followed by electrophoresis. As many as 13 bands were present in profiles from labeled whole cells. Of the surface-labeled bands, eight corresponded to bands in a radiolabeled outer membrane preparation. The outer membrane protein profile of *B. avium* was compared with profiles from other *Bordetella* spp., including 20 *B. avium*-like and 16 *B. bronchiseptica* strains isolated from turkeys. The outer membrane protein profile of *B. avium* was distinctly different from those of the other bordetellae. The effect of variations in the growth medium on the expression of outer membrane proteins of *B. avium* was examined. Expression of 22K, 26K, 56K, and 73K proteins was decreased or eliminated by addition of 50 mM $MgSO_4$ to the medium.**

*Bordetella avium* is the etiologic agent of turkey bordetellosis, a highly contagious upper respiratory disease of turkey poults, characterized by oculonasal discharge, sneezing, dyspnea, decreased weight gain, and tracheal collapse (5, 17, 42, 43). Uncomplicated bordetellosis in turkeys generally results in high morbidity and low mortality, but disease under field conditions is often more severe, with increased mortality due to increased stress (45) and infection by secondary pathogens (42). Like other members of the genus *Bordetella*, *B. avium* exhibits a tropism for ciliated epithelium of the upper respiratory tract (5, 6). Colonization of the trachea results in inflammation, with loss of epithelium and distortion of the mucosa and tracheal rings. Two other related bacteria are frequently isolated from the upper respiratory tracts of turkeys. Isolates identified as *Bordetella bronchiseptica* appear nonpathogenic in experimentally infected birds (25). The other group of organisms is phenotypically similar to *B. avium* but is avirulent and has been temporarily designated *B. avium*-like by Jackwood et al. (26).

Little is known about the bacterial surface structures of *B. avium* and their involvement in the pathogenesis of turkey bordetellosis. Surface appendages include peritrichous flagella and fimbriae (24, 27). *B. avium* possesses a poorly characterized hemagglutinin which, along with fimbriae, may play a role in adhesion to ciliated respiratory epithelial cells (8, 24). Outer membrane proteins (OMPs) from a limited number of *B. avium* isolates have been examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22). No apparent differences in OMP profiles from virulent *B. avium* strains were observed. Antibodies against at least eight OMPs were found in sera and tracheal washings from experimentally infected turkeys (7, 21). Reactivity to a 21,000-molecular-weight OMP (21K OMP) was especially intense in both sera and tracheal washings.

Initial work has indicated the potential importance of *B. avium* OMPs in the pathogenesis of turkey bordetellosis. In this study, OMP profiles from numerous *B. avium* isolates were obtained by detergent extraction of cell envelopes and compared with profiles from other *Bordetella* spp., including *B. bronchiseptica* and *B. avium*-like organisms isolated from turkeys. The OMPs of *B. avium* were evaluated for cell surface accessibility to radioiodination and noncovalent association with the underlying peptidoglycan layer. Finally, the effect of growth conditions on OMP expression by *B. avium* was determined by varying incubation time and growth medium.

## MATERIALS AND METHODS

**Bacterial strains.** Bacterial strains used for this study are listed in Table 1. In addition to these strains, OMPs from 41 *B. avium*, 18 *B. avium*-like, and 16 *B. bronchiseptica* isolates from turkeys with upper respiratory disease in Iowa, California, North Carolina, Ohio, Minnesota, and Wisconsin were examined. Stock cultures of all strains were maintained at −80°C during the course of this study.

**Media and growth conditions.** *B. avium* isolates were grown on brain heart infusion (Difco Laboratories, Detroit, Mich.) agar (BHIA) containing 1.5% agar (BiTek agar; Difco) for 24 h at 37°C and passaged to BHIA or brain heart infusion broth (BHIB). To evaluate the effect of incubation time on OMP expression, *B. avium* 75 was incubated at 37°C for 24, 36, or 48 h on BHIA or in BHIB prior to isolation of OMPs. To evaluate the effect of growth medium on expression of OMPs, strain 75 was grown on Bordet-Gengou agar (Difco) containing 15% defibrinated sheep blood and 1%

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TABLE 1. Bacterial strains used in this study

| Organism and strain | Virulence[a] | Origin (reference) |
|---|---|---|
| *B. avium* | | |
| 75 | + | L. H. Arp, Iowa (5) |
| ATCC 35086 (type strain) | + | American Type Culture Collection (27) |
| 838 | + | L. H. Arp, Iowa (10) |
| ATCC 31770 (vaccine strain) | +/− | American Scientific Laboratories, Wis. (13) |
| NCD | + | D. G. Simmons, N.C. (44) |
| NCD-1 (avirulent strain derived from NCD) | − | D. G. Simmons, N.C. (31) |
| W | + | D. G. Simmons, N.C. (44) |
| 197 | + | Y. M. Saif, Ohio (25) |
| F9000336 | NT | R. P. Chin, Calif. |
| *B. avium*-like | | |
| 023 | − | Y. M. Saif, Ohio (25) |
| 101 | − | Y. M. Saif, Ohio (25) |
| *B. pertussis* | | |
| BP536 (virulent) | NT | E. Tuomanen, N.Y. (38) |
| BP537 (avirulent-phase variant of BP536) | NT | E. Tuomanen, N.Y. (38) |
| *B. bronchiseptica* | | |
| T91 | NT | Host species: swine, Iowa |
| 103 | − | Host species: turkey; Y. M. Saif, Ohio (25) |
| *B. parapertussis* 504 (virulent) | NT | E. Tuomanen, N.Y. |

[a] Presence (+) or absence (−) of virulence for turkey poults; +/−, mildly virulent for turkeys (25); NT, virulence not tested for turkey poults.

glycerol (BGA) and on BGA supplemented with 5 mM nicotinic acid (Sigma Chemical Co., St. Louis, Mo.) or 50 mM $MgSO_4$ (Fisher Scientific, Fair Lawn, N.J.) at 37°C for 36 h and used for OMP isolation.

Strains used for comparison of OMP profiles among different *Bordetella* spp. were passaged twice on BGA. *B. pertussis* strains were grown for 72 h, *B. parapertussis* and *B. bronchiseptica* isolates were grown for 48 h, and *B. avium* and *B. avium*-like strains were grown for 36 h on BGA at 37°C.

Bacteria were harvested from plates in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4) buffer (Sigma) or from broth and collected by centrifugation at 10,000 × *g*. The bacteria were washed once with 10 mM HEPES buffer, and approximately 1.5 g (wet weight) of cells was suspended in 15 ml of buffer and stored at −80°C. All cells were used within 2 weeks for isolation of OMPs or peptidoglycan-associated proteins.

**OMP preparation.** The OMP-enriched fractions were prepared by a modification of the procedure of Hellwig and Arp (21). Briefly, the cells were thawed, and phenylmethylsulfonyl fluoride (PMSF; Sigma) was added to a final concentration of 0.1 mM. The cells were disrupted by a sonicator (model 350; Branson Sonic Power Co., Danbury, Conn.) for 10 1-min bursts (50% cycle, power setting of 7), while being cooled in an ice-water bath. Whole cells and large debris were removed by centrifugation at 5,000 × *g* for 20 min, and the total membrane fraction was harvested from the supernatant by centrifugation at 100,000 × *g* for 60 min at 4°C. The membrane fraction was suspended in 1% (wt/vol) Sarkosyl (sodium lauryl sarcosine; International Biotechnologies, Inc., New Haven, Conn.) in 10 mM HEPES (pH 7.4) buffer containing 0.1 mM PMSF for 30 min at room temperature. The detergent-insoluble material containing the OMP-enriched fraction was harvested by centrifugation at 100,000 × *g* for 60 min at 4°C. The final insoluble pellet was suspended in deionized water to a concentration of 1.0 to 8.0 mg of protein per ml and stored at −80°C. Protein concentrations were determined by the bicinchoninic acid assay (37) (Pierce Chemical Company, Rockford, Ill.) by using bovine serum albumin as a standard.

**Radioiodination.** Whole cells and OMPs from *B. avium* 75 were radiolabeled with $^{125}I$ essentially as described by Richardson and Parker (39). Bacteria to be radiolabeled were grown on BHIA or in BHIB for 36 h at 37°C. Whole cells for surface radioiodination were washed twice with phosphate-buffered saline (PBS; 0.0132 M phosphate, 0.15 M NaCl, pH 7.3) and resuspended to 0.1 g (wet weight) per ml of PBS. The OMPs were isolated from bacteria grown on BHIA or in BHIB as described above and diluted to 50 μg of protein per ml of PBS. Iodo-beads (four beads per whole-cell reaction and two beads per OMP reaction) were used as the catalyst for radioiodination. The Iodo-beads were rinsed following the procedure from the supplier (Pierce) and added to polypropylene tubes containing 1 mCi of $Na^{125}I$ (Amersham Corp., Arlington Heights, Ill.). After the beads were incubated at room temperature for 5 min, 1 ml of whole-cell or OMP suspension was added to each tube, and radioiodination was allowed to proceed for 5 min. The reaction was stopped by removal of the whole-cell and OMP suspension from the Iodo-beads. Unreacted $^{125}I$ was removed from whole cells by centrifugation (39) and from OMP preparations by using a desalting column (Econopac-Pac 10DG; Bio-Rad Laboratories, Richmond, Calif.).

**SDS-PAGE.** Discontinuous SDS-PAGE with 6 to 20% gradient gels was performed according to the procedure of Laemmli (29). Electrophoresis was carried out on a vertical slab apparatus (Bio-Rad) by following the procedure in the supplier's catalog. Proteins were solubilized in sample buffer containing 2% SDS, 5% 2-mercaptoethanol, 20% glycerol, 0.001% bromophenol blue, and 0.0625 M Tris (pH 6.8) at 100°C for 5 min, unless otherwise stated. The effect of the reducing agent was examined by comparing migration of proteins solubilized in the above sample buffer with those solubilized in an identical buffer without 2-mercaptoethanol. Each lane was loaded with either 5 to 20 μg of protein or ca. 40,000 cpm. After electrophoresis, gels were either stained with Coomassie brilliant blue R250 for visualization of protein bands or fixed with 40% methanol–10% acetic acid, dried onto Whatman 3MM paper, and exposed to X-ray film (X-OMAT AR; Eastman Kodak Co., Rochester, N.Y.). The following proteins were used as molecular size standards (Life Technologies, Inc., Gaithersburg, Md.): lysozyme (14,300), β-lactoglobulin (18,400), carbonic anhydrase (29,000), ovalbumin (43,000), bovine serum albumin (68,000), phosphorylase *b* (97,400), and myosin (H chain) (200,000). Apparent molecular weights were determined by comparison with the protein standards according to standard procedures (19).

Peptidoglycan-associated proteins were isolated as described by Armstrong and Parker (2). A total-cell membrane pellet obtained from sonicated whole cells was suspended in 15 mM Tris (pH 8.0) buffer containing 2% SDS, and equal fractions were heated at 37, 50, 60, 80, or 100°C for 15 min. The insoluble material consisting of crude peptidoglycan and associated complexes was pelleted by centrifugation at 100,000 × *g* for 1 h at 20°C. Each pellet was washed twice in

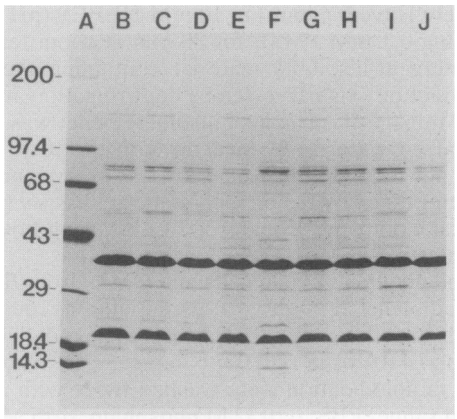


FIG. 1. SDS-PAGE analysis of OMPs from *B. avium* isolates grown on BHIA for 36 h at 37°C. Lanes: B, isolate 75; C, isolate 838; D, type strain ATCC 35086; E, vaccine strain ATCC 31770; F, isolate NCD; G, isolate NCD-1, an avirulent derivative of NCD; H, isolate W; I, isolate 197; J, isolate F9000336. Ten micrograms of protein was applied to each lane. Lane A, molecular size standards (in thousands), as described in the text.

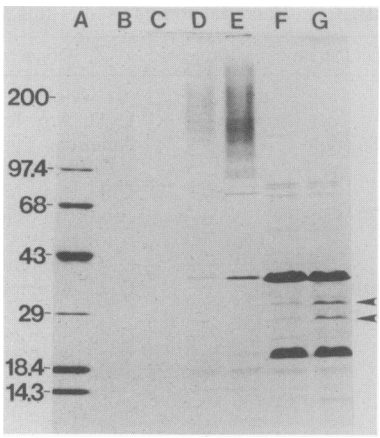


FIG. 2. SDS-PAGE of OMP-enriched fractions from *B. avium* 75 solubilized at different temperatures and for different times in sample buffer (as described in text) prior to electrophoresis. Solubilization conditions: lane B, room temperature for 5 min; lane C, 37°C for 5 min; lane D, 60°C for 5 min; lane E, 80°C for 5 min; lane F, 100°C for 5 min; lane G, 100°C for 30 min. Ten micrograms of protein was applied to each lane. Arrowheads indicate the 27K and 31K OMPs. Lane A, molecular size standards (in thousands), as described in the text.

10 mM HEPES (pH 7.4) buffer containing 0.1 mM PMSF and was suspended in 0.5 ml of deionized water. The samples (10 μl per lane) were treated by being heated in SDS-PAGE sample buffer at 100°C for 5 min and electrophoresed on a polyacrylamide gel.

## RESULTS

**SDS-PAGE of Sarkosyl-insoluble proteins of *B. avium*.** Sarkosyl-insoluble OMP fractions from 50 *B. avium* isolates grown on BHIA for 36 h were compared by SDS-PAGE. Two bands representing the 21K and 37K OMPs were the most prominent bands for all isolates. At least 13 less intensely stained bands (13.5K, 15K, 18K, 23K, 27K, 31K, 41K, 43K, 54K, 73K, 80K, 83K, and 143K) were apparent in the OMP profile from strain 75 (Fig. 1, lane B). No differences in the relative concentration or mobility of the major 21K and 37K bands were observed between strains, and little variation was noted in the less intensely stained bands (Fig. 1). Similar OMP profiles were observed for strain NCD and its avirulent derivative NCD-1 (Fig. 1, lanes F and G). In addition to the strains for which the results are shown in Fig. 1, 23 other *B. avium* isolates were examined, and all had identical or nearly identical OMP profiles (data not shown).

Because strains initially used for isolation of OMPs in our laboratory had been obtained from infected birds at least six years previously, we included 19 isolates from field outbreaks of upper respiratory disease in turkeys during 1989 and 1990 to determine whether OMP profiles from recent isolates differ from those of earlier isolates. No differences in the OMP profiles were noted between the two groups. The profile from strain F9000336 is representative of OMPs observed from recent field isolates (Fig. 1, lane J).

The solubility of strain 75 OMPs was examined by varying incubation temperature or time in SDS-PAGE sample buffer prior to electrophoresis. The majority of the OMPs were not solubilized in sample buffer incubated at room temperature or 37°C, as indicated by the relatively low amount of protein observed in lanes B and C of Fig. 2. Increased protein staining in the high-molecular-weight region of the gel was noted at solubilization temperatures of 60 and 80°C (Fig. 2, lanes D and E). The major 21K and 37K bands were visible when samples were solubilized at 80 and 60°C, respectively. The major bands increased in intensity with solubilization temperatures up to 100°C, but incubation for 30 min at 100°C in sample buffer prior to electrophoresis had no apparent effect on the intensity of either band. The 27K and 31K bands were not apparent when the OMP preparation was heated below 100°C and increased in intensity when the solubilization time was lengthened to 30 min (indicated by arrowheads in Fig. 2). Omission of the reducing agent 2-mercaptoethanol from the SDS-PAGE sample buffer appeared to have no effect on OMP mobility (data not shown).

**Peptidoglycan-associated proteins.** Noncovalent association of certain OMPs with the underlying peptidoglycan layer can affect solubility of these proteins. Total cell envelope fractions from strain 75 were incubated in 2% SDS at 37, 50, 60, 80, or 100°C for 15 min to release proteins that were noncovalently associated with the crude peptidoglycan. The 21K, 27K, and 31K proteins were associated with the peptidoglycan at lower temperatures but were totally dissociated when treated in SDS at temperatures in excess of 60°C (Fig. 3, lane F). The major 37K OMP was almost completely released from the peptidoglycan by incubation at 80°C and was totally solubilized by treatment in SDS at 100°C prior to SDS-PAGE (Fig. 3, lanes F and G). Other OMPs were not as closely associated with the peptidoglycan and were released from the crude peptidoglycan fraction by incubation at room temperature.

**SDS-PAGE of radiolabeled proteins.** Examination of radioiodinated OMPs from *B. avium* 75 by SDS-PAGE routinely showed nine bands that corresponded to the 13.5K, 15K, 18K, 21K, 27K, 31K, 37K, 54K, and 80K OMP bands seen in Coomassie blue-stained gel (Fig. 4, lanes B and D). The only consistent difference between radiolabeled OMPs from cells grown on BHIA and in BHIB was an increased intensity of the lower-molecular-weight bands from cells grown in BHIB. The same bands from BHIB-grown cells were observed to be more prominent in the Coomassie blue-stained gel; the increased intensity of the radiolabeled bands was probably due to an increased amount of protein (Fig. 5). If

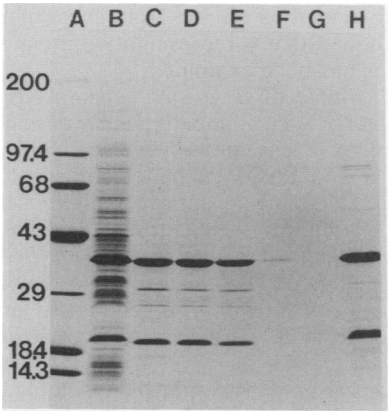


FIG. 3. SDS-PAGE analysis of peptidoglycan-associated proteins. Cell envelope preparations from *B. avium* 75 were treated at different temperatures in 2% SDS for 15 min. The insoluble material containing peptidoglycan and associated protein was incubated in SDS-PAGE sample buffer at 100°C for 5 min before electrophoresis. Cell envelope fractions were treated with no SDS (lane B) or with SDS at 37, 50, 60, 80, or 100°C (lanes C through G, respectively) prior to SDS-PAGE. Lane H, Sarkosyl-insoluble OMP preparation; lane A, molecular size standards (in thousands), as described in the text.

the X-ray film was overexposed, the 41K, 43K, and 143K OMP bands in the radiolabeled OMP preparation could also be observed (data not shown). The most prominent band from surface-radiolabeled whole cells grown in either medium corresponded to the 37K major OMP (indicated by an arrowhead in Fig. 4). In contrast, the major 21K OMP was poorly labeled relative to the intensity of radiolabel in the purified outer membrane preparation (Fig. 4, lanes A and C).

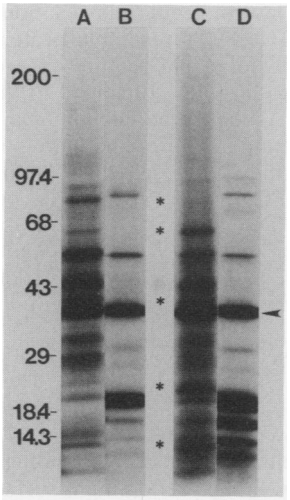


FIG. 4. SDS-PAGE analysis of radioiodinated whole cells and outer membranes of *B. avium* 75. Samples were radioiodinated and subjected to electrophoresis after solubilization in sample buffer at 100°C for 5 min. After electrophoresis, the gel was exposed to X-ray film. Lanes: A and C, $^{125}$I-labeled whole cells grown on BHIA and in BHIB, respectively; B and D, labeled Sarkosyl-insoluble extracts of cell envelopes from cells grown on BHIA and in BHIB, respectively. Asterisks indicate bands observed for surface-labeled whole cells but not for labeled outer membrane preparations. The arrowhead indicates the major 37K OMP. The positions of the molecular size standards (in thousands) are indicated on the left.

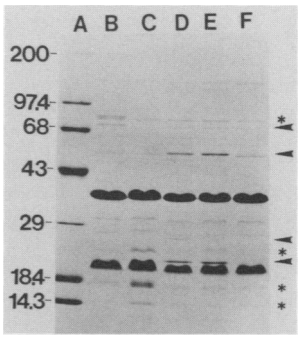


FIG. 5. SDS-PAGE profiles of Sarkosyl-insoluble OMPs isolated from *B. avium* 75 grown for 36 h at 37°C on the following media: BHIA (lane B), BHIB (lane C), BGA (lane D), BGA containing 5 mM nicotinic acid (lane E), and BGA containing 50 mM $MgSO_4$ (lane F). Ten micrograms of protein was applied to each lane. Asterisks indicate OMPs which appear to differ in intensity because of growth in different media. Arrowheads show the 22K, 26K, 56K, and 73K proteins which are apparent for cells grown on BGA but not on BGA supplemented with 50 mM $MgSO_4$. Lane A, molecular size standards (in thousands), as described in text.

Surface-labeled bands that corresponded to other OMPs included the 13.5K, 15K, 18K, 41K, 43K, and 54K bands, while the 27K and 31K OMP bands comigrated with two areas of diffuse bands from the labeled cells. Bands present in surface-labeled cells that did not correspond to OMPs included the 14K, 22K, 39K, 64K, and 79K bands (indicated by asterisks in Fig. 4). Profiles of surface-labeled proteins were comparable for cells grown on both media, with one exception: a 79K band was observed only for cells grown on BHIA.

**Effect of growth conditions on expression of *B. avium* OMPs.** The OMPs isolated from strain 75 grown on various media were compared to determine the effect of different growth conditions on OMP expression. The major 21K and 37K OMPs were the most prominent bands observed and were present in relatively the same concentrations in profiles from cells grown in any of the media. At least eight bands were observed to differ in intensity because of growth in various media (indicated by asterisks and arrowheads in Fig. 5). The 22K, 26K, 56K, and 73K bands present in the OMP profile from cells grown on BGA were not apparent in the profile from cells grown on BGA containing 50 mM $MgSO_4$ (indicated by arrowheads in Fig. 5). Addition of 5 mM nicotinic acid to the medium did not appear to affect OMP expression.

The effect of incubation time on OMP expression was examined by using *B. avium* 75. Sarkosyl-insoluble protein profiles from cells grown on BHIA and in BHIB for 24, 36, and 48 h showed no apparent differences due to incubation time (data not shown).

**Comparison of Sarkosyl-insoluble protein profiles from *Bordetella* spp.** Sarkosyl-insoluble proteins of *Bordetella* spp., including *B. avium*-like and *B. bronchiseptica* isolates from turkey upper respiratory tracts, were compared with OMPs of *B. avium* by SDS-PAGE. All strains were passaged twice on BGA prior to isolation of OMPs. Two major bands, one ranging from 36K to 40K and a second with a molecular weight of 20,000 to 21,000, were observed for OMP profiles isolated from each species (Fig. 6). A second major OMP band with a molecular weight of 20,000 to 21,000 was present in each lane. A minor 18K band was present in all species, but comparison of the remaining bands in the *B. avium* OMP

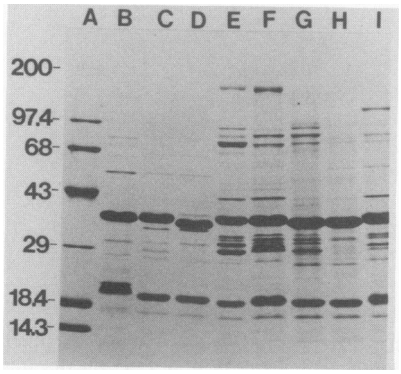


FIG. 6. SDS-PAGE analysis of Sarkosyl-insoluble OMPs from *Bordetella* spp. grown on BGA. Lanes: B, *B. avium* 75; C, *B. avium*-like 023; D, *B. avium*-like 101; E, *B. bronchiseptica* 103; F, *B. bronchiseptica* T91; G, *B. pertussis* BP536; H, *B. pertussis* BP537; I, *B. parapertussis* 504. Lane A, molecular size standards (in thousands), as described in the text.

profile with bands from other species showed few similarities.

The 20 *B. avium*-like isolates examined could be separated into two groups on the basis of different OMP profiles. The first group contained nine isolates with a major 38K band and was represented by strain 023 (Fig. 6). The remaining isolates formed a second group with a major 36K band and were represented by *B. avium*-like 101. Despite the difference in the 36K and 38K bands, the majority of the minor bands and the major 21K band from the two groups had identical mobilities. No differences in colonial morphology and standard laboratory tests between the two groups of *B. avium*-like isolates were seen (data not shown). Though certain bands in *B. avium*-like strains 023 and 101 and *B. avium* 75 OMP profiles had similar mobilities, the profiles were distinctly different.

The OMP profiles of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* strains resembled those previously described (3, 14, 41). *B. bronchiseptica* isolates from turkeys formed an extremely homogeneous group on the basis of Sarkosyl-insoluble OMPs, of which the profile of strain 103 is representative (Fig. 6, lane E). The *B. bronchiseptica* 103 profile was clearly different from *B. avium* and *B. avium*-like profiles but possessed various OMP bands that comigrated with bands present in profiles from *B. bronchiseptica* T91, *B. parapertussis* 504, and *B. pertussis* isolates BP536 and BP537.

## DISCUSSION

The protein profiles of Sarkosyl-insoluble fractions from *B. avium* isolates are strikingly similar. This result is in agreement with previous studies involving total cellular proteins (23), soluble cell proteins (27), cell envelope proteins (25), and OMPs (22) from *B. avium*, which has indicated little difference in protein profiles between isolates. Homogeneity of OMP profiles within a species appears to be characteristic of members of the genus *Bordetella* (34, 36, 41). In certain gram-negative bacteria, OMP profiles differ according to serotypes or biotypes (9, 35), but homogeneity of cell envelope proteins is seen for *B. pertussis*, even for strains from different serotypes (3). Serotypes have been shown to exist in *B. avium* (27); unfortunately, little is known about them, and a serotyping scheme is not available. By including numerous isolates from geographically diverse areas, it is hoped that a representative cross section of *B. avium* strains has been examined.

The OMP profile of *B. avium* appears typical of gram-negative bacteria, with a limited number of major bands and numerous minor or less intensely stained bands (11, 32). The major 21K and 37K OMPs had identical electrophoretic mobilities for all isolates examined. Major OMP bands with comparable mobilities were present in other *Bordetella* species; however, other similarities with *B. avium* OMP profiles were limited. This result was not unexpected since *B. avium* has been shown to be genetically and phenotypically distinct from *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* (27).

Initial comparison of Triton X-100-insoluble OMP profiles of *B. avium* and a *B. avium*-like isolate showed differences, but 23K, 81K, 102K, and 123K bands were present with both organisms (22). In this study, the Sarkosyl-insoluble protein profiles were distinct for each group of organisms. These differences in OMP profiles support the proposal by Jackwood et al. that *B. avium* and *B. avium*-like isolates might be different species (26). However, more phenotypic and genetic analysis is needed to determine the taxonomic relationship of the two organisms. Two OMP profile types were observed for *B. avium*-like organisms. *B. avium*-like isolates have been shown to revert between two colony types during in vitro growth (12, 25); however, colony type did not appear to correlate with the OMP pattern (data not shown).

The *B. avium* OMPs appeared to have variable solubilities in SDS-PAGE sample buffer. Heating at increased temperature dissociates protein-protein complexes normally found in the outer membrane and allows migration of solubilized proteins at a lower apparent molecular weight (32). The noncovalent association of the 21K and 37K OMPs with the peptidoglycan might also affect the solubility of these proteins. The extended incubation time required for complete solubilization of the 27K and 31K bands indicates possible strong intra- or intermolecular associations of these OMPs. The 27K and 31K OMPs resemble proteins found in other gram-negative bacteria that require extended solubilization times at 100°C to be fully converted to the heat-modifiable form (20, 35). As observed for the major 21K and 37K OMPs, the 27K and 31K proteins were peptidoglycan associated, because they required solubilization temperatures of greater than 60°C in 2% SDS to be released from the peptidoglycan. The apparent lack of extensive disulfide bonding in the OMPs of *B. avium* was indicated by the inability of the reducing agent to affect protein migration and has been observed for the membrane proteins of *B. pertussis* (2).

Surface-exposed proteins of *B. avium* were identified by radiolabeling whole cells with $^{125}I$. The most prominent bands had molecular weights ranging from 37,000 to 64,000 for bacteria grown in BHIB and from 37,000 to 79,000 for BHIA-grown cells. A band corresponding to the major 37K OMP routinely was the predominant surface-labeled protein in all preparations. This is not surprising since large amounts of the 37K protein in Sarkosyl-insoluble preparations indicate that the protein is most likely a prevalent component of the outer membrane. In contrast, the major 21K protein was poorly radiolabeled relative to the intensity of numerous other proteins in both whole-cell preparations. This might indicate that the 21K protein is not surface exposed or that the protein contains no exposed tyrosine residues. Amino acid analysis has shown that the 21K protein contains tyrosine residues, but it is possible that these amino acids are

buried and not accessible to radioiodination (30). Sarkosyl extraction of the total envelope fraction may expose the tyrosine residues of the 21K protein because the protein is strongly labeled in the OMP-enriched fractions. The majority of bands from the radiolabeled whole cells corresponded to bands in the labeled OMP preparations, with the exception of five bands (indicated by asterisks in Fig. 4). The five bands may represent proteins in the outer membrane which are solubilized during Sarkosyl extraction and, therefore, are not present in the OMP profiles. The bands, especially the minor bands, could correspond to proteins which are secreted by the whole cells or are exposed to radioiodination because of release by any lysed cells in the whole-cell preparations (39).

Comparison of OMP profiles from strain 75 grown on different media indicated that expression of the major 21K and 37K OMPs is constitutive, at least on the media examined. It is not known whether the 21K and 37K OMPs are expressed in vivo, but antibodies to OMPs with similar molecular weights have been demonstrated in sera and tracheal washings from experimentally infected turkeys (21). In addition, serum antibodies to 100K, 97K, 55K, 31K, 18K, and 14K OMPs were present. Several minor OMPs described in this study share similar mobilities with these immunoreactive OMPs, but a direct comparison has not been made.

Expression of certain OMPs (3, 14, 34, 36, 41, 47) and other virulence-associated factors (40, 47) of *B. pertussis* is affected by two forms of regulation, antigenic modulation and phase variation. Antigenic modulation is a coordinate, reversible phenotypic change seen when *B. pertussis* is grown in media containing certain ions and nutrients (28, 33). Phase variation results in similar phenotypic changes but is due to a metastable frameshift mutation in the *bvg* or *vir* locus (46, 47). The *bvg* locus contains two genes (*bvgA* and *bvgS*) which encode proteins that sense environmental stimuli and coordinately regulate transcription of the virulence-associated genes, including certain OMP genes of *B. pertussis* (1). The *bvg* loci have been demonstrated for *B. bronchiseptica* and *B. parapertussis* (18), and sequences homologous to the *bvgS* gene have recently been identified for *B. avium* by Gentry-Weeks and coworkers (16). Spontaneous phase variants of *B. avium* which did not express the dermonecrotic toxin and the 27K, 38K, 48K, and 93K OMPs were isolated (16). The same proteins, which were detected by using Western blot analysis, were not expressed in bacteria grown in the presence of $MgSO_4$ or nicotinic acid, two nutrients that induce phenotypic modulation in *B. pertussis* (15, 16). In this study, production of the 22K, 26K, 56K, and 73K OMPs was reduced or eliminated when strain 75 was grown in medium containing $MgSO_4$ but not nicotinic acid. The inability of nicotinic acid to modulate expression of various OMPs of strain 75 might indicate that this factor functions in a strain-dependent fashion in *B. avium*. Modulation by nicotinic acid has been shown to be strain dependent in *B. pertussis* (33). It is possible that the 26K OMP is identical to the 27K OMP described by Gentry-Weeks and coworkers (16), but no apparent similarity exists between the other modulated OMPs. The differences observed may be due to the different detection methods used in each study.

The major 37K OMP of *B. avium* shares certain characteristics with the major 40K OMP of *B. pertussis* which functions as an anion-selective porin (4). The proteins had similar mobilities in SDS-PAGE, and both were the most prominent bands in Sarkosyl-insoluble OMP profiles from *B. avium* and *B. pertussis*. The 40K protein has been shown to be constitutively expressed in several *B. pertussis* strains grown on different media and in phenotypically modulated and avirulent-phase cells (3, 14). Further characterization by several investigators has shown that the 40K protein is exposed to surface radioiodination (2, 3, 36), is not affected by the reducing agent 2-mercaptoethanol, and is noncovalently associated with the peptidoglycan layer (2). Though we have shown that the 37K OMP of *B. avium* possesses these characteristics, we have not attempted to directly compare the two OMPs, and more work is needed to determine the structure and function of the major 37K OMP of *B. avium* and its relationship, if any, with the 40K porin protein of *B. pertussis*.

We have characterized the proteins in the outer membrane of *B. avium* and have compared them with OMPs from other *Bordetella* spp. It is likely that many of the OMPs of *B. avium* described are, in fact, true OMPs because they share characteristics, such as Sarkosyl insolubility, surface exposure to radioiodination, and noncovalent association with the peptidoglycan layer, with OMPs of other gram-negative bacteria. More work is needed to further characterize the OMPs of *B. avium* and their involvement in the pathogenesis of turkey bordetellosis. We have shown that expression of certain OMPs depends on in vitro growth conditions. It is essential that the differences in OMP expression be taken into consideration in further experimental studies and in vaccine production, because the antigenicity of the organism might be significantly affected by these changes.

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## REFERENCES

1. **Arico, B., J. F. Miller, C. Roy, S. Stibitz, D. Monack, S. Falkow, R. Gross, and R. Rappuoli.** 1989. Sequences required for expression of *Bordetella pertussis* virulence factors share homology with prokaryotic signal transduction proteins. Proc. Natl. Acad. Sci. USA **86**:6671–6675.
2. **Armstrong, S. K., and C. D. Parker.** 1986. Heat-modifiable envelope proteins of *Bordetella pertussis*. Infect. Immun. **54**: 109–117.
3. **Armstrong, S. K., and C. D. Parker.** 1986. Surface proteins of *Bordetella pertussis*: comparison of virulent and avirulent strains and effects of phenotypic modulation. Infect. Immun. **54**:308–314.
4. **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.
5. **Arp, L. H., and N. F. Cheville.** 1984. Tracheal lesions in young turkeys infected with *Bordetella avium*. Am. J. Vet. Res. **45**:2196–2200.
6. **Arp, L. H., and J. A. Fagerland.** 1987. Ultrastructural pathology of *Bordetella avium* infection in turkeys. Vet. Pathol. **24**:411–418.
7. **Arp, L. H., and D. H. Hellwig.** 1988. Passive immunization versus adhesion of *Bordetella avium* to the tracheal mucosa of turkeys. Avian Dis. **32**:494–450.
8. **Arp, L. H., R. D. Leyh, and R. W. Griffith.** 1988. Adherence of *Bordetella avium* to tracheal mucosa of turkeys: correlation with hemagglutination. Am. J. Vet. Res. **49**:693–696.
9. **Barenkamp, S. J., R. S. Munson, Jr., and D. M. Granoff.** 1981. Subtyping isolates of *Haemophilus influenzae* type b by outer-membrane protein profiles. J. Infect. Dis. **143**:668–676.
10. **Barnes, H. J., and M. S. Hofstad.** 1983. Susceptibility of turkey poults from vaccinated and unvaccinated hens to alcaligenes rhinotracheitis (turkey coryza). Avian Dis. **27**:378–392.

11. **Benz, R.** 1988. Structure and function of porins from gram-negative bacteria. Annu. Rev. Microbiol. **42:**359–393.
12. **Berkhoff, H. A., and G. D. Riddle.** 1984. Differentiation of *Alcaligenes*-like bacteria of avian origin and comparison with *Alcaligenes* spp. reference strains. J. Clin. Microbiol. **19:**477–481.
13. **Burke, D. S., and M. M. Jensen.** 1980. Immunization against turkey coryza by colonization with mutants of *Alcaligenes faecalis*. Avian Dis. **24:**726–733.
14. **Ezzel, 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.
15. **Gentry-Weeks, C. R., B. T. Cookson, W. E. Goldman, R. B. Rimler, S. B. Porter, and R. Curtiss III.** 1988. Dermonecrotic toxin and tracheal cytotoxin, putative virulence factors of *Bordetella avium*. Infect. Immun. **56:**1698–1707.
16. **Gentry-Weeks, C. R., D. L. Provence, J. M. Keith, and R. Curtiss III.** 1991. Isolation and characterization of *Bordetella avium* phase variants. Infect. Immun. **59:**4026–4033.
17. **Gray, J. G., J. F. Roberts, R. C. Dillman, and D. G. Simmons.** 1983. Pathogenesis of change in the upper respiratory tracts of turkeys experimentally infected with an *Alcaligenes faecalis* isolate. Infect. Immun. **42:**350–355.
18. **Gross, R., and R. Rappuoli.** 1988. Positive regulation of pertussis toxin expression. Proc. Natl. Acad. Sci. USA **85:**3913–3917.
19. **Hames, B. D.** 1981. An introduction to polyacrylamide gel electrophoresis, p. 1–91. *In* B. D. Hames and D. Rickwood (ed.), Gel electrophoresis of proteins. IRL Press, Oxford.
20. **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.
21. **Hellwig, D. H., and L. H. Arp.** 1990. Identification of *Bordetella avium* antigens recognized after experimental inoculation in turkeys. Am. J. Vet. Res. **51:**1188–1191.
22. **Hellwig, D. H., L. H. Arp, and J. A. Fagerland.** 1988. A comparison of outer membrane proteins and surface characteristics of adhesive and non-adhesive phenotypes of *Bordetella avium*. Avian Dis. **32:**787–792.
23. **Hinz, K.-H., G. Glunder, and K. J. Romer.** 1983. A comparative study of avian *Bordetella*-like strains, *Bordetella bronchiseptica*, *Alcaligenes faecalis* and other related nonfermentatable bacteria. Avian Pathol. **12:**263–276.
24. **Jackwood, M. W., and Y. M. Saif.** 1987. Pili of *Bordetella avium*: expression, characterization, and role in adherence. Avian Dis. **31:**277–286.
25. **Jackwood, M. W., Y. M. Saif, P. D. Moorhead, and R. N. Dearth.** 1985. Further characterization of the agent causing coryza in turkeys. Avian Dis. **29:**690–705.
26. **Jackwood, M. W., M. Sasser, and Y. M. Saif.** 1986. Contribution to the taxonomy of the turkey coryza agent: cellular fatty acid analysis of the bacterium. Avian Dis. **30:**172–178.
27. **Kersters, K., K.-H. Hinz, A. Hertle, P. Segers, A. Lievens, O. Siegmann, and J. De Ley.** 1984. *Bordetella avium* sp. nov., isolated from the respiratory tracts of turkeys and other birds. Int. J. Syst. Bacteriol. **34:**56–70.
28. **Lacey, B. W.** 1960. Antigenic modulation of *Bordetella pertussis*. J. Hyg. **58:**57–93.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
30. **Leyh, R. D., and R. W. Griffith.** Unpublished data.
31. **Luginbuhl, G. H., J. M. Rader, and D. G. Simmons.** 1984. Evidence for plasmid DNA in *Alcaligenes faecalis*. Am. J. Vet. Res. **45:**1679–1681.
32. **Lugtenberg, B., and L. van Alphen.** 1983. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. Biochim. Biophys. Acta **737:**51–115.
33. **Melton, A. R., and A. A. Weiss.** 1989. Environmental regulation of expression of virulence determinants in *Bordetella pertussis*. J. Bacteriol. **171:**6206–6212.
34. **Parton, R., and A. C. Wardlaw.** 1975. Cell-envelope proteins of *Bordetella pertussis*. J. Med. Microbiol. **8:**47–57.
35. **Rapp, V. J., R. S. Munson, Jr., and R. F. Ross.** 1986. Outer membrane protein profiles of *Haemophilus pleuropneumoniae*. Infect. Immun. **52:**414–420.
36. **Redhead, K.** 1983. Variability in the surface exposure of the outer membrane proteins of *Bordetella pertussis*. FEMS Microbiol. Lett. **17:**35–39.
37. **Redinbaugh, M. G., and R. B. Turley.** 1986. Adaptation of the bicinchoninic acid protein assay for use with microtiter plates and sucrose gradient fractions. Anal. Biochem. **153:**267–271.
38. **Relman, D. A., M. Domenighini, E. Tuomanen, R. Rappuoli, and S. Falkow.** 1989. Filamentous hemagglutinin of *Bordetella pertussis*: nucleotide sequence and crucial role in adherence. Proc. Natl. Acad. Sci. USA **86:**2637–2641.
39. **Richardson, K., and C. D. Parker.** 1985. Identification and characterization of *Vibrio cholerae* surface proteins by radioiodination. Infect. Immun. **48:**87–93.
40. **Robinson, A., C. J. Duggleby, A. R. Gorringe, and I. Livey.** 1987. Antigenic variation in *Bordetella pertussis*, p. 147–161. *In* M. A. Horwitz (ed.), Bacteria-host cell interaction. Alan R. Liss, Inc., New York.
41. **Robinson, A., and D. C. Hawkins.** 1983. Structure and biological properties of solubilized envelope proteins of *Bordetella pertussis*. Infect. Immun. **39:**590–598.
42. **Saif, Y. M., P. D. Moorhead, R. N. Dearth, and D. G. Jackwood.** 1980. Observations on *Alcaligenes faecalis* infection in turkeys. Avian Dis. **24:**665–684.
43. **Simmons, D. G., J. G. Gray, L. P. Rose, R. C. Dillman, and S. E. Miller.** 1979. Isolation of an etiologic agent of acute respiratory disease (rhinotracheitis) of turkey poults. Avian Dis. **23:**194–203.
44. **Simmons, D. G., L. P. Rose, K. A. Brogden, and R. B. Rimler.** 1984. Partial characterization of the hemagglutinin of *Alcaligenes faecalis*. Avian Dis. **28:**700–709.
45. **Slavik, M. F., J. K. Skeeles, J. N. Beasley, G. C. Harris, P. Roblee, and D. Hellwig.** 1981. Effect of humidity on infection of turkeys with *Alcaligenes faecalis*. Avian Dis. **25:**936–942.
46. **Stibtz, S., W. Aaronson, D. Monack, and S. Falkow.** 1989. Phase variation in *Bordetella pertussis* by frameshift mutation in a gene for a novel two-component system. Nature (London) **338:**266–269.
47. **Weiss, A. A., and S. Falkow.** 1984. Genetic analysis of phase change in *Bordetella pertussis*. Infect. Immun. **43:**263–269.