Characteristics of Major Outer Membrane Proteins of *Haemophilus influenzae*

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Several properties of *Haemophilus influenzae* outer membrane proteins were analyzed to define related proteins in various isolates. *H. influenzae* type b 760705 had six major outer membrane proteins with the following characteristics. Protein a (*M*., 47,000) demonstrated heat modifiability in sodium dodecyl sulfate; its apparent molecular weight was 34,000 at temperatures below 60°C. This protein was extracted from cell envelopes by using Triton X-100–10 mM MgCl₂; in cell envelope preparations, the protein was degraded by trypsin. Proteins b (*M*., 41,000) and c (*M*., 40,000) were insensitive to trypsin degradation, were not heat modifiable in sodium dodecyl sulfate, and were peptidoglycan associated in 0.5% Triton X-100–0.2% sodium dodecyl sulfate. The amount of protein b was reduced in ultrasonically obtained cell envelopes. Protein d (*M*., 37,000) was heat modifiable in sodium dodecyl sulfate with an *M*., of 28,000 at temperatures below 100°C and was degraded by trypsin, leaving a membrane-bound fragment of *M*., 27,000. Both the intact and degraded proteins were immunologically cross-reactive with the heat-modifiable OmpA protein of *Escherichia coli* K-12. Protein d was absent in LiCl-EDTA extracts of cells. Protein e (*M*., 30,000), invariably present in all *H. influenzae* strains tested, was insensitive to trypsin and absent in LiCl-EDTA extracts of cells. Protein k (*M*., 58,000) was extracted from cell envelopes with 2% Triton X-100–10 mM MgCl₂ and, in cell envelopes, appeared to be sensitive to trypsin degradation. Proteins with similar properties to those of proteins a to k were found in 10 other *H. influenzae* strains, reference strains with serotype a, c, d, e, and f capsules, and 18 of 20 nonencapsulated strains. Their relative molecular weights, however, varied.

*Haemophilus influenzae* colonizes upper respiratory tracts, invades respiratory mucous membranes, and causes various systemic diseases, such as menigitis, epiglottitis, and cellulitis. Young children (0 to 4 years old) are most susceptible (27). These invasive diseases are caused almost exclusively by strains with serotype b capsules which consist of polyribitolribosylphosphate (5). The capsule of *H. influenzae* type b is regarded as an important virulence factor (16, 22, 23, 26). Therefore, the purified polysaccharide is potentially suitable for vaccine purposes, but, unfortunately, it is poorly effective in children younger than 18 months old, who have the highest risk for invasive diseases caused by *H. influenzae* (H. Käyhly, H. Peltola, and P. H. Mäkelä, Fourth International Conference on Immunity and Immunisation in Cerebrospinal Meningitis, Sienna, Italy, abstr. no. 17, 1981; 20). In adults, a rise in the bactericidal titer against type b polysaccharide, as well as against outer membrane protein and lipopolysaccharide, has been found after vaccination with conjugates consisting of polysaccharide and outer membrane complexes (1).

Since the usefulness of these outer membrane components as vaccine constituents depends on their general occurrence in disease isolates, characterization of outer membrane components is required. Loeb and Smith (12) have shown that the outer membranes of both encapsulated and nonencapsulated strains of *H. influenzae* have various major proteins, named a to f based on their relative positions in sodium dodecyl sulfate (SDS)-polyacrylamide gels. The patterns of these protein bands in SDS-polyacrylamide gels are strongly variable, especially among nontypable strains (12). Type b strains appear to have eight different patterns for the major outer membrane proteins (13). Barenkamp et al. analyzed the protein patterns of *H. influenzae* type b strains (from several regions of the United States) more systematically and found 21 different patterns, 6 of which were predominant (2, 3, 9).

Since protein patterns in gels are heavily
influenced by the conditions under which the SDS-polyacrylamide gel electrophoresis is carried out, the reported protein patterns are difficult to compare. Moreover, for the typing of \( H. \) \( \text{influenzae} \) isolates with SDS-polyacrylamide gel electrophoresis, definition of the various bands is required. Therefore, we analyzed biochemical and immunological properties of major outer membrane proteins of various disease isolates.

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**MATERIALS AND METHODS**

**Strains and growth conditions.** \( H. \) \( \text{influenzae} \) strains isolated from cerebrospinal fluid, throats, ears, sputum, blood, and eyes of patients were sent to us by bacteriological laboratories in The Netherlands. \( H. \) \( \text{influenzae} \) type b 760705 from cerebrospinal fluid and nontypable strain 1481 from the sputum of a patient with an upper respiratory tract infection were used as representative examples in most of the experiments. Strain H113 was a gift of M. Loeb (12). All strains were subcultured on GC agar base plates (Oxoid Ltd) supplemented with (per liter): 10 g of hemoglobin (Difco Laboratories), 5 g of glucose, 1 g of \( \text{l-glutamic acid} \), 3 g of \( \text{l-cysteine} \), 10 mg of ferrinitrate, and dialysate from 10 g of bakers yeast and examined for NAD and hemin requirements (11). Serotyping of the capsular polysaccharides was performed by coagglutination with Formalin-fixed cells of \( \text{Staphylococcus aureus} \) carrying protein A (Cowan I, NTCC 8530), coupled with rabbit antisera which were specific for the polysaccharide serotypes a to f as described previously (6). The antisera were raised and absorbed as described previously (6). Cells were grown overnight with aeration in brain heart infusion broth (Difco), supplemented with NAD and hemin (each, 10 \( \mu \)g/ml; Sigma Chemical Co.) and harvested by centrifugation (10 min at 10,000 \( \times \) g).

**Isolation and analysis of membranes.** Cell envelopes were obtained after ultrasonic disruption of the cells by differential centrifugation as described previously (15). Outer and cytoplasmic membranes were isolated according to a modification of the method of Heerikhuizen et al. (28), which basically consists of the standard method of Osborn et al. (18). All procedures were performed at 0 to 4°C. Cells obtained from 200 ml of culture were suspended in 4 ml of 200 mM Tris-hydrochloride (pH 8.0)–1 mM MgCl\(_2\)–0.2 mM dithiothreitol. At \( t = 0 \) min, 6 ml of 200 mM Tris-hydrochloride (pH 8.0)–0.2 mM dithiothreitol was added, followed after 1 min, by addition of 100 \( \mu \)l of 100 mM EDTA and after 2 min, by addition of 0.5 ml of 1 M sucrose was added. At \( t = 3.5 \) min, 400 \( \mu \)l of lysozyme solution (0.55 mg/ml) was added, followed by 20 ml of water at \( t = 4 \) min. Spheroplast formation, judged by phase-contrast microscopy, was >90%. Spheroplasts were collected by low-speed centrifugation (20 min at 5,000 \( \times \) g) and lysed osmotically by being suspended in 20 volumes of 10 mM Tris-hydrochloride (pH 7.8)–0.2 mM dithiothreitol. RNase and DNase (each, 100 \( \mu \)g/ml) were added, and after brief sonication (10 s, 30 W), the complete lysate was loaded on a continuous, linear sucrose gradient (20 to 60% wt/wt) in 10 mM N-2-hydroxyethylpiperazine-N'2-ethane sulfonic acid (HEPES [pH 7.4])–0.2 mM dithiothreitol. After centrifugation for 16 h at 21,000 rpm in a Beckman SW27 rotor, two bands were visible. The gradient was fractionated, and the optical density at 200 nm and the specific gravity were determined (with an optical refractometer for each of the fractions [Fig. 1]). Peak fractions were pooled. The upper (cytoplasmic) band had a specific gravity of 1.18 g/cm\(^3\), and the lower (outer membrane) band had a specific gravity of 1.24 g/cm\(^3\). NADH-oxidase was determined as the marker enzyme for cytoplasmic membrane as described previously (18). More than 90% of the enzyme activity was associated with the upper band. After dilution of the gradient fractions to a final sucrose concentration of 10%, the membranes were collected by ultracentrifugation (2 h at 200,000 \( \times \) g), washed with 2 mM Tris-hydrochloride (pH 7.8), and centrifuged (1 h at 200,000

![FIG. 1. Optical densities (O.D.) of isopycnic centrifugation fractions of osmotically lysed spheroplasts from 100 ml of exponentially grown \( H. \) \( \text{influenzae} \) type b 760705 cells. Similar results were obtained with the nonencapsulated strain 1481. sg, Specific gravity; OM, outer membrane fraction; CM, cytoplasmic membrane fraction.](http://jb.asm.org/)
× g). The pellet was resuspended in the same buffer.

Outer membrane complexes were obtained with the LiCl-EDTA extraction method developed for Neisseria (8) or by extraction of complete cell envelopes with Triton X-100–Mg2+ as described previously by Schnaitman (25).

**SDS-polyacrylamide gel electrophoresis.** The proteins of total cell envelopes and isolated membrane (complexes) were analyzed on an 11% SDS-polyacrylamide gel by the method of Lugtenberg et al. (15). Where indicated, the running gel was supplemented with 6 M urea (19). Membrane complexes were solubilized in sample buffer (0.0625 M Tris-hydrochloride [pH 6.8]–10% glycerol–2% SDS–5% 2-mercaptoethanol–0.001% bromphenol blue) and heated for 10 min in a boiling water bath before they were applied to the gel (unless otherwise indicated). Gels were fixed and stained with 0.05% Coomassie brilliant blue in 10% acetic acid–25% methanol in water and destained in 10% acetic acid–25% methanol. Molecular weights of the proteins were determined relative to phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).

**Heat modifiability of outer membrane proteins.** Heat modifiability of cell envelope proteins in SDS was examined by incubating cell envelopes for 10 min at various temperatures between 30 and 100°C in sample buffer before they were applied to the gels.

**Sensitivity of cell envelope proteins.** Sensitivity of cell envelope proteins to proteolytic degradation was determined by incubation with trypsin (100 μg/ml in 10 mM Tris-hydrochloride [pH 8.0]) for 15 min at 37°C. Subsequently, the cell envelopes were cooled to 0°C, recovered from the incubation mixture by centrifugation (30 min at 225,000 × g), washed twice with 10 mM Tris-hydrochloride (pH 7.8)–0.3 M NaCl, and finally washed with 2 mM Tris-hydrochloride (pH 7.8). The protein patterns on the gels were analyzed as described above.

**Peptidoglycan-associated proteins.** Peptidoglycan-associated proteins were identified as described by Lugtenberg et al. (14) for Escherichia coli. In short, cell envelopes were incubated at various temperatures between 30 and 100°C, with the sample buffer for electrophoresis. Alternatively, cell envelopes were extracted for 1 h at 37°C with 0.0625 M Tris-hydrochloride (pH 6.8) containing 0.5% Triton X-100 and 0.2% SDS. The peptidoglycan-associated proteins were recovered in the pellet after centrifugation for 30 min at 40,000 × g.

**Immunological cross-reactivity of outer membrane proteins.** Homology among the heat-modifiable protein and the porins of E. coli K-12 and proteins of H. influenzae was examined immunologically on slices of SDS-polyacrylamide gels (gel immunoradioassay [GIRA; 21]), with specific rabbit antiserum raised against the purified OmpA, OmpC, and OmpF proteins of E. coli K-12, which were obtained from N. Overbeeke (State University, Utrecht, The Netherlands).

**RESULTS**

**Protein composition of outer and cytoplasmic membranes.** The results of protein composition analyses after various isolation procedures are shown in Fig. 2. Outer membranes obtained from spheroplasts contained fewer protein bands than did cytoplasmic membranes or complete cells (compare lanes 3, 4, and 5, respectively). In the outer membrane fraction, several more or less predominant proteins with molecular weights between 28,000 and 58,000 were visible, for which the nomenclature of Loeb et al. (13) was adapted, since the pattern of the outer membrane proteins of H. influenzae type b 760705 (lane 3) corresponded with that of their reference strain, H113 (lane 11). These major proteins are: a, Mr, 47,000; b, Mr, 41,000; c, Mr, 40,000; d, Mr, 37,000; and e, Mr, 30,000. Proteins b and c were poorly separated, independent of the heating of the samples or the presence of urea in the gel. Since another protein with molecular weight of 58,000 was sometimes a major outer membrane protein, we included this protein as protein k. Loeb et al. (13) have described a protein with a molecular weight of 28,000 (protein f), but we could only identify the non-heat-modified form of protein d, protein d*, at that position on the gel (see below). After electrophoresis of membranes on SDS-urea gels, an extra band was resolved between proteins b, c, and d. This protein was found in all the H. influenzae envelope preparations. Other proteins, not shown, are also detected by the immunological procedure described above. One of these proteins appears to be the 58,000-Mr outer membrane protein common to the outer membranes of various strains of H. influenzae (lane 3; lane 4 contains only isolated outer membranes).

**FIG. 2.** An SDS-polyacrylamide gel protein analysis of various cell fractions of H. influenzae 760705. Lanes: 2 and 10, cell envelope; 3, outer membrane (from spheroplasts); 4, cytoplasmic membrane (from spheroplasts); 5, cells; 6, Triton X-100–MgCl2 extract of cell envelopes; and 7, LiCl-EDTA extract of cells. Molecular weight marker proteins (lanes 1, 8, and 9) and strain H113 (lane 11) (donated by M. R. Loeb) cell envelope proteins have been included for reference. d*, Non-heat-modified form of protein d.
influenzae strains which we analyzed (protein 1) (cf. Fig. 4B). Comparison of patterns of outer membrane proteins with those of proteins from cell envelopes (lanes 2 and 3) revealed that protein b was present in smaller amounts in the cell envelope fraction.

To characterize outer membrane proteins more easily than with the spheroplast lysis isolation procedure, outer membrane material was collected according to various well-known isolation procedures. Extraction of cell envelopes was with Triton X-100–MgCl₂ to solubilize cytoplasmic membranes. The insoluble fraction contained most of the outer membrane proteins, but appeared to lack proteins a and k (Fig. 2, cf. lanes 2, 3, and 6). LiCl-EDTA extracts of cells (lane 7) also were not representative for the outer membrane, since the major proteins d, e, and k and several minor proteins were present in diminished amounts. Proteins a, b, and c, on the contrary, were strongly associated with this fraction.

Properties of outer membrane proteins. The outer membrane of H. influenzae type b contains two heat-modifiable proteins (proteins a and d) (13). We studied the transition tempera-

ture at which these proteins are modified to identify the corresponding bands in the gel. The results for H. influenzae type b 760705 are summarized in Fig. 3. At 100°C (lane 9), protein a had an apparent molecular weight of 47,000, and protein d had an apparent molecular weight of 37,000. Below 60°C, the electrophoretic mobility of protein a had a position with an apparent molecular weight of 34,000 (protein a*), and a transition for protein d occurred between 80 and 100°C. The non-heat-modified form of protein d (protein d*) had an apparent molecular weight of 28,000. Samples boiled for less than 10 min in the presence of SDS were heat modified incompletely with respect to protein d, and thus both protein d and d* were observed. The heat-modifiable and not heat-modifiable proteins of various encapsulated and nonencapsulated strains of H. influenzae were compared. The protein patterns are shown in Fig. 4. The patterns of the proteins on SDS-polyacrylamide gels were strongly heterogeneous (Fig. 4A). For some strains only one major band was visible where proteins b, c, and d were expected (lanes 5, 7, and 14). Electrophoresis in SDS-urea gels resolved a second band in strain 1481 (Fig. 4B, lane 5). The proteins at this band and at similar bands for the other strains were heat modifiable (Fig. 4C). The band in the protein d region in lane 14 was also a double band; in the samples which were not heated, three major bands were visible in lanes 12 and 16. In strain 780910 (lane 7) a heat-modifiable major protein in the protein d region is lacking. In addition to heat-modifiable protein d, all isolates except strain 1608 (lane 18) had a protein in the electrophoretic position of protein a.

The presence of peptidoglycan-associated proteins in H. influenzae was tested by solubilization of cell envelopes of the type b strain 760705 and the nontypable strain 1481 in SDS at temperatures between 30 and 100°C. None were found. Addition of either 10 mM EDTA or 10 mM MgCl₂ in the buffer at an extraction temperature of 37°C did not result in cosedimentation of any protein with the peptidoglycan layer. Extraction with 0.0625 Tris-hydrochloride–0.5% Triton X-100–0.2% SDS resulted in selective recovery of protein c and a small fraction of protein d in the pellet after centrifugation, whereas the extracted proteins were recovered in the supernatant. So, H. influenzae did not contain noncovalently peptidoglycan-associated proteins in SDS as was found for many Enterobacteriaceae and Pseudomonas spp. (10, 14), but protein c was weakly peptidoglycan associated in Triton-SDS mixtures.
FIG. 4. SDS-polyacrylamide gel electrophoretic analysis of the cell envelope proteins of 18 nontypable H. influenzae isolates from cerebrospinal fluid (lanes 4 to 9) and sputum (lanes 10 to 18). Three H. influenzae b strains were included for reference (lanes 1 to 3). Heated samples were used both on routine gels (A) and gels containing 6 M urea (B), and samples treated at 30°C in sample buffer are shown in (C). In the latter cases, only the relevant part of the gel is shown.

ic determinants as occur in pore proteins of E. coli. However, none of the outer membrane proteins of H. influenzae reacted in GIRAs with the antisera directed against the pore proteins of E. coli.

Immunological cross-reactivity between the heat-modifiable proteins d (and d*) of various H. influenzae strains and the heat-modifiable protein of E. coli K-12 (OmpA protein) were found in GIRAs when antiserum raised against the purified E. coli protein was used. Fig. 5 shows the result for strain 760705, which indicates that protein d was the only cross-reactive protein.

Sensitivity for proteolytic digestion of outer membrane proteins was tested by treating membranes with trypsin. Fig. 5A (lane 4) shows, with strain 760705 as an example, that proteins a, d, k, and several minor proteins were degraded. New bands appeared in the gel, among which a major band with a molecular weight of 27,000. This is most likely a membrane-associated fragment of protein d, since it reacted to some extent
FIG. 5. Biochemical and immunochemical properties of outer membrane proteins of *H. influenzae* b 760705. (A) Protein staining of SDS-polyacrylamide gel and (B) GIRA with slices of the same gel with antiserum raised against the heat-modifiable (OmpA) protein of *E. coli* K-12. The gel was run with cell envelope proteins, heated for 10 min at 100°C (lane 1) and 30°C (lane 2) and with outer membrane proteins, heated (lane 3) and outer membrane proteins after incubation with trypsin (lane 4).

with the antiserum against the OmpA protein of *E. coli* (Fig. 5B).

**DISCUSSION**

The outer membrane of *H. influenzae* contains several major proteins (2, 3, 9, 12, 13). This was confirmed when we analyzed the protein pattern of outer membranes obtained from spheroplasts (Fig. 2). These proteins were not found in cytoplasmic membranes. It should be noted that cytoplasmic membranes contain proteins which were absent in fractions of cell envelopes obtained by ultrasonic disintegration of cells. This phenomenon may be due to the presence of some cytoplasmic proteins in the cytoplasmic membrane fraction or to the loss of these proteins from the cell envelopes during the isolation procedure; this was not analyzed further.

Two well-known procedures for the isolation of outer membrane material (Triton X-100 extraction of cell envelopes and LiCl-EDTA extraction of cells) resulted in selective loss of outer membrane proteins (Fig. 2). Also, although after ultrasonic disintegration of cells, reduced amounts of protein b were recovered in cell envelope fractions, this procedure still appeared to be the fastest and most efficient method for the mass screening of strains. When, in addition, cell envelopes are extracted with sarcosyl in the presence of MgCl₂, outer membrane proteins are completely recovered in the insoluble fraction (reference 2 and unpublished observations). Triton X-100-MgCl₂ or LiCl-EDTA extracts, on the other hand, may be useful for the preparative isolation of various individual outer membrane proteins.

Table 1 summarizes the characteristics of the major outer membrane proteins of *H. influenzae* b 760705. Outer membrane proteins a, d, e, and k were characterized based on solubility in Tri-

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<tr>
<th>Nomenclature of protein</th>
<th>M, 10⁶</th>
<th>Characteristics</th>
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<tbody>
<tr>
<td>a</td>
<td>43–50 (47)⁶</td>
<td>Heat modifiable⁶, extracted by Triton X-100-MgCl₂, sensitive to trypsin</td>
</tr>
<tr>
<td>b⁶</td>
<td>39–42 (41)</td>
<td>Reduced in cell envelopes, insensitive to trypsin, not heat modifiable</td>
</tr>
<tr>
<td>c⁶</td>
<td>39–42 (40)</td>
<td>Insensitive to trypsin, not heat modifiable, peptidoglycan associated in 0.5% Triton X-100-0.2% SDS</td>
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<tr>
<td>d</td>
<td>37–39 (37)</td>
<td>Heat modifiable⁶, sensitive to trypsin (leaving a membrane-bound fragment of about 27,000), immunologically related to the comparable (OmpA) protein of <em>E. coli</em> K-12, absent in LiCl-EDTA extracts of cells Present in all strains, with the same molecular weight, not heat modifiable, insensitive to trypsin, absent in LiCl-EDTA extracts</td>
</tr>
<tr>
<td>e</td>
<td>30 (30)</td>
<td>Extracted in Triton X-100-MgCl₂, sensitive to trypsin, not heat modifiable</td>
</tr>
<tr>
<td>k</td>
<td>58 (58)</td>
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⁶ Numbers in parentheses are M, values for *H. influenzae* b 760705.

⁶ The non-heat-modified form had an apparent molecular weight of about 34,000.

⁶ Proteins b and c comigrate in gels.

⁶ The non-heat-modified form had an apparent molecular weight of 28,000 to 34,000.
ton X-100–MgCl₂, heat modifiability in SDS, sensitivity to trypsin, and cross-reactivity with *E. coli* heat-modifiable protein. Proteins b and c were the only major proteins which could not be characterized in this way because they did not react. Experiments are under way to obtain specific antisera which can be applied in several immunological tests. None of the outer membrane proteins had biochemical properties which are characteristic for pore proteins as found for *Enterobacteriaceae* (17), i.e., none were (i) pep-tidoglycan associated in 2% SDS at 60°C, although protein c appeared to be peptidoglycan associated in the presence of 0.5% Triton X-100–0.2% SDS at a low temperature (37°C), (ii) visible as dimers and trimers if non-heat-modified samples were applied to SDS gels, or (iii) cross-reactive with the porins of *E. coli* K-12. However, in *Neisseria gonorrhoeae* (7), porins are also not peptidoglycan associated. Although the heat-modifiable proteins of *H. influenzae* and *E. coli* were cross-reactive immunologically, the lack of cross-reaction between the porins of *E. coli* and some proteins of *H. influenzae* is not unexpected, since porins are less well conserved in evolution than heat-modifiable proteins (4).

Heat-modifiable protein d plays an important role in the definition of various protein subtypes. Insufficient heating of the samples in SDS resulted in variable amounts of both the modified and unmodified forms (2, 3, 12, 13). Treatment for 10 min in a boiling water bath is therefore required as a standard procedure.

SDS-polyacrylamide gel electrophoresis on SDS-urea gels appeared to be a useful addition for the analysis of cell envelope proteins, since a protein band with a molecular weight of 39,000 was resolved which comigrated with proteins b and c in gels without urea. This outer membrane protein was resolved in many *H. influenzae* strains investigated, consistently yielding the same molecular weight.

Proteins with properties similar to those of *H. influenzae* type b 760705 (heat modifiability, sensitivity to trypsin, and immunological cross-reactivity) were identified in a variety of both encapsulated and nonencapsulated strains, even though they had different molecular weights (with the exception of protein e). This implies that these proteins may be useful for vaccine purposes, since they share common physical properties or are immunologically cross-reactive. Such studies are currently in progress in several laboratories (1, 24). In this respect, it is interesting that antisera raised in rabbits against cells of *H. influenzae* type b 760705 reacted in GIRA not only with major proteins of the homologous strain but also with proteins of various other *H. influenzae* strains, indicating that they also have antigenic sites in common (unpublished data).

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**LITERATURE CITED**


