Isolation of a Major Cell Envelope Protein from Fusobacterium nucleatum

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A major, heat-modifiable cell envelope protein was identified in Fusobacterium nucleatum FDC 364 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This protein, designated HM-1, had apparent molecular weights of 38,500 and 50,000 when heated in sodium dodecyl sulfate at 50 and 100°C, respectively. Whole cells were labeled with 125I, and the results suggested that the HM-1 protein may be exposed on the bacterial surface. The HM-1 protein was isolated in association with the peptidoglycan by extraction of whole cells or cell envelopes with 2% sodium dodecyl sulfate at 55°C. Heating the peptidoglycan-HM-1 protein complex in the detergent at 100°C resulted in the quantitative release of the protein. Isoelectric focusing experiments and amino acid analysis revealed that the HM-1 protein had a basic character and was moderately hydrophilic. Various strains of F. nucleatum as well as three oral fusiform isolates contained a serologically related protein. The abundance and location of the HM-1 protein in F. nucleatum suggest that it has the potential to participate in cell surface-related interactions of this bacterium.

Members of the genus Fusobacterium constitute a group of obligately anaerobic, gram-negative bacteria which commonly inhabit both animals and humans. It has been reported that fusobacteria are often recovered from clinically significant anaerobic infections, which include orofacial, peritonsillar, lung, brain, hepatic, and intraabdominal abscesses, aspiration pneumonia, empyema, septicemia, and endocarditis (2, 11, 18). One species, Fusobacterium nucleatum, represents one of the predominant members of the cultivable oral flora of the human adult. Socransky and co-workers (42) have shown that the most frequently detected microbial species (86% incidence) in 43 subgingival sites in 15 periodontitis patients was F. nucleatum, 2nd in a separate study, Moore et al. (32) revealed that this species was the most common gram-negative isolate, constituting 8.2% of all the isolates from 77 subgingival samples, occurring at least once in 52% of the specimens. As these investigators have stated, F. nucleatum is a common pathogen in other body sites; its virulence may be mediated through the production of collagenase and large amounts of butyric acid and thus is a reasonable suspect for contributions to the etiology of periodontal disease. In the oral cavity, this bacterium increases in number with the maturation of dental plaque (26), possibly through direct interactions with the oral streptococci (25). The accumulation of gram-negative anaerobic bacteria in dental plaque is associated with the onset of periodontal disease (39), and patients with periodontal disease often demonstrate significantly elevated serum antibody titers to strains of F. nucleatum (4, 17). In addition, F. nucleatum exhibits, in vitro, various biological activities related to oral pathology and inflammation (5, 12, 35, 43, 44).

Cell surface proteins of gram-negative bacteria are often intricately involved in the adherence, aggregation, substrate transport, and antigenic character of that cell (see reference 15 for a review). These proteins have been extensively characterized in the enteric bacteria (15) as well as in other gram-negative species representing the genera Pseudomonas (19) and Neisseria (22). In these cells, outer membrane proteins function as specific substrate-binding proteins (20, 45), passive diffusion pores (7, 33), cell surface antigens (13, 23), and mitogens (10, 30). Few investigations concerning the outer membrane of the fusobacteria have been undertaken despite their increased numbers in dental plaque and possible role in periodontal disease. In this study, the major cell envelope protein of F. nucleatum, designated HM-1, was isolated and characterized, and its properties are discussed in relation to the outer membrane proteins of other gram-negative bacteria and the possible role of the protein in cell surface interactions of this bacterium.

MATERIALS AND METHODS

Bacterial strains and culturing conditions. The strains of F. nucleatum used in this study and their sources are listed in Table 1. Cells were routinely cultured in screw cap tubes or bottles containing brain heart infusion broth (Difco Laboratories) supplemented with 0.2% yeast extract (Difco), 0.5 mg of l-cysteine hydrochloride per ml, and freshly prepared 0.5% sodium bicarbonate in a hydrogen-carbon dioxide atmosphere (GasPak; BBL Microbiology Systems) at 37°C for 48 h. Clinical strains were recovered from periodontal pocket material; the samples were collected with sterile curettes, placed immediately into prereduced culture medium, and incubated anaerobically for 18 h. Dilutions of these enrichment cultures were then plated on the selective medium of Walker and co-workers (49). Single colonies were selected after 48 to 72 h of growth. The clones were serotyped with an anti-whole cell serum prepared against F. nucleatum 364, and their cell envelope polypeptide patterns were examined. Based upon these criteria, three strains (from a total of two patients) were obtained. Stock cultures were maintained frozen in 20% glycerol at −70°C. It has been reported that bacterial polypeptide profiles can be used to type oral isolates at the species level (31).

Preparation of cell envelope. Cell envelope and soluble protein fractions were isolated as reported previously (14).
Briefly, cells harvested from a 4-liter culture (48-h cultures) were washed in 200 ml of 10 mM Tris-hydrochloride, pH 8.0, and disrupted by sonication. Unbroken cells were removed by centrifugation at 12,100 × g for 10 min (Sorvall SS-34 rotor), and the envelope fraction was sedimented by centrifugation at 85,000 × g for 60 min (Spinco 50 Ti rotor). The protein that remained in suspension after the high-speed centrifugation was designated the soluble protein fraction and contained both cytoplasmic and periplasmic protein.

Isolation of the peptidoglycan and HM-1 protein. The cell envelope fraction was prepared from a 6-liter culture of \textit{F. nucleatum} 364 as described above and then extracted with 16 ml of a solution containing 2% sodium dodecyl sulfate (SDS) and 10% glycerol in 10 mM Tris-hydrochloride (pH 7.4) for 45 min in a water bath adjusted to 55°C (38). The extraction mixture was centrifuged at 85,000 × g for 30 min, and the pellet was reextracted with the same volume of SDS-glycerol buffer. The resulting pellet (SDS-insoluble fraction), representing peptidoglycan-protein complex, was washed five times with deionized water to remove residual detergent and was used in this form in some experiments. To isolate the HM-1 protein, the SDS-insoluble fraction (peptidoglycan-protein complex) was suspended in 10 ml of SDS-glycerol extraction buffer and heated in a boiling water bath for 15 min. This preparation was centrifuged at 85,000 × g for 30 min to separate the insoluble peptidoglycan from the released protein (HM-1 protein fraction). The pellet material was washed five times with deionized water and finally lyophilized. From 3.4 g of cells (dry weight), 15.3 mg of material was obtained and judged to be relatively protein-free based upon total protein determinations, SDS-gel electrophoresis, and amino acid analysis.

The supernatant fraction (HM-1 protein fraction) from the initial SDS-glycerol extraction at 100°C contained the majority of the protein present in the original SDS-insoluble fraction (peptidoglycan-protein complex). This fraction (10 ml) was dialyzed against 1 liter of a buffer containing 10 mM sodium phosphate (pH 7.0), 1% SDS, and 1 mM dithiothreitol for 2 h at room temperature. After dialysis, glycerol was added to the sample (final concentration, 2%), and the sample was applied to a column of Sepharose 6B (5 by 60 cm; 900-ml bed volume) previously equilibrated in the same buffer. Fractions (5 ml) were collected at a flow rate of 0.5 ml/min. The absorbance of each fraction was measured at 280 nm (Beckman DU spectrophotometer), and 25 μl of each of those fractions that corresponded to protein peaks was analyzed on SDS gels. Those fractions that contained the HM-1 protein were pooled and precipitated with acetone (final concentration, 60%) at room temperature. The precipitate was suspended in 1 ml of deionized water and reprecipitated. This procedure was repeated two additional times to remove the SDS, and the final precipitate was suspended in deionized water, lyophilized, and stored at −70°C.

Peptidoglycan sacculi were isolated for 125I-labeling experiments essentially as described above for the isolation of peptidoglycan-protein complex, except that the starting material was whole, washed cells rather than the cell envelope fraction.

Radioactive labeling of intact cells and peptidoglycan sacculi. Whole cells of \textit{F. nucleatum} 364 were washed three times in phosphate-buffered saline (PBS; 10 mM sodium phosphate [pH 7.2], 0.85% NaCl) and resuspended in PBS at 1:500 ml/ml for the isolation of peptidoglycan-protein complex, except that the starting material was whole, washed cells rather than the cell envelope fraction.

\textbf{Radioactive labeling of intact cells and peptidoglycan sacculi. Whole cells of \textit{F. nucleatum} 364 were washed three times in phosphate-buffered saline (PBS; 10 mM sodium phosphate [pH 7.2], 0.85% NaCl) and resuspended in PBS at 1,000 Klett units. Portions of these suspensions, 200μl in each case, were labeled with 100μCi of 125I (16.2 μCi/μg; Amersham Corp.) by the lactoperoxidase method as described by Bjorck and Kronvall (6).}

\textbf{Electron microscopy. Samples (5 μl) were placed on copper grids coated with carbon-Formvar. The excess liquid was drawn off, and the grids were washed in filtered, deionized water. A drop of 1% ammonium molybdate in 0.5% ammonium acetate was placed on the grid, and the grids were dried thoroughly under vacuum. Grids were examined in a Philips EM300 electron microscope operated at an acceleration voltage of 60 KV.}

\textbf{Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis and autoradiography were performed on 17.5% slab gels according to a previously published procedure (16). Isoelectric focusing experiments were performed in the presence of urea and a nonionic detergent, Nonidet P-40, according to Ames and Nakaido (1). Gels were cast in acid-washed glass tubes (3 mm [inside diameter] by 130 mm [length]) and contained 2% ampholines, pH range 9 to 11 and 7 to 9, in a ratio of 3:2 (vol/vol). The anode and cathode buffers were 10 mM H3PO4 and 20 mM NaOH, respectively, and electrophoresis was performed at 300 V for 18 h followed by 500 V for 2 h. Gels were stained and destained as previously described (1). Electrophoretic blotting of proteins to nitrocellulose was performed by the procedure of Towbin and co-workers (46).}

\textbf{Protein chemistry. Total protein concentration was determined by the method of Lowry et al. (28), employing bovine serum albumin as a standard. For amino acid analysis, 1 mg of protein was hydrolyzed in 1 ml of 6 N hydrochloric acid in evacuated tubes at 105°C for 24 and 72 h. Peptidoglycan (2 mg) was hydrolyzed in 6 N hydrochloric acid for 14 and 18 h, and both amino acids and amino sugars were determined on the amino acid analyzer. Analyses were performed on a Mark model M7800 amino acid analyzer. Protein hydrophobicity was calculated from the amino acid composition by the methods of Capaldi and Vanderkooi (9, 47) and Hatch (21).}

\textbf{Immunological methods. A 100-ml culture of \textit{F. nucleatum} 364 was grown for 48 h, harvested, washed twice in PBS, and resuspended in sterile PBS at an absorbance of 0.8 at 540 mM. The cells were heat killed at 55°C for 45 min, dispensed in 1-ml samples, and stored frozen at −20°C. New Zealand albino rabbits were injected subcutaneously in the back with 1 ml of the cell suspension. Injections were made twice a}
week for 3 weeks, and 1 week later blood was collected by cardiac puncture. The serum was collected, titred by hemagglutination inhibition, and stored at −20°C. Protein HM-1 was isolated as described above, and antiserum was produced as described for whole cells. Protein (100 μg) per injection was mixed 1:1 (vol/vol) with complete Freund adjuvant.

RESULTS

Cell envelope polypeptides of *F. nucleatum*. *F. nucleatum* 10953, 25586, and 364 were grown under anaerobic conditions for 48 h, and the cell envelope fractions were recovered. The polypeptide composition of the envelope fractions were then compared by SDS-polyacrylamide gel electrophoresis (Fig. 1). In initial experiments, samples were prepared for analysis by heating at 100°C in the presence of SDS, and as observed, complex polypeptide profiles were obtained for each strain (Fig. 1, lanes A, C, and E). On the basis of apparent molecular size, specific polypeptides were common to some strains and were lacking in others; however, the most striking feature was the presence in each strain of a predominant polypeptide which had an apparent molecular weight of ca. 55,000 to 50,000 (see arrows in Fig. 1, lanes A, C, and E). When the cell envelope fractions were heated at 50°C before electrophoresis, the major 50,000 polypeptide was lost, and a previously unobserved polypeptide having increased electrophoretic mobility was obtained in each case (Fig. 1, arrows in lanes B, D, and F). The apparent molecular weights of the new polypeptides, after the solubilization of samples at 50°C, were 41,000 (strain 10953) and 38,500 (strains 25586 and 364). Since major heat-modifiable outer membrane proteins were known to exist in other gram-negative bacteria (15, 19, 37), an attempt was made to determine the relationship between the 50,000- and 38,500-molecular-weight polypeptides in *F. nucleatum* 364.

Identification and localization of the major heat-modifiable protein. Several heat-modifiable outer membrane proteins, such as OmpC and OmpF in *Escherichia coli*, can be recovered in association with the peptidoglycan (29, 38). A similar association of the 50,000-molecular-weight protein of *F. nucleatum* with the peptidoglycan would permit the rapid isolation of this protein. Therefore, the cell envelope fraction from *F. nucleatum* 364 was extracted with 2% SDS–10 mM Tris-hydrochloride (pH 7.4)–10% glycerol at 55°C, a procedure previously employed for the extraction of the OmpF protein of *E. coli* (38). The SDS-insoluble material (peptidoglycan-protein complex), which was collected by centrifugation, was then analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2, lane A). The SDS-insoluble preparation was significantly enriched for the 38,500-molecular-weight protein. Alternatively, only a trace level of this protein was detected in the SDS-soluble extract (data not shown). When the SDS-insoluble material (Fig. 2, lane A) was heated at 100°C before gel electrophoresis, the major detectable protein had an apparent molecular weight of 50,000 (Fig. 2, lane B). These results suggested that the major cell envelope protein of *F. nucleatum* demonstrated a heat-modifiable property. This protein was designated HM-1.

To localize the HM-1 protein, whole cells rather than isolated envelopes were extracted with the SDS-glycerol buffer at 55°C, yielding large rod-shaped fragments of peptidoglycan (sacculi). The appearance of these sacculi could be observed in negative-stained preparations (Fig. 3B). The peptidoglycan sacculi were labeled with 125I, heated at 50°C, and subjected to gel electrophoresis. Autoradiography revealed the presence of the HM-1 protein (Fig. 4, lane A). Treatment of the 125I-labeled peptidoglycan sacculi with trypsin resulted in the release of the HM-1 protein (Fig. 4, lane B). However, it appeared that the trypsin cleaved a small fragment (molecular weight, 3,500) of the HM-1 protein as evidenced by a reduction in the apparent molecular weight of the HM-1 protein.
weight. Similar results were obtained with other proteases, such as pronase. However, peptidoglycan backbone-degrading enzymes such as lysozyme and lysostaphin failed to release the HM-1 protein. When whole cells of \textit{F. nucleatum} 364 were labeled with $^{125}$I, the HM-1 protein was the major radioactive product (Fig. 4, lane C). Radiolabeled whole cells contained additional $^{125}$I-labeled proteins which were absent in the peptidoglycan preparation. These results suggested that the HM-1 protein may reside in the outer membrane, with part of the protein in association with the peptidoglycan and part exposed on the cell surface.

**Purification and partial characterization of the HM-1 protein.** As demonstrated above, the HM-1 protein could be recovered in association with the peptidoglycan by extraction of cell envelopes or whole cells at 55°C, thus affording an initial concentration step for the purification of this protein. The SDS-insoluble fraction (peptidoglycan-protein complex) was resuspended in the SDS-glycerol buffer and heated at 100°C to release the HM-1 protein from the peptidoglycan. The protein is efficiently extracted by this procedure; however, it is irreversibly heat denatured since it exhibits the slower electrophoretic mobility even when solubilized at 50°C (data not shown). Repeated freezing to −20°C and thawing followed by heat treatment at 50°C did not reverse the effects of the high-temperature extraction. The SDS-insoluble material after extraction at 100°C (peptidoglycan fraction) contained only trace amounts of total protein. Chemical analysis of this material indicated that the sample consisted primarily of peptidoglycan, since amino acid analysis revealed the presence of muramic acid, glucosamine, the amino acids alanine and lanthionine, and an unidentified acidic, ninhydrin-positive component in a molar ratio of 2:1:1:1. The absence of contaminating protein was supported by the finding that only trace levels of other amino acids could be detected.

The HM-1 protein fraction, free from the peptidoglycan, was then dialyzed against 10 mM sodium phosphate buffer (pH 7.0) containing 1% SDS and 1 mM dithiothreitol and applied to a preparative Sepharose 6B column which was previously equilibrated in the same buffer. Fractions (5 ml) were collected, and the absorbance was measured at 280 nm (Fig. 5). Samples of each peak fraction were examined by electrophoresis (Fig. 5, insert), and those fractions containing the purified HM-1 protein were pooled (fractions 97 to 105). Beginning with 3.4 g of cells (dry weight), 3.4 mg of protein was recovered. Other gel filtration media were also
used in attempts to improve protein separation; however, the HM-1 protein, along with other contaminating proteins, eluted in the void volume of Sephadex G200, and chromatography on Bio-Gel P300 did not improve the separation which was achieved on the Sepharose gel. Elution of the HM-1 protein in the void volume of the Sephadex G200 column was apparently due to aggregation of the protein in SDS-buffer at room temperature. Resolution, during purification by chromatographic methods, was hampered by the insolubility of the protein in aqueous buffers and organic solvents. Attempts were also made to recover the protein from the peptidoglycan by nondenaturing (low temperature) treatments, such as high-salt extraction; however, these methods were unsuccessful.

A partial amino acid composition of the HM-1 protein is presented in Table 2, and several interesting features are evident. The protein appears to lack methionine and proline and has a basic character, containing ca. 33% basic residues. The basic nature of the protein was confirmed by isoelectric focusing, which revealed an isoelectric point in the range of 9.5 to 9.8 as determined in urea gels. The protein also appeared to be unusually hydrophilic when compared with other integral membrane proteins having a polarity index of 55 to 63% (depending upon the method used) and a ratio of polar to apolar residues of 2.5.

**Strain distribution of the HM-1 protein.** Antiserum was prepared with heat-killed cells of *F. nucleatum* 364, and this serum was reacted with cell envelopes isolated from the various strains listed in Table 1. In immunodiffusion experiments, the anti-whole cell serum showed at least three major precipitin bands with the cell envelope fraction from *F. nucleatum* 364. No reaction was observed when preimmune serum was employed. The cell envelope antigens did not cross-react with lipopolysaccharide isolated from the same strain. However, extensive cross-reactions were observed between several of the cell envelope antigens of *F. nucleatum* 364 and the envelope preparations from all other strains tested (data not shown). The envelope antigens from *F.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues/polypeptide&lt;sup&gt;a&lt;/sup&gt; (mol/mol)</th>
<th>Nearest integer&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>Lysine</td>
<td>37.4</td>
<td>37</td>
</tr>
<tr>
<td>Histidine</td>
<td>30.4</td>
<td>30</td>
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<tr>
<td>Arginine</td>
<td>14.5</td>
<td>15</td>
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<tr>
<td>Aspartic acid</td>
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<td>17</td>
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<tr>
<td>Threonine</td>
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<tr>
<td>Serine</td>
<td>14.1</td>
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<tr>
<td>Glutamic acid</td>
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<tr>
<td>Proline</td>
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<tr>
<td>Glycine</td>
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<tr>
<td>Half-cystine</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
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<tr>
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<tr>
<td>Tryptophan</td>
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<sup>a</sup> 24-h hydrolysis; based on 5 isoleucinyl residues per polypeptide chain.<br/>
<sup>b</sup> Total of 249.<br/>
<sup>c</sup> ND, Not determined.
nucleatum) 364 showed complete homology with those from F. nucleatum EUJ-6 and EIF-4. It has been reported that these two strains demonstrate close to 100% DNA homology (Y. Salin and J. L. Johnson, J. Dent. Res. [Spec. Issue A] 60:415, 1981). Based upon DNA homologies, immunodiffusion studies, and poly peptide profiles, it appeared that F. nucleatum EUJ-6, EIF-4, and 364 were identical. Anti-HM-1 serum was used to identify the HM-1 protein, or a serologically related protein, in those strains of F. nucleatum listed in Table 1. Peptidoglycan-protein complexes (SDS-insoluble fractions) were prepared and subjected to SDS-polyacrylamide gel electrophoresis. Protein was transferred to nitrocellulose by electrophoretic blotting, and the blots were incubated with anti-HM-1 serum. The results are shown in Fig. 6. When the protein samples were solubilized at 100°C, a major polypeptide with a molecular weight of 50,000 to 55,000 was detected in each strain (see arrow in Fig. 6). Several other proteins were also detected on the immunoblot and were probably the result of antibodies produced against minor protein contaminants in the HM-1 antigen preparation or fragments of the HM-1 protein produced during the isolation of the antigen.

**DISCUSSION**

In this study, a major heat-modifiable, peptidoglycan-associated cell envelope protein (HM-1) was identified, isolated, and partially characterized from F. nucleatum. The HM-1 protein of F. nucleatum appears to be related to the matrix protein class of peptidoglycan-associated proteins since it is noncovalently bound to the peptidoglycan, demonstrates heat-modifiable properties, and is apparently exposed on the surface of the cell. However, a more detailed investigation of the location of the HM-1 protein will have to be initiated to confirm its exposure on the cell surface in light of recently published data (27). Coincidentally, the HM-1 protein also exhibits properties which are similar to those of the OmpA protein, a major outer membrane protein which is not attached to the peptidoglycan in E. coli (37). Outer membrane proteins which are similar to the OmpA protein have been characterized in various genera of gram-negative bacteria (3) and have been identified in other oral gram-negative species (16). Like the OmpA protein, the HM-1 protein is heat modifiable and demonstrates an increase in molecular weight from 38,500 to ca. 50,000 when heated in the presence of SDS at 50 and 100°C, respectively. The HM-1 protein may also be relatively trypsin resistant when in complex with the peptidoglycan. Under these conditions, a fragment of the protein (molecular weight, 3,500) is removed, and the remainder of the protein is left intact. Trypsin treatment of the OmpA protein of E. coli yields similar results, except that the protection is mediated by lipopolysaccharide (41). Although the HM-1 protein of F. nucleatum exhibits properties which are similar to those of several major outer membrane proteins in E. coli, compositionally this protein is quite distinct. The amino acid compositions of the matrix proteins of E. coli, Salmonella typhimurium, and Neisseria gonorrhoeae have been reported, and they indicate a high degree of relatedness (7). By comparison, the HM-1 protein is considerably more basic and hydrophilic in nature.

The highly basic character of the HM-1 protein suggests that it has the potential to interact with negatively charged molecules such as lipopolysaccharide and phospholipids, a property which may have important implications in relation to the assembly and organization of this protein in the gram-negative outer membrane and pathogenic mechanisms based upon interactions of the protein with host cell membranes. Accordingly, the functions of the HM-1 protein are currently being pursued, especially in regard to its role as a passive diffusion pore-forming protein and as a possible cell surface receptor in corn cob formation. It seems likely that the HM-1 protein could have a generalized function in F. nucleatum since this protein, or a serologically related one, is found in all strains examined to date (36) and one of the most predominant envelope proteins in these cells. Although the HM-1 protein exhibits molecular size heterogeneity, this is not an unusual occurrence, since clinical human isolates of E. coli contain OmpA, OmpC, and OmpF proteins which demonstrate considerable heterogeneity (3, 36). Since the HM-1 protein was isolated by the same procedure employed by Rosenbusch (38) for the recovery of the matrix proteins, its apparent mode of binding to the peptidoglycan could be similar. However, the association appears to be more hydrophobic than ionic since the HM-1 protein could not be extracted with various concentrations of sodium chloride. The peptidoglycan of F. nucleatum is typical of gram-negative peptidoglycans, except for the replacement of diaminopimelic acid by lanthionine, a diamino, dicarboxylic sulfur-containing amino acid (24, 48). The composition of the peptidoglycan of F. nucleatum 364, as reported in this study, was consistent with published findings for this species. Although lanthionine was present in this peptidoglycan, it was not a constituent of the HM-1 protein.

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LITERATURE CITED


CELL ENVELOPE PROTEIN OF *F. NUCLEATUM* 393


