Purification and Partial Characterization of a Major Outer-membrane Protein of *Fusobacterium nucleatum*

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The major outer-membrane proteins of 40–41 kDa were identified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in *Fusobacterium nucleatum* strains ATCC 10953, ATCC 25586, F3, F6 and Fev1. The proteins were purified by preparative gel electrophoresis. Their behaviour in gel filtration and gel electrophoresis, their sensitivity to proteolytic enzymes, and their amino acid composition were investigated. The purified proteins were partly sequenced from the N-terminal end. A 36.5 kDa portion was protected against extrinsic proteolytic (trypsin, chymotrypsin or pronase) digestion of whole cells. This polypeptide was isolated and partially sequenced from the N-terminal end. From these data and data from extrinsic iodination it was concluded that the N-terminal end of the protein is probably exposed on the surface of the cell. A database search revealed amino acid sequence similarity in an Ala-Pro-rich region of outer-membrane protein A (OmpA) in other Gram-negative bacteria.

INTRODUCTION

The Gram-negative anaerobic bacterium *Fusobacterium nucleatum* is one of the most common inhabitants of subgingival plaque (Socransky, 1970), and it possibly plays a role in the development of periodontal disease (Dzink *et al.*, 1988; Socransky *et al.*, 1988). The outer membrane of *F. nucleatum* shares many features with those of other Gram-negative bacteria (Bakken & Jensen, 1986; DiRienzo & Spieler, 1983). Extensive work on the structure and function of the outer membrane in Gram-negative bacteria has shown that its protein components can act as receptors for bacteriophages, form channels through the membrane and contribute to the structure of the cells (see Lugtenberg & Van Alphen, 1983), and are involved in the pathogenicity of these organisms (Buchanan & Pearce, 1979; Blake & Gotschlich, 1982). Takada *et al.* (1988) reported on an outer-membrane protein from *F. nucleatum* ATCC 10953 with a molecular mass of 37 kDa in its native state. This protein was able to form pores in an artificial liposome membrane and to exhibit immunobiological activities (Takada *et al.*, 1988). Recently, Kaufman & DiRienzo (1989) showed that this protein functions as a receptor in the coaggregation between *F. nucleatum* ATCC 10953 and *Streptococcus sanguis* CC5A. Understanding the interactions between parasite and host requires knowledge at the molecular level of the structures involved. In this study we present data on major outer-membrane proteins of some *F. nucleatum* strains (ATCC 10953, ATCC 25586, F3, F6 and Fev1) with molecular masses of 40–41 kDa in the denatured state. These proteins are presumably the same as those described by Kaufman & DiRienzo (1989) and by Takada *et al.* (1988) for strain ATCC 10953. It has been suggested that these proteins have a transmembrane position (Bakken & Jensen, 1986) and they share some sequence similarity in a Pro-Ala-rich region with OmpA in other Gram-negative bacteria.
METHODS

Bacteria. *F. nucleatum* Fev1 was originally provided by Dr S. E. Mergenhagen, Bethesda, Maryland, USA; strains ATCC 10953 and ATCC 25586 (the type strain) were from the American Type Culture Collection; strains F3 and F6 were provided by T. Hofstad, Bergen, Norway. Bacteria were grown anaerobically and harvested in the experimental phase of growth as described previously (Vasstrand et al., 1979).

Isolation of outer-membrane proteins. Outer membranes were isolated by selective solubilization of inner membranes in a total membrane fraction, using the non-ionic detergent Triton X-100 (Schnaitman, 1971; Bakken & Jensen, 1986). The Triton X-100 insoluble material, representing outer membrane in association with the peptidoglycan (Bakken & Jensen, 1986), was extracted by the differential heat extraction method of Rosenbusch (1974). Briefly, after incubation in 2%(w/v) SDS, 2%(v/v) 2-mercaptoethanol, 10 mM-Tris/HCl (pH 7.4) and 10%(v/v) glyceraldehyde at 22°C, the outer-membrane proteins still associated with the peptidoglycan were pelleted by centrifugation at 10000 g, and the proteins were then solubilized by heating in the same buffer at 100°C for 30 min, unless stated otherwise.

Gel filtration on Sepharose CL-6B. Proteins extracted with SDS were applied to a 2.5 x 40 cm column of Sepharose CL-6B (Pharmacia). The elution buffer contained 10 mM-Tris/HCl (pH 7.4), 2%(w/v) SDS, 10%(v/v) glyceraldehyde and 3 mM-NaCl. Fractions of 2 ml were collected.

Gel filtration on Sephacryl S-200. Two columns were used in series, 1.5 x 75 cm and 2.5 x 83 cm, respectively. The buffer contained 10 mM-Tris/HCl (pH 7.4), 2%(w/v) SDS, 10%(v/v) glyceraldehyde and 0.5 M-NaCl, and chromatography was performed at 37°C.

Maleylation of bacterial cells. Maleic anhydride treatment (Butler et al., 1969) was performed in 0.1 m-sodium hydrogen carbonate buffer (pH 8.0). Freshly prepared 1 m-maleic anhydride in dioxan was added in six aliquots at 5 min intervals. After each addition the pH was adjusted to 8.0 with NaOH. After the final addition the suspension was left on ice overnight. The insoluble material was removed by centrifugation at 10000 g and contained nearly all outer-membrane protein material.

SDS-PAGE, and electroelution of proteins. Electrophoresis was performed as described by Laemmlli (1970) on 12.5% polyacrylamide gels unless stated otherwise. For protein detection the gels were stained with Coomassie brilliant blue R250 or silver stained (Marshall & Latner, 1981) with the modifications described previously (Bakken & Jensen, 1986). Silver staining for detection of lipopolysaccharides (LPS) was performed according to Tsai & Frash (1982). On a preparative scale, gels 2-3 mm thick were used and 1-1.5 ml sample, containing about 500 μg protein, was applied to one lane about 100 mm wide; molecular mass standards were applied to narrow lanes, one on each side. Polypeptides were visualized either by staining the gel in Coomassie brilliant blue R250 solution or by a 30 min incubation at room temperature in 4 m-sodium acetate (Higgins & Dahmus, 1979). The area containing the actual protein in question was then excised, and the protein was electrophoretically eluted. The buffer used contained 50 mM-Tris, 60 mM-boric acid (pH 8.0) and 1%(w/v) SDS.

Two-dimensional gel electrophoresis. Purified outer-membrane proteins from intact bacterial cells and from trypsin-treated whole cells were analysed by isoelectric focusing in the presence of 9.5 M-urea (O'Farrell, 1975). Since some of the samples contained SDS, Nonidet P-40 was added in a 1:8 ratio (Ames & Nikaido, 1976). Pl markers (Pharmacia) were treated in the same manner. The first-dimension gels were subsequently analysed in the second dimension as described by O'Farrell (1975).

Treatment with proteolytic enzymes. Protease digestion of intact bacterial cells and isolated outer membranes was performed in 0.1 M-Tris/HCl (pH 7.4). Trypsin, chymotrypsin and pronase were used at enzyme-to-substrate ratios of 1:50 (w/w) as described by Schweizer et al. (1978).

Amino acid composition. Purified protein was hydrolysed in 6 M-HCl at 105°C for 20 h. Amino acid analyses were performed as previously described (Vasstrand et al., 1980).

Polarity indexes were calculated according to Capaldi & Vanderkooi (1972) and ratios of polar to apolar residues according to Hatch (1965).

Amino acid sequencing. Analyses were performed with an Applied Biosystems 470A microsequenator according to Hewick et al. (1981). The analyses of strain Fev1 were carried out at the Comprehensive Cancer Center, University of Southern California, California USA. Strains ATCC 10953, ATCC 25586, F3 and F6 were analysed at the Department of Biochemistry, University of Oslo, Norway.

Sequence similarity search. To compare the sequenced part of the major outer-membrane protein with known sequences the IBJ/Pustell Sequence Analysis Program, Version 1.80, was used.

Other methods. Bacteria were radio-iodinated by the method of Marchalonis (1969). Antigenic relationships between the various species was tested by immunoblotting with polyclonal antisera, raised in rabbits, against the 40 kDa protein of strain Fev1, against the 41 kDa protein of strain ATCC 25586, and against whole Fev1 cells. Electrophoretic transfer of gels was carried out on a semi-dry-blotting apparatus (LKB), with the buffers and further procedure as recommended by Towbin et al. (1979). Goat anti-rabbit IgG conjugates with horseradish peroxidase (HRP) (Bio-Rad), was used as second antibody. HRP colour development solution, with 4-chloro-1-naphthol (Bio-Rad) was used to develop the immunoblots.
RESULTS

Gel filtration

Attempts to isolate individual protein-species present in the SDS-containing solutions by chromatography on Sepharose CL-6B were unsuccessful, mainly because most of the proteins did not enter the gel. Solubility was greatly improved by maleylation, and a high degree of purification of maleylated 40 kDa protein was achieved using the Sepharose CL-6B column (Fig. 1). SDS-PAGE showed that very little outer-membrane protein was solubilized by the maleylation itself, but most proteins were now readily solubilized in SDS at room temperature. On the Sepharose CL-6B column the maleylated major protein ran just ahead of the cytochrome c standard, i.e. as a monomer (Fig. 1). On SDS-PAGE the protein appeared to have a molecular mass of about 54 kDa (Fig. 1, insets). The increase in molecular mass most probably reflects the amount of maleyl groups bound, and an effect on conformation and binding of SDS. Demaleylation was achieved by lowering the pH to 3.5 and incubating the sample at 37 °C for 15 h. However, staining the gels for LPS showed that LPS was very poorly or not at all separated from the maleylated protein (not shown).

Chromatography of SDS-solubilized proteins was successfully performed on Sephacryl S-200, run at 37 °C (Fig. 2). However, as is clear from the silver-stained gel (inset B), the bulk of LPS (60–80 kDa) runs with the 40 kDa protein. It is not known whether this takes place because of similar effective sizes or because LPS is associated with the 40 kDa protein under these conditions. Most efficient purification was achieved by preparative SDS-PAGE (Fig. 3). Apparently pure 40 kDa protein could be obtained starting either from peptidoglycan-associated outer-membrane proteins or from fractions from Sephacryl S-200 (see above) from which the 40 kDa protein was precipitated with 80% (v/v) acetone. Heat-modifiability was again observed with the major proteins isolated from strains ATCC 25568 (Fig. 4), ATCC 10953, F3 and F6 (not shown), when these proteins were isolated under conditions where the temperature never exceeded 37 °C. Strains ATCC 10953, ATCC 25586, F3 and F6 possessed two proteins with molecular masses of about 35 kDa and 37 kDa at 37 °C, which had the same (or nearly the same) mobility at 100 °C (40–41 kDa). Since these proteins have identical N-terminal amino acid sequences (see below), they are probably modified forms of the same protein, or related proteins. The portion of the protein that was protected against proteolytic enzymes seemed to confer the heat-modifiable property (Fig. 4, bands d and e).

Localization of the major protein in the outer membrane

The surface exposure of the major protein was investigated by two methods: by extrinsic radio-labelling of cells with 125I (Fig. 5, lanes 1 and 3), and by proteolytic digestion of the whole cells (Fig. 5, lanes 2 and 4) and outer membranes. A fragment of about 3.5 kDa was cleaved from the major protein (Figs. 3–5). Prolonged incubation of cells or isolated outer membranes with trypsin gave no further degradation of the major protein. On the other hand, trypsin readily digested the purified protein (not shown). Pronase and chymotrypsin gave apparently identical results, i.e. a polypeptide of approximately 36-5 kDa. This protein was protected from digestion when located in the membrane, but was readily degraded further once isolated (not shown).

By comparing lanes 2 and 4 in Fig. 5, it is apparent that most of the radiolabelling was removed from the 40 kDa FeVl protein by trypsin treatment of the whole cells. As judged from densitometric tracing of bands in Fig. 5, about 25% of the radioactive iodine remained associated with the protected 36-5 kDa polypeptide.

Isolation of the tryptic fragment

The large fragment of 36-5 kDa was readily isolated both on the Sepharose CL-6B column run at room temperature (Fig. 6) and by preparative gel electrophoresis. Apparently the protease-sensitive part rendered the protein less soluble in 2% (w/v) SDS.
Fig. 1. Chromatography on Sepharose CL-6B of maleylated outer-membrane proteins from *F. nucleatum* Fev1. Inset A, SDS-PAGE analysis of fraction numbers 44, 80 and 92. Inset B, SDS-PAGE analysis of fraction numbers 70, 72–81 and 83. The position of the 40 kDa protein is indicated on the right. ST, molecular mass standards. The first and last peak, represented by fraction numbers 44 and 92, respectively, contained very little protein. The gels were stained with Coomassie brilliant blue R250.

Fig. 2. Chromatography on Sephacryl S-200 of SDS-solubilized outer-membrane proteins from *F. nucleatum* Fev1. Insets A and B, SDS-PAGE of fraction numbers 27, 29, 31, 33, 35, 37 and 39. The gel in inset A, stained with Coomassie brilliant blue R250, was dominated by the 40 kDa protein (lanes with fraction numbers 33–39); the gel in inset B was silver-stained for protein (the heavy staining in the 60–80 kDa region is due to the presence of LPS). Molecular mass standards (kDa) are indicated on the right of inset B.
Fig. 3. SDS-PAGE analysis of isolated major outer-membrane protein of *F. nucleatum* Fev1. Lane 1, typical starting material for electro-elution of the outer-membrane protein: SDS-solubilized, peptido-glycan-free outer-membrane preparation (heated in 2%, w/v, SDS at 100 °C for 30 min). Lane 2, purified (by electro-elution) 40 kDa protein. Lane 3, trypsin-insensitive fragment of the 40 kDa protein (obtained when whole bacterial cells were treated with proteolytic enzymes and isolated by gel filtration on Sepharose CL-6B). The molecular mass of the fragment (tr40 kDa) was 36.5 kDa. Lane 1 is stained with Coomassie brilliant blue; lanes 2 and 3 are silver-stained for protein.

Fig. 4. Heat-modifiability of the major outer-membrane protein in *F. nucleatum* ATCC 25586 (SDS-PAGE analysis of outer-membrane). (a) ST, molecular mass standards (as in Figs 1 and 2); lane 1, Triton X-100 insoluble fraction heated at 37 °C for 5 min; lane 2, Triton X-100 insoluble fraction heated at 100 °C for 5 min; lane 3, Triton X-100 insoluble fraction of trypsin-digested whole bacterial cells heated at 37 °C for 5 min; lane 4, as lane 3, but heated at 100 °C for 5 min. (b) The protein bands indicated by the small letters on the gel in (a) were cut out of the gel, heated at 100 °C for 5 min and analysed by SDS-PAGE. The proteins indicated with *a* and *b* have molecular masses of about 35 and 37 kDa respectively, when heated at 37 °C. They showed the same mobility after heating at 100 °C, namely 41 kDa. When heated at 100 °C, the large fragment after trypsin-digestion (protein band *d*) migrated the same distance as *e*. The gels were stained with Coomassie brilliant blue R250.
Fig. 5. Localization of the major outer-membrane protein (SDS-PAGE analysis of outer-membrane proteins from cells of *F. nucleatum* Fev1 labelled with $^{125}$I-lactoperoxidase and treated with pronase). Lane 1, isolated outer membrane; lane 2, Triton X-100 insoluble fraction, isolated from pronase-digested cells; lanes 3 and 4, $^{125}$I-labelled cells, isolated as 1 and 2, respectively. Lanes 1 and 2 were stained with Coomassie brilliant blue R250; proteins in lanes 3 and 4 were detected by autoradiography. Only the area of interest (25-55 kDa) is shown.

Fig. 6. Isolation of the tryptic fragment from *F. nucleatum* Fev1 by chromatography on Sepharose CL-6B. ———, SDS-extracted proteins; ————, rerun of fraction numbers 60-80. Inset, SDS-PAGE analysis of the rerun fractions 65-79. The positions of molecular mass standards are indicated on the left. A protein of 36.5 kDa was isolated. The gel was stained with Coomassie brilliant blue R250.

**Amino acid composition**

The purified major outer-membrane proteins from the various strains were subjected to amino acid analysis. Table 1 shows the results from a single analysis of each protein species. Amino acid analysis of different batches of outer-membrane proteins gave consistent results from one experiment to another, indicating that the correct values are close to those given in Table 1. The
Major outer membrane protein of F. nucleatum

Table 1. Amino acid composition (mole percentages) and polarity of the major outer-membrane protein of F. nucleatum Fev1, ATCC 10953, ATCC 25586, F3 and F6

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<th>F3</th>
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Amino acid compositions of the various 40–41 kDa species were very similar. Largest differences were found between the charged amino acids, but the sums of aspartic acid and glutamic acid, and of arginine and lysine, are close among the various species. By isoelectric focusing the PI was determined to be about 10 (not shown). In order to explain such a basic nature of the proteins, one has to anticipate that the glutamic acid and aspartic acid residues are amidated to a considerable extent.

The polarity index (Capaldi & Vanderkooi, 1972) of the proteins was calculated to be in the region of 50%, and the ratio of polar to apolar residues (Hatch, 1965) was about 2:0 (see Table 1). No hexosamines were found in the preparations of the 40 kDa protein from strain Fev1, whereas the amino acid analysis indicated that the 41 kDa protein from strain ATCC 25586 contained a small amount of (modified) galactosamine.

Amino acid sequencing

Electrophoretically purified major proteins were subjected to N-terminal amino acid sequence analysis (Fig. 7). Only a few amino acids differed near the N-terminus of the various protein species. The N-terminal amino acid of the 41 kDa protein of strain ATCC 25586 could not be determined. This protein contained some amino acid substitutions in addition to an additional N-terminal amino acid residue. The tryptic fragment of the 40 kDa protein of strain Fev1, which was purified by chromatography on a Sepharose CL-6B column, overlapped with the sequence of the 40 kDa protein of strain Fev1 between residues 28 and 36. The identity of the amino acid residues in positions 26 and 27 is uncertain. The peptide containing 27 amino acid residues, which was removed from the N-terminus, is equivalent to a fragment of 3393 Da, which fits well with the shortening of the peptide as observed by SDS-PAGE (Figs 3–5).
Strain 10..20..25
Fev 1
ATCC 10953
F6
F3
ATCC 25586
30..35..40..45..50
Fev I
Fev I/Trypt
Glu-Val-Ala-Trp-Arg-Pro-Asn-
Gly-Val-Val-Glu-Val-Glu-

Fig. 7. N-terminal amino acid sequences of the 40-41 kDa outer-membrane proteins of *F. nucleatum* strains Fev1, ATCC 10953, F6, F3 and ATCC 25586. Fev1/Trypt represents the 36.5 kDa fragment of Fev1, isolated after trypsin treatment of whole cells. The bars indicate the extent to which sequence analysis was performed. ND, Not determined.

Bacterium Sequence similarity

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Fig. 8. Comparison of amino acid sequences of the 40–41 kDa protein of *F. nucleatum* strains and the OmpA protein of *Escherichia coli*, *Enterobacter aerogenes*, *Shigella dysenteriae* and *Salmonella typhimurium*.

Antigenic properties

Antigenic cross-reaction was observed between all the major proteins isolated from the various strains. When run at 37 °C the 35 kDa and 37 kDa protein bands of strains ATCC 10953, ATCC 25586, F3 and F6 reacted (not shown).

Sequence similarity search

The 40–41 kDa proteins of the strains of *F. nucleatum* showed similarity to a Pro-Ala-rich region of OmpA in *Escherichia coli*, *Enterobacter aerogenes*, *Shigella dysenteriae* and *Salmonella typhimurium* (Fig. 8).

DISCUSSION

Outer membranes were successfully isolated from cell envelopes from all the *F. nucleatum* strains by selective solubilization of inner membranes, using Triton X-100 (see Fig. 2 in Bakken & Jensen, 1986). Several attempts were made to purify individual protein species from these outer membranes, using different detergent and salt extraction procedures, without much success. The detergents used were CHAPS, Zwittergent, Sarkosyl and Triton X-114. These were used alone or in combinations, with and without MgCl2 or EDTA.

Data presented in this study and in earlier work (Bakken & Jensen, 1986) show that the dominant protein of the outer membrane of the different strains of *F. nucleatum* shares many characteristics with major outer-membrane proteins from other Gram-negative bacteria. The protein appears to be both associated with the peptidoglycan and exposed at the surface, and thus spans the outer membrane. With exception of the protein of strain Fev1, it is heat modifi-
Major outer membrane protein of F. nucleatum

The part of the protein exposed at the cell surface contains three Lys-Glu ionic pairs, and also the Pro-Ala-Pro-Thr-Pro-Ala-Pro region, which is very similar to that found in OmpAs. An outer-membrane protein (PIII) of Neisseria gonorrhoeae also contains an Ala-Pro repeat (Gotschlich et al., 1987). Such sequences may assume the conformation of a polyproline helix and attention has been drawn to the structure of the hinge region of immunoglobulins (Chen et al., 1980). Proline tends to induce bends in the protein backbone, and such bends may make important antigenic determinants. The importance of this sequence for binding of antibodies will be studied and further sequencing of these proteins in F. nucleatum is at present being carried out in our laboratory.

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REFERENCES


