Outer Membrane Proteins of *Fusobacterium nucleatum* Fev1

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(Received 13 May 1985; revised 27 November 1985)

Outer membrane enriched material from six strains of *Fusobacterium nucleatum* was analysed by SDS-PAGE. The protein profiles of all the strains were dominated by proteins with molecular masses of about 40 kDa, and a very high degree of homology in relation to apparent molecular masses was observed. In all strains except Fev1, one of the most dominant proteins exhibited heat modifiable properties, having an apparent molecular mass of about 38 kDa and 42 kDa when heated in SDS at 50 and 100 °C, respectively. None of the proteins of the outer membrane of *F. nucleatum* Fev1 demonstrated such heat modifiable properties. The 40 kDa protein, and several other proteins, appear to be both exposed on the cell surface and peptidoglycan associated.

INTRODUCTION

The genus *Fusobacterium* consists of obligately anaerobic Gram-negative rods having as their habitat the mucous membranes of man and animals; diseases caused by these bacteria show characteristic inflammation followed by ulceration and necrosis. *F. nucleatum* is a member of the oral microflora. It is regularly found in human dental plaque associated with periodontal disease (Socransky, 1970; Van Palenstein-Helderman, 1975), and it is always present in diseases like necrotizing ulcerative gingivitis (ANUG) and cancrum oris (Hofstad, 1981).

It is assumed that components of the outer membrane, among them proteins, are involved in the pathogenesis of infection by Gram-negative bacteria (Buchanan & Pearce, 1979; Hofstra *et al.*, 1980). Besides the polysaccharide portion of LPS, a number of outer membrane proteins can be exposed on the surface of the cell, and these may serve as diffusion pores, specific substrate binding site proteins and mitogens (DiRienzo *et al.*, 1978; Lugtenberg & Van Alphen, 1983). They may also serve as antigens and have been considered as vaccine candidates (Blaser *et al.*, 1984; Buchanan & Pearce, 1979; Gilleland *et al.*, 1984). The outer membrane proteins of fusobacteria have hitherto received little attention. Evidence has been presented indicating that outer membrane proteins are involved in corn-cob formation between *F. nucleatum* and *Streptococcus sanguis* (Lancy *et al.*, 1983). The capability of *F. nucleatum* to bind to erythrocytes of many species (Falkler & Hawley, 1977) is seemingly mediated by a fusobacterial haemagglutinin and evidence has been presented that this is a cell surface exposed protein or is dependent on such a protein for function (Dehazya & Coles, 1980, 1982). These molecular interactions presumably do not involve filamentous surface structures, since examination of cells and cell fragments by various techniques failed to demonstrate the presence of pili or flagella (Dahlén *et al.*, 1978). A group reactive protein antigen, designated P2, was isolated by Kristoffersen (1969*a,b*). DiRienzo & Rosan (1984) presented data on a heat-modifiable protein isolated from the envelope of *F. nucleatum* strain 364.

As a first approach towards an understanding of the role of the outer membrane proteins of *F. nucleatum* in pathogenicity, we decided to investigate the composition and localization of proteins in the cell wall. Various techniques for preparation of outer membrane enriched material were compared with respect to their suitability, and the protein composition was analysed by SDS-PAGE.

METHODS

Bacteria. *F. nucleatum* strains F1, F3, F6 and F14 were gifts from T. Hofstad, Bergen; ATCC 10953 was from the American Type Culture Collection, Rockville, Md., USA, and Fev1 was provided by S. E. Mergenhagen, Bethesda, Md. Bacteria were grown anaerobically in the medium described by Vasstrand *et al.* (1979), using ascorbic acid as the reducing agent, and were harvested in the mid to late exponential phase of growth.

Selective solubilization of inner membranes. The harvested cells were washed in cold 0.03 M-Tris/HCl, pH 8.1, resuspended in the same buffer and broken by one passage through a French pressure cell at about 3000 lbf in⁻² (20.7 MPa). After centrifugation at 2000 *g* for 5 min to remove unbroken cells (a very small pellet), the cell envelopes were sedimented by centrifugation at 10000 *g* for 15 min at 4 °C. The envelope fraction was washed twice by resuspension in 10 mM-HEPES/10 mM-MgCl₂ buffer, pH 7.4, followed by centrifugation as above. The non-ionic detergent Triton X-100 was added to 2% (v/v) concentration and the mixture incubated at room temperature for 20 min. After centrifugation at 10000 *g* for 15 min at 4 °C, the pellet was washed twice in 10 mM-HEPES/10 mM-MgCl₂. The protein content of the Triton X-100 insoluble material was estimated to be 30% of the dry weight (Table 1).

Sucrose density gradient centrifugation. The procedure used was in essence that described by Ito *et al.* (1977). Concentrations of sucrose between 50 and 60% (w/v) were used to separate the inner and the outer membranes according to their buoyant densities.

Radioiodination of bacteria. Labelling with ¹²⁵I was done according to the method of Marchalonis (1969). To 200 µl cell suspension (5 × 10⁸ cells ml⁻¹) in 0.02 M-phosphate buffer, 0.9% (w/v) NaCl, pH 7.1, was added 500 µCi (18.5 MBq) Na¹²⁵I, 5 µg lactoperoxidase and 8 × 10⁻⁵ M-H₂O₂. The reaction was stopped after 10 min with 10 µl 1 M-2-mercaptoethanol (2-ME), followed by 800 µl cell suspension. The cells were sedimented (10000 *g* for 10 min) and Triton X-100 insoluble cell envelope material was prepared as described above. After analysis by SDS-PAGE, the gels were laid onto Kodak X-Omat films for autoradiography.

SDS-PAGE. Membrane proteins were separated by SDS-PAGE according to the method of Laemmli (1970). The concentration of polyacrylamide in the separating gel was 15% (w/v) unless otherwise stated. The proteins were stacked in 4% polyacrylamide gel. Samples were heated in the sample buffer at 100 °C for 5 min, or at different temperatures in experiments to determine the degree of heat modifiability and peptidoglycan association. Samples containing 10–30 µg or 0.1–1 µg protein were applied to gels when they were stained with Coomassie brilliant blue R250 or silver, respectively. Silver staining was done according to Marshall & Latner (1981), with the modifications that all steps were done at 50 °C, employing ammonia in the diamine step, and 2% (v/v) glutaraldehyde. Bio-Rad low molecular weight protein standards were used as size markers.

Peptidoglycan association. Membrane fractions were suspended in a buffer containing 10 mM-Tris/HCl, pH 7.4, 10% (v/v) glycerol, 2% (w/v) SDS and 0.7% (v/v) 2-ME. The samples were incubated at 50 °C for 30 min then centrifuged at 10000 *g* for 15 min at 4 °C. The resulting pellet was either resuspended in the same extracting buffer and treated in the same manner, or it was resuspended in the sample buffer used for SDS-PAGE and boiled for 5 min before gel electrophoresis. The gels were scanned with a Zeineh scanning densitometer, soft laser 630 nm, adjusting in each case the intensity of the most dense peak to about 80% of full scale.

Electron microscopy. The procedure described previously (Vasstrand & Jensen, 1984) was followed throughout.

Other methods. Protein was estimated by a modified Lowry method as outlined by Markwell *et al.* (1978), and from amino acid analysis following hydrolysis in 4 M-HCl, 105 °C, 16 h, under vacuum (Vasstrand *et al.*, 1980). Heptose was determined by the cysteine/H₂SO₄ method according to Dische (1953). The difference in the absorbance between 510 and 540 nm was corrected for nonspecific absorbance in the blank and used as a relative measure of the concentration of heptose.

Lactate dehydrogenase and succinate dehydrogenase activities were analysed according to Owen *et al.* (1982), and ATPase according to Lanzetta *et al.* (1979).

RESULTS

The cell envelope was dominated by a few proteins (Fig. 1, lane 2). About 40% of the protein content of the cell envelope was soluble in Triton X-100 (Table 1; Fig. 1, lane 4), but the dominant proteins in the envelope fraction with molecular masses of 40 and 44 kDa were not soluble in this detergent (Fig. 1, lane 3). The amounts of lactate dehydrogenase and succinate dehydrogenase found in the envelope preparations were too low for them to be utilized as marker enzymes for the inner membrane, but ATPase was found exclusively in the Triton X-100 soluble fraction (Table 1). Heptose, a constituent of the LPS of the cell wall (Hofstad, 1979), was found exclusively in the Triton X-100 insoluble (outer membrane) fraction (Table 1). Attempts to isolate pure outer and inner membranes by isopycnic centrifugation on sucrose gradients (Ito *et al.*, 1977) failed. SDS-PAGE of heavy and light bands obtained by this procedure showed that

Fig. 1. SDS-PAGE analysis of proteins in *F. nucleatum* Fev1. Lane 1, whole bacterial cells; lane 2, isolated cell walls from French pressed cells; lane 3, Triton X-100 insoluble cell wall fraction; lane 4, Triton X-100 soluble cell wall fraction. Molecular mass standards (kDa) are indicated on the left. The gel was silver stained; 0.5 to 1.0 µg protein was applied to each lane.

Table 1. *Analysis of cell envelope fractions obtained by Triton X-100 extraction*

Cell envelopes were prepared from 2.2 l of cells (OD_{650} 0.51; wet weight 3.87 g). The values presented are the means of two (heptose and ATPase) or three (protein and peptidoglycan) analyses.

Fraction	Dry weight (mg)	Protein + peptidoglycan* (mg)	Peptidoglycan† (mg)	Heptose (%)	ATPase‡
Cell envelope	369	196	32	ND	ND
Triton soluble	97	43	0	0	2.0
Triton insoluble	215	83	22	100	0.0

ND, Not determined.

* From amino acid analysis.

† From amino acid analysis, calculated from muramic acid and lanthionine.

‡ Expressed as $\mu\text{mol ATP (mg protein)}^{-1} \text{ h}^{-1}$ at 37 °C.

Fig. 2. Electron microscopy of *F. nucleatum* Fev1. Sections through, intact cells (a), French pressed cells (b) and Triton X-100 insoluble material (c). CM, cytoplasmic membrane; OM, outer membrane, P, peptidoglycan layer. Bars, 100 nm.

the fractions were cross-contaminated by several proteins, and only approximately 65% of the heptose was recovered from the heavy and 35% from the light fractions.

The preparation of the cell wall fractions was also followed by electron microscopy. The three electron dense layers of the cell wall, corresponding to the outer membrane, the peptidoglycan and the cytoplasmic membrane, were observed both in whole cells (Fig. 2a) and in isolated cell walls (Fig. 2b). Less than 1% of the cells remained unbroken after the French press procedure.

Fig. 3. SDS-PAGE analysis of outer membrane proteins (Triton X-100 insoluble cell wall) of six strains of *F. nucleatum*. The samples were heated for 5 min at 50 °C (lanes 1, 3, 5, 7, 9 and 11) or 100 °C (lanes 2, 4, 6, 8, 10 and 12). Proteins with changed mobility and the 40 kDa protein of Fev1 are arrowed. Molecular mass standards (kDa) are indicated on the left. The gel contained 12.5% acrylamide and was stained with Coomassie brilliant blue.

The inner membrane could not be observed in the Triton X-100 insoluble cell wall material, whereas the double track layer of the outer membrane and the thin dark line, presumably representing the peptidoglycan, were still clearly visible (Fig. 2c). The peptidoglycan specific components lanthionine (Vasstrand *et al.*, 1979) and muramic acid were found exclusively associated with the outer membrane fraction (Table 1). The Triton X-100 insoluble material retained its nature as a membrane in close association with the peptidoglycan (Fig. 2c), and was not merely aggregates of proteins insoluble in the detergent.

Comparison between various strains of *F. nucleatum*

The polypeptide composition of the outer membranes (i.e. Triton X-100 insoluble fractions) of six strains of *F. nucleatum* was compared. Gel profiles of outer membrane proteins were very similar in all the strains (Fig. 3), as were the patterns of inner membrane proteins (data not shown). When heated at 50 °C before electrophoresis, some proteins exhibited an increased mobility (Fig. 3). This was particularly evident with the proteins of molecular mass about 40 kDa in all strains except Fev1, for which the profiles were identical at 50 and 100 °C. The results with strains, F1, F3, F6, F14, and 10953 were very similar to those described previously (Di Rienzo & Rosan, 1984) for 10953 and some other strains of *F. nucleatum*.

Fig. 4. SDS-PAGE analysis of outer membrane proteins from ^{125}I -lactoperoxidase labelled cells. Outer membrane proteins were isolated by selective solubilization of inner membranes with Triton X-100. Lanes 1-5: Triton X-100 insoluble fractions heated at different temperatures (37, 50, 60, 70 and 100 °C, respectively) before gel electrophoresis. Lane 6: proteins insoluble after heating at 50 °C for 30 min in 2% SDS. Lane 7: Triton X-100 soluble cell wall fraction. Protein bands were detected by autoradiography. Molecular mass standards (kDa) are indicated on the left.

Radioiodination of surface exposed proteins

The surface exposure of proteins of the outer membrane of *F. nucleatum* Fev1 was investigated by extrinsic radiolabelling of live cells with ^{125}I , by the lactoperoxidase method (Marchalonis, 1969). Outer membrane enriched material was isolated from these cells by Triton X-100 extraction of cell envelopes and analysed by SDS-PAGE and autoradiography (Fig. 4). Several proteins were isotopically labelled; again the 40 kDa protein was dominant (Fig. 4, lane 5). Also proteins with apparent molecular masses of 70, 50, 37, 32, 29, 19, 17 and 16 kDa were labelled to some extent (Fig. 4), and, according to this criterion, exposed on the cell surface. In particular, the 37 kDa protein was strongly labelled. On the other hand, no radioactive bands were evident in the Triton X-100 soluble cell wall material (lane 7), or from the cytoplasmic fraction (not shown), suggesting that the labelling indeed took place on the cell surface. Moreover, exactly the

Fig. 5. Peptidoglycan associated proteins. The SDS-PAGE gels were stained with Coomassie blue and scanned. The scans show peptidoglycan-protein complexes after one (a), two (b) and three (c) extractions with 2% SDS at 50 °C. The numbers on the peaks indicate molecular masses (kDa).

same profile was obtained if labelling was done for 2 min or for 10 min. When live *F. nucleatum* Fev1 cells were dansylated prior to SDS-PAGE analysis of the whole cells, a very similar protein profile to that seen in Fig. 4, lane 5, was observed under UV light. The ^{125}I -labelled outer membrane proteins were analysed with respect to heat modifiable properties (Fig. 4, lanes 1 to 5). The relative amounts of the 40 kDa protein increased on going from 37 via 50 to 60 °C (see below).

Association of peptidoglycan with the outer membrane proteins

The peptidoglycan associated proteins of *Escherichia coli* are not released from the peptidoglycan by heating at 60 °C in 2% SDS (Rosenbusch, 1974), and this method has been used to investigate peptidoglycan association in other Gram-negative bacteria. Gels stained with silver or Coomassie blue showed that the 40 kDa protein was only partly soluble in the sample buffer at 37 °C (not shown). Fig. 4, lane 6, shows the ^{125}I -labelled proteins that were not solubilized upon heating at 50 °C for 30 min in 2% SDS. In addition to the 40 kDa protein, several minor protein bands became visible. Compared to the outer membrane fraction (Fig. 4, lane 5) the pellet fraction was concentrated about eight-fold to obtain suitable amounts on the gel. From the relatively large increase in abundance of the 17 kDa protein compared to that of the 40 kDa protein (Fig. 4, lanes 5 and 6) one would expect the 17 kDa protein to be particularly strongly bound to the peptidoglycan.

Peptidoglycan association was also determined using unlabelled cells by repeating the Rosenbusch procedure (50 °C, 2% SDS, 30 min) on the insoluble peptidoglycan-protein complex, and taking samples for SDS-PAGE analysis after each treatment. For convenient comparison of the relative strength of association, the Coomassie blue stained gels were scanned (Fig. 5). The 40 kDa protein was still present after three extractions, but its relative abundance decreased compared to those of other proteins with apparent molecular masses of 50, 34–35, 32 and 17 kDa.

DISCUSSION

Whereas isopycnic centrifugation on sucrose gradients failed to separate pure outer and inner membranes, selective extraction of inner membrane proteins by Triton X-100 seemed to be very efficient. Selective solubilization of inner membranes by various detergents has provided a method for isolating outer membrane proteins of a wide variety of Gram-negative bacteria (Koval & Murray, 1981; Moriyon & Berman, 1982; Owen *et al.*, 1982; Schnaitman, 1971). Very little cross-contamination of proteins in the Triton X-100 soluble and insoluble fractions was

found (Fig. 1), and the cytoplasmic membrane marker ATPase was found exclusively in Triton X-100 soluble material and the LPS constituent heptose exclusively in the Triton X-100 insoluble material (Table 1). Moreover, electron micrographs indicated that the Triton X-100 insoluble material was predominantly outer membrane in close association with the peptidoglycan. Accordingly, extrinsic ^{125}I -labelled material was recovered exclusively from the Triton X-100 insoluble fraction, suggesting that the labelling actually took place on the cell surface, and that the detergent did not solubilize outer membrane proteins. The present work thus indicated that *F. nucleatum* Fev1 cells have an outer membrane that is quite similar in many respects to those of other Gram-negative organisms. Good separation of inner and outer membranes by isopycnic centrifugation usually depends on efficient formation of spheroplasts (Owen *et al.*, 1982). The effect of lysozyme on *F. nucleatum* Fev1 seems to be rather atypical (Vasstrand & Jensen, 1984), and we suspect that the reason for the failure to obtain pure outer and inner membranes by this procedure is to be found in this step.

Some of the outer membrane proteins are evidently among the most abundant proteins in the cell. A 40 kDa protein was responsible for the major band and this dominated the SDS-PAGE profile of the cell envelope, and was also clearly seen when whole cells were applied to the gels (Fig. 1).

The polypeptide composition of the outer membranes of the six strains of *F. nucleatum* examined (F1, F3, F6, F14, 10953 and Fev1) showed a high degree of homology in relation to molecular masses. There was, however, a clear difference when heat modifiable properties were examined. Thus, in all strains except Fev1, one of the most abundant outer membrane proteins exhibited heat modifiable properties. None of the outer membrane proteins of *F. nucleatum* Fev1 exhibited any detectable heat modifiable properties even after boiling for 30 min. This seems to be a strain specific property. DiRienzo & Rosan (1984) have recently published data on a major envelope protein from *F. nucleatum* strain 364 with an apparent molecular mass of 50 kDa and 35.5 kDa at 100 °C and 50 °C, respectively. Similar results were reported for strain 10953 (DiRienzo & Spieler, 1983). Our results indicated that the shift in apparent molecular mass of the heat modifiable proteins of strain 10953, as well as strains F1, F3, F6, and F14, was from about 38 kDa to about 42 kDa. Such differences in the actual values observed could be due to the different techniques used (Lugtenberg & Van Alphen, 1983). The 40 kDa protein of *F. nucleatum* Fev1 was exposed at the cell surface, as determined by the ^{125}I -lactoperoxidase method, and was peptidoglycan associated as defined by Rosenbusch (1974). It should be emphasized, however, that the Rosenbusch procedure does not by itself establish that the protein is actually bound to the peptidoglycan, rather than being present as an insoluble protein aggregate. However, purified 40 kDa protein, freed from peptidoglycan by centrifugation after heating to 50 °C or higher, was soluble in 2% SDS at room temperature.

The 40 kDa protein was often seen in gels as a doublet (e.g. Fig. 1, lane 3 and Fig. 4, lane 5). The presence of similar doublets in the corresponding proteins in the other strains was also indicated (Fig. 3). Further separation of proteins in the 40 kDa region will be attempted by two-dimensional electrophoresis.

In addition to the major 40 kDa protein, several other proteins were apparently exposed on the cell surface (Fig. 4). Some of these surface exposed proteins were also peptidoglycan associated, in particular a 32 kDa and a 17 kDa protein. It is thus possible that these proteins, and the 40 kDa protein, are involved in formation of pores or channels (DiRienzo *et al.*, 1978; Lugtenberg & Van Alphen, 1983), which seems to be universally found in the outer membrane of Gram-negative bacteria. Variations with growth conditions in the amount of these proteins, and of the outer membrane proteins in general, are under investigation.

It has been suggested that the D-diaminopimelic acid residue of the peptidoglycan plays an important role in its binding to the matrix protein in *E. coli* (DiRienzo *et al.*, 1978). Lanthionine, the monosulphur analogue of diaminopimelic acid, replaces diaminopimelic acid in *F. nucleatum* Fev1 (Vasstrand *et al.*, 1979, 1982); 85% is recovered as *meso*-lanthionine and 15% as DL-lanthionine (Vasstrand *et al.*, 1979). The question arises whether these isomers of lanthionine play any role in the association between the 40 kDa protein and the peptidoglycan in this organism.

We are grateful to T. Thorsen for help with the radioiodination experiments, P. G. Krüger for help and guidance during electron microscopy, E. Vasstrand for valuable discussions and B. Høgh for excellent technical assistance. This investigation was supported by the Norwegian Research Council for Science and the Humanites.

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