Hexagonal Periodicity in the Outer Membrane of *Bacteroides buccae*

By EERO KEROSUO,^{1,2*} MARKUS HAAPASALO,¹ HELENA RANTA^{1,4}
AND KARI LOUNATMAA^{2,3}

Departments of ¹Cariology, ²Electron Microscopy and ³General Microbiology, University of Helsinki, Mannerheimintie 172, SF-00280, Helsinki, Finland

⁴National Public Health Institute, Mannerheimintie 166, SF-00280, Helsinki, Finland

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In *Bacteroides buccae*, a hexagonally arranged periodic structure was found in the outer membrane (OM), in addition to hexagonal lattices present in its external surface layer (S-layer). This crystalline OM protein (COMP) was present as patches on the concave fracture face (the outer leaflet) of the OM in freeze-fractured cells. Occasionally, hexagonally arranged structures could also be seen on the convex fracture face of the OM as 'fingerprints' of the COMP. The OM proteins were isolated and analysed by gel electrophoresis. The major band protein had an apparent molecular mass of 17 kDa. Whether the minor band proteins are also components in the structure of the COMP remains to be elucidated. Other oral Gram-negative anaerobic rods studied did not show any periodicity in their OM.

INTRODUCTION

Periodically arranged surface layers, composed of protein or glycoprotein, are found on the cell envelope of numerous bacteria including a few human pathogens (for a review, see Sleytr & Messner, 1983). The ultrastructure of these protein layers has been studied by electron microscopy using thin sectioning, negative staining (Lounatmaa, 1985) and freeze-etching techniques (Sleytr & Glauert, 1975), and the periodic structure has been identified as a surface layer (S-layer) external to the peptidoglycan layer in Gram-positive bacteria and external to the outer membrane (OM) in Gram-negative bacteria (Sleytr & Messner, 1983). By the freeze-fracture technique the OM of *Escherichia coli* and *Salmonella typhimurium* has been shown to be densely packed with intramembranous particles (IMP) (van Gool & Nanninga, 1971; Smit *et al.*, 1975). These IMP are apparently porins, which are the most intensively studied OM proteins in these two species (for a review, see Nikaido & Vaara, 1985). When the OM proteins of *E. coli* are treated with sodium dodecyl sulphate (SDS) the porins appear as hexagonal crystals (Rosenbusch, 1974) and a matrix protein has been shown to form a periodic monolayer (Steven *et al.*, 1977). In intact cells of *E. coli* a similar arrangement has not been found (van Gool & Nanninga, 1971; Nikaido & Vaara, 1985). There are few reports to show crystalline arrangement of the OM proteins in intact cells. In *Chlamydia trachomatis* hexagonally arranged granular subunits were first found by negative staining (Zhang *et al.*, 1980) and later by the cryochamber and shadow-cast technique (Chang *et al.*, 1982). Using the freeze-fracture technique, Every and Skerman (1983) found hexagonally arranged crater-like structures on the concave fracture face of the OM of *Bacteroides nodosus*.

The aim of this study was to describe the ultrastructure of the OM of an oral anaerobic rod, *Bacteroides buccae*, previously shown to possess a crystalline S-layer (Kornman & Holt, 1981; Haapasalo *et al.*, 1985; Sjögren *et al.*, 1985). The freeze-fracture studies of one *B. buccae* strain,

Abbreviations: CM, cytoplasmic membrane; COMP, crystalline outer membrane protein; IMP, intramembranous particles; OM, outer membrane; S-layer, surface layer.

ES57, revealed the presence of a crystalline outer membrane protein. For comparison, other *B. buccae* strains and also other oral Gram-negative anaerobic rods were studied.

METHODS

Strains. Thirteen strains originally isolated from the human oral cavity were studied (Table 1). Laboratory strains were isolated from human dental root canal infections and identified as described previously (Haapasalo *et al.*, 1985, 1986).

Culture methods. Strains from the American Type Culture Collection were obtained as freeze-dried cultures. Laboratory isolates were kept in glycerin-milk at -70°C . All strains were cultured on MCG agar, containing bacteriological agar no. 1 (Oxoid), 5% (v/v) horse blood, 0.5% (w/v) yeast extract, 0.5 mg menadione l^{-1} , 500 mg cysteine l^{-1} and 0.2% (w/v) glucose (Haapasalo *et al.*, 1986) in an anaerobic chamber (Anaerobic System, model 1024, Forma Scientific) in an atmosphere of $\text{H}_2/\text{CO}_2/\text{N}_2$ (10:5:85, by vol.) at 37°C . Three-day-old cultures after the third or fourth transfer were used for ultrastructural studies.

Electron microscopy. The bacterial cells were collected with a glass rod from the plates into 0.1 M-sodium phosphate buffer (pH 7.2).

For thin sections, the samples were prepared as previously described (Lounatmaa *et al.*, 1976).

For freeze-fracturing, glycerol was added to a final concentration of 30% (v/v) to facilitate the fracturing through the OM (Lounatmaa & Nanninga, 1976). The pellet collected by centrifugation (9980 g, 20°C , 2 min) was frozen in liquid Freon 22 cooled by liquid nitrogen. The fracturing, in a Balzers BAF 400T freeze-etching unit, was done at -120°C and the platinum shadowing at an angle of 40° .

For negative staining, the buffer suspension was allowed to sediment on a grid with a resin film (Piolofilm 2295/10, Polaron Equipment Ltd) coated with an additional thin carbon layer and the staining was performed with 2% (w/v) phosphotungstic acid (pH 6.5).

The electron micrographs were taken with JEM-100CX (negative staining) and with JEM-1200EX (thin sections and freeze-fracture replicas) transmission electron microscopes at 80 and 60 kV, respectively.

Separation of cell wall components. Three-day-old cultures of *B. buccae* ES57 on MCG agar were collected and gently washed with phosphate buffered saline (PBS: 0.85% NaCl, 0.15 M-sodium phosphate, pH 7.2). The S-layer was separated from the cells by carefully pipetting the bacterial mass, in PBS, in and out of a 10 ml pipette through a narrow tip (diameter 1.2 mm) 20 times. After centrifugation (9980 g, 5 min, 20°C) the supernatant containing crude S-layer protein was further centrifuged (72 000 g, 30 min, 4°C). The partly purified S-layer protein was collected from the supernatant and analysed by SDS-PAGE.

After mechanical removal of the S-layer the cells were resuspended in 0.75 M-sucrose (10 mM-Tris, pH 7.8) and the OM proteins were isolated by the procedure described by Osborn *et al.* (1972) with the modifications of a twofold concentration of lysozyme (20 mg ml^{-1}) and a prolonged period of spheroplast formation (Sarvas, 1985). The partly purified OM protein fraction was collected from the sucrose gradient and analysed by SDS-PAGE.

SDS-PAGE. This was done as described by Laemmli (1970).

RESULTS

Patches consisting of hexagonally arranged IMP were frequently found on the concave fracture faces of the OM of the seven strains of *B. buccae* (Table 1; Figs 1 and 2). The diameter of the patches varied between 50 and 70 nm but occasionally much larger areas of hexagonally arranged IMP were seen. The size of the patches also varied in the same specimen, being larger at the division zone of the cell (Fig. 1). The centre-to-centre spacing between the individual IMP was always about 8 nm (Figs 1 and 2). Randomly distributed IMP were observed between adjacent patches and the orientation of the axes of symmetry seemed to be random in adjacent patches (Figs 1 and 2). Usually no periodicity was seen on the convex fracture face of the OM. Occasionally, however, the convex face too showed patches of hexagonally arranged structures and when the inner leaflet of the OM was fractured away another periodic structure was seen (Fig. 3) instead of the typical rough appearance of the convex face of the cytoplasmic membrane.

In thin-sectioned cells of *B. buccae* strains the typical cell envelope structures of Gram-negative bacteria were seen and the presence of an S-layer was confirmed (Fig. 4). No periodicity could be visualized either in the OM or in the S-layer by this technique.

The freeze-fracture technique sometimes revealed the cross-fractured S-layer as a row of 'pearls' around the cell. The S-layer was not in direct contact with the OM, but there was a gap

Fig. 1. A freeze-fractured cell of *B. buccae* ES57. All four fracture faces are seen: the convex face of the CM ($\widehat{CM}$), the convex face of the OM ($\widehat{OM}$), the concave face of the CM ($\widecheck{CM}$) and the concave face of the OM ($\widecheck{OM}$); CC, the cell content. The hexagonally arranged IMP form numerous small patches on the $\widecheck{OM}$. Note the division area with larger patches and the cross-fractured S-layer (S) appearing like a necklace around the cell. The direction of shadowing is indicated by an arrow in this and subsequent fracture micrographs. The scale bar represents 0.2 μ m in this and subsequent micrographs.

Table 1. Sources and sites of isolation of the strains

Strain	Source/received from	Site of isolation
<i>B. buccae</i>		
ATCC 33574 [†]	ATCC*	Gingival sulcus
ES42	Own isolate	Root canal
ES57	Own isolate	Root canal
ES14B	Own isolate	Root canal
ATCC 33691 [†]	ATCC	Subgingival plaque
NP333 [‡]	H. Shah	Dental plaque
WPH78 [‡]	H. Shah	Dental plaque
<i>B. gingivalis</i>		
ES30	Own isolate	Root canal
<i>B. intermedius</i>		
ES63	Own isolate	Root canal
<i>B. oris</i>		
ATCC 33573 [†]	ATCC	Gingival sulcus
ATCC 27518	ATCC	Human wound
ES2354	Own isolate	Root canal
<i>Fusobacterium nucleatum</i>		
ESF4	Own isolate	Root canal

T, type strain.

* American Type Culture Collection.

[†] Formerly designated as *B. capillus* (Kornman & Holt, 1981; Johnson & Holdeman, 1985).

[‡] Formerly designated as *B. pentosaceus* (Shah & Collins, 1981; Johnson & Holdeman, 1985).

Fig. 2

Fig. 3

Fig. 2. A freeze-fractured cell of *Bacteroides buccae* ES57. Hexagonally arranged IMP are seen as patches on the concave fracture face of the OM ($\bar{OM}$). Between the patches the particles are randomly distributed. CM, the concave fracture face of the CM.

Fig. 3. A freeze-fractured cell of *B. buccae* ES57. A small area of hexagonally arranged structure (white arrow) is visible on the convex fracture face of the OM ($\hat{OM}$). The inner leaflet of the OM is partly broken revealing another net-like structure (short black arrow).

Fig. 4

Fig. 5

Fig. 4. The cell envelope of a thin-sectioned cell of *B. buccae* ES57. The cytoplasmic membrane (CM), the peptidoglycan layer (PG), the outer membrane (OM), and the S-layer (S) are clearly seen.

Fig. 5. A freeze-fractured cell of *B. oris* ATCC 27518. Randomly distributed IMP on the concave fracture face of the OM ($\bar{OM}$) are visible.

Fig. 6. SDS-PAGE analysis of the cell envelope proteins of *B. buccae* ES57. Lanes: A, J, N and O, protein standards; B, whole cells; C, cells after removal of S-layer protein by pipetting the bacterial mass, in PBS, in a 10 ml pipette 20 times; D, crude S-layer protein after centrifugation (9980 *g*, 5 min); E, broken cells after mechanical removal of S-layer protein; F, crude OM-CM; G and K, pure S-layer protein after ultracentrifugation (72000 *g*, 30 min); H and L, partly purified CM from the sucrose gradient; I and M, partly purified OM from the sucrose gradient.

(about 20 nm) between these two outermost cell envelope structures (Fig. 1), corresponding to the alignment of these structures in a thin-sectioned cell (Fig. 4).

No periodic structures were found when the strains of *B. gingivalis*, *B. intermedius*, *B. oris* and *Fusobacterium nucleatum* (Table 1) were freeze-fractured. The IMP were randomly distributed also on the concave fracture face of the OM (Fig. 5).

On SDS-PAGE the partly purified OM preparation of *B. buccae* ES57 showed one major band with an apparent molecular mass of about 17 kDa (Fig. 6, lane I) and a few other, minor, bands with higher molecular masses. The partly purified S-layer preparation (Fig. 6, lane G) showed three major bands, one of about 70 kDa and a double band of about 16 kDa. The 70 kDa band was also present in the whole-cell preparation (Fig. 6, lane B) but not in the OM preparation (Fig. 6, lane I).

DISCUSSION

The OM of Gram-negative bacteria is an asymmetrical membrane composed of lipopolysaccharide and a large amount of protein (Nikaido & Vaara, 1985). When the cell is freeze-fractured, numerous IMP are seen on the concave fracture face of the OM. Since in mutants of *S. typhimurium* and *E. coli* lacking one or more of the major OM proteins these IMP are absent or reduced in density in micrographs, they are considered to be composed of complexes of the OM proteins (Smit *et al.*, 1975; Verkleij *et al.*, 1977; Lounatmaa, 1979). Consequently, the hexagonally arranged IMP found in this study on the concave fracture faces of the OM (Figs 1 and 2) are assumed to be complexes of proteins, thus tentatively named crystalline outer membrane protein (COMP). A schematic illustration is shown in Fig. 7.

Fig. 7. Schematic illustration of the ultrastructure of the cell envelope of *B. buccae* ES57 as visualized by the freeze-fracture technique. The dimensions are arbitrary. The surface of the OM (SOM) and the lipopolysaccharide covering the cell (not indicated) are not visualized by this technique. $\widehat{OM}$, convex face of the OM; PG, peptidoglycan; $\widehat{CM}$, convex face of the CM; $\check{CM}$, concave face of the CM; $\check{OM}$, concave face of the OM; COMP, crystalline outer membrane protein; S, S-layer; CFS, cross-fractured S-layer; CC, cell content.

The crystalline structures (Fig. 3) which were occasionally found on the convex fracture face of the OM are probably 'fingerprints' of the COMP from the outer leaflet of the OM. It is not known whether the other crystalline structure exposed after the incomplete breakage of the inner leaflet of the OM (Fig. 3) represents peptidoglycan.

The centre-to-centre spacing (about 8 nm) of the COMP of *B. buccae* found in this study differs distinctly from those reported for crystalline OM lattices in *C. trachomatis* (17.5 nm) (Chang *et al.*, 1982) and *B. nodosus* (20 nm) (Every & Skerman, 1983).

For the first time, both the S-layer and the COMP were found in the same strains. In *B. nodosus*, a hexagonal protein lattice in the OM was detected only in a strain lacking the S-layer (Every & Skerman, 1983) and the *C. trachomatis* strain studied by Chang *et al.* (1982) did not possess an S-layer.

The structural relationship between the S-layer and the COMP is interesting. The centre-to-centre spacing found in the COMP (about 8 nm) is very close to that found in the smaller S-layer lattice (7.7 nm) in the same species (Sjögren *et al.*, 1985). However, it cannot be excluded that the cell-wall components, interpreted as S-layer by Sjögren *et al.* (1985) are supported by OM fragments. Thus, it is possible that the 7.7 nm lattice in fact represents the COMP lattice found in this study. The 21.5 nm lattice represents the S-layer, which has also been visualized by freeze-

etching (Ranta *et al.*, 1983). Fracturing without etching rarely exposes S-layer surfaces (Sleytr & Messner, 1983); thus in this study the S-layer appeared cross-fractured (Fig. 1). The SDS-PAGE analysis showed that the band of about 17 kDa was present among the OM proteins and not even as a minor band among the S-layer proteins (Fig. 6, lanes G and I). It is possible that the 17 kDa polypeptide found in this study is the main component of the COMP. Whether the minor band proteins found (Fig. 6, lane I) are also components of the structure of the COMP remains to be elucidated.

Several OM proteins are known to be associated with resistance to antibiotics (Nikaido & Vaara, 1985) and with important virulence factors (Paakkanen *et al.*, 1979; Carter *et al.*, 1980) but these proteins are not known to form crystals (Kapperud *et al.*, 1985; Zaleska *et al.*, 1985). So far, the S-layers are the most intensively studied crystalline protein layers but their function is still poorly understood. In several species the S-layer proteins are known to have a molecular-sieve function, which protects the cell against lytic enzymes (Sleytr & Messner, 1983). However, S-layer was not found to be related to immunoprotection in *B. nodosus* (Every & Skerman, 1983). Also, the function of the COMP found in this study is not clear.

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