Ultrastructure, Serogrouping and Localization of Surface Antigens of *Bacteroides intermedius*

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The surface ultrastructure of 21 strains of *Bacteroides intermedius* was investigated by electron microscopy. Rat monoclonal antibodies (mAbs) were used to define serogroups and to detect the location of surface antigens. All 21 isolates had capsules as demonstrated by the use of wet and dry Indian ink stains. Negative staining of whole cells with 1% (w/v) methylamine tungstate showed that all 21 isolates carried clumped peritrichous fibrils with strain dependent morphology, density and length ($\leq 0.75 \mu\text{m}$). Fibrils on 11 of 13 fresh clinical isolates were more conspicuously clumped and easily visible, whereas those on 6 of 8 laboratory strains were indistinct and were at the limits of the resolution of the negative staining technique. Staining with ruthenium red (RR), followed by thin sectioning, revealed a dense, amorphous RR staining layer (RRL), up to $24.8 \pm 3.0 \text{ nm}$ thick, adjacent to the outer membrane on all of 15 strains examined. All isolates had a less dense RR staining matrix (RRM) extending away from the RRL. The structure of the RRM varied between strains. Four rat mAbs (37BI6.1, 38BI1, 39BI1.1 and 40BI3.2) were used to serogroup the 21 strains of *B. intermedius*. Immunonegative staining revealed that the mAbs were not directed against fibrils. Antigens recognized by mAb 37BI6.1 and mAb 39BI1.1 were located on the surfaces of cells, beneath fibrils, and on extracellular vesicles. mAb 38BI1 recognized an antigen which was most accessible on lysed cells, and non-specific binding of mAb 40BI3.2 to grids prevented its localization on the cell surface.

INTRODUCTION

Bacteroides intermedius can be isolated from both healthy and diseased human subgingival sites, and there is a growing body of evidence implicating it as a potential causative agent of periodontal disease (Slots, 1979, 1986; Zambon *et al.*, 1981; Moore *et al.*, 1985). The precise role of *B. intermedius* in this process remains unclear. However, its ability to colonize the subgingival area, whether by adhering to host tissue or to other bacterial members of the subgingival microflora, is likely to play a significant part in determining its pathogenic potential. It has been suggested that surface components such as fimbriae, capsules and lipopolysaccharides may be important in the adhesion of bacteria to oral surfaces (Slots & Genco, 1984; Mayrand & Holt, 1988).

Little is known about the surface properties of *B. intermedius*. Some studies have indicated that surface structures detected by electron microscopy differ between oral black-pigmented *Bacteroides* species (Listgarten & Lai, 1979; Woo *et al.*, 1979; Okuda *et al.*, 1981; Handley &

Abbreviations: BS, borate buffered saline; mAb, monoclonal antibody; RR, ruthenium red; RRL, ruthenium red staining layer; RRM, ruthenium red staining matrix.

Tipler, 1986). Listgarten & Lai (1979) suggested that the morphological differences they observed may be useful to differentiate between certain species or subspecies, and Okuda *et al.* (1981) proposed that such differences may lead to species interacting differently with mammalian cells. These studies have usually examined only one or two strains of *B. intermedius*. Yamamoto *et al.* (1982) found that not all of nine strains of *B. intermedius* examined possessed 'pili-like' structures, as visualized by negative staining. However, it is probable that their negative staining technique failed to reveal all these structures, as more strains carried them when viewed in shadowed preparations. Handley & Tipler (1986) described the dense masses of peritrichous fibres seen on two strains of *B. intermedius*, and some other *Bacteroides* species, as fibrils; these were distinct from fimbriae in that they clumped copiously and had no measurable width.

Staining of some *Bacteroides* spp. with ruthenium red (RR) has been used to visualize polyanionic surface components, probably acidic polysaccharides (Luft, 1971), but these have usually included only a few strains of *B. intermedius* (Woo *et al.*, 1979; Okuda *et al.*, 1981). Yamamoto *et al.* (1982), however, found that the appearance of the RR staining layer (RRL) of *B. intermedius* varied between strains.

In addition to these ultrastructural differences, antigenic heterogeneity in *B. intermedius* has been reported (Gmür & Guggenheim, 1983; Gmür & Wyss, 1985), and *B. intermedius* isolates have been divided into three serogroups, which may be correlated with genotype (Gmür & Wyss, 1985). The ultrastructural localization of the antigens used in serogrouping is unknown.

As previously mentioned, it has been suggested that some surface components may be important in the adhesion of bacteria to oral surfaces (Slots & Genco, 1984; Mayrand & Holt, 1988). Also, these cell components have the potential to determine host antibody response and the susceptibility of bacteria to complement and phagocytosis. A survey of the surface ultrastructure of a larger number of *B. intermedius* strains would characterize the species more completely, and would form a basis for future studies aimed towards understanding the adhesive and other surface properties of the organism. Therefore, in this study the surface ultrastructure of 21 strains of *B. intermedius* was examined. In addition, the serogroup of each strain was determined, and the structural location of the antigens used in serogrouping was investigated by immunoelectron microscopy.

METHODS

Bacterial strains. Twenty-one isolates of *B. intermedius* were studied. Six strains were included in a previous study (Gmür & Wyss, 1985). Of these, strains OMZ 227 (M107-74), OMZ 248, OMZ 326 (FDC 581) and OMZ 327 (FDC 377) were originally isolated from subgingival plaque. Strain OMZ 277 (ATCC 25261) was isolated from a laryngotomy wound, and OMZ 311 (3b) was isolated from an unspecified oral site. Strains BH 18/23 and BH 20/30, periodontal pocket and supragingival plaque isolates respectively, were provided by G. Bowden (Department of Oral Biology, University of Manitoba, Canada). These eight organisms have been isolated for a number of years, and some are well recognized reference strains; they will all be referred to as laboratory strains. Thirteen recently isolated clinical strains were also included for study. These were isolated from periodontal pockets of adults with rapidly progressing periodontitis and were provided by D. B. Drucker (Department of Cell and Structural Biology, University of Manchester, UK). All strains were confirmed as *B. intermedius* with the API 20A identification kit.

Growth conditions. All strains were grown in modified FUM medium (Gmür & Guggenheim, 1983), which was prepared and sterilized as described by Loesche *et al.* (1972). FUM contained (per litre of distilled water): 10 g tryptone, 5 g yeast extract, 3 g glucose, 2 mg haemin, 1 mg menadione, 0.5 g cysteine.HCl, 0.9 g NaCl, 0.5 g Na₂CO₃, 1 g KNO₃, 0.45 g KH₂PO₄, 0.45 g K₂HPO₄, 0.9 g (NH₄)₂SO₄, 0.188 g MgSO₄·7H₂O, and had a pH of 7.1. After sterilization, 5% (v/v) sterile horse serum was added. Columbia agar base (Oxoid CM311) supplemented with 5% (v/v) horse blood was used as a solid medium. For all procedures, 1 ml of an overnight broth culture was transferred to 9 ml fresh FUM, which was incubated for no more than 48 h. Cultures were grown in prereduced media at 37 °C in a Don Whitley anaerobic cabinet (mark II) under an atmosphere of 80% N₂, 10% H₂ and 10% CO₂. All strains of *B. intermedius* were stored in glycerol at -70 °C.

Capsule detection. Capsules were detected by negative staining with Indian ink. A broth suspension of bacteria was mixed on a glass slide with 10% (w/v) glucose solution and a drop of Indian ink. This mixture was spread thinly across the surface of the slide, allowed to dry, fixed with ethanol and stained with ammonium crystal violet.

Wet films were also prepared by mixing the bacterial suspension with 10% (w/v) glucose and Indian ink on a glass slide, placing a coverslip on the mixture and then blotting out the excess. Bacteria were examined using a Leitz Dialux 20 light microscope.

Negative staining for electron microscopy. Cultures of *B. intermedius*, grown for up to 48 h in FUM medium, were washed twice in water and were stained using the method described by Handley & Tipler (1986). The washed suspensions were placed on Formvar covered copper grids (400 mesh, Agar Aids) which had been carbon-coated and plasma-glowed in a Nanotech 300S coating unit. Bacteria were then negatively stained with 1% (w/v) methylamine tungstate (EMscope Laboratories) or 1% (w/v) ammonium molybdate (Agar Aids). Micrographs were taken on a Hitachi 600 electron microscope.

Ruthenium red staining. A modification of the method described by Luft (1971) was used to visualize polyanionic components associated with the surface of *B. intermedius*. Cultures grown for 16–48 h in FUM medium were washed three times in 0.2 M-sodium cacodylate buffer (pH 7.3) and were resuspended in a mixture containing equal volumes of 0.2 M-cacodylate buffer (pH 7.3), 3.6% (v/v) glutaraldehyde, and a solution of RR (10 mg ml⁻¹, 1500 p.p.m.). Fixation in this mixture took place on a rotating turntable for 1 h at room temperature. Following three further washes in 0.2 M-cacodylate buffer (pH 7.3), bacteria were resuspended in equal volumes of 4% (w/v) osmium tetroxide, 0.2 M-cacodylate buffer (pH 7.3) and RR, and were rotated overnight at room temperature. The suspensions were then washed three times in 0.2 M-cacodylate buffer (pH 7.3) and were dehydrated through a graded series of ethanol, with concentrations ranging from 30% (v/v) to 100% (v/v). Controls were prepared by replacing RR with distilled water throughout the procedure. Specimens were embedded in LR White resin (London Resin Co.) and sections cut on a Reichert OMU4 ultramicrotome. Thin sections of control preparations were post-stained with a saturated solution of uranyl acetate in 50% (v/v) aqueous alcohol, and lead citrate (Reynolds, 1963). Micrographs were taken on a Hitachi 600 electron microscope.

ELISA. The whole cell ELISA technique described by Gmür & Guggenheim (1983) was used to determine the antigenic reactivity groups of the 21 strains of *B. intermedius*. Hybridoma culture supernatants of rat monoclonal antibodies (mAbs) 37BI6.1, 38BI1, 39BI1.1 and 40BI3.2 (mAb37, mAb38, mAb39 and mAb40) were stored at -20 °C. *B. intermedius* strains were grown in FUM for 16–48 h and were washed twice in 0.9% (w/v) NaCl. They were resuspended in phosphate buffered saline (pH 7.2) to an optical density at 550 nm of 0.5 (2–3 × 10⁸ organisms ml⁻¹). Flat-bottomed microtitre plates were pretreated for 30 min at room temperature with 0.1 ml 0.1% (v/v) glutaraldehyde in borate buffered saline (BS: 0.14 M-boric acid, 0.025 M-sodium tetraborate, 0.075 M-NaCl, pH 8.1). Bacterial suspension (0.2 ml) was added to each well and the plates were centrifuged for 20 min at 1300 g. The bound bacteria were then fixed by the addition of 0.1 ml 0.1% (v/v) glutaraldehyde in BS for 30 min at room temperature. The plates were washed twice with BS and were stored at 4 °C, after the addition to each well of 0.2 ml BS containing 0.1% (w/v) bovine serum albumin.

mAbs were diluted in BS containing 0.5% (w/v) bovine serum albumin and 0.05% (v/v) Tween 20. The initial dilution was 1 in 10 followed by serial doubling dilutions up to 1 in 10240. Antigen-coated microtitre plates were washed twice with BS and 0.2 ml of the appropriate mAb dilution was added. After incubation at 4 °C overnight the wells were washed four times with ELISA wash solution (0.9% w/v, NaCl plus 0.05% v/v, Tween 20). Horseradish-peroxidase-conjugated rabbit anti-rat IgG (P+S Biochemicals) was diluted 1 in 2000 in BS containing 0.5% (w/v) bovine serum albumin and 0.05% (v/v) Tween 20, and 0.1 ml was added to each well. The plates were incubated at 37 °C for 90 min following which they were washed eight times with ELISA wash solution, and the wells were filled with 0.2 ml ELISA substrate solution (3.7 mM-1,2-phenylene-diamine, 0.012% v/v, H₂O₂ in 24.3 mM-citric acid, 25.7 mM-Na₂HPO₄, pH 5.1). After incubation in the dark at room temperature, 0.05 ml 2.5 M-H₂SO₄ was added to each well to stop the reaction, and the highest dilution of mAb producing a detectable colour reaction was recorded.

Immunonegative staining. FUM cultures (16–24 h) of *B. intermedius* strains OMZ 227, OMZ 248, OMZ 277, BH 18/23, MUI 32, MUI 41, MUI 44, MUI 57 and MUI 65 were washed twice in BS containing 0.5% (w/v) bovine serum albumin and 0.05% (v/v) Tween 20, pH 8.6. The same buffer solution was used to dilute the mAbs. Bacteria were placed on Formvar covered nickel grids (400 mesh, Agar Aids) which had been carbon-coated and plasma-glowed in a Nanotech 300S coating unit. For all further treatments and washings the grids were suspended upside-down on 0.02 ml drops of the appropriate solution, which had been placed on a strip of Parafilm. The grids were first treated for 5 min with BS containing 0.5% (w/v) bovine serum albumin and 0.05% (v/v) Tween 20. They were blotted and were then placed on a drop of mAb dilution, usually 1 in 5 or 1 in 10, and binding took place for 30 min at room temperature. The grids were washed in buffer five times, blotting between each wash. Goat anti-rat IgG conjugated to 10 nm gold particles (Janssen) was diluted 1 in 10 in BS containing 0.5% (w/v) bovine serum albumin and 0.05% (v/v) Tween 20, and the grids were suspended on this solution for 30 min. Following five washes in water, the grids were stained with 1% (w/v) methylamine tungstate. Controls were run in which the mAb was replaced by buffer. Some strains were also tested against mAbs with which they had not reacted in the ELISA system. Micrographs were taken on a Hitachi 600 electron microscope.

RESULTS

Capsule production

All of the 21 *B. intermedius* isolates were capsulate when examined in both dry and wet Indian ink preparations. Capsule sizes ranged from equal to the cell width to about three times the cell width. No bacterial cells without capsules were detected, although the coarseness of the Indian ink stain, combined with the poor staining of many of these strains with crystal violet, may have made non-capsulate cells difficult to see. Capsules were detected on cells in the exponential and stationary phases of growth.

Negative staining for electron microscopy

The structures seen on *B. intermedius* strains are defined as fibrils. These have no measurable width along their length as they clump together, often producing a tapered appearance, and individual fibrils are rarely observed (Handley & Tipler, 1986). They are distinct from fimbriae, which have a measurable width (3–14 nm) and a variable length of up to 20 µm (Ottow, 1975). Fibril production by the strains examined in this study did not appear to be subject to phase variation and, unless otherwise stated, the results discussed are those for 16–24 h cultures which were in the early to mid-stationary growth phase.

All 21 isolates of *B. intermedius* screened in this study carried peritrichous, usually flexible, clumped fibrils. However, the density, length and ultrastructure of the fibrils were strain dependent.

Thirteen isolates (OMZ 277, BH 18/23 and 11 of the 13 fresh clinical isolates) had very clumped, easily detectable, peritrichous fibrils (Fig. 1*a*). All cells in a population were fibrillar. Fig. 1*a* demonstrates the copious clumping of these fibrils, and their subsequent splaying out, producing tapered structures whose ends could not be seen clearly. Thus, fibril length was difficult to measure accurately. The longest fibrils detected were on strain MUI 77 (Fig. 1*a*) and were up to 0.75 µm long.

Fibrils between adjacent cells of *B. intermedius* strains frequently interacted in the way seen in Fig. 1*b*). They were stretched between cells and were apparently fused.

The remaining eight isolates of *B. intermedius* (OMZ 227, OMZ 248, OMZ 311, OMZ 326, OMZ 327, BH 20/30, MUI 1 and MUI 65) produced fibrils which appeared indistinct against the background stain, lacked structure and showed less tendency to clump together (Fig. 1*c*, strain OMZ 227). These fibrils were easily discernible on heavily stained cells, but were difficult to see when staining was light. Therefore, it was impossible to detect if all cells carried fibrils. In addition, staining the same cultures with 1% (w/v) ammonium molybdate often failed to reveal surface structures on any cells in a population.

Neither the type of fibril produced, nor fibril length appeared to be related to capsule width. All isolates produced extracellular vesicles (Fig. 1). When grown in FUM for 16–24 h most strains produced few of them. By 48 h, vesicle production had increased markedly, and broth cultures had usually lost viability. Fibrils could be seen attached to large extracellular vesicles, but were rarely seen free in areas surrounding bacterial cells.

Ruthenium red staining. Fifteen isolates were fixed and stained with RR before thin sectioning. All strains carried an amorphous, electron dense RRL adjacent to the outer membrane, shown clearly in Fig. 2*a*). On a few isolates this layer was indistinct (Fig. 2*b*) and was thus difficult to measure. On the remaining isolates the width of the RRL varied between strains, and ranged from 15.8 ± 1.9 nm on strain MUI 44 to 24.8 ± 3.0 nm on strain MUI 24.

Fig. 1. Negative staining of whole cells of *B. intermedius* with 1% (w/v) methylamine tungstate. (a) *B. intermedius* MUI 77. This clinical isolate carries dense, peritrichous, clumped fibrils extending up to 0.75 µm from the cell surface. (b) *B. intermedius* MUI 32 carries similar fibrils to MUI 77, and they can be seen extending between adjacent cells, apparently fused together. (c) *B. intermedius* OMZ 227: a laboratory strain carrying peritrichous fibrils (arrowed) which are difficult to resolve and are most visible in areas of darker stain. All strains produced extracellular vesicles. All bar markers represent 100 nm.

Table 1. Patterns of reactivity of strains of *B. intermedius* with mAbs

Serogroup	Reaction with mAb:				Strains in serogroup
	37B16.1	38B11	39B11.1	40B13.2	
I	+	+	+	+	BH 20/30, MUI 1, MUI 32, MUI 36, MUI 65
II	+	+	+	—	BH 18/23, MUI 4, MUI 15, MUI 21, MUI 24
III	+	+	—*	—	MUI 37, MUI 41, MUI 44, MUI 57, MUI 77

* The weakly reacting antigen described by Gmür & Guggenheim (1983) was not detected in this study.

In addition, all isolates had a RR staining matrix (RRM) external to the RRL, the density and amount of which varied between strains (Fig. 2). The RRM of strains OMZ 326, MUI 21 and MUI 24 (Fig. 2a) comprised small amounts of fragmented RR stained components loosely attached to the RRL. On strains BH 18/23, OMZ 311 (Fig. 2b), MUI 1 and MUI 57 the RRM formed a delicate intercellular meshwork of sparse, branched fibres. A loose-edged matrix of intermediate density, which did not usually form distinct fibres (Fig. 2c), was observed outside the RRL of six other strains (BH 20/30, MUI 4, MUI 15, MUI 32, MUI 44 and MUI 77). Finally, most cells of strains OMZ 248 (Fig. 2d) and OMZ 327 carried a RRM which varied considerably in width but was of apparently uniform density. An RRL could be discerned on some cells, but on others it was probably obscured by the large amounts of external RRM. No layers comparable to the RRL or RRM were seen external to the outer membrane on post-stained control preparations.

It is not known whether the RRM represents the fibrils or capsules of these strains. The fibrillar structures do not appear to be preserved by RR. For example, the RRM of strain MUI 32 (Fig. 2c) has no fibrillar structure when compared with the negative stain of the same strain (Fig. 1b).

ELISA

Gmür & Wyss (1985) found that *B. intermedius* strains belong to one of three serogroups, as assessed by their reaction patterns with four mAbs. They had previously determined that strains OMZ 248, OMZ 311, and OMZ 326 belong to serogroup I (positive reactions with all four mAbs), OMZ 277 to serogroup II (positive reactions with mAb37, mAb38 and mAb39), and strains OMZ 227 and OMZ 327 to serogroup III. *B. intermedius* in serogroup III were described by Gmür & Wyss as reacting strongly with mAb37 and mAb38, negatively with mAb40, and very weakly with a great excess of mAb39 (determined spectrophotometrically). This weak reaction of mAb39 with strains OMZ 227 and OMZ 327 was not detected visually by our ELISA technique using an antibody dilution of 1/2. Any strains having the same reactivity pattern as isolates OMZ 227 and OMZ 327 were placed with them in serogroup III.

The remaining fifteen isolates of *B. intermedius* divided evenly between the three serogroups (Table 1). The titres against mAb37 and mAb38 ranged from 1/640 to 1/10240. Against mAb39 the range was 1/160 to 1/5120. Strains BH 18/23, MUI 4 and MUI 15 had relatively low titres against this antibody (1/160 to 1/320), although the colour reaction in the ELISA test was strong. Finally, titres against mAb40 ranged from 1/1280 to 1/10240.

Strains with similar morphology, either after negative staining with 1% methylamine tungstate or after staining with RR, were not found in the same serogroup, indicating that these structures may not bear the antigens used in serogrouping.

Fig. 2. *B. intermedius* isolates after fixation, staining with RR, and thin sectioning. (a) *B. intermedius* MUI 24 carries a distinct, thick RRL adjacent to the outer membrane, with small amounts of fragmented RRM loosely attached to it. (b) *B. intermedius* OMZ 311 is a laboratory strain whose RRM forms into fine fibrous processes extending away from the narrow RRL. (c) *B. intermedius* MUI 32. A loose edged RRM of intermediate density, which does not form fibrous processes, can be seen external to the RRL. (d) *B. intermedius* OMZ 248 carries a dense RRM of variable thickness which partially obscures the RRL beneath it. All bar markers represent 100 nm.

Immunonegative staining

Nine strains of *B. intermedius* were used to localize the antigens recognized by the four mAbs. Controls in which mAb was replaced by buffer had only a few isolated gold particles randomly scattered over the grids. Strains which did not react in the ELISA test with mAb39 or mAb40 were also included as negative controls. No non-specific binding of mAb39 took place. However, with all strains the distribution of mAb40 was similar regardless of the ELISA result: gold particles were spread diffusely and non-specifically in wide areas around the bacterial cells. Therefore the antigen recognized by mAb40 could not be localized.

The antigens recognized by mAb37 and mAb39 had similar distributions. These antibodies had high affinities for components on the cell surface (Fig. 3a) and extracellular vesicles (Fig. 3b). Neither antibody labelled fibrillar components. All cells within a population were labelled by the mAbs, although the amount of label per cell varied.

When isolates were reacted with mAb38 gold label was rarely associated with the cell surface. Whole cells were labelled with only a few gold particles, whereas mAb38 had greatest affinity for disrupted cells (Fig. 3c). In the areas surrounding bacterial cells gold label was seen on amorphous extracellular material and cell debris, and on some extracellular vesicles.

DISCUSSION

All 21 strains of *B. intermedius* examined in this study carried fibrils. The term fibril was first introduced to describe the surface structures seen on both oral streptococci (Handley *et al.*, 1984, 1985) and *Bacteroides* spp. (Handley & Tipler, 1986) that were distinct from fimbriae.

Other studies have found strains of *B. intermedius* that apparently lacked surface structures (Woo *et al.*, 1979; Yamamoto *et al.*, 1982). This could be due to the negative stain used. This study showed that ammonium molybdate, which was used by Woo *et al.* (1979), failed to reveal fibrils on some strains which were fibrillar when stained with methylamine tungstate. Yamamoto *et al.* (1982) observed branched 'pili-like' structures on more strains of *B. intermedius* after shadowing than on negatively stained preparations. This suggests that the negative staining technique may have been at fault. In addition, the strains included in these earlier studies were classified as *B. melaninogenicus* subsp. *intermedius*, which has subsequently been divided into *Bacteroides corporis* and *B. intermedius* (Johnson & Holdeman, 1983).

The results of this study do not confirm those of Okuda *et al.* (1981), who examined a range of *Bacteroides* species and stated that surface structures were remarkably consistent among representative strains of each species or subspecies. Although all strains of *B. intermedius* in this study carried fibrils, their density, length and morphology varied considerably between strains. In addition, most of the fresh clinical isolates carried much more easily resolvable fibrils than those on the majority of laboratory isolates. This difference may be due to the repeated subculture and long-term storage of the laboratory isolates. The bacterial capsule, or glycocalyx, can be lost or changed with *in vitro* passage (Kasper *et al.*, 1980; Costerton *et al.*, 1981) and the same may be true of fibrils.

Alternatively, as the clinical strains were isolated from a limited geographical area, it is possible that the isolation rates of certain fibrillar types may vary geographically, in the same way that *Escherichia coli* lipopolysaccharide and capsular types are known to vary (Grüneberg *et al.*, 1968; Sarff *et al.*, 1975).

Extracellular vesicle production by the strains of *B. intermedius* examined in this study increased with increasing incubation time. Previous studies have described the production of extracellular vesicles by *Bacteroides* spp. (Listgarten & Lai, 1979; Handley & Tipler, 1986;

Fig. 3. Immunonegative staining of *B. intermedius* with mAbs attached to a 10 nm gold immunoconjugate. (a) mAb37 bound to the cell surface of *B. intermedius* OMZ 277 beneath the layer of peritrichous fibrils. (b) mAb39 labelling extracellular vesicles of *B. intermedius* OMZ 248. (c) mAb38 reacted with *B. intermedius* BH 18/23. Little labelling of whole cells took place but the antibody had a high affinity for disrupted cell contents and cell debris. All bar markers represent 100 nm.

McKee *et al.*, 1986; Grenier & Mayrand, 1987). This phenomenon may be widespread among Gram-negative bacteria, as it has been described in other genera (Knox *et al.*, 1966; Hoekstra *et al.*, 1976; Lai *et al.*, 1981; Thompson *et al.*, 1985). Increased vesicle production under conditions of growth limitation or environmental stress has also been described previously (Knox *et al.*, 1966; Thompson *et al.*, 1985; McKee *et al.*, 1986). It has been suggested that vesicles carry proteases and other toxic materials, and may thus be potential virulence factors (Nowotny *et al.*, 1982; Thompson *et al.*, 1985; Grenier & Mayrand, 1987; Mayrand & Holt, 1988). Although *B. intermedius* has been shown to produce some proteolytic enzymes (Lantz *et al.*, 1985; van Steenberg & de Graaff, 1986) the role of vesicle production by this species is unknown.

Structural heterogeneity of the RR staining components external to the cell wall of *B. intermedius* has been reported previously. Yamamoto *et al.* (1982) described two types of RR stained structure; an electron dense layer adjacent to the outer membrane and a fibrous meshwork which formed an intercellular matrix, structures which resembled the RRL and RRM described in this study. Yamamoto *et al.* (1982) reported that strains of *B. intermedius* carried either or both of these structures, three of their strains having no electron dense layer adjacent to the outer membrane. However, in the present study all of the 15 strains examined carried an RRL, and all had an RRM of variable density external to this layer.

Other studies have described an electron lucent layer between the outer membrane and the RRL of *B. intermedius* isolates which had been grown on agar (Woo *et al.*, 1979; Okuda *et al.*, 1981). Such a layer was not observed on any of the 15 strains examined in this study, nor on any examined by Yamamoto *et al.* (1982), all of which were grown in broth.

The dense, 40 nm thick RRL outside the cell wall of *Bacteroides fragilis* has been called a capsule (Kasper, 1976; Kasper *et al.*, 1977; Mansheim & Kasper, 1977). The use of this term to describe the RRL of the *B. intermedius* strains examined in this study would be confusing, as the RRL could not account for the wide capsules seen on these strains in the light microscope after staining with Indian ink. The RRM external to the RRL observed on these strains may be responsible for the Indian ink exclusion zone. However, comparative measurements on capsules and RRM cannot be used to establish this, as many capsular polysaccharides collapse when stained with RR unless they are first stabilized with specific antibodies (Bayer & Thurow, 1977; Lambe *et al.*, 1984). The term glycocalyx has been used to describe all structures such as capsules, RRLs and RRM which surround bacterial cells (Costerton *et al.*, 1981), and use of this term may be less confusing until more is known about the biochemical identity of the surface structures of *B. intermedius*.

So far the relationship between the capsule seen by light microscopy, the RRM seen after sectioning and fibrils seen by negative staining of whole cells is unknown. Although there was no correlation between the morphology of the RRM and the morphology of fibrils on the strains of *B. intermedius* examined in this study, it is possible that the two are the same cellular component. The fibrils found on *Streptococcus salivarius* HB are glycoproteins (Weerkamp & Jacobs, 1982) that can be seen by both negative staining with 1% methylamine tungstate and by RR staining followed by thin sectioning (Handley *et al.*, 1988). Preliminary results in this laboratory have shown that the fibrils of *B. intermedius* strains may contain protein in addition to polysaccharide, as they can be removed by trypsin treatment of whole cells.

The presence of a RRL adjacent to the cell wall may be quite common in both Gram-positive and Gram-negative bacteria. It has been observed on *B. fragilis* and *Bacteroides ovatus* (Reid & Patrick, 1984; Patrick *et al.*, 1986), on *Streptococcus salivarius* (Handley *et al.*, 1988), as well as on *Streptococcus sanguis* (Hesketh *et al.*, 1987) and *Streptococcus milleri* strains (P. S. Handley, unpublished data).

Since RR stains polyanions of high charge density (Luft, 1971) the *B. intermedius* strains carry a matrix of charged polysaccharide outside the cell wall which may function in adhesion or interbacterial coaggregation. Considerable strain to strain variation was seen in the morphology of the RR stained layers, as well as in the fibrils, of the strains examined in this study. This may indicate considerable biochemical variation in the outer layer of strains of *B. intermedius*, and future studies may also reveal corresponding functional variations.

Serologically, *B. intermedius* isolates are heterogeneous (Gmür and Wyss, 1985; Nakazawa *et*

al., 1988). Using techniques of biochemical analysis, Gmür & Wyss (1985) suggested that the antigens recognized by mAb37, mAb38, mAb39 and mAb40 are located on the outer membrane or in the layer covering this structure. The immunonegative staining in this study showed that none of the mAbs was directed against the fibrillar layer, which was surprising considering the density of this layer on the immunizing strain, OMZ 248. If this proves to be due to a lack of immunogenicity of this outer fibrillar layer of *B. intermedius*, it may be a distinct advantage in evading host defences *in vivo*.

It has been suggested that the antigens recognized by mAb37 and mAb39 may be found in the polysaccharide portions of different lipopolysaccharide molecules (Gmür & Wyss, 1985). Immunonegative staining supported this proposal, as these two mAbs labelled the cell surface, beneath the fibrillar layer, and the surfaces of extracellular vesicles.

Gmür & Guggenheim (1983) found that the antigen recognized by mAb38 was expressed on all saccharolytic black-pigmented *Bacteroides* strains tested. Biochemical studies indicated that this antigen is a protein with lipophilic groups, which was assumed to be associated with the outer membrane and could be extracted with it (Gmür & Wyss, 1985). In this study immunonegative staining revealed only small amounts of this antigen exposed on the cell surface. Most mAb38 bound to disrupted cell contents and to cell debris. Thus, as the antigen recognized by this antibody can be extracted with the outer membrane, it is possible that it is usually found on the inner leaflet of the outer membrane and is only rarely expressed on the cell surface. The increased amount of the antigen recognized by mAb38 detected here after cell lysis may also help to explain the uneven expression of the antigen found by Gmür & Wyss (1985) when examining strains of *B. intermedius* by immunofluorescence. They found that, for some strains, a minority of cells showed strong immunofluorescence with mAb38, while the majority of cells appeared unlabelled. Other strains, which presumably lysed more readily, stained homogeneously.

There are two genotypes of *B. intermedius* exhibiting less than 45% DNA homology (van Steenberg *et al.*, 1982; Johnson & Holdeman, 1983), but which are metabolically very similar. It has been suggested that the two genotypes represent different species (Johnson & Holdeman, 1983), which may differ in their capacity to cause periodontal disease (Moore *et al.*, 1983, 1985). Phenotypic markers have not previously been available to distinguish between them. There is evidence to suggest that the antigen recognized by mAb40 may be a marker, being carried only by strains of genotype I (Gmür & Wyss, 1985). In this study immunonegative staining could not localize this antigen, and the structural study has not been able to identify structural subgroups which could correlate with the two different genotypes. However, functional differences may be revealed in future studies.

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