Surface structures, haemagglutination and cell surface hydrophobicity of *Bacteroides fragilis* strains

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Nineteen strains of *Bacteroides fragilis* were examined by negative staining for surface structures. One strain (ATCC 23745) possessed peritrichous fibrils, 16 strains carried peritrichous fimbriae and two strains carried no surface structures. The fimbriae had a diameter of 2.1 ± 0.25 nm and appeared to be 'curly'. Only a small proportion (4 to 41%, depending on the strain) of cells in a population carried fimbriae or fibrils. Strain A312 showed phase variation of fimbriae as expression of fimbriae was repressed at 20 °C and in early exponential phase at 37 °C. The fibrils on strain ATCC 23745 did not exhibit phase variation in response to changes in incubation temperature, growth phase or growth in two different media. Capsules were demonstrated by the Indian ink method on 18 of the 19 strains, varying in size from strain to strain and within the same population. Cultures often contained both capsulate and noncapsulate cells. All strains possessed an electron dense ruthenium red staining layer between 7.9 and 23.9 nm in width attached to the outer membrane. Cell surface hydrophobicity quantified by the hexadecane partition assay gave low values ranging from 6.6 to 52.1%. Only a few strains were able to haemagglutinate and these were only weakly active. There was no correlation between cell surface hydrophobicity, haemagglutinating activity and surface structures.

Introduction

Bacteroides fragilis is the most common Gram-negative anaerobe isolated from clinical infections (Onderdonk *et al.*, 1977) such as bacteraemia, wound, intra-abdominal and urogenital infections (Lindberg *et al.*, 1979). Adhesion to epithelial surfaces is considered to be a prerequisite for pathogenicity for most bacteria, but the cell surface components that mediate in adhesion of *B. fragilis* have not been unequivocally identified.

Fimbriae are proteinaceous, filamentous nonflagellar appendages (Duguid & Old, 1980) with a diameter of less than 10 nm and they mediate adhesion (Ottow, 1975). Some strains of *B. fragilis* carry fimbriae (Pruzzo *et al.*, 1984; Shinjo & Kiyoyama, 1984; van Doorn *et al.*, 1987), but descriptions are not always detailed or apply only to a few strains. Other species of *Bacteroides* can carry peritrichous fimbriae (Handley & Tipler, 1986), including *B. gingivalis* which was shown to possess 'curly' fimbriae (Yoshimura *et al.*, 1984). In contrast to fimbriae, some other species of *Bacteroides* carry fibrils. Fibrils are shorter than fimbriae (usually less than 400 nm long), with no measurable width and they tend to

clump to give a tapered appearance (Handley *et al.*, 1985). Fibrils have been described on different species of oral streptococci (Handley *et al.*, 1984, 1985) and have been shown to be responsible for adhesion to epithelial cells and to other bacteria (Weerkamp *et al.*, 1986). Fibrils have also been described on other species of *Bacteroides* (Handley & Tipler, 1986; Devine *et al.*, 1989), but not so far on *B. fragilis* strains.

Several roles have been suggested for the capsule of *B. fragilis*. It has been shown to be important in virulence as capsular material can cause abscesses (Onderdonk *et al.*, 1977) and capsules may be involved in adhesion (Onderdonk *et al.*, 1978) and resistance to phagocytosis (Connolly *et al.*, 1984; Lindberg & Weintraub, 1985). However, not all strains of *B. fragilis* are capsulate (Brook *et al.*, 1984; Pruzzo *et al.*, 1984) and cultures may contain capsulate and noncapsulate subpopulations (Babb & Cummins, 1978; Patrick & Reid, 1983).

Ruthenium red (RR) stains polyanionic polymers with a high charge density (Luft, 1971) and is generally used as a stain for acidic polysaccharides. When used in combination with osmium tetroxide fixation, RR staining has shown that the surface of *B. fragilis* is covered by a RR staining layer (RRL) a thin dense layer external to the outer membrane. The majority of *B. fragilis* strains

Abbreviations: RR, ruthenium red; RRL, ruthenium red staining layer; RBCs, red blood cells.

appear to possess a RRL (Kasper *et al.*, 1979), but its role in adhesion and pathogenicity is unclear. It is a separate structure from the capsule seen in Indian ink films (Patrick *et al.*, 1986) and has been implicated as being involved in haemagglutination (Patrick *et al.*, 1988).

High cell surface hydrophobicity correlates with the adhesion of a number of oral organisms to teeth (Weiss *et al.*, 1982) and also with the adhesion of enteropathogenic *Escherichia coli* to intestinal brush border membranes (Cantey *et al.*, 1981). It is not known whether hydrophobicity plays an important role in adhesion of *B. fragilis* as most other *Bacteroides* species are hydrophilic (Handley & Tipler, 1986).

Methods

Bacterial strains. Strains ATCC 23745 and NCTC 9343 were obtained from the National Collection of Type Cultures. The remaining seventeen strains were all clinical isolates from abscesses: strain 2/79 was kindly provided by Dr. H. Shah (London Hospital Medical College, UK); strains MRI 2, MRI 3 and MRI 6 to MRI 10 were isolated at the Manchester Royal Infirmary, UK; strains A4, A200, A334, A363, A459, A312, SCI 7, SCI 9 and SCI 13 were kindly provided by Dr D. B. Drucker (Dept of Cell and Structural Biology, University of Manchester, UK).

All strains were identified as *B. fragilis* using the API 20A kit for the identification of anaerobes.

Media and growth conditions. Cultures were grown in an anaerobic cabinet (Don Whitley mark II) at 37 °C in an atmosphere of N₂/H₂/CO₂ (8:1:1 by vol.) on Columbia agar (Oxoid CM311) supplemented with 5% (v/v) horse blood.

All strains were grown in modified brain heart infusion (BHI) broth, pH 8, containing (per litre of distilled water): 37 g BHI powder (Oxoid CM225), 5 g yeast extract, 1 g NaHCO₃, 1 g KNO₃ and 500 µg menadione. Some strains were also grown in FUM medium which was prepared and sterilized as described by Loesche *et al.* (1972). FUM, pH 7.1, 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 hydrochloride, 0.45 g KH₂PO₄, 0.45 g K₂HPO₄, 0.9 g (NH₄)₂SO₄ and 0.188 g MgSO₄ · 7H₂O.

Negative staining for fimbriae and fibrils. Strains grown for 24 h in modified BHI broth were washed three times with water and negatively stained as described by Handley & Tipler (1986). A drop of bacterial suspension was placed on a grid (400 mesh, Agar Aids) which had been covered with a thin carbon film, or Formvar which had been carbon-coated, and plasma-glowed in a Nanotech 300S coating unit to produce a hydrophilic surface. The bacteria were then negatively stained with a drop of 2% (w/v) methylamine tungstate (Emscope). Grids were examined under a Hitachi 600 electron microscope and the presence of fimbriae and fibrils was noted. As some staining was not even it was anticipated that there may be some counting error. Therefore four grids prepared from 24 h cultures of ATCC 23745 were counted to determine the standard deviation for each count.

Negative staining by a spray method. Several spraying methods have been described, some of which have been reviewed by Horne (1965*a, b*) and Haschemeyer & Myers (1972). A spray technique was used to check that the drop method of preparing grids as described above did not select out subpopulations which adhered preferentially to the grid when the excess suspension was blotted off, thus giving abnormally high or low fimbriate counts.

Strain A312 was grown and washed as above and mixed with an equal volume of 1% methylamine tungstate. A fine aerosol of the suspension was sprayed using an artists' air-brush (DeVilbiss Co.) onto Formvar-coated grids which had been carbon-coated and plasma-glowed. The grids were then allowed to air-dry before being examined under the electron microscope.

Detection of capsules. Capsules were detected by negative staining with Indian ink. A drop of a 24 h broth culture was mixed on a microscope slide with a drop of 10% (w/v) glucose and a drop of Indian ink. This was spread thinly over the slide, allowed to air-dry, fixed with ethanol and stained with ammonium crystal violet. Wet preparations were also made by mixing the bacterial suspension, glucose and ink on a slide, placing a coverslip on the mixture and blotting off the excess. The films were examined under a Leitz Dialux 20 light microscope.

Phase variation. The effect of growth phase, incubation temperature and medium on expression of fibrils and fimbriae was investigated. Strains ATCC 23745 (fibrillar) and A312 (fimbriate) were grown in modified BHI broth with samples taken at hourly intervals for negative staining throughout the growth cycle, which was monitored as the OD₆₆₀. The two strains were also incubated in modified BHI at 20, 37 and 42 °C. Finally, cultures were grown for 24 h in modified BHI and FUM media. Samples were washed with water and negatively stained with 1% methylamine tungstate.

Ruthenium red fixation. Acidic polysaccharides were stained using a modification of the method of Luft (1971). Cultures grown for 24 h in modified BHI were washed three times in 0.2 M-sodium cacodylate buffer, pH 7.3. The cells were then resuspended in equal volumes of 3.6% (v/v) glutaraldehyde, RR solution (10 mg ml⁻¹) (Johnson and Matthey Chemicals) and 0.2 M-cacodylate buffer. After 1 h at room temperature on a turntable, the cells were washed three times in cacodylate buffer and fixed overnight, rotating at room temperature, in a mixture of equal parts of 4% (w/v) osmium tetroxide, RR solution and 0.2 M-cacodylate buffer. The suspensions were then washed three times in 0.2 M-cacodylate buffer. Controls were prepared by substituting distilled water for the RR solution. The cells were then dehydrated in a graded series of ethanol concentrations (30 to 100%) and then embedded in LR White resin (London Resin Co.). After polymerization at 60 °C for 20 h sections were cut on a Reichert OMU4 ultramicrotome. Micrographs were taken on a Hitachi 600 electron microscope.

Hexadecane assay. Rosenberg *et al.* (1980) developed a simple, rapid assay to determine the relative surface hydrophobicity of bacteria by their interaction with hexadecane. The organisms were grown for 24 h in modified BHI, then washed three times in Sörensen's phosphate buffer, pH 7.2. The cells were resuspended in buffer to an OD₄₄₀ of 0.5. A sample (3 ml) was transferred to each of six test-tubes. Hexadecane (200 µl) was layered on top and the layers mixed by vigorous vortexing for 60 s. The layers were allowed to partition while standing for 15 min. The OD₄₄₀ of the aqueous phase of six replicates was used to calculate the mean percentage hydrophobicity (Handley *et al.*, 1987). Each strain was assayed twice to monitor variation between batches.

Strain A312 was also partitioned and the aqueous layer negatively stained to investigate whether fimbriate cells preferentially adhered to the hexadecane, as fimbriae are often composed of hydrophobic amino acids. The count was compared with that made from the unpartitioned suspension.

Haemagglutination. Chicken, horse, rabbit and human bloods were used to investigate the haemagglutinating activity of the strains. Blood was collected freshly each week and stored at 10% (v/v) concentration in Alsever's solution at 4 °C. When needed, blood (20 ml) was washed twice in phosphate-buffered saline (PBS; 8 g NaCl l⁻¹, 1.21 g K₂HPO₄ · 3H₂O l⁻¹, 0.34 g KH₂PO₄ l⁻¹, pH 7.2) and 0.2 ml of packed

red blood cells (RBCs) resuspended to give a 2% (v/v) suspension in PBS. Bacterial cultures were grown for 24 h in modified BHI then washed in PBS, pH 7.4. The cells were resuspended to an OD₆₀₀ of 1.0 (3×10^9 cells ml⁻¹). The haemagglutination was carried out in round-bottomed microtitre trays. PBS was prepared with 1% (w/v) BSA to act as a nonspecific protein blocker which prevented the RBCs autoagglutinating, and 50 µl of this was placed in each well. An equal volume of bacterial suspension was placed in the first well and double-diluted across the tray. To each well was added 25 µl of 2% RBCs. The tray was shaken for 30 s at room temperature on a plate shaker and left at room temperature. Trays were examined under a plate microscope for haemagglutination.

Results

Negative staining for fimbriae and fibrils

Strain ATCC 23745 was the only strain to carry peritrichous fibrils. They were clumped and flexible (Fig. 1*a*) and their ends tapered so no width or length was measurable. The fibrils appeared to be repelled from the surface of nonfibrillar cells (Fig. 1*b*). In addition, the fibrils seemed to attract each other as they appeared to be attached to fibrils from other cells across spaces between fibrillar cells (Fig. 1*c*). Fibrils were carried by only 12.3%

of cells from stationary phase broth cultures (Table 1). Four grids were counted to determine the variation introduced by counting error and uneven staining. The standard deviation of the four counts was only ± 1.4 .

Sixteen strains possessed fimbriae (2.1 ± 0.25 nm in width). They were flexible structures which appeared to be 'curly' (Fig. 2), were carried on 4 to 41% of cells in stationary phase culture depending on the strain (Table 1), and their number varied between cells in a population. There was no difference in counts of fimbriate cells of A312 made from grids prepared by the spray method compared with the usual drop method, so there was no preferential adherence of fimbriate cells to the grid to distort the counts.

No structures were found on two (A4 and SCI 13) of the nineteen strains.

Capsule staining

Only one strain, A334, was noncapsulate. Of the capsulate strains, ATCC 23745, NCTC 9343, SCI 9, SCI 7, A459, A363, A312, A4 and 2/79 all had both capsulate and noncapsulate cells in the same population.

Table 1. Expression of surface structures and surface hydrophobicity of *B. fragilis* strains

Strain	Percentage of cells carrying:		Hydrophobicity† (%)	RRL width‡ (nm)
	Fibrils	Fimbriae*		
23745	12.3	—	7.3 (5.4)	23.9 (4.2)
A459	—	41	38.0 (2.7)	7.9 (1.7)
A312	—	35	31.5 (1.8)	20.7 (3.1)
MRI 8	—	30	28.5 (1.4)	13.3 (2.2)
SCI 7	—	20	47.1 (1.9)	14.9 (3.2)
MRI 2	—	19	12.1 (1.3)	15.3 (2.9)
MRI 10	—	16	52.1 (5.4)	19.5 (3.1)
2/79	—	14	10.9 (1.3)	21.3 (3.9)
MRI 9	—	11	9.8 (2.5)	21.8 (4.2)
MRI 7	—	11	17.4 (1.4)	19.3 (2.1)
A363	—	10	14.8 (5.7)	11.6 (2.2)
MRI 3	—	10	9.2 (1.0)	13.1 (1.9)
SCI 9	—	8	13.1 (1.4)	15.4 (1.9)
9343	—	7	16.6 (3.1)	20.7 (4.3)
A200	—	7	6.6 (2.7)	15.0 (2.2)
MRI 6	—	6	8.2 (0.7)	14.9 (1.6)
A334	—	4	51.9 (1.8)	18.1 (4.5)
A4	—	—	10.7 (2.6)	14.1 (3.2)
SCI 13	—	—	25.1 (3.1)	19.5 (2.9)

* Counts on 100 cells were made from a 24 hour broth culture, negatively stained.

† Hydrophobicity values calculated are means (SD values in parentheses) from six replicates from two separate batches of cells.

‡ Values represent the mean thickness (SD values in parentheses) of the RRL on ten different cells.

Fig. 1. Laboratory strain ATCC 23745 negatively stained with 1% methylamine tungstate. (a) Clumped fibrils forming a matrix around the cell. Bar, 0.5 μ m. (b) Zones around nonfibrillar cells were often seen which may have been due to a repulsion between fibrils and the cell surface. Bar, 0.5 μ m. (c) Fibrils fused across spaces between cells. Bar, 0.5 μ m. (d) Staining of ATCC 23745 with RR revealed the RRL of width 23.86 ± 4.24 nm. Fibrils did not stain. Bar, 0.1 μ m. (e) Strain A363 stained with RR revealed the RRL with loose amorphous material coming off the cell surface. Bar, 0.1 μ m.

Fig. 2. *B. fragilis* A312 negatively stained with methylamine tungstate, showing the 'curly' appearance of the fimbriae (arrowed). Fimbriae appeared to clump at the base and spread at the ends to show the individual thin structures. Bar, 0.1 µm.

The largest capsules were seen on strain ATCC 23745, although this strain and strains A363, A312, A4, NCTC 9343, MRI 7 and MRI 9 showed a range of capsule sizes. The proportion (over 90%) of capsulate cells of ATCC 23745 did not vary with growth phase.

Phase variation

Fibril expression on *B. fragilis* ATCC 23745 did not vary significantly with growth phase, incubation temperature or medium. However, the expression of fimbriae by strain A312 did show phase variation, decreasing from 35% of cells in the inoculum to almost zero in early exponential phase, and increasing again to 34% in late exponential phase. Expression of fimbriae was also completely repressed at all stages of growth by incubation at 20 °C. In contrast, fimbrial expression was not affected by incubation at 42 °C. When cells were examined after growth in FUM or BHI, no variation in expression was observed between the two types of broth culture.

Ruthenium red fixation

All strains carried an amorphous electron dense RRL adjacent to the outer membrane (Fig. 1*d*). The width of this layer ranged from 7.9 ± 1.7 nm on strain A459 to 23.9 ± 4.2 nm on ATCC 23745 (Table 1). Fimbriae,

fibrils or capsules were not observed after RR staining, although some cells did show loosely attached fragments of RR staining material close to the RRL (Fig. 1*e*). No structure equivalent to the RRL was seen in the nonstained control sections.

Cell surface hydrophobicity

The most hydrophobic strain was MRI 10 (52.1%), and the most hydrophilic was strain A200 (6.6%) (Table 1). The remaining strains fell into a range between these values and no clear groupings were visible.

When the bacteria in the aqueous phase were negatively stained after partitioning of A312, there was no difference in the proportion of fimbriate cells when compared to the original unassayed suspension. Therefore fimbriate bacteria were not being preferentially partitioned with the hexadecane layer.

Haemagglutination

Only one strain of *B. fragilis* (A459) was able to strongly haemagglutinate, and it agglutinated all four blood types tested. No other strain agglutinated horse or human RBCs. All strains except NCTC 9343, A4, SCI 9, 2/79, MRI 3, MRI 8 and MRI 9 were able to haemagglutinate rabbit blood (Table 2), although most strains were only able to haemagglutinate weakly at bacterial concentra-

Table 2. Haemagglutination by strains of *B. fragilis*

++++, Haemagglutination at 9.37×10^7 bacteria ml⁻¹; + + + +, at 1.87×10^8 ml⁻¹; + + +, at 3.75×10^8 ml⁻¹; + +, at 7.5×10^8 ml⁻¹; +, at 1.5×10^9 ml⁻¹.

Strain	Structure type*	Haemagglutination of RBCs from:			
		Chicken	Rabbit	Horse	Human group O
23745	Fib	+	+	-	-
A459	F	++++	++++	+++	++++
A312	F	-	+	-	-
MRI 8	F	-	-	-	-
SCI 7	F	-	+	-	-
MRI 2	F	-	+++	-	-
MRI 10	F	+	+	-	-
2/79	F	-	-	-	-
MRI 9	F	-	-	-	-
MRI 7	F	-	+	-	-
A363	F	+	-	-	-
MRI 3	F	-	-	-	-
SCI 9	F	-	-	-	-
9343	F	-	-	-	-
A200	F	-	++	-	-
MRI 6	F	-	+	-	-
A334	F	-	+	-	-
A4	B	-	-	-	-
SCI 13	B	-	+	-	-

* F, fimbriate strain; Fib, fibrillar strain; B, bold strain.

tions of 1.5×10^9 bacteria ml⁻¹. For strain A459, chicken blood was the most positive, being agglutinated at a bacterial concentration of 9.37×10^7 bacteria ml⁻¹. Thus there was no correlation between surface structure expression, cell surface hydrophobicity and the ability to haemagglutinate.

Discussion

This study has shown that *B. fragilis* consists of three structural subgroups, as strains were found to carry fimbriae, fibrils or no surface structures.

Fibrils have not previously been reported on *B. fragilis*, but have been found on other *Bacteroides* species (Handley & Tipler, 1986; Weiss *et al.*, 1988; Devine *et al.*, 1989). Fibrils were found only on strain ATCC 23745, and appear to be atypical for *B. fragilis*. The majority of the strains possessed fimbriae with a mean diameter of 2.1 nm, which fall into the 'very thin' category proposed by Paranchych & Frost (1988).

The fimbriae observed in this study appeared in some preparations to be 'curly', the term used by Yoshimura *et al.* (1984) to describe the fimbriae of *B. gingivalis* 381. However, further high resolution electron microscopy studies are necessary to clarify their ultrastructure in

detail. Thin curly fimbriae are not restricted to *Bacteroides*. They have also been observed on *Haemophilus influenzae* (van Alphen *et al.*, 1988) and the K99 adhesin of *E. coli* was described as having an 'open helical' structure (Jacobs & de Graaf, 1985).

Fibril expression by *B. fragilis* ATCC 23745 was not subject to phase variation, but fimbrial expression by A312 showed repression at 20 °C and in early exponential phase. This supports work by van Doorne *et al.* (1987), who showed by Western blotting that the expression of *B. fragilis* fimbriae was repressed at 20 °C. However, even at maximum expression the majority of *B. fragilis* cells were nonfimbriate.

Strain ATCC 23745 has a range of capsule sizes, and both capsulate and noncapsulate cells are present in a population (Patrick *et al.*, 1986); this was seen with the capsulate strains studied here. Only 12.3% of ATCC 23745 cells carried fibrils whereas 90% of cells carried capsules, so capsules and fibrils represent distinct cell surface polymers and fibrils are not artefacts representing the collapsed capsule.

The RRL has previously been called the capsule by Kasper (1976) and Kasper *et al.* (1980). However, the noncapsulate *B. fragilis* strain A334, which showed no halo by the Indian ink method, possessed a RRL. Therefore the two techniques are not detecting the same

structure and the term capsule should be reserved only for the halo seen by light microscopy. All strains in this study had a RRL which was carried by all cells within a population even when a subpopulation was noncapsulate. The function of the RRL is not known, although its thickness may be able to influence the level of cell surface hydrophobicity (Harty & Handley, 1989), but no such correlation was observed in this study.

Although RR did not preserve the capsules of *B. fragilis* strains in this study, Patrick *et al.* (1986) did detect a RR staining matrix on ATCC 23745 and NCTC 9343 which they suggested was the capsule. RR has also been shown to stain the glycoprotein fibrils of *S. salivarius* (Handley *et al.*, 1988), but fibrils of *B. fragilis* ATCC 23745 were found not to stain with RR in this study.

The *B. fragilis* strains studied here showed fairly low cell surface hydrophobicity values. Capsules are usually hydrophilic and may therefore contribute to the low hydrophobicity of these strains. It has been proposed that hydrophobic properties may be carried on some types of fimbriae (Boedeker *et al.*, 1979; Honda *et al.*, 1984; Sherman *et al.*, 1985), but there was no correlation between surface structure type and hydrophobicity for these strains. Even for fimbriate and fibrillar strains, the majority of cells in a population did not carry surface structures, and therefore the effect of fibrils and fimbriae on cell surface hydrophobicity is likely to be limited.

Several previous papers have discussed the ability of *B. fragilis* to agglutinate RBCs. Haemagglutination by *B. fragilis* has been proposed to be a property of the capsule (Riley & Mee, 1984), electron dense layer (Patrick *et al.*, 1988) and fimbriae (Pruzzo *et al.*, 1984). This study, in common with the work published by Vel *et al.* (1986), Shinjo & Kiyoyama (1984) and Pruzzo *et al.* (1989), found no correlation between surface structures and the ability to agglutinate RBCs. From all the conflicting data listed above it may be possible that more than one adhesin may be involved or that an outer membrane protein may be responsible for haemagglutination.

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