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Adherence and invasion of *Bacteroidales* isolated from the human intestinal tract

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ABSTRACT

Members of the genera *Bacteroides* and *Parabacteroides* are important constituents of both human and animal intestinal microbiota, and are significant facultative pathogens. In this study, the ability of *Bacteroides* spp. and *Parabacteroides distasonis* isolated from both diarrhoeal and normal stools (n = 114) to adhere to and invade HEp-2 cells was evaluated. The presence of putative virulence factors such as capsule and fimbriae was also investigated. Adherence to HEp-2 cells was observed in 75.4% of the strains, which displayed non-localized clusters. Invasion was observed in 37.5% and 26% of the strains isolated from diarrhoeal and non-diarrhoeal stools, respectively. All strains displayed a capsule, whereas none of them showed fimbriae-like structures. This is the first report of the ability of *Bacteroides* spp. and *P. distasonis* to adhere to and invade cultured HEp-2 epithelial cells.

Keywords adherence, *Bacteroides* spp., capsule, HEp-2 cells, invasion, *Parabacteroides* spp.

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INTRODUCTION

Intestinal bacteria belonging to the order *Bacteroidales* are anaerobic commensals constituting only 1–2% of the human gastrointestinal microbiota, yet they are the most important human anaerobic pathogens [1]. Adhesion to the epithelial surface is considered to be a prerequisite for pathogenicity in most bacteria, and this attachment may be selective for different cell types [2]. Establishing a site in the host is critical to the role of *Bacteroides* spp. and *Parabacteroides* spp. [3], both as commensals located in the mucosal surfaces of the intestinal epithelium and as pathogens causing abscesses or other infectious processes. These organisms contain a variety of cell surface molecules that are either critical or advantageous for colonization, including adhesins, haemagglutinins, a polysaccharide capsule, fimbriae and proteases [4,5].

Bacteroides fragilis is one of the most important species of intestinal *Bacteroidales* and possesses a complex capsular polysaccharide composed of at least eight different polysaccharides, which appear to be antigenically diverse [6–8]. Also, *B. fragilis* may express three different types of capsule, large or small, and an electron-dense layer. This heterogeneous nature of encapsulating structures within individual strains could explain the controversial observations in the literature with respect to the *B. fragilis* surface structures related to adhesion [9,10]. Similar surface structures have been seen in other intestinal species, such as *B. thetaiotaomicron* [11].

Outer membrane proteins (OMPs) function as a dynamic interface between the cell and its surroundings, and their functions include maintenance of cell structure, passive and active transport, adhesion, and binding of a variety of

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substances [12]. OMPs of several Gram-negative microorganisms contribute to the invasion of mammalian cells. For example, adhesion of enteropathogenic *Escherichia coli* to epithelial cells is mediated by a OMP encoded by the *eae* gene [13], and the invasion of *Yersinia* spp. is mediated by an OMP encoded by the *inv* gene [14].

Concerning anaerobes, there is little information about pore-forming OMPs and virtually none about the importance of these proteins in adhesion, invasion and antimicrobial resistance. OmpA was shown to be the major OMP in *Bacteroides* spp., and may be important in the attachment of the organism to mucus glycans [15,16].

Although they are not of intestinal origin, the HeLa and HEp-2 cell lines have been useful in assessing the adherence of *E. coli* isolated from diarrhoeal and non-diarrhoeal stools in different geographical regions [17,18] and have been used extensively for this purpose. In *B. fragilis*, adherence to and histopathological effects on intestinal cells such as Caco-2 and WiDr have been shown, but with heterogeneous adherence patterns [19,20]. Therefore, an investigation was undertaken to determine whether the HEp-2 cell line would be useful in assessing the adherence and invasion capabilities of several *Bacteroides* spp. and *Parabacteroides* spp., and to determine whether the presence of specific factors (including capsule and fimbriae) could be correlated with adhesion or invasion. This is the first report of the ability of *Bacteroides* spp. to adhere to and invade HEp-2 cells.

MATERIALS AND METHODS

Strains and culture conditions

In total, 114 strains of intestinal *Bacteroidales* were tested: 64 isolated from 15 children with diarrhoea, and 50 from 24 children without diarrhoea (age range from 2 months to 8 years) (six and ten *Parabacteroides distasonis*, 39 and 27 *B. fragilis*, eight and six *B. vulgatus*, six and one *B. uniformis*, five and two *B. ovatus*, zero and two *B. eggerthii*, and zero and two *B. thetaiotaomicron*, respectively). Bacteria were isolated between April and December 2000, identified by established methods [21] and stored in skimmed milk at −80°C, at the Anaerobe Laboratory of the Department of Microbiology (University of São Paulo-USP). The Ethics Commission of the Institute of Biomedical Sciences, USP, approved this study (Process number 158). Organisms were grown in brain–heart infusion (BHI) broth (Difco Laboratories, Detroit, MI, USA), under anaerobiosis at 37°C, for 24 h in all experiments.

Adherence assay

Adherence assays using 1×10^5 HEp-2 cells were performed as previously described [22]. Briefly, 960 μL of Dulbecco's modified Eagle's medium (Cultilab, Campinas, SP, Brazil) with fetal bovine serum (2% v/v) were added to the wells of tissue culture plates and inoculated with 40 μL of bacterial culture (*c.* 1.5×10^8 cells/mL). Plates were incubated (3 h in 5% CO_2), washed three times with 0.1 M phosphate-buffered saline (PBS) (136 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), fixed (absolute methanol), and stained with May–Grünwald–Giemsa stains. The enteropathogenic *E. coli* strain E2348/69 displaying a localized adhesion pattern was used as a control. All assays were performed in duplicate.

Invasion assay

Invasion assays were performed as described by Nakagawa *et al.* [23] with modifications. A bacterial suspension (100 μL of a 24-h culture, *c.* 1.5×10^8 cells/mL) was added to *c.* 1×10^5 epithelial cells, and the plates were incubated (2 h in 5% CO_2). Non-adherent bacteria were killed by incubation (1 h in 5% CO_2, at 37°C) in 1 mL of Dulbecco's modified Eagle's medium containing 2% fetal bovine serum with either metronidazole at 200 mg/L (for *B. fragilis*) or gentamicin at 300 mg/L (for *E. coli*). The antibiotics used at these concentrations did not affect the morphology of the epithelial cells. Then, the cells were washed in PBS (three times) and lysed in 400 μL of Triton X-100 (1%, v/v). After mixing, the suspension was transferred to tubes containing 1.6 mL of BHI broth, and aliquots of 100 μL were plated on Brucella blood agar (Difco Laboratories, UK), and incubated anaerobically (37°C, 48 h), and the number of CFUs was determined. An enteroinvasive *E. coli* strain, serotype O124:NM, and the non-invasive *E. coli* strain HB101 were used respectively as positive and negative controls. All assays were performed in duplicate. Invasion was expressed as the percentage of bacteria recovered from the initial inoculum after antibiotic treatment and lysis of epithelial cells according to Tang *et al.* [24].

Antiserum production

Specific antisera were raised in rabbits against *B. fragilis* P60f and *B. vulgatus* P15f, respectively, which proved to be the most invasive strains (data not shown; see Results). Sera were obtained from two female rabbits (2 months old, *c.* 2 kg), which were injected in the right ear vein with 500 μL of an overnight culture of *B. fragilis* P60f and *B. vulgatus* P15f (*c.* 1.8×10^7 bacteria/mL). Three more intravenous injections of increasing volume (1, 2 and 4 mL of culture) were given over the following 8 days. Post-immunization sera were obtained 10 days after the last injection and kept in aliquots at −20°C until use.

Serum absorption

Anti-*B. fragilis* P60f and anti-*B. vulgatus* P15f sera were absorbed with non-invasive strains (*B. fragilis* S15g and *B. vulgatus* S56e, respectively). Five hundred microlitres of whole serum diluted in PBS were added to a pellet of bacteria obtained after 48 h of growth in 200 mL of BHI broth. After vortexing, the mixtures of bacteria and antisera were incubated overnight at 4°C with shaking. After centrifugation,

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the absorbed sera were recovered. The procedure was repeated four times.

Confocal microscopy to detect invasion

After antibiotic treatment in the invasion assay, cells were washed in PBS and fixed with paraformaldehyde (4%, v/v) at 4°C overnight. Slides were washed with PBS and incubated with whole anti-*B. fragilis* and anti-*B. vulgatus* serum (diluted 1 : 100 in PBS containing 0.01% Tween-20 and 0.5% bovine serum albumin (BSA) for 30 min at 37°C. The cells were then washed in PBS for 5 min and incubated with phycoerythrin-conjugated anti-rabbit IgG (Sigma, St Louis, MI, USA) diluted 1 : 100 in PBS containing 0.05% Tween-20 and 0.5% BSA for 30 min at 37°C. After three washes in PBS, the slides were incubated with 3,3′-dihexyloxacarbocyanine iodide ($DIOC_6(3)$) (5 mg/L final concentration) for staining of bacterial and HEp-2 cell membranes. After incubation, the slides were washed in PBS and embedded in Mowiol (Calbiochem, San Diego, CA, USA), covered with coverslips, and stored at 4°C until use. Samples were analysed in an LSM510 META laser scanning confocal microscope (Carl Zeiss, Jena, Germany). The excitation wavelengths were 488 nm (argon laser) for $DiOC_6(3)$ (green fluorescence) and 543 nm (HeNe laser) for Propidium Iodide (PI) and phycoerythrin (red fluorescence). Fluorescent images were obtained with a 100×/1.3 oil Plan Apochromatic objective lens, and the laser power and the irradiation time were minimized to avoid photobleaching and possible photodynamic effects. The images were analysed with LSM 5 IMAGE software (Carl Zeiss).

Agglutination assays

Agglutination assays were performed to assess the antiserum titres. Suspensions of *P. distasonis* S63a, *B. fragilis* S15g, *B. fragilis* S16a, *B. fragilis* S18c, *B. distasonis* S43b, *B. ovatus* S43e, *B. vulgatus* S53c, *B. vulgatus* S56e, *B. thetaiotaomicron* S69c, *B. eggerthii* S72e, *B. thetaiotaomicron* S72g, *B. vulgatus* P15f, *B. fragilis* P60a, *B. fragilis* P60f, *B. uniformis* P65d and *B. fragilis* ATCC 43858 were washed and resuspended in PBS (*c.* 1.5×10^8 bacteria/mL). Ten microlitres of washed bacterial suspension was added to wells containing 100 μL of anti-*B. fragilis* and anti-*B. vulgatus* whole serum (1 : 5). The agglutination results were read semiquantitatively.

Immunogold labelling

Immunogold labelling of *B. fragilis* P60f, *B. vulgatus* P15f, *B. ovatus* S43e and *B. fragilis* ATCC 43858 was performed as described by Nara *et al.* [25] with modifications. Broth cultures were used for analysis. For whole cell immunogold labelling, nickel 400-mesh grids coated with formvar and carbon were floated on droplets of cells suspended in PBS and incubated for 1 min. Excess liquid was blotted off. The grids were incubated for 1 h at 37°C with absorbed anti-*B. fragilis* and anti-*B. vulgatus* antiserum, respectively, diluted (1 : 100) in PBS containing 0.01% Tween-20 and 1.5% BSA. After incubation, the grids were washed with PBS and incubated for 30 min with 5-nm-gold-conjugated goat anti-rabbit IgG (diluted 1 : 100). The grids were further washed with PBS and water, stained with 2% uranyl acetate for 1 min, and examined with a Zeiss EM 109 transmission electron microscope (TEM) operated at 80 kV.

Detection of capsule

Capsules were detected by Hiss staining with modifications [4]. A drop of bacterial suspension, from a culture in peptone–yeast extract with glucose (1%, w/v), was deposited on a microscope slide, covered with crystal violet, and heated until vapour emission. Each slide was washed with copper sulphate (20%, w/v) and allowed to air-dry. Capsules were identified under light microscopy (100×) as clear areas around the bacteria.

Detection of fimbriae

The presence of fimbriae was investigated by negative staining with phosphotungstic acid (2%) and uranyl acetate (2%), and examined with a Zeiss EM 109 TEM operated at 80 kV [25].

Statistical analyses

All statistical analyses were performed with GRAPHPAD INSTAT statistical analysis software (version 3.05, Graphpad Software, San Diego, CA, USA). The data were calculated from two experiments, and analysed using the chi-square test and Fisher's exact test. A p-value of less than 0.05 was considered significant.

RESULTS

Adhesion assay

Adherence to HEp-2 cells was observed with 86 of the 114 strains tested. Of these, 49 accounted for 76.5% of the isolates from diarrhoeal stools ($n = 64$) and, at a very similar rate, 37 accounted for 74% of the non-diarrhoeal stools ($n = 50$). Adherent bacteria displayed non-localized clusters, and were easily differentiated from the enteropathogenic *E. coli* strain E2348/69, which displayed a localized adhesion pattern (Fig. 1a–d).

Invasion assay

Thirty-seven of the 86 adherent strains were able to invade HEp-2 cells. None of the non-adherent strains was able to invade HEp-2 cells. Of the adherent strains, 24 accounted for 37.5% of the isolates from diarrhoeal stools and 13 for 26% of the non-diarrhoeal stools. Nevertheless, the strains isolated from children with diarrhoea appeared to be more invasive than the strains of the corresponding species isolated from control children, but the difference was not statistically significant ($p = 0.229$). *B. fragilis* (mean: 4.78×10^3 CFU/mL) and *B. vulgatus* (mean: 12.88×10^3 CFU/mL) isolated from diarrhoeal stools were the most invasive species (Table 1).

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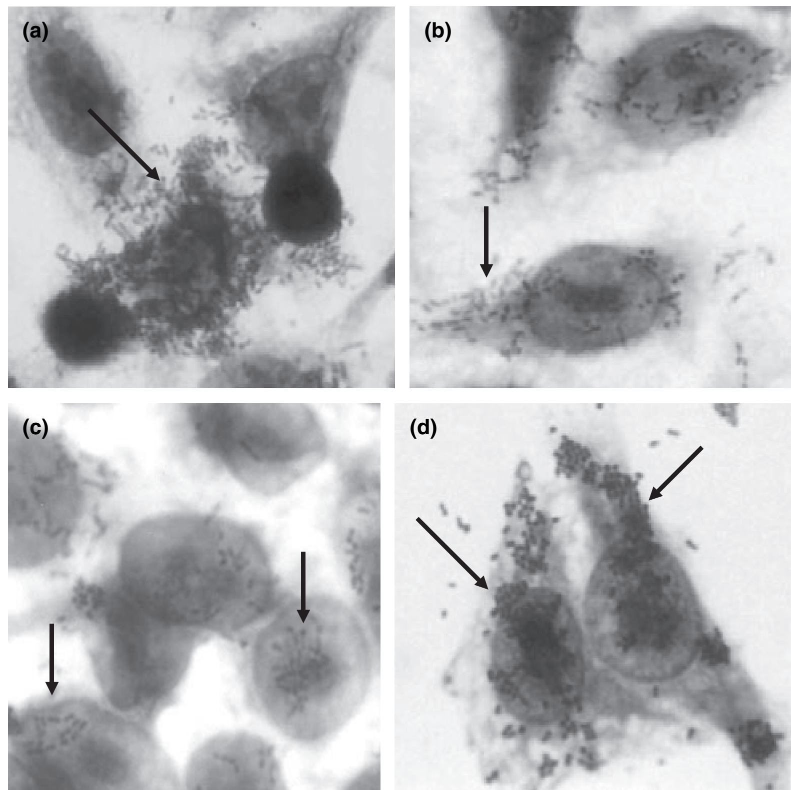

Fig. 1. Adherence to HEp-2 cells of members of the intestinal *Bacteriodales*. (a) *Bacteroides fragilis* P60c; (b) *B. ovatus* P23e; (c) *B. vulgatus* P23l and (d) *Escherichia coli* O127:H6 (E2348/69) (enteropathogenic). Arrows show the bacterial adherence. Magnification: 1000×.

Table 1. Adhesion and invasion assays of 114 strains of the intestinal *Bacteroidales* isolated from children with and without diarrhoea

	Diarrhoea				No diarrhoea			
Species	No. of isolates	Adhesion-positive (%)	Invasion-positive (%)	CFU/mL[a]	No. of isolates	Adhesion-positive (%)	Invasion-positive (%)	CFU/mL[a]
Parabacteroides distasonis	6	4 (66.6)	2 (33.3)	1.14×10^3	10	7 (70)	2 (20)	0.06×10^3
Bacteroides fragilis	39	30 (77)	13 (33.3)	4.78×10^3	27	20 (74.1)	6 (22.2)	1.68×10^3
B. vulgatus	8	6 (75)	4 (50)	12.88×10^3	6	5 (83.3)	2 (33.3)	2.36×10^3
B. uniformis	6	5 (83.3)	5 (83.3)	3.02×10^3	1	1	0	0
B. ovatus	5	4 (80)	0	0	2	2	2	0.14×10^3
B. eggerthii	0	0	0	0	2	1	0	0
B. thetaiotaomicron	0	0	0	0	2	1	1	0.12×10^3
Total	64	49 (76.5)	24 (37.5)		50	37 (74)	13 (26)	
Escherichia coli	1	1[b]	1[c]	8.8×10^3				
E. coli[d]	1	0	0	0				

[a]Mean of two assays.
[b]*E. coli* E2348/69 (enteropathogenic) for adhesion assay.
[c]*E. coli* O124 NM (enteroinvasive) for invasion assay.
[d]*E. coli* HB101, positive and negative controls, respectively.

Antiserum production and characterization

B. fragilis P60f and *B. vulgatus* P15f were the most active in the invasion assay, and were selected for antiserum production. Antibody titres were measured by agglutination assays. The anti-*B. fragilis* P60f antiserum agglutinated the cognate strain and *B. fragilis* S15g, *B. ovatus* S43e, *B. fragilis* P60a and *B. uniformis* P65d. Both sera were able to recognize invasive and non-invasive strains.

The specific antisera were absorbed with the non-invasive strains *B. fragilis* S15g and *B. vulgatus* S56e, respectively. After absorption, anti-*B. fragilis* P60f serum was able to agglutinate *B. fragilis* P60f, but not the four other strains. Anti-*B. vulgatus* P15f serum did not agglutinate the cognate strain, but did agglutinate *B. fragilis* P60f. Neither whole nor absorbed antisera were able to agglutinate *E. coli* K12 (data not shown).

Confocal microscopy using fluorescent stain

Strains of intestinal *Bacteroidales* were internalized into HEp-2 cells after 2 h of incubation as accumulated clusters in the cytoplasm and uniformly around the membrane (Fig. 2). Confocal microscopy showed that *B. fragilis* P60f and *B. vulgatus* P15f, labelled with their respective cognate antisera, occurred in clusters and were dispersed around cellular nuclei. Labelled *B. fragilis* P60f is shown in Fig. 2a, with the bacteria in red (phycoerythrin) and the membranes in green ($DiOC_6(3)$) (Fig. 2b), and the overlap image with orange–red fluorescence (Fig. 2c).

Immunogold labelling

Absorbed anti-*B. fragilis* and anti-*B. vulgatus* sera were immunogold labelled. The antisera were added to suspensions of *B. fragilis* ATCC 43858 (non-invasive), *B. ovatus* S43e (invasive), *B. fragilis* P60f (invasive) and *B. vulgatus* P15f (invasive) (Fig. 3). Interestingly, neither serum recognized *B. fragilis* ATCC 43858, as only background amounts of label were detected (Fig. 3a), and both sera recognized *B. ovatus* S43e (Fig. 3b). Anti-*B. fragilis* and anti-*B. vulgatus* sera exhibited more binding in the extracellular structures of *B. fragilis* P60f and *B. vulgatus* P15f (Fig. 3c,d).

Detection of capsule

A capsule was observed in all the strains tested by light microscopy (data not shown) and was seen as an electron-dense layer in the TEM (Fig. 4). The bacteria did not show any fimbria-like structures, although vesicles on the bacterial surface were observed (Fig. 4a,b).

DISCUSSION

Several factors have been related to the virulence of *Bacteroides* spp. and *Parabacteroides* spp., such as production of fimbriae, sialidases and other adhesins, but the role of these factors in adherence is still unclear. Also, each member of these species synthesizes significant amounts of multiple capsular polysaccharides, which are structurally different, suggesting distinct functional properties [6,11,19,20,26]. All of the strains analysed in this study showed vesicles and capsule, according to the criteria of Domingues *et al.* [27]

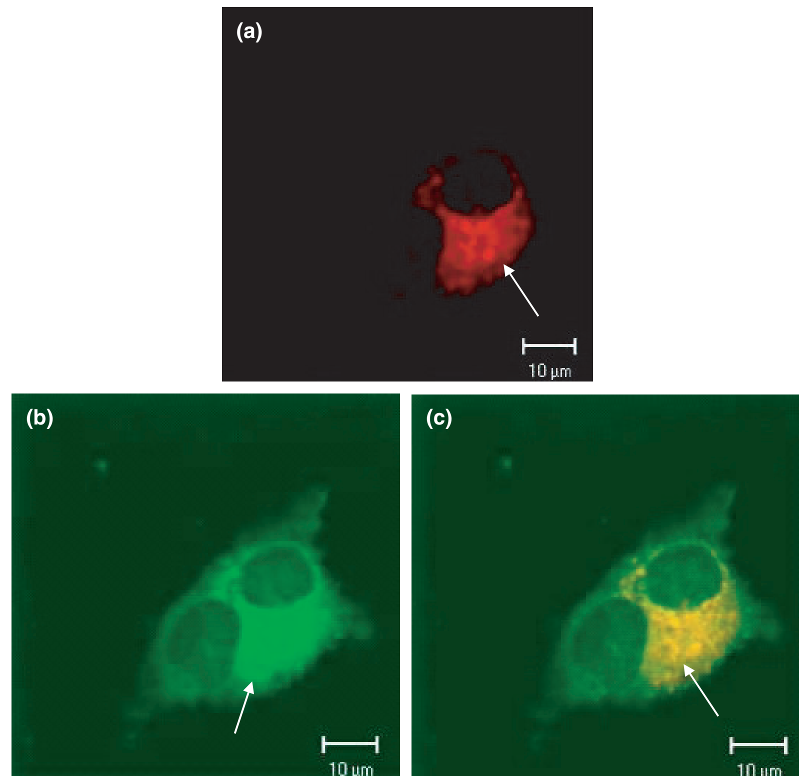

Fig. 2. Bacterial invasion observed by confocal microscopy. Arrows show: (a) *Bacteroides fragilis* P60f labelled with anti-*B. fragilis* antibody and phycoerythrin-conjugated goat anti-rabbit IgG (red); (b) membranes stained with 3,3′-dihexyloxacarbocyanine iodide (green); and (c) overlap image showing invasive bacteria in the HEp-2 cells (orange–red). Images were captured and analyzed with a laser confocal microscope. Magnification: 1000×. Bars = 10 µm.

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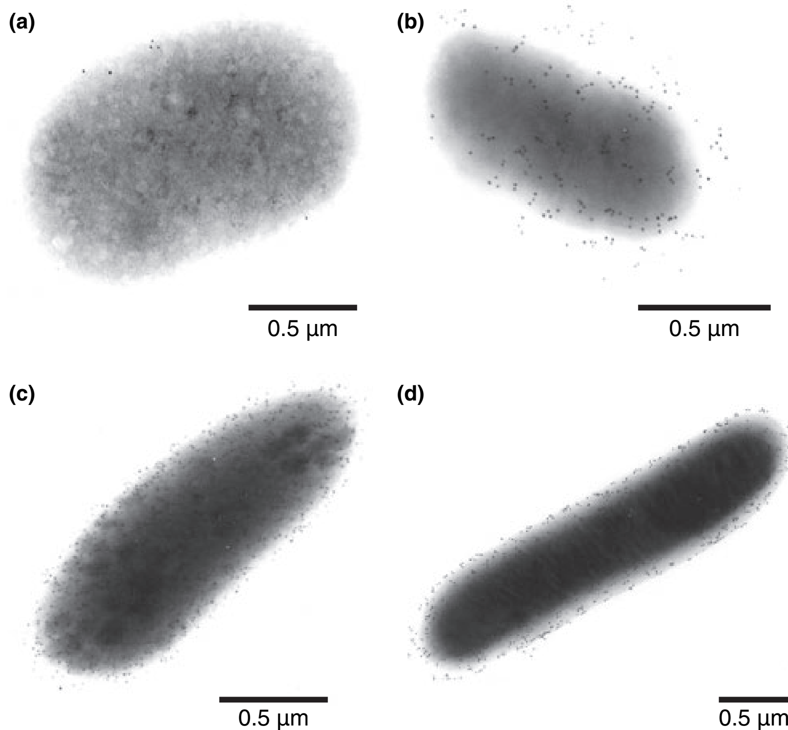

Fig. 3. Bacterial immunogold labelling. (a) *Bacteroides fragilis* ATCC 43858 (non-invasive) labelled with anti-*B. fragilis* antibodies (magnification: 84 000×). (b) *B. ovatus* S43e (invasive) labelled with anti-*B. vulgatus* antibodies (magnification: 114 000×). (c) *B. fragilis* P60f labelled with anti-*B. fragilis* antibodies (magnification: 90 000×). (d) *B. fragilis* P60f labelled with anti-*B. vulgatus* antibodies (magnification: 48 000×). Bars = 0.5 µm.

and Patrick *et al.* [9]. Surface vesicles have been observed in oral bacteria, e.g. *Porphyromonas gingivalis*, and it has been suggested that there is an association between those structures and biological activities, such as proteolysis, haemagglutination and co-aggregation [28].

Encapsulated *Bacteroides* species are most often found in abdominal abscesses and the bloodstream, but have been reported in isolates from resident intestinal microbiota [20]. Capsules were found in this study in all strains from diarrhoeal and non-diarrhoeal stools. Although a direct correlation between the presence of capsule and adhesion or invasion was not seen, it may be that capsule is a necessary but not sufficient factor in these processes. The capsular polymer can first protect *B. fragilis* cells from phagocytosis and then, probably when produced in larger amounts, stabilize adhesion of cells to epithelia. In strains that colonize the intestine, the simultaneous presence of two different adhesins could be needed for bacteria to resist the expulsive action of intestinal peristalsis. This force is certainly much higher than the expulsive actions to which bacteria are exposed in other parts of the body [29].

Several studies have demonstrated the pathogenicity of encapsulated anaerobes and their ability to induce abscesses in experimental animals even when inoculated alone. Onderdonk *et al.* [30] found a correlation between the formation of intra-abdominal abscesses in mice and rats caused by *B. fragilis* strains and the presence of capsule; they also found that encapsulated *B. fragilis* strains adhered better to rat mesothelium than did non-encapsulated strains [31].

In the 86 faecal isolates that were able to adhere to epithelial cells, no fimbria-like structures were observed, but it may be possible that more than one adhesin is involved and/or that OMP(s) could play a role in the adhesion process. Pruzzo *et al.* [29] speculated that knowledge about fimbria-like structures is poor because they require precise growth conditions to be assembled, and low iron concentrations and temperature influence their synthesis.

Most anaerobic pathogens are not primarily invasive. Gram-positive anaerobic bacilli (e.g. *Clostridium tetani* and *Clostridium perfringens*) and most Gram-negative anaerobic bacilli do not invade intact healthy epithelial or mucosal surfaces [32]. Currently, no specific invasion mechanisms have been identified, although *B. fragilis* does have many factors (fimbriae, haemagglutinin, capsule, lipopolysaccharide, outer membrane vesicles, and enzymatic activities) that

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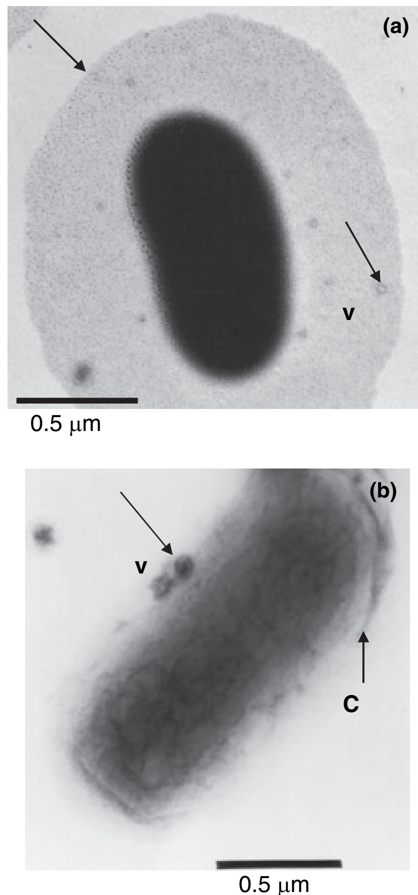

Fig. 4. Transmission electron microscopy of *Bacteroides* strains. Arrows show: (a) the presence of an electron-dense area and vesicles (v) on *Bacteroides fragilis* S15g (magnification 27 400×); and (b) the presence of a thin shell of capsule (c) and of vesicles (v) on the *B. vulgatus* P15f surface and outside (uranyl acetate negative stain). The electron-dense material corresponds to the genomic DNA (magnification: 46 000×). Bars = 0.5 μm.

can perturb host defence mechanisms and initiate tissue destruction [5].

The invasion rates of the *Bacteroides* spp. and *P. distasonis* strains were low in comparison to those of *Porphyromonas gingivalis* [23] and other enteric pathogens such as *Salmonella*, *Shigella* and *Escherichia* [33,34]. Nevertheless, the strains isolated from children with diarrhoea appeared to be more invasive than those from control children.

The Serény test is often performed to evaluate invasive enteropathogens. None of the strains tested was positive in the Serény test as described by Wood *et al.* [35] (data not shown). This observation raises the possibility that *Bacteroides* spp. and *Parabacteroides* spp. could invade epithelial cells by mechanisms other than those found in enteroinvasive *E. coli* and *Shigella*, which yield positive Serény test results. In this study, immunological characterization did not distinguish between the invasive and non-invasive strains. Immunoblotting experiments with invasive strains of *B. fragilis* and *B. vulgatus* using absorbed sera suggested that some proteins were unique to the invasive strains (data not shown), but other studies will be necessary to analyse their possible involvement in the invasion process.

Confocal microscopic analysis of invasion provided further proof that intestinal *Bacteroidales* strains were internalized in HEp-2 cells after 2 h of incubation and accumulated in cytoplasmic clusters inside cells, uniformly around the membrane. In this study, *B. fragilis* and *B. vulgatus* labelled with anti-*B. fragilis* and anti-*B. vulgatus* antisera, respectively, were able to invade epithelial cells. Both bacteria displayed the same profile in confocal microscopy (Fig. 2).

The immunogold-labelled antibody was evenly distributed on the bacterial surface, and the more invasive strains (*B. fragilis* P60f, *B. vulgatus* P15f and *B. ovatus* S43e) were more densely labelled, suggesting that some extracellular structure was more prevalent in these strains and might be associated with adhesion or invasiveness.

This first study demonstrates that *Bacteroides* spp. and *P. distasonis* have the potential to adhere to and invade HEp-2 cells. Although HEp-2 cells are not of intestinal origin, they were chosen for this study because the ability of bacteria to invade these cells is considered to be a good indicator of virulence. In conclusion, these data show differences in invasion of HEp-2 cells by species of the intestinal *Bacteroidales* isolated from diarrhoeal or normal stools. Further studies to characterize the mechanisms involved in the invasion of epithelial cells by *Bacteroides* spp. and *Parabacteroides* spp. are underway.

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TRANSPARENCY DECLARATION

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