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Bile salts enhance bacterial co-aggregation, bacterial-intestinal epithelial cell adhesion, biofilm formation and antimicrobial resistance of *Bacteroides fragilis*

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Abstract

Bacteroides fragilis is the most common anaerobic bacterium isolated from human intestinal tract infections. Before *B. fragilis* interacts with the intestinal epithelial cells, it is exposed to bile salts at physiological concentrations of 0.1–1.3%. The aim of this study was to determine how pre-treatment with bile salts affected *B. fragilis* cells and their interaction with intestinal epithelial cells. *B. fragilis* NCTC9343 was treated with conjugated bile salts (BSC) or non-conjugated bile salts (BSM). Cellular ultrastructure was assessed by electron microscopy, gene expression was quantified by comparative quantitative real-time RT-PCR. Adhesion to the HT-29 human intestinal cell line and to PVC microtitre plates (biofilm formation) was determined. Exposure to 0.15% BSC or BSM resulted in overproduction of fimbria-like appendages and outer membrane vesicles, and increased expression of genes encoding RND-type efflux pumps and the major outer membrane protein, OmpA. Bile salt-treated bacteria had increased resistance to structurally unrelated antimicrobial agents and showed a significant increase in bacterial co-aggregation, adhesion to intestinal epithelial cells and biofilm formation. These data suggest that bile salts could enhance intestinal colonization by *B. fragilis* via several mechanisms, and could therefore be significant to host–pathogen interactions.
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1. Introduction

Bacteroides fragilis is the most prevalent anaerobic bacterium isolated from human intestinal infections. Factors contributing to its ability to persist in high numbers in the gut, both as a commensal and as an infectious opportunistic pathogen, include high bile tolerance, an exceptional capability to use a wide range of dietary polysaccharides and the capacity to create variable surface antigenicities to evade host immune responses and antibiotics [1,2]. To date, extensive studies on the interactions between bacteria and bile have mostly been conducted in Gram-positive food-associated bacteria [3–7]. A few studies have also been conducted in aerobic Gram-negative bacteria [3,8–10] and none have been conducted in significant intestinal anaerobic bacteria such as *B. fragilis*.

Adherence of bacteria to the surface layer cells of the host enables commensal bacteria and potential pathogens to overcome flushing mechanisms which cleanse mucous membranes [11]. Although adhesion is essential for maintaining members of the normal microflora in their host, it is also the crucial first stage in any infectious disease [11]. The mechanisms by which bacterial cells may adhere

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to epithelial cells or colonize cellular secretions (mainly mucin) are often unknown, although different types of interactions including pili/fimbria- and adhesin-mediated attachment are often involved [11]. Several in vivo studies have demonstrated that 99.9% of bacterial species, including *B. fragilis* can naturally attach to a surface in the form of a biofilm. A biofilm is a structured community of bacterial cells enveloped in a self-produced polymeric matrix and adherent to an inert or living surface [12–15]. Over the past decade bacteria in the form of biofilms have been recognized as important causes of a variety of human infections, including infections of prosthetic devices, endocarditis, dental caries, pneumonia in cystic fibrosis and prostatitis [14,16,17].

In order for a pathogen to successfully colonize the human intestine, it has to overcome the present bile salts. Physiological concentrations of bile in the human intestine can range from 2 to 23 mM (i.e. 0.1–1.3%) depending on the particular anatomical area studied [10,18]. In humans, bile exists in two forms: conjugated and non-conjugated or free bile salts. The conjugated bile salts containing a linked amino acid are synthesized from cholesterol in the liver and are transported to the intestine where they are split (de-conjugated) by hydrolase enzymes into their component free bile salts and amino acids [19]. Most intestinal bacteria can synthesize bile salt hydrolases (BSHs) [6,7,19].

Bile salts have a detergent-like activity, which can permealizes bacterial membranes and can eventually lead to membrane collapse and cell damage [4–6]. Factors that enable the bacterium (e.g. *B. fragilis*) to tolerate bile salts are an important element in its ability to survive in the gut. Stellwag and Hylemon [20] identified a BSH enzyme in *B. fragilis* and demonstrated its activity. To our knowledge, no extensive bile studies have been conducted in *B. fragilis*. Since *B. fragilis* has a high tolerance for bile salts, we hypothesized that the interaction of *B. fragilis* with bile salts affected its subsequent interaction with intestinal epithelial cells and its persistence in the gut. Bacteria have a variety of mechanisms to rid themselves of potentially noxious agents and one of the mechanisms involved is expulsion via an extensive network of efflux pumps. Studies in aerobic Gram-negative bacteria have demonstrated that RND-family efflux pumps can transport bile salts, thus contributing to tolerance of these substances. Also bile salts can modulate expression of these pumps [8–10,21]. Since we recently described and characterized 16 RND-family efflux pumps in *B. fragilis* [22,23], we were interested in exploring whether their expression was effected by the presence of bile salts. In addition, since bile salts act on the membrane, we were interested in investigating their effects on cellular ultrastructure and on expression levels of *ompA*, encoding the most abundant outer membrane protein in *B. fragilis*. The aim of this study was to determine the effect of bile salt treatment on the cell structure of *B. fragilis* NCTC 9343 and the resultant inter-bacterial and bacterial-intestinal epithelial cell interactions, and biofilm formation.

2. Results

2.1. Putative bile salt hydrolases and pilus genes in *B. fragilis*

Two putative BSH genes were identified on the *B. fragilis* genome sequence: Gene BF1508 had 32.7% amino acid identity to *Clostridium perfringens* choloylglycine hydrolase and 35.9% amino acid identity to *Brucella melitensis* choloylglycine hydrolase (this gene was described previously [20]); gene BF3586 had 54.51% amino acid identity to *B. thetaiotaomicron* choloylglycine hydrolase. Both genes were detectable by PCR but only BF1508 gave a detectable transcript in NCTC9343. Two genes (BF3402 and BF3229) encoding putative homologues of the pilus assembly protein PilF were identified. Both these genes had approximately 50% amino acid identity to a *B. theteiotaomicrn* conserved protein.

2.2. Growth rates and survival

Growth kinetic studies demonstrated that *B. fragilis* NCTC9343 had a longer lag phase, slower growth rate and lower maximum growth in the presence of 0.15% bile salt mixture (BSM) (Fig. 1A). *B. fragilis* NCTC9343 grown in the presence of 5% BSM had fewer colony forming units ($\leqslant$50 cfu) than cells grown with media alone or in the presence of 5% bile salt conjugate (BSC) (confluent growth $\approx$5000 cfu), demonstrating that survival in 5% bile salts was also significantly reduced by $\geqslant$100-fold (Fig. 1B).

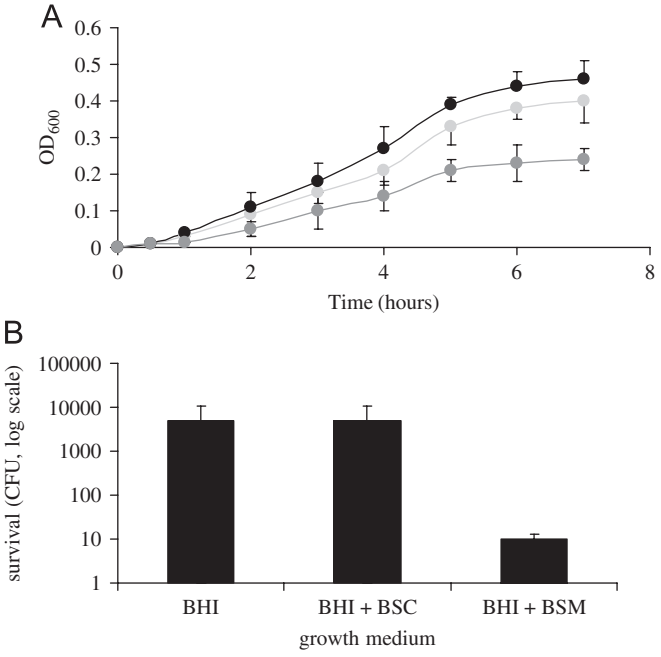

Fig. 1. (A) Growth rates of *B. fragilis* NCTC 9343 with or without 0.15% bile salt treatment. Black is untreated, light gray is treated with 0.15% bile salt conjugate (BSC) and dark gray is treated with 0.15% bile salt mixture (BSM), (B) survival after 12 h with or without exposure to 5% BSC or 5% BSM. An average of five data sets was performed for each experiment.

Viability of cells in 10% BSC was found to be low, comparable to that in 5% BSM (data not shown).

2.3. Bile salt-induced gene expression changes

Exposure of *B. fragilis* NCTC 9343 to either 0.15% BSC or 0.15% BSM resulted in overexpression of *bmeB5*, *bmeB6*, *bmeB15* and *bmeB16*, *ompA1* and reduced expression of *bmeB1* and *bmeB14*. Increase in expression of *bmeB4*, *bmeB12* and the cholylglycine hydrolase gene BF1508 was also observed in the presence of 0.15% BSC only. Increase in expression of *bmeB8*, *BmeB10*, *BmeB11* and *bmeB13* and reduced expression of *bmeB2* was observed in the presence of 0.15% BSM only (Table 1). These data indicate that expression of 13 of the 16 RND-family efflux pumps was affected (either increased or decreased) by exposure to conjugated or non-conjuagted bile salts. The pumps *bmeB5*, *bmeB6*, *bmeB15* and *bmeB16* appear to be involved in transport or some other adaptive mechanism for survival in both conjugated and non-conjugated bile salts, while some of the other pumps appear selectively involved in response to either conjugated or non-conjugated bile salts. It is possible that reduced expression of *bmeB1* and *bmeB14* occurred as a response to create a balance in efflux pump expression.

Table 1
Comparative gene expression in NCTC9343 grown in brain heart infusion broth (BHI); BHI + 0.15% bile salt conjugate (BSC) and BHI + 0.15% bile salt mixture (BSM) (fold-changes)

	Expression (mean fold-change ± SD)[a]		
Growth condition Gene	BHI	BHI + 0.15% BSC	BHI + 0.15% BSM
bmeB1	1.0	***0.15 ± 0.06***[b]	***0.45 ± 0.11***
bmeB2	1.0	1.37 ± 0.42	***0.48 ± 0.09***
bmeB4	1.0	**2.48 ± 0.93**[c]	1.15 ± 0.36
bmeB5	1.0	**9.45 ± 2.25**	**5.92 ± 1.25**
bmeB6	1.0	**4.78 ± 1.67**	**7.84 ± 1.07**
bmeB8	1.0	1.06 ± 0.70	**3.84 ± 0.87**
bmeB10	1.0	0.86 ± 0.19	**4.41 ± 0.75**
bmeB11	1.0	1.64 ± 0.52	**62.68 ± 9.89**
bmeB12	1.0	**4.00 ± 1.14**	0.94 ± 0.09
bmeB13	1.0	0.84 ± 0.07	**6.68 ± 1.89**
bmeB14	1.0	***0.16 ± 0.05***	***0.47 ± 0.35***
bmeB15	1.0	**18.13 ± 4.47**	**200.85 ± 12.97**
bmeB16	1.0	**19.56 ± 3.23**	**69.55 ± 7.76**
BSH	1.0	**2.10 ± 0.44**	**1.36 ± 0.28**
ompA1	1.0	**10.78 ± 2.10**	**24.08 ± 6.35**

[a] Expression changes are presented as the averages ± standard deviation (SD) obtained from five replicates.
[b] Reduction in expression ⩽ 0.5-fold is denoted in bold-italics.
[c] Induction values ⩾ 2-fold are denoted in boldface type.

2.4. Antimicrobial susceptibility and uptake of crystal violet (CV)

The minimum inhibitory concentrations (MICs) of both BSC and BSM on *B. fragilis* were $\geqslant 130\,mg\,L^{-1}$. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) reduced these values to 106 and $64\,\mu g\,mL^{-1}$, respectively. MICs of other antimicrobial agents were increased in NCTC9343 pre-exposed to 0.15% BSC or BSM (Table 2). Addition of CCCP significantly reduced these MICs ($P < 0.05$), suggesting the presence of an energy (PMF) dependent resistance mechanism. Bacteria pre-exposed to 0.15% BSC or BSM also accumulated significantly less crystal violet (CV) than the unexposed cells. Addition of CCCP increased CV accumulation significantly more in the bile salt-exposed bacterium than in the unexposed cells (Table 2), suggesting an energy dependent agent accumulation mechanism.

Both the susceptibility and uptake data, along with the data confirming overexpression of *bmeB* efflux pumps with bile exposure, strongly implicate the involvement of the *bmeB* efflux pumps in transport of various antimicrobial agents and dye, especially in response to bile salt exposure.

2.5. Morphological adaptation in response to bile salt exposure

Scanning electron microscopy (SEM) demonstrated that exposure to 0.15% BSC caused no significant change in cell size compared to untreated cells, with no detectable change in the cell surface. These bacteria were smooth and similar in size to untreated cells with an average length of 1.97 ± 0.29 μm (Fig. 2A). Although cells exposed to 5% BSC had the same viability as untreated cells, they were marginally smaller (average length 1.64 ± 0.27 μm). Exposure to 0.15% BSM caused marked changes in cell

Table 2
Antimicrobial susceptibilities and CV uptake of bile salt-treated vs. untreated *B. fragilis* NCTC9343

Growth medium[a]	MICs ($\mu g\,mL^{-1}$)								CV uptake ($\mu g\,mL^{-1}$)[b]	
	Cefoxitin	Cefoperazone	Ciprofloxacin	Clindamycin	Imipenem	Metronidazole	Ethidium	SDS	−CCCP	+CCCP
BHI	2.3	24.5	3.7	0.39	0.16	1.3	112.3	95.73	3.50 ± 0.57	4.85 ± 0.74
BHIBSC	5.5	66.9	4.7	0.42	0.20	2.2	181.6	⩾102.882	2.33 ± 0.43	4.81 ± 0.65
BHIBSM	4.2	52.0	5.3	0.60	0.28	2.0	181.6	⩾102.882	2.29 ± 0.41	4.82 ± 0.69

[a] BHI, Brain heart infusion broth; BHIBSC = BHI + 0.15% bile salt conjugate; BHIBSM = BHI + 0.15% bile salt mixture ($n = 5$ each).
[b] A total of $5\,\mu g\,mL^{-1}$ of CV was added to the uptake culture tube. Uptakes are presented as the averages ± standard deviation (SD) obtained for five replicates.

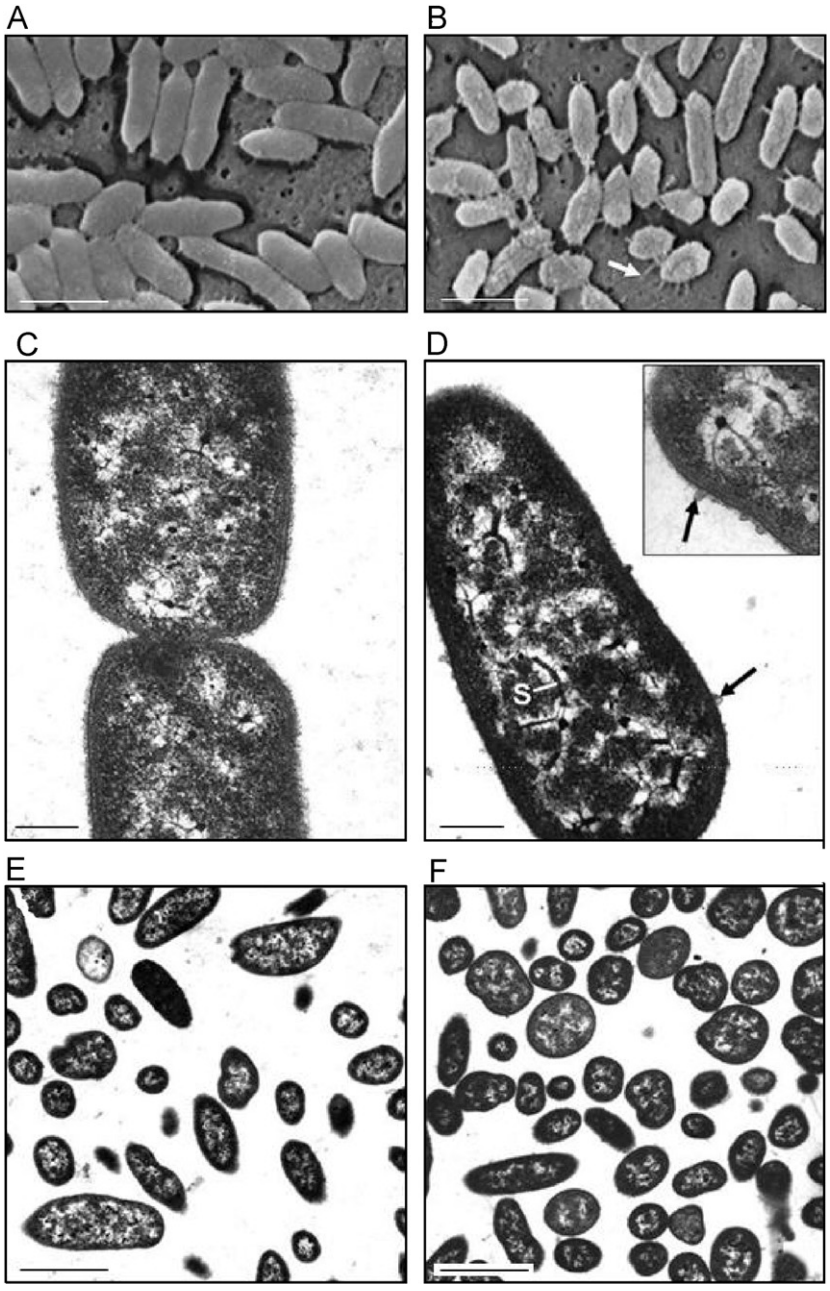

Fig. 2. Morphology of untreated vs. bile salt-treated NCTC 9343. (A) SEM of 0.15% bile salt conjugate (BSC)-treated cells. Scale bar = 2.0 μm. (B) SEM of 0.15% bile salt mixture (BSM)-treated cells. The white arrow indicates the fimbria-like appendages on the surface of the 0.15% BSM-treated cells. Scale bar = 2.0 μm. (C, E) TEM of 0.15% BSC-treated cells. Scale bar on C = 0.2 μm, scale bar on E = 1.0 μm. (D, F) TEM of 0.15% BSM-treated cells. The black arrows indicate the circular surface structures (outer membrane vesicles) on 0.15% BSM-treated cells and S indicates the electron dense regions formed by the nucleic acids and ribosomes in the 0.15% BSM-treated cells. Scale bar on $D = 0.2$ μm, scale bar on $F = 1.0$ μm.

morphology. Most of the cells were significantly reduced in size with an average length of 1.12 ± 0.17 μm. These cells also produced numerous fimbria-like appendages which extruded from the cell surface and adhered to the substrate and to neighboring cells (Fig. 2B).

Transmission electron microscopy (TEM) demonstrated that cells grown with or without bile salt exposure had a certain characteristic common to all viable cells—no enlargement of the periplasmic space (dead cells have an enlarged periplamic space with greatly compromised membrane permeability). The outer membrane, peptidoglycan layer and inner membrane appeared well defined in cells grown in media with or without added bile salts. Cells grown with or without 0.15% BSC had a full cytoplasm (containing a distinct nucleoid and ribosome-rich peripheral cytoplasm); the cell envelope, including the periplasmic compartment, appeared intact (Fig. 2C,E). The nuclear apparatus exhibited a dispersed network of fibrils (approximately 20–30 Å in width), possibly representing the deoxyribonucleic acid portion of chromatin material and

enclosed within an area of low electron density. Cells grown with 0.15% BSM exhibited alterations in the nuclear apparatus. The nuclear fibrils appeared somewhat thicker (approximately 80–100 Å wide) with many more electron dense segments, which occurred as long dark strands (Fig. 2D). Furthermore, these cells had a more electron-dense ribosomal cytoplasm (Fig. 2F), which suggested that they may be dividing more rapidly [24]. These cells also exhibited surface structures with circular cross-sectional profiles blebbing from the outer membrane, and often seen detached from the cell (Fig. 2D inset). These structures had an obvious electron-dense membrane and more electron-translucent lumen and are outer membrane vesicles. However, there was no evidence of breaks in the outer membranes both under low- and high-power magnification. As observed with cells treated with 0.15% BSC, the cell envelope of 0.15% BSM-treated cells appeared continuous even at magnifications as high as 400k ×. Cells grown with 5% BSC were intermediary between cells grown without bile salts and those with 0.15% BSM; i.e., they had a more ribosome-rich cytoplasm, but did not overproduce fimbria-like appendages or exhibit electron-dense segments of nuclear material (data not shown).

2.6. Bacterial co-aggreagtion, adhesion to intestinal epithelial cells and biofilm formation

There was significantly higher adhesion of bile salt-treated *B. fragilis* cells to HT-29 intestinal epithelial cells than untreated *B. fragilis* ($P<0.05$) (Fig. 3A). Untreated bacteria occurred as single or double cells and adhered to mammalian cells in chains, whereas cells treated with either BSC or BSM occurred as co-aggregates of numerous bacteria attached to one another and adhered to the HT-29 cells in defined clusters (Fig. 3A-inserts). There was a small but statistically insignificant difference between adhesion of BSC- and BSM-treated cells. Bile salt-treated cells had increased biofilm formation as shown by their significantly higher binding to polyvinyl chloride (PVC) microtitre plates compared with untreated cells ($P<0.05$) (Fig. 3B). There was also a significant difference in adhesion between BSC- and BSM-treated cells. Similar biofilm assay data

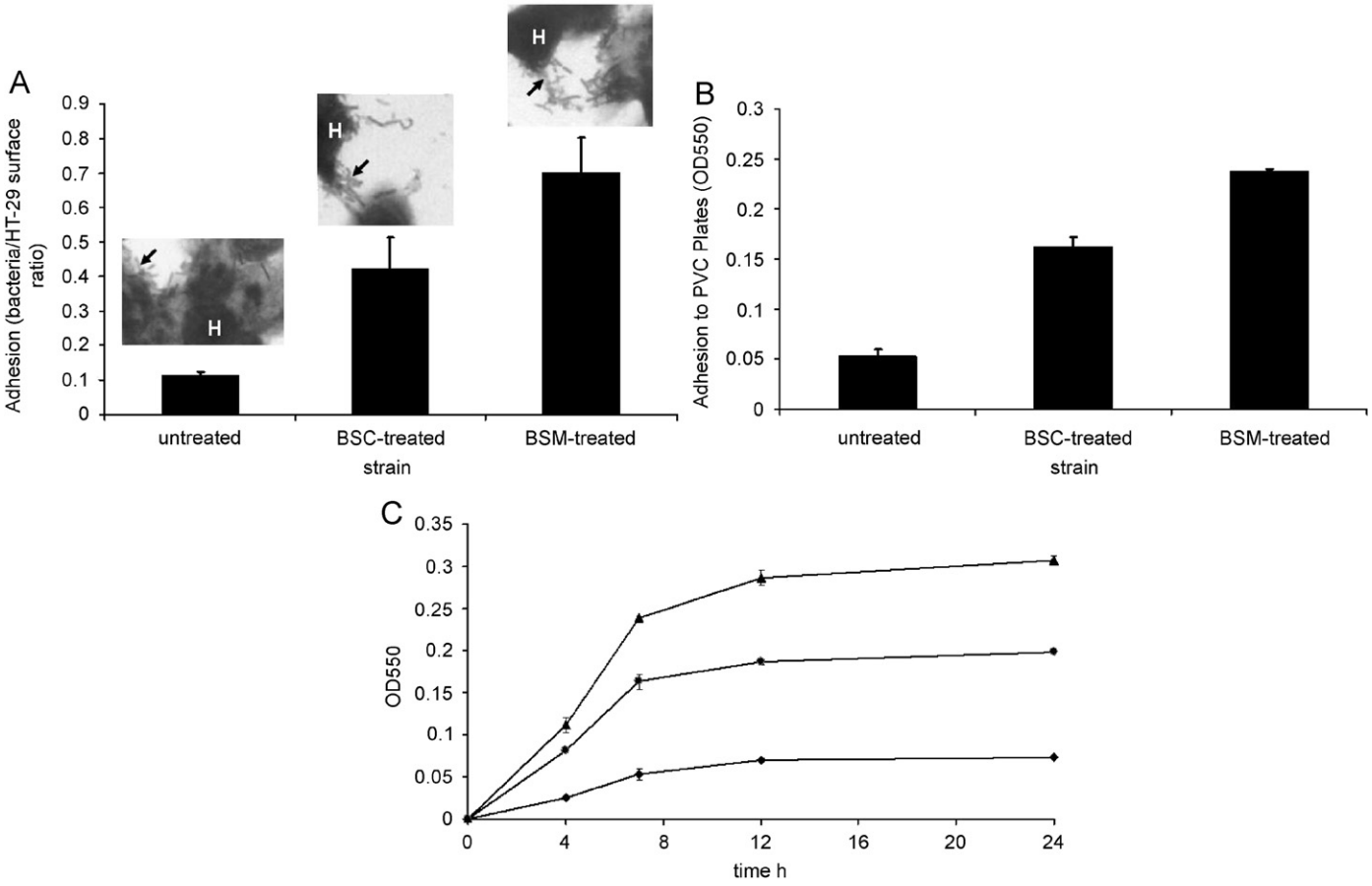

Fig. 3. Bacterial adhesion to HT-29 human intestinal cells (A) and biofilm formation (B). Compared with the untreated, bacteria treated with bile salt conjugate (BSC) or bile salt mixture (BSM) had increased adhesion to HT-29 cells and enhanced biofilm formation. HT-29 adhesion data were represented ratios of the HT-29 cell surface covered by bacteria ± standard deviation. Untreated bacteria adhered in chains whereas BSC or BSM-treated bacteria adhered in clusters (A-inserts). Arrows indicate bacterial cells; H = HT-29 intestinal epithelial cells. Biofilm formation was measured as the mean OD_{550} of bacteria bound to PVC microtitre plates ± standard deviation from five experiments. Biofilm formation kinetics (C) showed that untreated cells formed significantly less biofilms than bile salt-treated cells. Diamonds = untreated; circles = BSC-treated; triangles = BSM-treated. Data are expressed as OD_{550} ± standard deviation. Error bars were small and sometimes not visible.

were obtained when the bound bacteria were stained with basic fuchsin instead of CV (data not shown).

2.7. Biofilm growth curve

For both untreated and bile salt-treated cells, biofilm formation showed a steady increase over a 12 h period. Between the 12 h and the 24 h time point, biofilm formation levelled off to steady state kinetics (Fig. 3C). Although they did not grow faster than the untreated cells, bile salt-treated cells attained a significantly higher biofilm formation between 4 and 24 h.

3. Discussion

Although *B. fragilis* is the most prevalent anaerobic bacterium isolated from human intestinal infections, there is no description of factors contributing to its colonization success. When *B. fragilis* infects the intestine it is exposed to bile salts which are present at high molar concentrations. It is well known that bile salts have detergent-like activity that can permealize bacterial membranes and can lead to membrane collapse. Several bacteria have adapted mechanisms to evade this antimicrobial activity of bile salts. Studies in other bacteria have shown that bile salts can enhance expression of genes which enable the extrusion of bile salts from the bacterium [8,9,21], and one study has reported that bile acids can stimulate biofilm formation in *Vibrio cholerae* [13]. This is the first study to provide evidence that bile salts can stimulate bacterial co-aggregation, bacterial adhesion to HT-29 human cells and biofilm formation of *B. fragilis*. Several studies have demonstrated that some bacteria form biofilms during infection [14,16,17]. Bile salt-treated cells did not show a faster growth rate than untreated cells and with growth rates standardized, the treated cells attained much higher biofilm OD_{550} vales which suggested that the differences in binding to PVC plates were due to differences in biofilm formation and not growth rate. Although in vivo biofilm formation by *B. fragilis* has not been demonstrated the finding in the current study that *B. fragilis* bile production was enhanced by bile salts support the possibility that *B. fragilis* could grow as a biofilm during colonization of the human intestine.

B. fragilis treated with bile salts overproduced fimbria and cellular vesicles and had increased expression of RND-type efflux pump genes. These changes were absent in bacteria pre-exposed to bile salts for 1 min before experiments exhibited similar properties as those of untreated bacteria, confirming that they where not merely due to the presence of bile.

Studies in *E. coli* have demonstrated that bile salts can cause fimbria overproduction and increase bacterial adhesion to human epithelial cells [25,26]. Therefore, overproduction of fimbria-like appendages observed in the current study was possibly a response of *B. fragilis* to bile salt exposure as a signal for attachment to host cells and to other bacteria. These appendages probably corresponded to overexpressed pili genes. The *B. fragilis* genome carries at least two genes encoding the putative pilus assembly protein PilF (http://www.sanger.ac.uk/Projects/B_fragilis/). However, these genes will be characterized in further work. A study by Murphy and Kirkman [14] demonstrated a role of pili in biofilm formation by *Haemophilus influenzae*. The current study observed a positive correlation between over-production of fimbria-like appendages and increased biofilm formation suggesting a possible association between the two. Some individual bacteria in the untreated cell population also had fimbria-like appendages, although far fewer than in the treated cells, untreated cells were also able to bind moderately to intestinal epithelial cells. It is likely that *B. fragilis* may produce basal levels of colonization factors which are overproduced upon stimulation by various colonization signals in this case bile salts.

In other Gram-negative bacteria, stress has been shown to cause a loss in cytoplasmic content which can sometimes lead to separation of membranes and eventually cell death [27–29]. However, in this study the cells maintained membrane integrity and showed no signs of membrane breaks under both low- and high-power electron microscopy as well as an intact periplasmic space. Reduction in cellular size of cells treated with 0.15% BSM was possibly due to the fact that although the cells maintained their integrity, they were also under some minor stress due to this treatment. Cells treated with 0.15% BSM also exhibited numerous outer membrane vesicles, which were rarely seen under other conditions. These vesicles were not characterized in this study, but they were probably analogous to the surface blebs or extracellular vesicles previously observed in *B. fragilis* and shown to have a haemagglutinin function [30] and sialidase activity [31] as well as carrying endotoxin to target cells, making them a possible signaling factor in both virulence and commensalism. Similar outer membrane vesicles have been observed in *B. gingivalis* and *Pseudomonas fragi* and *Xenorhabdus nematophilus*, where it has been suggested that they may serve as a vehicle for toxins and attachment to host cells [9,27,32]. Furthermore, similar vesicles have been shown to carry quinolone signaling molecules in *Pseudomonas aeruginosa* for intercellular signaling [22]. The electron-dense segments of DNA observed with 0.15% BSM possibly correspond to regions of DNA condensation or increased binding of ribosome proteins to the DNA. These regions could be analogous to the beading of DNA that was observed in *Salmonella* stressed by high pH [33]. This also suggests that the stressed cells were dividing rapidly [24]. At a concentration of 5% BSC, the cells were still able to grow as well as in standard media but started to show signs of stress. Therefore, *B. fragilis* can withstand physiological concentrations of both conjugated and non-conjugated bile salts, and can also tolerate super-physiological concentrations of conjugated bile salts ($\approx$5%).

A few studies in other Gram-negative bacteria and fungi have demonstrated the role of both RND-family and non RND-family efflux transporters in transport of bile salts as well as the linkage of bile salt resistance to multiple antibiotic resistance [9,21,34,35]. We had previously identified sixteen RND-family efflux pump genes (*bmeB1-16*) in *B. fragilis* [22,23]. The current study investigated the association of these pumps with *B. fragilis* bile salt adaptation. In the absence of any information about which pump(s) were involved, *B. fragilis* NCTC9343 was exposed bile salts and expression changes of all *bmeB* efflux pump genes determined. These data demonstrated a correlation between bile salt exposure of *B. fragilis* and increased expression of several *bmeB* efflux pumps. Our other studies deleting one or more efflux pump genes showed a small decrease in MICs of bile salts and antimicrobial agents, which suggested that these agents were substrates of these efflux pumps (unpublished data). *OmpA* is the most abundant outer membrane protein in *B. fragilis* which was previously found to have more structural than transport roles [36,37]. The current data demonstrate that the *ompA* gene was overexpressed in cells exposed to either 0.15% BSC or BSM, which was possibly a compensatory adaptive response to replace any damaged membrane proteins and restore membrane structure. A study by Torres et al. [38] demonstrated that *OmpA* of *Escherichia coli* stimulates dendritic cell activation and is required for binding to the $CaCO_2$ intestinal cell line, while Teng et al. [39] demonstrated that an *ompA* deletant of *E. coli* had reduced expression of type 1 fimbriae and decreased binding to human brain microvascular endothelial cells (HBMEC). In view of the two studies mentioned above and the positive correlation between increased adhesion and *ompA* expression observed in the current study, it is possible that *B. fragilis OmpA* could be associated with adhesion to human intestinal epithelial cells but further work is required to investigate this. It is likely that there is a large population of proteins differentially expressed during adaptation of *B. fragilis* to bile salts. Leverrier et al. [29] identified 24 distinct polypeptides in *Propionibacterium freudenreichii* that were either induced during bile salt adaptation and/or comprised the bile salts stress proteins (BSSPs). It is possible that *ompA* and *bmeB* genes found to be differentially expressed in this study are part of the BSSPs group in *B. fragilis*. A study by Prouty et al. [10] demonstrated that bile-salt induced gene expression was via a regulator-mediated route. It is possible that the changes we observed were due to changes in a regulator gene(s), so our further work will focus on characterization of the effects of bile salts on *B. fragilis* regulator genes.

4. Conclusion

Taken together, these data suggest that bile salts could enhance colonization of the human gut by *B. fragilis* via several mechanisms and could therefore be a significant contributor to host–pathogen interactions. Previous studies have extensively demonstrated the adverse effects of bile salts on bacterial membranes. The current study contributes to the increasing evidence that bile salts can also stimulate production of bacterial factors that favor host colonization. Furthermore, as the role of biofilms in human infection becomes better defined, characterization of biofilms may be important in understanding the pathogenesis of *B. fragilis* infections.

5. Materials and methods

5.1. Bacteria and growth conditions

B. fragilis NCTC9343 (ATCC25285) was used in this study. Bacteria were routinely grown in brain heart infusion broth supplemented with 0.5% yeast extract and 15 μg hemin ml^{-1} (BHIS) or Brucella-blood agar under anaerobic conditions at 37 °C. For bile salt assays, porcine bile extract i.e. a BSC, containing glycine and taurine conjugates of hyodeoxycholic acid (TDCA) plus other bile salts, and bile salts i.e. a bile salt mixture (BSM), containing 50% each of cholic acid (CA) sodium salt and deoxycholic acid (DCA) sodium salt, both from Sigma (Sigma; St. Louise, MO), were added at a final amounts of 0.15% or 5% (wt/vol). Porcine bile was chosen over oxgall because it is more similar to human bile with respect to bile salt/cholesterol, phospholipid/cholesterol and glycine to taurine ratios [4].

5.11. Identification of putative bile salt hydrolase (BSH) and pilus genes

A TBLASTN search against the recently available *B. fragilis* genomic database for *B. fragilis* NCTC9343 (http://www.sanger.ac.uk/Projects/B_fragilis)] was performed and primers designed to detect the gene with PCR assays.

5.3. DNA procedures

Total cellular chromosomal DNA was isolated with the DNeasy tissue kit, according to the manufacturers' instructions (Qiagen; Valencia, CA). BSH genes were detected by PCR with the following primers: BF1508-forward, 5′-TAG AAG GTG GTA CGC ATC-3′ and BF1508-reverse, 5′-GCT GTC AGA CCT TCA CGA-3′; BF3386-forward, 5′-CCG TCG TAA TGA TAC TCG-3′ and BF3586-reverse, 5′-CAT CAG TGA TAG CCA TGT GC-3′. PCR conditions used for both genes included an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min.

5.4. RNA procedures

Total cellular RNA was isolated from logarithmic growth phase cells (OD_{600} 0.4) with the RNeasy-RNA Protect® kit (Qiagen, Valencia, CA).

Gene expression was quantified by real-time comparative quantitative RT-PCR with the Quantitect® SYBR® Green one-step RT-PCR kit (Qiagen, Valencia, CA) on the SmartCycler® (Cepheid, Sunnyvale, CA). Final amounts of 250 ng of RNA were converted to cDNA and reactions were normalized with 16SrRNA. Primers used for *bmeB* efflux pump genes have been described previously [22]. Primers used for cholylglycine hydrolase genes were as shown above. Primers used for *ompA* were *ompA*-forward, 5′-GGA TAT GAC GGT GTT GCC AG-3′ and *ompA*-reverse, 5′-TAG CAG CAG CCA TGT CAT TC-3′. Expression was quantified by the $\Delta\Delta C_t$ approximation method [22]. Experiments were repeated five times. Data were analyzed by Student's *t*-test and a value of $P \leqslant 0.05$ was considered significant. A $\geqslant$2-fold difference in expression was considered significant.

5.5. Growth kinetics and survival

Bile salt amounts were chosen in order to include a range of concentrations that spanned the physiological intestinal concentration. Growth of *B. fragilis* NCTC9343 in either BHI, BHI + 0.15% BSC or BHI + 0.15% BSM was measured spectrophotometrically by determining the optical density at 600 nm at 0, 0.5, 1, 2, 3, 4, 5, 6 and 7 h. Survival (viable cfu mL^{-1}) was measured by diluting 12 h cultures (1:100) of *B. fragilis* NCTC9343 grown in BHI, BHI + 5% BSC or BHI + 5% BSM and plating 100 μL aliquots on Brucella-blood agar plates. Plates were incubated anaerobically at 37 °C for 48 h, after which colonies were counted and cfu mL^{-1} determined.

5.6. Antimicrobial susceptibility with or without bile exposure

The MIC of BSC and BSM on *B. fragilis* NCTC9343 as well as those of eight antimicrobial agents (cefoxitin, cefoperazone, imipenem, metronidazole, norfloxacin, ethidium bromide and SDS) on *B. fragilis* NCTC9343 pre-exposed to 0.15% BSC or BSM were determined. The MICs were determined with the spiral gradient endpoint (SGE) method [40]. Susceptibility assays were repeated three independent times.

The effects of adding the efflux pump inhibitor (EPI); CCCP was determined by measuring the decrease in MICs after incorporation of this agent into the assay plate at a final concentration of 25 μg mL^{-1} (an amount previously determined not to be inhibitory to the strain).

5.7. Uptake of CV

Uptake of CV was assayed as described elsewhere [8]. Briefly, *B. fragilis* strain NCTC9343 was grown to mid log phase ($OD_{600} \approx 0.5$) in BHI broth with or without 0.15% BSC or BSM (10 ml), washed twice with phosphate-buffered saline (PBS), and finally resuspended in 10 ml of PBS. A final concentration of 5 μg mL^{-1} of CV was added to the cells and the cultures incubated at 37 °C for 180 min constant stirring, 1 ml aliquots were removed at regular time intervals of 0, 30, 60, 90 and 180. The aliquots were centrifuged and the amount of CV remaining in the supernatant was estimated by measuring absorbance at 590 nm (OD_{590} of 1 ml 5 μg mL^{-1} CV was used as the total amount). In some experiments, in order to determine the effect of EPI on uptake, 25 μg mL^{-1} CCCP was added prior to the addition of CV [22].

5.8. Morphological studies

Electron microscopy analysis was performed essentially as described elsewhere [7,12]. Briefly, *B. fragilis* NCTC9343 was grown for 12 h in BHI, BHI + 0.15% or 5% BSC, BHI + 0.15% or 5% BSM. Cultures were washed three times in 1 × PBS and finally resuspended in 100 μL PBS. Four hundred microliters of 2% gluteraldehyde (Ted Pella Inc.; Redding, CA) in sterile distilled water was then added and the suspension fixed at room temperature for 30 min. For SEM, the suspension was placed by gentle filtration on 0.1-μm-pore-size membranes (MF disk MCE philic; Millipore, Billerica, MA) and immediately rinsed three times with 1 × PBS. Samples were dehydrated with ethanol (10%, 25%, 50%, 75%, 90% and 2 × 100% for 10 min each and finally 100% for 1 h), critical point dried by the CO_2 method [29,41], and coated with gold. Cells were examined and photographed with a Cambridge 360 scanning electron microscope operating at 10 kV. Average cell length was calculated from at least 10 measurements for each observation.

For TEM, the suspension was centrifuged, resuspended in osmium tetroxide and dehydrated in graded ethanol as described above, embedded in epon, sectioned thinly (60–70 nm) on a Reichert-Jung Ultracult E ultramicrotome and picked up on formvar coated copper grids. The grids were stained with uranyl acetate and examined and photographed with a Jeol XC100 and the Philips EM420 transmission electron microscope both operating at 80 kV.

5.9. Assay for adhesion to intestinal epithelial cell lines

Bacterial adhesion to mammalian cells was performed as described elsewhere [42]. Briefly, 1 mL of an HT-29 cell suspension (ca. 1.5×105 cells mL^{-1}) was transferred to 24-well plates (TPP, Switzerland) containing sterile circular cover slips (13 mm diameter). Plates were incubated at 37 °C, in atmosphere of 5% CO_2 to obtain a sub-confluent monolayer. After which, 900 mL of Dulbecco MEM medium (DMEM—Cultilab) with 2% fetal bovine serum (FBS) were added, and 100 mL of the bacterial growth (ca. 1.5×108 cells mL^{-1}) were transferred into the wells and incubated for 3 h. Unattached bacteria were separated from the epithelial cells by two cycles of suspension in a buffer of physiological saline (0.9%) with 10 mg mL^{-1} choline chloride. Afterwards, cells were fixed with absolute methanol and stained with May Grünwald's (1:2) on

Sørensen buffer (KH_2PO_4, $NaHPO_4$, pH 7.3), for 10 min followed by staining with Giemsa (1:3), for 20 min. Then, the cover slips were allowed to dry at room temperature and placed on glass slide. The mean number of adhering bacteria per epithelial cell was counted using an interference contrast microscope. An enteropathogenic *E. coli* (EPEC) E2348/69 displaying a localized adhesion (LA) pattern was used as a positive control for the adhesion experiments. All assays were repeated five independent times.

5.10. Quantification of bacterial adhesion to HT-29 cells

The number of adherent bacteria was determined on five random selections of photomicrographs of each sample by a commonly used stereological method [43,44]. Each photomicrograph was analyzed on a Weibel stereological table by using a line test probe containing 84 line segments and 168 test points. The ratio of the mammalian cell surface covered by bacteria was determined by dividing the number of points on the bacterial cells by the number of points on the HT-29 cells.

Assay for adhesion to PVC microtitre plates (biofilm formation). The biofilm assay was performed essentially as described elsewhere [12,15]. Briefly, bile salt-treated vs. untreated NCTC9343 was diluted 1:1000 in fresh BHIS broth and 100 μL aliquots were added in quadruplicate to wells of PVC microtitre plates. The bacteria were grown anaerobically for 7 h at 37 °C under static conditions. The plates were washed three times with 1 × PBS and 100 μL of 0.01% w/v CV was added the plates were incubated for 20 min to stain the bacteria. The stain was removed and the plates washed four times with 1 × PBS. The adherent bacteria and stain were solubilsed with 33% acetic acid and the OD_{550} was determined. Each plate included four wells which contained sterile broth instead of bacteria but were treated identically otherwise. The OD_{550} was standardized against these wells. The absorbance was equivalent to the number of adherent bacteria. Experiments were repeated five times.

5.11. Biofilm growth assay

Biofilm growth kinetics were performed essentially as described elsewhere [14]. Briefly, biofilm formation of bacteria grown and diluted as described above was monitored over a 24 h period. Biofilm formation was quantified at 0, 1.5, 4, 7, 12, and 24 h time points.

5.12. Statistical analyses of bacterial adhesion assays

For both adhesion assays, data were analysed by Student's *t*-test. This test was more appropriate than ANOVA because the values for untreated bacteria were low and thus had low variances so that the assumption of equal variance used with ANOVA could not be used and a *t*-test was used without assuming equal variance. Data are expressed as mean ± standard deviation. A P value $\leqslant 0.05$ was considered significant.

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