Cell surface properties of *Clostridium difficile*: haemagglutination, relative hydrophobicity and charge

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Five well characterised strains of *Clostridium difficile* of differing virulence and two *Escherichia coli* strains, a verotoxigenic O157:H7 isolate and a urinary isolate, were examined for cell surface hydrophobicity and charge, and haemagglutinating ability. Phase partition in hexadecane or octan-1-ol was similar for *C. difficile* and *E. coli*, as was retention by hydrophobic interaction chromatography (HIC), indicating moderate hydrophobicity. The salt agglutination test showed *E. coli* to be hydrophobic and *C. difficile* to be hydrophilic. Relative hydrophobicity determined by HIC when charge effects were not nullified, i.e., to reflect more closely conditions *in vivo*, showed *C. difficile* to bind less well. Growth of *C. difficile* in caecal emulsions to simulate conditions *in vivo* did not alter the cell surface hydrophobicity. The phase partition method for charge determination indicated that *E. coli* and *C. difficile* had a net negative charge, although this was weaker for *C. difficile* than *E. coli*. However, although *E. coli* exhibited a net negative charge as determined by immuno-gold electronmicroscopy (IGEM), in keeping with the results of the phase partition method, *C. difficile* was shown to be predominantly positively charged by IGEM, and by movement in a charged field as determined by paper electrophoresis and a novel method based on light microscope observation. A cell-wall deficient mutant of *C. difficile* was weakly positively charged, showing that most of the charge resides in the cell wall.

Introduction

Clostridium difficile is an important nosocomial enteric pathogen, causing pseudomembranous colitis and many cases of antibiotic-associated diarrhoea [1, 2]. Various established and putative virulence factors have been described [3]. These are mainly toxins A and B [4], but also include factors such as capsule production [5], which may confer protection against phagocytosis, and production of general and tissue degradative proteases, e.g., collagenase and hyaluronidase [6, 7]. Apart from the toxins most work has centred on adherence as a colonisation and putative virulence factors. *C. difficile* can adhere to the colonic mucus of man [8] and hamsters [9], and there is a positive correlation between the ability to adhere *in vivo* and virulence [9]. More recently, *C. difficile* has been shown to adhere to the cultured intestinal cell lines Caco-2 and HT29-MTXC

[10]. This cell-culture adhesion is associated with 27-kDa and 40-kDa *C. difficile* proteins, which have now been cloned [11]. *C. difficile* has been shown to produce fimbriae [12], which are potential mediators of adhesion, although these do not appear to be related to the 27-kDa and 40-kDa putative adhesion proteins [10]. However, the nature of the host receptor for *C. difficile* in any of these systems, if a specific receptor be involved, is unknown. Also, only limited information is available on the physicochemical characteristics of the bacterial cell surface of *C. difficile* that may be involved in the adhesion process, and none after growth in media that more closely resemble the situation *in vivo*. This study was undertaken to identify and characterise two physicochemical parameters that may be involved in mediating adhesion, namely cell surface hydrophobicity and cell surface charge, and to use the commonly applied haemagglutination assay for the identification of putative carbohydrate receptors.

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Materials and methods

Bacterial strains and culture conditions

Five well characterised strains of *C. difficile* and two

Escherichia coli strains were used. The *C. difficile* strains were M-1, a non-toxigenic avirulent strain [13]; BAT, a weakly virulent toxigenic strain [14]; B-1, a highly virulent toxigenic strain [14]; VPI 10463, a highly virulent toxigenic strain (made available by Dr D. Lyster, Virginia Polytechnic Institute); and 8864, a less virulent cytotoxigenic strain that fails to produce toxin A [15]. Strain B-1 adheres strongly to hamster gut mucus, and strains M-1 and BAT adhere poorly [9]. The *E. coli* strain U1 was isolated from urine (Department of Microbiology, Northwick Park Hospital) and strain O157:H7 is a verotoxigenic strain isolated from faeces (Enteric Reference Laboratory, CPHL, Colindale Avenue, London). The bacteria were cultured in Brain Heart Infusion (BHI) Broth (Unipath) for 18 h at 37°C, in an atmosphere of CO₂ 10%, H₂ 10% and N₂ 80% for *C. difficile* in an anaerobic cabinet (Forma Scientific, Marietta, OH, USA) and aerobically for *E. coli*. The broths were then centrifuged at 1500 *g* for 20 min at 4°C, the pellets were resuspended in 10 ml of fresh BHI broth, and 0.1 ml was used to seed 10 ml of the following growth media: filtrates (filter pore size 0.45 µm) of an aqueous 5% suspension of caecal contents from hamsters pre-treated with clindamycin or from untreated hamsters and BHI broth. Inoculated material was incubated for 18 h at 37°C under anaerobic conditions for *C. difficile* and aerobically for *E. coli*. The broths and emulsions were centrifuged at 1500 *g* for 20 min at 4°C, washed twice in Tris-buffered saline (TBS; 50 mM NaCl, 0.1 M Tris-HCl, pH 7.2), resuspended in 10 ml of TBS and standardised by addition of TBS to an optical density of 0.9 at 580 nm (OD₅₈₀ of 0.9 = 10⁷ cfu/ml for *C. difficile* and 10⁹ cfu/ml for *E. coli*) for all experiments other than labelling with charged gold.

Animals and preparation of caecal filtrates

Adult female Syrian hamsters (*Mesocricetus auratus*) weighing 150–200 g, obtained from breeding colonies at the National Institute for Medical Research, London, were housed individually in sterilised filter-lidded polycarbonate boxes (Stephen Clark Fabrications Ltd, Alva, Scotland) and given autoclaved bedding, feed and tap water [9]. Some animals were given one intraperitoneal injection of 0.5 ml of a solution of clindamycin phosphate (Upjohn, Crawley, West Sussex) 10 mg/ml. Thirteen days later the animals were killed, their caeca were removed, and the caecal contents from untreated and clindamycin-treated hamsters were mixed separately with distilled water to give 1 in 20 w:v suspensions which were sterilised by centrifugation and filtration as described in detail previously [16]. These were termed normal caecal content filtrates (NH-CCF) and filtrates from clindamycin-treated hamsters (CTH-CCF).

Hydrophobicity

Three methods were used to determine the hydro-

phobicity of *C. difficile* strains; bacterial adhesion to hydrocarbons (BATH) [17], the salt aggregation test (SAT) [18], and hydrophobic interaction chromatography (HIC) [19]. *E. coli* strains U1 and O157:H7 were used as positive controls in all three methods as the majority of *E. coli* from human sources are hydrophobic [20]. Before use, these isolates were confirmed as hydrophobic by the SAT test, which is a good measure of hydrophobicity for *E. coli* [18]. For BATH and SAT the bacteria were harvested from NH-CCF, CTH-CCF and BHI broths. For HIC the bacteria were harvested only from BHI broths. All experiments were done in triplicate and values given are means of triplicate experiments.

BATH method. Three ml of each bacterial suspension were added to 3 ml of octan-1-ol (BDH) and separately to 3 ml of hexadecane (BDH), in sterile tubes and vortex mixed for 2 min, then left to stand at room temperature until the two phases had separated (*c.* 1 h). The absorbance of the bacterial aqueous phase was measured at 580 nm. Controls consisted of 3 ml of phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M phosphate buffer, pH 7.2) in place of the octan-1-ol or hexadecane to establish the absorbance of the initial bacterial suspension. The percentage hydrophobicity was calculated by the formula

$$\frac{\text{ODi} - \text{ODE}}{\text{ODi}} \times 100$$

where ODi is the initial reading of the bacterial suspension and ODe is the reading of the aqueous phase.

The condition of the bacterial cells in the aqueous phase after partitioning in the hexadecane system was examined by electronmicroscopy as described below for determination of surface charge distribution, but without reaction with charged colloidal gold.

SAT test. Concentrations of ammonium sulphate in sodium phosphate buffer (pH 6.8, 0.02 M), ranging from 4 M to 0.02 M in increments of 0.02 M, were prepared before the experiment. Ten µl of each bacterial suspension were spotted on glass slides containing 10 µl of the different salt concentrations in the micro-method adaptation of the SAT [13], and then rocked gently until aggregation was observed (*c.* 2 min). The last dilution at which aggregation occurred was recorded.

HIC test. Sterile 5-ml plastic syringes were plugged with siliconised glass wool and packed to a height of 20 mm with octyl-Sepharose CL-4B (Sigma) in an equal volume of 0.01 M phosphate buffer (pH 6.8) containing Triton X-100 0.1% v/v (equilibration buffer). The column was washed with 20 bed-volumes of sterile 4 M NaCl solution. The bacterial strains *C. difficile* B-1 and *E. coli* U1 were suspended in 2 ml of the same solution and absorbance at 580 nm was

measured, then a 2 ml suspension was loaded on separate columns for each strain. Elution was with 6 ml of equilibration buffer. The absorbance readings of the 2-ml eluates were measured at 580 nm, the first 2-ml void volume being discarded. The percentage hydrophobicity was calculated as described above for the BATH method, where ODi was the reading of the 2-ml bacterial suspensions before loading on to the column and ODe was the reading of the last 2-ml eluates. In addition, the HIC method was repeated for all test strains in solutions of lower ionic strength (i.e. PBS), as a measure of interaction when both charge and hydrophobicity are involved.

Net cell surface charge

The overall net bacterial cell surface charge of the *C. difficile* strains was assessed by three methods; charged partition [21], paper electrophoresis [22] and a novel method of observation by microscopy of movement in a charged field (OM-MCF).

Charged partition. For this method poly-ethyleneglycol (PEG) (Sigma) 4.4% w/v and dextran (Sigma) 6.2% w/v were prepared in 0.03 M Tris-HCl, pH 7.0, to a total volume of 100 ml and left to separate overnight at 4 °C in a separating funnel. The dextran-rich bottom phase and the PEG-rich top phase were collected and stored separately at 4 °C until needed. The charged system was prepared by adding sodium sulphate to equal volumes of the two phases to give a final salt concentration of 0.1 M. The two phases were re-mixed, left to separate and stored as above. To determine the net bacterial charge 3 ml of each phase were dispensed into a sterile tube, 1 ml of a suspension of *C. difficile* strain B-1 (see above), after growth in BHI, was added, and the mixture was vortex mixed for 2 min and left to separate into the two phases. An uncharged system was prepared as a control with NaCl replacing the sodium sulphate. The optical density of each phase was then measured at 580 nm. This was repeated for *E. coli* strain U1.

Paper electrophoresis method. The bacterial suspension after growth in BHI was spotted on the centre of dry, sterile filter paper (Whatman) strips (1 × 8 cm), laid on a horizontal gel electrophoresis bed with PBS as running buffer. The ends of the paper strips were immersed in the buffer. A current of 5 mA was applied for c. 5 min, the paper strip removed and carefully inverted on the surface of a Columbia agar plate with horse blood (CBA, Unipath) 10%. This was repeated for each of the bacterial strains and the agar plates were incubated overnight at 37 °C anaerobically.

OM-MCF method. Bacteria harvested from CBA after growth for 48 h or BHI after growth for 18 h were resuspended in 40 mM Tris-HCl in saline 0.85%, pH 7.0. Fifty µl of the suspension were applied to the centre of a conventional glass coverslip to make

contact with two filter paper strips soaked in the suspension medium that ran from the centre to each end of the slide. These served as wicks which made contact to termini. This apparatus was placed under a light microscope and a glass coverslip and immersion oil were applied in the conventional manner. The positive and negative leads of a power pack (BioRad) were connected to the termini and while the bacteria were viewed under phase contrast the power pack was switched on (current 10 mA) and the direction of movement of the bacteria was recorded. As the microscope optics cause lateral inversion of the image a movement to the negative or positive terminal is indicative of a negative or positive charge, respectively.

To determine whether or not any charge seen was due entirely to cell-wall constituents a cell-wall deficient mutant (autoplast) of strain M-1 was made as described for the generation of autoplasts for *C. perfringens* [23]. In these experiments the suspension medium contained sucrose to a final concentration of 0.4 M to maintain autoplast integrity. The parent M-1 strain was examined in a similar suspension medium as a control.

In all experiments the polarity was reversed to confirm the bacterial charge, and negatively charged carboxylated microspheres (1–6 µ diameter; Poly-sciences Ltd, Northampton) were used as controls.

Cell surface charge distribution

Charged colloidal gold was used to assess the topographical distribution of anionic and cationic charges on the cell surface of *C. difficile* strain B-1 and *E. coli* O157:H7 by electronmicroscopy. The cultures were harvested from BHI broth after growth for 18 h and pelleted by centrifugation at 1500 g for 20 min, washed once in PBS and re-pelleted. Cationic colloidal gold (Biocell) with a mean diameter of 10 nm, was diluted in PBS with bovine serum albumin (BSA) 1% (pH 7.0) to give a final concentration of 1 in 10 and left at room temperature for 1 h. The bacteria were pelleted by centrifugation at 1500 g for 10 min at 4 °C and washed with PBS. A droplet (25 µl) of each bacterial suspension was placed on 200 mesh formvar-carbon coated gold grids and left for c. 5 min, to allow adherence of the cells to the grid. The grid was blotted dry and floated on glutaraldehyde 3% in 0.1 M phosphate buffer (pH 7) for at least 2 min to fix the cells and then washed three times by floating on distilled water. The grid was then blotted dry and observed with a Jeol 1200EX transmission electron-microscope. Exactly the same procedure was used for anionic gold labelling except that the anionic gold had to be prepared as it is not commercially available. The anionic gold was made by conjugating 10 nm colloidal gold (Biocell) to acetylated BSA, (Promega, 1 mg/ml solution). The pH of the colloidal gold was adjusted with 0.2 M K₂CO₃ to pH 9.0 and acetylated BSA

40 µg/ml (with allowance for a 10% excess) was added to the colloidal gold, which was then centrifuged at 24 000 *g* at 4°C for 30 min. The minimal amount of acetylated BSA needed to stabilise the gold from aggregation by salt was determined as described by Roth [24]. The conjugate was resuspended in PBS and stored at 4°C after the addition of 2×10^{-3} M azide. At least 25 cells from unstained grids were examined to determine the mean labelling per cell. Unstained grids were used to determine the mean labelling density as negative stain obscures some of the gold particles. Cells were then negatively stained with potassium phosphotungstate 1% (pH 6.5) for 10 s to improve contrast for photography.

Haemagglutination assay

In addition to bacterial suspensions prepared after growth in BHI, NH-CCF and CTH-CCF, the test strains were also harvested in PBS, after anaerobic incubation on CBA for 48 h, pelleted and washed twice in PBS and standardised in PBS to an OD₅₈₀ of 0.9. Five-ml blood samples from normal horse, sheep, turkey, goose, chicken (Tissue Cultures Services, Botolph Claydon, Bucks), hamster, pig, rat, goat, mouse, guinea-pig and rabbit (obtained from animals housed on site) and human blood group A⁺, O⁺ and B⁺ (obtained from healthy volunteers) were stored in Alsever's solution (0.03 M sodium citrate, 0.11 M glucose, 0.07 M NaCl, pH 6.1) at 4°C. Uninoculated NH-CCF and CTH-CCF and sham harvests from CBA plates were used as medium controls and *E. coli* strain U1 which is known to haemagglutinate (data not shown) as a positive control. Before use the red blood cells (RBC) were washed twice with TBS, resuspended in the buffer to give an RBC suspension of 1% v/v, and 50 µl of the RBC suspension for each blood type were added to 50 µl of two-fold serially diluted bacterial suspensions

in 96-well, round-bottomed microtitration plates (Sero-Well; Sterilin, Stone, Staffs). The plates were set up in triplicate with one plate incubated at room temperature, one at 37°C and one at 4°C. RBC suspensions (1% v/v in TBS) were also treated with *Vibrio cholerae* neuraminidase (Behring) 0.1 unit/ml for 2 h at 37°C to expose backbone sequences. In addition, human blood group B RBCs were also incubated with α-fucosidase (Sigma) 0.2 units/ml for 2 h at room temperature to remove fucose moieties and reveal Gal α1-3 B-like structures. The RBCs were then pelleted by centrifugation at 1500 *g* for 5 min, washed twice in TBS buffer and resuspended to give a 1% suspension. Control RBCs were processed in the same way, with TBS in place of the neuraminidase and α-fucosidase.

Statistical analysis

Results were compared for statistical difference by a one-way analysis of variance.

Results

Hydrophobicity

The results are presented in Table 1. After culture in BHI broths the partition method of assessing hydrophobicity indicated greater hydrophobicity for all strains when hexadecane rather than octan-1-ol was used as the non-aqueous phase, this difference being greatest for *C. difficile* strain M-1. In all cases the percentage hydrophobic values for *C. difficile* by the partition method were similar to those of *E. coli*, with the exception of *C. difficile* strain 8864 which was less hydrophobic. Analysis by electronmicroscopy of *C. difficile* cells isolated from the aqueous phase after partition in hexadecane revealed cell-wall damage as indicated by complete uptake of negative stain. In

Table 1. Hydrophobicity of *C. difficile* and *E. coli* and the effects of culture in different media

Bacterial strain	Preparation	Mean (SD)* percentage hydrophobicity by partition method		Salt agglutination test (lowest M value showing agglutination) (SD)		Hydrophobic interaction chromatograph (percent of cells retained) (SD)	
		octan-1-ol	hexadecane			PBS	4 M NaCl
<i>C. difficile</i> VPI 10463	a	43% (0.6)	49% (0.6)	3.1 (0.1)		55% (1.0)	...
	b	44% (1.5)	52% (0.6)	2.3 (0.3)			
	c	40% (0.6)	47% (1.0)	3.1 (0.1)			
B-1	a	32% (1.0)	46% (1.0)	3.0 (0.0)		43% (3.0)	72% (2.0)
	b	28% (0.6)	46% (1.0)	3.0 (0.1)			
	c	30% (1.0)	49% (0.6)	2.0 (0.0)			
BAT	a	30% (1.0)	43% (0.6)	2.2 (0.2)		41% (1.5)	...
	b	33% (1.0)	45% (0.0)	2.0 (0.0)			
	c	29% (0.6)	42% (0.5)	3.0 (0.0)			
8864	a	20% (1.0)	28% (1.1)	3.1 (0.1)		31% (2.5)	...
	b	22% (1.5)	25% (0.0)	3.0 (0.0)			
	c	25% (1.5)	22% (1.1)	3.0 (0.0)			
M-1	a	27% (1.0)	51% (1.1)	4.1 (0.1)		41% (2.6)	...
	b	26% (1.1)	53% (0.6)	3.1 (0.1)			
	c	28% (1.0)	49% (1.5)	4.0 (0.0)			
<i>E. coli</i> U1	a	36% (1.5)	48% (0.1)	0.5% (0.1)		65% (2.0)	66% (2.0)
O157:H7	a	38% (2.5)	50.7% (5.1)	0.9% (0.2)		85% (2.0)	...

a, BHI broth; b, filtrate of normal hamster caecal contents (NH-CCF); c, filtrate of clindamycin-pre-treated hamster caecal contents (CTH-CCF); PBS, phosphate-buffered saline.

*SD of triplicate mean values.

contrast, the results of the salt agglutination test clearly distinguished between the two species with all of the *C. difficile* strains agglutinating only at high salt concentrations (hydrophilic) and the two *E. coli* strains agglutinating at low salt concentrations (hydrophobic). However, HIC (with solutions of high ionic strength to remove the effect of charge) showed *C. difficile* strain B-1 to be as hydrophobic as *E. coli* strain U1. The HIC test conducted in the presence of solutions of lower ionic strength indicated that both *E. coli* test strains were retained by the octyl-sepharose better than any of the *C. difficile* strains.

To determine whether or not growth of *C. difficile* in conditions more reflective of those found within the gut would alter cell surface hydrophobicity the strains were re-examined by the salt agglutination test and partition method after growth in caecal emulsions. In all cases the findings were similar to those obtained after growth in BHI broth (Table 1).

Net cell surface charge

In the partition method of net cell surface charge determination (Table 2) *C. difficile* B-1 (chosen as a representative strain), and *E. coli* strain U1 were significantly more attracted to the positively charged PEG phase than to the negatively charged dextran phase ($p < 0.001$), indicative of a net negative charge at pH 7. However, this partitioning was significantly less than for the negatively charged *E. coli* (OD₅₈₀ 0.41 versus 0.19; $p < 0.001$), with the OD₅₈₀ absorbance value of 0.19 for *C. difficile* in the PEG phase being similar to that of 0.14 in the uncharged system ($p = 0.06$). For *E. coli*, the difference between the charged and uncharged PEG system was significant ($p = 0.002$). In contrast, overall movement determined by paper electrophoresis of all five *C. difficile* strains in a charged field was towards the cathode, indicative of a net positive charge. However, a few cells had moved to the anode, showing that some cells present carried an overall negative charge. The OM-MCF method showed all *C. difficile* strains had a net positive charge. The autoplasts of strain M-1 moved very slowly compared to the other strains and the M-1 parent strain control in sucrose, indicative of a weak net positive charge.

Cell surface charge distribution

There was a fairly even distribution of gold particles on the cell surface of *C. difficile* B-1 after interaction with anionic colloidal gold (Fig. 1a) with 83.3% of the cells labelled at a mean of 74.6 particles/cell (range 7–210), whereas there was little labelling of *E. coli* O157:H7 (Fig. 1b), with 39.0% of cells labelled (mean 23.0 particles; range 1–204). In contrast, few *C. difficile* cells were labelled with cationic colloidal gold (Fig. 1c) and these had few gold particles associated with them (35.0% of cells labelled with a mean of 5.2 particles/cell; range 1–23). The majority of *E. coli* O157:H7 cells showed heavy labelling with cationic colloidal gold (Fig. 1d) with 96.1% of cells labelled with a mean of 50.1 particles/cell; range 1–161.

Haemagglutination

After being washed twice none of the five *C. difficile* strains assayed haemagglutinated any of the 15 RBC types from 13 different animal species at any of the test temperatures. This same pattern of lack of haemagglutination was also seen after the RBCs had been treated with neuraminidase or α -fucosidase. *C. difficile* washed only once after growth in caecal content filtrates and on CBA plates agglutinated porcine, rabbit and goat RBCs at 37°C. However, this pattern of haemagglutination was also evident with uninoculated caecal content filtrates and sham harvests from CBA agar plates. The *E. coli* strain U1 positive control haemagglutinated rabbit, sheep and human (A⁺, B⁺, O⁺ blood group) RBCs.

Discussion

The interaction of bacteria with cell surfaces or secreted products of the host is considered to be the end result of a complex set of interactions involving both attractive and repulsive forces operating between target cells or cell products and the bacterial cell surface [25]. In addition to the accepted contribution of adhesin–receptor interactions, there is some evidence to implicate hydrophobic interactions and net charge [26]. Additional factors include access to receptors and chemotaxis of the bacterial cell. Of the physicochemical factors, electrostatic forces are considered to be the

Table 2. Net cell surface charge determined by partition between polyethylene-glycol and dextran for *C. difficile* B-1 and *E. coli* strain U1

System	Mean OD ₅₈₀ (SD) of triplicate experiments			
	Polyethylene-glycol		Dextran	
	<i>C. difficile</i>	<i>E. coli</i>	<i>C. difficile</i>	<i>E. coli</i>
Charged	0.19 (0.01)	0.41 (0.01)	0.08 (0.006)	0.04 (0.007)
Uncharged	0.14 (0.01)	0.17 (0.007)	0.18 (0.006)	0.16 (0.007)

*OD₅₈₀ triplicate mean value $\pm$ SD.

Fig. 1. Electronmicroscopic analysis of cell surface charge of *C. difficile* strain B-1 and *E. coli* O157:H7. Detection of positive charge on **a**, *C. difficile*, **b**, *E. coli* with 10 nm anionic colloidal gold, and of negative charge on **c**, *C. difficile*, **d**, *E. coli*, with 10 nm cationic colloidal gold. A few *E. coli* cells bound anionic colloidal gold (not shown).

ones that operate over the greatest distances [27] and, therefore, likely to dominate the initial interaction between a pathogen and the negatively charged host cell. These can be divided into long-range Van der

Waals interactions and medium-range electrostatic interactions. Mathematical descriptions of these and other physicochemical interactions are to be found in a review by Busscher and Weerkamp [28]. It is evident

from the studies presented here that *C. difficile* isolates from different sources and of different virulence carry a net positive cell surface charge with movement to the cathode in a charged field (paper electrophoresis and OM-MCF methods). In contrast, the charged partition method showed movement of *C. difficile* to the positive phase indicative of a net negative cell surface charge. However, this degree of partitioning was much less than for the *E. coli* strain which was negatively charged as determined by binding to charged gold particles. This observation is in keeping with the net negative charge of most bacteria [29]. Further support for a net positive charge for *C. difficile* comes from the electronmicroscopic observations of interaction with charged gold particles which showed that the vast majority of cells carried only a positive charge, and that the charge was evenly distributed over the cell surface. However, a few cells possessed some negative charge. The extent to which these cells may have a predominant weakly negative charge is difficult to determine, although direct evidence that a few such cells in a population exist comes from the fact that a small number of cells migrated to the anode in the paper electrophoresis method. The implication of the above finding is that there may be net charge heterogeneity within a population of *C. difficile* cells. The slow migration of autoplasts of strain M-1 towards the cathode showed that most of the contribution to net charge came from cell-wall components. The decreased rate of migration was not due to the viscosity of the autoplast suspension medium or differences in its conductivity as the parent strain retained an indistinguishable rate of migration in the conventional or sucrose-containing suspension media. Therefore, intracellular components of *C. difficile* also impart a positive charge to the cells that contributes to the net charge.

Interestingly, net electrostatic charge has been correlated with virulence for the fish pathogen *Aeromonas salmonicida* [30]. For this pathogen there is intra-species variation in net charge, the virulent strains possessing a net negative charge and adhering to positively charged fish cells in tissue culture better than positively charged avirulent strains.

It is generally accepted that hydrophobicity is important in the interaction of bacteria with host cells, with high bacterial cell surface hydrophobicity correlating with adhesion of oral organisms to oral cavity surfaces [31], and adhesion of gut pathogens, e.g., enteropathogenic *E. coli* adhesion to intestinal brush border membranes [32]. There has been only one previous study reporting on the hydrophobic properties of *C. difficile* [33]. That study used relative adherence to polystyrene as an indicator of hydrophobicity, and concluded that all the isolates examined were hydrophobic. The present studies with *C. difficile* strain B-1 and hydrophobic interaction chromatography confirm the hydrophobic nature of *C. difficile*.

The partition methods gave values of intermediate hydrophobicity for all strains examined (i.e., values falling between 15% and 80%), with lower values overall for octan-1-ol as the organic phase than for hexadecane. The extent to which these values truly reflect the hydrophobicity of the intact cell is difficult to determine as electronmicroscopic examination of *C. difficile* recovered from the aqueous or organic phases showed that they had suffered extensive damage. In contrast, the SAT method gave the clearest distinction between *C. difficile* and *E. coli*, showing *E. coli* to be hydrophobic and *C. difficile* to be highly hydrophilic. Unfortunately, the SAT test is most reliable for detecting strongly hydrophobic microorganisms [34] and its accuracy for bacteria of intermediate hydrophobicity is unclear, although there is a good correlation between HIC and SAT determinations for *E. coli* [18]. We cannot explain why *C. difficile* strain B-1 was hydrophilic when assessed by the SAT but moderately hydrophobic when assessed by HIC.

It is known that a number of factors can affect the hydrophobicity of a bacterial cell surface, such as expression of bacterial surface components [35, 36], and growth conditions [37]. It is also known that bacterial surface components can be altered or surface architecture disorganised, or both, by antibiotics, particularly those such as clindamycin which perturb protein synthesis [38]. Therefore, in order to examine the possibility that the cell surface hydrophobicity of *C. difficile* differed following culture in conditions simulating those *in vivo*, the bacteria were examined from untreated and clindamycin-treated animals. These growth conditions had no apparent effect on the cell surface hydrophobicity of *C. difficile* as determined by either of the partition methods or the salt aggregation method.

The extent to which the presence of capsular material on *C. difficile* [5] contributes to hydrophobicity is unknown, as conflicting results have been found for other gram-positive bacteria [35, 39].

It is possible that a positive cell surface charge may contribute to the known resistance of *C. difficile* to phagocytosis [40] as decreasing negative charge has been correlated with resistance to phagocytosis [41] and conversely increases in negative cell surface charge are associated with increased susceptibility to phagocytosis [42].

In an attempt to assess the degree of binding when both charge and hydrophobic interactions were simultaneously involved, hydrophobic interaction chromatography with a relatively low ionic strength solution was performed. Interestingly, when the effect of electrostatic charge interactions was retained the degree of binding for *C. difficile* strain B-1 was lower than when electrostatic interactions were mini-

mised by the use of high ionic strength solutions. No such difference was seen with the test *E. coli* strain used. The implication is that, at pH 7.0, the net cell surface charge of *C. difficile* serves to reduce the amount of binding expected from hydrophobic interactions alone. Due to the weak negative charge of the octyl-sepharose CL-4B an increase in binding of *C. difficile* would have been expected. These results highlight the problem of simplistic extrapolations from charge and hydrophobicity results alone.

It was surprising that haemagglutination by *C. difficile* could not be demonstrated under any of the experimental procedures used. This is particularly so because Karjalainen *et al.* [11] have recently reported haemagglutination by *C. difficile* of human O⁺, A⁺, B⁺, sheep, rabbit and horse erythrocytes. These differences could be explained by differences in methodology as Karjalainen *et al.* [11] noted a low degree of haemagglutination; they used a slide agglutination assay whereas the microwell assay for haemagglutination was used in this study. Also, it is unclear from their study whether or not the bacteria grown on agar were washed, and to what degree, before they were examined for haemagglutinating ability. This is important as this study has shown that agar components may contribute to haemagglutination, and a similar observation has been made by others when studying haemagglutination by *Helicobacter pylori* [43].

In conclusion, although there is a little intra-species variability, *C. difficile* cells are of medium hydrophobicity and possess a net positive cell surface charge. These characteristics may contribute to resistance to phagocytosis and also contribute to their ability to adhere to gut mucus and underlying cells.

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