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Identification of cell wall proteins of *Bacteroides fragilis* to which bacteriophage B40-8 binds specifically

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Bacteriophage infecting *Bacteroides fragilis*, one of the most abundant bacteria in the human colon, have been proposed as indicators of virological faecal pollution. The first identification of a receptor for a bacteriophage in *B. fragilis* is reported here. First, resistant mutants were characterized following phage inactivation, and it was shown that cell wall proteins are involved in phage binding. Then the proteins involved were identified by various approaches: (i) comparison of the protein profiles of wild-type *B. fragilis* HSP40 and phage-resistant mutants; (ii) application of a modification of the virus overlay protein blot assay (VOPBA). At least two proteins of *B. fragilis*, with apparent molecular masses of 35 ± 5 kDa and 65 ± 5 kDa, bind to B40-8. This result was later confirmed by running a complex consisting of this phage bound to radiolabelled proteins of *B. fragilis* on an immunoaffinity column loaded with a specific antibody against the phage. Cell proteins retained in the column also coincided with the proteins that differed in the profiles of resistant mutants. Finally, to identify the potential function of these two proteins, their N-terminal sequences were determined and compared to published sequences, but no homologies were found.

Keywords: bacteriophage receptor, *Bacteroides fragilis*, cell wall proteins, VOPBA

INTRODUCTION

Bacteroides fragilis is a Gram-negative rod, obligate anaerobe and one of the most abundant bacteria in the human colon (Moore & Holdeman, 1974; Moore & Moore, 1995). Bacteriophages of *B. fragilis* HSP40 (ATCC 51477) fulfil most of the requirements for being a good indicator of virological faecal contamination (International Association of Water Pollution Research Control, 1991): they have a human origin, cannot replicate outside the colon, are a homogeneous group and are easier to detect than enteric viruses. Therefore, they have been proposed as model micro-organisms for assessing the presence of human viruses in waters (International Association of Water Pollution Research Control, 1991; Jofre *et al*., 1995; Tartera & Jofre, 1987). Adsorption of phages infecting *B. fragilis* (Keller & Traub, 1974) is less efficient than the adsorption of most phages infecting *Escherichia coli* (Adams, 1959). Knowledge of the receptors involved in the interactions between *B. fragilis* and its phages may improve the methods for phage detection in environmental samples and facilitate their use as an indicator of virological faecal pollution.

In this study, the lytic bacteriophage B40-8 was used as a model to examine the interactions of phages with *B. fragilis*. B40-8 is a member of the *Syphoviridae* family, which was first isolated by Tartera and Jofre (Tartera & Jofre, 1987; Tartera, 1986). Moreover, the most abundant bacteriophages infecting *B. fragilis* HSP40 isolated from sewage and sewage polluted waters are similar to B40-8 in both morphology (Lasobras *et al*., 1997) and genome (Puig *et al*., 2000).

The adsorption of phages to their bacterial host is mediated by specific interactions of phage proteins with molecules or structures on the bacterial surface. Phages may bind to several cell structures, such as flagella or capsules, or more frequently to molecules on the cell

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Abbreviation: VOPBA, virus overlay protein blot assay.

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wall, such as lipopolysaccharides or proteins (Beumer *et al*., 1984; Eriksson & Lindberg, 1977; Osborn & Wu, 1980; Schwartz, 1980; Wendlinger *et al*., 1996). Most studies on phage–bacteria interactions have been performed in *E. coli* or related species (Heller, 1992). These studies were facilitated by the availability of mutants that lack the phage receptors. However, this is not the case for *B. fragilis*, which moreover is an atypical Gram-negative bacterium, phylogenetically separate from most Gram-negative rods (Weisburg *et al*., 1985), with a much more complex cell wall (Salyers, 1984).

The purpose of this study was to identify the receptors on *B. fragilis* HSP40 to which the phage B40-8 binds. Virus overlay protein blot assays (VOPBAs) (Crane *et al*., 1991; Gershoni *et al*., 1986; Karger & Mettenleiter, 1996) demonstrated that two proteins of 35 ± 5 kDa and 60 ± 5 kDa of *B. fragilis* bind to B40-8. This result was consistent with the protein profiles of resistant mutants, and it was later confirmed by running phage B40-8 bound to radiolabelled proteins of *B. fragilis* HSP40 through an immunoaffinity column.

METHODS

Organisms and culture conditions. *B. fragilis* HSP40 (ATCC 51477) had been isolated previously from a hospital source (Tartera & Jofre, 1987). The basal medium used for bacterial growth and phage propagation was BPRM broth (*Bacteroides* phage recovery medium) (Tartera *et al*., 1992). *B. fragilis* was grown at 37 °C under anaerobic conditions (Gaspak system BBL, anaerobic jars; Oxoid) or in screwcap tubes filled with broth. Stock cultures were maintained at 4 °C in these conditions. Bacteriophage B40-8 was isolated from urban sewage and propagated on *B. fragilis* as described previously (Tartera *et al*., 1992). For titration and inactivation experiments, phage suspensions were diluted in phosphate buffer (Clowes & Hayes, 1968). *E. coli* CN13 (Payment & Franco, 1993) grown on tryptone medium at 37 °C was used as a negative control strain in inactivation assays.

Isolation of spontaneous mutants. Spontaneous mutants of *B. fragilis* resistant to phage B40-8 were isolated as follows. First, a mixture containing 10^8 bacteria plus 10^8 infectious phages in 10 ml BPRM broth was incubated for 16 h at 37 °C. This mixture was then diluted 10-fold and spread on BPRM agar plates. Colonies of presumptive phage-resistant mutants appeared after 48 h anaerobic incubation at 37 °C, several of which were selected and tested for their resistance by spotting B40-8 phage onto a lawn of cells of a particular mutant.

Isolation of cell surface membrane structures. Two series of experiments were carried out. In the first, the native structure of proteins was maintained throughout the experiment; in the second, denaturing conditions were used.

When native conditions were maintained, all the steps were performed at 4 °C. A batch culture of 1 litre of bacteria was harvested by centrifugation (10000 ***g***, 10 min) during exponential growth phase (OD_{620} 0·6–0·9). Cell pellets were resuspended in 200 ml Tris buffer (10 mM Tris/HCl + 0·3 % NaCl, pH 8) and washed twice by centrifugation (10000 ***g***, 10 min). The washed pellets were resuspended in 30 ml Tris buffer and passed three times through a French Press. Cell debris and unbroken cells were removed by centrifugation at 10000 ***g*** for 10 min. The resulting supernatant solution was centrifuged at 31000 ***g*** for 2 h. Supernatant (soluble fraction) was then removed, and membrane pellet (cell wall fraction) was resuspended in 10 ml Tris buffer and stored at −20 °C.

In the second series of experiments, denaturing conditions were used and a treatment of protein solubilization from cell wall fraction was carried out. Cultures of 300 ml of the strain, which had reached an OD_{620} of 0·6, were centrifuged at 10000 ***g*** for 10 min. Pellets were resuspended in 100 ml HEPES buffer (25 mM HEPES, 4 mM EDTA, 4 mM EGTA, pH 7·4), and washed twice by centrifugation (10000 ***g***, 10 min). The final pellet was resuspended in the same buffer containing 1 U aprotinin ml^{-1}, 2 mM PMSF, 2 μM leupeptin and 2 μM pepstatin A as protease inhibitors, and it was passed three times through a French Press as described above. Cell debris and unbroken cells were removed by centrifugation at 10000 ***g*** for 10 min. The resulting supernatant was centrifuged at 31000 ***g*** for 2 h. Membrane pellet (cell wall protein fraction) was resuspended in 1·8 ml HEPES buffer containing protease inhibitors. Triton X-100 was then added to the suspension, at a final concentration of 1 %, and the mixture was shaken at 4 °C for 90 min. Finally, the mixture was centrifuged at 150000 ***g*** for 1 h 30 min at 4 °C. Soluble cell wall proteins were obtained in the supernatant, which was stored at −20 °C.

Outer membranes were obtained by solubilizing cytoplasmic membranes from a cell wall fraction suspension with an equal volume of 1·5 % sodium lauryl sarcosinate for 20 min at room temperature (Filip *et al*., 1973). The outer-membrane fraction was pelleted by centrifugation at 16000 ***g*** for 1 h at 4 °C. This process was repeated three times. The final outer-membrane extraction was resuspended in MilliQ water and stored at −20 °C.

Protein concentrations were determined by the Bicinchoninic Acid Protein Assay Kit (Sigma Procedure TPRO-562) with BSA as a standard.

Phage binding kinetics. To determine the kinetics of adsorption, a volume of stock of bacteriophage B40-8 was added to 10 ml *B. fragilis* (OD_{620} 0·3, approximately 3×10^8 c.f.u. ml^{-1}) to a final concentration of 10^6 p.f.u. ml^{-1}. The culture was maintained at 37 °C. Sample (0·1 ml) was removed at 0, 20 and 40 min, diluted into 10 ml PBS and 1 ml was removed and centrifuged in an Eppendorf microcentrifuge at 16000 ***g*** for 3 min. The supernatants were diluted with PBS, and assayed on *B. fragilis* by the double agar layer method (Adams, 1959). Controls without *B. fragilis* cells were performed. The phage adsorption of the resistant mutant RM12 was done in the same way: the phages were added to 10 ml of an RM12 culture and the free phages were quantified on *B. fragilis* HSP40. The adsorption rate constant (K) was calculated as described by Adams (1959).

In a second series of experiments, five different concentrations of proteins from the cell wall protein fraction of *B. fragilis*, diluted in BPRM, were used: 0, 0·1, 0·2, 0·4 and 0·8 mg protein ml^{-1}. Membranes and soluble fraction from *E. coli* CN13 were used as a negative control. B40-8 (10^4 p.f.u. ml^{-1}) was added to the different concentrations of proteins and incubated for 40 min at 37 °C in order to allow phage–receptor contact. Samples were centrifuged at 16000 ***g*** for 30 min and the supernatant was removed. The phages not adsorbed to the receptor were counted and the number was compared to the original titre.

Effect of proteinase K treatment of cells and cell wall protein fraction on phage binding. Cells were grown on BPRM and harvested at a OD_{620} of 0·5–0·6. The pellet was washed three times in 10 ml Tris/HCl (0·01 M, pH 7·8) buffer and finally resuspended in 10 ml of the same buffer. To one half of the

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suspension, proteinase K (Merck) was added to a final concentration of 80 μg ml^{-1}. The other half, without proteinase K, was used as a negative control. Tubes were then incubated at 45–50 °C. Samples were removed after 0, 10, 20, 30 and 40 min incubation, mixed with 25 μl EDTA (0·5 M, pH 8·0) and kept at 4 °C to stop proteinase K activity. B40-8 was then added at the same time to all the samples, at a final concentration of 10^4 p.f.u. ml^{-1}, and incubated at 37 °C for 40 min to allow adsorption to host cell receptor. Samples were centrifuged at 16000 ***g*** for 5 min at 4 °C, and supernatant was removed and titrated to enumerate the p.f.u. of non-adsorbed phages.

Furthermore, cell wall protein fractions were diluted in Tris/HCl (0·01 M, pH 7·8) at different concentrations, which ranged from 0 up to 7·4 mg protein ml^{-1}, and treated as described above.

Modification of the VOPBA procedure. VOPBA (Crane *et al.*, 1991; Gershoni *et al.*, 1986; Karger & Mettenleiter, 1996) was adapted to detect proteins on the surface of *B. fragilis* to which bacteriophage B40-8 binds.

In the first set of experiments, under native conditions (Goldenberg, 1989), samples containing cell wall protein fractions (100 μg) of *B. fragilis* were subjected to electrophoresis on a native 7·5 % polyacrylamide electrophoresis gel at 4 °C. Proteins were electrotransferred at 4 °C to Bio-Rad nitrocellulose membranes (0·45 μm, 162-0115). These membranes were blocked with a solution of TBS-Tween buffer (10 mM Tris, 140 mM NaCl, 0·1 % Tween 20) + 3 % BSA overnight at 4 °C and washed in TBS-Tween for 5 min. One millilitre of a stock of B40-8, which contained 10^{11} p.f.u. ml^{-1}, was added and incubated as a primary element, rolling at low speed for 1 h at 37 °C in a hybridization oven. Afterwards, membranes were washed twice in TBS-Tween buffer for 5 min each time. From this point on, the procedure was similar to a Western blot. Polyclonal antibodies against B40-8, at a dilution of 1:4000 in TBS-Tween, were incubated for 1 h at room temperature. These polyclonal antibodies were detected with mouse anti-rabbit alkaline-phosphatase-conjugated secondary antibodies (goat anti-rabbit IgG whole molecule, alkaline phosphatase conjugate; Sigma).

In order to identify the proteins contained in the band developed under native conditions, cell wall protein fractions of *B. fragilis* were electrophoresed under native conditions, but this time, in order to localize the binding proteins in the gel, only the first and last lanes of the gel were electrotransferred to the nitrocellulose membrane, and treated as described above. The remaining gel was saved and kept at 4 °C, until the band corresponding to the binding protein was localized on the nitrocellulose membrane. At that point, the area of the gel corresponding to those proteins was cut out and electroeluted to remove the proteins from the gel. After electroelution, the sample was electrophoresed on an SDS-PAGE gel. We were thus able to count the proteins running in the band corresponding to the zone to which B40-8 had bound.

In another series of experiments, denatured proteins were used as sample. The cell wall protein fractions of wild-type *B. fragilis* that were soluble in 1 % Triton X-100 (200 μg) were separated by SDS-PAGE (7·5 or 12 % polyacrylamide gels) (Laemmli, 1970) and treated as described above.

Detection of bacteriophage B40-8 by PCR. Pieces of the nitrocellulose membrane containing membrane proteins of *B. fragilis* to which phages supposedly bound were cut out and analysed by PCR using specific primers (Puig & Girones, 1999).

Identification by affinity chromatography of radiolabelled cell wall proteins. *B. fragilis* was grown on a defined minimal medium (Cheng *et al.*, 1995) containing 0·5 % glucose as the sole carbon source. When the culture had reached an OD_{620} of 0·15, L-[^{35}S]methionine [Amersham Life Science SJ1015; 500 μCi (18·5 MBq)] was added to a final concentration of 15 μCi ml^{-1} (555 kBq ml^{-1}) and growth was continued until the culture had reached an OD_{620} of 0·4–0·5. Cells were harvested by centrifugation at 3000 ***g*** for 10 min and resuspended in 1 ml disruption buffer (Madrid, 1992). After 1 cycle of freeze–thaw at −80 °C, samples were placed in an ultrasonic bath for 7 min at 200 W. The last step consisted of passing the sample 15 times through a syringe with a 21G needle. This suspension was centrifuged at 10000 ***g*** for 3 min and the supernatant was removed and centrifuged at 16000 ***g*** for 2 h at 4 °C. The radiolabelled proteins were resuspended in 50 μl 10 mM phosphate buffer (pH 7·2). B40-8 suspension (150 μl; 10^{10} p.f.u. ml^{-1}) was added to the labelled proteins and incubated for 40 min at 37 °C. These samples, which contain phage bound to labelled proteins, were passed through a Protein A affinity column.

The column was prepared according to the protocol described by Harlow & Lane (1988). Polyclonal rabbit antibodies against B40-8 were linked to protein A acrylic beads (Sigma P-1052). After loading the sample, the column was washed with 10 ml 10 mM phosphate buffer (pH 7·2). Phages and bound proteins were eluted using 300 μl of a solution containing 3·5 M $MgCl_2$ and 10 mM phosphate buffer (pH 7·2). The phage-containing fractions were dialysed overnight at 4 °C against MilliQ water, and electrophoresed on an SDS-PAGE 12 % gel. Proteins were electrotransferred to nitrocellulose membranes and developed autoradiographically.

N-terminal protein sequencing. Soluble proteins from cell wall samples were electrophoresed and electrotransferred to a PVDF membrane, which was stained with Coomassie brilliant blue. The bands corresponding to the cell wall proteins of *B. fragilis* to which bacteriophage B40-8 had bound were cut out and sequenced. An LF 3000 Series sequencer from Beckman Instruments, based on Edman degradation and coupled to a PTH (phenylthiohydantoin) amino acid HPLC analyser (System Gold, Beckman), was used for protein N-terminal amino acid sequencing.

RESULTS AND DISCUSSION

Phage-binding kinetics and specific phage binding to proteins

The first step in phage infection is the adsorption of the virus onto its host. We have verified that the adsorption of *B. fragilis* bacteriophage B40-8 to its host strain HSP40 is not very efficient, as reported for other bacteriophages infecting *B. fragilis* (Keller & Traub, 1974).

Adsorption of phage B40-8 to whole cells of *B. fragilis* followed first-order kinetics and 56 % of the initial number of bacteriophages were adsorbed after 40 min contact (Fig. 1). This adsorption corresponds to an adsorption rate constant (K) of $6{\cdot}8 \times 10^{-12}$ cm^{-3} min^{-1}, significantly lower than the K reported for T2 on *E. coli* (3×10^9 cm^{-3} min^{-1}) (Adams, 1959). One explanation could be the low expression of the phage receptor (Booth *et al.*, 1979). Therefore, increasing the expression of receptors, as in media containing maltose used to

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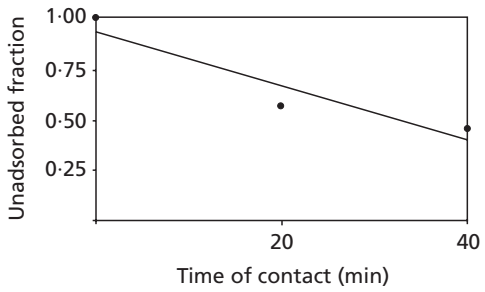

Fig. 1. Adsorption of phage B40-8 at 37 °C on *B. fragilis* HSP40 cells growing in BPRM.

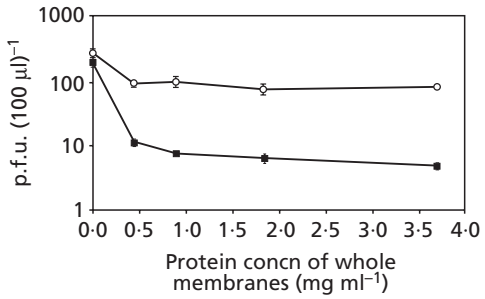

Fig. 2. Effect of treatment of cell wall protein fractions from *B. fragilis* HSP40 with proteinase K on adsorption of phage B40-8. ○, Cell wall protein fractions treated with proteinase K; ■, cell wall protein fractions untreated.

infect *E. coli* with bacteriophage λ (Sambrook *et al.*, 1989), may improve the adsorption and consequently the recovery of bacteriophages from water samples, and this was the major reason why we attempted to identify the phage receptor.

Adsorption of phage B40-8 was inhibited by treatment of the cell wall protein fraction with proteinase K (Fig. 2). Moreover, *B. fragilis* cells treated for more than 20 min with proteinase K adsorbed fewer phages than untreated cells (data not shown). Hence, the receptor for B40-8 on *B. fragilis* is likely to be a cell wall protein. The finding that proteinase K inhibited binding after only 10 min may indicate that the protein or proteins are outer-membrane proteins.

Differences in the protein profiles of spontaneous resistant mutants and wild-type *B. fragilis* HSP40

We attempted to identify the host proteins to which B40-8 binds by comparing the outer-membrane composition of bacteriophage B40-8 resistant mutants with those of the wild-type host. This approach had been successful with other bacteria and phages (Scheider *et al.*, 1993; Yokota *et al.*, 1994). Spontaneous mutants resistant to B40-8 infection were easily obtained. Protein profiles of the outer membranes of wild-type, spontaneous resistant mutants and revertants of these mutants were determined by SDS-PAGE and densitograms of relevant fractions of the gel (Fig. 3).

The comparison of the densitograms of the outer-membrane protein profile of two spontaneous resistant

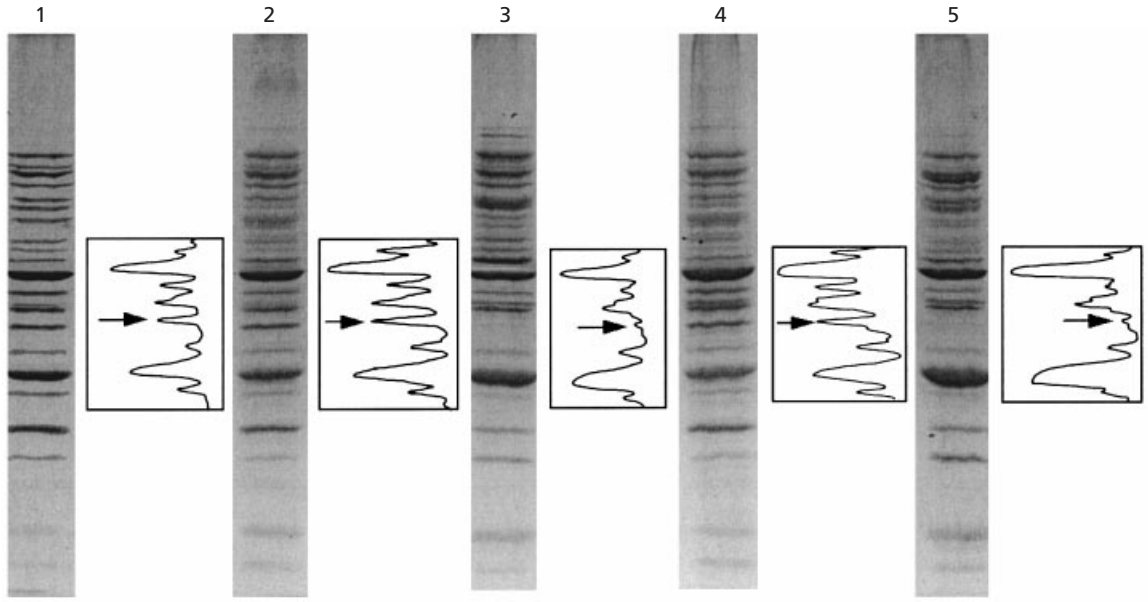

Fig. 3. Outer-membrane protein profile and densitograms from wild-type *B. fragilis* HSP40, spontaneous mutant strains resistant to B40-8 infection and revertants to wild-type phenotype (sensitive) of these mutants. Lanes: 1, *B. fragilis* HSP40 strain; 2, revertant mutant RRM03; 3, spontaneous resistant mutant RM03; 4, revertant mutant RRM12; 5, spontaneous resistant mutant RM12.

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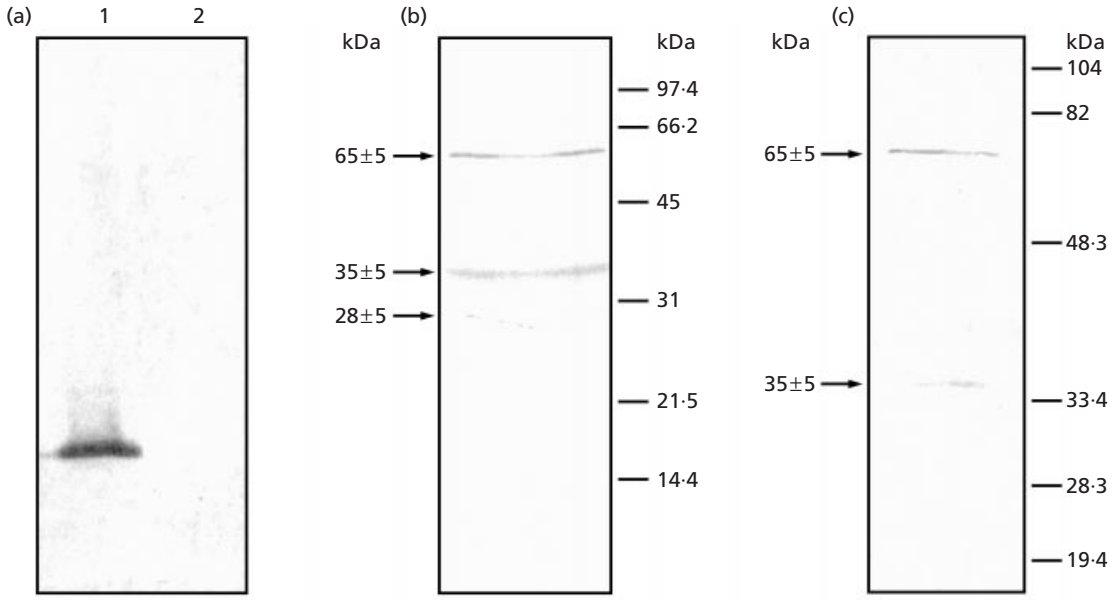

Fig. 4. (a) Adsorption of phage B40-8 to cell wall proteins of *B. fragilis* HSP40 (lane 1) and to cell wall proteins of the negative control (*E. coli* CN13) (lane 2). The gel was run under native conditions and 100 μg total protein was loaded in each lane. The bacteriophage B40-8 of *B. fragilis* was used as a primary binding element. (b) SDS-PAGE gel (12 %) of the electroeluted proteins corresponding to the area of B40-8 immunodetection following binding to *B. fragilis* HSP40 cell wall proteins. (c) Adsorption of B40-8 to solubilized cell wall proteins of *B. fragilis* HSP40.

mutants (RM03 and RM12) of *B. fragilis* and the wild-type showed a decrease in the density of a band corresponding to a protein with an apparent molecular mass of 55 ± 5 kDa. The protein profiles of the revertants (RRM03 and RRM12) from these mutants, which recovered sensitivity to phages, were similar to those of the wild-type. Moreover, when we tested the adsorption of the mutants on *B. fragilis*, the adsorption rate of RM12 was only of 11 % compared to the 56 % obtained for the wild-type after 30 min incubation.

Identification of the binding proteins by VOPBA

The study followed a biochemical approach, in order to identify membrane proteins of *B. fragilis* to which bacteriophage B40-8 specifically binds.

For that purpose, we used a modification of the VOPBA. This assay consists of a modification of the Western blot protocol where, instead of an antibody, a virus is used as a primary ligand protein. VOPBA has been successfully used in the identification of proteins that bind animal viruses (Crane *et al.*, 1991; Gershoni *et al.*, 1986; Karger & Mettenleiter, 1996; Salas-Benito & del Angel, 1997). The method presented here uses bacteriophage B40-8 as the primary ligand.

First, we applied VOPBA under native conditions, using phage B40-8 as the first ligand and antibody against the phage as the second ligand.

These experiments revealed that phage B40-8 bound to a protein with the same electrophoretic mobility (Fig. 4). The same protocol was applied to outer-membrane proteins of *E. coli* used as control. In this case, B40-8 phages did not bind to any of the proteins on the native gel (Fig. 4).

Since electrophoresis under native conditions does not guarantee that one single band corresponds to a unique protein, the band was later cut out and run under denaturing conditions. Three proteins were detected, with apparent molecular masses of 65 ± 5 kDa, 35 ± 5 kDa and 28 ± 5 kDa (Fig. 4).

The VOPBA procedure was repeated with previously solubilized cell wall proteins, and PAGE was performed under denaturing conditions. We could thus use higher concentrations of proteins than when cell wall protein fractions were examined. Two clear proteins of apparent molecular mass of 65 ± 5 kDa and 35 ± 5 kDa were revealed (Fig. 4). When soluble proteins from *E. coli* were studied as control, no bands were observed.

The results obtained under denaturing conditions confirm those obtained under native conditions, indicating that B40-8 binds to at least two proteins of 65 ± 5 kDa and 35 ± 5 kDa. From now on these proteins will be called BactA and BactB, respectively.

The protein of 28 ± 5 kDa found in the complex to which the phages bind under native conditions does not bind specifically to bacteriophages after denaturation.

Since BactA and BactB are able to bind the bacteriophage under native and denaturing conditions, the binding site is likely to be a linear epitope of the protein rather than a conformational epitope.

These results indicate that VOPBA is a fast procedure

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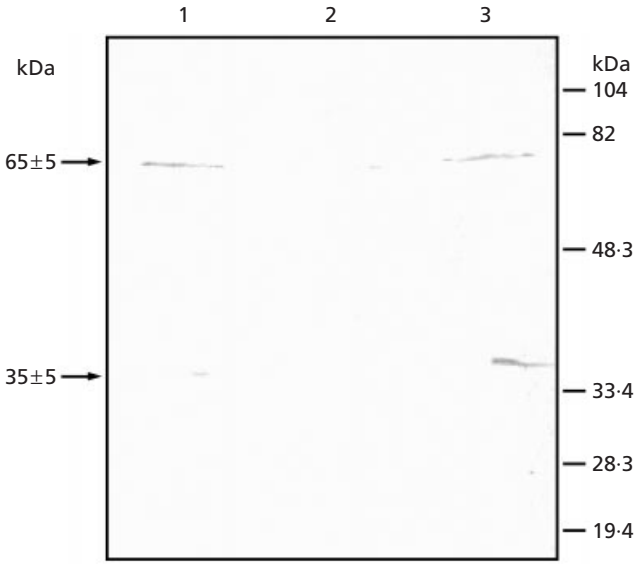

Fig. 5. Modification of the VOPBA method applied to soluble cell wall proteins, in 1% Triton X-100, from *B. fragilis* HSP40 wild-type (lane 1), a spontaneous mutant (lane 2) and a revertant (lane 3). Two hundred micrograms of protein was loaded in each lane.

for identifying phage receptors in bacterial hosts for which no mutants are available.

Assessment of the specificity of the union between phage B40-8 and *B. fragilis* as determined by VOPBA

To rule out the possibility that the results of the VOPBA procedure were due to cross-reactivity of the antibody against phage B40-8 with a cell wall protein of the host, the nature of the material bound to the protein bands was assayed by PCR. The bands corresponding to BactA, BactB and a third one, used as a negative control, were electroeluted and analysed to detect the presence of phage DNA by PCR, using specific primers for phage B40-8. The results showed that the bands corresponding to BactA and BactB contained phage DNA, while the band used as a negative control did not.

Study of the occurrence in resistant mutants of the phage-binding proteins by VOPBA

Some of the spontaneous resistant mutants showed very low levels of a protein that corresponds to BactA. Revertants from these mutants, which recovered sensitivity to B40-8, showed wild-type levels of BactA protein.

To confirm that the changes in the protein profile of the resistant mutant (RM12) corresponded to BactA or BactB, soluble cell wall proteins (treated with 1% Triton X-100) from the resistant mutant were electrophoresed on SDS-PAGE and electro-transferred onto nitrocellulose membranes, and the presence of the phage-binding proteins was determined by VOPBA (Fig. 5). The signal corresponding to the 35 ± 5 kDa protein disappeared almost completely, while a decrease in the signal corresponding to the 65 ± 5 kDa protein was observed. On the other hand, when a revertant (RRM12) was analysed, the signal for these two proteins was recovered.

Identification of binding proteins by immunoaffinity chromatography of radiolabelled proteins of *B. fragilis*

The binding of BactA and BactB to B40-8 was further confirmed by their retention in an immunoaffinity column chromatography designed for isolating phage-binding protein complexes. Radiolabelled proteins from the bacterial host *B. fragilis* were placed in contact with bacteriophage B40-8. The phage–protein receptor complex was separated from the rest of the bacterial proteins using an immunoaffinity chromatography column which retained the phages but not the *B. fragilis* proteins. The fraction containing both bacteriophages and the bound bacterial host proteins was subjected to SDS-PAGE. After electrotransfer to nitrocellulose membranes, these were developed autoradiographically and three proteins were detected. The apparent molecular masses were 65 ± 5 kDa, 38 ± 5 kDa (BactA and BactB) and 25 ± 5 kDa (data not shown), which coincide with those detected by VOPBA under native conditions.

Therefore, it was confirmed by different approaches that B40-8 receptors on the surface of *B. fragilis* consist of at least two proteins, BactA and BactB.

N-terminal sequences of BactA and BactB

N-terminal sequences of BactA and BactB were obtained and compared with those in the databases (Non-reductant GenBank, CDS translations, PDB, SWISS-PROT, Spupdate and PIR).

Results for sequences of proteins BactA (65 ± 5 kDa) and BactB (35 ± 5 kDa) were as follows: BactA (65 kDa), MEEIKYIEPAAL(H)DEML(A)L(R)N- (accession no. A59325); and BactB (35 kDa), MKKTG(I/V)(N/G)-AFNVI (A/N)TMANVLAF- (accession no. B59325).

There were no similarities between them, nor did the comparison of these two sequences with those published in databases reveal any significant homology.

Consequently the proteins could not be associated with any known function. However, this is not surprising since *B. fragilis* belongs to the *Bacteroides–Cytophaga–Flavobacterium* branch of the eubacterial phylogenetic tree (Weisburg *et al.*, 1985), which has been less extensively studied than other groups. Nonetheless, the reason for this lack of homology could also be the low number of amino acids sequenced. When the search was performed against the *Porphyromonas gingivalis* complete genome database (The Institute for Genomic Research, website at http://www.tigr.org), a weak homology to proteins with unknown function was found. *P. gingivalis* belongs to the same group as *B.*

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fragilis, and its chromosome was the first of this group to be sequenced. In spite of the lack of homology of these sequences, the N-terminal sequence of these proteins may allow us to perform reverse genetics, and localize and try to complete the sequence of those proteins.

In summary, our results indicate that at least two membrane proteins, BactA and BactB, with apparent molecular masses of 65 ± 5 kDa and 35 ± 5 kDa, are involved in the adsorption of bacteriophage B40-8 to the surface of *B. fragilis*.

Further studies of mutants which lack these proteins may identify their function and improve our understanding of the cell wall of *B. fragilis*. Moreover, this knowledge may contribute to the development of better methods for the detection of bacteriophages in the environment, and to confirming the feasibility of using bacteriophages as indicators of virological faecal pollution in waters.

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