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Identification of two new *Helicobacter pylori* surface proteins involved in attachment to epithelial cell lines

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Helicobacter pylori causes the development of gastritis, gastric ulcers and adenocarcinomas in humans. The establishment of infection is influenced by adherence to the gastric epithelium, and several bacterial adhesins and host cell receptors have been identified. *H. pylori* recognize the Lewis[b] receptor through the BabA adhesin but also readily adhere to epithelia in the absence of the Lewis[b] epitope, demonstrating the relevance of additional adhesive interactions. This study presents a novel method of identifying bacterial adhesins. Nickel beads were coated with *H. pylori*-derived, recombinantly expressed ORF proteins, and epithelial cells from the human stomach, intestine or urinary tract were allowed to adhere to those beads. The binding of epithelial cells to the protein-coated nickel beads was confirmed by electron microscopy or flow cytometry using antibodies directed towards the His-tags. Among the five ORFs tested, two new adhesive proteins (HP1188 and HP1430) were identified. Both were expressed on the surface of virulent *H. pylori*, with the HP1188 protein being most abundant. The purified HP1188 and HP1430 proteins bound more strongly to gastric than to other epithelial cell lines, suggesting that they may be involved in the colonization of the human gastric mucosa. In conclusion, this method facilitates the identification of ORFs of microbial origin involved in cellular interactions such as adherence.

INTRODUCTION

Adherence to mucosal surfaces guides the tissue tropism of many pathogens. During the complex interaction with the mucosa, many different adhesive surface molecules may be expressed, thus allowing the pathogen to bind secreted host molecules, epithelial cell receptors or inflammatory cells. In view of this complexity, it can be quite difficult to identify the individual adhesins and to understand their contribution to disease pathogenesis.

Several approaches may be taken to study how individual adhesive proteins contribute to the attachment of mucosal pathogens. Blocking with soluble receptors is a classical way of hindering attachment of one adhesin class to cell-bound receptors and may lead to a reduction or complete inhibition of adherence if the ligand recognizing the receptor is sufficiently important for binding. Antibodies to bacterial surface adhesins may also prove valuable in the selective blocking of specific adhesins, and mutational inactivation of discrete adhesin genes may provide additional information. However, these approaches are unlikely to give distinct results for micro-organisms such as *Helicobacter pylori* that

express multiple strong adhesins on their surface, and where additional adhesive proteins may become involved subsequent to the initial attachment (Nishihara *et al.*, 1999; Palovuori *et al.*, 2000; Su *et al.*, 1998).

In this study, a novel method was developed to identify bacterial adhesins using *H. pylori* as a model. The complete genome sequence of *H. pylori* was used to select a sample of ORFs with structural characteristics of membrane proteins. The candidate proteins, recombinantly expressed with a six histidine amino acid tag, were bound to nickel beads and used to study adhesive interactions with epithelial cell lines derived from the stomach or other mucosal environments. Two new adhesins were shown to interact strongly with gastric epithelial cell lines and to be expressed on the surface of virulent *H. pylori* strains. We conclude that the nickel bead assay is a useful tool for the testing of unknown bacterial surface proteins as novel adhesins.

H. pylori is the causative agent of chronic gastritis and peptic ulcers in humans. Chronic infection is associated with the development of gastric adenocarcinoma and gastric lymphoma (Blaser, 1990, 1992; Lee *et al.*, 1993; Warren & Marshall, 1983) and *H. pylori* was recently designated a class 1 carcinogen (Logan, 1994). This genetically diverse bacterial

Abbreviation: SEM, scanning electron microscopy.

species (Akopyanz *et al.*, 1992) colonizes the stomach of at least half of all humans (Graham *et al.*, 1988), making it one of the most common human pathogens. Infection is thought to require attachment and bacterial adherence factors that promote the colonization of the human gastric epithelium, thus contributing to the virulence of *H. pylori* (Clyne & Drumm, 1996; Thomsen *et al.*, 1990; Wadstrom *et al.*, 1996, 1997).

Several adhesins are involved in *H. pylori* adherence, including the surface protein BabA and the lipoproteins AlpA and AlpB (Odenbreit *et al.*, 1999). The blood-group antigen-binding adhesin, BabA, recognizes the Lewis[b] saccharides on gastric epithelial cells (Boren *et al.*, 1993, 1994; Falk *et al.*, 1995), and is mainly expressed by type I isolates (Ilver *et al.*, 1998). BabA and BabB are members of a paralogous family of outer-membrane proteins. Pride *et al.* (2001) have found the presence of well-conserved allele groups in BabA and BabB diversity regions, which may imply an important functional role in adherence. Colonization with type II *H. pylori* may involve other adhesins as the Lewis[b] antigen is not needed for *H. pylori* type II adherence to gastric epithelial cells or epithelial tumour cell lines, and no correlation to ABO blood group has been observed (Clyne & Drumm, 1997; Heneghan *et al.*, 2000). Furthermore, Lewis X structures in *H. pylori* LPS may mediate adhesion through binding to lectins in the gastric epithelium (Edwards *et al.*, 2000). Other adhesin–receptor interactions are involved in epithelial cell adherence of *H. pylori*; however, their molecular nature remains undefined.

METHODS

Reagents. Nickel beads (Probond Resin) were purchased from Invitrogen, IPTG from ICN Biomedicals (Aurora) and BSA from Sigma. GAB-CAMP plates were prepared according to Soltesz *et al.* (1992) and contained Bacto-Agar, 0·5 % cysteine hydrochloride, 8·5 % hematinized horse blood, 10 % inactivated horse serum, 3·5 % Iso-Vitalex (all BD Biosciencies) and antibiotics vancomycin, nalidixic acid and amphotericin B. Lysozyme, arginine, Benzonase, Tris/HCl, MgCl$_2$, PCR reagents, restriction enzymes, T4 ligase, and EDTA were from

Merck. PefaBloc (which eliminates DNA, as does Benzonase) was from Interbiotech. Vent polymerase was from New England Biolabs.

Cloning of the *H. pylori* ORF proteins. DNA fragments encoding different *H. pylori* proteins were obtained by PCR amplification using the *H. pylori* X47-2AL (ORV 2001) strain as a source of DNA. Restriction sites used for the cloning were included in the 5′ and 3′ PCR primers (Table 1). PCR amplification conditions were as follows: 97 °C for 30 s, 55 °C for 1 min and 72 °C for 50 s for 25 cycles, and Vent DNA polymerase was used. The PCR product was cloned into the pET28c vector (Novagen) using restriction endonucleases and T4 DNA ligase according to the manufacturer's instructions, and electrocompetent *Escherichia coli* BL21 λDE3 were used for transformation. The constructs were characterized by restriction mapping analysis and DNA sequencing at the 5′ and 3′ ends of the vector cloning site. For small-scale expression, bacteria were grown in LB. Expression was induced with 1 mM IPTG for 3 h and the protein was detected by PAGE followed by Coomassie staining and by Western blot analysis using an anti-His-tag monoclonal antibody (Invitrogen). A 10 ml culture of individual positive clones was divided into 0·5 ml aliquots and kept frozen (−20 °C) after addition of an equivalent volume of glycerol.

Purification of recombinant His-tagged *H. pylori* ORF proteins. One millilitre of frozen bacteria was used to inoculate 50 ml LB medium containing 25 μg kanamycin ml^{-1} in a 250 ml Erlenmeyer flask, and incubated at 37 °C for 2 h or until the OD$_{600}$ reached 0·4–1·0. The culture was placed at 4 °C overnight, and 10 ml of the overnight preculture was used to inoculate 240 ml LB medium containing 25 μg kanamycin ml^{-1} with the initial OD$_{600}$ of about 0·02–0·04. The cells were grown to an OD$_{600}$ of 1·0 (about 2 h at 37 °C), induced with 1 mM IPTG and grown for 3 h at 37 °C.

Cells were harvested by centrifugation at 5000 **g** for 15 min at 4 °C, resuspended in 50 mM Tris/HCl pH 8·0, 2 mM EDTA (250 ml for 1 l of culture) and centrifuged at 12 000 **g** for 20 min. The supernatant was discarded and the pellets were stored at −45 °C. The bacterial pellets were thawed, resuspended in 95 ml 50 mM Tris/HCl pH 8·0, and Pefabloc and lysozyme were added to final concentrations of 100 μM and 100 μg ml^{-1}, respectively. The mixture was then incubated at 5 °C for 30 min. Benzonase was added at 1 U ml^{-1} final concentration in the presence of 10 mM MgCl$_2$ to ensure total digestion of DNA. The suspension was subjected to sonication (Branson Sonfier 450) for three cycles of 2 min each at maximum output. After centrifugation (20 000 **g**, 20 min), Tris/HCl (300 mM, pH 8·0) 3 M NaCl and 2 M imidazole were added to the supernatant to give a final solution of 50 mM pH 8·0, 0·5 M and 10 mM, respectively. Hi-trap chelating columns (1 ml; Pharmacia) were used for purification according to the manufacturer's instructions.

Table 1. DNA sequences of the *orf* gene primers

Primer	Sequence*
5′ HP1430 *Eco*RI	CGC<u>GAATTC</u>ACGGATAACAACCAAAAC
3′ HP1430 *Xho*I	CCC<u>CTCGAG</u>TCAAAAAGAATGGGCATG
5′ HP1188 *Bam*HI	CGC<u>GGATCC</u>AAGAGAGTTAGAGAAC
3′ HP1188 *Xho*I	CCC<u>CTCGAG</u>TCAGCAAATATTTTTTTG
5′ HP1256 *Eco*RI	GG<u>GAATTC</u>TTACAGGCCATTTATAACG
3′ HP1256 *Xho*I	CCC<u>CTCGAG</u>TTAGACCTTTAAGATCG
5′ HP1145 *Bam*HI	CGC<u>GGATCC</u>GAAGACAAAGAAGTCTTGATCTA
3′ HP1145 *Xho*I	CCG<u>CTCGAG</u>TTAGTTCTTCTTAAAGAGTTG
5′ BabB *Bam*HI	CGC<u>GGATCC</u>GAATCCAATTTAATCCAAAAAGG
3′ BabB *Xho*I	CCG<u>CTCGAG</u>TTAGTAAGCGAACACATA

*Underlining indicates the restriction enzyme site for the respective restriction enzymes.

His-tagged protein was eluted with Tris/HCl, NaCl and 500 mM imidazole, and eluted fractions were monitored as the absorbance at 280 nm. Fractions corresponding to the protein peak were pooled, dialysed against PBS containing 0·5 M arginine, filtered through a 0·22 μm membrane and stored at −45 °C.

H. pylori strains and culture conditions. *H. pylori* strain NCTC 11637 (CagA+/VacA+) was obtained from the American Type Culture Collection and cultured on GAB-CAMP plates in a microaerobic atmosphere (5–6 %, v/v) at 35 °C. The *H. pylori* strain X47-2AL that was used for the cloning of the various ORF proteins is a streptomycin-resistant strain adapted to mice by serial passage. The X47-2AL *H. pylori* strain was isolated originally from a domestic cat and was adapted to Swiss-Webster mice by sequential *in vivo* passages of *H. pylori*. It was kindly provided by J. Fox (Massachusetts Institute of Technology, Cambridge, MA, USA).

Immunization of mice with recombinant H. pylori proteins. Eight-week-old outbred OF1 mice (Iffa Credo) were immunized four times, 1 week apart, by the rectal route with 25 μg recombinant *H. pylori* protein in the presence of 2 μg *E. coli* heat-labile toxin (Sigma) in a total volume of 25 μl. Serum was taken 2 weeks after the last immunization and used to analyse the expression of each protein on the *H. pylori* surface.

Cell cultures. AGS (gastric epithelium), HT29 (ileal epithelium), CaCo2 (colonic epithelium), A498 (renal epithelium) and J82 (urinary bladder epithelium) cell lines were used in this study. All cell lines were obtained from the American Tissue Culture Collection. A498, HT29, CaCo2 and J82 cells were grown in RPMI 1640 containing 10 % fetal calf serum (FCS), 1 % non-essential amino acids, 1 % sodium pyruvate, 50 μg ml^{-1} gentamicin (GIBCO BRL, Life Technologies). AGS cells were grown in F-12 medium containing 10 % FCS, 1 % non-essential amino acids, 1 % sodium pyruvate, 50 μg ml^{-1} gentamicin (GIBCO BRL, Life Technologies). The tissue culture flasks were incubated at 37 °C with 95 % humidity in 5 % CO_2. The medium was changed every 3 days and the cells were harvested every 7 days when confluent. Prior to harvesting, the cells were washed twice with 5 ml 50 mM EDTA in PBS. Three millilitres of this solution was added and the cells were harvested when detached from the culture bottles.

Protein coating of nickel beads. Nickel beads were coated with His-tagged recombinant proteins. Twenty microlitres of Probond Resin (containing approximately 10 μl beads) was washed twice in PBS and resuspended in a volume of PBS that would give a final volume of 100 μl after the addition of recombinant proteins. The beads were incubated overnight at 4 °C and the protein content was analysed using a modified Lowry assay (Bio-Rad). The bead–peptide solution was then washed three times in PBS and the pellet was resuspended in R10 (RPMI 1640 with 10 % FCS and 50 μg ml^{-1} of gentamicin).

Interaction of epithelial cells with protein-coated nickel beads. Single cell suspensions (1 × 10^6 ml^{-1}) of the epithelial cell lines in RPMI 1640 or F-12 (for AGS cells) with 50 μg ml^{-1} gentamicin were mixed with the bead–peptide preparation and incubated for 30 min at 37 °C with shaking every 10 min. Medium containing 10 % FCS (900 μl) was added to each tube and the mixture was transferred to a 24-well culture plate. The cells and resin-bound proteins were left to interact at 37 °C, 95 % humidity, 5 % CO_2 for 24 h. Cells binding to the beads were inspected under a light microscope or by scanning electron microscopy (SEM).

SEM. Nickel beads coated with various recombinant proteins were incubated with the cells as described above. After 24 h, 500 μl of the medium was removed and approximately one-third of the remaining mixture of beads and cells was harvested by centrifugation at 500 r.p.m. for 5 min in a cytospin centrifuge (Cytospin 3, Shandon Life Sciences

International) and collected on a carbon tab (12 mm, Ted Pella). The sample was then fixed in 1 % glutaraldehyde (Sigma Aldrich Chemie) and 1 % formalin (Sigma Aldrich Chemie) for 15 min, dehydrated in a number of steps using 50 %, 75 %, 95 % and finally 99·5 % ethanol sequentially for a period of 5 min each and then left overnight in 99·5 % ethanol at 4 °C.

When totally dehydrated, the sample was dried in a critical point dryer (Balzer CPD 030). The alcohol was first replaced by liquid CO_2 at 10 °C. Once saturated with CO_2, the chamber was heated to 8–9 °C above the critical point of CO_2. The CO_2 was then vented at a rate of 1–2 l min^{-1} until atmospheric pressure was reached. The sample was then removed and placed in a sputter-coating machine (Polaron E5150). Coating was performed at 5 °C with a current of 15–20 mA. The samples were sputter-coated with gold at a thickness of 15 nm and stored in a low humidity environment until viewed through the scanning electron microscope (Philips SEM 515).

Binding of H. pylori proteins to epithelial cell lines. Epithelial cells (0·2 × 10^6) were incubated with the ORF proteins (2 μg) in a total volume of 200 μl PBS containing 1 % BSA (PBS-BSA) for 45 min on ice, washed twice in ice-cold PBS-BSA and incubated for another 45 min on ice with 2 μg anti-His(C-term)-FITC conjugated antibody (Invitrogen) in 200 μl PBS-BSA. Prior to the analysis by flow cytometry, the cells were washed once in PBS-BSA. They were analysed in a Beckton-Dickinson FACS-Calibur instrument.

Surface expression of ORF proteins by H. pylori. NCTC 11637 *H. pylori* were grown on GAB-CAMP plates and 10^9 cells were suspended in 1 ml of ice-cold PBS. Around 5 × 10^6 bacteria were incubated with antibodies to each ORF protein or normal mouse serum (Dakopatts) to detect membrane expression of the proteins. Serum was diluted 100-fold in PBS-BSA and incubated at room temperature with bacteria in a total volume of 500 μl for 45 min. The bacteria were harvested by centrifugation at 6000 *g* for 5 min and washed with PBS-BSA. FITC-conjugated swine-anti-mouse antibody (10 μg) (Dakopatts) was added, and the bacteria were incubated for 45 min at room temperature, washed and analysed by flow cytometry.

RESULTS

Cloning and expression of ORF proteins

Five ORFs were selected from the *H. pylori* genome sequence using algorithms for predicted type I or type II signal sequences and membrane-associated proteins (PSORT). These five proteins were chosen due to high yields in the expression system and ease of purification to homogeneity. Each ORF was cloned into the pET28c vector (Table 1), and transformed *E. coli* BL21 were used for expression and purification of the recombinant proteins. The predicted sizes of the recombinant proteins were confirmed by PAGE analysis and the proteins were identified by N-terminal sequencing and Western blots (not shown).

There were some homologies to antigens from other micro-organisms and known virulence factors. The 185 aa 19 kDa ORF 89 (HP1256 in TIGR database) showed homologies with *Haemophilus influenzae* ribosome releasing factor (*frr*) and the 179 aa, 20 kDa ORF 161 (HP1245) showed sequence homology with *E. coli* single-strand DNA binding protein (*ssb*). The 689 aa, 77·4 kDa ORF 175 (HP1430) showed sequence homology with *Bacillus subtilis* conserved hypothetical ATP-binding protein. The 103 aa, 11·7 kDa ORF 34

(HP1145) and the 269 aa, 30·6 kDa ORF 155 (HP1188) both lacked homology to known sequences. The recombinant ORFs were expressed in *E. coli* BL21 with a six-histidine tag. One of the ORFs encoded the known adhesin BabB, which was also expressed as described above, and was used as a positive control.

Coating of nickel beads with His-tagged proteins

The His-tagged recombinant proteins were used to coat nickel beads at concentrations ranging from 3 to 30 μg. Bound protein was quantified after elution according to the Lowry method. The different proteins bound with similar efficiency to the nickel beads, with the HP1188 and HP1430 proteins in the lower and the HP1145, HP1256 and HP1245 proteins in the higher range (Fig. 1). Subsequently, 10 μg of protein and 20 μl of the nickel beads were used in the cell adhesion assay.

Binding of gastric epithelial cells to nickel beads coated with recombinant protein

The ability of the protein-coated nickel beads to interact with epithelial cells was studied using the gastric AGS cell line. The beads were incubated with a single cell suspension of the AGS cells at 37 °C for 24 h and studied under a light microscope and by SEM. Results for the positive control adhesin BabB are shown in Fig. 2. After 24 h of incubation, the beads coated with the BabB protein were covered with adherent cells. Two of the proteins (HP1188 and HP1430) were shown to mediate adherence with similar efficency to the BabB control. Beads coated with the HP1188 and HP1430 proteins were
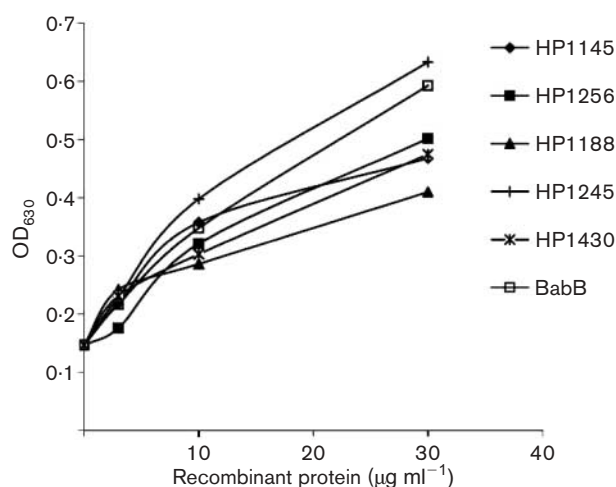

Fig. 1. Colorimetric analysis of protein bound to nickel beads. Approximately 5000 nickel beads were incubated with His-tagged recombinant *H. pylori* proteins at a concentration range of 3−30 μg per 20 μl beads. The beads were washed and stripped of bound protein by the addition of 0·5 mM EDTA and the supernatant was analysed for protein content using the Lowry method.

covered with a confluent layer of AGS cells (Fig. 2). The remaining proteins did not mediate cell attachment and there was no unspecific binding to uncoated beads.

After 1 h, initial cell clustering in discrete regions was observed on beads coated with BabB, HP1188 and HP1430. After 24 h, the cells had become flattened and covered the entire bead surface (not shown). These results demonstrated that the proteins HP1188 and HP1430 were strong adhesins for gastric epithelial cells whereas the remaining proteins lacked these adhesive properties.

Cellular spectrum of the adhesins

Epithelial cell lines from the small or large intestine (HT29 or CaCo2), urinary bladder (J82) or kidney (A498) were allowed to react with the protein-coated nickel beads. All cell lines bound to the HP1188 and HP1430 protein-coated beads (Fig. 3). There was no adherence to beads coated with the other proteins (data not shown).

The HT29 and A498 cells bound strongly to the HP1188- and HP1430-coated beads, forming a confluent layer after 24 h. The J82 cells showed weak adherence, with few cells bound after 24 h, and the CaCo2 cells bound sparsely (Fig. 3A). All
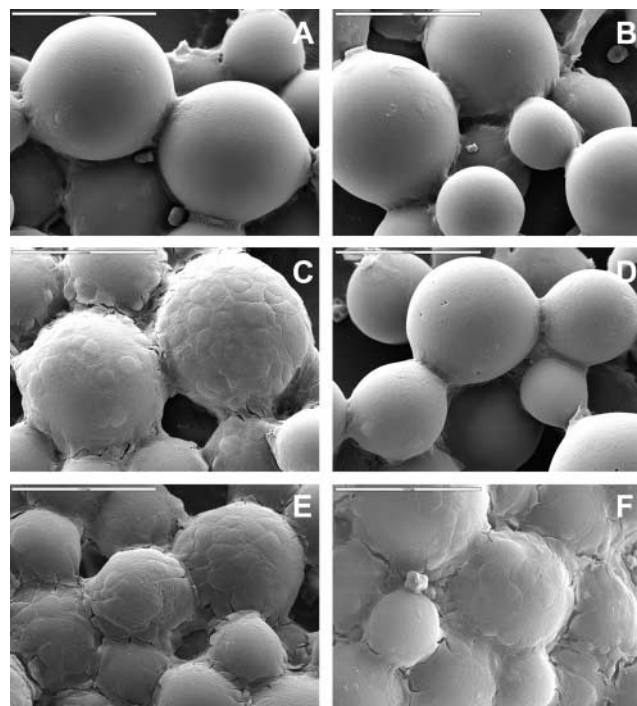
Fig. 2. SEM of AGS cells bound to nickel beads coated with His-tagged recombinant *H. pylori* proteins. The proteins were: (A) HP1145, (B) HP1256, (C) HP1188, (D) HP1245, (E) HP1430 and (F) BabB. The cells (10^6) were allowed to interact with 10^4 beads for 24 h at 37 °C, and beads and cells were collected for SEM. The AGS cells adhered to beads coated with the HP1188 and HP1430 proteins. After 24 h an almost confluent cell layer was formed. The AGS cells did not adhere to beads coated with the HP1145, HP1256 or HP1245 proteins. Bars, 100 μm.

HP1188   HP1430

Fig. 3. Cellular spectrum of the adhesins. Nickel beads coated with the HP1188 and HP1430 proteins were incubated at 37 °C for 24 h with (A) J82, bladder epithelium; (B) HT29, small intestine; (C) A498, renal pelvic epithelium; (D) AGS, gastric epithelium; or (E) CaCo2, colonic epithelium, and thereafter prepared for SEM. Uncoated nickel beads are shown in (F). Bars in panels HP1188 (F), HP1430 (E) and HP1430 (F) represent 200, 100 and 50 µm, respectively.

of these cell lines bound less strongly to the protein HP1188 coated beads than did the AGS cells. These results demonstrated that the HP1188 and HP1430 proteins mediate adherence to several epithelial cell lines, of different tissue origin. The most impressive adherence was observed for the AGS cells derived from the human stomach, followed by the HT29 and A498 cells. The AGS cells in Fig. 3(D) bound profusely to beads, and thus did not flatten to the same extent as in Fig. 2, where less cells bound and flattened to cover the beads.

Binding of recombinant proteins to epithelial cells, as detected by flow cytometry

Experiments were performed to compare the properties of the soluble proteins with those exposed on the coated nickel. The binding of soluble proteins to each cell line was quantified by flow cytometry using a monoclonal antibody reacting with the His-tag of the recombinant proteins (Fig. 4). This permitted us to use the same antibody to detect all proteins.
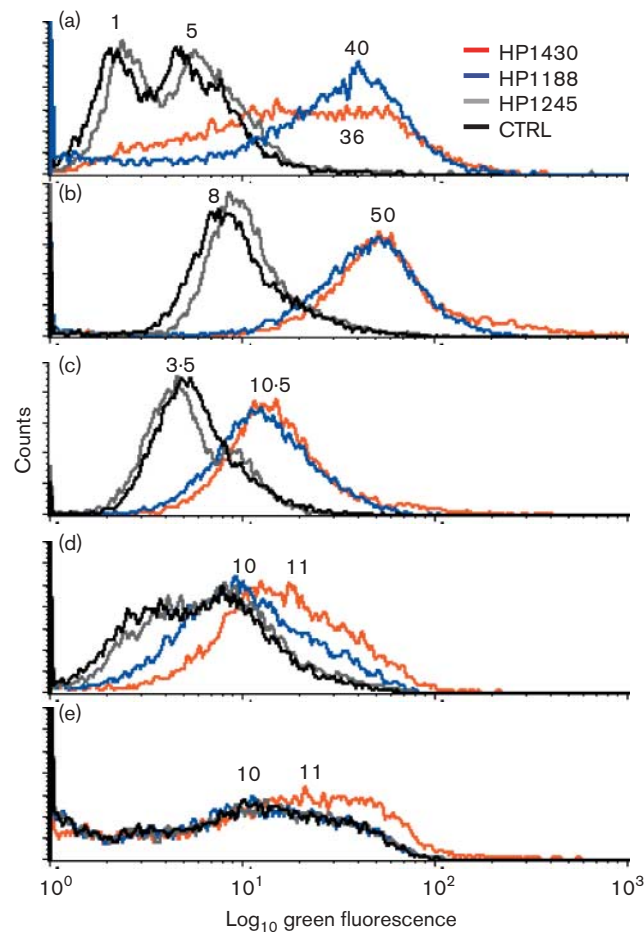
The HP1188 and HP1430 proteins were shown to bind most efficiently to the cells. Binding to AGS, A498 and J82 cells could be detected at lower protein concentrations (2 µg per 100 000 cells) than binding to HT29 and CaCo2 cells. Peak channel values are included above each peak as a logarithmic value (Fig. 4). Binding of the other proteins was not detected

at similar protein concentrations. The results showed that the HP1188 and HP1430 proteins bound to cells from the gastric and urinary tract, but not the small intestine or colonic cell lines.

Surface expression of the ORF proteins by *H. pylori*

Polyclonal antibodies were used to study the surface expression of the HP1188 and HP1430 proteins by *H. pylori*. The clinical *H. pylori* isolate NCTC 11637 was incubated with antibodies raised against each protein and examined by flow cytometry after staining with a FITC-labelled secondary antibody. The HP1188 protein was strongly expressed on the bacterial surface (Fig. 5), and the HP1430 protein was detected, but to a lesser extent. Surface expression of other ORF proteins was weaker but detectable above the background, defined by normal mouse serum. These results suggested that all the proteins studied were present on the surface of virulent *H. pylori* but that the HP1188 protein was the most strongly expressed.

DISCUSSION

Mucosal pathogens like *H. pylori* use adhesive interactions to target sites of infection. A number of adhesive ligands have been identified and in some cases the corresponding recep-

Fig. 4. Flow cytometer analysis of recombinant protein binding to epithelial cell lines. The cells (10^5) were incubated with 2 µg of HP1430, HP1188 or HP1245 recombinant protein, and binding was visualized using an FITC-labelled anti-His-tag antibody. Cells studied: (a) AGS (gastric epithelium); (b) A498 (renal pelvic epithelium), (c) J82 (urinary bladder epithelium), (d) HT29 (ileal epitheilum) and (e) CaCo2 (colonal epithelium). Logarithmic peak channel values are given above each peak.

tors are known, but these adhesive interactions are not sufficient to explain the attachment of *H. pylori* to the gastric mucosa.

This study describes a new method to identify bacterial adhesins using *H. pylori* as an example. ORFs with no known function were selected from the *H. pylori* genome sequence, and the corresponding proteins were recombinantly expressed and bound to nickel beads through His-tags. The coated beads were then allowed to interact with epithelial cell lines from different tissues, including the gastric mucosa. Two new adhesive ORF proteins were identified (HP1188 and HP1430) and they showed similar binding capacity to the BabB adhesin, which was used as a positive control. Indeed, BabB was one of the ORFs tested in the nickel bead assay, and only later was it identified. We conclude that the
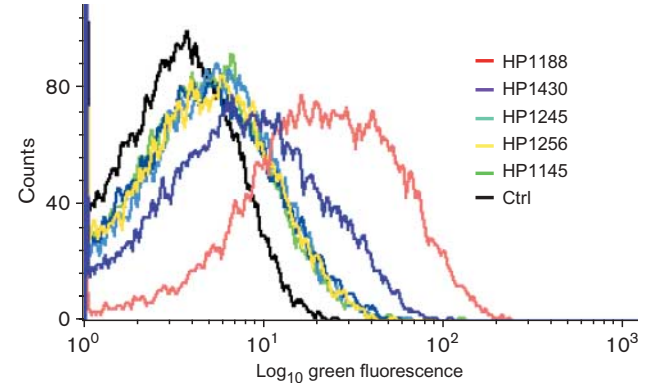

Fig. 5. Flow cytometer analysis of ORF proteins expressed on the membrane surface of *H. pylori* NCTC11637. Around 5×10^6 bacteria per sample were incubated with mouse antiserum raised against the various *H. pylori*-derived ORFs or with normal mouse serum (Ctrl). Antibody binding was detected by a FITC-labelled swine-anti-mouse polyclonal antibody in a flow cytometer.

coupling of His-tagged proteins allows for genome-based screening of ORFs as potential adhesin candidates, and for the analysis of their cellular spectrum.

The study was not designed to extensively screen *H. pylori*-derived recombinant proteins for their role in epithelial adhesion, but to exemplify how the method can be used. Yet the limited screening of five ORFs proved useful in that three adhesive proteins were found. The two new adhesive proteins HP1188 and HP1430 had no previously known function, and HP1188 protein showed no homology with any known adhesins. They had no mutual sequence homology and did not resemble the BabB adhesin, despite the functional similarity. The new adhesins adhered most strongly to the gastric cell line, as expected if this binding were relevant for the *in vivo* localization of *H. pylori*. The remaining three proteins showed little or no cell binding capacity. This was unrelated to their weak surface expression in whole bacteria, since they were freely available to bind cells on the nickel beads. The new ORF adhesins, in contrast, were not just present in the genome of the TIGR strain, but were detected on the surface of virulent *H. pylori*. The HP1188 protein was strongly expressed on *H. pylori* NCTC 11637 and HP1430 protein was expressed to a lesser extent, but was clearly present. Analysis of the amino acid sequence of the HP1188 protein revealed a putative prokaryotic membrane lipoprotein attachment motif at amino acid position 59–65 (Klein *et al.*, 1988) as well as a putative *N*-myristoylation site at amino acid position 57–63 (Towler *et al.*, 1988), which further strengthens the notion that the HP1188 protein is attached to the bacterial cell membrane. Also the HP1430 protein had nine putative *N*-myristoylation sites, which would direct the protein to lipid membranes.

The nickel bead assay thus permitted the analysis of single molecules and their interactions with host cells. Therefore it is possible to dissect the complex interactions in which the

entire bacterium expressing multiple adhesins is involved. The coated nickel beads provided a particulate stimulus and may resemble intact bacteria. The assay may be used to study the cellular changes that follow the adhesive interactions of individual proteins with the host cell. In addition, the beads may provide a useful tool for our understanding of mucosal inflammation and other tissue changes that result from *H. pylori* infection. Cells that bound to the beads remained viable and continued to proliferate. In fact, the HP1188 and HP1430 proteins seemed to make gastric cells thrive on the nickel beads. The proteins bound the gastric AGS cells with rapid kinetics, resulting in massive cell numbers on the bead surface after 24 h. This assay may therefore be useful to study the cellular responses and eventually to study mechanisms of proliferation and potentially the development of neoplastic growth.

Several tentative receptors for *H. pylori* adhesins have been identified on the gastric epithelium. Highly sulphated glucosaminoglycans such as heparan sulphate are potent receptors for *H. pylori* (Ascencio *et al.*, 1993), and sulphated glycolipids, present on gastric epithelial cells have been identified as a potential receptor structure (Kamisago *et al.*, 1996; Saitoh *et al.*, 1991). There was no difference in fluorescence intensity between HP1188 and HP1430 on AGS cells although *H. pylori* express larger amounts of HP1188. One obvious reason would be that HP1430 bind epithelial cells with a stronger affinity than HP1188, thus compensating for lesser expression on the bacterial surface membrane. The HP1188 and HP1430 proteins bound strongly to epithelial cell lines derived from a wide range of human tissues. The gastric cells showed strong interactions with BabB, HP1188 and HP1430 protein on the nickel beads, and intermediate interactions were seen with H29 (jejunal) and uroepithelial cells (A498 and J82). The colon-derived CaCo2 cells interacted poorly with the HP1188 and HP1430 proteins. We propose that this differential binding is explained by specific interactions between the proteins and corresponding receptors. These receptors remain to be identified.

Bacteria are known to trigger inflammatory responses in epithelial cells (Svanborg *et al.*, 1999; Godaly *et al.*, 1997; Hedges *et al.*, 1992). For example, cytokines and chemokines are released upon bacterial stimulation and set up the local cellular network by recruitment of inflammatory cells or lymphocytes. *H. pylori* and other mucosal pathogens stimulate epithelial cells to secrete IL-8 and other chemokines (Agace *et al.*, 1993; Peek *et al.*, 1995). Furthermore, bacteria upregulate ICAM-1 expression on epithelial cells (Agace *et al.*, 1995) through translocation of NF-κB (Mori *et al.*, 2000). Bacterial ligands may activate the cells through different signalling pathways, for example the release of ceramide from the glycosphingolipid receptors as has been shown for *E. coli*-induced epithelial inflammation (Hedlund *et al.*, 1998, 1996) and may require TLR4 as a co-receptor (Frendeus *et al.*, 2001; Hedlund *et al.*, 2001). *H. pylori* also bind glycolipid receptors,

and could thus utilize these mechanisms. This model system may be used to investigate the mediators involved in the growth of gastric epithelial cells and in carcinogenesis.

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