

MOLECULAR CHARACTERISATION AND DIAGNOSIS

Identification of an immunogenic 18-kDa protein of *Helicobacter pylori* by alkaline phosphatase gene fusions

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The use of alkaline phosphatase fusion methodology to identify *Helicobacter pylori* exported proteins enabled the identification of an open reading frame (ORF) encoding a highly immunogenic, previously uncharacterised exported protein. The predicted amino-acid sequence displays a typical N-terminal signal peptide and contains regions of C-terminal hydrophobicity consistent with a membrane-associated protein. Southern blot analysis revealed that the gene encoding the protein was absent in several *Helicobacter* spp. and a combination of PCR and sequence analysis of the amplified gene showed that it is highly conserved amongst isolates of *H. pylori*. To obtain pure recombinant protein, the gene encoding the protein was cloned and expressed as a β -galactosidase (β -gal) fusion in *Escherichia coli* and the protein was purified by affinity chromatography and proteolytic cleavage of the β -gal portion. The purified protein, which has an apparent mol. wt of 18 kDa, was recognised by antibody present in 71% of sera from patients infected with *H. pylori*, but in only 16% of sera from patients with unrelated or no gastrointestinal disease, by Western blot assays. These results indicate that the 18-kDa protein from *H. pylori* is immunogenic and is expressed *in vivo*.

Introduction

Helicobacter pylori is a gram-negative, spiral bacterium whose discovery and subsequent characterisation has revolutionised understanding of gastrointestinal diseases. It is now accepted as the primary aetiological agent in chronic gastritis, and is strongly associated with both peptic and duodenal ulceration [1]. Evidence supporting the role of *H. pylori* infection as a precursor of gastric carcinoma and mucosa-associated lymph tissue (MALT) lymphoma continues to accumulate [2], making *H. pylori* a significant human pathogen.

The recent release of two *H. pylori* genome sequences [3, 4] has accelerated and simplified research in this field, but there is still a large gap in the understanding of the mechanism of *H. pylori* pathogenesis. Despite a vigorous humoral and cellular immune response, the bacteria continue to evade the host's defence mechanisms and persist in the hostile environment of the stomach. Antibodies against various *H. pylori* proteins

can be detected in sera from patients [5], but the identity of many of these proteins remains to be established. Some immunodominant proteins of *H. pylori* that have been well-characterised include the urease subunits [6], the vacuolating cytotoxin (VacA) and cytotoxin-associated protein (CagA) [7], and proteins involved in flagella structure [8]. The discovery that the *H. pylori* genome harbours a pathogenicity island [9] is a major step toward the identification of virulence factors.

The observation in animal models that immunisation with various cell-derived antigens can confer protection against establishment of *H. pylori* infection [10] has led to the search for protective antigens that could be used in a subunit vaccine. Secreted or membrane-associated proteins often play a major role in the pathogenesis of infection [11] and are potentially useful candidates for vaccine development. The lack of a simple antibiotic treatment regimen for *H. pylori* infection and the emergence of antibiotic-resistant strains make the development of a vaccine a desirable goal. Secreted bacterial proteins are also of therapeutic interest, as their cellular location makes them more accessible to drug therapy than cytosolic proteins.

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Gene fusion methodology based on alkaline phosphatase (PhoA) has been used successfully to identify exported proteins in various bacterial systems [12] and has been used in this laboratory to identify *H. pylori* exported proteins [13]. The pJEM11 plasmid vector was first described by Lim *et al.* [14] and was designed to allow gene fusions between a truncated *phoA* gene and heterologous genomic DNA. This enables the identification of open reading frames (ORFs) containing a promoter and signal peptide in frame with *phoA*, which are detected when enzymically active PhoA is transported across the cytoplasmic membrane and turns the recombinant colonies blue in the presence of a chromogenic substrate. The aim of the present study was to identify, by a genetic approach, surface-located or secreted proteins of *H. pylori* that might be involved in pathogenesis and assess their immunogenicity during infection. This report describes the use of *phoA* gene fusions to identify *H. pylori* genes encoding exported proteins, immunological screening of these fusion proteins, and the expression, purification and investigation of an 18-kDa protein for its distribution among *H. pylori* clinical isolates and its immunogenicity against a panel of sera from patients with or without *H. pylori* infection.

Materials and methods

Bacterial strains, plasmids and culture media

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was routinely grown on Luria Bertani (LB) medium under aerobic conditions at 37°C. *H. pylori* isolates (kindly supplied by P. W. O'Toole, Massey University) were grown on Columbia agar plates (Difco) supplemented with horse

blood 10% in a micro-aerobic atmosphere at 37°C. Genomic DNA was purified by standard procedures [16] and plasmid DNA was prepared with the Bresatec plasmid isolation kit (Bresatec, Australia). Plasmids were maintained in culture containing ampicillin 100 µg or kanamycin 30 µg. PhoA activity was detected by plating the recombinants on LB media containing 5-bromo-4-chlor-3-indolyl phosphate (BCIP) (Sigma) 40 µg/ml.

Construction of a plasmid genomic library

A library of *H. pylori* genomic DNA was constructed in the plasmid vector pJEM11 (kindly supplied by B. Gicquel, Pasteur Institute, Paris, France) as described previously [13]. Briefly, *H. pylori* was partially digested with *Sau3A* and fragments ranging from 200 to 2000 bp were purified and ligated into the *Bam*HI site of the dephosphorylated pJEM11 vector. The ligation mix was used to transform *E. coli* DH10B by electroporation and the resulting transformations were plated on LB containing the substrate BCIP and kanamycin. Restriction digest analysis of 20 clones revealed that 19 (95%) contained an insert and c. 2–3% of transformants were blue on BCIP.

DNA sequencing and analysis

The DNA inserts from pJEM11 plasmid constructs were partially or completely sequenced by automated sequencing with an ABI Prism 337 DNA Sequencer (Perkin Elmer). Oligonucleotide primers were designed to the non-coding strand of *phoA* (primer pJEM6) and the coding strand of the terminator sequence (primer pJEM7) on the pJEM11 vector – pJEM6: 5' GCAG TAATATCGCCCTGAGCAGC 3'; pJEM7: 5' TTAA

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype/phenotype	Source [Reference]
Strains		
<i>E. coli</i> DH10B	<i>F'</i> <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>)f80 <i>φ</i> <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ(<i>ara,leu</i>)7697 <i>galU</i> <i>galK</i> λ- <i>rpsL</i> <i>nupG</i>	Life Technologies
<i>E. coli</i> XLI-blue	<i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>hsdR17</i> <i>supE44</i> <i>relA1</i> <i>lac[F'</i> <i>proAB</i> <i>lacIqZ</i> Δ <i>M15</i> <i>Tn10</i> (<i>tet'</i>)	[16]
<i>E. coli</i> BL21 (DE3)	<i>hsdS</i> <i>gal</i> (λ <i>clts857</i> <i>ind1</i> <i>Sam7</i> <i>nin5</i> <i>lacUV5-T7</i> gene 1)	[16]
<i>H. pylori</i> 17874	<i>H. pylori</i> type strain, Cag A ⁺ /Vac A ⁺ (Type I), identical to NCTC 11637	Culture collection, University of Gothenburg, Sweden
<i>H. pylori</i> MU005	Clinical isolate, Auckland, Cag ⁺ /VacA ⁺ (Type I)	[15]
<i>H. pylori</i> MU007	Clinical isolate, Auckland, Cag ⁺ /VacA ⁺ (Type I)	[15]
<i>H. pylori</i> MU008	Clinical isolate, Auckland, CagA ⁻ /VacA ⁻ (Type II)	[15]
<i>H. pylori</i> MU015	Clinical isolate, Auckland, CagA ⁻ /VacA ⁻ (Type II)	[15]
<i>H. pylori</i> MU016	Clinical isolate, Auckland, Cag ⁺ /VacA ⁺ (Type I)	[15]
<i>H. pylori</i> MU038	Clinical isolate, Auckland, CagA ⁻ /VacA ⁻ (Type II)	[15]
<i>H. pylori</i> MU064	Clinical isolate, Auckland, Cag ⁺ /VacA ⁺ (Type I)	[15]
<i>H. pylori</i> MU091	Clinical isolate, Auckland, CagA ⁻ /VacA ⁻ (Type II)	[15]
<i>H. felis</i>	Cat clinical isolate	P. W. O'Toole
<i>H. mustelae</i> 4298	Laboratory passaged strain	P. W. O'Toole*
Plasmids		
pJEM11	<i>E. coli</i> -mycobacterial shuttle vector carrying a truncated <i>phoA</i> gene	[14]
PJHp20	pJEM11 carrying part of a <i>H. pylori</i> gene encoding an 18-kDa antigen	This study
pXa1	Expression vector (P _{lac} :f _{ori} :)	Boehringer Mannheim
pXa-1085	pXa1 vector containing the 20/1–20/2 PCR amplification product	This study

*Originally obtained from J. G. Fox, Massachusetts Institute of Technology, MA, USA.

TTGGGGACCCTAGAGGTCC 3'. Primers were designed and tested for suitability by GeneWorks (Release 2.45, IntelliGenetics, CA, USA). Sequencing contigs were built up with the GeneWorks Sequencing Project document. DNA and protein databases were searched with the BLAST [17] and FASTA servers to identify similarities between the insert DNA and previously identified sequences. The BLAST + BEAUTY algorithm [18] was also used to search databases for sequence similarities and structural motifs. After the release of the entire *H. pylori* genome sequence [3,4] the *H. pylori* genome databases (HPDB) were searched to identify sequence similarities with the annotated ORFs. The GeneWorks program was used for all other sequence analyses.

Isolation of periplasmic space proteins

For fractionation of the intracellular, periplasmic and extracellular protein, plasmids were transformed into *E. coli* strain BL21 (DE3). The periplasmic space proteins were isolated by the method of Neu and Heppel [19]. Briefly, the cells were grown in supplemented M63 media to late-log phase (OD 0.7–0.9) and harvested by centrifugation. The supernate was stored as the extracellular fraction. The cells were washed with 10 mM NaCl, then resuspended and agitated gently in sucrose 25% solution in 1.5 mM EDTA and collected by centrifugation. After adding ice-cold water to shock the cells, the preparation was filtered through a 0.45- μ m membrane to collect the periplasmic space fraction. The cells were then washed in 10 mM Tris-HCl, 1 mM EDTA and sonicated three times for 1 min with 30-s intervals to release the intracellular protein.

Detection of alkaline phosphatase and β -galactosidase activity

The alkaline phosphatase assays on protein extracts were based on a method originally described by Brickman and Beckwith [20]. Briefly, each protein sample was assayed in duplicate in 1 M Tris, pH 8. As a control, 1 μ l of a 1 in 100 dilution (equal to 0.0014 U) of *E. coli* alkaline phosphatase (Pharmacia Biotech, Sweden) was assayed. The tubes were equilibrated at 37°C and 100 μ l of 20 mM phosphatase substrate *p*-nitrophenylphosphate (Sigma) were added to each tube at 37°C. After 40 min, 100 μ l of stop solution (1 M K₂HPO₄) was added and the absorbance was measured at 420 nm. The assay of β -galactosidase activity in protein samples was based on a method described by Miller [21]. Each sample was assayed in duplicate in 1 \times Z buffer (100 mM Na₂HPO₄·7H₂O, 350 mM NaH₂PO₄·H₂O, 10 mM KCl, 2 mM MgSO₄, pH 7). After equilibration at 30°C for 5 min, the reaction was started by the addition of 200 μ l of σ -nitrophenyl- β -D-galactoside. (ONPG; Sigma) and the time taken for sufficient yellow colour to appear was recorded. The reaction was stopped by adding 500 μ l of 1 M Na₂CO₃. One unit of β -galactosidase was calculated as the

amount of enzyme that liberated 1 μ mol of σ -nitrophenol per min at 30°C, pH 7, under the above conditions.

Expression and purification of the 18-kDa protein

A 444-bp fragment of the gene encoding the putative exported protein (ORF HP1085) devoid of the signal sequence was amplified from *H. pylori* CCUG 17874 genomic DNA by PCR with the following primers: 20/1, 5' TAATGAATTCACCTTACACGCTAGAC 3'; 20/2, 5' TTGAGGTACCGGGACTTGATAAGC 3'. The PCR produced a truncated HP1085 sequence beginning five residues from the putative cleavage point of the signal sequence with an *Eco*RI site incorporated into the 5' end and a *Kpn*I site at the 3' end. The PCR fragment was cloned into the *Eco*RI–*Kpn*I sites of pXa1 (Boehringer Mannheim) to create pXa-1085. PCR amplification consisted of an initial denaturation step (95°C, 5 min) followed by 30 amplification cycles of denaturation (95°C, 30 s), annealing (50°C, 30 s) and primer extension (72°C, 1 min). Expression of the fusion protein was induced by the addition of 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG) into the culture medium. The protein was purified by affinity chromatography with an APTG (4-aminophenyl- β -D-thiogalactopyranoside) column according to the manufacturer's instructions (Boehringer Mannheim). The β -gal portion of the fusion was removed by proteolytic cleavage with factor Xa (biotin-labelled) which was subsequently removed with streptavidin gel. The cleaved β -gal portion was then removed by a second round of APTG affinity chromatography, yielding pure recombinant protein. The pure protein was electrophoresed on an SDS-PAGE gel and blotted on to a polyvinylidene difluoride (PVDF) membrane (NEN, Boston, MA, USA), stained with Coomassie Brilliant Blue (BioRad) 0.25% and excised for N-terminal sequencing. The N-terminal amino-acid sequence analysis was performed with a pulse liquid phase sequencer (Model 477A, Applied Biosystems).

PCR and Southern blot analysis

Genomic DNA prepared from eight *H. pylori* clinical isolates, *H. pylori* CCUG 17874, *H. mustelae* 4298 and *H. felis* was used as the template in the PCR with primers 20/1 and 20/2 to amplify the 444-bp fragment of HP1085. The PCR conditions were as described above and PCR products were sequenced by automated sequencing. For Southern blot experiments, genomic DNA from *Helicobacter* spp. was digested with *Hind*III and transferred from agarose gels to a positively charged membrane (Amersham) by the Southern blot technique [16]. PCR primers 20/1 and 20/2 were used to produce a 444-bp product from *H. pylori* 17874, which was purified from an agarose gel followed by phenol-chloroform extraction for use as a probe in Southern blots. The probe was labelled with

DIG and quantified according to the manufacturer's instructions (Boehringer Mannheim). After pre-hybridisation, the blot was hybridised with 25 ng of probe/ml of hybridisation solution (DIG Easy Hyb, Boehringer Mannheim) overnight at 42°C. The blot was washed under moderate stringency with 1 × SSC at 60°C and developed with anti-DIG antibody conjugated to alkaline phosphatase. After incubation for 15 min in substrate, the blot was developed by autoradiography.

Sera

Sera were collected from 32 patients at the Wakefield Gastroenterology Centre, Wellington. Of these, 21 were infected with *H. pylori* as diagnosed by either the CLO test, urease breath test (UBT) or by testing for anti-*H. pylori* antibodies in serum (Quikview Kit). Control sera were obtained from 11 asymptomatic volunteers who were determined to be *H. pylori* negative by Quikview and CLO test (three) or by Quikview only (eight). Serum samples were stored at -70°C until the immunoblots were performed. This study was approved by the Wellington Ethics Committee, Central Regional Health Authority, New Zealand.

SDS-PAGE and Western blot analysis

Electrophoresis of proteins was performed routinely on SDS-polyacrylamide gels by standard methods with a BioRad Mini Protean II apparatus according to the manufacturer's instructions. For Western blotting, c. 10 µg of purified protein/lane were run on an SDS-PAGE 12% gel and transferred to a PVDF membrane by a TransBlot semi-dry transfer cell (BioRad) according to the manufacturer's instructions. The blocking solution was PBS (pH 7.4) supplemented with milk powder 2% and Tween-20 0.01%. Blots were incubated in sera diluted 1 in 200 in PBS for 2 h at room temperature (RT). After being washed, the

membranes were incubated at room temperature for 2 h in anti-human IgG alkaline phosphatase-conjugated secondary antibody (Sigma) diluted 1 in 20 000 in PBS. The colour reaction was developed with BCIP 0.05 mg/ml and nitroblue tetrazolium 0.01% (NBT). Protein concentrations were determined by the BCA Protein Estimation Assay (Pierce, Rockford, USA) according to the manufacturer's instructions, with bovine serum albumin as standard.

Statistical analyses

The significance of the bands formed on immunoblots to the *H. pylori* 18-kDa protein for *H. pylori*-positive and -negative patient sera was evaluated by Fisher's exact test.

Results

Identification of an ORF encoding an immunogenic protein

An *H. pylori* genomic library was constructed in the plasmid vector pJEM11. After plating out on the substrate BCIP, 2–3% of the recombinant clones turned blue, indicating that these contained genes encoding exported proteins [13]. Sequence data from 15 blue recombinant clones demonstrated that, in each case, the gene fused to *phoA* contained an N-terminal sequence characteristic of a signal peptide. Searches of the *H. pylori* genome database with these sequences revealed that, in most case, the ORF identified had been designated a signal peptide by Signal-P analysis as reported by Tomb *et al.* [3] (Table 2). To reduce *E. coli* background for screening with patient antibodies, the fusion proteins were partially purified by isolation of the periplasmic space protein. Enzyme assays were carried out to confirm PhoA activity in the periplasm and β-gal activity confined to the intracellular fraction:

Table 2. Open reading frames (ORFs) identified by sequencing of pJEM11 *H. pylori* insert DNA from recombinant blue colonies

HP ORF [†] (fused in-frame to <i>phoA</i>)	Description
HP 0021*	Hypothetical protein
HP 0144	Cytochrome C oxidase subunit, membrane-bound (fixN)
HP 0375	Hypothetical protein
HP 0415*	Conserved hypothetical integral membrane protein
HP 0567*	Cell envelope protein
HP 0726*	Hypothetical protein (OMP)
HP 0758	Conserved hypothetical integral membrane protein
HP 0780*	Hypothetical protein
HP 0896*	Outer-membrane protein (OMP19)
HP 1085*	Hypothetical protein (OMPLP)
HP 1117*	Conserved hypothetical secreted protein
HP 1136*	ATP synthase FO, subunit b (atpF)
HP 1185*	Conserved hypothetical integral membrane protein
HP 1511*	Brute force ORF
HP 1568*	Hypothetical protein

*Signal peptide designated to this gene product

[†]The ORFs are listed according to the nomenclature and description designated in the published genome sequence [3].

>95% of PhoA activity was found in the periplasmic fraction, with <5% in the intracellular fraction and no detectable PhoA activity in the extracellular milieu. All β -gal activity was found in the intracellular fraction, indicating that no intracellular protein had contaminated the periplasmic fraction (data not shown). These results also indicated that the fusion protein was exported across the cytoplasmic membrane and, therefore, contained functional expression and exportation signals fused to alkaline phosphatase. The periplasmic space fractions from eight strongly blue recombinant pJEM11 clones were screened with pooled sera from five patients with *H. pylori* infection to detect B-cell epitopes in the *H. pylori* proteins. A fusion protein was identified that reacted with this pool of sera on Western blots. When tested against the individual sera, the protein was recognised by sera from all five patients infected with *H. pylori* (data not shown). The plasmid encoding this fusion protein was designated pJHp20 and selected from the pJEM11 library for further analysis. DNA sequencing and Western blotting revealed that the plasmid pJHp20 contained a 1.1-kb *H. pylori* insert that included a 170-bp fragment of the HP1085 [3] ORF fused in-frame with the *phoA* gene (Fig. 1) and expressed a fusion protein *c.* 5 kDa larger than native alkaline phosphatase on Western blots (data not shown). Sequence analysis with a network program, Signal-P [22], revealed that the gene contained classical signal peptide features with a potential cleavage point at residue 23. The signal sequence consisted of a positively charged N domain followed by a hydrophobic stretch (H domain) of 15 residues at the N-terminus (Fig. 2).

Distribution of the HP1085 gene in *Helicobacter* species

In the PCR, the HP1085 gene was detected as a 444-bp product in all 10 *H. pylori* isolates tested, but was not amplified from one strain each of *H. mustelae* or *H. felis*. Southern blot experiments were performed to confirm the absence of the HP1085 gene in chromosomal DNA from other *Helicobacter* spp. with the 444-bp PCR amplification product amplified from *H. pylori*

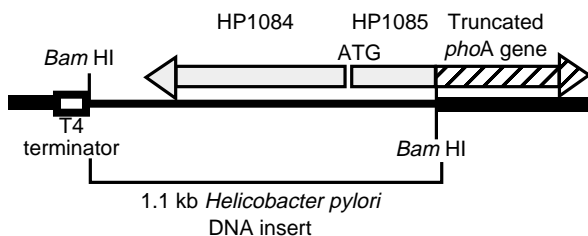


Fig. 1. Schematic diagram of pJHp20. The plasmid is an *E. coli*-mycobacterial shuttle vector (pJEM11) that carries the *aph* gene for kanamycin resistance. It contains a 1.1-kb *H. pylori* insert fused to the truncated alkaline phosphatase (*phoA*) gene (striped). Thick black line represents vector DNA; thin black line, *H. pylori* insert DNA.

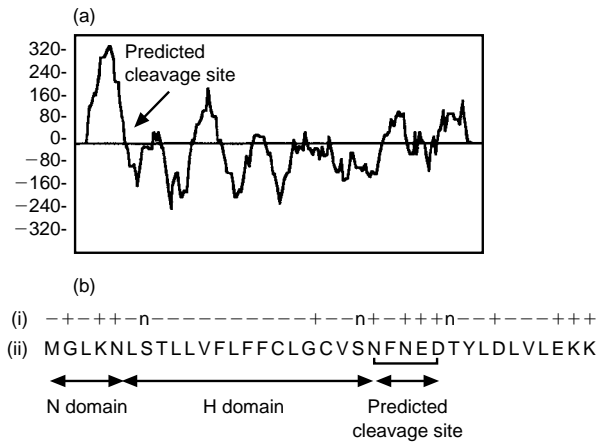


Fig. 2. (a) Kyte-Doolittle plot of the 18-kDa protein. Hydrophobic regions are shown above the line and hydrophilic below. (b) Signal sequence features of the N-terminal region of the 18-kDa protein showing the predicted domains and cleavage point (i) amino-acid hydropathy, (ii) amino-acid sequence. -, hydrophobic; +, hydrophilic; n, neutral.

CCUG 17874 as a probe. No hybridisation was observed with chromosomal DNA from *H. felis* or *H. mustelae*, but a strong signal was detected with *H. pylori* 17874 and clinical isolate no. 7 (data not shown). The results of this limited screen indicate that the gene may be specific to *H. pylori*. Furthermore, sequencing of the PCR product from five *H. pylori* clinical isolates nos. 7, 8, 38, 64 and 91) demonstrated that the gene is highly conserved, with only 0.5–3% nucleotide variation in the gene sequence compared with the type strain CCUG 17874. Also, a comparison of the 18-kDa protein from the two *H. pylori* strains for which the genome has been sequenced [3, 4] revealed that there was 96.5% identity in the DNA sequence between HP1085 (strain 26695) and its counterpart JHP0340 (strain J99). The protein has not been assigned a function in either database. Searches of DNA and protein databases to identify sequence similarities with HP1085 revealed no homologies, suggesting that this gene may be unique to *H. pylori*.

Purification and immunogenicity of the 18-kDa protein

To overexpress the entire 18-kDa protein, the HP1085 gene was expressed as a β -gal fusion which was purified by affinity chromatography and proteolytic cleavage of the β -gal portion. The size of the recombinant protein released is in agreement with the calculated M_r of the polypeptide deduced from the DNA sequence (*c.* 18 kDa). N-Terminal sequencing of the purified recombinant protein confirmed its identity (data not shown). The immunogenicity of the mature protein was then determined by immunoblotting against a panel of sera. A total of 21 serum samples from *H. pylori*-infected patients and 11 from non-infected controls were collected and used in individual im-

munoblot assays to determine the frequency of recognition of the purified protein by antibodies from infected patients. Of the 21 *H. pylori*-positive samples, 15 recognised the purified OMP in Western blots (71%), demonstrating that the protein is immunogenic during the infection process. Only two (18%) of the 11 sera from control patients recognised the *H. pylori* 18-kDa protein ($p = 0.008$) (Fig. 3).

Discussion

Several proteins secreted by or on the surface of *H. pylori* have been described as virulence factors. These include the vacuolating cytotoxin (VacA), a secreted 87-kDa protein present in a subset of *H. pylori* strains associated with the more serious clinical outcomes of *H. pylori* infection [23]. Various other secreted or surface-located proteins involved in motility [24], adhesion [25] and iron uptake [26] have been identified also. Progress in identifying secreted or membrane proteins by standard biochemical techniques has been hampered by the presence of the highly immunogenic urease and GroEL homologue proteins. These normally intrinsic cytoplasmic proteins found on the surface of the bacterium are thought to be released by autolysis

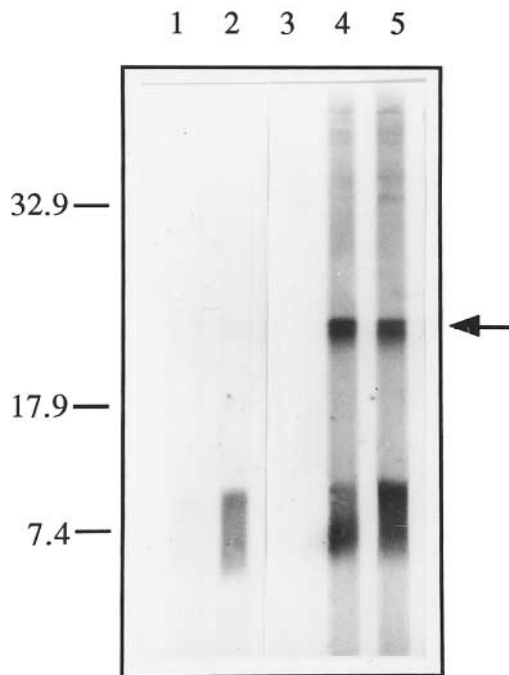


Fig. 3. Immunoblot of the 18-kDa protein from *H. pylori* CCUG 17874. The protein was purified by affinity chromatography and analysed by SDS-12.5% PAGE, followed by blotting and Western screening with a BioRad mini-protean II multi-screen apparatus. Lanes 1 and 2, sera from *H. pylori* non-infected patients; 3, PBS control; 4 and 5, sera from *H. pylori*-infected patients. Arrow refers to the position of the purified 18-kDa *H. pylori* protein. Numbers refer to the molecular masses (kDa) of the standard proteins.

[11, 27], or a novel programmed release mechanism [28].

The aim of the present study was to identify, by a genetic approach, surface-located or secreted proteins of *H. pylori* that stimulate an immune response during infection. Several translational fusion systems for the identification of bacterial export signals have been developed for enteric pathogens [29–31] and, more recently, *H. pylori* [32]. Localisation of proteins at the bacterial surface is the result of complex targeting processes, involving exportation signals and interaction with bacterial membranes. In most cases, cell membrane-associated and secreted proteins of gram-negative bacteria contain a characteristic signal peptide that directs their export across the cytoplasmic membrane via the general secretory pathway [33]. To identify exported proteins of *H. pylori*, a library was constructed based on PhoA fusion methodology [13], originally developed to identify exported proteins in mycobacteria [14]. With this system, *H. pylori* sequences were identified that encode signals for the export of a large number of *H. pylori* proteins [13].

Screening of a number of these *H. pylori* PhoA fusion proteins with sera from infected patients led to the identification of one recombinant fusion protein that was strongly recognised by patients' antibodies. Sequencing revealed that the gene partially encoded an 18-kDa putative outer-membrane protein (OMP) [3] and contained a classical signal sequence and potential transmembrane domains. The full gene encoding the 18-kDa protein was cloned, expressed as a β -Gal fusion and the recombinant protein was purified by affinity chromatography. When a combination of Southern blot analysis and PCR was used, the gene was not found in *H. felis* or *H. mustelae*, but as only one strain of each species was screened, it is possible that the gene may be present in other strains or that it might be detected under less stringent conditions. Nevertheless, these results, and the absence of any homology to sequences in the databases, suggest that the gene may be specific to *H. pylori*. It is present in different clinical isolates from New Zealand and the geographically diverse type strains CCUG 17874, 26695 [3] and J99 [4]. A comparison of the DNA sequence of HP1085 from five *H. pylori* isolates and the three type strains showed a variation of up to 3.5% in the DNA sequence, suggesting that the gene is highly conserved. This is consistent with the 2–5% variation frequently observed when nucleotide sequences of conserved genes from different *H. pylori* isolates are compared. This was observed in the case of the urease genes and the heat-shock protein gene *hspB* [34].

Infection with *H. pylori* in man results in a strong humoral immune response, characterised by high levels of both secretory IgA and serum IgG antibodies to *H. pylori* immunodominant antigens. Western blot analysis of *H. pylori* protein with patients' sera as the primary

antibody has demonstrated that several immunodominant proteins are involved in the infection, but many of these remain to be identified. To investigate the role of the 18-kDa protein in the antibody response, the purified recombinant protein was used in Western blot assays against patients' sera. Sera from the majority of infected patients (71%) recognised the protein, compared with only 16% those from uninfected controls. One patient in the study, diagnosed as negative for *H. pylori* infection but positive for antibodies to the 18-kDa protein on Western blot, had been diagnosed with an active duodenal ulcer 18 years previously. Therefore, this result may indicate long-lasting immunity to a previous infection or exposure to *H. pylori*. However, the possibility cannot be excluded that the initial diagnostic tests used (CLO test and Quikview) were unable to detect a low grade infection with the bacterium in the two patients positive on Western blot but diagnosed as negative for the infection.

It is known that not all *H. pylori*-infected patients produce antibodies against important *H. pylori* virulence factors such as the urease B subunit (UreB) [35] and heat-shock protein A (HspA) [36]. Recognition of these antigens and other outer-membrane proteins [35] by antibody from *H. pylori*-infected individuals varies from 35% (HspA) to 75% (UreB). The antibody titres in the remaining 29% of patients in the present study may have been relatively low, and therefore, below the sensitivity of Western blotting. Host factors may have some influence on the level of antibody to *H. pylori* antigens, as age has shown to be an important marker for seropositivity to the HspA antigen [36]. It is possible that the detection of antibody in only 71% of patients in this study is reflected by an absence of the 18-kDa antigen in some strains of *H. pylori*, as demonstrated with the immunodominant CagA antigen [37]. However, the evidence presented in this study suggests that the 18-kDa antigen is conserved among isolates. Alternatively, antibodies to this antigen may be present in all infected sera, but the immunoreactive epitopes may not be conserved among *H. pylori* strains and, therefore, no reactivity to the recombinant proteins derived from *H. pylori* strain 17874 would be observed.

Serology is used extensively to diagnose *H. pylori* infection, and is particularly useful as an initial non-invasive diagnostic tool. Sensitivities of ELISA-based methods are generally high, but these assays can be plagued by low specificity due to cross-reactivity [38]. The advantage of the immunoblot technique in serological assays is its accurate detection of antibodies that react with defined antigens from an infectious agent. Therefore, immunoblot analysis has been investigated as a more reliable alternative for serodiagnosis of *H. pylori* [39] and, in this study, gave an accuracy of 72.2%. It is known that not all *H. pylori*-infected patients produce antibodies against important *H. pylori* proteins such as urease [40], fumarate

reductase [41] and heat-shock protein A [42]. Variable IgG antibody responses to immunogenic iron-repressible OMPs (IROMPs) from *H. pylori* have also been described [43].

Recent work with the Rhesus monkey as a model of *H. pylori* infection has suggested that neither secretory nor serum IgA plays a protective role against infection [44] and this has led to investigations into the role of IgG. Experimental data have shown that administration of IgG is sufficient to confer passive protection against many diseases, including gastrointestinal infections [45]. Oral immunisation of mice with *H. pylori* bacterial sonicates, along with cholera toxin, results in the induction of specific IgG-secreting cells in the gastric mucosa and the proliferation of such antibody-secreting cells correlates with both the presence of IgG in gastric secretions and protection against *H. felis* infection [46]. Furthermore, administration of the GroES homologue of *H. pylori* increased the production of specific anti-*H. pylori* IgG1 in mice and concurrently protected against experimental *H. felis* infection [47]. Thus, induction of a strong IgG response to *H. pylori* antigens appears to be a feasible strategy in the development of vaccines to protect against *H. pylori* infection.

In summary, this report describes the use of alkaline phosphatase fusion technology to identify a number of putative exported proteins of *H. pylori*, one of which was shown to encode a highly immunogenic 18-kDa protein. The organisation of the amino-acid sequence is consistent with an exported protein with regions of hydrophobicity in the C-terminal domain that could be involved in membrane anchorage of the protein [48]. The 18-kDa protein was frequently recognised by antibodies in the sera of patients infected with *H. pylori*, but showed only a weak reaction with two negative patients. The finding that sera from the majority of patients positive for *H. pylori* infection recognise the 18-kDa protein described in this paper indicates that it is expressed *in vivo*, is probably surface-exposed and is, therefore, worth investigating as a possible candidate for inclusion in a subunit vaccine. Further investigations are currently under way to assess its ability to produce an immune response that may protect against *H. pylori* infection in a murine animal model.

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