Characterization of the low-pH responses of *Helicobacter pylori* using genomic DNA arrays

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***Helicobacter pylori* is unique among bacterial pathogens in its ability to persist in the acidic environment of the human stomach. To identify *H. pylori* genes responsive to low pH, the authors assembled a high-density array of PCR-amplified random genomic DNA. Hybridization of radiolabelled cDNA probes, prepared using total RNA from bacteria exposed to buffer at either pH 4.0 or pH 7.0, allowed both qualitative and quantitative information on differential gene expression to be obtained. A previously described low-pH-induced gene, *cagA*, was identified together with several novel genes that may have relevance to the survival and persistence of *H. pylori* in the gastric environment. These include genes encoding enzymes involved in LPS and phospholipid synthesis and *secF*, encoding a component of the protein export machinery. A hypothetical protein unique to *H. pylori* (HP0681) was also found to be acid induced. Genes down-regulated at pH 4.0 include those encoding a sugar nucleotide biosynthesis protein, a flagellar protein and an outer-membrane protein. Differential gene expression was confirmed by total RNA slot-blot hybridization.**

Keywords: acid-induced gene expression, genomic DNA array, pathogenicity

INTRODUCTION

Helicobacter pylori is unique among bacterial pathogens in that it can persist in the acidic environment of the human stomach. Although most of the *H. pylori* population in the stomach is found in the mucous layer close to the gastric epithelial cell surface where the pH is thought to be neutral (Thomsen *et al.*, 1990), the bacterium is able to tolerate extreme acidity in the gastric lumen following ingestion and is likely to encounter gastric acid periodically during the course of infection. *H. pylori* produces large quantities of urease, which neutralizes the acidic environment locally around the organism by hydrolysis of urea to ammonia. *In vitro*, urease is required for survival at pH 4.0 and below, whereas at higher acidic pH, mechanisms independent of urease are protective (see McGowan *et al.*, 1996). Further evidence of other specialized acid defence mechanisms is provided by the existence of urease-positive, acid-sensitive mutants (Bijlsma *et al.*, 1998).

To investigate these urease-independent mechanisms, McGowan and co-workers used subtractive RNA hybridization and identified a gene which was induced at pH 4.0 and involved in LPS O-antigen biosynthesis.

Mutation of this gene resulted in increased acid sensitivity, suggesting that structural changes to LPS may be an important acid-induced adaptation (McGowan *et al.*, 1998). Proteome analysis of *H. pylori* has shown that decreasing the pH of the growth medium from pH 7.0 to 5.0 reduces the amount of VacA and HtrA, a vacuolating cytotoxin and a serine protease, respectively, possibly as a result of increased secretion of these proteins (Jungblut *et al.*, 2000).

Other acid-induced genes of *H. pylori* include *cagA*, encoding the cytotoxin-associated protein of unknown function, which is induced at pH 6.0 (Karita *et al.*, 1996), and HSP70 (DnaK), induced and surface-expressed following brief exposure to pH 2.0. Induction of HSP70 results in a change in host receptor binding specificity and this may provide a survival advantage at low pH (Huesca *et al.*, 1998).

The aim of our study was to identify genes differentially expressed following exposure to pH 4.0 that may potentially have a role in the pathogenesis of infection. To achieve this we assembled a high-density array of PCR-amplified random genomic DNA derived from a library of strain NCTC 11637 (Clayton *et al.*, 1995).

Radiolabelled cDNA probes were prepared from total RNA of bacteria exposed to either pH 4.0 or pH 7.0 and hybridized to the genomic DNA array. This system, used previously to study competence gene expression in *Streptococcus pneumoniae* (Rimini *et al.*, 2000), allowed us to identify *H. pylori* genes which are up- and down-regulated in response to an acidic environment. Amongst the differentially expressed genes identified by array hybridization, those having a possible role in pathogenesis were further investigated by total RNA slot-blot hybridization to confirm induction or repression by low pH.

METHODS

Bacterial strains and culture conditions. *H. pylori* strain 26695 (Tomb *et al.*, 1997) was grown on *Helicobacter* selective agar, consisting of Blood Agar Base no. 2 (Oxoid) supplemented with 7% lysed defibrinated horse blood (Sigma-Aldrich) and Dent's selective supplement (Oxoid) in a microaerobic atmosphere at 37 °C. A microaerobic atmosphere was generated either by using a CampyGen sachet (Oxoid) in a gas pack jar or by incubation in an incubator equilibrated with 10% CO₂, 85% N₂ and 5% O₂. For exposure to low pH, strain 26695 was inoculated from 48 h agar cultures into brain heart infusion (BHI) broth (Oxoid) containing 10% fetal calf serum, and incubated overnight on a rotary shaker (150 r.p.m.) in a microaerobic atmosphere at 37 °C.

RNA preparation. To isolate total RNA from bacteria exposed to low pH, *H. pylori* 26695 was incubated for 30 min either in citrate buffer at pH 4.0 or in phosphate-buffered saline (pH 7.0). Bacterial cells were harvested by centrifugation at 4000 r.p.m. for 10 min at 20 °C, and total RNA purified using the Ribolyser-Blue kit (Hybaid). The quality of the RNA was evaluated on 0.7% non-denaturing agarose gels and concentration determined by absorbance at 260 nm. Genomic DNA was eliminated by digestion with DNase I (Life Technologies) and this was confirmed by *ureB*-specific primer PCR (Table 1), sensitive to DNA concentrations of 1 µg.

Preparation of complex cDNA probe. Radiolabelled single-stranded cDNA was prepared by reverse transcription of total RNA isolated from bacteria exposed to either pH 4.0 or pH 7.0. The reaction, which consisted of 12.5 µg total RNA, 5 ng random hexamers (Amersham Pharmacia), 5 µl [³²P]dCTP [3000 Ci mmol⁻¹ (111 TBq mmol⁻¹; Amersham Pharmacia), 2.5 µl 0.1 M dithiothreitol, 1.25 µl of a mixture of 1 mM dATP, dGTP, dTTP and 0.01 mM dCTP, 10 U RNase inhibitor (Boehringer Mannheim) and 250 U SuperScript II reverse transcriptase (Life Technologies), in a total volume of 25 µl, was incubated at 42 °C for 90 min. Following inactivation of reverse transcriptase, cDNA probes were purified using Sephadex G-50 spin columns (Amersham Pharmacia). To examine the quality and length of complex probes, aliquots of each probe were analysed on 6% polyacrylamide-TBE gels (Novex Electrophoresis) and visualized using a Molecular Dynamics phosphor imager (Storm 860, Amersham Pharmacia). To prevent masking of neighbouring spots by rRNA and tRNA hybridization, 30 µg each of unlabelled *Escherichia coli* 16S 23S rRNA and tRNA (Sigma-Aldrich) was added to the probes to block hybridization to RNA clones on the array. A luciferase cDNA probe was prepared by oligo-dT priming using 1 µg luciferase mRNA (Promega) and Superscript II reverse transcriptase (Life Technologies) as previously described (Kittler *et al.*, 2000).

Preparation of high-density genomic DNA array. A pBluescript genomic library of *H. pylori* NCTC 11637 (consisting of 3354 clones of mean insert size 1.0 kb) (Clayton *et al.*, 1995) was used as template for 96-well PCR amplification using *Taq* polymerase (Perkin Elmer), *Pfu* polymerase (Stratagene) and M13 forward and reverse primers (Amersham Pharmacia) in 50 µl containing 0.2 mM dNTPs, and 1 × Promega PCR buffer. The M13 forward and reverse primers which flank a T7 primer binding site were selected in order to amplify a common segment of vector DNA from each clone. The PCR consisted of 30 cycles of 1 min of denaturation at 94 °C, 30 s of annealing at 50 °C, and 2 min of extension at 72 °C. One column from each 96-well plate was analysed by agarose gel electrophoresis to determine the success rate of amplification. Products were transferred to 384-well plates and arrayed in duplicate onto positively charged 11 × 15 cm nylon membranes (Boehringer Mannheim) using an automated 384-pin gridding device (Genetix Q-bot). The 384-well plates containing the PCR products were retained for sequencing. The membranes were denatured (0.5 M NaOH, 100 mM NaCl), neutralized (1 M Tris pH 7.2, 100 mM NaCl) and UV cross-linked using a Stratalinker (Stratagene). The luciferase gene of *Coleoptera* was amplified by PCR and gridded at determined positions on the array to allow orientation of the hybridized image. To determine the variability of robotic arraying of PCR products, duplicate arrays were hybridized with a T7 oligonucleotide probe internal to the PCR products. These experiments showed that there was less than 5% variation in the amount of DNA spotted at a particular coordinate between duplicate arrays.

End-labelling of T7 oligonucleotide with [³²P]dATP. T7 primer (40 pmol; Amersham Pharmacia) was end-labelled with [³²P]dATP in a 50 µl reaction containing 5 µl [³²P]dATP (3000 Ci mmol⁻¹; 111 TBq mmol⁻¹) and 20 U polynucleotide kinase (New England Biolabs). The reaction was incubated at 37 °C for 1 h and unincorporated nucleotides removed using Sephadex G25 spin columns (Amersham Pharmacia).

Hybridization. Six copies of the genomic DNA array were generated: four for duplicate hybridizations with the pH 4.0 and pH 7.0 cDNA probes, and two for duplicate hybridizations with a T7 oligonucleotide probe internal to all PCR products in order to determine the relative amounts of DNA present on the array. Prehybridization and hybridization were carried out at 45 °C in DIG Easy Hyb solution (Boehringer Mannheim) in a rotisserie oven. Prehybridization was carried out for a minimum of 30 min and hybridization for 72 h. Membranes hybridized with cDNA probes were washed three times for 15 min using 2 × SSC, 0.1% SDS at 60 °C. Membranes hybridized with the T7 oligonucleotide were washed three times for 15 min with 6 × SSC, 0.5% SDS at 45 °C. All membranes were exposed to a phosphor storage screen for 48 h and image data were captured on the Molecular Dynamics phosphor imager.

Image analysis. Image data files were analysed and compared using the Glaxo Wellcome proprietary dGENT software package. The median spot intensity of each image was used to normalize for differences in specific activity between probes. Only spots showing at least a 1.3-fold difference in hybridization intensity between the pH 4.0 and pH 7.0 cDNA probes and no significant difference between T7 oligonucleotide hybridization intensities were reported. In addition, only differential hybridizing spots showing a minimum 95% consistency between duplicates were considered significant.

Identification of differentially expressed spots. Differentially expressed spots were identified by sequencing of the cor-

Table 1. Oligonucleotide PCR primers

Primer	Sequence (5'–3')	Target gene TIGR HP no.*
EA20F	ATGAAAAAGATTAGCAGAAAAGAA	<i>ureB</i> , HP0072
EA20R	CTAGAAAATGCTAAAGAGTTGCGC	<i>ureB</i> , HP0072
EA41F2	TTGAAAGAGTTTGCTTATAG	HP0366
EA41R2	CTCTCAAAAGTTTTTAAACGC	HP0366
EA42F	ACGAAAGAGAACTTGAATGGC	HP0681
EA42R	CACCCCCATTTAGCGCAAAGC	HP0681
EA43F	TTAAGATCTCTGTGGATAACG	HP1052
EA43R	TTAACCAACATGGAATTGAGC	HP1052
HP1459F	CAAGCACTCCAGAAGAGAGGC	HP1459
HP1459R	GCAACTCATTGTATTCTTGGC	HP1459
EA46F	ACAAATATGATAGAGACTTGG	HP1588
EA46R	CGACTTCTTATCTCCATTGGC	HP1588
EA50F	TAAAGATCCGAATGTGTTGCG	HP0871
EA50R	ATAAGACAAAGGAGTTATCGC	HP0871
RC5F	ATTATTCAAACGAACTAG	HP1549
RC5R	ACTTGACCGCTCTATAC	HP1549
EA60F	ACGCCGCGTGGAGGATG	16S rRNA
EA60R	GCTCCCCACGCTTTCGC	16S rRNA

* Refers to the genome sequence of *H. pylori* 26695 (Tomb *et al.*, 1997); TIGR, The Institute for Genomic Research.

responding PCR product and subsequent database searches using BLAST tools (Altschul *et al.*, 1997). Sequencing was performed with T7 or T3 primers (Amersham Pharmacia) using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) as recommended by the supplier. Samples were analysed on an ABI model 377 automated sequencer.

RNA slot-blot hybridizations. For confirmation of differential gene expression by slot-blot hybridization, independent RNA samples were prepared as described above. Samples (5–10 µg) of total RNA were mixed with 3 vols denaturation solution and heated at 65 °C for 15 min. After cooling on ice, 2 vols ice-cold 20 × SSC was added and samples applied to Hybond-N (Amersham Pharmacia) using a slot-blot filtration manifold (Amersham Pharmacia). Membranes were pre-wet in 10 × SSC and slots washed with 0.5 ml 10 × SSC before and after addition of sample. Membranes were blotted dry and baked at 80 °C for 2 h. DNA probes were generated by PCR using primers (Table 1) based on DNA sequences obtained from the TIGR website (<http://www.tigr.org/tdb/CMR/ghp/htmls/SplashPage.html>). Probes were labelled with [α -³²P]dCTP (3000 Ci mmol⁻¹; 111 TBq mmol⁻¹) using a Rediprime random prime labelling kit (Amersham Pharmacia) and hybridized with the blots at 42 °C for 16 h in buffer containing formamide (AMS Biotechnology). Membranes were washed twice with 2 × SSC, 0.1% SDS at 42 °C and then with 0.1 × SSC, 0.1% SDS at 55 °C and analysed using the Molecular Dynamics phosphor imager.

RESULTS

Construction of the *H. pylori* genomic DNA array

In order to generate a *H. pylori* array, a pBluescript genomic DNA library of strain NCTC 11637 consisting of 3354 clones with a mean insert size of 1.0 kb (Clayton *et al.*, 1995) was amplified by 96-well PCR using vector-

specific primers. This library was selected because the 1.0 kb inserts correspond approximately to the size of a *H. pylori* ORF (Tomb *et al.*, 1997). Agarose gel electrophoresis of one column from each 96-well plate indicated that approximately 81% of the amplifications were successful. As the *H. pylori* genome is 1.67 Mb, the generated array was expected to provide approximately 1.6-fold coverage of the genome. A series of hybridizations of duplicate arrays with a T7 oligonucleotide probe internal to each PCR product showed that there was at least 95% consistency between arrays in the amount of PCR product spotted.

Genes up-regulated at low pH

An image showing genomic DNA arrays hybridized with pH 4.0 and pH 7.0 cDNA probes and analysed using proprietary software is shown in Fig. 1. Comparison of the hybridization patterns of the pH 4.0 and pH 7.0 cDNA probes identified 25 clones showing an increase in signal intensity of 1.3–2.2 fold with the pH 4.0 probe compared to the pH 7.0 probe. Analysis of duplicate arrays hybridized with the T7 oligonucleotide showed that these differences were not due to differences in the quantity of DNA. The PCR-amplified genomic DNA fragments corresponding to these 25 clones were recovered from the stored 384-well plates and sequenced. Four PCR products failed to give meaningful sequence information and were not progressed further. Since a PCR-amplified random genomic DNA library was used for array construction, it was possible to have multiple detections of the same clone. BLAST analysis of the sequences of the 21 remaining PCR products showed

Fig. 1. Close-up images of duplicate genomic DNA arrays hybridized with (a) complex cDNA probe derived from pH 4.0 RNA and (b) T7 oligonucleotide probe. The intense hybridizing spots in (a) contain rRNA genes.

that four genes were represented more than once (three genes were present in duplicate PCR products and one gene in triplicate). One PCR product contained the known acid-induced gene *cagA* (Karita *et al.*, 1996). As our primary interest is bacterial pathogenesis, of the 16 remaining genes we chose to confirm acid-induction of only those genes we considered likely to play a role in the pathogenesis of *H. pylori* infection. The inclusion or exclusion of putative acid-induced genes from further analysis was based primarily on sequence similarity between the *H. pylori* gene product and known pathogenicity factors present in the databases. Total RNA slot-blot hybridizations were performed using PCR-amplified DNA probes for 11 of the 16 putative acid-induced genes. These hybridizations were carried out using fresh RNA samples isolated in independent low-pH exposure experiments. Analysis of these hybridizations confirmed pH 4.0-induced upregulation of 7 of the 11 genes tested. These genes are listed in Table 2.

HP0681, induced at least 2.2-fold at pH 4.0, is considered unique to *H. pylori* as it exhibits no similarity with sequences in the public databases. There is a paralogue of HP0681 present in both sequenced strains: HP1289 in strain 26695 (Tomb *et al.*, 1997; Alm *et al.*, 1999). These paralogues share 69% identity in a 157 amino acid overlap. Both HP0681 and HP1289 are preceded by members of another two-member paralogous family (HP0682 and HP1288), also unique to *H. pylori* and containing predicted N-terminal signal peptides for Sec-dependent secretion (Tomb *et al.*, 1997). The relatively

short HP0681–0682 and HP1288–1289 intergenic distances suggests that these gene pairs may be co-transcribed.

HP1052, the expression of which is increased 1.6-fold at pH 4.0 (Table 2), is an orthologue of *envA* (*lpxC*) of *E. coli* encoding UDP-3-O-acyl *N*-acetylglucosamine deacetylase. This enzyme, ubiquitous in Gram-negative bacteria, catalyses the second step of lipid A biosynthesis and is a point of regulation for LPS biosynthesis (Sorensen *et al.*, 1996). Analysis of the predicted amino acid sequence of HP1052 using BLASTP (Altschul *et al.*, 1997) identifies the putative UDP-3-O-acyl *N*-acetylglucosamine deacetylase of *Campylobacter jejuni* as the closest match, with 54% identity in a 294 amino acid overlap. The predicted *H. pylori* enzyme shares 34% identity (in a 297 amino acid overlap) with its counterpart in *Escherichia coli*. The transcript level of HP0871, encoding CDP-diacylglycerol pyrophosphatase, is also increased at pH 4.0. This enzyme is involved in synthesis of phospholipids which are components essential for the structure and function of bacterial membranes.

Two enzymes concerned with ribonucleotide biosynthesis and modification are also upregulated at pH 4.0 (HP1459, HP0919). HP1459 encodes an orthologue of *C. jejuni* pseudouridine synthase, required for synthesis of the modified ribonucleotide, pseudouridine, in large-subunit rRNA. The HP0919 gene product shows significant similarity with the carbamoyl-phosphate synthase enzyme of *C. jejuni* involved in the biosynthesis of pyrimidine ribonucleotides.

Table 2. Genes up- or down-regulated at pH 4.0, closest orthologue, and putative function

Gene no. in strain		Description of <i>H. pylori</i> gene product or closest orthologue (E-value from BLASTX analysis)	Organism	Function/other information	Accession no.‡	Induction ratio	
						Array§	Slot-blot
26695*	J99†						
Up-regulated							
0681	0622	Hypothetical 19.2 kDa protein (0.0)	<i>H. pylori</i>	Unknown function	A64605	2.2	4.4
0919	0853	Carbamoyl-phosphate synthase large chain (0.0)	<i>C. jejuni</i>	Carbamoyl-phosphate synthase; pyrimidine ribonucleotide biosynthesis	AL139074.2	1.9	1.8
1588	1494	Conserved hypothetical 28.4 kDa protein (9e-21)	<i>E. coli</i>	Unknown function; paralogues HP1587, 1589	C64721	1.8	3.2
1459	1352	Putative ribosomal pseudouridine synthase b (<i>rluB</i>) (2e-68)	<i>C. jejuni</i>	Synthesis of pseudouridine from uracil in 23S rRNA	AL139079.2	1.8	1.4
1052	0373	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase (<i>lpxC</i>) (2e-91)	<i>C. jejuni</i>	Lipid A biosynthesis	AL139074.2	1.6	1.8
0871	0805	CDP-diacylglycerol pyrophosphatase (<i>cdh</i>) (1e-102)	<i>E. coli</i>	Phospholipid biosynthesis	P06282	1.4	1.2
0547	0495	Cytotoxin-associated immunodominant antigen (<i>cagA</i>) (0.0)	<i>H. pylori</i>	Unknown function, located on <i>cag</i> pathogenicity island	P55746	1.4	ND
1549	1450	Probable protein-export membrane protein (<i>secF</i>) (8e-63)	<i>C. jejuni</i>	Protein secretion	AL139077.2	1.3	2.0
Down-regulated							
0366	1015	Probable sugar nucleotide biosynthesis protein (7e-87)	<i>C. jejuni</i>	?Protein glycosylation	AL139078.2	-6.2	-3.9
0753	0690	Flagellar protein (<i>flis</i>) (4e-39)	<i>C. jejuni</i>	?Chaperone for flagellar export	AL139075.2	-2.0	-1.2
0229	0214	Outer-membrane protein (<i>bopA</i>) (0.0)	<i>H. pylori</i>	Porin	E64548	-1.9	-2.7

* According to the genome sequence of strain 26695 (HP prefixes omitted) (Tomb *et al.*, 1997).

† According to the genome sequence of strain J99 (HP prefixes omitted) (Alm *et al.*, 1999).

‡ Accession numbers refer to the GenBank/EMBL, SWISS-PROT or PIR databases.

§ Induction ratios expressed as maximum fold increase in hybridization intensity between duplicate spots on genomic DNA arrays hybridized with pH 4.0 cDNA probe compared to that hybridized with pH 7.0 cDNA probe.

|| Induction ratios expressed as maximum fold increase in hybridization intensity between pH 4.0 and pH 7.0 total RNA slot-blots hybridized with gene-specific DNA probe; ND, not done.

Also identified as acid-induced was *secF* (HP1549), encoding a component of the cytoplasmic membrane protein secretion apparatus. This gene was induced 1.3-fold at pH 4.0 compared to pH 7.0. SecF is one of six components of the Sec-dependent protein export pathway in *E. coli*. The *H. pylori* genome contains four of the six *E. coli* components (Tomb *et al.*, 1997), together with orthologues of the two leader peptidases required in *E. coli* for cleavage of signal sequences from exported proteins. The short intergenic distance between *secF* and *secD* in both sequenced strains of *H. pylori* (Tomb *et al.*, 1997; Alm *et al.*, 1999) suggests that, in common with *E. coli*, these genes may be co-transcribed. SecF and SecD are thought to act at a similar step in protein secretion but their precise function is unclear.

The other acid-induced gene identified in this study, HP1588, encodes a protein with an orthologue in *E. coli*, the function of which is unknown. Interestingly, this gene is a member of a paralogous family which includes the adjacent gene HP1587. In our array hybridizations, HP1587 was also identified as acid-induced and showed a similar induction ratio to that of HP1588, suggesting that these genes may be co-transcribed.

Genes down-regulated at low pH

Our analysis also identified 34 differentially hybridizing spots which showed significantly decreased signal intensity (1.4–6.2 fold) at pH 4.0 compared to pH 7.0. Sequence analysis of the 34 PCR-amplified genomic DNA fragments identified four encoding 23S–5S rRNA,

indicating that addition of unlabelled rRNA to the probe had been only partially successful in blocking hybridization to rRNA clones. Of the 30 remaining PCR products, three genes were multiply represented. Taking into account this redundancy, we identified 14 genes putatively down-regulated at pH 4.0. Of the seven of these which were considered to have a likely role in pathogenesis and were tested by total RNA slot-blotting, three were confirmed using RNA extracted in independent low-pH experiments (Fig. 2, Table 2). The four other genes analysed by slot-blot hybridization showed no change in expression level at pH 4.0 (not shown).

The expression of HP0366, encoding a probable sugar nucleotide biosynthesis protein, is down-regulated at pH 4.0. The predicted amino acid sequence of this gene shows significant similarity to proteins involved in polysaccharide biosynthesis. Another orthologue, FlmB of *Caulobacter crescentus*, appears to be involved in the post-translational modification of flagellins, essential for proper assembly of the flagellar filament (Leclerc *et al.*, 1998). Studies are under way to determine the function of HP0366 in *H. pylori*.

Two other genes were found to be down-regulated at pH 4.0: HP0753, encoding a flagellar protein, and HP0229, which encodes the HopA porin. This porin is a member of a closely related family of porins (HopA to HopD) whose precise function is unknown (Exner *et al.*, 1995). Although a change in expression of an outer-membrane protein in response to environmental conditions is not surprising given its proximity to the

Fig. 2. Total RNA slot-blot hybridization of acid-responsive genes: (a) genes up-regulated at pH 4.0; (b) genes down-regulated at pH 4.0.

external milieu, the effect of down-regulation of HopA on cell physiology requires further investigation.

DISCUSSION

By hybridization of total RNA-derived cDNA probes to a random genomic DNA array, we were able to identify genes of *H. pylori* differentially expressed at pH 4.0, compared to pH 7.0. To construct our array we used as template for PCR a random genomic library of *H. pylori* NCTC 11637. This library was constructed for an independent genome sequencing project before publication of the genome sequence of strain 26695 and it was our intention to use this resource to derive functional information.

Among the up-regulated genes detected in our experiment was *cagA*, the immunodominant cytotoxin-associated gene, known to be inducible at acidic pH (Karita *et al.*, 1996). Karita and co-workers reported a 1.7-fold increase in *cagA* transcription at pH 6.0 compared to pH 7.0. We detected a similar increase in *cagA* transcription at pH 4.0.

Several other acid-inducible genes were identified, including those encoding proteins involved in the structure and synthesis of cell surface components (HP1052, HP0871) and enzymes involved in ribonucleotide biosynthesis and modification (HP0919, HP1459). Other novel acid-induced genes identified in this work include *secF* (HP1549), encoding a component of the protein secretion machinery, and HP0681, a gene unique to *H. pylori*.

The mRNA induction ratios we observed following exposure of *H. pylori* to pH 4.0 were relatively low compared to those reported in other studies of differential gene expression in bacteria. For example, a 20-fold increase in transcription of one gene was observed during competence development in *Streptococcus pneumoniae* (Rimini *et al.*, 2000), and induction ratios of several hundred fold were reported in a study of global gene expression in *B. subtilis* grown in anaerobic conditions (Ye *et al.*, 2000). In our study, the fact that several of the genes showing low fold changes in gene expression using the array could be confirmed using conventional total RNA slot-blot hybridization demonstrates the sensitivity of our system. It should also be emphasized that the slot-blot hybridizations used RNA samples prepared in independent acid-exposure experiments, demonstrating that our data are reproducible. Our data also show that the *in vitro* response of *H. pylori* to pH 4.0 comprises a subtle change in the pattern of gene expression. These subtle changes may still be significant *in vivo*: the *atrB* gene of *Salmonella enterica* serovar Typhimurium, an essential component of the acid tolerance response, is induced only 1.4-fold at pH 5.8 *in vitro* (Foster & Bearson, 1994).

Of the 18 putative acid-responsive genes identified in the array studies, eight failed to be confirmed by RNA slot-blot hybridization. This may be due to the relatively low sensitivity of the Northern hybridization technique or because the genes concerned produce short-half-life mRNA not consistently isolated in all RNA samples. We used total RNA slot-blot hybridization as it is considered

the standard for mRNA quantitation although a more sensitive method such as RT-PCR may have improved our confirmation rate.

For those genes which were confirmed as differentially expressed, the induction ratios determined by densitometric analysis of the slot-blot hybridizations correlated well in most cases with those calculated using the array. Quantitative differences were apparent, however, between the array and slot-blot data for some genes (e.g. HP0681, HP1588). This may result from the fact that independent RNA samples were used, perhaps reflecting slight differences in the exposure time between individual experiments. An alternative reason for the difference is that different probe types were used in the two experiments, an explanation given previously in studies where discrepancies of much greater magnitude (10-fold) were apparent, even between experiments using the same RNA sample (Rimini *et al.*, 2000).

Our finding that the lipid A biosynthetic enzyme UDP-3-O-acyl *N*-acetylglucosamine deacetylase (HP1052) is acid-induced is consistent with previously published data which demonstrated that the LPS biosynthesis enzyme WbcJ, involved in O-antigen production in *H. pylori*, is induced at pH 4.0 (McGowan *et al.*, 1998). We did not detect induction of *wbcJ* in our studies, either using the array or by DNA probe hybridization to pH 4.0 RNA on slot-blots. The reason for this is unclear but probably reflects differences in the *in vitro* conditions or duration of the acid exposure. The fact that an isogenic *wbcJ* mutant shows increased sensitivity to acid stress does suggest, however, that the ability to alter the structure of LPS is an important acid survival mechanism. It is suggested that LPS may contribute to the barrier function of the outer membrane, reducing proton influx and thus promoting maintenance of a favourable periplasmic and intracellular pH (McGowan *et al.*, 1998). Indeed, the phenotype of an *E. coli* UDP-3-O-acyl *N*-acetylglucosamine deacetylase (*envA*) null mutant includes increased sensitivity to antibiotics as a result of hyperpermeability (Grunstrom *et al.*, 1980). The levels of the *E. coli* enzyme increase during periods of lipid A depletion, suggesting that control of expression of the deacetylase regulates the rate of lipid A biosynthesis (Sorensen *et al.*, 1996). Although this control is exerted at the level of translation in *E. coli*, it is feasible that transcriptional control of UDP-3-O-acyl *N*-acetylglucosamine deacetylase may regulate LPS biosynthesis in *H. pylori*. Given that the *E. coli* enzyme is required for the normal barrier function of the outer membrane and that expression of the enzyme is regulated in response to levels of lipid A, it may be that transcriptional up-regulation of this enzyme in *H. pylori* is a direct response to acidic pH and reflects a need to maintain an effective barrier against proton influx. Indeed, any acid-induced changes in gene expression with the potential to alter the surface properties of the bacterium are of interest as these may be relevant to its ability to maintain a favourable intracellular pH during exposure to gastric acid. The induction of the phospholipid biosynthetic enzyme CDP-diacylglycerol pyrophosphatase at low pH

may also reflect a need to maintain an effective membrane barrier. Induction of LPS and phospholipid biosynthetic enzymes was reported in *S. enterica* serovar Typhimurium following exposure to an antimicrobial protein from neutrophils (Qi *et al.*, 1995) and perhaps represents a general response to stress conditions.

Induction of SecF may also be an adaptive response to acidic conditions. Co-overexpression of SecF and SecD in *E. coli* results in increased secretion efficiency, suggesting that these proteins could be up-regulated at low pH in *H. pylori* to enhance secretion of a protective extracellular or cell envelope protein.

The identification of a ribonucleotide-modifying enzyme induced at low pH is also interesting as it is suggested that modified nucleotides such as pseudouridine, found clustered in or close to functionally important regions of the ribosome, may be important for regulating the activity of ribosomes (see Bjork, 1996). It is tempting to speculate that in *H. pylori*, ribosome modification resulting from increased expression of pseudouridine synthase results in an alteration in ribosome activity favouring synthesis of proteins required for survival in acid.

The identification of a *H. pylori*-specific acid-induced gene (HP0681) is particularly interesting as such genes could contribute to the unique ability of the bacterium to survive exposure to gastric acid. The association of HP0681 with a predicted Sec-dependent secreted protein is intriguing given that *secF* is also acid-induced and the association between secreted proteins (e.g. CagA; Covacci *et al.*, 1993) and virulence (Finlay & Falkow, 1997). The studies reported here were designed to identify possible acid-responsive genes which may play a role in survival *in vivo*. Further studies are under way to investigate the function of HP0681 and specifically its role in survival at low pH.

In addition to genes induced by acidic conditions, we identified three genes whose expression was decreased at pH 4.0. Their putative translation products were a putative sugar nucleotide biosynthesis protein (HP0366), a flagellar protein (HP0753) and the HopA porin (HP0229). The significance of down-regulation of these genes requires further investigation.

In summary, we have studied *H. pylori* gene expression in response to an environment of low pH by hybridization of total RNA-derived cDNA probes to high-density genomic DNA arrays. A total of 11 acid-responsive genes were identified, including eight which showed increased expression at pH 4.0 compared with pH 7.0, and three which showed decreased expression. These genes are worthy of further study as they may be relevant to the survival and persistence of *H. pylori* *in vivo*.

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