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Transcriptional profiling of *Helicobacter pylori* Fur- and iron-regulated gene expression

Florian D. Ernst,[1,2,3] Stefan Bereswill,[2]† Barbara Waidner,[2] Jeroen Stoof,[1] Ulrike Mäder,[3]‡ Johannes G. Kusters,[1] Ernst J. Kuipers,[1] Manfred Kist,[2] Arnoud H. M. van Vliet[1] and Georg Homuth[3]‡

Correspondence
Arnoud H. M. van Vliet
a.h.m.vanvliet@erasmusmc.nl

[1]Department of Gastroenterology and Hepatology, Erasmus MC – University Medical Center Rotterdam, Dijkzigt L-455, Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands

[2]Department of Microbiology and Hygiene, Institute of Medical Microbiology and Hygiene, University Hospital of Freiburg, D-79140 Freiburg, Germany

[3]Institut für Mikrobiologie und Molekularbiologie, Ernst-Moritz-Arndt-Universität Greifswald, D-17487 Greifswald, Germany

Intracellular iron homeostasis is a necessity for almost all living organisms, since both iron restriction and iron overload can result in cell death. The ferric uptake regulator protein, Fur, controls iron homeostasis in most Gram-negative bacteria. In the human gastric pathogen *Helicobacter pylori*, Fur is thought to have acquired extra functions to compensate for the relative paucity of regulatory genes. To identify *H. pylori* genes regulated by iron and Fur, we used DNA array-based transcriptional profiling with RNA isolated from *H. pylori* 26695 wild-type and *fur* mutant cells grown in iron-restricted and iron-replete conditions. Sixteen genes encoding proteins involved in metal metabolism, nitrogen metabolism, motility, cell wall synthesis and cofactor synthesis displayed iron-dependent Fur-repressed expression. Conversely, 16 genes encoding proteins involved in iron storage, respiration, energy metabolism, chemotaxis, and oxygen scavenging displayed iron-induced Fur-dependent expression. Several Fur-regulated genes have been previously shown to be essential for acid resistance or gastric colonization in animal models, such as those encoding the hydrogenase and superoxide dismutase enzymes. Overall, there was a partial overlap between the sets of genes regulated by Fur and those previously identified as growth-phase, iron or acid regulated. Regulatory patterns were confirmed for five selected genes using Northern hybridization. In conclusion, *H. pylori* Fur is a versatile regulator involved in many pathways essential for gastric colonization. These findings further delineate the central role of Fur in regulating the unique capacity of *H. pylori* to colonize the human stomach.

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INTRODUCTION

Infection with the human pathogen *Helicobacter pylori* results in persistent gastritis which can develop into peptic ulcer disease and adenocarcinoma of the distal stomach (Blaser & Berg, 2001). Approximately half of the world's human population is colonized by *H. pylori*, leading to significant morbidity and mortality. For these reasons, the infection is considered an important public health problem with serious economic consequences. The only known habitat of *H. pylori* is the mucus layer overlaying the epithelial cells in the human stomach. Colonization of this acidic and variable environmental niche has necessitated the development of adaptive stress responses by *H. pylori*.

In the gastric environment, changes in iron availability represent one of the important environmental stimuli for *H. pylori*. Iron is an essential element for almost all living organisms, as it is a cofactor of many enzymes and acts as a catalyst in electron transport processes. However, in the presence of oxygen, iron potentiates the formation of toxic oxygen radicals. Therefore regulation of intracellular iron homeostasis, as mediated by the ferric uptake regulator (Fur) protein, is of critical importance (Andrews *et al.*, 2003). Regulation of gene expression via Fur has been extensively investigated in several Gram-negative and Gram-positive bacteria, where it is involved in the regulation of

†Present address: Institut für Mikrobiologie und Hygiene, Campus Charité Mitte, Berlin, Germany.

‡Present address: School of Cell and Molecular Biosciences, Newcastle University Medical School, Newcastle-upon-Tyne, UK.

The complete MIAME information for the identification of Fur- and iron-regulated gene expression in the *Helicobacter pylori* dataset is shown in Supplementary Table S1, available online as supplementary data with the online version of this paper at http://mic.sgmjournals.org.

many cellular processes, including iron metabolism, oxidative stress defence and central intermediary metabolism (Andrews *et al.*, 2003; Hantke, 2001). However, while the absence of Fur affects many cellular processes, several of the regulatory phenomena described mostly in *E. coli* are only indirectly affected by Fur (Masse & Gottesman, 2002).

Fur is a transcriptional repressor protein, which displays iron-dependent binding to conserved DNA sequences (Fur boxes) located in the promoters of iron-regulated genes (Hantke, 2001). In most bacteria, including *H. pylori*, the iron-complexed form of Fur binds to promoters of iron-uptake genes, thus repressing iron uptake in iron-replete conditions (Delany *et al.*, 2001b; van Vliet *et al.*, 2002a). However, *H. pylori* Fur has acquired the thus far unique ability also to bind the *pfr* promoter in its iron-free form, thus repressing expression of iron-storage proteins in iron-restricted conditions (Bereswill *et al.*, 2000; Delany *et al.*, 2001a; Waidner *et al.*, 2002).

The relative paucity of transcriptional regulators in *H. pylori*, combined with the necessity to respond to environmental stresses, may have resulted in *H. pylori* Fur being involved in the regulation of other adaptive responses. Other than regulation of iron metabolism, *H. pylori* Fur has also been implicated in the regulation of acid resistance (Bijlsma *et al.*, 2002; Bury-Mone *et al.*, 2004; van Vliet *et al.*, 2004), nitrogen metabolism (van Vliet *et al.*, 2001, 2003) and oxidative stress resistance (Barnard *et al.*, 2004; Cooksley *et al.*, 2003; Harris *et al.*, 2002). Fur-mediated regulation is also required for gastric colonization by *H. pylori*, as demonstrated in a mouse model of infection (Bury-Mone *et al.*, 2004).

DNA array technology has proved to be a powerful technique for the study of global gene regulation in many organisms (Conway & Schoolnik, 2003), and has also been successfully applied to study alterations in *H. pylori* gene expression (Bury-Mone *et al.*, 2004; Forsyth *et al.*, 2002; Kim *et al.*, 2004; Merrell *et al.*, 2003a, 2003b; Thompson *et al.*, 2003; Wen *et al.*, 2003) and genetic variation between isolates (Israel *et al.*, 2001a; Salama *et al.*, 2000). In this study, we have applied DNA array technology to growth experiments with the well-characterized *H. pylori* reference strain 26695, both to define the *H. pylori* responses to variation in iron availability and to identify new members of the *H. pylori* Fur regulon.

METHODS

Bacterial strains, plasmids, media and growth conditions. The *H. pylori* strains used in this study were reference strain 26695 (Tomb *et al.*, 1997) and its isogenic *fur* mutant (Bijlsma *et al.*, 2002; van Vliet *et al.*, 2002a). *H. pylori* strains were routinely cultured on Dent agar at 37 °C under microaerophilic conditions (10 % CO_2, 5 % O_2 and 85 % N_2). Broth cultures were grown in brucella broth (Difco) supplemented with 3 % newborn calf serum (Gibco) (BBN). Broth cultures were continuously shaken at 40 r.p.m. under microaerophilic conditions. Iron restriction was achieved by supplementing BBN with desferal (deferoxamine mesylate, Sigma) to a final concentration of 20 μM. Iron-replete conditions were achieved by supplementing desferal-treated BBN with ferric chloride (Sigma) to a final concentration of 100 μM (van Vliet *et al.*, 2002a).

Purification and analysis of RNA. Total RNA was isolated using Trizol (Gibco), according to the manufacturer's instructions. The amount of RNA was determined spectrophotometrically. RNA electrophoresis, blotting, hybridization with DIG-labelled RNA probes, and detection of bound probe were carried out as described previously (Homuth *et al.*, 1997). Directly after transfer, the membranes were stained with methylene blue to confirm the integrity of the RNA samples and to confirm loading of equal amounts of RNA based on the relative intensities of the 16S and 23S rRNA (van Vliet *et al.*, 2001). Chemiluminescence was detected using a Lumi-Imager (Roche Diagnostics), and chemiluminographs were quantified using the Lumi-Analyse software package (Roche Diagnostics). The DIG-labelled specific RNA probes were synthesized by *in vitro* transcription using T7 RNA polymerase (Roche Diagnostics), and PCR products were amplified using the primers listed in Table 1.

Table 1. Oligonucleotide primers used in this study

Primer sequences were derived from the *H. pylori* 26695 genome sequence (Tomb *et al.*, 1997). Lowercase letters indicate a 5′ extension with T7 promoter sequence for the creation of an antisense RNA probe.

Primer name	Sequence (5′–3′)
AmiE-F1	AGTAGCAGCCCAGATACTGT
AmiE-R-T7	ctaatacgactcactatagggagaTCGCTACCGCTACATAACAT
HP1432-F1	GGCACACCATGAACAACAAC
HP1432-R-T7	ctaatacgactcactatagggagaTGTTGGTTTTGTTGTTGCGC
Hp0388-F1	TGATGACATGCTGGAGCGAT
HP0388-R-T7	ctaatacgactcactatagggagaTCCACATGCTTAAACCCCAC
SerB-F1	TGACTCCACGCTAGTCAATG
SerB-R-T7	ctaatacgactcactatagggagaGGGCTAAATCAGGCTCATTG
Pfr-F1	AGACATCATTAAGTTGC
Pfr-R-T7	ctaatacgactcactatagggagaAGATTTCCTGCTTTTAG

Synthesis of labelled cDNA for transcriptome analysis. *H. pylori* strain 26695 and its isogenic *fur* mutant were grown in iron-restricted and iron-replete conditions (van Vliet *et al.*, 2002a), and total RNA was isolated from cells grown for 20 h and checked by Northern hybridization using an *amiE*-specific probe (Fig. 1). For annealing of the specific oligonucleotide primers complementary to the mRNAs specified by all *H. pylori* genes, 1 μg total RNA (concentration determined photometrically) was hybridized to 4 μl cDNA primer mix (0·05 pmol μl^{-1}) (Eurogentec) in hybridization buffer (10 mM Tris/HCl, pH 7·9, 1 mM EDTA, 250 mM KCl) in a total volume of 30 μl (1 h, 42 °C). After annealing, 30 μl of reverse transcription premix [12 μl 5× First Strand Buffer (Gibco-BRL), 6 μl 0·1 mM DTT (Gibco-BRL), 2 μl 10 mM dATP, 2 μl 10 mM dGTP, 2 μl 10 mM dTTP, 4·5 μl [α-^{33}P]dCTP (10 μCi μl^{-1}, Amersham Pharmacia), 1·5 μl reverse transcriptase (Superscript II; Gibco-BRL)] was added, and reverse transcription was carried out for 1·5 h at 42 °C. Next, 2 μl 0·5 M EDTA was added to stop all reactions. Alkaline hydrolysis of the RNA was performed by addition of 6 μl 3·0 M NaOH and incubation of the solution for 30 min at 65 °C, followed by 15 min at room temperature. The solution was neutralized with 20 μl 1 M Tris/HCl, pH 8·0, and 6 μl 2N HCl. Finally, the cDNA was precipitated overnight at −20 °C after the addition of 10 μl 3 M sodium acetate, pH 5·2, and 400 μl ethanol. The cDNA was pelleted by centrifugation at 17 600 ***g*** for 15 min at 4 °C, washed with 70 % (v/v) ethanol, dried, and resuspended in 100 μl sterile water. Labelling efficiency was determined by liquid scintillation measurement.

Hybridization of labelled cDNA to DNA macroarrays. *H. pylori* arrays (Eurogentec): nylon membranes carrying PCR products which represented 97 % of all *H. pylori* 26695 and J99 protein-encoding genes (n=1578), were incubated for 10 min in 50 ml saline sodium phosphate EDTA (SSPE) buffer (0·18 M NaCl, 10 mM sodium phosphate, pH 7·7, 1 mM EDTA). Prehybridization was carried out in 10 ml hybridization solution [5× Denhardt solution, 5× SSC, where 1× SSC is 0·15 M NaCl plus 0·015 M sodium citrate, 0·5 % SDS, 100 μg denatured salmon sperm DNA (Sigma) ml^{-1}] for 2 h at 65 °C. Subsequently, hybridization was performed for 20 h at 65 °C in 5 ml hybridization solution containing the labelled cDNA probe which had been boiled for 5 min and rapidly cooled on ice before hybridization. Arrays were washed twice with 200 ml 2× SSC and 0·1 % SDS (5 min at room temperature and 20 min at 65 °C) and once with 200 ml 0·2× SSC and 0·1 % SDS. Finally, arrays were air-dried for 2 min, sealed in plastic bags, and exposed to PhosphorImager screens. The transcriptome analysis was carried out twice, using two independently isolated sets of RNA preparations and two different array batches. Exposed PhosphorImager screens were scanned with a Storm 860 PhosphorImager (Molecular Dynamics) at a resolution of 50 μm and a colour depth of 16 bit. To remove the labelled cDNA from the arrays prior to subsequent hybridizations, the membranes were incubated three times (2, 5 and 60 min) in 300 ml boiling stripping buffer (10 mM Tris/HCl, pH 8·0; 1 mM EDTA; 1 % SDS). Exposure of the arrays after stripping revealed that the complete activity was successfully removed from the membrane. Using this method, it was possible to use the macroarrays five times without significant loss in quality.

Analysis of transcriptome data. For quantification of the hybridization signals and background values, the ArrayVision software (Imaging Research) was used (Eymann *et al.*, 2002). Subsequently, a quality score was calculated for each spot reflecting the ratio between the signal intensity and the background intensity. This quality score was utilized to identify hybridization signals close to the detection limit. Data normalization and data anlysis were done with the GeneSpring software (Silicon Genetics). After background subtraction, normalized intensity values of the individual spots were calculated (median normalization). Only genes specifying signals which significantly exceeded the background signal level (determined by the quality scores) under at least one condition were included in further data analysis. The average of the normalized intensity values

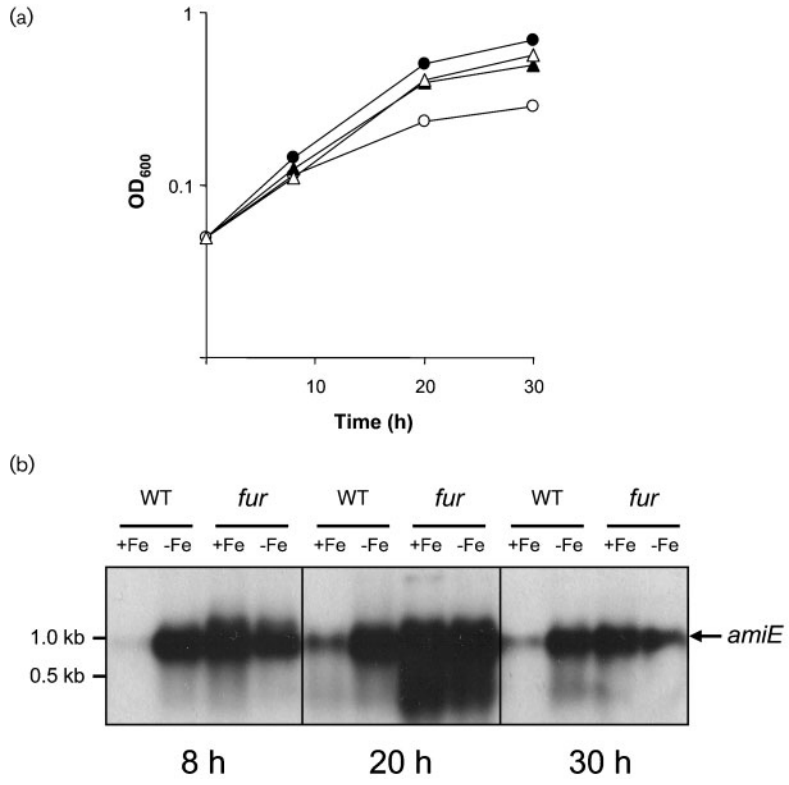

Fig. 1. Selection of the growth phase of cultures of *H. pylori* 26695 wild-type isogenic *fur* mutant for isolation of RNA and subsequent transcriptome analysis. (a) Growth curves of *H. pylori* 26695 wild-type (circles) and *fur* mutant (triangles) grown in iron-restricted (open symbols) and iron-replete conditions (closed symbols). Growth is expressed as OD_{600}. (b) Verification of iron- and Fur-responsive regulation at 8, 20 and 30 h of growth in iron-restricted (−Fe) and iron-replete (+Fe) conditions using the iron- and Fur-repressed *amiE* gene. Northern hybridization of RNA isolated from *H. pylori* 26695 (WT) and its isogenic *fur* mutant (*fur*) with a probe specific for the *amiE* gene. The position of the two relevant RNA marker sizes (in kb) is indicated on the left.

of the duplicate spots of each gene was used to calculate the expression level ratios. Induction or repression ratios $\geqslant 2$ in both experiments were considered as significant and used in subsequent analysis (Eymann *et al.*, 2002).

Final evaluation of the macroarray data included the consideration of putative operon structures derived from the genome sequence as well as previously known operons. Genes exhibiting significant expression ratios were analysed for their transcriptional organization using the PyloriGene database (http://genolist.pasteur.fr/PyloriGene/) (Boneca *et al.*, 2003). The complete dataset is shown in Supplementary Table S1, which is available online as supplementary data with the online version of this paper at http://mic.sgmjournals.org.

Furbox Analyses. Sequences of Fur-regulated genes (Table 2) were obtained from the *H. pylori* 26695 genome sequence using the PyloriGene database (http://genolist.pasteur.fr/PyloriGene/). Sequences included the intergenic regions upstream of the regulated gene when applicable. Genes were designated as being located downstream of co-transcribed genes (*fliP*, *murB*, *ispE*, *pdxJ* and *hp0241*) when there was less than 10 bp between the stop codon of the preceding gene and the start codon of the following gene. All genes included in this analyses had putative ribosome-binding sites (RBS) located upstream of the translational start codon.

To find putative binding sequences for iron-cofactored Fur, promoter sequences were analysed for the presence of consecutive NAT triplets (van Vliet *et al.*, 2002b) using the GeneRunner program (http://www.generunner.com). Putative binding sequences for apo-Fur were identified by aligning the promoter sequences with the Pfr boxes I and II (Delany *et al.*, 2001a) using the Clone Manager 7 Suite (Scientific and Educational Software).

RESULTS

Identification of iron- and Fur-regulated *H. pylori* genes by transcriptional profiling

For characterizing Fur- and iron-regulated gene expression, *H. pylori* strain 26695 was selected, as this allowed direct comparison with the available genome sequence (Tomb *et al.*, 1997). In addition, *H. pylori* 26695 has been extensively characterized for the role of Fur and iron in the regulation of genes putatively involved in iron transport (van Vliet *et al.*, 2002a). The *H. pylori* 26695 isogenic *fur* mutant used in this study contains the *Campylobacter coli* chloramphenicol resistance cassette in the unique *Bcl*I restriction inside the *fur* coding region, and was characterized previously with regard to acid resistance (Bijlsma *et al.*, 2002), iron uptake (van Vliet *et al.*, 2002a) and iron- and acid-responsive regulation (Bury-Mone *et al.*, 2004; van Vliet *et al.*, 2004).

We selected a single time-point (20 h) to compare gene expression, as this is when *H. pylori* 26695 reaches the late exponential phase (Fig. 1a). To confirm that the 20 h time-point was representative for identification of iron- and Fur-regulated genes, RNA samples isolated at 8, 20 and 30 h post-inoculation were hybridized with a probe specific for the *amiE* gene (Fig. 1b). The *amiE* gene was previously demonstrated to be iron- and Fur-repressed (van Vliet *et al.*, 2003), and this regulation is apparent at each of the three time-points (Fig. 1b). The *amiE* mRNA, with a size of $\sim$1 kb, was detected in the wild-type strain only in iron-restricted conditions, but was constitutively expressed in the *fur* mutant (Fig. 1b). Although the *amiE* gene has also been reported to be growth-phase regulated (Merrell *et al.*, 2003b; Thompson *et al.*, 2003), this was not apparent in the conditions used in this study.

RNA for array testing was isolated from two independent cultures of *H. pylori* 26695 and its isogenic *fur* mutant, grown in iron-restricted and iron-replete conditions. Subsequently the RNA samples were used for transcriptional profiling using the Eurogentec *H. pylori* DNA array, which contains 97 % of all ORFs of *H. pylori* strain 26695. To exclude potential artefacts, only genes with a signal to noise ratio >3 were included in the subsequent data analysis. In total, 1248 out of 1551 genes (80·5 %) fullfilled these criteria for at least one of the conditions in both array experiments, and this percentage of genes exhibiting significant expression signals is relatively high compared to that reported in previous studies ($\sim$50 %) (Ang *et al.*, 2001; Merrell *et al.*, 2003a; Thompson *et al.*, 2003). In total, data for iron regulation were available for 1241 genes in the wild-type strain, and for 964 genes in the *fur* mutant. Data for Fur regulation were available for 994 genes in iron-restricted conditions, and 909 genes in iron-replete conditions.

For each of the 1248 genes, iron regulation was assessed by calculating the ratio between expression levels in iron-restricted conditions ($-$Fe) and the expression levels in iron-replete conditions ($+$Fe). To assess Fur regulation, the ratio between expression levels in the wild-type strain (WT) was compared with the expression levels in the *fur* mutant strain (*fur*/WT ratio). Since *H. pylori* Fur affects transcription in both iron-replete and iron-restricted conditions, the *fur*/WT ratio was calculated for both $-$Fe and $+$Fe conditions. Genes were considered to be regulated by either iron or Fur when the repression or induction ratio was >2 in both independent RNA preparations. Genes regulated by Fur, together with the different ratios, are presented in Table 2.

In total, 61 genes (4·9 %) displayed iron-repressed expression in the wild-type strain, whereas 36 genes (2·9 %) displayed iron-induced expression. Of these 97 iron-regulated genes, only 10 still displayed iron-dependent regulation in the *fur* mutant, with data for 22 genes not being available in the *fur* mutant. This underlines the central role of Fur in iron-regulated gene expression in *H. pylori*. Sixteen genes displayed derepressed expression in the *fur* mutant in iron-replete conditions, and thus these genes probably are regulated by the iron-complexed form of Fur. Conversely, 16 genes displayed derepressed expression in iron-restricted conditions, possibly representing repression by the iron-free form of Fur.

Fur-repressed genes

In most Gram-negative bacteria, Fur binds its target promoters in an iron-dependent fashion, in other words, only

Table 2. Fur-regulated genes of *Helicobacter pylori* strain 26695

The Gene number shown is from the complete genome sequences of *H. pylori* strain 26695 (Tomb *et al.*, 1997) and strain J99 (Alm *et al.*, 1999). The Predicted function column shows the functions and functional categories as defined on the PyloriGene database (Boneca *et al.*, 2003). Values in the Ratio columns show the ratio of expression levels in *H. pylori* wild-type (WT) or fur mutant (*fur*) strain, in iron-restricted (−Fe) or iron-replete (+Fe) conditions. The value shown is the average ratio of two independent array experiments. Values in italic type indicate significant down-regulation of expression; values in bold type indicate significant upregulation of expression. Significant regulation was defined as at least twofold changes in the mRNA levels in both independent array experiments. ND, not detectable: the signal on the array was below the detection threshold in both array experiments.

Gene number for strains:		Predicted function	Ratio			
26695	J99		WT−Fe/WT+Fe	*fur*−Fe/*fur*+Fe	*fur*−Fe/WT−Fe	*fur*+Fe/WT+Fe
Repressed by iron-bound Fur						
Biosynthesis of cofactors, prosthetic groups and carriers						
HP1406	JHP1298	Biotin synthetase (*bioB*)	2·6	0·6	1·4	**6·7**
HP1443	JHP1336	4-disphosphocytidyl-2-C-methyl-D-erythritol kinase (*ispE*)	**3·7**	0·6	1·5	**9·2**
HP1583	JHP1490	Pyridoxal phosphate biosynthetic protein A (*pdxA*)	**9·5**	2·3	0·9	**4·0**
Cell envelope and surface structures						
HP0685	JHP0625	Flagellar biosynthetic protein (*fliP*)	**2·4**	0·8	1·3	**4·2**
HP1418	JHP1313	UDP-*N*-acetylenolpyruvoylglucosamine reductase (*murB*)	**2·8**	0·6	1·6	**7·8**
Cellular processes						
HP0115	JHP0107	Flagellin B (*flaB*)	ND	*0·4*	ND	**3·0**
HP0870	JHP0804	Flagellar hook (*flgE*)	ND	*0·4*	ND	**4·2**
Energy metabolism						
HP0294	JHP0279	Aliphatic amidase (*amiE*)	**2·2**	0·9	0·8	**2·1**
HP1238	JHP1159	Formamidase (*amiF*)	0·9	0·5	1·7	**3·5**
Hypothetical proteins						
HP0906	JHP0842	*H. pylori* predicted coding region HP0906	0·8	*0·3*	1·4	**3·0**
Protein synthesis						
HP1431	JHP1322	16S rRNA (adenosine-N_6,N_6-)-dimethyltransferase (*ksgA*)	**3·5**	1·4	1·4	**3·7**
Transport and binding proteins						
HP0686	JHP0626	Iron(III) dicitrate transport protein (*fecA1*)	**8·5**	1·2	0·6	**4·1**
HP0807	JHP0743	Iron(III) dicitrate transport protein (*fecA2*)	**30·2**	1·7	0·5	**8·3**
HP0876	JHP0810	Iron-regulated outer-membrane protein (*frpB1*)	**5·5**	0·7	0·9	**6·8**
HP1339	JHP1258	Biopolymer transport protein (*exbB2*)	1·6	1·4	2·1	**2·2**
HP1432	JHP1321	Histidine- and glutamine-rich protein	**4·0**	0·7	1·8	**9·5**
Repressed by iron-free Fur						
Amino acid biosynthesis						
HP0652	JHP0597	Phosphoserine phosphatase (*serB*)	0·7	2·3	**8·6**	2·9
Cellular processes						
HP0389	JHP0992	Superoxide dismutase (*sodB*)	*0·2*	0·6	**8·7**	1·9
HP0616	JHP0559	Chemotaxis protein (*cheV2*)	*0·2*	1·8	**14·1**	1·9
HP0922	JHP0856	Toxin-like outer-membrane protein/VacA paralogue	1·1	2·3	**2·4**	1·3
Energy metabolism						
HP0631	JHP0574	Quinone-reactive Ni/Fe hydrogenase, small subunit (*hydA*)	*0·4*	1·2	**3·4**	1·2
HP0632	JHP0575	Quinone-reactive Ni/Fe hydrogenase, large subunit (*hydB*)	*0·3*	0·9	**3·2**	1·2
HP0633	JHP0576	Quinone-reactive Ni/Fe hydrogenase, cytochrome *b* subunit (*hydC*)	*0·3*	1·0	**4·2**	1·2
HP1227	JHP1148	Cytochrome c_{553}	0·4	1·0	**4·3**	1·5
Hypothetical proteins/unknown function						
HP0241	JHP0226	Predicted coding region HP0241	*0·3*	1·1	**2·6**	0·7
HP0388	JHP0993	Conserved hypothetical protein	*0·3*	0·9	**4·9**	1·4
HP0629	JHP0572	Predicted coding region HP0629	0·8	1·3	**2·6**	1·7
HP1094	None*	Predicted coding region HP1094		ND	**2·2**	**3·2**
HP1502	JHP1395	Predicted coding region HP1502	0·9	1·7	**3·6**	1·8
HP1524	JHP1413	Predicted coding region HP1524	*0·4*	0·9	**2·3**	0·8

Table 2. cont.

Gene number for strains:		Predicted function	Ratio			
26695	J99		WT−Fe/ WT+Fe	*fur*−Fe/ *fur*+Fe	*fur*−Fe/ WT−Fe	*fur*+Fe/ WT+Fe
Protein synthesis						
HP1253	JHP1174	Tryptophanyl-tRNA synthetase (*trpS*)	ND	1·2	**3·0**	1·0
Transport and binding protein						
HP0653	JHP0598	Non-heme iron-containing ferritin (*pfr*)	*0·2*	**2·5**	**53·1**	**2·3**
Fur-induced						
Cell envelope and surface structures						
HP0638	JHP0581	Outer-membrane protein (*omp13*; *oipA*)	1·0	ND	*0·2*	*0·1*
HP0855	None*	Alginate *O*-acetylation protein (*algI*)	**5·8**	ND	*0·3*	ND
HP1494	JHP1387	UDP-MurNac-tripeptide synthetase (*murE*)	**4·3**	2·1	*0·5*	0·9
DNA metabolism, restriction and modification						
HP0260	JHP0244	Adenine-specific DNA methyltransferase (*mod*)	1·8	1·0	*0·4*	0·9
HP1323	JHP1243	Ribonuclease HII (*rnhB*)	**4·4**	1·6	*0·4*	0·9
Hypothetical proteins/unknown function						
HP0207	JHP0193	ATP-binding protein (*mpr*)	**4·0**	**2·6**	*0·4*	0·5
HP0236	JHP0221	Predicted coding region HP0236	1·4	0·8	*0·4*	0·8
HP0248	JHP0233	Conserved hypothetical protein	2·6	0·9	*0·4*	1·1
HP0322	JHP0305	Poly-E-rich protein	*0·3*	0·8	0·9	*0·4*
HP0424	None*	Predicted coding region HP0424	2·0	ND	*0·4*	ND
HP0773	JHP0710	Predicted coding region HP0773	2·6	1·8	*0·5*	0·7
HP1117	JHP1045	Cysteine-rich protein X (*hcpX*)	1·8	1·5	0·9	*0·4*
HP1142	JHP1070	Predicted coding region HP1142	*0·5*	ND	ND	*0·4*
Protein synthesis						
HP1141	JHP1069	Methionyl-tRNA formyltransferase (*fmt*)	**2·3**	1·3	*0·4*	0·7
HP1160	JHP1087	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase	**2·6**	1·2	*0·4*	0·8
Transport and binding proteins						
HP1172	JHP1099	Glutamine ABC transporter, periplasmic glutamine-binding protein (*glnH*)	**3·2**	1·5	*0·4*	0·7

*This gene is absent in the *H. pylori* J99 complete genome sequence.

in iron-replete conditions. Uniquely, the iron-free form of *H. pylori* Fur is also capable of repressing transcription, thus allowing differential expression of genes depending on iron availability in the cytoplasm (Delany *et al.*, 2001a). Surprisingly, not all Fur-regulated genes identified in this study displayed iron-responsive expression (Table 2).

(i) Genes repressed by the iron-complexed form of Fur (Fe-Fur). Of the 16 genes demonstrating derepressed expression under iron-replete conditions in the *fur* mutant, 10 also demonstrated iron-repressed expression in the wild-type strain (Table 2), with four displaying iron-independent expression; for two genes, data were not available. As predicted in previous studies (Delany *et al.*, 2001a, 2001b; Fassbinder *et al.*, 2000; van Vliet *et al.*, 2002a), several of these genes (*fecA1*, *fecA2*, *frpB1* and *exbB2*) encode homologues of iron transport and binding proteins, and probably play a role in the uptake and transport of iron to the cytoplasm. In addition, the *hp1432* gene, encoding a nickel-binding histidine- and glutamine-rich protein (Gilbert *et al.*, 1995), is also regulated by Fur, and this regulation was confirmed by Northern hybridization (Fig. 2) and RNA slotblot hybridization (data not shown). This gene has also been classified as nickel- and NikR-activated (Contreras *et al.*, 2003), and acid-induced (Merrell *et al.*, 2003a).

The *fur* mutation also influenced several other classes of genes (Table 2). These included genes putatively involved in: i) biosynthesis of cofactors and prosthetic groups, including biotin (*bioB*), isoprenoid (*ispE*) and pyridoxal phosphate (*pdxA*); ii) production of cell envelope and surface structures, such as the *murB* peptidoglycan synthesis gene and the *flaB* and *fliP* flagellar biosynthesis genes (Josenhans *et al.*, 2000); iii) energy metabolism, with both the paralogous amidases *amiE* and *amiF* (Skouloubris *et al.*, 2001; van Vliet *et al.*, 2003); iv) protein synthesis, in which the 16S rRNA dimethyltransferase gene *ksgA* is putatively involved. Finally, expression of the hypothetical protein HP906 was repressed by Fe-Fur. For two of the

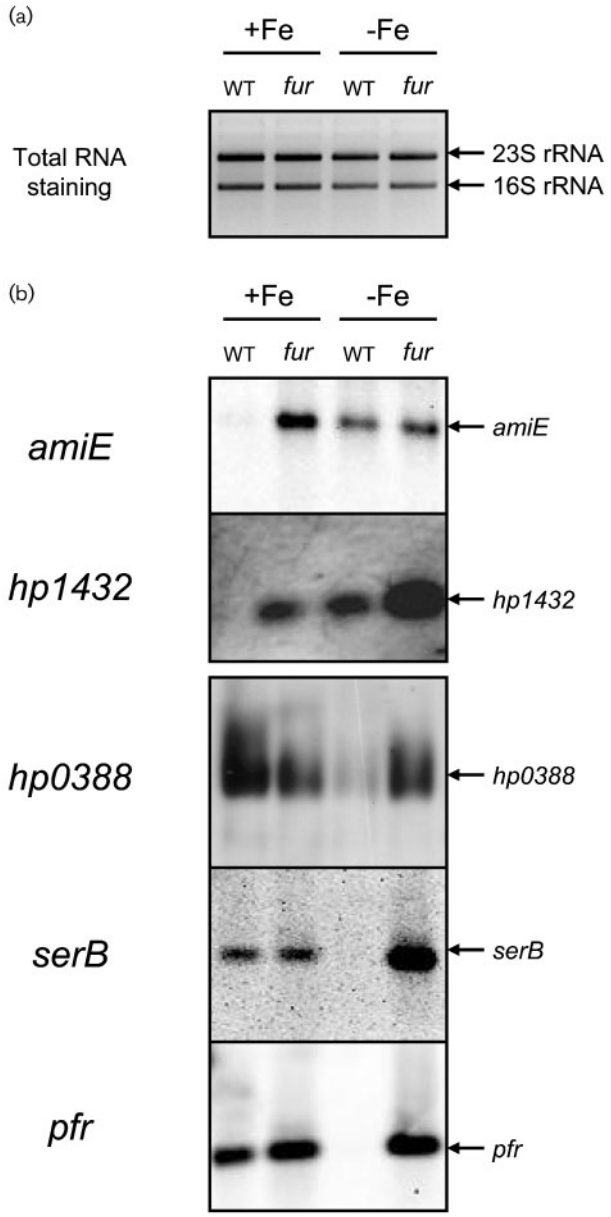

Fig. 2. Confirmation of Fur- and iron-responsive regulation of a subset of genes selected from Table 2. (a) Staining of transferred RNA by methylene blue to allow for comparison of RNA amounts. (b) Northern hybridization with probes specific for five genes using RNA purified from *H. pylori* wild-type (WT) and *fur* mutant (*fur*) cells grown in iron-restricted (−Fe) and iron-replete (+Fe) conditions. Probes used are indicated on the left; the specific mRNA is indicated on the right.

iron-repressed Fur-regulated genes (*amiE* and *hp1432*), the transcriptional pattern was confirmed using Northern hybridization (Fig. 2).

(ii) Genes repressed by the iron-free form of Fur. Sixteen genes demonstrated increased expression in the *fur* mutant, when compared to the wild-type strain grown in iron-restricted conditions (Table 2). This unique form of regulation has so far only been reported for the *pfr* gene (Bereswill *et al.*, 2000; Delany *et al.*, 2001a; Waidner *et al.*, 2002), but is probably more widespread in *H. pylori*. Of the 16 genes demonstrating derepressed expression under iron-restricted conditions in the *fur* mutant, nine genes also demonstrated iron-induced expression in the wild-type strain (Table 3), with five genes displaying iron-independent expression; for two genes, data were not available.

Several genes associated with energy and oxygen metabolism displayed Fur-mediated repression of transcription, including the nickel/iron-cofactored hydrogenase subunit genes (*hydABC*) (Olson *et al.*, 2001), a putative cytochrome c553, and the *sodB* gene encoding the iron-cofactored superoxide dismutase (Pesci & Pickett, 1994; Seyler *et al.*, 2001; Spiegelhalder *et al.*, 1993). Further genes regulated by the iron-free form of Fur included the chemotaxis gene *cheV2*, the *hp0922* gene encoding a toxin-like outer-membrane protein, the *serB* gene, which is cotranscribed with *pfr*, the tryptophanyl-tRNA synthetase gene *trpS*, and five genes encoding hypothetical proteins (Table 2). For three of the iron-induced Fur-regulated genes (*pfr*, *serB* and *hp0388*), the transcriptional pattern was confirmed using Northern hybridization (Fig. 2).

(iii) Fur-induced genes. Sixteen genes displayed decreased expression in the *fur* mutant when compared to the wild-type strain (Table 2). This inverse regulation is atypical for a repressor like Fur, and is likely to represent indirect regulation. This cluster of genes included the *oipA* gene encoding an outer-membrane protein, the *murE* gene involved in peptidoglycan synthesis, a DNA methyltransferase (*mod*), the ribonuclease HII-encoding *rnhB* gene, the periplasmic binding protein of the glutamine ABC transporter, two genes involved in protein synthesis, and eight genes encoding hypothetical proteins.

Identification of putative binding sequences for Fe-Fur and apo-Fur

All Fur-repressed genes listed in Table 2 were further investigated for the presence of putative Fur boxes in their respective promoters (Fig. 3). Firstly, the putative promoter region was identified for each gene using the PyloriGene database (see Methods for details). Putative binding sites for Fe-Fur were identified as up to six consecutive nAT triplets, with n representing any nucleotide (Delany *et al.*, 2001b; van Vliet *et al.*, 2002b). Each of the promoters included in this analysis contained a putative Fur box, with identities to the $(nAT)_6$ sequence ranging from 6 to 11 per 12 residues (Fig. 3a).

The identification of binding sites of apo-Fur is currently hampered by the absence of a consensus sequence, since only the *pfr* promoter has been analysed to date (Delany *et al.*, 2001a). In this promoter there are two high-affinity sites for apo-Fur, designated Pfr box I and Pfr box II (Delany

Table 3. Iron-regulated (Fur-independent) genes of *Helicobacter pylori* strain 26695

The gene number shown is from the complete genome sequences of *H. pylori* strain 26695 (Tomb *et al.*, 1997) and strain J99 (Alm *et al.*, 1999). The Predicted function column shows the function and functional category as defined on the PyloriGene database (Boneca *et al.*, 2003). Values in the Ratio columns show the ratio of expression levels in *H. pylori* wild-type (WT) or fur mutant (*fur*) strain, in iron-restricted (−Fe) or iron-replete (+Fe) conditions. The value shown is the average ratio of two independent array experiments. Values in italic type indicate significant down-regulation of expression; values in bold type indicate significant upregulation of expression. Significant regulation was defined as at least twofold changes in the mRNA levels in both independent array experiments. ND, not detectable: the signal on the array was below the detection threshold in both array experiments.

Gene number for strain:		Predicted function	Ratio			
26695	J99		WT−Fe/ WT+Fe	*fur*−Fe/ *fur*+Fe	*fur*−Fe/ WT−Fe	*fur*+Fe/ WT+Fe
Iron-repressed						
Biosynthesis of cofactors, prosthetic groups and carriers						
HP0625	JHP0569	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthetase (*ispG*)	**2·7**	2·6	0·7	0·8
HP0798	JHP0734	Molybdenum cofactor biosynthesis protein C (*moaC*)	**4·2**	ND	ND	ND
HP0804	JHP0740	GTP cyclohydrolase II/3,4-diOH-2-butanone 4-phosphate synthase (*ribBA*)	**2·5**	ND	0·5	ND
HP0841	JHP0779	Pantothenate metabolism flavoprotein (*dfp*)	**2·9**	**2·5**	0·9	ND
Cell envelope and surface structures						
HP0279	JHP0264	Lipopolysaccharide heptosyltransferase-1 (*rfaC*)	**2·6**	ND	ND	ND
HP0648	HP0593	UDP-*N*-acetylglucosamine enolpyruvyl transferase (*murZ*)	**2·7**	1·0	0·5	1·1
HP1429	JHP1324	Polysialic acid capsule expression protein (*kpsF*)	**9·0**	**4·7**	1·0	ND
HP1456	JHP1349	Membrane-associated lipoprotein (*lpp20*)	**2·7**	1·8	0·7	1·0
Cellular processes						
HP0246	JHP0231	Flagellar basal-body P-ring protein (*flgI*)	**2·3**	1·4	0·7	1·1
HP0752	JHP0689	Flagellar hook-associated protein 2 (*fliD*)	**4·2**	2·2	0·6	1·2
HP0792	JHP0728	Predicted DNA transformation competence protein (*comM*)	**2·7**	2·0	0·7	0·8
HP1030	JHP0394	fliY protein (*fliY*)	**2·6**	ND	0·5	ND
HP1031	JHP0393	Flagellar motor switch protein (*fliM*)	**4·5**	2·1	0·4	ND
HP1069	JHP0356	Cell division protein (*ftsH2*)	**2·6**	2·1	0·7	1·0
HP1420	JHP1315	Flagellar export protein ATP synthase (*fliI*)	**4·1**	**2·1**	0·4	0·7
DNA metabolism, restriction and modification						
HP1114	JHP1041	Excinuclease ABC subunit B (*uvrB*)	**3·2**	2·0	0·6	1·0
HP1478	JHP1371	DNA helicase II (*uvrD*)	**3·2**	2·5	0·6	ND
Energy metabolism						
HP1103	JHP1029	Glucokinase (*glk*)	**2·2**	ND	ND	ND
Fatty acid and phospholipid metabolism and biosynthesis						
HP0201	JHP0187	Fatty acid/phospholipid synthesis protein (*plsX*)	**2·8**	3·3	0·6	0·6
Hypothetical proteins/unknown function						
HP0066	JHP0061	Conserved hypothetical ATP-binding protein	**2·7**	ND	0·8	ND
HP0258	JHP0242	Conserved hypothetical integral membrane protein	**2·7**	ND	ND	ND
HP0346	None*	Predicted coding region HP0346	**5·6**	ND	ND	ND
HP0347	JHP0321	Conserved hypothetical protein	**3·1**	ND	ND	ND
HP0356	JHP0330	Predicted coding region HP0356	**2·1**	ND	ND	ND
HP0726	JHP0663	Predicted coding region HP0726	**3·3**	**3·5**	0·7	ND
HP0806	JHP0742	Predicted coding region HP0806	**4·0**	1·5	0·5	ND
HP1335	JHP1254	Conserved hypothetical protein	**4·2**	**2·3**	0·4	ND
HP1336	JHP1255	Predicted coding region HP1336	**2·7**	2·3	0·7	ND
HP1343	JHP1262	Conserved hypothetical integral membrane protein	**2·2**	ND	ND	0·7
HP1424	JHP1319	Predicted coding region HP1424	**3·5**	**3·3**	0·7	ND
HP1428	JHP1325	Conserved hypothetical protein	**4·8**	**3·9**	1·0	ND
HP1430	JHP1323	Conserved hypothetical ATP-binding protein	**4·6**	7·6	1·0	1·0
HP1454	JHP1347	Predicted coding region HP1454	**3·4**	2·3	0·5	0·8
HP1467	JHP1360	Predicted coding region HP1467	**2·4**	1·6	0·7	1·1
HP1567	JHP1475	Conserved hypothetical GTP-binding protein	**2·3**	ND	ND	ND

Table 3. cont.

Gene number for strain:		Predicted function	Ratio			
26695	J99		WT−Fe/ WT+Fe	*fur*−Fe/ *fur*+Fe	*fur*−Fe/ WT−Fe	*fur*+Fe/ WT+Fe
Protein synthesis						
HP1201	JHP1124	Ribosomal protein L1 (*rpl1*)	**3·5**	2·6	0·7	0·9
HP1480	JHP1373	Seryl-tRNA synthetase (*serS*)	**3·0**	2·2	0·6	0·7
HP1547	JHP1452	Leucyl-tRNA synthetase (*leuS*)	**2·6**	1·9	0·6	0·8
Purines, pyrimidines, nucleosides and nucleotides						
HP0043	JHP0037	Mannose-6-phosphate isomerase (*pmi*) or (*algA*)	**2·7**	ND	0·8	ND
HP0854	JHP0790	GMP reductase (*guaC*)	**4·4**	**4·4**	0·6	ND
Regulatory functions						
HP0278	JHP0263	Guanosine pentaphosphate phosphohydrolase (*gppA*)	**3·3**	2·2	0·5	0·7
Transport and binding proteins						
HP0724	JHP0660	Anaerobic C4-dicarboxylate transport protein (*dcuA*)	**2·3**	3·4	2·0	ND
HP0818	JHP0754/7†	Osmoprotection protein (*proWX*)	**3·2**	ND	0·5	ND
HP1400	JHP1426	Iron(III) dicitrate transport protein (*fecA3*)	**2·5**	3·2	0·9	0·7
HP1491	JHP1384	Phosphate permease	**2·7**	2·0	0·7	0·9
Iron-induced						
Cell envelope and surface structures						
HP0229	JHP0214	Outer-membrane protein (*omp6*; *hopA*)	*0·1*	0·4	2·2	0·7
HP0492	JHP0444	Neuraminyllactose-binding haemagglutinin homologue/paralogue of HpaA	*0·5*	0·6	1·5	1·1
Cellular processes						
HP0103	JHP0095	Methyl-accepting chemotaxis protein (*tlpB*)	*0·4*	0·6	1·1	0·6
HP0522	JHP0471	*cag* pathogenicity island protein (*cag3*)	*0·5*	0·9	1·2	0·6
HP0523	JHP0472	*cag* pathogenicity island protein (*cag4*)	*0·3*	ND	ND	0·6
HP0547	JHP0495	*cag* pathogenicity island protein (*cag26*; *cagA*)	*0·2*	0·5	1·0	0·5
HP0875	JHP0809	Catalase (*katA*)	*0·4*	0·5	1·7	1·4
HP0887	JHP0819	Vacuolating cytotoxin (*vacA*)	*0·2*	*0·3*	3·7	2·0
DNA metabolism, restriction and modification						
HP0091	JHP0084	Type II restriction enzyme R protein (*hsdR*)	*0·4*	ND	ND	ND
HP0481	JHP0433	Type II adenine specific DNA methyltransferase (MFOKI)	*0·4*	ND	ND	0·7
Hypothetical proteins/unknown function						
HP0097	JHP0089	Predicted coding region HP0097	*0·4*	0·7	1·4	0·8
HP0102	JHP0094	Predicted coding region HP0102	*0·4*	ND	ND	ND
HP0119	None*	Predicted coding region HP0119	*0·5*	0·9	1·3	0·7
HP0120	None*	Predicted coding region HP0120	*0·5*	1·0	1·4	0·7
HP0130	JHP0119	Predicted coding region HP0130	*0·4*	0·7	1·0	0·6
HP0377	JHP1004	Thiol : disulfide interchange protein (dsbC), putative	*0·4*	ND	ND	0·5
HP0762	JHP0699	Predicted coding region HP0762	*0·4*	0·9	1·3	0·6
HP0938	JHP0873	Predicted coding region HP0938	*0·4*	ND	ND	1·0
HP1143	JHP1071	Predicted coding region HP1143	*0·5*	ND	ND	0·6
HP1175	JHP1102	Conserved hypothetical integral membrane protein	*0·5*	0·5	1·1	1·0
Protein fate						
HP0109	JHP0101	Chaperone and heat-shock protein 70 (*dnaK*)	*0·4*	0·7	1·1	0·6
HP0264	JHP0249	ATP-dependent protease binding subunit (*clpB*)	*0·4*	0·6	0·9	0·6
HP0470	JHP0422	Oligoendopeptidase F (*pepF*)	*0·4*	0·8	1·2	0·7
Purines, pyrimidines, nucleosides and nucleotides						
HP0404	JHP0977	Predicted ADP hydrolase of the HIT protein family (HINT)	*0·4*	0·5	ND	0·8
Transport and binding proteins						
HP1082	JHP0343	Multidrug resistance protein (*msbA*)	*0·5*	1·0	1·3	0·7
Other						
HP0472	JHP0424	Outer-membrane protein (*omp11*)	ND	*0·3*	1·1	ND
HP0708	JHP0647	Predicted coding region HP0708	0·5	*0·3*	0·8	1·3

Table 3. cont.

Gene number for strain:		Predicted function	Ratio			
26695	J99		WT−Fe/ WT+Fe	*fur*−Fe/ *fur*+Fe	*fur*−Fe/ WT−Fe	*fur*+Fe/ WT+Fe
HP0909	JHP0845	Predicted coding region HP0909 (pseudogene)	0·6	*0·5*	1·6	1·2
HP1458	JHP1351	Thioredoxin	0·6	*0·3*	1·0	1·3
HP0004	JHP0004	Carbonic anhydrase (*icfA*)	1·9	**3·6**	1·2	ND
HP0220	JHP0101	Synthesis of [Fe-S] cluster (*nifS*)	2·3	**3·6**	1·1	0·7
HP0438	JHP0249	IS*605* transposase (*tnpB*)	1·4	**2·1**	1·3	0·9
HP1095	JHP0422	IS*605* transposase (*tnpB*)	1·6	**2·5**	1·6	1·0
HP1387	JHP1438	DNA polymerase III epsilon subunit (*dnaQ*)	2·7	**3·4**	1·2	ND
HP1534	JHP0977	IS*605* transposase (*tnpB*)	2·0	**2·7**	1·7	1·1

*This gene is absent in the *H. pylori* J99 complete genome sequence (Alm *et al.*, 1999).
†The HP0818 (*proWX*) gene is a single in *H. pylori* strain 26695, but consists of two genes (JHP0754 and JHP0757) in *H. pylori* strain J99.

et al., 2001a). Therefore these boxes were aligned with the promoters of the genes putatively regulated by apo-Fur (Fig. 3b). All promoters contained sequences similar to each of the Pfr boxes, although this identity ranged from 17 to 24 per 41 residues (Pfr box I) and 15 to 22 per 37 residues (Pfr box II), respectively (Fig. 3b).

Iron-responsive regulation independent of Fur

While only approximately half of the Fur-repressed genes displayed iron-responsive expression, several other genes displayed iron-responsive expression which was not significantly altered in the *fur* mutant (Table 3). Forty-five genes displayed iron-repressed expression in the wild-type strain, i.e. higher mRNA levels in iron-restricted conditions, whereas twenty-five genes displayed iron-induced expression. As with the Fur-repressed genes, genes belonging to several functional classes were affected by iron restriction when compared to iron-replete conditions.

(i) Iron-repressed genes. This group of iron-repressed genes includes five motility-associated genes, the *fliD*, *fliI*, *fliM*, *fliY* and *flgI* genes, encoding components of the flagellum of *H. pylori* (O'Toole *et al.*, 2000). Their regulation by iron may explain the effect of iron restriction and acid exposure on the motility of *H. pylori* (Merrell *et al.*, 2003b). In addition to motility-associated genes, iron repressed the expression of genes involved with the cell envelope and surface structures (*lpp20*), cell division (*ftsH*), peptidoglycan synthesis (*murZ*), LPS (*kpsF*, *rfaC*) and phospholipid synthesis (*plsX*). Other membrane-associated structures repressed by iron included a putative phosphate permease and one of the ferric citrate outer membrane receptors (*fecA*3). In addition to these genes, genes involved in protein synthesis, stress response, nucleotide metabolism and modification, and cofactor biosynthesis were also induced by iron restriction, as were several genes encoding hypothetical proteins (Table 3).

(ii) Iron-induced genes. Many genes subject to Fur-independent, iron-induced transcriptional regulation encode major virulence factors of *H. pylori*. These include the VacA vacuolating cytotoxin, the CagA cytotoxin, and the HopA and HP0492 outer-membrane proteins. Also included in this category are genes encoding proteins functioning in stress response, such as the KatA catalase and the chaperones DnaK and ClpB (Table 3). Chemotaxis may also be iron responsive via the *tlpB* gene, which encodes a methyl-accepting chemotaxis protein. Finally, two genes involved in nucleotide metabolism/modification and eight genes encoding hypothetical proteins displayed Fur-independent, iron-induced expression.

(iii) Abberantly regulated genes. Seven genes displayed iron-regulated expression in the *fur* mutant only, but were transcribed in an iron-independent manner in the wild-type strain. This cluster includes the HP0004 gene encoding carbonic anhydrase, the HP1458 gene encoding a putative thioredoxin, which is transcribed at higher levels in iron-replete conditions in the *fur* mutant, the HP0220 *nifS* gene, which is involved in the formation of Fe-S clusters (Olson *et al.*, 2000), and three copies of the IS*605* transposase (*tnpB*), whose expression is decreased in iron-replete conditions (Table 3).

DISCUSSION

In many bacteria, the Fur repressor is the central regulator of iron homeostasis (Andrews *et al.*, 2003; Hantke, 2001). Fur mediates its iron homeostasis function via careful regulation of iron-acquisition and iron-storage systems: in iron-restricted conditions, iron-uptake systems are expressed and iron storage is repressed, but conversely in iron-replete conditions, iron-storage systems are expressed and iron uptake is abolished (Bereswill *et al.*, 2000; Delany *et al.*, 2001a; van Vliet *et al.*, 2002a). The switch between repression and induction of iron uptake is coupled to iron

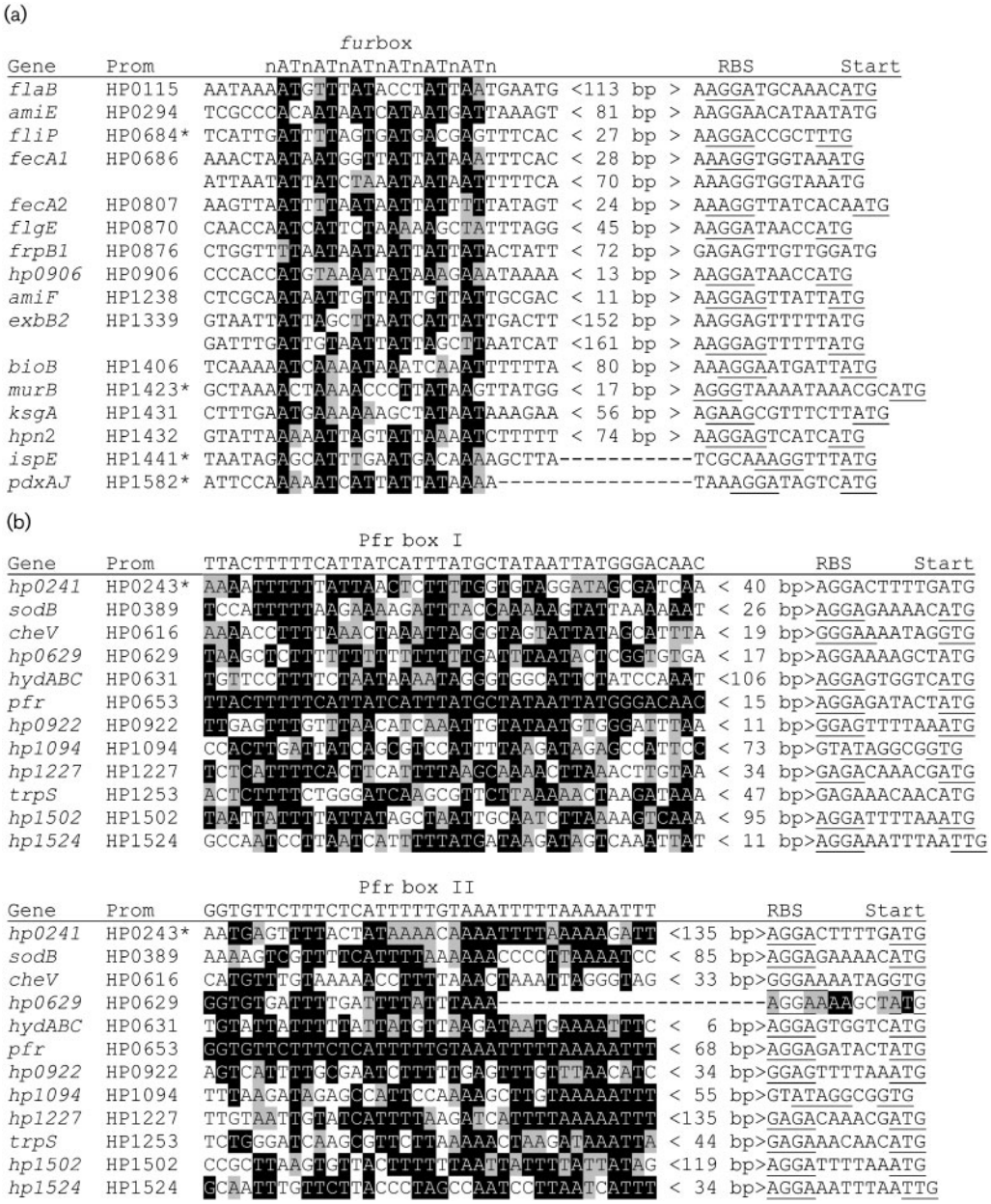

Fig. 3. Identification of putative binding sequences for Fe-Fur and for apo-Fur. (a) Putative Fe-Fur-repressed promoter sequences were searched for the presence of consecutive nAT triples, indicative of binding sequences for Fur (van Vliet *et al*., 2002b). Residues with black background are identical to the $(NAT)_6$ Furbox, whereas residues with grey background represent A/T and T/A substitutions. (b) Putative apo-Fur-regulated promoters were aligned with the two high-affinity apo-Fur binding sequences identified in the *H. pylori pfr* promoter (Pfr box I and Pfr box II) (Delany *et al*., 2001a). Residues with black background are identical to the respective Pfr box, whereas residues with grey background represent A/T and T/A substitutions. For all aligned promoters, the position respective to the underlined ribosome-binding site (RBS) and underlined translational start codon (Start) are given. Designations above the alignments: Gene, gene designation given in Table 2; Prom, putative promoter of regulated gene. An asterisk indicates that the regulated gene is likely to be transcribed as a member of an operon, and the putative promoter of the gene at the beginning of the operon was analysed for the presence of a binding sequence for Fur-Fe or apo-Fur.

availability in the cytoplasm: when iron is available, a Fur dimer forms a complex with ferrous iron and binds to Fur-binding sequences (Fur boxes) in the promoters of iron-uptake genes (Hantke, 2001). This situation is, however, not as clear for the switch in the repression and induction of ferritin-mediated iron storage: while iron

induction of ferritin expression is found in several bacteria, the role of Fur in this process is not universal.

In this study, transcriptional profiling was used to identify *H. pylori* genes that are regulated by Fur and iron at the transcriptional level. Recent studies focusing on the effects of iron restriction, growth phase and acidic pH on gene expression in *H. pylori* have indicated that many genes classified in different functional categories are affected by these conditions (Allan *et al.*, 2001; Ang *et al.*, 2001; Kim *et al.*, 2004; Merrell *et al.*, 2003a, 2003b; Thompson *et al.*, 2003; Wen *et al.*, 2003). For 1248 genes, data were obtained on their regulation by iron or by Fur. In our study using the wild-type *H. pylori* strain 26695, 97 genes displayed iron-responsive regulation and 43 genes displayed Fur-dependent regulation.

Genes regulated by Fe-Fur and apo-Fur are classified in several functional categories (Table 2), indicative of the role of Fur as global regulator in *H. pylori*. This is consistent with the phenotypes reported for the *fur* mutant thus far, which displays increased iron uptake (van Vliet *et al.*, 2002a), decreased acid resistance (Bijlsma *et al.*, 2002), and attenuation in a mouse model of *H. pylori* infection (Bury-Mone *et al.*, 2004). Rather surprisingly, while mutation of *fur* affects many cellular processes, the *fur* mutant is not significantly affected in growth under *in vitro* conditions (Fig. 1a).

Other than the genes functioning in metal metabolism, many of the genes regulated by Fe-Fur and apo-Fur have not been investigated previously and require experimental confirmation of their predicted function. However, based on homology, several of the proteins encoded by Fur-regulated genes are predicted to be iron-cofactored, like the biotin synthetase BioB (Sanyal *et al.*, 1994). The *E. coli* BioB protein also requires pyridoxal phosphate (Ollagnier-De-Choudens *et al.*, 2002), as synthesized by the PdxA protein, and in *H. pylori* this gene displays similar regulation to the *bioB* gene (Table 2). Furthermore, in *E. coli*, the *ksgA* gene is cotranscribed with the *pdxA* gene, and both are growth-phase regulated (Pease *et al.*, 2002), while in *H. pylori* both genes are subjected to regulation by Fe-Fur (Table 2). Other Fe-Fur-regulated genes include the *flaB* and *flgE* genes, and taken together with the iron-responsive regulation of several *fli* genes (Table 2), this may explain the effect of iron on the motility of *H. pylori* (Merrell *et al.*, 2003b). Finally, genes regulated by apo-Fur encode iron-cofactored enzymes like hydrogenase and superoxide dismutase (Table 2), and this form of regulation may ensure that these enzymes are only expressed when iron is available. Comparison with Fur and iron regulons in other bacteria is hampered by the lack of operon structure in the *H. pylori* genome sequence. The most closely related bacterium is *Campylobacter jejuni*, and recently its Fur and iron regulons were determined (Palyada *et al.*, 2004). Interestingly, both in *H. pylori* and *C. jejuni*, motility-associated genes were affected by iron and the mutation of *fur*, suggesting a common mechanism behind the iron-responsive regulation of motility.

Iron-responsive genes were also recently identified in the mouse-adapted *H. pylori* strain SS1 (Merrell *et al.*, 2003b; Thompson *et al.*, 2003), and show partial overlap with the iron-responsive genes in our study. Unfortunately, a direct comparison with the two related studies is hampered by the use of different strains of *H. pylori* and differences in the experimental set-up. An important difference may be that in the previously published studies (Merrell *et al.*, 2003b; Thompson *et al.*, 2003), iron restriction was achieved via the use of 2,2-dipyridyl, which has a high affinity for ferrous iron and is membrane permeable, whereas in our study we used desferal, which is a siderophore-based iron chelator that removes ferric iron from the medium and makes it unavailable for *H. pylori* (van Vliet *et al.*, 2002a). Comparison of the datasets is further complicated by the difference in *H. pylori* strains used. The complete genome sequence of *H. pylori* 26695, the strain used in this study, is available (Tomb *et al.*, 1997), whereas the other two studies were based on *H. pylori* strain SS1 (Merrell *et al.*, 2003b; Thompson *et al.*, 2003), whose genome sequence is not yet known. Thus the gene order, promoter sequences and regulatory responses of *H. pylori* SS1 are unknown and may differ significantly from those in *H. pylori* 26695 (Alm *et al.*, 1999; Israel *et al.*, 2001a, 2001b; Salama *et al.*, 2000; Tomb *et al.*, 1997). However, the majority of Fur-regulated genes identified in our study display iron-responsive regulation (Table 2) and cluster mostly in the group of stationary-phase induced genes (Merrell *et al.*, 2003b). This is consistent with our experimental set-up, since we used late-exponential-phase cells to isolate RNA for the transcriptome studies (Fig. 1a).

Rather surprising was the relative lack of operon structure in the transcriptome data. While several of the genes identified in this study are predicted to be transcribed as part of a multicistronic mRNA, this was not apparent from the array data. An example of this is the *pdxA* gene, which is predicted to constitute an operon with the upstream *pdxJ* gene. However, expression of the *pdxJ* gene seems not to be affected by the *fur* mutation or by iron restriction (see Supplementary Table S1). This may be partially due to differential mRNA degradation, as was described for the urease operon (Akada *et al.*, 2000), and it is interesting to see that a ribonuclease gene (*rnhB*) is included in the list of iron-regulated genes (Table 3).

Despite its small genome, *H. pylori* is a highly successful colonizer of the human gastric mucosa, and is present for life unless eradicated by antibiotic treatment (Blaser & Berg, 2001). Its potential to adapt to hostile environmental niches with changing conditions is apparent, despite the relative paucity of transcriptional regulators. One of the possibilities explaining such adaptive capacity with relatively few regulators is that these regulators have broadened their regulatory potential, and this seems to be the case for *H. pylori* Fur. This protein, well known for its central role in

iron homeostasis in bacteria, controls the expression of different pathways involved in normal metabolism, stress resistance, motility and virulence. This central role in these important pathways makes it a prime candidate for further study on the role of bacterial adaptation in long-term colonization of hostile environmental niches.

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REFERENCES

Akada, J. K., Shirai, M., Takeuchi, H., Tsuda, M. & Nakazawa, T. (2000). Identification of the urease operon in *Helicobacter pylori* and its control by mRNA decay in response to pH. *Mol Microbiol* **36**, 1071–1084.

Allan, E., Clayton, C. L., McLaren, A., Wallace, D. M. & Wren, B. W. (2001). Characterization of the low-pH responses of *Helicobacter pylori* using genomic DNA arrays. *Microbiology* **147**, 2285–2292.

Alm, R. A., Ling, L. S., Moir, D. T. & 20 other authors (1999). Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature* **397**, 176–180.

Andrews, S. C., Robinson, A. K. & Rodriguez-Quinones, F. (2003). Bacterial iron homeostasis. *FEMS Microbiol Rev* **27**, 215–237.

Ang, S., Lee, C. Z., Peck, K., Sindici, M., Matrubutham, U., Gleeson, M. A. & Wang, J. T. (2001). Acid-induced gene expression in *Helicobacter pylori*: study in genomic scale by microarray. *Infect Immun* **69**, 1679–1686.

Barnard, F. M., Loughlin, M. F., Fainberg, H. P., Messenger, M. P., Ussery, D. W., Williams, P. & Jenks, P. J. (2004). Global regulation of virulence and the stress response by CsrA in the highly adapted human gastric pathogen *Helicobacter pylori*. *Mol Microbiol* **51**, 15–32.

Bereswill, S., Greiner, S., van Vliet, A. H. M., Waidner, B., Fassbinder, F., Schiltz, E., Kusters, J. G. & Kist, M. (2000). Regulation of ferritin-mediated cytoplasmic iron storage by the Ferric Uptake Regulator homolog (Fur) of *Helicobacter pylori*. *J Bacteriol* **182**, 5948–5953.

Bijlsma, J. J. E., Waidner, B., van Vliet, A. H. M. & 7 other authors (2002). The Ferric Uptake Regulator (Fur) homologue of *Helicobacter pylori* is involved in acid resistance. *Infect Immun* **70**, 606–611.

Blaser, M. J. & Berg, D. E. (2001). *Helicobacter pylori* genetic diversity and risk of human disease. *J Clin Invest* **107**, 767–773.

Boneca, I. G., de Reuse, H., Epinat, J. C., Pupin, M., Labigne, A. & Moszer, I. (2003). A revised annotation and comparative analysis of *Helicobacter pylori* genomes. *Nucleic Acids Res* **31**, 1704–1714.

Bury-Mone, S., Thiberge, J. M., Contreras, M., Maitournam, A., Labigne, A. & De Reuse, H. (2004). Responsiveness to acidity via metal ion regulators mediates virulence in the gastric pathogen *Helicobacter pylori*. *Mol Microbiol* **53**, 623–638.

Contreras, M., Thiberge, J. M., Mandrand-Berthelot, M. A. & Labigne, A. (2003). Characterization of the roles of NikR, a nickel-responsive pleiotropic autoregulator of *Helicobacter pylori*. *Mol Microbiol* **49**, 947–963.

Conway, T. & Schoolnik, G. K. (2003). Microarray expression profiling: capturing a genome-wide portrait of the transcriptome. *Mol Microbiol* **47**, 879–889.

Cooksley, C., Jenks, P. J., Green, A., Cockayne, A., Logan, R. P. & Hardie, K. R. (2003). NapA protects *Helicobacter pylori* from oxidative stress damage, and its production is influenced by the ferric uptake regulator. *J Med Microbiol* **52**, 461–469.

Delany, I., Spohn, G., Rappuoli, R. & Scarlato, V. (2001a). The Fur repressor controls transcription of iron-activated and -repressed genes in *Helicobacter pylori*. *Mol Microbiol* **42**, 1297–1309.

Delany, I., Pacheco, A. B. F., Spohn, G., Rappuoli, R. & Scarlato, V. (2001b). Iron-dependent transcription of the *frpB* gene of *Helicobacter pylori* is controlled by the Fur protein. *J Bacteriol* **183**, 4932–4937.

Eymann, C., Homuth, G., Scharf, C. & Hecker, M. (2002). *Bacillus subtilis* functional genomics: global characterization of the stringent response by proteome and transcriptome analysis. *J Bacteriol* **184**, 2500–2520.

Fassbinder, F., van Vliet, A. H. M., Gimmel, V., Kusters, J. G., Kist, M. & Bereswill, S. (2000). Identification of iron-regulated genes of *Helicobacter pylori* by a modified Fur Titration Assay (FURTA-Hp). *FEMS Microbiol Lett* **184**, 225–229.

Forsyth, M. H., Cao, P., Garcia, P. P., Hall, J. D. & Cover, T. L. (2002). Genome-wide transcriptional profiling in a histidine kinase mutant of *Helicobacter pylori* identifies members of a regulon. *J Bacteriol* **184**, 4630–4635.

Gilbert, J. V., Ramakrishna, J., Sunderman, F. W., Jr, Wright, A. & Plaut, A. G. (1995). Protein Hpn: cloning and characterization of a histidine-rich metal-binding polypeptide in *Helicobacter pylori* and *Helicobacter mustelae*. *Infect Immun* **63**, 2682–2688.

Hantke, K. (2001). Iron and metal regulation in bacteria. *Curr Opin Microbiol* **4**, 172–177.

Harris, A. G., Hinds, F. E., Beckhouse, A. G., Kolesnikow, T. & Hazell, S. L. (2002). Resistance to hydrogen peroxide in *Helicobacter pylori*: role of catalase (KatA) and Fur, and functional analysis of a novel gene product designated 'KatA-associated protein', KapA (HP0874). *Microbiology* **148**, 3813–3825.

Homuth, G., Masuda, S., Mogk, A., Kobayashi, Y. & Schumann, W. (1997). The *dnaK* operon of *Bacillus subtilis* is heptacistronic. *J Bacteriol* **179**, 1153–1164.

Israel, D. A., Salama, N., Arnold, C. N. & 8 other authors (2001a). *Helicobacter pylori* strain-specific differences in genetic content, identified by microarray, influence host inflammatory responses. *J Clin Invest* **107**, 611–620.

Israel, D. A., Salama, N., Krishna, U., Rieger, U. M., Atherton, J. C., Falkow, S. & Peek, R. M., Jr (2001b). *Helicobacter pylori* genetic diversity within the gastric niche of a single human host. *Proc Natl Acad Sci U S A* **98**, 14625–14630.

Josenhans, C., Eaton, K. A., Thevenot, T. & Suerbaum, S. (2000). Switching of flagellar motility in *Helicobacter pylori* by reversible length variation of a short homopolymeric sequence repeat in *fliP*, a gene encoding a basal body protein. *Infect Immun* **68**, 4598–4603.

Kim, N., Marcus, E. A., Wen, Y., Weeks, D. L., Scott, D. R., Jung, H. C., Song, I. S. & Sachs, G. (2004). Genes of *Helicobacter pylori* regulated by attachment to AGS cells. *Infect Immun* **72**, 2358–2368.

Masse, E. & Gottesman, S. (2002). A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. *Proc Natl Acad Sci U S A* **99**, 4620–4625.

Merrell, D. S., Goodrich, M. L., Otto, G., Tompkins, L. S. & Falkow, S. (2003a). pH-regulated gene expression of the gastric pathogen *Helicobacter pylori*. *Infect Immun* **71**, 3529–3539.

Merrell, D. S., Thompson, L. J., Kim, C. C., Mitchell, H., Tompkins, L. S., Lee, A. & Falkow, S. (2003b). Growth-phase-dependent response of *Helicobacter pylori* to iron starvation. *Infect Immun* **71**, 6510–6525.

Ollagnier-De-Choudens, S., Mulliez, E., Hewitson, K. S. & Fontecave, M. (2002). Biotin synthase is a pyridoxal phosphate-dependent cysteine desulfurase. *Biochemistry* **41**, 9145–9152.

Olson, J. W., Agar, J. N., Johnson, M. K. & Maier, R. J. (2000). Characterization of the NifU and NifS Fe-S cluster formation proteins essential for viability in *Helicobacter pylori*. *Biochemistry* **39**, 16213–16219.

Olson, J. W., Mehta, N. S. & Maier, R. J. (2001). Requirement of nickel metabolism proteins HypA and HypB for full activity of both hydrogenase and urease in *Helicobacter pylori*. *Mol Microbiol* **39**, 176–182.

O'Toole, P. W., Lane, M. C. & Porwollik, S. (2000). *Helicobacter pylori* motility. *Microbes Infect* **2**, 1207–1214.

Palyada, K., Threadgill, D. & Stintzi, A. (2004). Iron acquisition and regulation in *Campylobacter jejuni*. *J Bacteriol* **186**, 4714–4729.

Pease, A. J., Roa, B. R., Luo, W. & Winkler, M. E. (2002). Positive growth rate-dependent regulation of the *pdxA*, *ksgA*, and *pdxB* genes of *Escherichia coli* K-12. *J Bacteriol* **184**, 1359–1369.

Pesci, E. C. & Pickett, C. L. (1994). Genetic organization and enzymatic activity of a superoxide dismutase from the micro-aerophilic human pathogen, *Helicobacter pylori*. *Gene* **143**, 111–116.

Salama, N., Guillemin, K., McDaniel, T. K., Sherlock, G., Tompkins, L. & Falkow, S. (2000). A whole-genome microarray reveals genetic diversity among *Helicobacter pylori* strains. *Proc Natl Acad Sci U S A* **97**, 14668–14673.

Sanyal, I., Cohen, G. & Flint, D. H. (1994). Biotin synthase: purification, characterization as a [2Fe-2S]cluster protein, and in vitro activity of the *Escherichia coli bioB* gene product. *Biochemistry* **33**, 3625–3631.

Seyler, R. W., Jr, Olson, J. W. & Maier, R. J. (2001). Superoxide dismutase-deficient mutants of *Helicobacter pylori* are hypersensitive to oxidative stress and defective in host colonization. *Infect Immun* **69**, 4034–4040.

Skouloubris, S., Labigne, A. & De Reuse, H. (2001). The AmiE aliphatic amidase and AmiF formamidase of *Helicobacter pylori*: natural evolution of two enzyme paralogues. *Mol Microbiol* **40**, 596–609.

Spiegelhalder, C., Gerstenecker, B., Kersten, A., Schiltz, E. & Kist, M. (1993). Purification of *Helicobacter pylori* superoxide dismutase and cloning and sequencing of the gene. *Infect Immun* **61**, 5315–5325.

Thompson, L. J., Merrell, D. S., Neilan, B. A., Mitchell, H., Lee, A. & Falkow, S. (2003). Gene expression profiling of *Helicobacter pylori* reveals a growth-phase-dependent switch in virulence gene expression. *Infect Immun* **71**, 2643–2655.

Tomb, J. F., White, O., Kerlavage, A. R. & 39 other authors (1997). The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**, 539–547.

van Vliet, A. H. M., Kuipers, E. J., Waidner, B. & 7 other authors (2001). Nickel-responsive induction of urease expression in *Helicobacter pylori* is mediated at the transcriptional level. *Infect Immun* **69**, 4891–4897.

van Vliet, A. H. M., Stoof, J., Vlasblom, R. & 10 other authors (2002a). The role of the Ferric Uptake Regulator (Fur) in regulation of *Helicobacter pylori* iron uptake. *Helicobacter* **7**, 237–244.

van Vliet, A. H. M., Ketley, J. M., Park, S. F. & Penn, C. W. (2002b). The role of iron in *Campylobacter* gene regulation, metabolism and oxidative stress defense. *FEMS Microbiol Rev* **26**, 173–186.

van Vliet, A. H. M., Stoof, J., Poppelaars, S. W., Bereswill, S., Homuth, G., Kist, M., Kuipers, E. J. & Kusters, J. G. (2003). Differential regulation of amidase- and formamidase-mediated ammonia production by the *Helicobacter pylori* Fur repressor. *J Biol Chem* **278**, 9052–9057.

van Vliet, A. H. M., Kuipers, E. J., Stoof, J., Poppelaars, S. W. & Kusters, J. G. (2004). Acid-responsive gene induction of ammonia-producing enzymes in *Helicobacter pylori* is mediated via a metal-responsive repressor cascade. *Infect Immun* **72**, 766–773.

Waidner, B., Greiner, S., Odenbreit, S. & 11 other authors (2002). Essential role of ferritin Pfr in *Helicobacter pylori* iron metabolism and gastric colonization. *Infect Immun* **70**, 3923–3929.

Wen, Y., Marcus, E. A., Matrubutham, U., Gleeson, M. A., Scott, D. R. & Sachs, G. (2003). Acid-adaptive genes of *Helicobacter pylori*. *Infect Immun* **71**, 5921–5939.