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)

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***Helicobacter pylori* infection elicits an aggressive inflammatory response that the bacterium is able to resist by virtue of its well-adapted antioxidant defence mechanisms. Catalase (KatA) appears to be a key enzyme in this resistance. Upstream of *kata*, a low-affinity ferric uptake regulator (Fur)-box has been identified. Downstream of *kata*, an ORF (HP0874) with no known function has also been identified. Non-polar isogenic mutants of *kata*, *fur* and HP0874 were constructed by allelic exchange. The impact of these mutations on the catalase activities and bacterial viability following exposure to hydrogen peroxide was studied. Concurrently, the effect of variation in the iron content of the media used to grow the cells was determined. The data showed that catalase-deficient isolates of *H. pylori* were hypersensitive to hydrogen peroxide, whereas wild-type cells could resist ~100 mM hydrogen peroxide. Fur-deficient mutants and cells grown on low-iron-containing medium showed a distinct reduction in catalase activity and increased sensitivity to hydrogen peroxide. The data suggest a direct or indirect effect of Fur and iron on the activity of catalase. HP0874-deficient mutants showed no reduction in catalase activity but showed an increased sensitivity to hydrogen peroxide. That is, the protein encoded by HP0874 appears to have a role in resistance to hydrogen peroxide not directly related to catalase activity. This is the first report of a functional relationship of the product of this ORF. There is evidence of protein-protein interaction between KatA and the product encoded by HP0874, and the name 'KatA-associated protein' (KapA) is proposed.**

Keywords: influence of iron on catalase activity, oxidative stress response

INTRODUCTION

Helicobacter pylori is one of the most common agents of infectious disease, infecting between 20 and 90% of populations in developed and developing countries, respectively (Feldman *et al.*, 1997). *H. pylori* causes acute (active) or chronic gastritis (Dixon, 1994; Hazell

et al., 1987) and can result in the development of peptic ulcer disease (Graham, 1989) and gastric carcinoma (Parsonnet *et al.*, 1991; Uemura *et al.*, 2001; Wotherspoon *et al.*, 1991).

Gastritis is characterized by an infiltration of plasma cells, lymphocytes, neutrophils and monocytes into the lamina propria and epithelium (Crabtree, 1996; Dixon, 1994; Hazell *et al.*, 1987; Ernst, 1999). Infiltrating inflammatory cells release reactive oxygen species (ROS) (Ernst, 1999). *H. pylori* is able to withstand ROS, including the oxidative burst due to the frustrated phagocytosis, as a result of the well-adapted antioxidant defences (Hazell *et al.*, 2001; Ramarao *et al.*, 2000).

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Abbreviations: CSA, *Campylobacter*-selective agar; Fur, ferric uptake regulator; ROS, reactive oxygen species.

Hydrogen peroxide has the potential to cause widespread cellular damage; however, it is the rapid formation of other ROS from hydrogen peroxide, including hydroxyl radicals, which poses more of a threat (Cadenas, 1989; Farr *et al.*, 1988; Nunoshiba *et al.*, 1999). While no enzymic protection against the hydroxyl radicals exists, catalase (EC 1.11.1.6) is responsible for the removal of hydrogen peroxide, whether generated exogenously or endogenously.

H. pylori catalase is expressed in high levels, accounting for approximately 1% of the cell's total protein (Hazell *et al.*, 1991). The characteristic 'explosive' reaction observed when *H. pylori* is exposed to hydrogen peroxide is used as an identification test (Harris & Hazell, 2000). *In vitro*, *H. pylori* can stimulate the neutrophilic oxidative burst in gastric cell lines (Mooney *et al.*, 1991). Catalase appears to be important to the *in vitro* survival of *H. pylori* in the presence of professional phagocytes (Ramarao *et al.*, 2000), although not essential *in vitro* under standard growth conditions (indicating that endogenously generated hydrogen peroxide is not a problem) (Manos *et al.*, 1997; Odenbreit *et al.*, 1996; Westblom *et al.*, 1992).

Iron, in the presence of hydrogen peroxide, acts as a catalyst to generate more ROS. The ferric uptake regulator (Fur) and Fur homologues, such as PerR, are involved in the regulation of enzymes involved in the detoxification of ROS (Herbig & Helmann, 2001), for example *sodA* of *Escherichia coli* (Naik & Hassan, 1992). Fur is an iron-dependent positive regulator of *katA* in *Staphylococcus aureus* (Horsburgh *et al.*, 2001) and a negative regulator of catalase-peroxidase (*catC*) of *Streptomyces coelicolor* (Hahn *et al.*, 2000). PerR of *Bacillus subtilis* is involved in sensing hydrogen peroxide (Herbig & Helmann, 2001), and several organisms possess PerR, including *Campylobacter jejuni* (Van Vliet *et al.*, 1999); however, *H. pylori* appears to lack PerR (Alm *et al.*, 1999; Tomb *et al.*, 1997).

The catalase of *H. pylori* (KatA) has been characterized (Hazell *et al.*, 1991) and subsequently sequenced (Manos *et al.*, 1997; Odenbreit *et al.*, 1996). A putative Fur-box upstream of the coding region suggested a possible role for Fur in the regulation of KatA (Manos *et al.*, 1997; Odenbreit *et al.*, 1996). Delany *et al.* (2001b) demonstrated that the putative Fur-box upstream of *katA* was modulating the expression of *frpB*. However, a low-affinity Fur-binding region was identified closer to, but not overlapping, the -35 and -10 sites of *katA*. Delany *et al.* (2001b) were unable to detect any transcriptional difference in the expression of *katA* between the wild-type and a *fur*-deficient mutant of *H. pylori*. Despite these data, the potential for iron and Fur to influence catalase expression or activity in *H. pylori* is worthy of further investigation.

Approximately 150 bp downstream of *katA*, an ORF of unknown function has been identified (Alm *et al.*, 1999; Manos *et al.*, 1997; Odenbreit *et al.*, 1996; Tomb *et al.*, 1997). This ORF, designated HP0874 by TIGR (Tomb *et al.*, 1997), appears to be conserved in all

strains of *H. pylori* (Salama *et al.*, 2000) with the only apparent homologue being a putative gene encoding a protein of unknown function in *Pasteurella multocida* (<http://www.tigr.org>) (May *et al.*, 2001).

Putative stem-loop structures in the intergenic regions between HP0874 and *katA* suggest that both *katA* and HP0874 are transcribed independently. However, there are no recognizable RNA polymerase binding sites upstream of HP0874 (Manos, 1998; Odenbreit *et al.*, 1996). Using a yeast two-hybrid model, Rain *et al.* (2001) have indicated that the protein encoded by HP0874 interacts with KatA. This possible interaction suggests a role for HP0874, directly or indirectly, in the oxidative stress response of *H. pylori*. When HP0874 was disrupted using transposon mutagenesis (Odenbreit *et al.*, 1996), no differences in the catalase activities of cell-free extracts were observed, resulting in the conclusion that HP0874 does not affect catalase activity.

The *in vitro* response of *H. pylori* to hydrogen peroxide has been the subject of limited investigation, despite the purification and molecular characterization of KatA (Alm *et al.*, 1999; Hazell *et al.*, 1991; Manos *et al.*, 1997; Odenbreit *et al.*, 1996; Tomb *et al.*, 1997). Further, studies of the role of Fur and Fur-like homologues and the importance of iron in the regulation of the oxidative stress response of *H. pylori* have been limited. The aims of the current investigation were to study the roles of KatA, HP0874 and Fur and extracellular iron in the response of *H. pylori* to hydrogen-peroxide-induced stress. We present data indicating a role for HP0874 in resistance to oxidative damage and propose the name 'KatA-associated protein' (*kapa*).

METHODS

Bacteria and routine culture conditions. *Helicobacter pylori* strain 26695 was obtained from the culture collection at the School of Microbiology and Immunology, University of New South Wales. Cultures of *H. pylori* were maintained on *Campylobacter*-selective agar (CSA) and incubated for 36–48 h in a CO₂ incubator (Heraeus Instruments) at 37 °C with a reduced oxygen environment (micro-aerobic) consisting of 10% CO₂ in air and 95% relative humidity. Culture purity was confirmed by phase-contrast microscopy, and rapid positive urease and catalase activities as determined by the addition of cells to urease reagent (Hazell *et al.*, 1987) and hydrogen peroxide, respectively.

Cultures of *Escherichia coli* DH5 α were grown on Luria-Bertani agar or broth as described by Sambrook *et al.* (1989). The agar was supplemented with antibiotics as appropriate (50 μ g ampicillin ml⁻¹ or 50 μ g kanamycin ml⁻¹).

A summary of bacteria used in the current study and their genotypes are shown in Table 1.

Media preparation. Cultures of *H. pylori* were maintained on CSA consisting of blood agar base No. II (Oxoid) 5% (v/v) defibrinated horse blood (Oxoid), 4.2 mg amphotericin B ml⁻¹ (ER Squibb and Sons) and Skirrow's selective supplement (Skirrow, 1977). CSA using Columbia agar base (Oxoid) in preference to blood agar base no. II was used for the transformation of *H. pylori*, with kanamycin (20 μ g ml⁻¹) supplementing the media as appropriate.

Preparation of iron-depleted media. IsoSensitest agar and broth (Oxoid) are chemically defined media used in antibiotic sensitivity testing and support growth of *H. pylori* if supplemented with serum (Hazell *et al.*, 1989). These media were prepared as per the manufacturer's instructions. In these experiments, we used Chelex to remove iron and other ions, and reconstituted the medium with the ions, apart from iron, according to the published formulation of IsoSensitest medium. We chose Chelex over other iron chelators as it facilitated the physical removal of iron from the medium, eliminating the potential for *H. pylori* to extract the iron from the chelated complexes *in situ*, a potential problem when other chelators such as Desferal or EGTA are used.

Horse serum (100 ml) (Oxoid) was mixed with 25 g Chelex (Sigma) with continual stirring at 4 °C for 16 h to remove divalent cations. The Chelex was regenerated and the process of removing divalent cations repeated. After removal of divalent cations, the horse serum was filter-sterilized through a 0.22 µm filter and stored frozen until required. The IsoSensitest broth (1 l) was depleted of divalent cations using Chelex as outlined above. Agar (15 g l⁻¹) was added to the broth, sterilized by autoclaving and allowed to cool to 48 °C before the addition of 100 ml of divalent-cation-depleted horse serum, Skirrow's selective supplement and amphotericin B. The medium was supplemented with 1 mg CuSO₄, CoSO₄·7H₂O, ZnSO₄·7H₂O and MgSO₄·7H₂O l⁻¹, 2 mg MnCl₂ l⁻¹ and 100 mg calcium gluconate l⁻¹ replacing cations removed by Chelex, in accordance with the chemical definition of IsoSensitest. The medium was used within 24 h of preparation.

Preparation of iron-supplemented media. Iron-supplemented medium was made by the addition of FeSO₄ (100 mM) to IsoSensitest agar. The medium was supplemented with sterile horse serum (5% v/v), Skirrow's selective supplement and amphotericin B, and used within 24 h of preparation.

Atomic absorbance spectroscopy. To quantify the amount of iron present in each of the media used to culture *H. pylori*, atomic absorbance spectroscopy was carried out on 20 ml samples of broth prior to the addition of agar. This procedure was performed using a series of FeSO₄ standards ranging from 0.1 p.p.m. Fe to 100 p.p.m. Fe as a reference. The spectra were obtained using a Varian SpectrAA-200 (Varian) and a hollow cathode lamp for Fe (Photron) as per the manufacturer's instructions. All spectra were determined twice. The presence of erythrocytes in the blood-based media prohibited the determination of iron content.

Protein concentration determination. The protein concentration of each sample was determined using the bicinchonic acid method based on a microtitre plate assay (Pierce). The concentration of each sample was calculated based on the values obtained for bovine serum albumin standards, these being 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mg protein ml⁻¹.

Catalase activities of cell-free extracts. Cell-free extracts of *H. pylori* were obtained using the freeze-thaw method of lysis (Mendz *et al.*, 1998). The catalase activities of the cell-free extracts of *H. pylori* 26695 wild-type and 26695 *fur::aphA3* grown on different media were determined using the method of Beers & Sizer (1952). The data, expressed as µmol hydrogen peroxide decomposed min⁻¹ (mg protein)⁻¹, were determined a total of nine times for each sample. The limit of sensitivity for the test was 1 unit (mg protein)⁻¹.

Measuring of hydrogen peroxide concentration. The hydrogen peroxide was diluted so the absorbance measured at 240 nm in a quartz cuvette, with a path length of 1 cm, was

between 0.1 and 0.9. The concentration of hydrogen peroxide was calculated using the absorption coefficient of 43.48 l mol⁻¹ cm⁻¹ (Beers & Sizer, 1952; Hazell *et al.*, 1991).

Time-dependent killing by hydrogen peroxide. Fresh 24 h cultures of *H. pylori* 26695 wild-type, *kata*, HP0874 (*kapA*) and *fur* isogenic mutants, grown on CSA (blood-based media), IsoSensitest agar (serum-based), iron-depleted or iron-supplemented IsoSensitest Agar were harvested in 0.9% (w/v) NaCl (physiological saline). The cells were washed once and resuspended in physiological saline. The optical densities of the cell suspensions, measured at 600 nm, were adjusted to 0.7. Cells were used immediately.

At time zero, 200 µl of the cell suspension was added to 1.8 ml of either 98 or 49 mM hydrogen peroxide. Immediately after the addition of the cell suspension, 100 µl was removed and combined with 100 µl 1% (w/v) bovine liver catalase (Sigma) to dismutate the hydrogen peroxide. Four 10-fold dilutions were made in physiological saline, which were cultured in 10 µl spots, in triplicate, onto 'dry' CSA plates. Every 4 min, the process was repeated, for a total of 60 min. The agar plates were incubated for 3–5 days, after which individual colonies were counted. Negative controls, where water was used in place of hydrogen peroxide, were carried out with all strains grown on all four different media. The response to hydrogen peroxide by the isogenic mutants was determined at least twice for each medium.

Molecular manipulation of *H. pylori*. Genomic DNA from *H. pylori* 26695 was extracted using the Wizard DNA preparation kit (Promega). Plasmids were extracted from *E. coli* using an alkaline lysis-based kit (Quantum mini prep, Bio-Rad). All restriction digests and other molecular manipulations were carried out according to the manufacturer's instructions. RNA was extracted from *H. pylori* using the RNeasy RNA extraction kit (Qiagen), and the quality of RNA was assessed by formaldehyde gel electrophoresis as described by Sambrook *et al.* (1989). Sequencing was carried out using the BigDye Terminator kit from ABI PRISM (Applied Biosystems). The separation of labelled fragments of DNA was carried out using a 377 sequencer (Applied Biosystems) automated sequencing facility, University of New South Wales. Results were obtained using the INHERIT software package (Applied Biosystems). RT-PCR was carried out using the Enhanced Avian RT-PCR kit (Sigma-Aldrich).

RT-PCR. RT-PCR was carried out according to the manufacturer's instructions. The primer sequences designed for RT-PCR are listed in Table 1. Primer pairs (KatA 557F, KatA 1242R; KatA 1001F, KapA 103R; KapA 423F, KapA 824R; Fig. 1) were designed to allow the detection of transcripts specific for *kata*, specific for *kapA* and for the intragenic region between the two genes. The primers were annealed stepwise (70 °C for 2 min; 65, 60, 55, 50 and 45 °C for 1 min each step) before the reverse transcriptase was added. The high temperatures at the beginning of the stepwise annealing denatures the stem-loop structures in the RNA (Tillet *et al.*, 2000). The RT reaction was performed at 45 °C for 30 min, followed by five cycles of 50 °C for 1 min, 53 °C for 1 min and 56 °C for 1 min. A control without the reverse transcriptase was used to determine whether the RNA was free of DNA. PCR followed using the following parameters: 94 °C for 4 min and a cycle of 94 °C for 10 s, 50 °C for 10 s and 72 °C for 1 min, repeated 35 times. A final elongation step of 72 °C for 7 min followed. RT-PCR products were analysed using agarose gel electrophoresis.

Polymerase chain reaction. Fragments of DNA to be cloned into vectors were amplified using PCR. All PCRs were carried

Table 1. Bacterial strains, plasmids and primer sequences used in the current study

Strain, plasmid or primer	Characteristics or sequence	Source
<i>Helicobacter pylori</i>		
26695 wild-type	Wild-type, genome sequenced	Eaton <i>et al.</i> (1991); Tomb <i>et al.</i> (1997)
26695 <i>katA::aphA3</i>	Allelic exchange product of 26695 when transformed with pΔKatA; catalase-deficient	This study
26695 <i>kapA::aphA3</i>	Allelic exchange product of 26695 when transformed with pΔKapA	This study
26695 <i>fur::aphA3</i>	Allelic exchange product of 26695 when transformed with pΔFur	This study
<i>Escherichia coli</i>		
DH5α	F ⁻ <i>supE44</i> Δ(<i>lacZYA-argF</i>)U169 (φ80 <i>lacZ</i> ΔM15) <i>hsdR17</i> (r _k ⁻ m _k ⁺) <i>recA1 endA1 gyrA96 thi-1 relA1</i>	Life Technologies
Plasmids		
pGem-T easy	Ap ^r , cloning vector	Promega
pUC19	Ap ^r , cloning vector	New England Biolabs
pKSF	Ap ^r Km ^r , source of <i>aphA3</i>	Copass <i>et al.</i> (1997)
pKatA	Ap ^r , 1863 bp PCR fragment containing <i>H. pylori katA</i> cloned into pGem-T easy	This study
pΔKatA	Ap ^r Km ^r , <i>katA::aphA3</i> . 1.2 kb <i>aphA3</i> from pKSF ligated into unique <i>ClaI</i> site of <i>katA</i>	This study
pKapA	Ap ^r , 1918 bp PCR fragment containing <i>H. pylori kapA</i> (HP0874) directionally cloned into <i>SacI</i> and <i>SalI</i> sites of pUC19	This study
pKapA II	Ap ^r , construct with <i>PstI</i> site removed from vector backbone by double digest using <i>SapI</i> and <i>SalI</i> , resulting in unique <i>PstI</i> site in <i>kapA</i>	This study
pKapA III	Ap ^r , unique <i>PstI</i> site in <i>kapA</i> (HP0874) destroyed, unique <i>XhoI</i> site created	This study
pΔKapA	Ap ^r Km ^r , <i>kapA::aphA3</i> , 1.2 kb <i>aphA3</i> from pKSF ligated into unique <i>XhoI</i> site of <i>kapA</i> (HP0874) [in pKapA (HP0874) III]	This study
pFur	Ap ^r , 1.65 kb PCR fragment containing <i>H. pylori fur</i> directionally cloned into <i>PstI</i> and <i>BamHI</i> sites of pUC19	This study
pΔFur	Ap ^r Km ^r , <i>fur::aphA3</i> ; 1.2 kb <i>aphA3</i> from pKSF ligated to unique <i>BclI</i> site in <i>fur</i>	This study
Primers		
HpKatA F	GacgtcgacCGGCTTGGTGTCTAGATTACG; recognition site for <i>SalI</i> <u>underlined</u>	This study
HpKatA R	GacgtcgacTGCAGAATCAAAATCAATGCTG; recognition site for <i>SalI</i> <u>underlined</u>	This study
HpKapA F	GtggagctcCGATTCCATCCGTTTGATGTTAC; recognition site for <i>SacI</i> <u>underlined</u>	This study
HpKapA R	GaggtcgacCTCCATAAAAGGTAGCATCACCAAC; recognition site for <i>SalI</i> <u>underlined</u>	This study
<i>XhoI</i> Linker I	AGATCTCGAGTGCA; recognition site for <i>XhoI</i> <u>underlined</u>	This study
<i>XhoI</i> Linker II	CTCGAGATCTTGCA; recognition site for <i>XhoI</i> <u>underlined</u>	This study
HpFur F	TagcctcgagTGATACGCATTATAACCTTACTAGC; recognition site for <i>PstI</i> <u>underlined</u>	This study
HpFur R	AgatggatccTCAAATTAAATTATGAGATTGGTG; recognition site for <i>BamHI</i> <u>underlined</u>	This study
Kan I F	CcatcgatctcgagCAGCGCGCTTATCAATATATC; recognition sites for <i>ClaI</i> in bold and <i>XhoI</i> <u>underlined</u>	Copass <i>et al.</i> (1997)
Kan II F	TaagatctgatcaCAGCGCGCTTATCAATATATC; recognition sites for <i>BclI</i> <u>underlined</u>	Copass <i>et al.</i> (1997)
Kan I R	CcatcgatctcgagTTTTAGACATCTAAATCTAGCTAC; recognition sites for <i>ClaI</i> in bold and <i>XhoI</i> <u>underlined</u>	Copass <i>et al.</i> (1997)
Kan II R	TcgcgctgatcaTTTTAGACATCTAAATCTAGGTAC; recognition sites for <i>BclI</i> <u>underlined</u>	Copass <i>et al.</i> (1997)
KatA 557 F	TCCCTAAATCTTTCCGCC; forward primer used to sequence <i>katA</i> from <i>H. pylori</i> 26695 <i>katA::aphA3</i> ; also used in RT-PCR	This study
KatA 1001 F	GAAAGAGTTTGAAGTGTTGG; reverse primer used to sequence <i>katA</i> from <i>H. pylori</i> 26695 <i>katA::aphA3</i> ; also used in RT-PCR	This study

Table 1. (cont.)

Strain, plasmid or primer	Characteristics or sequence	Source
KatA 1249 R	CCACACTTCAAACCTCTTTC; forward primer used to sequence <i>kapA</i> (HP0874) from <i>H. pylori</i> 26695 <i>kapA</i> :: <i>aphA3</i> ; also used in RT-PCR	This study
KapA 103 R	TTGCAGCACTCCCTTTGTC; reverse primer used in RT-PCR of <i>kapA</i> (HP0874) and KatA intragenic region from <i>H. pylori</i> 26695	This study
KapA 423 F	AACATTGCCAACCAGCTC; forward primer used in RT-PCR of <i>kapA</i> (HP0874) from <i>H. pylori</i> 26695	This study
KapA 824 R	CCTAAATCCCTACCATCC; reverse primer used to sequence <i>kapA</i> (HP0874) from <i>H. pylori</i> 26695 <i>kapA</i> :: <i>aphA3</i> ; also used in RT-PCR	This study
Fur 120 F	GTCATGGCTAATCAGCTTGG; forward primer used to sequence <i>fur</i> from <i>H. pylori</i> 26695 <i>fur</i> :: <i>aphA3</i>	This study
Fur 405 R	CACACACCTAAGCCCTGAAG; reverse primer used to sequence <i>fur</i> from <i>H. pylori</i> 26695 <i>fur</i> :: <i>aphA3</i>	This study
M13/pUC F	CGCCAGGGTTTTCCAGTCACGAC	New England Biolabs
M13/pUC R	AGCGGATAACAATTCACACAGGA	New England Biolabs

* Primer sequences: upper-case letters indicate nucleotides present in template DNA, and lower-case letters indicate nucleotides added for cloning purposes.

Fig. 1. Schematic diagram of the insertional deactivation of *katA* (HP0875), *kapA* (HP0874) and *fur* (HP1027). Thick arrows indicate the direction of transcription, and smaller arrows indicate the position and direction of the PCR primers used in the cloning and sequencing of these genes. Restriction enzyme abbreviations: B, *BclI*; C, *Clal*; P, *PstI*; X, *XhoI*. See Table 1 for sequences of primers and *XhoI* linkers.

out in 20 µl volumes and consisted of the appropriate volume of PCR buffer, 2.125 mM MgCl₂, dNTPs (final concentration 0.2 mM each dNTP), 10–100 ng template DNA, 10 pmol each primer and 0.5 U of a (9:1) *Taq/Pfu* DNA polymerase mix. All primers used in the current study are listed in Table 1 and were synthesized at Genset Pacific Oligos (Lismore, Australia) and

obtained using previously published sequences for *katA*, HP0874 (*kapA*), *fur* (Tomb *et al.*, 1997) and *aphA3* (Copass *et al.*, 1997). The reactions were carried out in an Applied Biosystems thermal cycler using identical cycling parameters. These parameters were as follows: 80 °C, until the DNA polymerase had been added, 94 °C for 4 min, followed by 30

cycles of 94 °C for 10 s, 50 °C for 10 s with elongation at 68 °C for 1 min per kb to be extended. A final extension period of 8 min at 68 °C was performed before PCR fragments were analysed by agarose gel electrophoresis.

Construction of pKatA and pKata. Genomic DNA from *H. pylori* 26695 was extracted and *katA* amplified using the PCR primers HpKatA F and HpKatA R. The 1863 bp PCR fragment was cloned into the multiple cloning site of pGEM-T Easy (Promega) and transformed into chemically competent *E. coli* DH5 α , as described by Hanahan (1983). The construct, pKatA, was 4896 bp in length, and a unique site recognized by the restriction endonuclease *Clal* was found 819 bp downstream of the ATG start codon of *katA*. PCR was used to amplify the gene encoding kanamycin resistance, kanamycin phosphotransferase, *aphA3* (Km^r) with primers Kan I F and Kan I R, designed to be in-frame, in the same orientation and lacking transcriptional terminator sequences, using pKSF as the template (Copass *et al.*, 1997). The PCR product was digested by *Clal* (New England Biolabs) and ligated into the unique *Clal* site of pKatA, using T₄ DNA ligase (New England Biolabs). Restriction mapping, PCR and nucleotide sequencing were used to confirm the new construct, pKataA, and to determine the orientation of *aphA3* relative to *katA* (Fig. 1).

Construction of pKapA, pKapA II, pKapA III and pAKapA. HP0874 (*kapA*) was amplified using primers HpKapA F and HpKapA R (1918 bp) and genomic DNA extracted from *H. pylori* 26695 as a template. The PCR product was directionally cloned into the *SacI* and *Sall* restriction sites in the multiple cloning site of pUC19. The resulting plasmid, pKapA, was digested with *SapI* and *Sall* to remove the *PstI* site in the vector backbone. The non-compatible sticky ends produced from the double digest were filled in using a Klenow fragment, as described by Sambrook *et al.* (1989). The linear plasmid was gel-purified and allowed to self-ligate to form a new construct termed pKapA II. The removal of the *PstI* site from the vector resulted in a single *PstI* site, 147 bp downstream from the ATG start codon of HP0874 (*kapA*). A new restriction site was created by digestion of pKapA II with *PstI* and ligation with an equimolar linker mix (*XhoI* linker 1 and *XhoI* linker 2). Upon ligation to the linker, the *PstI* site in the pKapA II was destroyed, and a unique *XhoI* restriction site was created. To ensure successful construction of the unique *XhoI* site, *PstI* was incorporated into the ligation reaction, further ensuring that only plasmids with the *XhoI* restriction site were transformed into *E. coli*. The construct was termed pKapA III. PCR was used to amplify *aphA3* using the primers Kan I F and Kan I R with pKSF as the template (Copass *et al.*, 1997). The PCR product was digested by *XhoI* and inserted into the unique *XhoI* restriction site in pKapA III. The resulting construct, pAKapA, and orientation of *aphA3* relative to HP0874 (*kapA*) were confirmed by restriction mapping, PCR and nucleotide sequencing.

Construction of pFur and pAFur. A 1.65 kb fragment containing *fur* was amplified from *H. pylori* 26695 genomic DNA using the primers HpFur F and HpFur R. Recognition sites for *PstI* and *BamHI* were incorporated into the 5' end of these primers to allow directional insertion into the pUC19 vector. The construct containing *fur* was termed pFur. A unique site recognized by the restriction endonuclease *BclI* was present 291 bp downstream of the ATG start codon of *fur*. The kanamycin resistance cassette, *aphA3*, was amplified by PCR using the primers Kan II F and Kan II R. The PCR product was digested with *BclI* and ligated in-frame and lacking transcriptional terminator sequences into the unique *BclI* restriction

site within the ORF of the *fur* gene. The ligation product, termed pAFur, was transformed into chemically competent *E. coli*. PCR and nucleotide sequencing were used to confirm that *aphA3* was in the same orientation as *fur*.

Transformation of *H. pylori*. *H. pylori* was grown on CSA made using Columbia agar base (Oxoid) for 24 h at 37 °C in a micro-aerobic environment. Cells were harvested with Brain Heart Infusion (Oxoid), centrifuged at room temperature for 30 s at 15 000 *g* and resuspended in a minimal volume of 0.9% (w/v) NaCl. The resultant cell suspension was placed onto dry CSA plates in well-separated 10 μ l aliquots and allowed to incubate at room temperature for 15 min, before being incubated in a CO₂ incubator for 5 h. Ten microlitres of plasmid DNA (varied concentrations), prepared using the Quantum midi prep (Bio-Rad) was mixed with the bacterial 'spots' growing on CSA. The cells were incubated for a further 24 h before transferring the bacteria to CSA (Columbia agar base) containing 20 μ g kanamycin ml⁻¹. The cells were incubated for 24–72 h until colonies were present. DNA from the isolates was subjected to PCR and nucleotide sequencing to confirm that homologous recombination had occurred.

Statistical analysis. Statistical comparisons of the catalase activities of the Fur isogenic mutants and wild-type cells grown on the same media were carried out using the Mann–Whitney *U*-test. A statistical analysis between the same strains grown on different media was carried out using the Kruskal–Wallis analysis (SPSS).

RESULTS

RT-PCR of *katA* and *kapA* (HP0874)

RT-PCR was used to determine whether *katA* and *kapA* were part of a multi-cistronic operon. Products were observed when primers were directed towards the 3' end of *katA* (KatA 557F; KatA 1249R) and *kapA* (KapA 423F; KapA 824R). No product was detected, however, with primers KatA 1001F and KapA 103R, designed to detect a transcript that spanned the intragenic region, if it existed (Fig. 2a). This would indicate that *katA* and HP0874 are not part of a multi-cistronic operon, as there does not appear to be a single transcript for the two genes. The primer pairs produced products of the desired size when genomic DNA of *H. pylori* was used as a template in a PCR (not shown).

Construction of isogenic mutants of *katA*, *kapA* (HP0874) and *fur*

Isogenic mutants of *katA*, HP0874 (*kapA*) and *fur* were all created by allelic exchange. To validate the construction of the isogenic mutants, genomic DNA was extracted from the kanamycin-resistant isolates and subjected to PCR using the oligonucleotide primers used to clone the genes into the cloning vectors (Table 1). As determined by the size of the PCR products, each of the genes, *katA*, HP0874 (*kapA*) and *fur*, was successfully disrupted by *aphA3* (Fig. 3). Furthermore, the presence of *aphA3* in the isogenic mutants was confirmed by PCR using the primers Kan I F and Kan I R (Fig. 3). The PCR products of *H. pylori* 26695 *katA::aphA3*, *H. pylori* 26695 *kapA::aphA3* and *H. pylori* 26695 *fur::aphA3* were sequenced using the oligonucleotide primers out-

Fig. 2. (a) Agarose gel of RT-PCR of RNA extracted from *H. pylori* 26695 wild-type. Lanes: 1 and 2, primers KatA 557 F and KatA 1249R, using RNA as the template (the 692 bp product showing the presence of the *katA* transcript); 3 and 4, primers KatA 1001F and KapA 103R, using RNA as template [absence of a product (predicted size of 770 bp) indicating that no single transcript exists for *katA* and *kapA*]; 5 and 6, primers KapA 423F and KapA 824R, using RNA as template (the 401 bp product indicating that a transcript is present for *kapA*); 7 and 8, primers KatA 557 F and KatA 1249R, using RNA as the template (reverse transcriptase was omitted from the reaction). The absence of any bands indicates that the RNA samples contain no genomic DNA; 9, primers KatA 557 F and KatA 1249R, using genomic DNA as a template (positive control); 10, 1 kb marker. (b) Agarose gel of RT-PCR of RNA extracted from *H. pylori* 26695 wild-type and KatA-deficient mutant. Lanes: 1, λ HindIII digest; 2, 401 bp product produced using primers KapA 423F and KapA 824R from RNA extract from wild-type *H. pylori*; 3, 401 bp product produced using primers KapA 423F and KapA 824R from RNA extract from KatA-deficient strain of *H. pylori*, indicating that transcription of the KapA is occurring in the KatA isogenic mutant; 4 and 5, primers KapA 423F and KapA 824R used in an RT-PCR where the reverse transcriptase was omitted, indicating that RNA samples were free of DNA; 6, primers KapA 423F and KapA 824R using genomic DNA extracted from wild-type *H. pylori* 26695 (this lane was a positive control).

lined in Table 1, confirming the orientation of *aphA3* in each of these genes.

A PCR using the M13F and M13R sequencing primers (specific for plasmid DNA) was carried out to confirm that the DNA isolated from the isogenic mutants

Fig. 3. Confirmation of *katA*, *kapA* (HP0874) and *fur* allelic exchange isogenic mutants. (a) A 0.8% (w/v) agarose gel stained with ethidium bromide (lane, template, primers): 1, 26695 wild-type; HpKatA F and HpKatA R; 2, 26695 *katA::aphA3*; HpKatA F and HpKatA R; 3, 26695 Wt; HpKapA F and HpKapA R; 4, 26695 *kapA::aphA3*; HpKapA F and HpKapA R; 5, 26695 Wt; HpFur F and HpFur R; 6, 26695 *fur::aphA3*; HpFur F and HpFur R; 7, λ HindIII, DNA marker. (b) A 0.8% (w/v) agarose gel stained with ethidium bromide (lane, template, primers): 1, 26695 wild-type; Kan I F and Kan I R; 2, 26695 *katA::aphA3*; Kan I F and Kan I R; 3, 26695 Wt; Kan I F and Kan I R; 4, 26695 *kapA::aphA3*; Kan I F and Kan I R; 5, 26695 Wt; Kan I F and Kan I R; 6, 26695 *fur::aphA3*; Kan I F and Kan I R; 7, λ HindIII, DNA marker.

contained no DNA from the suicide vectors. No PCR product was obtained (data not shown).

RT-PCR of *kapA* (HP0874) in *katA* isogenic mutant and wild-type

In order to determine whether the isogenic mutant of *katA* was transcriptionally non-polar, RT-PCR was carried out on the RNA extracted from the wild-type *H. pylori* 26695 and the *katA* isogenic mutant (Fig. 2b). The primers used to determine whether *kapA* (HP0874) was expressed (KapA 423F; KapA 824R) produced a product 401 bp in length when RNA extracted from the wild-type and the *katA* isogenic mutant were used as templates in the RT-PCR. The same reaction, with the omission of the reverse transcriptase, was also carried out. No product was produced, indicating the absence of DNA from the sample of RNA from the samples. These results indicate that the insertion of *aphA3* into *katA* is non-polar, as transcription of the downstream gene is unaffected.

Fig. 4. Box plots of catalase activities [μmol hydrogen peroxide degraded min^{-1} (mg protein) $^{-1}$] of cell-free extracts of *H. pylori* 26695 wild-type and 26695 *fur::aphA3* when grown on IsoSensitest, iron-supplemented, iron-depleted and blood-based media. Values under each box are the median value for the group (represented by the line in the middle of the box).

Atomic absorbance spectroscopy of media

Atomic absorbance spectroscopy was used to determine the amount of iron present in the medium. The medium that had been treated with Chelex (iron depleted) had a total Fe^{2+} content of 0.255 mg l^{-1} . IsoSensitest supplemented with 5% horse serum (standard preparation) had a total Fe^{2+} content of 1.0 mg l^{-1} , approximately four times that of the iron-depleted medium. The total Fe^{2+} content of the iron-supplemented medium was 6.587 mg l^{-1} , approximately 6.5 times greater than the IsoSensitest media and 26 times greater than the iron-depleted medium. Atomic absorbance spectroscopy was not applied to CSA, due to the presence of erythrocytes.

Catalase activities of cell-free extracts

Catalase activities of cell-free extracts of wild-type and *Fur*-deficient mutants of *H. pylori* were measured to determine whether *Fur* and environmental iron (iron content of the medium) affected the catalase activity. Box plots of the catalase activities are presented in Fig. 4. There were statistically significant differences between the catalase activities of the wild-type strain and the *Fur*-deficient mutant (Mann–Whitney *U*-test at a confidence level of 5%), regardless of the medium used to culture the bacteria. The catalase activities of the *Fur*-deficient mutant were substantially lower than that of the wild-type cells.

The iron concentration of the media also affected the catalase activities of the cell (Fig. 4). Wild-type strain 26695 grown on the four different media shows that the differences observed between each the four groups are statistically significant (Kruskal–Wallis; $P \leq 0.05$). Media with limited iron resulted in lower levels of catalase expression, whereas media with ‘normal’ levels of iron promoted catalase activity. Excessive levels of iron did

Table 2. Catalase activity of cell-free extracts of *H. pylori* 26695 wild-type and the *H. pylori* KapA-deficient and *H. pylori* KatA-deficient mutant

Activity was determined spectroscopically by the method of Beers & Sizer (1952). The limit of sensitivity for the assay is 1 unit per milligram of protein. The results are means $\pm$ SE.

	Catalase activity [$\mu\text{mol H}_2\text{O}_2$ decomposed min^{-1} (mg protein) $^{-1}$]
26695 wild-type	445.43 ± 25.55
KapA-deficient mutant	467.30 ± 10.10
KatA-deficient mutant	00.00 ± 00.00

not promote catalase activity, with no significant difference in activity between cells grown on iron-supplemented medium compared with cells grown on a medium containing ‘normal’ levels of iron. Cell-free extracts prepared from cells grown on blood-based medium displayed lower catalase activities than cells grown on serum-based medium.

To test the interaction between HP0874 (KapA) and catalase, the catalase activities of the wild-type cells and the HP0874-deficient mutants were compared. Inactivation of HP0874 had no apparent effect on catalase activity, which is in accord with the results of Odenbreit *et al.* (1996) (Table 2).

Response of *H. pylori* to hydrogen peroxide

To determine how important KatA, HP0874 (KapA) and *Fur* were to resistance of oxidative stress in *H. pylori*, time-course experiments, exposing *H. pylori* to hydrogen peroxide, were performed. As a negative control, the bacteria were cultured on the four different media and exposed to water for 1 h, instead of hydrogen peroxide. There were no significant reductions in viability in any of the strains under the control conditions over the period of exposure (Fig. 5a).

When the strains of *H. pylori* grown on IsoSensitest medium were exposed to $\sim 100 \text{ mM}$ hydrogen peroxide, the KatA-deficient mutant experienced a rapid decline in viability (Fig. 5b). There was a decrease in viability of the HP0874 (KapA)-deficient mutant when exposed to $\sim 100 \text{ mM}$ hydrogen peroxide, and this was characterized by an initial lag period followed by a rapid decrease in viability (Fig. 5b). There was no apparent reduction in the viability of the *Fur*-deficient mutant and the control (26695 wild-type) (Fig. 5b).

H. pylori grown on iron-supplemented media and exposed to $\sim 100 \text{ mM}$ hydrogen peroxide (Fig. 5c) resulted in similar patterns to those of cells grown on IsoSensitest media without iron supplementation.

Growing the *H. pylori* strains on iron-limited media followed by exposure of the cells to $\sim 100 \text{ mM}$ hydrogen peroxide resulted in a rapid decrease in cellular viability

Fig. 5. Response of *H. pylori* 26695 wild-type (■), 26695 *katA::aphA3* (▽), 26695 *kapA::aphA3* (◇) and 26695 *fur::aphA3* (◆) to: (a) water over time (negative control); (b) 98 mM hydrogen peroxide when grown on IsoSensitest medium supplemented with 5% (v/v) horse serum; (c) 98 mM hydrogen peroxide when grown on iron-supplemented medium; (d) 98 mM hydrogen peroxide when grown on iron-limited medium; (e) 49 mM hydrogen peroxide when grown on iron-depleted medium; (f) 98 mM hydrogen peroxide when grown on blood-based medium. The error bars represent the standard deviation of each group.

for the *katA* isogenic mutant (Fig. 5d). Both HP0874 (KapA)- and Fur-deficient mutants experienced significant reductions in viability compared to the wild-type cells. The initial lag observed with the HP0874 (KapA)-deficient mutant, as noted above, was less apparent under these conditions. Rather, a decrease in viability during the first 20 min of exposure, which tapered off over the following 40 min, was observed. The viability of the Fur-deficient mutant decreased similarly to the HP0874 (KapA)-deficient mutant.

Exposure of cells grown on blood-based media to ~100 mM hydrogen peroxide was fatal to the KatA-deficient mutant (Fig. 5f). The HP0874 (*kapA*) isogenic mutant exhibited an initial 4 min lag followed by a rapid loss of cellular viability (4 logs or 99.99%) over the

initial 20 min, the rate decreasing over the remaining 40 min. The Fur-deficient mutant also experienced a significant drop in viability, 4 logs (99.99%), over the 60 min of exposure.

DISCUSSION

H. pylori has a potent set of antioxidant defences, including enzymes such as alkyl hydroperoxide reductase (Ahp), thioredoxin-linked thiol peroxidase (scavengase) (Alm *et al.*, 1999; Cha *et al.*, 1995; Stojiljkovic *et al.*, 1994; Wan *et al.*, 1997), superoxide dismutase (SodB) (Seyler *et al.*, 2001; Spiegelhalter *et al.*, 1993) and catalase (KatA) (Hazell *et al.*, 1991). See Hazell *et al.* (2001) for a review. We have undertaken a study examining catalase and the potential interaction

between *katA* and Fur, iron and a gene (HP0874) encoding a product that we have designated the KatA-associated protein (*kapA*). Not surprisingly, the *katA* isogenic mutant was shown to be hypersensitive to hydrogen peroxide. In contrast, wild-type cells were able to survive exposure to physiologically high concentrations of hydrogen peroxide (~ 100 mM). A catalase-deficient mutant of *Campylobacter coli* displayed a similar sensitivity when exposed to relatively low levels of hydrogen peroxide (~ 1 mM) (Grant & Park, 1995). That *H. pylori* wild-type was able to survive such high concentrations of hydrogen peroxide is indicative of the importance of catalase to the survival of the bacterium when exposed to ROS.

The catalase of *H. pylori* requires a haem prosthetic group. *H. pylori* has a number of iron-acquisition systems (Velayudhan *et al.*, 2000), and isogenic mutants deficient in FeoB, a high-affinity ferrous transporter, while capable of growth *in vitro*, were unable to colonize the murine gastric mucosa (Velayudhan *et al.*, 2000). In addition, iron can play an important role in the regulation of the genes encoding antioxidant defence enzymes via Fur, PerR and other iron-dependent mechanisms. Our data indicate that the iron concentration had a significant impact on catalase activity (Fig. 4). The significant drop in catalase activity of *H. pylori* wild-type cells grown on iron-limited media may relate to KatA being an iron-dependent enzyme. In an iron-limited environment, the cell's physiology might be focused towards iron acquisition with less catalase required or less iron available to form haem.

The Fur homologue of *H. pylori* was originally characterized by Bereswill and associates (Bereswill *et al.*, 1998, 2000). Fur is an iron-dependent regulatory protein, regulating genes involved in iron uptake (Stojiljkovic *et al.*, 1994). In certain bacteria, Fur also regulates, either positively or negatively, the acid-tolerance response, chemotaxis, motility, and the oxidative stress response (Hahn *et al.*, 2000; Hassett *et al.*, 1996; Horsburgh *et al.*, 2001; Naik & Hassan, 1992; Niederhoffer *et al.*, 1990; Stojiljkovic *et al.*, 1994). Fur upregulates catalase (KatB) from *Pseudomonas aeruginosa* and *Staphylococcus aureus* KatA (Horsburgh *et al.*, 2001). Conversely, Fur downregulates the KatA in *P. aeruginosa* (Hassett *et al.*, 1996) and *C. jejuni* (Van Vliet *et al.*, 1999, 1998). These proteins are upregulated in the presence of iron in Fur mutants, indicating Fur-independent iron regulation of KatA in these bacteria, with the possible involvement of the iron-storage molecule bacterioferritin (Ma *et al.*, 1999; Wai *et al.*, 1996).

That Fur has different effects on the regulation of catalase in different bacteria is of interest. Part of the difference could relate to the global regulation of oxidative stress in different bacteria. For instance, in *E. coli*, *fur* is regulated by OxyR and SoxRS (Zheng *et al.*, 1999). *H. pylori* lacks OxyR, SoxRS, RpoS, LexA and PerR homologues (Tomb *et al.*, 1997), which may relate to the adaptation of the bacterium to life on the inflamed gastric mucosa. In such circumstances, regulation of KatA may be attuned more to differences in the flux of

iron in an environment of near-constant oxidative stress. Delany *et al.* (2001a) demonstrated that Fur interacted with iron within the promoter of an iron-activated gene of *H. pylori* and suggested a model in which the iron status of the Fur protein differentially alters its affinity for operators in either iron-repressed or iron-activated genes.

A putative Fur-box has been identified upstream of *katA* (Manos *et al.*, 1997; Odenbreit *et al.*, 1996). Delany *et al.* (2001b) demonstrated, using Dnase I footprinting, two Fur-boxes in the intergenic region between *katA* and *frpB*. The putative Fur-box immediately upstream of *katA* was a low-affinity or type 2 Fur-box. Delaney *et al.* (2001b) were unable to identify any differences between the levels of specific transcript for *katA* in wild-type and Fur-deficient mutants. The cell-free extracts from the Fur-deficient mutant created in our study had a significantly lower catalase activity than the wild-type cells. Both wild-type and Fur-deficient mutants had high levels of catalase, differences that may not have been detected at the genetic level. Another explanation is the pleiotropic effect that disrupting Fur might have on the cell's physiology. Fur may impact on the synthesis of haem prosthetic groups required for the enzymic function. Massé & Gottesman (2002) have recently shown in *E. coli* that iron, and ultimately Fur, regulates a small RNA molecule, RyhB, that in turn regulates a number of different genes involved in iron metabolism and other cellular enzymes such as succinate dehydrogenase and superoxide dismutase (*sodB*). The only region of the *H. pylori* J99 genome having any similarity to *ryhB* was the 5' end of the anthranilate synthase component I (*trpE*) (Altschul *et al.*, 1997). The possibility remains, however, that unidentified systems, such as small RNA molecules involved in the post-transcriptional regulation of protein expression, may be influencing the catalase activity of the Fur-deficient mutants.

We previously demonstrated that as the relative erythrocyte concentration of a medium increased, the catalase activity of *H. pylori* decreased (Hazell *et al.*, 1991). In this study, we have shown that Fur-deficient mutants of *H. pylori* appear to be more sensitive to hydrogen peroxide when grown on a blood medium (Fig. 5e), with a similar sensitivity to mutants grown on medium with depleted free iron (Fig. 5d). Thus, growth on whole blood mimics growth on iron-depleted media.

Downstream of *katA* is an ORF with no known function (Manos *et al.*, 1997; Odenbreit *et al.*, 1996). There were initially suggestions that this gene, HP0874 (*kapA*), was co-expressed with *katA*. Odenbreit *et al.* (1996) used transposon shuttle mutagenesis to disrupt HP0874 and the intergenic region between *katA* and HP0874 (*kapA*). The catalase activities of these mutants were no different from the wild-type cells. These results failed to clarify the issue relating to possible co-transcription. We have shown, using RT-PCR, that no co-transcription of *katA* and HP0874 occurs (Fig. 2). These results indicate, despite the absence of any recognizable RNA polymerase binding sites in the intergenic region, that *katA* and HP0874 (*kapA*) are expressed independently of each

other. Further, disrupting the *katA* did not affect the expression of HP0874 (*kapA*) (Fig. 2b).

Exposing HP0874 (*kapA*) isogenic mutants to a high concentration of hydrogen peroxide resulted in cell death irrespective of the presence of active catalase. This was in contrast to the results of exposing the wild-type cells to hydrogen peroxide. These results indicate that HP0874 (KapA) plays a role in resistance to hydrogen peroxide. The rate at which the HP0874 (*kapA*) isogenic mutant lost viability was different to that of the *katA* isogenic mutant. Upon exposure to ~100 mM hydrogen peroxide, the HP0874 (KapA)-deficient mutant typically experienced a lag period followed by a rapid decrease in cell viability, and then the rate of loss decreased. It is critical to note that the HP0874 (*kapA*) mutants were catalase-positive (Table 2). This point is important as it was evident that catalase was rapidly depleting hydrogen peroxide in these experiments. Under these conditions, catalase could mask the importance of HP0874 (KapA). For this reason, the concentrations of hydrogen peroxide used in these experiments were high by comparison with physiological concentrations. That is, when catalase-positive HP0874 (KapA)-deficient mutants were exposed to hydrogen peroxide, the actual hydrogen peroxide concentration was rapidly and continuously decreasing. Therefore, the effective hydrogen peroxide concentration was much lower than 100 mM.

There are a number of possible functions for HP0874 (KapA), such as a chaperone, a transporter protein or a peroxide-sensing protein. Hydrogen peroxide can generate other peroxides (organic peroxides) (Farr *et al.*, 1988). There are two known mechanisms for removing organic peroxides in *H. pylori*, alkyl hydroperoxide reductase (Alm *et al.*, 1999; Hazell *et al.*, 2001; Olczak *et al.*, 2002; Tomb *et al.*, 1997) and scavengase (Wan *et al.*, 1997). Using 'identify function of unknown proteins', a BioNavigator macro (#26487) (<http://www.bionavigator.com/>), utilizing the programs HMMpfam, Garnier, Motifs (GCG), BlockSearcher and BlastP revealed that there were two small regions of HP0874 that have some similarity to the aldehyde ferredoxin oxidoreductase of the thermophilic archaeon *Pyrococcus furiosus* (Chan *et al.*, 1995) (*E* value 0.44) (data not shown). Aldehyde ferredoxin oxidoreductase is a tungsten-requiring enzyme responsible for catalysing the reversible oxidation of aldehydes to their corresponding carboxylic acid with the reduction of the ferredoxin. Ferredoxin NADP⁺ reductases (FNR) have been identified as components of the oxidative stress response system of *E. coli*. FNR-deficient mutants display abnormal sensitivities to hydrogen peroxide (Krapp *et al.*, 1997). Notwithstanding these features, HP0874 (KapA) appears unique. The HP0874 is conserved in all strains of *H. pylori* (Salama *et al.*, 2000) with the only substantive homologue being a putative gene encoding a protein of unknown function in *Pasteurella multocida* (May *et al.*, 2001) (<http://www.tigr.org>).

Rain *et al.* (2001) noted a protein–protein interaction between KatA and HP0874 (KapA). The authors stated

that recognition of protein–protein interactions 'allows prediction of protein function'. These data add to our observation that HP0874 has a role in the resistance to hydrogen peroxide, even if further functional analyses are required. Based on our data and the above finding, we have proposed the name KatA-associated protein, *kapA*, for the gene and associated protein.

In conclusion, Fur knockouts and cells grown on a low-iron-containing medium showed a reduction in catalase activity and increased sensitivity to hydrogen peroxide. KapA-deficient mutants showed no reduction in catalase activity but did show an increase in sensitivity to hydrogen peroxide. Our data indicate that KatA is essential for the survival of *H. pylori* in the presence of hydrogen peroxide. Furthermore, KapA is an important component of the bacterium's resistance to oxidative damage, especially hydrogen peroxide. The evidence indicates that Fur and iron, either directly or indirectly, regulate catalase activity in *H. pylori*.

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