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Up-expression of NapA and other oxidative stress proteins is a compensatory response to loss of major *Helicobacter pylori* stress resistance factors

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Abstract
Twenty-six *Helicobacter pylori* targeted mutant strains with deficiencies in oxidative stress combating proteins, including 12 double mutant strains were analyzed via physiological and proteomic approaches to distinguish the major expression changes caused by the mutations. Mutations were introduced into both a Mtz^S and a Mtz^R strain background. Most of the mutations caused increased growth sensitivity of the strains to oxygen, and they all exhibited clear compensatory up-expression of oxidative stress resistance proteins enabling survival of the bacterium. The most frequent up-expressed oxidative stress resistance factor (observed in 16 of the mutants) was the iron-sequestering protein NapA, linking iron sequestration with oxidative stress resistance. The up-expression of individual proteins in mutants ranged from 2 to 10 fold that of the wild type strain, even when incubated in a low O_2 environment. For example, a considerably higher level of catalase expression (4 fold of that in the wild-type strain) was observed in *ahpC napA* and *ahpC sodB* double mutants. A Fur mutant up-expressed ferritin (Pfr) protein 20-fold. In some mutant strains the bacterial DNA is protected from oxidative stress damage apparently via overexpression of oxidative stress-combating proteins such as NapA, catalase or MdaB (an NADPH quinone reductase). Our results show that *H. pylori* has a variety of ways to compensate for loss of major oxidative stress combating factors.

Keywords: *Oxidative stress, ulcer, reactive oxygen species, detoxification, hydroxyl radical*

Introduction

Helicobacter pylori is a gastric pathogen which resides in more than 50% of humans [1]. Upon persistent infection, the host phagocytic cells undergo an oxidative burst response resulting in the formation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot}$), and hydroxyl radical ($^{\cdot}OH$) [2]. One key to survival of *H. pylori* both in the lab as well as within the human gastric mucosa is displaying a resistance to these toxic oxygen-derived metabolites. This process is mediated by proteins like catalase (KatA), alkylhydroperoxide reductase (AhpC), and superoxide dismutase (SodB); these proteins dissipate hydrogen peroxide, organic peroxides and superoxide, respectively. Additional stress-related proteins sequester toxic levels of iron (neutrophile activating protein, termed NapA), or reduce the quinone pool using NADPH as the reductant (termed MdaB protein). Disruption of *sodB*, *ahpC*, or *mdaB* in *H. pylori* results in an oxidative stress sensitive phenotype and severely affects the bacterium's ability to colonize the host stomach [3–6], whereas katA [7] disruption has a lesser effect, and the *napA* affect [8] *in vivo* has not been tested.

Previously, we observed that disruption of AhpC in *H. pylori* resulted in expression of another antioxidant

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factor (NapA) at levels about 5-fold greater than that made in the wild-type [3]. Overexpression of NapA in the *ahpC* mutant strain occurred even under minimum oxidative stress conditions (i.e. 1% O_2 environment). It was proposed that a genetic change had occurred to allow synthesis of more NapA protein, increasing the cell's ability to withstand the oxidizing conditions. Similarly, mutation of both *ahpC* and *napA* together resulted in recovery of strains that expressed a much higher level of MdaB, a protein that was subsequently shown to be an important enzyme (an NADPH quinone reductase) in combating oxidative stress [6]. These phenotypic changes could be the result of transient regulation of the newly-expressed component due to the first mutation or they could be the result of some secondary mutation, as strains with the original (AhpC) mutation that did not exhibit the secondary up-expression effect could also be recovered. The latter were the less predominant class of *ahpC* mutants, termed as type II [3].

These observations prompted us to further assess the ability of *H. pylori* to express compensatory changes in response to removal of important oxidative stress resistance factors. We studied a series of gene-directed mutants that lack the expression of one or in many cases two major protein(s) involved in oxidative stress resistance. A proteomic-based approach was used to identify highly expressed proteins compared to the parent strain. We found that the compensatory responses to loss of oxidative stress resistance proteins via up-expression of other resistance factors is a common response phenomena in both metornidazole resistant (43504) and sensitive (SS1) strains of *H. pylori*. The specific compensatory factors vary depending on the original mutation and involve KatA, SodB and NapA. Among all the mutants, neutrophile activating protein (NapA) is the most frequently expressed protein indicating a crucial link between iron metabolism and oxidative stress resistance.

Experimental procedures

Bacterial strains and growth conditions

Genetic manipulations were performed with *Escherichia coli* strain Top10 (Invitrogen, Carlsbad, Calif) and DH5α. *E. coli* was cultured aerobically on Luria-Bertani (LB) medium at 37°C. *H. pylori* strain ATCC 43504 or SS1 [9] was used as a background parental strain. Cells were grown microaerobically at 37°C in a CO_2 incubator (model 3130; Forma Scientific) containing 5% CO_2 and low levels of oxygen (1% partial pressure). The balance of the gas was N_2, and this atmosphere is hereafter referred to as the 1% O_2 condition. Blood agar plates were used for growth of *H. pylori* as described before [3]. Kanamycin (50 $\mu g\,ml^{-1}$) and chloramphenicol (40 $\mu g\,ml^{-1}$) were used for selection of mutated, antibiotic resistant strains.

DNA techniques

All techniques were preformed as previously described [10]. Primers were designed to amplify genes encoding Fur (FurR: 5′-ATCGCAGCGATAAAGGCGTG3′ and FurF: 5′-ACCCGCATGATTATAACGGC3′) and KatA (KatA22: 5′-CCACAATGTGATTACG-GCCG-3′ and KatA12: 5′-TCCATAAGAGAACAA-GCCCC-3′). Primers were synthesized by Integrated DNA Technologies, Coralville, Iowa. The PCR fragments were amplified by a Perkin–Elmer 2400 thermal cycler with *Tag* DNA polymerase (Fisher) and cloned into pGEMT cloning vector (Promega) in order to generate pFur and pKatA plasmids. Subsequently, antibiotic cassettes were inserted at the *Bcl*I and *Hind* III sites within the *fur* and *katA* sequence of pFur and pKatA, respectively. The chloramphenicol cassette was inserted into *Bal*I site of previously constructed pAhpC plasmid [3]. Plasmid DNA were isolated with QiaPrep Spin mini kit (Qiagen). PCR products and DNA fragments were purified from agarose gels with the QIAquick gel extraction kit (Qiagen).

Construction of H. pylori *mutants*

Single mutation strains were obtained through either electroporation or natural transformation via allelic exchange. These strains were recovered by screening for transformants in a microaerobic environment (1% O_2 condition). In order to create double mutants, single mutant strains were spread onto an agar plate and incubated for 5 h. Then, 10 μl of plasmid DNA containing the interrupted gene were spotted directly onto the inoculated area and incubated for another 16 h (under1% O_2 partial pressure). Transformed cells were then transferred onto a new plate containing both antibiotics. Cells resistant to antibiotics were selected for further analysis. All mutations were confirmed by PCR amplification using Taq polymerase with DNA isolated from each strain. In all cases the increase in PCR product (based upon the size of the inserted cassette) was confirmed.

Sensitivity to oxygen

Cells were grown for 48 h in the 1% O_2 condition and then harvested and resuspended in PBS to OD_{600} of 0.1. Five milliliters of this suspension was placed in a petri dish and exposed to air at 37°C. At each time point, the dilutions were made and the cells were plated on BA plates containing relevant antibiotics. These plates were then incubated at 1% O_2 and the number of viable colonies was determined [5].

Gel electrophoresis and protein sequencing

Cells grown at the 1% O_2 condition were harvested and resuspended in PBS buffer. Cells were broken by sonication (W-380 Heat System-Ultrasonics, Inc). Crude extracts were collected by centrifugation at 10,000*g* for 10 min. The protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Five micrograms of each protein extract was used for loading each lane of the gel. Densitometric scanning [3] of all up-expressed proteins was used to determine the factor by which the intensity of the band was increased. Peptides associated with a significant increase in the intensity of their respective bands were transblotted onto a polyvinylidene difluoride (PVDF) type membrane [3]. Protein sequencing reactions were performed by the Microchemical Facility at the Emory University. Later, the sequence was compared with the *H. pylori* TIGR database (www.tigr.org/tifr-scirpts/CMR2/GenomePage3.spl?database = ghp) for identification.

DNA damage/fragmentation analysis by electrophoresis

Wild-type and mutant cells were harvested and suspended in PBS buffer to an OD_{600} of 0.5. The cell suspension was equally divided into two portions—one portion was immediately prepared for DNA analysis, while the other portion was exposed to air for 4 h (labeled air exposed sample) before further preparation. A measured quantity of 500 μl of the sample was centrifuged for 1 min at 15000*g*, the pellet washed in Tris-EDTA (TE) buffer (50 mM Tris HCl, 5 mM EDTA, pH 8) at 4°C and finally suspended in 10 μl of TE. This suspension was then added to 50 μl of 1% low melting point agarose (Fisher). Agarose and cells were mixed thoroughly and 60 μl blocks were made by pipeting the mixture onto parafilm. After solidification, the blocks were placed in a lysing solution (0.25 mM EDTA, 0.5% (w/v) sarkosyl, 0.5 mg ml^{-1} proteinase K) and incubated at 55°C for 1 h, followed by overnight incubation at room temperature. The next day, the blocks were washed three times for 10 min each in cold TE buffer. Agarose plugs were then submerged in a 0.8% agarose gel and the gel was subjected to electrophoresis for 7 h at 30 V. It was then stained for 30 min with EtBr (0.5 μg ml^{-1}) and destained in dH_2O and visualized under UV light.

Sequencing of fur and upstream region of napA

In order to sequence the *fur* gene, three forward and one reverse primers were designed. FurF1 (5′ ACCCGCATGATTATAACGGC 3′), FurF2 (5′ TCCATTTTAGAGCGCTTGAG 3′), FurF3 (5′ CATTGAATTTGCAGACCCTG 3′) and FurR (5′ GTGCGGAAATAGCGACGCTA 3′. The combination of primers FurF1 and FurR gave a PCR product of 1050 bp. Twenty microlitres of PCR reaction from strains 43504 where AhpC type I mutant and AhpC type II mutant were used as templates, were gel purified and subjected to DNA sequencing. Twelve microlitres of primers FurF1, FurF2, FurF3 and FurR were used for sequencing. Three sequencing reactions were performed with each primer and analyzed for any possible mutations. Also, primers NapAF and NapAR were used for sequencing the upstream region of *napA*. PCR reactions containing the desired product were submitted to the Molecular Genetics Instrumentation Facility at the University of Georgia.

Results

Construction and primary characterization of in vitro *phenotypes associated with oxidative stress resistant mutants*

Genes like *ahpC* (HP1563), *sodB* (HP 0389), *katA* (HP0875), *napA* (HP0243), *bcp* (HP0136), *mdaB* (HP0630) and *tpx* (HP0390) [12,13] are known for playing roles in *H. pylori*'s resistance to oxidative stress [3–6]. Mutations in these genes represent an additional stress imposed on an O_2 -dependent bacterial cell. In order to measure the protein profile in the absence of one or more of these oxidative stress related genes, we cloned each gene and constructed the respective mutant strain. Previously, we obtained single mutants with a mutation in each gene mentioned above [3–6]. In the present study, we were able to obtain individual strains with most combinations of the possible double mutations (Table I) by incubation of transformants for an extended time in a 1% O_2 containing atmosphere. Nevertheless, attempts to construct an *ahpC mdaB* double mutant were not successful. Attempts to make this mutant involved introducing the plasmid construct with the *ahpC* or *mdaB* gene interrupted with an antibiotic cassette into an *H. pylori* strain carrying a mutation in either *mdaB* or *ahpC*, respectively. This approach was carried out by both natural transformation and electroporation. However, none of these attempts resulted in transformants even after incubation at the 1% O_2 condition for 10 days.

All the strains carrying double mutations in major oxidative stress resistance proteins showed slower growth when compared to the single mutants and the wild-type strain. Among them, the *ahpC sodB* double mutant strain grew the slowest. This indicated that even in a low O_2 (1% partial pressure) condition, these double mutants suffer serious oxidative stress. We measured the sensitivity (i.e. viability) of these strains to atmospheric oxygen levels (see Figure 1). As noted previously [6], wild-type cells can survive more than 10 h exposure to air, whereas no cells of the *mdaB* or *ahpC* single mutant strains could survive 8 or 6 h of air

Table I. Strains and plasmids.

Strains and plasmids	Description	Source or reference
Plasmids		
PGEMT	cloning vector	Promega
pG2	*aphA3* inserted into *Nco*I site of pKSsodB-3	[5]
pAhpCKan	*aphA3* inserted into *Afl*II site of pAhpC	[3]
pAhpCCm	*cat* insertion into *Bal*I site of pAhpC	(This study)
pNapACm	*cat* inserted in *Sac*I site of pNA⊗S	[3]
pKatACm	*cat* inserted into *Hind*III site of pKatF	(This study)
pKatAKan	*aphA3* inserted into *Hind*III site of pkatF	(This study)
pGEM-*mdaB*-Cm	*cat* cassette inserted into *Eco*47II of pGEM-*mdaB*	[6]
pTpxKan	*aphA3* inserted into *Bst*API site of pTpx	[4]
pFurKan	*aph*A3 inserted into *Bcl*I site of pFur	(This study)
pFurCm	*cat* inserted into *Bcl*I site of pFur	(This study)
Strains		
H. pylori		
ATCC 43504	parent strain for all *H. pylori* strains	ATCC[a]
ahpC	*aphA3* insertion within *ahpC* (*ahpC*::Kan)	[3]
napA	*cat* insertion within *napA* (*napA*::Cm)	[3]
mdaB	*cat* insertion within *mdaB* (*mdaB*::Cm)	[6]
sodB (HPG2)	*aphA3* insertion within *sodB* (*sodB*::Kan)	[5]
tpx	*aphA3* insertion within *tpx* (*tpx*::Kan)	[4]
katA	*aphA3* insertion within *katA* (*katA*::Kan)	(This study)
fur	*aphA3* insertion within *fur* (*fur*::Kan)	(This study)
fur	*cat* insertion within *fur* (*fur*::Cm)	(This study)
ahpC napA	*ahpC*::Kan *napA*::Cm	[3]
katA ahpC	*katA*::Kan *ahpC*::Cm	(This study)
ahpC sodB	*ahpC*::Cm *sodB*::Kan	(This study)
sodB napA	*sodB*::Kan *napA*::Cm	(This study)
sodB mdaB	*sodB*::Kan *mdaB*::Cm	(This study)
fur napA	*fur*::Kan *napA*::Cm	(This study)
fur ahpC	*fur*::Kan *ahpC*::Cm	(This study)
fur katA	*fur*::Cm *katA*::Kan	(This study)
fur sodB	*fur*::Cm *sodB*::Kan	(This study)
Strain SS1		
ahpC	*aphA3* insertion within *ahpC* (*ahpC*::Kan)	[3]
ahpC	*cat* insertion within *ahpC* (*ahpC*::Cm)	(This study)
mdaB	*cat* insertion within *mdaB* (*mdaB*::Cm)	[6]
sodB	*aphA3* insertion within *sodB* (*sodB*::Kan)	[5]
tpx	*aphA3* insertion within *tpx* (*tpx*::Kan)	[4]
fur	*aphA3* insertion within *fur* (*fur*::Kan)	(This study)
fur	*cat* insertion within *fur* (*fur*::Cm)	(This study)
fur ahpC	*ahpC fur*::Kan *ahpC*::Cm	(This study)
fur katA	*katA fur*::Kan *katA*::Cm	(This study)
fur sodB	*sodB fur*::Cm *sodB*::Kan	(This study)
Strain 26695		
ahpC	*cat* insertion within *ahpC* (*ahpC*::Cm)	(This study)
Strain 11639		
ahpC	*cat* insertion within *ahpC* (*ahpC*::Cm)	(This study)

Strains containing single mutations were obtained by transforming wild-type strain with the cloning plasmid carrying the gene of interest interrupted with the appropriate antibiotic cassette (*aphA3* or *cat*). Double mutant strains were isolated by transforming single mutant strains of *H. pylori* with the plasmid containing a second gene of interest interrupted with another antibiotic cassette (*cat* or *aphA3*). [a]ATCC, American Type Culture Collection.

exposure, respectively [3,6]. The majority of the double mutants showed a further decrease in survival time upon exposure to air. For example, *katA ahpC*, *ahpC sodB* and *sodB napA* mutants were no longer viable after 4 h of exposure to air (Figure 1). These results indicated that mutations in two major resistance factors impose a strong oxidative stress effect on cells, leading to lower survival ability for the majority of double mutants and to possible lethality in the case of *ahpC mdaB* double mutations.

Overexpression of catalase as a compensatory response to loss of other major oxidative stress proteins

Catalase is known for its crucial role in combating oxidative stress conditions and specifically in dissipating H_2O_2. The enzyme is essential for *H. pylori* survival in the presence of hydrogen peroxide [13]. It has also been shown that catalase is important for *H. pylori* survival at the surface of the phagocytic cells during the oxidative burst

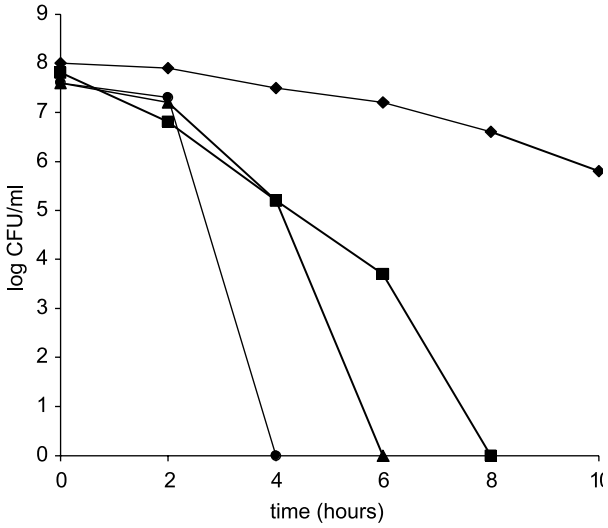

Figure 1. Survival curve of nongrowing *H. pylori* strains incubated under atmospheric oxygen. Wild type 43504 (diamonds), *mdaB* mutant (squares), *ahpC* mutant (triangles), *katA ahpC* double mutant (circles) cells grown under 1% oxygen were harvested and suspended in PBS (OD_{600} of 0.1) and incubated at 37°C under atmospheric conditions. Samples were plated at times indicated and the plates incubated in a 1% oxygen environment for viability (i.e. colony) counts.

response upon infection-mediated inflammation [14]. *H. pylori* expresses a large amount of catalase that is easily identified on SDS-PAGE using the cell extracts as a starting material. The identity of the KatA band on SDS-PAGE can be confirmed by loss of the band in the *katA* mutant strains (Figure 2) and by direct N-terminal sequencing of the KatA band excised from the gel. To study the relationship between catalase and other oxidative stress related proteins such as AhpC, SodB and NapA, we measured the KatA protein expression level in various mutants (Figure 2). Single mutant strains in *ahpC*, *sodB*, or *napA* as well as double mutants (*sodB napA*) did not cause a significant change of the KatA expression level. However, we observed an increased level of catalase protein in *ahpC napA* and *ahpC sodB* double mutants (Figure 2). In both strains

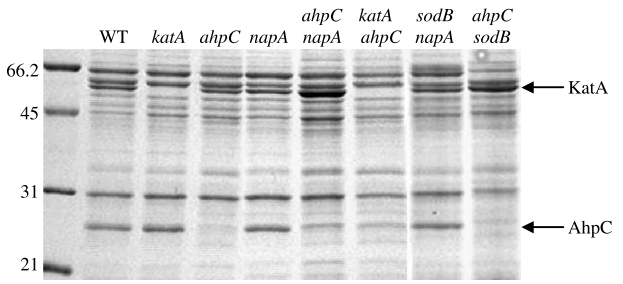

Figure 2. SDS-PAGE of *H. pylori* proteins in strain 43504 showing up-expression of KatA. Each lane contains 5 μg of crude extract of different strains as indicated on the top. The locations of KatA and AhpC proteins are marked with arrows on right. The first lane shows protein standards ranging from 21 to 66.2 kDa.

the expression level of catalase protein was four times that of the wild-type (Table II). The activity of catalase in both strains increased accordingly (data not shown). Clearly, overexpression of KatA is a compensatory response to loss of two other major oxygen stress-related proteins. Interestingly, the proteomic profile of wild-type cells grown at elevated levels of oxygen (i.e. 12% partial pressure) also indicated an upregulation of catalase by approximately 4 fold (data not shown), compared to 1%O_2 - incubated cells. Again, this clearly shows that the mutation itself can impose a stress equivalent to the one inflicted by the exposure to a "high" level of oxygen.

Factors linking iron metabolism to oxidative stress

Although essential for metabolism in nearly all organisms, iron can become toxic to the cell when in excess or if not sequestered. One reason for this toxicity is the close relationship between iron metabolism and generation of oxidative stress agents. Thus, the cell's ability to control iron homeostasis is a very important part of its defense against oxidative stress. Accordingly, the ferric uptake regulator (Fur, HP1027) plays an important role in adjusting the level of iron uptake by *H. pylori* [15–18]. In order to analyze the *H. pylori* protein expression pattern when a strain lacks a functional Fur, we created a *fur* single mutant and double mutants in which *fur* is mutated in combination with another oxidative stress resistance gene. The proteomic profiles of the *fur* mutants showed a consistent presence of an intense Pfr protein (ferritin, 19 kDa), which is in agreement with previous results showing that a mutation in *fur* caused an increase in cellular level of Pfr [19]. The intensity of the ferritin band in a *fur* single mutant was determined to be 20 fold of that in the wild-type (Table II). Increased expression of Pfr is also apparent in other (double) mutant strains like *fur ahpC* (20 fold), *fur katA* (10 fold), *fur sodB* (10 fold) and *fur napA* (4 fold).

NapA is an iron-binding protein [20], and its expression might be regulated by Fur [8], CsrA [21] and/or some other (unknown) regulator. A large increase of NapA protein was not observed in a *fur* (single) mutant (less than 2 fold increase, Figure 3). Only when a mutation in *fur* is accompanied by mutation in another (oxidative stress) component like AhpC (in *fur ahpC*) or KatA (in *fur katA*), was a significant increase of NapA observed (Table II). Previously, we observed that NapA was overexpressed 5 fold in an *ahpC* single mutant when compared to the parent strain [3]. In the current study, we extend this observation to show that up-expression of NapA is a general compensatory response to loss of many oxidative stress resistance factors. For example, the level of NapA expression in strains lacking a functional

Table II. Proteomic response to loss of oxidative stress resistance factors in *H. pylori*.

		Proteomic response	
		Overexpression fold‡	
Mutant strains*	Overexpressed protein†	43504	SS1
ahpC	NapA	5	4
katA	NapA	4	NR¶
	AhpC		
SodB	NapA	2	
	AhpC		2
tpx	NapA	3	2
katA ahpC	NapA	8	NR¶
ahpC napA	KatA	4	NR¶
	MdaB	3	
ahpC sodB	Kat	4	
	NapA	6	NR¶
sodB mdaB	NapA	2	
	Pfr	8	NR¶
fur	Pfr	20	20
	NapA		4
fur napA	Pfr	4	NR¶
fur ahpC			
	SodB	5	4
	Pfr	20	20
	NapA	10	8
fur katA			
	Pfr	10	10
	NapA	3	3
	AhpC		2
fur sodB	Pfr	10	10
	NapA	2	2

The over-expression fold is the intensity of a specific protein band in the mutant strain divided by that in the wild-type strain. The data are averaged from at least three experiments and rounded to the nearest integer. Changes less than 2 fold are considered insignificant and are not included. In addition, we also observed upexpression of KatA (10 fold) in *ahpC* mutant in strain 26695 and upexpression of SodB (3 fold) in *ahpC* mutant in strain 11639. * *H. pylori* strains carrying single or double mutation in oxidative stress resistance genes. † Proteins that are over-expressed in the mutant strain compared to the WT. These proteins are identified on SDS-PAGE by direct N-terminal sequencing of the protein band. ‡ The intensity of the protein bands was measured by densitometric scanning. ¶ Mutants could not be recovered (NR).

thiolperoxidase (Tpx), SodB or catalase (KatA) is 3, 2, or 4 times that of the wild-type strain, respectively (Table II). A much stronger up-expression of NapA (8 fold) can be observed when the cell is lacking two crucial oxidative stress resistance proteins such as KatA and AhpC at the same time. Nearly the same significant raise in the amount of NapA (6 fold) can be observed in an *ahpC sodB* mutant (Figure 3, Table II). These results

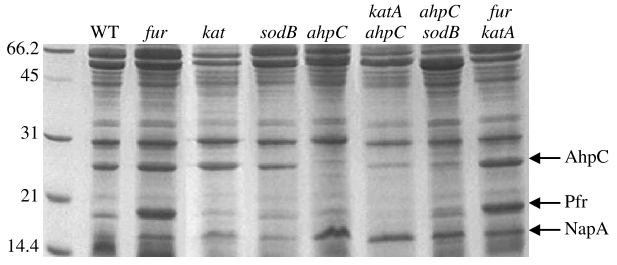

Figure 3. Expression levels of NapA and Pfr in selected strains. The wild-type 43504 (WT) and representative mutant strains are indicated on the top, and the position of NapA, Pfr and AhpC proteins are marked with arrows.

indicate that NapA protein expression increases when other oxidative stress resistance components are scarce, providing a direct link between iron sequestration and oxidative stress.

DNA protection as an outcome of the compensatory response

Reactive oxygen species (ROS) will accumulate within a living cell when that cell lacks major oxidative stress resistance factors. Accumulation of those radicals can then cause damage to one of the most fundamental macromolecules of the cell - DNA. Therefore, we addressed whether up-expression of some major oxidative stress resistance factors had a role in protecting DNA damage in cells exposed to air. Strains lacking proteins involved in resistance to oxidative stress were exposed to air and the extent of DNA degradation was measured by gel electrophoresis (Figure 4). One strain with a high up-expression of NapA is the *katA ahpC* mutant strain (Table II). Before exposure to air, the *katA ahpC* strain showed higher

DNA damage than the wild-type, but after 4 h both strains lost similar amounts of intact DNA (figure 4A). The up-expression of NapA in *katA ahpC* strain could be one of the responsible factors for this protective phenotype. Furthermore, strains like the single mutant *ahpC* or the double mutant *ahpC napA* had almost no or very little amount of DNA degradation (Figure 4B). This effect on DNA protection could be due to high levels of compensatory proteins in these strains (high NapA levels in the *ahpC* mutant and high KatA and MdaB expression in the *ahpC napA* mutant). Overall, mutant strains demonstrating a weak or no compensatory response were significantly more prone to DNA damage than the strains with significant up-expression of NapA, KatA and MdaB (some data not shown).

Search of mutations yielding an up-expression phenotype

In the *ahpC* type I mutant strain the NapA level was 5 fold higher than that in the wild-type. This was not the case, however, for type II *ahpC* mutants [3]. In searching for sites carrying a possible mutation to explain the type I and II phenotypes, we first sequenced the DNA region upstream of *napA* in both types of mutants. However, no difference in the nucleotide sequence of the promoter region was observed in the mutants when compared to the wild-type. Similarly, the sequence of *fur* (entire sequence plus 285 bp upstream) in both types of mutants was the same as for the wild-type. We then noticed a gene (HP0244) as a possible regulator [22] located 209 bp upstream of *napA*. Mutants in this putative sensor encoding gene were consistent with it playing a regulatory role [22]. We sequenced HP0244 in the *ahpC* type II mutant and compared it with the wild type *H. pylori* strain 43504. Once more, no sequence differences were found between these strains. This allowed us to eliminate the histidine kinase gene as a potential site of mutation to explain the differential expression effects on NapA in *ahpC* mutants.

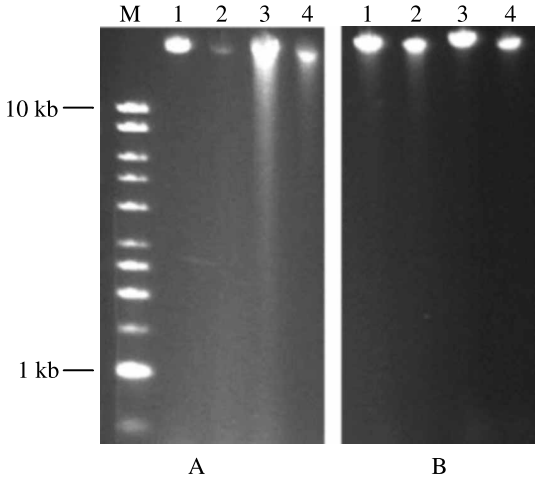

Figure 4. Detection of DNA degradation by 0.8% agarose gel electrophoresis. Each well contained 10 μl of whole cells at OD_{600} of 0.5 embodied in 1% low melting point agarose. PanelA: M, 1kb DNA ladder, 1, wild-type 43504 cells without air exposure, 2, wild-type after 4 h exposure to air, 3, *katA ahpC* double mutant without air exposure, 4, *katA ahpC* exposed to air for 4 h. PanelB: 1, *ahpC* single mutant without air exposure, 2, *ahpC* single mutant after 4 h of exposure to air, 3, *ahpC napA* double mutant with no exposure to air, 4, *ahpC napA* after 4 h of exposure to air.

Compensatory phenotype of mutant strains generated in other (metronidazole sensitive) background strains

H. pylori displays a vast genetic diversity between its strains [23] and strains resistant to metronidazole have been shown to exhibit oxidative stress related phenotypes due to the lower activity of NADH oxidase [24,25]. Since strain 43504 is resistant to metronidazole, we addressed whether similar compensatory responses to mutations in oxidative stress resistance genes described above can also occur in strains sensitive to metronidazole (like strain SS1). In strain SS1, we were able to mutate genes encoding the following proteins: AhpC, SodB, Tpx and Fur. NapA protein was up-expressed in all of these mutants to a similar level as described above for strain 43504 (Table II). In addition to up-expression of NapA, a strain with a mutated SodB showed an increased level of alkyl hydroperoxide reducatase protein (AhpC) by approximately 2 fold. In a *fur* mutant an increased level of Pfr (20 fold) was observed, as well as an increase of NapA, by approximately 4 fold. With strain SS1 as the background, we were not able to obtain a NapA single mutant, suggesting this gene may play an indispensable role in this strain. Also, we were unable to recover any double mutant strains carrying mutations in two major oxidative stress resistance genes at the same time. However, we did obtain double mutants that contain a mutation in *fur* and in one major oxidative stress resistance gene together. These included double mutants *fur ahpC*, *fur katA* and *fur sodB*. Like strain 43504, all these mutated strains showed an elevated level of Pfr and NapA; one strain (*fur ahpC)* overexpressed SodB (4 fold), and another (*fur katA*) showed in addition higher level of AhpC by about 2 fold. We also extended the observations to include other Mtz^S strains like 26695 and 11639; the SodB protein was upexpressed in *ahpC* mutant in strain 11639 by 3 fold, while in strain 26695 the KatA level increased by approximately 10 fold.

Discussion

Bacteria have intriguing ability to adapt to environmental stresses such as oxidative stress [26]. These responses result in a phenotype allowing relief from the lethality of the stress, permitting survival of the population. In several bacterial systems, mutation in a major oxidative stress resistance gene caused increased expression of other resistance factors. For

example, upon mutagenesis of oxidative stress genes in *Streptococcus mutans*, a new ferritin-like oxidative stress related protein (Dpr) with a role in iron sequestering was identified [28,29]. Also, in *Bacillus subtilis*, a mutation in *ahpC* increased transcription of both *mrgA* (a homolog of Dps) and catalase [29]. As another example, knock out of KatG (catalase-peroxidase) was compensated for by up-regulation of AhpC [30], illustrating overlapping defensive capacities of KatG and AhpC. An increase in AhpC was also seen in strains rendered metronidazole resistant upon addition of metronidazole [31]. All these examples are manifestations of microorganisms' endless efforts to adapt and cope with the environment imposed upon them. Therefore, studying stress resistance mutants is useful for both establishing functional relationships between major resistance factors as well as for identifying new stress related factors. In this work, we observed the up-expression of proteins upon mutagenesis of major oxidative stress components, revealing both the general adaptability of *H. pylori*, as well as the specific new proteins that allow the bacterium to survive/combat stress conditions.

A number of single and double mutant *H. pylori* strains lacking major oxidative stress factors were obtained. We were able to construct strains carrying combinations of mutations in almost every oxidative stress gene in background strain 43504 except for *ahpC* and *mdaB* genes together. The double mutant apparently cannot compensate for the severe effect of introducing both mutations. Many other double mutant combinations could be obtained and they are hypersensitive to oxygen; their survival time upon exposure to air is much shorter than that for the wild-type or for the single mutants.

To survive the severe oxidative stress imposed by mutation, most of the mutated strains showed the compensatory response by up-expressing other stress related factors. For example, an increase in catalase was most obvious in the double mutants *ahpC sodB* and in the *ahpC napA* strain. In the absence of proteins like AhpC and SodB, the cell is presumably exposed to toxic levels of oxygen radicals or related intermediates—products like superoxide anions, peroxides and hydroxyl radicals. By oxidizing iron sulfur clusters of other proteins like fumarase or aconitase, superoxides can cause release of iron [32]. Released iron can bind negatively charged DNA and in the presence of H_2O_2 undergo Fenton reaction. This can result in formation of the most harmful and highly reactive species—hydroxyl radicals [33]. These radicals will ultimately cause lethal damage to the cell—damage to the DNA, proteins and lipids [34–38].

Our results showed that mutations in *ahpC sodB* together could be partially compensated for with increased levels of NapA and KatA. Catalase was also upexpressed in the *ahpC napA* double mutant where not only would lipid peroxides accumulate, but also would free iron. Thus with higher production of catalase, an *ahpC napA* mutant can diminish the amount of H_2O_2. Of note is the fact that when the wild-type cells were exposed to a high (12%) level of oxygen, KatA protein was clearly up-expressed. This suggests that up-expression of KatA is the result of regulation occurring upon sensing the increased oxidative stress, whether this is imposed by loss of certain stress-combating factors or by exposure to high O_2.

When Fur is nonfunctional, the cell will synthesize a higher level of Pfr [19]. Thus many mutants containing the *fur* mutation in combination with a mutation in another oxidative stress related gene like *ahpC*, *katA*, *sodB*, or *napA* also showed increased levels of Pfr. Since Fur is known to repress the synthesis of Pfr during iron starvation [19], we can conclude that increased level of Pfr in every *fur* mutant is likely due to the Fur-dependent regulation. NapA up-expression, on the other hand, is not obvious in the *fur* mutant in strain 43504, although Cooksley proposed that *H. pylori* NapA is in part regulated by Fur [8]. Only when a *fur* mutation is combined with another mutation in an oxidative stress factor such as catalase or alkyl hydroperoxide reductase was NapA increased. We also noticed that Pfr level was up-expressed in a *sodB mdaB* mutant (Table II), but the mechanism behind this unexpected phenotype is not known.

Among the series of mutants we generated, NapA up-expression is the most frequently occurring change. NapA was over-expressed 2–5 fold when a single oxidative stress resistance gene (*katA, sodB, tpx, ahpC*) was disrupted. Much higher levels (6–10 fold increase) of NapA expression were observed in double mutants. These results support the earlier notion that the (iron binding) role of NapA must be one of the most important facets of the *H. pylori* oxidative stress response. Functionally similar to ferritin, NapA is capable of binding nearly 500 atoms of iron [20].

Strains resistant to metronidazole have been suggested to have a tendency to display more sensitivity to oxidative stress conditions. This may result from the cell's weak ability to convert O_2 to H_2O due to a deficiency in NADH oxidase [24]. It is noteworthy that a strain resistant to metronidazole displayed a concomitant up-expression of AhpC in the presence of the drug [31]. We used several background strains in our study: 43504 (Mtz^R), SS1(Mtz^S), 26695 (Mtz^S) and 11639(Mtz^S). Single mutations in genes encoding oxidative stress resistance proteins were obtained in both 43504 and SS1strains and the phenotypes in the two backgrounds were similar. The studies on other Mtz^S strains were in accord with the general tendency to compensate, albeit some mutant strains obtained in 43504 and SS1 were not recovered in other background strains.

Many double mutations in oxidative stress resistance genes could not be obtained in SS1 strain. Single mutants could only be accompanied by another mutation in *fur*. These double mutants yielded similar results to the mutants obtained in 43504 background strain. From these results we were able to conclude that compensatory responses described here occur in both metronidazole resistant as well as metronidazole sensitive strains.

Exposure to air can generate superoxides whose accumulation can then lead to release of free iron; this in turn, can give rise to formation of hydroxyl radicals. We observed less DNA damage in strains highly expressing the compensatory stress-combating proteins like NapA, KatA and MdaB. Although their actions differ from each other, the end result of all three of these proteins is to fulfill one common goal—to protect *H. pylori* from damage derived from oxidative stress.

The relative stability of *H. pylori*'s environment (the gastric mucosa) and the scarcity of regulatory genes (revealed by the sequence) suggest that *H. pylori* may have a rather limited stress-response [40]. Accordingly, we thought that NapA overexpression in *ahpC* mutants could be due to a secondary mutation, rather than due to regulation. However, we did not see any mutation (in the NapA promoter region or within the sequence encoding Fur and histidine kinase genes) that is potentially responsible for the compensatory phenotype. Recent findings have shown that gene regulation occurs frequently in *H. pylori* as a response to stress [40–42]. Thus, it is also possible that some of the compensatory changes observed in this study are the result of regulation of gene expression. There are examples that cell adjustments occurring upon mutation-imposed stress can result from either mutation or regulation [30,43,44]. Further studies will help us better understand the mechanism underlying the adaptability of *H. pylori* to oxidative stress.

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