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Oxidative stress defense mechanisms to counter iron-promoted DNA damage in *Helicobacter pylori*

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Abstract

Iron, a key element in Fenton chemistry, causes oxygen-related toxicity to cells of most living organisms. *Helicobacter pylori* is a microaerophilic bacterium that infects human gastric mucosa and causes a series of gastric diseases. Exposure of *H. pylori* cells to air for 2 h elevated the level of free iron by about 4-fold as measured by electron paramagnetic resonance spectroscopy. *H. pylori* cells accumulated more free iron as they approached stationary phase growth, and they concomitantly suffered more DNA damage as indicated by DNA fragmentation analysis. Relationships between the intracellular free iron level, specific oxidative stress enzymes, and DNA damage were identified, and new roles for three oxidative stress-combating enzymes in *H. pylori* are proposed. Mutant cells defective in either catalase (KatA), in superoxide dismutase (SodB) or in alkyl hydroperoxide reductase (AhpC) were more sensitive to oxidative stress conditions; and they accumulated more free (toxic) iron; and they suffered more DNA fragmentation compared to wild type cells. A significant proportion of cells of *sodB, ahpC,* or *katA* mutant strains developed into the stress-induced coccoid form or lysed; they also contained significantly higher amounts of 8-oxo-guanine associated with their DNA, compared to wild type cells.

Keywords: *Oxidative stress, iron-promoted DNA damage, Helicobacte*r, superoxide dismutase, alkyl hydroperoxide reductase

Introduction

Helicobacter pylori is a microaerophilic bacterium that infects the gastric mucosa of the human stomach and plays important roles in the pathogenesis of gastritis, peptic ulcer disease, and gastric cancer [1]. During the process of colonizing the host, *H. pylori* induces a strong inflammatory response of the host cells, leading to generation of a number of reactive oxygen species (ROS) [2–5]. However, *H. pylori* can survive and colonize persistently in the harsh conditions within the gastric mucosa. Therefore, mechanisms for detoxification of ROS are of particular interest in understanding *H. pylori* pathogenesis. Recent studies demonstrated that *H. pylori* is equipped with a variety of enzymatic systems to combat the toxic effects of ROS, including catalase (KatA), superoxide dismutase (SOD) and alkyl hydroperoxide reductase (AhpC) [6–9]. Disruption of the corresponding gene for any one of these enzymes severely affects the bacterium's ability to colonize the host [7,10,11].

H. pylori express a high level of catalase that catalyzes decomposition of H_2O_2 into H_2O and O_2. *H. pylori* catalase has some properties distinct from other typical catalases in that it has a pI $>$ 9, and it is highly stable at very high concentration of H_2O_2 [12]. *H. pylori* cells were shown to be resistant to a high concentration ($\sim$100 mM) of H_2O_2, and this resistance was abolished

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in *katA* mutants [9]. *H. pylori* has a dimeric iron-SOD enzyme encoded by the *sodB* gene. Similar to other bacterial iron-cofactored SODs, *H. pylori* SOD catalyzes dismutation of superoxide anions. *H. pylori sodB* mutants were shown to be devoid of SOD activity, to be more sensitive to O_2, and to have a higher frequency of spontaneous mutation [7]. *H. pylori* also express an abundant amount of AhpC enzyme that reduces organic hydroperoxides (ROOH, also extended to include HOOH) into the corresponding non-toxic alcohol (ROH) [6]. The elimination of organic hydroperoxides is particularly important for living cells because organic hydroperoxides can initiate a lipid peroxidation chain reaction and consequently propagate free radicals, leading to both DNA and membrane damage [13]. AhpC is a component of a large family of thiol-specific antioxidant proteins, with roles that are not generally well understood [14,15]. *H. pylori* AhpC was shown to be very important in oxidative stress resistance [8] and pathogenesis [10], and plays a novel role in protecting catalase from organic peroxide-mediated inactivation [16].

As the most abundant transition metal in cells, iron is an essential element for almost all living organisms, due to its primary function in many important enzymes mediating one-electron redox reactions [17]. On the other hand, when present in the free form, iron catalyzes decomposition of H_2O_2 to generate hydroxyl radical [$OH^{\cdot}$] by Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + [OH^{\cdot}] + OH^{-}$) [18]. Hydroxyl radical is a highly reactive oxygen species that can damage many macromolecules in cells [19,20]. Among the targets of [$OH^{\cdot}$], DNA is of unique importance [21], as it carries genetic information and is present in only a single copy. One important form of DNA damage is strand breakage caused by the attack of [$OH^{\cdot}$] to the carbon-4 of the sugar moiety [22,23]. Another form of DNA modification that results from oxidative reactions of ROS or [$OH^{\cdot}$] radicals is 8-oxo-guanine, the most frequently occurring mutagenic lesion [24,25].

In this study, we examined the relationship between the intracellular free iron and DNA damage in *H. pylori* cells, and investigate the individual roles of KatA, SodB and AhpC in preventing accumulation of free iron and subsequent oxidative DNA damage.

Experimental procedures

Biochemicals

Unless otherwise stated, all biochemicals and reagents were from Sigma Chemicals, USA.

H. pylori culture and construction of mutants. *H. pylori* strain ATCC43504 or the isogenic mutants were cultured on Brucella agar (BA) plates supplemented with 10% defibrinated sheep blood or 5% fetal bovine serum. Cultures of *H. pylori* were grown microaerobically at 37°C in an incubator under continuously controlled levels of oxygen (5% CO_2, 1–7% O_2, and balance with N_2). For culturing mutants, chloramphenicol (50 μg/ml) or kanamycin (40 μg/ml) was added in the medium. As described previously [8], the *H. pylori* mutants were constructed by natural transformation via allelic exchange followed by selection for the antibiotic resistance marker. The disruption of the gene in the genome of the mutant strain was confirmed by polymerase chain reaction (PCR) by showing an increase in the expected size of the PCR product. The mutants used in this study include *katA* [16], *sodB* [7] and *ahpC* (type II) [8].

Air killing assay. The assay was used to examine the sensitivity of an *H. pylori* strain to O_2. *H. pylori* cells grown to late log phase were suspended in phosphate-buffered saline (PBS) and incubated at 37°C under normal atmospheric conditions (21% O_2). Samples were removed at various times, serially diluted and spread on BA plates. Colony counts were recorded after 3 days of incubation under the optimum O_2 condition (7% partial pressure for wild type, and 2% for the mutants).

Electron paramagnetic resonance (EPR) spectroscopy. In this study, EPR was applied to whole cells to assess intracellular free iron levels. Basically, we followed the protocol established for *E. coli* [26]. Briefly, a 5 ml cell suspension in PBS ($OD_{600\,nm} = 8$) was incubated with 20 mM desferrioxamine at 37°C for 15 min. The cells were then centrifuged, washed with cold 20 mM Tris–HCl (pH 7.4), resuspended in a final volume of 0.4 ml of the same buffer, and frozen in 3-mm quartz EPR tubes by immersion in liquid nitrogen. Samples were stored at −78°C for EPR analysis. X-band (~9.6 GHz) EPR spectra were recorded on a Bruker ESP-300E EPR spectrometer equipped with an ER-4116 dual-mode cavity and an Oxford Instruments ESR-9 flow cryostat. The relative amounts of intracellular free iron were assessed based on the doublyintegrated intensity of the $g = 4.3$ EPR signal in desferrioxamine-treated samples, after normalizing to the cell concentration.

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. Some cell suspensions were subjected to exposure to air for specified time periods as indicated in the figure legends. Analysis of DNA fragmentation was performed as described by Zirkle and Krieg [27],

with some modification as follows. A measure of 500 μl of the sample was centrifuged for 1 min at 15,000*g*, washed in Tris–EDTA (TE) buffer (50 mM Tris–HCl, 5 mM EDTA, pH 8) at 4°C and suspended in 10 μl of TE. This suspension was then added to 50 μl of 1% low melting point agarose (Fisher) at 37°C. Agarose and cells were mixed thoroughly and 60 μl blocks were made by pipeting the mixture onto a parafilm. After solidification, the blocks were placed in a lysing solution (0.25 mM EDTA, 0.5% (w/v) sarkosyl, 0.5 mg/ml proteinase K) and incubated at 55°C for 1 h, followed by overnight incubation at RT. The next day, the blocks were washed 3 times for 10 min each in cold TE buffer. Agarose plugs were then submerged in a 0.8% agarose gel. Samples were subjected to gel electrophoresis for 7 h at 30 V. Gels were then stained for 30 min with EtBr (0.5 μg/ml) and de-stained in H_2O and visualized under UV light.

Fluorescent staining of cells and quantification of 8-oxo guanine. We followed the protocol of Chen et al.[28] with some modifications. Approximately 10^6 cells were suspended into 30% methanol. A measure of 20 μl of the cell suspension was air-dried onto a gelatin-coated glass slide for 15 min and then covered with 100 μl of 25 mM Tris/HCl (pH 8), 50 mM glucose, 10 mM EDTA containing 2 mg lysozyme per ml for 5 min. The slides were immersed in methanol for 1 min, and then in acetone for 1 min followed by air-drying. 100 μl PBS containing 0.05% (w/v) Tween 20 and 2% (w/v) BSA was then applied to the sample on the slide for 20 min. Fluorescein-5-isothiocyanate (FITC)-conjugated avidin (0.5%; 1:200 dilution in PBS) was added, and the slide incubated in a moist chamber for 1 h. The slides were then extensively washed with PBS, stained with propidium iodide (PI; 12.5 μg/ml), and mounted in a gel-mount solution. The slides were examined with a Leica DM IRB fluorescence microscope. The fluorescent images were recorded by use of a Hamamatsu C4742-95 digital camera and the images were processed using Open Lab Image Software (Improvision®). The contrast adjustments for PI and FITC images were standardized and the FITC fluorescence of each defined region was divided by the PI fluorescence of the same region. The ratio averaged from at least 8 different regions from each of the strains was taken as a measure of the content of 8-oxoG.

Results

Increase of intracellular free iron under oxidative stress

Using an established protocol of EPR [26], we sought to monitor the iron status in whole cells of *H. pylori*. In this protocol, the cells were treated with desferrioxamine, a cell-permeable Fe(III)-chelator that promotes oxidation of all "free iron" to the Fe(III) form. Cells were then subjected to EPR analysis which detects the Fe(III) signal at $g = 4.3$. This assay has been shown to be specific for measuring intracellular free iron level without disrupting iron-containing proteins [29].

H. pylori wild type cells (grown for 30 h at 4% O_2) were suspended in PBS, exposed to air (21% O_2) for a specified time period, and then prepared for EPR analysis (Figure 1). Exposure to air for 2 h increased the Fe(III)-specific EPR signal ($g = 4.3$) about 4-fold over that of the control (without air-exposure). Longer time exposure to air resulted in a further increase in the free iron level, with the 4 h exposure sample being 6-fold higher than the control. This result clearly demonstrates that the level of intracellular free iron is increased under oxidative stress conditions.

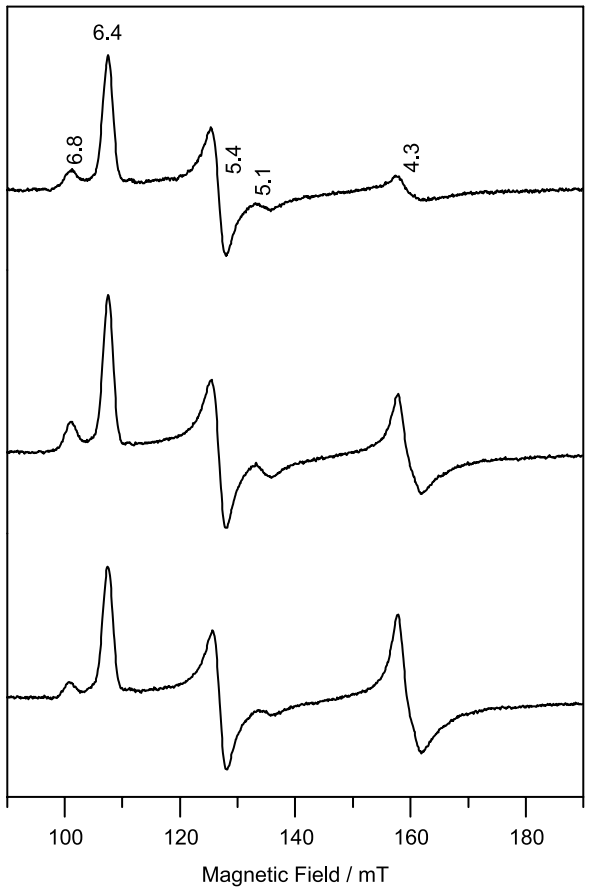

Figure 1. Low temperature EPR spectra of *H. pylori* cells with varying air-exposure times. The wild type cells of *H. pylori* strain ATCC43504 were grown at 4% O_2 for 30 h, and suspended in PBS. The cell suspensions were exposed to air for 0 (top spectrum), 2 (middle spectrum) or 4 h (bottom spectrum) before preparing for EPR samples as described under "Experimental Procedures" section. The EPR spectra were normalized to represent the same number of cells. Conditions of measurement: temperature, 4.2 K; microwave power, 10 mW; modulation amplitude, 0.63 mT; microwave frequency, 9.6 GHz. Selected *g*-values are indicated on the top spectrum. The value $g = 4.3$ represents the signal of intracellular free iron, and $g = 5.4$ and 6.4 signals are characteristic of catalase ferric heme resonance.

In *H. pylori*, catalase is a highly abundant protein that contains iron in the form of a heme prosthetic group. By using the same EPR protocol used here, we previously ascertained the catalase status (heme b-associated spectral properties) in the whole cells of *H. pylori* [16]. Wild type *H. pylori* cells showed features at *g* values of 6.4 and 5.4 which are characteristic of the low-field components of the catalase high spin ferric heme resonance. Figure 1 shows that upon exposure of cells to air for 2 or 4 h, there was no significant change on the EPR signals of catalase, compared to the control (without air-exposure). This suggested that the accumulated free iron was not released from catalase. This is in agreement with the current prevailing view that the superoxide anion oxidizes [Fe–S] clusters in certain vulnerable enzymes causing release of iron [20,30].

Intracellular free iron and DNA damage at different growth phases

To study the relationship between oxidative stress and cell aging, we determined the level of intracellular free iron and DNA damage at different growth phases of *H. pylori* (Figure 2). *H. pylori* wild type cells grown at 4% O_2 for 30 h contain significantly lower levels of intracellular free iron, as detected by EPR, compared to cells harvested when approaching stationary phase (Figure 2A). When grown for a longer time (approaching stationary phase), the level of intracellular free iron increased significantly, about 2-fold more for 54 h and about 3-fold more for 78 h cultures, compared to the result for the 30 h culture. The genomic DNA from the same samples of cells was analyzed on agarose gel electrophoresis; and the results (Figure 2B) revealed that the amount of fragmented DNA from aged cells was increased considerably. A significant correlation between the growth phase, the level of intracellular free iron and the level of DNA damage can be drawn from these results.

Status of intracellular free iron

In all the EPR measurements described so far, we used desferrioxamine to chelate the intracellular free iron. By chelating iron, desferrioxamine converts Fe(II), which is not EPR detectable, to Fe(III) that is EPR-detectable. In order to estimate the relative proportion of Fe(II) among the total free iron, we conducted EPR measurements on identical samples with or without desferrioxamine treatment. As shown in Figure 2A, the cells grown for 30 h contained the smallest amount of total free iron (solid line); and no $g = 4.3$ Fe(III) signal was observed in the absence of the chelator (dashed line), indicating that almost all the free iron was present in the Fe(II) state. The aged cells contained a higher amount of total free iron as

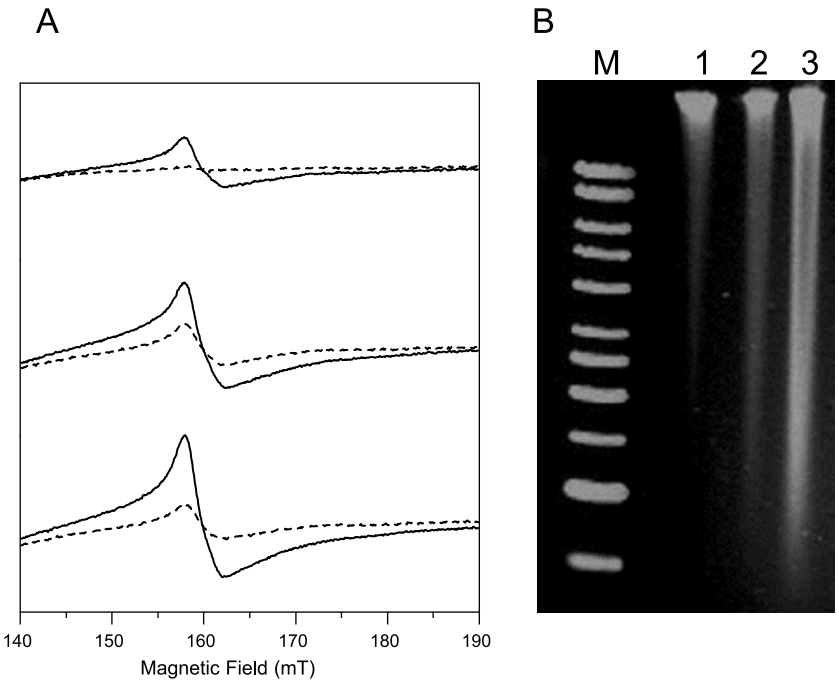

Figure 2. Levels of free iron (A) and DNA fragmentation pattern (B) in *H. pylori* cells at different growth phases. (A) EPR spectra in the region of the free Fe(III) resonance at $g = 4.3$ of *H. pylori* wild type cells grown at 4% O_2 for 30 (top), 54 (middle) or 78 h (bottom). The EPR measurements were conducted on identical samples with (solid lines) or without desferrioxamine treatment (dotted lines). The EPR conditions are the same as in Figure 1. (B) Agarose gel electrophoresis showing genomic DNA fragmentation of *H. pylori* wild type cells grown at 4% O_2 for 30, 54 or 78 h, labeled lane 1, 2, 3, respectively. Preparation of samples for DNA fragmentation analysis is described in "Materials and Methods" section.

indicated by the EPR signal in the presence of chelator (solid lines). A much smaller Fe(III) EPR signal was also observed in the absence of chelator (dashed lines), representing a minor proportion of Fe(III). These results indicated that the majority of the intracellular free iron is in the Fe(II) state in *H. pylori*, similar to that observed in *E. coli* [29], but different from that in yeast [31].

The level of Intracellular free iron and DNA fragmentation in mutant cells

H. pylori possess catalase (KatA), superoxide dismutase (SodB) and alkyl hydroperoxide reductase (AhpC) enzymes that have been shown to be important to oxidative stress resistance. To study the effects of these factors in preventing iron-promoted oxidative DNA damage, we examined mutants in each of these genes. While the optimal growth condition for the wild type *H. pylori* cells is 7% O_2, the mutants can only be grown in a low O_2 atmosphere (no more than 4% partial pressure O_2). In addition, these strains require longer incubation times to obtain confluent growth (more than 3 days to collect enough cells for assays). We determined the sensitivity of the mutant cells to killing by O_2, compared to wild type cells (Figure 3). Upon exposing approximately 10^8 cells to air for 10 h, about 10^6 viable cells of the wild type strain could be recovered. In contrast, the *sodB* or *ahpC* mutant cells were completely killed (no viable cells recovered) by exposure to air for 6 h. The *katA* mutant cells were completely killed by exposure to air for 8 h.

To determine the roles of KatA, SodB and AhpC in preventing the accumulation of intracellular free iron, we determined the levels of free iron within cells of each mutant strain by EPR. We measured the magnitude of the $g = 4.3$ EPR signals from the samples of each mutant strain, and compared them to that for the wild type strain. As shown in Figure 4, the *katA*, *sodB* or *ahpC* mutant cells contain about 3- to 4-fold higher amount of free iron than the wild type. These results indicate that the stress-combating enzymes play important roles in preventing the accumulation of intracellular free iron.

Next, we examined the amount of fragmented DNA present in the cells of mutant strains (Figure 5). For the control condition in this experiment, the wild type cells were grown under the optimum condition (at 7% O_2 for 1 day) to late log phase; this yielded cells with almost no DNA damage. The samples of each mutant strain (*katA*, *sodB*, *ahpC*), grown to late log phase at 2% O_2 for 2 days, exhibit much more degraded DNA, compared to the wild type cells. From these results, a significant correlation between the level of intracellular free iron and the level of DNA damage was observed.

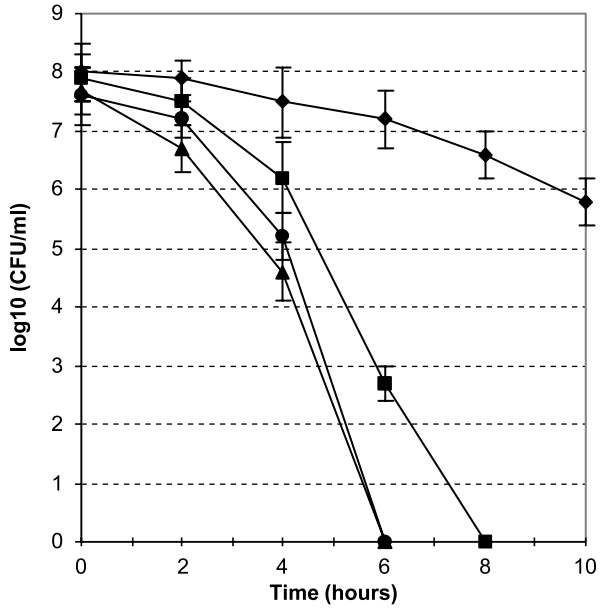

Figure 3. Oxygen sensitivity of *H. pylori* mutant strains. *H. pylori* cells grown under 2% oxygen to late log phase were suspended in PBS and incubated at 37°C under normal atmospheric conditions. Samples were removed at the times indicated in the *x* axis and were used for plate counts in a 2% oxygen environment. The data are the means of three experiments with standard deviation as indicated. Symbols: diamond, Wild type; square, *katA* mutant; circle, *sodB* mutant; triangle, *ahpC* mutant.

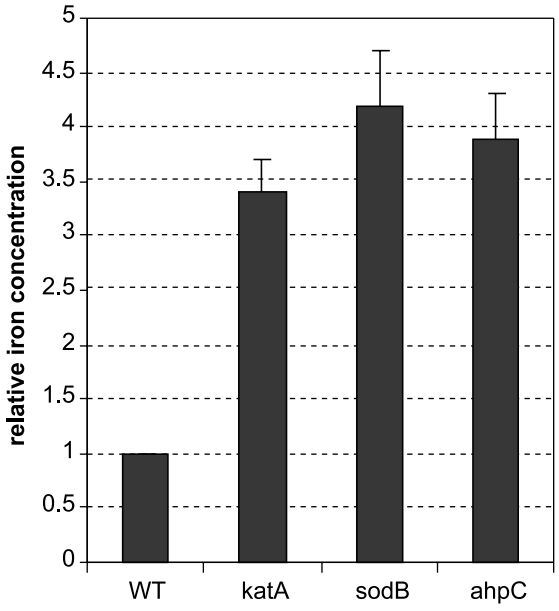

Figure 4. Levels of free iron in *H. pylori* cells of mutant strains. (A) The free iron concentration in the cells of *katA*, *sodB* or *ahpC* mutant was determined by measuring the magnitude of the $g = 4.3$ EPR signal, and the value was expressed as relative to that in wild type cells. The data were the averages of three experiments with standard deviation indicated.

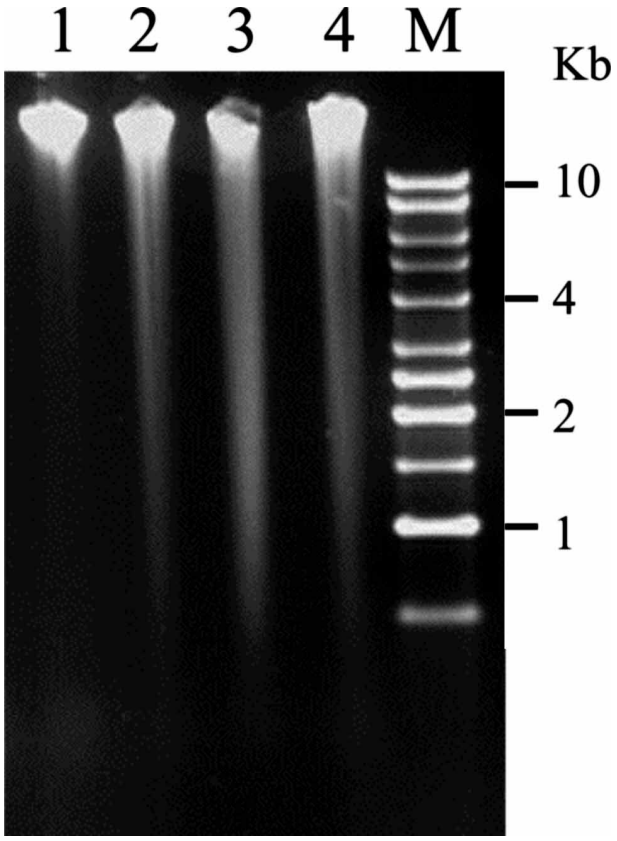

Figure 5. The level of DNA fragmentation of in *H. pylori* cells of mutant strains: WT (lane 1), *katA* (lane 2), *sodB* (lane 3) and *ahpC* (lane 4). Note: wild type cells were grown at 7% O_2 for 1 day, and the mutant strains were grown at 2% O_2 for 2 days. The conditions for DNA fragmentation analysis by agarose gel electrophoresis are same as that in Figure 2B.

Cell morphology and the level of 8-oxo guanine in mutant cells

To further investigate the roles of KatA, SodB and AhpC in protecting *H. pylori* cells from oxidative damage, we examined the level of 8-oxoG DNA lesions in the mutant cells by using the FITC-avidin/PI fluorescence microscopy. In this technique, because FITC-avidin specifically labels DNA lesions and the PI labels all DNA, the intensity ratio of FITC to PI staining reflects the level of 8-oxoG DNA lesions in the cells [28]. *H. pylori* wild type or mutant cells were grown under their optimum conditions (see above) before immunofluorescent staining of cells. Figure 6A and B show that the majority of wild type cells are in the bacillary form. However, a large fraction of the *sodB* cells are present as either the coccoid form, fragmented or lysed (Figure 6C and D), suggesting these cells suffer from persistent oxidative stress [32]. The luminosity of FITC and PI was measured from 8 sets of images, and the average ratio of luminosity of FITC/PI was calculated for the wild type and the *sodB* mutant cells; this represents the level of 8-oxoG DNA lesions in the cells (Table I). The ratio of FITC-avidin/PI fluorescence intensity in the *sodB* mutant cells was determined to be 1.7-fold higher than that in the wild type cells, demonstrating that the *sodB* mutant cells contain considerably higher levels of 8-oxoG DNA lesions. The statistical data on the cell morphology and 8-oxoG levels of the wild type and 3 mutant strains are given in Table I. The levels of 8-oxoG in all 3 mutant strains are significantly higher than that of wild type cells ($P < 0.001$).

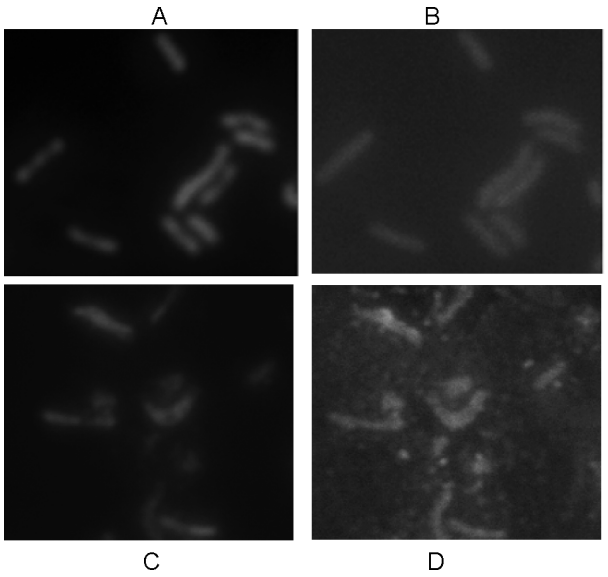

Figure 6. Detection of 8-oxoG by immunofluorescent staining. *H. pylori* WT (A and B) and *sodB* mutant (C and D) cells were fixed on the glass slide and stained with 8-oxoG -specific avidin- FITC conjugate (B and D) and propidium iodide (A and C) followed by examination via fluorescence microscopy. The contrast adjustment was normalized for all the images, and a representative set of images is shown here.

All of the above results were obtained from cells grown under optimum conditions with no additional stress such as air-exposure or H_2O_2 treatment. When additional stress (i.e. 2 h air-exposure) was applied to the wild type cells, there was a slight change in both cell morphology and the 8-oxoG levels. However, under the same stress condition, the majority of mutant cells (particularly *sodB* and *ahpC* mutant cells)

Table I. Cell morphology and the level of 8-oxo guanine in *H. pylori* cells.

Strains	Cell morphology* (% damaged cells)	8-oxoG level† (FITC/PI intensity ratio)
WT	<5	0.76 ± 0.04
KatA	~15	1.07 ± 0.04
SodB	~70	1.27 ± 0.05
AhpC	~65	1.29 ± 0.07

* Cell morphology was examined under fluorescent microscope, and the numbers are estimated percentage of damaged cells including coccoid and broken cells in the whole cell population.

† 8-oxoG level is expressed as mean intensity ratios of avidin-FITC/PI with standard deviation. According to statistical analysis with Student's *t* test, the data of each mutant strain are significantly different from that of the wild type ($P < 0.001$).

became lysed in the process of the immunofluorescent staining (data not shown); and, therefore, could not be examined using this method.

Discussion

It is well known that superoxide destroys many enzymes that contain [Fe–S] clusters such as dihydroxy acid dehydratase, aconitase and fumarase, releasing the cluster iron [30,33–35]. Here, we have shown that *H. pylori* wild type cells, when exposed to an oxidative stress condition (air-exposure) displayed a significant increase in their intracellular free iron levels. While facultative bacteria like *E. coli* thrive in the presence of a high concentration of O_2 (e.g. atomospheric levels), the microaerophile *H. pylori* is highly vulnerable to O_2 toxicity. The optimum condition for wild type *H. pylori* growth *in vitro* is 5–8% O_2, and the mutant cells defective in any one of a number of oxidative stress resistance factors (e.g. *sodB* or *ahpC* mutant) can only grow in low O_2 conditions (1–4% partial pressure O_2). Thus, in spite of the presence of several resistance factors such as SodB, AhpC, KatA, etc. the defense of *H. pylori* seems insufficient when challenged by ROS generated by exposure to air.

Similar to the phenomenon observed in *E. coli* [29] as well as in yeast cells [31], we showed that SodB plays an important role in preventing the accumulation of excess free iron in *H. pylori* cells. Thus, the *sodB* mutant cells accumulate a higher level of free iron than the parent strain. Considering the potential origin of the free iron, *H. pylori* harbors a number of proteins containing Fe–S clusters, such as hydrogenase, fumarate reductase, aconitase and ferredoxins [36]. It needs to be determined which of these enzymes are most vulnerable to the attack of superoxide generating the release of iron. Compared to other bacteria, *H. pylori* harbors abundant amounts of the catalase protein (2–3% of its total proteins) [16]. This pool of heme-protein is a possible target of superoxide damage to release free iron. However, our results suggest this is not the case.

Catalase is the major enzyme that dissipates H_2O_2 in *H. pylori* [5,9]. Loss of catalase activity would apparently lead to accumulation of H_2O_2 in the cells. Here, we observed a modest increase of free iron in *katA* mutant cells. Thus, the increases in both H_2O_2 and free iron, the substrates for the Fenton reaction, are likely responsible for the DNA damage observed in *katA* mutant cells.

We also showed that *ahpC* mutant cells, like the *sodB* mutant cells, accumulate a higher level of free iron than the parent strain. It is known that lipid peroxidation generates a range of substances that can damage DNA [37]. However, to our knowledge, this is the first time a role has been assigned to AhpC in preventing the accumulation of free iron. The main physiological role of AhpC in *H. pylori* appears to be distinct from its counterpart in *E. coli*. It was reported that *E. coli* lacks the polyunsaturated fatty acids [38], and that the major physiological substrate of *E. coli* AhpC is H_2O_2 [39]. *H. pylori* AhpC was shown *in vitro* to be capable of reducing a variety of hydroperoxides, including H_2O_2, t-butyl hydroperoxide, and linoleic acid hydroperoxide [6]. However, under most physiological conditions, dissipation of H_2O_2 is mainly the function of *H. pylori* catalase [5,9]. *H. pylori* contains considerable amounts of unsaturated fatty acids associated with their lipids [40,41]; and our recent results showed that *H. pylori ahpC* mutant cells contain higher amounts of lipid peroxides than the wild type [16]; this suggests that the major physiological role of AhpC in *H. pylori* is detoxification of lipid peroxides (LOOH). The accumulation of LOOH due to the loss of AhpC activity would be expected to facilitate one-electron reduction and oxygenation to produce epoxyllylic peroxyl radicals ($OLOO^{\cdot}$) and peroxyl radical ($LOO^{\cdot}$) [42]. It is likely that these products of the lipid peroxidation cycle attack the [Fe–S] cluster-containing proteins in *H. pylori* cells, generating the release of iron.

Increased levels of free iron promote oxidative DNA damage by catalyzing the Fenton reaction that in turn produces highly reactive hydroxyl radicals. We determined the relative levels of DNA damage in cells by use of a DNA fragmentation assay that measures DNA strand breaks, and also by immunofluoresent microscopy using FITC-avidin that specifically identifies 8-oxo-guanine in the DNA. To examine the roles of SodB, AhpC or KatA in protecting *H. pylori* cells from iron-promoted oxidative damage, we examined the intracellular free iron level and DNA damage in the mutant strains defective in each of these genes. A positive correlation between the free iron level and oxidative DNA damage was observed. From these results two conclusions can be drawn: (1) The majority of the observed DNA damage came from the attack of hydroxyl radicals that are generated due to the presence of excess free iron; and (2) SodB, AhpC and KatA proteins play major roles in preventing iron-promoted oxidative DNA damage in *H. pylori*.

The majority of *H. pylori* cells within a single culture display a bacillary appearance, unless they are subjected to nutrient limitation or stress. Either aging (when exponential growth ceases) or exposure to oxidative stress results in transformation of the cells from the bacillary form to a coccoid form [43,44]. It has been demonstrated that this morphology transformation resulted from oxidative damage to cellular components including proteins and DNA [32,45]. We observed that *H. pylori* wild type cells accumulate more free iron at stationary phase, and concomitantly suffer more DNA damage, indicating the aged cells are suffering an oxidative stress condition. The mutant

strains that are defective in any one of the genes—*sodB*, *ahpC* or *katA*, even at the exponential growth phase, contain more free iron and suffer more DNA damage than the wild type cells. Hence, while the majority of wild type cells are in the bacillary form, many mutant cells transformed to coccoid form or even lysed, suggesting these mutant strains suffer from persistent oxidative stress. Our work provides an integrated picture displaying connections between intracellular free iron, specific virulence factors that combat oxidative DNA damage, and cell morphology transformations in *H. pylori*.

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