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MicroReview

The diverse antioxidant systems of *Helicobacter pylori*

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Summary

The gastric pathogen *Helicobacter pylori* induces a strong inflammatory host response, yet the bacterium maintains long-term persistence in the host. *H. pylori* combats oxidative stress via a battery of diverse activities, some of which are unique or newly described. In addition to using the well-studied bacterial oxidative stress resistance enzymes superoxide dismutase and catalase, *H. pylori* depends on a family of peroxiredoxins (alkylhydroperoxide reductase, bacterioferritin co-migratory protein and a thiol-peroxidase) that function to detoxify organic peroxides. Newly described antioxidant proteins include a soluble NADPH quinone reductase (MdaB) and an iron sequestering protein (NapA) that has dual roles – host inflammation stimulation and minimizing reactive oxygen species production within *H. pylori*. An *H. pylori* arginase attenuates host inflammation, a thioredoxin required as a reductant for many oxidative stress enzymes is also a chaperon, and some novel properties of KatA and AhpC were discovered. To repair oxidative DNA damage, *H. pylori* uses an endonuclease (Nth), DNA recombination pathways and a newly described type of bacterial MutS2 that specifically recognizes 8-oxoguanine. A methionine sulphoxide reductase (Msr) plays a role in reducing the overall oxidized protein content of the cell, although it specifically targets oxidized Met residues. *H. pylori* possess few stress regulator proteins, but the key roles of a ferric uptake regulator (Fur) and a post-transcriptional regulator CsrA in antioxidant protein expression are described. The roles of all of these antioxidant systems have been addressed by a targeted mutant analysis approach and almost all are shown to be important in host colonization. The described antioxidant systems in *H. pylori* are expected to be relevant to many bacterial-associated diseases, as genes for most of the enzymes carrying out the newly described roles are present in a number of pathogenic bacteria.

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Introduction

Oxidative stress is a problem experienced by nearly every living organism. The partial reduction of molecular oxygen by the auto-oxidation of flavoproteins generates a mixture of superoxide (O_2^-) and hydrogen peroxide (H_2O_2), which represent major sources of intracellular reactive oxygen species (ROS) (Imlay, 2003). Superoxide destroys many enzymes that contain [Fe-S] clusters, resulting in the release of iron and subsequent inactivation of the enzyme (Fridovich, 1995). As a powerful oxidant, H_2O_2 attacks 4Fe-4S clusters and the sulphur atoms of cysteine and methionine residues, with potentially lethal consequences (Imlay, 2003). Most importantly, H_2O_2 reacts with intracellular free iron via the well-characterized Fenton reaction to produce hydroxyl radicals [OH•] (Halliwell and Gutteridge, 1989).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + [OH^{\bullet}] + OH^-$$

The product [OH•] is the most toxic of all ROS; it can damage many biomolecules, including DNA (Imlay and Linn, 1988; Imlay, 2003).

Reactive nitrogen intermediates (RNI) represent another class of oxidants that have damaging effects on living cells. RNI are the oxidation intermediates of the nitrogenous products of nitric oxide synthase (NOS), including nitric oxide (•NO), nitrogen dioxide (NO_2^-), S-nitrosothiols (RSNO) and peroxynitrite ($OONO^-$) (Fang, 1997; Nathan and Shiloh, 2000).

$$RNH2 \xrightarrow[NOS]{-5e} {}^{\bullet}NO \xrightarrow{-e} NO_2^- \xrightarrow{-e} NO_3^-$$
$$^{\bullet}NO + RSH \xrightarrow{-e} RSNO + H^+$$
$$^{\bullet}NO + O_2^- \xrightarrow{-e} OONO^-$$

$OONO^-$, the joint product of •NO and O_2^-, is an unstable but very powerful oxidant with properties similar to hydroxyl radical (Nathan and Shiloh, 2000).

The human gastric pathogen *Helicobacter pylori* plays important roles in the pathogenesis of gastritis, peptic ulcer disease and gastric cancer (Dunn *et al.*, 1997). *H. pylori* infects about one-half of the world's population

and usually persists lifelong unless eradicated by antibiotic treatment (Blaser and Atherton, 2004). *H. pylori* is a microaerophilic bacterium and is highly vulnerable to O_2 toxicity. The optimum condition for wild-type *H. pylori* growth *in vitro* is 5–8% O_2 (partial pressure).

During the process of colonizing the host, *H. pylori* induces a strong inflammatory response from infiltrating host cells. This defence is mediated by neutrophils and macrophages, culminating in generation of large amounts of ROS/RNS presented to the persistent pathogen. Production of ROS by gastric cells (Bagchi *et al*., 1996) and phagocytes (Ramarao *et al*., 2000) induced by *H. pylori* has been shown *in vitro*, and increased levels of ROS in the gastric mucosa have been measured in *H. pylori*-infected patients (Davies *et al*., 1994; Baik *et al*., 1996). Exposure of gastric epithelial cells to *H. pylori* resulted in an inflammatory reaction with production of ROS and nitric oxide (Nardone *et al*., 2004). Nevertheless, *H. pylori* survives these conditions and persistently colonizes the gastric mucosa. Therefore, mechanisms for detoxification of ROS and repair of damaged cell components in *H. pylori* are of particular interest in understanding *H. pylori* pathogenesis/persistence. A few years ago, the known *H. pylori* enzymatic systems to combat the ROS were few (Hazell *et al*., 2001). Recent studies have shown the oxidative stress response of *H. pylori* is much more vast, adaptable and interconnected than previously appreciated. Furthermore, mutations affecting almost all of these newly described enzymes appear to be as important to survival in the host as the well-studied traditional oxidative stress combating enzymes like catalase and superoxide dismutase.

Iron homeostasis, oxidative damage and morphological transformation

Iron is an essential element for almost all living organisms, due to its primary function in many important enzymes mediating one-electron redox reactions (Andrews *et al*., 2003). On the other hand, when present in the free form, iron rapidly promotes decomposition of H_2O_2 to generate hydroxyl radical [$OH^{\bullet}$] by the Fenton reaction (Halliwell and Gutteridge, 1989). Hydroxyl radicals are highly reactive oxygen species that damage many macromolecules, particularly DNA (Meneghini, 1997; Imlay, 2003). One important form of DNA damage is strand breakage caused by [$OH^{\bullet}$] attack on the carbon-4 of the sugar moiety (von Sonntag, 1984; Breen and Murphy, 1995). Another form of DNA modification that results from oxidative reactions of ROS or [$OH^{\bullet}$] radicals is 8-oxoguanine, the most frequently occurring mutagenic lesion (Cheng *et al*., 1992; Halliwell and Dizdaroglu, 1992).

Electron paramagnetic resonance spectroscopy showed that the intracellular free iron level within *H. pylori* cells is significantly higher under oxidative stress conditions. Mutants lacking any one of the key oxidative stress-combating enzymes contain more free iron than wild-type cells and suffer more DNA damage (i.e. fragmentation or 8-oxoguanine lesion) (Wang *et al*., 2005a). The increase of the intracellular free iron level in *H. pylori* is due to destruction of [Fe-S] cluster-containing proteins such as hydrogenase and aconitase. Not only $O_2^{\bullet}$, but also peroxyl radicals ($OLOO^{\bullet}$ and $LOO^{\bullet}$, lipid peroxidation products) can attack these proteins resulting in release of iron (Wang *et al*., 2006).

The majority of *H. pylori* cells within a culture remain rod-shaped unless they are subjected to nutrient limitation or stress. Ageing (when exponential growth ceases) or exposure to oxidative stress causes a shift from bacillus to a coccoid form that is viable but non-cultivable (Kusters *et al*., 1997; Donelli *et al*., 1998). While *H. pylori* bacilli generate superoxide, the coccoid form appears to generate the hydroxyl radical. The coccoid form of *H. pylori* cells exhibit markedly lower levels of activities of urease, superoxide dismutase and catalase (indicative of damaged proteins) and contain higher amounts of DNA damage as revealed by the generation of 8-oxoguanine and DNA fragmentation (Nakamura *et al*., 2000). The bacillary form of *H. pylori* undergoes transformation to the coccoid form not only under aerobic (21% O_2) condition but also under anaerobic ($< 1\%$ O_2) condition. Carbonylated proteins (protein damage by ROS) are more abundant in *H. pylori* cultured under non-optimum O_2 tension (aerobic or anaerobic) than in microaerobically (7% O_2) cultured cells, as are DNA damage and mutations (Park *et al*., 2004).

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is the best-studied enzyme for combating the effects of oxidative stress. SODs are ubiquitous in aerobic organisms, and gene homologues of SOD are also found in some anaerobes. This enzyme catalyses the dismutation of superoxide to H_2O_2, which is subsequently removed by catalase or peroxidase.

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

In contrast to *Escherichia coli*, which has three known SODs (Benov and Fridovich, 1994), *H. pylori* produces only a single SOD that contains iron, and is encoded by the gene *sodB* (Spiegelhalder *et al*., 1993; Pesci and Pickett, 1994). *H. pylori* SOD consists of two identical subunits of 24 kDa, and different isoforms of the SOD (due to sequence variations that cause different net electric charges of the protein) have been identified in different strains of *H. pylori* (Bereswill *et al*., 2000).

sodB null mutants show increased O_2 sensitivity in terms of both growth and viability. An important role for SOD in DNA protection was also indicated by the high

mutation frequency of *sodB* strains. Most importantly, SOD severely affects the colonization of the mouse stomach (Seyler *et al*., 2001). Similarly, in *Campylobacter coli*, which is also a microaerophile with a single SOD, *sodB* mutants were more sensitive to air for survival and had a decreased ability to colonize the chicken gut, but their growth rates *in vitro* under high-oxygen conditions were unaffected (Purdy *et al*., 1999).

By reducing the level of O_2^-, SOD indirectly prevents formation of peroxnitrite, the most toxic RNI. For example, a *sodC* mutant of *Salmonella typhimurium* is extremely susceptible to the combination of superoxide and nitric oxide (De Groote *et al*., 1997). *H. pylori* appears to generate larger amounts of superoxide and has a lower specific SOD activity than some other bacteria, e.g. *E. coli* (Nagata *et al*., 1998). Because NO levels in gastric juice are fairly high (Nagata *et al*., 1998), superoxide-dependent peroxynitrite toxicity may be an important factor in the defective colonization abilities of *H. pylori sodB* mutants.

Catalase (KatA) and KatA-associated protein (KapA)

Catalase is a ubiquitous, well-studied enzyme that catalyses the decomposition of H_2O_2 into water and oxygen to protect cells from the damaging effects of H_2O_2 (Nicholls *et al*., 2001).

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The dismutation of H_2O_2 usually occurs in two electron reactions. The first H_2O_2 molecule oxidizes the haem to form an intermediate compound I ($-Fe^{IV}=O$). Compound I then reacts with a second H_2O_2 molecule, returning to its original state ($-Fe^{III}$). Excess levels of H_2O_2 may promote the formation of an intermediate compound II ($-Fe^{IV}$-OH) due to one electron oxidation; this intermediate is not active in H_2O_2 removal. NADPH binding is important to maintain an active enzyme as such binding can prevent the formation of compound II.

Some of the catalytic properties of the *H. pylori* catalase are very similar to those of other classical catalases (Hazell *et al*., 1991). However, *H. pylori* catalase has some unique properties, e.g. it has a high isoelectric points (pI) value (> 9), is more resistant to cyanide and is stable at very high concentration of H_2O_2 (Hazell *et al*., 1991), as indicated by the fact that purified *H. pylori* catalase retains nearly full activity after treatment with a molar ratio of 1:3000 H_2O_2 (Wang *et al*., 2004). *H. pylori* catalase is uniquely sensitive to organic hydroperoxides due to perturbation of the haem environment in the enzyme and formation of a catalytically incompetent compound II species (Wang *et al*., 2004). The core structure of *H. pylori* catalase is very similar to that of other catalases but (unlike others) *H. pylori* catalase does not bind NADPH. Instead, it binds formic acid to promote the breakdown of the compound I intermediate and to prevent formation of inactive compound II (Loewen *et al*., 2004).

Helicobacter pylori catalase-deficient mutants are viable when cultured *in vitro* (Odenbreit *et al*., 1996; Manos *et al*., 1998; Harris *et al*., 2002), suggesting that endogenously generated H_2O_2 is not a significant problem for *H. pylori*. However, catalase is important for survival in the presence of extracellular ROS produced by professional phagocytes (Ramarao *et al*., 2000) and macrophage phagosomes (Basu *et al*., 2004). Wild-type *H. pylori* cells can withstand exposure to ~100 mM H_2O_2, but the *katA* mutants die within a few minutes under these conditions (Harris *et al*., 2002; 2003). A protein (KapA, for KatA-associated protein) encoded by a gene downstream of *katA* is involved in H_2O_2 resistance. Disruption of *kapA* in *H. pylori* does not affect catalase activity but increases sensitivity to H_2O_2 (Harris *et al*., 2002). *kapA* seems to be an *H. pylori*-specific gene, as no homologue has been found in any other related or unrelated species. Unlike *E. coli*, which has two catalases, one cytoplasmic and one periplasmic, *H. pylori* has only one catalase located in both the cytoplasm and periplasm (Harris and Hazell, 2003). Only periplasmic catalase activity is affected by the loss of KapA. KapA appears to be exported by the twin-arginine translocator (TAT) system. Potential interaction between KatA and KapA (Rain *et al*., 2001) might allow KapA to carry the fully assembled KatA into the periplasm (Harris and Hazell, 2003). Mouse studies indicate that both KatA and KapA are important in persistence of *H. pylori*, as both mutants colonized the gastric mucosa in the long-term infection experiments (24 weeks) at much lower levels than did the wild type (Harris *et al*., 2003).

Peroxiredoxins: AhpC, Tpx and BCP

Peroxiredoxins (Prx) are ubiquitous proteins that confer resistance to oxidative stress. These enzymes lack prosthetic groups and catalyse the reduction of hydrogen peroxide, peroxynitrite, and a wide range of organic hydroperoxides (ROOH) to their corresponding alcohols (Wood *et al*., 2003).

$$ROOH + 2e^- \rightarrow ROH + H_2O$$

Peroxiredoxins can be divided into two subgroups according to the number of the conserved cysteines (Cys) within the protein. Some peroxiredoxins contain only a single essential, N-terminal Cys residue per subunit (1-Cys Prx), and other peroxiredoxins contain an additional conserved Cys residue that links the two subunits via an intersubunit disulphide bond with the N-terminal Cys of the oxidized protein (2-Cys Prx). Bacterial AhpC (alkyl hydroperoxide reductase) has sequence homology to 2-Cys Prx of

higher organisms. AhpC is regarded as the founding member of the Prx family, and its homologues appear to be more widely distributed than SOD and catalase (Chae *et al*., 1994).

The *H. pylori* genome contains a gene (HP1563) encoding a 26 kDa AhpC protein. Unlike many other bacteria, which produce a flavoprotein AhpF dedicated solely to AhpC reduction, *H. pylori* does not have an *ahpF* homologue. Instead, *H. pylori* harbours thioredoxin-1 (Trx1) (HP0824) and thioredoxin reductase (TrxR) (HP0825) (homologues of *E. coli trxA* and *trxB* respectively) that are similar to the components of a eukaryotic peroxidase reduction system. Using the purified *H. pylori* AhpC, Trx1 and TrxR proteins, Baker *et al*. (2001) reconstructed the complete system to catalyse reduction of peroxides (ROOH). The electron transfer proceeds as follows:

$$\text{NADPH} \rightarrow \text{TrxR} \rightarrow \text{Trx1} \rightarrow \text{AhpC} \rightarrow \text{ROOH}$$

Helicobacter pylori AhpC exhibited broad specificity for hydroperoxide substrates, with similar rate constants in reducing hydrogen peroxide, t-butyl hydroperoxide and linoleic acid hydroperoxide (Baker *et al*., 2001).

Helicobacter pylori AhpC is apparently essential for growth under normal *in vitro* condition (Lundstrom and Bolin, 2000; Baker *et al*., 2001), but *ahpC* mutants can be obtained under extremely low O_2 condition (Olczak *et al*., 2002). Majority of *ahpC* mutants express abnormally high levels of neutrophil activating protein (NapA), an iron-binding protein, leading to the suggestion that NapA is an antioxidant protein that compensates for the loss of AhpC. The *ahpC* mutants were highly sensitive to oxidative stress and mutated more frequently than the wild-type strain (Olczak *et al*., 2002). The *ahpC* mutants of the mouse-adapted strain SS1 were unable to colonize the stomachs of mice, whereas most of the mice inoculated with the parental strain were infected with *H. pylori* (Olczak *et al*., 2003).

Peroxiredoxins serve also in protection against RNI. An *S. typhimurium ahpC* mutant was significantly more sensitive than the wild type to killing by RNI (Chen *et al*., 1998). The defect in peroxiredoxins Tsa1p and Tsa2p (AhpC homologues) in yeast renders hypersensitivity to peroxynitrite, and the *in vitro* peroxynitrite reductase activity of Tsa2 was demonstrated (Wong *et al*., 2002). AhpC proteins purified from *S. typhimurium*, *Mycobacterium tuberculosis* and *H. pylori* were shown to have the peroxynitrite reductase activity *in vitro* (Bryk *et al*., 2000). Thus, *H. pylori ahpC* mutants are expected to be sensitive to RNI, although this has not been tested. However, the AhpC of *Campylobacter jejuni* confers resistance to oxidative stress caused by organic peroxides and atmospheric oxygen (Baillon *et al*., 1999), but not to RNI (Elvers *et al*., 2004).

Physiological analysis of *H. pylori ahpC* mutants revealed a novel connection between two abundant and important antioxidant enzymes. The *ahpC* mutant strains have decreased catalase activity due to inactivation of catalase by organic hydroperoxides that accumulate in these mutants (Wang *et al*., 2004). Lipid hydroperoxide levels in *ahpC* mutants are about three times higher than in wild-type cells, suggesting that AhpC reduces organic hydroperoxide levels. A *C. jejuni ahpC* mutant was significantly more sensitive to cumene hydroperoxide, but not to H_2O_2 (Baillon *et al*., 1999), suggesting a major role of AhpC is to reduce organic hydroperoxides. In contrast, the major physiological substrate of AhpC in *E. coli* appears to be H_2O_2 rather than organic hydroperoxides (Seaver and Imlay, 2001).

Helicobacter pylori AhpC is more similar to mammalian peroxiredoxins than to bacterial AhpC (Chuang *et al*., 2006). The crystal structure (Papinutto *et al*., 2005) of *H. pylori* AhpC consists of a pentameric arrangement of homodimers [$(\alpha_2)_5$ decamer], similar to other members of 2-Cys Prx family. Interestingly, the structure of *H. pylori* AhpC protein could shift from low-molecular-weight oligomers with peroxide-reductase activity to high-molecular-weight complexes that have molecular chaperone function under oxidative stress (Chuang *et al*., 2006). This structural change occurs in *H. pylori* cells, possibly to prevent protein misfolding under oxidative stress. The molecular events triggering this switch are of major interest.

Helicobacter pylori genome contains genes for two other thiol-peroxidases, *tpx* (HP0390, also named *tagD*) and *bcp* (HP0136), both belonging to a 1-Cys Prx subgroup. Tpx was originally identified as an *E. coli* homologue of scavengase p20, which has a thioredoxin-linked peroxidase activity (Zhou *et al*., 1997). An *H. pylori tpx* mutant is more sensitive to killing by peroxide and superoxide than the wild-type strain and is less able to colonize mouse stomachs (Comtois *et al*., 2003; Olczak *et al*., 2003). BCP, originally identified in *E. coli* as a 'bacterioferritin co-migratory protein', is a new member of the 1-Cys Prx family. *E. coli* BCP has thioredoxin-dependent thiol-peroxidase activity, with the substrate linoleic acid hydroperoxide being preferred to H_2O_2 (Jeong *et al*., 2000). *H. pylori bcp* mutants exhibit moderately increased sensitivity to oxidative stress reagents and significantly reduced ability to colonize mouse stomachs within 3 weeks after inoculation (Wang *et al*., 2005b). Like its *E. coli* counterpart, purified *H. pylori* BCP has a thioredoxin-dependent thiol-peroxidase activity, with linoleic acid hydroperoxide being the preferred substrate. Whereas the *E. coli bcp* mutant is equally (hyper)sensitive to H_2O_2 and organic peroxides as the *ahpC* mutant (Jeong *et al*., 2000), an *H. pylori bcp* mutant is less sensitive to oxidative stress than the *ahpC* mutant, and AhpC seems

to play a greater oxidative stress-combating role than BCP (Wang *et al*., 2005b; 2006).

Lipid peroxidation contributes to cell injury by altering the basic physical properties and structural organization of membrane components. The growth of *H. pylori* displayed sensitivity to the added unsaturated free fatty acids due to their incorporation into phospholipids of the membrane, leading to membrane disfunction (Hazell and Graham, 1990; Khulusi *et al*., 1995; Sun *et al*., 2003). Both AhpC and BCP were shown to confer protection from unsaturated fatty acids-mediated toxicity; the mutant cells defective in AhpC, BCP or in both enzymes contained increased amounts of LOOH, implicating their role in preventing lipid peroxidation (Wang *et al*., 2006).

Thioredoxin system (Trx, TrxR) and arginase (RocF)

Unlike many other prokaryotes, *H. pylori* lacks the enzymes to generate glutathione and other thiol reductants such as glutaredoxin, but it does possess the thioredoxin (Trx) system. Trxs are key proteins for many crucial cellular functions, including oxidative stress management (Ritz and Beckwith, 2001). The redox protein Trx and the associated enzyme TrxR constitute a thiol-dependent reduction-oxidation system that can catalyse the reduction of specific proteins (e.g. a number of ROS detoxification enzymes) by NADPH (Holmgren, 1985).

Helicobacter pylori possess two Trxs (Trx1 and Trx2) and a Trx reductase (TrxR). Trx1 was considered as a stress response element in *H. pylori*, as its expression increased dramatically under conditions of oxidative stress (Windle *et al*., 2000). Trx1 and TrxR together form a reductase system for *H. pylori* AhpC (Baker *et al*., 2001) (see the section on Peroxiredoxins above). This reductase system is also active for reduction of BCP (Wang *et al*., 2005b) and Msr (methionine sulphoxide reductase, see below) (Alamuri and Maier, 2004). The function of Trx2 is not clear. However, a mutation in either Trx1 or Trx2 resulted in an increase in sensitivity to several forms of oxidative and nitrosative stress (Comtois *et al*., 2003). The phenotype of an *H. pylori trxR* mutant has not yet been reported.

Most recently, a novel role of Trx1 as an arginase chaperone and guardian against oxidative and nitrosative stresses was discovered. *H. pylori* Trx1 is able to re-nature urea- or heat-denatured arginase back to catalytically active state (McGee *et al*., 2006). Whether Trx1 acts as a guardian for other *H. pylori* enzymes is unknown. *H. pylori* arginase is also an oxidative stress-combating enzyme, because it inhibits nitric oxide production from host cells by consuming L-arginine, the common substrate for inducible nitric oxide synthase (iNOS) and arginase. Thus, wild-type *H. pylori* can survive co-culture with activated macrophages, whereas inactivation of the arginase gene (*rocF*) resulted in high-output NO production by macrophages, and killing of the mutant when co-cultured with macrophages (Gobert *et al*., 2001). When tested for the ability to colonize mouse stomach, a *rocF* mutant strain colonized mice equally well as wild type; however, another *rocF* mutant strain had moderately reduced colonization (McGee *et al*., 1999).

NADPH quinone reductase MdaB

Studies of oxidative stress resistance mutants in *H. pylori* (Olczak *et al*., 2002) identified a novel potential antioxidant protein (MdaB) by its up-expression apparently to compensate for the loss of other major antioxidant components. MdaB of *E. coli* was first identified as a modulator of drug activity and later as an NADPH-specific quinone reductase that catalyses the two-electron reduction of quinone to quinols (Hayashi *et al*., 1996). An *H. pylori mdaB* mutation increases sensitivity to H_2O_2, organic hydroperoxides and molecular oxygen, and significantly reduces colonization of mouse stomachs (Wang and Maier, 2004). Purified *H. pylori* MdaB is a flavoprotein that catalyses a two-electron transfer from NADPH to a variety of quinone molecules, including the two major quinone types present in *H. pylori* (Wang and Maier, 2004).

Although the roles of membrane-bound quinone oxidoreductases in respiratory metabolism are well studied, little is known about the biological role of soluble bacterial quinone reductases. Although MdaB homologues are present in many pathogenic bacteria (revealed by genome sequences), *H. pylori* MdaB is the first example of a soluble bacterial quinone reductase contributing to antioxidant defence. Another example of soluble quinone reductase in bacteria is ChrR (a chromate-reducing enzyme) of *Pseudomonas putida* that plays a significant role in bacterial defence against peroxide stress (Gonzalez *et al*., 2005). However, *P. putida* ChrR is not a homologue of bacterial MdaB, and ChrR is a flavin mononucleotide (FMN)-binding flavoprotein, whereas MdaB contains flavin adenine dinucleotide (FAD) as cofactor.

Only one eukaryotic soluble quinone reductase, also known as NAD(P)H quinone oxidoreductase (mammalian NQO1), is a well-studied enzyme, both structurally and mechanistically (Li *et al*., 1995). It catalyses the two-electron reduction of quinones to quinols, thus avoiding formation of cytotoxic semiquinones by competing for the detrimental one-electron reduction pathway (Siegel *et al*., 1997). NQO1 also has a direct role in scavenging superoxide (Siegel *et al*., 2004). Except for the highly conserved flavodoxin domain, bacterial MdaB has limited sequence homology to mammalian NQO1, whereas the overall sequence homology between MdaBs of *E. coli* and

H. pylori is about 50%. The crystal structure of *E. coli* MdaB exhibits a significant structural similarity to a number of flavoproteins including mammalian NQO1, suggesting it may function in antioxidant defence (Adams and Jia, 2006).

Neutrophil-activating protein NapA and ferritin Pfr

HP-NAP (NapA, HP0243 in the *H. pylori* genome) was first identified as a factor that mediates neutrophil activation. It plays a major role in recruiting human neutrophils and monocytes to the site of infection (Evans *et al*., 1995). HP-NAP is a major antigen in the human immune response and stimulates the production of reactive oxygen intermediates (ROI) by human neutrophils and monocytes (Evans *et al*., 1995; Satin *et al*., 2000). HP-NAP is located in the bacterial cytosol, and it can bind to carbohydrates on the external surface of the outer membrane, thereby mediating *H. pylori* binding both to host cells and to the stomach mucus (Teneberg *et al*., 1997; Namavar *et al*., 1998). NapA-induced ROI production involves a cascade of intracellular activation events, including increase of cytosolic calcium ion concentration and phosphorylation of proteins, leading to the assembly of the superoxide-forming NADPH oxidase on the neutrophil plasma membrane (Satin *et al*., 2000; Montecucco and de Bernard, 2003; Nishioka *et al*., 2003).

The primary sequence and structure of HP-NAP are similar to those of the proteins in the Dps family of iron-binding, DNA-protecting proteins (Grant *et al*., 1998). The 17 kDa NapA forms a dodecameric complex with a central cavity that sequesters large amounts of iron (nearly 500 atoms of iron per NapA protein) (Tonello *et al*., 1999; Zanotti *et al*., 2002). However, the synthesis of NapA in *H. pylori* is not altered in response to the iron content in the growth medium, leading to the proposal that the primary role of NapA *in vivo* may not be as a iron-binding protein (Dundon *et al*., 2001).

Two attributes of Dps family proteins account for their role in protecting DNA from oxidative damage. Through direct interaction, Dps and DNA form a highly ordered and stable nucleoprotein complex called a biocrystal so that DNA is 'sheltered' from the attack of the free oxidative radicals (Wolf *et al*., 1999); and Dps sequesters intracellular free ferrous ions, thus preventing the production of $OH^{\bullet}$ through Fenton reaction (Buda *et al*., 2003). Dps family proteins are present in many prokaryotes, such as *E. coli* (Zhao *et al*., 2002), *Listeria innocua* (Bozzi *et al*., 1997), *Streptococcus mutans* (Yamamoto *et al*., 2002), *Agrobacterium tumefaciens* (Ceci *et al*., 2003), *Mycobacterium smegmatis* (Gupta and Chatterji, 2003), *Porphyromonas gingivalis* (Ueshima *et al*., 2003), *C. jejuni* (Ishikawa *et al*., 2003) and *Helicobacter hepaticus* (Hong *et al*., 2006). All Dps proteins have been reported to bind iron ions but some lack the ability to bind DNA *in vitro* (Tonello *et al*., 1999; Ceci *et al*., 2003; Ishikawa *et al*., 2003). Nonetheless, they all play an important role in DNA protection under oxidative stress conditions.

The role of NapA in protecting *H. pylori* from oxidative stress damage was first indicated by the observation that loss of AhpC leads to a concomitant increase in NapA expression (Olczak *et al*., 2002). Most recent studies further demonstrated that increased NapA production is the most frequent change associated with the apparent compensatory response to loss of major oxidative stress resistance factors in *H. pylori*. For example, NapA is two to five times more abundant when a single oxidative stress resistance gene (*katA*, *sodB*, *tpx*, *ahpC*) is disrupted, and 6–10 times more abundant in double mutants (Olczak *et al*., 2005). Like other Dps proteins, *H. pylori* NapA production is maximal in stationary-phase cells, and an *H. pylori napA* mutant survives less well than the wild-type strain upon exposure to oxidative stress conditions (Cooksley *et al*., 2003). Although results from an *in vitro* DNA-binding assay suggested that *H. pylori* NapA does not bind DNA (Tonello *et al*., 1999), other data demonstrated that NapA colocalizes with the nucleoid, suggesting that it can interact with DNA *in vivo* (Cooksley *et al*., 2003). All these results suggest that *H. pylori* NapA have a similar function as Dps family proteins in protecting cells from oxidative stress damage. Such a role of NapA in the host environment (effect on colonization efficiency) has yet to be investigated. The ability to induce a series of signal transduction events in the eukaryotic host cells makes Hp NapA distinct from other proteins of Dps family.

Similar to *C. jejuni*, *H. pylori* contains a 'canonical' ferritin Pfr, which is the major iron storage protein playing a significant role in iron homeostasis (Bereswill *et al*., 1998; Waidner *et al*., 2002). A *pfr* mutant of *H. pylori* was completely defective for the colonization in the gerbil stomach, indicating that iron storage is a prerequisite for the successful establishment of *H. pylori* infection (Waidner *et al*., 2002). *H. pylori* Pfr confers resistance to killing by iron toxicity, but appeared not to be protective against oxidative stress (Bereswill *et al*., 1998). An *H. pylori pfr* mutant was shown to be more resistant, rather than sensitive, to paraquat than the wild type, which may be accounted for by the low iron content in the mutant cells (Waidner *et al*., 2002). *S. mutans* does not contain 'canonical' ferritins or bacterioferritins, but has a Dpr (for Dps-like peroxide resistance) that confers oxygen tolerance (Yamamoto *et al*., 2002; 2004). The role of ferritin as iron storage may not require rapid iron sensing, while Dps is considered to quickly sequester fluxes of free irons in order to prevent generation of oxygen related radicals during oxidative stress.

DNA repair proteins: endonuclease III (Nth), MutS and RuvC

As described above, *H. pylori* has a broad repertoire of ROS detoxification enzymes. When facing the oxidative burst elicited by the host immune systems, however, these oxidant-detoxification activities might not be sufficient to combat oxidative damage. As another line of defence against oxidative damage, *H. pylori* has other mechanisms for DNA damage repair, including universal proteins that are involved in DNA replication, recombination and repair such as RecA (Thompson and Blaser, 1995) and UvrABC (Thompson *et al*., 1998), and additional proteins specific for oxidative DNA damage repair that have only recently received much attention (see below).

The question of whether *H. pylori* DNA is subject to damage by the host-generated oxidative response was first addressed by studying *H. pylori nth* mutants unable to repair oxidized pyrimidines (O'Rourke *et al*., 2003). *nth* encodes endonuclease III (EndoIII), the enzyme responsible for excising lethal or mutagenic pyrimidine lesions such as thymine glycol, 5,6-dihydrothymine and urea. In addition to its DNA glycosylase activity, all EndoIII enzymes known in other systems possess an abasic (AP) lyase activity that cleaves the DNA backbone 3′ of the AP site. *H. pylori nth* mutants are more sensitive than the wild-type strain to killing by exposure to oxidative agents or activated macrophages and exhibit significantly reduced mouse colonization capacity, being eradicated 15 days after co-infection even when inoculated in a 1:9 wild type:mutant ratio (O'Rourke *et al*., 2003). This indicates that the deleterious DNA lesions reduce competitivity.

The DNA methyl-directed mismatch repair (MMR) system, consisting of three proteins MutS, MutL and MutH, functions in post-replication DNA repair and plays a central role in maintaining genetic stability. *H. pylori* does not have *mutL* or *mutH* genes, but contains a MutS homologue. Inactivation of the *mutS* gene in *H. pylori* has little effect on the mutation frequency, and about 25% of natural *H. pylori* isolates exhibit higher mutation frequencies than those found in MMR-defective *Enterobacteriaceae*, indicating that *H. pylori* lacks a MutSLH-dependent DNA mismatch repair system (Bjorkholm *et al*., 2001). A recent study (Pinto *et al*., 2005) showed that *H. pylori* MutS protein binds to DNA structures mimicking recombination intermediates, indicating a role for *H. pylori* MutS in inhibiting DNA strand exchange (suppression of DNA recombination). In another study (Wang *et al*., 2005c), *H. pylori mutS* mutants were found to be more sensitive than wild-type cells to oxidative stress, and to have elevated oxidative stress-induced mutation rates. Strikingly, most mutations generated in *mutS* strains under oxidative stress condition are G:C to T:A transversions, a signature of 8-oxoguanine. Indeed, *H. pylori mutS* cells contain a higher level (approximately three times) of 8-oxoguanine DNA lesions than wild-type cells. Furthermore, purified *H. pylori* MutS is highly active on double-stranded DNA containing 8-oxoguanine, supporting its role in repairing 8-oxoguanine. Finally, an *H. pylori mutS* strain exhibits a significantly decreased animal colonization efficiency, further supporting the idea that oxidative DNA damage repair plays an important role in bacterial survival/colonization in the host (Wang *et al*., 2005c).

Some types of DNA damage require repair by homologous DNA recombination. A component of DNA recombination machinery is the endonuclease RuvC, which is responsible for resolving the recombination intermediate 'Holliday Junction'. Inactivation of *ruvC* in *H. pylori* reduced the frequency of homologous recombination and increased sensitivity to DNA-damaging agents (Loughlin *et al*., 2003). The *H. pylori ruvC* mutants were also more susceptible to oxidative stress and reduced survival within macrophages, highlighting the role of DNA recombination in oxidative DNA damage repair. An *H. pylori ruvC* mutant was attenuated for colonization in the mouse, providing support for genetic recombination playing an important role in establishing infection (Loughlin *et al*., 2003).

Protein repair by methionine sulphoxide reductase

Protein oxidation occurs due to irreversible oxidation of sensitive amino acid residues and is a deleterious consequence of oxidative stress with a significant impact on the cell survival under oxidative stress conditions. Methionine (Met) is one of the most sensitive residues to oxidizing conditions and is converted to a reversible methionine sulphoxide (MetO) under mild oxidation and to an irreversible methionine sulphone upon prolonged oxidation (Vogt, 1995). The reduction of MetO to Met is catalysed by Trx-TrxR-dependent methionine sulphoxide reductase (Msr), an enzyme system that is highly conserved from eubacteria to humans (Boschi-Muller *et al*., 2005). The enzymes MsrA and MsrB catalyse the reduction of the two diastereomers S-sulphoxide and R-sulphoxide of Met(O) respectively (Boschi-Muller *et al*., 2005).

Msr in *H. pylori* (HP0224) is a 42 kDa protein with the MsrA and MsrB domains fused together, a feature observed in *Haemophilus influenzae*, *Neisseria gonorrhoeae* and *N. meningitides*, whereas MsrA and MsrB are two separate functional proteins in most of the other bacteria including *E. coli* (Ezraty *et al*., 2005). Inactivation of either the *H. pylori msrB* region (C-terminus) or complete inactivation of the two domains (*msr*) resulted in the loss of specific Msr activity, which compromises growth in the presence of chemical oxidants. Comparison of whole-

protein profiles from cells subjected to oxidative stress conditions showed increased abundance of carbonyl groups on protein side-chains in the mutant. Finally, both mutants showed reduced ability to colonize mouse stomachs, indicating that Msr is an important *H. pylori* persistence factor (Alamuri and Maier, 2004).

Several methionine-rich proteins protected by Msr in *H. pylori* have been identified, which include catalase (KatA), the heat shock protein chaperone (GroEL) and a 23 kDa protein annotated as site-specific recombinase (SSR). Repair of oxidized KatA and SSR by Msr was not reported in any other bacterial species. An *H. pylori msr* mutant showed significant decrease in its catalase activity and was particularly sensitive to hydrogen peroxide and peroxinitrite. Msr appears to target a few but physiologically significant proteins in the cell, the protection of which would indirectly confer resistance against oxidative stress (Alamuri and Maier, 2006).

In vitro, in the presence of Trx1 and TrxR, *H. pylori* Msr exhibits activity in reducing only the R-isomer of methyl-p-tolyl sulphoxide, a characteristic of an MsrB-type enzyme (Alamuri and Maier, 2006). However, in *N. gonorrhoeae*, both MsrA and MsrB domains could independently reduce the S- and R- isomers of methionine sulphoxide. *H. pylori* contains an inactive MsrA domain, but the closely related bacteria *H. hepaticus* and *C. jejuni* only contain *msrA* and lack any *msr*B homologues in their genome.

Regulation of antioxidant proteins

For successful and persistent infection in the host, pathogenic bacteria usually depend on the ability to respond to changing environmental conditions to co-ordinate the expression of virulence factors. *H. pylori* persistently colonizes the human gastric mucosa and most of the antioxidant proteins described above appear to be important virulence factors. However, *H. pylori* possess a surprisingly low number of regulators potentially involved in regulation of expression of antioxidant proteins; no homologues of the oxidative stress regulators present in other bacteria, including OxyR, SoxR, SoxS, RpoS, LexA and PerR, have been found in the *H. pylori* genome. This may reflect the fact that *H. pylori* survives in the relatively restricted niche of the human gastric mucosa, with an apparent lack of competition from other microorganisms (Berg *et al*., 1997). Nevertheless, recent studies using microarrays have shown that gene regulation occurs frequently in *H. pylori* as a response to stress such as acid, growth phase and iron starvation (Merrell *et al*., 2003a,b). Gene expression profiling using microarray for *H. pylori* in response to oxidative stress has not been reported. A proteomic analysis of proteins expression in *H. pylori* revealed that expression of AhpC was inhibited, rather than induced, under oxidative stress, which could account for the loss of survival and proliferation potential of the bacterium under such condition (Chuang *et al*., 2005).

Iron homeostasis and oxidative stress defence are intimately linked, and *H. pylori* contains the ferric uptake regulator (Fur). *sodB* expression in *H. pylori* is directly repressed by the iron-free form of Fur via its binding to the promoter sequence upstream of the *sodB* gene. Repression is terminated by iron supplementation, allowing expression of Fe-SOD to counter the iron-mediated formation of ROS (Ernst *et al*., 2005). This direct role of Fur contrasts with the regulation of iron-cofactored SOD in *E. coli*, which is mediated indirectly by Fur via the RyhB small RNA (Masse and Gottesman, 2002). Fur-deficient mutants and cells under iron starvation condition showed a reduction in catalase activity and increased sensitivity to hydrogen peroxide, suggesting a direct or indirect role for Fur in regulating catalase expression in response to iron (Harris *et al*., 2002). Fur is also, at least partly, involved in iron-responsive regulation of NapA expression (repression of NapA by iron starvation) (Cooksley *et al*., 2003). Reporter gene fusion studies revealed up-expression of *H. pylori msr* under conditions of peroxide stress, iron starvation, and in stationary phase of growth; however, this regulation appeared not to be mediated by Fur (Alamuri and Maier, 2006).

CsrA is a post-transcriptional regulator and functions as a global regulator for genes involved in motility, flagellar synthesis, gluconeogenesis, glycogen synthesis and biofilm formation in *E. coli* (Romeo, 1998; Baker *et al*., 2002). *H. pylori* CsrA was necessary for full motility and survival under oxidative stress. The mRNA transcript of NapA or AhpC was highly induced under oxidizing conditions in *H. pylori* wild type or in a *csrA* mutant. A corresponding increase in protein level, however, was not observed in the *csrA* mutant, suggesting a potential role of CsrA in mRNA stabilization (Barnard *et al*., 2004). CsrA was also shown to regulate other known regulators in *H. pylori* such as the metalloregulator Fur and the heat shock gene regulator HspR. This hierarchy in regulation indicates a surprisingly complex regulatory network exists in *H. pylori*; and with very limited sigma factors (RpoN, RpoD and FliA), post-transcriptional regulation perhaps plays a major role in the regulation of various stress response genes in *H. pylori* (Barnard *et al*., 2004).

Final comments

The *H. pylori* antioxidant defence is multifaceted and diverse, including some well-studied proteins present in many pathogens, along with some unique mechanisms and novel physiological roles to combat oxidative stress. The antioxidant proteins described above are listed in Table 1. The paradox is that despite this impressive

Table 1. *Helicobacter pylori* antioxidant proteins.

Gene	HP No.	kDa	Activity	Oxidative stress-related function	Colonization phenotype (reference)
sodB	0389	25	Superoxide dismutase	Dissipates superoxide	Defective (Seyler *et al*., 2001)
katA	0875	55	Catalase (high pI; highly resistant to H_2O_2, but sensitive to ROOH*)	Removes H_2O_2	Attenuated (Harris *et al*., 2003)
kapA	0874	33	KatA-associated protein	Assists KatA translocation into periplasm*	Attenuated (Harris *et al*., 2003)
ahpC	1563	26	Alkyl hydroperoxide reductase, Peroxynitrite reductase*	Detoxifies ROOH and $OONO^-$* Protects KatA* Molecular chaperon*	Defective (Olczak *et al*., 2003)
tpx	0390	18	Thiol-peroxidase	H_2O_2 scavenger?	Attenuated (Olczak *et al*., 2003)
bcp	0136	17	Thiol-peroxidase	Lipid peroxides scavenger?	Attenuated (Wang *et al*., 2005b)
trx1	0824	12	Thioredoxin	Thiol-dependent reduction system; Arginase chaperon*	Not tested
trxR	0825	34	Thioredoxin reductase	Thiol-dependent reduction system	Not tested
rocF	1399	37	Arginase	Inhibits NO production by host cells*	Normal or attenuated (McGee *et al*., 1999)
mdaB	0630	22	NADPH quinone reductase	Maintains redox balance (reduced quinone pool)*	Attenuated (Wang and Maier, 2004)
napA	0243	17	Iron-binding, Dps-like	Induces host inflammation;* Sequesters intracellular free iron*	Not tested
pfr	0653	19	Ferritin	Iron storage	Defective (Waidner *et al*., 2002)
nth	0585	25	Endonuclease III	Removes oxidized pyrimidines in DNA	Attenuated (O'Rourke *et al*., 2003)
mutS	0621	86	8-oxoguanine binding;* 'Holiday Junction' binding	Repairs oxidative DNA damage;* Suppresses DNA recombination*	Attenuated (Wang *et al*., 2005c)
ruvC	0877	17	Endonuclease	Resolves recombination intermediate	Attenuated (Loughlin *et al*., 2003)
msr	0224	42	Methionine sulphoxide reductase MsrB-type enzyme*	Repairs oxidized proteins; A major repair target is KatA*	Attenuated (Alamuri and Maier, 2004)
fur	1027	18	Ferric uptake regulator	Regulates iron uptake and metabolism; Regulates antioxidant genes*	Attenuated (Bury-Mone *et al*., 2004)
csrA	1442	8	Carbon storage regulator	Global regulation; Post-transcriptional regulator for antioxidant genes*	Attenuated (Barnard *et al*., 2004)

The activities or functions that are *H. pylori*-specific or newly described are highlighted with an asterisk (*). For colonization phenotypes, 'defective' means less than 5% of the wild-type colonization ability, whereas 'attenuated' means significantly less than wild-type colonization.

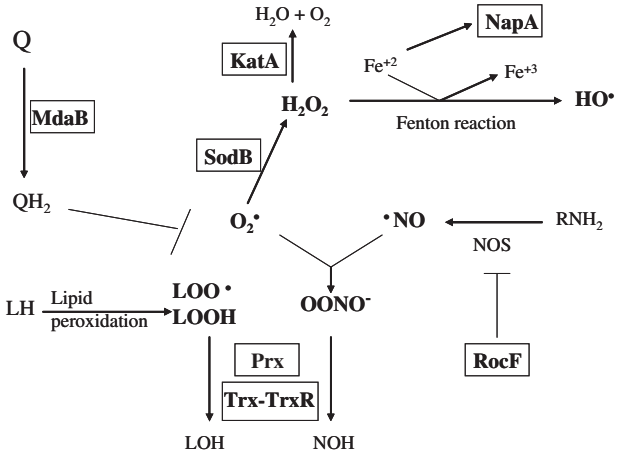

Fig. 1. Sources of ROS and the detoxification systems in *H. pylori*. The intracellular reactive oxygen species (ROS) are highlighted in bold; and the antioxidant proteins for ROS detoxification are boxed. $O_2^{\bullet}$ and H_2O_2, the major sources of ROS, are removed by SodB and KatA. Through Fenton reaction, H_2O_2 reacts with intracellular free iron to produce $HO^{\bullet}$, the most toxic ROS. NapA plays a central role in removing excess intracellular free iron, thus inhibiting its toxic effects. Under oxidative stress condition, lipid (LH) undergoes peroxidation, producing lipid peroxides (LOOH) and some radicals such as peroxyl radical ($LOO^{\bullet}$). LOOH are reduced to non-toxic alcohol form by various peroxiredoxins (Prx) including AhpC, Tpx and BCP. The function of Prx requires the Trx-TrxR reduction system. MdaB catalyses reduction of quinones (Q) to quinols (QH2), preventing formation of toxic semiquinone and maintaining the redox balance of the cell to inhibit the effects of ROS. Host gastric cells produce nitric oxide ($^{\bullet}NO$), whose production is inhibited by *H. pylori* arginase. $OONO^-$ is the joint product of $^{\bullet}NO$ and O_2^-. Enzymes responsible for detoxification of $OONO^-$ are SOD (indirectly) and AhpC (directly).

repertoire the bacterium remains oxygen sensitive (microaerophilic). Most likely, some of the susceptible *H. pylori* targets of oxidative stress cannot be repaired, or perhaps the bacterium contains macromolecules that facilitate O_2-initiated production of highly damaging reactive species. Whatever the mechanism, the bacterium obviously encounters severe stress in the gastric mucosa, and it mounts a sophisticated and interconnected battery of diverse enzymes that contribute to its persistence. Figure 1 illustrates the major sources of ROS *H. pylori* encounters and the systems *H. pylori* uses to detoxify them. In addition, as a second line of defence against oxidative stress, *H. pylori* is equipped with protein and DNA repair systems as listed in Table 1. Although some of the *H. pylori* antioxidant systems are known to be used by other pathogens, oftentimes the *H. pylori* studies have revealed a unique way of managing oxidative stress. The activities or functions of the antioxidant systems that are *H. pylori*-specific or newly described are highlighted with an asterisk in Table 1. These mechanisms range from protecting a single key oxidative stress enzyme (catalase) from oxidative inhibitors via the catalytic activity of peroxiredoxins, or repair of damaged proteins by Msr enzyme, to repairing 8-oxoguanine-containing DNA by bacterial MutS2, and even expressing proteins (AhpC, NapA, Trx1, arginase) with multiple functions. The antioxidant systems described above will likely be relevant to many bacterial-associated and especially inflammation-associated infectious diseases.

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