Role of the thioredoxin system and the thiol-peroxidases Tpx and Bcp in mediating resistance to oxidative and nitrosative stress in *Helicobacter pylori*

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*Helicobacter pylori* possesses two distinct thioredoxin proteins (Trx1 and Trx2) which may play important roles in the ability of this bacterium to survive oxidative stress. Trx1 has previously been shown to be an electron donor *in vitro* for alkyl-hydroperoxide reductase (AhpC), one of three members of the peroxiredoxin family of antioxidant peroxidases present in *H. pylori*. In this study, mutants in the *trxA1* and *trxA2* genes encoding Trx1 and Trx2, respectively, and in the *tpx* and *bcp* genes, which encode the remaining two members of the *H. pylori* peroxiredoxin family, were constructed in order to determine their roles in resistance to damage by reactive oxygen and nitrogen species. Mutation of *trxA1* led to a pronounced increase in sensitivity to oxygen, hydrogen peroxide and the superoxide generator paraquat, as well as to the nitric oxide (NO) releasers sodium nitroprusside (SNP) and S-nitrosoglutathione (GSNO), consistent with an *in vivo* role for Trx1 as a reductant for AhpC. A *trxA2* single mutant grew normally in an atmosphere of 2 % (v/v) O$_2$ but grew very poorly in 10 % (v/v) O$_2$. It showed slight increases in killing by hydrogen peroxide, paraquat, SNP and GSNO compared to the wild-type, but was significantly more sensitive to cumene hydroperoxide in disc-diffusion assays. A *trxA1 trxA2* double mutant was very sensitive to all of the oxidative and nitrosative stresses applied. Comparison of the phenotypes of the *tpx* and *bcp* mutants showed that Tpx plays a significant role in peroxide and superoxide resistance in *H. pylori*, while the role of Bcp is minimal. No evidence was obtained for a role for either Tpx or Bcp in resistance to the toxic effects of NO. The results show that a functional thioredoxin system is necessary for both oxidative and nitrosative stress resistance in *H. pylori* but, surprisingly, is not essential for viability despite the absence of glutathione and a glutaredoxin system in this bacterium.

**INTRODUCTION**

*Helicobacter pylori* is a ubiquitous human pathogen and is the causative agent of type B gastritis and the majority of cases of duodenal and gastric ulceration (Dixon, 2001). Moreover, long-term infection with the bacterium is a known risk-factor for the development of gastric cancer (Forman et al., 1991). *H. pylori* is a microaerophilic bacterium that is unable to grow at normal atmospheric oxygen concentrations, and is routinely cultured in gas atmospheres containing 3–10 % (v/v) O$_2$ and 5–10 % (v/v) CO$_2$ (Andersen & Wadström, 2001). The molecular basis of the sensitivity of *H. pylori* to molecular oxygen is now beginning to be understood. The bacterium is known to possess oxygen-sensitive essential enzymes such as the pyruvate and 2-oxoglutarate oxidoreductases, which contain labile FeS redox centres (Hughes et al., 1995, 1998), and *H. pylori* also appears to generate larger amounts of superoxide and has a lower specific activity of superoxide dismutase than some other bacteria, e.g. *Escherichia coli* (Nagata et al., 1998).

Oxidative and nitrosative stress resistance are key properties that enable pathogenic bacteria to survive the effects of the production of reactive oxygen and nitrogen species by the host (Storz & Zheng, 2000). *H. pylori* seems well equipped to deal with peroxide stress, as it contains an active catalase and a number of peroxidases, including a periplasmic cytochrome c peroxidase (Tomb et al., 1997; Kelly, 1998; Alm et al., 1999). Interestingly, it possesses several enzymes of the peroxiredoxin family which may play key roles. The best studied of these enzymes is alkyl-hydroperoxide reductase...
AhpC, Tpx and Bcp are all members of the peroxiredoxin family (Schröder & Ponting, 1998; Jeong et al., 2000), which use reduced thioredoxin as an electron donor for the catalytic reduction of their respective substrates. H. pylori possesses two distinct thioredoxins, Trx1 and Trx2 (Windle et al., 2000; Baker et al., 2001), encoded by HP0824 and HP1458, respectively, in strain 26695. A thioredoxin reductase is encoded by trxB (HP0825 in strain 26695; Tomb et al., 1997). The H. pylori Trx1 thioredoxin has been characterized biochemically (Windle et al., 2000). Experiments with the purified proteins in vitro have shown that Trx1 (but not Trx2) acts as the electron donor to AhpC (Baker et al., 2001). In view of the fact that AhpC plays a critical role in the H. pylori oxidative stress response, it would be predicted that Trx1 is also of central importance, but this and the possibility that Trx2 or another reductant can substitute in vivo has not been investigated. Indeed, the normal physiological role of Trx2 is unclear, as are the functions of the additional peroxidases known to be encoded in the genome of H. pylori. In this study, we have constructed mutants in the trxA1, trxA2, tpx and bcp genes in order to determine the role of the thioredoxins and thiol-peroxidase proteins of H. pylori in contributing to the resistance of this bacterium to the effects of oxidative and nitrosative stress. Phenotypic analyses of these mutants show that both Trx1 and Trx2 have roles in oxidative and nitrosative stress resistance. Tpx mutants were found to be more sensitive to killing by peroxide and superoxide (but not oxygen or NO) compared to the wild-type parent strain, while bcp mutants had a similar but much weaker phenotype.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** A list of bacterial strains and plasmids used in this study is shown in Table 1. H. pylori was routinely grown on Columbia agar (Oxoid), supplemented with 5% (v/v) lysose horse blood (hereafter called blood agar). A selective antibiotic supplement of vancomycin, polymyxin B and amphotericin B, each at a final concentration of 10 μg ml⁻¹, was added to all H. pylori growth media. Plates were incubated at 37 °C for 48 h in a Variable Atmosphere Incubator (VAIN; Don Whitley) in an atmosphere of 5% (v/v) CO₂, 85% (v/v) N₂ and 10% (v/v) O₂. For growth experiments at low oxygen concentrations, the gas atmosphere was changed to 5% (v/v) CO₂, 93% (v/v) N₂ and 2% (v/v) O₂. Bacterial cultures were grown microaerobically in brain–heart infusion (BHI) broth supplemented with 5% (v/v) fetal calf serum (BHI/FCS) and the above antibiotics, in 25 or 250 ml media contained in 50 or 500 ml conical flasks shaken orbitally at 120 r.p.m. To select for H. pylori mutants carrying antibiotic-resistant determinants, either kanamycin or chloramphenicol was added to media at a final concentration of 30 μg ml⁻¹. For growth experiments, H. pylori overnight BHI/FCS starter cultures were inoculated into fresh BHI/FCS broth to an initial OD₆₀₀ value of between 0·1 and 0·2; growth was monitored by regular optical density readings. E. coli strains for plasmid subcloning were grown on Luria–Bertani (LB) agar or in LB broth. Supplements of 100 μg ampicillin ml⁻¹, 30 μg chloramphenicol ml⁻¹ and 30 μg kanamycin ml⁻¹ were used as appropriate for the selection and maintenance of plasmids.

**DNA isolation and manipulation.** Plasmid DNA for screening clones, sequencing and gene disruption experiments was routinely isolated using anion exchange resin spin-columns (Qiagen), according to the manufacturer’s instructions. H. pylori total chromosomal DNA was extracted using a modified SDS lysis procedure (Marmur, 1961). Restriction endonucleases, T4 DNA ligase and Taq polymerases were purchased from Promega and Pfu polymerase from Stratagene, and used according to manufacturer’s instructions. Standard techniques were used for the cloning, transformation, preparation and restriction analysis of plasmid DNA from E. coli.

**Inactivation of H. pylori genes.** A set of PCR primers were designed to amplify the entire coding regions of the trxA1, trxA2, tpx and bcp genes for insertional activation after cloning of the products into pGEM T-easy or pGEM-3-Zf (Promega). The primers used were TrxA1C-F (5′-GCGGAATTCAGATCCTGCTTTAG-3′), TrxA1C-R (5′-CTGGAGAGACTTTGAGCGATA-3′), Tpx-F (5′-CTTAAGAGAAGAAGCTTCCGCGA-3′), Tpx-R (5′-CATCTGGCGCAAGTCTGCTAAG-3′), TrxA2a-F (5′-ATCTGAATTCAGCTTGATGG-3′) and TrxA2a-R (5′-CGCGTATCGAGCGCCCTTTTA-3′). The TrxA1C and TrxA2a primers introduced EcoRI or PstI restriction sites (shown in bold italics) for the cloning of the products into pGEM T-easy or pGEM-3-Zf (Promega). PCR products were covalently linked to the vector polymeric RNA using genomic DNA from strain 26695 as a template. pGEM-3Zf(-) was linearized with EcoRI or PstI and ligated with the products resulting from PCR with the above primers after digestion with the same restriction enzymes, generating plasmids pSLC1 and pSLC4 (Table 1). The TrxA2(b) primers amplified an internal fragment of trxA2 and were used for mutant verification. The remaining primers were used for cloning into pGEM T-easy. All the resulting plasmids were linearized at unique restriction sites within the H. pylori gene inserts, as detailed in Table 1. Protruding 5′ termini were in-filled using the Klenow fragment of the E. coli DNA polymerase I. For
Table 1. Strains and plasmids used in this study

<table>
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Insertional inactivation, a chloramphenicol acetyltransferase (cat) cassette or an aminoglycoside phosphotransferase gene (aphAIII) derived from *Campylobacter coli* (Wang & Taylor, 1990; Pittman et al., 2001) were used. The 749 bp cat cassette excised from pUCAT by HindIII digestion was used to inactivate trxA1 and trxA2. The cassette was purified using a Gel Extraction Kit (Qiagen) and blunt-end ligated into pSLC1 and pSLC4, respectively. Plasmids pSLC7 and pSLC10 were generated (see Table 1). To construct a plasmid suitable for generating a *H. pylori* trxA1 trxA2 double mutant, the aphAIII gene was amplified from pUKAN with Pfu polymerase, using primers Kan-F (5′-ACTGAGATCTACTATGAAAGCGCA-TATT-3′) and Kan-R (5′-CAATAGATCTTTTAGACATCTTA-AATCTAG-3′). pSLC3 was linearized at the unique BstXI site within the trxA2 gene, in-filled using the Klenow fragment and blunt-end ligated with the aphAIII gene. The resulting plasmid was designated pSLC9. Plasmids pSLC11 and pSLC12 were derived from pSLC5 and pSLC6 by insertion of aphAIII into the bcp and tpx genes, respectively.

Transformation of *H. pylori* 26695 was carried out with pSLC7, pSLC10, pSLC11 and pSLC12 to generate single gene mutants in trxA1 (strain SLC100), trxA2 (strain SLC200), bcp (strain SLC400) and tpx (strain SLC500), respectively. Strain SLC100 was transformed with pSLC9 to generate a trxA1 trxA2 double mutant (strain SLC300). *H. pylori* 1061 was transformed with pSLC8 to construct a non-polar trxA1 mutant. Natural transformations were carried out as described by Ferrero et al. (1992). Genetic DNA was extracted from putative recombinants, and correct insertion of the resistance cassettes was evaluated by PCR using the above primers.

Effect of oxidative and NO stress on cell viability. Wild-type and mutant *H. pylori* strains were grown on Columbia blood agar plates for 48–72 h, harvested and resuspended in BHI/FCS to an OD_{600} value of 1.0. Viable counts showed that this corresponded to about 10^7–10^8 c.f.u. ml^{-1}. Aliquots (2 ml) of this cell suspension were distributed to a series of 20 ml tissue-culture flasks, to which either no additions were made (control) or 50 mM HO_2, 10 mM methyl viologen (paraquat; a superoxide generator), 10 mM sodium nitroprusside (SNP; an NO^+ donor) or 5 mM S-nitrosoglutathione (GSNO; an NO^+ releaser and NO^+ donor) were added. The flasks were incubated at 37 °C under microaerobic conditions with orbital shaking at 120 r.p.m. One flask with no additions was also incubated under fully aerobic conditions (21%, O_2; 37 °C with orbital shaking at 250 r.p.m. to determine the effect of oxygen stress. Periodically, samples were taken from the flasks, serially diluted in BHI and then plated onto blood agar. Colonies were counted after 96−120 h incubation.

Cumene hydroperoxide disc-oxidation assay. *H. pylori* was grown on a blood agar plate for 48–72 h, after which time the growth was scraped off and resuspended in 1 ml BHI broth. Aliquots (100 μl) of this suspension were spread out onto fresh blood agar plates and sterile 5 mm diameter Whatman filter paper discs were placed in the centre of the plates. Five microlitres of a 10% (v/v) cumene hydroperoxide solution in DMSO was pipetted onto the discs and the plates were incubated for 48–72 h at 37 °C. The diameter of the zone of growth inhibition was measured. No growth inhibition was observed when DMSO alone was placed on
the filter discs. Six replicate assays were performed for each strain, and the data were subjected to Student’s *t*-test to evaluate their statistical significance.

**RESULTS**

**Mutagenesis of the trx, tpx and bcp genes**

Fig. 1 shows the genes analysed in this study and the strategy used for mutagenesis. PCR-amplified genes were insertionally inactivated at unique restriction sites using antibiotic-resistance cassettes and transferred into *H. pylori* 26695 by natural transformation. For each mutant, verification that correct chromosomal insertion of the cassettes had occurred by a double homologous recombination event was obtained by PCR analysis of parental and mutant genomic DNA (Fig. 1a–f).

Polar effects were a consideration in constructing mutants in the *trxA1–trxB* operon. In strain 26695, *trxA1* (HP0824) was successfully mutagenized using the *cat* cassette (Wang & Taylor, 1990), which may also prevent transcription of *trxB*. However, transformation of strain 26695 by pSLC8, containing a terminatorless *aphAIII* gene in *trxA1* (allowing read-through into *trxB*, driven by the *aphAIII* promoter), was unsuccessful, but such mutants were obtained using the more highly transformable *H. pylori* strain 1061 (Fig. 1b). One such mutant was designated SLC600, and it showed phenotypic properties identical to those of strain SLC100 when compared by each of the experiments described below (data not shown), indicating that any polar effects on *trxB* were minimal and did not contribute to the observed phenotype of the *trxA* mutants. A *trxA1* *trxA2* double mutant was also constructed by transformation of strain SLC100 (26695 *trxA1*::*cat*) with pSLC9, which contains the *C. coli* *aphAIII* kanamycin-resistance gene inserted at the unique *BsrXI* site in HP1458 (Fig. 1d). Mutants in the *bcp* and *tpx* genes were successfully constructed using the *aphAIII* gene (Fig. 1e, f).

Growth characteristics of the *trx*, *tpx* and *bcp* mutants: thioredoxin deficiency results in growth inhibition by molecular oxygen

Strains SLC100 (26695 *trxA1*::*cat*), SLC200 (26695 *trxA2*::*cat*), SLC300 (26695 *trxA1*::*cat* *trxA2*::*aphAIII*) and SLC600 (1061 *trxA1*::*aphAIII*) all grew poorly on blood agar plates incubated under standard microaerobic conditions.

**Fig. 1.** Mutagenesis of thioredoxin and thiol-peroxidase genes in *H. pylori*, and verification of mutant strains by PCR. Mutants were constructed by cloning *cat* or *aphAIII* cassettes (*) into unique restriction sites in the insert DNA of the relevant plasmids shown (see also Table 1), followed by transformation into *H. pylori*. (a) Agarose gel of PCR products obtained from parental strain 26695 (P) and *trxA1* mutant (M) genomic DNA, using primers *TrxA1*-F and *TrxA1*-R, showing an increase in size of ~750 bp of the mutant PCR product due to insertion of the *cat* cassette. (b) Agarose gel of PCR products obtained from strain 1061 (P) and *trxA1* mutant (M) genomic DNA, using primers *TrxA1*-K-F and *TrxA1*-K-R, showing an increase in size of ~1.5 kb of the mutant PCR product due to insertion of the *aphAIII* gene. (c) Agarose gel of PCR products obtained from parental strain 26695 (P) and *trxA2* mutant (M) genomic DNA, using primers *TrxA2* (b)-F and *TrxA2* (b)-R, showing an increase in size of ~750 bp of the mutant PCR product due to insertion of the *cat* cassette. (d) Agarose gel of PCR products obtained from strain 26695 (P) and *trxA1*::*cat* *trxA2*::*aphAIII* mutant (M) genomic DNA, using primers *TrxA2* (b)-F and *TrxA2* (b)-R, showing an increase in size of ~1.5 kb of the mutant PCR product due to insertion of the *aphAIII* gene. (e) Agarose gel of PCR products obtained from strain 26695 (P) and *bcp* mutant (M) genomic DNA, using primers *Bcp*-F and *Bcp*-R, showing an increase in size of ~1.5 kb of the mutant PCR product due to insertion of the *aphAIII* gene. (f) Agarose gel of PCR products obtained from strain 26695 (P) and *tpx* mutant (M) genomic DNA, using primers *Tpx*-F and *Tpx*-R, showing an increase in size of ~1.5 kb of the mutant PCR product due to insertion of the *aphAIII* gene.
conditions (with 10% v/v O2), forming smaller colonies than the respective 26695 or 1061 parent strains. The nature of the growth defects in the 26695 thioredoxin mutants was determined in BHI/FCS liquid batch cultures incubated in gas atmospheres containing either 10% or 2% (v/v) O2 (Fig. 2). The wild-type grew at a similar rate in either gas atmosphere (doubling time of 3 h), but reached slightly higher final cell densities when cultured under 10% (v/v) O2 (Fig. 2a). Strain SLC100 (trxA1::cat) showed an extended lag period and a significantly reduced exponential growth rate (doubling time of 6 h) at 10% (v/v) O2 but cultures eventually reached the same final cell densities as the wild-type parent strain (compare Fig. 2a, b). However, when cultured with 2% (v/v) O2 in the gas atmosphere, strain SLC100 grew as well as the wild-type (Fig. 2a, b), even reaching slightly higher final cell densities. At 10% (v/v) O2, strain SLC200 (trxA2::cat) showed a severe reduction in both the rate and extent of growth compared to its isogenic parent, but grew as well or better than the parent strain at 2% (v/v) O2 (Fig. 2a, c). As expected, the SLC300 trxA1 trxA2 double mutant showed a severe growth defect at 10% (v/v) O2 but not at 2% (v/v) O2 (Fig. 2d), similar to that of strain SLC200. The data clearly show that both thioredoxin 1 and 2 are important for the normal microaerobic growth of H. pylori, with a deficiency of either resulting in an increased sensitivity to molecular oxygen. The isolation of strain SLC300, however, indicates that neither thioredoxin is absolutely essential for viability.

In contrast to the thioredoxin mutants, the bcp and tpx mutants (strains SLC400 and SLC500, respectively) displayed only a slight reduction in growth rate compared to the parent strain when incubated under 10% (v/v) O2, and both mutant and wild-type cultures eventually reached the same final cell density (data not shown).

**Differential roles of thioredoxins 1 and 2 in protection against oxidative stress**

Fig. 3 shows the effects of exposure to air, hydrogen peroxide or superoxide stress on the viability of cell suspensions of the wild-type and trx mutants, in comparison to control cells incubated under standard microaerobic conditions at 10% (v/v) O2. H. pylori wild-type cells taken from 2–3-day-old plate cultures and resuspended to densities of 10^7–10^9 c.f.u. ml^-1 showed a biphasic loss of viability after exposure to 21% (v/v) O2 in air, 50 mM H2O2 or 10 mM paraquat. An initial gradual decline was followed by a phase in which the cells lost viability much more rapidly (Fig. 3b–d). In contrast, incubation under microaerobic conditions resulted in maintenance of the initial level of viability in each of the strains for the duration of the experiment (Fig. 3a). Strain SLC100 (trxA1) was killed much more rapidly than its isogenic parent by each of the oxidative stress treatments imposed (Fig. 3b–d) and was particularly sensitive to hydrogen peroxide killing (Fig. 3c). Strain SLC200 (trxA2) also showed an increase in sensitivity to killing by oxidative stress, but this was much less pronounced than with SLC100. As expected from these results, strain SLC300 (trxA1 trxA2) was slightly more sensitive than SLC100 to loss of viability upon exposure to oxygen, hydrogen peroxide or paraquat. The data show that a deficiency in TrxA1 results in greater sensitivity of H. pylori to killing by several types of oxidative stress compared to TrxA2 deficiency.

The experiment shown in Fig. 4(a) shows the sensitivity of the wild-type and trx mutants to growth inhibition by the
potent organic oxidant cumene hydroperoxide, as measured by a disc-diffusion assay. The trxA1 and trxA2 mutants both showed a statistically significant increase in the diameter of the zone of growth inhibition in this assay compared to the parent strain \((P < 0.001\) in each case), indicating a role for both thioredoxins in organic peroxide detoxification. The zone of growth inhibition was significantly greater in the trxA2 mutant compared to the trxA1 mutant \((P = 0.008)\). Combining these mutations was even more deleterious, and the trxA1 trxA2 double mutant was significantly more sensitive to cumene hydroperoxide compared to the trxA2 single mutant \((P = 0.001)\).

**The thiol peroxidase Tpx provides protection against peroxide and superoxide stress**

Fig. 5 shows an analogous experiment to that in Fig. 3, in which the oxidative stress phenotypes of the bcp and tpx mutants were examined by comparisons of the kinetics of killing after exposure to air, hydrogen peroxide or superoxide generated in situ by paraquat. In contrast to the thioredoxins, Tpx or Bcp deficiency did not affect the sensitivity of \(H. pylori\) to killing by oxygen (Fig. 5b) consistent with the similar microaerobic growth characteristics of the respective mutants compared to the wild-type, as noted above. However, a clear increase in the rate of killing of the tpx mutant was observed upon exposure to hydrogen peroxide (Fig. 5c) and paraquat (Fig. 5d) compared to the parent strain. The bcp mutant showed only a very slightly increased sensitivity to hydrogen peroxide killing, but an increase in sensitivity to superoxide stress was more evident (Fig. 5d). The more significant role of Tpx compared to Bcp was also evident in the cumene hydroperoxide disc-diffusion assay (Fig. 4), where much greater growth inhibition by this oxidant was observed with the tpx mutant compared to that seen with the bcp mutant.
Resistance to nitrosative stress is mediated mainly by thioredoxin 1 but not by Tpx or Bcp

Fig. 6 shows the results of an experiment to test the involvement of the thioredoxins and the thiol-peroxidases Tpx and Bcp in mediating resistance to the bactericidal effects of NO. Due to the high reactivity of this molecule, compounds that decompose to release NO in situ were employed. The kinetics of killing of the wild-type and mutant strains by two different NO-releasing agents, SNP (predominantly a nitrosating agent by donation of the nitrosonium cation, NO⁺) and GSNO (an NO releaser and NO⁺ donor), were compared. Strain 26695 was killed by both reagents, but the trxA1 mutant was killed much more rapidly than its wild-type parent while the trxA2 mutant was killed more rapidly than thetrxA1 single mutant, particularly by GSNO, indicating some involvement of thioredoxin 2. In contrast to these results, neither SNP nor GSNO affected the kinetics of killing of the tpx or bcp mutants in comparison to the wild-type (Fig. 6c, d), suggesting that the peroxiredoxins encoded by these genes are not involved in NO detoxification.

DISCUSSION

Thioredoxins are key proteins in many crucial cellular functions, including oxidative stress management (Ritz & Beckwith, 2001), and act as electron donors to a number of enzymes involved in the detoxification of reactive oxygen or nitrogen species, including the AhpC family of peroxiredoxins. Several recent studies have begun to elucidate the important role played by AhpC in the management of oxidative stress in H. pylori (Lundstrom & Bolin, 2000; Bryk et al., 2000; Baker et al., 2001; Olczak et al., 2002). The source of electrons for the catalytic activity of this enzyme has been shown to be thioredoxin 1 rather than thioredoxin 2 by biochemical studies in vitro (Baker et al., 2001), although it is possible other electron donors might contribute in vivo. In this study, we have shown that mutation of the genes encoding either thioredoxin results in an increase in sensitivity to several forms of oxidative and nitrosative stress. The phenotypes observed for the trxA1 and trxA2 single mutants and the trxA1 trxA2 double mutant were different, implying that Trx1 and Trx2 have distinct roles in H. pylori. However, changes in the relative abundance of these proteins under various stresses or in different mutant backgrounds might also be important, but although both thioredoxins have been identified on two-dimensional gels by proteomic techniques (Jungblut et al., 2000; Windle et al., 2000), there is as yet no information on how distinct oxidative or nitrosative stresses might differentially regulate their expression.

The H. pylori thioredoxins are likely to interact with many cellular proteins, but analysis of the effects of the trxA1 mutation fit in well with a major in vivo role for TrxA1 as the electron donor to AhpC. Like mutants in the ahpC gene (Olczak et al., 2002), the trxA1 mutant was more oxygen sensitive than the parent strain; it grew less well in 10% (v/v) O₂ compared to 2% (v/v) O₂ and cell suspensions were killed more rapidly than the parent strain after exposure to 21% (v/v) O₂. It also showed greater sensitivity to hydrogen peroxide, cumene hydroperoxide and the superoxide generator paraquat. Our results also demonstrate a particularly important role for TrxA1 in resistance to nitrosative stress. NO reacts rapidly in aqueous solution with superoxide to form the extremely toxic peroxynitrite (Hughes, 1999). Studies with purified AhpC from H. pylori have demonstrated that it can catalyse the reduction of peroxynitrite to nitrite (Bryk et al., 2000) and hence detoxify this compound. An increased sensitivity of the trxA1 mutant to NO releasers would thus be expected if a major source of electrons for the reduction of peroxynitrite by AhpC was TrxA1.
The role of Trx2 in oxidative stress management in *H. pylori* is less clear than with Trx1. It has been shown that, *in vitro*, Trx2 does not act as an electron donor to AhpC (Baker et al., 2001). Nevertheless, the *trxA2* mutant constructed in this study was more sensitive to both oxidative and nitrosative stress than the parent strain. One dramatic effect of the inactivation of *trxA2* was a much greater inhibition of growth at 10% (v/v) O2 compared to that seen with the *trxA1* mutant. This was particularly apparent in BHI liquid batch cultures, whereas growth on blood agar plates was affected less severely, possibly due to a protective effect of haem in removing reactive oxygen species. Some of the effects of the *trxA2* mutation were more apparent in a *trxA1* background. The two thioredoxins may be able to substitute for some functions *in vivo*, but it is likely that TrxA2 is acting as an electron donor for another reductase, peroxidase or peroxiredoxin that contributes to the removal of reactive oxygen or nitrogen species, in addition to the Trx1/AhpC system. Obvious candidates would be Tpx or Bcp, although these enzymes seem not to be important for NO detoxification as judged from the phenotypes of the cognate mutants. Studies with the purified proteins will be needed to clarify this.

In many bacteria, the thioredoxin system acts as the electron donor for ribonucleotide reductase (RNR; Jordan & Reichard, 1998). *H. pylori* contains only a single RNR, a heterodimeric oxygen-dependent class I type enzyme encoded by the *nrdA* and *nrdB* genes (Tomb et al., 1997; Alm et al., 1999). Reliance on this type of RNR would prevent *H. pylori* from growing strictly anaerobically, as is the case with its close relative *Campylobacter jejuni* (Sellars et al., 2002). The activity of this enzyme is therefore essential for DNA synthesis and thus for cell viability under normal microaerobic conditions. Our finding that it was possible to isolate a *trxA1 trxA2* double mutant must imply that the *H. pylori* RNR can be supplied with electrons from a source other than Trx1 or Trx2, which is interesting in view of the fact that the bacterium appears to be deficient in other reduction systems that might substitute for Trx, particularly glutathione and the glutaredoxin system (Tomb et al., 1997; Alm et al., 1999; Baker et al., 2001). Although the double mutant clearly had a very severe deficiency in oxidative stress resistance, compensatory up-regulation of a Trx-independent antioxidant system may be able to compensate to some extent, as has been reported in the case of *ahpC* mutants of *H. pylori* in which expression of the iron-binding NapA protein is increased (Olczak et al., 2002).

The role of the thiol peroxidase Tpx in oxidative stress resistance has also been investigated in this study. Wan et al. (1997) first identified the Tpx protein in *H. pylori* and the partially purified enzyme was shown to protect glutamine synthetase from oxidative inactivation and to possess thioredoxin-linked peroxidase activity, when assayed using *E. coli* Trx. Tpx is an abundant protein in *H. pylori* as judged by two-dimensional gel analyses (Junghut et al., 2000) and the *tpx* gene is divergently transcribed from *sodB*, encoding superoxide dismutase (Tomb et al., 1997; Wan et al., 1997). The increased sensitivity of the *tpx* mutant to hydrogen peroxide, cumene hydroperoxide and the superoxide generator parquat confirms a significant *in vivo* role for Tpx as an antioxidant protein in *H. pylori*. That the *tpx* mutant did not show an increased sensitivity to killing by nitrosative stress indicates a specific role for Tpx in detoxifying reactive oxygen species, contrasting with AhpC, which appears to be a more general peroxiredoxin.

*H. pylori* also contains another member of the peroxiredoxin family, the Bcp protein encoded by HP0136 in 26695. This is a homologue of the *E. coli* Bcp protein, which was shown by Jeong et al. (2000) to be a thioredoxin-dependent thiol peroxidase with a preference for certain organic peroxides such as linoleic acid hydroperoxide. Unlike an *E. coli* bcp
null-mutant, which had a prolonged lag phase in liquid batch cultures and a pronounced hypersensitivity to hydrogen peroxide and organic peroxides (Jeong et al., 2000), the H. pylori bcp mutant constructed in this study had only a weak phenotype. A slight perturbation in growth characteristics (slightly slower growth rate) compared to the wild-type was reproducibly noted, but the mutant was not significantly more hydrogen peroxide sensitive and only slightly more superoxide sensitive than the parent strain. Thus, Bcp is not a major contributor to general oxidative stress resistance in H. pylori, but instead could have a more specific role, for example, in the removal of fatty acid hydroperoxides produced during metabolism or by oxidative stress.

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REFERENCES


