

Comparative Roles of the Two *Helicobacter pylori* Thioredoxins in Preventing Macromolecule Damage

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Thioredoxins are highly conserved throughout a wide range of organisms, and they are essential for the survival of oxygen-sensitive cells. The gastric pathogen *Helicobacter pylori* uses the thioredoxin system to maintain its thiol/disulfide balance. There are two thioredoxins present in *H. pylori*, Trx1 and Trx2 (herein referred to as TrxA and TrxC). TrxA has been shown to be important as an electron donor for some antioxidant enzymes, but the function of TrxC remains unknown (L. M. Baker, A. Raudonikiene, P. S. Hoffman, and L. B. Poole, *J Bacteriol* 183:1961–1973, 2001; P. Alamuri and R. J. Maier, *J Bacteriol* 188:5839–5850, 2006). We demonstrate that both TrxA and TrxC are important in protecting *H. pylori* from oxidative stress. Individual $\Delta trxA$ and $\Delta trxC$ deletion mutant strains each show a greater abundance of lipid peroxides and suffer more DNA damage and more protein carbonylation than the parent. Both deletion mutants were much more sensitive to O₂-mediated viability loss than the parent. Unexpectedly, the oxidative DNA damage and protein carbonylation was more severe in the $\Delta trxC$ mutant than in the $\Delta trxA$ mutant; it had 20-fold- and 4-fold-more carbonylated protein content than the wild type and the $\Delta trxA$ strain, respectively, after 4 h of atmospheric O₂ stress. *trx* transcript abundance was altered by the deletion of the heterologous *trx* gene. The $\Delta trxC$ mutant lacked mouse colonization ability, while the ability to colonize mouse stomachs was significantly reduced in the $\Delta trxA$ mutant.

Oxidative stress is a problem encountered by most organisms, and anaerobic and microaerophilic microorganisms frequently have a limited tolerance for oxygen. Reactive oxygen species (ROS) are abundantly generated in the environment, which leads to every living organism, at least transiently, having contact with different reactive oxygen molecules. ROS are generated in the form of superoxide (O₂⁻), hydroxyl radical (OH·), hydrogen peroxide (H₂O₂), and hypochlorous acid (HOCl). All of these can be generated by irradiation (X rays, gamma rays, and ultraviolet radiation) or by normal metabolic processes, including autoxidation of reduced electron carriers, lipid peroxidation, and metal-catalyzed reactions (1). These forms of ROS can be detrimental to organisms, as they react with and damage macromolecules (DNA, lipids, and proteins) of the cell if not scavenged by antioxidant proteins. Pathogens have to combat an additional source of ROS, the oxidative burst generated by host immune cells (2–6).

Helicobacter pylori is a Gram-negative microaerophilic organism that colonizes the gastric mucosa and is the causative agent of peptic ulcers and chronic gastritis. It is chronically in contact with an array of ROS during colonization due to the host inflammatory response (2, 7–10). The oxidative-stress response of *H. pylori* is made up of many enzymatic systems, including superoxide dismutase (SodB), catalase (KatA), methionine sulfoxide reductase (Msr), alkyl hydroperoxide reductase (AhpC), thiol-peroxidase (Tpx), and bacterioferritin comigratory protein (Bcp) (11–20). These enzymes function in concert as the primary means of combatting oxidative stress in the cell. These systems, combined with the Trx system as the reductant, provide *H. pylori* with the tools needed to maintain a reduced environment in times of oxygen stress. *H. pylori* lacks genes for oxidative-stress regulators and does not contain the glutathione-glutaredoxin reduction system (GSH) (21). The GSH reduction system is often used in addition to the Trx system in bacteria to maintain a reduced state inside the cell (22). Organisms that lack GSH, such as *Lactobacillus casei*, *Bacillus subtilis*, *Bacteroides fragilis*, *Staphylococcus aureus*, and *H.*

pylori, presumably must rely on the Trx system to maintain thiol/disulfide balance in the cell (21, 23–26). The mechanisms for the maintenance of this balance in *H. pylori* have not been studied in great detail.

The Trx system is highly conserved throughout many organisms and is comprised of thioredoxin reductase (TrxR), NADPH, and Trx. *H. pylori* contains two genes that encode Trx, namely, TrxA (KEGG accession number HP0824) and TrxC (HP1458), but only a single gene that encodes the reductase TrxR (HP0825) (13). The first known function of TrxA was identified in *Escherichia coli*, and it was found to provide electrons to ribonucleotide reductase (RNR). RNR is an essential enzyme in the replication and repair of DNA, as it functions to catalyze the synthesis of deoxyribonucleotides from ribonucleotides. Evidence for this function in *H. pylori* has not been presented. However, *H. pylori* TrxA is expressed in greater amounts as a response to stress agents (27), and it is the electron donor for two important antioxidant enzymes, AhpC and Msr (13, 19). TrxA but not TrxC also acts as a chaperone in *H. pylori*, in which it renatures denatured arginase into its active form (28).

Notably, an *H. pylori* $\Delta trxA$ mutant was shown to be more sensitive to oxidative stress agents (such as paraquat, S-nitroso-

Received 25 February 2015 Returned for modification 19 April 2015

Accepted 30 April 2015

Accepted manuscript posted online 11 May 2015

Citation Kuhns LG, Wang G, Maier RJ. 2015. Comparative roles of the two *Helicobacter pylori* thioredoxins in preventing macromolecule damage. *Infect Immun* 83:2935–2943. doi:10.1128/IAI.00232-15.

Editor: S. R. Blanke

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doi:10.1128/IAI.00232-15

TABLE 1 List of primers used in this study

Primer ^a	Sequence (5' to 3')
<i>trxA</i> -1	TTGAAATGGTGGAGAGGAAC
<i>trxA</i> -4	CCGGTAGCGATAATCACGC
<i>trxA</i> -F	ATTTATGCGATCAGCCCGAG
<i>trxA</i> -R	AAGTCTTGCCATCTTCTGCC
<i>trxC</i> -1	CGCGATGCAAGAGGGCTTG
<i>trxC</i> -2	ATCCACTTTTCAATCTATATCAATCATT
<i>trxC</i> -3	CCCAGTTGTGCGACTGATAACGTTAT
<i>trxC</i> -4	CGCTTGAGCAACCCACC
<i>trxC</i> -F	ACACCATATGTCAGAAATGATTAACGG
<i>trxC</i> -R	ACACCTCGAGCAATAACGCTTTAGAG
<i>trxA</i> -F(rt)	GTGGGCCTTGTAAGATG
<i>trxA</i> -R(rt)	CTGATACCAATTTTGCGCTC
<i>trxC</i> -F(rt)	GGATTGCAGAAAGATTGAGC
<i>trxC</i> -R(rt)	CTTGCGGATGCCTAAG
<i>gyrA</i> -F(rt)	GCTAGGATCGTGGGTGATGT
<i>gyrA</i> -R(rt)	TGGCTTCAGTGAACGCATC

^a All primers were obtained from Integrated DNA Technologies.

glutathione, and O₂) than the wild type (strain 26695), while the Δ *trxC* mutant was much less sensitive to these agents than the Δ *trxA* strain (15). No specific roles (e.g., electron donor) for *H. pylori* TrxC in conferring resistance to oxidative stress or involvement in any cell process are known. We sought to further compare the antioxidant roles of TrxA and TrxC in *H. pylori* (strain 43504). We approached this by studying the effect of *trx* deletions on the subsequent state of macromolecules in the cell, with a focus on biomarker damage for oxidative stress. We determined the effects of oxygen on cell viability, total protein carbonyls, lipid hydroperoxides, and 8-oxoguanine (8-oxoG) content in the DNA of the strains and determined the colonization ability of the strains compared to that of the wild-type strain in a mouse model. The studies infer an important role for these reductant proteins in maintaining the macromolecule integrity and survival of *H. pylori* under oxidative-stress conditions, including *in vivo*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. pylori* 26695 (21) or ATCC 43504 was used for all *in vitro* studies, and strain X47 (29) was used for the mouse colonization assay. *H. pylori* cells were routinely grown on *Brucella* agar (BA; Oxoid) plates containing 10% defibrinated sheep blood (HemoStat Laboratories, Bogart, GA) and maintained in a 37°C humidified chamber under 5% CO₂ and low levels of oxygen (4%), with N₂ used as the balance. Chloramphenicol was added to the plates at a concentration of 30 µg/ml as needed.

Oxygen stress conditions. *H. pylori* 43504, the Δ *trxA* mutant, or the Δ *trxC* mutant from BA plates after 2 days of growth were resuspended in phosphate-buffered saline (PBS), pH 7.4, and exposed to either atmospheric oxygen (20%) or HOCl. To expose cells to atmospheric oxygen (20%), cells were resuspended in PBS to an optical density at 600 nm (OD₆₀₀) of 2 and placed at 37°C while being shaken at 60 rpm for up to 12 h. For viability determination, cells were taken at each 2-h time point; dilutions were made and plated on BA plates. All plates were then incubated at 4% O₂ for 5 days, and the number of viable cells was determined. Cells exposed to HOCl were resuspended in PBS to an OD₆₀₀ of 2, subjected to HOCl treatment (0 or 200 µM), and incubated at 37°C with shaking for 1 h at 200 rpm. Cells were then washed with PBS and harvested via centrifugation at 7,500 × g for 10 min at 4°C.

Construction of mutant strains. (i) **Construction of the Δ *trxA* mutant.** Primers *trxA*-F and *trxA*-R (Table 1) were used to PCR amplify an 864-bp fragment containing the *H. pylori* *trxA* gene (*hp0824*). The PCR

fragment was directly cloned into the pGEM-T vector (Promega) according to the manufacturer's instructions to generate pGEM-*trxA*. The host strain used for cloning was DH5 α . Subsequently, a chloramphenicol acetyltransferase (CAT) cassette was inserted at the unique NheI (nucleotide G130) site within the *trxA* sequence of pGEM-*trxA*. The allelic-exchange (chloramphenicol-resistant) transformants were isolated by selection of single colonies upon incubation under 4% O₂ partial-pressure conditions. The balance of the atmosphere was N₂ (91%) and CO₂ (5%). The disruption of the gene in the genome of the mutant strain was confirmed by PCR using primers *trxA*-1 and *trxA*-4, which lie outside the mutagenized region. The PCR product indicated an increase to the expected size, indicating insertion of the CAT cassette.

(ii) **Construction of the Δ *trxC* mutant.** The Δ *trxC* mutant strain was constructed by overlapping PCR. Genomic DNA and primers *trxC*-1 and *trxC*-2cat were used to PCR amplify a 388-bp region that contains part of the *hp1459* gene and part of the *hp1458* and *cat* (Cm^r) genes. Next, primers *trxC*-3 and *trxC*-4 were used to PCR amplify a 409-bp region containing part of the *cat* and *hp1458* genes and the *hp1457* gene. After each PCR, the products were gel purified and used for a final PCR step using primers *trxC*-1 and *trxC*-4. The final elongation step yielded a 1.5-kb product that was then used for (natural) transformation into strain 26695, 43504, or X47. The transformants (grown at 4% O₂) were transferred onto BA plates supplemented with 30 µg/ml chloramphenicol, and individual colonies appeared after 3 to 5 days of incubation at 4% O₂. The mutant strains grow at a rate similar to that of the wild type on BA at 4% O₂. The deletion of the Δ *trxC* mutant was confirmed by PCR using genomic DNA from each chosen clone. The PCR product was analyzed on a 0.7% Tris-acetate-EDTA (TAE) agarose gel. The clones were further confirmed via sequencing at the Georgia Genomics Facility (University of Georgia, Athens, GA). Construction of deletion mutants using overlapping PCR was also confirmed by reverse transcription-PCR (RT-PCR).

(iii) **Construction of complemented mutant strains.** *E. coli* strain DH5 α was used for all plasmid DNA manipulations. The genes *trxA* and *trxC* were PCR amplified from *H. pylori* genomic DNA using primers *trxA*-F and *trxA*-R or *trxC*-F and *trxC*-R. The PCR products were digested with NdeI and XhoI and ligated into the pPA plasmid digested with the same restriction enzymes. This created p*PatrxA* or p*PatrxC*, such that the genes were located downstream of the *ureA* promoter. Digestion of the plasmids with BglII and XhoI released the fragment. The fragments were ligated into pEM39kan, also digested with BglII and XhoI. The plasmid pEM39kan has been shown to efficiently insert DNA by homologous recombination into the region of the chromosome corresponding to the HP0405 site. Disruption of this gene has been shown to have no effect on the cell (30, 31). The plasmid was then naturally transformed into the *trxA* or *trxC* mutant strain and selected on BA containing 30 µg/ml kanamycin and 30 µg/ml chloramphenicol. This results in the insertion of *trxA* or *trxC* along with the *ureA* promoter at the HP0405 site in the chromosome. Successful recombination of *trxA* or *trxC* into the chromosome was confirmed via PCR and functional restoration by determining the viability of the complemented strains, the lipid peroxide level, the carbonylated protein content, and mouse colonization as described previously. Experiments in which the genetically complemented strains were used apply to cell survival (see Fig. 1), lipid peroxidation (see Fig. 3), protein carbonyl content (see Fig. 5), and mouse colonization (see Fig. 6).

Preparation of cell extracts. *H. pylori* was grown on BA plates for 2 days before being harvested. Cells were resuspended in sterile PBS, pH 7.4, to an optical density of 1 to 2 at 600 nm. Cells were then broken by sonication (Ultrasonics Heat Systems sonicator) in 3 sets of 10-s intervals (4-W output power and 40% duty cycle) and placed on ice. Cell debris was removed by centrifugation at 14,000 × g for 10 min at room temperature (25°C).

Real-time qPCR. The Aurum total RNA minikit (Bio-Rad) was used to extract total RNA from *H. pylori* wild-type, Δ *trxA*, and Δ *trxC* cells grown under 4% O₂. The Turbo DNA-free kit (Ambion) was used to degrade any remaining DNA. cDNA was then synthesized using the

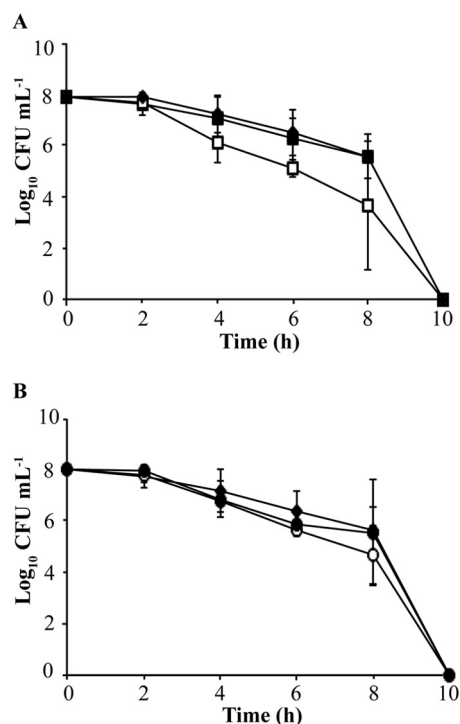


FIG 1 Survival of nongrowing cells incubated under atmospheric oxygen. (A) Filled diamonds, wild type; open squares, $\Delta trxA$ mutant; filled squares, $\Delta trxA$ complemented strain; (B) filled diamonds, wild type; open circles, $\Delta trxC$ mutant; filled circles, $\Delta trxC$ complemented strain. All strains were resuspended in sterile PBS and incubated under atmospheric oxygen for a 12-h period. Samples were taken at 2-h intervals, diluted, and plated onto BA plates. Dilutions were then incubated in a 4% partial-pressure O_2 atmosphere and colony counts determined. The means and standard deviations from results of 4 independent observations are plotted.

iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. The iQ SYBR green supermix (Bio-Rad) kit was used for quantitative PCR (qPCR) according to the manufacturer's instructions. Primers specific for *trxA* [*trxA*-F/R(rt)] or *trxC* [*trxC*-F/R(rt)] were used along with primers specific for the housekeeping gene *gyrA* (Table 1). Relative transcript abundance was calculated using the formula $2^{-\Delta C_T}$ (where C_T is threshold cycle) (32).

Determination of lipid hydroperoxides. Lipid hydroperoxides were extracted from the sample into chloroform as described in the work of Wang et al. (33), and the total amount of lipid hydroperoxides formed was quantified using a lipid hydroperoxide assay kit (Cayman Chemical, Ann Arbor, MI) by following the manufacturer's instructions. The chloroform extract was mixed with chloroform-methanol solvent and then combined with reagents 1 and 2 of the kit. Reagent 1 contained ferrous sulfate, which reacts with hydroperoxides to produce ferric ions. The resulting ferric ions react with ammonium thiocyanate, a chromogen that is detected at 500 nm with an extinction coefficient of $16,667 \text{ M}^{-1} \text{ cm}^{-1}$.

Fluorescent staining of cells and quantification of 8-oxoG. *H. pylori* 43504, the $\Delta trxA$ mutant, and the $\Delta trxC$ mutant were analyzed for the presence of 8-oxoG by fluorescent staining of the cells as described by Wang et al. (33). 8-oxoG is structurally similar to avidin's natural substrate biotin, so avidin binding to 8-oxoG can be used for detection of 8-oxoG (34). Eight sets of the immunofluorescent images were examined for the luminosity of fluorescein isothiocyanate (FITC) and propidium iodide (PI) as well as for cell morphology, and a representative set of images is shown in Fig. 4. The average ratio of the luminosity of FITC to PI was calculated from 8 sets of images for the wild-type and the mutant cells.

Isolation of carbonylated proteins. *H. pylori* 43504 cells grown for 2 days on BA plates were exposed to 20% O_2 for 2 or 4 h (as described above) and then washed twice (centrifugation at $10,000 \times g$ for 5 min at room temperature) in 50 mM potassium phosphate buffer, pH 6.7, with 1 mM EDTA. Cells were then resuspended in this buffer and cell extracts obtained, as described above. Samples were analyzed for contaminating nucleic acids by determining the ratio of the OD_{280} to the OD_{260} . The carbonylated proteins can be quantified from cell extracts by reacting them with 2,4-dinitrophenylhydrazine (DNPH) (35). DNPH (Sigma) at 10 mM dissolved in 2 M HCl was added to the sample tubes, and 2 M HCl was added to the control tubes. All samples were incubated for 1 h in the dark with vortexing every 15 min. After incubation with DNPH, 20% trichloroacetic acid (TCA; Sigma) was added to each tube. Samples were centrifuged at $10,000 \times g$ for 10 min, and the pellet was resuspended in 10% TCA. Samples were centrifuged again at $10,000 \times g$ for 10 min, and the pellet was resuspended in a 1:1 ethanol-ethyl acetate mixture and washed twice with this mixture. The remaining pellet was resuspended in 6 M guanidine hydrochloride (Fisher) and centrifuged at $10,000 \times g$ for 10 min, and the supernatant was transferred to the wells of a microtiter plate. Absorbance was then determined at 340 nm. The supernatant was also used to measure protein concentration of the samples by reading the absorbance at 280 nm and comparing this to a bovine serum albumin (BSA) standard curve.

Mouse colonization assay. *H. pylori* X47, the $\Delta trxA$ mutant, the $\Delta trxC$ mutant, the *trxA* complemented strain, and the *trxC* complemented strain were resuspended in sterile PBS, pH 7.5, and inoculated into 5- to 6-week-old female C57BL/6Ncr mice (NCI, Frederick, MD) via oral gavage with a 0.1-ml cell suspension. The 0.1 ml contained 1.7×10^8 viable cells. Three weeks after inoculation, mouse stomachs were removed and homogenized in PBS, pH 7.5 (36). The homogenized samples were then serially diluted, and 0.1 ml was plated onto BA plates containing 100 $\mu\text{g}/\text{ml}$ bacitracin, 10 $\mu\text{g}/\text{ml}$ vancomycin, and 10 $\mu\text{g}/\text{ml}$ amphotericin. The plates were incubated in a partial-pressure O_2 atmosphere (4%) for 5 to 7 days, the resulting colonies were counted, and numbers of CFU/g stomach were calculated. The data shown are the combined set from two separate experiments. The Institutional Animal Care and Use Committee gave prior approval for all animal work.

Statistical analysis. Results were analyzed using the unpaired *t* test and are presented as means \pm standard deviations (GraphPad). For the mouse colonization assay, the Wilcoxon signed-rank test was used to calculate statistical significance.

RESULTS

Oxygen stress tolerance of nongrowing cells. To elucidate the roles of the *trx* genes on the ability of *H. pylori* to survive under oxygen stress, we determined the oxygen sensitivity (i.e., viability) of the *trx* deletion mutants constructed with strain 43504. We have experienced greater success in the construction of mutant strains with 43504 than with other strains, so this strain was chosen as the parent. Nongrowing cells were incubated under atmospheric oxygen (20%) for a 12-h period. This condition is considered a stress environment for *H. pylori*, as the bacterium grows optimally under low oxygen (2 to 10%). As shown in Fig. 1, the wild-type and the complemented strains are only slightly affected by exposure to oxygen for an 8-h period, but the $\Delta trxA$ mutant shows a 100-fold loss in viability after 4 h of atmospheric oxygen exposure (Fig. 1A). The $\Delta trxC$ mutant also shows a loss of viability as a result of oxygen stress, but its sensitivity was not as severe as the $\Delta trxA$ mutant's. The $\Delta trxC$ mutant is 100-fold decreased in viability compared to that of the wild type upon 6 h of atmospheric oxygen exposure (Fig. 1B). These results are similar to those shown with the *trx* deletion mutants constructed with strain 26695 (15). Growth rates were not compared among strains, as these experiments were performed in nongrowing cells. The goal

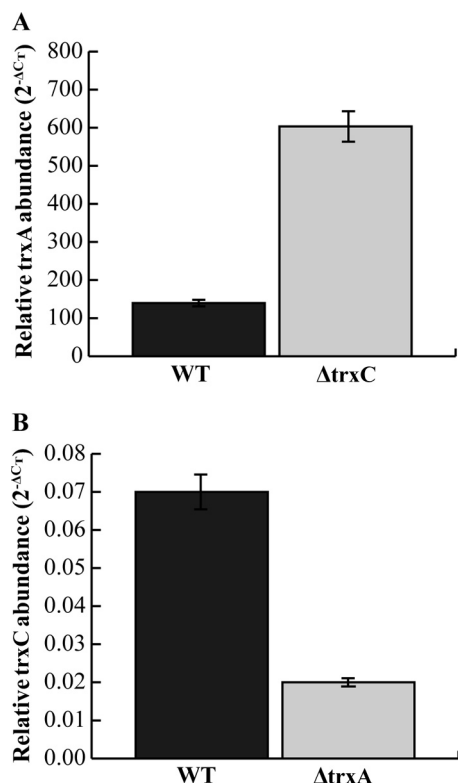


FIG 2 Relative *trx* transcript levels. (A) *trxA* abundance in the wild-type and Δ *trxC* strains; (B) *trxC* abundance in the wild-type (WT) and Δ *trxA* strains. Transcript abundance was determined by quantitative real-time PCR after cells were grown under conditions favorable for microaerophilic growth (4% O₂). The *gyrA* housekeeping gene was used as an internal control. Results shown are from two separate experiments sampled in triplicate. The experiment was repeated again with similar results.

was to assess/compare the viabilities of all *trx* strains while they were subjected to the same oxygen stress. Importantly, the viability of both strains was restored to wild-type levels upon complementation with the wild-type versions of both *trxA* and *trxC*. This makes conclusions about the roles of these enzymes from mutant strain analysis rigorous and indicates that both TrxA and TrxC play important roles in protecting the cell from oxidative stress. We conclude that *trxA* and *trxC* are essential for optimal growth under oxygen stress in *H. pylori* 43504.

Transcript abundance in the single *trx* gene deletion strains. *trx* transcript abundance was analyzed using quantitative real-time PCR (Fig. 2). There was a 6-fold increase in transcript abundance of *trxA* in the Δ *trxC* strain background compared to that in the wild type (Fig. 2A). However, a 4-fold decrease in the transcript abundance of *trxC* was observed in the Δ *trxA* background compared to its abundance in the wild type (Fig. 2B). It is noteworthy that the overall abundance of *trxC* was 1,000-fold less than that of *trxA* in the wild type. Transcript abundance differences of *trx* have previously been shown in other organisms that possess multiple thioredoxins (25, 37–39).

The amounts of lipid peroxides are greater in the Δ *trx* mutants than in the wild type under oxygen stress. Unsaturated phospholipids, glycolipids, and cholesterol in the cell membrane are all targets of oxidant attack. Reactive oxygen-mediated oxidation of these molecules results in the initiation of lipid peroxida-

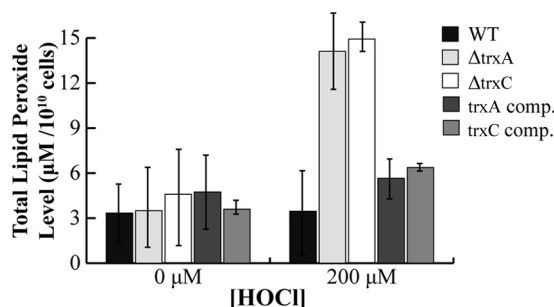


FIG 3 Lipid peroxide level in *H. pylori* *trx* mutants exposed to hypochlorous acid (HOCl) stress. *H. pylori* was grown on BA plates for 2 days and exposed to oxidant (or not), and cell extracts were obtained. The total amount of lipid peroxides was determined, and the hydroperoxide concentration in the sample was calculated. Data are presented as the averages from three independent experiments. Based on the Student *t* test, the Δ *trxA* and Δ *trxC* strains exposed to HOCl contain significantly greater amounts of hydroperoxide than the wild type at the 99% level of confidence. comp., complemented.

tion and subsequent free radical chain reaction under times of oxidative stress (40). Thus, quantification of LOOH is often used to assess the level of oxidative membrane damage in cells under stress.

H. pylori possesses two peroxiredoxins that are known to reduce such hydroperoxide-derivative molecules to their corresponding alcohol, AhpC, and bacterioferritin comigratory protein (BCP) (33). Since TrxA is required for the function of AhpC, we reasoned that the Δ *trxA* strain would contain a greater amount of lipid hydroperoxide than the wild type, especially under oxidative stress. Indeed, upon 200 μM HOCl exposure, we saw a 4-fold-greater lipid hydroperoxide level in the Δ *trxA* mutant than in the wild type (Fig. 3). The Δ *trxC* strain also had a 4-fold-greater level of lipid hydroperoxides than the parent strain (Fig. 3). The data from both mutants were shown to be statistically significantly greater than data for the wild type, with a 99% confidence level. This is similar to the lipid peroxide levels measured previously in the 43504 Δ *ahpC* mutant (3-fold higher than in the wild type) (33), and a rigorous comparison of all the strains—the two types of Δ *ahpC* strains plus the two Δ *trx* strains—with the parent strain and with multiple HOCl levels should reveal the genes most relevant to combating lipid peroxide formation. Importantly, complementation of either of the *trx* deletion strains with the wild-type version of the gene restored lipid peroxide levels to near wild-type levels (Fig. 3). The increase in lipid peroxides in the Δ *trxC* mutant is interesting because Trx2 had previously been ruled out as an electron donor to AhpC (13). The results suggest that both Trxs are involved in preventing lipid peroxidation.

Oxidative stress affects cell morphology and the level of 8-oxoG in *H. pylori* Δ *trx* mutants. Next, we examined the level of 8-oxoG DNA lesions in the mutant cells by using FITC-avidin/PI fluorescence microscopy. As FITC-avidin specifically labels DNA lesions while PI labels all DNA, the intensity ratio of stained FITC to PI reflects the level of 8-oxoG DNA lesions in the cells (41). This technique was successfully used in our lab to examine other oxidative-stress-related mutants of *H. pylori* (42, 43).

In this analysis, *H. pylori* wild-type or mutant cells were exposed to air for 4 h before subjecting them to immunofluorescent staining. Figure 4 shows that 4 h of exposure to air had no significant effect on the morphology of the wild-type cells, with ~90%

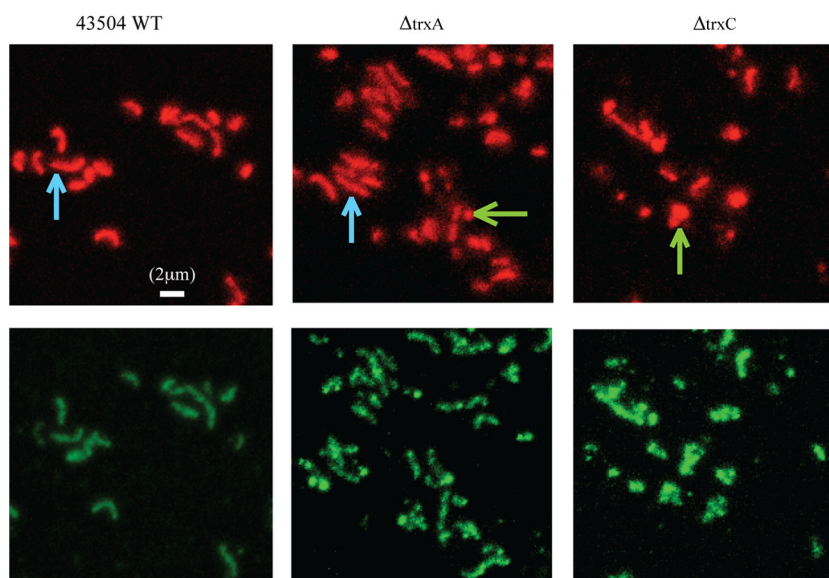


FIG 4 Detection of 8-oxoG by immunofluorescent staining. Wild-type *H. pylori*, Δ *trxA* mutant, and Δ *trxC* mutant cells were fixed on a glass slide and stained with 8-oxoG-specific avidin-FITC conjugate (lower panel) and propidium iodide (upper panel), followed by examination via fluorescence microscopy. The contrast adjustment was normalized for all the images, and a representative set of images is shown here. Blue arrows point to some examples of bacillary cells in the wild-type and Δ *trxA* strains, and green arrows highlight some coccoid or broken cells in the Δ *trxA* and Δ *trxC* mutant strains. A 2- μ m bar is given as a size scale.

of the cells being in the bacillary form. However, when similarly treated, \sim 45% of the Δ *trxA* cells and \sim 90% of the Δ *trxC* cells transformed to the coccoid form or were lysed, suggesting that these cells suffer from persistent oxidative stress (44). The ratio of FITC-avidin to PI fluorescence intensity in the Δ *trxA* and Δ *trxC* mutant cells was determined to be 1.7- and 2.4-fold higher, respectively, than that in the wild-type cells (Table 2). These results demonstrated that the Δ *trx* mutant cells exposed to oxidative stress contain considerably higher levels of 8-oxoG DNA lesions and that the Δ *trxC* mutant cells suffered more oxidative damage than the Δ *trxA* mutant cells.

In another experiment, *H. pylori* wild-type or Δ *trx* mutant cells were grown under optimum conditions with low O_2 (4% partial pressure in the atmosphere) before immunofluorescent staining was conducted. No significant difference between wild-type and Δ *trx* cells was observed either in cell morphology or in the level of 8-oxoG DNA lesions generated (data not shown).

Oxidative damage to proteins. To further assess the effect of the *trx* deletions on cells under oxidative stress, we estimated the

TABLE 2 Cell morphology and the level of 8-oxo guanine in *H. pylori* cells

Strain	% of damaged cells ^a	8-oxoG level (FITC/PI intensity ratio) ^b
Wild type	\sim 10	0.86 + 0.05
<i>trxA</i> mutant	\sim 45	1.45 + 0.06
<i>trxC</i> mutant	\sim 85	2.05 + 0.13

^a Cell morphology was examined by fluorescence microscopy, and the numbers are estimated percentages of damaged cells, including coccoid and broken cells, in the whole-cell population.

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

overall oxidative modification of proteins by measuring the presence of carbonyl groups. Protein carbonyls are often used as a biomarker for severe oxidative stress, and the accumulation of carbonylated proteins is linked to *E. coli* cell death (33, 45–50). Although some amino acid residues are precursors to carbonylation and can be repaired, the addition of a carbonyl group is an irreversible posttranslational modification (45). Figure 5 shows that at 20% O_2 exposure for 2 h, the Δ *trxA* and Δ *trxC* mutants contained 7- and 5-fold-greater amounts of carbonylated proteins, respectively, than the wild type. At 4 h, however, there was a greater amount of protein carbonyls in the Δ *trxC* mutant than in the Δ *trxA* mutant. A 7-fold-greater carbonyl level in the Δ *trxA* mutant than in the wild type was observed, while that in the Δ *trxC* mutant was increased 20-fold under 20% O_2 . Upon complementation, carbonyl levels were restored to wild-type levels. Taken together, these results indicate an increase in protein carbonylation in cells lacking the *trx* genes and indicate that these thioredoxins play roles in limiting protein damage.

The Δ *trxA* and Δ *trxC* mutants are decreased in mouse colonization ability. To determine whether *trxA* or *trxC* had an effect on the ability of *H. pylori* to colonize the stomach, we inoculated mice with wild-type X47, the Δ *trxA* mutant, the Δ *trxC* mutant, or each complemented strain. Three weeks after inoculation, the mouse stomachs were harvested and colony counts determined. As shown in Fig. 6, we recovered *H. pylori* from all 13 mice inoculated with the wild-type strain at numbers ranging between 10^3 and 10^6 CFU per gram of stomach. Eleven of the 13 mice inoculated with the Δ *trxA* mutant were *H. pylori* positive, but 2 of those 11 showed colonization numbers below 10^3 CFU/g stomach. All 13 mice inoculated with the Δ *trxC* mutant were *H. pylori* negative. Importantly, both the *trxA* and *trxC* complemented strains colonized the stomachs at levels similar to those of the wild type. The colonization efficiency of the Δ *trxA* mutant is significantly lower

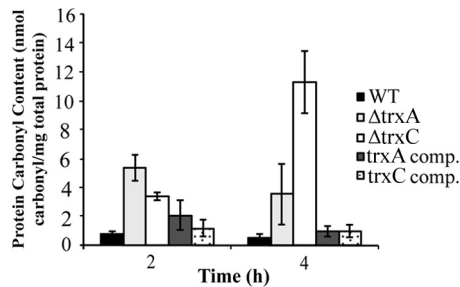


FIG 5 Protein carbonyl content in cells exposed to oxygen stress (20%) for 2 or 4 h. The wild-type, $\Delta trxA$ mutant, $\Delta trxC$ mutant, *trxA* complemented, or *trxC* complemented strain were exposed to 20% O_2 for 2 or 4 h, and extracts were reacted with DNPH. The carbonyl content was determined by measuring the absorbance at 340 nm. Total carbonyl content is expressed as nanomoles of carbonyl per milligram of total protein. Results shown are the averages of results from three independent experiments. The carbonyl content of both deletion strains was significantly greater than that of the wild type at both the 2- and 4-h time points ($P < 0.01$). In low O_2 (i.e., 4%), the carbonyl content of the mutant strains was not significantly greater than that of the wild type.

than that of the wild type at the 95% confidence level ($P < 0.05$), and that of the $\Delta trxC$ strain is lower at a $>99\%$ level of confidence. These results suggest that both *trxA* and (particularly) *trxC* are important for host colonization by the pathogen.

DISCUSSION

Maintaining the redox balance within *H. pylori* is important for cell viability, growth, and metabolism (51). This renders the Trx system essential for survival under oxidative stress, as this is the only disulfide reductase system present in *H. pylori*. The glutathione-glutaredoxin system, present in many other organisms for maintaining the cellular thiol/disulfide balance, is lacking in *H. pylori* (21). In addition, the Trx system is important because it is the electron donor for all three peroxidases, BCP, Tpx, and AhpC (13, 43). *H. pylori* TrxA contains the typical Trx motif found in most Trx proteins (CGPC), but TrxC contains an unusual motif (CPDC) (27). It is not known how this affects its activity or its roles. Alignment of the protein sequences of TrxA and TrxC show that the two proteins are only 30% identical. TrxC has been ruled out as an electron donor for the known oxidative-stress-combating reductases tested (AhpC and Msr) (13, 19). The TrxC of *H. pylori* is, however, still a functional disulfide reductase, as it has been shown to successfully reduce insulin *in vitro* when used as a substrate (13).

The O_2 exposure viability assay suggests that both Trxs have independent roles in protecting the cell from oxygen stress (Fig. 1). This is similar to what was concluded based on mutations in other organisms that contain more than one *trx* gene and no GSH reduction system. For example, *L. casei* is also a GSH-negative organism and has four *trx* genes. Disruption of either *trxA* or *trxC* resulted in moderate growth defects under aerobic conditions, and a *trxA trxC* double mutant of *L. casei* had severe growth defects (25). Still, the partial growth ability of the double mutant suggests that the other Trxs function to some extent. Similar results were shown for *B. fragilis*, which contains six *trx* genes. A single-mutation strain deleting one of the *trx* genes did not show any growth defect, but deletion of multiple genes in a single strain revealed a defective growth phenotype (52). A previous study using *H. pylori* 26695 as the parent strain showed defective growth

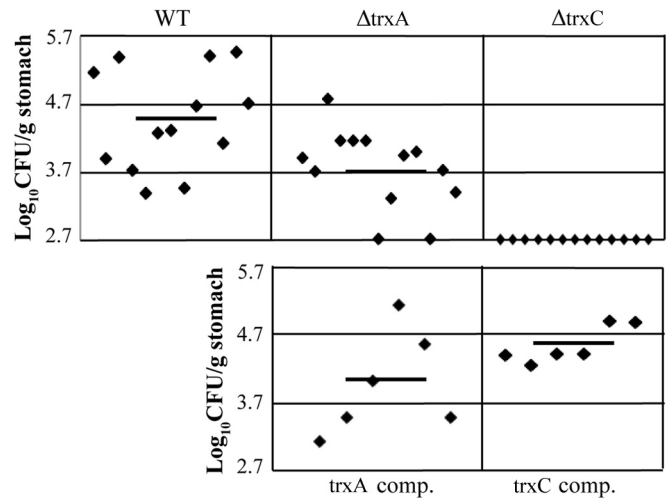


FIG 6 Mouse colonization assay of *H. pylori* X47 and *trx* strains. The mice were inoculated with a dose of 1.7×10^8 viable cells. Colonization of the mouse stomachs was determined 3 weeks postinoculation. Mouse stomachs were homogenized, and serial dilutions were plated. Data are presented as a scatter plot of numbers of CFU per gram of stomach as determined by plate counts. Each point represents a CFU count for one stomach, and the solid horizontal line represents the mean colonization for each strain. The baseline [$\log_{10}(\text{CFU g}^{-1}) = 2.7$] is the detection limit of the assay, which represents a count below 500 CFU g^{-1} stomach. Horizontal bars represent the average \log_{10} number of CFU/g stomach.

associated with a $\Delta trxA$ strain but a less severe defect in the $\Delta trxC$ strain. A $\Delta trxA trxC$ double mutant showed the largest growth defect (15). Our result indicates that TrxC is a major player in the protection of cells from oxygen stress and in their survival *in vivo*.

The $\Delta trxC$ strain contained more 8-oxoG than both the $\Delta trxA$ strain and the wild type (Fig. 4). This effect on DNA is a novel finding and has not been demonstrated in any other organism. Trxs are known to have important roles in the reduction of ribonucleotide reductase, an enzyme required for DNA synthesis (53). The function of *H. pylori* Trx2 is not known, but it is possible that it is involved in DNA replication and/or DNA repair. One indication that Trx2 may be involved in DNA replication is based upon the location of *trxC* in the chromosome. The *trxC* gene is located near *dnaE* (825 bp upstream), encoding the DNA polymerase III alpha subunit. This is similar to what was observed regarding the location of the essential *trxA* gene in *B. fragilis*; *trxA* is located directly downstream of *dnaE*. Phylogenetic analysis of TrxA grouped it with Trxs of *Campylobacter*, *Helicobacter*, and *Porphyromonas gingivalis*, indicating that the roles of TrxA and Trxs in these organisms may overlap (52). Trx2 may be involved in DNA repair in addition to DNA synthesis. Under oxygen stress conditions, DNA can become damaged by guanine oxidation (54). Oxidized guanine can then be incorporated into the genome, resulting in mispaired bases. *H. pylori* contains several proteins that function to excise 8-oxoG: MutT, MutY, and MutS (55). MutY is an iron-sulfur-containing glycosylase that excises mispaired adenines from the 8-oxoG (56). The Trx reductase system has been shown to be essential for iron binding in the iron-sulfur cluster protein IscA in *E. coli* (57). It is possible that *H. pylori* Trx2 aids in the iron binding of MutY.

We demonstrated an association between individual Trxs and host colonization ability for the first time in a bacterial system.

Previously, a *B. fragilis* Trx reductase (*trxB*) mutant was shown to be essential for survival in an *in vivo* mouse abscess model (24). This mutation renders the whole system incomplete (the reductase and Trx), so it does not provide insight as to the importance of Trx alone. We show that the *H. pylori* Δ *trxA* mutant is decreased in its ability to colonize the mouse stomach (Fig. 6). This phenotype is not surprising in light of the known functions of TrxA as the reductant for oxidized Msr and AhpC and its function as a chaperone for proper protein folding (13, 19, 28). However, the complete lack of colonization by the Δ *trxC* mutant (Fig. 6) was not expected. Based upon this result, it seems that *H. pylori* TrxC plays a large *in vivo* role. It may play a broad role like Trx proteins that function to provide electrons to DsbD, located in the cytoplasmic membrane (58). *H. pylori* contains several Dsb (disulfide bond) protein oxidases that allow for disulfide bond formation in the periplasm (59–62). This mechanism has been well characterized in *E. coli* but less so in *H. pylori* (63). In *E. coli*, DsbD functions as the acceptor of electrons from Trx and then transfers them to various periplasmic oxidoreductases and a cytochrome synthesis protein (DsbB, DsbA, DsbC, and CcmG). DsbD receives the electrons from cytoplasmic Trx proteins and has been shown to reduce electrons to proteins involved in disulfide bond isomerization, cytochrome *c* maturation, and possibly other processes (64). In *H. pylori*, homologues of DsbG, DsbC, DsbB (DsbI), and DsbD (CcdA) have been identified (60, 61). Both DsbC and DsbG have the Trx CXXC motif, and DsbG has been shown to have reductase activity. The reductase activity combined with a Trx motif indicates that DsbG functions as an oxidoreductase in the periplasm (59).

There are many virulence proteins located in the periplasm of *H. pylori* that, if damaged, would likely result in a lack of host colonization. While Trx maintains the methionine repair enzyme Msr reduced in the *H. pylori* cytoplasm, the players carrying out this role in the periplasm (where Msr is also known to function) are not known (19, 65). A membrane-bound Trx-like protein, PilB, provides electrons to Msr in the *Neisseria gonorrhoeae* periplasm, and the *N. gonorrhoeae* and *H. pylori* Msrs are highly homologous to each other (18, 66). Catalase is also an important periplasmic virulence protein in *H. pylori* (67). Catalase has been shown to be susceptible to oxidative inactivation and subsequent repair by Msr. Failure to repair a methionine sulfoxide-containing catalase could certainly result in a decrease in host colonization ability (68). It is noteworthy that during long-term colonization, a Δ *msr* mutant could not be recovered from a mouse stomach, indicating the importance of Msr in host colonization (18).

A proteomic analysis of *E. coli* Trx-targeted proteins revealed Trx to be involved in at least 26 different cellular processes, including transcriptional regulation, cell division, energy transduction, and several biosynthetic pathways (69). Trx is likely to be involved in multiple processes in *H. pylori* as well. Further work is needed to ascertain if any new protein-protein interactions occur that would aid in revealing new Trx roles, ones perhaps aiding peroxidase activity or macromolecule repair processes. While the specific roles of TrxC are not known, the importance of this protein in survival is now revealed. Both Trxs studied herein aid macromolecule integrity and homeostasis.

ACKNOWLEDGMENT

This work was supported by National Institutes of Health grant RO1 AI077569.

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