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Methionine Sulfoxide Reductase in *Helicobacter pylori*: Interaction with Methionine-Rich Proteins and Stress-Induced Expression

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The reductive repair of oxidized methionine residues performed by methionine sulfoxide reductase is important for the gastric pathogen *Helicobacter pylori* to maintain persistent stomach colonization. Methionine-containing proteins that are targeted for repair by Msr were identified from whole-cell extracts (after cells were exposed to O_2 stress) by using a coimmunoprecipitation approach. Proteins identified as Msr-interacting included catalase, GroEL, thioredoxin-1 (Trx1), and site-specific recombinase; with one exception (Trx1, the reductant for Msr) all these proteins have approximately twofold higher methionine (Met) content than other proteins. These Met-rich proteins were purified and were shown to individually form a cross-linked adduct with Msr. Catalase-specific activity in an *msr* strain was one-half that of the parent strain; this difference was only observed under oxidative stress conditions, and the activity was restored to nearly wild-type levels by adding Msr plus dithiothreitol to *msr* strain extracts. In agreement with the cross-linking study, pure Msr used Trx1 but not Trx2 as a reductant. Comparative structure modeling classified the *H. pylori* Msr in class II within the MsrB family, like the *Neisseria* enzymes. Pure *H. pylori* enzyme reduced only the R isomer of methyl *p*-tolyl-sulfoxide with an apparent K_m of 4.1 mM for the substrate. Stress conditions (peroxide, peroxynitrite, and iron starvation) all caused approximately 3- to 3.5-fold transcriptional up-regulation of *msr*. Neither the O_2 level during growth nor the use of background regulatory mutants had a significant effect on *msr* transcription. Late log and stationary phase cultures had the highest Msr protein levels and specific activity.

Protein oxidation is one of the consequences facing all organisms encountering oxidative stress. Reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hypochlorous acid oxidize either free amino acid residues or the residues within proteins. The oxidation of free residues renders them unusable in translation, whereas protein oxidation usually makes the protein nonfunctional (37). In many cases this oxidative damage cannot be remedied. However, the sulfur-containing amino acids cysteine and methionine can be enzymatically reduced or repaired to their native form. The oxidized form of methionine, methionine sulfoxide [Met(S)O], is reductively repaired by an enzyme termed methionine sulfoxide reductase (Msr). The reduction requires reduced thioredoxin and (indirectly) thioredoxin reductase (18). The enzymes MsrA and MsrB reduce the two isomers Met(S)O and Met(R)O of methionine sulfoxide, respectively (19, 21). Both enzymes are highly conserved in eubacteria and in eukaryotic cells including humans, and most organisms contain both forms of the enzyme (MsrA and MsrB) (7, 15).

Two major roles of Msr have been proposed. First, Msr activity can repair a few key proteins that then maintain function of other proteins, including housekeeping ones. For example, Msr targets key *Escherichia coli* protein Ffh (16), ribosomal protein L12 (9), or methionine-rich chaperones (1, 22). The net effect is maintenance of targeting, synthesis, and function (e.g., proper folding) of many proteins. Second, Msr-dependent reduction of some Met-containing proteins allows such proteins to serve as ROS quenchers or sinks by a continual oxidation-reduction cycle at the surface methionine residues. Such a cycle is thought to be especially important under stress conditions (38). One example of the latter is repair of the oxidized methionine residues in *E. coli* glutamine synthetase (23). Due to the multiple net positive effects of Msr-dependent Met repair, *msr* mutant strains of both bacteria and yeast are known to be highly sensitive to oxidative or nitrosative damage, and the enzyme is oftentimes upregulated by stress conditions (13, 20, 27, 33–36).

Helicobacter pylori is a microaerophilic human gastric pathogen, and a number of enzymes that detoxify ROS are important for survival of the bacterium in the host (30, 43). *H. pylori* Msr is a 42-kDa protein with fused MsrA- and MsrB-like domains (41), similar to the situation in a few other pathogens (*Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *Haemophilus influenzae*) (15). Inactivation of either *msrB* only (so that MsrA is still made) or of the entire gene (complete inactivation of both domains) resulted in *H. pylori* strains with an oxidative stress-sensitive phenotype. Also, neither mutant strain type could colonize mice for the longer time periods tested, indicating that Msr is an important persistence factor for the bacterium in the gastric mucosa (2).

Our understanding of some basic biochemical properties of the *H. pylori* enzyme is lacking. For example, many of the Met-containing direct targets for Msr-dependent repair are not known. Also, the enzyme's catalytic site and preferred substrate (Met R-S0 versus Met S-S0 isomer) are not known, nor has the thioredoxin reductant for Msr been identified. These aspects, as well as the conditions for *msr* up-regulation are addressed here.

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TABLE 1. List of primers used in this study

Primer[a]	Sequence (5′ to 3′)[b]	Restriction site
prommsrF	TGG**GAGCTC**ATGTGATTGGAACTTTTGTTGCCT	SacI
prommsrR	TGAATAACCTTTG**GGATTC**TTTTAATTTTAATGG TGTCCG	BamHI
PmsrF	ACTGG**CATATG**AAGGTATTATCTTATTTG	NdeI
PmsrR	GTG**CTCGAG**TTAATGCGACTTTTTATCATTGATG	XhoI
trx1F	TAGAGTAA**CATATG**AGTCACTATATTG	NdeI
trx1R	TCATGCTA**CTCGAG**CTAGCCTAAAAGTTTGTT	XhoI
trxrF	GCTAATAGC**CATATG**ATAGATTGCGCG	NdeI
trxrR	AGCTTGAT**CTCGAG**TTAATGGTGTTCTAAATA	XhoI
ssrF	TGATGATGCA**CTATG**TATTGTTTTTATTCTTTG	NdeI
ssrR	TTCGCG**CTCGAG**GTGGGATAAAAGATT	XhoI
trx2F	ATAAGGATT**CATATG**TCAGAAATGAGGAAC	NdeI
trx2R	TACCCC**CTCGAG**TTACAATAACGCTTTTAG	XhoI

[a] All primers were obtained from Integrated DNA Technologies.
[b] Restriction sites are in boldface.

MATERIALS AND METHODS

Bacterial strains and reagents used in this study. All cloning experiments were performed in *E. coli* strain Top10C. *H. pylori* strain SS1 was used in all experiments unless mentioned otherwise. All DNA modifying enzymes were purchased from Promega. Protein cross-linking reagents and a Seize X Protein A immunoprecipitation kit were purchased from Pierce (Illinois). Bacterial growth medium components were purchased from Difco Laboratories (Maryland). Rabbit antiserum against purified *H. pylori* KatA was generated by Cocalico Biologicals (California). Antibody against *E. coli* GroEL was purchased from Sigma, St. Louis, MO. The oxidized methionine substrates used in the in vitro Msr assays were purchased from Aldrich and Sigma, St. Louis, MO.

Plasmids and *xylE* assays. The 200-bp area containing the 3′ end of the hp0223 gene and the intergenic region of hp0223 and hp0224 (*msr*) were amplified using primers msrpromF and msrpromR (Table 1) and cloned into the SacI and BamHI sites upstream of the 980-bp promoterless *xylE* (*Pseudomonas putida*) gene of pTAD57 (31) to generate pSAP120. The 1,180-bp (P_{msr}-*xylE*) fragment was excised and introduced into the BamHI site of pHel3 to obtain pSAP131. Plasmid pSAP131 was transformed into *H. pylori* SS1 to obtain strain ALM 1018 (Table 2). For chromosomal fusions, the P_{msr}-*xylE* fragment was introduced into the hp405 region of the *H. pylori* genome using a previously described plasmid, peu39-cm (2), to generate *H. pylori* strain ALM 1014. To determine the possible regulator of *msr*, we transformed the same plasmid into independent isogenic *fur*, *nikR*, and *msr* mutants to obtain strains ALM 1032, ALM 1037, and ALM 1020, respectively (Table 2). All constructs were screened using PCR and were further sequenced at the University of Michigan DNA Sequencing Core facility. As a negative control, strain SS1 carrying the promoterless *xylE* in pHel3 was used. Cells were grown in Muller-Hinton medium with 10% serum in closed (serum stopper sealed) vials containing 4% partial pressure O_2 (5% CO_2 and the balance N_2). Cells were grown to logarithmic phase, and oxygen was added to bring the O_2 partial pressure to 12% O_2 in order to assess oxygen stress. For the other stress effects, cells maintained in 4% O_2 were supplemented separately with 300mM H_2O_2, 50mM *S*-nitrosoglutathione (GSNO), or 500mM $FeCl_3$, or 50mM 2,2-dipyridyl. Whole cells were collected from both pre- and poststress conditions, and *xylE* assays were performed as described elsewhere (31). Data reported are from five independent experiments with each experiment sampled in triplicate, for a total of 15 samples for each mean value shown. Data significance was determined by Student *t* tests (see the legend of Fig. 5).

Growth-phase-dependent expression. *H. pylori* strain SS1 cells were grown in a similar fashion as described above in Muller-Hinton medium supplemented with 5% calf serum but maintained in a constant 7% partial pressure oxygen atmosphere (5% CO_2; balance, N_2). Samples were collected every 8 h over an 80-h time period. Growth was monitored by measuring the optical density at 600 nm (OD_{600}) at each time point. Cells were disrupted by sonication (W-380 Heat System-Ultrasonics, Inc.). Seven micrograms of cell protein was resolved by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting was performed by use of anti-Msr antibody. Densitometric scanning (30) to determine the intensity of immunostained Msr protein bands was performed to assess the levels of expression. Cell extracts were centrifuged at 45,000 rpm for 2 h to separate the soluble protein from the membrane protein pellet. The pellet (membrane fraction) was suspended in 50 mM Tris-HCl (pH 7.4). Specific Msr activity was determined using MetSO and methyl *p*-tolyl sulfoxide as substrates as described elsewhere (2). One unit of Msr activity is equivalent to the number of nanomoles of NADPH oxidized/min/mg of membrane protein. Means ± standard deviations (SDs) are shown based on data from nine samples (three different experiments, each sampled in triplicate). As a control for immunoblotting-based growth phase expression of *ms*r, 5 μg of cell extract was loaded onto a separate gel, and the separated proteins were immunoblotted with anti-UreB antiserum. This was chosen as a control because UreB

TABLE 2. List of plasmids and strains used in this study

Plasmid or strain	Features and application	Reference or source
Plasmids		
pTAD57	Promoterless 980 bp *xylE* gene cloned into the SmaI site of pKS (in *Escherichia coli* DH5α)	31
pSAP112	*msr* (hp0224) cloned into the NdeI-XhoI site of pET21A for overexpression	This study
pSAP120	200-bp intergenic region of hp0223 and hp0224 (*msr*) cloned into the SacI and BamHI site of pTAD57 upstream of *xylE*	This study
pSAP123	The 1,180-bp PmsX (P_{msr}-*xylE*) fragment cloned into the EcoRV site of peu39cm used in induction studies	This study
pSAP131	The 1,180-bp PmsX fragment cloned into the BamHI site of pHel3 used in induction studies	This study
pSAP134	The 1,200-bp P_{ureA} and *xylE* gene fusion fragment cloned into the BamHI site of pHel3	This study
pSAP138	Thioredoxin (hp0824) cloned into NdeI-XhoI site of pET21A for overexpression	This study
pSAP139	Thioredoxin reductase (hp0825) cloned into NdeI-XhoI site of pET21A for overexpression	This study
pSAP159	Thioredoxin-2 (hp1458) cloned into the NdeI-XhoI site of pET21A for overexpression	This study
pSAP163	Site-specific recombinase (hp1009) cloned into NdeI-XhoI site of pET21A for overexpression of a C-terminus six-His-tagged SSR	This study
pILL690	*nikR* (hp1338) inactivated with *aph*A3	11
Strains		
ALM 1014	*H. pylori* SS1 carrying pSAP 123	
ALM 1018	*H. pylori* SS1 carrying pSAP131 for induction studies	
PBR 0126	*H. pylori* SS1 with pHel3 carrying promoterless *xylE*	6
ALM 1020	pSAP123 recombined in hp0405 locus of *msr*::*aphA3* in SS1	This study
ALM 1021	*H. pylori* SS1 carrying pSAP134 as a positive control for induction studies	This study
ALM 1032	pSAP123 recombined in the hp0405 locus of *fur*::*aphA3* in SS1	This study
ALM 1035	pILL690 transformed into SS1 to generate *nikR*::*aphA3*	This study
ALM 1037	pSAP123 recombined in hp0405 locus of ALM1035	This study

is an *H. pylori* protein whose expression is known not to be growth phase dependent (26).

Coimmunoprecipitation. The wild-type and *msr* strains were grown to mid-log phase in sealed vials at 4% partial pressure O_2, and half of the culture (for wild type) was removed for exposure to 12% partial pressure O_2 (for a 3-h period) to oxidize the cellular protein pool. This was done in order to enhance the chance of observing intimate interactions of target proteins with Msr. Cell extracts from all cultures were prepared identically and at the same time, and then the protein concentration was adjusted, and the extracts were incubated with different cleavable cross-linkers. The cross-linker-extract incubation was performed overnight in argon-sparged buffer. All three cross-linkers, heterobifunctional *N*-succinimidyl 3-(2-pyridyldithio) propionate, homobifunctional sulfo-disulfosuccinimidyl tartrate, and homobifunctional dithiobis (sulfosuccinimidyl propionate) were individually tested. The best yield of cross-linked products after immunoprecipitation was obtained using *N*-succinimidyl 3-(2-pyridyldithio) propionate. The source for antibody against *H. pylori* Msr is described elsewhere (2). Anti-Msr antibody was first purified by cross-precipitation with an *msr* strain extract to diminish nonspecific antibody-protein binding. A Seize X Protein A immunoprecipitation kit (catalogue no. 45215; Pierce) was used in the subsequent precipitation process. The purified anti-Msr antibody was first cross-linked to ImmunoPure Immobilized Protein A Plus beads using disuccinimidyl suberate. The slurry was incubated with previously cross-linked cell extracts with slow stirring overnight at 4°C. The slurry was later passed through Handee Spin Cup columns (provided with the kit) and washed initially with binding/wash buffer, followed by washing with stringent immunoglobulin G elution buffer. The cross-linked proteins were serially eluted with the elution buffer, and 10 μl of 1 M Tris-Cl, pH 8.0, was added to neutralize the protein elutions. The cross-linked protein complexes were cleaved by incubating either with dithiothreitol (DTT) or ammonium persulfate, depending on the cross-linker used in the previous step. The mixture was later resolved on a 4% to 20% gradient SDS-PAGE gel, and the bands were excised from the gel and submitted to The University of Georgia Proteomics facility for protein identification by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). All steps were simultaneously performed using cell extracts from both strain SS1 and the isogenic *msr* mutant.

Overexpression and protein purification. Proteins were purified either as recombinant proteins in *E. coli* (Msr, thioredoxin-1 [Trx1], Trx2, thioredoxin reductase [TrxR], and site-specific recombinase [SSR]) or from *H. pylori* strain SS1 (KatA or GroEL) for studying the protein-protein interactions or for determining the requirements for Msr activity in vitro. Other pure proteins (UreE and HypB) were described previously (3, 25).

MSR. Plasmid pSAP112 (Table 2) was transformed into *E. coli* BL21 Origami (Novagen). Twenty milliliters of overnight starter culture of the *E. coli* strain harboring pSAP112 was added to 2 liters of LB, and the culture was incubated at 37°C until it achieved an OD_{600} of 0.6. The culture was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 22°C for 3.5 h. Cells were harvested by centrifugation, and the pellet was suspended in phosphate buffer (50 mM sodium phosphate, 50 mM NaCl, pH 7.8) and then subjected to sonication. The sonicate was centrifuged at 45,000 rpm for 1.5 h, and the supernatant was loaded onto a Hi-Trap SP column. Protein was then eluted using buffer B (50 mM sodium phosphate and 1,000 mM NaCl) with a linear gradient from 50 mM to 1,000 mM NaCl. The fractions from the flowthrough were collected, applied to a Hi-Trap Q Sepharose column, and eluted by use of the same buffers. The flowthrough fractions were then applied to a 10/200 Sephacryl column for size exclusion purification. Peak fractions were collected and subjected to SDS-PAGE to assess purity. Protein concentration was determined using the Bradford assay.

Trx1. Plasmid pSAP138 (Table 2) was transformed into *E. coli* BL21 Rosetta (Novagen). Twenty milliliters of overnight starter culture of the *E. coli* BL21 Rosetta strain harboring pSAP138 was added to 2 liters of LB and incubated at 37°C until the culture reached an OD_{600} of 0.6. The culture was induced with 0.5 mM IPTG at 37°C for 3.5 h. Cells were harvested and resuspended in Tris-Cl buffer (25 mM Tris, 25 mM NaCl, pH 7.0) and subjected to sonication. The lysate was centrifuged at 45,000 rpm for 1.5 h, and the supernatant was loaded onto a Hi-Trap Q-Sepharose column. Protein was eluted using buffer B (25 mM Tris and 1,000 mM NaCl) with a linear gradient of 25 mM to 1,000 mM NaCl. Peak fractions were collected and dialyzed overnight against 3 liters of buffer C (25 mM Tris and 150 mM NaCl). The pooled protein was then run through a 10/200 Sephacryl column preequilibrated with buffer C.

Trx2. Plasmid pSAP159 (Table 2) was transformed into *E. coli* BL21 Origami (Novagen). Thirty milliliters of overnight starter culture of *E. coli* BL21 Rosetta strain harboring pSAP138 was added to 3 liters of LB and incubated at 37°C until the culture grew to an OD_{600} of 0.7. The culture was induced with 0.8 mM IPTG at 22°C for 4 h. Cells were harvested and later resuspended in buffer 1 (50 mM HEPES, 50 mM NaCl, pH 6.5), and the suspension was sonicated. The cell lysate was centrifuged at 45,000 rpm for 1.5 h. The supernatant was subjected to ammonium sulfate precipitation. Trx2 was precipitated with the least amount of contaminating proteins at 50% and 60% ammonium sulfate levels. The precipitate was resuspended in buffer 1 and dialyzed against buffer 1 (total volume, 3 liters) for 24 h. The dialyzed protein was loaded onto a Hi-Trap SP column. Protein was eluted using buffer B (50 mM HEPES and 1,000 mM NaCl) with a linear gradient from 50 mM to 1,000 mM NaCl. Peak fractions were collected and dialyzed overnight against 3 liters of buffer C (25 mM HEPES and 150 mM NaCl, pH 6.5). The protein was concentrated, and purity was analyzed on a 15% SDS-PAGE gel.

TrxR. Plasmid pSAP139 (Table 2) was transformed into *E. coli* BL21 Rosetta (Novagen). Twenty milliliters of overnight starter culture of *E. coli* BL21 Origami strain harboring pSAP112 was added to 2 liters of LB and incubated at 37°C until the culture reached an OD_{600} of 0.6. The culture was then induced with 0.5 mM IPTG at 37°C for 3 h. Cells were harvested and later resuspended in Tris-Cl buffer (25 mM Tris, 25 mM NaCl, pH 7.0) and sonicated. The lysate was centrifuged at 45,000 rpm for 1.5 h, and the supernatant was used for ammonium sulfate precipitation. TrxR was precipitated at 65% and 75% ammonium sulfate. The pellets were resuspended in buffer A (10 mM Tris–25 mM NaCl, pH 7.6), pooled, and dialyzed in the same buffer (total volume, 3 liters). The suspension was then subjected to Q-Sepharose chromatography using a linear 25 mM to 1 M NaCl gradient. The peak fractions were collected, concentrated, and applied to a 10/200 Sephacryl column equilibrated with 10 mM Tris (pH 7.5) containing 200 mM NaCl. Peak fractions were harvested and analyzed on the gels for purity assessment and then were stored at −80°C.

Catalase (KatA). Native KatA was purified from *H. pylori* strain SS1 as described earlier (42) using cells less than 2 days old harvested from blood agar plates.

GroEL. Native GroEL was purified from *H. pylori* strain SS1. Cells less than 2 days old from blood agar plates were harvested in buffer A (50 mM HEPES, 50 mM NaCl, pH 7.2). Cells were sonicated, and the cytosolic protein fraction was subjected to ammonium sulfate precipitation. GroEL was found in the 60% and 70% saturation fractions. The presence of GroEL at each purification step was monitored by using *E. coli* anti-GroEL antibody (Sigma). The ammonium sulfate fractions were dialyzed against buffer A (total volume, 4 liters) overnight, and the dialyzed protein was then subjected to ion exchange chromatography using a HiTrap Q column. Protein was eluted using 50 mM HEPES and a 50 mM to 1 M NaCl gradient. The fractions containing the protein were concentrated and later subjected to size exclusion chromatography using a 10/200 Sephacryl column preequilibrated with 50 mM HEPES (pH 7.2) containing 300 mM NaCl. Peak protein fractions were pooled, a sample was subjected to SDS-PAGE, and pure protein was stored in the appropriate buffer at −80°C. Monitoring the purification progress with anti-GroEL antibody was important in order to avoid fractions that contained UreB, a major contaminant protein that migrates with a similar molecular weight to GroEL.

SSR. Plasmid pSAP163 (Table 2) was transformed into *E. coli* BL21 Origami (Novagen). Twenty milliliters of overnight starter culture of *E. coli* BL21 Origami strain harboring pSAP163 was added to 2,000 ml of LB and incubated at 37°C until the culture reached an OD_{600} of 0.6. The culture was induced with 0.5 mM IPTG at 22°C for 3.5 h. Cells were harvested and resuspended in binding buffer (300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, pH 8.0); the suspension was sonicated, and the extract was subjected to ultracentrifugation (for 2 h) to obtain the soluble protein (supernatant). The soluble protein was passed through a preequilibrated column prepared with Ni-nitrilotriacetic acid-agarose resin (QIAGEN) and washed with washing buffer (binding buffer with 20 mM imidazole). Protein was eluted with elution buffer (binding buffer with 250 mM imidazole). Protein purity was assessed, and the pure protein fraction was dialyzed against 50 mM NaH_2PO_4 (pH 8.0) containing 150 mM NaCl for 24 h to eliminate residual imidazole. Protein concentration was assessed (by SDS-PAGE), and samples were stored as aliquots with 10% glycerol at −80°C.

Protein cross-linking assays. KatA, GroEL, or SSR were separately incubated in buffer along with 300 μM H_2O_2 for 15 min at 4°C to oxidize the surface amino acid residues of the protein. This oxidation step was to enhance their interaction with Msr prior to the addition of cross-linker. Msr was, in turn, oxidized in a similar manner to study its interaction with either Trx1 or Trx2. The protein samples were washed several times using Amicon YM-10K centricon tubes to remove excess peroxide, and the final concentration was determined using Bradford assay.

For all Msr-protein interaction reactions, a 10 μM concentration of each protein was mixed in a 1:1:1 ratio along with 30 μM lysozyme (to inhibit possible nonspecific binding), and the mixture was incubated with a noncleavable protein

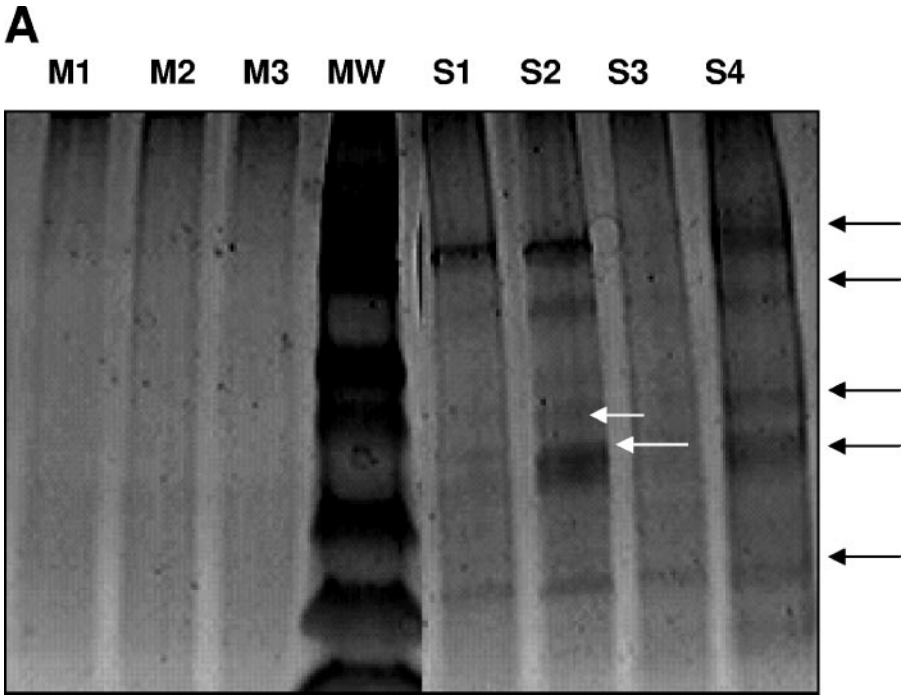

FIG. 1. (A) Sypro-Ruby-stained gel showing Msr-interacting proteins precipitated using CIP procedure. Lanes M1, M2, and M3 are elutions from the *msr* strain; lanes S1, S2, S3, and S4 are serial elutions of the proteins captured from SS1 (parent strain). The molecular masses (lane MW) of the standards used are 200, 116.3, 97.4, 66.3, 55.4, 36.5, 31.0, 21.5, 14.4, 6.0, 3.5, and 2.5 kDa. Arrows indicate different proteins identified using MALDI-TOF MS. TolB precursor and seryl t-RNA synthetase are indicated by the two arrows immediately to the right of lane S2. The clarity of the molecular mass marker had to be compromised for a better resolution of the eluted proteins. (B) Gel-based analysis of purity of potential Msr-interacting proteins. Catalase (KatA) and GroEL were purified from cell extracts of SS1. TrxR, Trx1, Msr, Trx2, and SSR were purified as recombinant proteins from *E. coli*. CE, cell extract. UN and IN denote protein collected from cells not induced and induced with IPTG, respectively. Lanes Q, Q-Sepharose fractions; lanes M, molecular mass markers. The molecular masses (in kDa) of the markers are as follows: 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4. Arrows in each panel identify the protein of interest.

cross-linker (3 mM dimethyl suberimidate) for 30 min at room temperature; later the reaction was quenched with 100 mM Tris-Cl (pH 7.5). The reaction mixture was run on three different gels (12% SDS-PAGE), and these were electroblotted onto separate nitrocellulose membranes. Immunoblotting was individually performed with antibodies against Msr (1:1,000), KatA (1:1,000), or GroEL (1:8,000). The membranes were subsequently incubated in the secondary antibody at a ratio of 1:1,500 for goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate for 1 h, and the substrates were added following consecutive membrane washes. Control experiments using a similar procedure (such as the oxidation procedure) but with pure HypB and pure UreE from *H. pylori* (proteins not rich in methionine residues) were important to ensure the specificity of the Msr-target protein results. After (attempted) cross-linking, these latter potential adducts were subjected to SDS-PAGE, and the blots were probed with anti-HypB (25) or anti-UreE (3) serum at dilutions of 1:2,000 and 1:1,000, respectively.

Catalase assays. Wild-type SS1 or *msr* strains were grown in closed vials at 4% partial pressure O_2 and shifted at late log phase of growth to 10% O_2 for 4 h. Viable counts were estimated by plating serial dilutions on blood agar plates. Catalase specific activity was determined on cell extracts from cells grown at 4% and 10% O_2 as described elsewhere (42). Catalase activity is reported as 1 unit is equivalent to 1 μmol of H_2O_2 decomposed/min/mg of cell protein. Results are the mean of 12 determinations; these were from four independent experiments, with each experiment sampled in triplicate. Cell extracts (5 μg of protein) from each strain (SS1 and *msr*) were subjected to SDS-PAGE, and the separated proteins were transferred to a nitrocellulose membrane for immunoblotting with anti-KatA antiserum.

Homology-based structural predictions. The amino acid sequence of the entire Msr protein was subjected to SWISS-MODEL in the first approach mode (http://www.expasy.org/swissmod). The known crystal structures of enzymes that share high sequence identity (with *H. pylori* Msr) upon pairwise alignments using protein databank entries 1L1Da (C-terminal methionine sulfoxide reductase domain of *N. gonnorrhoeae*) and 1L1Db *E. coli* (MsrA and MsrB) were used (39, 40). The models were analyzed manually for robustness, and the best fit model was selected for further analysis. The coordinate file was imported to a 3D-Mol viewer (a component of Vector NTI suite 8.0; Infomax Inc.) to visualize the residues with respect to the entire three-dimensional structure and to calculate the molecular surface (Conolly or Varshney method) and the distance between specific residues.

In vitro Msr activity. Methionine sulfoxide reductase activity assays were performed as described previously (28) by monitoring the oxidation of NADPH at 340 nm. Methyl-*p*-tolyl (S)-sulfoxide and methyl-*p*-tolyl (R)-sulfoxide were purchased from Sigma-Aldrich (catalogue no. 339997 and 343609, respectively). The reaction was conducted in prewarmed 50 mM Tris-HCl (pH 7.4). Msr activity was first studied using purified Msr (6 to 10 μg), a broad substrate (methyl tolyl sulfoxide) range of 0.5 mM to 14 mM, 50 mM DTT, and 100 μmol of NADPH. Later, the substrate concentration was reduced to the range of 0.5 to 12 mM, based on activity saturation results. Similarly, a range of thioredoxin (10 to 70 μM) and thioredoxin reductase (10 to 35 nmol) concentrations was used to establish saturation levels. For routine specific activity measurements and K_m determinations, Msr (6 to 10 μg), substrate (0.5 to 12 mM), thioredoxin (50 μM), TrxR (30 nmol), and NADPH (100 μmol) were used in a total reaction volume of 1 ml. A unit of activity is defined as 1 nmol of NADPH oxidized/min, as determined by the change in absorbance at 340 nm. Data presented are the means of 15 determinations; these were from five independent experiments, with each one sampled in triplicate.

RESULTS AND DISCUSSION

Identification of Msr-interacting proteins in *H. pylori*. Many important proteins are inactivated due to methionine oxidation; they have been identified in *E. coli* and in some eukaryotes (see reference 8 and the references therein). It is

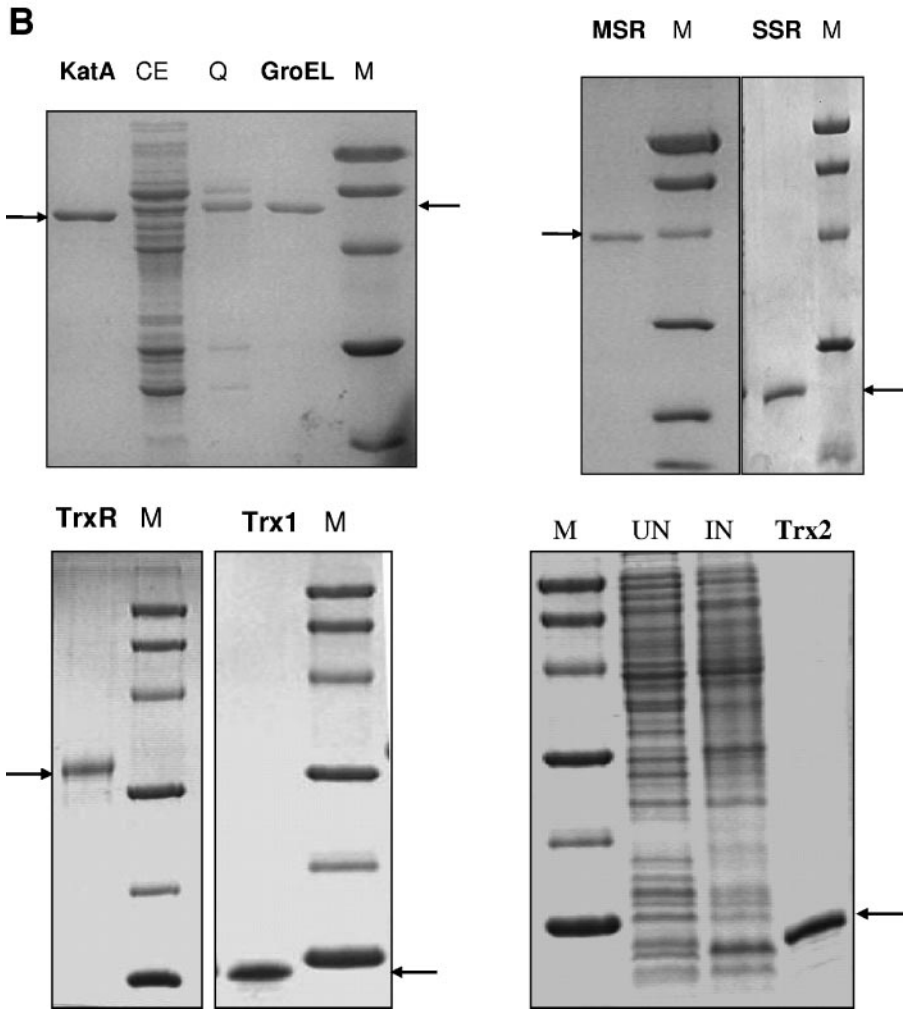

FIG. 1—*Continued*.

likely that a subset of these proteins embodies the direct targets of the MsrA/B protein repair system. However, the set of target proteins that interact with Msr (are targeted for Met reduction) in *H. pylori* are unknown.

The putative *H. pylori* Msr-interacting proteins were identified by combining cross-linking with a coimmunoprecipitation (CIP) approach. First, cells were exposed to oxidative stress conditions. The transient but intimate interactions of Msr with

TABLE 3. Proteins identified by MALDI-TOF MS[a]

Protein name	Protein CI (%)[b]	Total protein score[c]	Estimated size (kDa)	HP26695 gene no.[d]	% Met[e]	Function
GroEL	98.34	102	60.04	hp0010	3.7	Chaperone
Catalase	97.528	82	53.27	hp0875	3.4	Detoxification
Msr	99.03	97	41.30	hp0224	NA	Repair
SSR	96.02	89	22.24	hp1009	3.5	DNA Repair
Trx1	99.92	106	11.84	hp0824	NA	Electron transport
Seryl t-RNA synthase	34.12	27	47.44	hp1480	3.2	Protein synthesis
TolB precursor protein	0	36	47.71	hp1126	3.1	Colicin tolerance

[a] The proteins listed were observed in three different CIP experiments performed using cell extracts of *H. pylori* strain SS1. The bands were excised from the SYPRO-RUBY-stained gel and then identified using MALDI-TOF MS at The University of Georgia Proteomics Facility.
[b] Protein CI is a statistical calculation of how closely the acquired data match previous database searches. The closer the confidence percentage value is to 100%, the more likely the protein is correctly identified.
[c] Total protein score is calculated as $-10 \times \text{Log(P)}$, where P is the probability that the observed match is a random event. Only protein scores greater than 72 are significant ($P < 0.05$).
[d] Gene annotation and putative function are based on HP26695 (www.tigr.org).
[e] NA, not available.

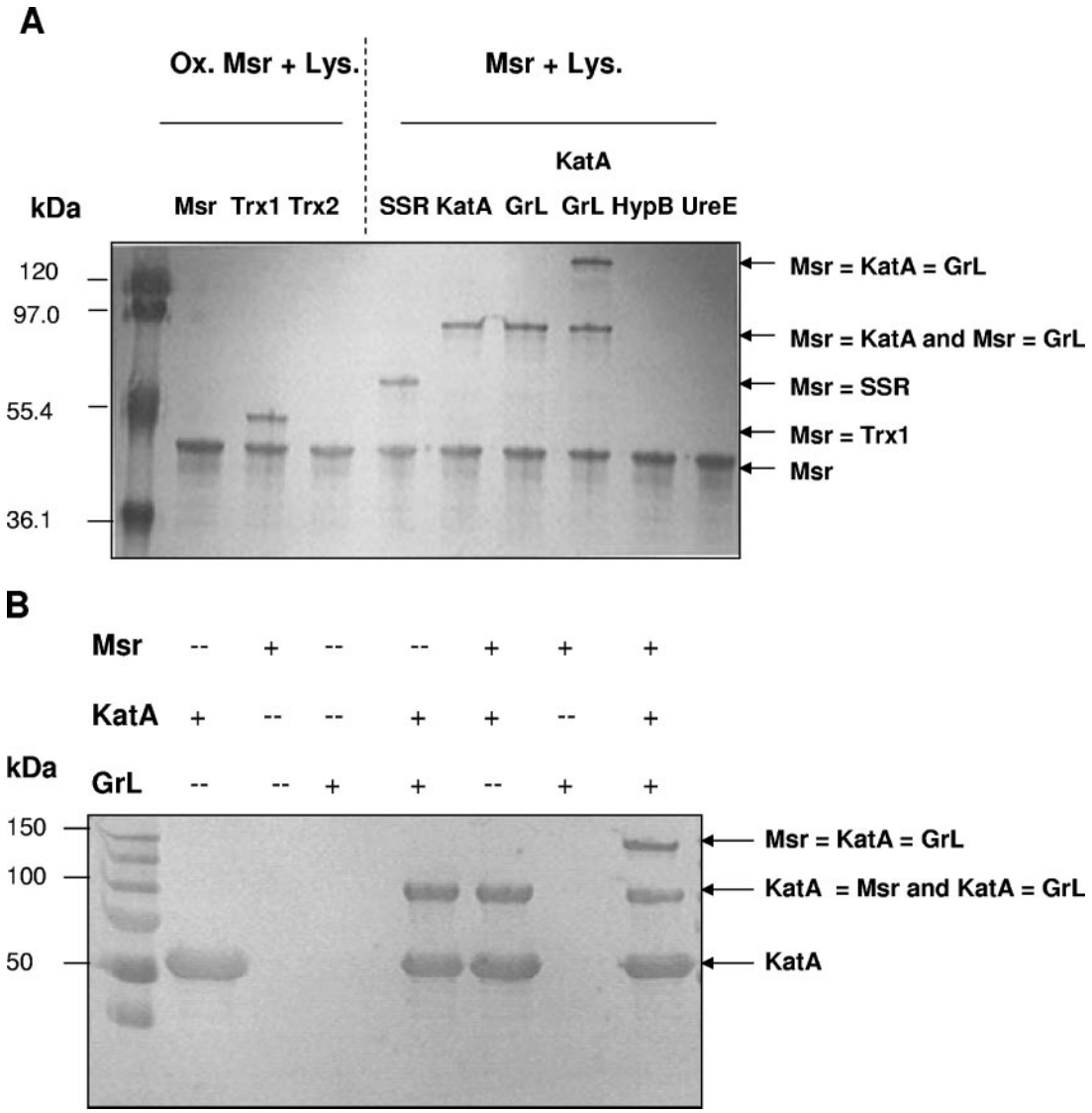

FIG. 2. (A) Immunoblot using anti-Msr antibody. Protein-protein interactions identified using Msr and its substrates (1:1) were studied using the noncleavable cross-linker, dimethyl suberimidate. Oxidized Msr (and oxidized lysozyme) were mixed with Msr (Msr), Trx1, and Trx2 on the left side of the gel. Native Msr (and oxidized lysozyme) were mixed with individual oxidized substrates in lanes labeled SSR, KatA, GrL (GroEL), or both KatA and GrL. Oxidized HypB and oxidized UreE were used as controls. Arrows at right indicate the complexes Msr-KatA-GroEL, Msr-KatA and Msr-GroEL, Msr-SSR, and Msr-Trx1. (B) Immunoblot using anti-KatA antibody. The same reactions used in panel A (pertinent to KatA) were resolved on a separate gel and immunostained with anti-KatA antibody. Arrows identify the following complexes: KatA-GroEL-Msr, KatA-Msr, KatA-GroEL, and KatA alone. The molecular sizes (in kDa) of prestained markers are given. (C) Immunoblot using anti-GroEL antibody. The same mixtures used in panel B were resolved on a separate gel and immunostained with antibody against GroEL. The cross-linked adducts formed in each case are identified by an arrow. The prestained marker set as used in panel B was used here. GrL, GroEL.

its oxidized protein substrates were then captured by adding a cleavable cross-linker to cell extracts. The yield of Msr-substrate complexes was then harvested by use of an immobile anti-Msr antibody, and the complexes were uncross-linked. The proteins that had formed adducts with Msr were identified by mass spectrometry. Two important control experiments were done to address the specificity of the observed Msr-protein interactions; extracts from an *msr* strain exposed to the oxidative conditions and an extract from wild-type cells that had not been exposed to oxidative conditions were both carried through the entire procedure.

Figure 1A shows SYPRO-RUBY-stained proteins from the wild type and the *msr* mutant, after the complete cross-link and CIP procedure; the proteins represent those that (when oxidized) had presumably intimately interacted with Msr. Proteins were clearly visible when the extract from wild-type cells (when exposed to oxidative stress, i.e., 12% partial pressure O_2) was used as the starting material for cross-linking and Msr-adduct immunoprecipitation. No proteins were obtained from the same procedure when the *msr* strain was used (Fig. 1A), indicating that only proteins interacting with Msr were precipitated using this approach for the wild type. Similarly, there was not much adduct yield when wild-type cells that had been maintained continually under low O_2 conditions (4% partial pres-

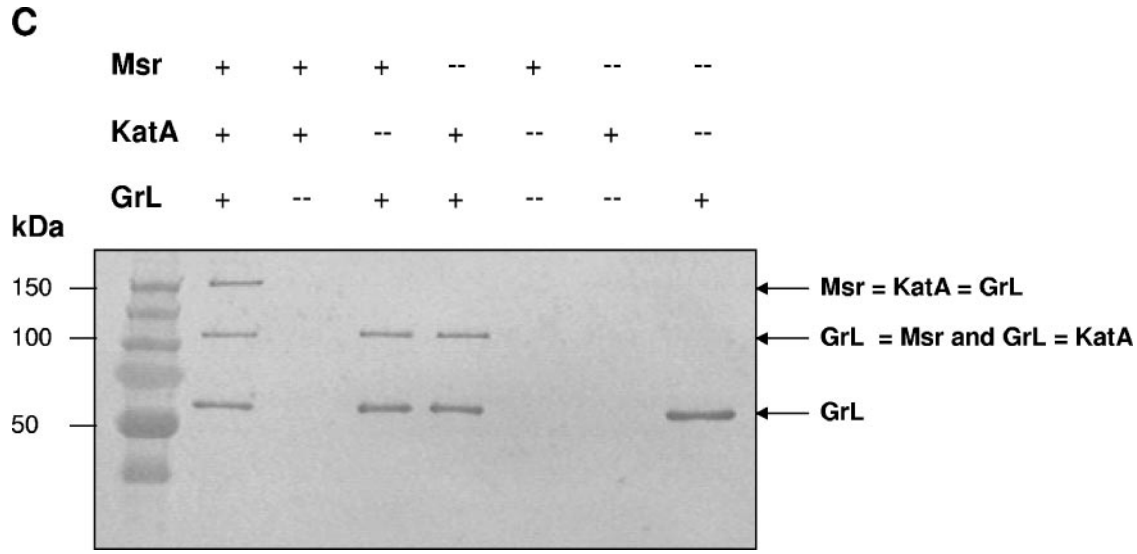

FIG. 2—*Continued*.

sure O_2) were used as the starting material (data not shown); this links the observed interactions to oxidative conditions. Table 3 lists the seven MALDI-TOF MS-identified proteins obtained from the procedure. Five of these had a high ion score (greater than 72) and significant confidence interval (CI) values. Of these five, catalase (KatA), SSR, and the chaperone protein GroEL contain approximately 3.2% to 3.5% methionine residues. It may be significant that this methionine percentage is higher than the average 1.7% methionine found in proteins (17). Another potential Msr-interacting protein (Table 3) identified was Trx1, an electron carrier predicted to be a reductant of Msr (5). In addition, Seryl-t-RNA synthetase and TolB protein precursor were identified as potential Msr-interacting proteins (Table 3). We did not pursue further studies on these latter two due to the lower CI values in the MS analysis. Still, the methionine content was elevated in these proteins (Table 3).

To confirm identification of some of the above potential Msr targets, we studied the interaction of individual purified proteins with Msr. Catalase, GroEL, Msr, SSR, TrxR, Trx1, and Trx2 were purified to at least 95% homogeneity (Fig. 1B). These potential pure substrates for Msr (GroEL, KatA, and SSR) were oxidized, and then their interaction with Msr was assessed by use of a noncleavable cross-linker (see Materials and Methods). Lysozyme (oxidized) was included in the reaction mixture at a threefold greater level than the test proteins to reduce nonspecific protein-protein interactions. For assessing catalase- or GroEL-Msr interactions, the cross-linked mixtures were subjected to three different SDS-PAGE gels, and the gels individually were blotted and probed separately with anti-Msr, anti-KatA, and anti-GroEL antibodies as previously described (Fig. 2A, B, and C, respectively). Msr interacted individually with KatA or with GroEL, as seen in the blot using anti-Msr antibody (Fig. 2A). The cross-linked interactions of Msr with KatA or with GroEL were apparent due to the identification of an approximately 100-kDa complex (Msr-KatA and Msr-GroEL) compared to the (noncomplexed) Msr in the blot. A similar banding pattern was identified when the same cross-linked sample was probed using anti-KatA (Fig. 2B, KatA-Msr) or anti-GroEL (Fig. 2C, GrL-Msr). The blots in Fig. 2B and C confirm that the adducts identified in Fig. 2A contain both Msr and its partner protein. The interaction of Msr with GroEL seen in this study supports a conclusion made previously based on a yeast two-hybrid screen to predict the protein-protein interactions in *H. pylori* (32). However, evidence for the interaction of Msr with KatA had not been previously obtained. Catalase and GroEL complexed together, also forming an approximately 100-kDa adduct (Fig. 2B and C, KatA-GrL and GrL-KatA, respectively).

Msr interacted only with the oxidized KatA and GroEL, as peroxide treatment was a prerequisite for obtaining these adducts (see Materials and Methods) (data not shown). A similar phenomenon was previously reported for observing *E. coli* GroEL-Msr interactions (22). A much higher molecular mass band (>150 kDa) was evident (Fig. 2A to C, Msr-KatA-GrL) when the three proteins (reduced Msr and oxidized KatA and oxidized GroEL) were mixed together with the cross-linker; thus GroEL may play a role in stabilizing a tripartite complex. Alternatively, Msr may always interact with the chaperone, and the presence of oxidized KatA may not interfere with that interaction. There was no evidence that the three proteins (Msr, KatA, and GroEL) formed higher-molecular-mass complexes (such as homodimers) with themselves (Fig. 2A to C).

Recognition of the oxidized recombinase protein SSR by pure Msr was also apparent based on an immunostained band of the expected size of 60 to 65 kDa (Fig. 2A, Msr-SSR). Like the others, this adduct formed even in the presence of a threefold greater amount (see Materials and Methods) of a control (lysozyme) protein. The cross-link approach combined with CIP identified only the Msr-Trx1 but not an Msr-Trx2 interaction in *H. pylori* (see text above). We further investigated this result by testing the ability of pure Trx1 and Trx2 to cross-link with oxidized Msr. Msr formed a cross-linked adduct only with Trx1 but not with Trx2 (Fig. 2A, Msr-Trx1). We tested the interaction of Msr individually with two additional pure *H. pylori* proteins for which we had antiserum available, HypB and

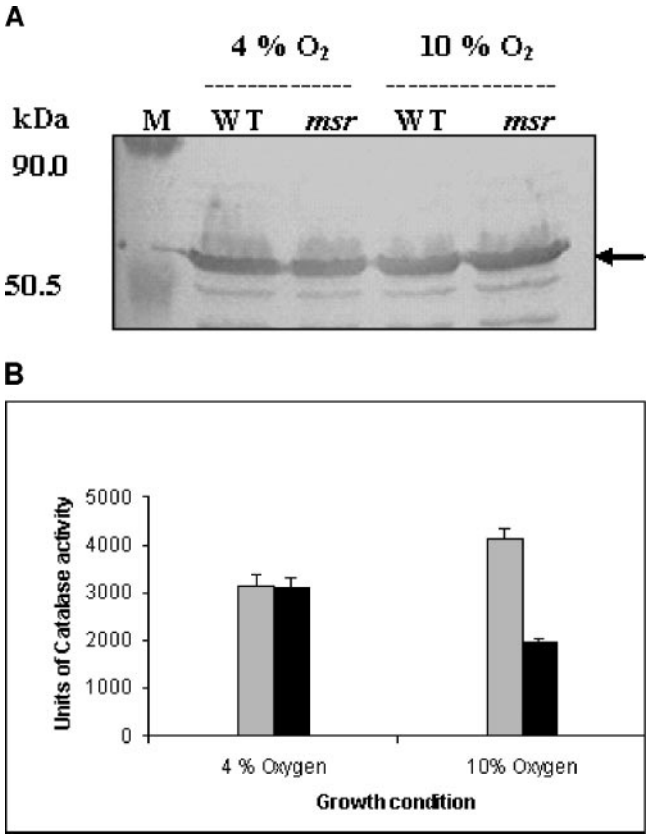

FIG. 3. (A) Relative catalase expression in SS1 and *msr* mutant. Cell extracts (5 μg) from the described conditions were run on a 12.5% SDS-PAGE gel, and immunoblotting was performed using anti-catalase antibody. The arrow indicates the immunostained catalase protein in all lanes. 4% O_2, cells grown in 4% oxygen, 10% O_2, cells grown in 4% oxygen and exposed for 3 h to 10% oxygen (see Materials and Methods); WT, SS1 strain; *msr*, *msr* mutant in SS1; M, molecular mass marker. The immunostained catalase (~55 kDa) is identified by an arrow. (B) Catalase activities in SS1 and *msr*. Specific activities of catalase are determined from extracts of cells grown in 4% oxygen or grown in 4% oxygen and then exposed to 10% oxygen for 3 h. One unit of activity is equivalent to the number of micromoles of H_2O_2 decomposed/min/mg of protein. Gray bars indicate the SS1 parent strain, and black bars indicate the *msr* mutant. The mean ± SD from 12 individual samples taken from four separate experiments (three replicates each) is shown here. The mutant results are significantly less than the wild type when both strains are in 10% O_2 ($P < 0.01$).

UreE. No Msr cross-linked adducts were formed to these (Fig. 2A, lanes HypB and UreE), indicating that the Msr interactions observed above are not likely to be artifacts of the cross-linking procedure.

The significance of Msr activity in reducing oxidatively damaged methionine residues of purified *E. coli* GroEL was previously demonstrated (22), but the interaction of Msr with catalase and with SSR are new findings. To understand the physiological significance of the catalase interaction, we compared both the relative expression and specific activity of catalase between the SS1 (parent strain) and *msr* mutant strains under non-O_2 stress versus under oxygen stress conditions. We observed that the levels of catalase expression (based on immunoblotting) in both strains were similar (Fig. 3A) but that the specific catalase activity of the mutant only was reduced by 50% due to oxygen stress (Fig. 3B). To investigate the possibility that the lower activity in the mutant is due to an inability to repair Met residues of catalase, pure Msr plus DTT was added to the *msr* strain extract. The resulting catalase activity (3,432 + 212 units) was about 85% of the wild type (4,144 + 197 units) from cells in the 10% O_2 exposure condition. The results are consistent with a role for Msr in maintaining catalase function. It is known that catalase is an important part of the oxidative stress defense in this pathogen; so this knowledge permits a better understanding of the severe oxidative stress resistance deficiency phenotype observed previously for an *H. pylori msr* mutant strain (2).

Intracellular organic peroxides formed during oxidative stress inhibited catalase activity in *H. pylori* (42). Alkyl hydroperoxide reductase activity prevented oxidative damage to catalase, presumably by removal of the accumulating organic peroxides (42). Our results taken together with the study of Wang et al. indicate there are at least two mechanisms that *H. pylori* has developed in order to maintain the function of a single ROS detoxification enzyme, catalase. GroEL and SSR are presumably other repair targets of Msr, but assays for these functions are very challenging. Chaperones (22), ribosomal proteins (9), and a signal recognition protein (16) are some previously identified targets for Msr-dependent repair in bacteria. Calmodulin (19) and alpha-1-proteinase inhibitor (10) are some well-studied targets for Msr in eukaryotes. Nevertheless, the present study extends the results to identify other interacting partners for Msr, and these additional proteins are proposed to be direct targets for repair in the gastric pathogen. In addition to their enzymatic role, these Msr-interacting methionine-rich proteins may serve as sinks for reactive oxygen and nitrogen intermediates.

Homology-based structure prediction of *H. pylori* Msr. *H. pylori* Msr is a 359-amino-acid single polypeptide that comprises two fused domains (MsrA, with residues 36 to 189, and MsrB, with residues 206 to 330) based on the sequence homology with known *E. coli* MsrA and *N. gonorrhoeae* MsrB proteins. The *H. pylori* protein contains two cysteine residues in each of its domains: Cys 44 and 184 in the MsrA domain and Cys 263 and 318 in the MsrB domain. However, which of these Cys residues could potentially reside on the surface (and thus participate in catalysis) is not known.

We applied homology-based crystal structure modeling to determine the potential sites of catalytic cysteines. Based on the Swiss-Prot analysis, we observed that the MsrB domain of *H. pylori* shared a high degree of sequence homology (identity of ~60%) including predicted folding to the *N. gonorrhoeae* enzyme known as MsrA/B (24). No significant identity was found between Msr of *H. pylori* and *E. coli* MsrA or MsrB proteins. *H. pylori* Cys 318 and Cys 263 (both within the MsrB domain) were surface exposed and in close proximity to each other (raw distances are preliminary) (Fig. 4). Cys 263 is part of the sequence GCGWP, and Cys 318 is part of the MsrB signature sequence, GGLRYCI. Based on the sequence homology of *H. pylori* Msr with that of *N. gonorrhoeae* or *N. meningitidis* and on the proximity of Cys residues to each other, this protein could be grouped into the same class (class II) of *N. meningitidis* or *N. gonorrhoeae* MsrB, wherein Cys 117 (Cys 318 for *H. pylori* Msr) is the catalytic Cys and Cys 63 (Cys 263 of *H. pylori* Msr) is the recycling residue. The latter forms a

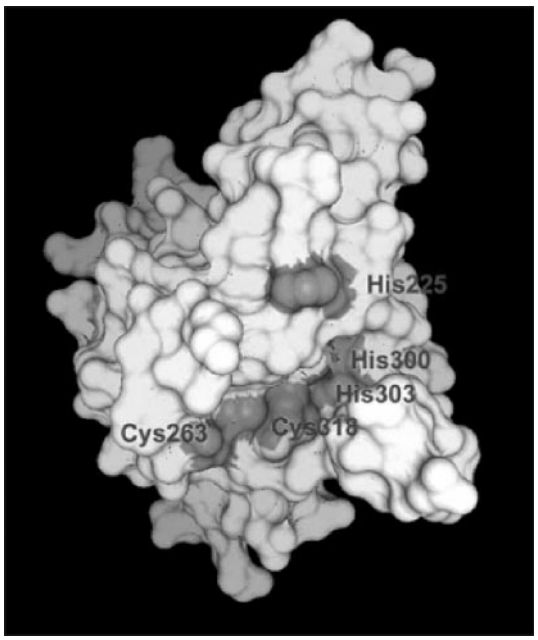

FIG. 4. Homology based 3-D model of Msr in *H. pylori.* The amino acid sequence of *H. pylori* Msr was aligned with known Msr proteins from different bacteria using the Swiss-Prot protein database. MsrAB from *Neisseria* (*N. gonorrhoeae* and *N. meningitidis*) shares a high percentage homology with *H. pylori* Msr and was used as a model to understand the folding of this protein. Surface-exposed cysteines and proximal histidines are labeled.

disulfide bond with the former residue (21). In addition, His residues (His 303 and His 300) that operate in the sulfoxide reductase reaction were also near the catalytic Cys residues. These results raised some interesting questions about the substrate preference (R or S isomer) of Msr in *H. pylori* and are discussed in the following section.

Substrate specificities of Msr. The results from protein modeling suggested that the *H. pylori* MsrB-like catalytic domain closely resembled the *Neisseria* MsrB, so that R-SO may be the preferred substrate for the *H. pylori* enzyme. The ability of *H. pylori* Msr to reduce the two (R and S-sulfoxide) isomers was directly tested. Msr in *H. pylori* preferentially reduced the Met R-SO isomer (Fig. 5). Indeed, no Met S-SO reduction could be detected. The ability of Msr to use the two possible reductants of Msr was assessed by conducting enzyme assays separately with pure Trx1 or Trx2. Only use of Trx1 resulted in Msr activity. The calculated K_m of Msr for the substrate methyl *p*-tolyl R-sulfoxide (using Trx1 as reductant) was 4.1 mM. The MsrA-like domain of *H. pylori* is apparently inactive, and this conclusion is supported by the previously described phenotype of an *msrB* domain-specific *H. pylori* mutant strain (2).

Induction of *msr*. (i) External stress-dependent expression. XylE reporter fusions to an *msr* promoter region were used to examine *msr* expression in cells exposed to various stress conditions or to iron availability regimes. We had earlier determined that a 200-bp intergenic region between hp0223 and hp0224 (*msr*) contained all the required promoter elements for the expression of *msr*, as this area was sufficient to complement the *msr* mutant strain (2). This intergenic promoter area was identified by a primer extension reaction that revealed that the 5′ end of the transcript was approximately 30 bp upstream of the ATG start codon (data not shown). We therefore used this fragment in the present study to examine *msr* expression both on the genome and on an introduced plasmid vector.

Cells were grown under optimum conditions of oxygen (4% partial pressure) to mid-log phase (OD_{600} of 0.5), and the cells were subjected to stress conditions of oxygen, peroxide, peroxynitrite, $FeCl_3$ supplementation, or iron starvation (i.e., chelation by use of 2′,2′-dipyridyl). Induction of *xylE* activity was determined before and after the addition of each of the above stress conditions. No significant effect on *msr* expression was

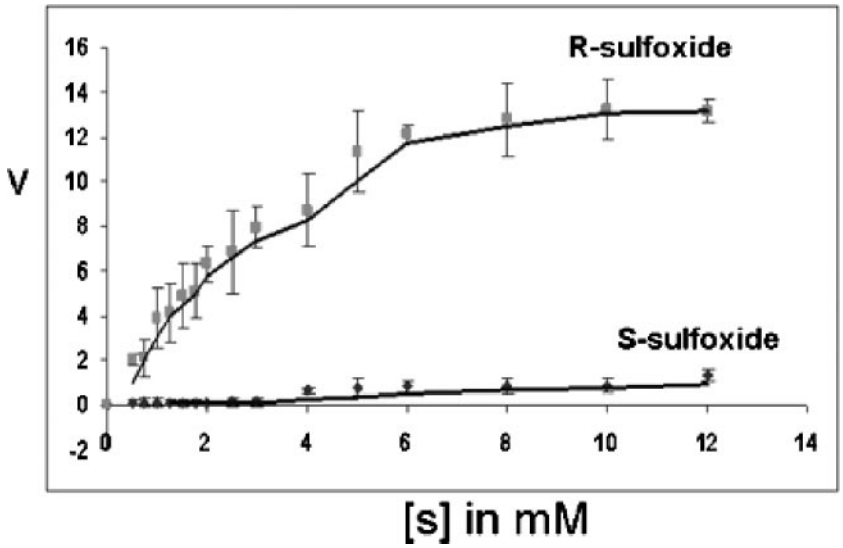

FIG. 5. In vitro Msr activity using R- and S-isomers of sulfoxide. The ability of Msr to reduce methyl *p*-tolyl R-sulfoxide and *p*-tolyl-S-sulfoxide was tested using purified reaction components (Msr, Trx1, and TrxR). Oxidation of NADPH was monitored at 340 nm as a measure of substrate reduction by Msr. V, nmoles of NADPH oxidized/min; [s] concentration (mM) of substrate used in this assay. Values are the means ± SD from four independent experiments, each performed in triplicate (total of 12 samples for each mean).

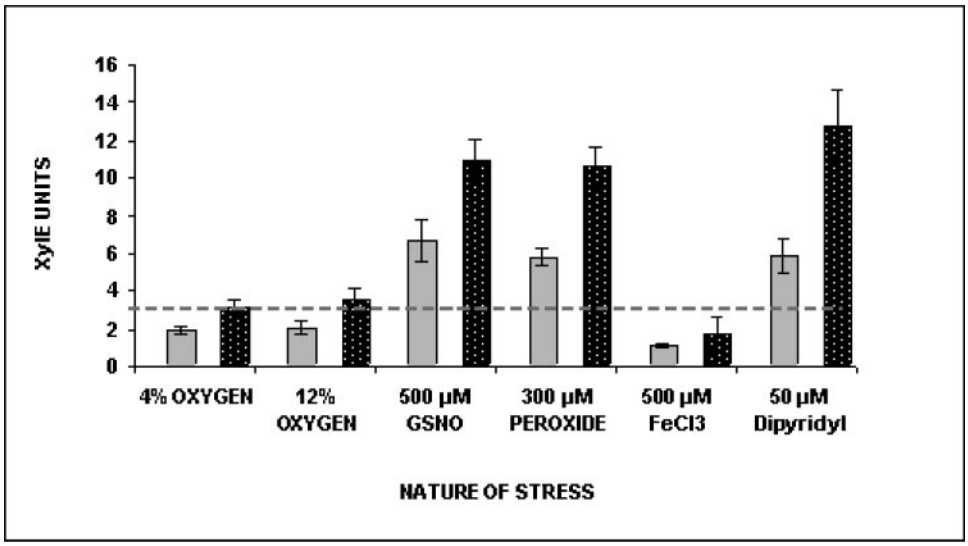

FIG. 6. *xylE* activities to monitor *msr* expression in *H. pylori.* Whole cells collected at 6 h postexposure to stress conditions were assayed for *xylE* activity. Gray bars indicate genomic fusions (P_{msr}-*xylE*) and black bars indicate fusions on the shuttle vector pHel3 (see Materials and Methods). Values are the means ± SD from 15 samples taken from five separate experiments (three replicates each) for each condition. One unit of *xylE* activity is equivalent to the number of micromoles of catechol oxidized/min/10^9 cells. The *S*-nitrosoglutathione (GSNO), peroxide, and iron-chelated conditions were all significantly greater ($P < 0.05$) for both the plasmid and the genomic expression than the non-stress-treated (4% O_2) samples, based on a Student's *t* test. Basal *xylE* activity (under 4% O_2 on the plasmid) is indicated by the dashed line.

observed by a shift to 12% O_2. An approximately threefold increase in *msr* expression (in a 6-h stress exposure period) was observed when either peroxide or peroxynitrite stress was applied. In addition, an approximately 3.5-fold increase in expression was observed under iron-chelated conditions (Fig. 6) compared to the same conditions but with iron. There was no transcriptional up-regulation observed when the cells were exposed to UV light (data not shown), and *msr* expression was slightly repressed (by less than twofold) when medium was supplemented with iron (Fig. 6). The induction of this gene due to peroxide or peroxynitrite is particularly interesting, since *H. pylori* lacks homologs of the known *E. coli* oxidative stress response regulators OxyR or SoxR; instead Fur and NikR are known regulators for some oxidative stress enzymes in this pathogen. To identify a potential regulator of *msr*, we further studied the expression of *msr* using the same reporter fusions (and protein levels were monitored by immunoblotting) individually in isogenic mutants of *fur*, *nikR*, and *msr* itself (the latter to test for possible autoregulation). There was no difference in the induction or expression pattern of *msr* in any of these backgrounds from that seen in the wild-type strain (data not shown). Also, as aconitase (*acn*) has been shown to be a (posttranscriptional) regulator of oxidative stress enzymes, an aconitase mutant strain was studied, too; the levels of Msr were the same in the *acn* mutant strain as for the wild type. Perhaps the regulation of *msr* in *H. pylori* is through an oxidative stress-mediated pathway or cascade that is yet to be identified.

From a microarray study it was reported (26) that an approximately threefold up-regulation of *msr* occurs by subjection of *H. pylori* to iron-starvation conditions. We observed a similar up-regulation by incubating cells in iron chelation conditions. We speculate that the up-regulation of *msr* in the absence of iron in *H. pylori* is perhaps a compensatory response by the pathogen to the known apo-Fur mediated repression of key ROS detoxification enzymes such as *napA* and *sodB* (both are abundant in *H. pylori* in iron-sufficient conditions) (12, 14). It seems plausible that *H. pylori* indirectly senses (either via Fur or through some other means) the iron-starved condition and consequently up-regulates *msr* to prevent the protein oxidation that would otherwise result from accumulation of ROS in the cell. The situation is likely exacerbated by the dysfunction of membrane-bound reductases that require iron as their cofactor and provide reductant for stress-combating enzymes; this would, of course, further impair the ROS detoxification ability of the cell. Increased up-regulation of *msr* in order to prevent the oxidation of cell protein would create an alternate non-iron-dependent defense mechanism against oxidative damage. A similar *msr* up-regulation would not be needed under the conditions of excess iron, as *napA*, *pfr*, and *sodB* (4, 12, 14) expression would provide a battery of oxidative stress defenses.

Growth phase-dependent expression. We determined the growth phase-dependent expression of *msr* by growing cells at a constant oxygen level (7% partial pressure O_2) over an 80-h period. Immunoblotting (using anti-Msr serum) performed on cell extracts obtained at various time points showed a 2- to 2.5-fold increase in Msr expression at both the 56 h and 72 h time points (late log phase to stationary phase of growth) (Fig. 7A) compared to early and mid-log phase expression. This growth phase-dependent up-regulation of Msr was also observed by enzyme activity assays (Fig. 7B). The increase in Msr activity in stationary phase can probably be attributed to nutrient (including iron) deprivation at the late phases of growth. This could perhaps be tested by supplementing the medium with iron, but the situation is confounded by the observed iron-dependent repression of *msr* (Fig. 6). Nevertheless, the increased *msr* expression in late log and stationary

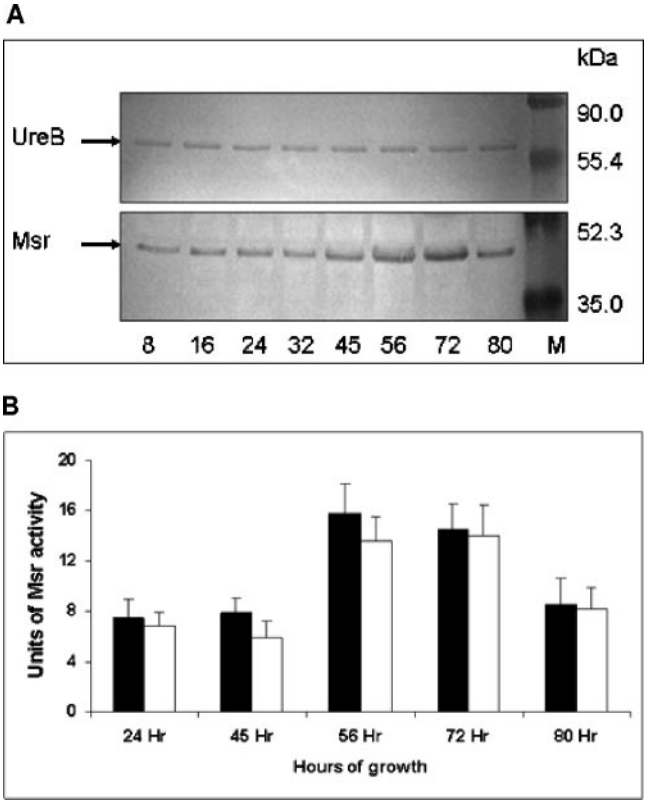

FIG. 7. Growth-phase-dependent expression of Msr. *H. pylori* strain SS1 was grown in Mueller-Hinton broth supplemented with 5% calf serum; 7% partial pressure oxygen was maintained in the atmosphere of the (sealed) bottles. Cells were collected from each time point, extracts were prepared identically for all time points, and cell protein from each sample was run on two separate 12.5% SDS-PAGE gels. Immunoblotting was performed using anti-Msr antibody and anti-UreB (as a control). The arrows identify immunostained UreB (~66 kDa) and Msr (~43 kDa) in the cell extracts (A). Membrane protein fractions were simultaneously collected from the cell extracts, and specific Msr activity was determined using MetSO or methyl *p*-tolyl sulfoxide as substrates. Values are the means ± SD from nine samples (from three separate experiments, each assay performed in triplicate) (B).Units of Msr activity are plotted against time of growth in hours. One unit of activity is equivalent to the number of nanomoles of NADPH oxidized/min/mg of membrane protein. Time samples at 56 and 72 h correspond to late log and stationary phases of growth, respectively. White bars indicate the assay using (equal mixture of R and S isomers) methyl *p*-tolyl sulfoxide, and black bars indicate (equal mixture of R and S isomers) MetSO as a substrate. The 56- and 72-h data are significantly greater than the other sampled time points at $P < 0.05$ (Student's *t* test).

phase is like the results reported for expression of *msrB* in *Saccharomyces cerevisiae* (27).

The conditions needed for high Msr expression in various bacteria is broad, ranging from nutrient starvation and growth phase dependence (in *E. coli* and *S. cerevisiae*) to radiation or antibiotics exposure (*Staphylococcus aureus*) (27, 29), (34). Exposure to chemical oxidants also caused increased Msr expression in yeast (33), and we observed this for *H. pylori*, too. It seems clear that *msr* is not regulated by the well-studied oxidative stress response regulators such as SoxR or OxyR (that sense superoxide and peroxide, respectively) but, instead, through other mechanisms. Under some of the above stress conditions, repair of the oxidatively damaged proteins (by Msr) is presumably one of the effective mechanisms to preserve the accumulated protein pool. In this study, we present some different environmental conditions such as iron starvation that aid in the up-regulation of *msr*. Of course, the precise nutrient conditions encountered by the pathogen in the host are not known, but the conditions can be expected to vary with degree of host colonization and infection. A further understanding of the dynamic regulation of *msr* and the complete physiological roles of the enzyme is desired.

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REFERENCES

1. **Abulimiti, A., X. Qiu, J. Chen, Y. Liu, and Z. Chang.** 2003. Reversible methionine sulfoxidation of *Mycobacterium tuberculosis* small heat shock protein Hsp16.3 and its possible role in scavenging oxidants. Biochem. Biophys. Res. Commun. **305:**87–93.
2. **Alamuri, P., and R. J. Maier.** 2004. Methionine sulphoxide reductase is an important antioxidant enzyme in the gastric pathogen *Helicobacter pylori*. Mol. Microbiol. **53:**1397–1406.
3. **Benoit, S., and R. J. Maier.** 2003. Dependence of *Helicobacter pylori* urease activity on the nickel-sequestering ability of the UreE accessory protein. J. Bacteriol. **185:**4787–4795.
4. **Bereswill, S., S. Greiner, A. H. van Vliet, B. Waidner, F. Fassbinder, E. Schiltz, J. G. Kusters, and M. Kist.** 2000. Regulation of ferritin-mediated cytoplasmic iron storage by the ferric uptake regulator homolog (Fur) of *Helicobacter pylori*. J. Bacteriol. **182:**5948–5953.
5. **Boschi-Muller, S., A. Olry, M. Antoine, and G. Branlant.** 2005. The enzymology and biochemistry of methionine sulfoxide reductases. Biochim. Biophys. Acta **1703:**231–238.
6. **Brahmachary, P., M. G. Dashti, J. W. Olson, and T. R. Hoover.** 2004. *Helicobacter pylori* FlgR is an enhancer-independent activator of sigma54-RNA polymerase holoenzyme. J. Bacteriol. **186:**4535–4542.
7. **Brot, N., H. Fliss, T. Coleman, and H. Weissbach.** 1984. Enzymatic reduction of methionine sulfoxide residues in proteins and peptides. Methods Enzymol. **107:**352–360.
8. **Brot, N., and H. Weissbach.** 2000. Peptide methionine sulfoxide reductase: biochemistry and physiological role. Biopolymers **55:**288–296.
9. **Caldwell, P., D. C. Luk, H. Weissbach, and N. Brot.** 1978. Oxidation of the methionine residues of *Escherichia coli* ribosomal protein L12 decreases the protein's biological activity. Proc. Natl. Acad. Sci. USA **75:**5349–5352.
10. **Carp, H., A. Janoff, W. Abrams, G. Weinbaum, R. T. Drew, H. Weissbach, and N. Brot.** 1983. Human methionine sulfoxide-peptide reductase, an enzyme capable of reactivating oxidized alpha-1-proteinase inhibitor in vitro. Am. Rev. Respir. Dis. **127:**301–305.
11. **Contreras, M., J. M. Thiberge, M. A. Mandrand-Berthelot, and A. Labigne.** 2003. Characterization of the roles of NikR, a nickel-responsive pleiotropic autoregulator of *Helicobacter pylori*. Mol. Microbiol. **49:**947–963.
12. **Cooksley, C., P. J. Jenks, A. Green, A. Cockayne, R. P. Logan, and K. R. Hardie.** 2003. NapA protects *Helicobacter pylori* from oxidative stress damage, and its production is influenced by the ferric uptake regulator. J. Med. Microbiol. **52:**461–469.
13. **Douglas, T., D. S. Daniel, B. K. Parida, C. Jagannath, and S. Dhandayuthapani.** 2004. Methionine sulfoxide reductase A (MsrA) deficiency affects the survival of *Mycobacterium smegmatis* within macrophages. J. Bacteriol. **186:**3590–3598.
14. **Ernst, F. D., G. Homuth, J. Stoof, U. Mader, B. Waidner, E. J. Kuipers, M. Kist, J. G. Kusters, S. Bereswill, and A. H. van Vliet.** 2005. Iron-responsive regulation of the *Helicobacter pylori* iron-cofactored superoxide dismutase SodB is mediated by Fur. J. Bacteriol. **187:**3687–3692.
15. **Ezraty, B., L. Aussel, and F. Barras.** 2005. Methionine sulfoxide reductases in prokaryotes. Biochim. Biophys. Acta **1703:**221–229.
16. **Ezraty, B., R. Grimaud, M. El Hassouni, D. Moinier, and F. Barras.** 2004. Methionine sulfoxide reductases protect Ffh from oxidative damages in *Escherichia coli*. EMBO J. **23:**1868–1877.
17. **Garrett, R. H., and C. M. Grisham.** 1995. Biochemistry. Saunders College Publishing, Philadelphia, Pa.
18. **Gonzalez Porque, P., A. Baldesten, and P. Reichard.** 1970. The involvement of the thioredoxin system in the reduction of methionine sulfoxide and sulfate. J. Biol. Chem. **245:**2371–2374.

19. **Grimaud, R., B. Ezraty, J. K. Mitchell, D. Lafitte, C. Briand, P. J. Derrick, and F. Barras.** 2001. Repair of oxidized proteins. Identification of a new methionine sulfoxide reductase. J. Biol. Chem. **276:**48915–48920.
20. **Hassouni, M. E., J. P. Chambost, D. Expert, F. Van Gijsegem, and F. Barras.** 1999. The minimal gene set member *msrA*, encoding peptide methionine sulfoxide reductase, is a virulence determinant of the plant pathogen *Erwinia chrysanthemi*. Proc. Natl. Acad. Sci. USA **96:**887–892.
21. **Kauffmann, B., A. Aubry, and F. Favier.** 2005. The three-dimensional structures of peptide methionine sulfoxide reductases: current knowledge and open questions. Biochim. Biophys. Acta **1703:**249–260.
22. **Khor, H. K., M. T. Fisher, and C. Schoneich.** 2004. Potential role of methionine sulfoxide in the inactivation of the chaperone GroEL by hypochlorous acid (HOCl) and peroxynitrite (ONOO−). J. Biol. Chem. **279:**19486–19493.
23. **Levine, R. L., B. S. Berlett, J. Moskovitz, L. Mosoni, and E. R. Stadtman.** 1999. Methionine residues may protect proteins from critical oxidative damage. Mech. Ageing Dev. **107:**323–332.
24. **Lowther, W. T., H. Weissbach, F. Etienne, N. Brot, and B. W. Matthews.** 2002. The mirrored methionine sulfoxide reductases of *Neisseria gonorrhoeae pilB*. Nat. Struct. Biol. **9:**348–352.
25. **Mehta, N., J. W. Olson, and R. J. Maier.** 2003. Characterization of *Helicobacter pylori* nickel metabolism accessory proteins needed for maturation of both urease and hydrogenase. J. Bacteriol. **185:**726–734.
26. **Merrell, D. S., L. J. Thompson, C. C. Kim, H. Mitchell, L. S. Tompkins, A. Lee, and S. Falkow.** 2003. Growth phase-dependent response of *Helicobacter pylori* to iron starvation. Infect. Immun. **71:**6510–6525.
27. **Moskovitz, J.** 2005. Methionine sulfoxide reductases: ubiquitous enzymes involved in antioxidant defense, protein regulation, and prevention of aging-associated diseases. Biochim. Biophys. Acta **1703:**213–219.
28. **Moskovitz, J., J. M. Poston, B. S. Berlett, N. J. Nosworthy, R. Szczepanowski, and E. R. Stadtman.** 2000. Identification and characterization of a putative active site for peptide methionine sulfoxide reductase (MsrA) and its substrate stereospecificity. J. Biol. Chem. **275:**14167–14172.
29. **Moskovitz, J., M. A. Rahman, J. Strassman, S. O. Yancey, S. R. Kushner, N. Brot, and H. Weissbach.** 1995. *Escherichia coli* peptide methionine sulfoxide reductase gene: regulation of expression and role in protecting against oxidative damage. J. Bacteriol. **177:**502–507.
30. **Olczak, A. A., J. W. Olson, and R. J. Maier.** 2002. Oxidative-stress resistance mutants of *Helicobacter pylori*. J. Bacteriol. **184:**3186–3193.
31. **Olson, J. W., and R. J. Maier.** 2002. Molecular hydrogen as an energy source for *Helicobacter pylori*. Science **298:**1788–1790.
32. **Rain, J. C., L. Selig, H. De Reuse, V. Battaglia, C. Reverdy, S. Simon, G. Lenzen, F. Petel, J. Wojcik, V. Schachter, Y. Chemama, A. Labigne, and P. Legrain.** 2001. The protein-protein interaction map of *Helicobacter pylori*. Nature **409:**211–215.
33. **Rodrigo, M. J., J. Moskovitz, F. Salamini, and D. Bartels.** 2002. Reverse genetic approaches in plants and yeast suggest a role for novel, evolutionarily conserved, selenoprotein-related genes in oxidative stress defense. Mol. Genet. Genomics **267:**613–621.
34. **Singh, V. K., and J. Moskovitz.** 2003. Multiple methionine sulfoxide reductase genes in *Staphylococcus aureus*: expression of activity and roles in tolerance of oxidative stress. Microbiology **149:**2739–2747.
35. **Singh, V. K., J. Moskovitz, B. J. Wilkinson, and R. K. Jayaswal.** 2001. Molecular characterization of a chromosomal locus in *Staphylococcus aureus* that contributes to oxidative defence and is highly induced by the cell-wall-active antibiotic oxacillin. Microbiology **147:**3037–3045.
36. **Skaar, E. P., D. M. Tobiason, J. Quick, R. C. Judd, H. Weissbach, F. Etienne, N. Brot, and H. S. Seifert.** 2002. The outer membrane localization of the *Neisseria gonorrhoeae* MsrA/B is involved in survival against reactive oxygen species. Proc. Natl. Acad. Sci. USA **99:**10108–10113.
37. **Stadtman, E. R., and R. L. Levine.** 2000. Protein oxidation. Ann. N. Y. Acad. Sci. **899:**191–208.
38. **Stadtman, E. R., J. Moskovitz, and R. L. Levine.** 2003. Oxidation of methionine residues of proteins: biological consequences. Antioxid. Redox Signal **5:**577–582.
39. **Tete-Favier, F., D. Cobessi, S. Boschi-Muller, S. Azza, G. Branlant, and A. Aubry.** 2000. Crystal structure of the *Escherichia coli* peptide methionine sulphoxide reductase at 1.9 Å resolution. Struct. Fold Des. **8:**1167–1178.
40. **Tete-Favier, F., D. Cobessi, G. A. Leonard, S. Azza, F. Talfournier, S. Boschi-Muller, G. Branlant, and A. Aubry.** 2000. Crystallization and preliminary X-ray diffraction studies of the peptide methionine sulfoxide reductase from *Escherichia coli*. Acta. Crystallogr. D **56:**1194–1197.
41. **Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzegerald, N. Lee, M. D. Adams, J. C. Venter, et al.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539–547.
42. **Wang, G., R. C. Conover, S. Benoit, A. A. Olczak, J. W. Olson, M. K. Johnson, and R. J. Maier.** 2004. Role of a bacterial organic hydroperoxide detoxification system in preventing catalase inactivation. J. Biol. Chem. **279:**51908–51914.
43. **Wang, G., and R. J. Maier.** 2004. An NADPH quinone reductase of *Helicobacter pylori* plays an important role in oxidative stress resistance and host colonization. Infect. Immun. **72:**1391–1396.