THE ROLE OF METHIONINE SULFOXIDE REDUCTASE IN *HELICOBACTER PYLORI*

by

PRAVEEN ALAMURI

(Under the Direction of Robert J Maier)

ABSTRACT

Msr catalyzes the reduction of oxidized methionine residues and is an important antioxidant enzyme in many bacterial pathogens as well as in humans. An *H. pylori msr* mutant could not reduce oxidized methionine substrates resulting in an oxidative stress sensitive phenotype. Under oxidative stress conditions the mutant had higher levels of oxidized proteins than the wild-type. An *msr* strain could not colonize the mouse stomach for the longer time periods tested (2, 3 and 4 weeks). A complemented *msr* strain had stress resistance properties and host colonization ability similar to the wild-type, associating the earlier phenotype to *msr* inactivation. The gene was also up-regulated in cells under peroxide stress or iron starvation conditions. Using a co-immunoprecipitation (CIP) approach I identified catalase, site specific recombinase, GroEL, and the known Msr reductant thioredoxin-1 as putative Msr interacting proteins in *H. pylori*. Under oxidative stress conditions the *msr* strain showed approximately 50 % of the wild-type catalase specific activity, indicating an Msr-dependent repair of oxidized catalase. Pure Msr only reduced the R-epimer of methionine sulfoxide, with a $K_{m(app.)}$ of 4.1 mM for the substrate.

MutS is a part of the DNA mismatch repair system in *E. coli*. An *H. pylori mutS* strain was sensitive to oxidative stress and had reduced colonization ability. Under stress conditions the mutant contained a greater amount of 8-OG in its DNA than did the wild-type. MutS bound to the Holliday junction DNA and to that containing the 8-oxo-guanine nucleotide with higher efficiency than MutS binding to DNA mismatch bases; this indicates an additional role for MutS as an enzyme involved in repairing oxidatively damaged DNA.

The [Fe-S] cluster synthesis proteins NifS/NifU are essential in *H. pylori*. The *nifS/nifU* operon was maximally expressed when the bacterium is in high oxygen or in iron supplemented conditions. Such expression was not observed in an isogenic *fur* mutant, suggesting a Fur dependent activation of the gene. Unlike for regulations of other genes, only iron-loaded Fur recognized the *nifS/nifU* promoter region. Similarly, the region recognized by Fur was farther upstream than the conventional Fur binding regions associated with the promoters of other Fur regulated genes.

INDEX WORDS: *H. pylori*, oxidative stress, Msr, NifS, MutS

THE ROLE OF METHIONINE SULFOXIDE REDUCTSAE IN *HELICOBACTER PYLORI*

by

PRAVEEN ALAMURI

B.S., OSMANIA UNIVERSITY, INDIA, 1996

M.S., RAMANAND TEERTH MARATHWADA UNIVERSITY, INDIA, 1998

M.S., GEORGIA STATE UNIVERSITY, 2001

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial
Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2006

THE ROLE OF METHIONINE SULFOXIDE REDUCTASE IN *HELICOBACTER PYLORI*

by

PRAVEEN ALAMURI

Major Professor:	Robert J Maier
Committee:	Eric Stabb Timothy Hoover William Whitman

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May, 2006

DEDICATION

This dissertation is dedicated to my parents, whose unconditional love, sacrifices and blessings helped me reach this stage in my career. You will have my love and deepest respect forever.

I affectionately dedicate this work to my wife Smitha, her love and respect was very supportive during arduous times.

ACKNOWLEDGEMENTS

My sincere thanks to Dr. Robert Maier for his encouragement, support and his faith in my abilities. His passion for research was a great inspiration and helped to bring out the best in me. I thank my committee Dr. Eric Stabb, Dr. Timothy Hoover, Dr. William Whitman for their support and suggestions. Thanks to Stephane Benoit for his council and humor. Thanks to Ge Wang for the opportunity to collaborate with him. Thanks to Mrs. Maier and Adriana for being such helpful colleagues. Thanks to Mike for his timely suggestions and the entire Maier lab crew for making it such a jovial and fun filled five years of learning experience. I will miss you all.

Thanks to my brother Pradeep, for encouraging me to pursue my doctoral degree here in the US and for being there in India for our parents and my friend Rajesh for his initial encouragement and support in making important decisions. Thanks are also due to Mr. Nitin Patel and his family for their selfless help; my experience in this country would not be the same without their help in the initial days. And to all the friends I made in Atlanta and here in Athens for their help.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS....v

LIST OF TABLES.... ix

LIST OF FIGURES x

CHAPTER

1 **Introduction and Literature Review**....1

Overview of microbiological characteristics....2

Pathogenesis of *H. pylori* infection 7

Oxidative stress 16

Oxidative stress and DNA damage 22

Protein oxidation 25

Oxidative stress resistance in *H. pylori*....27

Methionine oxidation and the Msr system 33

Scope of this study 50

References 52

2 **Methionine sulfoxide reductase is an important antioxidant enzyme in the gastric pathogen *Helicobacter pylori***....68

Abstract 69

Introduction 70

Materials and methods....72

Results 79

Discussion 91

Acknowledgements 93

References 94

3 **Methionine Sulfoxide Reductase in *Helicobacter pylori*: Interaction with methionine-rich proteins and stress-induced expression**..................................98

Abstract 99

Introduction 99

Materials and methods..101

Results and discussion..113

Acknowledgements 134

References 135

4 **The *Helicobacter pylori* MutS protein has a major role in the repair of oxidative DNA damage**…………………………………………………..….. 139

Abstract...140

Introduction 140

Materials and methods..142

Results 147

Discussion 161

Acknowledgements 165

References 166

5 **Iron sulfur cluster synthesis proteins in *H. pylori*** 171

References 175

6 **Regulation of the *Helicobacter pylori* Fe-S cluster synthesis protein NifS by iron, oxidative stress conditions and by Fur** 177

Abstract 178

Results and discussion 178

Acknowledgements 192

References 193

7 **Dissertation summary and future directions** 196

Physiological significance of Msr in *H. pylori* 197

Structure function relationship 199

Regulation of Msr 201

New role for MutS in *H. pylori* 202

Fur mediated activation of *nifS* 202

References 203

LIST OF TABLES

Page

Table 1.1: DNA repair enzymes in *H. pylori*. 32

Table 1.2: Permissive conditions of Msr induction. 43

Table 2.1: Primers used for inactivation, complementation and over expression of *msr*. 74

Table 2.2: Membrane-associated Msr activity. 84

Table 2.3: Growth of HP 26695, *msr*, *msrB* and merodiploid strains under various stress conditions. 85

Table 3.1. List of primers used in chapter 3. 103

Table 3.2.List of plasmids and strains used in chapter 3. 104

Table 3.3. Proteins identified by MALDI-TOF/MS. 116

Table 4.1. Mutation rates in *H. pylori* parental and *mutS* strains 151

Table 4.2.Mutation spectra in *H. pylori* parental and *mutS* strains. 153

Table 5.1. *H. pylori* proteins that are predicted to contain Fe-S clusters. 173

LIST OF FIGURES

Page

Figure 1.1: Schematic representation of *H. pylori* Msr 34

Figure 1.2: ClustalW alignment of MsrA domain 35

Figure 1.3: Clustal W alignment of MsrAB from various bacteria. 36

Figure 1.4: Oxidation of methionine..39

Figure 1.5: Genetic organization of *msrA* and *msrB* in eubacteria 41

Figure 1.6: Reaction mechanism of Msr catalyzed Methionine sulfoxide reduction 45

Figure 2.1A: Immunoblot of whole cell extracts from HP26695, *msrB* and *msr* 82

Figure 2.1B: Detection of Msr in cytoplasmic and membrane fractions......................................83

Figure 2.2: Stress tolerance (survival) of non-growing cells..87

Figure 2.3: Oxyblot of whole cell extracts of HP 26695 and *msr* strains.....................................90

Figure 3.1A: Sypro-Ruby stained gel showing Msr-interacting proteins precipitated using CIP procedure...115

Figure 3.1B: Gel-based analysis of purity of potential Msr-interacting proteins 119

Figure 3.2A: Immunoblot using anti-Msr antibody 120

Figure 3.2B: Immunoblot using anti-KatA antibody 121

Figure 3.2C: Immunoblot using anti-GroEL antibody 122

Figure 3.3A: Relative catalase expression in SS1 and *msr* mutant...124

Figure 3.3B: Catalase activities in SS1 and *msr* 124

Figure 3.4: Homology based structure model of Msr in *H. pylori* 127

Figure 3.5: *xylE* activities to monitor *msr* expression in *H. pylori* 129

Figure 3.6A: Growth phase dependent expression of Msr 132

Figure 3.6B: Growth phase dependent Msr activity 133

Figure 4.1: Sensitivity of *H. pylori mutS* mutant to oxidative stress 148

Figure 4.2: Stress survival of non growing SS1 and *mutS* strain 149

Figure 4.3: DNA binding ability of MutS...155

Figure 4.4: Detection of 8-oxoG by immunofluorescent staining 159

Figure 4.5: Mouse colonization assays of *H. pylori* SS1 and its isogenic mutant of *mutS*::CAT 160

Figure 6.1: Primer extension analysis of *nifS* transcript 180

Figure 6.2: XylE activities as a measure of *nifS* induction under various stress conditions........182

Figure 6.3: Purification of *H. pylori* Fur 184

Figure 6.4: Electrophoresis mobility shift assay using Fur and P_{nifS} 187

Figure 6.5: Competition assay to determine Fur-P_{nifS} affinity 191

Figure 7.1: Growth of WT and *msr* strains in oxygen 198

Figure 7.2: Kinetics of Msr activity 200

Chapter 1

Introduction and Literature Review

Drs Barry Marshall and Robin Warren of Australia received the Nobel Prize in Medicine in 2005 for their elegant work showing the association of the gastric pathogenic bacterium *Helicobacter pylori* with peptic and duodenal ulcers. Prior to this scientific and medical breakthrough in 1982, an imbalance between inherent gastric factors such as hydrochloric acid and pepsin secreted by the gastric mucosa, and several exogenous factors such as smoking, alcohol use or ingestion of non-steroidal anti-inflammatory drugs were believed to be responsible for peptic ulcers (66). When studies on the pathology of *H. pylori* were just starting, Warren correlated the presence of these helical bacteria with specific types of inflammation, chronic gastritis, and active chronic gastritis. He showed that large numbers of the curved bacterium were always associated with the gastric antrum of patients suffering from active gastritis (107). Based on the region of the stomach where these bacteria were found, the authors named it “pyloric campylobacter”. This bacterium was originally classified as *Campylobacter pyloridis*, which was later changed to *Campylobacter pylori* (106). The differences in its 16s rRNA sequences and the membrane fatty acid content justified the designation of a new genus *Helicobacter* and the gastric isolate was renamed *Helicobacter pylori* in 1989 (64, 169). *H. pylori* is now widely recognized as a causative agent of chronic inflammation of almost all stomach and peptic ulcers. This infection with *H. pylori* is one of the major risk-factors for gastric neoplasms, including lymphomas and gastric cancer (47). In 1994, the WHO classified *H. pylori* as a type-I carcinogen for humans.

OVERVIEW OF MICROBIOLOGICAL CHARACTERISTICS

Morphology

H. pylori is a gram-type negative, spiral, microaerophilic bacterium. It belongs to the epsilon subdivision of Proteobacteria, along with *Wolinella succinogenes*, and *Campylobacter* species. They demonstrate bluntly rounded ends in gastric biopsy specimens and are 2.5 to 5 µm long and 0.5 to 1.0 µm wide (43). When cultured in laboratory conditions, the bacteria are predominantly rods and become coccoid upon prolonged culture whether on solid or liquid medium. Coccoid forms appear as U-shaped bacilli with the two ends joined by a membranous structure (30). These forms are metabolically active and resistant to unfavorable environmental conditions like nutrient starvation or oxidative stress. However, they cannot be cultured in vitro.

A tuft of four to six unipolar sheathed flagella are essential for the motility of this bacterium. Each flagellum ends in a terminal bulb which is an extension of the flagellar sheath. The flagellar sheath is continuous with the outer membrane and is composed of phospholipids and lipopolysaccharides. The sheath protects the flagellum from gastric acidity that the bacterium frequently encounters (62).

Cell wall and Lipopolysaccharide

The cell surface of *H. pylori* is hydrophobic and negatively charged. The organization of its cell wall is very similar to that of other Gram-negative bacteria but certain differences do exist. For example, it contains a higher proportion of muropeptides, but with shorter glycan chain lengths than in *E. coli*. The low peptide cross-linking and short glycan chain length may result in peptidoglycan (PPG) that is weakly held together and perhaps aids the bacteria’s ability to alter morphology between spiral and coccoid forms.

H. pylori contains all the proteins required to synthesize the precursors for PPG assembly. It also has three high molecular weight and one low molecular weight penicillin binding proteins (PBPs) that are involved in the synthesis and modification of PPG. The high molecular weight PBPs have transglycosylase activities while the low molecular weight PBP acts as a carboxypeptidase and/or as an endopeptidase (91). However, based on the genome analysis it is difficult to predict whether murein turnover in *H. pylori* is similar to that observed in *E. coli* (40, 72)

Lipopolysaccharide (LPS) is an important structural, recognition, and viurlence component in Gram negative bacteria. *H. pylori* produces two types of LPS (rough and smooth) depending on the age of the culture or the growth media used. The smooth (S-LPS) is a high-molecular weight form and is produced by fresh isolates of *H. pylori*. It contains an O side chain, a core oligosaccharide and lipid A. Strains that have been repeatedly sub-cultured on solid media produce a low molecular weight, rough form (R-LPS), that lacks the O side chain (124). The core oligosaccharide contains a number of saccharides, including fucose, D-mannose, D-glucose and D-galactose. Structures for the lipid A components of both S-LPS and R-LPS have been elucidated. R-LPS has a larger proportion of lipid A than S-LPS, and both forms have lipid A that is under-phosphorylated and under-acylated when compared with that from enterobacterial lipid A (131). This is thought to be the reason for the low endotoxic property of *H. pylori* LPS.

Genome Overview

Genome sequences of two *H. pylori* strains are available. The first reported sequence was for *H. pylori* strain 26695 that was isolated from gastric ulcer patients before 1987. It has a genome size of 1.67 Mbp, with 1,590 coding sequences. The coding sequences represent about 91 % of the *H. pylori* chromosome (175). The second strain, J99, has a slightly smaller genome size of

1.64 Mbp with 1,495 coding sequences (~90.8%) (4), and the average G+C content of 39 % in both strains. The genome sizes of both strains are about one-third that of *E. coli* or *H. influenzae* genomes.

Out of the 1552 coding sequences in 26695, 1185 have orthologues found in other bacteria, 367 are *H. pylori* specific, and 69 are strain specific. Considerable variation exists between the two sequenced *H. pylori* genomes. Although both share up to 97% sequence homology and the variability occurs at the nucleotide level rather than at the amino acid level. The difference in the third base of the triplet codon contributes to this variation, though the primary amino acid sequence of both strains is highly conserved. *H. pylori* strains are naturally competent for DNA uptake and have a high recombination rate. These features are thought to contribute to the diversity amongst various strains (40, 101).

Metabolism and Respiration

The genome sequence and several independent physiological studies have provided evidence that *H. pylori* cannot catabolize carbohydrates. Glucose is the only sugar that can be utilized by both oxidative and fermentative pathways, but the bacterium is thought to be an obligate microaerophile. The intracellular phosphorylation of glucose is catalyzed by glucokinase that is encoded by HP 1103 that shares 59.5 % similarity to the *glk* gene of *E. coli* (101, 112).

The constitutive expression of genes encoding the enzymes of Entner-Doudoroff pathway suggests that it is a preferred alternative to glycolysis in this bacterium. In addition, Mendz and Hazell demonstrated the presence of all the enzymes for oxidative and non-oxidative steps of the pentose-phosphate pathway in *H. pylori*, including the ones for the biosynthesis of C_5 phosphorylated carbohydrates essential for nucleotide synthesis. Both pathways together provide NADPH as the major electron donor for metabolism in *H. pylori* (113).

The end product of glycolysis is pyruvate which under anaerobic conditions in *H. pylori* was shown to be metabolized to lactate, ethanol, and acetate and to acetate alone under aerobic conditions. In addition to pyruvate, lactate and succinate are also oxidized readily by this bacterium (112-115). From the genome sequences of both strains of *H. pylori* it is predicted that the primary dehydrogenases such as lactate dehydrogenase, glycerol-3-phosphate dehydrogenase, proline dehydrogenase, glycolate oxidase and amino-acid dehydrogenase are responsible for feeding electrons from the above substrates into the quinone pool. The involvement of malate: quinone oxidase was shown experimentally which suggest malate is also an electron donor (85). Genome sequences and analysis of lipid extracts of *H. pylori* revealed that this bacterium uses ubiquinones as the major quinone pool (102). It is possible that *H. pylori* also incorporate menaquinone from the host. From the quinone pool, electrons are passed to the bc_1 and 2Fe-2S protein complex to cytochrome c. Cytochrome c is a heme-protein (either periplasmic or membrane-anchored) and transfers electrons to oxygen via a type cbb_3 terminal oxidase (25). This oxidase has a high O_2 affinity.

In addition to oxygen, fumarate can also be used as a terminal electron acceptor by this bacterium. The reduction of fumarate is catalyzed by fumarate reductase and is an important energy transduction pathway in *H. pylori*. Apart from fumarate, there is no direct evidence that *H. pylori* is able to use alternative electron acceptors other than oxygen (61) and even anaerobic fumarate-dependent growth has not been reported.

H. pylori possesses a membrane-bound “uptake-type” hydrogenase involved in low potential electron generation and subsequent respiration that could contribute to energy generation in the cells provided with hydrogen (100). Respiration results in the formation of ATP from generation of a transmembrane proton-motive force. ATP synthase converts the proton electrochemical gradient across bacterial cytoplasmic membrane into ATP. The genes for the membrane-embedded F_0 proton-channeling complex and the catalytic F_1 are present in *H. pylori*. *H. pylori* maintains a constant proton motive force (PMF) at external pH values between 3 and 7. This PMF drives not only ATP synthesis, but also flagellar rotation and solute uptake (101, 175).

Nutritional Requirements

H. pylori requires a complex medium for growth in general laboratory conditions. Commonly used is a basal medium (either liquid or solid) with some form of supplementation such as whole blood, heme, serum, charcoal, cornstarch, or egg yolk emulsion. In addition, cyclodextrins support excellent growth of *H. pylori* and also aid in detoxifying the medium (67, 76, 103).

A defined medium first developed by Reynolds and Penn has facilitated a number of research studies over the last decade. This medium contains various amino acids, and vitamins (especially, B_{12} and thiamine) that aid the growth of this fastidious organism. *H. pylori* requires the amino acids arginine, histidine, leucine, isoleucine, valine, methionine and phenylalanine, which is consistent with the information from the genome sequence. It contains genes required to synthesize cofactors and vitamins and also has the ability to synthesize and hydrolyze polyphosphate for the storage and use of phosphorus (150).

H. pylori lacks the ability to synthesize purines from serine, glycine or formate. It does have homologs to genes required for purine salvage. In addition, genes required for de novo synthesis of UTP and CTP are evident from the genome sequence (40, 175).

Iron and nitrogen are important metal cofactors for the metabolism of *H. pylori*. This bacterium can incorporate nitrogen into amino acids from urea via the production of ammonia. *H. pylori* however lacks siderophores (small iron-chelating molecules). Experimental evidence suggests that this bacterium possesses a lactoferrin receptor, which would allow it to sequester iron directly (35). The results from *H. pylor*i sequence analysis indicate the presence of a system for iron uptake, which is analogous to ferric citrate *(fec*) uptake system of *E. coli*. Although genes similar to the *E. coli fec* locus have been identified, neither the sensor FecR nor regulatory FecI are observed based on the genome sequence. Similarly, the FecB dicitrate-binding and FecC transport proteins are absent (79, 175). The genes encoding the Fe-S cluster synthesis proteins NifS and NifU are present in *H. pylori*. These proteins aid in the synthesis of [2Fe-2S] or [4Fe-4S] clusters that are cofactors for enzymes such as aconitase, hydrogenase, MutY and components of electron transport complexes. Hence, the *nifS* and *nifU* genes are thought to be essential and mutants in these genes could not be obtained (141).

Genes involved in iron uptake, iron metabolism and iron storage are regulated by a bacterial ferric uptake regulator (Fur) homolog in *H. pylori* (60). Fur acts as a transcriptional repressor under iron deficient conditions. Fur was also shown to regulate genes involved in many different metabolic activities including the genes that encode antioxidant enzymes in this bacterium. Review of NifS/NifU related literature and experimental evidence of their regulation by Fur is given in Chapter 5.

PATHOGENESIS OF *H. PYLORI* INFECTION

Helicobacter pylori colonizes the human gastric mucosa at its interface with the gastric epithelial cells and long-term infection causes severe damage to the gastric tissue and hence the manifestation of *H. pylori* related diseases. The following section discusses the virulence factors in this pathogen, those factors causing gastric cell injury and conditions in host that contribute to ulcer and cancer risk.

Virulence factors in *H. pylori*

H. pylori cell components that either cause injury to the host or that combat host defense mechanisms and thus indirectly help in its persistence constitute the virulence factors. The well characterized factors are discussed below.

(1) Motility

Soon after *H. pylori* reaches the gastrointestinal tract, it encounters the harsh acidic conditions (pH ~2) in the lumen. This helical bacterium possesses a tuft of 4-6 unipolar flagella each of which is 30 µm long and 2.5 nm thick (62). They enable the bacterium to move in a cock-screw fashion from the lumen to the more favorable pH environment of the mucosa (32). This flagella-based motility is now known both as a colonization and a virulence factor since non-motile or non-flagellated strains could not colonize piglets or mice. The ultrastructure and genetics of flagella in *H. pylori* are very well studied. Each flagellum is made of a basal body, a hook, and a flagellar filament with a thick bulb at its tip. The filament protein is encoded by two genes; the major flagellin *flaA* (FlaA 56 kDa) and minor flagellin *flaB* (FlaB 57 kDa); they share 58% identity at the amino acid level (89, 95). The hook protein FlgE *(flgE)* connects the basal body to the flagellar filament (134). All these genes are under the control of the flagellar regulator, FlbA. Inactivation of *flbA* resulted in a strain that could not produce FlaA or FlaB proteins and had reduced levels of *flgE* mRNA, resulting in non-motile cells (153). Motility within the host mucus is essential for pathogens such as *H. pylori*, not only to escape the harsh (acid) environment but also to avoid attachment to host cells that may result in recognition by the immune response.

(2) Urease

H. pylori passes through the highly acidic lumen (pH 2-4) before reaching the slimy mucous and gastric epithelium where a neutral pH is maintained. The acidic lumen is one of the major barriers to *H. pylori* for successful colonization. This bacterium has a highly expressed Urease enzyme that degrades the available urea (5mM) in the lumen, producing a microenvironment of ammonia to protect itself from the acidity in the lumen. The 550-kDa enzyme urease catalyzes the hydrolysis of urea to produce ammonia and carbonate (46). In several animal models, urease negative strains could not colonize the stomachs at all, hence urease is regarded as a major virulence factor (17).

The genes encoding *H. pylori* urease and urease accessory proteins include *ureABIEFGH* (28, 92). With the exception of *ureI*, all the genes share homology with urease genes from bacteria such as *Klebsiella aerogenes* and *Ureaplasma urealyticum* (121, 130). The genes *ureA* and *ureB* encode the two structural units of the enzyme, and nickel ions are present in the active site of the mature enzyme. The accessory genes *ureI*, *ureE*, *ureF*, *ureG* and *ureH*, are essential for synthesis of a catalytically active urease (28, 92). These genes encode proteins that interact with the enzyme and aid in the delivery of nickel ions to the active site (120, 121). Urease activity is lost from whole cells at a pH below 5, whereas the cells remain intact, which raises questions about the enzyme's localization (120, 121). Fractionation studies have demonstrated that significant portions of urease are present both in the cytoplasm and associated with the outer membrane. Urease is also believed to be released as a result of autolysis of a fraction of individual bacterial cells and this may benefit the rest of the surviving population (45). This extracellular presence of urease makes it a good candidate for vaccine development.

(3) Hydrogenase

Hydrogen production due to colonic fermentations by the normal flora in humans serves as a good energy source for *H. pylori*. Hydrogenase (hp0630 gene product) (175) has a high affinity for H_2 ($K_m = 1.8\mu M$) and with the average hydrogen concentration in stomach around 43 µM, it is proposed to function as an energy substrate for *H. pylori*. Isogenic *hyd* mutants also demonstrated reduced ability to colonize mouse stomachs compared to the wild-type strains, supporting the conclusion that the lack of the ability to utilize H_2 affects the persistence of *H. pylori* in the host (142). Like urease, hydrogenase is also a nickel enzyme and some of the nickel sequestering proteins are common to maturation of both Ni-enzyme sinks.

(4) Adhesion and immune evasion

Bacterial cell surface structures define the recognition process by which bacterial cells interact inter-species, and with other species of bacteria, and to the host cell surfaces. Apparently to avoid being swept away by peristalsis or by the continuous turnover of mucus and epithelial cells, *H. pylori* adheres to the gastric epithelium via a number of adhesins that are described here (80, 81)

(A) Adhesins BabA and SabA: Once *H. pylori* reaches the stomach epithelial cells under the viscous mucous layer, it adheres to the gastric cells. BabA and SabA are two of the well studied adhesion proteins found on the surface of this bacterium. Boren et al first reported in 1993 that attachment of *H. pylori* to human gastric cells is dependent on the presence of a blood group antigen binding adhesin, BabA that binds to the fucosylated histo-blood group antigen Lewis^b. In vivo binding of *H. pylori* to the Lewis^b receptor was demonstrated using transgenic inbred mice expressing human Lewis^b antigen on grastric epithelia, showing attachment of BabA+ *H. pylori* and subsequent gastritis (18, 50)

A subset of *H. pylori* strains express an outer membrane protein **SabA** (hp0725). These are known for their ability to bind sialylated Lewis^x antigens expressed on the surface of immature cells of the gastric epithelium during chronic inflammation; hence they are called sialic acid-binding adhesins (SabA) (99). The binding of SabA to the sialylated neutrophil receptors was speculated to be one of the pivotal events in inducing an oxidative burst response in the host. This speculation was bolstered by a recent study that showed the inability of *sabA* mutants to activate neutrophils (179). This finding indicates SabA is a virulence factor and is likely significant in the more severe *H. pylori* pathogenesis symptoms.

(B) Hemagglutinins and other adhesins: The gene *hpaA* (hp 0410) encodes a hemagglutinin that binds to N-acetyl-neuraminidyl- (2, 3)- lactose and helps in the bacterial adherence to the human gastric cells. This adhesion is specific between the 25 kDa HpaA and the 3-linked sialic acid moiety on erythrocytes. This hemagglutination can be blocked with 3'-sialyl lactose and the glycoprotein fetuin (83). HpaA is synthesized in the cytoplasm and is exported by a dedicated protein export system (97). HpaA is shown to be essential for *H. pylori* colonization of the mouse stomach (24). Thus, these OMPs of *H. pylori* together constitute a repertoire of cell surface adhesins that help *H. pylori* overcome the physical factors in the stomach (such as peristaltic movement and mucous turnover) that could potentially eradicate the pathogen.

(C) Lewis Antigens and immune evasion: The O antigen of the S-LPS has extended chains with fucosylated and non-fucosylated N-acetyllactosamine units. Repeating units of O side chains mimic Type 2 Lewis blood group antigens (Le^x and Le^y) in structure (7). Since these blood group antigens are expressed in the gastric mucosa of normal individuals, this mimicry may camouflage the bacterium and thereby help in immune evasion and successful colonization of the host.

(5) CAG pathogenecity island

Strains of *H. pylori* can be grouped into two broad families referred as type I and type II based on the presence and expression of CagA antigen (cytotoxic associated antigen) and VacA (vacuolating toxin). The Cag pathogenicity island is a 40-kb genome segment that encodes 30 different genes and has 39-bp flanking DNA repeats (3). These genes have no known homologs in other *Helicobacter* species or in other bacteria, reflecting *H. pylori'*s specificity to the human gastric environment. The *cag* island also differs from the rest of the *H. pylori* genome in features such as the G+C content, flanking direct DNA repeats, insertion sequences and putative virulence genes that encode cytotoxic genes and apparatus of the type IV secretion system (TFSS) (135). The genes in this segment encode a 120-140 kDa highly immunogenic CagA protein that is injected into the mammalian cells by the TFSS (31). After translocation into the host cells, CagA is phosphorylated by the host Src family of kinases and induces rearrangements of the host cell cytoskeleton and affects spreading, migration, and adhesion of epithelial cells (123). This phenomenon can also be assessed in vitro by a change in epithelial cell morphology to the "humming bird" phenotype. CagA also induces a 20-fold increase in inflammatory IL-8 secretion by the epithelial cells and also promotes antiapoptotic pathways. This antiapoptotic pathway may aid bacterial persistence by slowing the turnover of the epithelial cells to which the bacteria are attached (16, 154). Thus, the *H. pylori* CagA protein interacts with major signal-transduction pathways of the epithelial cells to disrupt many normal host cell functions.

(6) Vacuolating Toxin (VacA)

In 1988 Leunk et al. first described a protein cytotoxin produced by *H. pylori* and secreted into the supernatants that could induce the formation of large cytoplasmic vacuoles in cultured eukaryotic cells (93). These vacuoles were seen to initiate in the perinuclear area and later fill the entire cytosol, leading to cell necrosis. This cytotoxin was identified as the gene product of hp0887 and was later purified as a single 95 kDa protein named VacA (33). VacA has a 33 amino acid N-terminus signal sequence, followed by a 37 kDa region rich in pleated β-sheet, a variable 57 kDa region that consists of a secreted cytotoxin region and a C-terminus outer membrane domain. The VacA toxin has several specific effects that are thought to contribute to *H. pylori* persistence in the gastric niche. Firstly, it forms pores in epithelial cell membranes, allowing exit of anions and urea, which is later hydrolyzed by *H. pylori* urease (82, 176). Secondly, it induces loosening of epithelial tight junctions, which essentially allows nutrients to cross the mucosal barrier (144). Recent studies have brought to light some immune suppression features of this protein: VacA blocks phagosome maturation in macrophages, selectively inhibits antigen presentation in T-cells, blocks T-cell proliferation and down-regulates the T-helper I cells. All this culminates in aiding survival and persistence of the pathogen in the hostile environment (14). Both *cagA* and *vacA* null mutants show colonization efficiency similar to the wild-type strains but the injury to the host cells in long colonization times is highly reduced.

(7) Antioxidant enzymes

The first line of defense by the human host is the peristaltic movement of the stomach cilia followed by chemical barriers like the acidic lumen, and slimy mucous layer. The factors described in earlier sections illustrate the ability of *H. pylori* to efficiently overcome these barriers and colonize the gastric tissue. However, the final response of the host is the inflammatory response orchestrated by the immune cells. Turnover of epithelial cells, phagocytosis, respiratory burst and generation of a highly toxic environment with reactive oxygen molecules constitute an inflammatory response. *H. pylori* has a well established antioxidant system comprising a battery of detoxifying enzymes such as Catalase, Superoxide dismutase, Alkylhydroxyperoxide reductase (and other peroxireductases) and the NADPH quinone oxidoreductase which together prevent macromolecular damage. A detailed illustration of their individual roles is discussed later.

Ulcer and cancer risk

H. pylori associated disease progression can be clearly grouped into various stages; it starts primarily with an infection, then gastritis followed by gastric ulcers that leads to a long-term and persistent infection; the end result may be gastric cancer. Gastric ulcers are normally preceded by corpus-predominant or pangastritis. This condition is associated with *H. pylori* colonization of the corpus and a low acid output. The impact of *H. pylori* on the gastric mucosa leads to accelerated epithelial cell exfoliation (shedding of cells), and compensatory cell proliferation by immune precursor cells. This impairs mucin and bicarbonate production, disturbing the integrity and barrier function of the gastric mucosa, thus making the human host more sensitive to acid and to further infection (12).

Several mechanisms were postulated to link *H. pylori* infection with gastric carcinogenesis. The above listed virulence and persistence factors in the pathogen play an important role in the development of the severe disease (143). Some observations of gastric abnormalities in *H. pylori*-infected individuals that link the bacterium to diseases are the following: 1) increased cellular proliferation and increased occurrence of apoptosis in gastric epithelial cells, 2) increased oxidative DNA damage and increased expression of inducible NO synthase in gastric tissue neutrophils and mononuclear cells, 3) increased ammonia production (urease activity) leading to gastric mucosal atrophy, 4) production of antigastric autoantibodies (indication of gastritis; the movement of immune cells to the gastric tissue), 5) increased production of N-nitroso compounds, 6) increased production of mutagenic free radicals, 7) decreased intragastric levels of the antioxidant ascorbic acid , 8) production of CagA and VacA which disturbs the integrity of epithelia, diffusion of nutrients across the cell membranes, suppression of Th-1 response, production of acid vacuoles (132). Some or all of these events occurring for over a decade induce clear disturbance in the human gastric cell configuration, mutagenic lesions in the DNA, and then can result in a malignant tumor formation termed gastric cancer (52).

Epidemiology

Our current state of knowledge indicates that acquisition of *H. pylori* seems to occur predominantly in childhood and that once acquired the infection usually persists life-long if untreated. The infection occurs worldwide but its extent in the population differs in various parts of the world, with a higher number of cases in the developing countries. Both males and females are infected and studies have shown that both sexes are infected at about the same rates with males perhaps being at higher risk (149).

H. pylori causes gastric inflammation, peptic ulcer disease, and has been linked to gastric cancer (13, 15, 53, 109, 145-147). The nature and severity of the disease depends on host characteristics, the specific bacterial strain and environmental factors. Possible sources of *H. pylori* infection are thought to be animals and water supplies (44, 119). Although the mode of transmission is not clearly understood, several routes have been described. The first, and perhaps least common, involves the introduction of endoscopes or tubes from an infected person to another individual. Endoscopists or gastroenterologists are thought to be more at risk for *H. pylori* infection (2, 43). *H. pylori* has been isolated from dental plaque and infected individuals often have *H. pylori* in their saliva, so oral-oral transmission may be a good possibility, but fecal-oral transmission also remains an unconfirmed possibility (119, 173, 174). *H. pylori* also converts to a coccoid form, which has been reported to survive for several years in river water and it has been suggested that water contaminated with these coccoid forms is a possible source of *H. pylori* infection (78).

Treatment

Therapy for an *H. pylori* infection involves ten days to two weeks of one or two effective antibiotics, such as amoxicillin, tetracycline, metronidazole, or clarithromycin (108). Other treatments include ranitidine bismuth citrate, bismuth subsalycilate, or a proton pump inhibitor (110). Acid suppression by a proton pump inhibitor in conjunction with the antibiotics helps alleviate ulcer-related symptoms (i.e., abdominal pain, nausea), helps heal gastric mucosal inflammation, and may enhance efficacy of the antibiotics against *H. pylori* at the gastric mucosal surface.

Currently, eight *H. pylori* treatment regimens are approved by the United States Food and Drug Administration (FDA). Eradication rates of the eight FDA-approved regimens range from 61% to 94% depending on the regimen used. Overall, triple therapy regimens (usually two antibiotics and a proton pump inhibitor) have shown better eradication rates than dual therapy. In addition, FDA studies have shown that long term (over 14 days) treatment results in better eradication rates (www.cdc.gov/ulcer/md.htm#treatment).

OXIDATIVE STRESS

Oxidative stress occurs due to an imbalance between prooxidants (superoxide: O_2^-, hydrogen peroxide H_2O_2, hydroxyl radicals $^{\cdot}OH$) and the antioxidant enzymes in a cell. Reactive oxygen species (ROS) are deleterious to any living cell as they oxidize the macromolecules (nucleic acids, proteins, lipids etc) and render them non- functional. Plants, animals, and human immune cells generate these toxic oxygen species to protect themselves from microbial invasion. For microorganisms, the source of ROS could either be from the metabolic activities within the organism, or imposed by the host, based on their primary inhabitation (pathogenic, comensal or free living) (168). Hence, the source of oxidative stress to microbes could broadly be categorized as endogenous and exogenous, with the latter likely of more concern to pathogenic organisms.

Endogenous source of ROS

Free ROS can frequently result from the normal biochemical processes occurring within a cell (68). These include respiration, metabolism, and exposure to oxygen, metals or UV radiation (42). Oxidative stress is an unavoidable by-product of the aerobic lifestyle, because O_2^- and H_2O_2 are formed whenever molecular oxygen chemically oxidizes electron carriers such as flavoproteins (11). Oxygen is a very efficient terminal electron acceptor during aerobic respiration but the leakage of electrons from the respiratory chain during the reduction of oxygen to a water molecule results in the generation of ROS. The reaction sequence shown below describes the events:

$$O_2 \xrightarrow{e^-} O_2^- \xrightarrow{e^-,\ 2H^+} H_2O_2 \xrightarrow{e^-,\ H^+} {}^{\cdot}OH \xrightarrow{e^-,\ H^+} H_2O$$

The four-electron reduction of O_2 to H_2O is sequentially catalyzed by a number of respiratory enzymes associated with the cell membrane. In the above reaction, redox agents carrying reducing equivalents from electron donors to the final acceptor oxygen may fail to reduce O_2 to H_2O completely. This results in the generation of superoxide ion, a highly reactive one electron reduction product (56, 57). Further, superoxide can contribute to the production of hydrogen peroxide resulting either from spontaneous reaction or a dismutation reaction catalyzed by superoxide dismutase (SOD) (59). Superoxide left unconverted may release iron from the iron-sulfur clusters. In this process, superoxide oxidizes the [4Fe-4S] center, resulting in release of iron. Reductants in the cell normally keep the free iron predominantly in the reduced form preventing it from reacting with H_2O_2 (58). However, the reaction of superoxide ion with free iron stimulates the Fenton reaction, a potential source of highly reactive hydroxyl radicals.

$$H^+ + \text{Fe (II)} + H_2O_2 \longrightarrow {}^{\cdot}OH + H_2O + \text{Fe (III)}. \ (178)$$

These toxic species can also be generated as by-products of various cellular reactions involving glucose oxidase, xanthine oxidase, and thiol groups and flavins.

In addition to direct toxicity mechanisms, superoxide, hydroxyl radicals and organic (t-butyl peroxide or cumene peroxide) and non-organic peroxides (H_2O_2) also pose a threat to bacterial macromolecules indirectly by rapidly reacting with the components of the cell. For example, many organic or unsaturated fatty acids like linoleic acid present in membrane lipids may be non-enzymatically converted to lipid hydroperoxides in the presence of ROS (105).

A number of microorganisms including *Enterococcus faecalis*, *Eschericia coli*, *Lactobacillus* spp., *Streptococcus pneumoniae*, also generate extracellular superoxide and hydrogen peroxide (186). These oxidants can exert a number of toxic effects on both host (in case of above pathogens) and on other microorganisms. For example, hydrogen peroxide producing Lactobacillus spp. present as part of the normal vaginal flora inhibits *Neisseria gonorrhoeae* in vitro (189). In contrast, O_2^- produced by *Mycoplasma pneumoniae* can indirectly accelerate DNA damage in the host by inactivating the host catalase enzyme (98). Similarly, H_2O_2 produced by *S. pneumoniae* was shown to induce tissue injury in the host during pneumococcal disease, as seen from mouse model studies.

Finally, ROS/RNS produced from human immune cells such as macrophages and various phagocytic immune cells (stimulated upon an inflammation at the site of infection), together form the exogenous source of toxic radicals against pathogenic microorganisms.

Exogenous source: Host immune cells and Oxidative burst

Reactive oxygen species play a central role in the pathophysiology of microbial infections. ROS and RNS (reactive nitrogen species) such as superoxide ions, hydrogen peroxide, hydroxyl radicals, and nitric oxide together constitute a series of strong microbiocidal compounds. These compounds exhibit a broad spectrum of biotoxicity and are part of the initial non-specific immune response of the host. Neutrophils are one of the professional phagocytes (in humans) that ingest bacteria into intracellular compartments called phagosomes where the pathogen is subjected to an arsenal of cytotoxic agents (117, 152). These oxidants are generated by four enzymes in the immune cells: NADPH oxidase, superoxide dismutase, nitric oxide synthase, and myeloperoxidase (referred to as eosinophil peroxidase in case of eosinophils).

1) NADPH oxidase (NoX)

Phagocytosing neutrophils undergo a burst of oxygen consumption (10-20 fold higher) caused by an NADPH oxidase complex that assembles at the phagosomal membrane during an inflammatory response. Electrons are transferred from cytoplasmic NADPH to oxygen on the phagosomal side of the membrane, generating superoxide along with a range of other reactive oxygen species (8). The reaction outlines the formation of superoxide ion.

$$2O_2 + NADPH \rightarrow 2O_2^{-} + NADPH^{+} + H^{+}$$

NoX is stimulated in phagocytes when they are activated by various stimuli, bacteria, and certain inflammatory polypeptides of the complement system. Upon activation, the enzyme complex is assembled into a single active oxidase form, involving the membrane associated subunits. The net result is the bombardment of the invading microbe with many toxic oxygen species including HOCl. Superoxide itself is a relatively mild oxidant with limited biologically toxic activity per-se. It inactivates iron/sulfur proteins. The antimicrobial importance of the NADPH-oxidase system of the host is exemplified in patients suffering from Chronic granulomatous disease (CGD), an inherited disorder in which phagocytes are unable to synthesize superoxide ions due to defects in the NADPH-oxidase complex (160).

2) Superoxide dismutase (SOD)

As the name suggests the enzyme catalyzes a dismutation reaction of the superoxide ion. It is called dismutation since the substrate O_2^- reacts with itself to form both an oxidized product – oxygen, and a reduced (product), the membrane permeable hydrogen peroxide (59).

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

This enzyme was first discovered by McCord and Fridovich. There are two types of SODs found in eukaryotes: a copper-zinc enzyme present in the cytoplasm and a manganese enzyme that is found in mitochondria. The Mn-SOD is inducible under conditions of stress (such as by tumor necrosis factor (TNF)), whereas the Cu-Zn-SOD is thought to remain constant in all conditions. This enzyme not only increases the rate of the spontaneous dismutation reaction, but it also enables the reaction to take place under low superoxide concentration. Hydrogen peroxide is a strong protein oxidizer that reacts slowly with reducing agents such as thiols, and is one of the primary sources of hydroxyl radical ($^{\cdot}OH$). It reacts with heme proteins and peroxidases to initiate several radical generating reactions and also lipid peroxidation. Hydroxyl radicals are generated by the interaction of H_2O_2 with iron leading to the previously outlined Fenton reaction (9). Hydroxyl radical is extremely reactive with most biological molecules, causing DNA modification, strand breaks, and inactivation of enzymes (58), (59).

3) Nitric oxide synthase (127)

NOS synthase catalyzes the production of nitric oxide (NO) from arginine, oxygen, and NADPH.

$$\text{Arginine} + \text{oxygen} + \text{NADPH} \rightarrow \text{NO} + \text{citrulline} + \text{NADP}^+$$

NOS is a highly complex enzyme containing FAD, FMN, heme and biopterin. There are two forms of NOS, the constitutive form that produces low amounts of NO and the inducible (iNOS) form that produces high NO upon stimulation of phagocytic cells by the invading microbes. Nitric oxide forms complexes with heme proteins and inactivates iron/sulfur centers. NO reacts rapidly with superoxide ion to form the highly oxidizing nitrogen species called peroxynitrite $ONOO^-$. Peroxinitrite is an unstable short lived, but very powerful oxidant with properties similar to hydroxyl radical. It hydroxylates and nitrates aromatic compounds, reacts rapidly with thiols, breaks down to nitrate and interacts with bicarbonate; the latter alters its reactivity. In addition peroxinitrite is a potent protein oxidizer, like hydrogen peroxide (77, 104).

4) Myeloperoxidase (MPO)

Phagocyte-derived H_2O_2 can also be converted to HOCl in the presence of chloride (or any halide ion) by the enzyme myeloperoxidase. HOCl is the most potent anti-bacterial oxidant known to be produced by neutrophils (69). Myeloperoxide is a heme enzyme containing as its prosthetic group the same heme that is found in hemoglobin (51).

$$Cl- + H_2O_2 \rightarrow OCl^- + H_2O$$

The hydrogen peroxide reactant here is the product of a dismutation reaction in the phagocyte. H_2O_2 and high concentrations of Cl^- in the stomach and body fluids together provide sufficient substrates for the catalysis of this reaction by activated phagocytes (60). Hypochlorous acid is membrane permeable and a strong oxidant of a wide range of biological compounds but it is more selective for cell targets than hydroxyl radical. Its preferred substrates are thiols and thioethers, it chlorinates phenols and unsaturated bonds, oxidizes iron centers, and cross links proteins (159). HOCl converts amines to chloramines that are milder and longer-lived oxidants than HOCl, and with more limited membrane permeability. Chloramines too react with thiols, thioethers, and iron centers.

MPO can also catalyze the reaction of $2O_2^-$ and HOCl to form ($^{\cdot}OH$) and is a significant source of ($^{\cdot}OH$). Thus, the series of reactions taken together make MPO an important enzyme in the phagosomal arsenal and hence, deficiency in MPO synthesis or activity in humans results in a documented delayed immune response to fungal or bacterial infections (69). Eosinophil peroxidase is an enzyme unique to eosinophils. Unlike MPO, eosinophil peroxidase cannot catalyze the oxidation of Cl^-; it uses Br^- and SCN^- as its substrates.

OXIDATIVE STRESS AND DNA DAMAGE

DNA damage is the result of extrinsic and intrinsic processes including ionizing radiation, UV light exposure and oxygen-derived free radicals that are consequences of various events/reactions as discussed in the above sections. The types of damage to DNA include base lesions, DNA sugar modifications, protein and DNA cross links, single-strand and double strand DNA breaks, all as a result of accumulating highly reactive free radicals. The intrinsic reactions that are likely contributors to ongoing DNA damage in vivo are oxidation, methylation, depurination and deamination (5, 156). Exposure of DNA to $^{\cdot}OH$ can lead to more than 20 different products including 8-hydroxy-2-deoxyguanosine (8-OG) that is an excellent marker to estimate the extent of DNA damage. Structural damage to the cellular DNA can be categorized into two broad groups; *DNA base damage* that results in the formation of 8-hydroxyadenine, 8-hydroxyguanine, 5-hydroxycytosine etc, and *DNA-sugar damage* leading to alterations of the sugar moiety. The products of the latter include 2, 5-dideoxypentose-4-ulose, 2, 3-dideoxypentose-4-ulose, etc (185).

Role of metals in oxidative DNA damage*:* Studies performed both on microbial and animal models have suggested a strong correlation between the presence of free iron or copper in the cell and increased oxidative DNA damage (166, 167). The significant ROS is hydroxyl radical that either oxidizes DNA or stimulates strand breakage. Superoxide releases [4Fe-4S] clusters from proteins in the cell, the free iron thus released is bound to the DNA and in the presence of hydrogen peroxide stimulates the Fenton reactions. In fact, a SOD mutant in *E. coli* contained much more oxidized DNA than the wild-type strain (87). A similar observation was made in *H. pylori* where cells in the stationary phase of growth were found to contain increased levels of free iron and these cells also showed increased DNA fragmentation than cells in the exponential phase of growth. In addition, *H. pylori* mutant strains that lack the key ROS detoxification enzymes such as SOD or peroxiredoxins show many fold higher 8-OG content than the wild-type strain (182). These examples directly correlate ROS (intrinsic or Fenton reaction mediated) and DNA damage.

Consequences of oxidative DNA damage: There are several different pathways leading from ROS mediated initial DNA base damage to subsequent mutation. The first is the chemical modification of DNA bases causing a change in their hydrogen bonding specificity, e.g.8-OG, thymine glycol and 2-hydroxy adenine. 8-OG has ambivalent base-pairing properties, being capable of pairing effectively with both C and A during DNA synthesis. Hence, it is an intrinsically highly mutagenic base analog, either as a free deoxynucleoside phosphate (8OGTP) or when integrated into the DNA. The incorporation of 8-OG into the DNA can occur in two different ways: (a) incorporation of 8-OGTP during DNA synthesis either opposite A or C; and (b) by direct oxidation of guanines in DNA. In addition 8-hydroxy adenine and pyrimidine fragmentation products can block replication in *E. coli* and were shown to be mutagenic (36, 37).

A second possible mechanism for mutagenesis of DNA is oxidative damage to polymerase-specific "hot-spots" which is likely a major contributor to DNA polymerase mediated mutagenesis. A third mechanism for an oxidatively-induced mutation is attributed to a conformational change in the DNA template that diminishes the accuracy of replication by DNA polymerases. The modified DNA is either repaired by the insertion/excision enzyme system or it is degraded by nucleases in the cell (38, 39, 185).

Enzymes involved in repair of damaged DNA: To combat the mutagenic consequences of 8-OG formation and to repair the base mismatch and strand breakage, organisms have developed a set of complementary elegant cellular defense mechanisms. The *E. coli* proteins involved in these functions have been well studied as model system and the respective mutants have been well characterized. The MutT, MutM, and MutY are the proteins involved in damage prevention with respect to 8-OG, and MutS is involved in base mismatch repair. MutT hydrolyzes 8-OGTP to 8-OGMP and prevents its incorporation into the replicating DNA by DNA polymerase III in *E. coli*. Hence this prevents the A-T to C-G transversions that are the result of 8-OG incorporation opposite A in the template. The *mutT* strain shows over 10, 000 fold higher levels of specific transversions with no effect on any other types of mutations (54).

MutM and MutY are glycosylases and function as base excision repair enzymes. MutT acts upon several modified purines. It excises 8-OG bases from the DNA to avoid any G-C to A-T transversions upon incorporation of A against 8-OG (27). MutY in turn only excises A from an A-G or an A-C mismatch. Strains containing inactivated *mutT* and *mutY* show several fold higher levels of G-C to A-T transversions as expected (118).

Another well studied protein (MutS) is a key part of the methyl directed DNA mismatch repair (MMR) system that contains MutL and MutH as the other components. This system was well characterized in *Saccharomyces cerevesiae* and *E. coli* (63). The MutS - MutL complex recognizes the mismatch in the DNA during the movement of the replication fork, whereas the methylation sensitive endonuclease MutH excises the mismatch nucleotide from the newly synthesized strand to avoid any further mutations in the downstream processes (122).

PROTEIN OXIDATION

Reactive oxygen species generated from autooxidation of electron transport carriers in the cell or due to external factors like the oxidative burst by the host immune cells are significant sources of protein oxidation in the cell.

Metal catalyzed protein oxidation: Under normal conditions (optimum oxygen concentration), ROS such as hydrogen peroxide or alkylperoxides are normally kept low by the oxidative stress defense system. However, proteins containing reactive metal centers are still prone to oxidation under these circumstances (26). Hydrogen peroxide and alkylperoxides react with the metals and generate hydroxyl and alkoxyl radicals respectively, via Fenton reaction. Almost all amino acids are prone to oxidation by the hydroxyl radicals, giving rise to their respective modified derivatives (163, 164).

ROS leading to protein oxidation include radical species hydroxyl ($^{\cdot}OH$), peroxyl ($RO_2^{\cdot}$), alkoxyl ($RO^{\cdot}$), hydroperoxyl ($HO_2^{\cdot}$), and non radical species such as H_2O_2, HOCl, and $ONOO^-$. Among the 20 amino acids, cysteine, tryptophan and methionine are highly susceptible to oxidation. ROS-mediated oxidation of proteins leads to the conversion of histidine residues to 2-oxohistidine, tryptophan to kynurenine, tyrosine to dihydroxy derivatives, methionines to methionine sulfoxide, leucine and valine to hydroxy derivatives and cysteine to disulfide derivatives. Of particular interest is the fact that oxidation of some amino acids (lysine, arginine and proline residues) leads to the formation of carbonyl groups (CO); these can be detected and quantified (164).

Detection of protein oxidation: The fact that carbonyl groups are major products of ROS-mediated protein oxidation reactions has led to the development of some highly sensitive methods for their detection. Carbonyl groups (aldehydes and ketones) are produced on protein side chains (Pro, Arg, Lys and Thr) upon their oxidation.

The highly sensitive assay for detection of protein carbonyls involve derivatization of CO with 2, 4-dinitrophenyl hydrazine (DNPH), which leads to a stable 2, 4-dinitrophenyl hydrazone (DNP) product. This stable DNP product can be detected either quantitatively or qualitatively. The DNP group absorbs UV light so the total carbonyl group in a given protein mixture can be estimated (34). Qualitative estimation of protein carbonyls in the cell can also be done by use of a procedure similar to western blot using commercially available antibodies against dintrophenyl hydrazones. Thus, the highly sensitive method (based on carbonyl content in oxidized proteins) serves as a critical biomarker in evaluating various age related diseases or to measure oxidative damage in the host cells. However, this technique has not been well developed to study oxidized protein profiles in bacteria (34).

Whereas much is known about DNA repair enzymes, our knowledge about the repair of oxidized proteins is relatively scarce. Of all the amino acids that are prone to oxidation, the sulfur containing amino acids cysteine and methionine are the only two that can be reduced to their native form. Whereas the reduction of disulfide bonds in cysteine is common and performed by well-studied proton donors, the reduction mechanism for methionines is quite unique. The oxidized sulfur in methionine (sulfoxide) is reduced to sulfur by the action of a thioredoxin dependent methionine sulfoxide reductase (Msr) system. The significance of Msr system has been well documented in many pathogenic bacteria, yeast and in higher eukaryotes including mammalian systems. It is a highly conserved protein in all strata of life (20-22).

OXIDATIVE STRESS RESISTANCE IN *H. PYLORI*

Mechanisms for detoxifying reactive oxygen species is an important survival aspect in the overall physiology of *H. pylori*. Although a strict aerobe, *H. pylori* is a microaerophile that can not survive above 12 % partial pressure oxygen in the laboratory. As human pathogen *H. pylori* encounters a severe oxidative stress environment in the host as was discussed in previous sections. It is thus not surprising that the bacterium has an impressive repertoire of enzymes responsible for ROS detoxification or for repairing oxidatively damaged macromolecules (mainly DNA and proteins).

(1) Superoxide dismutase (SOD)

H. pylori expresses superoxide dismutase that converts reactive superoxide into hydrogen peroxide. The gene product of *sodB* (hp 0389) in *H. pylori* is an iron-cofactored cytoplasmic SOD protein but has also been shown to be associated with the surface of the bacterium. Analysis of SOD from various strains of *H. pylori* show some strain specific attributes, but the enzyme is always correlated to virulence or colonization ability (148, 161). A *sodB* mutant in *H. pylori* is hypersensitive to oxidative stress (as evaluated by the use of chemical oxidants) and is severely deficient in mouse colonization (155).

(2) Catalase (KatA)

The *katA* gene product in *H. pylori* has a molecular weight of 58.6 kDa, and is responsible for the detoxification of hydrogen peroxide. A functional protein was purified and its ability to reduce hydrogen peroxide was also studied (75). Unlike in *E. coli* where the organism produces two catalase enzymes; one cytoplasmic and one periplasmic, *H. pylori* contains only one catalase enzyme located in the cytoplasm. Although there is evidence that catalase is also found on the surface of the bacterium, it was suggested to be the result of "altruistic autolysis" of a group of cells that also released other cytoplasmic proteins such as urease, and heat shock protein (70). The net affect of catalase in the cytoplasm is the prevention of Fenton reaction-mediated generation of hydroxyl radicals. In accordance with this activity, isogenic *katA* mutants of *H. pylori* could not detoxify H_2O_2 and were highly sensitive to peroxide-derived oxidative stress (136). The mutant strain when tested individually for its ability to colonize mouse stomachs showed decreased levels of (long-term) colonization (71).

(3) Peroxiredoxins

The enzymes Alkyl hydroperoxide reductase (AhpC) (hp1563), bacterioferritin comigratory protein (Bcp) (hp 0136) and thiol peroxidase (Tpx) (hp 390) together form a complementary group of peroxide reducing proteins in *H. pylori*. All these proteins exhibit a thioredoxin – thioredoxin reductase dependent activity (10, 181). Inactivation of each of these genes (*ahpC, bcp*, and *tpx*) rendered the pathogen sensitive to oxidative stress particularly that generated by organic peroxides such as cumene hydroperoxide, t-butyl hydroperoxide and to the superoxide generating oxidant, paraquat (137). Inactivation of any of these genes highly compromised the pathogen's ability to colonize the host in mouse model studies (138, 184). AhpC is one of the highly abundant proteins in *H. pylori* (84), and could thus have a significant role in reducing the lipid peroxides in the cell; consistent with this it the observation that the *ahpC* mutant is most sensitive to organic peroxide oxidative stress agents amongst all the individual peroxiredoxin family mutant strains tested. Though the roles of Bcp and Tpx may seem redundant, they may be differentially regulated (thus expressed at different times) based on the nature of stress conditions or degree of host infection. The significance of organic peroxide reductases is likely due to the presence of unsaturated fatty acids in the phospholipids of *H. pylori* cell membranes and the detoxification/reduction of lipid peroxides may be important for the viability of the pathogen. In addition the reduction of organic peroxides in *H. pylori* was shown to be important in protecting the heme B group in catalase from organic-peroxide mediated oxidative damage (181). All these results taken together emphasize the important role of peroxide reductases in oxidative stress resistance in *H. pylori*.

(4) NADPH quinone reductase

From several independent studies it was proposed that NADPH rather than NADP is the physiological electron donor in the respiratory chain in *H. pylori.* The NADPH dehydrogenase transfers electrons to the quinone pool. A novel protein MdaB (hp 0630) denoted as NADPH quinone reductase is a flavin-containing protein that catalyzes the two electron transfer from NADPH to quinones (175, 183). This enzyme showed around 10-fold higher preference for NADPH over NADH as the electron donor and reduced both ubiquinones and menaquinones with similar efficiencies. NADPH quinone reductases play an important role in combating oxidative stress in the cell. The two-electron reduction of quinones to quinols forms a reduced pool in *H. pylori* against the one-electron reduction of quinones to semiquinone radicals (by various reductases) that generate toxic superoxide radicals. A similar function of the *E. coli* MdaB and the eukaryotic counterpart DT-diapherose was previously documented (74). The *H. pylori mdaB* mutant was growth-sensitive to elevated oxygen concentrations and to the superoxide generator paraquat. Also, the *mdaB* mutant showed lower colonization efficiency than the wild type in mouse colonization studies, suggesting its influence on *H. pylori's* persistence in the host (183).

(5) Neutrophil activating protein (NapA)

The gene product of hp 0243 is a dodecameric DNA binding and iron sequestering protein NapA (177). The name is based on its ability to mediate neutrophil adhesion to endothelial cells and to bind to both mucin and neutrophil glycosphingolipids (170). It belongs to the Dps family of proteins that are shown to form a dodecameric structure tying the DNA into a compact structure and protecting it from iron related damage. A *napA* isogenic mutant of *H. pylori* is sensitive to oxidative stress (137). The involvement of this protein in oxidative stress was first identified when a subset of *ahpC* mutants in *H. pylori* upregulated NapA by 2-fold and showed relatively lower sensitivity to oxidative stress when compared to the majority of the *ahpC* isolates (137). This gene is regulated at the transcriptional level by the iron-dependent repressor Fur in *H. pylori*. (60). Fur negatively regulates *napA* by repressing its expression under iron deficient conditions and such expression is de-repressed under conditions of excess iron. Another interesting aspect of this gene is its greater than 3-fold up regulation in the stationary phase of growth (29). This presents an interesting phenomenon of gene regulation since *H. pylori* lacks a stationary phase sigma factor (175).

(6) Thioredoxin (Trx) and thioredoxin reductase (TrxR)

These two proteins in *H. pylori* form the essential thiol pool in the absence of the major ROS quencher glutathione. They perform the necessary disulfide reductase activity (10). The reductase system is essential for the survival of the pathogen and no targeted isogenic mutants in these could be obtained. The principal electron donor for the Trx – TrxR system is NADPH and they are crucial for reducing the oxidized reductases such as AhpC (10), and Bcp (184) in *H. pylori*.

(7) Compensatory response of *H. pylori* to the loss of antioxidant proteins

H. pylori compensates for the loss of major ROS detoxification enzymes in the cell. A proteomic study indicated that the inactivation (targeted disruption) of a key resistance factor resulted in recovery of strains that upregulated another oxidative stress combating enzyme, apparently to overcome the oxidative stress imposed by the original mutation. For example NapA was upregulated (5-fold) in a *sodB* mutant in most double mutants that had targeted disruptions in factors involved in hydrogen peroxide or superoxide detoxification (139).

All the above described proteins together constitute the ROS detoxification system in *H. pylori*'s defense against oxidative stress. In addition to these detoxifying enzymes, bacteria (including *H. pylori*) contain enzymes that repair oxidatively or otherwise damaged DNA or proteins.

(8) *H. pylori* enzymes involved in DNA repair

Helicobacter pylori contains the homologs of most of the DNA repair proteins that have been characterized in *E. coli* or in other eukaryotic systems (refer Table 1.1). However, *H. pylori* lacks the homologs of MutL and MutH (4, 175). There is evidence that a MutS homolog in *H. pylori* strongly recognizes and binds to the Holliday Junctions in DNA but its possible endonuclease activity in concert with any of the MutH homologs is not known. It would thus be very interesting to determine the specific role of MutS in the absence of the other components in the MMR complex. The table below outlines the different genes annotated as putative DNA repair enzymes in the *H. pylori* genome.

(9) *H. pylori* enzymes involved in protein repair

The gene product of hp0224 in *H. pylori* is annotated as a 42 kDa methionine sulfoxide reductase. The protein is 353 amino acids long with a translational fusion of MsrA and MsrB

Table 1.1. DNA repair enzymes in *H. pylori*. The predicted glycosylases and endonucleases involved in mutational repair in both the sequenced strains of *H. pylori* (90).

Gene	HP 26695	J99	Predicted function
mutS	0621	565	Mismatch recognition
mutY	0142	0130	A-G specific adenine glycosylase
nth	0585	0532	Glycosylase with AP lyase activity
magIII	0602	0549	3-MeA glycosylase
ogt	0676	0618	O-G methyltransferase
ung	1347	1266	Uracil DNa glycosylase

homologs. The N-terminus (a.a. 18 to 189) shares approximately 35 % identity with *E. coli MsrA* and the C-terminus (a.a.212 to 350) has approximately 60 % identity with *N. gonorrhoeae* and *H. influenzae* MsrB (http://us.expasy.org) (see figures 1.1, 1.2 and 1.3). Both MsrA and MsrB domains contain the putative catalytic residues as studied in Msr from other bacterial and eukaryotic systems. The putative MsrA domain contains the signature sequence of MsrA (**GGC**FWG) and the putative MsrB contains the signature sequence (K**GGLRY**C). Understanding the role of the Msr enzyme in *H. pylori* would give us a better understanding of the overall oxidative stress resistance in this pathogen. Msr is highly conserved in all studied aerobic and microaerobic microorganisms. The correlation between the ability to reduce oxidized methionines and the ability of the cell to combat oxidative stress has been consistently observed in bacterial pathogens and in higher eukaryotes. The following section summarizes the current literature available on the role of Msr in prokaryotes.

METHIONINE OXIDATION AND THE MSR SYSTEM

The maintenance of a cell function and homeostasis are based primarily on functions of proteins. Of course, the activities of many of these proteins are dynamic, and are controlled at various levels. Enzyme activity and/or structure often times need to be activated or modulated and their activity can also be blocked by the use of specific inhibitors. Another means of altering protein structure or function is by chemical modification of some of the amino acids (165, 180). Formation of disulfide bonds by oxidation/reduction of cysteines, phosphorylation, and methylation are well known examples of amino acid modifications. The last two decades have seen the emergence of yet another form of physiological protein modification; the oxidation – reduction of methionine residues. Along with cysteine and tryptophan, methionine (Met) is the most susceptible amino acid to oxidation.

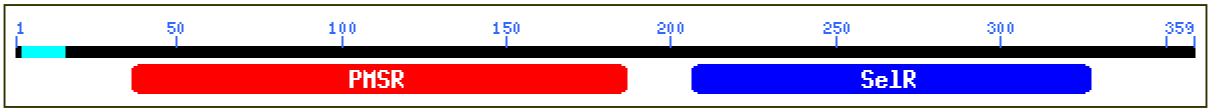


FIG. 1.1: Schematic representation of *H. pylori* Msr. It is a fused protein containing both MsrA and MsrB domains. The residues 36 to 190 are homologous to MsrA enzyme and the residues 210 to 330 are homologous to MsrB enzyme. The MsrB domain has been denoted as SelR which is a selenocysteine containing MsrB in humans.

```
EcoliMsrA         SLFDKKHLVSPADALPGRNTPMPVATLHAVNGHSMTNVPDGMEIAIFAMGCFWGVERLFW
HpyloriMsrAB      -------MKVLSYLKNFYLFLAIGAIMQASENMGSQHQKTDERVIYLAGGCFWGLEAYME
                         :   :            * ::* :. .  :   . .:  :* *****.*  :

EcoliMsrA         QLHGVYSTAAGYTGGYTPNPTYREVCSGDTGHAEAVRIVYDPSVISYEQLLQVFWENHDP
HpyloriMsrAB      RIYGVIDASSGYANGKTSSTNYEKLHESD--HAESVKVIYDPKKISLDKLLRYYFKVVDP
                  :::** .:::**:.* *....*.:: ..*  ***:*:::***. ** ::**: :::  **

EcoliMsrA         AQGMRQGNDHGTQYRSAIYPLTPEQDAAARASLERFQAAMLAADDDRHITTEIANATPFY
HpyloriMsrAB      VSVNKQGNDVGRQYRTGIYYVN-----SADKEVIDHALKALQKEVKGKIAIEVEPLKNYV
                  ..  :**** * ***:.** :.     :*  .:  .    *  : . :*: *:   . :

EcoliMsrA         YAEDDHQQYLHKNPYGYCG-----------------------------------------
HpyloriMsrAB      RAEEYHQDYLKKHPSGYCHIDLKKADEVIVDDDKYTKPSDEVLKKKLTKLQYEVTQNKHT
                   **: **:**:*:* ***

EcoliMsrA         ------------------------------------------------------------
HpyloriMsrAB      EKPFENEYYNKEEEGIYVDITTGEPLFSSADKYDSGCGWPSFSKPINKDVVKYEDDESLN

EcoliMsrA         -------------------------IGGIGVCLPPEA-----------------------
HpyloriMsrAB      RKRIEVLSRIGKAHLGHVFNDGPKELGGLRYCINSAALRFIPLKDMEKEGYGEFIPYIKK
                                           :**:  *: . *

EcoliMsrA         -------------
HpyloriMsrAB      GELKKYINDKKSH
```

FIG 1.2. ClustalW alignment of MsrA domain. The alignment of the putative MsrA domain in *H. pylori* (residues 36-190) with *E. coli* MsrA. The putative catalytic residues are shaded.

```
H._infl         ------------------------------------------------------------
H._pylori       ------------------------------------------------------------
N.gonnor        MKHRTFFSLCAKFGCLLALGACSPKIVDAGTATVPHTLSTLKTADNRPASVYLKKDKPTL
N._mening       MKHRTFFSLCAKFGCLLALGACSPKIVDAGAATVPHTLSTLKTADNRPASVYLKKDKPTL
E.coli_B        ------------------------------------------------------------

H._infl         ------------------------------------------------------------
H._pylori       ------------------------------------------------------------
N.gonnor        IKFWASWCPLCLSELGQAEKWAQDAKFSSANLITVASPGFLHEKKDGEFQKWYAGLNYPK
N._mening       IKFWASWCPLCLSELGQTEKWAQDAKFSSANLITVASPGFLHEKKDGDFQKWYAGLNYPK
E.coli_B        ------------------------------------------------------------

H._infl         ------------------------------------MKLSKTFLFITALCCATPTLAIQN
H._pylori       -------------------------------------------MKVLSYLKNFYLFLAIG
N.gonnor        LPVVTDNGGTIAQNLNISVYPSWALIGKDGDVQRIVKGSINEAQALALIRNPNADLGSLK
N._mening       LPVVTDNGGTIAQSLNISVYPSWALIGKDGDVQRIVKGSINEAQALALIRDPNADLGSLK
E.coli_B        ------------------------------------------------------------

H._infl         STSSSGEQKMAMENTQNIREIYLAGGCFWGMEAYMERIHGVKDAISGYANGNTEKTSYQM
H._pylori       AIMQASENMGSQHQKTDERVIYLAGGCFWGLEAYMERIYGVIDASSGYANGKTSSTNYEK
N.gonnor        HSFYKPDTQKKDSAIMNTRTIYLAGGCFWGLEAYFQRIDGVVDAVSGYANGNTENPSYED
N._mening       HSFYKPDTQKKDSKIMNTRTIYLAGGCFWGLEAYFQRIDGVVDAVSGYANGNTKNPSYED
E.coli_B        ------------------------------------------------------------

H._infl         IG--LTDHAETVKVTYDANQISLDKLLKYYFKVIDPTSVNKQGNDRGRQYRTGIYYQDGA
H._pylori       LH--ESDHAESVKVIYDPKKISLDKLLRYYFKVVDPVSVNKQGNDVGRQYRTGIYYVNSA
N.gonnor        VSYRHTGHAETVKVTYDADKLSLDDILQYYFRVVDPTSLNKQGNDTGTQYRSGVYYTDPA
N._mening       VSYRHTGHAETVKVTYDADKLSLDDILQYFFRVVDPTSLNKQGNDTGTQYRSGVYYTDPA
E.coli_B        ------------------------------------------------------------

H._infl         DKAVIGQALAQLQTKYKKPVQIEVQPLKNYIVAEEYHQDYLKKNPNGYCHIDITKADEPV
H._pylori       DKEVIDHALKALQKEVKGKIAIEVEPLKNYVRAEEYHQDYLKKHPSGYCHIDLKKADEVI
N.gonnor        EKAVIAAALKREQQKYQLPLVVENEPLKNFYDAEEYHQDYLIKNPNGYCHIDIRKADEPL
N._mening       EKAVIAAALKREQQKYQLPLVVENEPLKNFYDAEEYHQDYLIKNPNGYCHIDIRKADEPL
E.coli_B        ------------------------------------------------------------

H._infl         IDEKD------------YPKPSDAELKAKLTPLQYSVTQNKHTERSFSNEYWDNFQPGIY
H._pylori       VDDDK------------YTKPSDEVLKKKLTKLQYEVTQNKHTEKPFENEYYNKEEEGIY
N.gonnor        PGKTKAAPQGKGFDAATYKKPSDAELKRTLTEEQYQVTQNSATEYAFSHEYDHLFKPGIY
N._mening       PGKTKTAPQGKGFDAATYKKPSDAELKRTLTEEQYQVTQNSATEYAFSHEYDHLFKPGIY
E.coli_B        ----------------MANKPSAEELKKNLSEMQFYVTQNHGTEPPFTGRLLHNKRDGVY
                                   ***   ** .*:  *: ****  ** .*  .  .  . *:*
H._infl         VDITTGEPVFSSNDKFESGCGWPSFTKPIIKDVVHYETDNSFNMQRTEVLSRAGNAHLGH
H._pylori       VDITTGEPLFSSADKYDSGCGWPSFSKPINKDVVKYEDDESLNRKRIEVLSRIGKAHLGH
N.gonnor        VDVVSGEPLFSSADKYDSGCGWPSFTRPIDAKSVTEHDDFSFNMRRTEVRSRAADSHLGH
N._mening       VDVVSGEPLFSSADKYDSGCGWPSFTRPIDAKSVTEHDDFSYNMRRTEVRSHAADSHLGH
E.coli_B        HCLICDAPLFHSQTKYDSGCGWPSFYEPVSEESIRYIKDLSHGMQRIEIRCGNCDAHLGH
                  :  . *:* *  *::******** .*:  . :    * * . :* *: .   .:****
H._infl         VFDDGPKDKGGLRYCINSASIKFIPLAEMEKAGYGYLIQSIKK-------------
H._pylori       VFNDGPKELGGLRYCINSAALRFIPLKDMEKEGYGEFIPYIKKGELKKYINDKKSH
N.gonnor        VFPDGPRDKGGLRYCINGASLKFIPLEQMDAAGYGALKGEVK--------------
N._mening       VFPDGPRDKGGLRYCINGASLKFIPLEQMDAAGYGALKSKVK--------------
E.coli_B        VFPDGPQETG-ERYCVNSASLRFTDGENGEEING----------------------
                ** ***:  *  ***:*.*:::*    : :  .
```

FIG 1.3. Clustal W alignment of MsrAB from various bacteria. The MsrA and MsrB domains in the sequence are indicated separately. MsrAB from *H. influenzae* (H._infl), *H. pylori* (H._pylori), *N. gonnorhoeae* (N._gonnor), *N. meningitidis* (N._mening) and the *E. coli* MsrB (E. coli_B).The *H. pylori* MsrB domain (residues 206-330) is homologous to the *Neisseria* and *H. influenzae* MsrB domains. The putative catalytic fingerprints are shaded.

Upon mild oxidation, the sulfur moiety is oxidized to sulfoxide giving rise to a reversible **methionine sulfoxide (Met(O)** (163). Prolonged unchecked oxidation of methionine converts them to methionine sulfone (see fig. 1.4) which cannot be repaired. The latter form has rarely been found in biological systems. All biologically relevant oxidants such as superoxide, hydrogen peroxide, hydroxyl radicals, hypochlorous acid, and chloramines can oxide either free or protein bound Met residues. Until recently the oxidation of Met to Met(O) was seen as merely a oxidation-destroying process. The finding that oxidation of methionines may serve a regulatiory role came to light by the discovery of enzymatic reduction of Met(O). Also, oxidation of free Met to Met(O) eliminates its ability to serve as a methyl donor (180) .

Evidence for an enzyme system that could reduce free Met(O) to Met was demonstrated in yeast cells by Black et al (22). Then studies on *E. coli* cell ribosomal proteins L12 demonstrated that peptide bound Met(O) can also be reduced in the cell (21). The 50S ribosomal proteins in *E. coli* lack the residues, Cys, Lys, Tyr and His that are susceptible to oxidation, yet the proteins were inactivated upon exposure to hydrogen peroxide. The protein, however contains three Met residues, the oxidation of which inhibits the formation of a biologically active dimer. A search for an enzyme that could reduce protein bound methionine resulted in the discovery of a thioredoxin-thioredoxin reductase dependent Msr system (22, 23). These studies led to the identification of two enzymes in *E. coli* that could catalyze the reduction of Met(O) to Met; one that reduces free Met(O), but not Met(O) in peptides, and a second enzyme with broad specificity and could reduce free Met(O) as well as protein bound residues with equal efficiency. The latter enzyme was referred to as MsrA (22).

Substrate specificity of Msr

The oxidation of Met to Met(O) results in the formation of an asymmetric sulfur atom and consequently two diastereomers, Met-S-(O) and Met-R-(O). Studies using *E. coli msrA* strains showed that the methionine auxotroph could grow on either of the two forms of methionines. Subsequent in vitro assays confirmed that the *E. coli,* yeast, and bovine MsrA showed high specificity to Met-S-(O) and was speculated to contain an epimerase that produces the active isomer (127).

Later studies by Grimaud and co-workers (65) using in vitro oxidation-reduction of the methionine-rich calcium sensing protein calmodulin revealed a new enzyme, MsrB from *E. coli* that had specificity towards Met-R-(O). Calmodulin contains 16 surface exposed methionines, and oxidation of these residues generated a mixture of the two oxidized diastereomers (R-SO and S-SO). Oxidized calmodulin could be reduced in vitro by using MsrA, yet cell extracts from an *msrA* mutant reduced the remaining methionines. The enzyme was subsequently purified and characterized as MsrB. It reduces Met-R-(O), whether the residues are free or within proteins. The two proteins (MsrA and MsrB) do not share any sequence homology but have a similar mechanism of action. Many homologs of these two enzymes were found in other bacteria and in higher eukaryotes with varying sequence homologies (65).

Genetic features of *msrA* and *msrB* genes and induction under stress

The most interesting feature of *msrA* and *msrB* is that they do not share any homology either at primary sequence or at structural levels. Nevertheless their overlapping catalytic role in the cell suggests a case of convergent evolution. The arrangement of *msrA* and *msrB* in various microorganisms differ greatly (see fig. 1.5). For example, *E. coli* has two separate transcriptional units (*msrA* and one *msrB*) that are located at different loci on the chromosome.

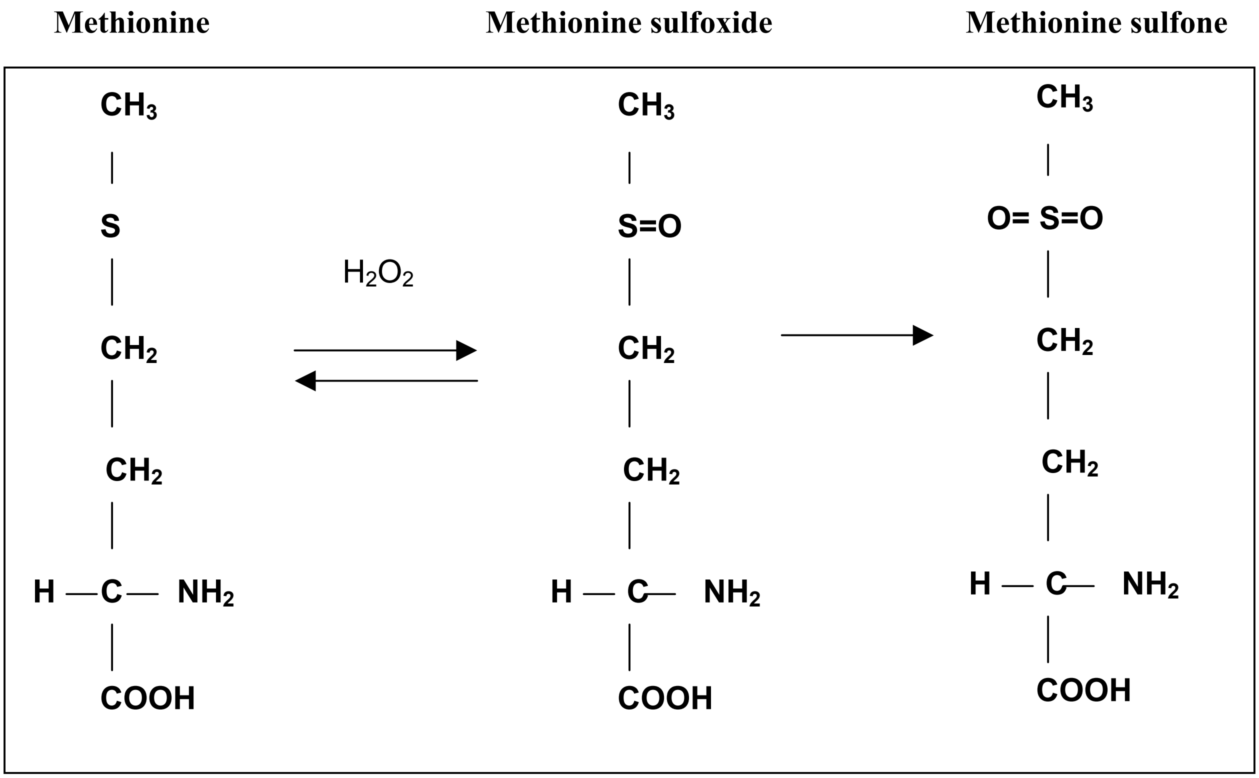


FIG 1.4. Structure of Methionine, methionine sulfoxide and methionine sulfone. Formation of methionine sulfoxide is a reversible reaction whereas further oxidation leads to an irreversible methionine sulfone (180).

The two genes appear to form an operon in *Bacillus subtilis* (42) as in *Staphylococcus aureus* where three *msrA*s are transcriptionally fused to one *msrB* (157). *Vibrio cholerae* presents a unique case where chromosome 1 contains one *msrA* and two *msrB* genes and chromosome 2 contains one of each. Another interesting feature of genetic redundancy is observed in *Rhizobium meliloti* that is predicted to have 3 genes each of *msrA* and *msrB*, with one *msrA* and one *msrB* gene carried on a plasmid. This redundancy might indicate in a differential regulation of genes based on the life style of the organism as a free living one or as a symbiont (48). When examined at the protein level *Neisseria gonorrhoeae, H. influenzae* and *H. pylori* Msr's have a different feature than other bacteria. These three human pathogens express a fused MsrA and MsrB with *N. gonorrhoeae* also having an extra thioredoxin domain at its N terminus and is shown to be essential for the Msr activity (96, 188).

The conditions for up regulation of *msrA* or *msrB* were studied in various bacteria and in yeast, and such conditions appeared to vary greatly. Since both gene products function as protein repair systems; it is not the ROS *per se* but perhaps the lesions caused by the ROS in the cell that induces the expression of these genes. For example, *msrA* was upregulated when *E. coli* cells were subjected to nitrogen starvation or in stationary phase of growth (128). One plausible explanation of this induction is that under low nitrogen conditions, the cell would preferably preserve the existing protein or amino acid pool by repairing/reducing the damaged proteins (by the use of Msr system). Similarly, in the stationary phase of growth the nutrients are presumably scarce, and may signal a need for protein or amino acid conservation. Hence neither MsrA nor MsrB were shown to be regulated by the classic *E. coli* oxidative stress response regulators OxyS and SoxR that respond to peroxide and superoxide stress, respectively. Table 1.2 summarizes the conditions under which the induction of *msrA* or *msrB* either at the level of mRNA or protein

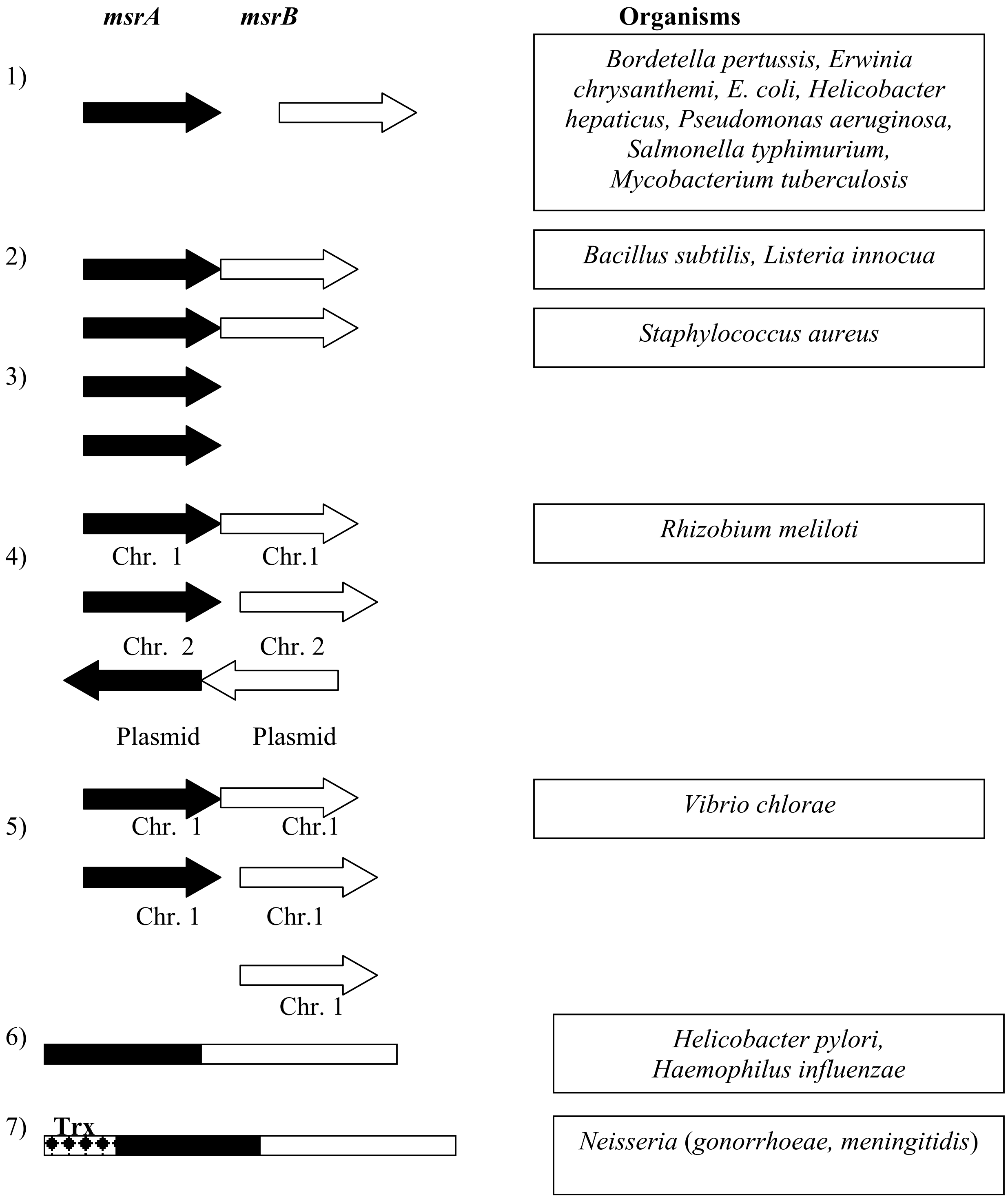


FIG. 1.5. Genetic organization of *msrA* and *msrB*. Organization of *msrA* (black arrow) and *msrB* (white arrow) in select eubacteria. (modified from (48, 65).

was expressed in various systems. One documented condition for up-regulation of *H. pylori msr* was under iron starvation conditions. Comparison of the *H. pylori* transcriptome under iron supplemented and iron chelated conditions showed an approximately three-fold up-regulation of the *msr* transcript under the latter condition (116). Although no further studies were performed addressing this, it indicates a unique condition under which the cells presumably needs Msr dependent protein repair.

Mechanism of action and different classes of Msr

The mechanism of reduction of Met-S-O and Met-R-O is similar, and has been very well studied using purified enzyme components from *E. coli*, *N. meningitidis,* and *N. gonorrhoeae* (6, 96, 133).

Cysteines are the catalytic residues in these and all of the known Msr enzymes and the reduction of substrate is universally catalyzed in a thioredoxin-thioredoxin reductase dependent mechanism (see fig. 1.6) (19). The mechanism includes three steps and is quite similar for both MsrA and MsrB: 1) formation of a sulfenic acid intermediate on the catalytic Cys residue (CysA), with concomitant release of 1 mol of Met per mol of enzyme, 2) formation of an intramonomeric disulfide bond between the catalytic Cys and the recycling Cys (CysB), with release of 1 mol of water and 3) reduction of the Msr disulfide bond by thioredoxin, leading to the regeneration of the reduced form of Msr. The oxidized thioredoxin is recycled (reduced) by the NADPH-mediated donation of protons by thioredoxin reductase. The fact that the catalytic mechanism takes place through the formation of a sulfenic acid chemistry is a general feature in the reduction of sulfoxides by thiols (140). Though the chemistry of sulfoxide reductase is similar for all known MsrA and MsrB enzymes, the participating Cys residues and the number of

Table 1.2: Permissive conditions of Msr induction. Growth or environmental conditions that cause induction of MsrA and MsrB levels as monitored by the expression of mRNA or protein or both, respectively.

Organism	**Induction conditions for MsrA and MsrB**
E. coli	Stationary phase, amino acid starvation and nitrogen starvation; checked for MsrA protein only (128)
S. aureus	Oxacillin (both MsrA and MsrB); RNA and protein level (129, 158)
S. cerevisiae	*msrA* mRNA under nitrogen starvation, AA starvation, diamide and γ irradiation. *msrB* mRNA: nitrogen starvation, stationary phase, heat shock and methyl methanesulfonate (125)
Arabidopsis thaliana	Dehydration and hydrogen peroxide exposure; checked for *msrB* mRNA only (151)
Mammals	Chronic dietary intake of soy protein – *msrA* mRNA expression. Selenium diet: MsrB mRNA and protein expression (129)

cysteines present in the protein varies greatly and is an important feature that further divides MsrA and MsrB into subclasses.

Both MsrA and MsrB enzymes are further sub-classified into different groups based on 1) the presence or absence of a third cysteine in the reaction mechanism, and 2) the placement of the recycling cysteines. In the case of MsrA, the *E. coli*, human, and bovine MsrA belong to the class I where the CysA (catalytic) is part of the fingerprint sequence GCFWG and is closer to the N-terminus. CysB (recycling) is part of the GYCG sequence. The CysC in this group is proposed to interact with the thioredoxin protein and undergoes disulfide reduction. The CysB and CysC are separated by a glycine rich linker (171, 172).

Msr from *Neisseria* spp., *H. pylori* and *M. tuberculosis* constitute class II. They contain CysA in the catalytic signature sequence GCFWG and the recycling CysB as part of GYCGH, but they lack CysC. The *M. tuberculosis* CysB (bold) on the other hand is part of a **C**YxCH sequence. The Class III MsrA contains enzymes from *Bacillus subtilis* and *Dienococcus radiodurans*. Here both CysA and CysB are part of GCFWC and are only separated by two other amino acids. This group too lacks the second recycling Cys residue, CysC (19, 86).

Inspection of primary structures of MsrBs revealed that about 50% of MsrBs possess a metal binding site formed by two CXXC motifs. These motifs form a Zn^{+}- binding site in *E. coli* and *Drosophila* MsrBs. Any structural change in the metal binding site affected the catalytic efficiency and thermal stability of these enzymes. The zinc binding feature distinguishes the *E. coli, Drosophila* and the mammalian MsrB into class I. The CXXC motif adds two more cysteines to the entire sequence in these two proteins. The *Neisseria* and *H. pylori* enzymes lack this zinc binding motif and are hence placed in Class II of MsrB (19, 86).

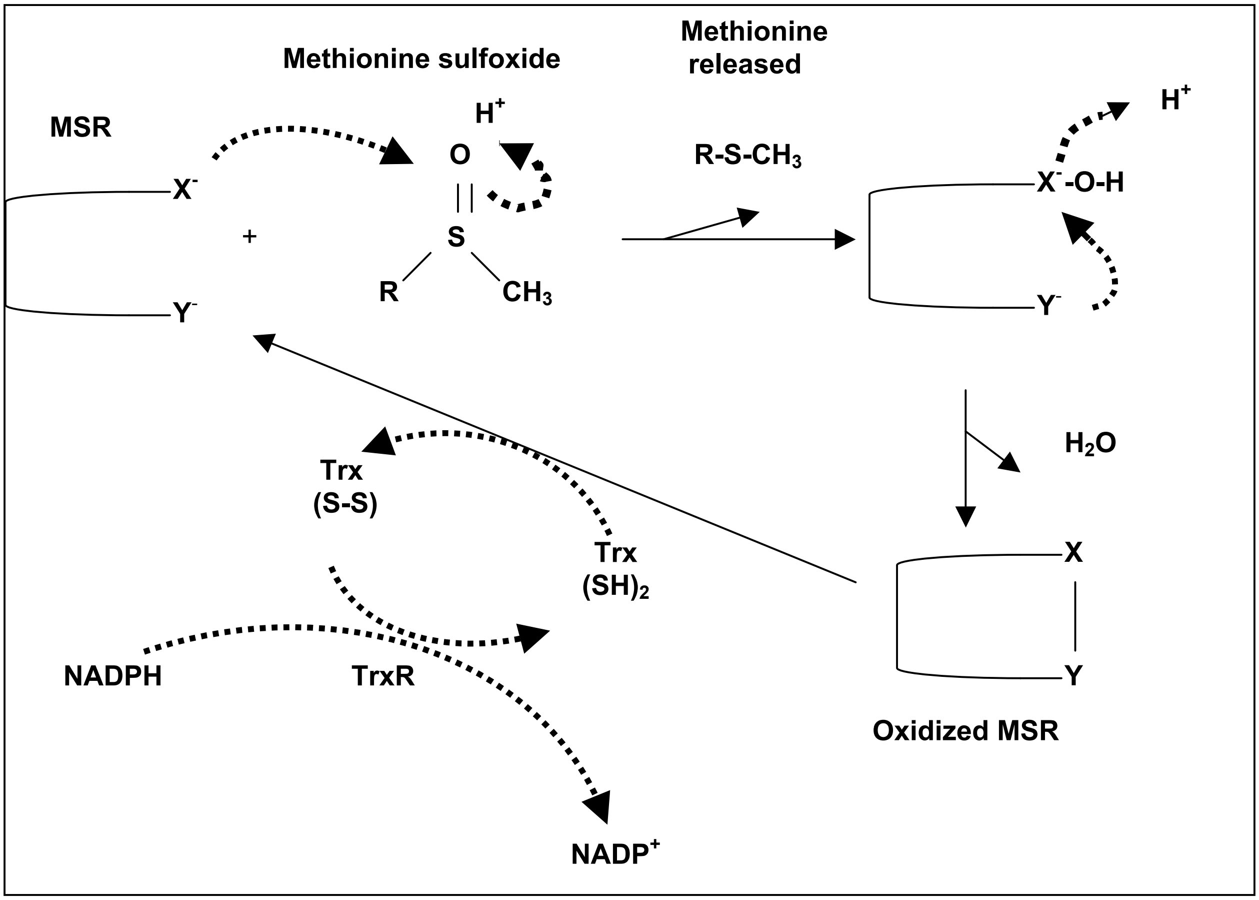


FIG. 1.6. Reaction mechanism of Msr catalyzed methionine sulfoxide reduction. Reduction of Met(O) to Met by Msr in a thioredoxin (Trx) – thioredoxin reductase (TrxR) dependent manner. NADPH is the electron donor to TrxR (adapted from 19, 21).

Physiological significance of MsrA and MsrB

MsrA activity was discovered for the first time in the context of oxidation of ribosomal proteins in *E. coli* and the resulting sensitivity of the bacterium to ROS (23). It is remarkable that despite the presence of numerous antioxidant enzymes in most bacteria, the absence of MsrA/MsrB invariably leads to increased susceptibility to oxidative conditions. There are three known hypotheses designed to better understand the need for methionine sulfoxide reductive repair (126, 165, 180). The first hypothesis is that some important processes in the cell are impaired due to oxidation of methionines so that the MsrA/B dependent repair thus becomes essential. In support of this theory was an elegant study by Ezraty and coworkers (49) in which the role of Msr in protein targeting in *E. coli* was discovered. The bacterial protein Ffh is a eukaryotic signal recognition particle (SRP54) homolog and it interacts with its target proteins and the 4.5S RNA at its methionine rich N-terminus (M-domain). It was observed that the *msrA* and *msrB* double mutant was unable to target the MalF protein to the inner membrane, especially under oxidative stress conditions. No such defect was observed in the wild type strain even under severe oxidative conditions. Mass spectrometric studies revealed a highly oxidized M-domain of Ffh only in the mutant, and an overproduction of the native Ffh restored the protein targeting defect, suggesting an Msr-dependent activation of oxidized Ffh (49).

The second hypothesis is that the accumulation of misfolded or altered (oxidized) proteins is toxic to the cell. Under these circumstances, the MsrA/B participates with the chaperones in protein repair in the cell or maintains chaperones in a functional form. Evidence in support of this theory was the Msr-dependent reduction of oxidized GroEL in *E. coli*. GroEL in *E. coli* consists of a double ring structure made of two heptamers stacked back to back. In the presence of peroxide, peroxynitrite or hypochlorous acid, GroEL underwent dissociation of the oligomers into monomers. All methionine residues in these monomers were oxidized and this was suspected to be the cause of the dissociation. When the oxidized GroEL was treated with MsrA and MsrB the structure and much of the function of GroEL was restored, indicating GroEL in *E. coli* as an important target of the Msr system (88, 111).

Another example of a chaperone being inactivated due to methionine oxidation comes from studies on *Mycobacterium tuberculosis*. Under oxidative stress conditions, the tetrameric chaperone Hsp16.3 is one of the highly induced proteins in the cell. Treatment of the pure protein with hydrogen peroxide allowed dissociation of Hsp16.3 into monomers due to the oxidation of susceptible methionine residues. Although no direct repair/reduction or interaction of Hsp16.3 with MsrA/B system was demonstrated, it is very likely that the oxidized chaperone is a potential substrate of the Msr system in this pathogen (1).

The third hypothesis states that methionine rich proteins provide a sink for quenching ROS in the cell. Levine and co-workers proposed that proteins rich in methionine could function as an alternative sink for ROS, and Msr-dependent recycling of their oxidized methionine residues would enable retention both of the sink and the active state of these proteins. Glutamine synthetase in *E. coli* is one such example where the protein that plays a role in general metabolism in the cell is rich in surface methionines and plays a ROS quenching role due to being reduced/repaired by the Msr system (94). These observations reinforce the physiological significance of Msr system.

Importance of *msrA* and *msrB* in bacterial pathogenecity

In recent years, both MsrA and MsrB emerged as important virulence factors for bacterial pathogens:

Erwinia chrysanthemi: *E. chrysanthemi* is a plant pathogen that secretes highly efficient plant cell wall degrading enzymes. Transposon mutagenesis was performed to isolate mutants that were unrelated to function of the degrading enzymes but still affected the pathogens' survival in the plant host. The gene *msrA* was one of the potential virulence factors identified, as the mutant was non-motile and lost its ability to persist in the host. Although increased susceptibility to oxidizing conditions was one speculation, no definitive role of the gene in the pathogens' survival was identified (48, 73).

Staphylococcus aureus: The *msrA1* is one of the three *msrA* genes present in *S. aureus*. Although inactivation of this gene rendered the cell sensitive to peroxide stress, no apparent up-regulation was observed when the cell was challenged with H_2O_2. Like the observation on *E. coli msr*, the *S. aureus* gene *msrA1* was upregulated in stationary phase of growth in response to various cell wall degrading antibiotics. No precise role for gene products of *msrA2* and *msrA3* is known but it is suggested that they are not significant in combating oxidative stress in *S. aureus.* (157, 158).

Mycobacterium smegmatis: Comparative studies of the wild-type and an isogenic *msrA* strain of *M. smegmatis* showed a growth-sensitive phenotype for the mutant under oxidative stress conditions. An important pathogenesis-related observation was the difference in survival of these strains in the phagocytes. In the phagocytic cells, the *msrA* mutants recruited the oxidative enzymes such as iNOS much faster than the wild type strains. This result suggests that MsrA affects the protein/cell signaling in the human immune cells and thus indirectly delays the recruitment of the host toxic ROS generators. This perhaps is one of the survival mechanisms of this pathogen within phagocytes (41).

Mycobacterium tuberculosis: An isogenic *msrA* mutant in this pathogen is hypersensitive to peroxide or peroxinitrite stress. MsrA also influences the survival of this pathogen in murine macrophages. Taken together with the repair of oxidized chaperone protein Hsp16.3, the inactivation of *msrA* in this pathogen might have a pleiotropic effect on the cell (1, 162).

Neisseria gonorrhoeae and ***N. meningitidis***: Both of these *Neisseria* are well known human pathogens, colonizing the urinary tract and the nasopharyngeal tract, respectively. The structure of Msr is of great interest regarding these pathogens, since Msr contains a disulfide reductase (thioredoxin) domain at the N-terminus of the protein. Cell fractionation studies showed that the complete protein is present on the outer surface of the cell and the truncated MsrA-MsrB domain remains in the cytoplasm, without the Trx domain. Inactivation of the Trx domain rendered these pathogens sensitive to oxidative stress. It is speculated that the extracellular version provided protection to this pathogen; alternatively, the Trx domain is perhaps essential in recycling the MsrA-B domains for their sulfoxide reductase activity and hence the cytoplasmic protein itself is non-functional (187). Although no direct evidence for the Msr enzyme in *Neisseria* virulence has been documented, it would not be surprising to see a mutant strain has compromised ability to persist in the host.

The cases discussed here present compelling evidence that MsrA and MsrB are essential for the survival of bacteria under oxidative stress conditions, and their loss always attenuates virulence of the bacterial pathogen (where tested). Msr in *H. pylori* shares significant sequence homology to these studied systems at the amino acid level (Fig 1.5 and 1.6). Since combating oxidative stress is an important persistence factor in this bacterium, I thought it would be interesting to study the role of Msr in *H. pylori*.

SCOPE OF THIS STUDY

Helicobacter pylori requires a highly active antioxidant repertoire for surviving the oxidative stress in human stomach. After combating acidity, an argument could be made that the next most important line of defense to enable gastric colonization by *H. pylori* are the ROS-combating enzymes. The absence of a thiol (such as glutathione) to quench the ROS in *H. pylori* suggests a need for an alternative mechanism to minimize oxidative stress conditions. The MsrA/B enzymes are well studied methionine repair systems that reduce oxidized methionines in the cell and this repaired methionine pool is now believed to be a powerful antioxidant reservoir. Inactivation of either *msrA/msrB* or both is shown to impair the oxidative stress resistance in many bacterial pathogens.

The Msr enzyme in *H. pylori* is a 353 amino acid long translational fusion of MsrA and MsrB domains, and the MsrB domain shares high homology with the MsrB domain of *Neisseria* enzymes. The close homology of *H. pylori* Msr with well studied enzymes and the predicted importance of such a protein repair system in *H. pylori* together present a strong rationale to characterize Msr in this pathogen. Firstly, a genetic approach was taken to study the physiological significance of Msr in *H. pylori* by comparing the sensitivity of the mutant to the wild type to oxidative stress. Using a mouse model, the colonizing efficiency of the mutant was determined. The methods used and the results obtained are discussed in Chapter 2.

Since little is known about the proteins that directly interact with or are targeted for repair by Msr in pathogenic microorganisms, the biochemical approach taken in Chapter 3 is designed to determine the potential in vivo substrates for this enzyme. Reporter gene fusions were made to the promoter of *msr* to test its induction under various endogenous and exogenous stress factors. In vitro activity of the pure enzyme was tested to determine the substrate binding domain in this protein. The results obtained from my studies will document a role for Msr in the oxidative stress resistance of *H. pylori* thus making it the first Msr system to be studied in a microaerophilic pathogen.

MutS is a part of the DNA mismatch repair enzyme system in *E. coli*. *H. pylori* contains a MutS homolog but it lacks the mismatch repair domain in its N-terminus. Chapter 3 describes both genetic and biochemical approaches taken to study an additional role of the *H. pylori* MutS protein in the oxidative stress resistance/damage repair in this pathogen.

The iron-sulfur cluster synthesis protein NifS-NifU is essential in *H. pylori*. Preliminary microarray studies to compare whole-genome expression in *H. pylori* at 2 % oxygen versus 12 % oxygen growth conditions showed a 5-fold up-regulation of *nifS* at the higher oxygen level. I made *xylE* fusions to the promoter of *nifS* to study its specific regulation under high oxygen, and in iron supplementation, and iron chelation conditions. Chapter 4 discusses these results and shows an interesting facet of *nifS* regulation by Fur in *H. pylori*.

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. **Akamatsu, T., K. Tabata, M. Hironga, H. Kawakami, and M. Uyeda.** 1996. Transmission of *Helicobacter pylori* infection via flexible fiberoptic endoscopy. Am J Infect Control **24:**396-401.

3. **Akopyants, N. S., S. W. Clifton, D. Kersulyte, J. E. Crabtree, B. E. Youree, C. A. Reece, N. O. Bukanov, E. S. Drazek, B. A. Roe, and D. E. Berg.** 1998. Analyses of the cag pathogenicity island of *Helicobacter pylori*. Mol Microbiol **28:**37-53.

4. **Alm, R. A., L. S. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smith, B. Noonan, B. C. Guild, B. L. deJonge, G. Carmel, P. J. Tummino, A. Caruso, M. Uria-Nickelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. F. Vovis, and T. J. Trust.** 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature **397:**176-80.

5. **Ames, B. N.** 1989. Endogenous oxidative DNA damage, aging, and cancer. Free Radic Res Commun **7:**121-8.

6. **Antoine, M., S. Boschi-Muller, and G. Branlant.** 2003. Kinetic characterization of the chemical steps involved in the catalytic mechanism of methionine sulfoxide reductase A from *Neisseria meningitidis*. J Biol Chem **278:**45352-7.

7. **Aspinall, G. O., M. A. Monteiro, H. Pang, E. J. Walsh, and A. P. Moran.** 1996. Lipopolysaccharide of the *Helicobacter pylori* type strain NCTC 11637 (ATCC 43504): structure of the O antigen chain and core oligosaccharide regions. Biochemistry **35:**2489-97.

8. **Babior, B. M.** 1999. NADPH oxidase: an update. Blood **93:**1464-76.

9. **Babior, B. M.** 2000. Phagocytes and oxidative stress. Am J Med **109:**33-44.

10. **Baker, L. M., A. Raudonikiene, P. S. Hoffman, and L. B. Poole.** 2001. Essential thioredoxin-dependent peroxiredoxin system from *Helicobacter pylori*: genetic and kinetic characterization. J Bacteriol **183:**1961-73.

11. **Bannister, J. V., W. H. Bannister, and G. Rotilio.** 1987. Aspects of the structure, function, and applications of superoxide dismutase. CRC Crit Rev Biochem **22:**111-80.

12. **Blaser, M. J.** 1998. *Helicobacter pylori* and gastric diseases. Biomed. J. **316:**1507-1510.

13. **Blaser, M. J.** 1990. *Helicobacter pylori* and the pathogenesis of gastroduodenal inflammation. J. Infect. Dis. **161:**626-633.

14. **Blaser, M. J., and J. C. Atherton.** 2004. *Helicobacter pylori* persistence: biology and disease. J Clin Invest **113:**321-33.

15. **Blaser, M. J., and J. Parsonnet.** 1994. Parasitism by the "slow" bacterium *Helicobacter pylori* leads to altered gastric homeostatis and neoplasia. J. Clin. Invest. **94:**4-8.

16. **Blaser, M. J., G. I. Perez-Perez, H. Kleanthous, T. L. Cover, R. M. Peek, P. H. Chyou, G. N. Stemmermann, and A. Nomura.** 1995. Infection with *Helicobacter pylori* strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. Cancer Res **55:**2111-5.

17. **Bode, G., P. Malfertheiner, G. Lehnhardt, M. Nilius, and H. Ditschuneit.** 1993. Ultrastructural localization of urease of *Helicobacter pylori*. Med Microbiol Immunol (Berl) **182:**233-42.

18. **Boren, T., P. Falk, K. A. Roth, G. Larson, and S. Normark.** 1993. Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. Science **262:**1892-5.

19. **Boschi-Muller, S., A. Olry, M. Antoine, and G. Branlant.** 2005. The enzymology and biochemistry of methionine sulfoxide reductases. Biochim Biophys Acta **1703:**231-8.

20. **Brot, N., H. Fliss, T. Coleman, and H. Weissbach.** 1984. Enzymatic reduction of methionine sulfoxide residues in proteins and peptides. Methods Enzymol **107:**352-60.

21. **Brot, N., and H. Weissbach.** 2000. Peptide methionine sulfoxide reductase: biochemistry and physiological role. Biopolymers **55:**288-96.

22. **Brot, N., L. Weissbach, J. Werth, and H. Weissbach.** 1981. Enzymatic reduction of protein-bound methionine sulfoxide. Proc Natl Acad Sci U S A **78:**2155-8.

23. **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 U S A **75:**5349-52.

24. **Carlsohn, E., J. Nystrom, I. Bolin, C. L. Nilsson, and A. M. Svennerholm.** 2006. HpaA is essential for *Helicobacter pylori* colonization in mice. Infect Immun **74:**920-6.

25. **Chen, M., L. P. Andersen, L. Zhai, and A. Kharazmi.** 1999. Characterization of the respiratory chain of *Helicobacter pylori*. FEMS Immunol Med Microbiol **24:**169-74.

26. **Chevion, M.** 1988. A site-specific mechanism for free radical induced biological damage: the essential role of redox-active transition metals. Free Radic Biol Med **5:**27-37.

27. **Chung, M. H., H. Kasai, D. S. Jones, H. Inoue, H. Ishikawa, E. Ohtsuka, and S. Nishimura.** 1991. An endonuclease activity of *Escherichia coli* that specifically removes 8-hydroxyguanine residues from DNA. Mutat Res **254:**1-12.

28. **Clayton, C. L., M. J. Pallen, H. Kleanthous, B. W. Wren, and S. Tabaqchali.** 1990. Nucleotide sequence of two genes from *Helicobacter pylori* encoding for urease subunits. Nucleic Acids Res **18:**362.

29. **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:**461-9.

30. **Costa, K., G. Bacher, G. Allmaier, M. G. Dominguez-Bello, L. Engstrand, P. Falk, M. A. de Pedro, and F. Garcia-del Portillo.** 1999. The morphological transition of *Helicobacter pylori* cells from spiral to coccoid is preceded by a substantial modification of the cell wall. J Bacteriol **181:**3710-5.

31. **Covacci, A., S. Censini, M. Bugnoli, R. Petracca, D. Burroni, G. Macchia, A. Massone, E. Papini, Z. Xiang, N. Figura, and et al.** 1993. Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. Proc Natl Acad Sci U S A **90:**5791-5.

32. **Cover, T. L., and M. J. Blaser.** 1999. *Helicobacter pylori* factors associated with disease. Gastroenterology **117:**257-61.

33. **Cover, T. L., and M. J. Blaser.** 1992. Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. J Biol Chem **267:**10570-5.

34. **Dalle-Donne, I., R. Rossi, D. Giustarini, A. Milzani, and R. Colombo.** 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta **329:**23-38.

35. **Dhaenens, L., F. Szczebara, and M. O. Husson.** 1997. Identification, characterization, and immunogenicity of the lactoferrin-binding protein from *Helicobacter pylori*. Infect Immun **65:**514-8.

36. **Dizdaroglu, M.** 1991. Chemical determination of free radical-induced damage to DNA. Free Radic Biol Med **10:**225-42.

37. **Dizdaroglu, M., Z. Nackerdien, B. C. Chao, E. Gajewski, and G. Rao.** 1991. Chemical nature of in vivo DNA base damage in hydrogen peroxide-treated mammalian cells. Arch Biochem Biophys **285:**388-90.

38. **Dizdaroglu, M., D. Schulte-Frohlinde, and C. von Sonntag.** 1975. Radiation chemistry of DNA, II. Strand breaks and sugar release by gamma-irradiation of DNA in aqueous solution. The effect of oxygen. Z Naturforsch [C] **30:**826-8.

39. **Dizdaroglu, M., C. von Sonntag, and D. Schulte-Frohlinde.** 1975. Letter: Strand breaks and sugar release by gamma-irradiation of DNA in aqueous solution. J Am Chem Soc **97:**2277-8.

40. **Doig, P., B. L. de Jonge, R. A. Alm, E. D. Brown, M. Uria-Nickelsen, B. Noonan, S. D. Mills, P. Tummino, G. Carmel, B. C. Guild, D. T. Moir, G. F. Vovis, and T. J. Trust.** 1999. *Helicobacter pylori* physiology predicted from genomic comparison of two strains. Microbiol Mol Biol Rev **63:**675-707.

41. **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:**3590-8.

42. **Dowds, B. C.** 1994. The oxidative stress response in Bacillus subtilis. FEMS Microbiol Lett **124:**255-63.

43. **Dunn, B. E., H. Cohen, and M. J. Blaser.** 1997. *Helicobacter pylori*. Clin Microbiol Rev **10:**720-41.

44. **Dunn, B. E., H. Cohen, and M. J. Blaser.** 1997. *Helicobacter pylori*. Clin. Microbiol. Rev. **10:**720-741.

45. **Dunn, B. E., N. B. Vakil, B. G. Schneider, M. M. Miller, J. B. Zitzer, T. Peutz, and S. H. Phadnis.** 1997. Localization of *Helicobacter pylori* urease and heat shock protein in human gastric biopsies. Infect Immun **65:**1181-8.

46. **Eaton, K. A., C. L. Brooks, D. R. Morgan, and S. Krakowka.** 1991. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect Immun **59:**2470-5.

47. **Ernst, P. B., and B. D. Gold.** 2000. The disease spectrum of *Helicobacter pylori*: the immunopathogenesis of gastroduodenal ulcer and gastric cancer. Annu Rev Microbiol **54:**615-40.

48. **Ezraty, B., L. Aussel, and F. Barras.** 2005. Methionine sulfoxide reductases in prokaryotes. Biochim Biophys Acta **1703:**221-9.

49. **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:**1868-77.

50. **Falk, P. G., L. Bry, J. Holgersson, and J. I. Gordon.** 1995. Expression of a human alpha-1,3/4-fucosyltransferase in the pit cell lineage of FVB/N mouse stomach results in production of Leb-containing glycoconjugates: a potential transgenic mouse model for studying *Helicobacter pylori* infection. Proc Natl Acad Sci U S A **92:**1515-9.

51. **Fenna, R., J. Zeng, and C. Davey.** 1995. Structure of the green heme in myeloperoxidase. Arch Biochem Biophys **316:**653-6.

52. **Fischbach, W., A. O. Chan, and B. C. Wong.** 2005. *Helicobacter pylori* and Gastric Malignancy. Helicobacter **10 Suppl 1:**34-9.

53. **Forman, D., D. G. Newell, F. Fullerton, J. W. Yarnell, A. R. Stacey, N. Wald, and F. Sitas.** 1991. Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from a prospective investigation. Br. Med. J. **302:**1302-1305.

54. **Fowler, R. G., and R. M. Schaaper.** 1997. The role of the mutT gene of *Escherichia coli* in maintaining replication fidelity. FEMS Microbiol Rev **21:**43-54.

55. **Fowler, R. G., S. J. White, C. Koyama, S. C. Moore, R. L. Dunn, and R. M. Schaaper.** 2003. Interactions among the *Escherichia coli* mutT, mutM, and mutY damage prevention pathways. DNA Repair (Amst) **2:**159-73.

56. **Fridovich, I.** 1999. Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? Ann N Y Acad Sci **893:**13-8.

57. **Fridovich, I.** 1998. Oxygen toxicity: a radical explanation. J Exp Biol **201:**1203-9.

58. **Fridovich, I.** 1997. Superoxide anion radical (O2-.), superoxide dismutases, and related matters. J Biol Chem **272:**18515-7.

59. **Fridovich, I.** 1995. Superoxide radical and superoxide dismutases. Annu Rev Biochem **64:**97-112.

60. **Furtmuller, P. G., U. Burner, and C. Obinger.** 1998. Reaction of myeloperoxidase compound I with chloride, bromide, iodide, and thiocyanate. Biochemistry **37:**17923-30.

61. **Ge, Z., Q. Jiang, M. S. Kalisiak, and D. E. Taylor.** 1997. Cloning and functional characterization of *Helicobacter pylori* fumarate reductase operon comprising three structural genes coding for subunits C, A and B. Gene **204:**227-34.

62. **Geis, G., S. Suerbaum, B. Forsthoff, H. Leying, and W. Opferkuch.** 1993. Ultrastructure and biochemical studies of the flagellar sheath of *Helicobacter pylori*. J Med Microbiol **38:**371-7.

63. **Goldfarb, T., and E. Alani.** 2005. Distinct roles for the Saccharomyces cerevisiae mismatch repair proteins in heteroduplex rejection, mismatch repair and nonhomologous tail removal. Genetics **169:**563-74.

64. **Goodwin, C. S., W. McConnell, R. K. McCullough, C. McCullough, R. Hill, M. A. Bronsdon, and G. Kasper.** 1989. Cellular fatty acid composition of *Campylobacter pylori* from primates and ferrets compared with those of other campylobacters. J. Clin. Microbiol. **27:**938-943.

65. **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:**48915-20.

66. **Grossman, M. I.** 1980. Peptic ulcer: the pathophysiological background. Scand J Gastroenterol Suppl **58:**7-16.

67. **Hachem, C. Y., J. E. Clarridge, D. G. Evans, and D. Y. Graham.** 1995. Comparison of agar based media for primary isolation of *Helicobacter pylori*. J Clin Pathol **48:**714-6.

68. **Halliwell, B., and J. M. Gutteridge.** 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J **219:**1-14.

69. **Hampton, M. B., A. J. Kettle, and C. C. Winterbourn.** 1998. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood **92:**3007-17.

70. **Harris, A. G., and S. L. Hazell.** 2003. Localisation of *Helicobacter pylori* catalase in both the periplasm and cytoplasm, and its dependence on the twin-arginine target protein, KapA, for activity. FEMS Microbiol Lett **229:**283-9.

71. **Harris, A. G., J. E. Wilson, S. J. Danon, M. F. Dixon, K. Donegan, and S. L. Hazell.** 2003. Catalase (KatA) and KatA-associated protein (KapA) are essential to persistent colonization in the *Helicobacter pylori* SS1 mouse model. Microbiology **149:**665-72.

72. **Harz, H., K. Burgdorf, and J. V. Holtje.** 1990. Isolation and separation of the glycan strands from murein of *Escherichia coli* by reversed-phase high-performance liquid chromatography. Anal Biochem **190:**120-8.

73. **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 U S A **96:**887-92.

74. **Hayashi, M., H. Ohzeki, H. Shimada, and T. Unemoto.** 1996. NADPH-specific quinone reductase is induced by 2-methylene-4-butyrolactone in *Escherichia coli*. Biochim Biophys Acta **1273:**165-70.

75. **Hazell, S. L., D. J. Evans, Jr., and D. Y. Graham.** 1991. *Helicobacter pylori* catalase. J Gen Microbiol **137 (Pt 1):**57-61.

76. **Hazell, S. L., and D. Y. Graham.** 1990. Unsaturated fatty acids and viability of Helicobacter (Campylobacter) pylori. J Clin Microbiol **28:**1060-1.

77. **Hevel, J. M., K. A. White, and M. A. Marletta.** 1991. Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein. J Biol Chem **266:**22789-91.

78. **Hulten, K., H. Enroth, T. Nystrom, and L. Engstrand.** 1998. Presence of Helicobacter species DNA in Swedish water. J Appl Microbiol **85:**282-6.

79. **Illingworth, D. S., K. S. Walter, P. L. Griffiths, and R. Barclay.** 1993. Siderophore production and iron-regulated envelope proteins of *Helicobacter pylori*. Zentralbl Bakteriol **280:**113-9.

80. **Israel, D. A., and R. M. Peek, Jr.** 2006. The role of persistence in *Helicobacter pylori* pathogenesis. Curr Opin Gastroenterol **22:**3-7.

81. **Israel, D. A., N. Salama, C. N. Arnold, S. F. Moss, T. Ando, H. P. Wirth, K. T. Tham, M. Camorlinga, M. J. Blaser, S. Falkow, and R. M. Peek, Jr.** 2001. *Helicobacter pylori* strain-specific differences in genetic content, identified by microarray, influence host inflammatory responses. J. Clin. Invest. **107:**611-620.

82. **Iwamoto, H., D. M. Czajkowsky, T. L. Cover, G. Szabo, and Z. Shao.** 1999. VacA from *Helicobacter pylori*: a hexameric chloride channel. FEBS Lett **450:**101-4.

83. **Jones, A. C., R. P. Logan, S. Foynes, A. Cockayne, B. W. Wren, and C. W. Penn.** 1997. A flagellar sheath protein of *Helicobacter pylori* is identical to HpaA, a putative N-acetylneuraminyllactose-binding hemagglutinin, but is not an adhesin for AGS cells. J Bacteriol **179:**5643-7.

84. **Jungblut, P. R., D. Bumann, G. Haas, U. Zimny-Arndt, P. Holland, S. Lamer, F. Siejak, A. Aebischer, and T. F. Meyer.** 2000. Comparative proteome analysis of *Helicobacter pylori*. Mol Microbiol **36:**710-25.

85. **Kather, B., K. Stingl, M. E. van der Rest, K. Altendorf, and D. Molenaar.** 2000. Another unusual type of citric acid cycle enzyme in *Helicobacter pylori*: the malate:quinone oxidoreductase. J Bacteriol **182:**3204-9.

86. **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:**249-60.

87. **Keyer, K., and J. A. Imlay.** 1996. Superoxide accelerates DNA damage by elevating free-iron levels. Proc Natl Acad Sci U S A **93:**13635-40.

88. **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:**19486-93.

89. **Kostrzynska, M., J. D. Betts, J. W. Austin, and T. J. Trust.** 1991. Identification, characterization, and spatial localization of two flagellin species in *Helicobacter pylori* flagella. J Bacteriol **173:**937-46.

90. **Kraft, C., and S. Suerbaum.** 2005. Mutation and recombination in *Helicobacter pylori*: mechanisms and role in generating strain diversity. Int J Med Microbiol **295:**299-305.

91. **Krishnamurthy, P., M. H. Parlow, J. Schneider, S. Burroughs, C. Wickland, N. B. Vakil, B. E. Dunn, and S. H. Phadnis.** 1999. Identification of a novel penicillin-binding protein from *Helicobacter pylori*. J Bacteriol **181:**5107-10.

92. **Labigne, A., V. Cussac, and P. Courcoux.** 1991. Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. J Bacteriol **173:**1920-31.

93. **Leunk, R. D., P. T. Johnson, B. C. David, W. G. Kraft, and D. R. Morgan.** 1988. Cytotoxic activity in broth-culture filtrates of Campylobacter pylori. J Med Microbiol **26:**93-9.

94. **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:**323-32.

95. **Leying, H., S. Suerbaum, G. Geis, and R. Haas.** 1992. Cloning and genetic characterization of a *Helicobacter pylori* flagellin gene. Mol Microbiol **6:**2863-74.

96. **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:**348-52.

97. **Lundstrom, A. M., K. Blom, V. Sundaeus, and I. Bolin.** 2001. HpaA shows variable surface localization but the gene expression is similar in different *Helicobacter pylori* strains. Microb Pathog **31:**243-53.

98. **Lynch, R. E., and B. C. Cole.** 1980. Mycoplasma pneumoniae: a prokaryote which consumes oxygen and generates superoxide but which lacks superoxide dismutase. Biochem Biophys Res Commun **96:**98-105.

99. **Mahdavi, J., B. Sonden, M. Hurtig, F. O. Olfat, L. Forsberg, N. Roche, J. Angstrom, T. Larsson, S. Teneberg, K. A. Karlsson, S. Altraja, T. Wadstrom, D. Kersulyte, D. E. Berg, A. Dubois, C. Petersson, K. E. Magnusson, T. Norberg, F. Lindh, B. B. Lundskog, A. Arnqvist, L. Hammarstrom, and T. Boren.** 2002. *Helicobacter pylori* SabA adhesin in persistent infection and chronic inflammation. Science **297:**573-8.

100. **Maier, R. J., C. Fu, J. Gilbert, F. Moshiri, J. Olson, and A. G. Plaut.** 1996. Hydrogen uptake hydrogenase in *Helicobacter pylori*. FEMS Microbiol Lett **141:**71-6.

101. **Marais, A., G. L. Mendz, S. L. Hazell, and F. Megraud.** 1999. Metabolism and genetics of *Helicobacter pylori*: the genome era. Microbiol Mol Biol Rev **63:**642-74.

102. **Marcelli, S. W., H. T. Chang, T. Chapman, P. A. Chalk, R. J. Miles, and R. K. Poole.** 1996. The respiratory chain of *Helicobacter pylori*: identification of cytochromes and the effects of oxygen on cytochrome and menaquinone levels. FEMS Microbiol Lett **138:**59-64.

103. **Marchini, A., M. d'Apolito, P. Massari, M. Atzeni, M. Copass, and R. Olivieri.** 1995. Cyclodextrins for growth of *Helicobacter pylori* and production of vacuolating cytotoxin. Arch Microbiol **164:**290-3.

104. **Marletta, M. A.** 1994. Nitric oxide synthase: aspects concerning structure and catalysis. Cell **78:**927-30.

105. **Marnett, L. J.** 2000. Oxyradicals and DNA damage. Carcinogenesis **21:**361-70.

106. **Marshall, B. J., R. W. McCallum, and C. Prakash.** 1987. Campylobacter pyloridis and gastritis. Gastroenterology **92:**2051.

107. **Marshall, B. J., and J. R. Warren.** 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet **1:**1311-5.

108. **Megraud, F., S. Hazell, and Y. Glupczynski.** 2001. Antibiotic susceptibility and resistance. ASM Press, Washington, D. C.

109. **Megraud, F., and H. Lamouliatte.** 1992. *Helicobacter pylori* and duodenal ulcer. Dig. Dis. Sci. **37:**769-772.

110. **Megraud, F., S. Hazell, and Y. Glupczynski.** 2001. Antibiotic susceptibility and resistance. ASM Press. Washington. D.C.

111. **Melkani, G. C., C. McNamara, G. Zardeneta, and J. A. Mendoza.** 2004. Hydrogen peroxide induces the dissociation of GroEL into monomers that can facilitate the reactivation of oxidatively inactivated rhodanese. Int J Biochem Cell Biol **36:**505-18.

112. **Mendz, G. L., and S. L. Hazell.** 1993. Glucose phosphorylation in *Helicobacter pylori*. Arch. Biochem. Biophys. **300:**522-525.

113. **Mendz, G. L., S. L. Hazell, and B. P. Burns.** 1994. The Entner-Doudoroff pathway in *Helicobacter pylori*. Arch. Biochem. Biophys. **312:**349-356.

114. **Mendz, G. L., S. L. Hazell, and B. P. Burns.** 1993. Glucose utilization and lactate production by *Helicobacter pylori*. J. Gen. Microbiol. **139:**3023-3028.

115. **Mendz, G. L., S. L. Hazell, and L. van Gorkom.** 1994. Pyruvate metabolism in *Helicobacter pylori*. Arch. Microbiol. **162:**187-192.

116. **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:**6510-25.

117. **Metchinkoff, E.** 1968. Immunity in infective diseases. NY Johnson Reprint Corporation.

118. **Michaels, M. L., J. Tchou, A. P. Grollman, and J. H. Miller.** 1992. A repair system for 8-oxo-7,8-dihydrodeoxyguanine. Biochemistry **31:**10964-8.

119. **Mitchell, H. M.** 2001. Epidemiology of infection. ASM Press, Washington, D. C.

120. **Mobley, H. L. T.** 2001. Urease. ASM Press, Washington, D. C.

121. **Mobley, H. L. T.** 2001. Urease. ASM Press. Washington. D.C.

122. **Modrich, P., and R. Lahue.** 1996. Mismatch repair in replication fidelity, genetic recombination, and cancer biology. Annu Rev Biochem **65:**101-33.

123. **Moese, S., M. Selbach, T. Kwok, V. Brinkmann, W. Konig, T. F. Meyer, and S. Backert.** 2004. *Helicobacter pylori* induces AGS cell motility and elongation via independent signaling pathways. Infect Immun **72:**3646-9.

124. **Moran, A. P., I. M. Helander, and T. U. Kosunen.** 1992. Compositional analysis of *Helicobacter pylori* rough-form lipopolysaccharides. J Bacteriol **174:**1370-7.

125. **Moskovitz, J.** 2005. Methionine sulfoxide reductases: ubiquitous enzymes involved in antioxidant defense, protein regulation, and prevention of aging-associated diseases. Biochim Biophys Acta **1703:**213-9.

126. **Moskovitz, J., B. S. Berlett, J. M. Poston, and E. R. Stadtman.** 1999. Methionine sulfoxide reductase in antioxidant defense. Methods Enzymol **300:**239-44.

127. **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:**14167-72.

128. **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:**502-7.

129. **Moskovitz, J., and E. R. Stadtman.** 2003. Selenium-deficient diet enhances protein oxidation and affects methionine sulfoxide reductase (MsrB) protein level in certain mouse tissues. Proc Natl Acad Sci U S A **100:**7486-90.

130. **Mulrooney, S. B., and R. P. Hausinger.** 1990. Sequence of the Klebsiella aerogenes urease genes and evidence for accessory proteins facilitating nickel incorporation. J Bacteriol **172:**5837-43.

131. **Muotiala, A., I. M. Helander, L. Pyhala, T. U. Kosunen, and A. P. Moran.** 1992. Low biological activity of *Helicobacter pylori* lipopolysaccharide. Infect Immun **60:**1714-6.

132. **Naumann, M.** 2005. Pathogenicity island-dependent effects of *Helicobacter pylori* on intracellular signal transduction in epithelial cells. Int J Med Microbiol **295:**335-41.

133. **Neiers, F., A. Kriznik, S. Boschi-Muller, and G. Branlant.** 2004. Evidence for a new sub-class of methionine sulfoxide reductases B with an alternative thioredoxin recognition signature. J Biol Chem **279:**42462-8.

134. **O'Toole, P. W., M. Kostrzynska, and T. J. Trust.** 1994. Non-motile mutants of *Helicobacter pylori* and *Helicobacter mustelae* defective in flagellar hook production. Mol. Microbiol. **14:**691-703.

135. **Odenbreit, S., J. Puls, B. Sedlmaier, E. Gerland, W. Fischer, and R. Haas.** 2000. Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. Science **287:**1497-500.

136. **Odenbreit, S., B. Wieland, and R. Haas.** 1996. Cloning and genetic characterization of *Helicobacter pylori* catalase and construction of a catalase-deficient mutant strain. J Bacteriol **178:**6960-7.

137. **Olczak, A. A., J. W. Olson, and R. J. Maier.** 2002. Oxidative-stress resistance mutants of *Helicobacter pylori*. J Bacteriol **184:**3186-93.

138. **Olczak, A. A., R. W. Seyler, Jr., J. W. Olson, and R. J. Maier.** 2003. Association of *Helicobacter pylori* antioxidant activities with host colonization proficiency. Infect Immun **71:**580-3.

139. **Olczak, A. A., G. Wang, and R. J. Maier.** 2005. Up-expression of NapA and other oxidative stress proteins is a compensatory response to loss of major *Helicobacter pylori* stress resistance factors. Free Radic Res **39:**1173-82.

140. **Olry, A., S. Boschi-Muller, and G. Branlant.** 2004. Kinetic Characterization of the Catalytic Mechanism of Methionine Sulfoxide Reductase B from *Neisseria meningitidis*. Biochemistry **43:**11616-22.

141. **Olson, J. W., J. N. Agar, M. K. Johnson, and R. J. Maier.** 2000. Characterization of the NifU and NifS Fe-S cluster formation proteins essential for viability in *Helicobacter pylori*. Biochemistry **39:**16213-9.

142. **Olson, J. W., and R. J. Maier.** 2002. Molecular hydrogen as an energy source for *Helicobacter pylori*. Science **298:**1788-90.

143. **Pakodi, F., O. M. Abdel-Salam, A. Debreceni, and G. Mozsik.** 2000. *Helicobacter pylori*. One bacterium and a broad spectrum of human disease! An overview. J Physiol Paris **94:**139-52.

144. **Papini, E., B. Satin, N. Norais, M. de Bernard, J. L. Telford, R. Rappuoli, and C. Montecucco.** 1998. Selective increase of the permeability of polarized epithelial cell monolayers by *Helicobacter pylori* vacuolating toxin. J Clin Invest **102:**813-20.

145. **Parsonnet, J., G. D. Friedman, D. P. Vandersteen, Y. Chang, J. H. Vogelman, N. Orentreich, and R. K. Sibley.** 1991. *Helicobacter pylori* infection and the risk of gastric carcinoma. N. Engl. J. Med. **325:**1127-1131.

146. **Parsonnet, J., G. D. Friedman, D. P. Vandersteen, Y. Change, J. H. Vogelman, N. Orentreich, and R. K. Sibley.** 1991. *Helicobacter pylori* infection and the risk of gastric carcinoma. N. Engl. J. Med. **325:**1127-1131.

147. **Parsonnet, J. S., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warnke, E. Jellum, N. Orentreich, J. H. Vogelman, and G. D. Friedman.** 1994. *Helicobacter pylori* infection and gastric lymphoma. N. Engl. J. Med. **330:**1267-1271.

148. **Pesci, E. C., and C. L. Pickett.** 1994. Genetic organization and enzymatic activity of a superoxide dismutase from the microaerophilic human pathogen, *Helicobacter pylori*. Gene **143:**111-6.

149. **Replogle, M. L., S. L. Glaser, R. A. Hiatt, and J. Parsonnet.** 1995. Biologic sex as a risk factor for *Helicobacter pylori* infection in healthy young adults. Am J Epidemiol **142:**856-63.

150. **Reynolds, D. J., and C. W. Penn.** 1994. Characteristics of *Helicobacter pylori* growth in a defined medium and determination of its amino acid requirements. Microbiology **140 (Pt 10):**2649-56.

151. **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:**613-21.

152. **Samuni, A., C. D. Black, C. M. Krishna, H. L. Malech, E. F. Bernstein, and A. Russo.** 1988. Hydroxyl radical production by stimulated neutrophils reappraised. J Biol Chem **263:**13797-801.

153. **Schmitz, A., C. Josenhans, and S. Suerbaum.** 1997. Cloning and characterization of the *Helicobacter pylori* flbA gene, which codes for a membrane protein involved in coordinated expression of flagellar genes. J Bacteriol **179:**987-97.

154. **Segal, E. D., J. Cha, J. Lo, S. Falkow, and L. S. Tompkins.** 1999. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. Proc Natl Acad Sci U S A **96:**14559-64.

155. **Seyler, R. W., Jr., J. W. Olson, and R. J. Maier.** 2001. Superoxide dismutase-deficient mutants of *Helicobacter pylori* are hypersensitive to oxidative stress and defective in host colonization. Infect Immun **69:**4034-40.

156. **Shigenaga, M. K., C. J. Gimeno, and B. N. Ames.** 1989. Urinary 8-hydroxy-2'-deoxyguanosine as a biological marker of in vivo oxidative DNA damage. Proc Natl Acad Sci U S A **86:**9697-701.

157. **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:**2739-47.

158. **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:**3037-45.

159. **Slungaard, A., and J. R. Mahoney, Jr.** 1991. Thiocyanate is the major substrate for eosinophil peroxidase in physiologic fluids. Implications for cytotoxicity. J Biol Chem **266:**4903-10.

160. **Smith, R. M., and J. T. Curnutte.** 1991. Molecular basis of chronic granulomatous disease. Blood **77:**673-86.

161. **Spiegelhalder, C., B. Gerstenecker, A. Kersten, E. Schiltz, and M. Kist.** 1993. Purification of *Helicobacter pylori* superoxide dismutase and cloning and sequencing of the gene. Infect Immun **61:**5315-25.

162. **St John, G., N. Brot, J. Ruan, H. Erdjument-Bromage, P. Tempst, H. Weissbach, and C. Nathan.** 2001. Peptide methionine sulfoxide reductase from *Escherichia coli* and *Mycobacterium tuberculosis* protects bacteria against oxidative damage from reactive nitrogen intermediates. Proc Natl Acad Sci U S A **98:**9901-6.

163. **Stadtman, E. R., and R. L. Levine.** 2003. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. Amino Acids **25:**207-18.

164. **Stadtman, E. R., and R. L. Levine.** 2000. Protein oxidation. Ann N Y Acad Sci **899:**191-208.

165. **Stadtman, E. R., J. Moskovitz, and R. L. Levine.** 2003. Oxidation of methionine residues of proteins: biological consequences. Antioxid Redox Signal **5:**577-82.

166. **Stohs, S. J.** 1995. The role of free radicals in toxicity and disease. J Basic Clin Physiol Pharmacol **6:**205-28.

167. **Stohs, S. J., and D. Bagchi.** 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radic Biol Med **18:**321-36.

168. **Storz, G., and J. A. Imlay.** 1999. Oxidative stress. Curr Opin Microbiol **2:**188-94.

169. **Taylor, D. E., M. Eaton, N. Chang, and S. M. Salama.** 1992. Construction of a *Helicobacter pylori* genome map and demonstration of diversity at the genome level. J. Bacteriol. **174:**6800-6806.

170. **Teneberg, S., H. Miller-Podraza, H. C. Lampert, D. J. Evans, Jr., D. G. Evans, D. Danielsson, and K. A. Karlsson.** 1997. Carbohydrate binding specificity of the neutrophil-activating protein of *Helicobacter pylori*. J Biol Chem **272:**19067-71.

171. **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 A resolution. Structure Fold Des **8:**1167-78.

172. **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 Biol Crystallogr **56 (Pt 9):**1194-7.

173. **Thomas, J. E., G. R. Gibson, M. K. Darboe, A. Dale, and L. T. Weaver.** 1992. Isolation of *Helicobacter pylori* from human faeces. Lancet **340:**1194-5.

174. **Thomas, J. E., G. R. Gibson, M. K. Darboe, A. Dale, and L. T. Weaver.** 1992. Isolation of *Helicobacter pylori* from human faeces. Lancet **340:**1194-1195.

175. **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, and et al.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-47.

176. **Tombola, F., L. Morbiato, G. Del Giudice, R. Rappuoli, M. Zoratti, and E. Papini.** 2001. The *Helicobacter pylori* VacA toxin is a urea permease that promotes urea diffusion across epithelia. J Clin Invest **108:**929-37.

177. **Tonello, F., W. G. Dundon, B. Satin, M. Molinari, G. Tognon, G. Grandi, G. Del Giudice, R. Rappuoli, and C. Montecucco.** 1999. The *Helicobacter pylori* neutrophil-activating protein is an iron-binding protein with dodecameric structure. Mol Microbiol **34:**238-46.

178. **Touati, D.** 2000. Iron and oxidative stress in bacteria. Arch Biochem Biophys **373:**1-6.

179. **Unemo, M., M. Aspholm-Hurtig, D. Ilver, J. Bergstrom, T. Boren, D. Danielsson, and S. Teneberg.** 2005. The sialic acid binding SabA adhesin of *Helicobacter pylori* is essential for nonopsonic activation of human neutrophils. J Biol Chem **280:**15390-7.

180. **Vogt, W.** 1995. Oxidation of methionyl residues in proteins: tools, targets, and reversal. Free Radic Biol Med **18:**93-105.

181. **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:**51908-14.

182. **Wang, G., R. C. Conover, A. A. Olczak, P. Alamuri, M. K. Johnson, and R. J. Maier.** 2005. Oxidative stress defense mechanisms to counter iron-promoted DNA damage in *Helicobacter pylori*. Free Radic Res **39:**1183-91.

183. **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:**1391-6.

184. **Wang, G., A. A. Olczak, J. P. Walton, and R. J. Maier.** 2005. Contribution of the *Helicobacter pylori* thiol peroxidase bacterioferritin comigratory protein to oxidative stress resistance and host colonization. Infect Immun **73:**378-84.

185. **Weitzman, S. A., P. W. Turk, D. H. Milkowski, and K. Kozlowski.** 1994. Free radical adducts induce alterations in DNA cytosine methylation. Proc Natl Acad Sci U S A **91:**1261-4.

186. **Whittenbury, R.** 1964. Hydrogen Peroxide Formation and Catalase Activity in the Lactic Acid Bacteria. J Gen Microbiol **35:**13-26.

187. **Wizemann, T. M., J. Moskovitz, B. J. Pearce, D. Cundell, C. G. Arvidson, M. So, H. Weissbach, H. R. Masure, N., and.** 1996. Peptide methionine sulfoxide reductase contributes to the maintenance of adhesins in three major pathogens. Proc Natl Acad Sci U S A **93:**7985-90.

188. **Wu, J., F. Neiers, S. Boschi-Muller, and G. Branlant.** 2005. The N-terminal domain of PILB from *Neisseria meningitidis* is a disulfide reductase that can recycle methionine sulfoxide reductases. J Biol Chem.

189. **Zheng, H. Y., T. M. Alcorn, and M. S. Cohen.** 1994. Effects of H2O2-producing *Lactobacilli* on *Neisseria gonorrhoeae* growth and catalase activity. J Infect Dis **170:**1209-15.

Chapter 2

Methionine sulfoxide reductase is an important antioxidant enzyme in the gastric pathogen *Helicobacter pylori*

Alamuri, P and R. J. Maier. 2004. Mol. Microbiol.**53**:1397-406
Reprinted here with permission of publisher. 08/10/2005

ABSTRACT

The ability of *H. pylori* to colonize the stomach requires that it combat oxidative stress responses imposed by the host. The role of methionine sulfoxide reductase, a methionine repair enzyme, in *H. pylori* stress resistance was evaluated by a mutant analysis approach. An *msr* mutant strain lacked immonologically detectable sulfoxide reductase protein and also showed no enzyme activity when provided with oxidized methionines as substrates. The mutant strain showed diminished growth compared to the parent strain in the presence of chemical oxidants, and showed rapid viability loss when exposed to oxidizing conditions. The stress resistance and enzyme activity could be recovered by complementing the mutant with a functional copy of the *msr* gene. Upon fractionation of parent strain and the complemented mutant cells into membranes and cytoplasmic proteins, most of the immunologically-detectable Msr was localized to the membrane, and this fraction contained all of the Msr activity. Qualitative detection of the whole cell protein pattern using 2,4-Dinitro phenyl hydrazine (DNPH) showed a far greater number of oxidized protein species in the mutant than in the parent strain when subjected to oxygen, peroxide or s-nitrosoglutathione (GSNO) induced stress. Importantly, no oxidized proteins were discerned in either strain upon incubation in anaerobic conditions. A mutant strain that synthesized a truncated Msr (corresponding to the MsrA domain) was slightly more resistant to oxidative stress than the *msr* strain. Mouse colonization studies showed Msr is an important colonization factor, especially for effective longer-term (14 and 21 day) colonization. Complementation of the mutant *msr* strain by chromosomal insertion of a functional gene restored mouse colonization ability.

INTRODUCTION

Helicobacter pylori is a microaerophilic bacterium that is one of the most common human gastrointestinal pathogens, colonizing almost two-thirds of the worlds population (13). It has been identified as the causative organism for chronic superficial gastritis and peptic ulcer disease in humans and is linked to the development of adenocarcinoma of the stomach (2). The clinical significance of this bacterium has bolstered molecular research involving both initial colonization determinants such as flagella synthesis (15) and enzymes such as urease (14) as well as later stage cell recognition factors and antigens that are important for maintenance of the bacterium within the host (9, 18, 22).

Oxidative stress and nitrosative stress are important factors that *H. pylori* must combat to colonize the human stomach, especially after inflammatory host responses are triggered. Therefore, the pathogenesis of *H. pylori* relies on its ability to survive harsh conditions in the stomach, which includes attack by phagocytes that release reactive oxygen species and nitrogen intermediates (ROS / RNI) (13). These reactive species can even cause measurable host cell damage. A repertoire of *H. pylori* antioxidant enzymes such as Superoxide dismutase (SOD), Alkyl hydroxyperoxide reductase C (AhpC), Catalase (KatA), Neutrophil activating protein (NapA), Thiol peroxidase (Tpx) and others (such as an NADPH quinine reductase) protect the bacteria from various ROS/RNI released by the host immune system (7, 8, 16, 27, 28, 32). Independent mutations in these genes render the bacterium highly sensitive to chemical oxidants *in vitro* and concomitantly diminish the ability of *H. pylori* to colonize the mouse stomach. Taken together, the results underscore the significance of a strong multi-faceted antioxidant system for the survival of this bacterium.

Another form of antioxidant defense mechanism not yet addressed in *H. pylori* is the repair of proteins damaged by oxidation. Upon oxidation, methionine and cysteine residues are the only amino acids that can be converted back to the reduced forms by their specific reductases (40). The reversible oxidation of these residues enables cells to use them as a temporary sink for ROS and RNI thereby helping to protect sensitive macromolecules from oxidative damage (20, 24, 36, 40). The enzyme methionine sulfoxide reductase (Msr) is encoded by two genes called *msrA* and *msrB* in most bacteria, wherein each gene encodes a protein that catalyzes the reversible oxidation-reduction of sulfur atoms associated with methionine residues. MsrB catalyzes the reduction of Met (R) O to methionine both within proteins and when present as free Met (R) O, whereas MsrA catalyzes the reduction of Met (S) O in a thioredoxin-thioredoxin reductase dependent reaction (3-5, 41).

A role for Msr in conferring resistance to various ROS and RNI has been previously shown in plant or human pathogens such as *Escherichia coli* (26), *Mycobacterium tuberculosis* (35), *Neisseria gonorrhoeae* (34), *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Saccharomyces cereviseae* (25), *Mycoplasma genitalium* (12), and *Erwinia chrysanthemi* (17). Inactivation of *msr* in these bacteria rendered the strains sensitive to oxidative or nitrosative stress and in most cases also impaired the ability of these pathogens to colonize their respective eukaryotic hosts (42). Based on the genome sequence of *H. pylori* the predicted Msr is composed of MsrA and MsrB proteins fused together as a single enzyme; such is also the case for some other human pathogens such as *N. gonorrhoeae,* and *H. influenzae.* For *H. pylori*, the gene is designated as *msr* (hp0224) in the published genome sequence of HP 26695.

Here we address the role of Msr in *H. pylori*; we asked if Msr plays a role in oxidative/nitrosative stress resistance in *H. pylori* and address its importance to stomach colonization. The gene *msr* was inactivated to create a complete knock-out strain and another one that still synthesizes an MsrA domain. Inactivation of this gene (in both cases) resulted in increased sensitivity to chemical oxidants. The comparison of the whole cell protein profile of the *msr* mutant to that of the parent strain showed that many proteins were susceptible to oxidation in the mutant strain but underwent little oxidation in the parent strain (HP 26695). In addition, the mutant's strains ability to colonize the host tissue was severely affected.

MATERIALS AND METHODS

Bacterial strains, media and plasmids used in this study. *Helicobacter pylori* 26695 was used as the parent strain. The Sydney strain, SS1 was used in mouse colonization studies. Cultures were grown at 37°C, under conditions of 5% CO_2 and 2 to 10% O_2 (varied as described) in a CO_2 incubator (Forma Scientific). Blood Agar (BA) plates contained Brucella agar (Difco), supplemented with 10% defibrinated sheep blood (Gibson) or 5% Bovine serum (Atlanta Biologicals) or in Muller Hinton broth (MH) (Difco) supplemented with 5% serum. Where necessary kanamycin or chloramphenicol were supplemented at a concentration of 30 μg/ml or 20 μg/ml.

Cloning was performed in *Escherichia coli* strain DH5α, grown in Luria-Bertani medium supplemented with either ampicillin (100 μg/ml) or kanamycin (25 μg/ml). Plasmids pGEM-T (Promega), pET21a (Novagen) and pBluescript KS(+) were used for cloning. Paraquat, hydrogen peroxide and *s*-nitrosoglutathione (GSNO), methyl p-tolyl (s) sulfoxide and methionine sulfoxide were all purchased from Sigma. Oxyblot-protein oxidation detection kit was purchased from Serologicals Ltd. (Atlanta, GA).

Inactivation of *msr* in *H. pylori*. A 1500 bp DNA fragment containing the *msr* gene with 300 bp upstream flanking sequence was amplified by PCR using primers msrF and msrR from the chromosomal DNA of HP 26695 and cloned into pGEM-T vector (Promega) to obtain pmsr. An *Eco*RV restriction site was created towards the 5'- end of the gene (nt. 124, in the region encoding MsrA domain) by overlapping PCR technique using primers msrnifF-msrmutR, msrmutF-, msrintR. The chromosomal kanamycin resistance gene *aph*A3 from *Campylobacter coli* was excised from plasmid pHP1 (37) via *Eco*RI digestion, gel purified (Qiagen), and blunted with T4 DNA polymerase. The *msr* gene was then inactivated by inserting a 1300 bp blunt *aph*A3 fragment into the *Eco*RV site towards the 5'-end (nt.124), giving rise to pmsrΔKm. To inactivate the putative *msrB* region of the gene, *msr* was amplified using msrBF and msrBR with a 400 bp 3' flanking sequence and cloned into pKS and the gene *aph*A3 was inserted at the *Afl*II site towards the 3'-end (nt. 922, in the region encoding the MsrB domain) to obtain pmsrBΔKm. The plasmids pmsrΔKm and pmsrBΔKm were then naturally transformed into HP 26695 to obtain Km^r *msr* mutants. Mutant strains were obtained via allelic exchange of the chromosomally encoded *msr* with the plasmids containing the inactivated gene. Mutants were selected on blood agar containing 30µg/ml kanamycin and incubated at 2% oxygen, by the use of gas environment controlled incubators. The mutant strains were subsequently screened by PCR to confirm the correct insertion of *aph*A3 at the corresponding gene on the chromosome and the size of the PCR products in the mutants was compared to that from the parent strain.

Detection of Msr in HP 26695, *msrB* and *msr* by immunoblotting. The gene *msr* was cloned into pET21a and the C-terminus his-tag version of the protein was over-expressed in *E. coli* BL21 *origami*.

Table 2.1: Primers used for inactivation, complementation and over expression of *msr*.

Designation	**Sequence 5’ → 3’**	**Site**
msrF	GGCATATGAAGGTATTATCTTATTTG	*Nde*I
msrN	GGCCTAGGTTAATGCGACTTTTTATCATTG	*Bam*HI
msrBF	TTCACCAGCAAGCGATAATCTCG	
msrBR	AGGGTAATGATGTGGGCAGG	
msrmutF	AGACGCAAGCTCTGATATCGCTAACGGCAAG	*Eco*RV
msrmutR	CTTGCCGTTAGCGATATCAGAGCTTGCGTCT	*Eco*RV
nifmsrF	ATAAAACGGCGTTTTTGGGCGAAG	
msrintR	ATCAATATGGCAATAACCACTAGGG	
msrcF	CCTTAAACCGCCATGATTGTG	
Hp 405 F	GAACGGGAATTCTTACGCC	
Cm R	AATGGGTTATCTCGGCGGTCACTC	

Msr 6x-His was purified using Ni-NTA Agarose affinity purification (Qiagen) followed by HiPrep® Sephcryl S-100 High Resolution Gel Filtration Chromatography (Phramacia Biotech). Rabbit anti-Msr antibody was raised against the purified recombinant Msr of *H. pylori* by Cocalico Biological Inc. To determine the protein synthesized in the mutants as compared to the parent strain, whole cell extracts from HP 26695, *msrB*, and *msr* were obtained by passing the cells through French Pressure Cell three times. Electrophoresis of the whole cell extracts was done immediately after centrifugation of cell lysates. To separate the membrane and cytoplasmic fraction (includes periplasmic fraction), the whole cell extracts were further centrifuged at 60,000 rpm in Beckman-Coulter Optima LE-80K ultra-centrifuge rotor type Ti-70 for 3 h. The purity of each fraction was assessed by monitoring hydogenase activity as a marker, a known membrane protein in *H. pylori*. Fractions were tested for hydrogenase activity under aerobic conditions using 100 µM methylene blue as the electron acceptor. The assays were performed as described elsewhere (21). Hydrogenase activity under aerobic conditions was calculated to be 0.61 ± 0.18 nmoles of H_2 oxidized/min/mg of protein for the cytoplasmic fraction whereas the membrane fraction showed a twenty-fold higher hydrogenase activity with 14.6 ± 1.68 nmoles of H_2 oxidized /min/mg of protein. These observations are in good agreement with the hydrogenase activities reported earlier in *H. pylori* (21). The fractions were later utilized for immunoblotting as well as methionine sulfoxide reductase activity. For immunoblotting, 7µg of cytoplasmic and membrane fractions from each of the strains was loaded onto a 12.5 % SDS PAGE. The proteins were then electro-transferred onto a nitrocellulose membrane (0.45µm-pore-size membrane; Osmonics) as described by Towbin *et al.,* (39). The membrane was then incubated with primary anti-Msr antiserum (1:1500 dilution) followed by incubation with secondary Goat-Anti-rabbit-Alkaline phosphatase (AP) conjugate antisera (1:2500 dilution) (ICN Biomedicals Inc.).

Determination of methionine sulfoxide reductase activity in HP 26695, *msr* mutants and merodiploid strain. Cytoplasmic and membrane extracts of HP 26695, *msrB*, *msr* and the merodiploid strains were tested separately for methionine sulfoxide reductase activity as described previously (34)**.** Briefly, 1 ml reaction mixture contained 100 mM $MgCl_2$, 200 mM KCl, 10 μg of protein in 10 mM Tris, 200 μM NADPH, 200 mM DTT and 200 μM substrate in Tris-Cl (pH 7.4). Reduction of the substrate and the Msr activity of the extracts were measured. Oxidation of NADPH was measured at 340 nm in Beckman DU-640B spectrophotometer. Enzyme activity was calculated as 1 unit=1 nmol of NAD(P)H oxidized/min/mg protein.

Growth in the presence of chemical oxidants. Disc inhibition assays were performed by plating HP 26695, *msr*, *msrB* and merodiploid cells separately on BA-serum plates. Approximately 10^8 cells were spread on the plate and 10 μl of the chemical oxidants, 2.0 mM paraquat, 300 mM H_2O_2, or 15 mM GSNO (s-nitrosoglutathione) were added separately onto sterile 7.5-mm filter paper disks on the individual plates and the plates were incubated at 2% oxygen for 72 hours. The diameter of zone of growth inhibition was recorded. Distilled water was added onto discs as a control.

Complementation of *msr* mutant. The gene msr along with its promoter region (1500 bp) was amplified using primers msrcF and msrN. This fragment was then cloned into the EcoRV site adjacent to the cat gene in pEU39cm. The plasmid pEU39cm has been shown to be an efficient tool for inserting DNA by homologous recombination into the region of the chromosome corresponding to strain HP 26695 hp0405. This gene encodes a NifS like protein and inactivation of this gene did not affect the growth rate, hydrogenase or the urease activities of the strain (29, 31)**.** The plasmid was then transformed into msr strain by natural transformation and selected on BA containing 30μg/ml chloramphenicol. This results in insertion of msr along with its promoter in hp0405 region on the chromosome to give a merodiploid (*msr*/pEU9cm-*msr*). Restoration of the functional Msr in the mutant strain was checked by immunoblotting the cell extract with anti-Msr antibody and also by determining the sensitivity of the merodiploid to GSNO, paraquat and hydrogen peroxide as described above. The membrane fractions of the merodiploid strain were also checked for Msr activity.

Sensitivity of non-growing cells. To measure sensitivity to O_2 , H_2O_2, and GSNO, cells were diluted to approximately 10^8 cells/ml (PBS) and sparged with anaerobic mixture containing 5% CO_2-10% H_2-85%-N_2 to flush out oxygen. 10^8 cells were then incubated at 2% partial pressure O_2 (O_2 was injected through the serum stopper), with 0.15 mM H_2O_2 and 2% oxygen or with 0.5 mM GSNO and 2% O_2 or exposed to atmospheric oxygen (serum-stopper removed). Samples were removed every 2 hours for a 12-hour incubation period and serial dilutions were plated on Brucella agar-serum plates. The plates were incubated in a 2% oxygen condition for 72 hours, and the colony numbers were determined for comparison. Mean Log_{10} CFU from five independent observations was determined and plotted against time in hours. For the SS1 survival curves, time points were taken at 4 hour intervals.

Detection of oxidized whole cell protein profiles. *Preparation of cell extracts.* HP 26695 and *msr* mutant strains were grown at 2% oxygen to late log phase on BA-serum plates. The cells were then suspended in sterile 25 ml MH broth with 5% calf serum. The tubes were tightly closed (sealed with serum stopper) and oxygen was injected to a final concentration of 2% partial pressure. H_2O_2 was added from a stock solution to the tube to obtain a final concentration of 0.3 mM in the culture. A similar procedure was followed to obtain cultures at 2% oxygen and 0.5 mM GSNO or 10% oxygen controlled atmosphere. Then the tubes were incubated at 37°C with shaking for 3 h. A dilution of cells was plated on BA-serum plates to determine the viable cell count before and after the treatment. Cells were centrifuged at 10,000 rpm in Avanti J-25 centrifuge at 4°C. The pellet was resuspended in 10 mM Tris-25 mM NaCl and 50 mM DTT pH 7.5. The cells were disrupted by sonication to obtain the extracts. The whole cell protein concentration was determined by Bradford assay (Bradford, 1976).

Derivitization of carbonyl groups. Oxyblot-Protein oxidation detection kit was purchased from Serologicals Ltd. (catalogue S-7150). 10 µg of extract from each treatment of parent strain and *msr* strain was mixed with 7 µl of 1x DNPH (dinitrophenyl hydrazine) solution (as described in the kit instructions). The tubes were incubated at 25°C for 30 minutes. Neutralization solution (7.5 µl) was added to the reaction mixture. The entire reaction mixture was then loaded on a 12.5% SDS-PAGE. Prederivitized protein markers (S-7150-3) provided in the kit were used.

Detection of oxidized proteins by Oxyblot. The proteins on the gel were electrotransferred onto a nitrocellulose membrane and blocked for 5 hours in PBS -0.5% Tween-20 and 1% BSA. The membrane was incubated in 1:250 dilution of Rabbit anti-DNPH antibody (S-7150-4) for 2 hours and then in Goat anti-Rabbit IgG Horse Raddish Peroxidase (HRP) conjugate (S-7150-5) (1:500) secondary antibody for 2 h. The appropriate substrate was added and the membrane was developed to visualize the oxidized proteins.

Mouse colonization assay. Mouse colonization was carried out in 5-6 week old, female, *Helicobacter*-free mouse C57BL/6J from Jackson Laboratories as described earlier (28, 30, 32)**.** *H. pylori* strain SS1 (19) was used for this purpose. Disruption of *msr* in SS1 was performed as described above for HP 26695. SS1 parent strain and the corresponding *msrB*, *msr* and merodiploid strains were all individually tested for their colonization efficiency. Cell suspensions were prepared by scraping plates after 48 hr growth on BA into PBS at an O. D. of 2.5 at 600 nm corresponding to 2.5 X 10^9 CFU/ml. The headspace above these suspensions was sparged with argon gas to maintain low oxygen exposure. Food, water and bedding material were removed 2 h prior to oral administration of the bacteria. Colonization was tested at 7, 14 and 21 days post inoculation. Twelve mice with each of the mutants (n=12 for both *msr* and *msrB*) and nine mice with strain SS1 (n=9) were infected by oral gavage with 150 µl of the individual cell suspensions containing 3.75×10^8 cells; this oral administration procedure was repeated two days after the first injection. Relative colonization abilities of parent SS1 strain and mutants was determined 7, 14, and 21 days after the second oral gavage administration, by procedures described earlier (30). As a control the merodiploid strain of SS1 (with a functional *msr* gene inserted into the chromosome of the *msr* strain) was inoculated in four mice and colonization was tested 14 day post (second) oral administration.

RESULTS

Inactivation of *msr* and immunoblotting experiments. Mutants were obtained by insertional inactivation of *msr* towards the 5' and 3' regions to separately inactivate *msrAB* and *msrB* regions of the gene and to determine the possible activity of the truncated protein (i.e., in the *msrB* strain). PCR confirmation of the mutant strains showed bands corresponding to 2.4 kb in the mutants as compared to 1.1 kb in HP26695 (data not shown). Sequence analysis of *msrB* showed that insertion of *aph*A3, a gene conferring kanamycin resistance, results in a stop codon 25 bp downstream from the *aph*A3 start site due to a frame-shift mutation. Immunoblots of cell extracts of strains (Fig 1A) showed that there was no protein formed in the *msrAB* strain where the gene was inactivated at the 5'- end (Fig. 2.1A, lane 2). A smaller ~30 kDa protein corresponding to the MsrA domain was observed (lane 5) in the extracts of the *msrB* strain, if the extracts were immediately subjected to gel electrophoresis after cell lysis (see experimental procedures), as compared to a 43 kDa Msr seen in HP 26695 (as expected from the sequence). As there was no Msr protein detected in the *msrAB* strain it will henceforth be referred to as *msr*.

Immunoblots were also performed on cell fractions (see experimental procedures) to address the localization of Msr (Fig. 2.1B). Hydrogenase is known to be membrane associated in *H. pylori* (21). To confirm the separation of the fractions (membrane versus cytoplasmic components) we used hydrogenase activity associated with each fraction as a marker. Both the parent strain and the merodiploid strain (the latter containing a functional *msr* gene introduced chromosomally into the mutant, see experimental procedures) produced an immunoreactive peptide at 43 kDa in both the membrane and cytoplasmic fractions. The intensity of the immunostained band is about equal for the two fractions (the cytoplasmic and membrane associated forms, see Fig. 2.1B), but based on the total protein recovery in the two fractions, the majority of Msr (~80%) is membrane associated. We could not detect immunologically-reactive Msr in the separated fractions of the *msrB* strain (data not shown) and we attribute this to instability of the protein upon breaking the cells.

Msr activity of membrane and cytoplasmic fractions of HP 26695 and *msr* mutants. Soluble and membrane-containing cell extracts of HP 26695, *msr* and *msrB* were each tested for their ability to reduce oxidized sulfur atoms when two different substrates were supplied; these are methionine sulfoxide and methyl p-tolyl (s) sulfoxide. The cytoplasmic fraction of each strain showed no detectable Msr activity; the background activity of the cytoplasmic fraction (NADPH oxidation without the sulfoxide substrate) was 25 ± 3 units and the cytoplasmic activity of various strains ranged from 24 to 28 units. However, the membrane fraction of the parent strain showed Msr activity clearly above the background (Table 2.2). This activity did not show preference for either one of the two oxidized sulfur substrates. The mutant strains however, lacked significant Msr activity in the membrane fraction (Table 2.2) even though an immunologically detectable protein (~30 kDa) was observed in the *msrB* strain.

Sensitivity of mutants to oxidative and nitrosative stress. In order to assess the importance of Msr to oxidative stress resistance, disk inhibition studies using paraquat, H_2O_2 and GSNO were performed. The mutants showed increased sensitivity compared to the parent strain to various chemical oxidants (Table 2.3). The *msrB* strain that appeared to be capable of synthesizing a partial Msr (related to MsrA domain) was sensitive to the oxidants (like the *msr* strain) but was less sensitive to stress agents than the *msr* strain. The difference in sensitivity observed between the *msrB* and *msr* strain was statistically significant due to the large number of independent replicate disk assays performed (see Table 2.3 legend). Therefore, some phenotypic function is ascribed to the truncated peptide corresponding to the MsrA domain.

Complementation of the *msr* strain. To make conclusions on the significance of *msr* in *H. pylori* based on the phenotype associated with the mutant strains, the mutation must be due to inactivation of *msr* only. Therefore, to associate the phenotype shown by the mutant to the inactivation of *msr*, we complemented the inactivated gene in the *msr* strain with a functional copy of *msr* introduced elsewhere in the genome. The complementation was confirmed by PCR where the merodiploid strain (*msr*/pEU39cm-*msr*) showed two bands when amplified from the genomic DNA, a 2.4 kb band corresponding to the inactivated gene at the original locus and the other 1.1 kb corresponding to the functional copy of the gene of interest (data not shown). A fully synthesized Msr from the membrane-associated fraction of the merodiploid strain was also confirmed by immunoblotting as seen in fig. 2.1B.

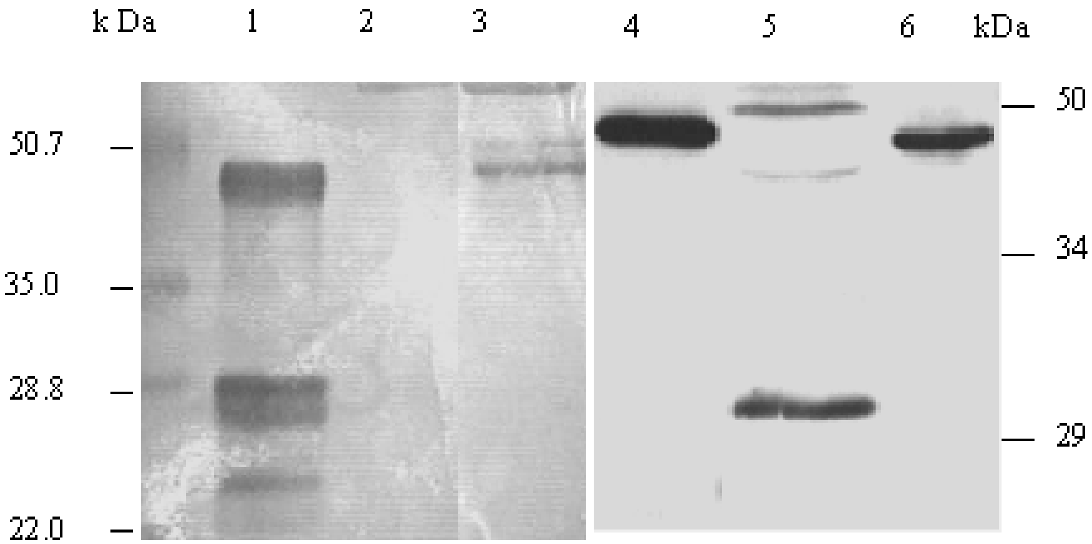


FIG. 2.1A. Immunoblot of whole cell extracts from HP26695, *msrB* and *msr*. Proteins were transferred onto a nitrocellulose membrane and probed with Rabbit anti-Msr antisera and then with Goatanti-rabbit HRP conjugate. The positions of molecular weight standards are indicated. Lanes 1 and 4: Purified Msr (43 kDa); Lane 2: *msr*, Lane 3 and 6: HP 26695 (43 kDa); Lane 5: *msrB* (~30 kDa). The two gels were probed with different anti-Msr serum samples (i.e., bleeds) that resulted in slightly different background signals. The 50 kDa band in the extracts of *msrB* (lane 5) is unrelated to Msr, and is observed in membrane-containing fractions in all the strains (see Fig. 2.1B).

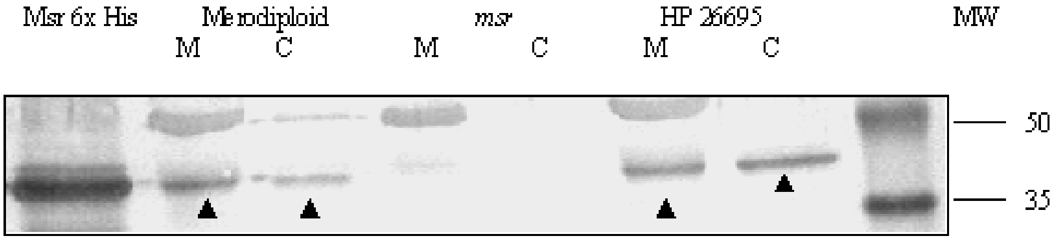


FIG. 2.1B. Detection of Msr in cytoplasmic and membrane fractions. The protein on the membrane was probed with Rabbit Anti-Msr antisera and Goat anti-rabbit Alkaline phosphatatse conjugate. Msr in each lane is indicated by an arrow. MW= Molecular weight standard, C = cytoplasm, and M=Membrane.

Table 2.2. **Membrane-associated Msr activity**. Msr activity was tested in parent strain, *msr* mutants and the merodiploid strains. Assay for Msr activity was performed on both the

cytoplasmic and membrane fractions of HP 26695, *msrB*, *msr* and the merodiploid strain, but only the membrane-associated activities are shown. Activity in the reaction mixture containing protein with NAD(P)H (without the substrates) was considered as background value. Specific activity of the membrane fractions after subtracting the background value (27.3 ± 2.8) from various strains is shown in the table. 1 unit of enzyme activity = 1 nmole of NAD(P)H oxidized/ min/mg of protein. Mean and SD values from ten independent replicates is shown.

Substrate	**HP 26695**	***msrB***	***msr***	**Merodiploid**
Methyl p-tolyl *(S)* sulfoxide	14.2 ± 1.1	0.8 ± 1.0	1.0 ± 2.3	14.0± 1.7
Methionine sulfoxide	13.6 ± 1.6	1.0 ± 1.5	0.5 ± 1.2	12.7 ± 1.4

Table 2.3: Growth of HP 26695, *msr*, *msrB* and merodiploid strains under various stress conditions. Mean ± SD of twelve independent observations (diameter of inhibition zone in mm)

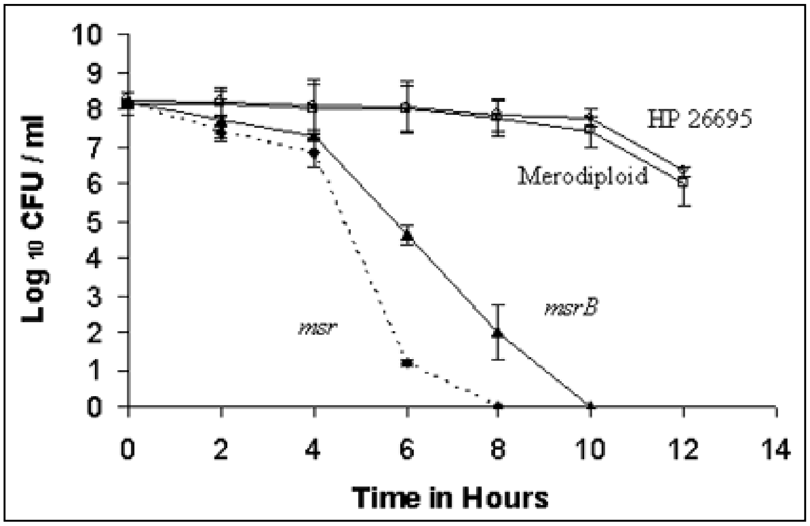
is given. An H_2O-treated control disc gave no inhibition for any strain. All of the stress agent results for the two mutant strains are significantly different (greater zone of inhibition) than both the parent strain or the merodiploid at the 99% level of confidence (α' = 0.01) according to the students' t – distribution test. The *msrB* results are significantly different for all stress reagents (smaller zone of inhibition) than the *msr* strain at the 95% level of confidence (α' = 0.05) or greater, with 22 degrees of freedom (n=12 for each strain).

Strain	**2.0 mM Paraquat**	**300 mM H_2O_2**	**15 mM GSNO**
HP 26695	6.2 ± 0.6	3.0 ± 0.3	4.5 ± 0.9
msr	18.6 ± 2.5	17.6 ± 0.6	10.8 ± 0.6
msrB	14 .0 ± 1.5	12.3 ± 1.3	7.4 ± 1.1
Merodiploid	7.0 ± 1.1	3.5 ± 0.7	5.2 ± 1.3

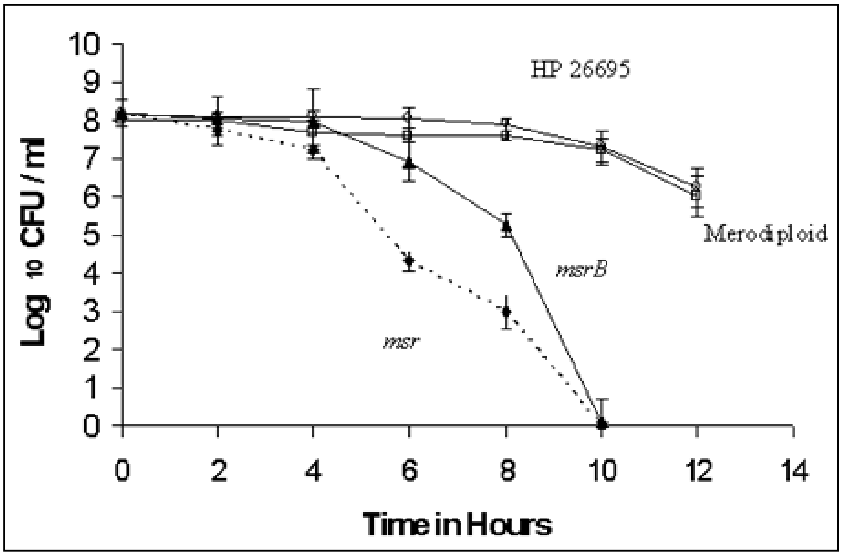
The sensitivity of this strain to paraquat, hydrogen peroxide and GSNO was also tested and it showed resistance like the parental HP 26695 strain (Table 2.3). The complemented strain also had Msr activity like the parent strain (Table 2.2); these results associate the observed characteristics of the mutant to loss of Msr activity.

Stress tolerance of non-growing cells. To more critically address the oxidative stress tolerance of the strains we measured the ability of non-growing cells to survive oxygen and chemical oxidants over a period of 12 h (Fig. 2.2). Both parent strain and the merodiploid strains were resistant to chemical oxidant-dependent killing for the first 8 hours, but showed loss of viability from an initial viable number of 10^8 cells to about 10^6 cells in the 12-h period of stress exposure. However, the *msr* strain was much more susceptible to the oxidants as seen by clear viability loss in the first 6 hours and no viable cells could be recovered at 8 h. This increased sensitivity to chemical oxidants and oxygen in the absence of Msr shows that the protein plays a significant role in protecting the cell from oxidative/nitrosative stress. Like the disk-sensitivity experiments, the *msrB* strain was slightly more resistant to the oxidants than the *msr* strain. This was true whether the experiments were done with HP 26695 as the parent or strain SS1 background (see below).

Detection of oxidized protein species in HP 26695 and *msr*. The comparative extent of protein carbonyl group formation in biological tissues has been a useful criterion for assaying oxidative modification of proteins; such a criterion has been used to study the oxidative repair capacity of both human brain and muscle tissues (1, 10, 11)**.** To help define the role that Msr plays in *H. pylori* we sought to estimate the protein carbonyls in the parent strain HP 26695 and the *msr* strain. Incubation of the cell extracts (see methods) with DNPH and immunoblotting the proteins with anti-DNPH antibody detected several prominent protein species with molecular masses ca. 95, 55, 43, 35, and many in the 20-30 kDa range that were oxidized in the absence of Msr (i.e., in

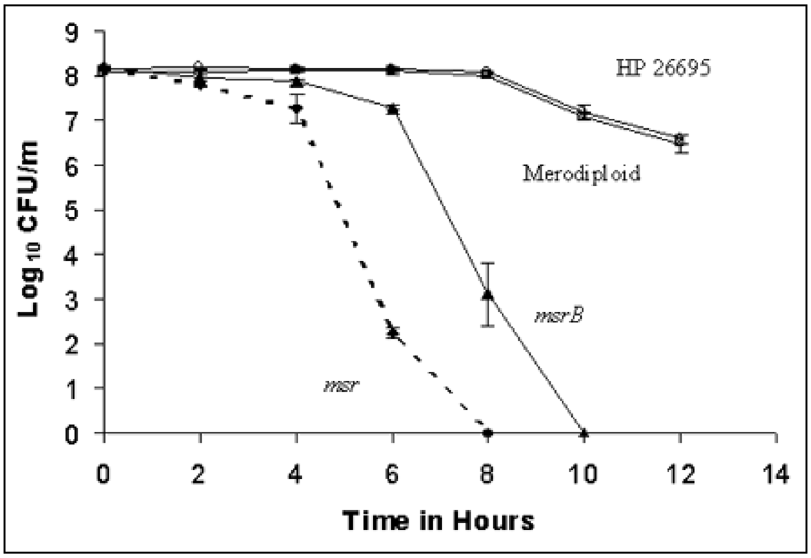


0.15 mM H_2O_2 and incubated at 2% partial pressure oxygen

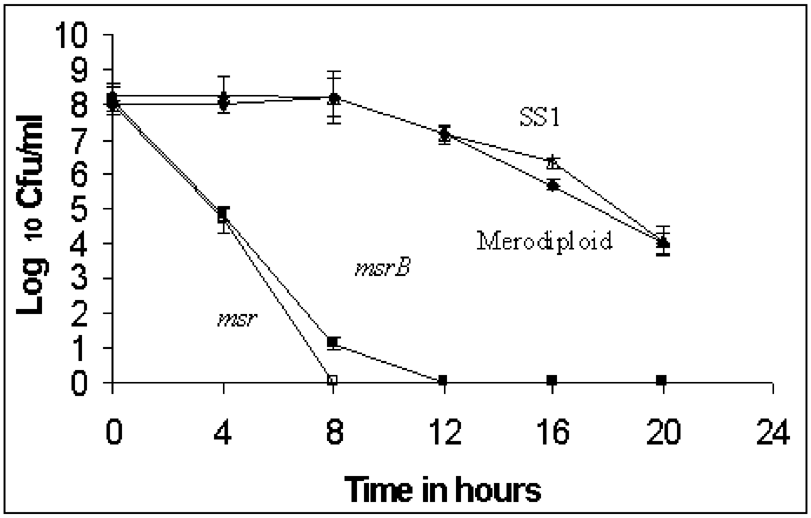


20 % oxygen

FIG. 2.2. Stress tolerance (survival) of non-growing cells. 10^8 cells/ml of HP 26695 (open circles), and *msr* (dotted lines), *msrB* (closed triangles), merodiploid strain (open squares) were inoculated in sterile PBS with the conditions mentioned below the graphs. Dilutions were plated from the culture at 2-h intervals over a 12-h period. Mean Log_{10} CFU/ml (y-axis) is plotted against time in hours (x-axis). Mean and standard deviation from eight independent observations is plotted.



0.5 mM GSNO and 2% oxygen



20% oxygen affects on SS1 strain and *msr* mutants in SS1background

the mutant strain) (Fig. 2.3B). The Ponceau-stained protein pattern was similar for both the strains (Fig. 2.3A). The specific oxidized protein species differed slightly depending on the particular stress conditions (oxygen, H_2O_2, or GSNO) and importantly no oxidized protein was detected in the mutant when it was incubated under anaerobic conditions without addition of chemical oxidants (see Fig. 2.3B lane 8). The oxidized protein profile for the *msrB* strain was not significantly different than from the *msr* strain (data not shown). The overall oxidized protein pattern observed in the mutant strains allows us to conclude that the repair function of Msr is important for maintenance of many proteins.

Mouse stomach colonization assays. Msr has been shown to be a virulence factor in some pathogens, based on the criteria that mutants deficient in the activity were unable to colonize the host tissues (42). To determine whether the repair activity of Msr was important to colonization by *H. pylori*, the relative abilities of the mouse adapted strain SS1, or *msr* mutants in the SS1 background were determined. A merodiploid strain in SS1 was also created as for strain 26695 described above. Sensitivity of HP 26695, the mutants and the merodiploid strain to various chemical oxidants was compared and it was observed that the mutants in SS1 showed a similar pattern in their sensitivity as we observed for the same mutations in strain 26695 (see Fig. 2.2D for oxygen affect, data not shown for H_2O_2 and GSNO affects). Colonization efficiency of strain SS1 (n=9 mice for each time point) and each of the *msr* mutant types (n=12 mice for each mutant for each time point) was determined at 7, 14, and 21 days post oral administration. Parent strain SS1 was recovered at Cfu's of (7 day) $5\text{x}10^3$ -- $2.2\text{x}10^5$, (14 day) $1.0 \text{ x } 10^4$ -- $5.0 \text{ x } 10^5$ and (21 day) $1.0 \text{ x } 10^5$-- $9.0 \text{ x } 10^5$ as the range per gm of stomach tissue from all inoculated mice.

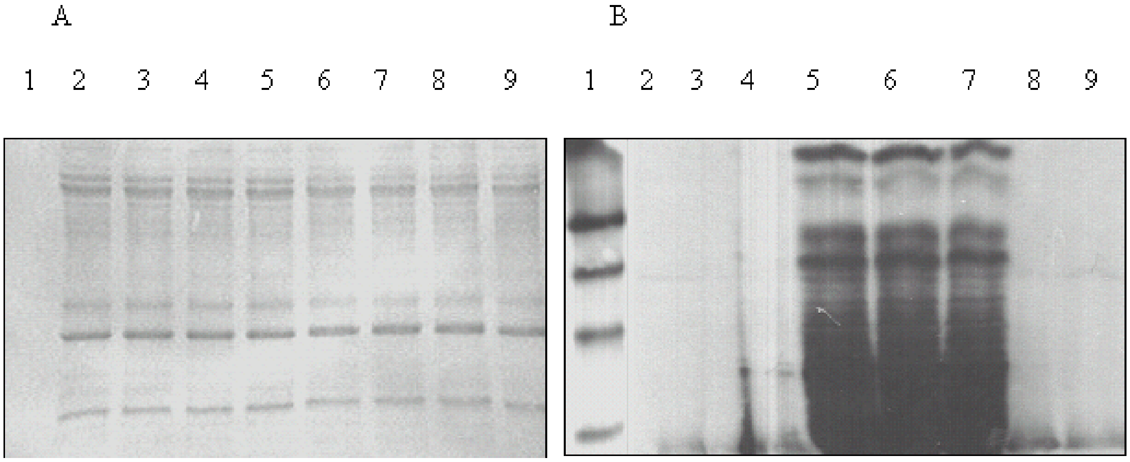


FIG 2.3. Oxyblot of whole cell extracts of HP 26695 and *msr* strains. Cells collected at mid-log phase were incubated for 3 hours under conditions described (see experimental procedures). Panel A: Nitrocellulose membrane stained with Ponceau. Panel B: Oxyblot film exposed for 30 secs. Lanes: **A1, B1**- Prederivitized marker (95 Da, 68 kDa, 43 kDa, 29 kDa, and 21 kDa); **A2, B2**: HP26695 incubated at 10% O_2; **A3, B3**: HP 26695incubated in 0.15 mM H_2O_2; **A4, B4**: HP 26695 incubated in 0.5 mM GSNO, **A5, B5**: *msr* at 10% O_2; **A6, B6**: *msr* incubated in 0.15 mM H_2O_2; **A7, B7**: *msr* incubated in 0.5 mM GSNO; **A8, B8**: *msr* incubated in 0% O_2; **A9, B9**: *msr* No DNPH added (negative control).

The mutant strains showed colonization only at the day 7 time point (3 colonization positives out of 12 mice each) and the colonization numbers were several fold less than that shown by the strain SS1. In both mutant cases (*msr* and *msrB)* the bacteria recovered ranged from 220 to about 500 Cfu / gm of mouse stomach. No bacteria were recovered from any of the stomachs harvested at the later time points for either of the mutant strains. It is noteworthy that *H. pylori* could be recovered at day 14 in the range of 1.6 x 10^3 – 2.0 x 10^5 Cfu / gm of stomach from all four mice that had been inoculated with the merodiploid strain. SS1 and mutant strains recovered from the stomach of the mice were confirmed both by microscopic examinations as well as by their ability to hydrolyze urea.

DISCUSSION

Methionine sulfoxide reductase in *H. pylori* strain 26695 is a 42 kDa protein encoded by a 1077 bp gene (hp0224). To be consistent with other studies we refer to this as Msr. A considerable amount of data has identified this protein as a conserved repair enzyme within bacteria and eukaryotes, including humans. Msr of *H. pylori* has 56% and 66% identity to the enzymes of the pathogens *N. gonorrhoeae* and *H. influenzae*, respectively. Like in *H. pylori* both of the latter pathogens express a single Msr, corresponding to the two fused domains MsrA and MsrB (24, 32). The role of Msr in *E. coli* and some pathogens has been well established to be a sulfoxide reductase of either methionines as individual residues or while in proteins. This serves to complement the role of other scavenging/reducing systems such as glutathione and thioredoxin. However, the absence of glutathione in *H. pylori* (33) likely makes the need for quenchers of reactive radicals involving a role for oxidation/reduction of methionine residues even more critical to this gastric bacterium.

Oxidation of proteins in the cell is one of the most important molecular consequences of oxidative stress (11)**,** but this aspect is not as well studied as mutation frequency or direct DNA damage for example. We sought to assess the oxidized protein patterns associated with loss of Msr, by an approach previously used only on human neurological and muscle tissue. The observation that the *msr* mutants showed a clearly greater number of oxidized protein species supports the conclusion that loss of methionine sulfoxide reductase results in a large increase of oxidized amino acids within many proteins, rather than specifically affecting a few proteins. However, the oxidized protein pattern we observed may not be due solely to methionine residues, as amino acids other than methionine are prone to oxidative modification (34); should the oxidation-reduction cycle of methionine residues be altered (by inactivation of *msr*) it is expected that the oxidative stress affecting all oxidizable amino acid residues would be greater. Consequently, the proteins observed as oxidized in the *msr* mutant would not be expected to be directly repaired by Msr.

From the immunoblotting experiments, we observed that Msr is localized both in membranes and in the cytoplasmic fraction of cell extracts. However, most of the methionine sulfoxide reductase activity was confined to the membrane-associated fraction. It was reported that Msr of *H. pylo*ri has an N-terminal signal sequence and that at least some of the protein is secreted outside of the cells or localized in small membrane vesicles not associated with whole cells (6)**.** A similar observation was made by Mintz and colleagues who studied the protein from the oral pathogen *Actinobacillus actinomycetemcomitans* (23)**.**

Our observations may be most like the results reported for Msr of *N. gonorrhoeae*, where the protein that contains a signal sequence and conferred protection against oxidative damage was located extra-cytoplasmically (to the outer membrane), whereas another form lacking the signal sequence was cytoplasmic and did not confer resistance to oxidative damage (34). We do not know if the cytoplasmic form we studied contains a signal sequence, but based on enzyme activity measurements it lacks significant activity, so some type of activation or processing of Msr may be dependent on localization. Based on SDS-PAGE analysis only, we are unable to detect a size difference between the two forms. Like the previous *N. gonorrhoeae* results, based on the location of the active form of Msr in *H. pylori*, the enzyme has the greatest potential for playing roles in protection of membrane associated, periplasmic, or outer membrane associated proteins, and important virulence-related proteins are thought to reside in all of those locales (38). One approach to help identify both its protective role with respect to its localization and its repair activity may be to identify the major proteins that undergo oxidation in various cell fractions of the *msr* strain.

ACKNOWLEDGEMENTS

This project was funded by NIH grant DK60061. Thanks to Maier, S. and Olczak, A. for helping with mouse stomach colonization assays. Thanks are also due to Drs. Benoit, S, Mehta, N and Wang Ge for their valuable suggestions and comments on this paper. This work was supported by the NIH grant DK60061 to RJM.

REFERENCES

1. **Aksenov, M. Y., M. V. Aksenova, D. A. Butterfield, J. W. Geddes, and W. R. Markesbery.** 2001. Protein oxidation in the brain in Alzheimer's disease. Neuroscience **103:**373-83.

2. **Blaser, M. J.** 1998. *Helicobacter pylori* and gastric diseases. Bmj **316:**1507-10.

3. **Brot, N., H. Fliss, T. Coleman, and H. Weissbach.** 1984. Enzymatic reduction of methionine sulfoxide residues in proteins and peptides. Methods Enzymol **107:**352-60.

4. **Brot, N., and H. Weissbach.** 2000. Peptide methionine sulfoxide reductase: biochemistry and physiological role. Biopolymers **55:**288-96.

5. **Brot, N., L. Weissbach, J. Werth, and H. Weissbach.** 1981. Enzymatic reduction of protein-bound methionine sulfoxide. Proc Natl Acad Sci U S A **78:**2155-8.

6. **Cao, P., M. S. McClain, M. H. Forsyth, and T. L. Cover.** 1998. Extracellular release of antigenic proteins by *Helicobacter pylori.* Infect Immun **66:**2984-6.

7. **Comtois, S. L., M. D. Gidley, and D. J. Kelly.** 2003. Role of the thioredoxin system and the thiol-peroxidases Tpx and Bcp in mediating resistance to oxidative and nitrosative stress in *Helicobacter pylori*. Microbiology **149:**121-9.

8. **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:**461-9.

9. **Cover, T. L., and M. J. Blaser.** 1999. *Helicobacter pylori* factors associated with disease. Gastroenterology **117:**257-61.

10. **Dalle-Donne, I., R. Rossi, D. Giustarini, N. Gagliano, P. Di Simplicio, R. Colombo, and A. Milzani.** 2002. Methionine oxidation as a major cause of the functional impairment of oxidized actin. Free Radic Biol Med **32:**927-37.

11. **Dalle-Donne, I., R. Rossi, D. Giustarini, A. Milzani, and R. Colombo.** 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta **329:**23-38.

12. **Dhandayuthapani, S., M. W. Blaylock, C. M. Bebear, W. G. Rasmussen, and J. B. Baseman.** 2001. Peptide methionine sulfoxide reductase (MsrA) is a virulence determinant in *Mycoplasma genitalium.* J Bacteriol **183:**5645-50.

13. **Dunn, B. E., H. Cohen, and M. J. Blaser.** 1997. *Helicobacter pylori*. Clin Microbiol Rev **10:**720-41.

14. **Eaton, K. A., C. L. Brooks, D. R. Morgan, and S. Krakowka.** 1991. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect Immun **59:**2470-5.

15. **Eaton, K. A., D. R. Morgan, and S. Krakowka.** 1992. Motility as a factor in the colonisation of gnotobiotic piglets by *Helicobacter pylori*. J Med Microbiol **37:**123-7.

16. **Harris, A. G., J. E. Wilson, S. J. Danon, M. F. Dixon, K. Donegan, and S. L. Hazell.** 2003. Catalase (KatA) and KatA-associated protein (KapA) are essential to persistent colonization in the *Helicobacter pylori* SS1 mouse model. Microbiology **149:**665-72.

17. **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 U S A **96:**887-92.

18. **Labigne, A., and H. de Reuse.** 1996. Determinants of *Helicobacter pylori* pathogenicity. Infect Agents Dis **5:**191-202.

19. **Lee, A., J. O'Rourke, M. C. De Ungria, B. Robertson, G. Daskalopoulos, and M. F. Dixon.** 1997. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. Gastroenterology **112:**1386-97.

20. **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:**323-32.

21. **Maier, R. J., C. Fu, J. Gilbert, F. Moshiri, J. Olson, and A. G. Plaut.** 1996. Hydrogen uptake hydrogenase in *Helicobacter pylori*. FEMS Microbiol Lett **141:**71-6.

22. **McGee, D. J., and H. L. Mobley.** 1999. Mechanisms of *Helicobacter pylori* infection: bacterial factors. Curr Top Microbiol Immunol **241:**155-80.

23. **Mintz, K. P., J. Moskovitz, H. Wu, and P. M. Fives-Taylor.** 2002. Peptide methionine sulfoxide reductase (MsrA) is not a major virulence determinant for the oral pathogen Actinobacillus actinomycetemcomitans. Microbiology **148:**3695-703.

24. **Moskovitz, J., B. S. Berlett, J. M. Poston, and E. R. Stadtman.** 1999. Methionine sulfoxide reductase in antioxidant defense. Methods Enzymol **300:**239-44.

25. **Moskovitz, J., B. S. Berlett, J. M. Poston, and E. R. Stadtman.** 1997. The yeast peptide-methionine sulfoxide reductase functions as an antioxidant in vivo. Proc Natl Acad Sci U S A **94:**9585-9.

26. **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:**502-7.

27. **Olczak, A. A., J. W. Olson, and R. J. Maier.** 2002. Oxidative-stress resistance mutants of *Helicobacter pylori*. J Bacteriol **184:**3186-93.

28. **Olczak, A. A., R. W. Seyler, Jr., J. W. Olson, and R. J. Maier.** 2003. Association of *Helicobacter pylori* antioxidant activities with host colonization proficiency. Infect Immun **71:**580-3.

29. **Olson, J. W., J. N. Agar, M. K. Johnson, and R. J. Maier.** 2000. Characterization of the NifU and NifS Fe-S cluster formation proteins essential for viability in *Helicobacter pylori*. Biochemistry **39:**16213-9.

30. **Olson, J. W., and R. J. Maier.** 2002. Molecular hydrogen as an energy source for *Helicobacter pylori*. Science **298:**1788-90.

31. **Olson, J. W., N. S. Mehta, and R. J. Maier.** 2001. Requirement of nickel metabolism proteins HypA and HypB for full activity of both hydrogenase and urease in *Helicobacter pylori*. Mol Microbiol **39:**176-82.

32. **Seyler, R. W., Jr., J. W. Olson, and R. J. Maier.** 2001. Superoxide dismutase-deficient mutants of *Helicobacter pylori* are hypersensitive to oxidative stress and defective in host colonization. Infect Immun **69:**4034-40.

33. **Shirin, H., J. T. Pinto, L. U. Liu, M. Merzianu, E. M. Sordillo, and S. F. Moss.** 2001. *Helicobacter pylori* decreases gastric mucosal glutathione. Cancer Lett **164:**127-33.

34. **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 U S A **99:**10108-13.

35. **St John, G., N. Brot, J. Ruan, H. Erdjument-Bromage, P. Tempst, H. Weissbach, and C. Nathan.** 2001. Peptide methionine sulfoxide reductase from *Escherichia coli* and *Mycobacterium tuberculosis* protects bacteria against oxidative damage from reactive nitrogen intermediates. Proc Natl Acad Sci U S A **98:**9901-6.

36. **Stadtman, E. R., J. Moskovitz, B. S. Berlett, and R. L. Levine.** 2002. Cyclic oxidation and reduction of protein methionine residues is an important antioxidant mechanism. Mol Cell Biochem **234-235:**3-9.

37. **Taylor, D. E., W. Yan, L. K. Ng, E. K. Manavathu, and P. Courvalin.** 1988. Genetic characterization of kanamycin resistance in *Campylobacter coli*. Ann Inst Pasteur Microbiol **139:**665-76.

38. **Testerman, T. L., D. J. McGee, and H. L. Mobley.** 2001. *Helicobacter pylori* growth and urease detection in the chemically defined medium Ham's F-12 nutrient mixture. J Clin Microbiol **39:**3842-50.

39. **Towbin, H., T. Staehelin, and J. Gordon.** 1992. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979. Biotechnology **24:**145-9.

40. **Vogt, W.** 1995. Oxidation of methionyl residues in proteins: tools, targets, and reversal. Free Radic Biol Med **18:**93-105.

41. **Weissbach, H., F. Etienne, T. Hoshi, S. H. Heinemann, W. T. Lowther, B. Matthews, G. St John, C. Nathan, and N. Brot.** 2002. Peptide methionine sulfoxide reductase: structure, mechanism of action, and biological function. Arch Biochem Biophys **397:**172-8.

42. **Wizemann, T. M., J. Moskovitz, B. J. Pearce, D. Cundell, C. G. Arvidson, M. So, H. Weissbach, H. R. Masure, N., and.** 1996. Peptide methionine sulfoxide reductase contributes to the maintenance of adhesins in three major pathogens. Proc Natl Acad Sci U S A **93:**7985-90.

Chapter 3

Methionine Sulfoxide Reductase in *Helicobacter pylori*: Interaction with methionine-rich proteins and stress-induced expression

---

Alamuri, P., and R. J. Maier
Submitted to Journal of Bacteriology

ABSTRACT

The reductive repair of oxidized methionine residues performed by methionine sulfoxide reductase is important for the gastric pathogen *H. pylori* to maintain persistent stomach colonization. Methionine-containing proteins that are targeted for repair by Msr were identified from whole cell extracts (after exposing cells to O_2 stress) by using a co-immunoprecipitation 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 two-fold higher methionine (Met) content than other proteins. These Met-rich proteins were purified and shown to individually form a crosslinked adduct with Msr. Catalase specific activity in an *msr* strain was one-half that of the parent strain; this difference was only observed in oxidative stress conditions, and the activity was restored to nearly wild type levels by adding Msr plus DTT to *msr* strain extracts. In agreement with the crosslinking study, pure Msr used Trx1, but not thioredoxin-2 as 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. Stress conditions (peroxide, peroxynitrite, iron starvation) all caused approximately 3--3.5 fold transcriptional up-regulation of *msr*. Neither O_2 level nor use of background regulatory mutants had a significant affect on *msr* transcription. Late log and stationary phase cultures had the highest Msr protein levels and specific activity.

INTRODUCTION

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 non-functional (37). In many cases this oxidative damage cannot be remedied. However, the sulfur containing amino acids cysteine and methionine can be enzymatically reduced/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 bacteria and eukaryotes 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 *E. coli* proteins Ffh (16), ribosomal protein L12 (9) or methionine rich chaperones (1, 22). The net affect is maintenance of targeting, synthesis, and function (e.g. proper folding) of many other cellular 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 affects 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 up regulated by stress conditions (13, 20, 27, 33-36).

H. 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), like the situation in a few other pathogens (*Neisseria gonorrhoeae*, *N. meningitidis*, and *Haemophilus influenzae,* see 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 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 are 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(S)O versus Met(R)O] 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.

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 crosslinking reagents and Seize® X Protein A Immunoprecipitation Kit were purchased from Pierce, IL. Bacterial growth media components were purchased from Difco Laboratories, MD. Rabbit antiserum against purified *H. pylori* KatA was generated by Cocalico Biologicals, CA. 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 hp0223 gene and the intergenic region of hp0223 and hp0224 (*msr*) were amplified using primers msrpromF and msrpromR (see Table 3.1) and cloned into the *Sac*I and *Bam*HI site upstream of the 980 bp promoterless *xylE* (*Pseudomonas putida*) gene of pTAD57 (31) to generate pSAP120. The 1180 bp (P_{msr}-*xylE*) fragment was excised and introduced into the *Bam*HI site of pHel3 to obtain pSAP131. Plasmid pSAP131 was transformed into HP SS1 to obtain strain ALM1018 (see 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 3.2). All constructs were screened using PCR and further sequenced at the University of Michigan DNA Sequencing Core facility. As a negative control 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 balance N_2). Cells were grown to logarithmic phase, and oxygen was then added to bring the O_2 partial pressure to 12% O_2 in order to assess oxygen stress, or cultures maintained in 4% O_2 were supplemented either with 300 μM H_2O_2, or 50 μM GSNO (for testing the oxidative stress conditions) or 500 μM $FeCl_3$, or 50 μM 2, 2-dipyridyl were added for studying the effect of excess iron or iron chelated conditions, respectively. Whole cells were collected from both pre- and post-stress 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 as determined by student t-tests are described in figure 5 legend.

Table. 3.1. List of primers used in this study. All primers were obtained from Integrated DNA Technologies.

Primer	**Sequence 5'→ 3'**	**Restriction Site**
prommsrF	tggGAGCTCatgtgattggaacttttgttgcct	*Sac*I
prommsrR	tgaataacctttgGGATTCttttaattttaatggtgtccg	*Bam*HI
PmsrF	actggCATATGaaggtattatcttatttg	*Nde*I
PmsrR	gtgCTCGAGttaatgcgactttttatcattgatg	*Xho*I
trx1F	tagagtaaCATATGagtcactatattg	*Nde*I
trx1R	tcatgctaCTCGAGctagcctaaaagtttgtt	*Xho*I
trxrF	gctaatagcCATATGatagattgcgcg	*Nde*I
trxrR	agc ttgatCTCGAGttaatggtgttctaaata	*Xho*I
ssrF	tgatgatgcaCTATGtattgtttttattctttg	*Nde*I
ssrR	ttcgcgCTCGAGgtgggataaaagatt	*Xho*I
trx2F	ataaggattCATATGtcagaaatgaggaac	*Nde*I
trx2R	taccccCTCGAGttacaataacgcttttag	*Xho*I

Table 3. 2. List of plasmids and strains used in this study.

Plasmids/strains	**Features and application**	**Reference**
pTAD57	Promoter less 980 bp *xylE* gene cloned into the *Sma*I site of pKS (in *E.coli* DH5α)	(31)
pSAP112	*msr (hp0224)* cloned into the *Nde*I - *Xho*I site of pET21A for overexpression	This study
pSAP120	200 bp intergenic region of hp0223 and hp 0224 (*msr*) cloned into the *Sac*I and *Bam*HI site of pTAD57 upstream of *xylE*	This study
pSAP123	The 1180 bp PmsX (P_{msr}-*xylE*) fragment cloned into *Eco*RV site of peu39cm used in induction studies	This study
pSAP131	The 1180 bp PmsX fragment cloned into *Bam*HI site of pHel3 used in induction studies	This study
pSAP134	The 1200 bp P_{ureA} and *xylE* gene fusion fragment cloned into *BamH*I site of pHel3	This study
pSAP138	thioredoxin (hp 0824) clone into *Nde*I - *Xho*I site of pET21A for overexpression	This study
pSAP139	thioredoxin reductase (hp 0825) clone into *Nde*I - *Xho*I site of pET21A for overexpression	This study
pSAP159	thioredoxin2 (hp 1458) cloned into *Nde*I - *Xho*I site of pET21A for overexpression	This study
pSAP163	Site specific recombinase (hp 1009) cloned into *Nde*I - *Xho*I site of pET21A for overexpression of a C-terminus 6X-His tagged SSR.	This study
pILL690	*nikR* (hp 1338) inactivated with *aph*A3	(11)
ALM 1014	HP SS1 carrying pSAP 123	
ALM 1018	HP SS1 carrying pSAP131 for induction studies	
	HP SS1 with pHel3 carrying promoterless *xylE*	(6)
ALM 1020	pSAP123 recombined in hp0405 locus of *msr:aphA3* in SS1	This study
ALM 1021	HP 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
ALM1035	pILL690 transformed into SS1 to generate *nikR*:*aphA3*	This study
ALM1037	pSAP123 recombined in *hp0405* locus of ALM1035	This study

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 hr time period. Growth was monitored by measuring OD_{600} at each time point. Cells were disrupted by sonication (W-380 Heat System-Ultrasonics, Inc.). 7 µg of cell protein was resolved on a 12.5% SDS-PAGE and immunoblots were performed by use of anti-Msr antibody. Densitometric scanning (30) to determine the intensity of immunostained Msr protein bands were 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 performed using MetSO and methyl p-tolyl sulfoxide as substrates as described elsewhere (2). 1 unit of Msr activity = nmol of NADPH oxidized/min/mg of membrane protein. Mean ± std. dev. are shown based on data from 9 samples (three different experiments, each sampled in triplicate). As a control for immunoblot based growth phase expression of *ms*r, 5 µg of cell extract was loaded onto a separate gel, and the separated proteins immunoblotted with anti-UreB antiserum. This was chosen as a control because UreB is a *H. pylori* protein whose expression is known not to be growth phase dependent (26).

Co-immunoprecipitation. The wild type and *msr* strain were grown to mid-log phase in sealed vials at 4 % partial pressure O_2 and half of the culture (for wild type) were removed for exposure to 12 % partial pressure O_2 (for a three hr 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 crosslinkers. The crosslinker-extract incubation was performed overnight in argon-sparged buffer. All three crosslinkers, heterobifunctional SPDP (N-Succinimidyl 3-(2-pyridyldithio) propionate, homobifunctional Sulfo-DST (Sulfo-Disulfosuccinimidyl tartrate), and homobifunctional DTSSP (Dithiobis (sulfosuccinimidylpropionate) were individually tested. The best yield of crosslinked products after immunoprecipitation was obtained using SPDP. 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 non-specific antibody-protein binding. Seize® X Protein A immunoprecipitation kit (Cat: 45215; Pierce) was used in the subsequent precipitation process. The purified anti-Msr antibody was first crosslinked to ImmunoPure Immobolized Protein A Plus beads using DSS (disuccinimidyl suberate). The slurry was incubated with previously crosslinked cell extracts with slow stirring overnight at 4º 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 IgG Elution buffer (EB). The crosslinked proteins were serially eluted with the EB, and 10 µl of 1 M Tris-Cl pH 8.0 was added to neutralize the protein elutions. The crosslinked protein complexes were cleaved by incubating either with DTT or ammonium persulfate, depending on the crosslinker used in the previous step. The mixture was later resolved on a 4%-20% gradient SDS-PAGE, and the bands were excised from the gel and submitted to The University of Georgia Proteomics facility for protein identification by MALDI–TOF/MS. All steps were simultaneously performed using cell extracts from both SS1 and the isogenic *ms*r mutant.

Overexpression and protein purification. Proteins were purified either as recombinant proteins in *E. coli* (Msr, Trx1, Trx2, TrxR and 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 (see Table 3.2) was transformed into *E. coli* BL21 origami (Novagen). 20 ml of overnight starter culture of the *E. coli* strain harboring pSAP112 was added to two liters of LB and the culture incubated at 37 ºC until it achieved an OD_{600} of 0.6. The culture was induced with 0.5 mM IPTG at 22 ºC 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 45000 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 1000 mM NaCl) with a linear gradient from 50 mM to 1000 mM NaCl. The fractions from the flow-through were collected, applied to a Hi-Trap Q sepharose column and eluted fractions obtained by use of the same buffers. The flow through 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.

Thioredoxin-1 (Trx1): Plasmid pSAP138 (see Table 3.2) was transformed into *E. coli* BL21 Rosetta (Novagen). 20 ml of overnight starter culture of *E. coli* BL21 Rosetta strain harboring pSAP138 was added to two liters of LB and incubated at 37 ºC until the culture reached an OD_{600} of 0.6. The culture was induced with 0.5 mM IPTG at 37 ºC for 3.5 hrs. 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 45000 rpm for 1.5 hrs and the supernatant was loaded onto a Hi-Trap Q-Sepharose column. Protein was eluted using buffer B (25 mM Tris and 1000 mM NaCl) with a linear gradient of 25 mM to 1000 mM NaCl. Peak fractions were collected and dialyzed overnight against three liters of buffer C (25 mM Tris and 150 mM NaCl). The pooled protein was then run through a 10/200 Sephacryl column pre-equilibrated with buffer C.

Thioredoxin-2 (Trx2): Plasmid pSAP159 (see Table 3.2) was transformed into *E. coli* BL21 Origami (Novagen). 30 ml of overnight starter culture of *E. coli* BL21 Rosetta strain harboring pSAP138 was added to three liters of LB and incubated at 37 ºC until the culture grew to OD_{600} of 0.7. The culture was induced with 0.8 mM IPTG at 22 ºC for 4 hrs. Cells were harvested and later re-suspended in buffer-1 (50 mM HEPES, 50 mM NaCl pH 6.5) and the suspension sonicated. The cell lysate was centrifuged at 45000 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 (3-liters total volume) for 24 h. The dialyzed protein was loaded onto a Hi-Trap SP column. Protein was eluted using buffer B (50 mM HEPES and 1000 mM NaCl) with a linear gradient from 50 mM to 1000 mM NaCl. Peak fractions were collected and dialyzed overnight against three 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.

Thioredoxin Reductase-1 (TrxR): Plasmid pSAP139 (see Table 3.2) was transformed into *E. coli* BL21 Rosetta (Novagen). 20 ml of overnight starter culture of *E. coli* BL21 origami strain harboring pSAP112 was added to two liters of LB and incubated at 37 ºC until the culture reached an OD_{600} of 0.6. The culture was then induced with 0.5 mM IPTG at 37 ºC for 3hrs. 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 45000 rpm for 1.5 hrs 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 (10mM Tris/25 mM NaCl, pH 7.6), pooled and dialyzed the same buffer (3-liters total volume). The suspension was then subjected to Q-sepharose chromatography using a linear 25mM - 1M NaCl gradient. The peak fractions were collected, concentrated and applied to a 10/200 Sephacryl column equilibrated with 10mM Tris (pH 7.5) containing 200mM NaCl. Peak fractions were harvested and analyzed on the gels for purity assessment and then stored at -80 ºC.

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

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

Site specific recombinase (SSR): Plasmid pSAP163 (see Table 3.2) was transformed into *E. coli* BL21 Origami (Novagen). 20 ml of overnight starter culture of *E. coli* BL21 Origami strain harboring pSAP163 was added to 2000 ml of LB and incubated at 37 ºC until the culture reached an OD_{600} of 0.6. The culture was induced with 0.5 mM IPTG at 22 ºC for 3.5 hrs. Cells were harvested, 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 pre-equilibrated column prepared with Ni-NTA-agarose resin (Qiagen) and washed with washing buffer (binding buffer with 20 mM imidazaole). Protein was eluted with elution buffer (binding buffer with 250 mM imidazole). Protein purity was assessed and the pure protein fraction 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 (on SDS-PAGE) and samples stored as aliquots with 10% glycerol at -80 ºC.

Protein crosslinking assays. KatA, GroEL or SSR were separately incubated in buffer along with 300 μM H_2O_2 for 15 min at 4ºC 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 crosslinker. 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, 10 μM of each protein was mixed in a 1:1:1 ratio along with 30 μM lysozyme (to inhibit possible non-specific binding) and the mixture was incubated with a non-cleavable protein crosslinker (3 mM Dimethyl Subermidate) for 30 min at room temperature and later the reaction was quenched with 100 mM Tris-Cl (pH7.5).The reaction mixture was run on three different gels (12% SDS-PAGE) and these were electroblotted onto separate nitrocellulose membranes. Immunoblots were individually performed with antibodies against Msr (1:1000) or KatA (1:1000) or GroEL (1: 8000). The membranes were subsequently incubated in the secondary antibody 1:1500 Goat Anti-Rabbit IgG- Alkaline phosphotase 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 specificity of the Msr-target protein results. After (attempting) crosslinking these latter potential adducts were subjected to SDS-PAGE and the blots probed with anti-HypB (25) or anti-UreE (3) serum at 1:2000 and 1:1000 dilution, 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 = 1 µmole of H_2O_2 decomposed/min/mg cell protein. Results are the mean of 12 determinations; these were from four independent experiments, with each experiment sampled in triplicate. Cell extract (5 microgram protein) from each strain (SS1 and msr) were subjected to SDS-PAGE and the separated proteins 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 respectively (39, 40). The models were analyzed manually for the robustness and the best fit model was selected for further analysis. The coordinate file was imported to 3D-Mol viewer (a component of Vector NTI suite 8.0, Infomax Inc.) to visualize the residues with respect to the entire 3-D 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 (cat no. 339997 and 343609, respectively). The reaction was conducted in prewarmed 50mM Tris-HCl (pH 7.4). Msr activity was first studied using purified Msr (6-10 μg), a broad substrate (methyl tolyl sulfoxide) range of 0.5mM 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) were used to establish saturation levels. For routine specific activity measurements and Km determinations, Msr (6—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 mean of 15 determinations; these were from five independent experiments 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 (8, and the references therein). It is 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 crosslinking with a co-immunoprecipitation (CIP) approach. First, cells were exposed to oxidative stress conditions. The transient but intimate interaction of Msr with its oxidized protein substrates were then captured by adding a cleavable crosslinker to cell extracts. The yield of Msr-substrate complexes were then harvested by use of an immobile anti-Msr antibody, and the complexes were un-crosslinked. The proteins that had formed adducts with Msr were identified by mass spectrometry. Two important control experiments were done to address 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 3.1A shows SYPRO-RUBY stained proteins from wild-type and the *msr* mutant, after the complete crosslink 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 crosslinking and Msr-adduct immunoprecipitation. No proteins were obtained from the same procedure when using the *msr* strain (Fig. 3.1A), 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 in low O_2 conditions (4% partial pressure O_2) were used as the starting material (data not shown); this links the observed interactions to oxidative conditions. Table 3.3 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 CI values. Of these five, catalase (KatA), site specific recombinase (SSR) and the chaperone protein GroEL (GRL) contain approximately 3.2% to 3.5% methionine residues. It may be significant that this methionine percentage is higher than the average 1.7% methionines found in proteins (17). Another potential Msr-interacting protein (see Table 3.3) identified was thioredoxin-1, 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 (final two in table 3.3). 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 (see Table 3.3).

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 about 95% homogeneity (Fig. 3.1 B). These potential pure substrates for Msr were oxidized and then their interaction with Msr was assessed by use of a non cleavable cross-linker (see methods). Lysozyme was included in the reaction mixture at 3-fold greater level than the test proteins to reduce non-specific protein-protein interactions. For assessing catalase- or GroEL-Msr interactions, the crosslinked mixtures were subjected to three different SDS-PAGE gels, the gels individually blotted and probed separately with anti-Msr, anti-KatA and anti-GroEL (Figs. 3.2A, B, and C) antibodies as described. Msr interacted individually with KatA or with GroEL as seen in the blot using anti-Msr antibody (Fig. 3.2A). The crosslinked

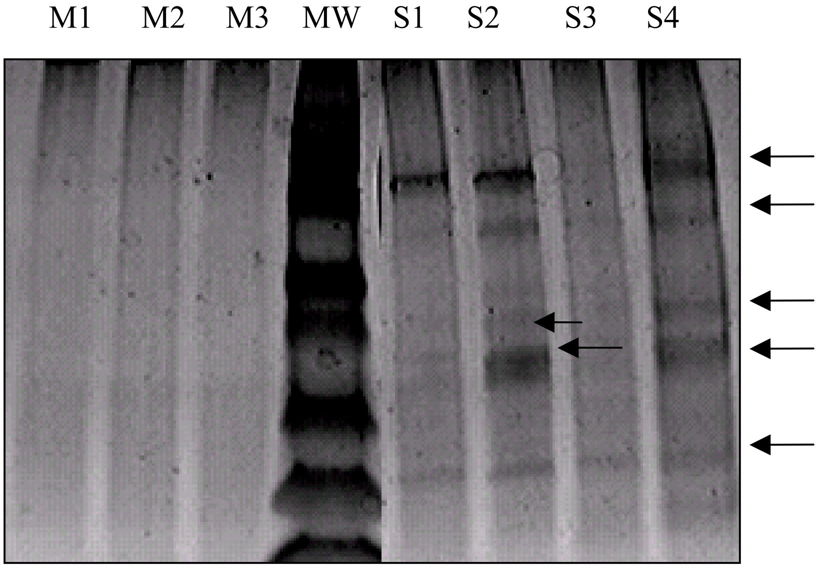


FIG. 3.1A. Sypro-Ruby stained gel showing Msr-interacting proteins precipitated using CIP procedure. Lanes: M1, M2, and M3 are elutions obtained by using the *msr* strain; S1, S2, S3, and S4 are serial elutions of the proteins captured using SS1 (parent strain). MW =Molecular weights (in kDa) of the 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. Arrows indicate different proteins identified using MALDI-TOF/MS. From the top arrow (lane S4), these are GroEL, KatA, Msr, SSR and Trx1. 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 weight marker had to be compromised for a better resolution of the eluted proteins.

Table 3.3: Proteins identified by MALDI-TOF/MS: 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.

Protein Name	[a] Protein C.I.%	[b] Total protein score	Estimated MW.	[c] Hp gene No.	% Met	Function
GroEL	98.34	102	60.04	hp 0010	3.7	Chaperone
Catalase	97.528	82	53.27	hp 0875	3.4	Detoxification
Msr	99.03	97	41.30	hp 0224	NA	Repair
Site specific recombinase	96.02	89	22.24	hp 1009	3.5	DNA Repair
Thioredoxin	99.92	106	11.84	hp 0824	NA	Electron transport
Seryl t-RNA synthase	34.12	27	47.44	hp 1480	3.2	Protein synthesis
TolB precursor protein	0	36	47.71	Hp 1126	3.1	Colicin tolerance

[a] Protein confidence interval (CI) is a statistical calculation of how closely the acquired data matches previous database searches. The closer the confidence percentage value is to 100 % the more likely the protein is correctly identified.

[b] Total protein score is -10*Log(P), where P is the probability that the observed match is a random event. Only protein scores greater than 72 are significant ($p<0.05$).

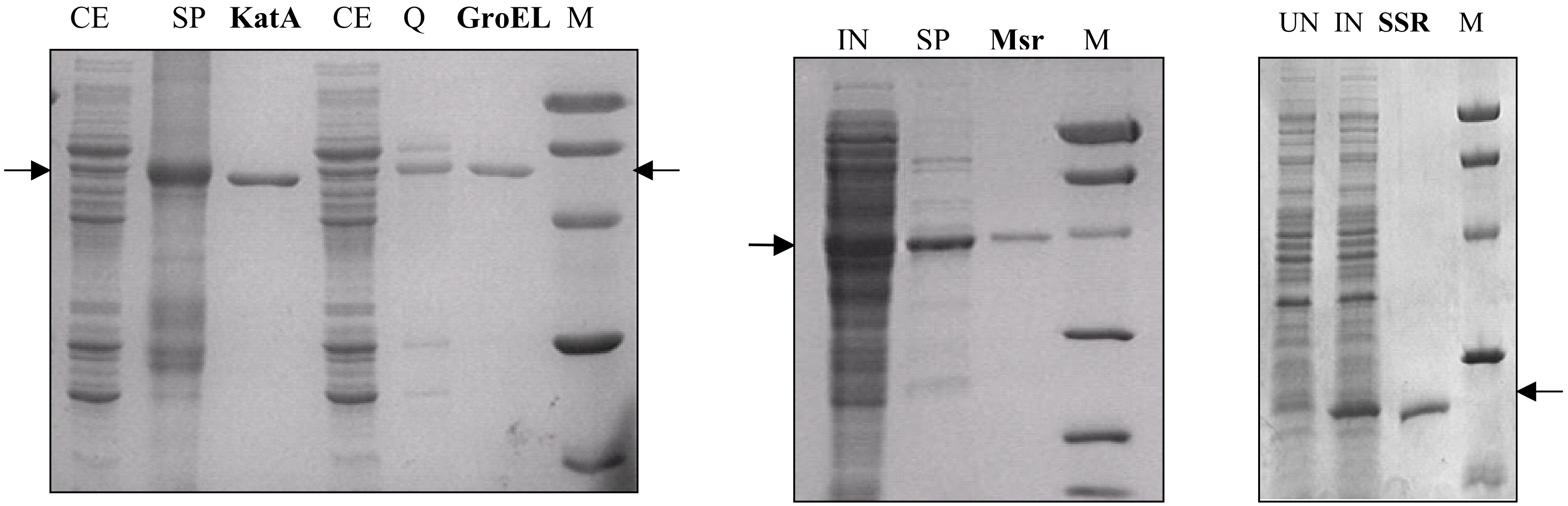
[c] Gene number based on the HP 26695 genome sequence (41).

interactions of Msr with KatA or with GroEL were apparent due to the identification of an approximately 100 kDa (lanes MK and MG in Fig. 3.2A) compared to the (non-complexed) Msr in the blot. A similar banding pattern was identified when the same crosslinked sample was probed using anti-KatA (Fig. 3.2B) or anti-GroEL (Fig. 3.2C). (MK in Fig. 3.2B and MG in Fig. 3.2C). The blots 3.2B and 3.2C confirm that the adducts identified in fig. 3.2A 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 interaction of Msr with KatA had not been previously obtained. Catalase and GroEL also complexed together forming an approx. 100 kDa adduct (lanes KG in fig. 3.2B and 3.2C).

Msr interacted only with the oxidized KatA and GroEL, as peroxide treatment was a pre-requisite for obtaining these adducts (see 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 kD) was evident (Lane MKG in Figs. 3.2A, 3.2B, and 3.2C) when the three proteins (reduced Msr and oxidized KatA and oxidized GroEL) were mixed together with the crosslinker; 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 weight complexes (such as homodimers) with themselves (see lanes M, lane K, lane G in Fig. 3.2A, B, and C, respectively).

Recognition of the oxidized recombinase protein SSR by pure Msr (Figure 3.2A, lane labeled MS) was also apparent based on an immunostained band of the expected size (third arrow from top, 60-65 kDa). Like the others, this adduct formed even in the presence of a 3-fold

FIG. 3.1B



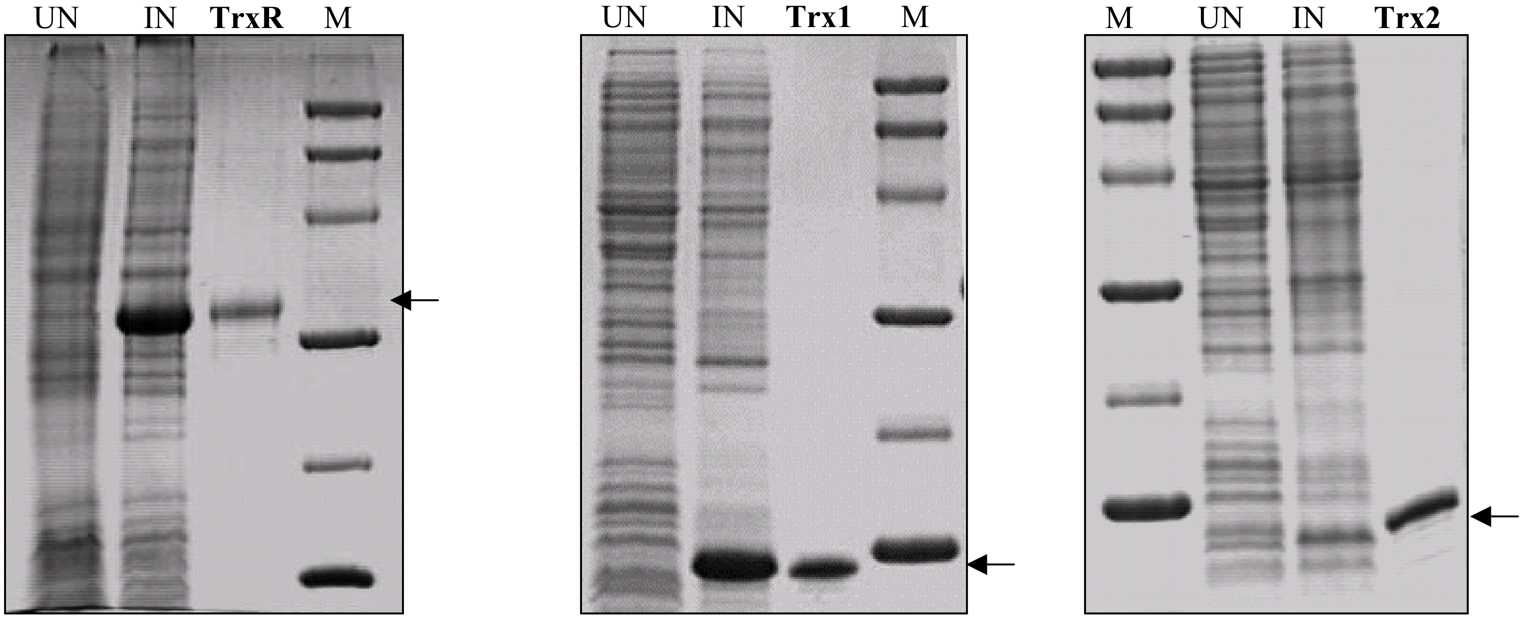


FIG. 3.1B. Gel-based analysis of purity of potential Msr-interacting proteins. Catalase (KatA) and GroEL were purified from cell extracts of SS1. Thioredoxin reductase (TrxR) and thioredoxin-1 (Trx1), Methionine sulfoxide reductase (Msr), thioredoxin-2 (Trx2) and site specific recombinase (SSR) were purified as recombinant proteins from *E. coli*. CE = cell free extracts; UN and IN denote protein collected from cells not induced and induced with IPTG, respectively**.** SP and Q denote HiTrap columns SP and Q-Sepharose fractions, respectively. M = molecular weight marker, the same marker was used in all the panels in this figure. The molecular mass in kDa is as follows: 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4. Arrows in each panel identify the protein of interest, and the protein names are in bold.

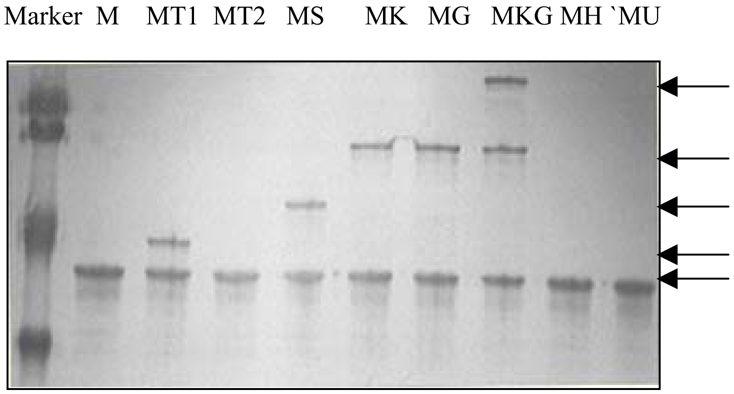


FIG 3.2A: Immunoblot using anti-Msr antibody: Protein-protein interactions identified using Msr and its substrates (1:1) studied using the non-cleavable crosslinker, DMS. Lanes: M= oxidized Msr alone; MT1 = oxidized Msr crosslinked with native Trx1; MT2 = oxidized Msr crosslinked with native Trx2; MS = Native Msr crosslinked with oxidized SSR; MK = Native Msr with oxidized KatA; MG = Native Msr with oxidized GroEL; MKG = Native Msr, oxidized KatA and oxidized GroEL. MH and MU are the control lanes with oxidized HypB and oxidized UreE with native Msr. The top four arrows identify complexes; Msr-KatA-GroEL, Msr-KatA or Msr-GroEL (same size, arrow 2), Msr-SSR, and Msr-Trx1, respectively. The fifth arrow is Msr alone. The molecular weights (in kDa) of the pre-stained marker are: 120, 97, 55.4, 36.1, (28.4, and 20.9).

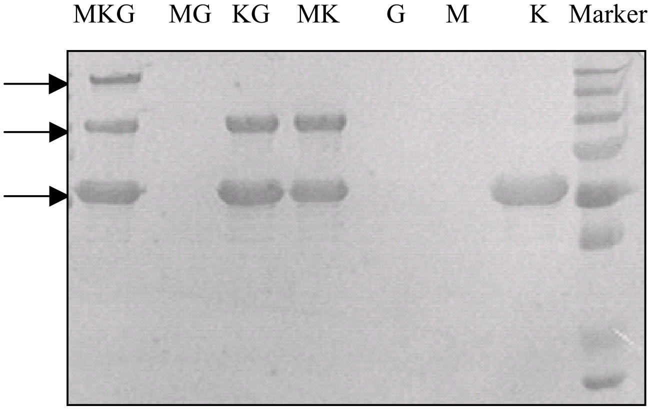


FIG.3.2B. Immunoblot using anti-KatA antibody: The same reactions used in Fig 2A (pertinent to KatA) were resolved on a separate gel and immunostained with anti-KatA antibody. Lanes: K = KatA; M = Msr; G = GroEL; MK = Oxidized KatA and native Msr; MG = Native Msr and oxidized GroEL; KG = Native KatA with GroEL; MKG = Oxidized KatA and GroEL crosslinked with native Msr. As expected, no (immunostained) bands are observed in M, G and MG. Arrows identify complexes KatA-GroEL-Msr (top arrow), KatA-GroEL and KatA-Msr (middle arrow, approximately same mobility), and KatA alone (lower arrow). The molecular weights (in kDa) of pre-stained marker are: 150, 100, 75, 50, 37, 25, 20, 15, and 10.

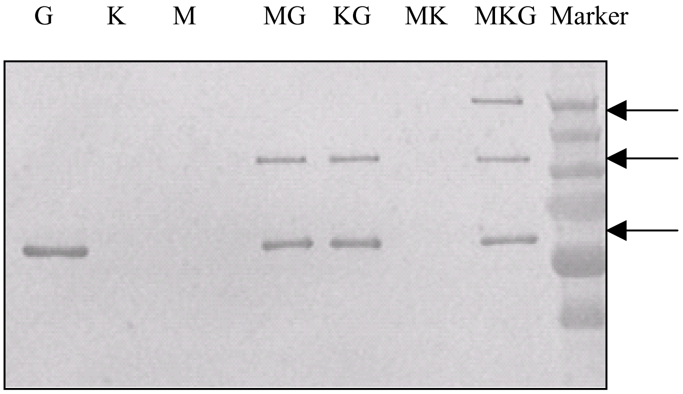


FIG. 3.2C. Immunoblot using anti-GroEL antibody: The same mixtures (used in 2B) were resolved on a separate gel and immunostained with antibody against GroEL. Lanes G = GroEL; K = KatA; M = Msr; KG = Native KatA and GroEL; MG = Native Msr and oxidized GroEL; MK = Native Msr and KatA; MKG = Oxidized GroEL and KatA cross-linked with native Msr. A shift in the molecular weight of the immunostained bands is indicated by an arrow in each case. Arrows indicate the complexes or GroEL alone (third arrow down). The same pre-stained marker as in Fig. 3.2B was used here.

greater amount (see 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 crosslinked adduct only with Trx1 but not with Trx2 (Figure 3.2A, Lanes MT1, MT2). We tested the interaction of Msr individually with two additional pure *H. pylori* proteins for which we had antiserum available, HypB and UreE. No Msr crosslinked adducts were formed to these (Fig. 3.2A, lanes on far right, labeled MH and MU), indicating that the Msr interactions observed above are not likely to be artifacts of the crosslinking 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 SS1 (parent strain) and *msr* mutant strain under non-O_2 stress versus under oxygen stress conditions. We observed that the level of catalase expression (based on immunoblots) in both strains were similar (Fig. 3.3A) but that the specific catalase activity of the mutant only was reduced by 50 % due to oxygen stress (Fig. 3.3B). 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 were added to the *msr* strain extract. The resulting catalase activity (3432 + 212 units) was about 85% of the wild type (4144 + 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

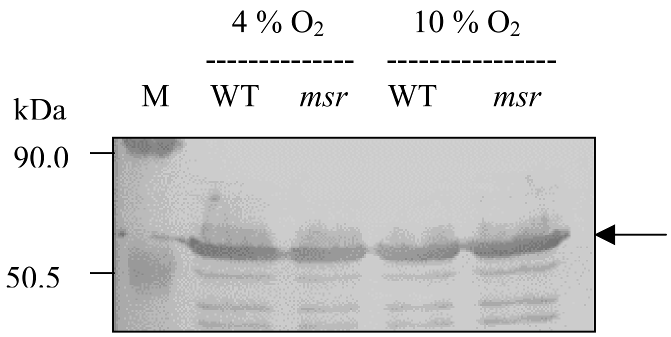


FIG.3.3A. Relative catalase expression in SS1 and *msr* mutant. Cell extracts (5 µg) from the described conditions were run on a 12.5% SDS-PAGE and an immunoblot was performed using anti-catalase antibody. The arrow indicates the immunostained catalase protein in all lanes. 4% O_2 = cells grown in 4% oxygen, 10% O_2 = cells grown in 4% oxygen and exposed for 3 h to 10 % oxygen (see methods). Lanes: WT = SS1 strain; *msr* = *msr* mutant in SS1; M = molecular weight marker. The immunostained catalase (~55 kDa) is identified by an arrow.

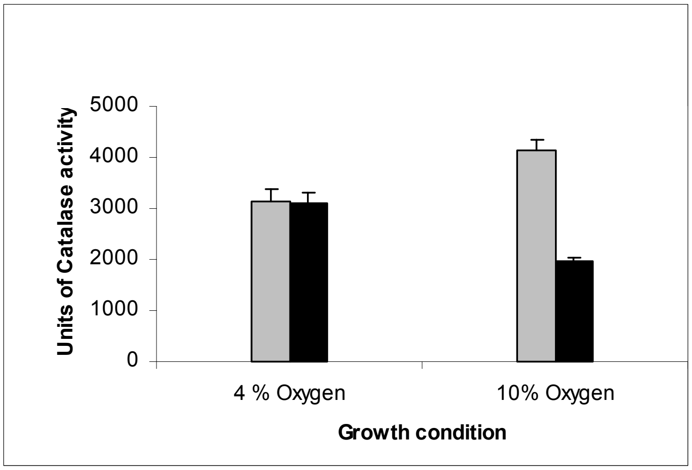


FIG. 3.3B. Catalase activities in SS1 and *msr*. Specific activities of catalase are from extracts of cells grown in 4% oxygen or grown in 4% oxygen and then exposed to 10% oxygen for 3 h. 1 unit= micromoles of H_2O_2 decomposed/min/mg of protein. Gray bars indicate SS1 parent strain and black bars indicate *msr* mutant. Mean and SD from 12 individual samples taken from 4 separate experiments (3 replicates each) is shown here. The mutant results are significantly less than the wild type when both strains are in 10% O_2 ($P < 0.01$).

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 that study indicate there are at least two mechanisms *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-long single polypeptide that comprises two fused domains (MsrA , with residues 36-189 and MsrB with residues 206-330) 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 (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 similarity (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) (see Fig. 3.4). 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 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 disulfide bond with the former residue (21). In addition, His residues (His 303, 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 (data not shown). 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 thioredoxin-1 or thioredoxin-2. Only use of thioredoxin-1 (Trx1) resulted in

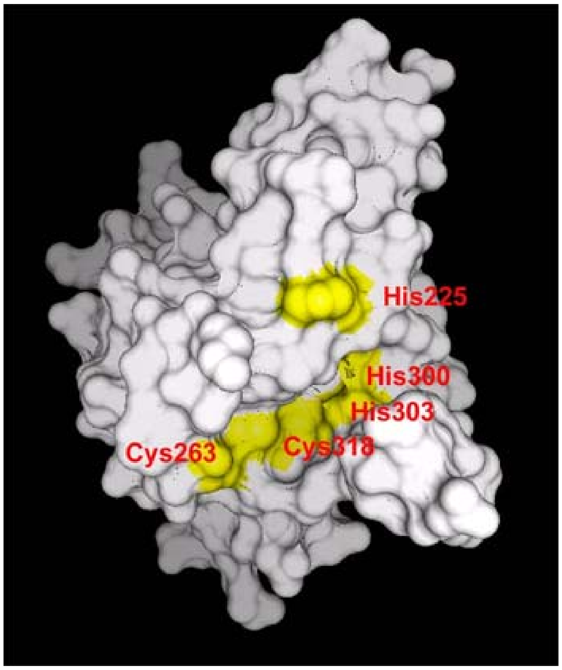


FIG. 3.4. Homology based structure model of Msr in *H. pylori.* The amino acid sequence of *H. pylori* Msr was aligned with known Msrs' from different bacteria using Swiss-Prot protein database. MsrAB from *Neisseria (gonorrhoeae* and *meningitidis*) shares a high percentage homology with *H. pylori* Msr and was used as a model to understand the folding of this protein. Surface exposed cysteines and proximal histidines are highlighted.

Msr activity. The calculated Km 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*

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 an 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 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 affect on *msr* expression was observed by a shift to 12 % O_2. An approximately 3-fold increase in *msr* expression (in a six-hour 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 (see Fig. 3.5) compared to the same conditions but with iron. There was no transcriptional up-

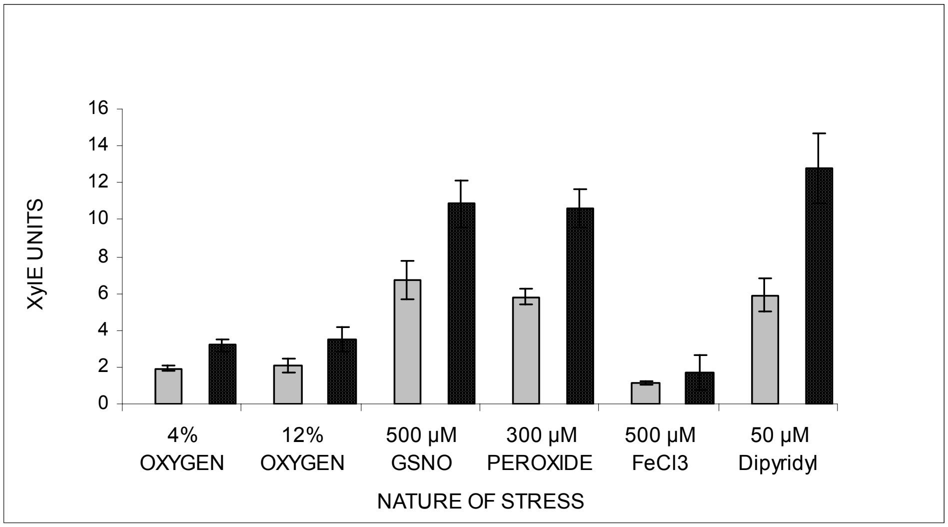


FIG.3.5. *xylE* activities to monitor *msr* expression in *H. pylori.* Whole cells collected 6 hr post exposure to stress conditions were assayed for *xylE* activity. Gray bars indicate genomic fusions (P_{msr}-xylE) and black bars indicate fusions on the shuttle vector pHel3 (see methods). Mean and SD from 15 samples taken from 5 separate experiments (3 replicates each) for each condition is shown. 1 unit of xylE activity = micromoles of catechol oxidized/min/10^9 cells. The 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.

expression observed when the cells were exposed to UV light (data not shown), and *msr* expression was slightly repressed (by less than 2-fold) when media was supplemented with iron (Fig 3.5). 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 immunoblots) individually in isogenic mutants of *fur*, *nikR*, and *msr* itself (the latter to test for possible autoregulation). There was no difference in the induction/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 (post-transcriptional) 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 3-fold 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-expresses *msr* to prevent 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-expression 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-expression would not be needed under the conditions of excess iron, as *napA*, *pfr*, *sodB* (4, 12, 14) expression would provide a battery of oxidative stress defense.

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 hr period. Immunoblots (using anti-Msr serum) performed on cell extracts obtained at various time points showed a 2-2.5 fold increase in Msr expression at both 56 hr and 72 hr time points (late log phase to stationary phase of growth) (see Fig. 3.6A) compared to early and mid-log phase expression. This growth phase dependent up-expression of Msr was also observed by enzyme activity assays (see Fig. 3.6B). 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 media with iron, but the situation is confounded by the observed iron-dependent repression of *msr* (Fig. 3.5). Nevertheless, the increased msr expression in late log and stationary phase is like the results reported for expression of *msrB* in *S. 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 (*S. 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

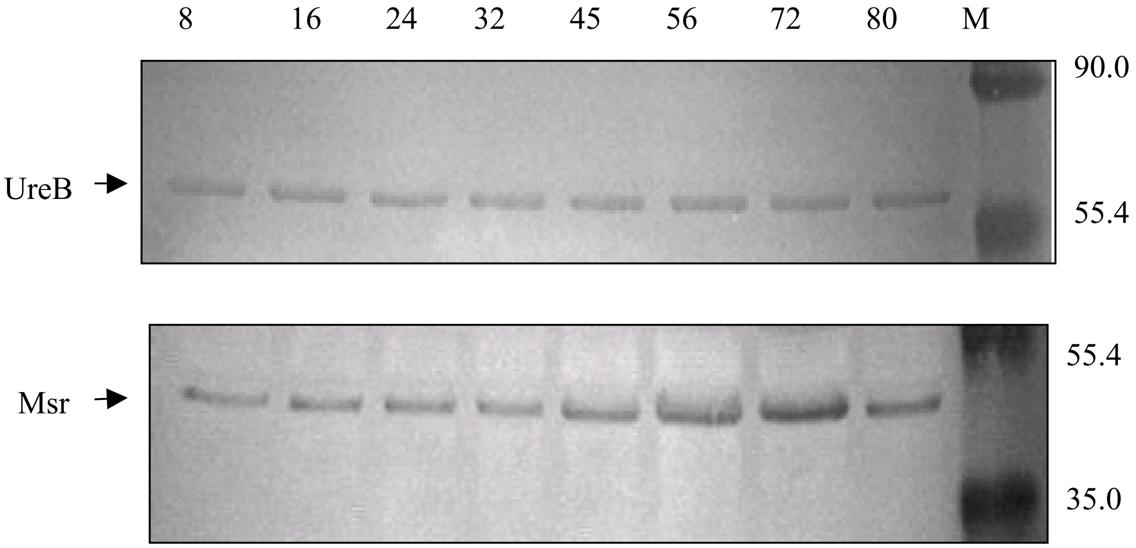


FIG.3.6A. Growth phase dependent expression of Msr: *H. pylori* strain SS1 was grown in Mueller Hinton broth supplemented with 5% calf serum; 7% partial pressure oxygen was maintained in the atmosphere of the (sealed) bottles. Cells were collected from each time point, extracts prepared identically for all time points, and cell protein from each sample was run on two separate 12.5 % SDS-PAGE. Immunoblots were performed using anti-Msr antibody and anti-UreB (as a control, see top panel). The arrows identify immunostained UreB (~66 kDa) and Msr (~43 kDa) in the cell extracts.

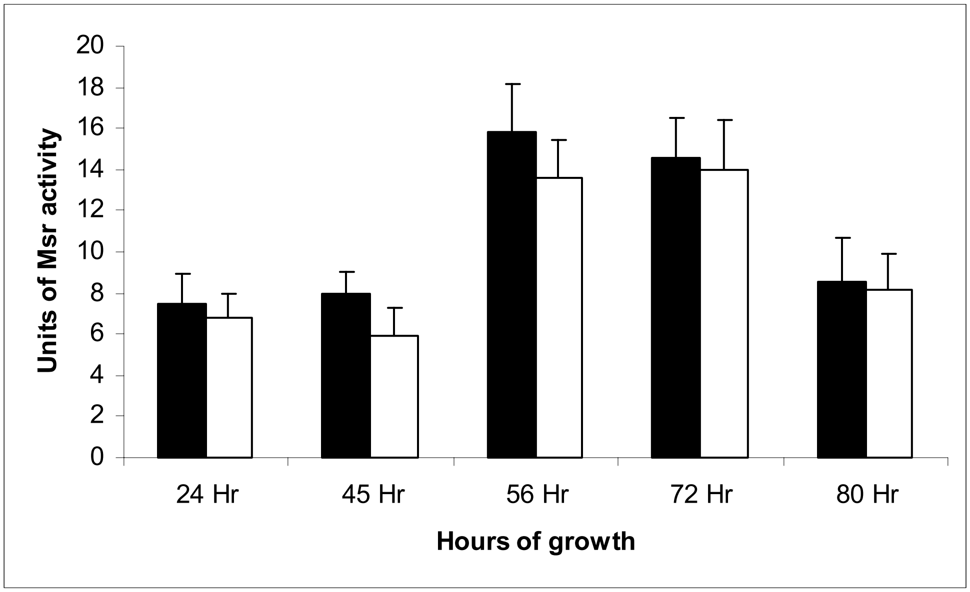


FIG.3.6B. Growth phase dependent Msr activity. Membrane protein fractions were simultaneously collected from the cell extracts and specific Msr activity was determined using MetSO or methyl p-tolyl sulfoxide (MPTSO) as substrates. Mean and SD from 9 samples (from 3 separate experiments, each assay performed in triplicate) are shown here. Units of Msr activity is plotted against time of growth in hours. 1 unit = nanomoles of NADPH oxidized/min/mg of membrane protein. Time samples at 56 and 72 h correspond to late log and stationary phase 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 substrate. The 56 and 72 h data is significantly greater than the other sampled time points at $P<0.05$ (student's t-test analysis).

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/infection. A further understanding of the dynamic regulation of *msr* and the complete physiological roles of the enzyme is desired.

ACKNOWLEDGEMENTS

We thank Dr. Praveen Nair for Msr structure modeling. We are thankful to Dr. Agnes Labigne's laboratory for the plasmid pILL690. Thanks are also due to Dr. Ge Wang (for *H. pylori katA* and acontiase mutants) and Adriana Olczak for (*H. pylori fur* mutant*).* We also thank The University of Georgia, Proteomics Facility for proteins identification using MALDI-TOF/MS analysis. This work was supported by NIH grant DK-60061 to RJM.

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:**1397-406.

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:**4787-95.

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:**5948-53.

5. **Boschi-Muller, S., A. Olry, M. Antoine, and G. Branlant.** 2005. The enzymology and biochemistry of methionine sulfoxide reductases. Biochim Biophys Acta **1703:**231-8.

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:**4535-42.

7. **Brot, N., H. Fliss, T. Coleman, and H. Weissbach.** 1984. Enzymatic reduction of methionine sulfoxide residues in proteins and peptides. Methods Enzymol **107:**352-60.

8. **Brot, N., and H. Weissbach.** 2000. Peptide methionine sulfoxide reductase: biochemistry and physiological role. Biopolymers **55:**288-96.

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 U S A **75:**5349-52.

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:**301-5.

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:**947-63.

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:**461-9.

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:**3590-8.

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:**3687-92.

15. **Ezraty, B., L. Aussel, and F. Barras.** 2005. Methionine sulfoxide reductases in prokaryotes. Biochim Biophys Acta **1703:**221-9.

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:**1868-77.

17. **Garrett, R. H., and Grisham, C.M.** 1995. Biochemistry. Saunders College Publishing.

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:**2371-4.

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:**48915-20.

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 chrysanthem*i. Proc Natl Acad Sci U S A **96:**887-92.

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:**249-60.

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:**19486-93.

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:**323-32.

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:**348-52.

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:**726-34.

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:**6510-25.

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:**213-9.

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:**14167-72.

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:**502-7.

30. **Olczak, A. A., J. W. Olson, and R. J. Maier.** 2002. Oxidative-stress resistance mutants of *Helicobacter pylori*. J Bacteriol **184:**3186-93.

31. **Olson, J. W., and R. J. Maier.** 2002. Molecular hydrogen as an energy source for *Helicobacter pylori*. Science **298:**1788-90.

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:**211-5.

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:**613-21.

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:**2739-47.

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:**3037-45.

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 U S A **99:**10108-13.

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:**577-82.

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 A resolution. Structure Fold Des **8:**1167-78.

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 Biol Crystallogr **56 (Pt 9):**1194-7.

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, and et al.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-47.

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:**51908-14.

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:**1391-6.

Chapter 4

The *Helicobacter pylori* MutS protein has a major role in the repair of oxidative DNA damage

---

Wang, G., Alamuri. P., Humayun, M.Z., Taylor, D.E., and R. J. Maier. 2005. Mol. Microbiol. 58:166-76. Reprinted here with permission of publisher.

My contributions to this manuscript are figures 4.3, 4.4 and parts of 4.5. The remaining data is the work of Ge Wang

ABSTRACT

The human gastric pathogenic bacterium *Helicobacter pylori* lacks a MutSLH-like DNA mismatch repair system. Here, we have investigated the functional roles of a *mutS* homologue found in *H. pylori*, and show that it plays a major physiological role in repairing oxidative DNA damage. *H. pylori mutS* mutants are more sensitive than wild type cells to oxidative stress induced by agents such as H_2O_2, paraquat or oxygen. Exposure of *mutS* cells to oxidative stress results in a significant (~ 10 fold) elevation of mutagenesis. Strikingly, most mutations in *mutS* cells under oxidative stress condition are G:C to T:A transversions, a signature of 8-oxoguanine (8-oxoG). Purified *H. pylori* MutS protein binds with a high specific affinity to double-stranded DNA containing 8-oxoG as well as to DNA Holliday junction structures, but only weakly to double-stranded DNA containing a G:A mismatch. Under oxidative stress conditions, *mutS* cells accumulate higher levels (~ 3 fold) of 8-oxoG DNA lesions than wild type cells. Finally, we observe that *mutS* mutant cells have reduced colonization capacity in comparison to wild type cells in a mouse infection model.

INTRODUCTION

Helicobacter pylori is one of the most common human gastrointestinal pathogens, and has been recognized as an etiological agent for stomach diseases ranging from gastritis to ulcers and gastric cancer (10). *H. pylori* displays a high level of genetic diversity, with strains differing not only in the types of individual genes, but also in the DNA sequences of the same genes. Mechanisms for the observed genetic variability may include high mutation rates for point mutations (5), inter-strain recombination (19, 44), the presence of extensive repetitive DNA in the genome (2), and high levels of gene transfer (36). Such genetic diversity impacts virulence, and may also impact host range of the pathogen (6, 50).

DNA methyl-directed mismatch repair (MMR) system plays a central role in maintaining genetic stability. The basic features of this system have been highly conserved during evolution (25). The best characterized *Escherichia coli* MMR system consists of three proteins: MutS, MutL, and MutH. MutS is the mismatch recognition protein, MutL couples mismatch recognition by MutS to downstream processing steps, and MutH is a methylation-sensitive endonuclease that targets repair to the newly synthesized DNA strand. In eukaryotes, there are multiple MutS and MutL homologues (18, 20). Based on the genome sequence analysis, *H. pylori* does not have *mutL* or *mutH* genes, but both sequenced strains do have a *mutS* homologue (1, 45). Phylogenetic analysis (13) indicated that *H. pylori* MutS belongs to the MutS2 subfamily of proteins that usually have no function in mismatch repair. In addition, we observed that transition mutations (A:T to G:C, and G:C to A:T) account for the majority of the inter-strain microdiversity of *H. pylori* (47). Such (transition) mutations are attributed to a loss of MMR function in *E. coli* (17). In addition, Bjorkholm et al. (5) observed that about 25% of clinical *H. pylori* isolates exhibit mutation frequencies that were higher than those found in MMR-defective *Enterobacteriaceae.* Furthermore, inactivation of the *mutS* gene in *H. pylori* had no substantial effect on the mutation frequency, indicating that *H. pylori* lacks a MutSLH-dependent DNA mismatch repair system.

During the process of colonization in the host, *H. pylori* induces an inflammatory oxidative stress response in the inflammatory cells in the mucosa leading to the generation of a number of reactive oxygen species (ROS) (3, 4, 32, 46); these would be expected to cause serious damage to the DNA of both host and bacterial cells. *H. pylori* is equipped with efficient ROS-detoxification systems (29, 32, 38, 48); however, these still may not be sufficient to avoid oxidative damage to DNA. Recent data (28) demonstrate that the host effectively induces lethal and pre-mutagenic oxidative DNA adducts in the *H. pylori* genome, and that the oxidative DNA damage repair mechanism of *H. pylori* plays an important role in permitting host colonization.

This study was initiated to investigate the functions of the *mutS* homologue in *H. pylori*. Our data show that *mutS* mutants are much more sensitive to oxidative stress than wild type cells, and that oxidative stress induces predominantly G:C→T:A mutations in *mutS*-defective cells but not in wild type cells. These findings indicate that the major role of MutS in *H. pylori* is to repair oxidative DNA lesions, particularly 8-oxoG. Consistent with this, we show that the purified *H. pylori* MutS protein binds to DNA substrates containing 8-oxoG, and that *H. pylori* *mutS* mutants contain much higher levels of 8-oxoG in their DNA as compared to wild type cells. Furthermore, we show that loss of MutS function results in a reduced colonization capacity in a mouse infection model.

MATERIALS AND METHODS

***H. pylori* growth conditions and assays for sensitivity to oxidative stress.** *H. pylori* was cultured on Brucella agar (BA) or Brain-heart-infusion / yeast extract (BHI-YE) agar plates supplemented with 10% defibrinated sheep blood or 5% fetal bovine serum. Cultures of *H. pylori* were grown microaerobically at 37 ^{o}C in a 5% CO_2 incubator under continuously controlled levels of oxygen (4% partial pressure). For paper disk sensitivity assay, sterile filter paper disks (7.5 mm in diameter) were applied to BA plates that had been streaked for confluent growth of *H. pylori* strains. Ten μl of the oxidazing agent were applied to each disk. After the plates were incubated under 4% O_2 condition for 48 h, the clear zones surrounding the disks (the distances from the edge of the disk to the end of the clear zone) were measured. For air survival assay, grown *H. pylori* cells were suspended in phosphate-buffered saline (PBS) and incubated at 37 ^{o}C under normal atmospheric conditions. Samples were removed at various times, serially diluted, and spread on BA plates. Colony counts were recorded after 3 days of incubation under 4% O_2 condition.

Construction of *H. pylori mutS* mutant. A 2.52 kb fragment containing the *H. pylori mutS* gene was PCR amplified from genomic DNA of strain 26695 and cloned into pGEM-T vector to generate pGEM-*mutS*. Subsequently, a chloramphenicol acetyl transferase (CAT) cassette was inserted within the *mutS* sequence of pGEM-*mutS* at the *Hind*III sites. The disrupted *mutS* gene was then introduced into *H. pylori* by natural transformation *via* allelic exchange and chloramphenicol-resistant colonies were isolated. The disruption of the gene in the genome of the mutant strain was confirmed by PCR showing an increase in the expected size of the PCR product and by direct sequencing of the PCR fragment.

Determination of mutation rate and mutation specificity. *H. pylori* cells were grown in a 4% O_2-containing environment in BHI-YE broth to late-log phase (viable cell number of about 10^9 cells/ml). For determination of mutation frequency under oxidative stress conditions, cell cultures were exposed to air for 4 h at 37 °C, while this step was omitted for the control experiments (normal condition, without air-exposure). The cell culture was diluted 10^{-4} in BHI-YE broth (to approximately 10^4 to 10^5 cells/ml) and divided into 20 independent 0.5-ml aliquots. These aliquots were then each allowed to grow (at 4% O_2) for 2 days to obtain parallel, independent cultures. The number of rifampicin resistant mutants that emerged in each culture was determined by plating the entire culture on BHI-YE agar plates containing 20μg/ml rifampicin. The total number of cells was determined by plating an appropriate dilution of three cultures on nonselective medium. Colonies on both selective and nonselective plates were counted after incubation for 4 days. The mutation rate was calculated by the Lea-Coulson method of median as described (34, 49). To determine mutation specificity, independent cultures were grown and plated as described above. To ensure that all mutations represent independent events (but are not the descendents of the same mutation), only one mutant colony was picked from each culture. From selected independent mutants, a 330 bp DNA fragment from the *rpoB* gene, or a 850 bp fragment from the *rdxA* gene was PCR-amplified using the primers described previously (49). The PCR fragments were sequenced, and the mutations responsible for resistance were identified by comparison to the corresponding wild-type sequence.

Overexpression and purification of *H. pylori* MutS protein. Based on our analysis of the *mutS* sequences from different *H. pylori* strains, we corrected the annotation of the translational start codon of the gene. The complete *mutS* gene (~2.5 kb) was amplified by PCR (primers MutSF: 5'CGCGCGGCATATGATGAATAATAATAATAC3' and MutSR: 5'ATACGCGCTCGAGCTTCAATTTAACGATTTT3') from strain 26695 and cloned into pET-21a vector to generate pET-MutS-6His. The recombinant plasmid was transformed into *E. coli* BL21 Rossetta strain. Expression of the MutS protein was induced by adding 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG) followed by further incubation for 3 h. The MutS protein was purified using a nickel-nitrilotriacetic acid (Ni-NTA) affinity column (Qiagen) following vendor's instructions and analyzed by SDS polyacrylamide gel electrophoresis. To eliminate possible contamination of *E. coli* MutY protein (~ 40 kDa), the MutS protein (> 80 kDa) was further purified by gel filtration chromatography using a Sephacryl S-200 column (Amersham Biosciences) and eluted with 25 mM sodium phosphate buffer (pH7.5). Protein concentrations were determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.).

Gel mobility shift assay for detection of MutS-DNA binding. Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA), and the oligonucleotides containing 8-oxoG were from Synthegen (Houston, TX). The Oligonucleotides were 5'-end labelled using [γ-^{32}P] ATP and T4 polynucleotide kinase, and then purified by ethanol precipitation. DNA substrates used for MutS binding in this study are shown in Fig.2B. dsDNA substrates were prepared by annealing molar equivalents of unlabeled or ^{32}P-labeled oligonucleotides with appropriate unlabeled complementary oligonucleotides as follows: heating at 90°C for 5 min followed by slow cooling overnight to room temperature. Residual single-stranded DNA was purified away from dsDNA using benzoylated naphthoylated DEAE-cellulose (Sigma). dsDNA was then concentrated using a YM-10 microcon centrifugal filter (Amicon Bioseparations) (22). The HJ structure was created by annealing one ^{32}P-labeled strand with three unlabeled strands.

DNA binding assays were performed by incubating each ^{32}P labelled DNA substrate (10 nM) with purified MutS protein at indicated concentrations, and 50 μg/ml sonicated calf thymus DNA in binding buffer (25 mM Tris-acetate, pH 8.0, 10 mM KCl, 8 mM Mg-acetate, 1 mM dithiothreitol and 3.5% [w/v] PEG8000) at 30°C for 10 min. For the competition assays, different concentrations of unlabeled DNA substrates were added in the binding reaction. After adding the gel-loading dye, the reaction mixture was subjected to electrophoresis on a 5% native polyacrylamide gel. The gel was subsequently dried, and analyzed using a PhosphoImager and ImageQuant software.

Fluorescent staining of cells and quantification of 8-oxoG. Approximately 10^6 cells in PBS were exposed to air for 4 h followed by incubation for another 4 h in 4% O_2. Cells were collected by centrifugation and re-suspended into cold 80% methanol. 20 μl of the cell suspension was air-dried onto a gelatin-coated glass slide for 15 min and then covered with 100 μl of 25 mM Tris/HCl (pH 8), 50 mM glucose, 10mM EDTA containing 2 mg lysozyme ml $^{-1}$ for 5 min. The slides were then immersed in methanol for 1 min, and then in acetone for 1 min followed by airdrying. 100 μl PBS containing 0.05% (w/v) Tween 20 and 2% (w/v) BSA was then applied to the sample on the slide for 20 min. Fluorescein-5-isothiocyanate (FITC)-conjugated avidin (0.5%; 1:200 dilution in PBS; Sigma) was added, and the slide incubated in a moist chamber for 1 h. The slides were then extensively washed with PBS, stained with propidium iodide (PI; 12.5 μg/ml), and mounted in a gel-mount solution. The slides were examined with a Leica DM IRB fluorescence microscope. The fluorescent images were recorded by use of a Hamamatsu C4742-95 digital camera and the images were processed using Open Lab Image Software (Improvision ®). The contrast adjustments for PI and FITC images were standardized and the FITC fluorescence of each defined region was divided by the PI fluorescence of the same region. The ratio averaged from at least 8 different regions from each of the strains was taken as an indicator of the content of 8-oxoG.

Mouse colonization. Mouse colonization assays were performed essentially as described previously (38, 48). Briefly, the wild type SS1 or SS1:*mutS* mutant cells were harvested after 48 h of growth on BA plates (37 °C, 4% oxygen) and suspended in PBS to an OD_{600} of 1.7. Headspace in the tube was sparged with argon gas to minimize oxygen exposure. These suspensions were administered to C57BL/6J mice (1.5 x 10^8 *H. pylori* cells / mouse). After 3 weeks, the mice were sacrificed and the stomachs were removed, weighed, and homogenized in argon-sparged PBS to avoid O_2 exposure. Stomach homogenate dilutions were plated on BA plates supplemented with bacitracin (100 μg/ml), vancomycin (10 μg/ml) and amphotericin B (10 μg/ml), and the plates were rapidly transported into an incubator containing sustained 2% partial pressure O_2. After incubation for 5 to 7 days the presence of *H. pylori* colonies was examined.

RESULTS

Sensitivity of *H. pylori mutS* mutants to oxidative stress. To test whether *H. pylori* MutS has a function in DNA damage repair, we created *H. pylori mutS* mutants in strains 26695 (genome sequence strain) and SS1 (mouse-adapted strain), and tested their sensitivity to various DNA damaging agents. No significant difference in sensitivity to UV light or to an alkylating agent (methyl methanesulfonate) was observed between the *mutS* mutants and their isogenic wild type strains (data not shown). However, *H. pylori mutS* mutants were more sensitive to oxidative stress than the wild type cells. In a paper-disk assay, the growth inhibition zones for the *mutS* mutant in the presence of H_2O_2 or paraquat were about 3- and 5-fold larger, respectively, than those for the wild type (Fig.4.1). Upon exposing approximately 10^8 cells to air for 10 h, about 10^6 viable cells of the wild type strain could be recovered. In contrast, the *mutS* mutant cells were completely killed (no viable cells recovered) by exposure to air for 10 h (Fig.4.2). These results suggest that *H. pylori* MutS may be involved in the repair of DNA damage induced by oxidative stress.

Mutation rate and mutation specificity in *H. pylori mutS* mutants. The spontaneous Rif^R mutation rates of the *mutS* mutant strains were determined and compared to those of their isogenic wild type strains. Under normal laboratory conditions for *H. pylori* culture, no significant difference in Rif^R mutation rate was observed between the wild type and the isogenic *mutS* mutant strains (Table 4.1). However, exposure of 26695*mutS* and SS1*mutS* cells to air for

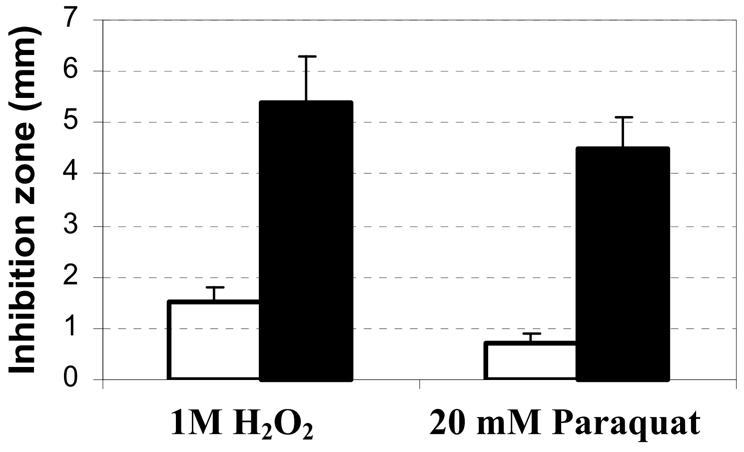


FIG. 4.1. Sensitivity of *H. pylori mutS* mutant to oxidative stress. Zones of growth inhibition were measured (in mm starting from the edge of the disk) around filter paper disks saturated with 10 µl of the indicated compounds. Results are the average of five independent experiments with standard deviations. Symbols: open bars, *H. pylori* SS1 wild type; filled bars, *H. pylori* SS1:*mutS* strain.

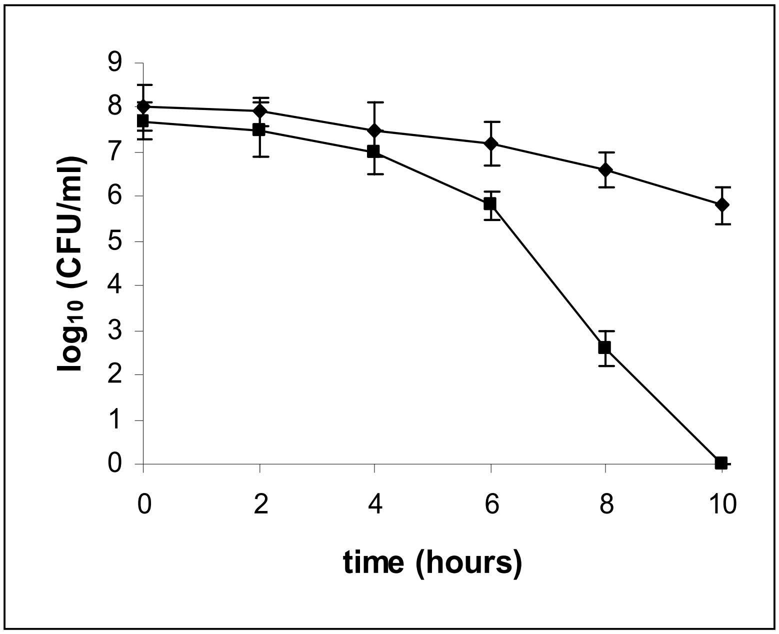


FIG. 4.2. Stress survival of non growing SS1 and *mutS* strain. *H. pylori* cell suspensions in PBS were incubated at 37^{o}C under normal atmospheric conditions (21% oxygen). Samples were removed at the times indicated in the x axis and were used for plate counts in a 4% oxygen environment. The data are the means of three experiments with standard deviation as indicated. Symbols: diamond, wild type strain SS1; square, *H. pylori* SS1: *mutS* strain.

According to Student t- distribution test, the results of the *mutS* mutant for both H_2O_2 and paraquat are significantly different (greater inhibition zone) from those of the parent strain at the 99% level of confidence. 4 hours increased the mutation rate about 9 and 16 fold respectively over that for the corresponding wild type strains (Table 4.1).

To determine whether the disruption of *mutS* leads to a change in the specificity of mutations relative to that found in wild type cells, we examined the spectra of mutations in the *rpoB* (Rif^R) and *rdxA* (Mtz^R) marker genes (49). A Rif^R phenotype in *H. pylori* results from mutations in the *rpoB*, the gene coding for RNA polymerase subunit β. A majority of the Rif^R (*rpoB*) mutations map to a small region of the *rpoB* gene and lead to changes in the rifampicin-binding site of the enzyme. Resistance to metronidazole (Mtz^R phenotype) results mainly from mutations in *rdxA*, the gene encoding a nitroreductase that converts the non-toxic metronidazole to the toxic hydroxylamine derivative in wild type cells. Any mutation within the *rdxA* gene that leads to loss of the enzyme function will give rise to an Mtz^R phenotype.

We subjected 26695 wild type and *mutS* cells to air for 1 h (O_2 stress), and isolated a number of Rif^R and Mtz^R mutants. We sequenced a 330 bp *rpoB* PCR fragment from 15 independently arising Rif^R mutants each from wild type and *mutS* cells. Similarly, we sequenced an 850 bp *rdxA* fragment from 10 independently arising Mtz^R mutants each from wild type and *mutS* cells (Table 2, upper panels). In wild type cells, 9 of the 15 Rif^R mutants are transitions, and the remaining 6 are transversions. In contrast, in the *mutS* cells, 14 of the 15 Rif^R mutants are transversions, with 13 being G:C-to-T:A mutations. In wild type cells, Mtz^R mutations are predominantly frameshifts at simple nucleotide repeats (8 of 10 mutants). In *mutS* cells however, 7 of 10 Mtz^R mutants are G:C-to-T:A transversions. This unique mutation specificity is distinct from that described for *E. coli mutS* mutant in which the increase in mutagenesis is

Table 4.1. Mutation rates in *H. pylori* parental and *mutS* strains

Strain	Mutation rate (x 10^8) [a]	
	No O_2 Stress	O_2 Stress [b]
26695	2.7 (0.6)	14 (3.2)
26695:*mutS*	3.1 (0.8)	130 (28)
SS1	4.3 (1.2)	11 (2.1)
SS1:*mutS*	7.8 (2.3)	180 (37)

[a] Mutation rates are determined as mutation to rifampicin resistance per cell division (x 10^8). Data are calculated from an experimentally determined median number of mutants from 20 independent cultures. The standard deviation for each data set is shown in parentheses.

[b] Cell suspension was exposed to air for 4 hours before plating for mutation rate assay.

predominantly due to transitions (15, 17). As a control, we also determined DNA sequences of 15 Rif^R mutants each isolated from wild type or *mutS* cells that were not subjected to O_2 stress (Table 4.2, lower panels). Five out of 15 Rif^R mutants from the wild type are transversions, and the remaining 10 are transitions. A similar spectrum was observed for the *mutS* cells in the absence of O_2 stress: 6/15 transversions and 9/15 transitions. Thus, the unique G:C-to-T:A mutation specificity was only observed in the *mutS* mutant cells under O_2 stress condition.

Binding of *H. pylori* MutS protein to anomalous DNA. That G:C to T:A transversion mutations, a signature of 8-oxoG damage, increase significantly in *H. pylori mutS* cells suggests that a major role of *H. pylori* MutS may be the repair of 8-oxoG damage in DNA. To determine whether purified *H. pylori* MutS protein can bind to DNA containing 8-oxoG, we over-expressed Hp MutS protein (with His tag) and purified it to near homogeneity using a nickel-nitrilotriacetic acid (Ni-NTA) affinity column followed by gel filtration chromatography (Fig.4.3A).

We performed gel mobility shift assays to test the binding affinity of Hp MutS to homoduplex DNA (G:C), double stranded DNA (dsDNA) containing a mismatch (G:A), dsDNA containing 8-oxoG lesions (GO:C or GO:A), and to Holliday junction (HJ; four way junction) DNA (Fig.4.3B-E). Hp MutS (0 – 100 nM) was incubated with 10 nM ^{32}P-labeled DNA substrates. Fig.2C and 2D show that *H. pylori* MutS does not bind to the normal homoduplex (G:C) DNA; it binds to the duplex containing a G:A mismatch very weakly; and it binds to DNA containing a GO:A or GO:C pair as well as the HJ structure with a high affinity. For most of the DNA substrates, addition of increasing amounts of the MutS protein resulted in the formation of increasing amounts of the MutS-DNA complex. However, this dose-dependency effect is not evident for GO:A substrate, suggesting that this substrate may be a preferred one at the lower concentration of MutS. Under our experimental conditions, we found that 10 nM DNA substrate

Table 4. 2. Mutation spectra in *H. pylori* parental and *mutS* strains.

Strains	Mutations	Occurrence
26695 (O$_2$ stress) Rif^R	A-to-G (Q527R)	1/15
	G-to-A (D530N)	2/15
	A-to-G (D530G)	3/15
	A-to-T (D530V)	3/15
	C-to-T (H540Y)	1/15
	C-to-A (Q527K)	1/15
	C-to-A (H540N)	1/15
	C-to-T (L525P)	2/15
	T-to-A (I586N)	1/15
26695:*mutS* (O$_2$ stress) Rif^R	C-to-A (L525I)	6/15
	C-to-A (Q527K)	5/15
	C-to-A (H540N)	2/15
	A-to-T (I586P)	1/15
	A-to-G (D530G)	1/15
26695 (O$_2$ stress) Mtz^R	C-to-T (R16C)	1/10
	+ A (a)	5/10
	- T (b)	2/10
	G-to-T (E175stop)	1/10
	- A (c)	1/10
26695:*mutS* (O$_2$ stress) Mtz^R	+ A (a)	3/10
	C-to-A (P44Q)	5/10
	C-to-A (Q50K)	2/10
26695 (No O$_2$ stress) Rif^R	C-to-A (Q527K)	1/15
	A-to-T (Q527L)	1/15
	G-to-A (D530N)	2/15
	G-to-T (D530Y)	1/15

	A-to-T (D530V)	1/15
	A-to-G (D530G)	2/15
	C-to-T (H540Y)	2/15
	C-to-A (H540N)	1/15
	A-to-G (H540R))	2/15
	C-to-T (R543C)	1/15
	C-to-T (S545L)	1/15
26695:*mutS* (No O_2 stress) Rif^R	C-to-A (Q527K)	1/15
	A-to-T (Q527L)	1/15
	G-to-A (D530N)	1/15
	G-to-T (D530Y)	1/15
	A-to-T (D530V)	1/15
	A-to-G (D530G)	1/15
	C-to-T (H540Y)	3/15
	C-to-A (H540N)	1/15
	A-to-G (H540R)	1/15
	C-to-T (R543C)	1/15
	G-to-T (R543L)	1/15
	C-to-T (S545L)	2/15

The changes in amino acid sequences caused by mutations are indicated in parenthesis.

(a) Addition of an A at a run of 7 As, leading to frameshift at codon 64 and translation stop at codon 73.

(b) Deletion of a T at a run of 4 Ts, leading to frameshift at codon 72 and translation stop at codon 76.

(c) Deletion of an A at a run of 3 As, leading to frameshift at codon 35 and translation stop at codon 55.

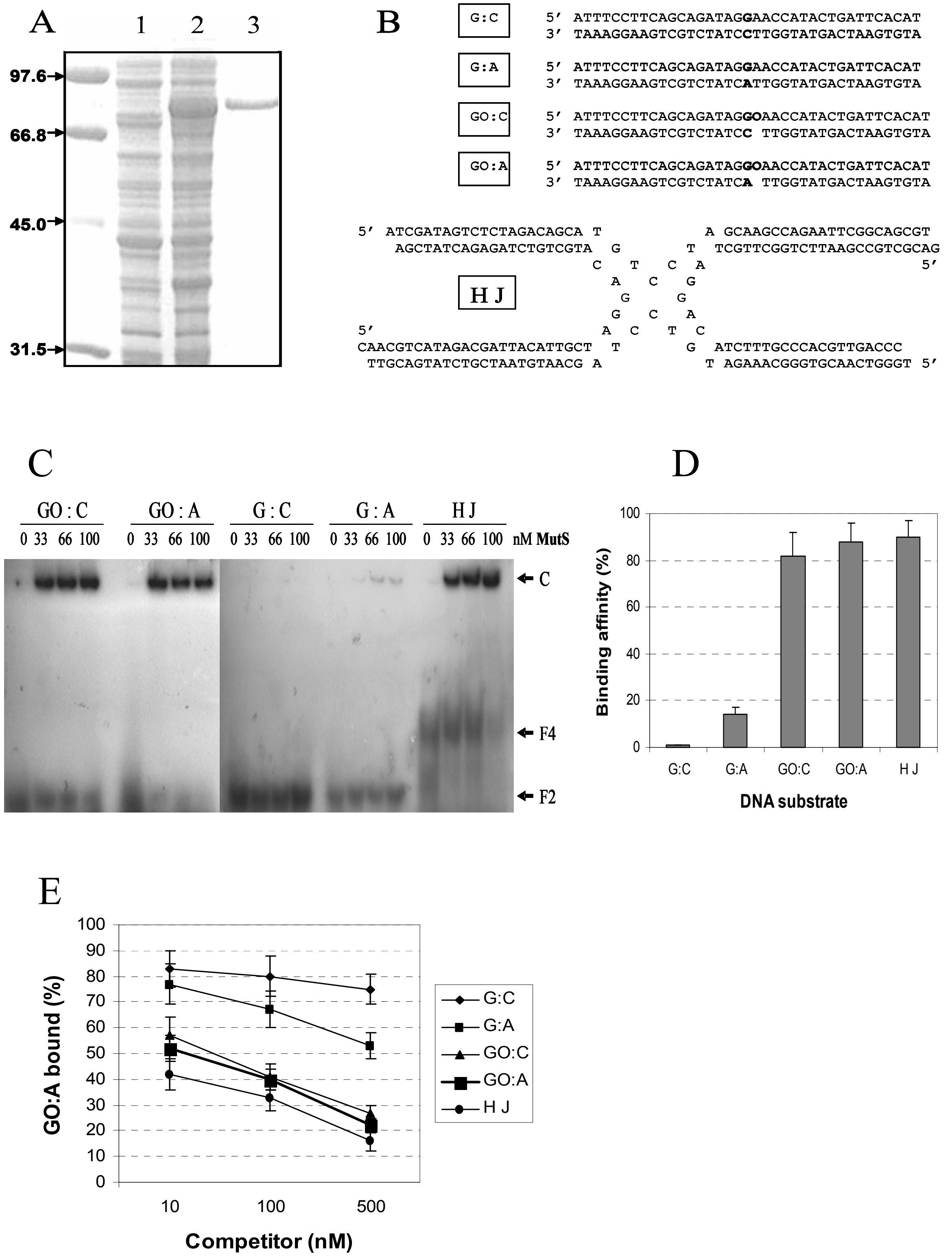
FIG. 4.3. DNA binding ability of MutS. (A) *H. pylori* MutS was overexpressed in *E.coli* and purified to near homogeneity as analyzed on 12% SDS-PAGE. Lanes 1: Uninduced culture; Lane 2: Induced with 800 μM IPTG; Lane 3: purified MutS.

(B) ^{32}P-labeled DNA substrates used for the binding assay: G:C, homoduplex DNA control; G:A, dsDNA containing a G:A mispair; 8-oxoG:C, 8-oxoG:C mispair; 8-oxoG:A, 8-oxoG:A mispair; HJ, Holliday junction.

(C) Formation of specific MutS-DNA complex analyzed by gel shift assay. Hp MutS (0 – 100 nM) was incubated with 10 nM ^{32}P-labeled DNA substrates. The bands of the MutS-DNA complex (C) and the free DNA substrates (F2, dsDNA; F4, four stranded DNA) are marked.

(D) Quantitation of specific complex formation at 100 nM Hp MutS. Binding affinity represents the percent of total DNA substrate present as DNA-MutS complex [C/(C+F)]. The data are averages of three experiments (Panel C showing a representative gel) with standard deviations.

(E) Competition assay. 100 nM MutS was incubated with 10 nM ^{32}P-labeled 8-oxoG:A DNA substrate and varying concentrations (10, 100, 500 nM) of unlabeled G:C, G:A, 8-oxoG:C, 8-oxoG:A, and HJ competitor DNAs, and then the amount of MutS-DNA complex was determined using gel shift assays. The values of y axis (% 8-oxoG:A bound) represent the density ratios of band C/(C+F). The data are averages of three experiments with standard deviations.


A
1
2
3
97.6
66.8
45.0
31.5
B
G:C
5′ ATTTCCTTCAGCAGATAGGAACCATACTGATTCACAT
3′ TAAAGGAAGTCGTCTATCCTTGGTATGACTAAGTGTA
G:A
5′ ATTTCCTTCAGCAGATAGGAACCATACTGATTCACAT
3′ TAAAGGAAGTCGTCTATCATTGGTATGACTAAGTGTA
GO:C
5′ ATTTCCTTCAGCAGATAGGOAACCATACTGATTCACAT
3′ TAAAGGAAGTCGTCTATCC TTGGTATGACTAAGTGTA
GO:A
5′ ATTTCCTTCAGCAGATAGGOAACCATACTGATTCACAT
3′ TAAAGGAAGTCGTCTATCA TTGGTATGACTAAGTGTA
H J
5′ ATCGATAGTCTCTAGACAGCA T A GCAAGCCAGAATTCGGCAGCGT
AGCTATCAGAGATCTGTCGTA G T TCGTTCGGTCTTAAGCCGTCGCAG
C T C A 5′
A C G
G G
G C A
5′ A C T C
CAACGTCATAGACGATTACATTGCT T G ATCTTTGCCCACGTTGACCC
TTGCAGTATCTGCTAATGTAACG A T AGAAACGGGTGCAACTGGGT 5′
C
GO : C
GO : A
G : C
G : A
H J
0 33 66 100
0 33 66 100
0 33 66 100
0 33 66 100
0 33 66 100
nM MutS
C
F4
F2
D
Binding affinity (%)
0
20
40
60
80
100
G:C
G:A
GO:C
GO:A
H J
DNA substrate
E
GO:A bound (%)
0
10
20
30
40
50
60
70
80
90
100
10
100
500
Competitor (nM)
G:C
G:A
GO:C
GO:A
H J

and 30 - 100 nM MutS protein were optimal for detecting the specific binding complex. At much higher concentrations (μM range) of DNA and MutS protein, some non-specific binding to the normal (single-stranded or double stranded) DNA was observed (data not shown). To further characterize the specificity of binding of Hp MutS to these substrates, we performed a competition assay with 100 nM MutS, 10 nM labelled 8-oxoG:A substrate, and increasing amounts of unlabeled G:C, G:A, GO:C, GO:A duplexes, as well as HJ substrates (Fig.4.3E).

The G:C and G:A substrates showed a very weak ability to compete for binding with GO:A, while the GO:A, GO:C, and HJ substrates were strong competitors. Based on these results, the relative affinity of Hp MutS for these substrates was G:C < G:A << GO:C, GO:A $\leq$ HJ.

Levels of 8-oxoG in *H. pylori* wild type and *mutS* mutant cells. To further investigate the role of *H. pylori* MutS in repair of 8-oxoG in DNA, we examined the level of 8-oxoG DNA lesions in the *mutS* mutant cells by using the FITC-avidin/PI fluorescence microscopy technique. As 8-oxoG is structurally similar to avidin's natural substrate biotin, avidin binds to 8-oxoG with a high specificity and has been used successfully for detection of 8-oxoG (43). Thus, in this technique, because FITC-avidin specifically labels DNA lesions and the PI labels all DNA, the intensity ratio of FITC to PI staining reflects the level of 8-oxoG DNA lesions in the cells (8). In this analysis, *H. pylori* wild type or *mutS* cells were exposed to air for 4 h, and were allowed to recover (repair) for 4h by incubation in 4% O_2 before immunofluorescent staining. Fig.4.4A and 3B show that 4h exposure to air had no significant effect on the morphology of the wild type cells, with a majority of the cells being in the bacillary form. However, when similarly treated, a majority of the *mutS* cells transformed to the coccoid form (Fig.4.4C and 4.4D), suggesting these cells suffer from persistent oxidative stress (30). The luminosity of FITC and PI was measured from 8 sets of images, and the average ratio of luminosity of FITC/PI was calculated for the wild type and the *mutS* mutant cells; this represents the level of 8-oxoG DNA lesions in the cells. The ratio of FITC-avidin / PI fluorescence intensity in the *mutS* mutant cells was determined to be 3-fold higher than that in the wild type cells. In another experiment, *H. pylori* wild type or *mutS* mutant cells were exposed to air for 4 h followed immediately by immunofluorescent staining (i.e. without recovery time). Similar results were obtained, suggesting that additional time is not necessary for the wild type cells to repair oxidative damage. These results demonstrated that the *mutS* mutant cells exposed to oxidative stress contain considerably higher levels of 8-oxoG DNA lesions. In contrast, no significant difference between wild type and *mutS* cells was observed in either cell morphology or the level of 8-oxoG DNA lesions under normal microaerophilic laboratory growth conditions (data not shown).

Decreased efficiency of mouse colonization of *H. pylori mutS* mutant strain. To determine whether loss of MutS activity has a significant effect on *H. pylori* colonization in the host, the relative abilities of the wild type SS1 and the SS1:*mutS* mutant strains to colonize the mouse stomach were compared (Fig.4.5). In one experiment, ten C57BL/6J mice were inoculated with the wild type and 11 mice were inoculated with the mutant strain, and the colonization of *H. pylori* cells in the mouse stomachs was examined 3 weeks after inoculation (Fig.4.5A). *H. pylori* was recovered from all 10 mice that had been inoculated with the wild type strain, with the bacterial numbers ranging from 10^5 – 10^6 CFU/g stomach. In contrast, 3 of 11 mice that were inoculated with the *mutS* mutant strain were free of *H. pylori*. In addition, the colonization titers for the 8 *H. pylori*-positive mouse stomachs were all below 10^5 CFU/g stomach (Fig.4.5A). According to Wilcoxin rank test analysis, the ranges of colonization values of the mutant strain is significantly lower than that of the wild type at the 99% confidence level ($P<0.01$). The entire mouse colonization assay was performed an additional time, with a similar

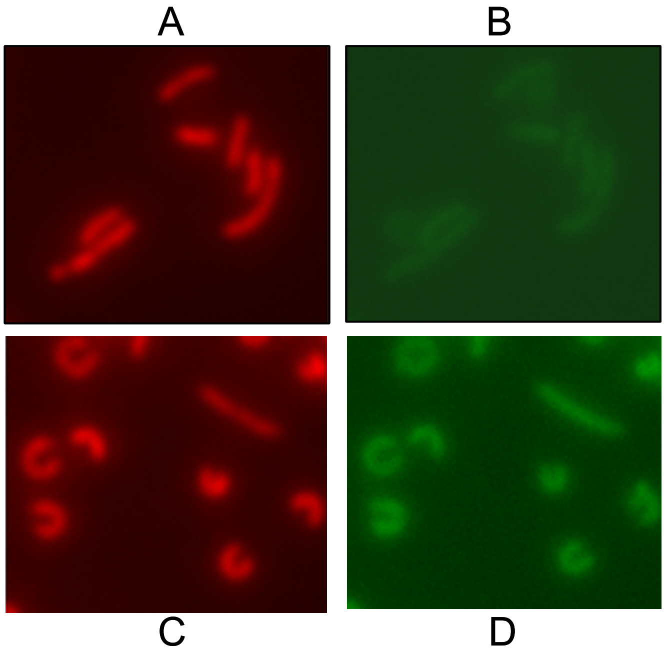
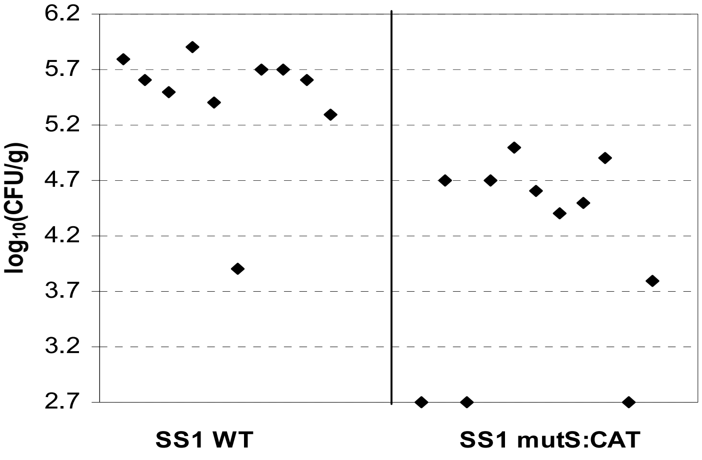
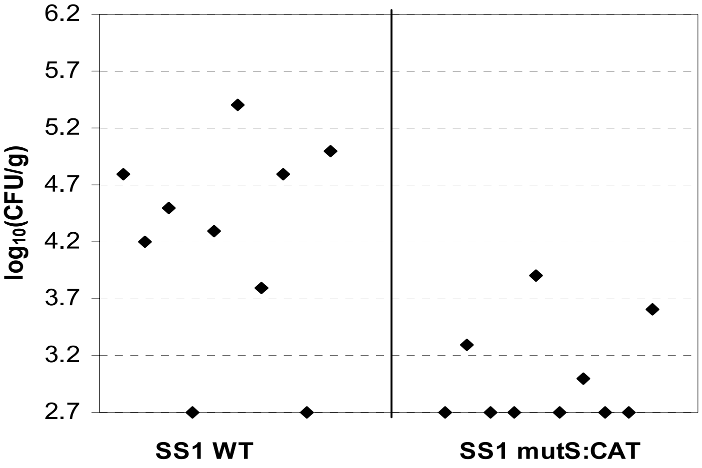


FIG. 4.4 Detection of 8-oxoG by immunofluorescent staining. *H. pylori* SS1 (A and B) and SS1:*mutS* mutant (C and D) cells were exposed to air for 4 h followed by a recovery incubation at 4% O_2 condition for 4 h. The cells were fixed on the glass slide and stained with 8-oxoG - specific avidin-FITC conjugate (B and D) and propidium iodide (A and C) followed by examination via fluorescence microscopy. The contrast adjustment was normalized for all the images, and a representative set of images is shown here.



A. Experiment 1



B. Experiment 2

FIG. 4.5. Mouse colonization assays of *H. pylori* SS1 and its isogenic mutant of *mutS*::CAT. The mice were inoculated with *H. pylori* two times (two days apart) with a dose of 1.5 x 10^8 viable cells. Colonization of *H. pylori* in mouse stomachs was examined 3 weeks after the first inoculation. Stomachs were homogenized in a low O_2 environment (see text). Data are presented as a scatter plot of colony forming units per gram of stomach as determined by plate counts. Each point represents the CFU count from one stomach. The base line [$\log_{10}$ (CFU/g) =2.7] is the detection limit of the assay, which represents a count below 500 CFU/g stomach. Panel A and B are two separate experiments performed at different times with different batch of mice.

difference observed between the parent and mutant strain, although the overall colonization efficiencies (for both wild type and mutant) were lower than those obtained in the first experiment (Fig.4.5B). Nevertheless, these results suggest that MutS plays a significant role in survival/colonization in the host probably because of its role in repairing oxidative DNA damage.

DISCUSSION

Post-replication mismatch correction plays an important role in maintaining replication fidelity in *E. coli* as well as in a number of other well-studied model organisms. In *E. coli*, loss of the MMR system, which consists of MutS, MutL, and MutH proteins, leads to an almost 1000-fold increase in spontaneous mutagenesis (37). *H. pylori* has a *mutS* homologue, but lacks the homologues of *mutH* or *mutL* genes. The absence of a full complement of genes required for an "*E. coli* like" MMR pathway may mean that *H. pylori* lacks a functional mismatch repair system (47). Consistent with this possibility, *H. pylori* clinical isolates display high spontaneous mutation frequencies, and furthermore, mutation frequency does not appear to be affected by the loss of the *mutS* gene (5). In addition to an indispensable role in MMR, the *E. coli mutS* gene has an essential role in suppressing homeologous recombination (33), and may play an additional role in the repair of 8-oxoG in DNA (51, 52). Several studies on eukaryotic MutS homologues also demonstrated their involvement in the repair of oxidative DNA damage (9, 11, 12, 22, 27). Here, we have asked if the *H. pylori mutS* homologue has a role in DNA damage repair. Many lines of evidence from our experiments indicate that the *H. pylori* MutS has a major role in the repair of oxidative DNA damage.

First, in cells disrupted for the *mutS* gene, there is a significant increase in sensitivity to oxidative stress, as indicated by sensitivity to H_2O_2, paraquat and oxygen (Fig.3.1 and 3.2).

Unlike wild type cells which show a normal bacillary morphology when exposed to air for 4 h, *mutS*-disrupted cells transform to a coccoid state under the same conditions (Fig.4.4), an indication of severely compromised ability to cope with oxidative stress. A 4-h exposure of *mutS*-disrupted cells to air leads to a 9- to 16-fold increase in mutation rate over that observed for the corresponding wild type cells (Table 4.1). Strikingly, the specificity of mutations induced in *mutS*-disrupted cells exposed to air for 1 h is profoundly different from that observed in similarly treated wild type cells (Table 4.2). Thus, in the *rpoB* as well as the *rdxA* marker genes, the predominant mutation in *mutS*-disrupted cells (as contrasted to wild type cells) proved to be a G:C→T:A transversion, a narrow specificity that is typically observed in *E. coli* cells defective for MutM (the major repair glycosylase that acts on 8-oxoG residues in DNA) or for MutY (which codes for an adenine glycosylase specific to adenines opposite 8-oxoG) (24). In the eukaryote *S. cerevisiae*, which is known to lack a *mutY* homologue, a complex of *MSH2* and *MSH6* proteins that recognizes 8-oxoG:A mispair has been proposed to serve as the functional equivalent of *mutY* (27). Although an increase in G:C→T:A mutations in *H. pylori mutS*-defective cells can result from the loss of either MutM-like or MutY-like activity, the observation that 8-oxoG levels in DNA increase in *mutS*-defective cells (Fig.4.4) suggests that the defect is in the repair of 8-oxoG. This idea is further supported by the observation that the *H. pylori* genome does have a *mutY* homologue, but not a *mutM* homologue (1, 45, 47), implying that other proteins are used for 8-oxoG repair. Finally, purified *H. pylori* MutS protein is able to bind to dsDNA containing an 8-oxoG with a high affinity (Fig 4.3), consistent with the possibility that it plays a role in the repair of 8-oxoG. Thus all lines of evidence presented here make a cumulative case for the proposal that a major function of the *H. pylori mutS* gene is the repair of oxidative damage in DNA.

The *H. pylori mutS* gene belongs to the MutS2 family of proteins that have a high degree of homology to the carboxy-terminal two-thirds of the MMR-related *mutS* gene product (sometimes called MutS1). MutS2 proteins however, lack the N-terminal 300 residues generally found in MutS1 proteins, but instead have a ~250 residue C-terminal extension that is not present in MutS1 (26). The C-terminal 90 residues in MutS2 proteins constitute the highly conserved *smr* domain (small *mutS*-related protein). In organisms that lack MutS2, such as *E. coli*, *smr* sequences are found in the form of a small protein coded by an independent *smr* gene. Conversely, organisms coding for MutS2 (such as *H. pylori*) usually do not have an independent *smr* gene. Malik and Henikof (21) hypothesized that the *smr* domain of the MutS2 protein may have an endonuclease activity. Such a possibility is supported by a recent finding of Fukui *et al.*, who reported that the *Thermus thermophilus* MutS2 protein has an endonuclease activity (14). Thus, it is possible that the *H. pylori* MutS protein has both DNA damage recognition and incision functions. Whether the *H. pylori mutS* gene product has an endonuclease activity is among the many interesting questions that remain to be investigated. Rossolillo and Albertini found that the loss of a *mutS2* (*yshD*) gene in *Bacillus subtilis* leads to a small increase in G:C→T:A mutations, leading to the speculation that *yshD* may be involved in a base excision repair pathway for oxidative DNA damage (35). On the basis of the literature summarized above, and the results presented here, we propose that a major function of the conserved MutS2 family of proteins may be the repair of 8-oxoG damage in DNA.

Homologues of MutS proteins are known to play multiple roles in DNA repair and recombination by virtue of their ability to recognize DNA structural anomalies including base mispairs, insertion-deletion loops, and HJ structures (18, 20, 25, 37). Upon completion of this work we noticed a recent paper (31) showing that *H. pylori* MutS protein binds to DNA structures mimicking recombination intermediates, indicating a possible role for *H. pylori mutS* in inhibiting DNA strand exchange. Our gel mobility shift assay results also show that Hp MutS binds to HJ structures with a high affinity, confirming their finding. *H. pylori* MutS, as an example of MutS2 proteins, lacks the N-terminal domain responsible for mismatch recognition identified in the MutS1 proteins (40). Thus, MutS2 proteins probably recognize anomalous DNA structures rather than binding directly to mismatched bases, as suggested for hMSH4-hMSH5 proteins (41). It remains to be determined which domain in the MutS2 protein is responsible for recognition/binding to anomalous DNA structures. Our MutS-DNA binding results suggest that the same domain of MutS is responsible for binding to 8-oxoG-containing duplex DNA and HJ structure, as they compete with each other for binding. The results of Pinto et al. (31) suggest that *H. pylori* MutS has an important role in suppression of DNA recombination, whereas our results here reveal another major role for *H. pylori* MutS in the repair of oxidative DNA damage. Further studies are required to determine whether MutS2 has a direct role in repair of oxidative damage (*i. e.*, a MutM-like activity) or an indirect role via recombination-mediated repair.

Repair of oxidative bacterial DNA damage is thought to be important to bacterial pathogenesis (42). For example, *Salmonella typhimurium* or *Listeria monocytogenes* mutants unable to express DNA repair enzymes are attenuated in virulence characteristics (7, 23). Also, DNA adenine methylase (45), which is required for the DNA mismatch repair system to function in *E. coli* and *S. typhimurium*, was shown to play an essential role in *Salmonella* virulence (16). In contrast, consistent with the idea that an "*E. coli*-like" mismatch repair system is not functional in *H. pylori*, the DNA adenine methylase in *H. pylori* (*Hpy*IIIM) does not appear to be involved in mismatch repair (39). *H. pylori* induces a strong inflammatory response in the host, triggering the release of high levels of host-derived toxic oxygen species. These reactive oxygen species have many macromolecular targets in the bacterium, including DNA. During the evolution of the pathogen in its gastric niche, *H. pylori* MutS might have adapted to play a major role in repair of oxidative DNA damage. Recent data of O'Rourke et al. (28) support the hypothesis that bacterial DNA damage constitutes a host defence mechanism, and that the repair of oxidative DNA damage (the function of the endonuclease III in their case) in *H. pylori* contributes to its ability to colonize the host effectively. Our data demonstrate that *mutS*-disrupted *H. pylori* cells are less able to colonize hosts as compared to wild type cells (Fig.4). We propose the loss of the *mutS* gene in *H. pylori* leads to persistent oxidative DNA damage to the bacterium and consequently cell death within the host.

ACKNOWLEDGEMENTS

This work was mainly supported by NIH grant (to R.J.M.) 1-RO1-DK60061. M.Z.H. is supported by USPHS grants CA73026 and GM58253. D.E.T. is supported by Canadian Bacterial Diseases Network and AHFMR. We thank the people of Dr. Boris Striepen lab (UGA) and Dr. Timothy Hoover lab (UGA) for their technical support on fluorescence microscopy and gel shift assay, respectively. We thank Sue Maier for her expertise and assistance on mouse colonization assays.

REFERENCES

1. **Alm, R. A., L. S. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smith, B. Noonan, B. C. Guild, B. L. deJonge, G. Carmel, P. J. Tummino, A. Caruso, M. Uria-Nickelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. F. Vovis, and T. J. Trust.** 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature **397:**176-80.

2. **Aras, R. A., J. Kang, A. I. Tschumi, Y. Harasaki, and M. J. Blaser.** 2003. Extensive repetitive DNA facilitates prokaryotic genome plasticity. Proc Natl Acad Sci U S A **100:**13579-84.

3. **Bagchi, D., G. Bhattacharya, and S. J. Stohs.** 1996. Production of reactive oxygen species by gastric cells in association with *Helicobacter pylori*. Free Radic Res **24:**439-50.

4. **Baik, S. C., H. S. Youn, M. H. Chung, W. K. Lee, M. J. Cho, G. H. Ko, C. K. Park, H. Kasai, and K. H. Rhee.** 1996. Increased oxidative DNA damage in *Helicobacter pylori*-infected human gastric mucosa. Cancer Res **56:**1279-82.

5. **Bjorkholm, B., M. Sjolund, P. G. Falk, O. G. Berg, L. Engstrand, and D. I. Andersson.** 2001. Mutation frequency and biological cost of antibiotic resistance in *Helicobacter pylori*. Proc Natl Acad Sci U S A **98:**14607-12.

6. **Blaser, M. J., and D. E. Berg.** 2001. *Helicobacter pylori* genetic diversity and risk of human disease. J Clin Invest **107:**767-73.

7. **Buchmeier, N. A., C. J. Lipps, M. Y. So, and F. Heffron.** 1993. Recombination-deficient mutants of *Salmonella typhimurium* are avirulent and sensitive to the oxidative burst of macrophages. Mol Microbiol **7:**933-6.

8. **Chen, S. K., M. H. Tsai, J. J. Hwang, W. P. Chang, and W. P. Chan.** 2001. Determination of 8-oxoguanine in individual cell nucleus of gamma-irradiated mammalian cells. Radiat Res **155:**832-6.

9. **DeWeese, T. L., J. M. Shipman, N. A. Larrier, N. M. Buckley, L. R. Kidd, J. D. Groopman, R. G. Cutler, H. te Riele, and W. G. Nelson.** 1998. Mouse embryonic stem cells carrying one or two defective Msh2 alleles respond abnormally to oxidative stress inflicted by low-level radiation. Proc Natl Acad Sci U S A **95:**11915-20.

10. **Dunn, B. E., H. Cohen, and M. J. Blaser.** 1997. *Helicobacter pylori*. Clin Microbiol Rev **10:**720-41.

11. **Dzierzbicki, P., P. Koprowski, M. U. Fikus, E. Malc, and Z. Ciesla.** 2004. Repair of oxidative damage in mitochondrial DNA of *Saccharomyces cerevisiae*: involvement of the MSH1-dependent pathway. DNA Repair (Amst) **3:**403-11.

12. **Earley, M. C., and G. F. Crouse.** 1998. The role of mismatch repair in the prevention of base pair mutations in *Saccharomyces cerevisiae.* Proc Natl Acad Sci U S A **95:**15487-91.

13. **Eisen, J. A.** 1998. A phylogenomic study of the MutS family of proteins. Nucleic Acids Res **26:**4291-300.

14. **Fukui, K., R. Masui, and S. Kuramitsu.** 2004. *Thermus thermophilus* MutS2, a MutS paralogue, possesses an endonuclease activity promoted by MutL. J Biochem (Tokyo) **135:**375-84.

15. **Garibyan, L., T. Huang, M. Kim, E. Wolff, A. Nguyen, T. Nguyen, A. Diep, K. Hu, A. Iverson, H. Yang, and J. H. Miller.** 2003. Use of the rpoB gene to determine the specificity of base substitution mutations on the *Escherichia coli* chromosome. DNA Repair (Amst) **2:**593-608.

16. **Heithoff, D. M., R. L. Sinsheimer, D. A. Low, and M. J. Mahan.** 1999. An essential role for DNA adenine methylation in bacterial virulence. Science **284:**967-70.

17. **Horst, J. P., T. H. Wu, and M. G. Marinus.** 1999. *Escherichia coli* mutator genes. Trends Microbiol **7:**29-36.

18. **Jiricny, J.** 1998. Eukaryotic mismatch repair: an update. Mutat Res **409:**107-21.

19. **Kersulyte, D., H. Chalkauskas, and D. E. Berg.** 1999. Emergence of recombinant strains of *Helicobacter pylori* during human infection. Mol Microbiol **31:**31-43.

20. **Kolodner, R. D., and G. T. Marsischky.** 1999. Eukaryotic DNA mismatch repair. Curr Opin Genet Dev **9:**89-96.

21. **Malik, H. S., and S. Henikoff.** 2000. Dual recognition-incision enzymes might be involved in mismatch repair and meiosis. Trends Biochem Sci **25:**414-8.

22. **Mazurek, A., M. Berardini, and R. Fishel.** 2002. Activation of human MutS homologs by 8-oxo-guanine DNA damage. J Biol Chem **277:**8260-6.

23. **Merino, D., H. Reglier-Poupet, P. Berche, and A. Charbit.** 2002. A hypermutator phenotype attenuates the virulence of Listeria monocytogenes in a mouse model. Mol Microbiol **44:**877-87.

24. **Michaels, M. L., C. Cruz, A. P. Grollman, and J. H. Miller.** 1992. Evidence that MutY and MutM combine to prevent mutations by an oxidatively damaged form of guanine in DNA. Proc Natl Acad Sci U S A **89:**7022-5.

25. **Modrich, P., and R. Lahue.** 1996. Mismatch repair in replication fidelity, genetic recombination, and cancer biology. Annu Rev Biochem **65:**101-33.

26. **Moreira, D., and H. Philippe.** 1999. Smr: a bacterial and eukaryotic homologue of the C-terminal region of the MutS2 family. Trends Biochem Sci **24:**298-300.

27. **Ni, T. T., G. T. Marsischky, and R. D. Kolodner.** 1999. MSH2 and MSH6 are required for removal of adenine misincorporated opposite 8-oxo-guanine in S. cerevisiae. Mol Cell **4:**439-44.

28. **O'Rourke, E. J., C. Chevalier, A. V. Pinto, J. M. Thiberge, L. Ielpi, A. Labigne, and J. P. Radicella.** 2003. Pathogen DNA as target for host-generated oxidative stress: role for repair of bacterial DNA damage in *Helicobacter pylori* colonization. Proc Natl Acad Sci U S A **100:**2789-94.

29. **Olczak, A. A., J. W. Olson, and R. J. Maier.** 2002. Oxidative-stress resistance mutants of *Helicobacter pylori*. J Bacteriol **184:**3186-93.

30. **Park, A. M., Q. Li, K. Nagata, T. Tamura, K. Shimono, E. F. Sato, and M. Inoue.** 2004. Oxygen tension regulates reactive oxygen generation and mutation of *Helicobacter pylori*. Free Radic Biol Med **36:**1126-33.

31. **Pinto, A. V., A. Mathieu, S. Marsin, X. Veaute, L. Ielpi, A. Labigne, and J. P. Radicella.** 2005. Suppression of homologous and homeologous recombination by the bacterial MutS2 protein. Mol Cell **17:**113-20.

32. **Ramarao, N., S. D. Gray-Owen, and T. F. Meyer.** 2000. *Helicobacter pylori* induces but survives the extracellular release of oxygen radicals from professional phagocytes using its catalase activity. Mol Microbiol **38:**103-13.

33. **Rayssiguier, C., D. S. Thaler, and M. Radman.** 1989. The barrier to recombination between *Escherichia coli* and Salmonella typhimurium is disrupted in mismatch-repair mutants. Nature **342:**396-401.

34. **Rosche, W. A., and P. L. Foster.** 2000. Determining mutation rates in bacterial populations. Methods **20:**4-17.

35. **Rossolillo, P., and A. M. Albertini.** 2001. Functional analysis of the *Bacillus subtilis* yshD gene, a mutS paralogue. Mol Gen Genet **264:**809-18.

36. **Salama, N., K. Guillemin, T. K. McDaniel, G. Sherlock, L. Tompkins, and S. Falkow.** 2000. A whole-genome microarray reveals genetic diversity among *Helicobacter pylori* strains. Proc Natl Acad Sci U S A **97:**14668-73.

37. **Schofield, M. J., and P. Hsieh.** 2003. DNA mismatch repair: molecular mechanisms and biological function. Annu Rev Microbiol **57:**579-608.

38. **Seyler, R. W., Jr., J. W. Olson, and R. J. Maier.** 2001. Superoxide dismutase-deficient mutants of *Helicobacter pylori* are hypersensitive to oxidative stress and defective in host colonization. Infect Immun **69:**4034-40.

39. **Simala-Grant, J. L., E. Lam, M. Keelan, and D. E. Taylor.** 2004. Characterization of the DNA adenine 5'-GATC-3' methylase HpyIIIM from *Helicobacter pylori*. Curr Microbiol **49:**47-54.

40. **Sixma, T. K.** 2001. DNA mismatch repair: MutS structures bound to mismatches. Curr Opin Struct Biol **11:**47-52.

41. **Snowden, T., S. Acharya, C. Butz, M. Berardini, and R. Fishel.** 2004. hMSH4-hMSH5 recognizes Holliday Junctions and forms a meiosis-specific sliding clamp that embraces homologous chromosomes. Mol Cell **15:**437-51.

42. **Storz, G., and Zheng, M.** 2000. Oxidative stress, p. 47-60. *In* G. Storz, and Hengge-Aronis, R. (ed.), Bacterial stress responses. ASM Press, Washington, D.C.

43. **Struthers, L., R. Patel, J. Clark, and S. Thomas.** 1998. Direct detection of 8-oxodeoxyguanosine and 8-oxoguanine by avidin and its analogues. Anal Biochem **255:**20-31.

44. **Suerbaum, S., J. M. Smith, K. Bapumia, G. Morelli, N. H. Smith, E. Kunstmann, I. Dyrek, and M. Achtman.** 1998. Free recombination within *Helicobacter pylori*. Proc Natl Acad Sci U S A **95:**12619-24.

45. **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, and J. C. Venter.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-47.

46. **Touati, E., V. Michel, J. M. Thiberge, N. Wuscher, M. Huerre, and A. Labigne.** 2003. Chronic *Helicobacter pylori* infections induce gastric mutations in mice. Gastroenterology **124:**1408-19.

47. **Wang, G., M. Z. Humayun, and D. E. Taylor.** 1999. Mutation as an origin of genetic variability in *Helicobacter pylori*. Trends in Microbiology **7:**488-493.

48. **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:**1391-6.

49. **Wang, G., T. J. M. Wilson, Q. Jiang, and D. E. Taylor.** 2001. Spontaneous Mutations That Confer Antibiotic Resistance in *Helicobacter pylori*. Antimicrob. Agents Chemother. **45:**727-733.

50. **Webb, G. F., and M. J. Blaser.** 2002. Dynamics of bacterial phenotype selection in a colonized host. Proc Natl Acad Sci U S A **99:**3135-40.

51. **Wyrzykowski, J., and M. R. Volkert.** 2003. The *Escherichia coli* methyl-directed mismatch repair system repairs base pairs containing oxidative lesions. J Bacteriol **185:**1701-4.

52. **Zhao, J., and M. E. Winkler.** 2000. Reduction of GC --> TA transversion mutation by overexpression of MutS in *Escherichia coli* K-12. J Bacteriol **182:**5025-8.

Chapter 5

Iron Sulfur Cluster Synthesis Proteins in *H. pylori*

Iron sulfur clusters [Fe-S] are ubiquitous prosthetic groups that are essential to sustain fundamental life processes across all strata of life (10). These clusters are contained in proteins that have a wide range of characteristics: electron transfer, substrate binding/activation, iron/sulfur storage, regulation of gene expression, and enzyme activity (10). Iron-sulfur clusters have the ability to delocalize electron density over both Fe and S atoms thereby allowing for many different structures and oxidation states (10).

There are 3 major systems that have been identified to synthesize [Fe-S] clusters: Isc, Nif, and Suf (10). The IscS/IscU system in *Azotobacter vinelandii* and *Pseudomonas putida* have been shown to perform a general housekeeping function in terms of generation of [Fe-S] clusters and maturation of [Fe-S] proteins (3, 13). The Suf system has many overlapping roles with the Isc system in *E. coli* but plays a very specialized role during iron-starvation conditions in the maturation of oxidative stress related [Fe-S] proteins. The Nif pathway is composed of NifS (cystine desulfurase) and NifU (3 domain [Fe-S] scaffolding protein) and is responsible for generation of [Fe-S] clusters needed for maturation of the nitrogenase Fe and MoFe components under nitrogen fixing conditions (9). Since the availability of whole genome sequences, it is clear that many organisms have multiple [Fe-S] cluster formation proteins. *Helicobacter pylori* is different in that it does not contain the [Fe-S] cluster formation system, Isc or Suf, but it does have the NifS/NifU proteins even though it does not have any nitrogenase proteins (17). This would suggest that *H. pylori* uses the Nif proteins to play the general housekeeping roles that would normally be played by the Isc and Suf proteins. This is novel because NifS/NifU have been shown to be unable to replace the function of IscS/IscU to generate [Fe-S] clusters for proteins other than for nitrogenase (11).

NifU functions in conjunction with NifS to assemble transient [2Fe-2S] and [4Fe-4s] clusters for transfer into apoproteins (7, 19). NifS catalyses the elimination of sulfur from L-cystine to yield L-alanine and elemental sulfur (20). This sulfur is then used in conjunction with free Fe^{2+} to assemble clusters using NifU as a scaffold. *H. pylori* NifU (HP0221) is a homodimer with 3 conserved domains within each monomer and it contains a number of highly conserved cysteine residues within each domain. The N-terminal region has 3 conserved cysteines and is highly homologous to the N-terminal region of many IscU proteins. NifU is thought to play a role in assembling "transient" [Fe-S] clusters for their transfer to apo-nitrogenase (19). The central domain contains 4 conserved cysteines that hold a "permanent" [2Fe-2S] cluster proposed to be important in maintaining the redox balance of the protein (1). The C-terminal domain has 2 conserved cysteines in the thioredoxin-like motif, -Cys-X-X-Cys. This domain is thought to function as an additional scaffold for the formation of [Fe-S] clusters but its role is abundantly less clear than that of the N-terminal domain (16).

The *H. pylori* NifU and Nfu proteins are the only putative [Fe-S] scaffolding proteins predicted by sequence homology. Still, [Fe-S] clusters are essential for many proteins in *H. pylori*. *H. pylori* has at least 16 proteins with many various functions that require [Fe-S] clusters: aconitase (transcriptional regulator, [4Fe-4S]), hydrogenase (2[4Fe-4S], 1[3Fe-3S]), 3 ferredoxin proteins ([2Fe-2S]), MutY (DNA repair enzyme, [4Fe-4S]), and others listed in table 5.1 (15). A NifU mutation is shown to be lethal under all conditions tested and thus the gene may be essential in *H. pylori*; NifU is probably the dominant [Fe-S] scaffolding protein in the cell (15).

Table 5.1. ***H. pylori*** **proteins that are predicted to contain Fe-S clusters.** (Edited from (15)

TIGR no.	Protein	Type
HP 0799	aconitase	4Fe-4S
HP 0126	phosphogluconate dehydratase	4Fe-4S
HP 0631	hydrogenase	2 (4Fe-4S)
	(small subunit)	1 (3Fe-3S)
HP 0191	fumarate reductase (iron-sulfur subunit)	1 (2Fe-2S) 1 (3Fe-3S), 1 (4Fe-4S)
HP 0142*	MutY	4Fe-4S
HP 0221	NifU	2Fe-2S
HP 1109	pyruvate-ferredoxin oxidoreductase (subunit d)	2 (4Fe-4S)
HP 0588	2-oxoglutarate:acceptor oxidoreductase(subunit d)	2-(4Fe-4S)
HP 1508	ferredoxin	2Fe-2S
HP 0588	ferredoxin	2Fe-2S
HP 0227	ferredoxin	2Fe-2S
HP 1356	quinolinate synthetase	4Fe-4S
HP 1540	ubiquinol cytochrome *c* oxidoreductase	2Fe-2S
HP 1260-HP 1273, HP 0037	NADH-Q oxidoreductase (complex I)	up to 9 clusters (both 2 and 4 Fe)

Fur in *H. pylori*: A Ferric uptake regulator (Fur) is present in many Gram negative and Gram-positive bacteria; it is known as a regulator of iron homeostasis. Fur binds to an A-T rich (TT**AAATAAT**AAT**TA**GT) consensus sequence known as the Fur-box in the promoter regions and regulates multiple gene expression in an iron-dependent manner. For example, In *E. coli*, (4, 14) and *Bradyrhizobium japonicum* (8) Fur acts as an activator under iron-replete conditions and as a repressor under iron-depleted conditions. The *H. pylori* homolog of Fur (hp 1027) (17)was shown to regulate genes involved in many cellular activities including iron transport, iron storage, urea metabolism and combating oxidative stress, and it acts as an iron dependent repressor (5, 18). For example, in the case of two genes (*sodB* and *pfr*) iron-free Fur represses the transcription by binding to the promoter region. In the conditions of excess iron, iron bound-Fur releases from the promoter and de-represses the expression (2, 6). In contrary to these observations and the accepted dogma for Fur-regulation in *H. pylori*, preliminary evidence from recent transcriptome and proteome analysis of *H. pylori* indicate that the expression of certain genes is much lower in the *fur* mutant strain than in the wild type suggesting a Fur dependent activation mechanism (5, 12). Due to the complexity of these different modes of regulation by Fur, and considering the fact that *H. pylori* has very few known regulators in its genome, an analysis of Fur mediated regulation was deemed to be fruitful. Therefore, analysis of Fur's role in *nifS*/*nifU* transcription provides a greater understanding of *H. pylori*'s adaptation to various environmental (stress) conditions.

REFERENCES

1. **Agar, J. N., P. Yuvaniyama, R. F. Jack, V. L. Cash, A. D. Smith, D. R. Dean, and M. K. Johnson**. 2000. Modular organization and identification of a mononuclear iron-binding site within the NifU protein. J Biol Inorg Chem **5**:167-77.

2. **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**:5948-53.

3. **Carl, B., A. Arnold, B. Hauer, and S. Fetzner.** 2004. Sequence and transcriptional analysis of a gene cluster of *Pseudomonas putida* 86 involved in quinoline degradation. Gene **331**:177-88.

4. **Dubrac, S., and D. Touati.** 2000. Fur positive regulation of iron superoxide dismutase in *Escherichia coli*: functional analysis of the sodB promoter. J Bacteriol **182**:3802-8.

5. **Ernst, F. D., S. Bereswill, B. Waidner, J. Stoof, U. Mader, J. G. Kusters, E. J. Kuipers, M. Kist, A. H. van Vliet, and G. Homuth.** 2005. Transcriptional profiling of *Helicobacter pylori* Fur- and iron-regulated gene expression. Microbiology **151**:533-46.

6. **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**:3687-92.

7. **Frazzon, J., J. R. Fick, and D. R. Dean**. 2002. Biosynthesis of iron-sulphur clusters is a complex and highly conserved process. Biochem Soc Trans **30**:680-5.

8. **Friedman, Y. E., and M. R. O'Brian.** 2004. The ferric uptake regulator (Fur) protein from *Bradyrhizobium japonicum* is an iron-responsive transcriptional repressor in vitro. J Biol Chem **279**:32100-5.

9. **Jacobson, M. R., V. L. Cash, M. C. Weiss, N. F. Laird, W. E. Newton, and D. R. Dean.** 1989. Biochemical and genetic analysis of the nifUSVWZM cluster from *Azotobacter vinelandii.* Mol Gen Genet **219**:49-57.

10. **Johnson, D. C., D. R. Dean, A. D. Smith, and M. K. Johnson.** 2005. Structure, Function, and Formation of Biological Iron-Sulfur Clusters. Annu Rev Biochem **74**:247-281.

11. **Johnson, D. C., P. C. Dos Santos, and D. R. Dean.** 2005. NifU and NifS are required for the maturation of nitrogenase and cannot replace the function of isc-gene products in *Azotobacter vinelandii*. Biochem Soc Trans **33**:90-3.

12. **Lee, H. W., Y. H. Choe, D. K. Kim, S. Y. Jung, and N. G. Lee.** 2004. Proteomic analysis of a ferric uptake regulator mutant of *Helicobacter pylor*i: regulation of *Helicobacter pylori* gene expression by ferric uptake regulator and iron. Proteomics **4**:2014-27.

13. **Nakamura, M., K. Saeki, and Y. Takahashi**. 1999. Hyperproduction of recombinant ferredoxins in *Escherichia coli* by coexpression of the ORF1-ORF2-iscS-iscU-iscA-hscB-hs cA-fdx-ORF3 gene cluster. J Biochem (Tokyo) **126**:10-8.

14. **Niederhoffer, E. C., C. M. Naranjo, K. L. Bradley, and J. A. Fee.** 1990. Control of *Escherichia coli* superoxide dismutase (*sodA* and *sodB*) genes by the ferric uptake regulation (*fur*) locus. J Bacteriol **172**:1930-8.

15. **Olson, J. W., J. N. Agar, M. K. Johnson, and R. J. Maier.** 2000. Characterization of the NifU and NifS Fe-S cluster formation proteins essential for viability in *Helicobacter pylori*. Biochemistry **39**:16213-9.

16. **Smith, A. D., G. N. Jameson, P. C. Dos Santos, J. N. Agar, S. Naik, C. Krebs, J. Frazzon, D. R. Dean, B. H. Huynh, and M. K. Johnson**. 2005. NifS-mediated assembly of [4Fe-4S] clusters in the N- and C-terminal domains of the NifU scaffold protein. Biochemistry **44**:12955-69.

17. **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, and et al**. 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori.* Nature **388**:539-47.

18. **van Vliet, A. H., J. Stoof, R. Vlasblom, S. A. Wainwright, N. J. Hughes, D. J. Kelly, S. Bereswill, J. J. Bijlsma, T. Hoogenboezem, C. M. Vandenbroucke-Grauls, M. Kist, E. J. Kuipers, and J. G. Kusters**. 2002. The role of the Ferric Uptake Regulator (Fur) in regulation of *Helicobacter pylori* iron uptake. Helicobacter **7**:237-44.

19. **Yuvaniyama, P., J. N. Agar, V. L. Cash, M. K. Johnson, and D. R. Dean.** 2000. NifS-directed assembly of a transient [2Fe-2S] cluster within the NifU protein. Proc Natl Acad Sci U S A **97**:599-604.

20. **Zheng, L., R. H. White, V. L. Cash, R. F. Jack, and D. R. Dean**. 1993. Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis. Proc Natl Acad Sci U S A **90**:2754-8.

Chapter 6

Regulation of the *Helicobacter pylori* Fe-S Cluster Synthesis Protein NifS By Iron, Oxidative Stress Conditions and by Fur

---

Alamuri, P., Mehta, N.S., Burk, A.C, and R. J. Maier*

Submitted to Journal of Bacteriology

ABSTRACT

Transcription of both chromosomal and extra-chromosomally-introduced *nifS* was regulated (up-expressed) by oxygen or by supplemental iron conditions. This up-regulation was not observed in a *fur* mutant strain background, or when an iron chelator was added. Iron-bound Fur (but not apo-Fur) recognized the *nifS* promoter, and Fur bound significantly further upstream (-155 bp to -190 bp and -210 to -240 bp) in the promoter than documented *H. pylori* Fur binding regions. This binding was stronger than Fur recognition of *flgE* or *napA* promoters, and includes a Fur-recognition sequence common to *H. pylori pfr* and *sodB* upstream areas. Studies on Fur regulated genes in *H. pylori* have indicated that apo-Fur acts as a repressor, but our results demonstrate iron-bound Fur activates transcription.

RESULTS AND DISCUSSION

The human gastric pathogen *Helicobacter pylori* is well adapted to colonize a unique niche, the stomach mucosa (3). In the mucosa it is subject to severe oxidative stress from the oxidative burst of the host immune system that results in production of a variety of reactive oxygen intermediates (ROI) that damage macromolecules of the bacterium (21). Due to the Fenton reaction, the ROI are especially a problem in the presence of excess free intracellular iron (7). Considering the persistent nature of *H. pylori* it is not surprising that the pathogen has a repertoire of enzymes involved in detoxification of the oxidative agents or in repairing the oxidized macromolecules in the cell (1, 17, 18, 22, 24).

Although combating oxidative stress is key to *H. pylori* survival, we know little about regulation of expression of the specific genes involved. To aid in understanding the overall oxygen stress-modulated gene expression in *H. pylori*, we compared the global gene expression of *H. pylori* grown at 2% versus 12 % oxygen using a microarray approach. Preliminary results from these studies showed an up-expression of expected genes such as thioredoxin reductase, thioredoxin, superoxide dismutase, and thiol peroxidase, all enzymes known to be directly involved in combating oxidative stress. In addition, amongst the other highly up-regulated genes was one encoding the Fe-S cluster synthesis protein NifS. We observed an approximate five-fold up-regulation of the *nifS*/*nifU* operon (hp0220-0221) after a 2 hr shift from 2% to 12% O_2 (ASM 2005 Abstract 05-GM-A-1982-ASM). NifS belongs to the crucial IscS family of proteins that are involved in Fe-S cluster formation (12). The NifS proteins provide sulfur donation via an L-cysteine desulfurase activity. The cluster maturation proteins are usually considered to be essential "housekeeping" ones, as nearly all organisms have multiple proteins that require Fe-S clusters for their function. Thus, inactivation of the *H. pylori nifS*/*nifU* (hp0220-0221) was previously shown to be lethal to the bacterium (12, 13).

In this study we performed promoter-reporter gene fusions to determine the regulation of *nifS/nifU* operon under conditions of excess oxygen or iron. We first mapped the 5' end of the *nifS*/*nifU* transcript using a 28 bases long oligo specific to 5'-*nifS* (Fig. 6.1). A primer extension product between 66 and 82 bases long was generated and the 5' of the transcript was concluded to be approximately 38--54 bases upstream of the ATG start codon of *nifS* (hp0220). For the reporter gene fusions, a 300 bp (spanning both the intergenic region of hp0219 and hp0220 and part of the hp0219) comprising all of the promoter elements of *nifS*/*nifU* was amplified by PCR using the following; primers forward primer NIFPF 5'-*GAGCTC*GCCCATTATCATTACCGCTCT-3' and reverse primer NIFPR 5'-CGCCGGC*GGATCC*TCAAAATTTACATAG-3' with the *Sac*I site in the forward primer and *Bam*HI site in the reverse primer italicized**.** The promoter fragment was introduced upstream of a 980 bp long promoterless *xylE* gene (encoding Catechol, 2, 3- dioxygenase) from

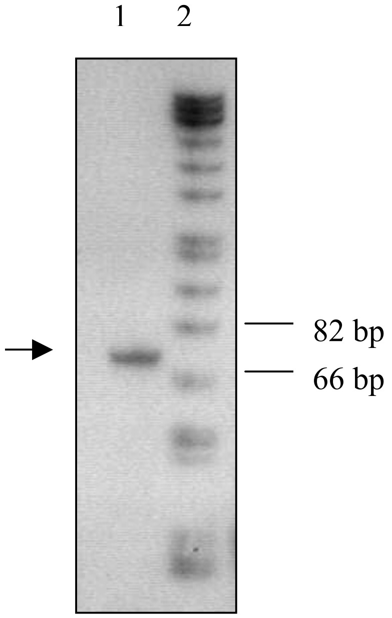


FIG. 6.1. Primer extension analysis of *nifS* transcript. A primer extension system-AMV reverse transcriptase kit (Promega Madison, WI. cat. no. E3030) was used to obtain the primer extension product of the *nifS* transcript. A 28-mer oligo (5'-TAAATTCGTTGTAACAAGGTT AATATTC–3') complementary to the bases spanning the ATG start codon (TTG for *nifS*) was end labeled with ^{32}P-ATP and RNA from *H. pylori* was used as the substrate for the primer extension reaction. The reaction was carried out as per the manufacturer's instructions and the products were subjected to PAGE (6% polyacrylamide). Lane 1: the primer extension product of *nifS* transcript. Lane 2: DNA ladder**.**

Pseudomonas putida. The 1280 bp long P_{nifS} -*xylE* cassette was cloned into either previously described peu39cm (1) or the *H. pylori* shuttle vector pHel3 (19) to study the regulation chromosomally or extra-chromosomally, respectively. The plasmids with the fusions were transformed into wild-type HP43504 or into an isogenic *fur* mutant to study the role of Fur in the regulation of the *nifS/nifU* operon.

The *xylE* assays were performed as described elsewhere (19). Briefly, thirty six hour old cells were inoculated into Muller Hinton broth supplemented with 10% bovine serum and grown in 2% partial pressure oxygen (5% CO_2, balance N_2). Stress conditions (excess oxygen, excess iron or iron chelation) were applied at the logarithmic phase of growth. XylE activities of cells grown at 2% oxygen were compared with those after stress conditions were applied (Fig. 6.2). The *nifS*/*nifU* operon was upregulated four-fold in 12% oxygen and 3-fold in excess iron, compared to the expression in 2% oxygen or 2% oxygen with iron chelation conditions, respectively. However, a similar up-expression by high O_2 or high iron was not observed in a *fur* mutant background, indicating that Fur in *H. pylori* perhaps acts either as a direct or an indirect transcriptional activator of the *nifS* operon. To our knowledge this is the first evidence that Fur in *H. pylori* is an activator, in contrast to its widely accepted role as an iron-dependent repressor (5, 10). The genome-wide response of *H. pylori* to iron-starvation is highly significant (16). The ferric uptake regulator (Fur) in *H. pylori* is a key iron sensing protein that has been shown to directly regulate synthesis of ferritin and SOD, and to influence the expression of iron transport genes in *H. pylori* (2). Based on a genome wide transcriptional profile, it was concluded that expression of many *H. pylori* genes even outside iron metabolism are regulated by Fur (9).

The question of whether Fur acts by directly binding to the promoter sequences of the *nifS*/*nifU* operon was addressed using gel retardation assays. The *H. pylori* Fur was purified as a

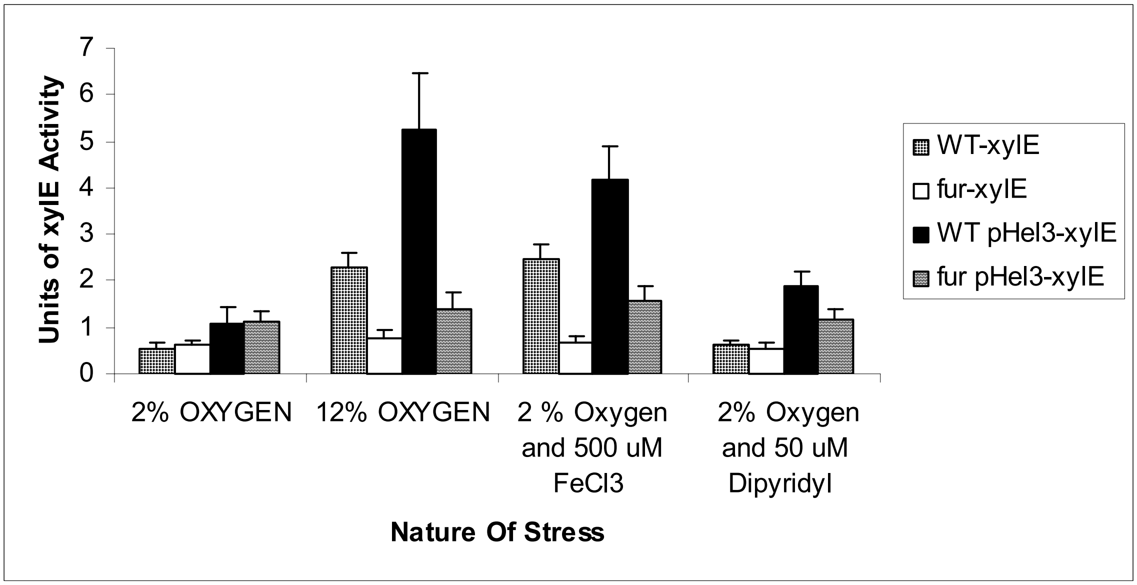


FIG. 6.2. XylE activities as a measure of *nifS* induction under various stress conditions. Cells were grown at 2% oxygen partial pressure until logarithmic phase (OD_{600} ~0.4) and oxygen was added to 12% partial pressure in closed gas system, or 2% O_2 atmosphere maintained, but the medium (see text) supplemented with 500 µM $FeCl_3$ or 2% O_2 with 75 µM of iron chelator 2, 2- dipyridyl to study the effect of oxygen stress, iron and iron starvation on transcription of the gene. WT-xylE and fur-xylE denote P_{nifS}-*xylE* fusion in the hp405 region in the genome; pHel3-*xyl*E denotes $P_{nifS} -$ *xylE* fusion on the shuttle vector. Simultaneous experiments were conducted in wild-type and *fur* mutant strain background. The mean and SD from five independent experiments are shown here with 3 replicates for each experiment (total of 15 samples for each mean). All wild-type results for both the O_2 added and the iron stress conditions are significantly greater than the 2% O_2 conditions ($P < 0.01$), and the results in the *fur* strain are significantly less ($P<0.01$) than for that same stress condition (12% O_2 or supplemented iron) for the wild-type strain. The iron chelator condition is not statistically different than the 2% O_2 condition. 1 unit of xylE = µmol of catechol oxidized / min / 10^9 cells).

recombinant protein in *E. coli* BL21 Rosetta. The purity of the 18 kDa Fur was assessed on a 15% SDS-PAGE (Fig. 6.3). The DNA substrate for the assay was prepared by end-labeling the 300 bp promoter fragment with [γ-^{32}P]-ATP (Amersham, cat.no. PB10168) using T4 polynucleotide kinase (Promega). Fur-P_{nifS} binding assays were carried out in standard EMSA buffer (20). Pure Fur (200 nM) plus (iron substituted) $MnCl_2$ bound the 300 bp P_{nifS} fragment (Fig. 6.4A). The effect of 150 µM EDTA (Fig. 6.4B), on the Fur-P_{nifS} binding complex was assessed. We observed that EDTA prevented the binding of Fur to P_{nifS} even at the highest concentration (1000 nM) of Fur. The results indicate that iron-loaded Fur is the active form. We next performed a titration assay of Fur-P_{nifS} binding by using a fixed concentration (50 pM) of radio-labeled *nifS* promoter and varying concentrations (0-1000 nM) of Fur in the presence of 100 µM $MnCl_2$. An initially linear increase in the binding was observed with the added incremental increases in Fur concentration; the binding was saturated at about 1000 nM. The half dissociation concentration (K_d) at which 50% of the DNA was bound was calculated to be 360 nM (Fig. 6.4C). For comparison the binding affinity of apo-Fur (in the absence of iron or manganese) for the *H. pylori sodB* promoter is 260 nM (10). We consistently observed Fur binding to sequences that included up to 300 bp upstream of the start codon, but no Fur binding was observed when a sequence containing 138 bp immediate upstream region was used (Fig. 6.4D).

The entire 300 bp fragment was analyzed for potential Fur-recognition sequences using a DNase protection assay. We used radio-labeled oligos NIFSPF and NIFSPR to amplify the promoter region and it was incubated with increasing concentrations of Fur (0-10 µM) in the presence or absence of 100 µM $MnCl_2$. 1 µl of 0.05 U/µl DNase enzyme (Promega cat. No. M6101) was added to the reaction and the reaction mixture was incubated at room temperature

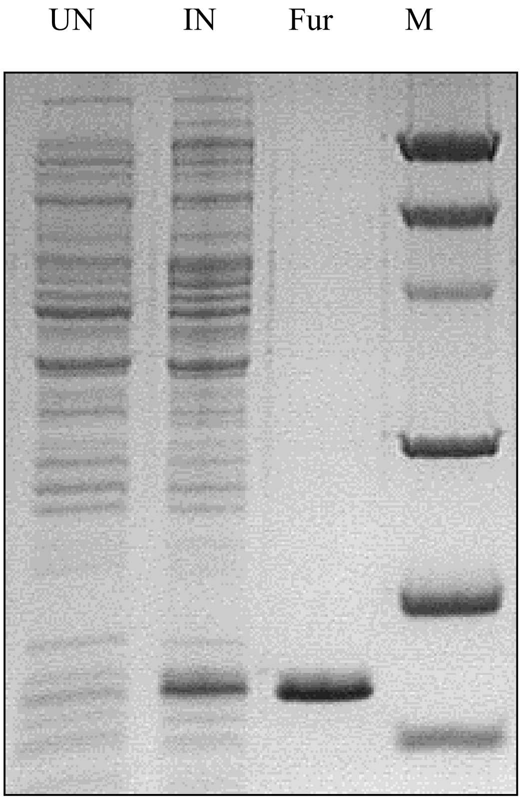
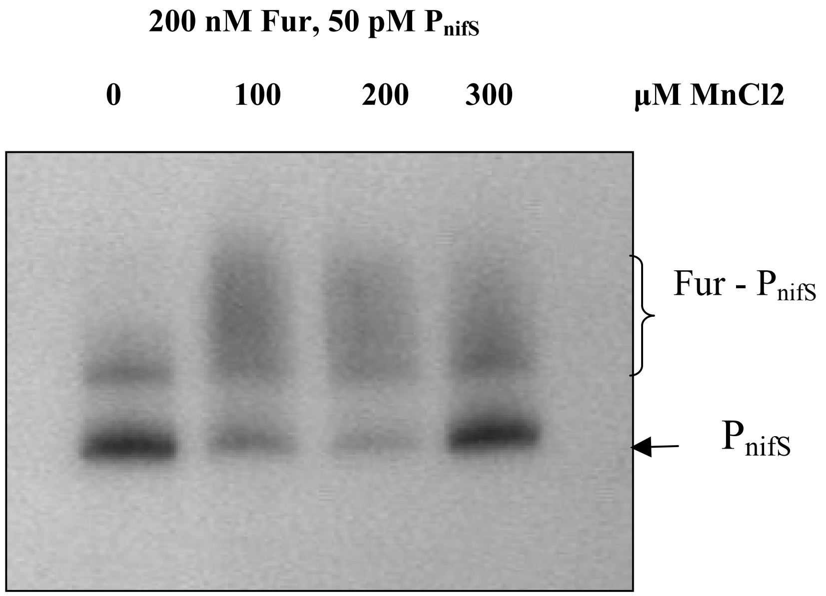
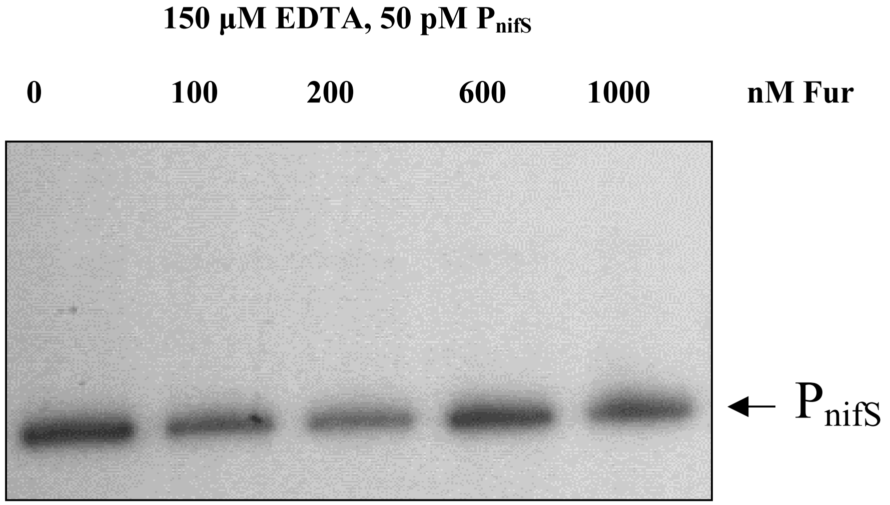


FIG. 6.3. Purification of *H. pylori* Fur. The entire gene (hp1027) from *H. pylori* encoding Fur was introduced downstream of the T7 promoter in pET21A vector (Novagen) and over-expressed in *E. coli* BL21 Rosetta strain by inducing with 0.5 mM IPTG (SIGMA) at 30 ºC for 3 hours. The cytoplasmic protein from crude extracts prepared from the IPTG-induced cells was obtained by ultracentrifugation (45,000 rpm for 2h). The purification was performed using FPLC; the cytoplasmic protein was first passed through a HiTrap SP-column for ion exchange based purification using a salt gradient of 50 mM – 1000 mM NaCl (obtained by using Buffer A: 50 mM sodium phosphate, 50 mM NaCl, pH 8.0 and Buffer B: 50 mM sodium phosphate, 1000 mM NaCl, pH 8.0). Peak fractions containing Fur protein (from the ion-exchange procedure) were collected and further purified based on size exclusion by using a Sephacryl-200 column (Buffer C: 50 mM sodium phosphate, 200 mM NaCl, pH 8.0). UN = uninduced, IN = induced with 0.5 mM IPTG, Fur = Purified Fur, M = Molecular weight marker (Weights in kDa from the top band are: 97.4, 66.2, 45.0, 31.5, 21.0, and 14.4).

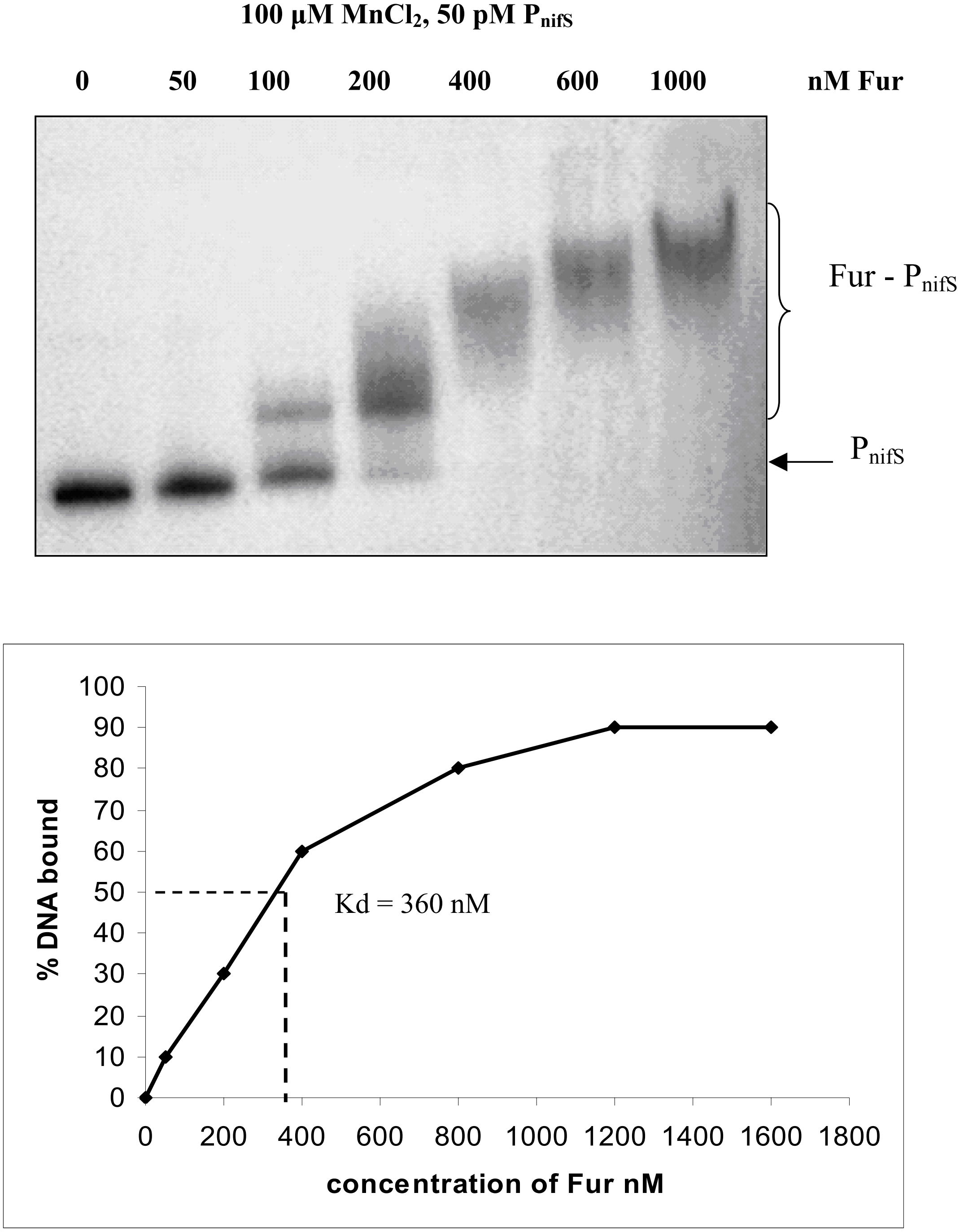
6.4A)


200 nM Fur, 50 pM P_{nifS}
0
100
200
300
µM MnCl2
Fur - P_{nifS}
P_{nifS}

6.4B)


150 µM EDTA, 50 pM P_{nifS}
0
100
200
600
1000
nM Fur
P_{nifS}

6.4C)


100 µM MnCl2, 50 pM PnifS
0
50
100
200
400
600
1000
nM Fur
Fur - PnifS
PnifS
100
90
80
70
60
50
40
30
20
10
0
% DNA bound
Kd = 360 nM
0
200
400
600
800
1000
1200
1400
1600
1800
concentration of Fur nM

6.4D)

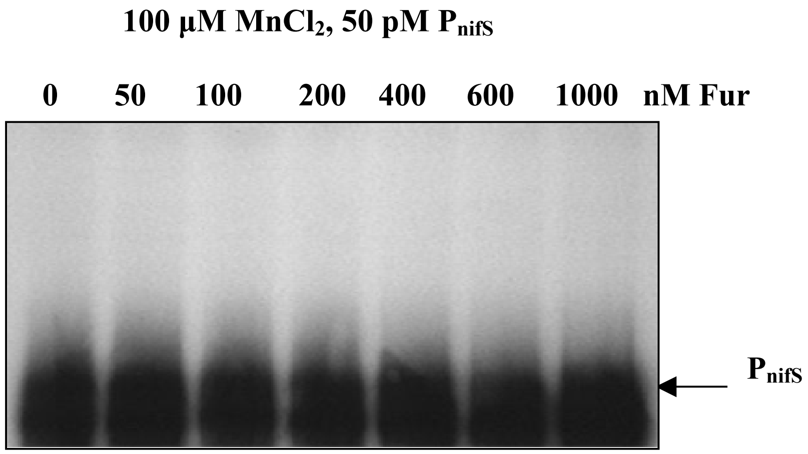


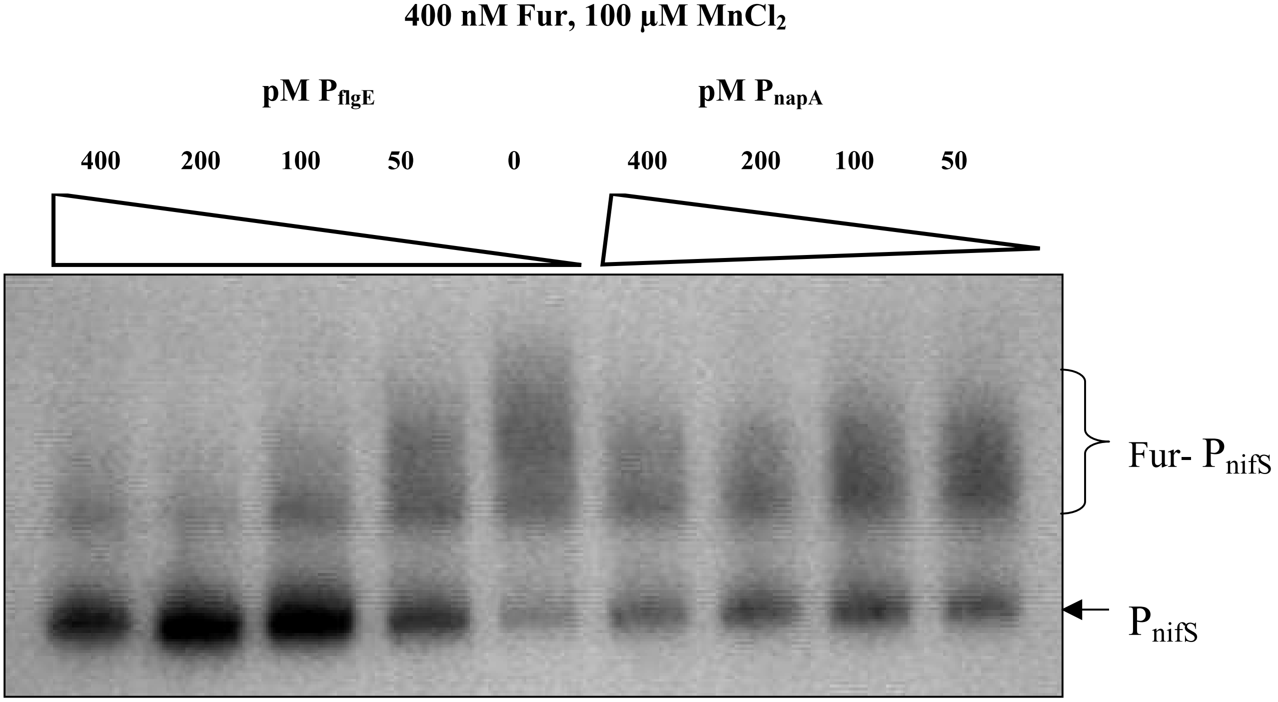
FIG. 6.4. Electrophoresis mobility shift assay. The Fur – P$_{nifS}$ interaction was studied using the 300 bp radio-labeled P$_{nifS}$ fragment and pure Fur protein. 50 pM of P$_{nifS}$ was assayed with various MnCl$_2$ concentrations and constant Fur levels (A); or with increasing concentration of Fur (0-1000 nM) in the presence of 150 µM EDTA (B); the Fur-P$_{nifS}$ binding was determined with 100 µM of MnCl$_2$ and varying concentrations of Fur; the percent DNA bound was determined using phosphoimager scanning, and then the K$_d$ value was calculated from all the (bound and unbound) values (C). A 150 bp interior region of the gene encoding 16s rRNA and pure thioredoxin reductase as controls representing non-specific DNA and protein, respectively were used as controls; these exhibited no shift (data not shown). The region of Fur recognition in P$_{nifS}$ (in the presence of MnCl$_2$) was narrowed by use of a 138 bp non-Fur-binding fragment upstream of the start codon rather than the 300 bp fragment used in other experiments. The inability of this fragment to bind Fur is shown. (D).

for 45 s. The reaction was stopped by adding 3 µl of stop solution and the sample was purified using Qiagen spin columns; the DNA was eluted using 10 µl H_2O and 6 µl of denaturing loading dye was then added to the eluant. The entire (approximately 16 µl) was subjected to PAGE (6% polyacrylamide gel containing 8 M urea) for several hours at 1200 V. The two approximate areas protected from DNase activity were -155 to -190 bp and -210 to -240 bp in the nifS promoter (data not shown); within both of these areas are conserved Fur boxes that correspond to the identified Fur binding areas of *H. pylori pfr* and *sodB* promoters (2, 7,10). No DNase protection was detected in the absence of $MnCl_2$ even at the highest concentration of Fur (data not shown). The *xylE* assays combined with the EDTA experiment and the $MnCl_2$ dependent binding assays together indicate that, unlike the previously reported *H. pylori* Fur-interacting promoters (2, 5, 10), Fur acts as a transcriptional activator of *nifS* in an iron-dependent fashion.

To compare the affinity of P_{nifS}-Fur to other promoters, we carried out a competition assay using *flgE* and *napA* promoters, ones which were previously shown to be repressed by apo-Fur (9, 23). The competition assay was performed in the presence of $MnCl_2$ (Fig. 6.5A) using 400 nM Fur, 50 pM labeled P_{nifS} and a range (0-400 pM) of 200-bp long *flgE* or *napA* promoter DNA. We observed that at about four time's excess concentration (compared to P_{nifS}), P_{flgE} reduced the binding of Fur to P_{nifS} by more than 50%. There was little or no inhibition of P_{nifS}-Fur interaction by P_{napA}. Cooksley et al (4) showed that *napA* in *H. pylori* is repressed by Fur, but the expression is induced when the cells are grown in medium supplemented with $FeCl_3$, and in a *fur* mutant strain *napA* expression did not depend on iron levels (4). In our study, the competition experiment was performed in the presence of $MnCl_2$ (a preferable substitute for iron, as previously described, see 11) that is needed for the Fur-P_{nifS} binding. For *napA* regulation it seems that apo-Fur is the active form that recognizes the promoter, hence it is not surprising that the *napA* promoter could not successfully inhibit the Fur-P_{nifS} complex. When a similar experiment was performed using cold PnifS as the competitor DNA, the binding of Fur to radio-labeled PnifS was decreased to approximately 25% (compared to binding in the absence competitor) in the presence of 200 pM of competitor promoter (Fig 6.5B); this suggests a specific binding of Fur to P_{nifS}.

Fur in *H. pylori* has been well studied as a transcriptional repressor, and the iron-dependent transcriptional induction activity has been most well studied for regulation of *sodB* and *pfr.* Apo-fur recognizes the Fur-box consensus sequence upstream of the promoter region of these genes and inhibits the transcription by blocking the movement of RNA polymerase (10). In conditions of excess free-iron in the cell, the transcription of Fur-regulated genes occurs by the derepression by the iron-bound Fur (2, 5, 10, 23) . Thus, Fur in *H. pylori* acts as a classical transcriptional repressor in contrast to its role as a positive regulator exemplified in the case of the *E. coli sodB* (8); in the latter case Fur was required for the transcription of the gene encoding Fe-SOD both under anaerobic and aerobic conditions of growth (in a RhyB dependent mechanism) (14, 15)**.** Although sequences more than 150 bp upstream have been proposed to be potential Fur binding areas, documented *H. pylori* Fur binding has been observed no more than 70-80 bp upstream of the transcription start site (4, 6, 7). The upstream binding we observed may be related to the different type of regulation (positive by iron-bound form) observed herein. In a recent study regarding the transcriptional profiling of *H. pylori* iron-regulated gene expression (9), the authors observed a large set of genes whose regulation was modulated by the intracellular levels of iron, and another subset of genes (*mod, murE*, and *rnhB*) that were down-regulated in a *fur* mutant. The latter result was interpreted as an aberration from the accepted

6.5A)


400 nM Fur, 100 µM MnCl$_2$
pM P$_{flgE}$
pM P$_{napA}$
400
200
100
50
0
400
200
100
50
Fur- P$_{nifS}$
P$_{nifS}$

6.5B)

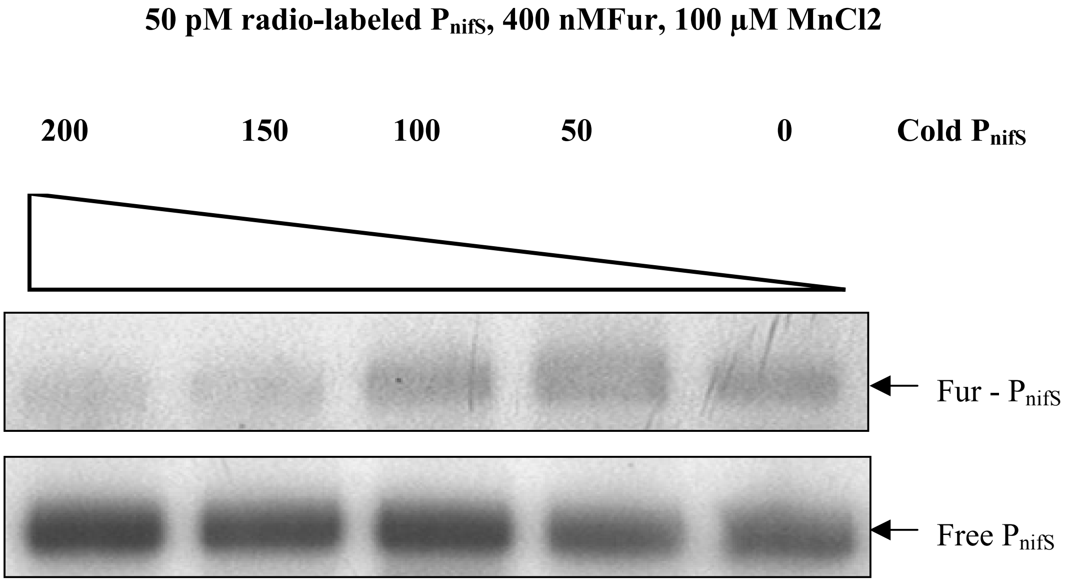


FIG. 6.5. Competition assay to determine Fur-P$_{nifS}$ affinity. The competitive effect of P$_{flgE}$ and P$_{napA}$ on Fur-P$_{nifS}$ (P$_{nifS}$ at 50 pM) binding in the presence of 100 µM MnCl$_2$ was determined using increasing concentrations (0-400 pM) of competitor promoter DNA (A). The effect of cold P$_{nifS}$ (0-200 pM) on Fur binding to the radio-labeled *nifS* promoter DNA (B).

model of Fur-dependent regulation and was speculated to be an indirect form of Fur-dependent regulation. In addition, *nifS* was observed to be among another set of genes that included carbonic anhydrase and a thioredoxin that were further aberrantly expressed (9); iron levels did not significantly affect that expression in the wild type. However, their transcriptome study used a higher O_2 level in the supplemental iron experiment as well as a different parent strain than our study.

In our study, we report that the *nifS*/*nifU* operon of *H. pylori* is up-regulated in high oxygen or in high iron conditions, and the observed up-regulation was not observed in an isogenic *fur* mutant. The oxygen affect is most likely related to the recently reported damaging affect of O_2 and related ROI on release of free iron from Fe-containing proteins in *H. pylori* (25). The net result is a significant increase of intracellular free iron which we expect would be recognized by Fur. The connection between increased oxygen stress and NifS expression is presumably related to the increased need for Fe-S cluster synthesis at a time when Fe-S proteins are oxidatively damaged. Further analysis of the *nifS* and other *H. pylori* iron-regulated promoters are needed to understand the full role of Fur.

ACKNOWLEDGEMENTS

We thank Adriana Olczak for providing the *fur* mutant. This work was supported by NIH grant 1-RO1-DK60061 to R.J.M.

REFERENCES

1. **Alamuri, P., and R. J. Maier.** 2004. Methionine sulphoxide reductase is an important antioxidant enzyme in the gastric pathogen *Helicobacter pylor*i. Mol. Microbiol. **53:**1397-1406.

2. **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.

3. **Blaser, M. J.** 1997. The versatility of *Helicobacter pylor*i in the adaptation to the human stomach. J. Physiol. Pharmacol. **48:**307-314.

4. **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.

5. **Delany, I., A. B. Pacheco, G. Spohn, R. Rappuoli, and V. Scarlato.** 2001. Iron-dependent transcription of the *frpB* gene of *Helicobacter pylori* is controlled by the Fur repressor protein. J. Bacteriol. **183:**4932-4937.

6. **Delany, I., G. Spohn, A. B. Pacheco, R. Ieva, C. Alaimo, R. Rappuoli, and V. Scarlato.** 2002. Autoregulation of *Helicobacter pylori* Fur revealed by functional analysis of the iron-binding site. Mol. Microbiol. **46:**1107-1122.

7. **Delany, I., G. Spohn, R. Rappuoli, and V. Scarlato.** 2001. The Fur repressor controls transcription of iron-activated and -repressed genes in *Helicobacter pylori*. Mol. Microbiol. **42:**1297-1309.

8. **Dubrac, S., and D. Touati.** 2000. Fur positive regulation of iron superoxide dismutase in *Escherichia coli*: functional analysis of the *sodB* promoter. J. Bacteriol. **182:**3802-3808.

9. **Ernst, F. D., S. Bereswill, B. Waidner, J. Stoof, U. Mader, J. G. Kusters, E. J. Kuipers, M. Kist, A. H. van Vliet, and G. Homuth.** 2005. Transcriptional profiling of *Helicobacter pylori* Fur- and iron-regulated gene expression. Microbiology **151:**533-546.

10. **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.

11. **Escolar, L., J. Perez-Martin, and V. de Lorenzo.** 1999. Opening the iron box: transcriptional metalloregulation by the Fur protein. J. Bacteriol. **181:**6223-6229.

12. **Johnson, D. C., D. R. Dean, A. D. Smith, and M. K. Johnson.** 2005. Structure, Function, and Formation of Biological Iron-Sulfur Clusters. Annu. Rev. Biochem. **74:**247-281.

13. **Johnson, D. C., P. C. Dos Santos, and D. R. Dean.** 2005. NifU and NifS are required for the maturation of nitrogenase and cannot replace the function of isc-gene products in *Azotobacter vinelandii*. Biochem. Soc. Trans. **33:**90-93.

14. **Masse, E., and S. Gottesman.** 2002. A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **99:**4620-4625.

15. **Masse, E., C. K. Vanderpool, and S. Gottesman.** 2005. Effect of RyhB small RNA on global iron use in *Escherichia coli*. J. Bacteriol. **187:**6962-6971.

16. **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.

17. **Olczak, A. A., J. W. Olson, and R. J. Maier.** 2002. Oxidative-stress resistance mutants of *Helicobacter pylori*. J. Bacteriol. **184:**3186-3193.

18. **Olczak, A. A., R. W. Seyler, Jr., J. W. Olson, and R. J. Maier.** 2003. Association of *Helicobacter pylori* antioxidant activities with host colonization proficiency. Infect. Immun. **71:**580-583.

19. **Olson, J. W., and R. J. Maier.** 2002. Molecular hydrogen as an energy source for *Helicobacter pylori*. Science **298:**1788-1790.

20. **Pereira, L., and T. R. Hoover.** 2005. Stable accumulation of sigma54 in *Helicobacter pylori* requires the novel protein HP0958. J. Bacteriol. **187:**4463-4469.

21. **Sachs, G., D. L. Weeks, K. Melchers, and D. R. Scott.** 2003. The gastric biology of *Helicobacter pylori.* Annu. Rev. Physiol. **65:**349-369.

22. **Seyler, R. W., Jr., J. W. Olson, and R. J. Maier.** 2001. Superoxide dismutase-deficient mutants of *Helicobacter pylori* are hypersensitive to oxidative stress and defective in host colonization. Infect. Immun. **69:**4034-4040.

23. **van Vliet, A. H., J. Stoof, R. Vlasblom, S. A. Wainwright, N. J. Hughes, D. J. Kelly, S. Bereswill, J. J. Bijlsma, T. Hoogenboezem, C. M. Vandenbroucke-Grauls, M. Kist, E. J. Kuipers, and J. G. Kusters.** 2002. The role of the Ferric Uptake Regulator (Fur) in regulation of *Helicobacter pylori* iron uptake. Helicobacter **7:**237-244.

24. **Wang, G., P. Alamuri, M. Z. Humayun, D. E. Taylor, and R. J. Maier.** 2005. The *Helicobacter pylori* MutS protein confers protection from oxidative DNA damage. Mol. Microbiol. **58:**166-176.
25. **Wang, G., R. C.Conover., A.A. Olczak, P., and M. K. J. Alamuri, and R. J. Maier.** 2005. Oxidative stress defense mechanisms to counter iron-promoted DNA damage in *Helicobacter pylori*. Free. Rad. Res.**30:**1183-1191.

Chapter 7

Dissertation summary and future directions

Helicobacter pylori is an etiological agent for peptic and duodenal ulcers in humans. Since the discovery of this bacterium in 1981, many lines of study were focused on understanding its physiology and factors associated with its pathogenesis. Amongst the most fascinating features of this microaerophilic, gram-negative type bacterium is its ability to persist in the hostile conditions present in the human stomach. Acute and prolonged inflammatory response and the resulting injury to the gastric epithelium is the hallmark of *H. pylori* related disease (4, 5, 7). Bacterial factors such as urease, cell surface adhesins, cytotoxic proteins and the repertoire of antioxidant enzymes in this pathogen contribute to its virulence and its long term persistence in the host (12).

Antioxidant enzymes such as superoxide dismutase, catalase, alkylhydroperoxide reductase are some key enzymes that detoxify the toxic reactive oxygen species (ROS) (10, 13, 14). These enzymes in *H. pylori* form the first line of defense against oxidative stress conditions. However, oxidation of macromolecules (protein and DNA) is one unavoidable consequence of ROS exposure. Oxidation of either free amino acids or those integrated into proteins renders the residues unusable and hence the proteins non-functional and even toxic. Oxidation of DNA induces mutations in the genome that also could be lethal to the cell. In this study I characterized the enzyme methionine sulfoxide reductase (Msr), a peptide repair enzyme that catalyzes reduction of the oxidized form of methionine, methionine sulfoxide. The reversible oxidation of methionines allows aerobic and microaerophilic organisms to use these residues as a recycling sink for ROS and this makes Msr an important antioxidant enzyme in many bacteria and in eukaryotes (6, 9).

PHYSIOLOGICAL SIGNIFICANCE OF MSR IN *H. PYLORI*

A) The mutant analysis approach: To understand the role of Msr in *H. pylori*, I compared the tolerance ability of the wild type and the *msr* strain to oxidizing conditions. Loss of Msr activity rendered the mutant strain growth sensitive above 6% partial pressure oxygen (see fig 7.1) or in the presence of chemical oxidants. This oxygen sensitivity of the mutant strain was directly related to a higher level of oxidized protein compared to the wild type. This consequence was expected in the mutant strain as a result of the accumulation of ROS resulting in severe protein oxidation. The significance of Msr was evident in vivo wherein the *msr* strain could not persist for longer incubation periods (past one week) in mouse stomachs (2). The observed phenotype of the *msr* strain is slightly different from that seen for an *ahpC* or a *sodB* mutant of *H. pylori*. Although the latter strains were hypersensitive to oxygen (could not grow above 1% oxygen partial pressure) (13, 14), they were completely negative in mouse colonization. These results taken together indicate that the absence of the detoxifying enzymes would result in multiple types of macromolecule damage (DNA, lipid and protein) where as protein oxidation would be the direct primary effect by the absence of Msr.

B) Biochemical approach: In this study I used an unbiased co-immunoprecipitation (CIP) approach and identified a few methionine rich proteins GroEL, catalase (KatA) and site specific recombinase (SSR) that may be targeted for Msr-dependent repair in *H. pylori*. Also, formation of adducts with Msr by these proteins only in their oxidized form not only underlines the functional specificity of the Msr enzyme but also substantiates the relevance of the findings from

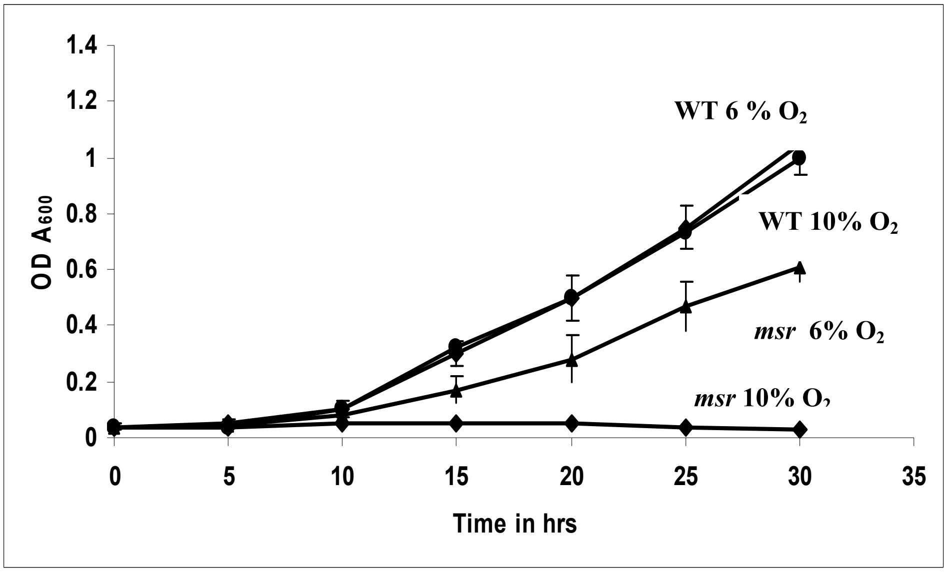


FIG. 7.1. Growth of WT and *msr* strains in oxygen. The *msr* mutant shows decreased growth in 6% oxygen grown conditions but growth is completely compromised in10% partial pressure oxygen. The wild type strain however did not show any difference in growth patter in either oxygen condition

the CIP procedure. Similar to its function in *E. coli* and *M. tuberculosis* (1, 11), Msr in *H. pylori* too protects GroEL from oxidative damage and thus perhaps indirectly aids in protein maintenance in this pathogen. As is the case with glutamine synthetase in *E. coli, H. pylori* KatA and SSR methionine residue turnover may play a role in ROS quenching. Msr likely aids in combating oxidative damage in part by reducing/reactivating KatA and SSR in the cell to maintain ROS detoxification (H_2O_2 dissipation) and DNA recombination repair functions, respectively. Consistent with these hypotheses, the specific activity of catalase was reduced in the *msr* strain especially under oxidizing conditions. This indicates a role for Msr in repair of oxidized methionines within catalase. The catalase activity could be restored to nearly wild-type levels upon the addition of pure Msr to the msr mutant cell extract. However, finding that Msr reduced specific surface exposed methionine residues of these proteins would link the physiological and crosslinking observations to a precise biochemical role for Msr; this would be an excellent next step for this project.

STRUCTURE FUNCTION RELATIONSHIP

The structure-function relationship of the *H. pylori* Msr is also very interesting. The Msr enzyme in *H. pylori* is a translational fusion of MsrA (N-terminus) and MsrB (C-terminus). MsrA and MsrB are two separate enzymes specific for the S- and R-isomers of methionine sulfoxide in several other eubacteria and in eukaryotes. A homology based structure prediction of *H. pylori* Msr and the in vitro Msr assay indicated that the MsrA domain of this enzyme is apparently non functional, while all the sulfoxide reductase activity was attributed to the MsrB domain. Hence only the reduction of R-isomer of the oxidized methionine substrates was observed in an in vitro assay using the purified reaction components (Fig. 7.2). The observed reductase activity by the

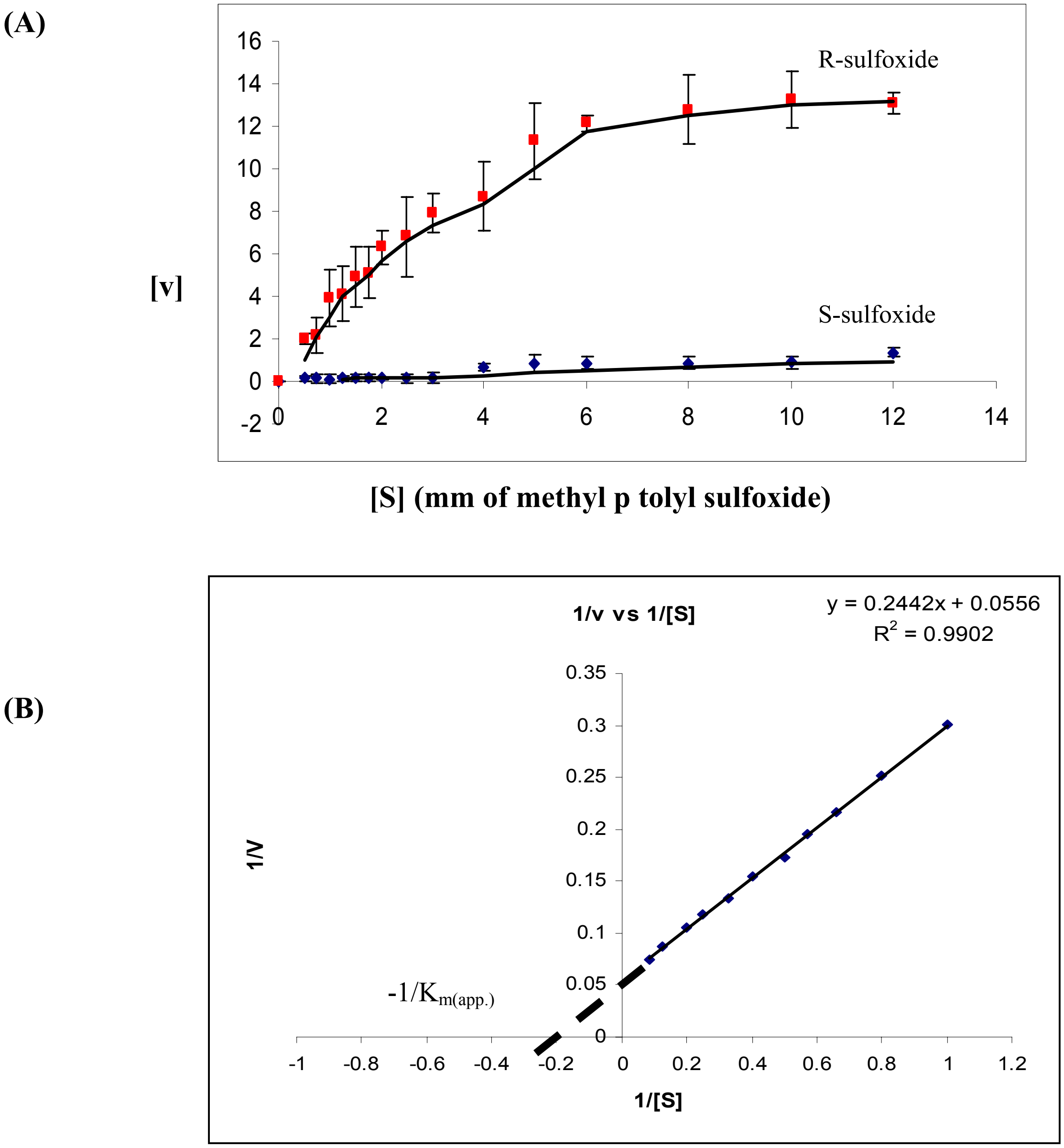


FIG. 7.2. **Kinetics of Msr activity. (A)** Methionine sulfoxide reductase activity using purified Msr, S and R isomers of methyl p-tolyl Sulfoxide (0.25-12 mM), Thioredoxin-1 (50 µM), Thioredoxin reductase (30 nmol) and 100 µmol NADPH in a 1ml reaction volume. Msr activity as a measure of nmol of NADPH oxidized/min/mg of protein. *H. pylori* Msr could reduce only R-sulfoxide and used only Trx1 as its reductant. **(B)** K $_{m(app.)}$ of methyl p tolyl R-sulfoxide was determined by plotting the 1/v versus 1/[s] values obtained from Fig. 6.3A.

MsrB domain agrees with the oxidative stress sensitive phenotype of the *msrB* mutant (chapter 2). Interestingly, the phenotypic characteristics (both in vitro and in vivo) of the *msrB* mutant were similar to that exhibited by the *msr* strain (where both MsrA and MsrB domains were inactivated), further confirming the inactive state of the MsrA domain of this enzyme.

Among all the characterized Msr enzymes that have fused MsrA and MsrB domains, only the *H. pylori* enzyme shows this disagreement in structure-functional relationship. The plausible reasons for this anomaly are; **a)** an improper folding of the MsrA domain, so that the catalytic and the recycling cysteines are not brought together, thus preventing the essential disulfide bond formation (see requirements for Msr activity), **b)** thioredoxin (reductant of Msr) perhaps fails to recognize the cysteines taking part in the Msr activity and hence the lack of cysteine recycling renders the MsrA domain inactive.

To further understand the role of each of the participating cysteine residues in Msr activity, the cysteines within the functional MsrB domain could be mutated and each form of the purified Msr protein could be tested for activity. Also, in vitro Msr assays using Met rich oxidized proteins from *H. pylori* as substrates may help understand a possible role of MsrA domain in this enzyme.

REGULATION OF MSR

The conditions that up-regulated *msr* in various systems (both bacterial and eukaryotes) include growth phase, nutrient limitation, excess selenium in diet, UV radiation, mutagenic agents etc. In this study I observed a unique iron starvation condition that caused cells to up-regulate *msr* expression. These results suggest that intracellular factors may have a stronger influence on the expression of *msr* than the original agent (ROS) that caused the damage.

The cellular localization of Msr (seen in both soluble and insoluble fractions) and the ability of cells to sense the condition of low iron in the medium together indicate that in addition to being a protein repair enzyme in *H. pylori*, Msr could also play a role in iron-sensing; alternatively it may be regulated by a protein that plays a key role in iron sensing/metabolism. Further studies to determine the regulator of *msr* would enable us to understand regulation of stress combating factors in this pathogen.

NEW ROLE FOR MUTS IN *H. PYLORI*

In this study we presented evidence for an additional role for the MutS protein in *H. pylori* as a protein involved in oxidative stress defense in *H. pylori* lacks the homologs of MutH and MutL enzymes which comprise part of the well studied *E. coli* mis-match repair system. In a DNA binding assay, the MutS protein bound the DNA substrate containing the oxidized purine (8-oxoguanine) with higher affinity over the DNA oligo that contains a mis-match base pair. Consistent with this observation were the higher levels of 8-oxoguanine in the *mutS* strain than seen in the wild type. It is however not known if the MutS protein also has base excision activity; studies along these lines would further our understanding about the physiological role of this protein in *H. pylori* (15).

FUR MEDIATED ACTIVATION OF *nifS*

In this study I presented a unique case of Fur mediated up-regulation of *nifS* in *H. pylori*. Using *xylE* gene fusions I observed that the *nifS* gene was up-regulated in conditions of high oxygen or excess iron only in a wild-type strain of *H. pylori*; such an up-regulation was not observed in an isogenic *fur* mutant. Considering the well accepted role of Fur as an iron-dependent repressor (3, 8), Fur dependent up-regulation of *nifS* is an unusual observation worth further investigation.

Studies on regulation of other Fur-dependent genes would enable us to understand some additional roles of Fur in *H. pylori*.

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:**1397-406.

3. **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:**5948-53.

4. **Blaser, M. J.** 1990. *Helicobacter pylori* and the pathogenesis of gastroduodenal inflammation. J Infect Dis **161:**626-33.

5. **Blaser, M. J., and J. C. Atherton.** 2004. *Helicobacter pylori* persistence: biology and disease. J Clin Invest **113:**321-33.

6. **Brot, N., H. Fliss, T. Coleman, and H. Weissbach.** 1984. Enzymatic reduction of methionine sulfoxide residues in proteins and peptides. Methods Enzymol **107:**352-60.

7. **Cover, T. L., and M. J. Blaser.** 1992. *Helicobacter pylori* and gastroduodenal disease. Annu. Rev. Med. **43:**135-145.

8. **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:**3687-92.

9. **Ezraty, B., L. Aussel, and F. Barras.** 2005. Methionine sulfoxide reductases in prokaryotes. Biochim Biophys Acta **1703:**221-9.

10. **Harris, A. G., J. E. Wilson, S. J. Danon, M. F. Dixon, K. Donegan, and S. L. Hazell.** 2003. Catalase (KatA) and KatA-associated protein (KapA) are essential to persistent colonization in the *Helicobacter pylori* SS1 mouse model. Microbiology **149:**665-72.

11. **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:**19486-93.

12. **McGee, D. J., and H. L. Mobley.** 1999. Mechanisms of *Helicobacter pylori* infection: bacterial factors. Curr Top Microbiol Immunol **241:**155-80.

13. **Olczak, A. A., J. W. Olson, and R. J. Maier.** 2002. Oxidative-stress resistance mutants of *Helicobacter pylori*. J Bacteriol **184:**3186-93.

14. **Seyler, R. W., Jr., J. W. Olson, and R. J. Maier.** 2001. Superoxide dismutase-deficient mutants of *Helicobacter pylori* are hypersensitive to oxidative stress and defective in host colonization. Infect Immun **69:**4034-40.

16. **Wang, G., P. Alamuri, M. Z. Humayun, D. E. Taylor, and R. J. Maier.** 2005. The *Helicobacter pylori* MutS protein confers protection from oxidative DNA damage. Mol Microbiol **58:**166-76.