Superoxide Dismutase-Deficient Mutants of *Helicobacter pylori* Are Hypersensitive to Oxidative Stress and Defective in Host Colonization

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Superoxide dismutase (SOD) is a nearly ubiquitous enzyme among organisms that are exposed to oxic environments. The single SOD of *Helicobacter pylori*, encoded by the *sodB* gene, has been suspected to be a virulence factor for this pathogenic microaerophile, but mutations in this gene have not been reported previously. We have isolated mutants with interruptions in the *sodB* gene and have characterized them with respect to their response to oxidative stress and ability to colonize the mouse stomach. The *sodB* mutants are devoid of SOD activity, based on activity staining in nondenaturing gels and quantitative assays of cell extracts. Though wild-type *H. pylori* is microaerophilic, the mutants are even more sensitive to O₂ for both growth and viability. While the wild-type strain is routinely grown at 12% O₂, growth of the mutant strains is severely inhibited at above 5 to 6% O₂. The effect of O₂ on viability was determined by subjecting nongrowing cells to atmospheric levels of O₂ and plating for survivors at 2-h time intervals. Wild-type cell viability dropped by about 1 order of magnitude after 6 h, while viability of the *sodB* mutant decreased by more than 6 orders of magnitude at the same time point. The mutants are also more sensitive to H₂O₂, and this sensitivity is exacerbated by increased O₂ concentrations. Since oxidative stress has been correlated with DNA damage, the frequency of spontaneous mutation to rifampin resistance was studied. The frequency of mutagenesis of an *sodB* mutant strain is about 15-fold greater than that of the wild-type strain. In the mouse colonization model, only 1 out of 23 mice inoculated with an SOD-deficient mutant of a mouse-adapted strain became *H. pylori* positive, while 15 out of 17 mice inoculated with the wild-type strain were shown to harbor the organism. Therefore, SOD is a virulence factor which affects the ability of this organism to colonize the mouse stomach and is important for the growth and survival of *H. pylori* under conditions of oxidative stress.

The gastric pathogen *Helicobacter pylori* is a curved, microaerophilic proteobacterium that has been implicated as a causal agent of peptic ulcers and a risk factor for adenocarcinoma. The only known natural hosts of *H. pylori* are primates, in whose gastric mucosa the organism can persist for years. During such infections, disease symptoms may or may not occur, though gastric inflammation is apparently ubiquitous. The pathogenesis of *H. pylori* relies on its persistence in surviving a harsh environment, including acidity, peristalsis, and attack by phagocytic cells and their released reactive oxygen species (28).

Using a variety of model systems (reviewed in reference 8), *H. pylori* virulence factors have been identified that affect the organism's ability to colonize the host. Such factors include motility and urease activity. Urease activity is critical for the establishment of infection (9) and is thought to enable colonization by producing ammonia, which helps neutralize the acidic microenvironment surrounding the organism (28), though this mechanism is not likely to be the only role for urease (10). Motility is required by the bacterium for colonization of the gastric mucosa (11), since *H. pylori* must move from the acidic environment of the stomach through the mucus, where the pH is higher. Other factors, such as adhesins for

recognizing host cell receptors in the gastric epithelium, metabolic enzymes required for growth in vivo, and host defense resistance mechanisms, are likely to be required for colonization, as well (28).

Mechanisms for the detoxification of reactive oxygen species are of particular interest in *H. pylori*. Despite the fact that this organism is an obligate aerobe, it is unable to grow in atmospheric concentrations of oxygen. Microaerophilic organisms, like *H. pylori*, are particularly vulnerable to the detrimental effects of oxygen and oxidative stress (20). Nevertheless, they do possess some of the enzymatic machinery needed to eliminate or minimize toxic oxygen-derived products. Organisms that grow in oxic environments must have mechanisms to handle reactive oxygen species (e.g., superoxide anions, peroxides, and hydroxyl radicals) that are by-products of oxygen metabolism (16, 17). In addition to internally generated reactive oxygen species, the successful pathogen must also deal with reactive oxygen species that are generated by phagocytic cells of the host immune response. In *H. pylori*, genes encoding superoxide dismutase (SOD), catalase, and several putative peroxidases have been identified (1, 31, 33, 37). Of these genes, only *katA* (catalase) mutants have been characterized (31). Although catalase-defective mutants are no different from the parent in their binding to epithelial cells, this enzyme may be important in detoxification of reactive oxygen species produced by the host immune response. Genes encoding an alkyl hydroperoxide reductase, a thiol-specific peroxidase, and other potential detoxification enzymes were identified (6, 28, 37), but

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
DH5 α	<i>E. coli</i> cloning strain	Lab stock
ATCC 43504	<i>H. pylori</i> Kan ^r Rif ^r ; wild-type strain	Lab stock
SS1	<i>H. pylori</i> Kan ^r Rif ^r ; mouse-adapted strain	D. McGee
HPG2	43504 <i>sodB::aphA3/SOD⁻</i> Kan ^r ; <i>aphA3</i> at <i>NcoI</i> site	This study
HPH2	43504 <i>sodB::aphA3/SOD⁻</i> Kan ^r ; <i>aphA3</i> at <i>SphI</i> site	This study
HP0388K	43504 HP0388: <i>aphA3/Kan^r</i> ; gene directly downstream of <i>sodB</i>	This study
SSG2	SS1 <i>sodB::aphA3/SOD⁻</i> Kan ^r ; <i>aphA3</i> at <i>NcoI</i> site	This study
Plasmids		
pBluescript KS(+)	Commercial cloning vector	Stratagene
pHP1	Source of <i>aphA3</i> cassette	29
pKSsodB-3	pKS+ (<i>sodB</i>)	This study
pG2	pKSsodB-3 (<i>aphA3</i>) at unique <i>NcoI</i> site	This study
pH2	pKSsodB-3 (<i>aphA3</i>) at unique <i>SphI</i> site	This study
pKS0388	pKS+ (HP0388); gene directly downstream of <i>sodB</i>	This study
pKS388K	pKS0388 (<i>aphA3</i>) at unique <i>NsiI</i> site	This study

mutations in these genes have not been reported or characterized.

Severe toxic effects of superoxide occur when this anion oxidizes the [4Fe-4S] clusters of redox enzymes, such as aconitase, 6-phosphogluconate dehydratase, and fumarase (reviewed in reference 13). This reaction not only disrupts the function of the target enzyme but frees Fe(II), which can bind to the negatively charged DNA and act as a catalyst for hydroxyl radical formation from H₂O₂ via the Fenton reaction (18, 19, 27). Perhaps the most destructive potential of superoxide is realized by production of the highly reactive hydroxyl radical. When produced in close proximity to DNA, this radical can damage the DNA, inducing mutations. Therefore, an important protective role of SOD, an enzyme that catalyzes the dismutation of superoxide anions (25), is ultimately its prevention of intracellular hydroxyl radical formation.

H. pylori expresses a dimeric SOD similar to other bacterial iron-cofactored (cytoplasmic) SODs, but the enzyme seems to be associated primarily with the surfaces of the cells (36). The enzyme has been purified, and the gene was cloned and sequenced (33). Recently, the distribution of Fe-SODs among *H. pylori* strains was studied (4). Different isoforms of the enzyme, apparently representing stable but strain-specific attributes, were identified. Though SOD has been suggested to be a likely virulence factor in *H. pylori* (6, 28), *sodB* mutants have not been generated to directly test this suggestion. In *Campylobacter coli*, which is also a microaerophile with a single SOD gene, *sodB* mutants were shown to have a decreased ability to colonize the chicken gut and were more sensitive to air for survival, but their growth rates in high-oxygen conditions (in shaken flasks) were unaffected (34).

Mouse colonization assays have been used previously to assay the importance of various genes for stomach colonization in *H. pylori* (8, 15). To better understand the microaerophily and pathogenesis of *H. pylori*, we constructed mutant strains with interrupted *sodB* genes and have characterized them with respect to growth and viability under various O₂ conditions, oxidative stress tolerance, and mouse colonization abilities.

MATERIALS AND METHODS

Bacterial strains, growth, and media. *Helicobacter pylori* ATCC 43504 was used as the wild-type strain. The Sydney strain, SS1 (22), was used in mouse colonization studies. Other *Helicobacter* strains were constructed as described below and are listed in Table 1. Cultures were grown at 37°C, under conditions of 5% CO₂ and 2 to 12% O₂ (varied as described) in a CO₂ incubator (Forma Scientific). Blood agar (BA) plates contained Brucella agar (Difco), supplemented with 10% defibrinated sheep blood (Gibson). Where necessary, kanamycin was supplemented at a concentration of 25 µg/ml.

Cloning was performed in *Escherichia coli* DH5 α , grown on Luria-Bertani medium (24) supplemented with either ampicillin (100 µg/ml) or kanamycin (25 µg/ml) as required.

Cloning of the *sodB* gene of *H. pylori* ATCC 43504. To clone the *sodB* gene from strain ATCC 43504, oligonucleotide primers which flank the gene were designed using the sequence published for strain 26695 (37), with mismatches that introduced restriction endonuclease (*EcoRI*) sites. The upstream primer had the sequence 5'-GTC TTG AAT TCA ACA AAG AAG-3', and the downstream primer sequence was 5'-CTA ATT GAA TTC CTA TAT TGC-3'. High-fidelity PCR was performed using *Pfu* DNA polymerase (Stratagene) as recommended by the manufacturer. Chromosomal DNA from strain ATCC 43504 was used as the template. PCR products were phosphorylated with polynucleotide kinase and cloned into the *SmaI* site of pBluescript KS(+) (Stratagene) to create pKSsodB-3. Table 1 describes the plasmids used in this study. The sequence of the cloned region was determined by the University of Georgia Molecular Genetics Instrumentation Facility.

Construction of *H. pylori* mutants. The kanamycin resistance gene *aphA3* from *C. coli* was excised from the plasmid pHP1 (29) via *EcoRI* digestion, gel purified (Qiagen), and blunted with T4 DNA polymerase. This fragment was cloned into pKSsodB-3, which had been cut with either *NcoI* or *SphI* (both sites are unique and within the coding region of *sodB*), and blunted with T4 DNA polymerase. To construct *sodB* mutants, these plasmids, pG2 (*aphA3* at the *NcoI* site) and pH2 (*aphA3* at the *SphI* site), were used to electrotransform *H. pylori* to the Kan^r phenotype by a protocol provided by D. McGee (personal communication). Briefly, *H. pylori*, grown on BA plates for 48 h, was harvested, washed with 10 ml of cold 15% glycerol-9% sucrose, and resuspended in 500 µl of the same buffer. Fifty-microliter aliquots were held on ice with 2 µl of plasmid (approximately 1 µg) for 10 min and then electroporated (25 µF, 800 Ω, 2.5 kV, 12.5 ms). Mueller-Hinton broth (100 µl) (Difco) was added, and the mixture was then spotted onto BA plates. Plates were incubated at 37°C for 2 days under conditions of 2% oxygen. Growth was scraped from these plates and streaked onto BA plates containing kanamycin, and incubation was continued for 5 to 8 days.

An approach similar to that used to isolate the *sodB* mutants was taken to obtain *H. pylori* strains with mutations in the downstream gene, known as gene HP0388 in strain 26695 (37). Oligonucleotides that flank the downstream gene (5'-TGT TGA ATT CAA GAC ACT CTG-3' upstream with the *EcoRI* site and 5'-CCC ATT TAA ACA GCG CTT CC-3' downstream with the *Eco47III* site) were used to amplify the gene, which was then cut with *EcoRI* and *Eco47III* and

TABLE 2. Growth^a of wild-type and mutant *H. pylori* strains under various oxygen concentrations

Strain	Growth under O ₂ concn (%) of:						
	2	3	4	5	6	8	12
43504	+++	++++	+++	+++	+++	+++	+++
HPG2	+++	++	+	+/-	+/-	+/-	-
HP388K	+++	NT	NT	NT	NT	NT	+++

^a Growth was scored after 48 h as follows: -, no growth; +/-, slight growth at the site of heaviest inoculum (see the text); +, significant growth at the site of heaviest inoculum; ++, significant growth and development of isolated, pinpoint colonies; +++, healthy growth with individual colonies up to ~1 mm; +++, healthy growth with colonies of >1 mm; NT, not tested.

cloned into pBluescript KS(+), which had been cut with *EcoRI* and *HincII*, to form pKS0388. Subsequently, HP0388 was interrupted by insertion of the *aphA3* cassette into the unique *NsiI* site to form pKS388K, which was used to transform *H. pylori*.

Sensitivity of growth to oxygen. *H. pylori* strains were streaked for isolated colonies onto BA plates and incubated at 37°C with concentrations of oxygen at 2, 3, 4, 5, 6, 8, or 12%. Growth was scored after 48 h as described in Table 2. Oxygen concentrations were maintained via a Forma 3230 variable oxygen incubator. This instrument continuously measures oxygen levels by thermal conductivity and adjusts to the set concentration by injection of nitrogen gas.

Sensitivity to oxygen for viability. To measure the toxicity of oxygen on wild-type and mutant strains of *H. pylori*, cells grown for 48 h on BA plates at a 2% oxygen concentration were suspended in 150 mM NaCl to an optical density at 600 nm (OD₆₀₀) of 0.1. Five milliliters of the suspension was stored under conditions of 5% CO₂-10% H₂-85% N₂ or stored in a covered petri plate under normal atmospheric conditions. Both sets of cells were stored at 37°C. Cell populations were measured via plate counts after various times of incubation.

Hydrogen peroxide sensitivity. Sterile 7.5-mm-pore-size-filter paper disks were applied to BA plates which had been streaked for confluent growth. H₂O₂, 30% (wt/wt) (20 µl), was applied to each disk, and plates were incubated at 37°C for 48 h before the zones of growth inhibition were measured. Incubation was carried out in an incubator under controlled O₂ concentrations which were varied from 2 to 12% partial pressure. The distance from the edge of the disk to the edge of the zone of growth was recorded. No zones of inhibition were observed when H₂O was added to the disks instead of H₂O₂.

Superoxide dismutase activity. Qualitative activities of cell extracts were assayed in nondenaturing gels by a method based on that of Beauchamp and Fridovich (2). Cells were harvested from BA plates after 48 h of growth, washed in 1.0 ml of lysis buffer (400 mM NaCl, 10 mM Tris [pH 8.0], 10 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM EDTA, 0.1 mM dithiothreitol, 10% [vol/vol] glycerol), and resuspended in 0.5 ml of lysis buffer. Cells were lysed via sonication (two 15-s pulses at an 80% micro-tip maximum with a W-380 Heat Systems-Ultrasonic, Inc., sonicator) on ice. Lysates were cleared by centrifugation at 4°C for 15 min and assayed for protein concentration using a kit (Pierce) based on Coomassie binding. Samples (100 µg) were applied to nondenaturing, 12.5% acrylamide gels and run until the dye front reached the bottom of the gel. Gels were soaked in 0.2% nitroblue tetrazolium for 20 min and then transferred to O₂⁻-generating buffer (100 ml of 36 mM potassium phosphate [pH 7.8], freshly supplemented with 325 mg of *N,N,N',N'*-tetramethylethylenediamine and 1 mg of riboflavin) for about 15 min under fluorescent light (10 to 20 cm from a 40-W light) until the gels turned blue and the contrast between the blue background and the achromatic (SOD) bands was easily discerned.

SOD activities were quantified by the method of McCord and Fridovich (25), with minor modifications as described by Maier and Moshiri (23). This method is based on the inhibition of superoxide-mediated cytochrome *c* reduction by extracts containing SOD. One unit of activity is the amount required to inhibit the reduction of cytochrome *c* by 50%. Data presented are the averages of three replicates ± the standard deviations.

Determination of spontaneous mutagenesis frequency. The frequency of mutagenesis was estimated by a protocol based on that of Sisson et al. (35). Wild-type or mutant cells that had been grown on BA plates at 2% O₂ were suspended in phosphate-buffered saline (PBS) at an OD₆₀₀ of ~25 (approximately 10¹⁰ CFU/ml). One-hundred-microliter samples (neat and 10⁻¹ dilutions) were plated on BA plates containing 2, 5, or 10 µg of rifampin/ml. Dilutions of each suspension were used for total viable cell determinations. All plates were incubated in 2% O₂. Mutation frequencies were calculated as the number of Rif^r colonies/10⁸ viable cells.

Mouse colonization studies. Mouse colonization studies were carried out with 5-to-6-week-old, female C57BL/6J mice from Jackson Laboratories. *H. pylori* SS1

(22) (a gift from D. McGee) and *sodB* mutants in this strain were each passaged on plates the same number of times (three times for experiment 1 and nine times for experiment 2) for each experiment. Mice were starved for 2 h prior to oral gavage with 100 µl of bacterial suspension. Suspensions were prepared by scraping 48-h growth from BA plates or BA plates with kanamycin into PBS at an OD₆₀₀ of 1.7 (~10⁹ CFU/ml). The head space above these suspensions was sparged with Ar gas to maintain low oxygen exposure during the time interval (approximately 15 min) between harvest and gavage. After 20 (experiment 1) or 21 (experiment 2) days, the mice were starved for 3 h and sacrificed, and the stomachs were excised. Stomachs were cut into four pieces each and homogenized in 5 ml of Ar-sparged PBS in 10-ml Dounce hand homogenizers. Samples (100 µl) were plated in duplicate, both neat and at 10⁻¹ dilutions. Plates were incubated for 5 to 7 days at 37°C in a 2% O₂ partial pressure atmosphere before colonies were counted.

RESULTS

Cloning and sequencing of the *sodB* gene of *H. pylori* ATCC 43504. The nucleotide and deduced amino acid sequences reported for *sodB* genes of different *H. pylori* strains vary slightly (1, 4, 36, 37). To compare the sequences for our wild-type strain, ATCC 43504, with those of other strains, we amplified the gene from chromosomal DNA via high-fidelity PCR using oligonucleotides complementary to strain 26695. The resulting fragment was cloned into pBluescript KS(+) (Stratagene) and sequenced. Compared with the sequence of 26695, we found 21 nucleotide differences within the coding region (data not shown), which confer only three amino acid changes. Compared to the amino acid sequence for ATCC 43504 reported by Bereswill et al. (4), the predicted primary amino acid structure for our strain 43504 differs by one amino acid. We found an aspartic acid encoded at position 54; Bereswill et al. (4) found an alanine at this position. Our predicted protein sequence is identical to that of strains 60190 (36) and 151 (4). Both of these strains belong to the B isoform group of Fe-SODs, described by Bereswill et al. (4).

Inactivation of *sodB* and the gene directly downstream, HP0388, in *H. pylori*. To inactivate *sodB*, the kanamycin resistance determinant from *C. coli* was cloned into either the unique *NcoI* site or the unique *SphI* site of the *sodB* gene carried on the pBluescript KS(+) vector. The resulting plasmids were used to transform *H. pylori* via electroporation. Homologous recombination resulted in kanamycin-resistant, SOD-deficient strains. Transconjugants were incubated for 7 to 8 days at 2% O₂ before small colonies could be recovered. All attempts to recover mutants with incubation at 5 or 12% concentrations of O₂ failed. PCR amplification of the *sodB* gene from each of the mutant strains confirmed the insertion of DNA consistent with the presence of the *aphA3* gene. HP0388 was inactivated in a similar fashion except that the *aphA3* cassette was inserted into the *NsiI* site of that gene.

FIG. 1. Superoxide dismutase activity of *H. pylori* cleared cell extracts in nondenaturing, activity-staining gels. Each lane was loaded with ~100 mg of protein from either wild-type cells (A), HPG2 *sodB* cells (B), or HPH2 *sodB* cells (C).

Superoxide dismutase activity. SOD activity was assayed in cell extracts both qualitatively on nondenaturing gels and quantitatively to compare specific activities in extracts from cells grown under two different oxygen stresses. In agreement with the results of Spiegelhalter et al. (36), only a single band of SOD activity was detected for wild-type *H. pylori* cells (Fig. 1). This band was absent in both *sodB* mutants (Fig. 1) but present in the HP0388 (downstream gene) mutant (data not shown). Quantitative assays of SOD activity, based on inhibition of cytochrome *c* reduction, revealed similar levels of activity in wild-type cell extracts whether the organism was grown at 2 or 12%-partial-pressure oxygen (Fig. 2). The *sodB* mutant had no detectable activity, and cells with interruptions in the downstream gene (HP0388) had levels comparable to that of the wild-type strain.

FIG. 2. Quantitative assay of superoxide dismutase activity in cleared cell extracts of *H. pylori* strains. O_2 concentrations during growth were as indicated for wild-type cells (2 and 12%). The *sodB* mutant was grown in 2% O_2 , and the HP0388 mutant was grown in 12% O_2 . Error bars represent the standard deviations of results for three replicate experiments.

FIG. 3. Survival of nongrowing *H. pylori* cells under atmospheric oxygen and anoxic environments. Wild-type (triangles) or *sodB* mutant (circles) cells grown under 2% O_2 were suspended in 150 mM NaCl and incubated in either atmospheric oxygen (closed symbols) or without oxygen (open symbols). Samples were removed at the times indicated on the x axis and used for plate counts in a 2% O_2 environment.

Growth under various oxygen concentrations. The ability of *H. pylori* mutants to grow under various oxygen concentrations was determined by comparing growth on BA plates in an oxygen-controlled environment to that of the isogenic wild-type strain (Table 2). When incubated at 2% partial-pressure O_2 , growth of all strains was similar. However, at increasing O_2 concentrations, the *sodB* mutant exhibited a decreased ability to grow. Above 3 to 4% O_2 , the mutant failed to form colonies on plates and only minimal growth was seen around the point of heaviest inoculum (initial streak on the plates). In contrast, the wild-type strain grew well at all O_2 levels (2 to 12%), although it showed slightly greater growth in an O_2 concentration of 12% partial pressure than at 2% O_2 . Growth of the HP0388 mutant was no different from that of the wild type at either 2 or 12% partial pressure O_2 .

Oxygen tolerance of nongrowing cells. To determine whether the impaired growth of the *sodB* mutant at higher O_2 levels was due to oxygen-dependent cell death, we measured the ability of nongrowing cells to survive periods of oxygen exposure (Fig. 3). A slight drop in viability was noted over the first few hours for both wild-type and mutant strains. However, after a 6-h period in an air-exposed container, the population of the wild-type cells decreased by about 1 order of magnitude (from 10^7 to 10^6 CFU/ml), while the mutant population decreased by more than 6 orders of magnitude. The decrease in wild type viability in air was not due to air exposure, since cells incubated in an anoxic environment showed a similar decrease in viability. Between hours 2 and 6, the mutant strain's viability in air rapidly decreased compared with that of cells incubated in an anoxic environment. The mutant population decreased to only about 20 CFU/ml after 5 h of incubation in the air atmosphere, and in three separate experiments no colonies were recovered after 6 h of incubation in air.

Susceptibility to hydrogen peroxide. Decreased SOD activity has been shown to increase sensitivity to hydrogen peroxide for *E. coli* (5). To test whether *H. pylori* *sodB* mutants are more susceptible to this oxidative stress, filter paper disks were applied to plates streaked for confluent growth. Hydrogen per-

TABLE 3. Hydrogen peroxide sensitivities^a of *H. pylori* strains at various oxygen concentrations

Strain	Growth inhibition zone (mm) at O ₂ concn (%) of:			
	2	3	4	5
43504	0	3	5	5
HPG2	10	17	19	ND ^b

^a Sensitivity assayed as a zone of inhibition of growth, measured from the edge of the disk (containing 20 μ l of 30% H₂O₂) to the edge of growth.

^b Not determined; growth was too light to accurately determine a zone of inhibition.

oxide was added to the disks, and the plates were incubated under various oxygen concentrations. Susceptibilities to hydrogen peroxide were measured as zones of inhibition of growth around the disks (Table 3). At each oxygen concentration, the zone of inhibition was considerably larger for the *sodB* mutants than for the wild type. Additionally, the zone size was affected by the O₂ concentration in the incubation atmosphere for both strains. The highest concentrations of O₂ (up to 12%) caused the largest zones of H₂O₂-dependent growth inhibition.

Determination of mutation frequency. To test whether mutations in *sodB* increase DNA damage in *H. pylori*, the mutation frequency was assayed by quantifying the appearance of spontaneous forward mutation to rifampin resistance (Rif^r). In four experiments, in which the rifampin concentration ranged from 2 to 10 μ g/ml, the wild-type strain exhibited mutation frequencies ranging from 0.84 to 1.69 Rif colonies per 10⁸ viable cells (Fig. 4). The SOD-deficient mutant, HPG2, gave frequencies ranging from 12.3 to 29.0 mutants per 10⁸ viable cells among the four experiments. In each experiment, the mutation frequency of the *sodB* mutant strain was between 14.0 and 17.4 times higher than that of the wild type.

FIG. 4. Spontaneous rifampicin resistance in wild-type and *sodB H. pylori*. Resistance was assayed by colony counts at three concentrations of rifampicin (as indicated) after incubation at 2% O₂ for 48 h. The data shown for the 5- μ g/ml Rif level is the mean for two separate experiments. Results for the 2- and 10- μ g/ml Rif levels are from single experiments.

TABLE 4. Mouse colonization abilities^a of wild-type strains and *sodB* mutant strains

Experiment no.	Colonization of stomachs by:	
	Wild type	<i>sodB</i> mutant
1	7/8	1/8
2	8/9	0/15

^a Reported as number of mouse stomachs from which *H. pylori* was recovered/total number of stomachs assayed. Colonization for the wild type ranged from 1.7×10^3 to 8.9×10^5 viable cells/g of stomach. The mutant colonization (one stomach from experiment 1) was 1.7×10^3 viable cells/g of stomach, but the other stomachs that had been inoculated with the *sodB* mutant strain had no colonies on the plates, even from those receiving 0.1 ml plated directly from the 5-ml stomach homogenate solution.

Mouse colonization of *sodB* mutants. SOD has been implicated as a virulence factor in other organisms, including *Campylobacter coli*, where SOD was shown to be a colonization factor (34). To determine whether a deficiency in SOD activity is important for *H. pylori* colonization, the relative abilities of wild-type and *sodB*-deficient strains to colonize the mouse stomach were evaluated in two separate experiments. In each of the two experiments, with mice inoculated with the mouse-adapted strain SS1, *H. pylori* was recovered from the stomachs of 15 out of 17 animals (Table 4). However, only one of 23 mice that were inoculated with SSG2 (*sodB* mutant) was found to harbor the strain 3 weeks after oral administration. Colonies isolated from this mouse stomach were confirmed to be the *sodB* mutant strain based on their kanamycin resistance and their inability to grow at 12% O₂. Both the wild-type and mutant strains were replated an identical number of times after the initial isolation of the mouse-colonizing strain. This step was done to reduce the possibility that repeated subculturing of the mutant led to the decrease in mouse infectivity that was observed. Also, the decrease in mutant colonization is not due to O₂ exposure of the stomach isolates, since the stomachs were homogenized in low-O₂ conditions, and the cells plated from the stomach homogenate dilutions were rapidly transported to the incubator containing 2% O₂.

DISCUSSION

SOD activity appears to be a common trait among organisms that live in and consume oxygen (26). This activity is a primary defense against the ubiquitous respiratory production of superoxide (17). In combination with hydrogen peroxide, another partially reduced O₂ by-product of respiration (16), superoxide gives rise to highly reactive hydroxyl radicals that can damage nearby macromolecules like DNA or membrane lipids. Because microaerophilic organisms may be especially sensitive to the reactive forms of oxygen (20), and because this oxygen sensitivity is likely to be especially important in pathogenic organisms that must confront the oxidative attack of the host immune system, we wished to investigate the properties of *H. pylori* that are deficient in SOD activity. We demonstrate that *sodB* mutants of this microaerophile are even more sensitive than the parent strain to oxygen and oxygen metabolites and that they are deficient in colonization of the mouse stomach.

The growth ability of the *sodB* mutant was decreased relative to that of the wild-type strain at O₂ levels above 2%. We observed some slight growth of the mutant in incubators held

at O₂ levels between 3 and 8%, although no isolated colonies appeared under these conditions. This observation suggests the presence of low-O₂ microenvironments around the point of heaviest inoculation. Similar low-O₂ microenvironments surrounding cells grown under different O₂ concentrations may help to explain why we saw no difference in SOD activity levels between cells harvested from growth at 2 or 12% O₂. Alternatively, SOD activities may be constitutive or regulated by factors that were not varied in our experiments.

We saw an increase in hydrogen peroxide sensitivity as O₂ levels were increased for both the wild-type and *sodB* strains. For each concentration, the mutant was more sensitive than the wild type. This oxygen-dependent peroxide sensitivity and hypersensitivity of the mutant are consistent with observations with *E. coli*, for which a major toxic effect of superoxide is its generation of catalysts for hydroxyl radical formation from hydrogen peroxide (19).

In contrast to *E. coli*, which has three known SODs (3), *H. pylori* has only one, an iron-cofactored species (36). Fe-SODs are normally associated with the cytoplasm (14) and are ordinarily involved in protection against internally generated superoxide. The SOD of *H. pylori* seems to be associated with the cell surface (36), consistent with a role in protection against exogenously produced superoxide like that incurred during an oxidative burst via a host inflammatory response. Use of an iron-cofactored SOD for protection from exogenous superoxide has been noted for *C. jejuni*, for which interruptions in *sodB* decreased the rate of survival inside host cells (32).

Our mouse studies indicate that *H. pylori* colonization is greatly affected by *sodB* mutations. However, from these experiments we cannot determine whether the decreased viability of the *sodB* mutants under oxidative conditions prohibits their survival before they can establish residency in the mucosa or whether they are more sensitive and fall prey to the oxidative burst of a host immune response while residing in the mucin. Because we did confirm that one mouse became colonized by strain HPG2, we cannot report that *sodB* is required for colonization. Due to animal variability in a model system such as this, we assume that atypical conditions which allowed growth and survival of *H. pylori* existed in this single mouse.

To investigate whether the phenotype of the *sodB* mutants was due to polar disruption of downstream gene expression, we isolated isogenic mutants with *aphA3* cassette interruptions in this gene. HP0388 is the designation for the gene that is 54 bp directly downstream of *sodB* in strain 26695 (37). The putative gene product has protein sequence homology to a hypothetical protein of unknown function from *Haemophilus influenzae* (HI0319) and to a putative methyl transferase in *E. coli* (EC1870) (<http://www.tigr.org>). The intergenic region, between *sodB* and HP0388, contains a putative transcriptional terminator, a 38-bp region with a 13-base inverted repeat (with one mismatch), followed by seven thymidine residues over the next nine bases. Unlike mutations in *sodB*, mutations in HP0388 do not affect SOD activity or growth in 12% O₂. Based on these data, we suggest that the phenotypes reported here for *sodB* mutants are not the result of polar effects. Though a slight possibility exists that the virulence defect is due to this hypothetical product, which is possibly under the same transcriptional control as *sodB*, the obvious problem of an oxida-

tive stress-related deficiency due to the *sodB* mutation is the likely cause for the decreased colonization.

In addition to [4Fe-4S] damage and subsequent hydroxyl radical damage, superoxide toxicity may include the reduction or oxidation of other compounds (12) and interaction with nitric oxide (NO) to form peroxynitrite (30). Because NO levels in gastric juice are fairly high (30), superoxide-dependent peroxynitrite toxicity may be an important factor in the defective colonization abilities of *sodB* mutants. Exposure to NO causes a transient decrease in respiration in *E. coli* but a sustained inhibition of respiration in *H. pylori* (30). Nagata et al. (30) have presented evidence that this inhibition is due to peroxynitrite production and is linked to higher superoxide levels in *H. pylori*. These data are consistent with the idea that *sodB* mutants of *H. pylori* may be compromised in respiration, another factor that could affect the ability of this organism to survive in its host.

Known *H. pylori* virulence factors include a variety of proteins that are involved in its pathogenesis, such as VacA and Cag (21). Another group of virulence factors is clearly important for colonization of *H. pylori* in the gastric mucosa. These include urease, motility factors (flagellin), and SOD. Because of this organism's microaerophilic nature and the increased levels of reactive oxygen in the infected host (7), we expect that other factors involved in the response to oxidative stress are likely to be required for virulence.

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