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Interruption of the *gpxA* Gene Increases the Sensitivity of *Neisseria meningitidis* to Paraquat

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Antioxidant enzymes are thought to be important for the survival of pathogenic *Neisseria* species. We have further characterized the glutathione peroxidase homolog gene (*gpxA*), which we recently isolated from *Neisseria meningitidis* FAM20 (T. D. E. Moore and P. F. Sparling, Infect. Immun. 63:1603–1607, 1995). GpxA was found to be produced constitutively in vivo. An isogenic, omega insertion mutant in the *gpxA* gene was constructed and characterized. The *gpxA* insertion mutant was much more sensitive to the oxidative stress caused by paraquat and slightly more sensitive to hydrogen peroxide. This is the first demonstration of a phenotype arising from a mutation of a glutathione peroxidase homolog gene in a prokaryotic organism. Protection of the cell by GpxA from the effects of oxidative stress caused by aerobic metabolism may contribute to the ability of *Neisseria meningitidis* to cause disease in the human host.

Aerobic organisms are protected from reactive oxygen species by both low-molecular-weight antioxidants (e.g., ascorbate and glutathione) and enzymatic defenses (1, 14). *Escherichia coli* has multiple protective enzymes, including superoxide dismutases (SOD) (MnSOD, FeSOD, and CuZnSOD) (3, 28, 35, 42), catalases (HPI and HPII) (7, 34), alkyhydroperoxidase (39), and thiol peroxidase (7, 8). To provide complete protection, these enzymes may be expressed in different compartments of the bacterial cell or at different growth phases and are induced by reactive oxygen species.

Neisseria meningitidis is a gram-negative bacterial pathogen of humans causing a rapid and often fatal meningitidis, primarily in children and young adults. Like *E. coli*, meningococci also produce both SOD and catalase activities (2). Recently, we isolated a gene encoding a glutathione peroxidase homolog, *gpxA*, from *N. meningitidis* (36). The *gpxA* gene from *N. meningitidis* FAM20 was found to be 49 to 57% identical at the amino acid level to seven other glutathione peroxidase family members over a 49-amino-acid region which is conserved among various organisms (exon3) (5, 36). The *gpxA* sequence was present in 7 *N. meningitidis* strains tested but was absent in 10 *Neisseria gonorrhoeae* strains and 6 nonpathogenic *Neisseria* species. Since the *gpxA* sequence was not found in either the nonpathogenic strains or the closely related gonococcus (which causes a less severe disease than the meningococcus), we were interested in studying *gpxA* further.

Overexpression and purification of recombinant *gpxA*. The *gpxA* gene was subcloned into the pET30a (Table 1) expression vector under the control of the T7 promoter. PCR was used to isolate and add restriction sites to the *gpxA* open reading frame. Specifically, each 50-μl reaction contained 68 ng of pUNCH910 as the template, 1 mM primers *Nco*I 5′-GGGC CCATGGGTATTTACGATTTTCA-3′ and *Hin*dIII 5′-CT CAAGCTTGGCGGCAGATTACAGCAA-3′, and 5 U of Taq polymerase. One-fiftieth of the PCR mixture was added to 50 ng of pCRII (Invitrogen) and ligated, and then OneShot cells (INVαF′; Invitrogen) were transformed and plated to Luria broth plates containing 30 μg of kanamycin per ml. The resulting construct, pUNCH915, was transformed into the expression strain BL21, which contains the DE3 lysogen allowing for expression of *gpxA* driven by the T7 promoter after induction by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The expression of *gpxA* in this construct is shown in Fig. 1. The fusion protein was predicted to be 25% larger than the native protein, consistent with the change in mobility from 20 kDa (for the predicted native protein) to 25 kDa (observed for the fusion protein). The recombinant fusion protein was purified on a denaturing affinity column. In this way, 40 mg of protein was purified from 1 liter of cell culture (Fig. 1). The purified GpxA was used to generate polyclonal antibodies. Purified protein was sent to Immuno-Dynamics (San Diego, Calif.), where two rabbits were injected with 200 μg of protein and Freund's adjuvant three times. One rabbit produced polyclonal antibodies which reacted with the GpxA protein.

Insertional inactivation of *gpxA*. The 2-kb Ω fragment of pHP45Ω (38) (containing transcription and translation terminators and genes for spectinomycin and streptomycin resistance) was ligated into a unique *Mlu*I site within the *gpxA* gene on pUNCH911 (36) (Table 1). The resulting construct was linearized and reintroduced into the chromosome of meningococcal strain FAM20 by transformation and allelic replacement by previously published procedures (4, 9), creating FAM80. Spectinomycin-resistant transformants were assumed to carry homologously recombined insertionally inactivated *gpxA*::Ω, since the plasmid cannot be maintained autonomously in *N. meningitidis*. Southern analysis was performed using genomic DNA (0.125 μg/lane) digested to completion with *Cla*I (Boehringer Mannheim and New England Biolabs) and probes labeled with digoxigenin (Boehringer Mannheim). Chromosomal DNA from FAM20 and FAM80 was hybridized to the 2-kb Ω fragment and to a 374-bp fragment within the *gpxA* gene. The *gpxA* probe hybridized to a 4-kb *Cla*I band in FAM20 and a 6-kb *Cla*I band in FAM80. The increase in size corresponded to the 2-kb Ω fragment inserted into the gene (Fig. 2A). When the Ω fragment was used as a probe, no hybridization was detected with FAM20 genomic DNA. The Ω fragment hybridized to a 6-kb *Cla*I band in FAM80, however,

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties[a]	Source or reference
N. meningitidis		
FAM20	FAM 18 derivative; Nal^r	11
FAM80	FAM20 *gpxA*::Ω interposon; Str^r Spc^r Nal^r	This work
E. coli		
DH5α-MCR	ϕ80 Δ*lacZ* ΔM15	Bethesda Research Laboratories
BL21	DE3 lysogen	Novagen
INVαF	ϕ80 Δ*lacZ* ΔM15	Invitrogen
Plasmids		
pCRII	Kan^r, Amp^r	Invitrogen
pET30a	Kan^r	Novagen
pHP45	Ω-Spc^r, Str^r	38
pUNCH911	*gpxA*	36
pUNCH912	Ω::*gpxA*	This work
pUNCH914	*gpxA* in pCRII	This work
pUNCH915	*gpxA* in pET30	This work

[a] Nal^r, nalidixic acid resistance (20 μg/ml); Amp^r, ampicillin resistance (100 μg/ml); Kan^r, kanamycin resistance (30 μg/ml); Str^r, streptomycin resistance (20 μg/ml); Spc^r, spectinomycin resistance (100 μg/ml).

indicating that recombination had occurred by a double crossover into the desired locus (Fig. 2B).

Northern analysis of *gpxA* mRNA. Bacterial RNA was isolated using the RNeasy system spin columns and procedures (QIAGEN, Inc., Chatsworth, Calif.). Total RNA (2 μg/lane) was separated on a 1% formaldehyde gel in morpholine propanesulfonic acid (MOPS) buffer by using published procedures (37). The digoxigenin-labeled probe hybridized to a single band in the total RNA from the wild-type strain FAM20, and the size of the band was consistent with the 521-nt open reading frame of *gpxA*. The native band comigrated with the band produced in *E. coli* expressing the *gpxA* gene from pUNCH911 (Table 1). The same probe was hybridized with total RNA from the mutant strain FAM80 RNA, but no band was detected (Fig. 2C).

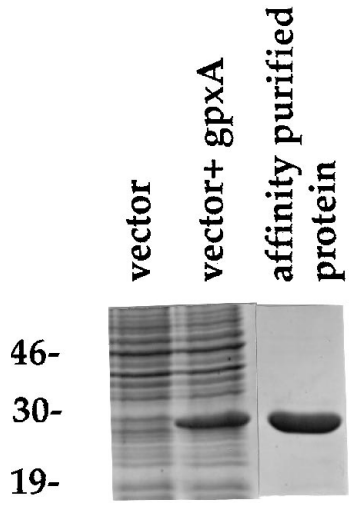

FIG. 1. Overexpression and affinity purification of recombinant GpxA. Shown are the results of SDS-PAGE followed by Coomassie blue staining of induced whole-cell lysates containing pET30a (vector), induced whole-cell lysates containing pUNCH915 (vector + gpxA), and recombinant GpxA containing the His tag which had been affinity purified on a nickel column (affinity purified protein). Molecular masses (in kilodaltons) are indicated to the left of the gel.

Western (immunoblot) analysis of FAM80. Using the polyclonal antibodies raised in rabbits to recombinant GpxA, we detected a 20-kDa band in either whole-cell lysates (Fig. 2D) or the soluble fraction (data not shown) of FAM20. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gels and then transferred to nitrocellulose membranes. Membranes were incubated with the polyclonal antibodies at a 1:1,000 dilution overnight. The M_r of the band was consistent with the predicted size of GpxA (177 amino acids, 20 kDa) and with the mobility of the *E. coli* glutathione peroxidase homolog BtuE (183 amino acids, 22 kDa) (10). There were also two bands that cross-reacted with either the secondary antibody (middle band) or the polyclonal antibody (bottom band) (Fig. 2D). The 20 kDa band was specifically absent from FAM80 whole-cell lysates.

Growth rate of FAM80 and regulation of GpxA. Growth curves were plotted to determine whether the *gpxA* mutant had a growth defect such as that found in *E. coli* SOD$^-$ mutants (6). The meningococcal strains FAM20 (wild type) and FAM80 (*gpxA* insertion mutant) were grown on GC base medium (Difco) plates overnight and inoculated into 10 ml GC broth to an A_{600} between 0.05 and 0.08. The liquid cultures were grown in duplicate at 37°C in 250-ml Erlenmeyer flasks with shaking at 200 rpm. Aliquots (10 μl) were immediately diluted into 1 ml of 25°C GC broth without supplements I or II added, diluted further, and then plated at several dilutions to enumerate the viable cells.

The growth rates of wild-type and mutant strains were identical as shown in Fig. 3. Cells at various stages of growth were harvested and subjected to Western analysis (Fig. 3). GpxA seemed to be made constitutively during the various growth phases.

Effect of *gpxA* mutation on growth and sensitivity of FAM80 to oxidative stress. The enzymes of the glutathione peroxidase family are thought to protect cells from oxidative stress or to repair damage caused by the reactive oxygen species. A glutathione peroxidase-homolog from plants has been shown to increase the tolerance of *E. coli* cells to paraquat (23). Sensitivity of wild-type FAM20 and *gpxA* mutant FAM80 to paraquat, hydrogen peroxide, and xanthine oxidase plus hypoxanthine was studied. Paraquat passes through the cell wall (27) and generates O_2^- inside the cell (20–22). Superoxide and hydrogen peroxide are unavoidable natural by-products of aerobic metabolism (25). Xanthine oxidase in the presence of either of its substrates, hypoxanthine or xanthine, generates hydrogen peroxide (80%) and superoxide (20%) outside the cell (16). The xanthine oxidase system has been used to mimic some effects of exposure to human neutrophils (41).

For paraquat sensitivity assays, the meningococcal strains FAM20 (wild type) and FAM80 (*gpxA* insertion mutant) were grown as described above until late log phase (approximately 5 h). The number of viable cells was determined, and 6 μl of FAM20 and FAM80 cell suspensions were diluted into 6 μl to 3 ml of GC broth (approx. 2×10^6 CFU/ml) containing 0, 0.05, 0.1, or 0.15 mM paraquat in 5-ml disposable borosilicate tubes. Assays were performed in triplicate. The cultures were incubated at 37°C for 19 h without shaking. Viable cell counts were determined for each of the triplicate cultures. The strains were also exposed to various concentrations of hydrogen peroxide and xanthine oxidase in the same manner that they were exposed to paraquat. The hydrogen peroxide and xanthine oxidase sensitivity assays were performed exactly as the paraquat sensitivity assays were with one exception: the 6 μl of FAM20

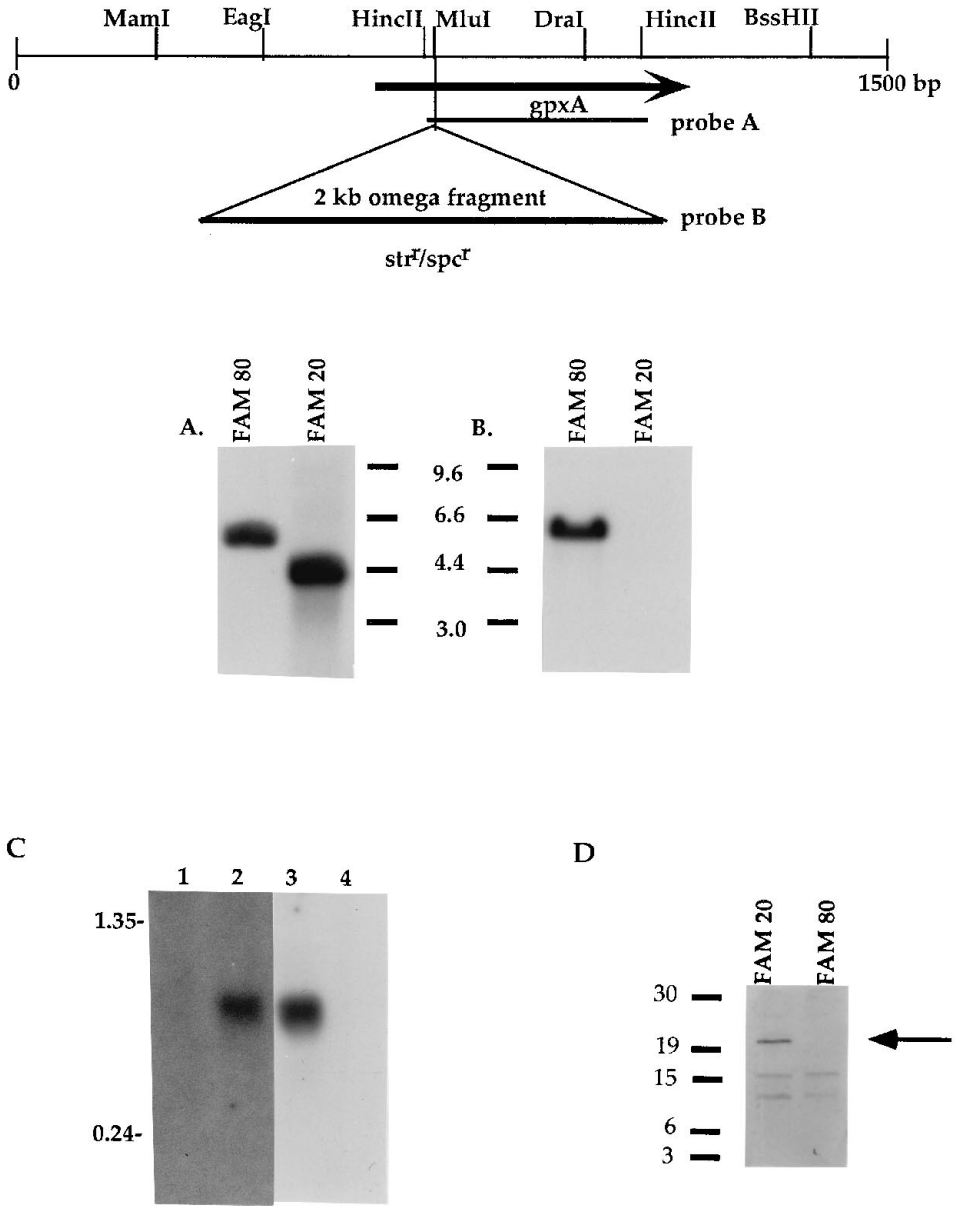

FIG. 2. Restriction map of pUNCH910 with an arrow indicating the *gpxA* gene and direction of transcription. Meningococcal *Cla*I-digested DNA was hybridized with (A) a 374-bp *Hinc*II fragment within the *gpxA* gene (indicated by a thin solid bar) or (B) the 2-kb omega fragment (indicated by the thick solid bar on the restriction map). FAM20, wild-type DNA; FAM80, *gpxA*::Ω DNA. Molecular size markers (in kilobase pairs) are shown. (C) Northern analysis of *gpxA* mRNA. RNA was isolated from *N. meningitidis* FAM80 (lane 1), *N. meningitidis* FAM20 (lane 2), *E. coli* DH5α-MCR cells transformed with plasmid pUNCH911 (lane 3), and *E. coli* DH5α-MCR cells (lane 4). The membranes were hybridized to the 374-bp *Hinc*II fragment from within the *gpxA* gene. RNA standards are indicated on the left in kilobases. (D) Western analysis of GpxA in whole-cell lysates. The unique 20-kDa protein, which is present in FAM20 but absent from the *gpxA* insertion mutant FAM80 is indicated by an arrow. Molecular mass standards (in kilodaltons) are shown on the left.

and FAM80 cell suspensions treated with xanthine oxidase were diluted into 6 μl to 3 ml of GC broth plus 1 mM hypoxanthine.

Statistical analysis was performed by using Sigma Stat and Sigma Plot (Jandel Scientific Software, San Rafael, Calif.). To determine *P* values, the Mann-Whitney ranked sum test was used for the paraquat and xanthine oxidase experiments and a paired *t*-test was used for the hydrogen peroxide experimental analysis. The predicted molecular weight was calculated for the GpxA protein using the MacVector software (IBI, Eastman Kodak Co., New Haven, Conn.).

By this assay, FAM80 was hypersensitive to paraquat compared with FAM20 (Fig. 4A). Since paraquat passes through the cell membrane and generates superoxide, *gpxA* apparently protected the cells against superoxide-mediated damage. The mutant strain was slightly more susceptible to oxidative stress caused by hydrogen peroxide (Fig. 4B). The mutant and wild-type strains were similar in their susceptibilities to xanthine oxidase, although FAM80 was slightly more sensitive at the 20 mU concentration (Fig. 4C). These results suggest that *gpxA* protects cells from oxidative stress. Excess superoxide and/or H_2O_2 can damage DNA, proteins, and the cell membrane (17, 26). The results also indicate that while the cytoplasmic protein GpxA may be able to protect the cell from the effects of

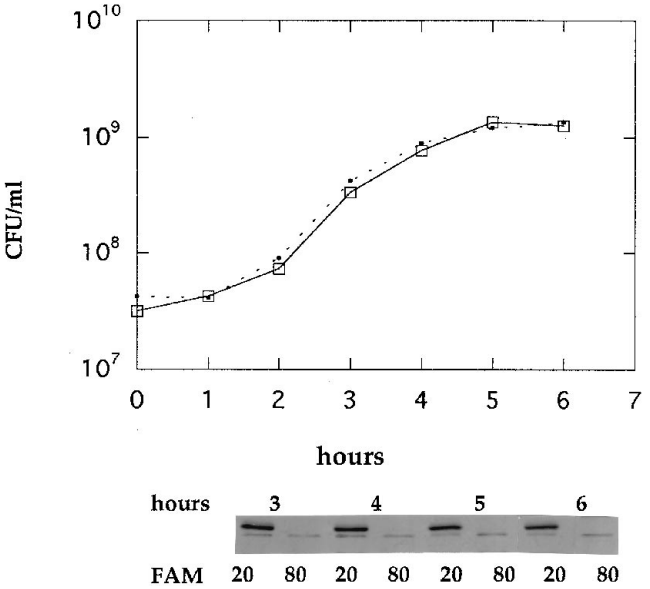

FIG. 3. Growth rates of FAM20 and FAM80. Each point shown in the growth curves in figure is the average CFU per milliliter of two cultures for each strain and are representative of three separate experiments. FAM20 is indicated by the dashed lines and solid dots, and FAM80 is indicated by solid lines and open squares. Western analysis of the growing cultures is shown below the graph. Samples of both FAM20 and FAM80 were collected at 3-, 4-, 5-, and 6-h time points. The whole-cell lysates then underwent SDS-PAGE and Western analysis with the anti-GpxA polyclonal antibody as described in the text.

superoxide generated by paraquat in the cytoplasm, it does not seem to protect the cells from damage by the superoxide generated by xanthine oxidase outside the cell.

Western analysis of the same FAM20 triplicate cultures used to study viability after exposure to various concentrations of paraquat (0 to 0.15 mM) did not indicate any increase in the level of GpxA in response to either paraquat or xanthine oxidase (data not shown). However, a slight change in protein levels cannot be ruled out by Western analysis.

All aerobic organisms must be prepared to manage the H_2O_2 and O_2^- which are generated as by-products of aerobic metabolism and which can in turn generate HO · , a powerful oxidant that damages DNA, proteins, and membranes (17, 19, 24, 25). Free iron is both required and dangerous to aerobic cells and organisms because of the ability of Fe^{2+} to react with H_2O_2 to form HO · , and this is the reason free iron is so tightly regulated (26, 40). In respiring cells, excess H_2O_2 contributes to the formation of HO · by reacting with Fe^{2+}, while excess O_2^- contributes not only by its metabolism to H_2O_2 but also because of its ability to catalyze the reduction of Fe^{3+} to Fe^{2+} (26, 30).

Superoxide can cause direct protein damage in the cell by inactivating enzymes with iron-sulfur centers (13, 18, 31, 32). It has recently been shown that O_2^- may cause damage by another mechanism which involves increasing the pool of free iron by oxidatively excising iron from the iron-sulfur clusters of enzymes (29, 33). Excess O_2^-, therefore, is not only a toxic oxidant molecule but it also increases the level of another dangerous substance, iron (29). Superoxide may also directly damage the cell membrane, but it is not clear if the cell envelope lesions generated in *E. coli* SOD mutants are due to the direct action of O_2^- or to that of a metabolite (12, 25, 33).

Our results suggest that a glutathione peroxidase homolog protects the cell in some way against the effects of oxidative

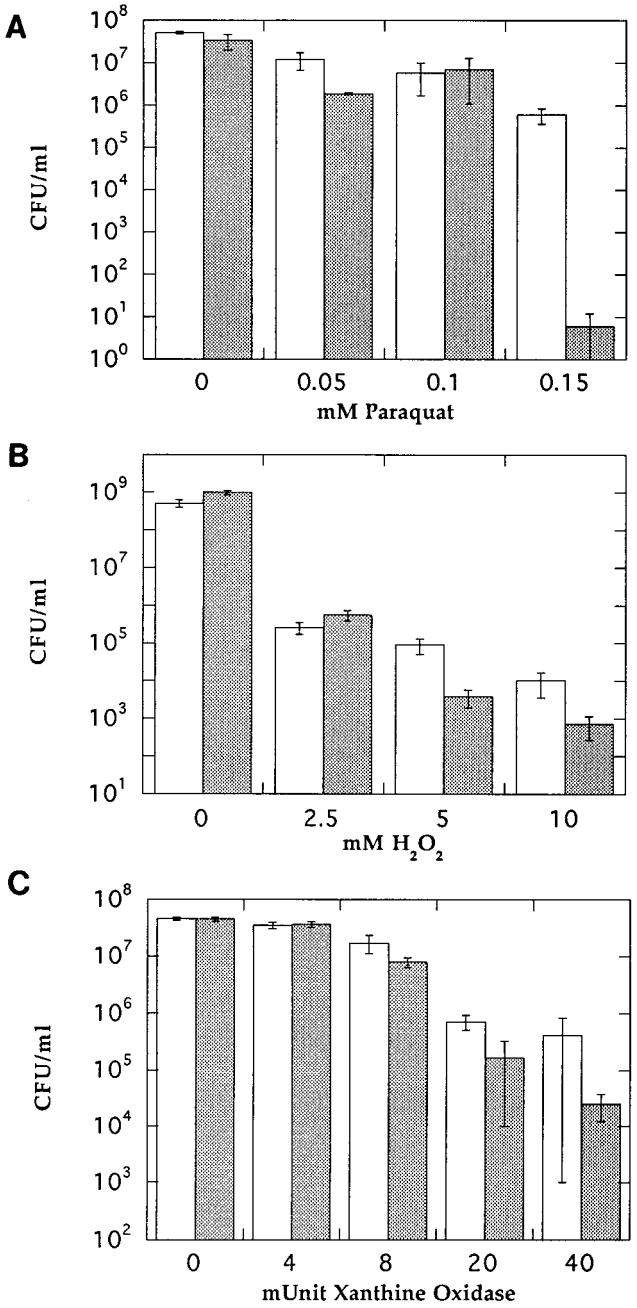

FIG. 4. (A) Effects of exposure to paraquat on *N. meningitidis* strains FAM20 (open bars) and FAM80 (shaded bars). Both the *gpxA* mutant (FAM80) and the wild-type strain (FAM20) were exposed to medium containing 0, 0.05, 0.1, or 0.15 mM paraquat. Each datum point represents the mean of three separate experiments, each done in triplicate on different days; error bars indicate standard errors. For the cells exposed to 0.15 mM paraquat $P = 0.0001$. (B) Effects of exposure to hydrogen peroxide on *N. meningitidis* strains FAM20 (open bars) and FAM80 (shaded bars). Both the *gpxA* mutant (FAM80) and the wild-type strains (FAM20) were exposed to medium containing 0, 2.5, 5, or 10% hydrogen peroxide. The graph is the result of two separate experiments, each done in triplicate on different days; error bars indicate standard errors. For the cells exposed to 5 mM hydrogen peroxide, $P = 0.059$. (C) Effects of exposure to xanthine oxidase/hypoxanthine on *N. meningitidis* strains FAM20 (open bars) and FAM80 (shaded bars). The strains were exposed to medium containing 0, 4, 8, or 20 mU of xanthine oxidase. Each datum point represents the mean of four separate experiments, each done in triplicate on different days; error bars indicate standard errors. For the cells exposed to 20 mU of xanthine oxidase, $P = 0.004$.

stress inside the cell. This protection could be against the effects of oxidative stress on DNA, on required proteins, or possibly on the cell membrane. GpxA could be protecting cellular components from the effects of an increase in the free iron concentration inside the cell due to oxidative excision of iron from Fe-S centers of proteins by O_2^-.

Glutathione peroxidases are thought to protect cells against hydroperoxides and/or to reduce lipid hydroperoxides in membranes. These enzymes catalyze the reduction of organic hydroperoxides (ROOH) and hydrogen peroxide coupled with the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) according to the following equation: ROOH + 2GSH→GSSG + ROH + H_2O. The family of eukaryotic glutathione peroxidases contains three distinct evolutionary branches which differ in peroxide-substrate specificity and possibly in the thiol hydrogen donor used by the enzyme (5). The eukaryotic enzymes have been studied extensively, but little is known about prokaryotic members of this extended family. No phenotype has been attributed to the *btuE* gene of *E. coli*, which also is a glutathione peroxidase homolog (10, 15).

In this study, we have shown that inactivation of the *gpxA* gene in *N. meningitidis* results in hypersensitivity to oxidative stress. A *gpxA* insertion mutant was considerably more sensitive to O_2^- generated inside the cell by paraquat, but it had a similar sensitivity to xanthine oxidase-generated H_2O_2 and external O_2^-. The mutant strain was slightly more susceptible to damage by hydrogen peroxide. The interruption of the *gpxA* gene of *N. meningitidis* did not affect aerobic growth. GpxA protected cells from the effects of paraquat and seemed to be constitutively expressed. It will be interesting to determine if there is a similar phenotype of a *btuE* mutant strain in *E. coli*. The mechanism for this protective effect of GpxA and its importance to the biology of infection remains to be determined.

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