Isolation and Identification of a Glutathione Peroxidase Homolog Gene, *gpxA*, Present in *Neisseria meningitidis* but Absent in *Neisseria gonorrhoeae*

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Antioxidant enzymes are thought to be important for the survival of pathogenic *Neisseria* species. We isolated a glutathione peroxidase-related gene (*gpxA*) from *Neisseria meningitidis* FAM20. The *N. meningitidis* glutathione peroxidase homolog was 49 to 57% identical to seven other glutathione peroxidase family members over a 49-amino-acid region which is conserved among various species. The *gpxA* sequence was present in all 7 meningococcal strains tested but absent in 10 gonococcal strains and 6 nonpathogenic neisserial strains as determined by Southern hybridization. The homology of *gpxA* to mammalian glutathione peroxidases and the presence of this gene specifically in the meningococcus suggest that it is important in the cellular metabolism or defense processes particular to this pathogen.

Neisseria meningitidis is a bacterial pathogen that can cause a serious and sometimes fatal disease in humans. Since *N. meningitidis* is medically important, the mechanisms of infection and proliferation of this organism once inside its human host are of interest. Part of the host response to this pathogen includes the evolution of O₂^{•−} and H₂O₂ from neutrophils. The levels of superoxide dismutase, catalase, and diamino benzene peroxidase activities in *N. meningitidis*, *Neisseria gonorrhoeae*, and commensal nonpathogenic neisserial strains have been compared. *N. meningitidis* has readily detectable levels of all three of these enzymes, while superoxide dismutase activity is reported to be absent from *N. gonorrhoeae* (3, 28). The diamino benzene peroxidase activity is KCN inhibitable, indicating a heme peroxidase. This activity is probably due to catalase, since a catalase mutant is devoid of both catalase and diamino benzene peroxidase activities (23). The ability of various *Neisseria* species to withstand oxidative attack may be due in part to high intracellular levels of superoxide dismutase, catalase, or peroxidase activity (7). Other means of protection may include the high level of glutathione which is observed in *Neisseria* species (3).

We report here the isolation and identification for the first time of the gene for a glutathione peroxidase (GPX) homolog (GPXH) from *N. meningitidis* FAM20, designated *gpxA*. Members of the GPX family are found in many eukaryotes, including humans and other mammals, plants, and parasitic worms (6, 7, 10, 16, 21). These enzymes are best studied in mammals, in which they are thought to provide protection to cells from attack by reactive oxygen species and/or to repair membranes damaged by oxygen radicals (16).

There has previously been only one other report of a GPXH gene in a prokaryotic organism, the *btuE* gene from *Escherichia coli* (11, 18). Although *btuE* was isolated and identified as part of the vitamin B₁₂ transport operon, the deduced protein sequence has significant homology only to the GPX family of enzymes (18). However, *BtuE* has not yet been shown to possess either transport or GPX activity.

The two pathogenic *Neisseria* species *N. meningitidis* and *N.*

gonorrhoeae are very similar organisms with the exceptions that meningococci produce a capsule, superoxide dismutase activity (3), and RTX related-proteins (32–34). Although the organisms are very similar genetically, *N. gonorrhoeae* usually infects the genital-urinary tract while *N. meningitidis* causes meningitis. We show that they also differ with respect to the presence of the *gpxA* gene.

Bacterial strains and growth conditions. All *Neisseria* strains

TABLE 1. Bacterial strains used

Species and strain	Serogroup ^a	Source or reference
<i>E. coli</i> DH5α-MCR	NA	Bethesda Research Laboratories
<i>N. meningitidis</i>		
FAM20	C	13
8013N	C	Xavier Nassif, Institut Pasteur
JB501	C	5
JB504	B	5
JB505	B	John Black, University of North Carolina Hospitals
JB512	C	5
Z2087	A	Mark Achtman, Max Planck, Berlin, Germany
<i>N. gonorrhoeae</i>		
MS11	PIB	25
FA1090	PIB	27
FA19	PIA	26
UU1008	ND	Zell McGee, University of Utah
F62	PIB	8
FA1135	PIA	John Black, University of North Carolina Hospitals
L2370	PIB	1
L4401	PIB	1
L4933	PIB	1
L8800	PIB	1
<i>M. catarrhalis</i>	NA	Joan Knapp, Neisseria Reference Lab
<i>N. flavescens</i>	NA	Joan Knapp, Neisseria Reference Lab
<i>N. lactamica</i>	NA	Joan Knapp, Neisseria Reference Lab
<i>N. sicca</i>	NA	Joan Knapp, Neisseria Reference Lab
<i>N. mucosa</i>	NA	Joan Knapp, Neisseria Reference Lab
<i>N. subflava</i>	NA	Joan Knapp, Neisseria Reference Lab

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^a NA, not applicable; ND, not determined.

TABLE 2. Plasmids used

Plasmid	Description	Vector	Reference
pSF4000	<i>hlyCABD</i> Cmp ^r	pACYC	35
pWAM300	<i>hlyC</i> Amp ^r	pUC19	17
pUNCH909	<i>hlyABD</i> Cmp ^r	pACYC	This work
pUNCH910	<i>gpxA</i> Amp ^r , 1.5-kb insert	pBS	This work (Fig. 3)
pUNCH911	<i>gpxA</i> Amp ^r , 1.0-kb insert	pBS	This work (Fig. 1)

(Table 1) were grown on GC base medium (Difco Laboratories, Detroit, Mich.) containing Kellogg's supplements I and II (24) in an atmosphere of 5% CO₂. *E. coli* DH5α-MCR strains were grown in Luria broth or Luria broth containing 100 µg of ampicillin per ml or 17 µg of chloramphenicol per ml or both as needed. Blood agar base medium for the hemolysin assay contains 40 g of BBL blood agar base (Becton Dickinson), 2 g of glucose, 5 g of yeast extract (Difco), 1 mM CaCl₂, and 50 ml of defibrinated sheep blood per liter (17).

Molecular cloning and characterization of *gpxA*. The GPX gene was isolated by a strategy actually designed to select for the *N. meningitidis* *hlyC* homolog, as follows. Plasmid pSF4000 contains the entire *E. coli* hemolysin A operon which confers a hemolytic phenotype to nonhemolytic strains of *E. coli* (35). This operon contains the *hlyCABD* genes, which are the acti-

vation, structural, and transporter genes of hemolysin A (22, 35). Plasmid pSF4000 was digested with *Bam*HI to release a fragment containing the *hlyC* coding region. The remaining fragment was religated to give plasmid pUNCH909 (Table 2). This plasmid contains *hlyABD* but does not confer a hemolytic phenotype unless *hlyC* is provided on a compatible plasmid. Electroporation-competent DH5α-MCR cells containing pUNCH909 were transformed with the *N. meningitidis* genomic plasmid library. Transformants were plated onto blood agar containing ampicillin and chloramphenicol, to select for both plasmids. The blood agar plates were incubated at 37°C for 15 h and then at 25°C for 4 days. Several colonies exhibited weak zones of hemolysis after 4 days and were selected for further study. Plasmids from these weakly hemolytic transformants were isolated. Restriction analysis of these plasmids indicated that two clones containing 1.5-kb inserts were identical, and they were named pUNCH910 (see Fig. 4). The hemolytic activity was localized to a smaller subclone containing a 1-kb fragment (pUNCH911; Fig. 1). This plasmid was subcloned, sequenced in its entirety, and found to contain a GPXH.

The sequence of the isolated DNA fragment revealed an open reading frame whose deduced amino acid sequence is homologous to that of GPXs (Fig. 1 and 2). The putative Shine-Dalgarno sequence is underlined, and the start codon is

EagI		
CGGCCGCGACAAATAAGTTTTCGGGTATGAAATGAGCAACTGCACCCGTTTTCGGGTGCACTTTTATTCGCGGATTGGGACGGGAATGGAAAAGGATGT	-93	
		SD
GGATTTTATTTTAAATCGGCTATACAATTGCTGCTTTCATGCGCTCTGAAGCAGCTTTCAGATGGCATCGGTTTACATCAGATAAGGATAAAA	-1	
		←→
ATG GGT ATT TAC GAT TTT CAA ATG AAA GAT GCG GAA GGC AAT GCG GTT GAT TTG TCA GGC	60	
M G I Y D F Q M K D A E G N A V D L S G	20	
TAT CGC GGC AAG GTT CTG CTG ATT GTC AAC ACG GCA ACG CGT TGC GGT CTG ACC CCG CAA TAC GAG GCT TTG CAG	135	
Y R G K V L L I V N T A T R C G L T P Q Y E A L Q	45	
AAG CTG TAT GCA CAA TAT ACC GCA GAA GGC TTG GAG ATT TTG GAT TTT CCG TGC AAC CAG TTC CGC GAA CAG GCT	210	
K L Y A Q Y T A E G L E I L D F P C N Q F R E Q A	70	
CCC GAA AGC AGC GGA GAA ATT GCC CAA GTG TGT ATG ATG AAG TTT GGC ACG AAG TTT AAG ATT TTC GAC AAA ATC	285	
P E S S G E I A Q V C M M K F G T K F K I F D K I	95	
GAA GTC AAT GGA GCA AAT ACC GCG CCC TTG TAT GCC TAC CTG AAA TOC GTC AAA CCG CAA GAT AAA GGC AAT CAT	360	
E V N G A N T A P L Y A Y L K S V K P Q D K G N H	120	
CTG TTT AAA GAT TTC GTG TTG AAG CTC GCA GCT TTG GGA GAG AAG CGC GAC GAA GGC GAC ATC AAA TGG AAC TTT	435	
L F K D F V L K L A A L G E K R D E G D I K W N F	145	
ACC AAA TTC CTC GTC AAC CGC GAC GGC GAA GTG GTC GAG CGT TTT GCG OCT AGT GTT ACG CCC GAA GAA ATC GAA	510	
T K F L V N R D G E V V E R F A P S V T P E E I E	170	
GCC GAT ATT CGG GCA TTG CTG TAA TTGTCCGTTTTCGGGGTTTATTCGGAATATCGGCAACACCGCTTTCGGGGAAGATGGGCAATCCGGC	602	
A D I R A L L Stop	177	
		BssHII
GGTGGGAGCAGGGTATCCAAAGTCAGGGCGGACGCATCGAGTGTGGGGTAGCCTTCAAACGGGACCGAATAACCGCTGTTTCGGTTCATACGGATTCCGGG	702	
CGTGCGCGCCAGAGGGAAGTGATTTTGTTCGGCAATGTGCCGAAGGGAAGCCCGTCAGCACGGGGATTTTCGCCGTGCGGAAACATGGTTGGCAAC	802	
GGCGGAAATATCATAAGACGGATC	826	

FIG. 1. Nucleic acid and deduced amino acid sequences of pUNCH911 containing *gpxA* isolated from the *N. meningitidis* FAM20 genomic library. The deduced 177-amino-acid sequence is shown under the nucleic acid sequence. A solid bar underlines the Shine-Dalgarno (SD) sequence, and the arrows indicate the *Neisseria* uptake consensus sequence (14, 19). Two restriction sites are shown for orientation.

FIG. 2. The predicted *N. meningitidis* GPXH sequence is compared with those of three other GPX proteins over their entire length. Residues which are identical in all four sequences are boxed. Residues important in other species for active-site structure and function are marked with asterisks. #, seleno-cysteines; Nmen, *N. meningitidis*; Nicotin, *Nicotiniana tabacum* (10); Sman, *S. mansoni* (29); PHGPX, phospholipid hydroperoxide GPX (6).

indicated. The spacing between the Shine-Dalgarno sequence and the start codon is appropriate, and the -3 base is adenine. The fragment contains a 10-bp *Neisseria* uptake consensus sequence (GCCGTCTGAA) (14, 19), which is indicated by a two-headed arrow in Fig. 1.

The GPX open reading frame was scanned for codon usage and was found to be consistent with typical neisserial genes (data not shown) (36). The open reading frame predicts a 177-amino-acid protein, which is compared along its entire length with three other GPXs in Fig. 2. Brigelius-Flohe et al. found that a 49-amino-acid region corresponding to exon 3 in porcine phospholipid hydroperoxide GPX and *Schistosoma mansoni* GPX is highly conserved throughout the GPX family (6). The *N. meningitidis* homolog is 49 to 57% identical at the amino acid level to the exon 3 region compared with the seven other GPX family members shown in Fig. 3. This exon 3 region corresponds to amino acids 22 through 69 of the predicted *N. meningitidis* sequence. The active-site residues Cys-35, Trp-143, and Gln-69, which are conserved in all of the other members and which are important for active-site structure, are conserved in the meningococcal homolog as well (15).

Plasmid library construction. A plasmid library was constructed from *N. meningitidis* genomic DNA. Routine DNA manipulations were done according to published procedures (4). Chromosomal DNA was partially digested with *Sau3AI*. Fragments of 1.5 to 3.5 kb in size were cut from an agarose gel,

purified, and ligated to *Bam*HI-digested pBluescript KS (Stratagene) which had been previously treated with shrimp alkaline phosphatase (United States Biochemicals). Phosphatase reactions were performed according to the manufacturer's instructions. DH5 α -MCR electroporation-competent cells containing pUNCH909 (Table 2) were prepared according to published procedures (12). A 40- μ l aliquot of cells and 2- μ l ligation reaction mixtures were mixed and placed into 0.2-mm electroporation cuvettes and electroporated on a Gene-Pulsar at 2.5 kV, 25 μ F, and 200 Ω .

DNA sequence and Southern analyses. For Southern analysis, genomic DNA was digested to completion with *Clai*I (Boehringer Mannheim and New England Biolabs), separated on a 0.8% agarose gel, transferred to a nylon membrane, and fixed by UV cross-linking. All membranes were hybridized with digoxigenin-labeled probes labeled according to the manufacturer's instructions (Genius; Boehringer Mannheim). Membranes were prehybridized at 42°C for 2 h in a mixture of 5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, 0.2% sodium dodecyl sulfate, 5% Genius blocking reagent, and 0.1% *N*-lauroylsarcosine. Membranes were hybridized overnight at 42°C in the same mixture with the addition of the probe. All membranes were washed according to the manufacturer's instructions and visualized with Lumiphos 530 (Boehringer Mannheim).

Double-stranded, polyethylene glycol-purified restriction

Nmen-GPXH	RGKVLIVNTATRCGLTPQ.YEALQKLYAQYTAEGLEILDFPCNQFREQ	100%
Ecoli-BtuE	A-N-----V-SK-----.-Q-ENIQKAWVDR-FMV-G-----L--	55%
Nemat-GPXH	-N-----V--Y-AY-M-.RDFNPILSNSNGT-N--G-----YL-	49%
Porcine-PHGPX	--Y-CIVT-V-SQ--K-EVN-TQ-VD-H-R-AEC--R--A-----GR-	49%
Nicotin-GPXH	K----I---V-SQ---NSN-TD-TEI-KK-KDQ-----A-----GG-	57%
Rat-GPX	-----E-V-SL---T-TRD-TEMND-QKRLGPR--VV-G-----GH-	49%
Sman-GPX	--H-C---V-CK--A-DKN-RQ--EMHTRLVPR--VV-G-----GG-	49%
HumPlas-PGPX	A--YV-E--V-SY---G-.IE-NA-QEELAPF--V--G-----GK-	51%

FIG. 3. The predicted *N. meningitidis* GPXH sequence is compared with those of seven other GPX proteins over the region corresponding to exon 3 of porcine phospholipid hydroperoxide GPX and *S. mansoni* GPX. Dashes indicate residues identical to the *N. meningitidis* predicted protein sequence; dots indicate gaps introduced to maximize alignment. The percentage of identical residues with the *N. meningitidis* sequence is indicated at the right. Abbreviations: Nmen, *N. meningitidis*; Ecoli, *E. coli* (18); Nemat, nematode (9); Nicotin, *N. tabacum* (10); Sman, *S. mansoni* (29); HumPlas, human plasma (31); GPXH, cysteine-containing GPX homolog; PHGPX, phospholipid hydroperoxide GPX (6); PGPX, plasma GPX. Rat data are from reference 20.

FIG. 4. Restriction map of the 1.5-kb fragment (pUNCH910) isolated from *N. meningitidis* FAM20. The thick line corresponds to the *gpxA* coding region, while the arrow indicates the 374-bp *HincII* fragment which was used to probe *ClaI*-digested genomic DNA from six *N. meningitidis* strains, six *N. gonorrhoeae* strains, and six nonpathogenic neisserial strains on the Southern blot below. Sizes of the bands are indicated on the left. Meningococcal strain lanes: 1, FAM20; 2, JB501; 3, JB504; 4, JB505; 5, JB512; 6, Z2087. Gonococcal strain lanes: 7, FA19; 8, F62; 9, FA1090; 10, F1135; 11, MS11; 12, UU1008. Nonpathogenic commensal strain lanes: 13, *M. catarrhalis*; 14, *N. flavescens*; 15, *N. mucosa*; 16, *N. lactamica*; 17, *N. sicca*; 18, *N. subflava*.

fragment subclones were used for DNA sequencing. DNA was sequenced either at the University of North Carolina at Chapel Hill automated DNA sequencing facility on a model 373A DNA sequencer (Applied Biosystems) with a *Taq* Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems) or with [³⁵S]dATP and a Sequenase (version 2.0) kit (United States Biochemicals). Sequence assembly and editing were performed with the MacVector and AssemblyLIGN programs (IBI). Database screening was done with the basic local alignment search tool (BLAST) program (2).

Molecular epidemiology of *gpxA*. A survey of 23 neisserial strains was performed to assess any possible correlation between the presence of *gpxA* and pathogenicity or disease. This limited epidemiologic analysis included 6 nonpathogenic strains, 10 gonococcal strains, and 7 meningococcal strains. A 374-bp *HincII* DNA fragment completely within the *gpxA* coding region hybridized to the meningococcal strains (FAM20, JB501, JB504, JB505, JB512, and Z2087 [Fig. 4] and 8013N [data not shown]) but did not hybridize either to the gonococcal strains (FA1090, FA19, UU1008, MS11, F62, and FA1135 [Fig. 4] and L2370, L4401, L4933, and L8800 [data not shown]) or to the nonpathogenic, commensal strains (*Neisseria subflava*, *Neisseria mucosa*, *Neisseria catarrhalis*, *Neisseria lactamica*, *Neisseria flavescens*, and *Moraxella catarrhalis* [Fig. 4]). In summary, all seven of the *N. meningitidis* strains but none of the *N. gonorrhoeae* strains or commensal species showed any evidence of possessing a GPXH.

GPX enzymes catalyze the reduction of hydroperoxides, with reduced glutathione acting as the hydrogen donor. Two distinct types of GPXs have been well characterized. The first is GPX, which exists as a tetramer and reduces both H₂O₂ and low-molecular-weight water-soluble hydroperoxides (6). In contrast, phospholipid glutathione GPX is a monomeric enzyme which has a similar kinetic mechanism but uses hydroperoxidized lipids within membranes as substrates (30). Members of the GPX family are thought to protect cells or cell membranes from toxic oxygen species (16). The GPXH found in the nematode *Brugia malayi* is a major surface glycoprotein which

is expressed only after introduction into the mammalian host, suggesting a role in pathogenicity (7, 9, 29).

It is unclear at this point exactly why *gpxA* was discovered in a complementation screen for homologs of the *E. coli* gene *hlyC*. The HlyC protein acetylates the hemolysin A protein, resulting in activation of the hemolysin (22). It is possible that the faint zone of hemolysis shown by the *E. coli* *gpxA* transformants is caused by an interaction between the enzyme and the heme released from erythrocytes.

We have isolated and identified a GPX-related gene from *N. meningitidis*. This gene is found in all seven of the *N. meningitidis* strains tested. However, neither the 10 *N. gonorrhoeae* strains nor the six commensal species tested showed any evidence of possessing a GPXH. While we have yet to discover an activity for *gpxA*, the striking conservation of the deduced protein sequence and the specificity of the gene for *N. meningitidis* encourage our pursuit of the function of *gpxA* in the biology of this human pathogen.

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