

Resistance of *Neisseria meningitidis* to the Toxic Effects of Heme Iron and Other Hydrophobic Agents Requires Expression of *ght*

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Several genetic systems that allow the use of iron-protoporphyrin IX (heme) have been described for the pathogenic bacterium *Neisseria meningitidis*. However, many questions about the process of heme acquisition and utilization remain to be answered. To isolate and analyze unidentified genes that play a role in heme iron uptake and utilization, a *Himar1* transposon mutant library was screened in *N. meningitidis* serogroup A strain IR4162. One locus identified by transposon mutagenesis conferred protection against heme toxicity. A mutant with a deletion in a gene termed *ght* (gene of hydrophobic agent tolerance) within this locus was susceptible to heme and other hydrophobic agents compared to the parental strain. Transcriptional analysis indicated that *ght* is cotranscribed with an upstream open reading frame NMA2149. Uncharacterized orthologues of *ght* were identified in many other gram-negative bacteria. We present genetic evidence for the importance of *ght* in resistance to hydrophobic agents and its potential role in interaction with other hydrophobic agent resistance mechanisms within *N. meningitidis*.

The success of a microorganism within its host often depends on that microbe's ability to scavenge iron. Host proteins, such as hemoglobin and myoglobin, contain iron in the form of iron-protoporphyrin IX (e.g., heme). It has been established that iron contained in heme can serve as the source of iron in a wide range of bacteria. Heme also performs a crucial role in many metabolic processes within the cell (20, 29, 33, 35). However, the internal presence of heme, or excess iron resulting from its degradation, can have detrimental effects because heme can also facilitate oxidative damage within the bacteria. The bacterial response to the detrimental effects of heme can be readily observed in stringent iron regulation of the expression of genes involved in heme and/or iron uptake (7, 9, 28). Heme has been shown to be toxic at low concentrations for *Staphylococcus aureus*, *Streptococcus faecalis*, and *Bacillus cereus* (15). Thus, bacteria must take up and utilize heme by mechanisms that also allow for protection against the toxic side effects caused by heme.

Heme uptake and utilization has been studied extensively in the meningococci. Two phase-variable TonB-dependent hemoglobin uptake systems have been described previously, the bipartite receptor, HpuAB, and the one-component receptor, HmbR (18, 30). In addition, a cytoplasmic heme oxygenase, HemO, has also been described, which allows for the extraction of iron from heme and subsequently provides a measure of protection against the toxicity of heme itself (37). However, no inner membrane transport system similar to those of other heme iron-utilizing bacteria has been thus far identified in meningococci. Moreover, the utilization of heme without a protein ligand and in a TonB-independent manner is known to

be possible, but the mechanism for this process has yet to be elucidated (32). A recent study on the growth of *Neisseria gonorrhoeae* under iron restriction has shown that spontaneous genetic changes can result in bacterial growth by the use of heme, dissociated from hemoglobin, as the sole iron source. This process occurs in an HpuAB-independent manner (3). Two classes of mutants were identified that could use heme through this process. One class was identified as a specific point mutation in the *pilQ* locus, the gene coding for the type IV pilus secretin (8), while the other class remains unidentified. The point mutation in *pilQ* is predicted to allow for increased entrance of heme, as well other hydrophobic agents (HAs), including antibiotics, through the mutated secretin. This study indicated that the passage of heme, itself a hydrophobic compound, and other HAs across the outer membrane may be possible through channel-like proteins similar to PilQ.

Toxicity of HAs and mechanisms of HA resistance (HA^r) have been extensively studied in the gonococci (2, 10, 11, 26, 36). The gonococcal Mtr efflux system is a member of the resistance-nodulation-division family of energy-dependent efflux pumps. It has been shown to be involved in the efflux of hydrophobic drugs, dyes, detergents, antibacterial peptides, and protoporphyrin IX/metalloporphyrins. The expression of the Mtr system has been found to be positively regulated by the AraC-like activator MtrA and negatively controlled by the repressor MtrR; mutations in *mtrR* result in high levels of HA^r. HA toxicity in the meningococci has been only recently studied. Two recent genome sequencing projects have identified the Mtr counterparts in *Neisseria meningitidis*, and a recent study of *mtr* regulation suggests that they are not under the control of MtrA and MtrR but are regulated by integration host factor in a transcriptional and posttranscriptional manner involving a 155- to 159-bp Correia element that is inserted upstream of the *mtrCDE* operon (21, 24, 34).

In this study, a transposon library was screened to identify

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additional genes involved in uptake and utilization of heme iron in *N. meningitidis*. We identified a gene (*ght*) that confers protection to meningococci against heme toxicity. We report that *ght* is important in the protection of meningococci against the toxicity of not only heme but other HAs as well through a process independent of PilQ or the MtrC-MtrD-MtrE efflux system. We also show that homologues of *ght* can be identified throughout the genomes of many gram-negative organisms and that these homologues retain discrete conserved regions of unknown functional importance. (A preliminary account of these results was presented at the 14th International Pathogenic *Neisseria* Conference held in Milwaukee, Wis., 5 to 10 September, 2004.)

MATERIALS AND METHODS

Bacterial strains and media. The *N. meningitidis*, *N. gonorrhoeae*, and *Escherichia coli* strains and plasmids used in these experiments are listed in Table 1. *Neisseria* strains were routinely cultured at 37°C under 5% CO₂ (vol/vol) atmosphere on GC medium base agar (Difco Laboratories, Detroit, MI) or in GC broth (1.5% Proteose peptone no. 3 [Difco], 0.4% K₂HPO₄ [Sigma-Aldrich, St. Louis, MO], 0.1% KH₂PO₄ [Sigma-Aldrich], 0.5% NaCl [Sigma-Aldrich]) with Kellogg's supplements I and II. In some cases, meningococci were grown in brain heart infusion broth (Difco) with 0.043% NaHCO₃ (wt/vol). *N. meningitidis* transconjugants were selected on modified Thayer-Martin II media (BBL), in which 100 µl of a 1.25-mg/ml chloramphenicol solution was spread on the plate and allowed to absorb. *E. coli* was grown at 37°C in LB (Luria-Bertani broth). Where indicated, the following antibiotics were used with meningococci: kanamycin at 50 µg/ml, erythromycin at 3 µg/ml, streptomycin at 750 µg/ml, chloramphenicol at 5 µg/ml, rifampin at 3 µg/ml, and tetracycline at 2 µg/ml. Work with *E. coli* used kanamycin at 50 µg/ml, erythromycin at 300 µg/ml, chloramphenicol at 30 µg/ml, and tetracycline at 10 µg/ml.

Transposon mutant library screening. A *Himar1* transposon mutant library created previously in *N. meningitidis* strain IR4162 (*hpuB::ermC* derivative of clinical strain IR4048) was screened for heme and hemoglobin utilization mutants (1). Ten thousand kanamycin-resistant mutants were screened individually for their heme iron utilization profile. Screening by a GCB-Desferal plate assay involved plating a suspension of bacteria on GC agar plates containing only supplement I (glucose supplement) of the Kellogg's supplements and 50 µM deferoxamine mesylate (Sigma-Aldrich) and placing filter disks (6-mm Whatman 3MM paper) impregnated with test compounds (10 µl of 5-mg/ml stock solution of hemin chloride [Porphyrin Products Inc., Logan, Utah] in 100 mM NaOH and a 10-mg/ml stock solution of human hemoglobin [Sigma-Aldrich] in sterile, deionized, distilled H₂O). Confluent zones of growth were measured around the hemin chloride disks, and the number of colonies around the hemoglobin disk was assessed. A mutant (276D) hypersusceptible to heme was identified by this process. DNA from mutant 276D was then used to transform parental strain IR4162 to establish linkage between the *Himar1*-associated kanamycin resistance and heme toxicity phenotypes. Genetic transformation of *N. meningitidis* strains and chromosomal DNA isolation were carried out as described by Richardson and Stojiljkovic (23).

Himar1 transposon mapping was carried out by ligation-mediated PCR as previously described (22). Ligation-mediated PCR products were cloned into pCR2.1-TOPO and sequenced. For Southern blot hybridization analysis of the *Himar1* insertions, chromosomal DNA from transposon mutant 276D and transformant strain IR5627 was digested with ClaI and probed with the 1.3-kb PstI fragment of pUC4-KSAC containing the *aphA-3* gene. The probe, prepared by restriction digest and purified by agarose gel separation, was labeled using a digoxigenin DNA labeling and detection kit according to the manufacturer's specifications (Roche, Basel, Switzerland).

Construction of insertional mutations in *N. meningitidis*. Plasmid DNA preparation, restriction endonuclease analysis, and ligations were carried out by standard methods (25). Inactivation of *ght* was accomplished by insertion of the nonpolar *aphA-3* kanamycin resistance cassette (19) into the gene. This was carried out by PCR amplifying the region of the genome containing *ght* from IR4162 genomic DNA using primers 5'NMA2148 (5'-CATGAAACTTATCTA TACCGTCATC-3') and 3'NMA2148 (5'-CCTTTTCGAGATCAAGGATGC-3'), which included the neisserial uptake sequence required for efficient transformation. The resulting 2.1-kb PCR product was cloned into pCR2.1-TOPO, creating pAWR2090. Plasmid pAWR2090 was then linearized using NruI. An

850-bp SmaI fragment carrying the nonpolar *aphA-3* cassette from pUC18K was then ligated to the linearized pAWR2090. The resulting plasmid pAWR3038 was then linearized with NotI and transformed into strain IR4048, and kanamycin-resistant colonies were selected. Insertion was verified in the resulting transformant (strain IR5571) by PCR. The heme hypersensitivity phenotype was verified by the disk assay on GC-deferrioxamine mesylate plates as stated above.

The gene directly downstream of *ght*, *gloA*, was inactivated by insertion of the *aphA-3* cassette. This was carried out by PCR amplifying the region of the genome containing *gloA* from IR4048 genomic DNA using two sets of primers: set 1, 5'XhoI-*gloA* (5'-CCCTCGAGCGGAAGGCGACGTAC-3') and 3'*gloA*-NsiI (5'-CATGCATAGTATGGAGTAAGCGCATT-3'); set 2, 5'*gloA*-NsiI (5'-CATGCATGCTCCGCGTGGGCAATCTC-3') and 3'XbaI-*GloA* (5'-CGAGATCTATGCCGCGCAGGAAAA-3'). PCR assays with both set 1 and set 2 primers produced fragments of ~0.6 and ~0.4 kb, respectively, which were cloned into pCR2.1-TOPO, creating pAWR3019 and pAWR3020. Both plasmids were linearized by NsiI, ligated together, and used as the template for PCR amplification to obtain a 1-kb fragment, which was then cloned into pCR2.1-TOPO to create plasmid pAWR3033. The 1-kb fragment from pAWR3033 was then excised with EcoRI and cloned into the EcoRI site in pBluescript II SK(-), forming plasmid pAWR3034. That plasmid was linearized by cutting at the engineered NsiI site and cloning in a 1.3-kb *aphA-3* PstI fragment from pUC4-KSAC, creating plasmid pAWR3035. pAWR3035 was then linearized with NotI and transformed into strain IR4048, and a Kan^r colony was selected, creating strain IR5579. Insertion was verified by PCR.

The gene directly upstream of *ght*, NMA2149, was inactivated by insertion of a nonpolar *aphA-3* cassette. This was carried out by PCR amplifying the region of the genome containing NMA2149 from IR4048 genomic DNA using two sets of primers: set 1, 5'NMA2149-NUS (5'-GCCGCTGAAGGTCTGCTCATGATGTAAAGC-3') and NMA2149-SmaI (5'-CCCCGGCAAACATCCGAACA CGATGCCG-3'); set 2, 5'NMA2149-SmaI (5'-CCCCGGCAGCGTTGTGTGCG TTACGTGGCG-3') and 3'NMA2149-NUS (5'-GCCGCTGAAGGTGTGAGG TTCAAATCATACG-3'). PCR assays from both set 1 and set 2 primers produced fragments of ~0.5 and ~0.4 kb, respectively, which were cloned into pCR2.1-TOPO, creating pAWR3090 and pAWR3091. Both plasmids were linearized by SmaI, ligated together, and used as the template for PCR amplification to obtain a 0.9-kb fragment, which was then cloned into pCR2.1-TOPO to create plasmid pAWR3092. That plasmid was linearized by cutting at the engineered SmaI site and cloning in a 0.85-kb nonpolar *aphA-3* SmaI fragment from pUC18K, creating plasmid pAWR3093. pAWR3093 was then linearized with NotI and transformed into strain IR5694, and a Kan^r Str^r colony was selected, creating strain IR5759. Insertion was verified by PCR.

A deletion of *ght* was constructed by PCR amplification of the upstream and downstream regions of *ght* from IR4048 genomic DNA using two sets of primers: set 1, 5'NMA2148 (5'-CATGAAACTTATCTATACCGTCATC-3') and 3'*gohT*-del-KpnI (5'-GGTACCATATCGGCTTTCTCAAGG-3'); set 2, 3'NMA2148 (5'-CCTTTTCGAGATCAAGGATGC-3') and 5'*gohT*del-KpnI (5'-GGTACCAACA ACAAATGGCAGACGTTTA-3'). Both PCR products (~300 bp and ~700 bp) were cloned into pCR2.1-TOPO, creating pAWR303055 and pAWR3057. Plasmid DNA from both plasmids were digested with KpnI and treated with phenol-chloroform, and the DNA was ethanol precipitated. The plasmid DNA was then mixed and ligated together prior to PCR amplification with primers 5'*gohST* PE and 5'XhoI-*gloA* to generate a ~1.4-kb PCR product containing the deletion of *ght*. The fragment containing the deletion of *ght* was then cloned into pCR2.1-TOPO, creating plasmid pAWR3063. Next, pAWR3063 was linearized with NotI and used to transform strain IR5694 for resistance to streptomycin. Strain IR5575, used as the host strain for deletion of *ght*, was created by first isolating a spontaneous streptomycin-resistant (Str^r) mutant of parent strain IR4048 by plating 1 × 10¹⁰ cells on GC media supplemented with streptomycin (750 µg/µl). Next, the *rpsL-ermC* construct from pFLOB4300 (13) was PCR amplified using primers M13(-21) Forward-SmaI (5'-TCCCCGGGTGTAAAACGACGGCCAGT-3') and M13 Reverse-SmaI (5'-TC CCCCAGGACGAAACAGCTATGACC-3') and cloned into pCR2.1-TOPO, creating pAWR3068. The *rpsL-ermC* construct was then excised with SmaI from pAWR3068 and cloned into the NruI site in pAWR2092, creating pAWR3070. This plasmid was linearized with NotI and transformed into IR3070, and erythromycin-resistant colonies were selected. Isolates were then patched onto GC agar with streptomycin to confirm the presence of the dominant streptomycin-sensitive cassette. The resulting Erm^r Str^r strain, IR5694, was used for the creation of *ght* deletion knock-in strain IR5700. Deletions were verified by DNA sequencing and PCR.

An insertional mutation in the *mtrD* gene of *N. meningitidis* was accomplished by transforming strains IR5575 (spontaneous Str^r parent strain) and IR5700 (Δ *ght*) with chromosomal DNA from *N. gonorrhoeae* strain KH14 (*mtrD::Kan^r*), creating strains IR5721 and IR5725, respectively, with selection for Kan^r transformants.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
<i>Neisseria</i> spp.		
KH14	<i>mtrD::aphA-3</i> derivative of FA19 (gonococcus)	10
276D	<i>ght::Himar 1</i> derivative of IR4162	This study
IR4048	<i>N. meningitidis</i> serogroup A clinical isolate	Laboratory collection
IR4162	<i>hpuB::ermC</i> derivative of IR4048	1
IR5571	<i>ght::aphA-3 gloA⁺</i> derivative of IR4048	This study
IR5627	<i>ght::Himar1</i> retransformation derivative of IR4162	This study
IR5575	spontaneous Str ^r derivative of IR4048	This study
IR5579	<i>gloA::aphA-3</i> derivative of IR4048	This study
IR5648	<i>PilQ::cm N. meningitidis</i> serogroup B	34a
IR5694	<i>ght::ermC-rpsL</i> derivative of IR5575	This study
IR5700	Δ <i>ght</i> derivative of IR5694	This study
IR5721	<i>mtrD::aphA-3</i> derivative of IR5575	This study
IR5725	<i>mtrD::aphA-3</i> derivative of IR5700	This study
IR5737	IR4048 containing unaltered pHT128	This study
IR5738	IR4048 containing pAWR3088	This study
IR5744	<i>ght::aphA-3 gloA⁺</i> derivative of IR5738	This study
IR5746	<i>ght::aphA-3 gloA⁺</i> derivative of IR5737	This study
IR5749	<i>pilQ::Cm^r</i> derivative of IR4048	This study
IR5751	<i>pilQ::Cm^r</i> derivative of IR5700	This study
IR5757	IR5694 containing pAWR3088	This study
IR5759	<i>NMA2149::aphA-3</i> derivative of IR5757	This study
IR5762	IR5757 harboring pAWR3088	This study
<i>E. coli</i>		
DH5 α	Cloning strain	Stratagene
TOP 10	Cloning strain	Invitrogen
Plasmids		
pCR 2.1-TOPO	Cloning vector, Amp ^r , Kan ^r	Invitrogen
pBS II SK-	Cloning vector, Amp ^r	Fermentas
pUC4-KSAC	Cloning vector, Kan ^r	Pharmacia
pUC18K	Cloning vector, nonpolar Kan ^r	19
pFLOB4300	pUC18 but <i>ermC rpsL</i> cassette	13
pHT128	Cm ^r , Tet ^r <i>N. meningitidis</i> cloning vector	33a
pRK2013	Mobilization helper plasmid	17
pWMZ1591a	3-kb <i>ermC-lacIOP</i> cassette cloned upstream of <i>hemO</i>	37
pAWR2090	2.1-kb fragment with <i>ght</i> cloned into pCR 2.1-TOPO	This study
pAWR2092	2.1-kb fragment with <i>ght</i> cloned into pBluescriptII-SK-	This study
pAWR3019	0.6-kb 5' <i>gloA</i> gene fragment cloned into pCR 2.1-TOPO	This study
pAWR3020	0.4-kb 3' <i>gloA</i> gene fragment cloned into pCR 2.1-TOPO	This study
pAWR3033	1.0-kb <i>gloA</i> fragment with internal NsiI site cloned into pCR 2.1-TOPO	This study
pAWR3034	1.0-kb <i>gloA</i> fragment with internal NsiI site cloned into pBS II SK-	This study
pAWR3035	Like pAWR3034, but <i>aphA-3</i> cassette cloned into NsiI site of <i>gloA</i>	This study
pAWR3038	Like pAWR2090, but nonpolar <i>aphA-3</i> cloned into <i>ght</i> NruI site	This study
pAWR3055	0.3-kb 5' <i>ght</i> deletion fragment in pCR 2.1-TOPO	This study
pAWR3057	0.7-kb 3' <i>ght</i> deletion fragment in pCR 2.1-TOPO	This study
pAWR3063	1-kb <i>ght</i> deletion fragment in pCR 2.1-TOPO	This study
pAWR3068	PCR of <i>ermC rpsL</i> from pFLOB4300 with SmaI ends in pCR 2.1-TOPO	This study
pAWR3070	Like pAWR2090, but with <i>ermC-rpsL</i> in <i>ght</i> NruI site	This study
pAWR3075	<i>ght</i> with ribosome binding site in pCR 2.1-TOPO	This study
pAWR3081	3-kb <i>ermC-lacIOP</i> NotI fragment from pWMZ1581a in pAWR3075	This study
pAWR3087	3-kb <i>ght-lacIOP</i> PCR fragment with ClaI ends in pCR 2.1-TOPO	This study
pAWR3088	3-kb <i>ght-lacIOP</i> cloned into the ClaI site of pHT128	This study
pAWR3090	0.5-kb 5' NMA2149 fragment in pCR 2.1-TOPO	This study
pAWR3091	0.4-kb 3' NMA2149 fragment in pCR 2.1-TOPO	This study
pAWR3092	0.9-kb NMA2149 fragment in pCR 2.1-TOPO	This study
pAWR3093	Like pAWR3092, but with nonpolar <i>aphA-3</i> clones in SmaI site	This study

An insertional mutant in the *pilQ* locus was constructed by transforming strain IR4048 and IR5700 with genomic DNA prepared from strain IR5648 (*pilQ::Cm^r* [34a]), creating strains IR5749 and IR5751, with selection for Cm^r transformants.

Complementation analysis. Complementation of *ght* was accomplished by the construction of an inducible *ght* on a plasmid utilizing an *ermC lacI^q lacOP lacOP* construct previously described (37). *ght* was PCR amplified from meningococcal

strain IR4048 chromosomal DNA using primers 5'NMA2148com (5'-CGTCG ACAGCCTTGAGAAAGCCGA-3') and 3' NcoINMA2148ex (5'-GCCCATGG AGTAAGCGCATTTTTG-3') and cloned into pCR2.1-TOPO, creating pAWR3075. The *ermC lacI^q lacOP lacOP* construct was liberated from pWMZ1591a by digestion with NotI and cloned into the NotI site in pAWR3075 upstream of *ght* and its native ribosome binding site, creating pAWR3078. The

ght-lacI^a lacOP lacOP construct was PCR amplified from plasmid pAWR3078 with primers 3'NMA2148ex-ClaI (5'-ATCGATGGAGTAAGCGCATTTTTG-3') and 3'lacI^a-ClaI (5'-ATCGATTCAGTCCCGCTTTCCAGTC-3') and cloned into pCR2.1-TOPO, creating pAWR3087. The ~3.0-kb *ght-lacI^a lacOP lacOP* insertion was excised from pAWR3087 with ClaI and ligated into ClaI-digested pHT128 (33a), creating pAWR3088. The complementing plasmid pAWR3088 and unaltered pHT128 were conjugated into *N. meningitidis* strain IR4048 by a triparental mating procedure. Briefly, suspensions were made from the donor and helper strains (with pRK2013) (17) in LB broth and from the recipient strain in GC broth from overnight plate growth. Fifty microliters of the donor suspension, 50 μ l of the helper suspension, and 25 μ l of the recipient suspension were added to the surface of a GC agar plate, gently mixed, and incubated overnight at 37°C and 5% CO₂ overnight. A suspension of overnight growth was made in GC broth and plated on modified Thayer-Martin II plates supplemented with chloramphenicol to select for meningococcal transconjugants. Transconjugants IR5737 (containing unaltered pHT128) and IR5738 (containing pAWR3088) were transformed with chromosomal DNA from IR5571 to create *ght::aphA-3* strains IR5746 and IR5744, respectively. Insertions were verified by PCR.

For an insertional mutant in NMA2149 which also expresses *ght* extrachromosomally, plasmid pAWR3088 was mated into strain IR5694 in the same manner it was transferred above, creating strain IR5757. That strain was then transformed with linearized pAWR3093, and a Kan^r Str^r colony was selected, creating strain IR5762. Insertions were verified by PCR.

Determination of MBCs and MICs. The minimum bactericidal concentrations (MBCs) of metalloporphyrin compounds (heme, PPIX, IX, Ga-PPIX, and Zn-PPIX) were determined as described previously (12, 31), while the MICs of other antimicrobial compounds were determined as described by Sparling et al. (27).

RT-PCR and PE experiments. RNA preparation for reverse transcriptase PCR (RT-PCR) was carried out by extracting RNA from an overnight culture of strain IR4048 using the Rneasy Midi Kit (QIAGEN, Hilden, Germany) according to the manufacturer's specifications. Contaminating DNA was digested by adding the RNase-free DNase set (QIAGEN). RT-PCR was carried out using the SuperScript one-step RT-PCR with platinum *Taq* kit (Invitrogen) according to the manufacturer's specifications. Primer 3'RTgohT (5'-GGCTCCGGTTGT ATCGGGAGAG-3'), which anneals within *ght*, was used for the reverse transcriptase reaction. Primers 3'RTgohT and 5'NcoI-2148ex (5'-GGCCATGGAC AATGAATTGTGGAT-3') were used for the PCR of only the *ght* portion of the transcript. Primers 3'RTgohT and 5'2149-BgIII-2 (5'-GGAGATCTTTCTCTA CTGCGGGGG-3') were used for the PCR of the region spanning both NMA2149 and *ght*. Primer extension (PE) products were produced using the SuperScript one-step RT-PCR with platinum *Taq* kit, but the PCR amplification step was omitted. Primer 3'2149-BgIII-1 (5'-GGAGATCTAACGGCATCCG TATTA-3'), which anneals within the NMA2149 transcript, was used for the PE reaction on 25 μ g of total RNA. The transcription start point (TSP) was determined by electrophoresis of the PE product on a 6% DNA sequencing gel using 6% Gene-Page Plus 7 M urea denaturing acrylamide blend (Amresco, Inc., Solon, Ohio) according to manufacturer's specifications. DNA sequencing reactions were carried out using SequiTherm EXCELII DNA sequencing kit according to the manufacturer's specifications (Epicenter, Madison, Wisconsin).

Analysis of sequence information. Putative proteins with similarity to *Ght* were identified through the Basic Local Alignment Search Tool program's protein-protein BLAST (BLASTP) searches at the National Center for Biotechnology Information (Bethesda, MD) website (<http://www.ncbi.nlm.nih.gov/BLAST/>) and at The Institute for Genomic Research (Rockville, MD) website (<http://tigrblast.tigr.org/cmr-blast/>). Protein alignments, percent identity, and divergence were calculated using the MegAlign program of the Lasergene software suite of sequence analysis tools, version 5.5 for Macintosh (DNASTAR, Madison, WI).

RESULTS

Identification of *ght* by transposon mutagenesis. An initial screen of 10,000 individual mutants in a *Himar1* transposon library of strain IR4162 (*hpuB::ermC*) was undertaken to identify candidate open reading frames (ORFs) whose gene products participate in heme and hemoglobin uptake and utilization in *N. meningitidis*. Mutants were screened by a GC-deferrioxamine mesylate (a ferric iron chelator) plate assay (37) to assess the ability of each mutant to use heme and hemoglobin as sole sources of iron. As verification of the ability of this

screen to identify genes that participate in heme and hemoglobin uptake, a mutation in the previously characterized heme iron uptake gene, *hmbR*, was identified in our screen. Moreover, we also identified mutants (data not presented) with mutations in *exbB* and *exbD* that are important in TonB-mediated energy delivery required for certain iron uptake and utilization processes (33). One mutant, 276D, showed a heme toxicity phenotype in that it was unable to utilize heme close to the filter disk and only a faint zone of growth a few millimeters away from the disk was evident (Fig. 1A). Hemoglobin utilization was also impaired in strain 276D. Only a few HmbR phase-on colonies of strain 276D were seen compared with the parental strain IR4162 (Fig. 1A).

Southern blot hybridization analysis revealed a single transposon insertion in strain 276D (data not presented). To verify linkage between the heme toxicity phenotype and the transposon insertion (containing the Kan^r marker), the parental strain, IR4162, was transformed with 276D chromosomal DNA. Ten Kan^r colonies were selected and tested for the heme toxicity phenotype. All 10 Kan^r transformants exhibited the heme toxicity phenotype displayed by donor strain 276D (data not presented), indicating linkage between the transposon and the heme toxicity phenotype.

Ligation-mediated PCR was performed to map the insertion of the *Himar1* transposon as described previously (22). Sequencing of PCR products and comparison to the genome sequence of the serogroup A *N. meningitidis* strain Z2491 revealed insertion of the transposon at nucleic acid residue 465 (data not presented) of ORF NMA2148, which was annotated by Parkhill et al. as encoding a putative periplasmic hypothetical protein (21). It was predicted to encode a 389-amino-acid polypeptide with a predicted molecular mass of 44.5 kDa (pI 7.46). Directly upstream of NMA2148, a small ORF of 315 bp, predicted to encode a 104-amino-acid hypothetical protein with a molecular mass of 11.4 kDa (pI of 10.7), was identified. Overlapping the 3' end of NMA2148 is *gloA* (Fig. 2), a glyoxalase I gene described previously (14). One additional transposon mutant (strain 15F) displayed an identical phenotype to 276D, which was subsequently determined to have a transposon insertion in NMA2148.

Analysis of the genome sequences of the serogroup B and C *N. meningitidis* genomes and the *N. gonorrhoeae* FA1090 genome sequence (www.genome.ou.edu) revealed that other meningococci and gonococci contain homologues of NMA2148 with 97.7% identity (Fig. 3). Interestingly, NMA2148 appears to have homologues in a diverse range of gram-negative bacteria; analysis of genetic databases at the National Center for Biotechnology Information and The Institute for Genomic Research identified more than 40 homologues among sequenced genomes; a selected few are listed in Fig. 3. These bacterial species include notable pathogens such as *E. coli* O157:H7, *Vibrio cholerae*, *Bordetella pertussis*, *Pasteurella multocida*, *Haemophilus influenzae*, *Salmonella enterica* serovar Typhimurium, and *Coxiella burnetii*. Several highly conserved domains appear to be present in the NMA2148-encoded ORF and its homologues; however, only two appear to belong to characterized domain families (Fig. 4). The first characterized domain is a C-terminal zinc-finger motif with a CX₂CX₁₀CX₂C amino acid residue arrangement and is completely conserved among all homologues. Additionally, many *ght* homologues contain a tet-

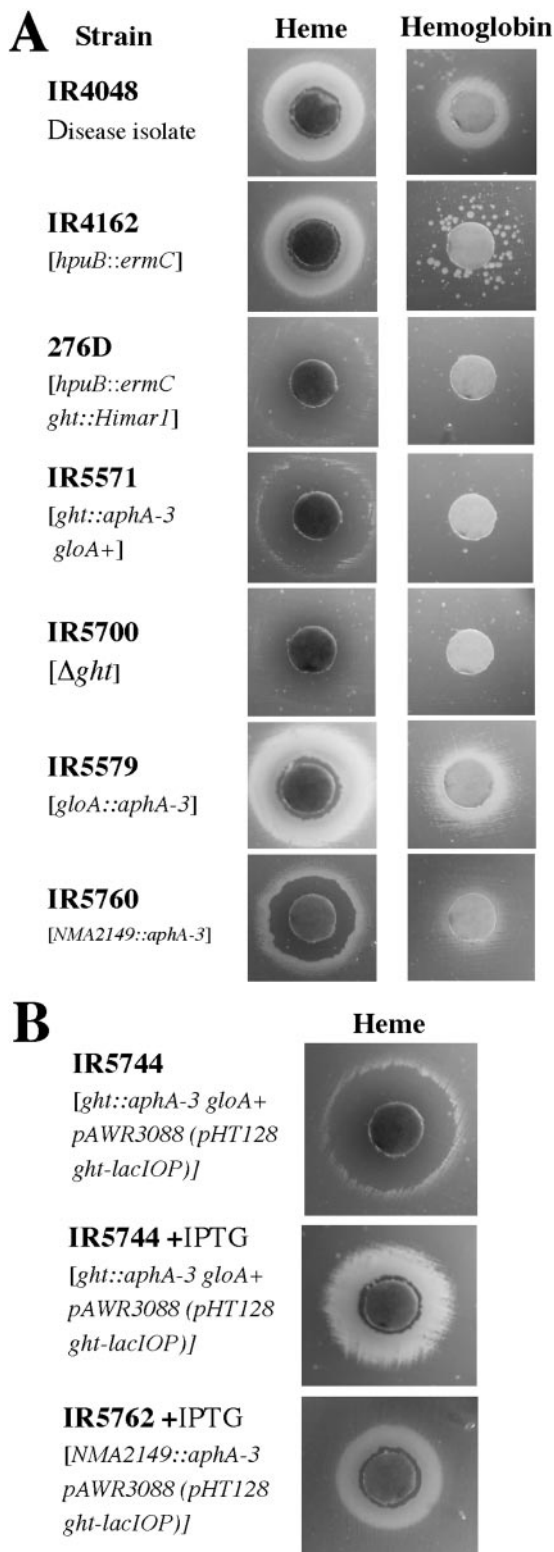


FIG. 1. Growth phenotypes of various mutants of *N. meningitidis* by disk assay on iron restriction plates. (A) Phenotypes of mutants grown in the presence of heme and hemoglobin are shown. (B) Complementation of *ght*. The phenotypes of *ght* strains carrying complementing plasmids with and without IPTG grown in the presence of heme are shown.

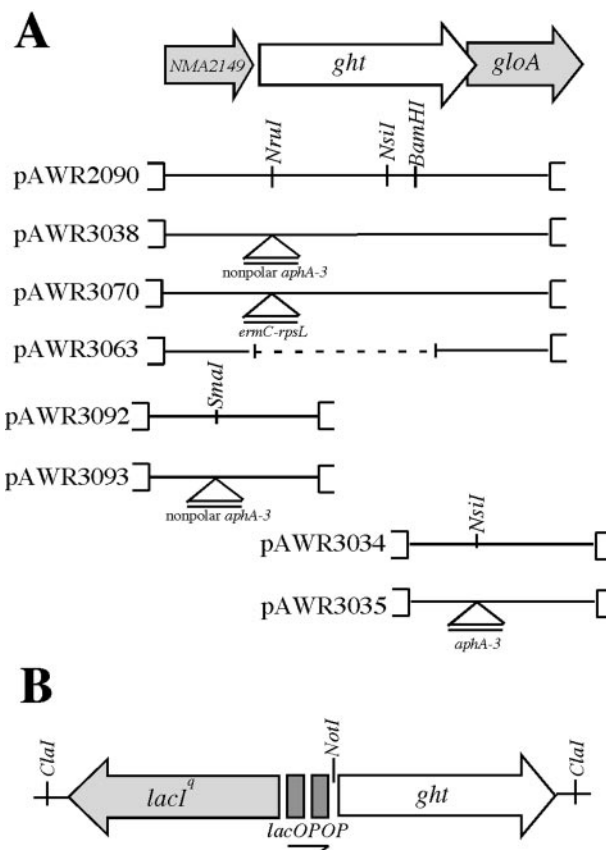


FIG. 2. Schematic representation of some plasmids frequently used in this study. (A) Visual representation of *N. meningitidis* *ght* locus and surrounding open reading frames. Plasmids used in gene replacements are shown below. Labeled triangles indicate the positions of antibiotic resistant cassettes used to generate insertion mutations in individual genes. The coding region of *ght* overlaps *gloA*, a glyoxylase I gene, by 34 bp. The dotted line in pAWR3070 indicates a deleted segment. (B) Schematic representation of the *lacIOPOP-ght* construct in pAWR3088, the inducible *ght*-complementing plasmid.

trapeptide repeat (TPR), suggesting a region involved in protein-protein interaction (6). The predicted NMA2148 product does contain a hydrophobic N-terminal region and periplasmic localization sequence (data not presented) that would indicate a periplasmic localization for the protein. Because of the lack of similarity to any described proteins and the hypersensitivity to heme and other HAs (see below) in the transposon mutant, ORF NMA2148 was named *ght* (gene of hydrophobic agent tolerance); the genomic organization of *ght* and flanking genes is shown in Fig. 2.

Transcriptional characterization of *ght*. The predicted stop codon of the ORF upstream of *ght*, NMA2149, is separated from *ght* by only 10 bases. RT-PCR analysis determined that an mRNA was transcribed from the NMA2149 region that spanned both NMA2149 and *ght*, indicating an operon structure for these determinants (Fig. 5A and B). Primer extension experiments were conducted to map the start site of *ght* transcription. A TSP was detected near the 5' end of NMA2149 (Fig. 5C). It was farther downstream than the TSP predicted by Parkhill et al. (21), lying in the predicted translational coding region of NMA2149. From sequence analysis, the actual trans-

		Ght	NMB0339	unann. NMC	NG1662	RSc0912	BP0953	CBU0530	STY1343	VC1912	YPO0225	b1280	SI367	PD1453	HI1223	PM0798
<i>N. meningitidis</i> A	Ght	100	97.7	97.7	35	33.7	29.3	28.3	28.3	28	27.2	27	26.5	24.7	24.4	
<i>N. meningitidis</i> B	NMB0339	2.4	100	99.5	34.2	32.6	28.8	27.8	28.3	27.8	27	26.7	25.7	24.2	23.9	
<i>N. meningitidis</i> C	unann.	2.4	0.5	100	98.5	34.4	32.9	29.3	28.3	28	27.2	27	26	24.7	24.4	
<i>N. gonorrhoeae</i>	NG1662	2.4	2.1	1.6	100	34.4	32.6	29.3	28	27.8	27.8	27	26.7	26	24.4	24.2
<i>R. solanacearum</i>	RSc0912	131.1	134.8	133.5	133.5	100	47.3	33.4	29.2	29.2	27.1	29.4	29.4	30.8	25.2	25.2
<i>B. pertussis</i>	BP0953	136.5	141.8	140.4	141.8	76.8	100	32	29.8	29.3	30	29.1	29.1	31.3	25.6	25.1
<i>Y. pestis</i>	YPO0225	159.4	162.8	159.4	159.4	123.2	137.3	100	40.4	40.6	39.6	39.7	39.4	37.9	33.2	34.3
<i>V. cholerae</i>	VC1912	166.3	170	166.3	168.1	146.4	149.9	109.3	100	54.2	78.7	93.3	93.1	37	48.1	49.6
<i>S. typhimurium</i>	STM1708	166.3	171.9	168.1	170	146.4	152.9	108.4	69.2	100	56.3	53.2	53	36.5	47.3	48.6
<i>C. burnetii</i>	CBU0530	168.1	170	168.1	170	160.3	148.4	112.3	25.2	64.5	100	77.6	77.4	37.3	48.8	49.4
<i>E. coli</i> K12	b1280	173.8	175.7	173.8	175.7	145	154.5	112.3	7	71.7	26.6	100	99.7	37.8	48.3	49.9
<i>S. flexneri</i>	SI367	175.7	177.7	175.7	177.7	145	154.5	113.2	7.3	72.3	27	0.3	100	37.5	48.1	49.6
<i>X. fastidiosa</i>	PD1453	179.8	186.1	184	184	136.5	141.3	119.4	122.7	124.9	121.6	119.4	120.5	100	30.4	30.9
<i>H. influenzae</i>	HI1223	194.7	196	194.7	197.2	174	178.8	140.4	84.8	87	82.6	84.1	84.8	154.8	100	74.7
<i>P. multocida</i>	PM0798	197.8	199	197.8	196	173.5	182.2	133.9	79.5	82.3	80.2	78.8	79.5	150.4	29.7	100

FIG. 3. Amino acid comparison between *N. meningitidis* serogroup A Ght and a some of its homologues: *Neisseria meningitidis* serogroup A strain Z2491, *Neisseria meningitidis* serogroup B strain MC58, *Neisseria meningitidis* serogroup C strain FAM18, *Neisseria gonorrhoeae* strain FA1090, *Ralstonia solanacearum* strain GMI1000, *Bordetella pertussis* strain Tohama I, *Yersinia pestis* CO92, *Vibrio cholerae* O1 biovar eltor strain N16961, *Salmonella enterica* serovar Typhimurium strain LT2, *Coxiella burnetii* strain RSA 493, *Escherichia coli* K-12 strain MG1655, *Shigella flexneri* 2a stain 2457T, *Xylella fastidiosa* Pierce's disease strain, *Haemophilus influenzae* strain RD KW20, and *Pasteurella multocida* strain PM70. Percent identity is shown in the upper right, while divergence is shown in the lower left. Comparison was done using available sequences from The Institute for Genomic Research and the National Center for Biotechnology Information and utilizing MegAlign in the Lasergene software suite of molecular biology tools (DNASTar). unann., unannotated genome.

lational start of NMA2149 may be as much as 168 bp downstream of the predicted start codon (the next ATG codon), making for a much smaller protein encoded by a truncated NMA2149. Analysis of the DNA sequence upstream of the mapped TSP revealed near consensus -10 and -35 hexamers that were separated by 18 nucleotides; the sequence of this promoter used for NMA2149-*ght* transcription is depicted in Fig. 5C.

Phenotypic characterization of *ght* mutants. The GCB-deferrioxamine mesylate plate assay was employed to test the ability of mutants in *ght* to use heme and heme-containing compounds as sources of iron. IR4048, a serogroup A clinical meningococcal isolate, is fully capable of utilizing heme and hemoglobin as sole iron sources (1), as illustrated by the large growth zones around both disks (Fig. 1A). Strain IR4162 (IR4048 *hpuB::ermC*), which was the parent strain in the *Himar1* transposon mutagenesis, utilizes heme as well as IR4048 but uses hemoglobin less efficiently (1). Thus, utilization of hemoglobin was relegated to those rare single colonies that originated from phase-off HmbR bacteria switching to phase-on, due to the mutation of *hpuB*. Interruption of *ght* by a nonpolar *aphA-3* cassette drastically altered the heme iron uptake in that the confluent heme zone disappeared and was replaced by a zone of inhibition with a very faint growth zone. In contrast, there was little visible growth around the hemoglobin disk, a characteristic exhibited by the original transposon mutant 276D (Fig. 1A). The heme growth zones could be restored by complementation with a extrachromosomal copy of *ght* on the broad-host-range vector pHT128 (Fig. 1B).

To assure that the interruption of *ght* in strain IR5571 did not have a polar effect on the downstream glyoxylase gene

gloA, a mutation of this gene was constructed. An isogenic *gloA* mutation did not affect heme or hemoglobin utilization (Fig. 1A). Additionally, a deletion of *ght*, except for the first 8 and last 35 nucleotides in the coding sequence, preserved the heme toxicity in the mutant strain (Fig. 2A). *gloA* expression in the *ght* deletion was verified by reverse transcriptase PCR (data not presented). Taken together, the heme toxicity property exhibited by mutant strain 276D is due to the mutation in *ght* and not due to altered expression of *gloA*.

To discern whether NMA2149, the ORF directly upstream of *ght*, plays a role in heme tolerance, a nonpolar mutation of NMA2149 was constructed. Insertional mutants of NMA2149 produced an intermediate heme toxicity phenotype (Fig. 1A); hemoglobin utilization was also slightly affected. However, when *ght* alone was expressed from a plasmid in a NMA2149 isogenic mutant, the resulting strain exhibited a normal heme utilization phenotype (Fig. 1B). Hemoglobin utilization was also unaffected (data not presented). This indicated that the mutation in NMA2149 still impacted *ght* expression. Based on these observations, we believe that the NMA2149 gene product plays little, if any, direct role in heme tolerance in meningococci.

HA susceptibility and growth analysis of a *ght* mutant. Bactericidal assays were carried out to better quantify the toxicity of heme against *ght* mutant strains and to assess whether any other metalloporphyrins or the heme precursor protoporphyrin IX (PPIX) displayed enhanced levels of toxicity. Heme was approximately 30-fold more toxic against the *ght* mutant strain (IR5511) compared to its parent strain (IR4162) (Table 2). However, both strains were equally sensitive to Zn-PPIX (zinc-protoporphyrin IX) and Ga-PPIX (gallium-protoporphyrin

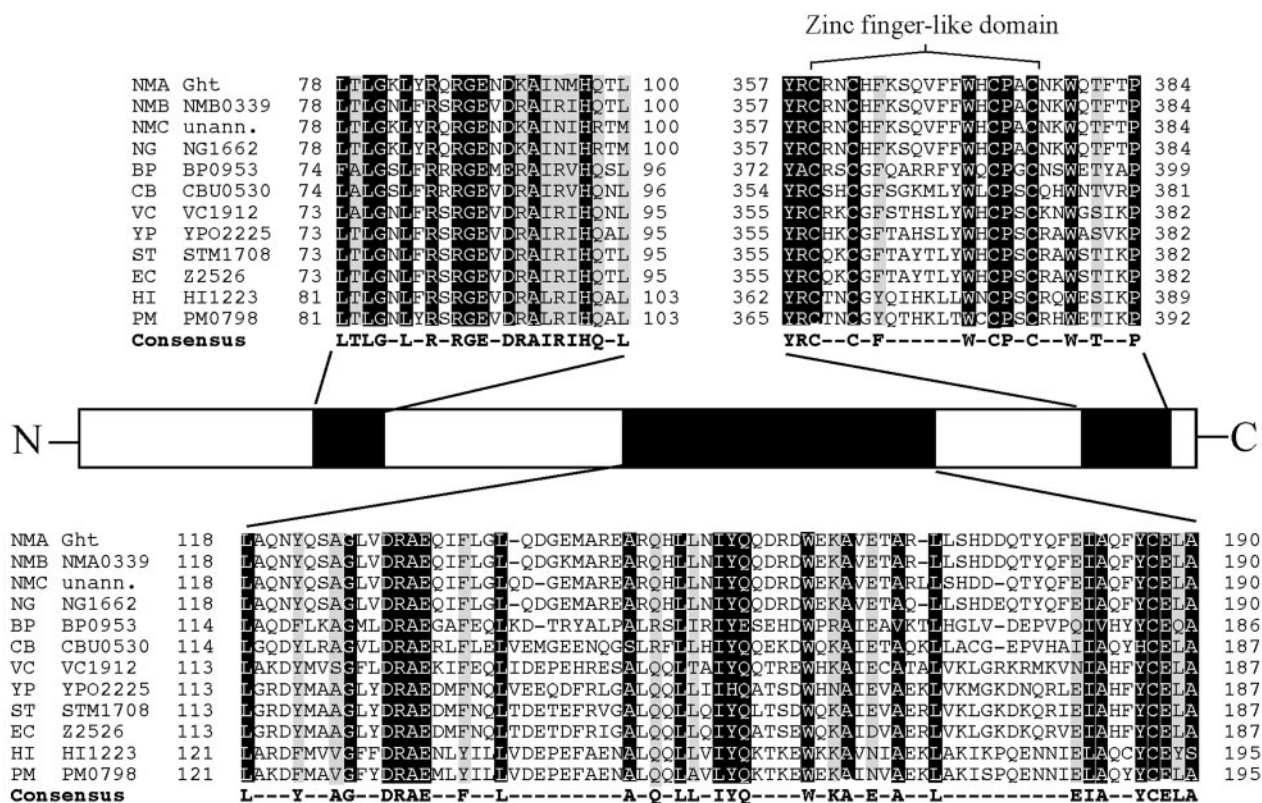


FIG. 4. Amino acid sequence comparison of the predicted *N. meningitidis* group A Ght protein with homologues from 12 human pathogens. Amino acid sequences were aligned by the ClustalW method using MegAlign in the Lasergene software suite (DNASTar). Residues conserved in 11 to 12 species are highlighted in black with white writing; residues conserved in 9 to 10 species are highlighted in gray with black writing. The consensus sequence of 9 to 12 matches is found below the alignment. NMA, *Neisseria meningitidis* group A Z2491; NMB, *Neisseria meningitidis* group B MC58; NMC, *Neisseria meningitidis* group C FAM18; NG, *Neisseria gonorrhoeae* FA1090; BP, *Bordetella pertussis* Tohama I; CB, *Coxiella burnetii* RSA 493; VC, *Vibrio cholerae* O1 biovar El Tor N16961; YP, *Yersinia pestis* CO92; ST, *Salmonella enterica* serovar Typhimurium LT2 SGSC1412; EC, *Escherichia coli* O157:H7 EDL933; HI, *Haemophilus influenzae* RD KW20; PM, *Pasteurella multocida* PM70; unann., unannotated genome.

IX). Unexpectedly, the mutant strain IR5571, but not parental strain IR4048, was very sensitive to PPIX, which lacks a metal ligand.

Hypersensitivity to low levels of PPIX in gonococci has been reported recently. One study indicated that two classes of mutants could utilize heme from hemoglobin for growth in the absence of a functional hemoglobin receptor (3). One class of mutants was determined to be a point mutation in the pilus secretion gene, *pilQ*, while the second was outside *pilQ* at an unidentified site. The similarity of the phenotype in the *ght* mutant strain IR5571 to that of the previously described *pilQ* mutant (3) led us to test whether mutations in *ght* altered levels of meningococcal HA toxicity. We found that a meningococcal *ght* deletion mutant (IR5700) had increased susceptibility to the HAs erythromycin, rifampin, and Triton X-100 (Table 3). We did not detect hypersensitivity to ampicillin (Table 3) and other hydrophilic antibiotics (data not presented). To eliminate direct involvement of Ght with PilQ-mediated HA hypersensitivity, we constructed an isogenic *pilQ* mutant and a double mutant, having insertional mutations in both *pilQ* and *ght*. The strains were then compared to the isogenic *ght* mutant and the wild-type strain for levels of HA susceptibility. A *pilQ* mutant had no effect on HA sensitivity compared to the wild-

type strain, and a double mutant bearing mutated *pilQ* and *ght* genes displayed levels of HA susceptibility identical to those of a strain bearing the *ght* mutation alone, indicating that the loss of Ght results in HA hypersensitivity of meningococci independent of PilQ (Table 3).

The gonococcal MtrC-MtrD-MtrE efflux system, which has orthologues in the meningococcal genomes (21, 24, 34), also functions in HA tolerance and was recently found to recognize PPIX and certain metalloporphyrins (2). This led us to test HA tolerance in meningococcal *ght* and *mtrD* (the cytoplasmic membrane transporter of the *mtr* efflux pump) (11, 24) mutants and a strain bearing a *ght-mtrD* double mutation (Table 3). As expected, an *mtrD* mutation increased meningococcal susceptibility to Triton X-100, erythromycin, and rifampin but not to heme, indicating that *ght* acts independently of the *mtr* system in meningococcal resistance to heme.

DISCUSSION

We initiated this transposon mutagenesis study to identify additional genes involved in the uptake and utilization of heme iron in the meningococci. By screening the *HimarI* mutant library, we identified *ght*, which when disrupted by the insertion

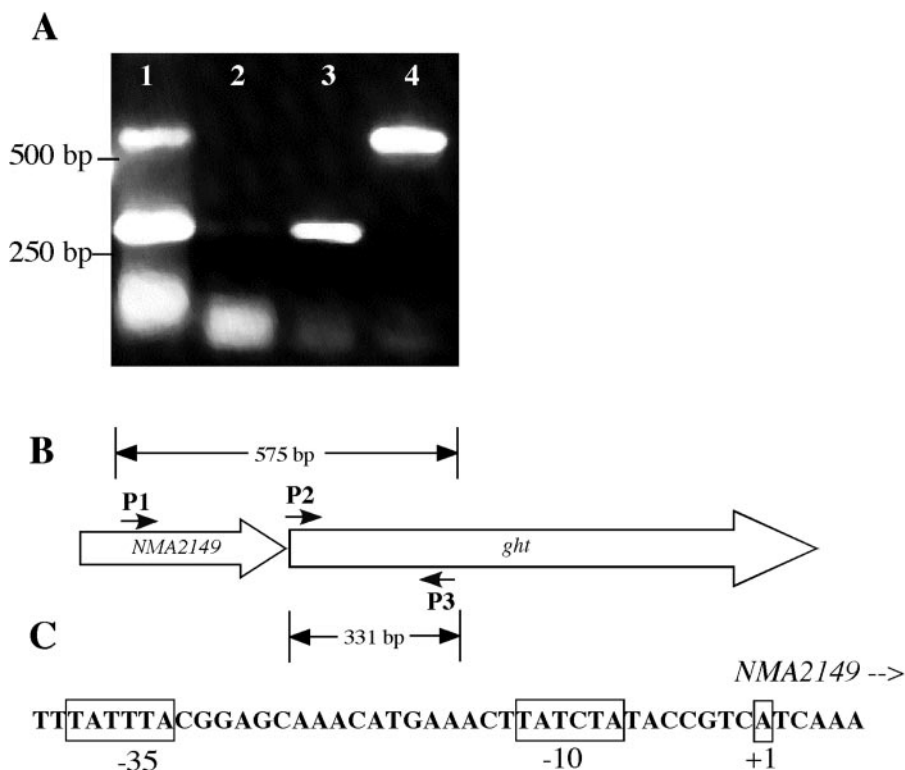


FIG. 5. Transcriptional characterization of *ght* region. (A) Reverse transcriptase PCR of the NMA2149 *ght* region. Lane 1, PCR using primers 5'2149-BglII-2 (P1), 5'NcoI-NMA2148ex (P2), and 3'RTgohT(P3) from IR4048 genomic DNA. Lane 2, PCR (minus RT) using the same primers as in lane 1 and IR4048 genomic RNA as a control for detecting DNA contamination. Lane 3, RT-PCR using primers 5'NcoI-NMA2148ex and 3'RTgohT. Lane 4, RT-PCR using primers 5'2149-BglII-2 and 3'RTgohT. (B) Representation of reverse transcriptase PCR products and their correspondence to the NMA2149 *ght* region. (C) Schematic of proposed -10, -35, and transcriptional start site upstream of NMA2149 as identified by primer extension.

of the transposon in a serogroup A meningococcus isolate, rendered the mutant highly susceptible to the toxic effects of heme iron. In addition to its hypersensitivity to heme, a *ght* mutant was also found to be highly susceptible to PPIX, the aporphyrin (non iron-loaded) moiety that is the precursor to heme in heme biosynthesis. This meningococcus isolate has not been previously shown to acquire PPIX specifically. Moreover, the meningococcus isolate has the necessary prerequisite genes for making its own PPIX (21, 34). However, susceptibility to both heme and PPIX is not unprecedented in the pathogenic neisseriae. As mentioned previously, two classes of mutants have been isolated in *Neisseria gonorrhoeae* that were more susceptible to both heme and PPIX. In addition to these porphyrin sensitivities, both classes of mutants were hypersensitive to other HAs as well (3). Subsequently, tests similar to those

carried out by Chen et al. (3) demonstrated that *ght* mutants are also hypersensitive to HAs such as Triton X-100 and rifampin in addition to heme and PPIX.

ght was found to be cotranscribed with the upstream ORF, NMA2149, which is not surprising, since NMA2149 and *ght* are separated by only 10 bp, and a TSP for this operon was mapped to the 5' region of NMA2149. The TSP is downstream of the start site predicted by Parkhill et al. (21). Although an interruption of NMA2149 with a nonpolar cassette appeared to decrease heme resistance, the phenotype could be complemented by the expression of *ght*. It is likely that the interruption of NMA2149 effects downstream expression of *ght* in *trans*, despite efforts to avoid disruption. This evidence suggests that NMA2149 plays no major role in HA tolerance, as mediated through *ght*. It is possible that NMA2149 may be required for optimal function of *ght*.

It is reasonable to assume that *gloA*, the locus directly downstream of *ght*, is also cotranscribed with NMA2149 and *ght*, since the 5' *gloA* coding sequence overlaps the 3' *ght* coding sequence by 37 bp. It is also for this reason that, when a deletion of *ght* was constructed, specific nucleotides were retained to conserve the start codon of *ght* and the 5' portion of *gloA* that overlaps *ght* in the event of translational coupling. Nevertheless, it was shown that a *gloA* mutation does not impact susceptibility to HAs.

Initial efforts to characterize *ght* concentrated only on its

TABLE 2. MBCs of porphyrins for IR4048 (parent strain) and IR5571 (*ght::aphA gloA*⁺)^a

Strain	MBC (mg/liter) of:			
	Heme	PPIX	Ga-PPIX	Zn-PPIX
IR4048	62	>500	32	<2
IR5571	2-4	2-4	32	<2

^a PPIX, protoporphyrin IX; Ga-PPIX, gallium protoporphyrin; Zn-PPIX, zinc protoporphyrin.

TABLE 3. Effect of *mtrD* and *pilQ* mutations on meningococcal strains with and without a deletion of *ght*

Strain	Relevant genotype	Source	MIC (mg/liter) of ^a :				
			Heme	ERY	AMP	TX100	RIF
IR4048	Parent strain	Clinical isolate	125	2.0	0.125	125	0.125
IR5700	Δ <i>ght</i>	Δ <i>ght</i> IR5575	15	1.0	0.125	30	0.0075
IR5721	<i>mtrD</i> ::Kan ^r	<i>mtrD</i> ::kan ^r IR4048	125	0.125	0.125	60	<0.003
IR5725	Δ <i>ght mtrD</i> ::Kan ^r	<i>mtrD</i> ::kan ^r IR5700	30	0.125	0.125	30	0.0075
IR5749	<i>pilQ</i> ::Cm ^r	<i>pilQ</i> ::cm ^r IR4048	125	2.0	0.125	125	0.06
IR5751	Δ <i>ght pilQ</i> ::Cm ^r	<i>pilQ</i> ::cm ^r IR5700	15	1.0	0.125	30	0.003

^a ERY, erythromycin; AMP, ampicillin; TX100, Triton X-100; RIF, rifampin. Results are mean values from three evaluations.

potential ability to participate in specific uptake and utilization of heme. Heme uptake in the meningococci involves outer membrane proteins that function to extract heme from heme-containing proteins and transport it into the periplasm where it is further relayed across the cytoplasmic membrane by an unidentified system. Once in the cytoplasm, the heme is degraded by a heme oxygenase to extract elemental iron; in the case of *N. meningitidis*, that heme oxygenase is HemO (37). The identity of the neisserial periplasmic transport system for heme has not been identified and was one of the targets of our mutagenesis. Initial studies attempted to prove a specific interaction between heme and Ght. We hypothesized that Ght may be functioning like a periplasmic binding protein of an ABC transporter system which has not been described for meningococci but has been assumed to exist (32). However, no such evidence was found from experiments that used heme-agarose affinity chromatography or spectrophotometric analysis (data not presented); these methods have been used previously to show direct interaction between neisserial proteins and heme (16). Moreover, Ght does not share similarity with other periplasmic heme binding proteins or any other described heme-binding proteins.

The similar hypersensitivity to certain HAs of strains bearing *ght* mutations, a point mutation in *pilQ*, and a yet unidentified mutation in the gonococci (3) is notable. Gonococcal strains bearing either the *pilQ* point mutation or the unidentified mutation could grow in the presence of hemoglobin as the sole source of iron despite lacking a functional HpuA or HpuB (3). This growth phenomenon could be abrogated by the addition of human serum albumin, indicating it was heme dissociated from hemoglobin that was the source of growth stimulation (3). The point mutation in the *pilQ* locus studied by Chen et al. (3) is presumed to allow increased entrance of hydrophobic compounds by alteration of PilQ structure, thus allowing passage of HAs through the altered secretin. PilQ had been thought to function by passively allowing only the extension and retraction of pilus structure; however, recent observations about the structure of PilQ suggest a more active role in pilus extension/retraction (4, 5). It has also been observed that wild-type PilQ may allow the passage of hydrophobic compounds across the outer membrane (J. Folster and W. Shafer, unpublished). Similar to both the *pilQ* point mutant and the uncharacterized mutant in *N. gonorrhoeae*, a *ght* mutant is also hypersensitive to Triton X-100 and rifampin and slightly sensitive to erythromycin. It is reasonable to hypothesize that *ght* may also play a role in stabilization of the pilus apparatus, specifically PilQ in the outer membrane. However, piliation was found to

be normal in a *ght* mutant (data not presented), and a mutation of *pilQ* was unable to rescue normal tolerance to HAs in a *ght* mutant, indicating that the increased HA susceptibility is not mediated by passage of HAs through the PilQ secretin. Efforts to transfer the nonpolar *ght*::Kan^r construct from the meningococci to the gonococci to better understand HA hypersensitivity and *ght*'s effects on piliation proved unsuccessful (data not presented), suggesting that *ght* may be an essential gene in the gonococci. Moreover, a meningococcal strain harboring mutations in HpuB and HmbR is not able to grow in the presence of hemoglobin as a sole iron source under the same conditions described by Chen et al. for their gonococcal mutants (3).

The function of Ght in relation to heme toxicity is independent of the meningococcal MtrC-MtrD-MtrE efflux system. In contrast to recent work with the gonococci (2), our data have shown that heme resistance in meningococci is not related to MtrC-MtrD-MtrE efflux activity. Ght is also unrelated to any currently described efflux system, but it is possible that Ght represents an integral part of a new class of efflux pump that is able to export HAs such as heme.

Maintenance of the integrity of the bacterial outer membrane is crucial to survival. The presence of *ght* orthologues in only gram-negative, but not gram-positive, bacteria and its proposed periplasmic localization indicate a ubiquitous role in gram-negative bacteria's membrane physiology. Ght may aid in sustaining the permeability of the outer membrane, specifically against the passage of chemically hydrophobic compounds independent of the PilQ secretin. This may be accomplished in one of two ways. Ght could maintain the integrity of the outer membrane itself; the proper formation and stability of the lipid bilayer is the first line of defense in providing a barrier that is permeable to nutrients but keeps out toxic compounds. The other way by which membrane integrity can be maintained is through protein complexes that span the outer membrane similar to PilQ. This may be accomplished by directly interacting with membrane-spanning pores or indirectly, like proper folding of outer membrane protein components. Whatever the mechanism is by which Ght protects against the toxicity of hydrophobic agents, it may only be elucidated by isolating other interacting members that aid in the function of Ght and by defining its cellular location. Discovery of Ght's mechanism of function may help in understanding how exogenous compounds are inhibited from penetrating the membrane defenses and how a potentially toxic molecule like heme is manipulated to benefit its bacterial consumer.

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