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Regulatory Role of the MisR/S Two-Component System in Hemoglobin Utilization in *Neisseria meningitidis*▽†

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Outer membrane iron receptors are some of the major surface entities that are critical for meningococcal pathogenesis. The gene encoding the meningococcal hemoglobin receptor, HmbR, is both independently transcribed and transcriptionally linked to the upstream gene *hemO*, which encodes a heme oxygenase. The MisR/S two-component system was previously determined to regulate *hmbR* transcription, and its *hemO* and *hmbR* regulatory mechanisms were characterized further here. The expression of *hemO* and *hmbR* was downregulated in *misR*/*S* mutants under both iron-replete and iron-restricted conditions, and the downregulation could be reversed by complementation. No significant changes in expression of other iron receptors were detected, suggesting that the MisR/S system specifically regulates *hmbR*. When hemoglobin was the sole iron source, growth defects were detected in the mutants. Primer extension analysis identified a promoter upstream of the *hemO*-associated Correia element (CE) and another promoter at the proximal end of CE, and processed transcripts previously identified for other cotranscribed CEs were also detected, suggesting that there may be posttranscriptional regulation. MisR directly interacts with sequences upstream of the CE and upstream of the *hmbR* Fur binding site and thus independently regulates *hemO* and *hmbR*. Analysis of transcriptional reporters of *hemO* and *hmbR* further demonstrated the positive role of the MisR/S system and showed that the transcription of *hmbR* initiated from *hemO* was significantly reduced. A comparison of the effects of the *misS* mutation under iron-replete and iron-depleted conditions suggested that activation by the MisR/S system and iron-mediated repression by Fur act independently. Thus, the expression of *hemO* and *hmbR* is coordinately controlled by multiple independent regulatory mechanisms, including the MisR/S two-component system.

Neisseria meningitidis, an obligate human pathogen, is a leading cause of meningitis and rapidly fatal sepsis, usually in otherwise healthy individuals. Normally a commensal inhabitant of the human nasopharyngeal mucosal surface, meningococcus can occasionally cross the epithelial layer and gain access to the bloodstream, and replication in the bloodstream can result in septicemia. Subsequently, meningococci may also cross the blood-brain barrier to cause meningitis. Iron is essential for almost all microorganisms, and thus the survival of bacterial pathogens in their hosts depends on their ability to scavenge iron (55). The free iron concentration in the human body is extremely low, and most of the iron is associated with various proteins. Heme and hemoglobin (Hb) are the most abundant sources of iron in the body, and the majority of iron in the blood is sequestered in hemoglobin (35, 43). Meningococci are equipped with several TonB-dependent receptors for acquiring iron from various sources available at different sites (37). Two outer membrane binary receptor complexes, TbpA/B and LbpA/B, are involved in human transferrin and lactoferrin acquisition, respectively (13). A two-component transport system, HpuA/B, enables meningococci to use iron from hemoglobin and the hemoglobin-haptoglobin complex (28), and another hemoglobin-binding outer membrane receptor, HmbR, is believed to extract heme from hemoglobin and subsequently transport it into the periplasm (47).

While HpuBA-dependent hemoglobin utilization was not able to distinguish between different sources of hemoglobin (48), the efficiency of utilization of different hemoglobins by HmbR-expressing *N. meningitidis* was shown to be species specific, and human hemoglobin was the best source of iron. An *hmbR* mutant was cleared from the bloodstream much more rapidly than the wild-type parent strain in an infant rat infection model, indicating the importance of HmbR-dependent hemoglobin utilization in survival in the bloodstream (47). *hmbR* is located downstream of *hemO*, encodes a heme oxygenase responsible for heme degradation (57), and is in an exchangeable meningococcal genomic island (23), indicating that this virulence factor was recently acquired. Interestingly, of the best-characterized meningococcal TonB-dependent iron receptors, only receptors targeting hemoglobin undergo phase variation, which is mediated through slip-strand mispairing of the poly(G) tract in the coding sequences of *hmbR* and *hpuA* (27, 38). It is believed that meningococci employ "on-off" phase variation to evade the host immune response. In addition, most outer membrane receptors for various iron sources are controlled by Fur-dependent iron regulation (11, 37). Fur interacts with ferrous iron and binds to the Fur box, which in most cases overlaps the promoters of iron-regulated genes, resulting in inhibition of transcription. Regulation of *hmbR* by iron availability (48) and by direct binding of the Fur protein to the *hmbR* promoter has been demonstrated (10, 44).

Two-component regulatory systems are some of the most

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common signal transduction mechanisms governing bacterial responses and adaptation to environmental changes (19). Many such systems act globally to coordinate expression of virulence determinants. These signal transduction systems generally consist of a sensor histidine kinase and a response regulator protein. When a signal is sensed, the histidine kinase undergoes autophosphorylation, and the phosphoryl group is subsequently transferred to the response regulator, modifying its activity. Mutation in the meningococcal MisR/S system has been shown to increase sensitivity to cationic antimicrobial peptides and polymyxin B (22), which correlates with the loss of all phosphoethanolamine decorations of the lipooligosaccharide inner core heptose II residue (50). In addition, a *misR* mutation was shown to cause attenuation of virulence in a murine model of meningococcal infection (34). Previous microarray studies of the MisR/S regulon have shown that inactivation of *misR* results in decreased expression of *hmbR* (51). Control of *hmbR* and *hemO* expression by the MisR/S system was further characterized in this study. The role of the MisR/S two-component system in regulating hemoglobin-dependent growth was demonstrated, and MisR was shown to specifically interact with the *hmbR* promoter sequence and the sequence upstream of the *hemO*-associated Correia element (CE). Thus, the expression of *hemO* and *hmbR*, in addition to responding to iron availability via Fur repression, is coordinately regulated by the MisR/S two-component system when as-yet-unidentified host signals are sensed.

MATERIALS AND METHODS

Plasmids, strains, and media. The strains and plasmids used in this study are listed in Table 1. *N. meningitidis* strain NMB (= CDC 8201085) is a serogroup B meningococcal strain that was originally isolated in 1982 from the cerebrospinal fluid of a patient with meningococcal meningitis in Pennsylvania. Meningococcal strains were grown with 5% CO_2 at 37°C on GC base (GCB) (Difco) agar containing 0.4% glucose and 0.68 mM $Fe(NO_3)_3$ or in GC broth supplemented with 0.4% glucose, 0.68 mM $Fe(NO_3)_3$, and 0.043% $NaHCO_3$. Brain heart infusion (BHI) medium (37 g/liter) with 1.25% fetal bovine serum was used when kanamycin selection was required. The *Escherichia coli* DH5α and Top10 strains were routinely grown in Luria-Bertani broth for cloning and propagation of plasmids. *N. meningitidis* was transformed by using the procedure of Janik et al. (20). *E. coli* strains were transformed by using chemical competence (Top10) or by electroporation (DH5α) with a GenePulser (Bio-Rad) used according to the manufacturer's protocol. The antibiotic concentrations used were as follows: for *N. meningitidis*, 80 μg/ml kanamycin and 3 μg/ml erythromycin; and for *E. coli* strains, 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 300 μg/ml erythromycin.

Construction of the *misR*::*aphA-3* mutant. A nonpolar *aphA-3* insertional mutation with deletion of a 510-bp fragment of *misR* (678 bp) was generated. A 760-bp PCR product containing the *misR* promoter region and 5′ coding sequence was generated using primers YT174-HindIII and YT185-KpnI, and the resulting DNA fragment was cloned into the pCR2.1 vector, yielding pTA174-185. Another 500-bp PCR product containing the 3′ sequence of *misR* and a 450-bp 5′ sequence of *misS* was amplified using primers YT186-BamHI and YT141, and the PCR product was cloned into the pCR2.1 vector, yielding pTA186-141. A 760-bp fragment of plasmid pYT174-185 was obtained by HindIII and KpnI digestion and then subcloned into the corresponding sites in the polylinker of pYT186-141, yielding pYT338. The *aphA-3* cassette released from pUC18K (32) by KpnI-BamHI digestion was inserted into the KpnI-BamHI sites of pYT338, yielding pSZ001. Removal of the *misR* internal sequence and the presence of a correctly oriented *aphA-3* cassette in the resulting pSZ001 plasmid were confirmed by performing colony PCRs and a sequence analysis. The construction procedure removed most of *misR* region and replaced it with a kanamycin resistance gene. To generate the meningococcal *misR* mutant, pSZ001 was linearized with ScaI to disrupt the *bla* gene, and the digestion mixture was used to transform meningococcal strains. Colonies were selected on BHI agar plates with kanamycin. Mutants were examined using colony PCR linking the *aphA-3* cassette to a chromosome-specific primer, DNA sequencing, and Southern blotting to confirm correct allelic exchange at the chromosomal *misRS* locus. In addition, Western blotting was performed to confirm the absence of the MisR protein in all *misR* and *misRS* mutants (data not shown).

Construction of the *hmbR*::*lacZ* transcriptional fusion. A 536-bp fragment of the *hmbR* promoter region was obtained by PCR amplification using primers hmbR-p-F2 and hmbR-p-R with chromosomal DNA of *N. meningitidis* serogroup B strain NMB as the template. The PCR product was cloned into pCR2.1 by TOPO cloning (Invitrogen) to construct pGM1. pGM1 was digested with EcoRI, and the released insert was purified and cloned into the EcoRI site of pYT328 (52) to generate a transcriptional fusion to the *lacZ* gene that was flanked by the meningococcal *NMB0428* and *NMB0430* coding sequences. The ligation reaction mixtures were transformed into *E. coli*, and erythromycin-resistant colonies were selected. Correct orientation of the promoter relative to the *lacZ* gene was confirmed by colony PCR using an outward primer (YT168) at the 5′ end of *lacZ* and a forward primer in the cloned promoter fragment. The resulting plasmid, pGM2, was digested with NcoI for linearization and transformed into *N. meningitidis* NMB and NMB310 (*misS*::*kan*). Transformants (strains GMT101 and GMT107, respectively) were selected on plates containing GC medium supplemented with erythromycin or BHI medium supplemented with erythromycin and kanamycin, and integration of the *hmbR*::*lacZ* fusion via homologous recombination into an irrelevant intergenic region was verified by PCR amplification using YT168 and chromosome-specific primer YT02.

Similar strategies were used to generate *hemO*::*lacZ* reporters and *hmbR*::*lacZ* reporters originating from the 5′ region of *hemO*. Primers hemO-pF1-ERI and hemO-pR1-ERI (418 bp), primers hemO-PF2-ERI and hemO-pR1-ERI (267 bp), primers hemO-pF1-ERI and hemO-pR3-ERI (918 bp), primers hemO-pF1-ERI and hemO-pR5-ERI (1,104 bp), and primers hemO-pF1-ERI and hmbR-pR-ERI (1,220 bp) were used to generate promoter fragments with flanking EcoRI sites using Phusion polymerase (New England Biolab) and the chromosomal DNA of strain NMB as the template. After EcoRI digestion, the fragments were purified and cloned into the EcoRI site of pYT328. The resulting plasmids were linearized with NcoI and used to transform meningococcal strain NMB. Correct transformants were identified by colony PCR as described above and designated O1101, O2002, O305, O501, and R1203, respectively. Reporters in the *misS* mutant background were generated by transformation of strain NMB310 and designated like the reporters of the wild-type strain but with the suffix s.

The *lacZ* fusion integrated into the native *hmbR* locus was constructed as follows. A ~1.4-kb PCR fragment obtained with primers hmbR-pF2 and hmbR-F3R was cloned into the pCR2.1 vector by TOPO cloning. The resulting plasmid, pPK-hmbR, was digested with HincII, which was followed by dephosphorylation with calf intestine alkaline phosphatase (CIP), and then it was ligated with the *lacZ-erm* cassette that was released from pAErmC′G (56) by BamHI digestion and filled in with Klenow DNA polymerase. The desired plasmid (designated phmbR103) with correct orientation of the *lacZ* cassette with respect to the *hmbR* coding sequence was identified by colony PCR using primers hmbR-pF2 and YT168. The plasmid was linearized with ScaI and then used to transform wild-type strain NMB and *misS* mutant strain NMB310 to obtain R103 and R103s, respectively. Integration into the *hmbR* locus via allelic exchange was confirmed by PCR using primers YT168 and hmbR-pg-1b, which annealed to sequences not included in the cloned fragment. A *lacZ* fusion at the native *hemO* locus was constructed in an analogous manner. A PCR product generated with primers hemO-pF1-ERI and hmbR-pR-ERI was cloned into the pSmartLCKan vector (Lucigen) by following the manufacturer's protocol. The resulting plasmid, pPK-hemO, was linearized at the unique BamHI site in the insert and ligated with the BamHI-digested *lacZ-erm* cassette. The orientation of the *lacZ* cassette was verified by PCR using primers YT168 and hemO-pF2-ERI. Plasmid phemO101 was used to obtain the reporter fusions O101 and O101s in the wild-type strain and the *misS* mutant, respectively.

β-Galactosidase assays. β-Galactosidase activity was assayed by the method of Miller (33), and experiments were performed in triplicate. The reporter strains were grown in GC broth with supplements at 37°C with aeration at 200 rpm to mid-log phase (optical density at 600 nm [OD_{600}], ~0.5). The cultures were then split into two 2-ml cultures and treated with and without 100 μM 2,2′-dipyridyl (Sigma) for the iron-depleted sample and the iron-replete control, respectively.

Hb utilization assay. Bacteria grown overnight on appropriate selection plates were collected and resuspended in GC broth without supplements at an OD_{600} of 0.1. Aliquots (100 μl) were plated onto GCB agar plates containing 100 μM deferoxamine mesylate (Desferal; Sigma). Filter disks (diameter, 0.25 in.) impregnated with 10 μl of human hemoglobin (Hb) (5, 3, and 1 mg/ml) or ferric nitrate (5 mg/ml) as a control were placed on the plates. The zones of growth around the disks were recorded after 48 h of incubation at 37°C in the presence of 5% CO_2. Six independent experiments were performed.

TABLE 1. Strains and plasmids used in this study

Strain	Genotype or description	Reference or source
N. meningitidis strains		
NMB	B:2b:P1.2,5:L2 (CDC8201085)	45
IR3261	NMB *hpuB::erm hmbR*^{off}	38
IR3287	NMB *hpuB::erm hmbR*^{on}	38
NMB310	NMB *misS*::*aphA-3*	52
YT391	NMB310 complemented with P_{lac}::*misS* at the *lctP-aspC* locus	42
SZT1001/YT0336	NMB *misR*::*aphA-3*	51
C1001	NMB *misR*::*aphA-3* complemented with P_{trc}::*misR* in the *iga* locus	51
SZT1003	NMB *misRS*::*aphA-3*	51
SZT1004	IR3261 *misR*::*aphA-3*	This study
SZT1005	IR3261 *misS*::*aphA-3*	This study
SZT1006	IR3261 *misRS*::*aphA-3*	This study
SZT1009	IR3287 *misR*::*aphA-3*	This study
SZT1010	IR3287 *misS*::*aphA-3*	This study
GMT101	P_{hmbR}::*lacZ*	This study
GMT107	P_{hmbR}::*lacZ* Δ*misS*	This study
O101	*lacZ-erm* cassette inserted into BamHI site of *hemO*	This study
O101s	*hemO*::*lacZ* Δ*misS*	This study
R103	*lacZ-erm* cassette inserted into HincII site of *hmbR*	This study
R103s	*hmbR*::*lacZ* Δ*misS*	This study
O1101	$P_{hemO}1$::*lacZ*	This study
O1101s	$P_{hemO}1$::*lacZ* Δ*misS*	This study
O2002	$P_{hemO}2$::*lacZ*	This study
O2002s	$P_{hemO}2$::*lacZ* Δ*misS*	This study
O305	P_{hemO}::*lacZ* with 918-bp promoter fragment	This study
O305s	P_{hemO}::*lacZ* with 918-bp promoter fragment, Δ*misS*	This study
O501	P_{hemO}::*lacZ* with 1,104-bp promoter fragment	This study
O501s	P_{hemO}::*lacZ* with 1,104-bp promoter fragment, Δ*misS*	This study
R1203	P_{hemO}::*lacZ* with 1,220-bp promoter fragment	This study
R1203s	P_{hmbR}::*lacZ* with 1,220-bp promoter fragment, Δ*misS*	This study
E. coli strains		
DH5α	Cloning strain	New England Biolabs
TOP10	Cloning strain	Invitrogen
Plasmids		
pUC18k	Source of *aphA-3* cassette	32
pCR2.1	TOPO cloning vector	Invitrogen
pSmartLCKan	Blunt-end cloning vector	Lucigen
pYT328	Cloning vector for chromosomal *lacZ* fusion	52
pYT338	5′ sequence (YT174-YT185) and 3′ sequence (YT186-YT141) of *misR* inserted into HindIII-KpnI site and EcoRI site of pCR2.1	This study
pSZ001	*aphA-3* cassette inserted into KpnI-BamHI site of pYT338	This study
pGM1	PCR product obtained with hmbR-pF2 and hmbR-PR in pCR2.1	This study
pGM2	EcoRI fragment of pGM1 inserted into EcoRI site of pYT328	This study
pPK-hemO	PCR fragment obtained with hemO-pF1-ERI and hmbR-pR-ERI cloned into pSmartLCKan	This study
phemO101	*lacZ-erm* cassette inserted into BamHI site of pPK-hemO	This study
pPK-hmbR	PCR fragment obtained with hmbR-pF2 and hmbR-F3R cloned into pCR2.1	This study
phmbR103	*lacZ-erm* cassette inserted into HincII site of pPK-hmbR	This study
pPK-hemO1101	PCR fragment obtained with hemO-pF1-ER and hemO-pR1-ER inserted into pYT328	This study
pPK-hemO2002	PCR fragment obtained with hemO-pF2-ER and hemO-pR1-ER inserted into pYT328	This study
pPK-hemO305	PCR fragment obtained with hemO-pF1-ER and hemO-pR3-ER inserted into pYT328	This study
pPK-hemO501	PCR fragment obtained with hemO-pF1-ER and hemO-pR5-ER inserted into pYT328	This study
pPK-hmbR1203	PCR fragment obtained with hemO-pF1-ER and hmbR-pR-ER inserted into pYT328	This study

Determination of the switching frequencies. Meningococcal strains that were *hmbR* phase off were isolated on nonselective GCB agar plates and then resuspended in GC broth. Aliquots (100 μl) of 10^{-1} to 10^{-3} serial dilutions were plated on GCB agar plates containing 100 μM deferoxamine mesylate (DFS) and 100 μg/ml of Hb (GCB-DFS-Hb plates), while aliquots of 10^{-5} to 10^{-8} serial dilutions were plated on nonselective GCB agar plates. The *hmbR* "off-to-on" switching frequencies were determined by dividing the number of colonies on GCB-DFS-Hb plates by the number of colonies on the nonselective GCB agar plates. Four independent measurements were obtained, and an analysis with Student's *t* test indicated that there was no statistically significant difference between the parent strain and the mutants.

RNA isolation and real-time qRT-PCR. Cultures of the wild-type strain and the mutants were grown and treated with dipyridyl as described above. Two volumes of RNAprotect reagent was added to cultures, and RNA was isolated using an RNeasy mini kit (Qiagen) by following the manufacturer's recommendations with on-column DNase digestion for 1 h at room temperature. PCR amplification of the RNA samples using primers hmbR-p-f and hmbR-p-r confirmed that there was no genomic DNA contamination. cDNA was obtained by reverse transcription (RT) of total RNA (1 μg) using a GeneAmp RNA PCR core kit (Applied Biosystems), and reactions without the reverse transcriptase were used as a negative control. The transcription of genes of interest was measured by real-time quantitative RT (qRT)-PCR using the SYBR green de-

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)
hmbR-p-F2	GCTTTATTGCGCCGAAGGATCCAATTTG
hmbR-p-R	ACCAGCGCGGCAATAGGGAGCAT
hmbR-pL-F	CAATCGGCTGGCTTTATTGCGCCGAAGGATCCAATTTG
hmbR-pL-R	AAAATACTGCCGACCAGCGCGGCAATAGGGAGCATTT
hmbR-pR-ER	CGGAATTCACCAGCGCGGCAATAGGGAGCATT
hemO-pF1-ERI	CGGAATTCCCGACTGCCGAACCCATTTGA
hemO-pF2-ERI	CGGAATTCAAATCAGGACAAGGCGGCGAAG
hemO-pR1-ERI	CGGAATTCCGCCGTGGTATCCGCCTTCA
hemO-pR3-ERI	CGGAATTCAACACGACTTTGTAGAATGCAAAG
hemO-pR5-ERI	CGGAATTCTAAGACAGTAATCCATGCAAACAAAGCCG
hemO-PE1	TGCTTGATTTTCGGTTTCACTCATATTTTTTCC
hemO-PE3	GTTCCGGTACTATTTGTACTGTCTGC
hmbR-F1	CCCGTTATGGCAACTTCAAC
hmbR-R1	GAAAGTCCGAGCGAAAAATG
hemO-F2	CTTGAAAGATTTGGGCGAAG
hemO-R2	CGCCGTTGTAATCGAGTTTT
NMB1436-F1	TTTACCCATTTCCTGCTTGC
NMB1436-R1	CGTTCGACGTTTTCCATACC
YT02	GGATACCGCAAGAACAGG
YT45	CGTAGATGACGATGCCCTGCTAACCG
YT46	GGCGGATGCTGGAGATGTGTACGTCG
YT141	GCAGGCGGCGGGCTTGGAGTTTG
YT168	GCGGATCAGTCCGGCGTAGAGG
YT173	CTTTGCATTCTACAAAGTCGTGTTG
YT174-HindIII	ACCCGGAAGCTTAACGCCTTTTCCC
YT185-KpnI	CCCGCTCAGGTACCCCTGTACGCCTGCTT
YT186-BamHI	AGTTGGGCGGATCCTCTCTGATTCAAA
rpsE F1/YT129	TGCTAAAGAGGGTAGTGGCG
rpsE R1/YT130	CAAACCATCTAATGTTGCACGT
lbpB-F1	GGAACATCGACCTTTTCCTG
lbpB-R1	TGCTTTTGCCGCTTCTTTAT
tbpB-F4	GCTGCCTGTGTTTTTGTTGA
tbpB-R4	TATCCGCCTTGGTCTTTTTG
YT150	GCGAAATCCTGACGGAATGG

tection method (SYBR green Supermix; Bio-Rad); the reaction conditions used have been described previously (50). The constitutive and highly expressed meningococcal ribosomal gene *rpsE*, which was not affected by the growth conditions, was used as an internal control in each experiment for normalization. The primers designed with Primer 3 software (http://fokker.wi.mit.edu/primer3/) (41) are listed in Table 2, and they were confirmed to yield similar amplification efficiencies and thus were suitable for the $2^{-\Delta\Delta C_t}$ method for determining the relative transcriptional differences between the mutant strains and the wild-type strain. The values for the wild-type strain grown in iron-replete conditions were used for calibration (30). RT negative control reactions were also analyzed to measure whether there was contaminating chromosomal DNA, and melting curve analyses were performed following each RT-PCR experiment to ensure that each reaction mixture contained only one specific product. Each qRT-PCR was performed in triplicate. Student's *t* test with a two-tailed hypothesis was used to determine the significance of differences ($P < 0.01$) between two variables in this study.

EMSA and DNase I protection assay. Both electrophoretic mobility shift assays (EMSA) and DNase I protection assays were performed using previously reported procedures (52). The *hmbR* promoter fragment (326 bp) was obtained by PCR amplification using primers YT173 and hmbR-p-r, while the probe for DNase I protection assays was prepared by PCR using ^{32}P-labeled primer hmbR-p-r. The *hemO* promoter fragment (418 bp) was obtained by PCR amplification using primers hemO-pF1-ERI and hemO-pR1-ERI, while the probe for the DNase I protection assays was prepared using ^{32}P-labeled primer hemO-pF1-ERI. The binding reactions were examined with phosphorylated (50 mM acetyl phosphate for 30 min at 37°C) or nonphosphorylated MisR. Competition with excess specific competitors (unlabeled probes) and nonspecific competitors (the 237-bp internal coding sequence of *misS* obtained by PCR amplification using primers YT150 and YT141) was performed to assess the specificity of the interaction.

Primer extension. Total RNA (30 μg) was used in primer extension reactions with ^{32}P-labeled *hemO*-specific primers (hemO-PE1, hemO-PE3, and hemO-pR1-ERI) using a previously described procedure (42). The corresponding DNA sequencing reactions were carried out using the same labeled oligonucleotides and PCR fragments of the promoter regions with a Thermo Sequenase dye primer manual cycle sequencing kit (USB). The extension products and the sequencing ladders were resolved on an 8% sequencing gel.

RESULTS

Expression of *hmbR* and *hemO* was reduced in the *misR*/*S* mutants. The HmbR hemoglobin receptor was identified as a member of the MisR/S regulon in a microarray study that compared the transcriptional profiles of the *misR*::*erm* mutant and the wild-type parent strain grown under iron-replete conditions (51). To better characterize the role of the MisR/S system in *hmbR* regulation under both iron-rich and iron-limited growth conditions, we measured *hmbR*-specific mRNA levels in wild-type strain NMB and compared these levels to the levels obtained for the corresponding Δ*misR*, Δ*misS*, and Δ*misRS* mutants by quantitative qRT-PCR. In addition, complemented Δ*misR* and Δ*misS* mutants were also included in the analysis. RNAs were isolated from mid-log-phase cultures grown in iron-replete medium and iron-depleted medium (treated with 100 μM dipyridyl for 45 min). Dipyridyl is a ferrous iron chelator that rapidly produced an iron-limiting environment by sequestering ferrous iron, a cofactor of the Fur protein that mediates iron regulation. The transcripts of the wild-type strain and the mutants were induced in dipyridyl-treated cultures, confirming the iron-restricted growth conditions and that the iron-dependent transcriptional control occurred in all of the mutants. When cultures were grown under iron-replete conditions, the levels of *hmbR* transcription in all of the *misRS* mutants were significantly reduced (Fig. 1A). Similarly, downregulation of *hmbR* was detected in all mutants under iron-restricted conditions (Fig. 1A). These results were consistent with previous data for an independent *misR* mutant (51) and suggested that the MisR/S system, either directly or indirectly, plays an activator role in *hmbR* expression regardless of the Fur repressor activity. The data showing that the *misS* mutant behaved like the *misR* mutant suggest that MisR activation via phosphoryl transfer from the MisS kinase is important for *hmbR* regulation. Complementation of the *misR* and *misS* strains, which placed the *misR* and *misS* genes under isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter control, rescued the *hmbR* expression under both growth conditions, confirming that the effect on *hmbR* expression was due to a defect in the two-component system.

hmbR is downstream of *hemO* (Fig. 2A) (57). Studies of *hemO* mutants of a serogroup C strain suggested that *hmbR* and *hemO* may be transcriptionally linked (57). Using cDNA generated by a reverse transcriptase reaction performed with a reverse primer complementary to the *hmbR* coding sequence, we obtained PCR products spanning the *hemO-hmbR* intergenic region, as well as positive PCR amplification of the *hemO* gene, confirming that there is also transcriptional coupling of *hmbR* and *hemO* in the serogroup B strain NMB (data not shown). Because the qRT-PCR primers for *hmbR* measured total *hmbR* transcripts, the significantly decreased *hmbR* expression suggested that *hemO* transcription would likely be affected by the *misR*/*S* mutations. Thus, we examined *hemO*

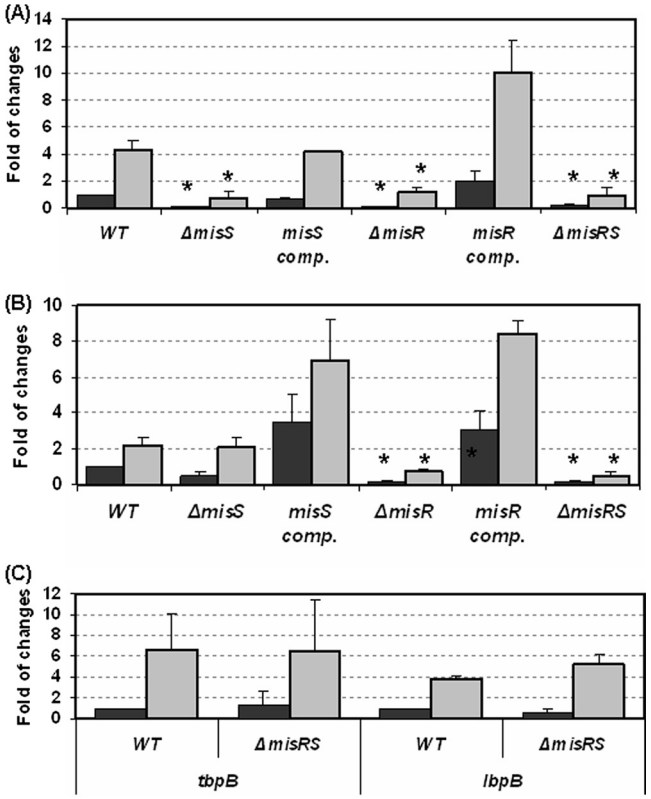

FIG. 1. qRT-PCR determination of relative transcriptional changes for *hmbR* (A), *hemO* (B), and *tbpB* and *lbpB* (C) in the *misR/S* mutants. Total RNAs were isolated from mid-log-phase cultures that were treated (gray bars) or not treated (black bars) with 100 μM dipyridyl for 45 min. The relative transcriptional differences between the mutants and the wild-type strain were calculated by the $2^{-\Delta\Delta C_t}$ method (30), using the transcriptional level of the wild-type strain under iron-rich conditions as the calibrator. Each qRT-PCR was performed in triplicate and was repeated with at least two independent RNA preparations. The asterisks indicate statistically significant differences between the wild-type strain and the mutant as determined by the Student *t* test with a two-tailed distribution ($P < 0.01$). *WT*, wild type; *comp*., complemented.

expression by using qRT-PCR, and, as shown in Fig. 1B, the transcription of *hemO* was indeed significantly reduced in the *misR* and *misRS* mutants, while the *misS* mutation resulted in a modest reduction in transcription. Again, the reduced *hemO* expression could be complemented by a second copy of the corresponding mutated gene, and the levels were higher than those of the wild-type strain, probably due to IPTG-induced overexpression of the two-component proteins. The fact that overexpressing either MisS or MisR appeared to enhance *hemO* expression in the complemented strains is consistent with a positive role for the two-component system.

To examine whether the MisR/S system specifically affected *hmbR* and *hemO* expression, mediating hemoglobin utilization in particular, the levels of transcription of *tbpB* and *lbpB* were also measured. *tbpB* and *lbpB* are the first genes in the *tbpB/A* and *lbpB/A* operons encoding the outer membrane receptors responsible for transferrin and lactoferrin acquisition, respectively. As shown in Fig. 1C, the *misRS* mutation resulted in no significant changes in expression of these genes. Several studies have demonstrated that these different iron acquisition receptors are all controlled by Fur-dependent repression, which is alleviated upon iron starvation (44, 48). The observation that mutations in the MisR/S system significantly affected only *hmbR* expression suggested that the regulatory effect of the MisR/S system does not broadly interfere with Fur-dependent regulation. Another Fur-dependent and iron-induced gene, *NMB1436*, which codes for a putative protein involved in oxidative stress protection (14), was also analyzed. As expected, the levels of the *NMB1436* transcript were reduced in the absence of iron, and no significant changes were detected in the *misS* or *misRS* mutants (0.98- ± 0.38-fold and 0.69- ± 0.24-fold, respectively). Thus, inactivating the MisR/S two-component system significantly and specifically downregulated the transcription of *hmbR* and *hemO*.

Mutation in the *misR/S* system affected hemoglobin utilization. Two TonB-dependent outer membrane receptors have been shown to mediate Hb utilization in *N. meningitidis*; HmbR enables uptake of hemoglobin, and the HpuA/B binary protein complex allows utilization of both hemoglobin and haptoglobin-Hb complexes (37). Disk diffusion assays were performed to examine whether the decreased *hmbR* and *hemO* expression in the two-component mutants affected hemoglobin utilization and consequently altered hemoglobin-dependent growth. The strains were plated onto GCB agar plates containing 100 μM Desferal to remove free iron, and specific iron sources were provided on disks. All strains utilized free iron equally well; however, when Hb was the sole iron source, all mutants showed reduced growth (Fig. 3A). To remove the contribution from the HpuB/A receptor, the *misR*, *misS*, and *misRS* mutations were introduced into a derivative of strain NMB (IR3261) that carries an *hpuB*::*erm* mutation to create strains SZT1004, SZT1005 and SZT1006, respectively. As shown in Fig. 3B, all mutants again showed an apparent defect in hemoglobin-dependent growth but not in free-iron-dependent growth.

Both meningococcal *hmbR* and *hpuB* are phase variable (27, 38) and are "phase off" in the serogroup B parental strain. When Hb was the sole iron source, all strains grew as single colonies due to the off-to-on switching of *hmbR* and/or *hpuB*. The off-to-on switching frequencies of the two wild-type strains, NMB and IR3261, and the six mutants were examined by plating either plate-grown cultures or broth cultures on standard GCB agar plates and GCB agar plates containing 100 μM Desferal and 100 μg/ml Hb, followed by a comparison of the CFU. No significant differences were detected between the parental wild-type strains and the corresponding mutants (data not shown), indicating that the observed growth phenotypes were not due to altered switching frequencies of the hemoglobin receptor genes. Finally, *misS* and *misR* mutations were created in an *hmbR* "on" derivative of IR3261 (IR3287) to generate SZT1009 and SZT1010, respectively, and both mutants displayed reduced Hb-dependent growth (Fig. 3C). The growth curves of all of the strains in standard GC broth were examined together with the plate growth assay results, which were recorded after 48 h of growth. Although slightly reduced growth rates were seen for most mutants, all mutants reached similar densities after 8 h of growth. Thus, the decreased *hemO* and *hmbR* expression in the meningococcal *misRS* mutants

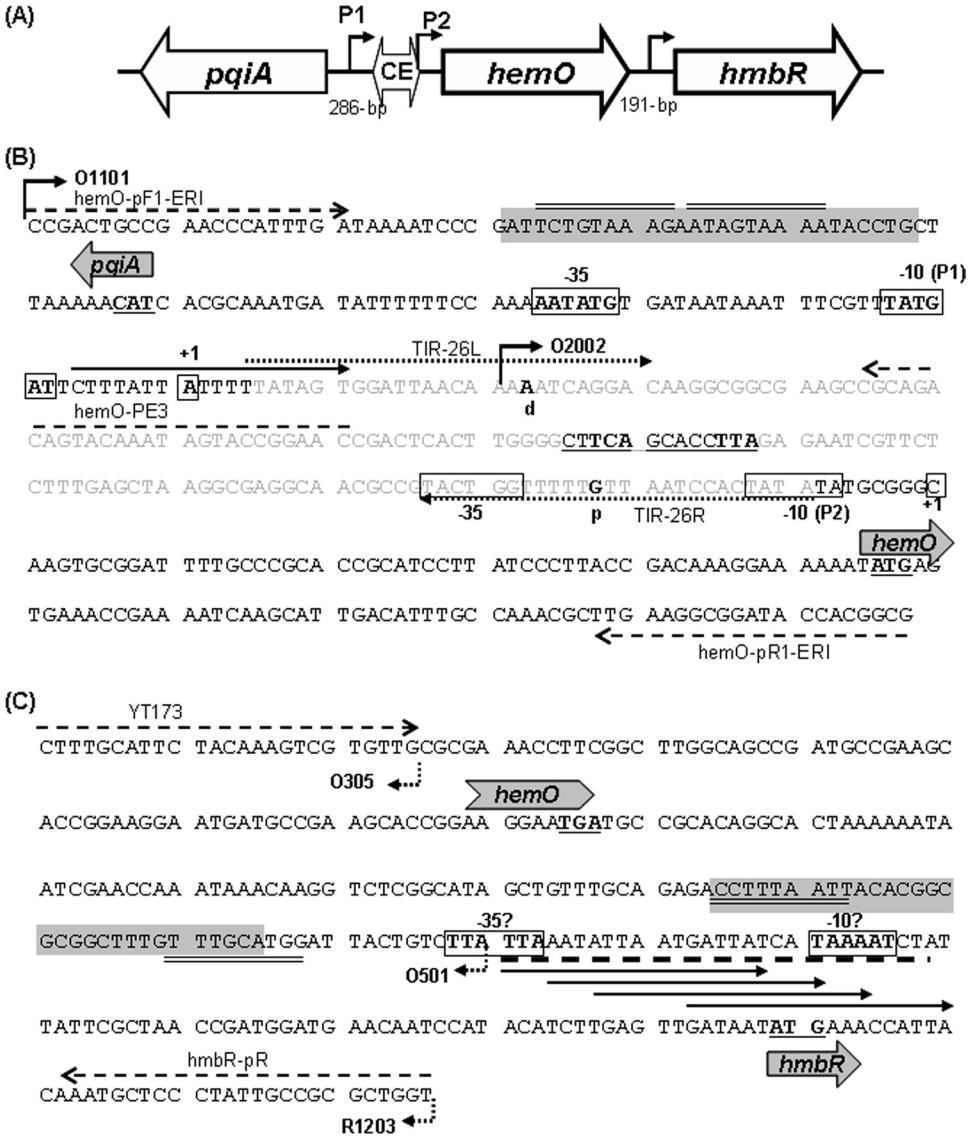

FIG. 2. (A) Organization of the *hemO-hmbR* locus in meningococcal strain NMB. The *hemO* gene is transcribed divergently from the *pqiA* (*hmp*) gene with a 286-bp intergenic space that includes a full-length Correia element (CE) (6), while *hemO* is separated from *hmbR* by a 191-bp intergenic region. The bent arrows represent the promoters of *hemO* and *hmbR*. The schematic diagram is not to scale. (B) Sequence of the 5′ region of *hemO*. The ATG start codons are in bold type and underlined, and the large arrows show the direction of transcription. The sequence of the CE is in gray type, the terminal inverted repeats (TIR) are indicated by dotted arrows, and the IHF binding sequence in the CE is underlined, with the nucleotides matching the consensus sequence nucleotides (7) in bold type. The putative transcript processing sites in the TIRs are indicated by bold type and labeled d and p. The putative Fur binding motif matching the $(NATWAT)_3$ motif is indicated by an arrow. The sequence protected by MisR in the DNase I protection assay is in bold type and shaded, and the sequences matching the MisR binding motif are indicated by double overlining. The 5′ ends of two *hemO*::*lacZ* fusions are indicated by bent arrows, and the hemO-pR1-ERI primer is the corresponding 3′ end. The hemO-pF1-ERI primer used to generate probes for DNase I protection assay and the hemO-PE3 primer used in primer extension are indicated by dashed arrows. The two transcriptional start sites and the two corresponding promoter elements are enclosed in boxes. (C) Nucleotide sequence of the *hmbR* promoter region. The stop codon of *hemO* and the start codon of *hmbR* are in bold type and underlined. The putative −10 and −35 hexamers are in bold type and enclosed in boxes. The sequence protected by MisR is shaded, and the two matching MisR consensus motifs are indicated by double underlining. The Fur-protected sequence reported by Delany et al. (10) is indicated by a dashed line. The primers used for the EMSA and DNase I protection assay, YT173 and hmbR-pR, are indicated by dashed arrows. The dotted bent arrows indicate the 3′ ends of promoter fragments cloned in reporter strains O305, O501, and R1203.

correlated with a diminished capacity to utilize Hb as the sole source of iron for growth.

To correlate the *hmbR* transcription with the results of the hemoglobin-dependent growth assay, which required use of the Fe^{3+} scavenger Desferal, we also examined the regulatory effect of the MisRS system under the iron-restricted conditions generated with Desferal, which may correspond to adaptation to iron starvation. Meningococcal strain IR3287, an *hmbR*^{on} derivative of serogroup B strain NMB, and the corresponding *misR* and *misS* mutants, SZT1009 and SZT1010, respectively,

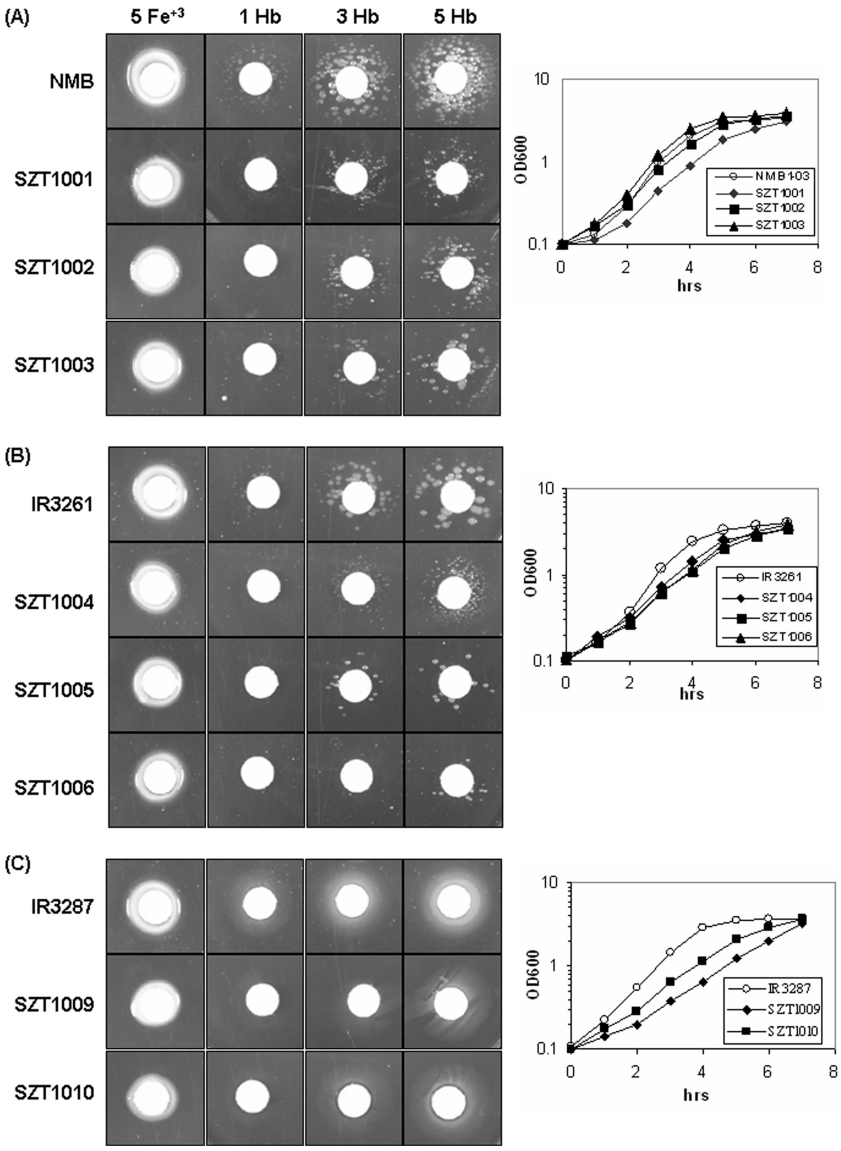

FIG. 3. Hemoglobin utilization assay. Bacteria grown overnight on appropriate selection plates were collected and resuspended at an OD_{600} of 0.1 in GC broth. Aliquots (100 μl) were plated onto GCB agar plates containing 100 μM Desferal. Filter paper disks were placed on the plates and then soaked with 10 μl of a human hemoglobin solution (1, 3, and 5 mg/ml [1 Hb, 3 Hb, and 5 Hb respectively]) or a ferric nitrate solution (5 mg/ml [5 Fe^{+3}]) as a control. The zones of growth around disks were measured after 48 h of incubation at 37°C. The results for one representative of six independent experiments are shown. The growth curves for all of the strains tested in standard GC broth are shown on the right. (A) *hmbR*^{off} *hpuA*^{off} background; (B) *hmbR*^{off} *hpuB*::*erm* background; (C) *hmbR*^{on} *hpuB*::*erm* background. NMB, IR3261, and IR3287 are wild-type parental strains. SZT1001, SZT1004, and SZT1009 are *misR*::*aphA-3* mutants. SZT1002, SZT1005, and SZT1010 are *misS*::*aphA-3* mutants. SZT1003 and SZT1006 are *misRS*::*aphA-3* double mutants.

were grown in the presence of 50 μM Desferal for 3 h, and qRT-PCR analyses of *hmbR* expression were performed with purified total RNAs. The overall changes in the *hmbR* expression patterns of these independent wild-type and mutant strains (see Fig. S1 in the supplemental material) were similar to those obtained with the dipyridyl treatment (Fig. 1A).

Multiple potential regulatory mechanisms are present in the *hemO-hmbR* locus. *hemO* is separated from *hmbR* by 191 bp in strain NMB and is transcribed divergently with a 286-bp intergenic region from a putative paraquat-inducible protein homologue gene, *pqiA* (Fig. 2A). Examination of the upstream sequences of *hemO* and *hmbR* revealed potential binding motifs of several global regulators. Searches of the upstream sequence of *hemO* using the $(NATWAT)_3$ meningococcal Fur binding motif (15) with a maximum of three mismatches identified a single putative Fur box sequence ~250 bp upstream of the *hemO* start codon (Fig. 2B), while four potential Fur boxes clustered within 100 bp of the ATG start site of *hmbR* coincided with the Fur binding site mapped by Delany et al. (10) (Fig. 2C). The more distal location of the Fur box with respect to the *hemO* coding sequence is due to the presence of a 156-bp full-length insertion element termed the Correia element (CE) (6) or Correia repeat-enclosed element (29). CEs are transposon-like elements prominent in neisserial genomes

that introduce TA dinucleotide duplications upon insertion, thus potentially creating divergent promoters at the ends of elements (5, 36). In addition, in the full-length element there is a functional binding site for integration host factor (IHF) that has been shown to interact with IHF proteins *in vitro* (5, 40). Finally, Mazzone et al. (31) and De Gregorio et al. (9) have reported that many CEs cotranscribed with the adjacent open reading frame (ORF) are posttranscriptionally processed at the terminal inverted repeats (TIRs) by RNase III. Either one or both TIRs that form a double-stranded RNA hairpin structure can be processed, and the stability of the processed products varies significantly (9).

qRT-PCR detected increased expression of *hemO* and *hmbR* after dipyridyl treatment (Fig. 1), indicating that both of these genes are repressed by iron. Because putative Fur boxes typically overlap the promoter elements of iron-repressed genes, we predicted that the *hemO* promoter maps upstream of the CE. However, transcriptional initiation originating from the ends of a CE has also been reported (4, 5, 36). Thus, we performed primer extension experiments using three primers that anneal either within or downstream of the CE to map the transcriptional start site of *hemO*. Using a primer annealed inside the CE (hemO-PE3), a transcriptional start site ($P_{hemO}1$) (Fig. 4A) was mapped to a location in the single predicted Fur box sequence, and an AATATG-17 bp-TA TGAT promoter element could be derived (Fig. 2B). A shorter extended product was detected (d site in Fig. 4A) that corresponded to the RNase III-cleaved distal site reported by Mazzone et al. (31). Another transcriptional start site ($P_{hemO}2$) (Fig. 4B) was detected using a primer that annealed within the *hemO* coding sequence (hemO-pR1-ER) at a location immediately downstream of the CE, and it may use an GTACTG-16 bp-TATATA promoter sequence, where the −10 hexamer contains the TA duplication of the proximal TIR (Fig. 2B). Similarly, we detected an additional major extended product (p site in Fig. 4B) that mapped to the G nucleotide of the TIR, which has been reported by Mazzone et al. (31) and De Gregorio et al. (9) to be an RNase III-processing site. Although the signals for both transcriptional start sites were relatively weak compared to those for the presumed RNase III-processed products, a third primer (hemO-PE1) located at the ATG start codon of *hemO* detected both start sites (data not shown). Thus, the transcription of *hemO* was driven by promoters located both upstream and downstream of the CE, and thus the Correia element was cotranscribed with *hemO*. In addition, the *hemO* mRNA was likely processed in a similar fashion, as described previously for other transcripts containing CE (9, 31, 40).

Despite the fact that a reasonable −10 and −35 promoter element (TTATTA-17 bp-TAAAAT) overlapping the potential Fur boxes was predicted, attempts to map the transcriptional start site of *hmbR* were unsuccessful (data not shown). A 4-bp substitution that changed the predicted −10 sequence from TAAAAT to TAAGCA was generated in reporter strain GM101 (see below) to probe its involvement in promoter activity. This mutation decreased the *hmbR* promoter activity (the activity was ~20% of the activity of the wild-type strain) (data not shown) under both iron-replete and iron-depleted conditions, confirming the importance of this sequence in the promoter function. In addition, the mutated promoter remained responsive to iron limitation, suggesting that the mutation did not interfere with iron regulation.

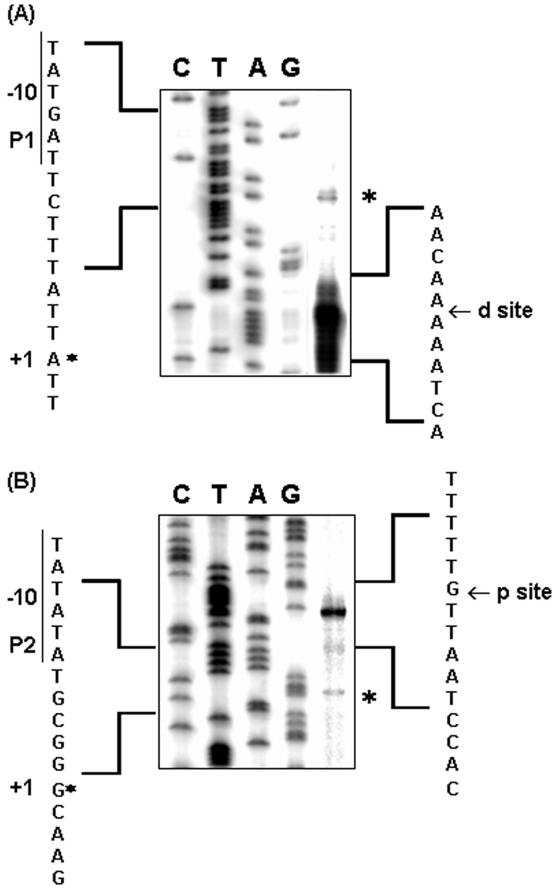

FIG. 4. (A) Primer extension analysis of *hemO* using the hemO-PE3 primer (A) and hemO-pR1-ERI primer (B). Lanes G, A, T, and C contained the dideoxy sequencing reaction mixtures. The asterisks indicate the transcriptional start sites, while the mRNA processing sites (d site and p site) as described by Mazzone et al. are indicated by arrows.

MisR response regulator directly interacts with both *hemO* and *hmbR* promoters. As the MisR/S two-component system was demonstrated to act as a positive regulator in the overall *hmbR* expression by qRT-PCR, we performed EMSA to determine whether MisR directly interacts with both the *hemO* and *hmbR* promoters. A direct interaction between MisR and the *hmbR* promoter has been found previously using electrophoretic mobility shift assays (EMSA) performed with purified His-tagged MisR proteins (52) to display a dose-dependent interaction (51), and the specificity of this interaction is further demonstrated in Fig. 5A. Unlabeled *hmbR* DNA specifically competed with the labeled probe for MisR binding, while excess nonspecific DNA did not interfere. Similarly, dose-dependent shifts of the *hemO* promoter with MisR were detected, and again, a competition EMSA demonstrated the specificity of this interaction (Fig. 5B).

The MisR-interacting sequences in the *hemO* and *hmbR* promoters were further defined by DNase I protection assays. MisR interacted with a ~30-bp sequence located upstream of the *hemO* promoter elements (Fig. 5C), consistent with the

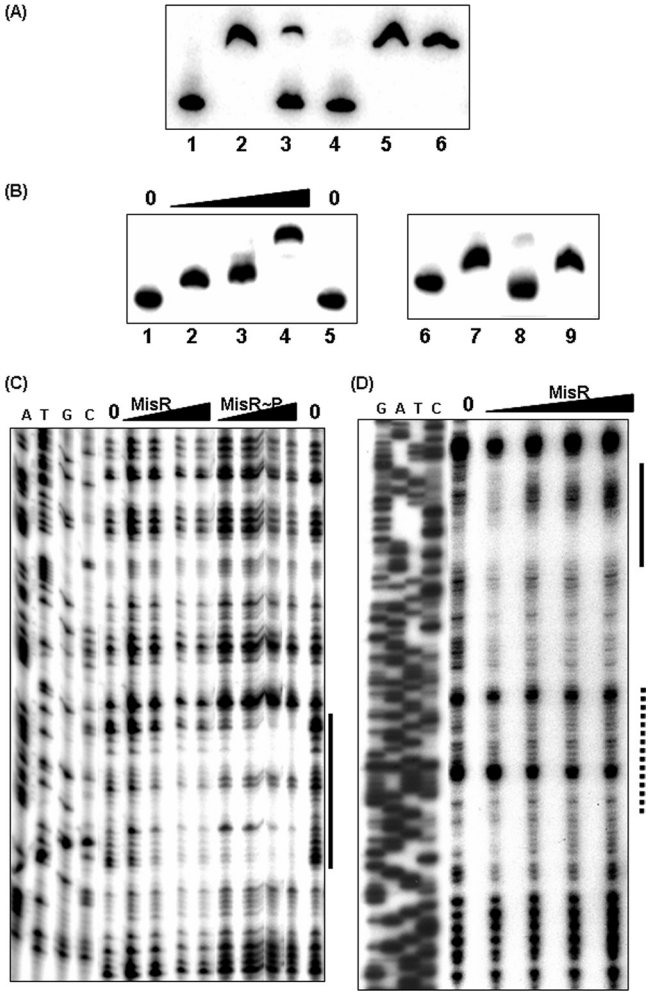

FIG. 5. (A) Competition EMSA experiments with the *hmbR* promoter and MisR. A 326-bp *hmbR* fragment (YT173-hmbR-pLR) was end labeled with [γ-^{32}P]ATP using T4 kinase and incubated with the MisR protein as described previously (52). Lane 1, DNA probe; lanes 2 to 6, DNA probe with MisR protein (77 pmol, 5 μM) (lane 2, no DNA competitor; lanes 3 and 4, probe with 1.5 and 2 μg specific DNA competitor, respectively; lanes 5 and 6, probe with 1.5 and 2 μg nonspecific DNA competitor, respectively). (B) (Left panel) Dose-dependent shift of the *hemO* promoter with MisR. Lanes 1 and 5, free probe; lanes 2 to 4, 0.6, 2.6, and 3.8 μM MisR protein, respectively. (Right panel) Competition EMSA with the *hemO* promoter. Lane 6, free probe; lanes 7 to 9, DNA probe with MisR protein (38 pmol, 2.5 μM). One microgram of cold specific DNA was added to lane 8, while 1 μg of nonspecific DNA was added to lane 9. (C and D) DNase I protection assays with the *hemO* promoter (C) and the *hmbR* promoter (D). The noncoding strand of the promoter fragment was end labeled with ^{32}P as described in Materials and Methods, incubated with increasing amounts of MisR for 20 min at 30°C, and then subjected to DNase I digestion in a 20-μl (total volume) reaction mixture. MisR~P was prepared by incubation with acetyl phosphate (50 mM) at 37°C for 30 min. For *hemO*, the amounts of MisR tested were 0, 93, 186, 232, and 278 pmol and the total amounts of MisR~P tested were 0, 93, 139, 232, and 371 pmol. For *hmbR*, the amounts of MisR tested were 0, 170, 340, 510, and 850 pmol. The results for dideoxy chain termination sequencing ladders corresponding to the probes are shown on the left (lanes A, T, G, and C). The solid line indicates the sequence protected by MisR, while the Fur protected region determined by Delany et al. (10) is indicated by a dotted line.

general location of a transcriptional activator (Fig. 2B). Analogously, MisR interacted with a ~30-bp sequence located upstream of the predicted −35 element of the *hmbR* promoter, and a hypersensitive DNase cleavage site was detected at higher MisR protein concentrations (Fig. 5D). Putative MisR binding motifs, (A/T)(A/T)TGTAA(A/G/C)G (51), were detected in the protected sequences of both promoters (Fig. 2B and 2C). Thus, in addition to the Fur-dependent iron regulation and the possible CE-mediated transcriptional and post-transcriptional regulation, the MisR/S two-component system also directly and positively regulates the expression of *hemO* and *hmbR*.

Transcriptional regulation of *hemO* and *hmbR* expression. As transcriptional coupling between *hemO* and *hmbR* was detected, we determined the contribution of each promoter's activity using *lacZ* transcriptional reporter fusions. Reporter fusions O1101, O2002, and GM101 were constructed as single copies at an irrelevant chromosomal location to determine the relative strengths of the $P_{hemO}1$ and $P_{hemO}2$ promoters and the proximal P_{hmbR} promoter, respectively (Fig. 6A). A reporter that comprised all three promoters, R1203, was also constructed to assess the overall *hmbR* expression (Fig. 6A). As shown in Fig. 6B, the $P_{hemO}1$ promoter was ~20-fold stronger than the proximal P_{hmbR} promoter (O1101 compared with GM101), indicating that the $P_{hemO}1$ promoter likely contributed the majority of transcription. The $P_{hemO}2$ promoter created by insertion of the CE (O2002) was relatively weak and thus did not contribute significantly to the overall expression of *hemO* and *hmbR*. Surprisingly, despite the presence of the $P_{hemO}1$ promoter, the R1203 fusion exhibited low transcriptional activity similar to that of the weak promoters (~6% of the strain O1101 activity), suggesting that the transcriptional read-through from *hemO* to *hmbR* was repressed or prematurely terminated. To ensure that the difference was not due to the location of the reporter fusions at a second site, we generated reporters at the native *hemO-hmbR* locus. The *lacZ-erm* cassette was inserted into the unique BamHI site in the *hemO* coding sequence to generate strain O101 and into the unique HincII site in *hmbR* to generate strain R103 (Fig. 6A). The significant difference in transcriptional activity between strains O1101 and R1203 was also observed for the corresponding strains O101 and R103 with insertions at the native locus (Fig. 6B), confirming that *hemO*-directed expression of *hmbR* was limited. Further, the transcription of *hmbR* detected in the R103 strain was not growth phase dependent (see Fig. S2 in the supplemental material), which ruled out the possibility that the change was caused by activities measured at different growth phases.

In order to further understand the regulatory mechanisms of the MisR/S system for controlling *hmbR* expression, we also compared the extents of *hemO-hmbR* transcription that were affected by the *misS* mutation. The reporter fusions were introduced into the *misS* mutant background. As shown in Fig. 6B, ~5-fold reductions were seen for all reporter fusions in the Δ*misS* background compared to the levels observed for the wild-type background. These reductions correlated with the qRT-PCR data and clearly demonstrated that the MisR/S system functions as a positive regulator of both *hemO* and *hmbR* expression. Introduction of the O2002 reporter fusion yielded identical activities in the wild-type strain and the *misS*

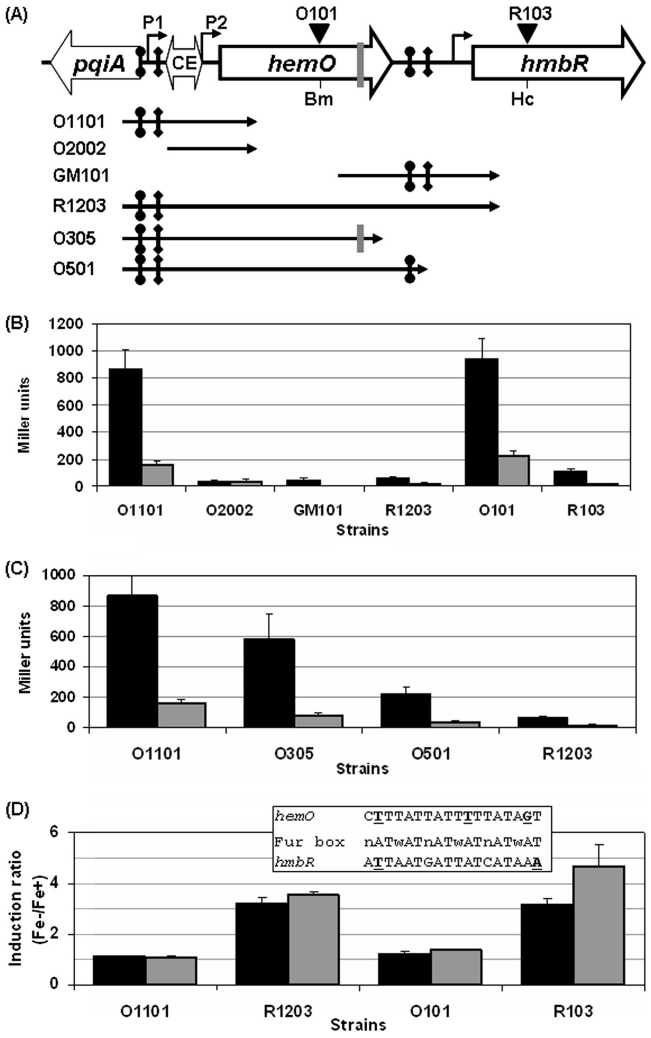

FIG. 6. (A) Schematic diagram of the *hemO-hmbR* locus. The MisR binding sites are indicated by vertical lines with circles at the ends, while the Fur binding sites are indicated by vertical lines with diamonds at the ends. The location of clustered repeats is indicated by a gray vertical line. The BamHI (Bm) and HincII (Hc) sites used for inserting the *lacZ-erm* cassette to generate the *hemO*::*lacZ* (O101) and *hmbR*::*lacZ* (R103) fusions, respectively, at the native locus are also indicated. The DNA fragments used to generate transcriptional *lacZ* reporter fusions at a permissive ectopic genomic location are indicated by black arrows. The schematic diagram is not drawn to scale. (B) Promoter activities of *hemO*::*lacZ* (O1101 and O2002) and *hmbR*::*lacZ* (GM101 and R1203) reporter strains integrated into a permissive chromosomal locus or the native locus (O101 and R103). Black bars, wild-type background; gray bars, *misS* background. (C) Comparison of reporter activities with various promoters. Black bars, wild-type background; gray bars, *misS* background. (D) Differential activation of *hemO*::*lacZ* (O1101 and O101) and *hmbR*::*lacZ* (R1203 and R103) fusions by iron limitation. Induction ratios were determined by dividing the promoter activity of the iron-depleted culture by the promoter activity of the iron-replete culture. Black bars, wild-type background; gray bars, *misS* background. (Inset) Alignment of the *hemO* Fur box and the best *hmbR* Fur box with the Fur box consensus sequence. Mismatched nucleotides are underlined and in bold type.

mutant because its promoter fragment did not contain the MisR binding site (Fig. 6A) and thus did not respond to the *misS* mutation.

To map the sequence motif conferring the reduction in transcriptional read-through, the sequence cloned in the R1203 reporter strain was analyzed, but no potential stem-loop-forming secondary structure that could function as a transcriptional terminator was detected (data not shown). A ~30-bp sequence near the 3′ end of the *hemO* coding sequence contains a clustered direct repeat, a dyad repeat, and a short inverted repeat, and thus a reporter strain, O305, was constructed to assess the influence of these motifs. Since the MisR and Fur binding motifs located in the *hemO-hmbR* intergenic region were also present in the R1203 reporter strain, another strain, O501, containing the MisR binding site but not the Fur binding site, was generated to examine the effect of these binding sequences (Fig. 6A). All of these fusions included the strong $P_{hemO}1$ promoter. As shown in Fig. 6C, both strain O305 and strain O501 displayed activities intermediate between those of strains O1101 and R1203. The activity of the O305 strain was reduced modestly (~68%) compared to that of strain O1101 and was ~11-fold higher than that of the R1203 strain, suggesting that the various repeats near the 3′ end of the *hemO* coding sequence were not the major factors interfering with transcriptional read-through. The activity of the O501 strain was ~25% of that of strain O1101 and was ~4-fold higher than that of strain R1203, implying that the MisR binding motif (as reflected in the strain O501 data) and the Fur box motif (as reflected in the strain R1203 data) in the *hemO-hmbR* intergenic region may have similar negative effects on the overall *hmbR* expression. However, the fact that a parallel gradual reduction in transcriptional activity from strain O1101s to strain 305s to strain 501s to strain R1203s was detected in the *misS* background (Fig. 6C) implied that the much reduced transcriptional activity in strain O501 compared to that in strain O1101 was not likely due to the MisR/S system. It is commonly believed that in a histidine kinase mutant a certain amount of nonphosphorylated response regulator is expressed. Thus, the remaining nonphosphorylated MisR in the *misS* mutant could elicit the negative effect. If this was the case, a difference in *hmbR* transcription was expected for the *misR* mutant compared to the *misS* mutant and the MisR/S system would function as a negative regulator of *hmbR*. However, the qRT-PCR data showed that the *misS* and *misR* mutations resulted in similar decreases in *hmbR* expression, suggesting that the nonphosphorylated MisR was not likely to contribute to the repression of transcriptional coupling of *hemO* and *hmbR*.

Fur elicited stronger iron-mediated repression of *hmbR* expression than of *hemO* expression. Because the absence of the *hmbR* Fur box in reporter strain O501 resulted in a 4-fold increase in *hmbR* expression (when O501 was compared to R1203) and another putative Fur box was present upstream of *hemO*, we also examined the effect of iron availability on the promoter activities of various reporter strains using a 45-min treatment with dipyridyl to generate iron-depleted conditions. As determined by qRT-PCR, the same treatment resulted in 4-fold and 2-fold induction of *hmbR* and *hemO* expression, respectively (Fig. 1). Surprisingly, although we indeed detected ~3-fold induction of the *lacZ* reporter activities of strains R1203 and R103 upon iron starvation (Fig. 6D), very limited induction was seen in reporter strains probed for *hemO* expression (O1101 and O101), which was consistent with the qRT-PCR pattern, in which a lower level of iron induction was

observed for *hemO*. The lack of strong iron-dependent induction of *hemO* suggests that iron-loaded Fur has limited binding affinity for the putative Fur box in the *hemO* promoter region or, alternatively, that insertion of a Correia element downstream interfered with the regulatory function of Fur. The reduced Fur repression accounts for the significantly higher level of *hemO* expression in iron-replete growth conditions used in standard reporter assays. In comparison, stronger repression of *hmbR* transcription that correlated with a probable higher affinity of Fur for the *hmbR* Fur boxes could result in a higher induction ratio for *hmbR* upon iron starvation. The diminished transcriptional read-through from *hemO* could potentially be caused by the binding of Fur to the *hmbR* Fur boxes. However, it is also possible that the mRNA stability of various reporter transcripts (i.e., posttranscriptional regulation) accounts for the difference in the apparent promoter activities. Alternatively, cryptic premature transcriptional termination may be responsible for the decrease in the amount of *hmbR* mRNA transcribed from *hemO*.

To examine whether the level of Fur repression was affected by the MisR/S system due to the close proximity of the binding motifs in the *hemO* and *hmbR* promoter regions, reporter strains with the *misS* background were treated similarly with or without dipyridyl, and the induction ratios (Fe negative/Fe positive) were determined. Reporter fusions for *hemO* and *hmbR* at either the native locus or the irrelevant site were examined. If Fur elicited stronger repression in the absence of MisR/S regulation, a higher induction ratio was expected for the *misS* mutant. As shown in Fig. 6D, there were not significant differences between the induction ratios for the wild-type strains and the *misS* mutants. Thus, it appeared that the MisR/S system-dependent activation and the iron-mediated Fur repression act independently.

DISCUSSION

Iron acquisition is essential for successful colonization and infection by many bacterial pathogens, and *N. meningitidis*, an obligate human pathogen, is capable of utilizing a diverse array of host iron sources, including hemoglobin. Two hemoglobin acquisition receptors, HmbR and HpuAB, have been characterized in *N. meningitidis*. The lack of *hmbR* in commensal organisms (38) and the characteristics of a pathogenicity island of the *hmbR* locus (17, 23) suggest that *hmbR* was acquired more recently by meningococci. It is believed that the functional redundancy and the ability to regulate expression of important virulence factors allow bacterial pathogens to minimize their exposure to immune recognition and responses. Hemoglobin receptors play an important role in the virulence of both Gram-negative and Gram-positive bacterial pathogens. For example, the HgbA hemoglobin receptor of *Haemophilus ducreyi* is required for virulence in both human and animal models of chancroid (2, 46), and *Staphylococcus aureus* mutants lacking the IsdB hemoglobin receptor display attenuated virulence in a murine model of abscess formation (49). The important contribution of *hmbR* and hemoglobin utilization to meningococcal virulence has been experimentally demonstrated by the findings that acquisition of iron via HmbR enhanced the ability of meningococci to replicate in the bloodstream of infant rats and the *hmbR* mutant was attenuated in an infant rat model of meningococcal infection (47). A recent investigation of the distribution of the *hmbR* gene among more than 700 disease and carriage meningococcal isolates from three separate isolate collections revealed a statistically significant association between disease and the presence of *hmbR* (17). In addition, all isolates belonging to six hyperinvasive lineages are *hmbR* positive (17). Thus, this study provides epidemiological evidence for the importance of *hmbR* in meningococcal virulence.

The regulation of Hb receptors in meningococci has been observed at two levels; one mechanism is phase variation (38) (a translational on-off switch), and the other is transcriptional regulation by Fur in response to iron availability (48). *hmbR* is downstream of *hemO*, and potential Fur box sequences can be identified in both *hemO* and *hmbR* promoter regions. Several experimental findings have documented the independent transcription of *hmbR* and the direct interaction of the *hmbR* promoter region with Fur. First, a Fur titration assay with *E. coli* showed that when multiple copies of the *hemO-hmbR* intergenic region were present, they were able to titrate Fur away from a Fur-regulated reporter gene (47). Second, purified gonococcal Fur proteins bind to the upstream region of *hmbR* (44), and DNase I protection assays have mapped the Fur binding site of the *hmbR* promoter region, which overlaps the predicted promoter element (10). Using *lacZ* reporter assays, we showed that the *hemO-hmbR* intergenic region has weak promoter activity and that both Fur and the MisR/S system exhibit direct regulation of the promoter. However, *hmbR* was repressed more strongly than *hemO* based on the different iron induction ratios (Fig. 6D). Sequence analysis using the Fur binding motif (15) with a maximum of three mismatches identified a single putative Fur box sequence upstream of *hemO* (Fig. 2B), while four potential Fur boxes clustered upstream of *hmbR* (Fig. 2C). Alignment of the single *hemO* Fur box and the best *hmbR* Fur box with the consensus sequence (Fig. 6D, inset) indicated that the *hemO* Fur box has an additional mismatch at the center of the motif. It is not clear whether this mismatch contributes to the weak repression of *hemO* or the clustering of several Fur boxes in the *hmbR* promoter region enhances the binding affinity of Fur for this region.

As meningococci reside in iron-restricted host environments, repression by Fur would likely be alleviated in many situations, and it is likely that there are other regulatory mechanisms that respond to different host microenvironments to control expression of important virulence factors. In the present study, we demonstrated that the transcription of *hmbR* and *hemO* is also regulated by the MisR/S two-component system. The results of the disk diffusion growth assays with Hb as the sole iron source support the hypothesis that the MisR/S system has an important role in Hb utilization. The strains were grown under iron-limiting conditions in the presence of the iron chelator Desferal, and relief of Fur repression is expected under these growth conditions. We detected phenotypic alteration of Hb utilization resulting from mutations in the MisR/S two-component system in meningococcal strain NMB that encodes the HpuAB receptor, as well as in the *hmbR*^{off} (NMB and IR3261) and *hmbR*^{on} (IR3287) backgrounds, indicating that the transcriptional control of *hmbR* by the MisR/S

system is an important regulatory mechanism in meningococcal Hb utilization.

Transcriptional coupling of *hemO* and *hmbR* was detected. A full-length Correia element is present upstream of *hemO* in all sequenced meningococcal genomes, as well as in the genomes of additional 15 strains analyzed by De Gregorio et al. (8); however, a CE is not present in the gonococcal FA1090 genome and in *Neisseria lactamica* strains (8). Correia elements account for ~2% of the *N. meningitidis* genome and are frequently inserted close to open reading frames (ORF). They fall into two major size classes, and a full-length element (154 to 158 bp) contains a 50-bp internal segment. The presence of CE may impact the expression of nearby downstream ORF in several ways. First, insertion of CE introduced a TA duplication and resulted in potential divergent outward promoters at the ends of elements (5). This has been demonstrated for *urvB* (4) and *lst* (36) in *Neisseria gonorrhoeae*, and we also identified a weak *hemO* promoter potentially associated with the duplicated TA dinucleotides. Second, in the unique 50-bp internal segment of the full-length element, a functional IHF binding site has been shown to interact with purified IHF proteins (5, 40). The IHF binding sequence in the *hemO*-associated CE is identical to that analyzed by Buisine et al. (5), which has been reported to have an apparent dissociation constant of ~5 nM (5). IHF is a DNA binding protein that induces DNA bending and is associated with regulation of a broad range of cellular functions, including DNA replication, recombination, and transcription (12). Deletion of the IHF binding site in the CE located upstream of the *mtrCDE* operon encoding an efflux pump in meningococci (40) enhanced expression of *mtrC* (40). As the *hemO*-associated CE is nearly identical to the CE located upstream of *mtrC*, similar IHF-mediated regulatory controls are likely for *hemO* expression. Finally, CE-containing transcripts were processed by RNase III, resulting in posttranscriptional regulation of the associated gene. Primer extension identified a strong promoter upstream of the CE, confirming that the CE is cotranscribed with *hemO*, and similarly processed transcripts were also detected, implying that there is possible posttranscriptional regulation of *hemO-hmbR* transcripts by RNase III.

We identified two MisR binding motifs, one located upstream of the $P_{hemO}1$ promoter and the other located upstream of the proximal P_{hmbR} promoter in the *hemO-hmbR* intergenic region. qRT-PCR experiments with *hmbR*-specific and *hemO*-specific primers, as well as *lacZ* reporter assays for either a permissive chromosomal site or the native locus, clearly showed that inactivation of the MisR/S system resulted in significantly decreased transcription of both *hemO* and *hmbR*. The MisR/S two-component system functions as an independent activator because without the positive regulatory input of the MisR/S system, the level of *hemO* and *hmbR* transcription did not reach the maximal level when Fur repression was alleviated by iron starvation. When meningococci encounter other iron sources and are able to obtain sufficient iron from the environment to elicit Fur repression of iron assimilation proteins, including the HmbR receptor, the MisR/S two-component system is positioned to upregulate *hmbR* and *hemO* expression when the activating signal is sensed, thus ensuring appropriate hemoglobin utilization capacity in certain host environments. Iron acquisition systems with secondary regulatory pathways that respond to particular iron-independent environmental signals and operate in concert with Fur have been described for several bacteria. The PchR regulator, which responds to extracellular pyochelin, regulates siderophore expression in *Pseudomonas aeruginosa* (18). In addition, the extracytoplasmic sigma factor has been demonstrated to control uptake systems for various iron sources, such as siderophores and hemin, in *E. coli* (3), *Pseudomonas putida* (24, 54), and *Bordetella pertussis* (53), in response to an extracytoplasmic inducing signal.

Interestingly, we observed a significant reduction in transcriptional read-through that minimized *hemO*-initiated *hmbR* expression. This was most likely mediated by an unidentified mechanism(s) operating near or in the *hemO-hmbR* intergenic sequence, since reporters carrying the intergenic sequence showed significantly decreased activities. The activities of various transcriptional *lacZ* reporter constructs in the wild-type and *misS* backgrounds suggested that binding of the MisR protein is not involved, while the involvement of Fur has not been tested. Decreased stability of *hemO-hmbR* mRNA upon RNase III processing might also contribute to or be responsible for the reduction. Another alternative explanation is premature Rho-dependent intracistronic transcription termination that was first described for another meningococcal phase-variable gene, *siaD* (*synD*) (26). It was reported that only ~5% of *siaD* mRNA was detected in unencapsulated variants (*siaD* phase off) compared to the encapsulated parental strain (16) because transcription of the out-of-frame *siaD* gene is prematurely terminated at a cryptic Rho-dependent site, a sequence segment with a higher cytosine content than guanosine content termed the transcription termination element, which is the target of Rho and is usually located upstream of multiple actual termination sites (1, 39). Further, this transcription elongation termination mechanism influences the phase variation frequency (26). As *hmbR* is phase off in strain NMB, it is probable that a similar Rho-dependent intracistronic termination mechanism operates on *hemO-hmbR* transcription and affects the overall *hmbR* expression.

Coordinated regulation of the heme catabolism protein and the heme uptake receptor is beneficial. Since excess heme is potentially toxic, downregulation of heme uptake when the heme catabolism capability is disrupted or reduced may be one of the mechanisms by which meningococci defend against heme toxicity. The transcriptional coupling of *hemO* and *hmbR* provides a means for such coordination. *hemO* is, however, transcribed at a much higher level than *hmbR* as a result of the reduction in transcriptional read-through. This correlates with the greater need for heme metabolism than for heme uptake. In addition, the immunogenic nature of the HmbR outer membrane receptor requires that it is absent (phase off) or expressed at a low level when it is not needed, while the cells require a certain amount of the heme-degrading enzyme, HemO. Another type of coordinated regulation is iron-mediated Fur-dependent repression, as indicated by the fact that transcription of both genes is induced under iron-limiting conditions and, again, weaker repression by Fur resulted in greater transcription of *hemO* in the iron-replete environment. This is consistent with the presence of other heme uptake mechanisms in meningococci. Finally, a third type of coordinated control of the two genes is mediated by the MisR/S two-component sys-

tem since MisR interacts directly with both *hemO* and *hmbR* promoters and expression of both genes decreased when the MisR/S system was inactivated. Despite the transcriptional linkage with *hemO*, the significant reduction in transcriptional read-through into *hmbR* might necessitate independent activation of the considerably weaker *hmbR* promoter by the MisR/S system, and the possible different affinities of MisR for *hemO* and *hmbR* promoters can differentially activate their expression. One can envision that higher-affinity binding of MisR to *hemO* than to *hmbR* ensures the presence of sufficient heme-degrading HemO prior to induction of the HmbR heme-hemoglobin uptake system. Finally, additional regulatory mechanisms due to insertion of the Correia element upstream of *hemO*, including the potential interaction with IHF and posttranscriptional processing by RNase III, as well as the potential Rho-dependent premature transcriptional termination mechanism influencing the phase variation frequency, further demonstrated that there is a complex regulatory network controlling the expression of *hemO* and *hmbR*, which allows regulation by various environmental signal inputs.

As the MisR/S system is not induced by hemoglobin, the rationale for specific positive regulation by the MisR/S two-component system awaits identification of the signal(s) activating this system. The intracellular phase of infection is not likely when enhanced *hmbR* expression is needed, because a meningococcal *hmbR* mutant has been shown to replicate more rapidly in epithelial cells than its wild-type parental strain (25). In addition, hemoglobin utilization in gonococci was shown to be dispensable for colonization of the female mouse genital tract (21). Hemoglobin is not readily accessible to meningococci due to its compartmentalization in erythrocytes, and the low level of Hb found in normal human serum as a result of spontaneous hemolysis is rapidly captured by haptoglobin (43). However, during the invasive phase of infection, local hemolysis may make free Hb available for assimilation. Thus, when a particular iron-limiting host environment is reached during infection, derepression of *hmbR* by Fur after the lack of iron is sensed might not be sufficient, and meningococci need to further increase the expression of *hmbR* through activation of the MisR/S two-component system, possibly to allow prompt adaptation to the new environment.

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