MisR/MisS Two-Component Regulon in *Neisseria meningitidis*†‡

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Two-component regulatory systems are involved in processes important for bacterial pathogenesis. Inactivation of the *misR/misS* system in *Neisseria meningitidis* results in the loss of phosphorylation of the lipooligosaccharide inner core and causes attenuation in a mouse model of meningococcal infection. One hundred seventeen (78 up-regulated and 39 down-regulated) potential regulatory targets of the MisR/MisS (MisR/S) system were identified by transcriptional profiling of the NMB0114/NMB0115 mutant and the parental wild-type meningococcal strain NMB. The regulatory effect was further confirmed in a subset of target genes by quantitative real-time PCR and β-galactosidase transcriptional fusion reporter assays. The MisR regulon includes genes encoding proteins necessary for protein folding in the bacterial cytoplasm and periplasm, transcriptional regulation, metabolism, iron assimilation, and type 1 protein transport. Mutation in the MisR/S system caused increased sensitivity to oxidative stress and also resulted in decreased susceptibility to complement-mediated killing by normal human serum. To identify the direct targets of MisR regulation, electrophoretic mobility shift assays were carried out using purified MisR-His6 protein. Among 22 genes examined, *misR* directly interacted with 14 promoter regions. Six promoters were further investigated by DNase I protection assays, and a MisR-binding consensus sequence was proposed. Thus, the direct regulatory targets of MisR and the minimal regulon of the meningococcal MisR/S two-component signal transduction system were characterized. These data indicate that the MisR/S system influences a wide range of biological functions in *N. meningitidis* either directly or via intermediate regulators.

Two-component regulatory systems are one of the most common bacterial signal transduction mechanisms controlling responses and adaptation to environmental changes (17). Many such systems act as global regulators in coordinating the expression of virulence determinants in bacterial pathogens. Often, these systems are composed of an inner membrane-bound histidine kinase that, upon sensing specific signals, undergoes autophosphorylation at a conserved histidine residue and a cytoplasmic response regulator partner that receives the phosphoryl group at an invariant aspartate residue (16) which consequently modulates DNA-binding activity.

*Neisseria meningitidis* is an obligate pathogen which inhabits the human nasopharynx but can rapidly disseminate to cause sepsis and meningitis during invasive infection (50, 57). Likely because of the restricted habitat of the meningococcus, the organism has a relatively small genome and few two-component systems, only four predicted pairs (39, 52), compared to other pathogens experiencing more-complex environments (36, 66). The persistence of these few functional two-component regulatory systems implies an important role in regulating meningococcal colonization and virulence. Sequence comparison suggests that one system (NMB0114/NMB0115) shares amino acid sequence similarities to NtrY/NtrX (COG5000/COG2204 domain family), which regulates nitrogen metabolism in *Azorhizobium caulinodans* (40), while the second pair, NMB1606/NMB1607, shares homology with pilS/pilR, which regulates piliation in *Pseudomonas aeruginosa* (15). The NMB1249/NMB1250 two-component system exhibits amino acid sequence similarities with NarQ/NarP, and the equivalent gonoococcal system has been shown to respond to anaerobic growth (30, 38). We have reported that inactivation of the fourth two-component system, encoded by NMB0595/NMB0594, designated *misR/misS*, results in the loss of phosphoethanolamine (PEA) substitutions on the lipooligosaccharide (LOS) inner core, with an increased sensitivity to cationic antimicrobial peptides (58). LOS or endotoxin is a major virulence factor of *N. meningitidis*, and structural changes in LOS are important in meningococcal pathogenesis. In addition, a *misR* response regulator mutant of a serogroup C meningococcal strain is avirulent in a mouse model of infection (34).

Microarray transcription profile comparison has frequently been used to obtain a general picture of a particular transcriptome. However, this strategy does not allow the delineation of direct versus indirect regulatory targets. A microarray study of the serogroup C *misR* mutant reported previously by Newcombe et al. (35) used RNAs isolated from cells grown on blood agar. A total of 281 genes were identified as being significantly up- or down-regulated in the mutant compared to those in the parent strain. However, these microarray observations were not validated by other biochemical or genetic means, nor were the direct regula-

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that the misRS operon is under the control of autoactivation, and this has been the only proven direct target of MisR (60). As phenotypic differences have been noted between the serogroup C misR mutant and the serogroup B misR mutant studied by our group (21, 35, 58, 60), we carried out transcriptional profile analyses of the NMBmisR mutant to independently identify MisR-regulated genes. Real-time reverse transcription (RT)-PCR and reporter assays were performed to confirm the potential regulatory effects of MisR on genes identified by microarray and to define the minimal regulon. Further, direct regulatory targets of MisR were identified by electrophoretic mobility shift assay (EMSA), and the MisR-binding sequences of several target genes were mapped by DNase I protection assays. With curation of the in vitro biochemical data and the bioinformatic analysis of the binding sequences, a MisR-binding motif is proposed.

**MATERIALS AND METHODS**

**Bacterial strains, medium, and reagents.** The strains and plasmids used in this study are listed in Table 1, while the primers used in this study are listed in Table S3 in the supplemental material. Meningococcal strain NMB (CDC 8201085) is a serogroup B N. meningitidis strain originally isolated from the cerebrospinal fluid of a patient with meningococcal meningitis in Pennsylvania in 1982. Meningococcal strains were grown with 3.5% CO2 at 37°C unless specified otherwise. Gonococcal strains were as follows: ampicillin, 100; kanamycin, 80; and erythromycin, 3.

**Construction of the misRS double mutant and complementation of the misRS mutation.** A 737-bp PCR product containing the 5′ region of misR and another 684-bp PCR product containing the 3′ region of misS were amplified separately.
using primer pairs YT174/HindIII-YT175/Smal and YT177/Smal-YT178/EcoRI. Two PCR products were digested with their respective restriction enzymes and then ligated with HindIII-EcoRI-restricted pUC18, yielding pYT334. The correct plasmid carrying the newly created Smal site was confirmed by restriction digestion and sequencing analysis. The apfA-3 cassette released from pUC18K by EcoRI-BamHI digestion and Klenow treatment was inserted into the Smal site of pYT334, yielding pYT336. Removal of the misR and misS internal sequence and the presence of a correctly oriented apfA-3 cassette in the resulting pYT336 plasmid were confirmed by a panel of colony PCRs and sequencing analysis. This construct removed most of the misR and misS sequences and contained the kanamycin resistance gene. To generate the meningococcal misRS mutant, pYT336 was linearized with Scal and the digestion mixture was used to transform the meningococcal strains. Kanamycin-resistant colonies were selected, and the mutation was confirmed as described above.

The pJK2DS81 plasmid (C. M. Kahler and J. Davies, unpublished data), which contains the Hermes-inducible expression cassette (27) with the spectinomycin marker flanked by gfp sequences on a low-copy suicide vector with a pSC101 origin, was used to complement the misR mutation. The coding sequence of misR was amplified using primers cspxRP and cspxRB containing flanking BamHI sites and cloned into the HincII site of pWSK29 (61) to create pJK2DS82. Both pJK2DS82 and pJK2DS81 were digested with BamHI and ligated, and chloramphenicol-resistance transformants, named pJK2DS83, were selected for further analysis. The pJK2DS83 plasmid contained the misR gene under the control of the RpsL promoter. MscL-linearized pJK2DS83 was used to transform meningococcal strain NMB, and spectinomycin-resistant transformants were isolated. A panel of PCR amplifications was performed on the resulting strain, CMK30, to confirm integration of the Hermes-misR expression cassette into gfp. DAP319 and DAP319F were used for the left border fragment DAP227 and DAP227F for the right flank, and DAP276 for the internal part of the cassette. Due to the difficulty of transforming the misR mutant, the misR::apfA-3 mutation was introduced into CMK30 and SpfKmRm transforms were selected. A panel of PCRs was done to ensure the presence of the misR::apfA-3 mutation at the white-type locus and that the second copy of the intact misR gene remained at the gfp locus.

Microarray. Bacterial strains grown in supplemented GC broth to mid-exponential phase were collected, and the total RNA was extracted using an RNeasy mini kit (Qiagen) according to the protocol recommended by the manufacturer. The RNA samples were further treated with DNase for 1 h at 37°C to remove contaminating chromosomal DNA and then purified through phenol-chloroform extraction and ethanol precipitated. The final RNA preparations were used as templates in standard PCR amplification of an unrelated chromosomal region to ensure the absence of DNA contamination. Reaction mixtures containing 40 ng total RNA and 0.1 pmol of a mixture of 3'-end-specific primers, which were used in generating the PCR products for printing slides (Eurogentec), were denatured at 90°C for 10 min, and then the primers were allowed to anneal to RNA on ice. Cy3- and Cy5-labeled cDNAs were generated using a CyScribe first-strand cDNA labeling kit (Amersham Pharmacia Biotech) and then purified by a QiAquick PCR purification kit (Qiagen). The percentage of incorporated label was estimated using the following extinction coefficients: DNA (6,000 M<sup>-1</sup> cm<sup>-1</sup> at an optical density at 260 nm [OD<sub>260</sub>]), Cy3 (150,000 M<sup>-1</sup> cm<sup>-1</sup> at OD<sub>280</sub>), and Cy5 (250,000 M<sup>-1</sup> cm<sup>-1</sup> at OD<sub>280</sub>). The labeled cDNAs were then mixed together prior to hybridization to the array slides. A prehybridization step was performed by incubating the microarray slide in a solution of 2.5 μL of salmon sperm DNA (10 mg/ml) diluted in 47.5 μL of Dig Easy solution (Roche) under a coverslip in a humidified chamber (Corning) for 1 h at 42°C. After prehybridization, the slide was rinsed in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.03 M sodium citrate) buffer, water, and then isopropanol and dried by brief low-speed centrifugation. The probes, resuspended to a total of 10 μL, were mixed with 3 μL of sperm DNA (10 mg/ml) and the solution was denatured at 95°C for 2 min and cooled on ice before the addition of 20 μL of Dig Easy solution. The hybridization was performed at 42°C overnight. The slide was washed in 0.2× SSC-0.1% sodium dodecyl sulfate for 5 min at 42°C, followed by consecutive rinses in 0.2× SSC-0.1% sodium dodecyl sulfate for 10 min at room temperature (two times) and 0.1× SSC for 5 min at room temperature (two times). The slide was dried by brief low-speed centrifugation and then scanned with a GMS 428 array scanner (Genetic Microsystems). Data analyses were performed with both Jaguar software (Affymetrix) and ImagiGene/Genesight software (Bio-discovery), which allow automatic gridding of the array and an attached database for flagging nonspecific spots that can be removed from later analyses. Measuring signal intensities detected for both fluorescent dyes and calculating the signal ratio determine relative amounts of a particular gene transcript in the two samples. Signal ratios of fluorescence were generated from each spot and then analyzed by Genesight (BioDiscovery). The standard twofold cutoff was used as a minimum value for induction and repression. The array glass slide (Eurogen-tech) contains 2,191 open reading frames (ORFs) of the serogroup A strain Z2491 and 73 specific ORFs of the serogroup B strain MC58 that were printed in duplicate. This represents ∼98% of all ORFs of the serogroup A and B genomes. One array analysis with fluorescence labels swapping between test and reference cdNA was done as a technical control, and three independent sets of wild-type and mutant RNA preparations were studied.

Construction of reporter strains and β-galactosidase reporter assays. The promoter fragments were obtained by PCR amplification and cloned into pCR2.1 by using a TOPO-TA cloning kit (Invitrogen). The inserts were released by EcoRI digestion, gel purified, and then ligated with pYT328, which had been digested with EcoRI and treated with shrimp alkaline phosphatase. Colony PCR using primer YT168, an outward primer at the 5’ end of lacZ, and the forward primer within the promoter identified clones with the correct orientation of the promoter to the lacZ gene. The NcoI-linearized plasmid constructs were used to transform the wild-type meningococcal strain NMB and the NMBmisS mutant. A panel of colony PCRs with a chromosome-specific primer and the lacZ-specific primer confirmed allelic exchanges into the expected locus. The wild-type reporter strains were transformed using the plate transformation method (20), with chromosomal DNA isolated from the NMBmisS mutant to generate reporter strains with the misRS double mutation. Transformants resistant to both erythromycin and kanamycin were selected on brain heart infusion plates and confirmed by colony PCR. The β-galactosidase activity assays were performed as described previously (60).

Protein purification and Western blots. The purification of the MisR-His protein and the Western blot procedure using polyclonal sera against the MisR-His protein have been described previously (60).

EMSAs and DNase I protection assay. Procedures for the EMSA and DNase I protection assay have been reported previously (60). Briefly, probes for the EMSA were generated by T4 kinase-mediated γ<sup>32</sup>P end labeling of PCR promoter fragments and purified using the QIAquick PCR purification kit (Qiagen). The binding reactions for mixtures consisting of 5 fmol labeled DNA and various amounts of protein in a binding buffer (20 mM HEPES, pH 7.9, 60 mM KC1, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM dithiothreitol, 0.3 mg/ml bovine serum albumin, 0.2 mg/ml salmon sperm DNA, and 10% glycerol) were carried out at 30°C for 20 min and analyzed with 6% polyacrylamide gels. The gels were dried under vacuum without prior fixing, and the resulting radioactive electrophoretic patterns were analyzed with a PhosphorImager (Molecular Dynamics). The binding reactions were examined with phosphorylated (50 μM acetyl phosphate for 5 min at 37°C) or nonphosphorylated MisR. Competition with excess specific competitors (unlabeled probes) and nonspecific competitors, a 593-bp internal coding sequence of misR, obtained by PCR amplification using primers YT45 and YT46, was performed to assess the specificity of the interaction.

For the DNase I protection assay, primers were end labeled using T4 polynucleotide kinase and γ<sup>32</sup>P and purified with the QIAquick nucleotide removal kit (Qiagen). PCR fragment primers were designed to contain a SmaI restriction site after the radionabeled primer and the corresponding reverse primer, and the labeled DNA products were purified with the QIAquick PCR purification kit. Using the binding conditions defined in the EMSA studies, the DNA-protein complex in the binding buffer without salmon sperm DNA was treated with diluted DNase I for 1 min at room temperature before being quenched with a phenol-chloroform extraction. Nucleic acids were recovered by ethanol precipitation and analyzed on 6% sequencing gels along with a sequencing ladder obtained with the same labeled primer.

Real-time quantitative RT-PCR assay. Reverse transcriptions were carried out with a GeneAmp kit by using 1 μg of total RNA according to the procedure suggested by the manufacturer (Applied Biosystems), and reactions without the reverse transcriptase were used as a negative control. The double-strand DNA-binding dye Sybr green detection method was employed to quantify the amount of mRNA from the RT reactions, and the assay conditions have been described previously (58). The primer sets that are listed in Table S3 in the supplemental material. All primer sets have been confirmed to yield similar amplification efficiencies. Melting curve analyses were performed following each RT-PCR experiment to ensure that each reaction mixture contained only a single specific product. rpsE encoding the constitutively and highly expressed meningococcal 30S ribosomal protein was used in each experiment as an endogenous reference gene for normalization. RT negative-control reactions were also analyzed by real-time RT-PCR to confirm that RT reactions were free of contaminating chromosomal DNA. The relative changes were calculated using the 2<sup>-ΔΔC<sub>T</sub></sup> method (32). Each set of RT-PCRs was examined in triplicate and was repeated with at least two independent RNA preparations. Student’s t test with a two-tailed hypothesis was used to determine the significant difference (P < 0.01) between two variables in this study.
Oxidative stress sensitivity assay and serum bactericidal assay. The sensitivities of meningococcal strains to hydrogen peroxide (H₂O₂) and paraquat were determined by disk diffusion growth inhibition assays. Briefly, cells from plate-grown cultures were suspended in GC broth to an OD₆₀₀ of 0.15. Fifty microliters of cell suspension was mixed with 5 ml of GC soft agar (GC broth with 0.5% agar) kept at 45°C, and the mixture was poured over a plate containing 15 ml of solidified GC agar. Disks of 6 mm diameter were placed onto soft agar, and 5-μl aliquots of tested agents were spotted on disks. Plates were incubated at 37°C for 24 h, and zones of growth inhibition were measured. Each condition was tested with three disks, and the assays were repeated three times. The procedure for the serum bactericidal assay has been described previously (24). The expression of Prce::misR in the complemented strain was induced by 1 mM IPTG (isopropylβ-D-thiogalactopyranoside) in broth culture.

Microarray analyses indicated that either MisR has a dual function as a repressor or alteration in the expression of several putative transcriptional regulators results in indirect MisR regulation. However, indirect transcriptional changes mediated by intermediate regulators under the control of misR and those caused by growth rate differences cannot be distinguished.

Characteristics of potential MisR regulatory targets. As shown in Table 2, the group of protein folding/stress response genes included dnaJ, fkpA, hslO, grpE, htpX, clpB, and dsbD. The significant changes observed in the transcription of dsbD and several genes involved in the general stress response may explain the pleiotropic effect of the misR mutation on growth. DsbD is an inner-membrane thioldisulfide interchange protein that maintains the periplasmic disulfide isomerase DsbC in its active form by using the reducing power from cytosolic thioredoxin (22); thus, the decreased expression of DsbD is expected to result in the accumulation of misfolded proteins in this compartment. ClpB is part of the Clp protease complex, which operates in concert with DnaJ/DnaK/GrpE chaperones to mediate the refolding and degradation of misfolded proteins in the cytoplasmic compartment of the cell. F kpA has homology to the C-terminal domain of the periplasmic F K506 binding protein-type peptidyl-prolyl cis-trans isomerase in E. coli and is transcribed divergently from hpr4 encoding a glycerate dehydrogenase, also up-regulated in the misR mutant. A gene cluster consisting of fkpA located upstream of eight pilS cassette was shown to up-regulate (see Table S1 in the supplemental material) in the array analysis. HslO, or Hsp33, belongs to a family of redox-sensing cytoplasmic heat shock proteins (Hsp33) (1, 19) and works with DnaJ/DnaK/GrpE to support the refolding of the substrate proteins (18). HtpX is a membrane-bound zinc metalloprotease with a cytosolic active site (47).

The next group of genes identified as potential MisR regulators includes three up-regulated putative transcriptional regulators and the misRS operon (60). NMA0738 contains domains that are found in a family of repressor proteins that includes the phase CI repressor and LexA. Interestingly, NMA0738 is near dnaK (NMA0736) and is divergently transcribed from a conserved hypothetical protein, NMA0737, which was also up-regulated. NMA1593 has one Rrf2-type DNA-binding domain and may encode a putative IscR, an iron-sulfur binding transcription factor of the iron-sulfur cluster assembly system, which is located immediately downstream (NMA1594 to NMA1598). However, no changes were detected in the transcription of the iron-sulfur cluster assembly genes. Finally, NMA1751 contains domains that are well conserved in the GntR regulator family. The identification of transcriptional regulators under the control of MisR suggested that MisR can indirectly regulate the expression of additional genes.

The gene prpB, prpC, and acnA, which are clustered and whose products participate in the 2-methylcitric acid cycle, were significantly induced in the misR mutant. Whether these genes constitute an operon was not clear since the expression of NMA2053, located between prpC and acnA, was not altered. The up-regulation of the prp genes whose products are involved in catabolism of the short fatty acid, propionic acid, in the misR mutant suggested that MisR influenced the expression of this alternative metabolic pathway.

Microarray analyses indicated that the expression levels of

RESULTS

Microarray identification of MisR-regulated genes. To assess the scope of MisR regulation, the transcriptome of the mutant was analyzed by a whole-genome microarray. Commercially available meningococcal genomic microarrays (Eurogentec, Belgium) based on the serogroup A genome (39) were used. The serogroup A genome is 91.2% similar to the serogroup B MC58 genome (52). ORFs unique to the MC58 genome that are found within the three major putative islands of horizontally transferred DNA were also included in the array. One of the islands contains the genes encoding the serogroup B capsule biosynthesis and transport proteins, and the other two contain genes encoding mostly hypothetical proteins (52). The total RNA of the wild-type parent strain NMB and the NMBmisR mutant was isolated from cultures grown in standard GC broth. cDNAs were generated using the CyScribe first-strand cDNA labeling kit with a mixture of 3′-end-specific primers. The individually labeled cDNAs were purified, combined, and hybridized with the array slides as described in Materials and Methods. Three independent RNA preparations of both the wild type and the NMBmisR mutant were examined, including one dye swap comparison. Genes that exhibited >2-fold change in two of three microarray experiments were designated as potential regulatory targets of the MisR response regulator (see Tables S1 and S2 in the supplemental material). A total of 78 genes were up-regulated and 39 genes were down-regulated in the NMBmisR mutant. As the mutants displayed increased doubling time compared to that of the wild-type parent strain, which can be corrected when complemented with a second copy of misR (data not shown), transcriptional changes due to the growth difference between the mutant and the wild-type strain were likely to be present in the microarray comparison. However, this pleiotropic phenotype is a likely indication that the regulatory targets of the MisRS two-component system are involved in important physiological functions of N. meningitidis.

As the microarray results did not differentiate between direct and indirect regulatory roles of MisR, a panel of 25 MisR-regulated genes was selected for further detailed biochemical characterization and could be classified into five important functional categories: protein folding/stress response, regulation, metabolism, iron assimilation, and transport/secretion (Table 2). While 12 genes were down-regulated in the NMBmisR null mutant, indicative of an activator role for MisR, the expression levels of 20 genes were increased. This observation indicated that either MisR has a dual function as a repressor or alteration in the expression of several putative transcriptional regulators results in indirect MisR regulation. However, indirect transcriptional changes mediated by intermediate regulators under the control of misR and those caused by growth rate differences cannot be distinguished.

Capsule ELISA. Serogroup B capsule-specific immunoglobulin M monoclonal antibody 2-2-B was used in the whole-cell enzyme-linked immunosorbent assay (ELISA) as previously described (59).
the iron-related genes, hmbR, bfrAB, and tlfH, were reduced in the misR mutant compared to those in the parent wild-type strain NMB (Table 2). BfrA and BfrB most likely form a transcriptional unit and are homologous bacterioferritin proteins that manage the intracellular storage of iron and protect the cell from iron-mediated oxidative stress (5). HmbR encodes a meningococcus-specific hemoglobin receptor (51). TdH contains a TonB box and is annotated to be a TonB-dependent outer-membrane iron-siderophore receptor; however, the specific substrate(s) has not been identified (52, 56).

The last group of genes identified encodes putative cell surface-localized proteins that include several transporters (e.g., the capsular polysaccharide transport protein CtrD) and a putative hemolysin. In particular, the HlyB and HlyD proteins together with TolC, an outer-membrane channel protein that is encoded in the same putative operon as hlyD, constitute the type I secretion machinery responsible for the secretion of several iron-inducible repeat-in-toxin (RTX) toxins homologues (53–55, 63).

**Validation of microarray by quantitative real-time PCR and β-galactosidase reporter assays.** Quantitative RT-PCR and lacZ transcriptional reporter fusion were employed as independent methods to assess the differential regulation of genes selected from the microarray experiments and to validate the microarray-based observations. Quantitative real-time RTPCRs were performed using three independent sets of wild-type and mutant RNAs, of which two were also examined by the microarray experiments. The transcription of all genes examined by quantitative RT-PCR correlated with the microarray data (Table 2). Using primer pairs annealed to the 5′ coding sequence upstream of the cassette insertion site, the expression of misR itself was one of the most significant reductions in the NMBmisR mutant, confirming that MisR functions as an autoactivator of the misR promoter (60).

To further confirm the importance of the MisR/MisS (MisR/S) two-component system in regulating the transcription of genes identified by the array study, transcriptional fusions of a β-galactosidase reporter were generated as a single copy on a permissive chromosomal locus. As the phosphorylation of MisR is critical in the activation of its regulatory function, the reporter was also inserted in the misS::aphA-3 mutant strain (60) to access the regulatory roles of MisR phosphorylation by MisS kinase. Additionally, reporter fusions of mtr, bfrA, hlyD, and misR were created in the misRS::aphA-3 double mutant background, and the resulting reporter activities were compared to those of the corresponding misS mutants to test whether nonphosphorylated MisR exhibited additional regulation. The double mutant with deletion-insertion
mutations in both misR and misS was constructed by replacing an ~1.6-kb coding sequence of misR and misS with an aphA-3 cassette as described in Materials and Methods. All mutations have been confirmed by colony PCRs, Southern blot analyses, and Western blot analyses (data not shown). As shown in Fig. 1, changes in the reporter activities of each gene examined were consistent with those obtained by arrays and quantitative RT-PCRs, although differences in the magnitude of changes were observed between the three methods. Comparable reporter activities were obtained in both the misS and the misRS mutants, indicating that the phosphorylation of MisR was critical in its regulatory activity, at least for the four genes studied.

Iron concentration is not the inducing signal of the MisRS two-component system. Iron availability is known to regulate proteins involved in iron metabolism through the function of the ferric uptake regulator, Fur (2, 7), and both hmbR (4, 51) and bfrAB (5) have been shown to be iron regulated. To determine whether iron availability is the signal activating the MisR regulon, the expression levels of the autoregulated misRS operon were compared under iron-replete and -deplete growth conditions. The wild-type strain NMB was grown to mid-log phase in the presence of 25 μM deferoxamine (Desferal) to deplete free iron, and total RNA was isolated and analyzed by real-time PCR. As controls, the expression levels of hmbR, tdhH, and bfrA were also examined. Consistent with published results (4, 5, 51), iron limitation caused an increase in hmbR expression and a down-regulation of bfrA, confirming that the cultures were iron starved (see Fig. S1 in the supplemental material). tdhH is not iron regulated (56), and tdhH expression was not altered with iron starvation (see Fig. S1 in the supplemental material). No changes were seen in the expression of misR (see Fig. S1 in the supplemental material), indicating that iron concentration was not involved in the control of the autoregulated misRS operon. This result indicated that the decrease in the expression of hmbR, bfrA, and tdhH observed in the NMBmisR mutant was not directly associated with changes in iron availability but was the result of an unknown inducing signal transmitted by MisS. Thus, an alternative environmental sensing mechanism involving the MisRS two-component regulatory system may regulate a subset of iron assimilation processes. Interestingly, among the TonB-dependent receptors, only hmbR and tdhH, but not the other iron receptors for transferrin, lactoferrin, or siderophores, were affected by the misR mutation, suggesting that the biological function(s) regulated by the misRS system may involve specific pathways of iron metabolism.

MisR specifically binds to a subset of regulated genes identified by microarray analysis. Response regulators typically affect gene expression by directly binding to specific sequences in the promoters of genes that they control; however, since altered expression of additional transcriptional regulators was identified, expression changes in the NMBmisR mutant may be indirectly related to the loss of MisR. To better delineate the direct regulatory effects of MisR, EMSAs were conducted on a subset of 22 genes, and the results indicated that MisR directly interacted with 14 promoter regions of these genes (Table 2 and Fig. 2). The remaining genes without detectable direct interaction with MisR may be indirectly regulated by MisR or, alternatively, were detected by microarray analysis due to growth differences. A relatively large probe size (~500 bp) was chosen to ensure the coverage of distant regulatory elements. However, it remains possible that MisR-binding sites were not included in the designed probes. Competition EMSAs were performed to demonstrate that the interaction ofMisR with these promoter fragments was specific (see Fig. 2 for results of bfrA, tdhH, and B0556 competition experiments; also data not shown). MisR directly interacted with the promoters of clpB, dnaJ, fkpA, bfrA, tdhH, hmbR, and three genes, NMA0738, NMA1593, and NMA1751, encoding the transcriptional regulators. In addition, MisR directly controlled the expression of hlyB and hlyD (Fig. 2). The autoregulatory mechanism of the misRS operon by MisR has been reported previously (60). The titration experiments whose results are shown in Fig. 2 indicated that generally phosphorylated MisR (MisR−P) exhibited higher affinity than nonphosphorylated MisR and that differences in binding affinity among the various promoters were present. A single protein complex species was observed for both MisR and MisR−P. As many response regulators interact with their target DNA as oligomers, the single shifted band of the MisR-DNA complex implied a potentially high cooperativity of MisR−P self-self interaction upon DNA binding. Although the expression of dsbD was significantly altered.
in the NMBmisR mutant (Table 2), MisR appears not to directly regulate the transcription of this gene since an interaction between MisR and the dsbD promoter was not demonstrated by EMSA (Fig. 2).

The expression of lgtG, the glucosyltransferase responsible for the glucose substitution of the HepII O-3 position of the LOS inner core, has been shown to increase in the NMBmisR mutant (58); however, a shift could not be detected with an lgtG probe that encompasses a 500-bp sequence upstream of lgtG (data not shown). Subsequently, a lacZ reporter strain constructed with the same fragment yielded minimal activity, indicating that there is no functional promoter immediately upstream of lgtG in strain NMB. Interestingly, the mtr gene encoding an amino acid transporter is transcribed in the same direction directly upstream of lgtG and was shown to be directly and negatively regulated by MisR (Fig. 1 and 2). Sequencing analysis of the intergenic region between mtr and lgtG in strain NMB indicated that an 89-bp deletion, in comparison to the MC58 genome, removes half of an inverted repeat motif located downstream of the mtr coding sequence and disrupts a potential stem-loop transcriptional terminator. Thus, the increase in lgtG expression likely resulted from a transcriptional coupling of mtr and lgtG expression in strain NMB and MisR influences lgtG expression indirectly.

Identification of the MisR-binding motif by DNase I protection assays. To understand how MisR regulates its targets, several MisR-regulated promoters have been studied by DNase I protection assays to map the MisR-binding sites. The promoters of hmbR, hlyB, and hylD (data not shown) and fkpA and mtr (Fig. 3) were examined. The sequence length protected by MisR−P was generally longer than those protected by nonphosphorylated MisR (see reference 60 for misR footprinting data; also data not shown), and this correlated with the affinity differences observed by EMSAs. Together with the previously reported footprinting data for the misR promoter (60), 12 binding sequences have been compiled and a conserved MisR-binding 9-bp core consensus motif, (A/T)(A/T)T GTAA(A/G/C)G, was derived (Fig. 4) using the BioProspector program (31). The importance of this motif correlated with a previous study of the misR promoter region (60). lacZ reporter assays have showed that deleting three bases at the 5′ end of this core motif significantly reduced the promoter activity to a level similar to that of a strain with only the −35 and −10 promoter elements, thus eliminating the activation by MisR. In addition, single base changes at either the first base (A/T→G) or the third base (T→A) of this core sequence (corresponding to bases 3 and 5 of the WebLogo plot in Fig. 4B) yielded a twofold reduction in promoter activity (60). Sequence searches indicated that either single- or multiple-core motifs with a maximum of two mismatches can be identified in the upstream sequence of MisR direct targets; however, the accuracy of MisR-binding sequence prediction will require confirmation with additional DNase I protection assays.

Inactivation of misRS results in sensitivity to oxidative stress. Gene products that are known to be involved in protection against oxidative stress, including bfrA/bfrB (5) and laz (65), were down-regulated in the misR mutant. In addition,
misRS mutation increased resistance to complement-mediated bactericidal activity of NHS. To ensure that the serum-resistant phenotype is caused by the misR mutation, the mutation was complemented with a second copy of misR under the control of the P_{lac} promoter and integrated into the igu locus. As shown in Fig. 5A, with induced expression of misR by IPTG (confirmed by Western blot analyses; data not shown), the complemented strain is more sensitive to 25% NHS than the mutant and behaved similarly to the wild-type parent strain, confirming that the misR mutation resulted in increased serum resistance.

Multiple mechanisms are utilized by meningococci to avoid killing by human complement (11, 24, 44). One of the major determinants to serum resistance is capsular polysaccharide. To test whether the level of capsule expression contributes to the increase in resistance, the total capsule expression of the misS and misR mutants was quantified by whole-cell ELISA and compared to that of the parent strain (Fig. 5B). The results indicated that the misRS mutants expressed ~40% more capsule than the wild-type parent strain. The capsule transport gene ctra was detected as up-regulated in the misR mutant by microarray analysis (see Table S1 in the supplemental material). To test whether the expression of capsule assembly genes was affected, we assessed the transcriptional levels of several capsule biosynthesis and transport genes, synA, ctra, ctnD, and ctnE (lipA), by real-time RT-PCR. Modest increases (~1.5-fold) were detected in the misR and misS mutants and could potentially result in an increase in capsule expression and lead to the serum resistance phenotype.

### DISCUSSION

The limited number of two-component regulatory systems in *N. meningitidis*, an obligate human pathogen, suggests that they regulate genes important for maintaining the lifestyle of the organism and possibly for virulence. For example, NarQ/NarP has been shown to regulate the denitrification pathway, which is important under oxygen-limited in vivo growth conditions, such as those present inside vacuoles containing gonococci or meningococci in human epithelial cells (30, 38).

Although interesting phenotypes associated with virulence have been observed for the MisR/S two-component system (34, 58), the regulatory targets of this system remain to be defined. Previous microarray studies of a misR mutant created in a meningococcal serogroup C strain grown on blood agar revealed a total of 281 genes that were up- or down-regulated compared to those of the parental strain. However, this microarray observation was not validated by other experimental methods, nor was information on direct targets of regulation provided. As phenotypic differences between the serogroup C and the serogroup B mutants have been noted previously (21, 35, 58, 60), we carried out transcriptional profile analyses of the NMBmisR mutant to independently identify misR-regulated genes. Our microarray data indicated that 117 genes displayed altered expression in the misR mutant, with 78 up-regulated and 39 down-regulated, respectively. The overlap of MisR regulatory targets between the study of the serogroup C misR mutant (15) and our study of the serogroup B NMBmisR mutant was approximately 30%. The limited overlap between these two studies may be due to the fact that the two strains...
may have different genotypes and were grown under different culturing conditions. Using quantitative real-time PCR, a β-galactosidase reporter assay, and an EMSA, we further characterized 25 potential MisR targets (Table 2). Fourteen promoters controlling the transcription of 15 genes were defined as direct targets of MisR, including several genes not seen in the other microarray study. Overall, the MisR/S regulon included genes involved in protein folding, chaperones, metabolism, iron assimilation, type I protein transport, and sensitivity to oxidative stress and human serum.

General protein folding machinery has been implicated in the virulence of many pathogens. Although it is likely that the transcriptional changes of some genes involved in protein folding were caused by the growth rate difference between the wild-type parent strain and the mutant, the MisR/S two-component system appears to directly regulate aspects of the cytoplasmic protein folding machinery, including *dnaJ*, *clpB*, and *fkpA*, as shown by EMSAs. In addition, the expression of *dsbD*, an integral membrane protein necessary for the protein isomerization pathway in the periplasm, also significantly decreased in the *misR* mutant. Differences in the genetic organization and regulation of chaperones between *Neisseria* spp. and other bacteria are significant. First, unlike genes in *E. coli*, which encode chaperones often organized as an operon, meningococcal *dnaJ*, *dnaK*, and *grpE* are independently transcribed at three distant chromosomal loci. Second, although *Neisseria* belongs to the betaproteobacteria that commonly utilize the HrcA/CIRCE system for chaperone regulation, no HrcA homologue has been found in the neisserial genomes (41). Instead, *dnaJ*, *dnaK*, and *grpE* are under the positive control of the heat shock sigma factor (RpoH) (14, 29) in gonococci and thus are influenced by the regulatory mechanisms controlling the amount and activity of RpoH itself. Our study indicates that MisR negatively regulates some of these chaperone genes. MisR was shown to bind the *dnaJ* promoter with low affinity, which appears to correlate with a modest up-regulation, while MisR bound the *clpB* promoter with high

TABLE 3. *misRS* mutation increases sensitivity to oxidative stress

<table>
<thead>
<tr>
<th>Strain</th>
<th>Zone of growth inhibition (mm) ± SD with*:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1% H₂O₂</td>
</tr>
<tr>
<td>NMB (wild type)</td>
<td>14.7 ± 0.6</td>
</tr>
<tr>
<td>NMBmisR mutant (<em>misR::aphA-3</em>)</td>
<td>18.0 ± 1.3*</td>
</tr>
<tr>
<td>YT0310 mutant (<em>misS::aphA-3</em>)</td>
<td>17.0 ± 1.0*</td>
</tr>
<tr>
<td>YT0336 mutant (<em>misRS::aphA-3</em>)</td>
<td>17.7 ± 0.6*</td>
</tr>
</tbody>
</table>

* Each value is an average of growth inhibition zones around three 6-mm filter disks obtained with the indicated concentrations of hydrogen peroxide and paraquat after a 24-hr incubation. Student’s t test showed significant differences compared to the parental wild-type strain (*, *P* < 0.05). Data were from one representative experiment.

FIG. 4. (A) Consensus alignment of MisR-binding sites. Regions protected from DNase I from *misR*, *hmbR*, *mtr*, *hlyB*, *hlyD*, and *fkpA* promoters are aligned by the BioProspector program (31). The bold letters indicate sequences identical to the 15-bp consensus motif. K, G or T; W, A or T; R, A or G; H, any bases except G; N, any bases. (B) Graphic representation of the consensus MisR-binding motif generated by the WebLogo program (6, 45). The overall height of the stack indicates the sequence conservation at that position, while the height of bases within the stack indicates the relative frequency of each base at that position.
affinity, resulting in a more significant increase in the transcription level when MisR was absent. This observation parallels that seen in Streptococcus pyogenes, where inactivation of the response regulator covR resulted in the derepression of dnaK, dnaJ, gprE, and groEL transcripts (12). In addition to this emerging mechanism of regulation of chaperone expression, dnaK and clpR were shown to be up-regulated in a gonococcal fur null mutant, and this regulation is independent of iron concentration (8). Thus, in addition to the general stress response (RpoH-dependent positive control) (14) and the Fur-mediated negative regulation (8), the MisRS two-component system is another negative regulatory mechanism of a subset of chaperone proteins in N. meningitidis. The biological significance of these interactions is as yet unclear; however, RpoH, the positive regulator of chaperone expression, was up-regulated, while NG0177, the equivalent of misR in Neisseria gonorrhoeae, was down-regulated when gonococci adhered to human epithelial cells (10), indicating a possible role for MisR in attachment and invasion.

Both Fur and the MisRS system are also involved in the control of the hemoglobin receptor hmbR. Fur has been shown to bind directly to the promoter region of hmbR and act as a repressor under iron-replete conditions (46). The array and real-time experiments reported here were performed with RNAs isolated from iron-replete cultures. Thus, the expression of hmbR was further decreased in the misR mutant, indicating that MisR functions as an activator in the presence of Fur repression. Whether MisR acts alone as an activator or functions through antagonizing the repressor effect of Fur remains to be clarified. On the other hand, the expression of the bfrA-bfrB operon is clearly regulated by iron availability (5), but no direct interaction of Fur with the bfrA promoter has been reported. We show that MisR interacts directly with the bfrA promoter. This represents the first example of a two-component regulatory system mediating the expression of iron storage proteins important in maintaining iron homeostasis. Similarly, as the expression of tdfH is not regulated by iron (56), the direct control by the MisR/S system might be the only regulatory mechanism modulating the expression of tdfH.

The EMSA data indicate that MisR indirectly regulates dsbD, the integral membrane component of the protein disulfide bond isomerization pathway in the periplasm. DsbD participates in correcting misfolded disulfide bonds of periplasmic proteins and is critical in providing the reducing power for cytochrome c assembly in the periplasm (26). Among all Dsb proteins, only DsbA has been shown to be a member of the Cpx two-component system regulon of E. coli (43). The regulatory mechanisms controlling other Dsb proteins have not been characterized. In addition to its involvement in the refolding of periplasmic proteins and cytochrome c assembly, DsbD can reoxidize the methionine sulfoxide reductase (MsrA/B) domains of PilB in N. gonorrhoeae, which repairs methionine damaged by reactive oxygen species (3), an important arsenal elicited by host defense. Further, the methionine sulfoxide reductase activity of PilB also contributes to the maintenance of adhesins in Streptococcus pneumoniae, N. gonorrhoeae, and E. coli (62). In correlation with the role of DsbD in maintaining the function of PilB and the down-regulation of azurin, which is involved in defense against hydrogen peroxide (65), we found that the misRS and misR mutants exhibited increased sensitivities to hydrogen peroxide and paraquat (Table 3).

One of the original phenotypes of the misR mutant in strain NMB was the disappearance of O-6-linked PEA at-tached to the HepII of the LOS inner core (58). However, the expression of lpt6, encoding the O-6 PEA transferase (23, 64), was unchanged in the misR mutant when examined by microarray and real-time PCR (data not shown). Without the transcriptional down-regulation of lpt6 expression, two models are being examined. In one model, Lpt6 may be misfolded or degraded in the periplasm, due to the impairment of the protein folding pathway dependent upon DsbD. Alternatively, Lpt6 activity may remain intact in the NMBmisR mutant, with the up-regulation of a putative PEA hydrolase removing all the HepII PEA substituents during the biosynthesis and assembly of the LOS. While plausible, such PEA hydrolase activity has yet to be observed in meningococci. Clearly, more studies are needed to address the mechanism leading to the loss of O-6 PEA phosphorylation. Another possible indirect target of MisR is a putative hemolysin
encoded by NMB1900. This gene has been shown to be 
up-regulated when meningococci attach to endothelial, but 
not epithelial, cells and is down-regulated when exposed to 
human serum (9, 28). However, its role in meningococcal 
pathogenesis has not been defined. Other exotoxin family 
proteins expressed by meningococci with unknown patho-
genic functions are the RTX toxins (FrpC). High levels of 
antibodies recognizing a recombinant FrpC protein were 
detected in convalescent-phase sera of patients recovering 
during disease (37). A type I secretion machinery homologous to HlyB/HlyD/TolC of E. coli is respon-
sible for the secretion of FrpC RTX toxin family pro-
teins, and this system is shown here to be directly 
and negatively controlled by the MisRS two-component sys-
tem.

The increased resistance of the misR mutants to killing 
by normal human serum bactericidal activity was striking 
(Fig. 5A). Multiple mechanisms are utilized by meningoc-
occi to avoid killing by human complement (11, 24, 44). 
Two main contributors to resistance are capsular polysac-
charide and LOS. Strain NMB in broth culture expresses a 
mixture of LOS structures, with all inner cores carrying O-6 
PEA groups and 70% carrying O-3-linked glucose. The misR 
mutant expresses a homogenous population of LOS struc-
tures, all of which lack the O-6 PEA of the inner-core HepII 
residue while being completely substituted with O-3-linked 
glucose (58). This effect has been shown to be the result of 
derepression of the O-3-linked glucosyltransferase gene, 
lgtG, in the NMBmisR mutant. Ram et al. (43a) have 
previously demonstrated that the O-6-linked PEA of the LOS 
inner core is a preferred target for binding complement 
component C4b, thus resulting in increased susceptibility to 
complement-mediated killing in serum bactericidal assays.

However, an lpt6 mutant in strain NMB did not display an 
increase in serum resistance (data not shown), indicating 
that the loss of O-6-linked PEA alone from the LOS inner 
core in the misRS mutant could not explain this phenotype.

An examination of the total capsule by whole-cell capsule 
ELISA indicated that the misR mutants expressed ~30 to 
50% more capsule than the wild-type parent strain (Fig. 5B).

This phenotype could potentially arise from the modest 
increase (~1.5-fold) in the transcription of the capsule bio-
synthesis and capsule transport genes in the misR and misS 
mutants. We have previously shown that small modulations 
in capsule expression of approximately 25% can result in 
detectable changes in serum resistance (25), indicating that 
these relatively small changes have important biological ef-
fects.

In summary, we have defined a minimal regulon of the 
MisRS two-component system through microarray, quantita-
tive real-time PCR, reporter fusion, EMSAs, and DNase I 
protection assays. The MisR-biding motif was clearly differ-ent from those of the PhoP and CpxR response regulators, 
which are potential functional homologues of MisR (13, 42).

The MisRS system directly or indirectly regulates genes in-
volved in a diverse array of functional categories, many with 
implicated roles in meningococcal pathogenesis. Additional 
data are needed to refine the MisR-binding motif and to iden-
tify critical nucleotides within the motif. The results reported 
here should enable a better understanding of the interesting 
regulatory circuitry of the important MisRS two-component 
system in N. meningitidis.

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