The MisR/MisS Two-Component Regulon in *Neisseria meningitidis*

Yih-Ling Tzeng,[1*] Charlene M. Kahler,[3] Xinjian Zhang,[1#] David S. Stephens[1,2]

[1]Department of Medicine, Emory University School of Medicine, Atlanta, GA, USA

[2]Laboratories of Bacterial Pathogenesis, Department of Veterans Affairs Medical Center, Decatur, GA, USA

[3]Discipline of Microbiology and Immunology, School of Biomedical, Biomolecular and Chemical Sciences, The University of Western Australia, Australia

#Current address: Department of Chemistry, Georgia State University, Atlanta, GA.

Running title: Regulon of meningococcal MisR/S two-component system

Key words: *Neisseria meningitidis*, two-component regulatory system, regulon, transcriptional regulation, MisR

*Corresponding author. Mailing address: Woodruff Memorial Research Building, Room 2101, 1639 Pierce Drive, Atlanta, GA 30322. Phone: (404) 727-8393. Fax: (404) 712-2278. Email: ytzeng@emory.edu

ABSTRACT

Two-component regulatory systems are involved in processes important for bacterial pathogenesis. Inactivation of the *misR/misS* system in *Neisseria meningitidis* results in 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/S system were identified by transcriptional profiling of the NMB*misR* 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 I protein transport. Mutation in the MisRS 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 (EMSA) were carried out using purified MisR-6xHis protein. Among 22 genes examined, MisR directly interacted with fourteen 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/MisS 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.

INTRODUCTION

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 (HK) that upon sensing specific signals undergoes autophosphorylation at a conserved histidine residue and a cytoplasmic response regulator (RR) partner that receives the phosphoryl group at an invariant aspartate residue (16) which consequently modulates DNA-binding activity.

N. 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), when 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 regulate nitrogen metabolism in *Azorhizobium caulinodans* (40), while the second pair, *NMB1606/NMB1607*, shares homology with *pilS*/*pilR* that regulate piliation in *Pseudomonas aeruginosa* (15). The NMB1249/NMB1250 two-component system exhibits amino acid sequence similarities with NarQ/NarP, and the equivalent gonococcal 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). Lipooligosaccharide 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 been frequently 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 significantly up- or down- regulated in the mutant compared to the parent strain. However, these microarray observations were not validated by other biochemical or genetic means, nor were the direct regulatory targets of MisR identified. We have previously shown 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 NMB*misR* mutant to independently identify MisR-regulated genes. Real time 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 EMSA, and the MisR-binding sequences of several target genes were mapped by DNaseI 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. Strains and plasmids used in this study are listed in Table 1, while primers used in this study are listed in Table S3. 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% CO_2 at 37^{o}C unless specified otherwise. GC base agar (Difco), supplemented with 0.4% glucose and 0.68 mM $Fe(NO_3)_3$, or GC broth with the same supplements and 0.043% $NaHCO_3$ were used. BHI medium (37 g/L brain heart infusion) with 1.25% fetal bovine serum was used when kanamycin selection was required. Antibiotic concentrations (μg/ml) used for *E. coli* strains were ampicillin, 100, kanamycin (Km), 50, and erythromycin (Erm), 300; and for *N. meningitidis* were kanamycin, 80, and erythromycin, 3. *E. coli* strain DH5α cultured on Luria Bertani (LB) medium was used for cloning and propagation of plasmids. Meningococci were transformed by the procedure of Janik *et al*. (20). *E. coli* strains were transformed by electroporation with GenePulser (BioRad) according to the manufacturer's protocol.

Construction of the *misRS* double mutant and complementation of the *misR* 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*Hind*III-YT175*Sma*I and YT177*Sma*I-YT178*EcoR*I. Two PCR products were digested with respective restriction enzymes and then ligated with *Hind*III-*EcoR*I restricted pUC18, yielding pYT334. The correct plasmid carrying the newly created *Sma*I site was confirmed by restriction digestion and sequencing analysis. The *aphA-3* cassette released from pUC18K by *EcoR*I-*BamH*I digestion and Klenow treatment was inserted into the *Sma*I site of pYT334,

yielding pYT336. Removal of the *misR* and *misS* internal sequence and the presence of a correctly oriented *aphA-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 *Sca*I 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 pJKD2581 plasmid (C.M. Kahler and J. Davies, unpublished data), which contains the Hermes (27) inducible expression cassette with the spectinomycin marker flanked by *iga* 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 cpxR5B and cpxR7B containing flanking *Bam*HI sites and cloned into the *Hinc*II site of pWSK29 (61) to create pJKD2582. Both pJKD2582 and pJKD2581 were digested with *Bam*HI, ligated and chloramphenicol resistant transformants, named pJKD2583, were selected for further analysis. The pJKD2583 plasmid contains the *misR* gene under the control of the P_{trc} promoter. *Msc*I linearized pJKD2583 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 *iga*: DAP139 and DAP119 for the left flank, DAP227 and DAP120 for the right flank and DAP486 and DAP276 for the internal part of the cassette. Due to the difficulty of transforming the *misR* mutant, the *misR::aphA-3* mutation was introduced into CMK30 and Sp^R/Kn^R transformants were selected. A panel of PCR reactions was done to ensure the presence of the *misR::aphA-3*

mutation at the wild type locus and that the second copy of the intact *misR* gene remained at the *iga* locus.

Microarray. Bacterial strains grown in supplemented GC broth to mid-exponential phase were collected and the total RNA extracted using the RNeasy midi 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 μg total RNA and 0.1 pmol of a mixture of 3′-specific primers, which were used in generating the PCR products for printing slides (Eurogentec), were first denatured at 70°C for 10 min and then the primers were allowed to anneal to RNA on ice. Cy3- and Cy5-labeled cDNA were generated using the CyScribe First-strand cDNA labeling kit (Amersham Pharmacia Biotech) and then purified by the Qiaquick PCR purification kit (Qiagen). The percentage of incorporated label was estimated using the following extinction coefficients: DNA (6,000 $M^{-1}cm^{-1}$ at OD_{260}), Cy3 (150,000 $M^{-1}cm^{-1}$ at OD_{550}) and Cy5 (250,000 $M^{-1}cm^{-1}$ at OD_{650}). The labelled 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 0.2x SSC buffer, water, 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 denatured at 95°C for 2 min and cooled on ice before the addition of 20 μL Dig easy solution. The hybridization was performed at 42°C

overnight. The slide was washed in 0.2x SSC/0.1% SDS for 5 min at 42°C, followed by consecutive rinses in 0.2x SSC/0.1% SDS for 10 min at room temperature (2x) and 0.1x SSC for 5 min at room temperature (2x). 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 Imagene/Genesight software (Biodiscovery), which allows automatic griding of the array and an attached database for flagging non-optimal spots that can be removed from later analyses. Measuring the signal intensities detected for both fluorophores and calculating the signal ratio determines relative amounts of a particular gene transcript in the two samples. Signal ratios of fluorescence were generated from each spot and then analysed by Genesight (BioDiscovery). The standard two-fold cut-off was used as a minimum value for induction and repression. The array glass slide (Eurogentec) contains 2191 ORFs from 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 open reading frames 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 using the TOPO-TA cloning kit (Invitrogen). The inserts were released by *EcoR*I digestion, gel purified, and then ligated with pYT328, which has been digested with *EcoR*I 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 correct orientation of the promoter to the *lacZ* gene. The *Nco*I-linearized plasmid constructs were used to transform the wild type

meningococcal strain NMB and the NMB*misS* mutant. A panel of colony PCRs with a chromosomal 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 NMB*misRS* mutant to generate reporter strains with the *misRS* double mutation. Transformants resistant to both Erm and Km were selected on BHI plates and confirmed by colony PCR. The β-galactosidase activity assays were performed as described (60).

Protein purification and Western blots. The purification of the MisR-$(His)_6$ protein and the Western blot procedure using polyclonal sera against the MisR-$(His)_6$ protein have been reported previously (60).

Electrophoretic mobility shift assay (EMSA) and DNase I protection assay. Procedures for both experiments have been reported previously (60). Briefly, probes for EMSA were generated by T4 kinase-mediated [γ-^{32}P] end-labeling of PCR promoter fragments and purified using Qiaquick PCR purification kit (Qiagen). The binding reactions consisting of 5 fmol labeled DNA and various amounts of protein in a binding buffer (20 mM HEPES, pH 7.9, 60 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.3 mg/mL BSA, 0.2 mg/mL salmon sperm DNA, and 10% glycerol) were carried out at 30^{o}C for 20 minutes and analyzed with 6% polyacrylamide gels. The gels were dried under vacuum without prior fixing and the resulting radioactive electrophoretic patterns analyzed with a PhosphoImager (Molecular Dynamics). The binding reactions were examined with phosphorylated (50 mM acetyl phosphate for 30 minutes at 37^{o}C) or nonphosphorylated MisR. Competition with excess specific competitors (unlabeled probes) and nonspecific competitors, 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 DNase I protection assay, primers were end-labeled using T4 polynucleotide kinase and [γ-^{32}P] and purified with the Qiaquick nucleotide removal kit (Qiagen). PCR fragments of the promoter region were then amplified with the radiolabeled primer and the corresponding reverse primer and the labeled DNA products purified with a Qiaquick PCR purification kit. Using the binding conditions defined in the EMSA studies, DNA-protein complex in the binding buffer without salmon sperm DNA was treated with diluted DNase for 1 minute at room temperature before quenching 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 Gene Amp kit using 1 µg of total RNA according to the procedure suggested by the manufactures (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). Primers used were listed in Table S3. 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 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 they were free of contaminating chromosomal DNA. The relative fold of changes was calculated using the $2^{-\Delta\Delta Ct}$

method (32). Each set of RT-PCR 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 (SBA). The sensitivities of meningococcal strains to hydrogen peroxide (H_2O_2) and paraquat were determined by disk diffusion growth inhibition assays. Briefly, cells from plate grown cultures were suspended in GC broth to an optical density of 0.15 at OD_{600}. Fifty μl of cell suspension were 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 hours and zones of growth inhibition were measured. Each condition was tested with three disks and the assays repeated three times. Procedure for the SBA has been reported previously (24). The expression of P*trc*::*misR* under of the complemented strain was induced by 1 mM IPTG in broth culture.

Capsule ELISA. Serogroup B capsule-specific IgM monoclonal antibody 2-2-B was used in the whole-cell enzyme-linked immunosorbent assay (ELISA) as previously described (59).

RESULTS

Microarray identification of MisR-regulated genes.

To assess the scope of MisR regulation, the transcriptome of the mutant was analyzed by 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 that of the serogroup B MC58 genome (52). Open reading frames (ORFs) unique to the MC58 genome that are encoded within the three major putative islands of horizontally transferred DNA (IHTs) 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 mostly hypothetical proteins (52). Total RNA of the wild type parent strain NMB and the NMB*misR* mutant were isolated from cultures grown in standard GC broth. cDNAs were generated using the CyScribe First-strand cDNA labeling kit with a mixture of 3′-specific primers. The individually labelled 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 NMB*misR* mutant were examined including one dye-swap comparison. Genes that exhibited greater than two-fold changes in two of three microarray experiments were designated as potential regulatory targets of the MisR response regulator (Tables S1 and S2). A total of 78 genes were up-regulated and 39 genes were down-regulated in the NMB*misR* mutant. As the mutants displayed increased doubling time compared to 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 or indirect regulatory roles of MisR, a panel of 25 MisR regulated genes was selected for further detailed biochemical characterization and could be classified into 5 important functional categories: protein folding/stress response, regulation, metabolism, iron assimilation and transport/secretion (Table 2). While 12 genes were down-regulated in the NMB*misR* null mutant, indicative of an activator role for MisR, the expression of 20 genes was increased. This observation indicated that either MisR has a dual function as a repressor, or that alteration in 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 difference can not 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 thiol:disulfide interchange protein that maintains the periplasmic disulfide isomerase DsbC in its active form 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 refolding and degradation of misfolded proteins in the cytoplasmic compartment of the cell. FkpA has homology to the C-terminal domain of the periplasmic FKBP-type peptidyl-prolyl cis-

trans isomerase in *E. coli* and is transcribed divergently from *hprA* encoding a glycerate dehydrogenase, also up regulated in the *misR* mutant. A gene cluster consisting of *fkpA* located upstream of eight *pilS* cassettes was up-regulated (Table S1) by the array analysis. HslO, or Hsp33, belongs to a family of redox sensing cytoplasmic heat shock proteins (Hsp33) (1, 19) and work with DnaJ/DnaK/GrpE to support the refolding of the substrate proteins (18). HtpX is a membrane bound zinc metallopeptidase with a cytosolic active site (47).

The next group of genes identified as potential MisR regulon 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 phage CI repressor and LexA. Interestingly, NMA0738 is near *dnaK* (NMA0736) and is divergently transcribed from a conserved hypothetical protein NMA0737 that 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 (NMA1594-1598). However, no changes were detected in the transcription of the iron-sulfur cluster assembly genes. Finally, NMA1751 contain 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 expression of additional genes.

The genes, *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 of the iron-related genes, *hmbR*, *bfrA/B* and *tdfH*, was reduced in the *misR* mutant when compared to the parent wild type strain NMB (Table 2). BfrA and BfrB, most likely form a transcriptional unit, and are homologous bacterioferritin proteins that manage intracellular storage of iron and protect the cell from iron-mediated oxidative stress (5). HmbR encodes a meningococcal specific hemoglobin receptor (51). TdfH 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 haemolysin. 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) toxin homologues (53-55, 63).

Validation of microarray by quantitative real time PCR (qRT-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 RT-PCRs were performed using three independent sets of wild type and mutant RNAs of which two were also examined by the microarray experiments. Transcription of all genes examined by qRT-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 NMB*misR* mutant, confirming MisR functions as an autoactivator of the *misR* promoter (60).

To further confirm the importance of the 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 phosphorylation of MisR is critical in 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 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 ~ 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 blots and Western blots (data not shown). As shown in Figure 1, changes in reporter activities of each gene examined was consistent with those obtained by arrays and qRT-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 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 ferric uptake regulator, Fur (2, 7), and both *hmbR* (4, 51) and *bfrA/B* (5) have been shown to be iron regulated. To determine if iron availability is the signal activating the MisR

regulon, expression of the autoregulated *misR/S* operon was compared between iron replete and deplete growth conditions. The wild type strain NMB was grown to mid-log phase in the presence of 25 μM desferal to deplete free iron and total RNA was isolated and analyzed by real time PCR. As controls, the expression of *hmbR*, *tdfH* and *bfrA* was also examined. Consistent with published results (4, 5, 51), iron limitation causes an increase in the *hmbR* expression and a down-regulation of *bfrA*, confirming that the cultures were iron starved (Figure S1). *TdfH* is not iron-regulated (56) and the *tdfH* expression was not altered with iron starvation (Figure S1). No changes were seen in the expression of *misR* (Figure S1), 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 *tdfH* observed in the NMB*misR* 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 MisR/S two component regulatory system may regulate a subset of iron assimilation processes. Interestingly, among the TonB-dependent receptors, only *hmbR* and *tdfH*, 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 *misR/S* 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 were identified, expression changes in the NMB*misR* 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 Figure 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 difference. A relatively large probe size (~500-bp) was chosen to ensure 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 of MisR with these promoter fragments was specific (see Figure 2 for *bfrA*, *tdfH* and *B0556* competition experiments and data not shown). MisR directly interacted with the promoters of *clpB*, *dnaJ, fkpA*, *bfrA*, *tdfH*, *hmbR*, and three genes, *NMA0738*, *NMA1593* and *NMA1751* encoding the transcriptional regulators. In addition, MisR directly controlled the expression of *hlyB* and *hlyD* (Figure 2). The autoregulatory mechanism of the *misR/S* operon by MisR has been reported previously (60). The titration experiments shown in Figure 2 indicated that generally MisR~P exhibited higher affinity than non-phosphorylated MisR, and 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 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 NMB*misR* 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 (Figure 2).

Expression of *lgtG*, the glucosyltransferase responsible for the glucose substitution of the HepII O-3 position of LOS inner core, has been shown to increase in the NMB*misR* mutant (58); however, a shift could not be detected with an *lgtG* probe that encompasses ~ 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 (Figures 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, hylD* (data not shown), *fkpA* and *mtr* (Figure 3) were examined.. The sequence length protected by MisR~P was generally longer than those protected by the nonphosphorylated MisR [see (60) for *misR* footprinting data and data not shown], and this correlated with the affinity differences observed by EMSA experiments. Together with the previously reported footprinting data of the *misR* promoter (60), 12 binding sequences have been compiled and a conserved MisR-binding 9-bp core consensus motif, (A/T)(A/T)TGTAA(A/G/C)G, was derived (Figure 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 Figure 4B) yielded a 2-fold 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 *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, DsbD, which provides reducing power to the peptide methionine sulfoxide reductase MsrA/B protein for repairing oxidative damage of periplasmic proteins (3, 48), was also significantly down-regulated in the *misR* mutant. These observations suggested that meningococcal *misRS* mutants might be more sensitive to the reactive oxygen species. The *in vitro* sensitivities of the mutants to hydrogen peroxide and paraquat were measured using disk diffusion assays as shown in Table 3. All three mutants exhibited enhanced sensitivities to both oxidative agents.

Inactivation of *misRS* results in resistance to the bactericidal activity of normal human serum and increased levels of capsular polysaccharide production.

To better correlate the avirulent phenotype reported by Newcombe et al (34) using a mouse model of infection, the ability of the serogroup B *misRS* mutants to resist complement-mediated bactericidal activity was examined using serum bactericidal assays with pooled normal human serum (NHS) (24). Surprisingly, the *misR* and the *misRS* mutants showed enhanced serum resistance when at 10% and 25% NHS, whereas the *misS* mutant showed an intermediate resistance at 25% NHS compared to the parental wild type strain (Figure 5A). An un-

encapsulated mutant was completely killed at 5% NHS. Thus, the *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 P_{trc} promoter and integrated into the *iga* locus. As shown in Figure 5A, with induced expression of *misR* by IPTG (confirmed by Western blots, 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, total capsule expression of the *misS* and *misR* mutants was quantified by whole cell ELISA and compared to the parent strain (Figure 5B). The results indicated that the *misRS* mutants expressed ~40% more capsule than the wild type parent strain. The capsule transport gene, *ctrD*, was detected as up-regulated in the *misR* mutant by microarray (Table S1). To test whether the expression of capsule assembly genes was affected, we assayed the transcriptional levels of several capsule biosynthesis and transport genes, *synA*, *ctrA*, *ctrD*, and *ctrE(lipA)*, by real time RT-PCR. Modest increases (~1.5-fold) were detected in the *misR* and *misS* mutants, and could potentially result in the 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/P has been shown to regulate the denitrification pathway which is important during 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 MisRS two-component system (34, 58), the regulatory targets of this system remained 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 when compared to 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 NMB*misR* 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 NMB*misR* mutant was approximately 30%. The limited overlap between 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, β-galactosidase reporter assay and EMSA, we further characterized 25 potential MisR targets (Table 2). Fourteen promoters controlling 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 virulence of many pathogens. Although it is likely that 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, 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 *E. coli* in which genes encoding chaperones are often organized as an operon, meningococcal *dnaJ*, *dnaK* and *grpE* are independently transcribed at three distant chromosomal loci. Second, although *Neisseria* belongs to the β-proteobacteria that commonly utilizes 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 σ^{32} (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 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 de-repression of *dnaK*, *dnaJ*, *grpE* and *groEL* transcripts (12). In addition to this emerging mechanism of regulation of chaperone expression, *dnaK* and *clpB* 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 *N. 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 MisR/S 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, expression of *hmbR* was further decreased in the *misR* mutant indicating that the 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 remain to be clarified. On the other hand, the expression of the *bfrA/B* 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 misformed 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 phosphoethanolamine (PEA) attached to the HepII of the LOS inner core (58). However, the expression of *lpt-6*, 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 *lpt-6* expression, two models are being examined. In one model, Lpt-6 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 NMB*misR* mutant, with the up-regulation of a putative PEA hydrolase removing all the Hep II PEA substituents during the biosynthesis and assembly of the LOS. While plausible, such PEA hydrolase activity has yet to be demonstrated in meningococci. Clearly, more studies are needed to address the mechanism leading to the loss of 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 attached 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 pathogenic functions are the RTX toxins (FrpC). High levels of antibodies recognizing a recombinant FrpC protein were detected in convalescent-phase sera of patients recovering from invasive meningococcal disease and were absent in the sera at hospital admission, indicating expression of FrpC like proteins during disease (37). A type I secretion machinery homologous to HlyB/HlyD/TolC of *E. coli* is responsible for the secretion of FrpC RTX toxin family proteins and this secretion system is shown here to be directly and negatively controlled by the MisRS two-component system.

The increased resistance of the *misRS* mutants to killing by normal human serum bactericidal activity was striking (Figure 5A). Multiple mechanisms are utilized by meningococci to avoid killing by human complement (11, 24, 44). Two main contributors to resistance are capsular polysaccharide and lipooligosaccharide (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 structures 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 de-repression of the O-3 linked glucosyltransferase, *lgtG*, in the NMB*misR* mutant. Ram et al. (44) has 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 *lpt-6* 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 total capsule by whole cell capsule ELISA indicated that the *misRS* mutants expressed ~30-50% more capsule than the wild type parent strain (Figure 5B). This phenotype could potentially arise from the modest increase (~1.5-fold) in the transcription of the capsule biosynthesis 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 effects.

In summary, we have defined a minimal regulon of the MisRS two-component system through microarray, quantitative real time PCR, reporter fusion, EMSAs and DNase I protection assays. The MisR binding motif was clearly different 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 involved in a diverse array of functional categories, many with implicated roles in meningococcal pathogenesis. Additional data is needed to refine the MisR-binding motif and to identify critical nucleotides within the motif. The results reported here should enable better understanding of the interesting regulatory circuitry of the important MisRS two-component system in *N. meningitidis*.

ACKNOWLEDGEMENTS

This work was supported by grants R01 AI061031 (Y.T.), R01 AI033517 (D.S.S. and C.K.) and R01 AI40247 (D.S.S.) from the National Institutes of Health. C.K. was supported by the National Health and Medical Research Council Project grant ID124465. We thank Larry Martin, Shaojia Bao and Soma Sannagrahi for excellent technical assistance and Lane Pucko for administrative assistance. We are grateful to John Davies for sharing the complementation vector.

REFERENCES

1. **Aslund, F., and J. Beckwith.** 1999. Bridge over troubled waters: sensing stress by disulfide bond formation. Cell **96:**751-3.
2. **Braun, V.** 2003. Iron uptake by *Escherichia coli*. Front. Biosci. **8:**s1409-21.
3. **Brot, N., J. F. Collet, L. C. Johnson, T. J. Jonsson, H. Weissbach, and W. T. Lowther.** 2006. The thioredoxin domain of *Neisseria gonorrhoeae* PilB can use electrons from DsbD to reduce downstream methionine sulfoxide reductases. J. Biol. Chem. **281:**32668-75.
4. **Chen, C. J., P. F. Sparling, L. A. Lewis, D. W. Dyer, and C. Elkins.** 1996. Identification and purification of a hemoglobin-binding outer membrane protein from *Neisseria gonorrhoeae*. Infection & Immunity **64:**5008-14.
5. **Chen, C. Y., and S. A. Morse.** 1999. *Neisseria gonorrhoeae* bacterioferritin: structural heterogeneity, involvement in iron storage and protection against oxidative stress. Microbiology **145 (Pt 10):**2967-75.
6. **Crooks, G. E., G. Hon, J. M. Chandonia, and S. E. Brenner.** 2004. WebLogo: a sequence logo generator. Genome Res. **14:**1188-90.
7. **Crosa, J. H.** 1997. Signal transduction and transcriptional and posttranscriptional control of iron-regulated genes in bacteria. Microbiol. Mol. Biol. Rev. **61:**319-36.
8. **Delany, I., R. Grifantini, E. Bartolini, R. Rappuoli, and V. Scarlato.** 2006. Effect of *Neisseria meningitidis fur* mutations on global control of gene transcription. J. Bacteriol. **188:**2483-92.

9. **Dietrich, G., S. Kurz, C. Hubner, C. Aepinus, S. Theiss, M. Guckenberger, U. Panzner, J. Weber, and M. Frosch.** 2003. Transcriptome analysis of *Neisseria meningitidis* during infection. J. Bacteriol. **185:**155-64.

10. **Du, Y., J. Lenz, and C. G. Arvidson.** 2005. Global gene expression and the role of sigma factors in *Neisseria gonorrhoeae* in interactions with epithelial cells. Infect. Immun. **73:**4834-45.

11. **Geoffroy, M. C., S. Floquet, A. Metais, X. Nassif, and V. Pelicic.** 2003. Large-scale analysis of the meningococcus genome by gene disruption: resistance to complement-mediated lysis. Genome Res **13:**391-8.

12. **Graham, M. R., L. M. Smoot, C. A. Migliaccio, K. Virtaneva, D. E. Sturdevant, S. F. Porcella, M. J. Federle, G. J. Adams, J. R. Scott, and J. M. Musser.** 2002. Virulence control in group A Streptococcus by a two-component gene regulatory system: global expression profiling and *in vivo* infection modeling. Proc. Natl. Acad. Sci. U S A **99:**13855-60.

13. **Groisman, E. A.** 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. J. Bacteriol. **183:**1835-42.

14. **Gunesekere, I. C., C. M. Kahler, D. R. Powell, L. A. Snyder, N. J. Saunders, J. I. Rood, and J. K. Davies.** 2006. Comparison of the RpoH-dependent regulon and general stress response in *Neisseria gonorrhoeae*. J. Bacteriol. **188:**4769-76.

15. **Hobbs, M., E. S. Collie, P. D. Free, S. P. Livingston, and J. S. Mattick.** 1993. PilS and PilR, a two-component transcriptional regulatory system controlling expression of type 4 fimbriae in *Pseudomonas aeruginosa*. Mol. Microbiol. **7:**669-82.

16. **Hoch, J. A.** 2000. Two-component and phosphorelay signal transduction. Curr. Opin. Microbiol. **3:**165-70.

17. **Hoch, J. A., and T. J. Silhavy.** 1995. Two-component signal transduction. American Society for Microbiology Press, Washington, DC.

18. **Hoffmann, J. H., K. Linke, P. C. Graf, H. Lilie, and U. Jakob.** 2004. Identification of a redox-regulated chaperone network. Embo J **23:**160-8.

19. **Jakob, U., W. Muse, M. Eser, and J. C. Bardwell.** 1999. Chaperone activity with a redox switch. Cell **96:**341-52.

20. **Janik, A., E. Juni, and G. A. Heym.** 1976. Genetic Transformation as a tool for detection of *Neisseria gonorrhoeae*. J. Clin. Microbiol. **4:**71-81.

21. **Johnson, C. R., J. Newcombe, S. Thorne, H. A. Borde, L. J. Eales-Reynolds, A. R. Gorringe, S. G. Funnell, and J. J. McFadden.** 2001. Generation and characterization of a PhoP homologue mutant of *Neisseria meningitidis*. Mol. Microbiol. **39:**1345-55.

22. **Kadokura, H., F. Katzen, and J. Beckwith.** 2003. Protein disulfide bond formation in prokaryotes. Annu. Rev. Biochem.

23. **Kahler, C. M., A. Datta, Y. L. Tzeng, R. W. Carlson, and D. S. Stephens.** 2005. Inner core assembly and structure of the lipooligosaccharide of *Neisseria meningitidis*: capacity of strain NMB to express all known immunotype epitopes. Glycobiology **15:**409-19.

24. **Kahler, C. M., L. E. Martin, G. C. Shih, M. M. Rahman, R. W. Carlson, and D. S. Stephens.** 1998. The (α2-->8)-linked polysialic acid capsule and lipooligosaccharide structure both contribute to the ability of serogroup B *Neisseria meningitidis* to resist the bactericidal activity of normal human serum. Infect. Immun. **66:**5939-47.

25. **Kahler, C. M., L. E. Martin, Y. L. Tzeng, Y. K. Miller, K. Sharkey, D. S. Stephens, and J. K. Davies.** 2001. Polymorphisms in pilin glycosylation locus of *Neisseria meningitidis* expressing class II pili. Infect. Immun. **69:**3597-604.

26. **Kranz, R. G., C. S. Beckett, and B. S. Goldman.** 2002. Genomic analyses of bacterial respiratory and cytochrome c assembly systems: *Bordetella* as a model for the system II cytochrome c biogenesis pathway. Res. Microbiol. **153:**1-6.

27. **Kupsch, E. M., D. Aubel, C. P. Gibbs, A. F. Kahrs, T. Rudel, and T. F. Meyer.** 1996. Construction of Hermes shuttle vectors: a versatile system useful for genetic complementation of transformable and non-transformable *Neisseria* mutants. Mol. Gen. Genet. **250:**558-69.

28. **Kurz, S., C. Hubner, C. Aepinus, S. Theiss, M. Guckenberger, U. Panzner, J. Weber, M. Frosch, and G. Dietrich.** 2003. Transcriptome-based antigen identification for *Neisseria meningitidis*. Vaccine **21:**768-75.

29. **Laskos, L., C. S. Ryan, J. A. Fyfe, and J. K. Davies.** 2004. The RpoH-mediated stress response in *Neisseria gonorrhoeae* is regulated at the level of activity. J. Bacteriol. **186:**8443-52.

30. **Lissenden, S., S. Mohan, T. Overton, T. Regan, H. Crooke, J. A. Cardinale, T. C. Householder, P. Adams, C. D. O'Conner, V. L. Clark, H. Smith, and J. A. Cole.** 2000. Identification of transcription activators that regulate gonococcal adaptation from aerobic to anaerobic or oxygen-limited growth. Mol. Microbiol. **37:**839-55.

31. **Liu, X., D. L. Brutlag, and J. S. Liu.** 2001. BioProspector: discovering conserved DNA motifs in upstream regulatory regions of co-expressed genes. Pac. Symp. Biocomput.**:**127-38.

32. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25:**402-8.

33. **Menard, R., P. J. Sansonetti, and C. Parsot.** 1993. Nonpolar mutagenesis of the *ipa* genes defines *IpaB*, *IpaC*, and *IpaD* as effectors of *Shigella flexneri* entry into epithelial cells. J. Bacteriol. **175:**5899-906.

34. **Newcombe, J., L. J. Eales-Reynolds, L. Wootton, A. R. Gorringe, S. G. Funnell, S. C. Taylor, and J. J. McFadden.** 2004. Infection with an avirulent *phoP* mutant of *Neisseria meningitidis* confers broad cross-reactive immunity. Infect. Immun. **72:**338-44.

35. **Newcombe, J., J. C. Jeynes, E. Mendoza, J. Hinds, G. L. Marsden, R. A. Stabler, M. Marti, and J. J. McFadden.** 2005. Phenotypic and transcriptional characterization of the meningococcal PhoPQ system, a magnesium-sensing two-component regulatory system that controls genes involved in remodeling the meningococcal cell surface. J. Bacteriol. **187:**4967-75.

36. **Oshima, T., H. Aiba, Y. Masuda, S. Kanaya, M. Sugiura, B. L. Wanner, H. Mori, and T. Mizuno.** 2002. Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. Mol. Microbiol. **46:**281-91.

37. **Osicka, R., J. Kalmusova, P. Krizova, and P. Sebo.** 2001. *Neisseria meningitidis* RTX protein FrpC induces high levels of serum antibodies during invasive disease: polymorphism of *frpC* alleles and purification of recombinant FrpC. Infect. Immun. **69:**5509-19.

38. **Overton, T. W., R. Whitehead, Y. Li, L. A. Snyder, N. J. Saunders, H. Smith, and J. A. Cole.** 2006. Coordinated regulation of the *Neisseria gonorrhoeae*-truncated

denitrification pathway by the nitric oxide-sensitive repressor, NsrR, and nitrite-insensitive NarQ-NarP. J. Biol. Chem. **281:**33115-26.

39. **Parkhill, J., M. Achtman, K. D. James, S. D. Bentley, C. Churcher, S. R. Klee, G. Morelli, D. Basham, D. Brown, T. Chillingworth, R. M. Davies, P. Davis, K. Devlin, T. Feltwell, N. Hamlin, S. Holroyd, K. Jagels, S. Leather, S. Moule, K. Mungall, M. A. Quail, M. A. Rajandream, K. M. Rutherford, M. Simmonds, J. Skelton, S. Whitehead, B. G. Spratt, and B. G. Barrell.** 2000. Complete DNA sequence of a serogroup A strain of *Neisseria meningitidis* Z2491. Nature **404:**502-6.

40. **Pawlowski, K., U. Klosse, and F. J. de Bruijn.** 1991. Characterization of a novel *Azorhizobium caulinodans* ORS571 two-component regulatory system, NtrY/NtrX, involved in nitrogen fixation and metabolism. Mol Gen Genet **231:**124-38.

41. **Permina, E. A., and M. S. Gelfand.** 2003. Heat shock (sigma32 and HrcA/CIRCE) regulons in beta-, gamma- and epsilon-proteobacteria. J Mol Microbiol Biotechnol **6:**174-81.

42. **Pogliano, J., A. S. Lynch, D. Belin, E. C. Lin, and J. Beckwith.** 1997. Regulation of *Escherichia coli* cell envelope proteins involved in protein folding and degradation by the Cpx two-component system. Genes Dev. **11:**1169-82.

43. **Raivio, T. L.** 2005. Envelope stress responses and Gram-negative bacterial pathogenesis. Mol. Microbiol. **56:**1119-28.

44. **Schneider, M. C., R. M. Exley, S. Ram, R. B. Sim, and C. M. Tang.** 2007. Interactions between *Neisseria meningitidis* and the complement system. Trends Microbiol. **15:**233-40.

45. **Schneider, T. D., and R. M. Stephens.** 1990. Sequence logos: a new way to display consensus sequences. Nucleic Acids Res. **18:**6097-100.

46. **Sebastian, S., S. Agarwal, J. R. Murphy, and C. A. Genco.** 2002. The gonococcal *fur* regulon: identification of additional genes involved in major catabolic, recombination, and secretory pathways. J. Bacteriol. **184:**3965-74.

47. **Shimohata, N., S. Chiba, N. Saikawa, K. Ito, and Y. Akiyama.** 2002. The Cpx stress response system of *Escherichia coli* senses plasma membrane proteins and controls HtpX, a membrane protease with a cytosolic active site. Genes Cells **7:**653-62.

48. **Skaar, E. P., D. M. Tobiason, J. Quick, R. C. Judd, H. Weissbach, F. Etienne, N. Brot, and H. S. Seifert.** 2002. The outer membrane localization of the *Neisseria gonorrhoeae* MsrA/B is involved in survival against reactive oxygen species. Proc. Natl. Acad. Sci. **99:**10108-13.

49. **Stephens, D. S., J. S. Swartley, S. Kathariou, and S. A. Morse.** 1991. Insertion of Tn*916* in *Neisseria meningitidis* resulting in loss of group B capsular polysaccharide. Infect. Immun. **59:**4097-102.

50. **Stephens, D. S., and S. M. Zimmer.** 2002. Pathogenesis, therapy, and prevention of meningococcal sepsis. Curr. Infect. Dis. Rep. **4:**377-386.

51. **Stojiljkovic, I., J. Larson, V. Hwa, S. Anic, and M. So.** 1996. HmbR outer membrane receptors of pathogenic *Neisseria spp.*: iron-regulated, hemoglobin-binding proteins with a high level of primary structure conservation. J. Bacteriol. **178:**4670-8.

52. **Tettelin, H., N. J. Saunders, J. Heidelberg, A. C. Jeffries, K. E. Nelson, J. A. Eisen, K. A. Ketchum, D. W. Hood, J. F. Peden, R. J. Dodson, W. C. Nelson, M. L. Gwinn, R. DeBoy, J. D. Peterson, E. K. Hickey, D. H. Haft, S. L. Salzberg, O. White, R. D.**

Fleischmann, B. A. Dougherty, T. Mason, A. Ciecko, D. S. Parksey, E. Blair, H. Cittone, E. B. Clark, M. D. Cotton, T. R. Utterback, H. Khouri, H. Qin, J. Vamathevan, J. Gill, V. Scarlato, V. Masignani, M. Pizza, G. Grandi, L. Sun, H. O. Smith, C. M. Fraser, E. R. Moxon, R. Rappuoli, and J. C. Venter. 2000. Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58. Science **287:**1809-15.

53. **Thompson, S. A., and P. F. Sparling.** 1993. The RTX cytotoxin-related FrpA protein of *Neisseria meningitidis* is secreted extracellularly by meningococci and by HlyBD+ *Escherichia coli*. Infect Immun **61:**2906-11.

54. **Thompson, S. A., L. L. Wang, and P. F. Sparling.** 1993. Cloning and nucleotide sequence of *frpC*, a second gene from *Neisseria meningitidis* encoding a protein similar to RTX cytotoxins. Mol. Microbiol. **9:**85-96.

55. **Thompson, S. A., L. L. Wang, A. West, and P. F. Sparling.** 1993. *Neisseria meningitidis* produces iron-regulated proteins related to the RTX family of exoproteins. J. Bacteriol. **175:**811-8.

56. **Turner, P. C., C. E. Thomas, I. Stojiljkovic, C. Elkins, G. Kizel, D. A. Ala'Aldeen, and P. F. Sparling.** 2001. Neisserial TonB-dependent outer-membrane proteins: detection, regulation and distribution of three putative candidates identified from the genome sequences. Microbiology **147:**1277-90.

57. **Tzeng, Y.-L., and D. S. Stephens.** 2000. Epidemiology and pathogenesis of *Neisseria meningitidis*. Microbes Infect. **6:**687-700.

58. **Tzeng, Y. L., A. Datta, K. D. Ambrose, J. K. Davies, R. W. Carlson, D. S. Stephens, and C. M. Kahler.** 2004. The MisR/MisS two-component regulatory system influences

inner core structure and immunotype of lipooligosaccharide in *Neisseria meningitidis*. J. Biol. Chem. **279:**35053-62.

59. **Tzeng, Y. L., J. S. Swartley, Y. K. Miller, R. E. Nisbet, L. J. Liu, J. H. Ahn, and D. S. Stephens.** 2001. Transcriptional regulation of divergent capsule biosynthesis and transport operon promoters in serogroup B *Neisseria meningitidis*. Infect. Immun. **69:**2502-11.

60. **Tzeng, Y. L., X. Zhou, S. Bao, S. Zhao, C. Noble, and D. S. Stephens.** 2006. Autoregulation of the MisR/MisS two-component signal transduction system in *Neisseria meningitidis*. J. Bacteriol. **188:**5055-65.

61. **Wang, R. F., and S. R. Kushner.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene **100:**195-9.

62. **Wizemann, T. M., J. Moskovitz, B. J. Pearce, D. Cundell, C. G. Arvidson, M. So, H. Weissbach, N. Brot, and H. R. Masure.** 1996. Peptide methionine sulfoxide reductase contributes to the maintenance of adhesins in three major pathogens. Proc Natl Acad Sci U S A **93:**7985-90.

63. **Wooldridge, K. G., M. Kizil, D. B. Wells, and D. A. Ala'aldeen.** 2005. Unusual genetic organization of a functional type I protein secretion system in *Neisseria meningitidis*. Infect. Immun. **73:**5554-67.

64. **Wright, J. C., D. W. Hood, G. A. Randle, K. Makepeace, A. D. Cox, J. Li, R. Chalmers, J. C. Richards, and E. R. Moxon.** 2004. *lpt6*, a gene required for addition of phosphoethanolamine to inner-core lipopolysaccharide of *Neisseria meningitidis* and *Haemophilus influenzae*. J. Bacteriol. **186:**6970-82.

65. **Wu, H. J., K. L. Seib, J. L. Edwards, M. A. Apicella, A. G. McEwan, and M. P. Jennings.** 2005. Azurin of pathogenic *Neisseria* spp. is involved in defense against hydrogen peroxide and survival within cervical epithelial cells. Infect. Immun. **73:**8444-8.

66. **Yamamoto, K., K. Hirao, T. Oshima, H. Aiba, R. Utsumi, and A. Ishihama.** 2005. Functional characterization *in vitro* of all two-component signal transduction systems from *Escherichia coli*. J. Biol. Chem. **280:**1448-56.

FIGURE LEGENDS

FIG. 1. β-Galactosidase reporter activities of MisR-regulated genes. The reporter fusion was integrated into a permissive chromosomal locus as a single copy. β-Galactosidase activities of wild type, *misS* and *misRS* mutants grown in GC broth to mid-log phase are expressed as Miller units and correspond to mean values of at least three independent experiments, each with triplicate measurements. Black bars, reporter strains in a wild type background; gray bars, reporter strains with a *misS::aphA-3* mutation; hatched bars, reporter strains with a *misRS::aphA-3* double mutation.

FIG. 2. EMSA assay of MisR-promoter interaction. ^{32}P-Labeled promoter fragments (~5 fmol) were generated by T4 kinase phosphorylation, mixed with increasing amounts of MisR~P (lanes 2-4) and MisR (lanes 5-7) for 20 minutes at 30°C and then subjected to gel electrophoresis. Free probe is shown in lane 1. MisR~P was generated by incubation with 50 mM acetyl phosphate for 30 minutes at 37°C. The amounts of MisR protein are 68 pmol (lanes 2 and 5), 136 pmol (lanes 3 and 6), and 272 pmol (lanes 4 and 7). The last three panels with 6 samples were representative competition experiments for *bfrA*, *tdfH* and *B556*. Lane1, probe alone; lane 2, probe and MisR; lanes 3 and 5, 2 μg specific and nonspecific competitor DNA, respectively; lanes 4 and 6, 4 μg specific and nonspecific competitor DNA, respectively.

FIG. 3. DNase I protection assays of the coding (A) and noncoding (B) strands of the *fkpA* promoter and the noncoding strand of the *mtr* promoter (C) by MisR~P. ^{32}P-End-labeled promoter fragments were incubated with increasing amounts of MisR~P for 20 minutes at 30°C and then subjected to DNase I digestion. MisR~P was prepared fleshly by reacting with 150 mM

acetyl phosphate at 37°C for 30 minutes. The amounts of MisR~P used in A and B are 0, 85, 170, 340, and 510 pmol, and in C are 0, 42.5, 85, 170, 340, 680, and 1360 pmol. Dideoxy chain-termination sequences corresponding to the probes are shown in the order of G, A, T, and C. The vertical bars indicate protected regions.

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 BioProspector program (31). The bold letters indicate sequence 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.

FIG. 5. (A) Serum bactericidal assays. Strains NMB (wild type, filled diamond), M7 (unencapsulated, filled square), NMB*misR* (*misR::aphA-3*, filled triangle), YT0310 (*misS::aphA-3*, open circle), YT0336 (*misRS::aphA-3*, asterisk) and C1001 (*misR::aphA-3/Ptrc::misR*, filled circle with dotted line) grown to log phase and resuspended in HEPES-MEM buffer were exposed to 5, 10, and 25% NHS. The viable cell counts (n=4) were determined after 30 min incubation (T = 30) and the percentages of survival calculated by comparing to the viable counts at T=0. *25% indicated treatment with 25% heat-inactivated serum for 30 minutes. Averages and standard deviations of at least three independent experiments are shown. Student's *t* test with a two-tailed hypothesis indicates significant differences between the wile type strain and three mutants at 10 and 25% serum levels ($P \leq 0.05$). (B) capsule-specific whole cell ELISA.

The OD_{405nm} readings of the serogroup B wild type strain NMB were used as 100% for normalizing the OD_{405nm} of the YT0310 and the NMB*misR* mutants. The nonencapsulated mutant M7 (*synA::Tn916*) was included as a negative control. (n =3).

1 Table 1. Strains and plasmids used in this study

Name	Description	Reference
Strains		
N. meningitidis		
NMB	B:2b:P1.2,5:L2 (CDC8201085)	(49)
NMB*misR*	NMB with *misR::erm* mutation	(58)
YT0310	NMB with *ΔmisS::aphA-3* mutation	(60)
YT0322	NMB with a chromosomal *misR::lacZ* fusion	(60)
CMK30	NMB with Ptrc::misR inserted into the iga locus	This study
C1001	CMK30 with *misR::aphA-3* mutation	This study
YT0336	NMB with *ΔmisRS::aphA-3* mutation	This study
YT0343	NMB with a chromosomal *mtr::lacZ* fusion	This study
YT0343s	YT0310 with a chromosomal *mtr::lacZ*	This study
YT0343rs	YT0343 with *ΔmisRS::aphA-3* mutation	This study
YT0344	NMB with a chromosomal *NMB1497(508bp)::lacZ* fusion	This study
YT0344s	YT0310 with a chromosomal *NMB1497::lacZ* fusion	This study
YT0346	NMB with a chromosomal *bfrA(486bp)::lacZ* fusion	This study
YT0346s	YT0310 with a chromosomal *bfrA::lacZ* fusion	This study
YT0346rs	YT0343 with *ΔmisRS::aphA-3* mutation	This study
YT0347	NMB with a chromosomal *hlyD(498bp)::lacZ* fusion	This study
YT0347s	YT0310 with a chromosomal *hlyD::lacZ* fusion	This study
YT0347rs	YT0343 with *ΔmisRS::aphA-3* mutation	This study
YT0349	NMB with a chromosomal *dnaJ::lacZ* fusion	This study

YT0349s	YT0310 with a chromosomal *dnaJ::lacZ* fusion	This study
YT0350	NMB with a chromosomal *dsbD(400bp)::lacZ* fusion	This study
YT0350s	YT0310 with a chromosomal *dsbD::lacZ* fusion	This study
YT0356	NMB with a chromosomal *hlyB::lacZ* fusion	This study
YT0356s	YT0310 with a chromosomal *hlyB::lacZ* fusion	This study
E. coli		
DH5α	Cloning strain	New England Biolabs
TOP10	Cloning strain	Invitrogen
Plasmids		
pCR2.1	TA cloning vector, Ap^R, Km^R	Invitrogen
pWSK29	Cloning vector	(61)
pUC18k	Source of *aphA-3* cassette	(33)
pYT328	Cloning vector for *lacZ* fusion in a chromosomal intergenic region	(60)
pYT334	pUC18 containing the 5′ sequence of misR and the 3′ sequence of *misS* with a newly created SmaI site	This study
pYT336	*aphA-3* cassette inserted into the SmaI site of pYT334	This study
pJKD2581	complementation vector that integrated into the *iga* locus	C. Kahler, unpublished
pJKD2582	pWSK29 (Cm^R) containing misR coding sequence	This study
pJKD2583	*Ptrc::misR* in pJKD2581 for complementation	This study

1

Table 2. Genes analyzed by qRT-PCR and EMSA

Functional catagory	ORF no. Z2491	Gene function and name	Fold change (array)[b]	Fold change (qRT-PCR)[c]	EMSA [d]
Protein folding/ stress response	NMA0209	Protein folding and stabilization, *dnaJ*	4.6 ± 2.2	5.5 ± 1.1	+
	NMA0273	fkbp-type peptidyl-prolyl cis-trans isomerase, *fkpA*	4.8 ± 2.2	10.0 ± 7.9	+
	NMA0441	Hsp33 protein; *hslO*	2.9 ± 0.5	nd	-
	NMA0744	Probable heat shock protein, *grpE*	3.5 ± 0.7	nd	-
	NMA1031	Putative membrane-bound zinc metallopeptidase, *htpX*	-3.0 ± 0.8	nd	-
	NMA1683	ATPase subunit of Clp protease, *clpB*	8.9 ± 4.1	11.6 ± 4.7	+
	NMA1719	thiol:disulfide interchange protein; *dsbD*	-5.6 ± 1.7	-13.9 ± 5.5	-
Regulation	NMA0738	Possible repressor protein	3.3 ± 1.0	4.1 ± 1.8	+
	NMA0797	Two-component histidine kinase, *misS*	-9.7 ± 4.0	-7.1± 3.4	nd
	NMA0798	Two-component response regulator, *misR*	-8.3 ± 1.1	-29.2 ± 7.0	+
	NMA1593	Possible *rrf2*-family DNA-binding protein	2.6 ± 0.7	3.2 ± 1.1	+
	NMA1751	Possible *gntR*-family transcriptional regulator	3.8 ± 1.6	2.7 ± 0.9	+
Metabolism	NMA0274	Glycerate dehydrogenase, *hprA*	4.1 ± 2.0	8.4 ± 6.9	+
	NMA1556	dihydrolipoamide dehydrogenase, *lpdA*	-2.3 ± 0.2	-2.3 ± 1.2	nd

	NMA2052	Aconitate hydrotase I, *acnA*	6.8 ± 2.4	18.3 ± 7.9	nd
	NMA2054	Citrate synthase, *prpB*	5.0 ± 2.3	16.0 ± 4.6	nd
Iron assimilation	NMA1377	Putative bacterioferritin A, *bfrA*	-4.1 ± 0.8	-8.0 ± 1.9	+
	NMA1700	TonB-dependent receptor, *tdfH*	-3.1 ± 0.6	-9.5 ± 2.2	+
	NMA1925	Hemoglobin receptor, *hmbR*	-2.3 ± 0.5	-13.4 ± 2.8	+
Transport/ secretion	NMA0277	Putative lipoprotein	3.5 ± 0.3	2.8 ± 1.5	+
	NMA0409	tryptophan transporter, *mtr*	3.5 ± 1.0	4.3 ± 2.1	+
	NMA0796	Conserved hypothetical protein, downstream of *misS*	-2.6 ± 0.8	-4.0 ± 3.4	nd
	NMA1620	Putative type I secretion protein, *hlyB*	2.5 ± 0.8	4.4 ± 0.9	+
	NMA1900	Putative haemolysin	-2.9 ± 0.6	-4.8 ± 1.8	-
	NMA1995/6	Putative type I secretion protein, *hlyD*	2.6 ± 0.4	4.1 ± 1.0	+

a: frame shift in the Z2491 genome

b: Mean values ± standard deviation of three microarray data, positive values indicate increased expression in the NMB*misR* mutant.

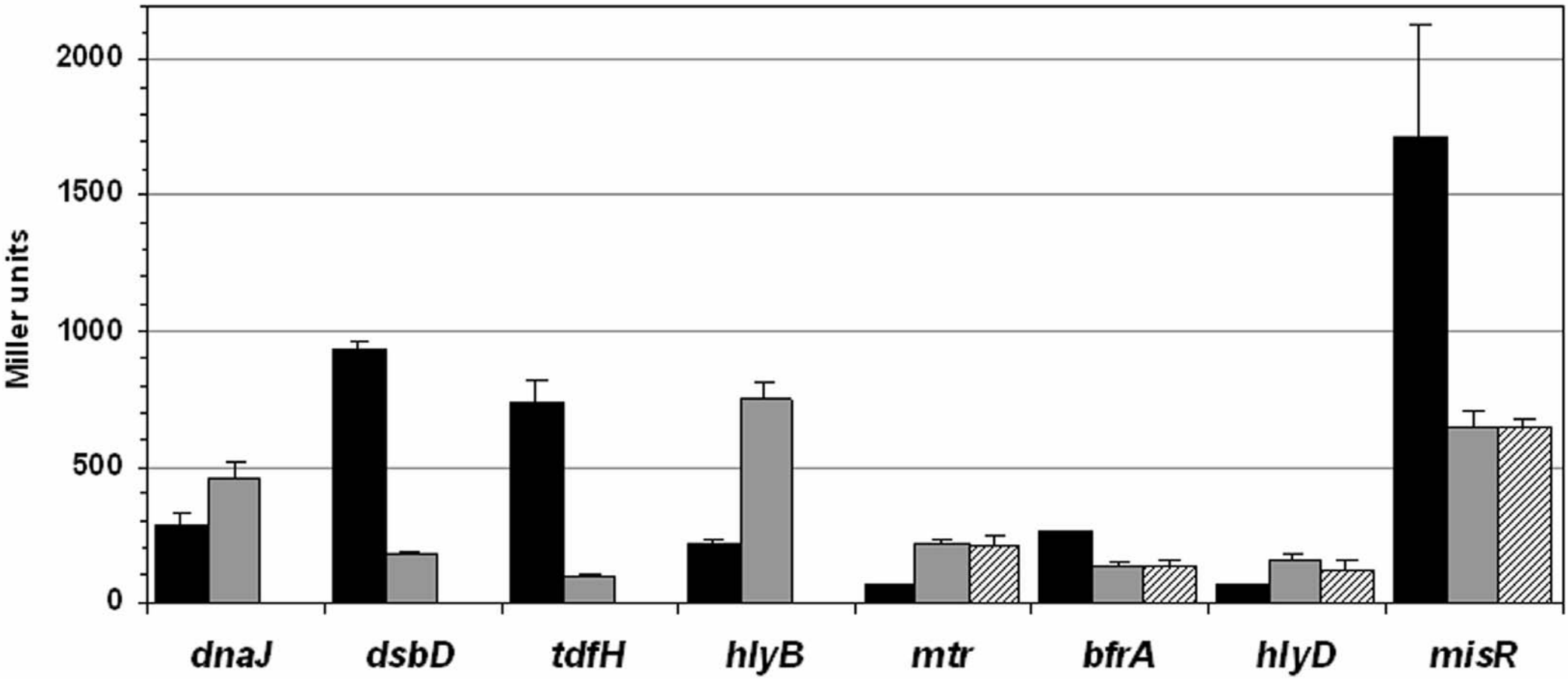
c: Mean values ± standard deviation of at least three independent qRT-PCR measurements using three sets of RNAs. Positive values indicate increased expression in the NMB*misR* mutant

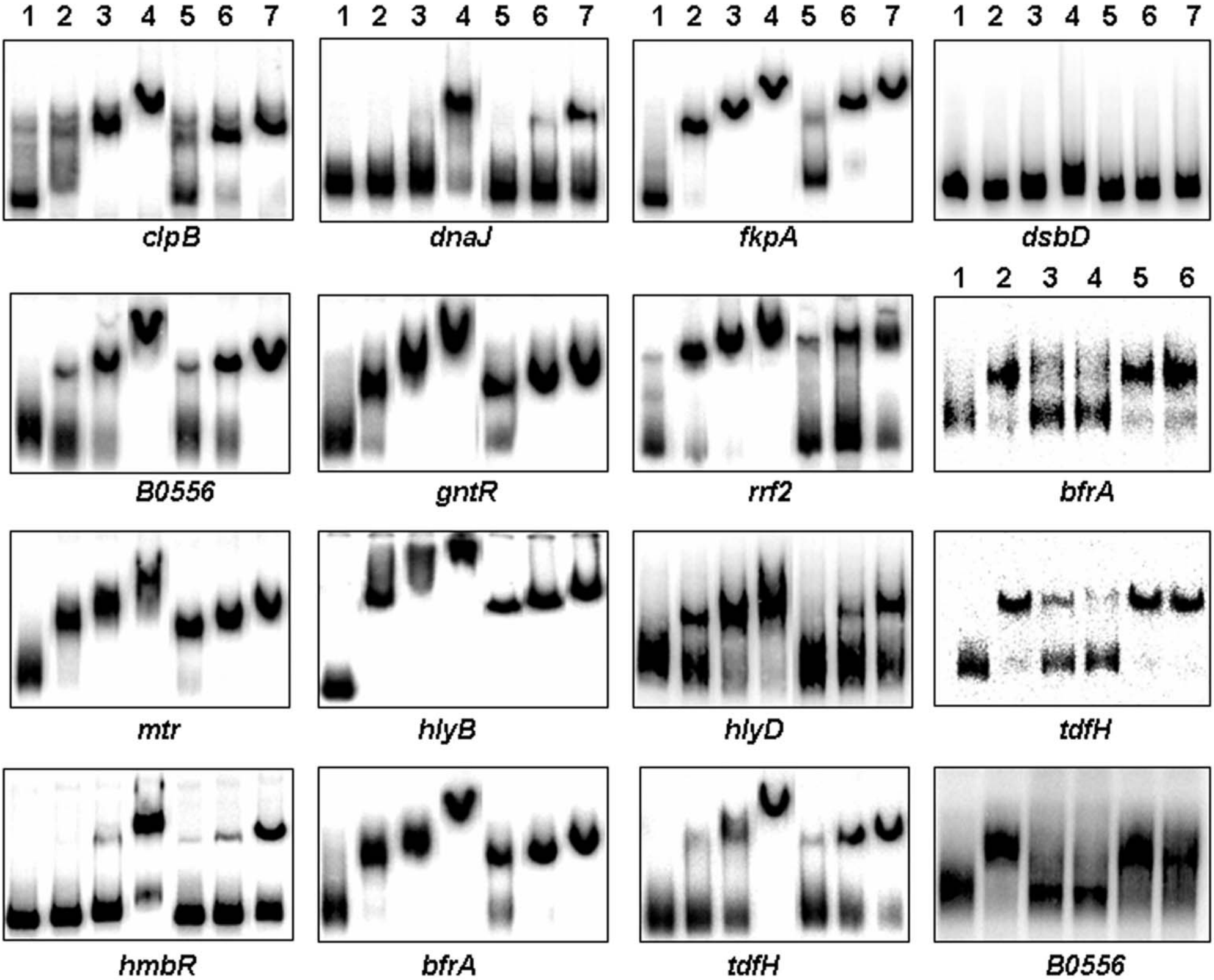
d: (+) positive band shifts observed; (-), no shift detected in all tested protein concentrations. nd, not done.

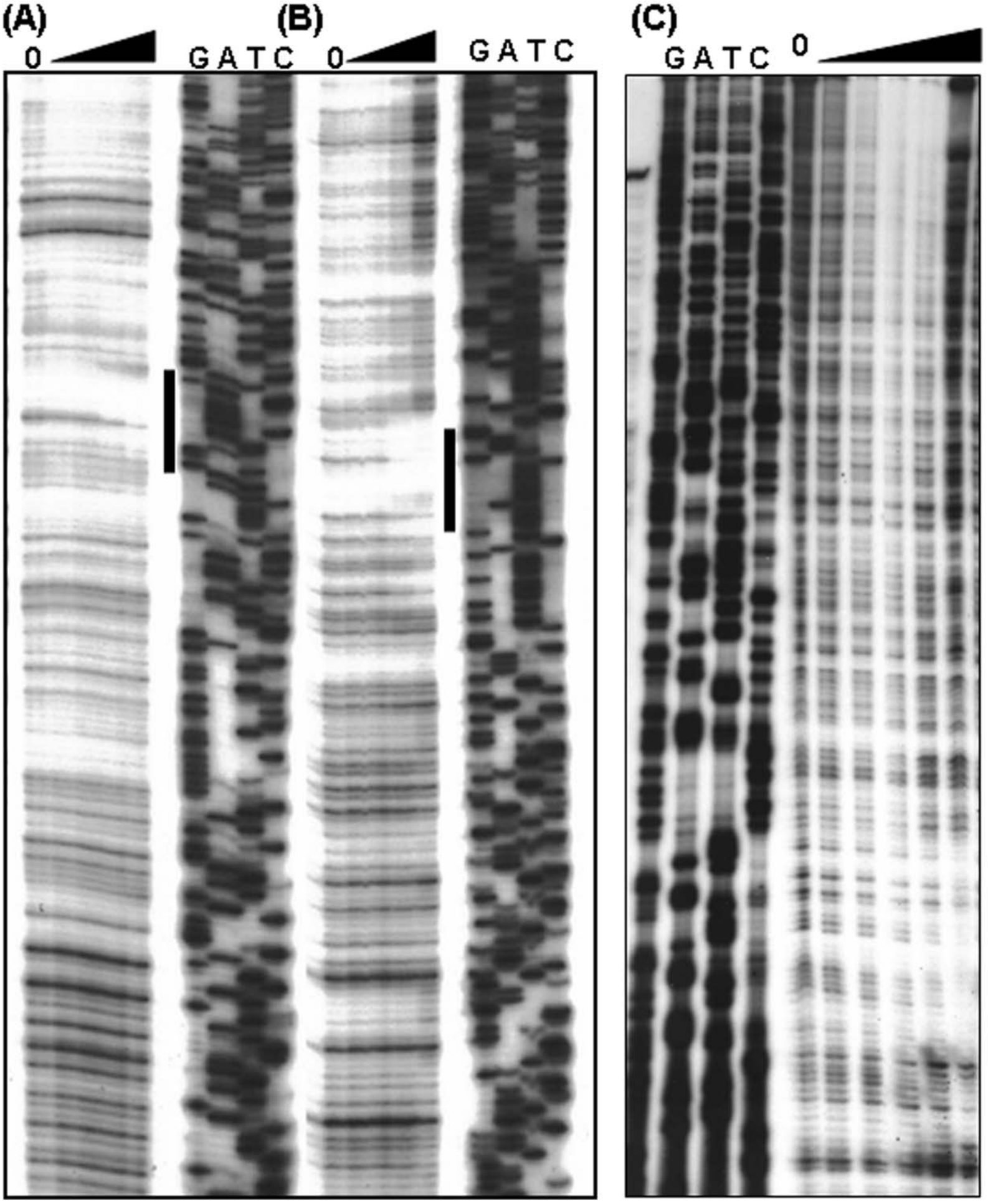
Table 3. The *misR/S* mutation increased sensitivity to oxidative stress

Strain	Zone of growth inhibition (mm) ± SD[a] with:			
	H_2O_2-0.1%	H_2O_2-0.25%	Paraquat (50 mM)	Paraquat (100 mM)
NMB (wild type)	14.7 ± 0.6	18.2 ± 0.3	8.3 ± 1.3	11.3 ± 0.6
NMBmisR (*misR::aphA-3*)	18.0 ± 1.3*	21.5 ± 1.3*	12.2 ± 0.6*	17.3 ± 1.2*
YT0310 (*misS::aphA-3*)	17.0 ± 1.0*	20.0 ± 1.0*	11.7 ± 0.6*	15.3 ± 0.6*
YT0336 (*misRS::aphA-3*)	17.7 ± 0.6*	22.2 ± 0.3*	13.3 ± 0.6*	17.3 ± 1.5*

[a] 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 24-hr incubation. A Students' t-test showed significant differences when compared to the parental wild type strain (*, $p < 0.05$). Data were from one representative experiment.


Miller units
2000
1500
1000
500
0
dnaJ
dsbD
tdfH
hlyB
mtr
bfrA
hlyD
misR

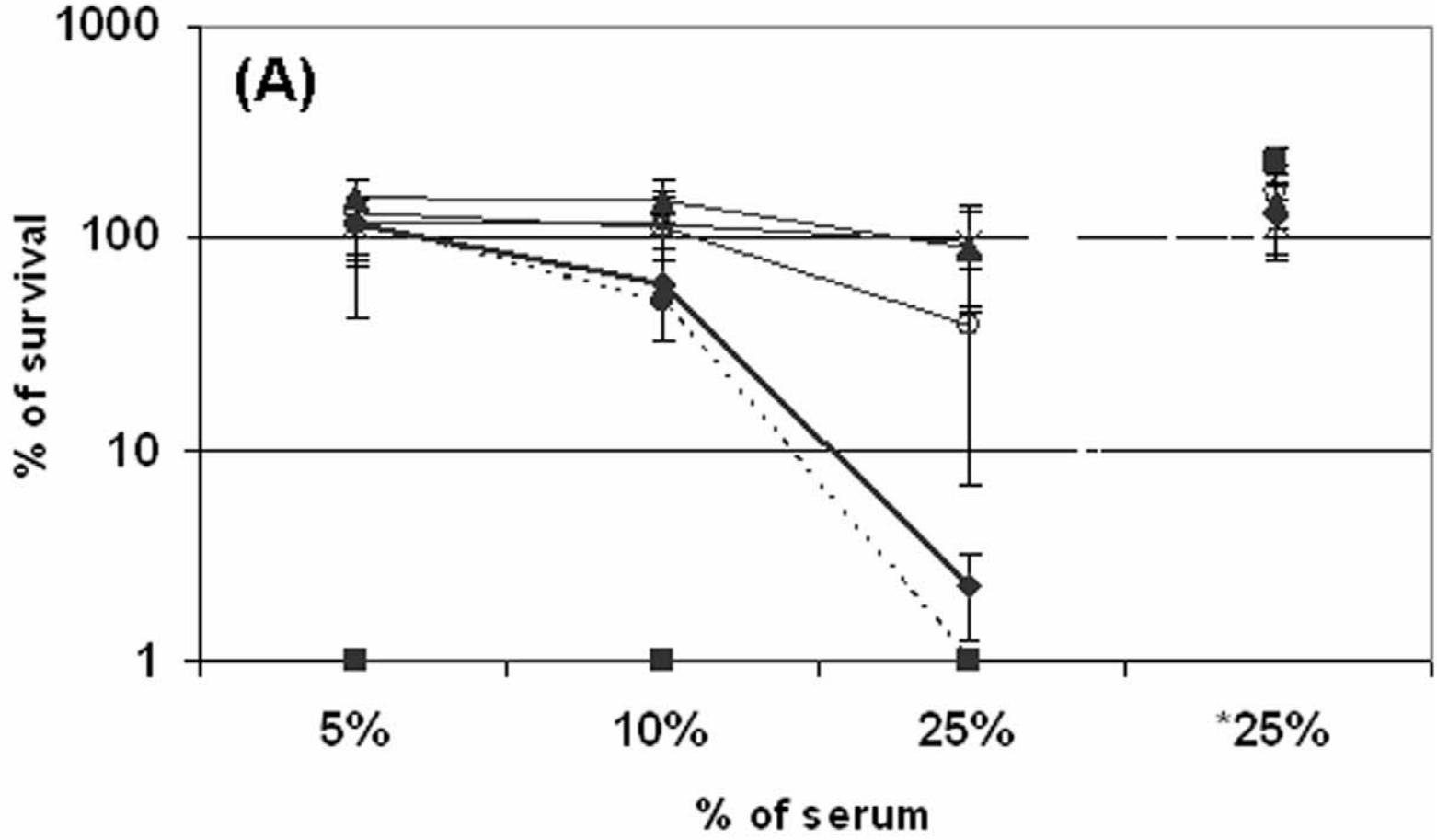

1 2 3 4 5 6 7
clpB
1 2 3 4 5 6 7
dnaJ
1 2 3 4 5 6 7
fkpA
1 2 3 4 5 6 7
dsbD
B0556
gntR
rrf2
1 2 3 4 5 6
bfrA
mtr
hlyB
hlyD
tdfH
hmbR
bfrA
tdfH
B0556

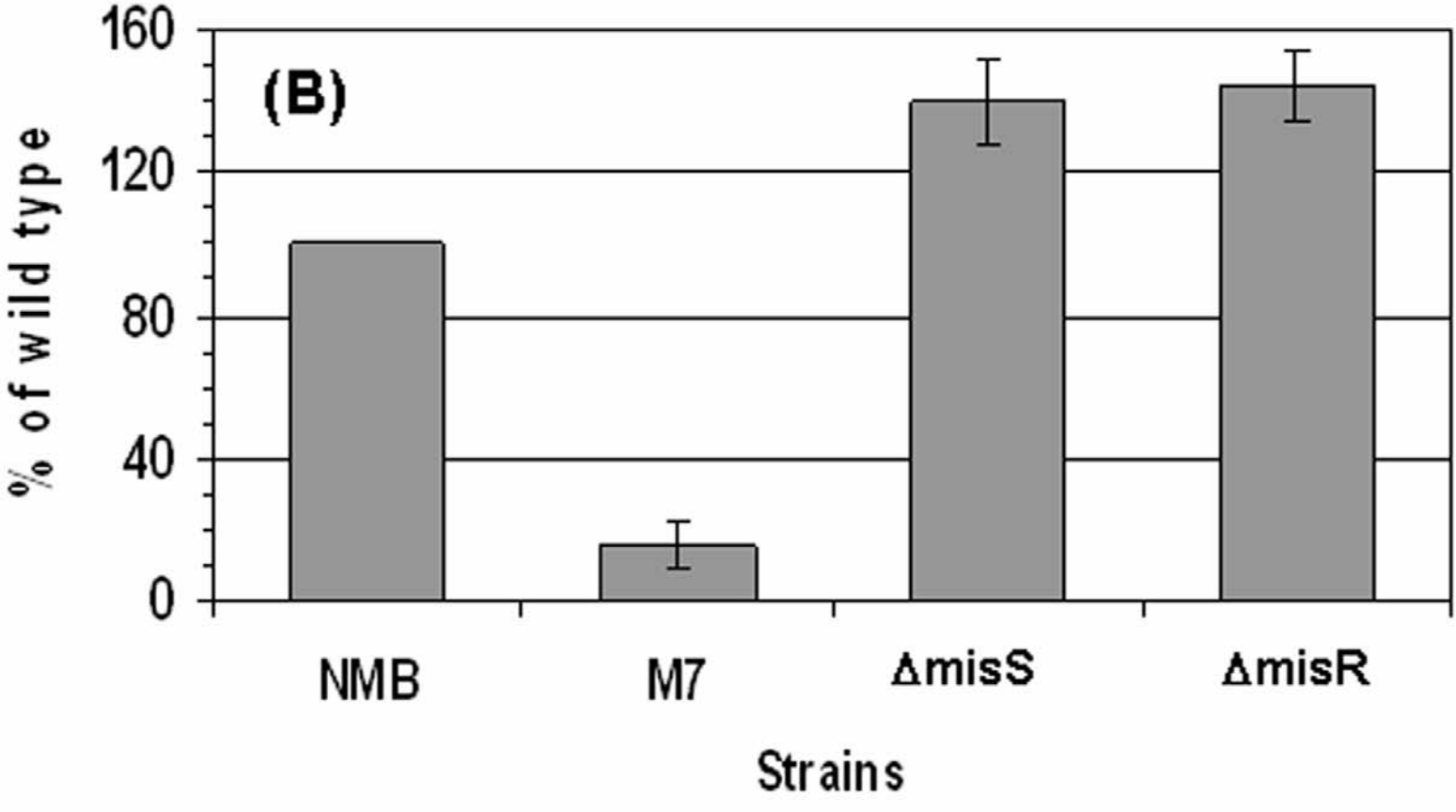

(A)
0
(B)
GATC
0
GATC
(C)
GATC
0

(A)

```
                                CCCGAAATGTAAAGCTCCATGCAAGACATTGCAAAAAACAG   >misR
ATGCAAACAAAGCCGCGCCGTGTAATTAAAGGTCTCTCT                                     >hmbR-1
TTTAATTACACGGCGCGGCTTTGTTTGCATGGATTA                                        >hmbR-2
      GCCGTAAAGTGTAAGGATTTGTAAGGTATTTGCGCC                                  >mtr-1
                GCCGTAAAGTGTAAGGATTTGTAAGGTATTTGCGCC                        >mtr-2
                 ATGGGAATTGTAAAGAGAAAGTATGGAAAA                             >hlyB-1
               CAGAACGCATTGTAAGGATATATATAT                                  >hlyB-2
   CGCAGTAAAATAATGAATATTTGTAACGACTTGGG                                      >hlyD-1
             CAATATAAATATGTAAAGGAATTTTAACGGAAAGCGCGGCAGC                    >hlyD-2
      TAAATATGTAAAGGAATTTTAACGGAAAGCGCGGCAGC                                >hlyD-3
      GACTGTTTTATTTTTTTCGTAAAGGTTCCATGCTTTTT                                >fkpA-1
   AGGCAACGCCGTACTGGTTTTTTGTTAATCCACTA                                      >fkpA-2
                    KWWWTGTAARGNNWH
```

(B)


(A)
1000
100
10
1
% of survival
5%
10%
25%
*25%
% of serum


(B)
160
120
80
40
0
% of wild type
NMB
M7
ΔmisS
ΔmisR
Strains