The Nitric Oxide (NO)-Sensing Repressor NsrR of *Neisseria meningitidis* Has a Compact Regulon of Genes Involved in NO Synthesis and Detoxification^{∇†}

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We have analyzed the extent of regulation by the nitric oxide (NO)-sensitive repressor NsrR from *Neisseria meningitidis* MC58, using microarray analysis. Target genes that appeared to be regulated by NsrR, based on a comparison between an *nsrR* mutant and a wild-type strain, were further investigated by quantitative real-time PCR, revealing a very compact set of genes, as follows: *norB* (encoding NO reductase), *dnrN* (encoding a protein putatively involved in the repair of nitrosative damage to iron-sulfur clusters), *aniA* (encoding nitrite reductase), *nirV* (a putative nitrite reductase assembly protein), and *mobA* (a gene associated with molybdenum metabolism in other species but with a frame shift in *N. meningitidis*). In all cases, NsrR acts as a repressor. The NO protection systems *norB* and *dnrN* are regulated by NO in an NsrR-dependent manner, whereas the NO protection system cytochrome *c'* (encoded by *cycP*) is not controlled by NO or NsrR, indicating that *N. meningitidis* expresses both constitutive and inducible NO protection systems. In addition, we present evidence to show that the anaerobic response regulator FNR is also sensitive to NO but less so than NsrR, resulting in complex regulation of promoters such as *aniA*, which is controlled by both FNR and NsrR: *aniA* was found to be maximally induced by intermediate NO concentrations, consistent with a regulatory system that allows expression during denitrification (in which NO accumulates) but is down-regulated as NO approaches toxic concentrations.

The bacterium *Neisseria meningitidis* is a human pathogen that causes two major diseases, meningitis and septicemia. The only natural habitat of *N. meningitidis* is the human pharynx, in which colonization is normally asymptomatic. Occasionally the organism causes invasive disease by spreading into the bloodstream to cause septicemia and crossing the blood–brain barrier to cause meningitis. *N. meningitidis* is one of the most important causes of death by infectious disease in young children and adolescents (41). *N. meningitidis* is exposed to the free-radical gas nitric oxide (NO), generated both internally by its own metabolism and externally by the human host tissue in its natural habitat. The tissue inhabited by *N. meningitidis* is rich in macrophages, which are a potent source of NO during infection (23, 24). We have found that *N. meningitidis* synthesizes NO detoxification proteins (NO reductase, NorB, and cytochrome *c'* [CycP]) that protect the organism from being killed by macrophage-generated NO (36). The environment in the pharyngeal mucosa is subject to various levels of oxygen availability, and the meningococcus has adapted and proliferates under conditions of oxygen limitation by using the alternative respiratory pathway of denitrification (30). In this process, nitrite is reduced to nitrous oxide, producing NO as a freely diffusible intermediate. The pathway is catalyzed by two enzymes, nitrite reductase, encoded by *aniA*, and NO reduc-

tase, encoded by *norB*. Thus, NorB has a dual role in both the protection against the toxicity of extracellular NO and the utilization of internally generated NO as part of a bacterial metabolic pathway. Furthermore, studies with tissue culture indicate that the activity of NorB has an impact on the signaling processes in human cells, affecting both apoptosis (38) and inflammatory cytokine production (35). The chief means of NO detoxification in *N. meningitidis* is the membrane-bound NO reductase NorB, with CycP having a secondary role (1). We have recently shown that the denitrification genes *aniA* and *norB* are both regulated by a repressor, NsrR, which is a sensor of NO (31).

NsrR was first identified as a nitrite-sensing repressor in the nitrifying bacterium *Nitrosomonas europaea* (3) and has subsequently been identified in a number of bacteria including *Escherichia coli* (4), *Bacillus subtilis* (26), *N. gonorrhoeae* (28), and *Salmonella enterica* serovar Typhimurium (14), as well as *N. meningitidis* (31). Rodionov et al. (32) predicted the role of NsrR in a number of alpha-, beta-, and gammaproteobacteria, in members of the order *Bacillales*, and in *Streptomyces* spp. prior to the experimental determination that this protein is the major NO-responsive transcriptional regulator among diverse bacterial taxa.

Recently, a global analysis of the NsrR regulon in *E. coli* was performed (11). In that study, it was found that nine operons containing 20 genes were negatively regulated by NsrR and that a similar number of genes was activated by the regulator. The regulon is larger than predicted bioinformatically; Rodionov et al. (32) had predicted four transcriptional units (containing five genes) regulated by NsrR in *E. coli* (*hcp/hcr*, *hmp*, *ytfE* [also known as *dnrN*], and *ygbA*). Given the potential for iden-

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TABLE 1. Strains, plasmids, and primers used in this study

Strains, plasmids, and oligonucleotides	Description or sequences (5'-3') ^a	Reference or origin
Strains		
<i>N. meningitidis</i>		
MC58	Wild-type serogroup B	25
<i>aniA</i> -Sp	Derivative of MC58 with the insertion of an Sp resistance cassette into <i>aniA</i>	30
<i>fur</i> -Em	Derivative of MC58 with the insertion of an Em resistance cassette into <i>fur</i>	30
<i>norB</i> -Sp	Derivative of MC58 with the insertion of an Sp resistance cassette into <i>norB</i>	1
<i>nsrR</i> -Sp	Derivative of MC58 with the insertion of an Sp resistance cassette into <i>nsrR</i>	31
<i>nsrR</i> -Tc	Derivative of MC58 with the insertion of a Tc resistance cassette into <i>nsrR</i>	This study
<i>nsrR</i> -Tc <i>aniA</i> -Sp	Derivative of MC58 <i>nsrR</i> -Tc with the insertion of an Sp resistance cassette into <i>aniA</i>	This study
<i>nsrR</i> -Tc <i>norB</i> -Sp	Derivative of MC58 <i>nsrR</i> -Tc with the insertion of an Sp resistance cassette into <i>norB</i>	This study
<i>nsrR</i> -Sp <i>nsrR</i> ⁺ and <i>nsrR</i> -Tc <i>nsrR</i> ⁺	Derivative of MC58 <i>nsrR</i> -Sp and <i>nsrR</i> -Tc with the insertion of the intact <i>nsrR</i> gene, with its promoter region from pKHE2: <i>nsrR</i> ⁺ between NMB102 and NMB103; Em ^r	This study
<i>E. coli</i> DH5α	F ⁻ <i>endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 Δ(lacZYA-argF)U169 deoR λ(φ80 dlacZ ΔM15)</i>	33
Plasmids		
pBLS-II KS	Cloning vector; ColE1 replicon, Ap ^r	Stratagene
pCMT18	Tc ^r donor vector	C. Tang
pCRBlunt II-TOPO	Cloning vector, Km ^r	Invitrogen
pGIT5.3	Derived from pCRII; carrying a <i>Neisseria</i> DNA uptake sequence, Cm ^r	T. Baldwin
pJR113	pGemT-easy carrying the <i>nsrR</i> gene disrupted by Sp ^r	31
pKHE2	Derived from pYHS25; with MCS to clone genes plus their native promoters into the intergenic region between the convergent genes NMB0102 and NMB0103; Em ^r	K. Heurlier, C. Tang, and K. Hardie, unpublished data
pKHE2: <i>nsrR</i> ⁺	pKHE2 carrying a 644-bp BamHI fragment with the <i>nsrR</i> gene and its upstream region; Em ^r	This study
pKHE25	pGIT5.3 carrying the flanking regions of the deleted <i>nsrR</i> gene replaced with Tc ^r	This study
Primers		
<i>nsrR</i> up-BamHI	AAAAGGATCCGGGATGTCGTTTCAA	
<i>nsrR</i> dwn-BamHI	AAAAGGATCCATTACTGCACCGCAA	
<i>aniA</i> dwn	GATATCAACCTTTACCGCTCC	
<i>nirV</i> -Stoprev	GTTATCGGCTTGTGCA	

^a BamHI restriction sites are underlined. Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol.

tifying novel NO-regulated genes and the power of global gene expression analysis for evaluating the extent of regulation in genomes, we decided to investigate the regulon of NsrR in *N. meningitidis*, in which Rodionov had predicted five NsrR-regulated gene clusters (*narQ/narP*, *dnrN*, *aniA* [also known as *nirK*], *norB*, and the *nos* [nitrous oxide-reductase] genes). Our experimental data fit well with these predictions and showed that NsrR has a small regulon in *N. meningitidis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown in Luria-Bertani (LB) medium (10 g Oxoid Bacto peptone, 5 g Oxoid yeast extract, 10 g sodium chloride per liter) or on LB agar plates at 37°C. *N. meningitidis* (MC58) derivative strains were routinely grown on Columbia blood agar (CBA; Columbia agar base plus 5% horse blood) plates at 37°C in the presence of 5% CO₂. Liquid cultures of *N. meningitidis* were started from a suspension in Mueller Hinton broth (MHB) of cells harvested from plates; aerobic growth was achieved in 7.5 ml MHB in 50-ml polypropylene Falcon tubes with shaking at 200 rpm. Microaerobic cultures were grown in 18 ml MHB in 25-ml polypropylene

Sterilin tubes, with shaking at 100 rpm, or in 1.1 ml MHB in 1.5-ml Eppendorf tubes with shaking at 150 rpm; denitrification conditions were obtained by adding 10 mM NaHCO₃ and 5 mM NaNO₂ to 22 ml MHB in 25-ml polypropylene Sterilin tubes and shaking at 100 rpm.

When required, antibiotics were added to the medium at the following concentrations: ampicillin, 100 µg ml⁻¹ (*E. coli*); tetracycline (Tc), 25 µg ml⁻¹ (*E. coli*) or 2.5 µg ml⁻¹ (*N. meningitidis*); spectinomycin (Sp), 50 µg ml⁻¹ (*E. coli* or *N. meningitidis*); kanamycin, 50 µg ml⁻¹ (*E. coli*); chloramphenicol, 25 µg ml⁻¹ (*E. coli*) or 2.5 µg ml⁻¹ (*N. meningitidis*); erythromycin (Em), 200 µg ml⁻¹ (*E. coli*) or 5 µg ml⁻¹ (*N. meningitidis*).

DNA manipulation and cloning procedures. Small-scale preparations of plasmid DNA were performed using a Miniprep kit (Qiagen). Chromosomal DNA was extracted from *N. meningitidis* as described previously (12). Restriction enzyme digestions, ligations, and agarose gel electrophoresis analyses were performed using standard methods (33). Restriction fragments were routinely purified from agarose gels, using a QIAquick kit (Qiagen). Transformation of *E. coli* strains was carried out by heat shock of calcium chloride-competent cells (16). For *N. meningitidis*, transformations were performed by incubating 10 µl of a dense suspension of *N. meningitidis* strains in MHB together with 10 µl of plasmidic or chromosomal DNA carrying the regions to cross over on a CBA plate for 4 h at 37°C in the presence of 5% CO₂, prior to plating onto CBA with the appropriate antibiotic for the selection of the recombination. To ensure that

mutant strains were disrupted only in the gene of interest and to prevent the isolation of phase variants, mutants were always backcrossed by retransforming the parental strain with chromosomal DNA isolated from mutant strains and by analyzing the phenotypes of three independent mutants derived in this way. Oligonucleotide primers were synthesized by MWG AG Biotech (United Kingdom) and are listed in Table 1 and in Table S1 in the supplemental material. Cloned PCR products were sequenced by MWG AG Biotech or by the technology facility of the University of York. The alignment of nucleotide sequences was performed by using ClustalW (<http://clustalw.genome.jp/>) software.

Plasmid and mutant constructions. To allow construction of the *nsrR aniA* and the *nsrR norB* double mutants, the Sp resistance cassette interrupting the deleted *nsrR* gene (31) was replaced with a Tc cassette. An inverse PCR using primers NoregInv1 and NoregInv2 (31) and High Fidelity polymerase (Roche) allowed the removal of the Sp cassette inserted in *nsrR* (*nsrR*-Sp) on pJR113 and the introduction of artificial HindIII sites. The ~4-kb DNA product obtained was digested with HindIII and rendered blunt by treatment with a DNA polymerase I Klenow fragment prior to ligation with a 2.5-kb Tc cassette excised from pCMT18 with EcoRV. A 3.5-kb NotI fragment carrying *nsrR'*-Tc was excised from the resulting plasmid and subcloned into pGIT5.3 digested with NotI, resulting in pKHE25 for the replacement of *nsrR* with *nsrR'*-Tc in *N. meningitidis* cultures.

A vector for the ectopic complementation of the *nsrR* mutation was constructed as follows. A 644-bp fragment encoding *nsrR* (NMB0437 [where the prefix NMA is *N. meningitidis* serogroup A, and NMB is *N. meningitidis* serogroup B]) and 198 bp of its upstream region were PCR amplified using *Pfu* polymerase (Promega) and primers *nsrR*up-BamHI and *nsrR*down-BamHI carrying engineered restriction sites for BamHI (Table 1), cloned into pCR-Blunt II-TOPO, and sequenced. The fragment was then excised using BamHI and cloned into pKHE2, digested with the same enzyme, resulting in pKHE2::*nsrR*⁺, which allows the insertion of *nsrR*⁺ and a selective Em cassette between NMB0102 and NMB0103 on the chromosome of *N. meningitidis*.

RNA extraction and cDNA synthesis. RNA was extracted for real-time (RT)-PCR experiments with cultures that reached an optical density at 600 nm (OD₆₀₀) of 0.5, i.e., after 3 to 4 h for aerobic cultures, after 7 h for microaerobic cultures in Eppendorf tubes, and after 8 h for microaerobic cultures in Sterilin tubes. Cultures were quenched for 10 s in a bath of ethanol plus dry ice and transferred on ice. Small-scale RNA preparations for RT-PCR analyses were performed with 1-ml samples of culture, using an RNeasy mini-kit (Qiagen), following the manufacturer's instructions. One microgram of RNA (as quantified by using a NanoDrop spectrophotometer) was used as a template for the synthesis of cDNA, using random hexamers and Superscript II reverse transcriptase (Invitrogen) at 42°C for 2 h. For large-scale preparations of RNA for microarray experiments, aerobic cultures were grown to an OD₆₀₀ of 0.7 to 1.0, and cultures were rapidly cooled with dry ice-ethanol and then stored on wet ice. Five-milliliter samples from cultures were harvested for RNA isolation, using an RNeasy mini-kit (Qiagen). Five milliliters of culture yielded 80 µl of RNA with a concentration of 250 to 500 ng/µl. RNA quality was checked by using a Bioanalyzer chip.

Microarray hybridization and data analysis. *N. meningitidis* microarray slides used for this study were obtained from Eurogentec (Belgium). Arrays consisted of glass slides containing PCR products of all open reading frames (ORFs) from *N. meningitidis* serogroup A strain Z2491 plus 73 ORFs from the *N. meningitidis* serogroup B strain MC58 (all in duplicate pairs). Negative controls (*Renilla* luciferase normalization control, spatial control, and three *E. coli* genes) were also included. A SuperScript indirect cDNA labeling system (Invitrogen Life Technologies, Carlsbad, CA) was used to label 10 µg of RNA, following the manufacturer's protocol. The product was resuspended in 50 µl of SlideHyb no. 1 hybridization buffer (Ambion, Inc., Austin, TX), denatured at 95 to 100°C for 2 min, and then pipetted onto the slides and coverslip (Corning, NY) before they were sealed into a hybridization chamber (Corning, NY). The sealed chambers were wrapped in aluminum foil and incubated at 42°C for 16 to 22 h. The arrays were subsequently washed for 5 min with 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% (wt/vol) sodium dodecyl sulfate, followed by a second 5-min wash in 0.5× SSC and 0.01% sodium dodecyl sulfate and a third 5-min wash in 0.05× SSC at room temperature. The slides were dried by centrifugation.

Arrays were read with an Axon 4000 scanner (Axon, Union city, CA) at 10 µm resolution and various photomultiplier tube voltage settings to obtain the maximal signal intensities with <1% probe saturation. The fluorescence intensities for each fluor and element on the array were captured using GenePix Pro 5.0 (Axon, Union city, CA) software. The local background value was subtracted from the value of each spot on the array. A pixel intensity cutoff value of 300 was established based on the pixel intensity values obtained from negative control

features within the array. Normalization of Cy3 and Cy5 signals was performed by adjusting the signal intensities of the two images (global normalization). Within-array normalization of the Cy3/Cy5 channel intensities was performed using the locally weighted linear regression (LOWESS) algorithm, incorporating signal intensities of all three replicated elements for each gene, which reduces differential dye effects (5, 42). The significance of the results was determined using SAM scatter plot analysis software (40).

Quantitative real-time RT-PCR analysis of gene expression. Transcript levels were measured by RT-PCR using Power SYBR Green PCR Master Mix and an ABI 7300 sequence analyzer (Applied Biosystems). Primers were designed using PrimerExpress (Applied Biosystems) and are listed in Table S1 in the supplemental material. Transcript levels were quantified using the threshold cycle method values (22) relative to the expression levels of the *metK* gene as a control.

Spermine NONOate treatment. Spermine NONOate (Z)-1-[N-(3-aminopropyl)-N-[4-(3-aminopropylammonio) butyl]-amino]diazene-1-ium-1,2-diolate; AG Scientific, Inc., San Diego, CA) was used as an NO donor with cultures that reached an OD₆₀₀ of ~0.5, as described previously (31), at final concentrations of 1, 2, 5, 10, 20, 35, 50 and 75 µM. Aliquots of 1 ml were harvested by centrifugation for RNA extraction after a 20-min incubation.

To estimate the concentration of NO released from spermine NONOate, 5 ml of cells grown microaerobically to an OD₆₀₀ of ~0.5 in MHB was transferred to a Clark-type oxygen electrode chamber. Spermine NONOate was added to final concentrations of 5, 20, and 50 µM, and NO concentration was measured by using an ISO-NOP Mark II 2-mm electrode (World Precision Instruments, Stevenage, United Kingdom). In parallel, the presence of oxygen was monitored by using a Clark-type oxygen electrode (Rank Bros., Bottissham, United Kingdom).

Microarray data accession number. Microarray data have been deposited in the Array Express database under accession number E-MEXP-1448.

RESULTS

Transcriptome analysis of the *N. meningitidis nsrR* mutant reveals a small regulon. To determine the extent of the regulation of NsrR, we performed a microarray analysis, comparing the wild-type *N. meningitidis* MC58 with its isogenic mutant *nsrR*-Sp. Fourteen genes were initially identified in which the effect of the *nsrR* mutation resulted in a greater than twofold change in expression. Each of these genes was further analyzed by RT-PCR, which yielded just four genes regulated more than twofold by both methods, plus a pseudogene (*mobA*) (Table 2; see Table S2 in the supplemental material). Complete microarray data sets are available in Table S3 in the supplemental material. The transcriptomic data confirm the negative control of NsrR over the denitrification cluster of the *norB* and *aniA* genes; in addition, NsrR exerts control over the expression of NMB1624 located immediately downstream of *aniA*. BLAST searches revealed that NMB1624 is similar to the *nirV* gene, which is cotranscribed downstream from the gene encoding copper-type nitrite reductase carried by *Rhodobacter sphaeroides* and is also found in other denitrifiers (19). The function of *nirV* is currently unknown, but it is likely to be involved in nitrite reductase function. Subsequently, NMB1624 will be referred to as *nirV*. As predicted (32), another gene that is strongly negatively controlled by NsrR is *dnrN* (NMB1365). DnrN is a di-iron protein that has been implicated in protection against NO damage. Recent literature has suggested that DnrN (also known as YtfE) in *E. coli* is involved in the repair of iron-sulfur clusters damaged by NO (20), while the DnrN homologue of *Ralstonia eutropha* (also known as NorA) is believed to act as a cytoplasmic NO buffer, protecting the cell from NO damage by lowering the cytoplasmic concentration of the radical (37). Another gene predicted to be controlled by NsrR in *N. meningitidis*, due to the presence of an NsrR recognition motif in its promoter, is *narQ* (32). However, *narQ*

TABLE 2. Regulation of the expression of *N. meningitidis* MC58 genes by NsrR and in response to NO

Gene	Gene product	Expression ratio (mean $\pm$ SE) ^b				Putative NsrR binding site ^c	Position (base) ^d
		<i>nsrR</i> versus wild type		<i>nsrR</i> complemented versus wild type (RT-PCR)	Following 50 μ M spermine NONOate treatment (RT-PCR)		
		Microarray	RT-PCR				
NMB1623	AniA ^a	5.89 $\pm$ 0.52	11.98 $\pm$ 2.33	0.837 $\pm$ 0.08	1.282 $\pm$ 0.07	taaG TTTCATAT ttt TaTGAAT tat	−106
NMB1624	NirV ^a	3.72 $\pm$ 0.38	4.65 $\pm$ 0.19	1.115 $\pm$ 0.09	1.508 $\pm$ 0.34		
NMB1622	NorB ^a	3.43 $\pm$ 0.18	23.17 $\pm$ 3.33	1.094 $\pm$ 0.12	32.8 $\pm$ 5.88	aac ATTTCATAT ttt TgTGAAT ttt	−41
NMB1365	DnrN ^a	2.87 $\pm$ 0.23	13.21 $\pm$ 1.98	1.036 $\pm$ 0.11	8.228 $\pm$ 0.55	ttg ATTCTT Aag TaTGAAT ggt	−50
NMB1248	MobA ^a	2.78 $\pm$ 0.86	2.92 $\pm$ 0.11	1.154 $\pm$ 0.15	2.51 $\pm$ 0.21	aaa ATTTCAGAT aa TaTGAAT tta	−102
NMB1249	NarQ	1.376 $\pm$ 0.228	2.08 $\pm$ 0.31	1.519 $\pm$ 0.33	1.483 $\pm$ 0.13	ttg ATTCTT Aag TaTGAAT ggt	−139
NMB1250	NarP	1.173 $\pm$ 0.123	1.34 $\pm$ 0.17	0.930 $\pm$ 0.09	1.177 $\pm$ 0.11		
NMB0923	CycP	1.594 $\pm$ 0.176	1.429 $\pm$ 0.33	1.113 $\pm$ 0.11	1.351 $\pm$ 0.12		

^a The gene was found to be regulated more than twofold by a microarray experiment and by RT-PCR.

^b Microarray experiments were carried out with three independent cultures, and each hybridization was analyzed from duplicate spots. All RT-PCRs were carried out with at least three independent cultures.

^c Motifs were found using the consensus sequence ATTCATATnnTnTGAAT, as described by Rodionov et al. (32). Capital letters indicate the consensus sequence; letters in bold type indicate bases that concur with the consensus.

^d Position relative to the translational start site of the gene.

and the cotranscribed gene *narP* were not shown to be strongly regulated by NsrR in our microarray (1.38 [$\pm$ 0.23]-fold and 1.17 [$\pm$ 0.12]-fold increased expression, respectively) analysis, while the expression of the NMB1248 gene (which encodes a truncated version of the molybdopterin-guanine dinucleotide biosynthesis protein A [*mobA*]), which is divergently transcribed from the same promoter region as *narQ*, is more than twofold repressed in the wild-type strain compared to that in the *nsrR*-deficient strain, according to microarray results (2.78 [$\pm$ 0.86]-fold) (Table 2). Rodionov et al. (32) predicted that the *nos* cluster of genes encoding nitrous oxide reductase in *Neisseria* species may also be regulated by NsrR. The effect of the *nsrR* mutation on *nos* expression in *N. meningitidis* was less than twofold (*nosR* expression was 1.46 [$\pm$ 0.15]-fold increased in an *nsrR* mutant, in our microarray experiment). It should be noted that the key structural gene for nitrous oxide reductase, *nosZ*, is absent from *N. meningitidis* and that the *nosR* regulator is severely truncated; this species is unable to reduce nitrous oxide.

Complementation of the *nsrR* mutation. To confirm that the phenotypes we attributed to the *nsrR* mutation were indeed NsrR dependent, we complemented the mutation by expressing in *trans* on the chromosome an intact copy of the *nsrR* gene (see Materials and Methods). The gene was under the control of its native promoter, i.e., the 198-bp upstream region that is intergenic between *nsrR* and its divergently transcribed neighboring gene NMB0438. The complementation was assessed by RT-PCR: for the *nsrR*-Sp mutant, no *nsrR* transcript could be detected, while for the wild type and the *nsrR*-Sp *nsrR*⁺ complemented mutant, transcripts are present (data not shown). Most likely because of its location closer to the origin of replication, the complementing copy of *nsrR* allows twofold more production of *nsrR* transcript. In addition, the expression of the *nsrR*⁺ gene in *trans* reverses the growth defect of the *nsrR*-Sp mutant aerobically (31) and restores wild-type levels of growth under denitrification conditions (data not shown).

In order to be able to construct different double mutants, we replaced the Sp cassette interrupting the *nsrR* gene of the

mutant with a Tc resistance cassette (see Materials and Methods). The resulting mutant has an aerobic growth defect similar to that of the *nsrR*-Sp mutant, compared to the growth pattern of the wild type, as observed previously (31) (data not shown). As expected, the *nsrR*-Tc mutant grows much faster than the wild type under denitrification conditions due to its derepression of *aniA* expression, as seen previously with the *nsrR*-Sp mutant (31).

NsrR response and NO response of target genes as quantified by RT-PCR. To quantify the control exerted by NsrR over the expression of the *norB*, *aniA*, and *nirV* genes in the denitrification cluster, RT-PCR experiments were carried out using mRNA prepared from aerobic cultures. All three genes were strongly up-regulated in response to the mutation in *nsrR*, an effect which was reversed on complementation with *nsrR* in *trans* (Table 2). To determine whether the repression by NsrR is relieved in response to the presence of NO, we performed another series of RT-PCR, this time using mRNA prepared from aerobic cultures of the wild type treated for 20 min with 50 μ M of spermine NONOate and comparing these results to those using an untreated control. This concentration of the NO donor derepressed the expression of *norB* about 30-fold, while it had no significant effect on the expression of *aniA* and *nirV* (Table 2). Overall, these results confirmed our previous observations using *lacZ* translational fusions, where the *norB* and *aniA* promoter fusions allowed increased β -galactosidase activities in an *nsrR* mutant compared to that in the wild type, while only *norB* expression increased with spermine NONOate treatment in the wild type (31).

The NsrR control over the potential NO stress response proteins DnrN and CycP (encoded by cytochrome *c'*) was also investigated. In confirmation of the microarray results, the expression of *dnrN* in an aerobic culture of the *nsrR* mutant is about 10-fold derepressed compared to that of the wild type, while there is no NsrR control over the expression of *cycP* (Table 2). The presence of NO derepressed the expression of *dnrN* in a similar manner but had no impact on the expression of *cycP* (Table 2).

The *norB*, *aniA*, and *dnrN* genes all possess NsrR binding motifs in their promoters (32). There is a putative NsrR binding motif located in the intergenic region between *mobA* and *narQP* (Table 2) (32), which might be involved in controlling the NsrR-dependent regulation of either of these transcriptional units. We used RT-PCR to compare levels of the *mobA*, *narQ*, and *narP* transcripts in the wild type with those in the *nsrR*-Sp mutant. Consistent with the results from microarray analysis, *mobA* expression was increased significantly (2.92 ± 0.11 -fold), whereas there is less impact on the expression of *narQ* (2.08 ± 0.31 -fold) and little change in the expression of *narP* (1.34 ± 0.17 -fold) (Table 2). Similarly, treatment of the sample with 50 μ M of spermine NONOate allowed a significant induction of *mobA* expression (2.51 ± 0.21 -fold) compared to that of *narQ* and *narP* (1.48 ± 0.13 -fold and 1.18 ± 0.11 -fold, respectively) (Table 2).

The *aniA* and *nirV* genes in the denitrification cluster form an operon. The NMB1624 (*nirV*) gene has been identified as a homologue of *nirV* of *Rhodobacter sphaeroides* 2.4.3 (19). Although *nirV* is relatively distant from *aniA* (124 bp downstream) and is not predicted to form an operon with it (using FGENESB software; Bacterial Operon and Gene Prediction), there is no obvious terminator of transcription between the two genes, and *nirV* expression follows exactly the same pattern of expression as *aniA* does in all experiments we performed. Furthermore, while the *aniA* promoter exhibits a conserved NsrR box, there is no such motif upstream of *nirV* (32; Table 2, our analysis). In *R. sphaeroides*, *nirV* is cotranscribed with the upstream nitrite reductase-encoding gene (19). Using RT-PCR, we found that *nirV* expression was up-regulated in an *nsrR* mutant but not in an *nsrR aniA* double mutant strain (in which the *aniA* gene is interrupted by an Sp cassette, including a terminator of transcription) (data not shown), indicating that *nirV* expression is dependent on the *aniA* promoter. Moreover, PCR using cDNA prepared from mRNA of the wild type and the *aniA*-Sp mutant using primer annealing downstream of the Sp resistance cassette and one annealing in the region of the stop codon of *nirV* confirms the presence of the 1.6-kb *aniA nirV* transcript that is absent in the *aniA*-Sp mutant (data not shown), supporting the notion that *aniA* and *nirV* form an operon.

The responses of NsrR and FNR to NO are concentration dependent and result in a biphasic expression of *aniA*. In order to determine the concentration of NO necessary to inactivate NsrR, we extracted mRNA from microaerobic cultures of wild-type *N. meningitidis* treated with increasing amounts of spermine NONOate. The microaerobic status of the cultures was verified in an independent experiment which showed that the cultures respired nitrite under these conditions (data not shown). With an NO electrode, we quantified the release of NO from these concentrations of NONOate in the presence of cells grown under the same conditions but maintained in a water-jacketed electrode chamber during the NONOate treatment. In this way, it was possible to obtain an estimation of the NO concentrations released by NONOate in this experiment. Over a 20-min incubation period in the presence of spermine NONOate, NO accumulated to a maximum of 50 ± 15 nM per μ M of spermine NONOate added (data not shown). Using a range of 0 to 50 μ M spermine NONOate, yields from 0 to 2 to 3 μ M NO were obtained.

FIG. 1. Effect of titration of NO on *N. meningitidis* NsrR and FNR-dependent gene expression. Expressions of (A) *norB*, (B) *dnrN*, (C) *aniA*, and (D) NMB0390 (an FNR-dependent control gene) in a wild-type *N. meningitidis* background and of (E) *aniA* in an *N. meningitidis nsrR-Tc norB*-Sp strain were assessed by RT-PCR quantification, which compared levels in wild-type cultures treated with 1, 2, 5, 10, 20, 35, or 50 μ M spermine NONOate for 20 min prior to total RNA extraction, relative to that of an untreated wild-type culture. Cultures were maintained in 1.1 ml in Eppendorf tubes, incubated at 37°C with shaking at 150 rpm. Each value is the mean $\pm$ standard error for three analyses of cDNA obtained from three independent cultures.

NsrR-dependent control of *norB*, *dnrN*, and *aniA* expression in the presence of 0 to 50 μ M spermine NONOate was assessed by RT-PCR using an untreated wild-type strain as the control. Higher concentrations (75 μ M spermine NONOate) have a toxic effect on cells (data not shown). The expression levels of *norB* and *dnrN* increase with the NO concentration over a range 0 to 20 μ M NONOate, after which expression begins to plateau (Fig. 1A and B). Previously, it was found that 50 μ M NONOate had no effect on *aniA* expression (Table 2) (31), but analysis over a range of NONOate concentrations, in the present study, showed that *aniA* expression is elevated in the presence of low concentrations of NO under microaerobic conditions (Fig. 1C). Maximal induction of *aniA* expression is seen at around 10 to 20 μ M spermine NONOate. As single *fnr* and double *nsrR fnr* mutants do not express any *aniA* (31), it is clear that FNR is crucial for *aniA* expression in *N. meningitidis*;

we therefore investigated the effect of NO on the FNR-dependent activation of gene expression, using NMB0390 as a target gene. NMB0390 encodes the maltose phosphorylase MapA and was shown to be controlled by FNR in a microarray study by Bartolini and colleagues (2). The expression of that gene decreased on treatment with spermine NONOate (Fig. 1D), indicating that *N. meningitidis* FNR may be sensitive to NO. This may explain the decrease in *aniA* expression with higher NONOate concentrations (Fig. 1C). To test this notion, we analyzed *aniA* expression in a *N. meningitidis* *nsrR*-Tc *norB*-Sp double mutant. The absence of NsrR should allow us to observe the impact of NO on the NsrR-independent activation of *aniA* expression. We used a strain that also lacked *norB* to prevent the rapid removal of NO via NorB, which is constitutively expressed in an *nsrR* mutant strain (31). In *N. meningitidis* *nsrR*-Tc *norB*-Sp, *aniA* is derepressed relative to that in the wild type in the absence of NO, but its expression is inhibited with increasing concentrations of spermine NONOate, consistent with the hypothesis that FNR is involved in the NO sensitivity of *aniA* expression (Fig. 1E).

As a control, we investigated the impact of NO on FUR-dependent expression in *N. meningitidis*. FUR is an iron-sensing repressor protein that has been implicated in the activation of the *aniA* gene expression (10) and shown to be a potential target of NO (8). To determine whether this regulator might be responsible for NO-dependent control of the *aniA* promoter, we analyzed the regulation of a major FUR target from *N. meningitidis* MC58, the NMB0460 gene (encoding a transferrin binding protein; the expression was 17-fold increased in a *fur* mutant; 9), in response to NO supplied as spermine NONOate over the range of 0 to 50 μ M. NO, had no appreciable impact on NMB0460 expression (data not shown), indicating that FUR is not important for NO-dependent control in this system.

DISCUSSION

Prior to this study, there was limited experimental evidence available to indicate the extent of regulation by NsrR within individual species. Our transcriptome analysis is in fairly good agreement with the bioinformatic predictions put forward by Rodionov et al. (32) and indicates that the NsrR regulon of *N. meningitidis* MC58 is very small (four genes plus one pseudogene). This differs from the situation in *E. coli*, in which it has been shown that NsrR controls the expression of approximately 40 genes (11). The finding that the NsrR regulon of *N. meningitidis* contains only a very small number of genes is in keeping with what we know about its regulation in response to other environmental variables, as measured by global transcriptome analysis of *Neisseria* species. Recent studies of regulation by the oxygen sensor-regulator FNR in *N. meningitidis* (2) and the oxidative stress sensor (OxyR) in *N. gonorrhoeae* (34) indicated that FNR controls nine transcriptional units and OxyR controls two operons (three genes). This compares with studies of *E. coli*, in which FNR controls more than 100 operons (27) and OxyR controls at least 20 genes (44). An exception to this pattern is regulation by the iron sensor-regulator Fur; the introduction of a *fur* mutation into *N. meningitidis* causes altered expression of more than 200 genes (15). In general, it seems that regulators that act globally in a highly

versatile organism, such as *E. coli*, are more prone to have more focused local activity against just a small number of regulated target genes in *N. meningitidis*, an organism that inhabits a single natural environment (the human upper respiratory tract) and contains only a small number of regulators.

We have been able to establish the range of spermine NONOate concentrations (and to a certain degree, the NO concentrations) over which the NsrR-dependent regulation of gene expression varies. For genes which are simply derepressed in an NsrR-dependent fashion (e.g., *norB* and *dnrN*), a concentration of around 20 μ M NONOate (which is equivalent to approximately 1 μ M NO) is sufficient to bring about considerable derepression. NO accumulates to around 1 μ M during the transition from aerobic growth to denitrification, and concentrations of around 1 μ M are sufficient to bring about a transient cessation of growth and inhibition of oxidase activity (30). The physiological concentrations of NO in human tissue are likely to be in the range of 0.1 to 1 μ M (21, 13, 17), indicating that the activation of gene expression in an NsrR-dependent/NO-dependent manner is tuned to be appropriate for the response to physiologically relevant NO concentrations.

norB and *dnrN* encode products that have been demonstrated to be involved in the response to NO: protecting the cell by reducing NO to N₂O (NorB [1]) and repairing iron-sulfur clusters damaged by NO (DnrN [9]). A third system for protecting *N. meningitidis* from NO is encoded by *cycP* (1), a gene that encodes CycP, which has been shown to be an NO-binding protein in *N. meningitidis* (18) and capable of protecting the bacteria *Rhodobacter capsulatus* (6) and *N. meningitidis* (1) from the toxic effects of NO. Our data demonstrate that there are NO-responsive (*norB* and *dnrN*) and constitutive (*cycP*) systems for protecting *N. meningitidis* against NO. Presumably, the constitutive CycP protects the organism against NO before other NO protection systems are able to be expressed, following exposure to a burst of NO. Indeed, in *N. gonorrhoeae*, CycP protects the bacterium during the transition to denitrification, a period in which NO accumulates prior to the expression of NO reductase (39).

The presence of NO may be perceived by *N. meningitidis* as a signal confirming the presence of a substrate for denitrification and, hence, may signal the expression of the nitrite reductase *aniA* gene. As AniA also leads to the production of NO, however, the cells have to ensure that endogenous NO never accumulates and reaches excessive, toxic concentrations. We show here that *N. meningitidis* uses an ingenious dual control of *aniA* expression by NsrR and FNR to stop nitrite reductase production before critical NO concentrations are achieved. (i) Under microaerobic conditions in the absence of NO, FNR allows some expression of *aniA*, despite the presence of the NsrR repressor (31). AniA is synthesized and nitrite respiration begins with a concomitant production of nitric oxide. (ii) As NO concentration increases (up to approximately 1 μ M, which is measurable on treatment with 20 μ M of spermine NONOate), NsrR becomes inactivated and the expression levels of the *norB*, *dnrN*, and *aniA* genes are derepressed (Fig. 1A, B, and C). NO concentrations are nontoxic, and genes encoding nitric oxide synthesizing activity (nitrite reductase, *aniA*), nitric oxide removal (*norB*), and detoxification (*dnrN*) are all expressed simultaneously. (iii) At higher NO concentrations (>1 μ M), FNR starts to be inactivated by NO, an effect which

does not influence the expression of *norB* or *dnrN* but causes a decrease in the expression of *aniA*. This switch to prevent excess nitrite reductase expression should protect the cell from NO as it approaches toxic concentrations. The sensitivities of NsrR and FNR to NO are appropriate to allow the optimal expression of denitrification/NO protection systems in response to varying NO.

FNR is normally regarded as an oxygen-sensitive activator protein, but its ability to respond to nitric oxide has been observed in *E. coli*, both in vitro (7) and in vivo (29). A recent report delineated a role for the FNR homologue ANR in controlling NO toxicity by preventing expression of the NO-synthesizing nitrite reductase in another denitrifying pathogen, *Pseudomonas aeruginosa* (43). Our work here confirms and extends this duality of function of the regulator FNR.

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