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Regulation of Denitrification Genes in *Neisseria meningitidis* by Nitric Oxide and the Repressor NsrR[▽]

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The human pathogen *Neisseria meningitidis* is capable of growth using the denitrification of nitrite to nitrous oxide under microaerobic conditions. This process is catalyzed by two reductases: nitrite reductase (encoded by *aniA*) and nitric oxide (NO) reductase (encoded by *norB*). Here, we show that in *N. meningitidis* MC58 *norB* is regulated by nitric oxide via the product of gene NMB0437 which encodes NsrR. NsrR is a repressor in the absence of NO, but *norB* expression is derepressed by NO in an NsrR-dependent manner. *nsrR*-deficient mutants grow by denitrification more rapidly than wild-type *N. meningitidis*, and this is coincident with the upregulation of both NO reductase and nitrite reductase even under aerobic conditions in the absence of nitrite or NO. The NsrR-dependent repression of *aniA* (unlike that of *norB*) is not lifted in the presence of NO. The role of NsrR in the control of expression of *aniA* is linked to the function of the anaerobic activator protein FNR: analysis of *nsrR* and *fnr* single and *nsrR fnr* double mutants carrying an *aniA* promoter *lacZ* fusion indicates that the role of NsrR is to prevent FNR-dependent *aniA* expression under aerobic conditions, indicating that FNR in *N. meningitidis* retains considerable activity aerobically.

The human pharynx is the only known natural habitat of *Neisseria meningitidis* (31). In the majority of cases, *N. meningitidis* colonization is asymptomatic, but occasionally invasive disease occurs following entry of meningococci into the bloodstream. *N. meningitidis* can grow aerobically, or, under oxygen limitation, growth is supported by denitrification using nitrite and nitric oxide as electron acceptors. Denitrification in this organism consists of the reduction of nitrite to nitric oxide via nitrite reductase AniA and reduction of nitric oxide to nitrous oxide via nitric oxide reductase NorB (1). The genes *aniA* and *norB* are divergently transcribed from one another and separated by an intergenic region of 370 base pairs (29). Nitric oxide (NO) in mammals is generated from arginine via the NO synthase enzymes (16). NO is present at high concentrations in the nasopharynx, as judged by its concentration in exhaled nasal breath (12). The ability of *N. meningitidis* to use NO is important for its lifestyle in several respects: (i) denitrification supports growth (23); (ii) NO is a toxic free radical gas, the toxicity of which is controlled by NO reductase in both pure culture (1) and tissue and organ culture models of *N. meningitidis* colonization (28); and (iii) bacterial NO reduction impacts upon host processes such as apoptosis (30).

Denitrification in *N. meningitidis*, and in its close relative *Neisseria gonorrhoeae*, is regulated by oxygen availability and also by the availability of nitrite and nitric oxide (10, 13, 23). The expression of the nitrite reductase gene *aniA* in *N. meningitidis* is controlled by oxygen via the transcriptional regulator FNR (fumarate and nitrate reduction regulator) and by nitrite via the two-component sensor-regulator NarQ/P (23). The expression of *norB*, on the other hand, appears to be consequent on the accumulation of NO in the culture medium. Here, we investigate the regulation of gene expression by NO and identify the NO-responsive regulator.

A number of different regulators have been implicated in the control of NO metabolism in bacteria. Regulators SoxR, FNR, and FUR, whose primary roles are considered to be the regulation of gene expression in response to superoxide stress, anaerobiosis, and iron limitation, respectively, have been shown to be capable of regulating gene expression in response to NO (5, 6, 19). Additionally, NO-specific regulators have been identified. NorR has been shown to regulate the expression of the flavorubredoxin NO detoxification system NorVW and other genes in *Escherichia coli* in direct response to NO (7). The regulator NsrR, which was originally identified as a nitrite-sensitive repressor in *Nitrosomonas europaea* (3), has homologues in *E. coli* and *Bacillus subtilis* which have been shown to be involved in control of gene expression in response to nitric oxide in these microorganisms (4, 17). The gene annotated as NMB0437 from the genome of *N. meningitidis* MC58 (29) was predicted by Rodionov et al. (24) to be an *nsrR* homologue. Here, we show that this gene from the pathogenic denitrifier *N. meningitidis* does indeed encode an *nsrR* homologue and that its gene product controls expression of denitrification genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All *N. meningitidis* strains used in this study were derived from *N. meningitidis* MC58 (Table 1). *N. meningitidis* strains were routinely cultured at 37°C in 5% CO_2 on Columbia horse blood agar plates or in liquid culture in Mueller-Hinton broth supplemented with 10 mM $NaHCO_3$. Aerobic culture was carried out in 7.5 ml of broth in a 50-ml Falcon tube with shaking at 200 rpm. Microaerobic culture was carried out using 20 ml of broth in a 25-ml McCartney bottle with shaking at 90 rpm and, where appropriate, supplemented with 5 mM $NaNO_2$. Antibiotics were used at the following concentrations: spectinomycin, 50 μg ml^{-1}; erythromycin, 100 μg ml^{-1}; and chloramphenicol, 2 μg ml^{-1}.

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TABLE 1. Strains, plasmids and oligonucleotide primers

Name	Description	Reference or source
N. meningitidis strains		
MC58	Wild-type serogroup B strain	14
nsrR	Derivative of MC58 with insertion of spectinomycin resistance cassettte into *nsrR*	This work
aniA	Derivative of MC58 with insertion of spectinomycin resistance cassette into *aniA*	23
norB	Derivative of MC58 with insertion of spectinomycin resistance cassette into *norB*	1
fnr	Derivative of MC58 with insertion of erythromycin resistance cassette into *fnr*	23
MC58 P*aniA-lacZ*	Derivative of MC58 containing an *aniA* promoter-*lacZ* fusion	23
aniA P*aniA-lacZ*	Derivative of *N. meningitidis aniA* containing an *aniA* promoter-*lacZ* fusion	1
nsrR P*aniA-lacZ*	Derivative of *N. meningitidis nsrR* containing an *aniA* promoter-*lacZ* fusion	This work
fnr P*aniA-lacZ*	Derivative of *N. meningitidis fnr* containing an *aniA* promoter-*lacZ* fusion	23
nsrR fnr P*aniA-lacZ*	Derivative of *N. meningitidis nsrR* transformed with *fnr* genomic DNA and containing an *aniA* promoter-*lacZ* fusion	This work
MC58 P*norB-lacZ*	Derivative of MC58 containing a *norB* promoter-*lacZ* fusion	This work
nsrR P*norB-lacZ*	Derivative of *N. meningitidis nsrR* mutant strain containing an *norB* promoter-*lacZ* fusion	This work
fnr P*norB-lacZ*	Derivative of *N. meningitidis fnr* containing an *norB* promoter-*lacZ* fusion	This work
nsrR fnr P*norB-lacZ*	Derivative of *N. meningitidis nsrR* transformed with *fnr* genomic DNA and containing an *norB* promoter-*lacZ* fusion	This work
Plasmids		
pHP45Ω	Contains Ω cassette encoding spectinomycin resistance	21
pLES94	Promoter-probe vector for neisserial promoters	27
pJR113	pGemT-easy vector containing *nsrR* with deletion and allelic disruption using Ω cassette	This work
pLES-P*aniA-lacZ*	*aniA-lacZ* translational fusion vector	23
pLES-P*norB-lacZ*	*norB-lacZ* translational fusion vector	This work
pCR-BluntII-TOPO	Vector for cloning blunt-ended PCR products	Invitrogen
Oligonucleotide primers		
NOregF1	5′-CAGGGTTGTGGCAAAAGCGGT-3′; for amplification of *nsrR* gene and flanking region	This work
NOregR1	5′-ACCACCTGACCCTGATGTGC-3′; for amplification of *nsrR* gene and flanking region	This work
NoregInv1	5′-AAAAGCTTCGTATCTGGACGGTTTCACGCTCC-3′; for inverse PCR deletion and allelic disruption of *nsrR* gene	This work
NoregInv2	5′-AAAAGCTTCCAGCGCATCGTCGTTGATGGC-3′; for inverse PCR deletion and allelic disruption of *nsrR* gene	This work
MFA7	5′-GGGATCCCGTTTCATAATGTTTTCCTTTTGT-3′; for amplification of *norB-aniA* promoter region	23
MFA8	5′-GGGATCCCGTCCCATTTTGAGAGCTCCTTTT-3′; for amplification of *norB-aniA* promoter region	23
MFA10	5′-GTGCTGCAAGGCGATTAAGTT-3′; for checking orientation of insertion of *norB-aniA* promoter in *lacZ* fusions	This work

E. coli was cultured in LB liquid medium (5 ml in 25-ml McCartney bottles shaken at 37°C and 200 rpm) or on LB agar plates. Antibiotics were used at the following concentrations: spectinomycin, 50 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}.

Construction of strains. Genetic mutation of gene *nsrR* (*N. meningitidis* MC58 gene NMB0437) was produced by allelic replacement with an insertionally inactivated cloned *N. meningitidis* MC58 gene. *nsrR* and its flanking region were amplified using primers NOregF1 and NOregR1 (Table 1), and the product was cloned into pGEM-T Easy (Promega Corp., Madison, WI). A partial deletion of the target gene was achieved by inverse PCR using primers (NoreginvF1 and NoreginvR1) that contain terminal HindIII sites. The resulting product was cut with HindIII, and the Ω cassette, encoding spectinomycin resistance from pHP45Ω (21), was ligated within the protein-coding region of *nsrR*. This plasmid (pJR113) was then transformed into *N. meningitidis* MC58 (26). Transformants were selected for by plating on Columbia horse blood agar containing spectinomycin, and correct chromosomal rearrangement was verified by PCR using primers NOregF1 and NOregR1. The *norB-aniA* promoter region (the intergenic region between NMB1622 and NMB1623 in *N. meningitidis* MC58) was amplified with MFA7 and MFA8 primers, and the product was cloned into pGEM-T Easy (Promega Corp., Madison, WI). The promoter region was extracted as a BamHI fragment and inserted upstream of the *lacZ* gene in pLES94 (27). Sequencing using primer MFA10, which complements the *lacZ* gene, was used to confirm orientation of the insert to yield an *norB-lacZ* fusion. Monoallelic promoter-*lacZ* fusions of the *aniA* and *norB* promoters were inserted into the *proAB* region of *N. meningitidis* MC58 as described previously (23). Chromosomal DNA from these strains was extracted and used to transform *nsrR*, *fnr*, and *aniA* strains of *N. meningitidis* MC58 to transfer the promoter *aniA-lacZ* or *norB-lacZ* reporter constructs. The double mutant *nsrR fnr* strains were constructed by using chromosomal DNA from the *fnr* mutant strain to transform the *nsrR* (P*aniA-lacZ*) and *nsrR* (P*norB-lacZ*) strains. All strains were verified for correct chromosomal rearrangement by PCR.

Growth and activity assays. Spermine NONOate (AG Scientific Inc., San Diego, CA) was resuspended in 0.1 M NaOH and was used to release NO into culture suspensions (half-life of 39 min at 37°C) at final concentrations of 10 to 50 μM. Spermine NONOate or nitrite was added to aerobic bacterial cultures in exponential phase, routinely with an optical density at 600 nm (OD_{600}) of 0.3 to 0.6(approximately 3 to 4 h of growth), and 1-ml aliquots were harvested by centrifugation at hourly intervals after additions; cell pellets were stored at −20°C overnight for β-galactosidase activity assays. At 2 h after the addition of spermine NONOate (or nitrite), cell suspensions were assayed for their ability to utilize NO. The amount of protein in 1 ml of bacterial cells was measured by bicinchoninic acid assay (Pierce, Rockford, IL) or Bradford assay (Bio-Rad GmbH, Munich, Germany). β-Galactosidase activity assays were carried out on cell pellets (15). NO uptake assays were conducted by placing a maximum of 2 mg of whole cells in 5 ml of Mueller-Hinton broth in an electrode chamber. The respiration of the cells was used to make the chamber anaerobic, and once no oxygen was left, aliquots of aqueous solution containing saturated NO solution were added to the culture. Saturated NO solution was made by sparging NO gas

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TABLE 2. Nitric oxide uptake rates in *N. meningitidis* in response to nitric oxide

Bacterial strain	NO reductase activity (nmol min^{-1} mg of $protein^{-1}$)	
	No treatment	Treatment with 50 μM spermine NONOate
N. meningitidis MC58	4.9 ± 0.6	54 ± 6
N. meningitidis nsrR	193 ± 12	188 ± 2
N. meningitidis norB	5.5 ± 0.4	5.1 ± 1

(Sigma-Aldrich, United Kingdom) through 1 M NaOH and into 10 ml of 1 M Tris, pH 8, and a sample was analyzed to check that the pH had not become acidic. The presence of oxygen in culture was measured using a Clark-type oxygen electrode (Rank Bros, Bottisham, United Kingdom), and NO concentration was measured by an ISO-NOP Mark II 2-mm electrode (World Precision Instruments, Stevenage, United Kingdom). Nitrite concentration in culture was measured by colorimetric assay (18). All activity and nitrite assays were performed in triplicate on at least three different occasions. Data sets shown are from one representative occasion, and the error bars represent one standard deviation of the mean.

Western blotting. Western blotting was carried out using antibodies raised in rabbits (Charles River Laboratories, France) against recombinant AniA protein overexpressed in *E. coli* and purified using standard biochemical techniques. Anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Sigma) was used as a secondary antibody, and the horseradish peroxidase activity was detected on X-ray film (SLS, United Kingdom) using a Super Signal Western Dura (Pierce) chemiluminescence kit.

RESULTS

Nitric oxide reductase expression in *N. meningitidis* is induced in response to nitric oxide. On transition to anaerobic conditions, NO reduction proceeds following the up-regulation and activity of the nitrite reductase *aniA* and appears to be a consequence of nitric oxide accumulation (23). In order to test the hypothesis that NO induces NO reductase expression, we incubated aerobically grown *N. meningitidis* with the NO releaser compound spermine NONOate. Spermine NONOate breaks down spontaneously at neutral pH to yield 1.5 molecules of NO per molecule of the NONOate with a half-life of 39 min at 37°C (AG Scientific Inc., San Diego, CA). Incubation with 50 μM spermine NONOate is sufficient to bring about induction of expression of a nitric oxide reductase activity (Table 2). The slow rate of NO removal in untreated cells is equivalent to the rate of NO disappearance from the electrode chamber in the absence of cells or with a cell suspension of a *norB*-deficient strain of *N. meningitidis* MC58 (Table 2). Under aerobic conditions NO breaks down into nitrite and nitrate, but neither of these anions was capable of inducing NO reductase at a 50 μM concentration (data not shown).

Mutagenesis of *nsrR* from *N. meningitidis*. NMB0437 (*nsrR*) was mutated by insertion of a spectinomycin resistance gene. Spectinomycin-resistant meningococcal transformants were checked for correct insertion of the antibiotic resistance gene by PCR. Chromosomal DNA from this original mutant strain was isolated and transformed into wild-type *N. meningitidis* MC58. The resultant spectinomycin-resistant colonies were screened for disruption of *nsrR* by PCR. A number of *nsrR* mutant strains independently derived by this procedure were analyzed and found to have identical phenotypes, indicating that the likelihood that the phenotype resulted from a second site mutation was extremely low.

NMB0437 is monocistronic: the adjacent genes NMB0436 and NMB0438 are both transcribed from the opposite strand of the *N. meningitidis* DNA. Quantitative real-time PCR studies confirm that the expression of these flanking genes was not altered in *nsrR* mutant strains compared to the *N. meningitidis* MC58 wild type (data not shown).

Growth of *N. meningitidis nsrR* versus wild type. To test whether the mutation in *nsrR* has an impact on growth by denitrification, *N. meningitidis* strains were grown under oxygen-limited conditions in the presence of 5 mM nitrite. *N. meningitidis nsrR* grew more rapidly than the wild type and consumed nitrite at a more rapid rate (Fig. 1). Under aerobic conditions, the *nsrR* mutant strain grew somewhat more slowly than the wild type and reached a lower final optical density (Fig. 1). These results suggest that in the *nsrR* mutant, the denitrification apparatus is expressed constitutively such that under oxygen limitation there is no lag in growth to allow for up-regulation of denitrification enzymes and no lag due to NO accumulation. Furthermore, under aerobic conditions, there may be a metabolic burden on the cell due to the expression of these enzymes that are not necessary for aerobic growth.

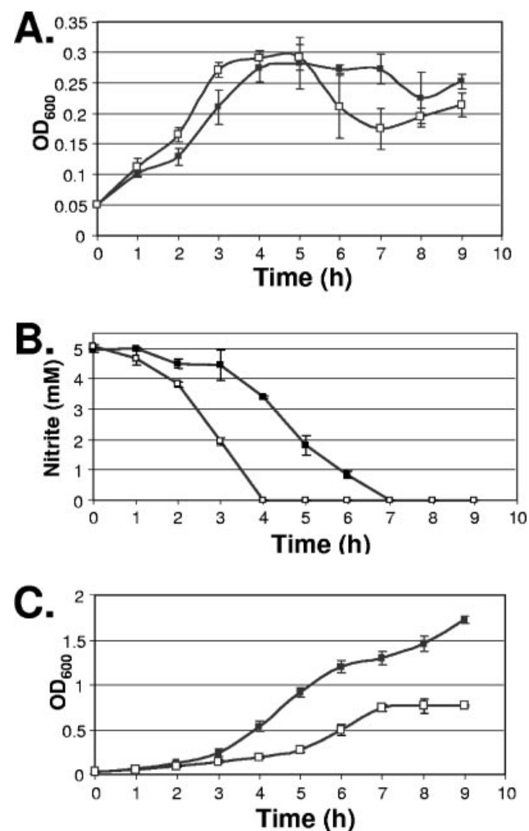

FIG. 1. Growth of wild-type and *nsrR* mutant strains of *N. meningitidis*. (A) Growth of strains was monitored (by OD_{600} values) during incubation of cultures under oxygen-limited conditions in the presence of 5 mM nitrite. (B) Disappearance of nitrite during growth under oxygen limitation. (C) Growth of strains under fully aerobic incubation conditions. Filled squares, wild-type *N. meningitidis* MC58; open squares, *N. meningitidis nsrR*-deficient strain.

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Denitrification enzymes are expressed aerobically and under oxygen limitation in an *nsrR* mutant. During aerobic growth of wild-type *N. meningitidis* and the *nsrR* mutant, nitrite is not utilized (data not shown). We have previously shown that oxygen is the preferred electron acceptor for respiration under these conditions (23), and therefore the lack of NO_2^- reduction does not necessarily imply that AniA is not being synthesized. Aerobically grown *N. meningitidis* wild type and the *nsrR* mutant were assayed for nitrite reductase activity under anaerobic conditions. While there was no nitrite reduction within ~1 h for the wild type, nitrite reduction occurred instantaneously for the *nsrR* mutant. Treatment with chloramphenicol to inhibit protein synthesis confirmed that nitrite reduction in the *nsrR* mutant was independent of de novo protein synthesis under anaerobic conditions (data not shown). Similarly, nitric oxide reduction was observed following aerobic growth of the *N. meningitidis nsrR* mutant strain but not the wild type (Table 2). Exogenous NO did not lead to further induction of NO reductase activity in the *nsrR* mutant, unlike the wild type, in which NO reductase expression is regulated by NO (Table 2).

In order to clarify the regulation of *aniA* and *norB* expression by NsrR and other regulatory proteins, we introduced *aniA* and *norB* promoter *lacZ* fusions into *N. meningitidis* MC58 and derivative strains with mutations in *nsrR*, *fnr*, and *aniA*.

***norB* expression is elevated in an *nsrR* mutant, and NsrR-dependent repression is lifted by NO.** The expression of *norB* was quantified by introducing a single copy of a *norB* promoter-*lacZ* fusion into the chromosome of *N. meningitidis* MC58 and an *nsrR* mutant. Following aerobic growth in the absence of nitric oxide, the β-galactosidase activity derived from this fusion construct was negligible in the wild type, but a significant activity was measured for the *nsrR* mutant strain bearing the fusion (Fig. 2), consistent with NsrR's being a repressor. Culturing *N. meningitidis* MC58 (P*norB-lacZ*) under conditions that allow denitrification to occur (i.e., oxygen limitation plus nitrite) or incubation of the same strain aerobically cultured in the presence of spermine NONOate led to elevated expression of *norB*, whereas these culture conditions had no effect on the levels of expression of the *norB-lacZ* reporter in the *nsrR* mutant strain (Fig. 2). Clearly, NsrR is functioning as a repressor, but the presence of NO is able to relieve the repression and allow NO reductase to be expressed. Under aerobic conditions, nitrite has no impact on P*norB-lacZ* expression in *N. meningitidis* wild type or the *nsrR* mutant strain (data not shown), whereas under microaerobic conditions nitrite increases expression in the wild type but not the *nsrR* mutant (Fig. 2), presumably due to NO accumulation as an intermediate in denitrification.

***aniA* expression is elevated in an *nsrR* mutant strain but is not strongly induced in response to NO.** The activity of an *aniA* promoter *lacZ* fusion was analyzed in *N. meningitidis* MC58 and in an *nsrR* mutant background. The promoter activity was judged to be increased approximately 5- to 10-fold in the absence of the *nsrR* gene (Fig. 3). The activity of the P*aniA-lacZ* fusion is increased in the *nsrR* mutant strain over the wild type when *N. meningitidis* is cultured aerobically or under oxygen limitation plus or minus nitrite. Clearly, *aniA* expression is still regulated by other environmental variables (oxygen availability and nitrite) in the absence of *nsrR*, whereas these variables do not affect expression of *norB* in an *nsrR* mutant strain.

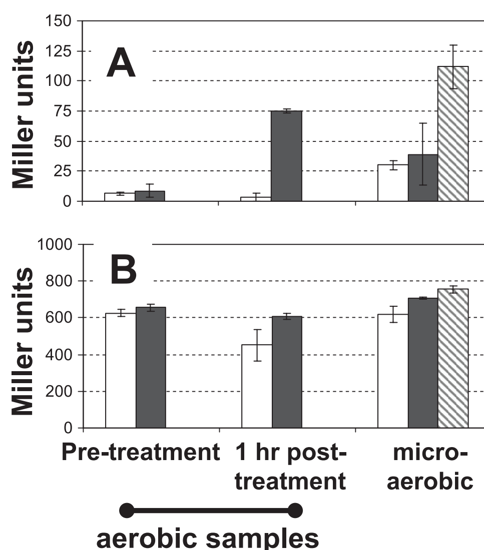

FIG. 2. Expression of *norB* monitored using a *norB* promoter *lacZ* fusion. β-Galactosidase activities were measured following aerobic growth or microaerobic growth. Aerobic samples were treated with 50 μM spermine NONOate when the OD_{600} was ≈0.3. The *x* axis indicates aerobic samples pretreatment and 1 h posttreatment with 50 μM spermine NONOate. Filled bars represent treated samples, and open bars represent control samples in which no NONOate was added. Likewise, microaerobically grown cultures were untreated (open bars), treated with spermine NONOate (black bars), or grown in the presence of 5 mM nitrite (striped bars). Panel A shows β-galactosidase activities in wild-type *N. meningitidis*, and panel B shows activities in the *nsrR*-deficient strain.

Treatment of *N. meningitidis* MC58 (P*aniA-lacZ*) (or indeed the *nsrR* mutant strain bearing this fusion construct) with spermine NONOate has little effect on the activity of β-galactosidase (Fig. 3), indicating that nitric oxide itself has little impact on expression of the *aniA* gene, even though the *aniA* promoter is strongly repressible by NsrR. The P*aniA-lacZ* fusion was introduced into an *aniA*-deficient strain of *N. meningitidis*, and this was used to confirm that *aniA* expression is controlled by both oxygen limitation and the presence of nitrite but is not reliant on the generation of NO (i.e., via nitrite reductase) (Fig. 3C). Western blotting of total cell extracts with antibodies raised against AniA confirmed that *aniA* expression is derepressed in an *nsrR* mutant strain but that NO has little effect on *aniA* expression (Fig. 3D). In the *N. meningitidis nsrR* mutant strain, *aniA* expression remains inducible by nitrite (Fig. 3B), whereas *norB* is not inducible by nitrite under denitrifying conditions (Fig. 2B), consistent with the idea that NarQP controls *aniA* in a nitrite-dependent manner. Rodionov (24) predicted that NsrR in *Neisseria* might control *narQP* expression. However, we have found, using real-time PCR, that *narQP* expression is unaffected by mutation of *nsrR* (data not shown). It is evident that the *norB* promoter is strongly regulated by NO in an NsrR-dependent manner but that the *aniA* promoter activity is barely affected by NO regardless of the presence or otherwise of an intact *nsrR* gene.

The role of NsrR in control of *aniA* expression involves prevention of FNR-dependent expression under aerobic conditions. NsrR represses *aniA* expression, but under no conditions that we have found does NO lift this repression. What,

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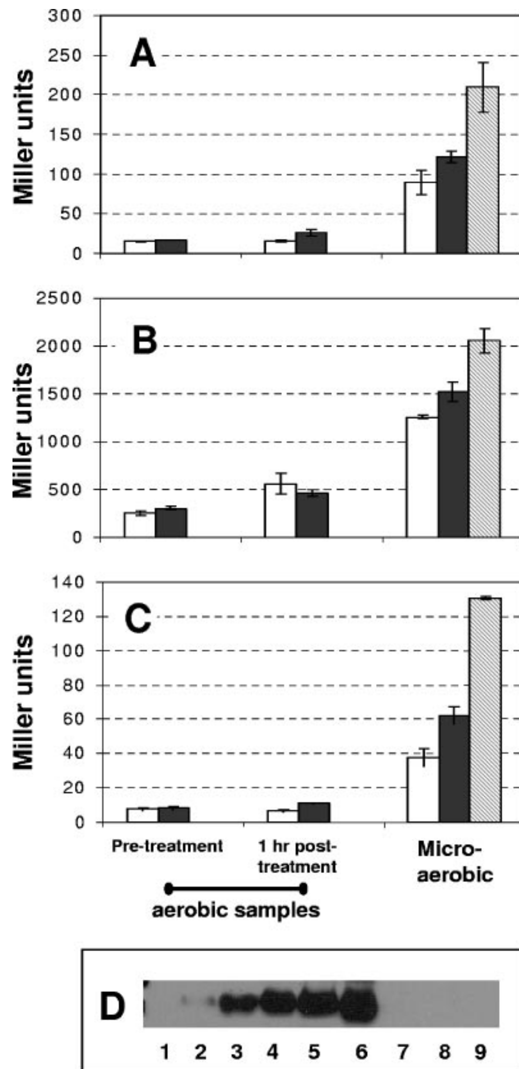

FIG. 3. Expression of *aniA* monitored using an *aniA* promoter *lacZ* fusion. β-Galactosidase activities were measured following aerobic growth or microaerobic growth. Aerobic samples were treated with 50 μM spermine NONOate when the OD_{600} was ≈0.3. The *x* axis indicates aerobic samples pretreatment and 1 h posttreatment with 50 μM spermine NONOate. Black bars represent treated samples, and open bars represent control samples in which no NONOate was added. Likewise, microaerobically grown cultures were untreated (open bars), treated with spermine NONOate (black bars), or grown in the presence of 5 mM nitrite (striped bars). Panel A shows β-galactosidase activities in wild-type *N. meningitidis*, panel B shows activities in the *nsrR*-deficient strain, and panel C shows activities in an *aniA*-deficient strain. Panel D shows expression of AniA monitored using Western blotting with antibodies raised to AniA. Lanes 1 to 3, wild-type *N. meningitidis*; lanes 4 to 6, *N. meningitidis nsrR* mutant strain; lanes 7 to 9, *N. meningitidis aniA* mutant strain. Lanes 1, 4, and 7 contain extracts from aerobically grown cultures; lanes 2, 5, and 8 contain extracts from aerobically grown cultures treated with 50 μM spermine NONOate; and lanes 3, 6, and 9 contain extracts from cultures grown under oxygen limitation with 5 mM nitrite.

therefore, is the functional significance of NsrR in the control of AniA expression? To address this question we examined the regulation of *aniA* expression in response to oxygen availability and nitrite in *N. meningitidis* MC58, an *nsrR* mutant, an *fnr* mutant, and an *nsrR fnr* double mutant. High levels of expression from the *aniA* promoter observed in the *nsrR* mutant strain (both aerobically and under oxygen limitation), in contrast with much lower levels of *aniA* expression in the *nsrR fnr* double mutant (Fig. 4), demonstrate that *aniA* expression in the *nsrR* mutant strain is FNR dependent. Oxygen limitation does elevate the level of expression of *aniA* in the *nsrR* mutant strain, yet there is clearly a very high background activity aerobically in the absence of NsrR. To confirm that the *aniA* promoter was active aerobically, the activity of P*aniA-lacZ* was monitored in cell suspensions of *N. meningitidis* MC58 and *nsrR* maintained at 60 to 80% air saturation in the chamber of a Clark oxygen electrode. This showed that the P*aniA-lacZ* fusion was active aerobically in the *nsrR* mutant but not the wild type (Fig. 4). This is consistent with the observation that FNR from *N. meningitidis* retains considerable activity as a transcriptional activator under aerobic culture conditions.

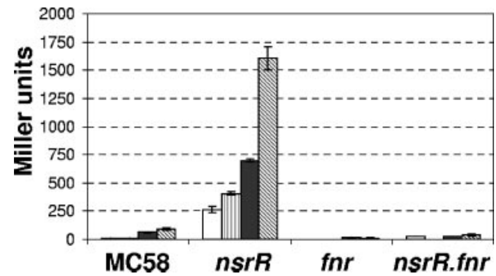

FIG. 4. β-Galactosidase activity of the *aniA* promoter in *N. meningitidis* MC58 and *nsrR*, *fnr*, and *nsrR fnr* mutant strains under aerobic and oxygen-limiting (with or without nitrite) conditions. The activity of P*aniA-lacZ* was measured following growth of each of these strains containing the *aniA* promoter-*lacZ* fusion. Open bars show activity under aerobic conditions, black bars show activity under oxygen-limiting conditions without nitrite, and diagonally striped bars show activity under oxygen-limiting conditions plus nitrite. Vertically striped bars show the activity of the promoter fusion in cell suspensions maintained at a controlled 60 to 80% air saturation in a Clark O_2 electrode chamber.

DISCUSSION

We have defined a new regulator of denitrification in the pathogenic denitrifying bacterium *N. meningitidis*. Analysis of *nsrR* mutant strains indicates that the *nsrR* gene encodes a repressor protein that prevents expression of both genes required for denitrification in this organism, namely, the nitrite reductase *aniA* and the nitric oxide reductase *norB*. We also report that the elevated expression of *norB* is achieved in response to NO (confirming prior indirect observations indicating that *norB* appeared be regulated by NO [23]) and that this NO responsiveness is dependent upon the presence of a functional *nsrR* gene. This is in keeping with findings for NsrR homologues in *Nitrosomonas europaea*, *E. coli*, and *B. subtilis* (3, 4, 17). Since the initial submission of this paper, NsrR has been shown to regulate denitrification in *N. gonorrhoeae* (20). In that paper, mutation of *nsrR* rendered *N. gonorrhoeae* insensitive to nitrite; however, we find that the *N. meningitidis nsrR* strain retains sensitivity to regulation of gene expression by nitrite (Fig. 3B), indicating that there are key differences in the regulatory networks of these two bacteria.

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NsrR was first identified as a repressor of the nitrite reductase *nirK* in the nitrifying bacterium *N. europaea* (3). In the presence of nitrite and a low pH, the repression of *nirK* via NsrR was lifted, leading these authors to conclude that NsrR is a nitrite-sensing transcriptional repressor. The physiological function of nitrite reduction in *N. europaea* was argued to be a defense mechanism against the toxic accumulation of nitrite, which is the product of the nitrification process in this organism (3). Although NsrR from *N. europaea* may respond to nitrite, an alternative interpretation, consistent with the pH dependence of the nitrite sensitivity, is that NsrR in fact senses nitric oxide, which can be generated chemically from nitrite under acidic conditions. Although this remains to be determined, the weight of evidence suggests that NsrR homologues from other microorganisms (*E. coli*, *B. subtilis*, and, here, *N. meningitidis*) are sensitive to nitric oxide rather than nitrite. *E. coli nsrR* (previously *yjeB*) was identified as a negative regulator of a number of genes that have been shown to be regulated by nitrosative stress (4). Focusing on the regulation of *ytfE*, the authors showed that the concentrations of NO necessary to bring about derepression of this NsrR-dependent promoter were 100 times lower than the concentration of nitrite required, supporting their conclusion that NsrR senses NO. In *B. subtilis* expression of flavohemoglobin (Hmp), a gene product that acts to detoxify nitric oxide, is activated by nitric oxide (100 μM) and nitrite (5 mM), and the nitric oxide scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide ablates this activation. In *nsrR* mutants *hmp* expression was derepressed and no longer sensitive to nitric oxide (17). Here, we have shown that the NO-dependent up-regulation of nitric oxide reductase (*norB*) in *N. meningitidis* is controlled by NsrR. Nitrite fails to activate *norB*, except under conditions when NO is generated as an intermediate of denitrification, providing further evidence that NsrR is responding to nitric oxide, not nitrite. The product of *norB* has a dual physiological function in *N. meningitidis*: NO detoxification (1) and supporting conservation of energy through respiration (20). As such, the role of regulator NsrR is, as in *N. europaea* and *E. coli*, to protect against toxicity, but it additionally acts to ensure optimal synthesis of key metabolic enzymes that allow *N. meningitidis* to exploit the variable availability of electron acceptors that support growth.

NO induces NO reductase activity and P*norB-lacZ* activity in the wild type but not in an *nsrR* mutant, whereas nitrite reductase expression is repressed by NsrR but is not affected by NO per se. Thus, it appears that NsrR can act as both an NO-responsive regulator (with *norB*) and a NO-independent regulator (with *aniA*). This difference in NO sensitivity for expression of *norB* and *aniA* may relate to different binding affinities of NsrR to the two predicted sites for NsrR binding in the *aniA*/*norB* promoter region that have been suggested from bioinformatic analysis (24). Modulation of gene expression at the *aniA* promoter relies on binding of FNR. Presumably, NsrR and FNR compete for binding at the promoter, and only when FNR is bound is the promoter active. This is in keeping with our finding that the derepression of P*aniA* in an *nsrR* mutant is quashed by introducing a mutation in *fnr* (i.e., expression of *aniA* relies on FNR).

In the absence of NsrR, *N. meningitidis* FNR retains considerable activity at the *aniA* promoter under aerobic conditions, and further FNR-dependent activation is achieved under oxygen limitation. FNR has been most extensively studied in *E. coli* where it acts a global regulator of gene expression in response to anaerobiosis (11). A recent transcriptomic study of *N. meningitidis* FNR indicated that it regulates a smaller set of genes than in *E. coli* (2). Our work here indicates that an additional distinction between FNR in these organisms is that the *N. meningitidis* protein retains transcriptional activation activity with oxygen concentrations of 60 to 80% of air saturation such that a corepressor protein is required to prevent excessive expression aerobically. An explanation of the apparent oxygen tolerance of *N. meningitidis* FNR may lie in the physiology of this bacterium. We have shown previously (23) that *N. meningitidis* is unable to grow under fully anaerobic conditions, due to the absence of an anaerobic ribonucleotide reductase, and thus has to tolerate a supply of molecular oxygen even under conditions when the bacterium is oxygen limited and using denitrification to grow. *N. meningitidis* FNR may thus have adapted to be more tolerant to molecular oxygen per se than its homologues in facultative anaerobes such as *E. coli*.

FNR is principally regarded as an oxygen-sensitive transcriptional activator, but it has been reported that FNR from *E. coli* can respond to NO, such that NO can cause down-regulation of FNR-activated genes. Similarly, the iron responsive regulator FUR, which has also been implicated in regulation of *aniA* and *norB* in *N. meningitidis* (8), may also be regulated by NO directly. However, since mutating *nsrR* ablates NO-dependent regulation, there is no evidence that FNR or FUR play significant roles in NO-dependent regulation of denitrification genes in *N. meningitidis*.

NsrR is a member of the Rrf2 family of transcriptional repressor proteins. The structure of MarA, which is a member of this family, has been solved bound to DNA, revealing that the protein has two DNA-binding helix-turn-helix domains (22). The NsrR proteins are most closely related to another repressor, the protein IscR, which has been characterized from *E. coli*. This protein senses the iron-sulfur cluster status of the cell, probably by reversible binding of an iron sulfur cluster (25). IscR and NsrR proteins contain three conserved cysteine residues that may be involved in the binding of an iron-sulfur cluster in both of these classes of protein. Nakano and colleagues indicated that NsrR from *Bacillus* may, indeed, contain a labile iron-sulfur cluster. Comparing the sequences of IscR/NsrR with MarA indicates that the sequence region containing the key predicted sensory cysteine residues is found between the two DNA-binding helix-turn-helix domains, suggesting that iron-sulfur cluster binding (IscR) or NO interaction with an iron-sulfur cluster (NsrR) could perturb the structure of the repressor proteins appropriately to impact upon DNA binding. Both NsrR and IscR appear to be dual functional. While some target genes of IscR are repressed specifically under anaerobic conditions, others are only repressed aerobically (9). Likewise, we have shown here that NsrR acts as a repressor in both an NO-dependent and an NO-independent manner at different target genes.

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