Identification of a Repressor of a Truncated Denitrification Pathway in *Moraxella catarrhalis*[▽]

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Growth of *Moraxella catarrhalis* in a biofilm resulted in marked upregulation of two open reading frames (ORFs), *aniA* and *norB*, predicted to encode a nitrite reductase and a nitric oxide reductase, respectively (W. Wang, L. Reitzer, D. A. Rasko, M. M. Pearson, R. J. Blick, C. Laurence, and E. J. Hansen, Infect. Immun. 75:4959–4971, 2007). An ORF designated *nsrR*, which was located between *aniA* and *norB*, was shown to encode a predicted transcriptional regulator. Inactivation of *nsrR* resulted in increased expression of *aniA* and *norB* in three different *M. catarrhalis* strains, as measured by both DNA microarray analysis and quantitative reverse transcriptase PCR. Provision of a wild-type *nsrR* gene in *trans* in an *nsrR* mutant resulted in decreased expression of the *AniA* protein. DNA microarray analysis revealed that two other ORFs (MC ORF 683 and MC ORF 1550) were also consistently upregulated in an *nsrR* mutant. Consumption of both nitrite and nitric oxide occurred more rapidly with cells of an *nsrR* mutant than with wild-type cells. However, growth of *nsrR* mutants was completely inhibited by a low level of sodium nitrite. This inhibition of growth by nitrite was significantly reversed by introduction of an *aniA* mutation into the *nsrR* mutant and was completely reversed by the presence of a wild-type *nsrR* gene in *trans*. *NsrR* regulation of the expression of *aniA* was sensitive to nitrite, whereas *NsrR* regulation of *norB* was sensitive to nitric oxide.

Moraxella catarrhalis was once thought to be a harmless commensal inhabitant of the human upper respiratory tract, but it is now acknowledged to be an organism that is capable of causing disease in both the upper and lower respiratory tracts of humans (36). In infants and very young children, *M. catarrhalis* is a common cause of acute otitis media (27, 35, 56). In adults, the disease caused by *M. catarrhalis* can take a much different and more serious form. This organism has been shown to be an important cause of infectious exacerbations of chronic obstructive pulmonary disease (COPD) (37, 38, 50). It was recently estimated that as many as 2 million to 4 million exacerbations of COPD in the United States can be attributed to *M. catarrhalis* each year (38). Worldwide, COPD is reported to be the fourth leading cause of death in developed countries, and, according to recent estimates, COPD is projected to become the third leading cause of death worldwide by 2020 (for a review, see reference 8).

The ability of *M. catarrhalis* to colonize the mucosal surface of the nasopharynx is crucial to its ability to cause disease in other anatomic regions because such colonization provides a foothold for *M. catarrhalis* in its human host. In fact, colonization of the nasopharynx by *M. catarrhalis* is common during infancy, and a high rate of colonization with this organism is associated with an increased risk of otitis media (10). The mechanisms essential for colonization of the nasopharyngeal mucosa by *M. catarrhalis* have not been elucidated to date,

although a number of *M. catarrhalis* gene products that may be involved in this process have been identified in the past few years (12, 21, 22, 29, 30, 34, 43, 44); essentially all of these gene products are surface-exposed proteins with demonstrated adhesive activity. In the human nasopharynx, it is likely that *M. catarrhalis* exists in a biofilm together with commensal bacteria on the mucosa, and there was a recent report describing *M. catarrhalis* biofilms detected on the mucosa of the middle ear in children with otitis media (16).

There have been only a few studies of biofilm formation by *M. catarrhalis* in vitro (6, 34, 41, 62), one of which identified genes which are upregulated when this organism grows in this manner (62). Among the genes whose expression was most highly upregulated during growth in a biofilm were several genes predicted to encode proteins involved in reducing nitrate (NO_3^-) to nitrous oxide (N_2O) (Fig. 1A). These upregulated genes included the *narGHJI* gene cluster encoding a nitrate reductase complex, *aniA* encoding a nitrite reductase (also described as the major anaerobically induced outer membrane protein [20]), and *norB* encoding a nitric oxide reductase (Fig. 1A). While the ability to reduce NO_3^- to nitrite (NO_2^-) is a well-established characteristic of *M. catarrhalis* that can be useful for identification of this organism (7), the presence of the other two enzymes mentioned above indicated that *M. catarrhalis* possesses the potential to express a truncated denitrification pathway, lacking only the ability to convert N_2O to gaseous nitrogen (N_2). Moreover, the ability to produce nitric oxide (NO) from NO_2^- and the ability to reduce NO to N_2O could provide *M. catarrhalis* with the ability to respire more efficiently under oxygen-limited conditions and to protect itself against NO generated by host defense mechanisms (42). Re-

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FIG. 1. Schematic diagrams of *M. catarrhalis* gene products involved in the truncated denitrification pathway and construction of relevant mutants. (A) The truncated denitrification pathway in *M. catarrhalis* involves the first three enzymatic steps shown. *M. catarrhalis* apparently lacks the ability to reduce N_2O to N_2 (indicated by brackets). (B to D) Schematic diagrams of the *M. catarrhalis* chromosomal locus containing the *norB*, *nsrR*, and *aniA* genes and flanking regions in (B) wild-type O35E strain, (C) the O35E *nsrR* mutant, and (D) the O35E *aniA* mutant. The relative positions of the different primers used for PCR are indicated by arrows.

cent studies with *Neisseria meningitidis*, another pathogen that initially colonizes the mucosa of the nasopharynx, have shown that its nitric oxide reductase can affect NO-based signaling processes in human cells (51, 55).

In this paper, we show that *M. catarrhalis* can reduce both NO_2^- and NO by virtue of its expression of the AniA and NorB proteins, respectively. We also show that an open reading frame (ORF) located between the *aniA* and *norB* genes encodes NsrR, a regulatory protein which controls the level of expression of both AniA and NorB. Inactivation of the *M. catarrhalis* *nsrR* gene resulted in overexpression of both AniA and NorB, which could be correlated with inhibition of growth by exogenously added NO_2^- . We also show that several other *M. catarrhalis* genes are regulated, directly or indirectly, by NsrR.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. catarrhalis* strains used in this study are listed in Table 1. The base medium employed in this study was brain heart infusion (BHI) (Difco, Detroit, MI) medium, and broth cultures were incubated at 37°C with aeration. BHI medium was supplemented with kanamycin (15 µg/ml) or spectinomycin (15 µg/ml) when appropriate. All BHI agar plates were incubated at 37°C in an atmosphere containing 95% air and 5% CO_2 .

To measure bacterial growth, bacteria were grown overnight on BHI agar and then suspended in BHI broth to a density of 260 Klett units. A 1-ml portion of the suspension was used to inoculate 20 ml of BHI broth in a 500-ml flask, which

was agitated (200 rpm) at 37°C. Growth was measured turbidimetrically every hour.

A freshly prepared solution of 2.5 M $NaNO_2$ (in water) was filter sterilized and was added (when needed) to BHI broth to a final concentration of 5 mM before the medium was used. To study the effect of NO on the expression of certain *M. catarrhalis* genes, spermine NONOate {N-4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl-1,3-propanediamine} was added to bacterial cultures to generate NO (46). Briefly, a freshly prepared solution of 25 mM spermine NONOate (in 0.1 M NaOH) was filter sterilized and was added to a logarithmic-phase culture to a final concentration of 50 µM. Growth was allowed to continue for 45 min, and then spermine NONOate was added again to the same final concentration. After an additional 45-min growth period, the cells were harvested for extraction of total RNA. Control cultures received equivalent portions of 0.1 M NaOH.

Whole-cell lysate preparation and Western blot analysis. Whole-cell lysates were prepared from BHI agar-grown cells as described previously (40). Western blot analysis was performed as described previously (60).

Construction of *M. catarrhalis* mutants. (i) *nsrR* mutants. DNA fragments containing nucleotide sequences located immediately 5' and 3' of the *nsrR* ORF were amplified by PCR with oligonucleotide primer pairs WW220/WW221 and WW222/WW223 (Fig. 1B and Table 2) using *M. catarrhalis* ATCC 43617 genomic DNA as the template. The two DNA fragments were used as templates for overlapping extension PCR (24) with primers WW220 and WW223. The resultant amplicon, designated $\Delta NSRR$ and containing an internal *SmaI* site, was digested with both *Bam*HI and *Sac*I and then ligated into the *M. catarrhalis* plasmid cloning vector pWW115 (61) that had been digested with the same restriction enzymes. The ligation reaction mixture was used to electroporate *M. catarrhalis* ETSU-9. The plasmid isolated from one of the spectinomycin-resistant clones was designated pWW123. The *kan* cartridge from pAC7 was amplified by PCR as described previously (62) and then cloned into the *SmaI* site in

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description or genotype	Reference or source
Strains		
7169	Wild-type strain	33
7169 <i>nsrR</i>	<i>nsrR</i> ::Kan ^r	This study
7169 <i>aniA</i>	<i>aniA</i> ::Kan ^r	This study
ATCC 43617	Wild-type strain	ATCC
ETSU-9	Wild-type strain	Steven Berk
ETSU-9 <i>aniA</i>	<i>aniA</i> ::Kan ^r	This study
ETSU-9 <i>nsrR</i>	<i>nsrR</i> ::Kan ^r	This study
ETSU-9 <i>nsrR</i> (pWW115)	ETSU-9 <i>nsrR</i> containing pWW115 vector	This study
ETSU-9 <i>nsrR</i> (pWW150)	ETSU-9 <i>nsrR</i> containing a cloned wild-type <i>nsrR</i> gene from ATCC 43617	This study
O35E	Wild-type strain	18
O35E <i>aniA</i>	<i>aniA</i> ::Kan ^r	This study
O35E <i>nsrR</i>	<i>nsrR</i> ::Kan ^r	This study
O35EΔ <i>nsrR</i>	<i>nsrR</i> deletion mutant	This study
O35EΔ <i>nsrR aniA</i>	<i>nsrR aniA</i> double mutant	This study
O35E <i>nsrR</i> (pWW115)	O35E <i>nsrR</i> containing pWW115 vector	This study
O35E <i>nsrR</i> (pWW150)	O35E <i>nsrR</i> containing a cloned wild-type <i>nsrR</i> gene from ATCC 43617	This study
Plasmids		
pWW115	Spec ^r , cloning vector for <i>M. catarrhalis</i>	61
pWW150	pWW115 containing the cloned wild-type ATCC 43617 <i>nsrR</i> gene	This study

the DNA insert in pWW123. The resultant recombinant plasmid, designated pWW124 and having the *kan* cartridge inserted in the same direction as the deleted *nsrR* gene, was used as the template for PCR amplification using primers WW220 and WW223. The resultant PCR product, designated Δ*NSRR*-KAN, was used to transform *M. catarrhalis* wild-type strains O35E, 7169, and ETSU-9. Kanamycin-resistant transformants were confirmed to be *nsrR* mutants (Fig. 1C) by performing anchored PCR using oligonucleotide primers WW210 and WW211 (Fig. 1B and Table 2), followed by nucleotide sequence analysis. A kanamycin-sensitive *nsrR* mutant was constructed by using the Δ*NSRR* DNA fragment described above to transform the kanamycin-resistant O35E *nsrR* mutant. One of the resultant kanamycin-sensitive transformants was confirmed to be an *nsrR* deletion mutant, designated O35EΔ*nsrR*, by nucleotide sequence analysis.

(ii) ***aniA* mutants.** DNA fragments containing nucleotide sequences located immediately 5' and 3' of the *aniA* ORF were amplified by PCR with primer pairs WW216/WW217 and WW218/WW219 (Fig. 1B and Table 2) using *M. catarrhalis* ATCC 43617 genomic DNA as the template. The two DNA fragments were used as templates for overlapping extension PCR (24) with primers WW216 and WW219. The resultant amplicon, designated Δ*ANIA* and containing an internal *Sma*I site, was digested with both *Bam*HI and *Sac*I and then ligated into pWW115 that had been digested with the same restriction enzymes. The ligation reaction mixture was used to electroporate *M. catarrhalis* ETSU-9. The plasmid isolated from one of the spectinomycin-resistant clones was designated

pWW121, and the *kan* cartridge described above was cloned into the *Sma*I site in the DNA insert in pWW121. The resultant recombinant plasmid, designated pWW122 and having the *kan* cartridge inserted in the same direction as the deleted *aniA* gene, was used as the template for PCR amplification using primers WW216 and WW219. The resultant PCR product, designated Δ*ANIA*-KAN, was used to transform *M. catarrhalis* wild-type strains O35E and ETSU-9. Kanamycin-resistant transformants were confirmed to be *aniA* mutants (Fig. 1D) by performing anchored PCR using oligonucleotide primers WW216 and WW211 (Fig. 1B and Table 2), followed by nucleotide sequence analysis.

(iii) ***aniA nsrR* mutant.** The PCR amplicon Δ*ANIA*-KAN described above was used to transform the *M. catarrhalis* O35EΔ*nsrR* mutant. One of the kanamycin-resistant transformants was confirmed to be an *nsrR aniA* double mutant by performing anchored PCR followed by nucleotide sequence analysis.

Cloning of the *M. catarrhalis nsrR* gene for use in complementation analysis. A DNA fragment containing the wild-type *nsrR* gene was PCR amplified by using oligonucleotide primers WW207 and WW210 (Fig. 1B) with *M. catarrhalis* ATCC 43617 genomic DNA as the template. The amplicon was ligated into the *Sma*I site in pWW115 and used to transform ETSU-9. A plasmid designated pWW150 containing the wild-type ATCC 43617 *nsrR* gene was isolated from one of the spectinomycin-resistant transformants.

RNA isolation and real-time qRT-PCR analysis. Total RNA was isolated from broth-grown and biofilm-grown cells as described previously (62). Quantitative reverse transcriptase PCR (qRT-PCR) was performed as described previously (62). The oligonucleotide primer pairs used for qRT-PCR analysis involving *norB* (MC ORF 679), *aniA* (MC ORF 681), and the endogenous control MC ORF 1234 have been described previously (62). Other oligonucleotide primers used in this study are listed in Table 2.

Identification of *NsrR*-regulated genes by DNA microarray analysis. Total RNA was isolated from *M. catarrhalis* wild-type strain O35E, 7169, and ETSU-9 cells and the *nsrR* mutants of these strains grown in BHI broth. DNA microarray analysis was performed as described previously (62) to identify genes whose expression was affected by *NsrR*. Independent experiments were performed to obtain a total of six sets of RNA samples (two samples per set) from three *M. catarrhalis* strain pairs (i.e., wild-type cells and *nsrR* mutant cells grown at the same time). These samples included one set from the 7169 strain pair, two sets from the ETSU-9 strain pair, and three sets from the O35E strain pair. "Dye swap" experiments were performed twice, once with ETSU-9 samples and once with O35E samples. Statistical analysis of the data was performed as described previously (62). *NsrR*-regulated gene expression in *M. catarrhalis* O35E was confirmed by qRT-PCR analysis. Two additional independent experiments were performed to isolate two sets of RNA samples from the *M. catarrhalis* O35E wild-type strain and *nsrR* mutant for qRT-PCR, and these analyses were performed twice for each set of RNA preparations as described previously (62).

Generation of polyclonal antibody. An oligopeptide (YTKGKYGEQLQFP DMEKAIREED) containing amino acids 236 to 258 of the predicted *M. catarrha-*

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence (5'–3')
WW207.....	TATCCCGGGTTCTCCTTATAGAAAGGT
WW210.....	GACACGCCCATCACAGACTAA
WW211.....	GCAACTCAACATGCTCATGAT
WW216.....	TTGGATCCGCGCACTGTCAACGACTTT ^a
WW217.....	GACAATCCATTGCCCCGGGTGGTTTGAGAAGTGCCT ^b
WW218.....	GGCAATGGATTGTCTGGTAA
WW219.....	CGACGAGTCTCCATTCTGCAATCTTAGGT ^c
WW220.....	GAGGATCCACCACTATATTTTCA ^a
WW221.....	GTTTATAGATTGCCCGGGTCAATGATGGCTGGATT ^b
WW222.....	CGGGCAATCTAAACACTCAATCTCA
WW223.....	TGGCGAGTCTCATATCAAGGGCTGTAGA ^c
WW247.....	TAGGATCCCAATCACACTTAGGATTATCA
683Fw.....	GGACATGATGGGATGGTC
683Rv.....	TAAGGCAGGTGCTGGCG
1550Fw.....	AGACACCATCAGCGTTAGCT
1550Rv.....	AAGGTGACCTCTCGGTGACAT

^a The *Bam*HI site is underlined.

^b The *Sma*I site is underlined.

^c The *Sac*I site is underlined.

lis ATCC 43617 AniA protein was synthesized by the Protein Technology Center at the University of Texas Southwestern Medical Center and was used to immunize mice for production of polyclonal AniA antibody.

Determination of NO, N₂O, and NO₂⁻ production and consumption. To determine NO production in the presence of NO₂⁻, wild-type, *nsrR* mutant, and *aniA* mutant *M. catarrhalis* O35E strains were grown in BHI broth to an optical density at 600 nm (OD₆₀₀) of 2.0. The cells were then washed and resuspended in fresh BHI broth to an OD₆₀₀ of 1.0, which was followed by addition of 5 mM (final concentration) NaNO₂. NO production was monitored using an ISO-NOPMC Mark II electrode (WPI Instruments, Sarasota, FL) run through an MLT1122 analog adapter system (AD Instruments, Colorado Springs, CO) with standard curves generated according to the manufacturer's instructions. To measure NO₂⁻ consumption, wild-type, *aniA*, *nsrR*, and *nsrR aniA* *M. catarrhalis* O35E strains were grown in BHI broth to an OD₆₀₀ of 2.0, washed, and resuspended in BHI broth to an OD₆₀₀ of 1.0. After addition of 5 mM (final concentration) NaNO₂, the concentration of NO₂⁻ remaining was determined using a standard Griess procedure. Briefly, culture supernatant fluid was prepared by pelleting cells after 5 min of incubation at 70°C. A 100-μl portion of this culture supernatant fluid was mixed with a 100-μl portion of a 1:1 mixture of 1% sulfanilamide (in 2.5% H₃PO₄) and 0.1% N-1-naphthylethylenediamine hydrochloride (in 2.5% H₃PO₄) and incubated at room temperature for 15 min. The NO₂⁻ concentration was determined by measuring the absorbance at 550 nm of the mixture and comparing the data to a standard curve. The NO-consuming activity of *M. catarrhalis* wild-type strain O35E and the isogenic O35E *nsrR* mutant was measured by growing cells to an OD₆₀₀ of 2.0 in BHI medium and then washing and resuspending the cells in BHI broth to an OD₆₀₀ of 1.0. Addition of 10 mM Prolino (half-life, 1.8 s; AG Scientific, San Diego, CA) was used to generate 20 μM NO. The concentration of dissolved NO remaining at different times was monitored using an ISO-NOPMC Mark II electrode (WPI Instruments). The production of N₂O by wild-type, *aniA*, *nsrR*, and *nsrR aniA* *M. catarrhalis* O35E strains was monitored using an oxygen-insensitive, N₂O-specific probe (N2O-50-3112; Unisense AS, Aarhus, Denmark) connected to a picoammeter (PA2000; Unisense AS) and run through an A/D converter (ADC 216; Unisense AS). Cells were grown in BHI broth to an OD₆₀₀ of 2.0 and resuspended in BHI medium to a final OD₆₀₀ of 1.0, and this was followed by addition of 5 mM (final concentration) NaNO₂.

Nucleotide sequence accession numbers. The GenBank accession numbers for the nucleotide sequences of the *norB*, *nsrR*, and *aniA* genes included in this study are as follows: for *M. catarrhalis* O35E, EU861986; for *M. catarrhalis* 7169, EU861987; and for *M. catarrhalis* ETSU-9, EU861988. The nucleotide sequences of the *norB*, *nsrR*, and *aniA* genes from *M. catarrhalis* ATCC 43617 have been deposited under accession number AX067454.

RESULTS

DNA sequence analysis of the locus containing the *aniA* and *norB* genes. It was previously shown that growth of *M. catarrhalis* in the biofilm state resulted in upregulation of numerous different genes, including several genes predicted to encode proteins involved in a dissimilatory nitrate reduction pathway (62). In addition to genes encoding components of a nitrate reductase complex, genes encoding a predicted nitrite reductase (*aniA*) and a predicted nitric oxide reductase (*norB*) were among these most highly upregulated genes (62). Examination of the *M. catarrhalis* ATCC 43617 genome (GenBank accession numbers AX067426 to AX067466) revealed that the *aniA* and *norB* genes are located close to each other and are transcribed in the same direction (Fig. 1B). The predicted *M. catarrhalis* AniA protein was 68% identical to the *Neisseria gonorrhoeae* AniA nitrite reductase (formerly designated the anaerobically induced major outer membrane protein) (20). The *M. catarrhalis* *norB* gene encoded a predicted protein that was similar (64 to 65% identity) to the NorB nitric oxide reductases of both *N. meningitidis* and *N. gonorrhoeae*.

A third ORF (MC ORF 680) was located between the *norB* and *aniA* genes and was transcribed in the opposite direction (Fig. 1B). The protein encoded by this ORF was designated

NsrR (for reasons described below). The 182-amino-acid NsrR protein most closely resembled (51% identity) a predicted BadM/Rrf2 transcriptional regulator found in *Psychrobacter* sp. strain PRwf-1 (GenBank accession no. YP_001279665.1). The *M. catarrhalis* NsrR protein was also similar (35% identity) to the *N. meningitidis* MC58 NsrR repressor, a protein which controls expression of genes encoding enzymes involved in dissimilatory nitrite reduction in this pathogen (46). Further examination of the *M. catarrhalis* NsrR protein sequence revealed that it contains the conserved domain of RirA, a novel iron-responsive transcriptional regulator (54, 57) found in nitrogen-fixing bacteria which may have a function similar to that of the Fur protein (for a review, see reference 49).

In a preliminary effort to determine the degree of conservation of the *aniA*, *nsrR*, and *norB* genes among *M. catarrhalis* strains, oligonucleotide primer pairs WW247/WW207 and WW210/WW211 (Fig. 1B and Table 2) were used to amplify overlapping DNA fragments using genomic DNA isolated from *M. catarrhalis* strains O35E, 7169, and ETSU-9 as the templates. Nucleotide sequence analysis showed that the predicted amino acid sequences of the AniA and NsrR proteins from these three strains were identical, whereas there were a few amino acid differences in the NorB proteins among these strains.

Construction of *M. catarrhalis* *nsrR* mutants. The DNA microarray-based study which showed that both *norB* and *aniA* were among the most upregulated genes in biofilm-grown *M. catarrhalis* cells also indicated that these two genes were among the most downregulated genes when this organism was grown under iron-limiting conditions (62). Although these two genes showed the same apparent pattern of regulation, it is apparent from their arrangement in the chromosome that they are not in an operon (Fig. 1B). To determine whether the NsrR protein might be involved in the regulation of expression of the *norB* and *aniA* genes, we constructed *nsrR* mutations, first in strain ETSU-9 and then later in strains O35E and 7169, as described in Materials and Methods.

qRT-PCR analysis of *aniA* expression in ETSU-9 wild-type and *nsrR* mutant cells. Total RNA samples obtained from *M. catarrhalis* ETSU-9 wild-type and *nsrR* mutant cells grown under planktonic conditions, under iron-limiting conditions, and in a biofilm as described previously (62) were used to measure expression of *aniA* by qRT-PCR. In the wild-type strain, expression of *aniA* was downregulated in iron-limiting media and was upregulated more than 10-fold in the biofilm (Fig. 2), in the same manner that was previously observed for *M. catarrhalis* ATCC 43617 (62). In contrast, expression of *aniA* was upregulated in the *nsrR* mutant under all three growth conditions, and the relative increase was greatest (more than 100-fold) in the biofilm-grown cells (Fig. 2). These results suggested that the *nsrR* gene might encode a regulatory protein that represses expression of *aniA* under aerobic growth conditions.

Effect of NsrR on expression of AniA. The *aniA* gene was inactivated in *M. catarrhalis* strains O35E and ETSU-9 as described in Materials and Methods (Fig. 1D). The *aniA* mutants were used initially as negative controls in a Western blot analysis in which AniA expression was measured in two different strain backgrounds. In addition, we cloned the wild-type *nsrR* gene from *M. catarrhalis* ATCC 43617 into pWW115. Recombinant plasmid pWW150 containing this *nsrR* gene was used to

FIG. 2. Expression of *aniA* by *M. catarrhalis* ETSU-9 wild-type and mutant strains under different growth conditions, as measured by qRT-PCR. Total RNA was isolated from wild-type ETSU-9 (wt) and ETSU-9 *nsrR* mutant (*nsrR*) cells grown in the planktonic state (PT) under iron limitation conditions in broth containing 30 μ M Desferal (DF30) and in a continuous-flow biofilm system (BF) as described previously (62). qRT-PCR was performed as described in Materials and Methods.

transform both the O35E and ETSU-9 *nsrR* mutants. Both of these mutants were also independently transformed with the plasmid vector pWW115.

The wild-type O35E cells (Fig. 3A, lane 1) and wild-type ETSU-9 cells (Fig. 3A, lane 6) expressed very low levels of AniA. In contrast, the O35E *nsrR* mutant (Fig. 3A, lane 2) and the ETSU-9 *nsrR* mutant (Fig. 3A, lane 7) expressed readily detectable amounts of AniA. As expected, the O35E *aniA* mutant (Fig. 3A, lane 3) and the ETSU-9 *aniA* mutant (Fig. 3A, lane 8) did not express AniA protein. A plasmid-borne wild-type *nsrR* gene eliminated detectable expression of AniA in O35E *nsrR*(pWW150) (Fig. 3A, lane 5) and in ETSU-9 *nsrR*(pWW150) (Fig. 3A, lane 10). The presence of the plasmid vector alone in O35E *nsrR*(pWW115) (Fig. 3A, lane 4) and in ETSU-9 *nsrR*(pWW115) (Fig. 3A, lane 9) had no apparent effect on the expression of AniA by these two mutants.

Effect of NO₂⁻ on the growth of wild-type, mutant, and complemented mutant strains of *M. catarrhalis*. To begin to explore the biological significance of the *M. catarrhalis* NsrR protein, the effects of different nitrogen compounds on the growth of *M. catarrhalis* were investigated. Inclusion of 5 or 10 mM NaNO₃ in the broth growth medium did not have any detectable effect on the growth of the wild-type O35E strain or its *nsrR* mutant (data not shown). The presence of 5 mM NaNO₂ had little or no effect on growth of wild-type O35E (Fig. 4A). Similarly, NO₂⁻ had at most a very modest effect on the growth of the O35E *aniA* mutant (Fig. 4B). In contrast, growth of the O35E *nsrR* mutant was completely inhibited by 5 mM NO₂⁻ (Fig. 4C). The same inhibitory effect of NO₂⁻ was observed with *nsrR* mutants of the 7169 and ETSU-9 strains (data not shown). This inhibition of growth by NO₂⁻ was significantly reversed by the presence of an *aniA* mutation in the O35E *nsrR* mutant background (Fig. 4D) and was completely reversed by the presence of a wild-type *nsrR* gene in *trans* (Fig. 4F). As expected, the presence of the vector pWW115 alone in the O35E *nsrR* mutant had no effect on its growth in the presence of NO₂⁻ (Fig. 4E).

Identification of NsrR-regulated *M. catarrhalis* genes. DNA microarray analysis was used to identify genes whose relative expression was upregulated at least twofold in all three *nsrR*

FIG. 3. Effect of NsrR on AniA protein expression in wild-type, mutant, and complemented mutant strains of *M. catarrhalis*. Whole-cell lysates of the strains were probed in a Western blot analysis with polyclonal AniA antiserum (A) or with the CopB-specific monoclonal antibody 10F3 (18) (B) as the primary antibody. Lane 1, wild-type strain O35E; lane 2, O35E *nsrR* mutant; lane 3, O35E *aniA* mutant; lane 4, O35E *nsrR*(pWW115); lane 5, O35E *nsrR*(pWW150); lane 6, wild-type ETSU-9; lane 7, ETSU-9 *nsrR* mutant; lane 8, ETSU-9 *aniA* mutant; lane 9, ETSU-9 *nsrR*(pWW115); lane 10, ETSU-9 *nsrR*(pWW150). The positions of the putative AniA monomers and dimers are indicated by arrows on the right in panel A. The CopB outer membrane protein was used as a loading control, and its position is indicated by an arrow on the right in panel B. The positions of molecular mass markers are indicated on the left in each panel.

mutants compared with the level of expression in the corresponding wild-type parental strains. The relative expression levels of four ORFs were shown to be increased at least twofold ($P < 0.002$) in all three *nsrR* mutants, as measured by DNA microarray analysis (Fig. 5A). These ORFs were *norB* (expression increased ~ 30 -fold), *aniA* (expression increased ~ 30 -fold), MC ORF 683 (expression increased ~ 10 -fold), and MC ORF 1550 (expression increased twofold). MC ORF 683 encodes a protein that has 39% identity with a hypothetical protein from *Pseudoalteromonas haloplanktis* (GENE ID 3708074 PSHAa1482). MC ORF 1550 encodes a protein with 67% identity to a photosynthetic reaction center (PRC)-barrel domain protein (GENE ID 5206683 PscPRwf_2012) from a *Psychrobacter* species. The PRC-barrel domain is a conserved domain found in PRC subunits (2) and in some proteins involved in RNA metabolism (2, 32). qRT-PCR analysis was performed to confirm that expression of these four ORFs was negatively regulated by NsrR. In the *M. catarrhalis* O35E *nsrR* mutant, the expression of all four ORFs was upregulated compared to the expression in wild-type parental strain O35E (Fig. 5B). Finally, a putative NsrR-binding site, as defined by Rodionov et al. (47), was present upstream from all four of these ORFs (data not shown).

Effects of different nitrogenous compounds on expression of *M. catarrhalis* NsrR-regulated genes. qRT-PCR analysis was performed to examine the effects of NO₂⁻ and NO on the relative expression levels of *aniA*, *norB*, MC ORF 683, and MC ORF 1550. It is important to note that the oligonucleotide primer pair used for detection of *aniA* transcripts (62) was located inside the 5' end of the *aniA* ORF but was upstream of the region that was deleted in the *aniA* mutant (Fig. 1D). Therefore, use of this primer pair in qRT-PCR could detect expression of the truncated *aniA* transcript in the *aniA* mutant. Transcription of *aniA* was increased about fivefold in both the O35E wild-type strain (Fig. 6A) and the O35E *aniA* mutant (Fig. 6B) in the presence of NO₂⁻. Addition of NO₂⁻ to cells of the wild-type O35E strain resulted in the production of a readily detectable level of NO, whereas the O35E *aniA* mutant produced little if any detectable NO under these same condi-

FIG. 4. Effect of NO₂⁻ on the growth of wild-type, mutant, and complemented mutant strains of *M. catarrhalis*. Cells of wild-type strain O35E (A), the O35E *aniA* mutant (B), the O35E *nsrR* mutant (C), the O35E Δ *nsrR aniA* mutant (D), O35E *nsrR*(pWW115) (E), and O35E *nsrR*(pWW150) (F) were grown in BHI medium (■) or in BHI medium containing 5 mM NaNO₂ (▲). Most of the data are means for three independent growth experiments; the exception is the data in panel B, which are the means for two independent experiments.

tions (Fig. 7A). Taken together, these results suggested that NsrR-regulated expression of *aniA* was sensitive to NO₂⁻.

Addition of NO₂⁻ resulted in elevated expression (i.e., two-fold) of *norB* in the O35E wild-type strain (Fig. 6A) but did not

increase expression of *norB* significantly in the O35E *aniA* mutant (Fig. 6B). Expression of the other two ORFs (MC ORF 683 and MC ORF 1550) in the putative NsrR regulon was not affected by the presence of NO₂⁻ (Fig. 6A and 6B). When the NO-generating agent spermine NONOate was added to a logarithmic-phase culture of *M. catarrhalis* wild-type strain O35E, expression of *norB* was increased approximately twofold after 90 min (Fig. 6C). In contrast, expression of *aniA* was not affected by NO (Fig. 6C). These results directly confirmed that the expression of *aniA* is not sensitive to NO, whereas the expression of *norB* is sensitive to NO.

Metabolism of nitrogenous compounds by *M. catarrhalis*.

The production of NO from NO₂⁻ by wild-type strain O35E but not by the O35E *aniA* mutant (Fig. 7A) confirmed that the *M. catarrhalis* AniA protein is indeed a nitrite reductase which can produce NO by reducing NO₂⁻. In addition, it was shown that the O35E *nsrR* mutant consumed NO₂⁻ much faster than the wild-type parent strain O35E (Fig. 7B). This was most likely the result of the elevated expression of AniA in the O35E *nsrR* mutant (Fig. 3A, lane 2) because the O35E *aniA* mutant did not consume NO₂⁻ during the same period of time (Fig. 7B). More importantly, inactivation of the *aniA* gene in the O35E *nsrR* mutant completely eliminated consumption of NO₂⁻ (Fig. 7B).

Examination of the consumption of NO by *M. catarrhalis* strains showed that the O35E *nsrR* mutant consumed NO much faster than the O35E wild-type strain (Fig. 7C). This most likely resulted from the increased expression of *norB* in the O35E *nsrR* mutant (Fig. 5). This very rapid consumption of NO by the *nsrR* mutant provides an explanation for the very low level of detectable NO produced from NO₂⁻ by this *nsrR* mutant (Fig. 7A).

FIG. 5. Identification of *M. catarrhalis* genes regulated by NsrR. (A) A DNA microarray analysis of total RNA isolated from wild-type strains O35E, 7169, and ETSU-9 and *nsrR* mutants of these strains was performed as described in Materials and Methods. Data for the genes consistently upregulated at least twofold ($P < 0.002$) in all three *nsrR* mutants are shown. The error bars indicate standard deviations. (B) qRT-PCR analysis of the relative levels of expression of *norB*, *aniA*, MC ORF 683, and MC ORF 1550 in *nsrR* mutant and wild-type cells of strain O35E. The endogenous control for the qRT-PCR measurements was expression of MC ORF 1234 (62). The maximum and minimum relative levels of expression are indicated by the error bars.

FIG. 6. Effects of nitrogenous compounds on expression of genes regulated by NsrR. (A and B) Total RNA extracted from cells of *M. catarrhalis* wild-type strain O35E (A) and the O35E *aniA* mutant (B) grown in the presence or absence of 5 mM NaNO_2 were used for qRT-PCR. (C) Total RNA extracted from logarithmic-phase *M. catarrhalis* O35E cells grown for 90 min in the presence or absence of the NO-generating compound spermine NONOate was used for qRT-PCR. The relative levels of expression of *norB* (bars 1), *aniA* (bars 2), MC ORF 683 (bars 3), and MC ORF 1550 (bars 4) are shown. The endogenous control for the qRT-PCR measurements was the expression of MC ORF 1234 (62). The maximum and minimum relative levels of expression are indicated by the error bars.

In a preliminary experiment, the *M. catarrhalis* O35E *nsrR* mutant generated a much higher level of N_2O than its parent strain (Fig. 7D) following the addition of NO_2^- . This *nsrR* mutant produced 2.5 mM N_2O from 5 mM NaNO_2 in about 50 min, whereas the wild-type parent strain generated about 0.4 mM N_2O in the same time period (data not shown). As expected, inactivation of the *aniA* gene resulted in no detectable level of N_2O production from nitrite by either the O35E *aniA* mutant or the O35E $\Delta\text{nsrR aniA}$ mutant (Fig. 7D).

DISCUSSION

Interest in the reduction of nitrogenous compounds (i.e., denitrification) by bacterial pathogens has increased in the past few years (for a review, see reference 42). Denitrification is essentially the reduction of soluble anionic nitrogen oxides to volatile uncharged gases (NO , N_2O , or N_2), which results in a net loss of nitrogen from the local environment. Bacteria often perform denitrification as a substitute for aerobic respiration, where a nitrogen oxide rather than molecular oxygen serves as the electron acceptor. Accordingly, denitrification is employed

to generate an electrochemical gradient across the cytoplasmic membrane via coupling sites that expel protons to the periplasmic space. In contrast to aerobic respiration, where molecular oxygen is associated with a terminal oxidase during its tetravalent reduction to water, denitrification involves the generation of many freely diffusible intermediates that interact with several independent reductases. The reaction generally results in the following sequential reduction: $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$. However, there is a great deal of diversity among denitrifying microbes with respect to the types of enzymes involved, as well as the completeness of the reaction (for a review, see reference 64).

While bacteria (e.g., some *Pseudomonas* species) that are able to live in several different environments (including a human host) have well-studied denitrification pathways that result in the conversion of NO_3^- to gaseous nitrogen (64), only recently has it been determined that some obligate human pathogens (e.g., *N. meningitidis*) also can reduce some nitrogenous compounds to the level of N_2O (3, 45). Indeed, under some conditions, nitrate reductase activity encoded by the *narGHJI* gene cluster of *Mycobacterium bovis* BCG can contribute to virulence in an animal model (63), and expression of a regulator (NnrA) of denitrification genes in *Brucella melitensis* is required for virulence in mice (15). Recent studies with *N. meningitidis* indicated that the nitric oxide reductase NorB is primarily responsible for the nitric oxide detoxification accomplished by this pathogen, as measured in both macrophages and a nasopharyngeal mucosa organ culture system (52).

M. catarrhalis is an obligate aerobe and cannot grow under anaerobic conditions (1, 23). The ability of this organism to reduce NO_3^- to the level of NO_2^- and beyond has been documented previously (for a review, see reference 7). However, only recently were the genes that likely encode the relevant enzymes identified in a study of global gene regulation in *M. catarrhalis* (62). Genes encoding a nitrate reductase complex (NarGHJI), a predicted nitrite reductase (AniA), and a predicted nitric oxide reductase (NorB) were all found to be highly upregulated when *M. catarrhalis* was grown in a biofilm in vitro (62). In the present study, we identified an ORF (*nsrR*) which, when inactivated, allowed increased expression of both *aniA* and *norB*. This effect was not strain specific but occurred in the three different *nsrR* mutants tested in this study (Fig. 2 and 5A). DNA microarray analysis of the transcriptomes of the same *nsrR* mutants showed that at least two other ORFs (MC ORF 683 and MC ORF 1550) were upregulated in the absence of NsrR expression (Fig. 5). Analysis of the regions immediately upstream from these four ORFs revealed, in all four instances, the presence of a sequence with homology to the NsrR-binding site defined previously in studies of other bacteria (47). While this putative NsrR regulon may appear to be rather small, the NsrR regulon in *N. meningitidis* (19) was recently shown to contain only five genes, including *aniA* and *norB*. In contrast, the NsrR regulon in *Escherichia coli* is much larger and contains at least 20 genes (11).

While both *aniA* and *norB* appear to be members of an NsrR regulon in *M. catarrhalis*, expression of these two genes can be differentially regulated in response to certain nitrogenous compounds. Expression of *aniA* was upregulated about fivefold in both the wild-type O35E strain (Fig. 6A) and the O35E *aniA* mutant (Fig. 6B) when NO_2^- was present, even though this

FIG. 7. Metabolism of nitrogenous compounds by *M. catarrhalis* wild-type strain O35E and the strain O35E mutants. (A) Production of NO from NO_2^- . Cell suspensions of the wild-type strain (WT), the *aniA* mutant, and the *nsrR* mutant in BHI medium supplemented with 5 mM NaNO_2 were monitored for 10 min for the presence of NO using an NO-specific electrode. The probe specificity was affirmed by the ability of an NO scavenger, Carboxy-PTIO, to quench the measurable signal (not shown). (B) Consumption of NO_2^- . Cell suspensions of the wild-type strain, the *aniA* mutant, the *nsrR* mutant, and the *nsrR aniA* mutant in BHI medium supplemented with 5 mM NaNO_2 were monitored for the presence of NO_2^- using the Griess reaction. (C) Consumption of NO. Cell suspensions of the wild-type strain and the *nsrR* mutant were exposed to 20 μM NO produced by addition of 10 mM ProliNO. The dissolved NO concentration was monitored using an NO-specific electrode. For reference, the NO-consuming activity of BHI medium was determined (dotted line). (D) Production of N_2O . Cell suspensions of the wild-type strain, the *aniA* mutant, the *nsrR* mutant, and the *nsrR aniA* mutant in BHI medium supplemented with 5 mM NaNO_2 (administered 2 min into the assay) were monitored for the presence of N_2O using an N_2O -specific electrode.

aniA mutant did not generate NO from NO_2^- (Fig. 7A). Nitrite was previously shown to at least partially relieve NsrR-dependent inhibition of *aniA* expression in *N. gonorrhoeae* (39) and in *Nitrosomonas europaea* (5). Moreover, similar to results obtained with the *N. meningitidis aniA* gene (46), chemically generated NO did not affect expression of the *M. catarrhalis aniA* gene (Fig. 6C).

The *M. catarrhalis nsrR* mutant expressed *norB* transcripts at a level that was more than an order of magnitude higher than the level of expression in the wild-type parental strain (Fig. 5). In the wild-type O35E strain, expression of *norB* was increased about twofold either when nitrite was added (Fig. 6A) or when NO was produced from spermine NONOate (Fig. 6C). The latter finding indicates that NO can relieve the NsrR-dependent repression of *norB* expression in *M. catarrhalis*. NO-sensitive NsrR repression of *norB* transcription was observed in both *N. meningitidis* (46) and *N. gonorrhoeae* (26). Because the *M. catarrhalis aniA* mutant did not generate NO from NO_2^- (Fig. 7A), addition of NO_2^- did not affect expression of *norB* in this mutant (Fig. 6B).

It has been established previously that growth of *M. catarrhalis* in the biofilm state results in a very high level of expression of the *aniA* gene (as well as the *narGHJI* and *norB* genes) (62), suggesting that there is probably a transcriptional regulator which upregulates expression of *aniA* in this growth environment, where oxygen is likely limiting. One candidate

activator is the transcriptional regulator of fumarate and nitrate reduction (FNR), which functions in response to oxygen (for a review, see reference 28) and which has previously been shown to be the transcriptional activator of the *aniA* gene in *N. gonorrhoeae* (25, 31). BLAST-based searching of the available nucleotide sequences of the *M. catarrhalis* ATCC 43617 genome (GenBank accession numbers AX067426 to AX067466) did not reveal any candidates for an *fnr* gene encoding a protein with the [4Fe-4S] cluster. In addition, a search of nucleotide sequences comprising most, if not all, of the *M. catarrhalis* 7169 genome did not reveal any homology with *fnr* from *N. meningitidis* MC58 at either the nucleotide or encoded protein level (Anthony Campagnari, personal communication). However, examination of the nucleotide sequence upstream of the *M. catarrhalis aniA* gene revealed the presence of a putative FNR-binding site. Interestingly, MC ORF 921 (62) encodes a protein with 26% identity to DnrD (gene id 5096479 dnrD), a regulator which lacks the [4Fe-4S] center and which belongs to a new subgroup of the FNR-CRP regulator family (58, 59). Whether the MC ORF 921-encoded protein or another regulator is involved in the activation of the expression of *aniA* under oxygen-limiting growth conditions remains to be determined.

An unexpected finding in this study was the complete inhibition of growth of the O35E *nsrR* mutant in the presence of 5 mM NaNO_2 (Fig. 4C); independently performed viability ex-

periments indicated that nitrite was bacteriostatic for the *nsrR* mutant (data not shown). The growth of this *nsrR* mutant was not affected by addition of 10 mM NaNO₃ (data not shown), likely because the *narGHJI* genes (encoding the nitrate reductase complex) are expressed at a very low level under well-aerated conditions (62), thereby limiting the rate at which NO₂⁻ could be produced. This nitrite-dependent growth inhibition of the *nsrR* mutant could be reversed by introducing a second mutation in the *aniA* gene (Fig. 4D) or by the presence of a wild-type *M. catarrhalis nsrR* gene in *trans* (Fig. 4F). The higher level of expression of the *aniA* and *norB* genes in the *nsrR* mutant resulted in enhanced consumption of both NO₂⁻ (Fig. 7B) and NO (Fig. 7C), together with elevated production of N₂O (Fig. 7D). Additional experiments involving exposure of suspensions of *M. catarrhalis* cells to both NO and N₂O independently revealed that NO had a modest bactericidal effect on both wild-type and *nsrR* mutant cells, whereas N₂O had no apparent effect on the viability of these cells (data not shown). At this point, the mechanism for the observed inhibition of growth of the *nsrR* mutants in the presence of NO₂⁻ remains to be determined. However, expression of NsrR may provide some protection for *M. catarrhalis* in the presence of a low level of nitrite in the environment.

Some bacteria express an NO-inducible flavohemoglobin protein (Hmp), a nitric oxide dioxygenase that plays an important role in NO detoxification by converting NO to NO₃⁻ aerobically (13, 17) or by converting NO to N₂O anaerobically (17). The expression of *Salmonella enterica* serovar Typhimurium Hmp was repressed by NsrR both aerobically and anaerobically (4, 14). In this enteric pathogen, Hmp is the enzyme primarily responsible for metabolism of NO under aerobic conditions and is required for virulence in a mouse model (4). In *E. coli*, expression of Hmp also was repressed by NsrR (11), and Hmp is required for NO consumption, for conferring resistance to nitrosative stress (17), and for protecting bacteria from killing within macrophages (53). The growth of wild-type *S. enterica* serovar Typhimurium and an *nsrR* mutant was not inhibited by the nitrosating agent *S*-nitrosoglutathione, whereas the growth of both an *nsrR hmp* double mutant and an *hmp* mutant was completely inhibited by *S*-nitrosoglutathione (14). The complete inhibition of growth of *M. catarrhalis nsrR* mutants by 5 mM NO₂⁻ (Fig. 4C) indicates that this bacterium does not have an Hmp protein for NO detoxification, and BLAST-based searching of the *M. catarrhalis* ATCC 43617 genome did not detect any genes encoding Hmp-like proteins. Instead, *M. catarrhalis* likely is dependent on the expression of NorB for NO detoxification.

N. meningitidis and *M. catarrhalis* appear to share some of the basic components of a truncated denitrification pathway (62), although only the former organism can grow anaerobically. The organization of the *norB*, *nsrR*, and *aniA* genes is different in these two pathogens; the *norB* and *aniA* ORFs are located adjacent to each other in the *N. meningitidis* MC58 genome (GenBank accession number NC_003112) but are transcribed divergently, and the *nsrR* gene is located elsewhere in the *N. meningitidis* genome. Interestingly, exogenously added nitrite appears to be toxic for an *M. catarrhalis nsrR* mutant under aerobic conditions, whereas an *N. meningitidis nsrR* mutant is able to grow more rapidly on nitrite under oxygen-limited conditions (46). In addition, in contrast to an

M. catarrhalis nsrR mutant (Fig. 4A and 4C), an *N. meningitidis nsrR* mutant grew more slowly than its wild-type parent strain under aerobic conditions (46). These results reinforce the fact that there is a fundamental difference between these two pathogens in terms of respiratory capability.

At least one of the *M. catarrhalis* gene products involved in this truncated denitrification pathway is expressed *in vivo* when this bacterium grows in its human host. The AniA protein, designated Msp78 (48), was recently shown to be present when *M. catarrhalis* grew in the respiratory tracts of patients with COPD who had acquired and then subsequently cleared an infection with this pathogen (48). Similarly, the AniA protein of *N. gonorrhoeae* was also shown to be expressed *in vivo* in humans (9). Ongoing studies in our laboratory are focused on further elucidation of the mechanisms controlling expression of the proteins involved in this truncated denitrification pathway and on the relevance of these enzymes to host-pathogen interactions.

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