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The *Moraxella catarrhalis* Nitric Oxide Reductase Is Essential for Nitric Oxide Detoxification▽

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***Moraxella catarrhalis* is a Gram-negative obligate aerobe that is an important cause of human respiratory tract infections. The *M. catarrhalis* genome encodes a predicted truncated denitrification pathway that reduces nitrate to nitrous oxide. We have previously shown that expression of both the *M. catarrhalis aniA* (encoding a nitrite reductase) and *norB* (encoding a putative nitric oxide reductase) genes is repressed by the transcriptional regulator NsrR under aerobic conditions and that *M. catarrhalis* O35E *nsrR* mutants are unable to grow in the presence of low concentrations of nitrite (W. Wang, et al., J. Bacteriol. 190:7762–7772, 2008). In this study, we constructed an *M. catarrhalis norB* mutant and showed that planktonic growth of this mutant is inhibited by low levels of nitrite, whether or not an *nsrR* mutation is present. To determine the importance of NorB in this truncated denitrification pathway, we analyzed the metabolism of nitrogen oxides by *norB*, *aniA norB*, and *nsrR norB* mutants. We found that *norB* mutants are unable to reduce nitric oxide and produce little or no nitrous oxide from nitrite. Furthermore, nitric oxide produced from nitrite by the AniA protein is bactericidal for a *Moraxella catarrhalis* O35E *norB* mutant but not for wild-type O35E bacteria under aerobic growth conditions *in vitro*, suggesting that nitric oxide catabolism in *M. catarrhalis* is accomplished primarily by the *norB* gene product. Measurement of bacterial protein *S*-nitrosylation directly implicates nitrosative stress resulting from AniA-dependent nitric oxide formation as a cause of the growth inhibition of *norB* and *nsrR* mutants by nitrite.**

Moraxella catarrhalis is an obligately aerobic Gram-negative bacterium that colonizes the human upper respiratory tract. For many decades, *Moraxella catarrhalis* was considered to be a harmless member of the normal flora and was known as *Neisseria catarrhalis* due to its morphological similarities to commensal *Neisseria* species (47). Recently, *M. catarrhalis* has been recognized as an important pathogen in both the upper and lower respiratory tracts (45). *M. catarrhalis* is the third leading bacterial cause of acute otitis media (32, 44, 67) in infants and very young children and the second most common bacterial cause of exacerbations of chronic obstructive pulmonary disease (COPD) in adults (43, 46, 58). It is estimated that 2 to 4 million exacerbations of COPD in the United States are attributable to *M. catarrhalis* infection each year (46). *M. catarrhalis* has been implicated in other infections, including community-acquired pneumonia (64), and extremely rarely may cause fatal bacteremia or pneumonia in patients with preexisting health conditions, such as immunodeficiency or impaired airway defenses (57).

Studies show that nasopharyngeal colonization with *M. catarrhalis* is common in infants and young children, and a high rate of colonization is associated with an increased risk of otitis media (16, 31). Recent surveys of nasopharyngeal colonization of *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae*, and *M. catarrhalis* showed that colonization with *M. catarrhalis* is highest among these pathogens surveyed in children between 2 and 12 years of age (42). *M. catarrhalis* frequently cocolonizes human nasopharyngeal mucosal surfaces with other bacteria, including *Streptococcus pneumoniae* (31, 42), *Staphylococcus aureus* (31), and *H. influenzae* (31, 68). Efforts to identify *M. catarrhalis* adhesins have uncovered several bacterial surface proteins that facilitate *M. catarrhalis* attachment to human epithelial cells *in vitro* (20, 27, 28, 36, 40, 41, 52, 54). Researchers have also identified several *M. catarrhalis* gene products that are important for growth under various *in vitro* conditions (1, 5, 10, 21, 41, 50). However, mechanisms of *M. catarrhalis* colonization of the nasopharyngeal mucosa remain to be fully elucidated. It was recently reported that *M. catarrhalis* forms biofilms on the middle ear mucosa in children with otitis media (24). It is likely that *M. catarrhalis* exists in biofilms together with other commensal bacteria in the human nasopharynx. In a chinchilla infection model, the persistence of *M. catarrhalis* within polymicrobial biofilms was shown to be facilitated by *H. influenzae* (4).

Gene expression during biofilm formation by *M. catarrhalis in vitro* has recently been examined (11, 41, 50, 71). Genes belonging to the *M. catarrhalis* truncated denitrification pathway (including the *narGHJI* cluster, *aniA*, and *norB*) (Fig. 1) were among the most highly upregulated genes in biofilm-grown cells (71). The *M. catarrhalis* transcriptional regulator NsrR represses the expression of both *aniA* and *norB* (Fig. 1) during aerobic growth. *M. catarrhalis nsrR* mutants are unable

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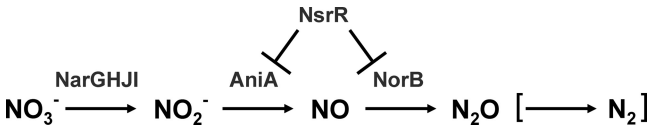

FIG. 1. Truncated denitrification pathway in *M. catarrhalis*. The truncated denitrification pathway in *M. catarrhalis* involves three enzymatic steps: reduction of nitrate (NO_3^-) to nitrite (NO_2^-) by the nitrate reductase complex NarGHJI, reduction of NO_2^- to nitric oxide (NO·) by the nitrite reductase AniA, and reduction of NO· to nitrous oxide (N_2O) by the nitric oxide reductase NorB. *M. catarrhalis* apparently lacks the ability to reduce N_2O to N_2 (indicated by brackets). The *M. catarrhalis* transcriptional regulator NsrR represses the expression of both AniA and NorB under aerobic growth conditions.

to grow in the presence of low concentrations of nitrite (72), but growth of an *M. catarrhalis nsrR* mutant in the presence of nitrite can be completely restored by disrupting the *aniA* gene to prevent the generation of nitric oxide (NO·) (72). These observations suggest that the reduction of nitrite to nitric oxide can be toxic for *M. catarrhalis*.

This study was undertaken to determine whether NorB metabolizes NO· to prevent its toxic effects. Here we show that the *M. catarrhalis norB* gene product reduces NO· to nitrous oxide (N_2O) and that *norB* is required for *M. catarrhalis* growth in the presence of low levels of nitrite, in either the presence or absence of an *nsrR* mutation. We also show that NO· generated by AniA from the reduction of nitrite is bactericidal for an *M. catarrhalis* O35E *norB* mutant but not for an isogenic wild-type strain, suggesting that *M. catarrhalis* relies primarily on NorB for NO· detoxification. The AniA-dependent generation of NO· from nitrite increases bacterial protein S nitrosylation levels in *M. catarrhalis* strains expressing AniA, and increased levels of AniA expression in *norB* and *nsrR* mutants correlate with elevated levels of protein S nitrosylation. This demonstrates that NO· generated by the truncated denitrification pathway can cause nitrosative stress for *M. catarrhalis*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. catarrhalis* strains used in this study are listed in Table 1. Bacterial culture conditions are as described previously (72). To measure the effect of nitrite on bacterial growth, a final concentration of 5 mM $NaNO_2$ was added to brain heart infusion (BHI) broth. Bacterial growth was monitored turbidimetrically every hour or by testing bacterial viability at the beginning (0 h) and end (6 h) of aerobic growth *in vitro*.

Whole-cell lysate preparation and Western blot analysis. Whole-cell lysates were prepared from BHI agar-grown cells as described previously (49). Western blot analysis was performed as described previously (69), except that the mouse polyclonal AniA antibody (72) and monoclonal antibody (MAb) 10F3 were used as primary antibodies to detect the *M. catarrhalis* AniA and CopB proteins, respectively.

Construction of *M. catarrhalis norB* mutants. The kanamycin-sensitive O35E Δ*nsrR* mutant and the kanamycin-resistant O35E *aniA* mutant were described previously (72). The kanamycin-sensitive O35E *aniA* deletion mutant, designated O35E Δ*aniA*, was constructed by transforming O35E *aniA* using the ΔANIA DNA fragment (72). One of the resulting kanamycin-sensitive transformants was confirmed to be a Δ*aniA* mutant by anchored PCR and sequence analysis (data not shown).

To construct *M. catarrhalis* O35E *norB* mutants, the oligonucleotide primer pairs WW247-WW248 and WW349-WW249 (Fig. 2A and Table 2) were used for PCR amplification using genomic DNA of *M. catarrhalis* ATCC 43617 as the template. The oligonucleotide primers WW248 and WW349 contain nucleotides (Table 2, underlined sequences) that are identical to the 5′ and the 3′ nucleotide residues of the *kan* cassette (71) from plasmid pAC7 (74). PCR amplification products were purified using a gel extraction kit (Qiagen) and, together with the *kan* cassette (71), were used as DNA templates for sequential overlapping extension PCR amplifications (29) (Table 3). The final amplicon, designated ΔNORB-KAN, was confirmed by DNA sequence analysis (data not shown) and used to transform wild-type O35E, the O35E Δ*aniA* mutant, the O35E Δ*nsrR* mutant, wild-type *M. catarrhalis* 7169, and ETSU-9. Kanamycin-resistant transformants were confirmed as the O35E *norB* mutant (Fig. 2B), O35E *aniA norB* mutant (Fig. 2C), O35E *nsrR norB* mutant (Fig. 2D), and ETSU-9 *norB* mutant (Fig. 2B) strains, respectively, by anchored PCR using the oligonucleotide primer pair WW247-WW217 (Fig. 2A) followed by sequence analysis (data not shown).

Repair of the *M. catarrhalis* O35E *norB* mutant. A DNA fragment containing the wild-type *norB* gene was amplified by PCR using primer pair WW247-WW207 (Fig. 2A) with genomic DNA of *M. catarrhalis* ATCC 43617 as the DNA template. This DNA fragment was used to transform the kanamycin-resistant O35E *norB* mutant. One of the kanamycin-sensitive transformants, designated O35E *norB* (*norB*), was confirmed to contain a wild-type ATCC 43617 *norB* gene by anchored PCR with primer pair WW220-WW217 (Fig. 2A), which was followed by nucleotide sequence analysis (data not shown).

Measurement of NO. consumption. Consumption of chemically generated NO· by *M. catarrhalis* cells was measured as described previously (72). Briefly, wild-type O35E, *norB* mutant, *nsrR norB* mutant, and *aniA norB* mutant *M. catarrhalis* cells were grown in BHI medium to an optical density at 600 nm (OD_{600}) of 2.0. Cells were washed and resuspended in freshly prepared BHI to an OD_{600} of 1.0. Approximately 3 ml of cells was assayed for NO· consumption in a sealed vessel. At approximately 0.5 min, the NO· -releasing reagent Proli-NO (half-life of 1.8 s) was added to the cell suspension to a final concentration of 10 μM, which releases a total of 20 μM NO· The concentration of dissolved NO· remaining over time was monitored using an ISO-NOPMC Mark II electrode (WPI Instruments).

Measurement of NO_2^- consumption. NO_2^- consumption by *M. catarrhalis* cells was measured as described previously (72). Briefly, after *M. catarrhalis* strains were resuspended in BHI to an OD_{600} of 1.0 as described immediately above, $NaNO_2$ was added to a final concentration of 5 mM. The concentration of remaining NO_2^- was determined using the Griess reaction as described previously (72).

Measurement of NO· and N_2O production. Production of NO· and N_2O by *M. catarrhalis* cells from the reduction of NO_2^- was measured as described previously (72), except that a lower concentration of NO_2^- (500 μM) was used to allow NO· and N_2O to be measured simultaneously. The NO· level was monitored as described above, and the N_2O level was monitored using an oxygen-insensitive, N_2O-specific probe (N2O-50-3112 [Unisense AS, Aarhus, Denmark]) connected to a PA2000 picoammeter (Unisense AS) and an analog-to-digital converter (A/D converter), ADC 216 (Unisense AS).

Detection of bacterial protein *S*-nitrosylation. A patented (Glythera Limited, United Kingdom) *S*-nitrosothiol (SNO) group binding reagent, designated SNOB, was used to detect protein *S*-nitrosylation. The SNOB reagents specifically bind SNO protein groups in a single chemical step. The biotin tag of the SNOB reagent allows the visualization of S-nitrosylated proteins using a streptavidin-horseradish peroxidase (HRP) conjugate in a Western blot assay, in which the intensities of protein bands reflect the relative levels of S nitrosylation. Briefly, an *M. catarrhalis* cell suspension (at a cell density of 260 Klett units) was added to 1 ml BHI containing SNOB reagent, with or without nitrite. The final

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description or genotype	Source or reference
Strains		
ATCC 43617	Wild-type strain	ATCC
O35E	Wild-type strain	26
ETSU-9	Wild-type strain	Steven Berk
O35E *norB*	*norB*::Kan^r	This study
O35E Δ*nsrR*	*nsrR* deletion mutant	72
O35E Δ*nsrR norB*	*nsrR norB* double mutant	This study
O35E Δ*aniA*	*aniA* deletion mutant	This study
O35E Δ*aniA norB*	*aniA norB* double mutant	This study
7169	Wild-type strain	Anthony Campagnari
Plasmids		
pWW115	Spec^r, cloning vector for *M. catarrhalis*	70
pWW149	pWW115 containing the wild-type ATCC 43617 *norB* gene	This study

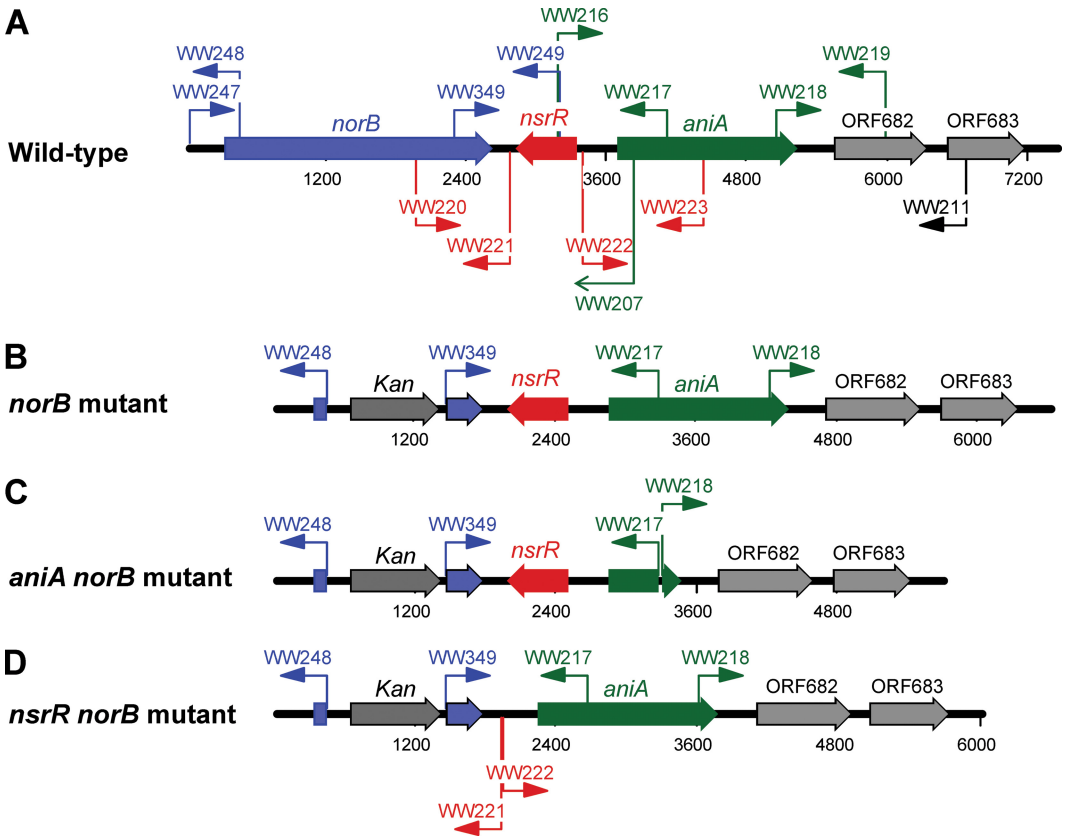

FIG. 2. Schematic representation of *M. catarrhalis* gene products involved in the truncated denitrification pathway and construction of relevant mutants. Schematic diagram of the *M. catarrhalis* chromosomal locus containing the *norB*, *nsrR*, and *aniA* genes and flanking regions in the wild-type O35E strain (A), the O35E *norB* mutant (B), the O35E *aniA norB* mutant (C), and the O35E *nsrR norB* mutant (D). The relative positions of the different primers used for PCR are indicated by the arrows.

concentrations of SNOB and nitrite were 1 mM and 3 mM (if added), respectively, and the final cell density was approximately 1 OD_{600} (5×10^8 CFU/ml). The mixtures were incubated at 30°C for 30 min, bacterial cells harvested by centrifugation, and cell pellets washed twice with ice-cold 1× phosphate-buffered saline (PBS) to remove unbound SNOB reagent. Whole-cell lysates (0.2 ml each) were prepared for Western blotting as described previously (14), except that the streptavidin–β-peroxidase (POD) conjugate (Roche) was used to detect the biotin tag of SNOB bound to S-nitrosylated proteins.

RESULTS

Construction of *M. catarrhalis norB* mutants. The wild-type *M. catarrhalis* O35E genetic locus containing the *norB*, *nsrR*, and *aniA* genes is shown in a schematic diagram (Fig. 2A). Mutations in *norB* (Fig. 2B) were introduced using a PCR amplicon, ΔNORB-KAN, into the wild-type *M. catarrhalis* O35E, ETSU-9, and 7169 strain backgrounds by allelic exchange to replace the DNA sequence between the oligonucleotide primers WW248 and WW349 with a *kan* resistance cassette from plasmid pAC7, as described in Materials and Methods. Additional O35E *norB* mutations were constructed in both the O35E Δ*aniA* mutant (described in Materials and Methods) and the O35E Δ*nsrR* mutant (72) backgrounds, resulting in an *M. catarrhalis* O35E *aniA norB* mutant (Fig. 2C) and an O35E *nsrR norB* mutant (Fig. 2D), respectively. All *norB* mutants were confirmed by anchored PCR and DNA sequence analysis.

***M. catarrhalis* NorB is required for NO consumption.** The levels of consumption of chemically generated NO· by wild-

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence (5′–3′)
WW247	TAGGATCCAATCACACTTAGGATTATCA
WW248	CGGAGCCTGCAGCCCGGGTGGTACGGTAAA TCTCAAA[a]
WW349	CTAGATTTAGATGTCGGGCTTTGTGCTGTTG ATTGT[b]
WW249	AGTTGAGCTCGCTTAAAGTCGTTGACAGTGC

[a] Nucleotides of WW248 that overlap primer WW195 are underlined.
[b] Nucleotides of WW349 that overlap primer WW196 are underlined.

TABLE 3. PCR extension

Primer pair	DNA template(s)	DNA polymerase	PCR-amplified DNA fragments
WW247-WW248	ATCC 43617 genomic DNA	Pfu	*up*
WW349-WW249	ATCC 43617 genomic DNA	Pfu	*down*
WW195-WW196	pAC7 plasmid DNA	Pfu	*kan*
WW247-WW196	*up* and *kan* PCR DNAs	ExTaq	*up-kan*
WW195-WW249	*kan* and *down* PCR DNAs	ExTaq	*kan-down*
WW247-WW249	*up-kan* and *kan-down* PCR DNAs	ExTaq	ΔNORB-KAN

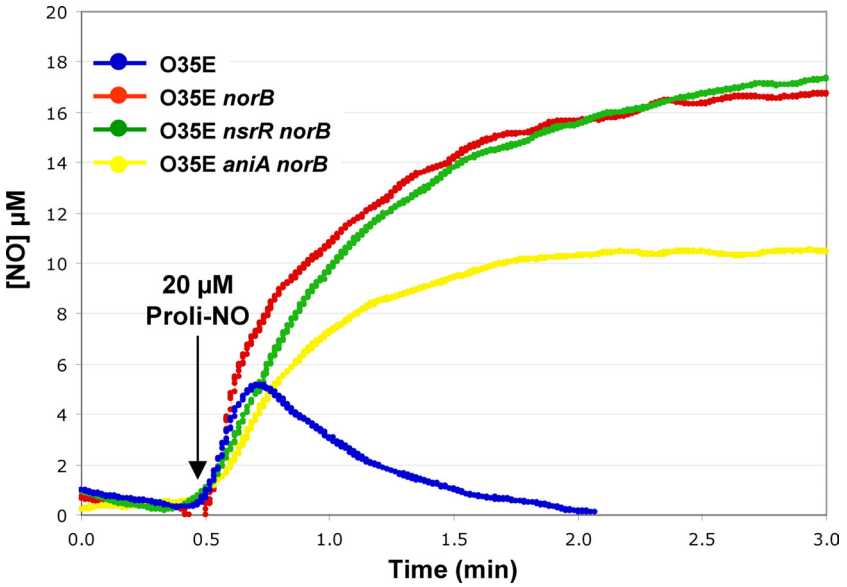

FIG. 3. Consumption of chemically generated NO· by the wild-type and mutant strains of *M. catarrhalis* O35E. Cell suspensions of the wild-type strain, the *norB* mutant, the *nsrR norB* mutant, and the *aniA norB* mutant were exposed to 20 μM NO· produced by the addition of 10 μM proline nitric oxide (Proli-NO). The dissolved NO· concentration was monitored using an NO· -specific electrode. For reference, the NO· -consuming activity of BHI medium was determined (data not shown). This experiment was performed three times, and representative data are shown.

type *M. catarrhalis* O35E and three *norB* mutant strains were compared as described in Materials and Methods. Wild-type O35E cells (Fig. 3, blue line) consumed NO·, as observed previously (72). The *norB* single mutant (Fig. 3, red line), the O35E *nsrR norB* mutant (Fig. 3, green line), and the O35E *aniA norB* mutant (Fig. 3, yellow line) strains failed to exhibit NO· consumption. After the addition of the NO· -releasing agent Proli-NO, NO· levels accumulated and were not consumed in the three O35E-derived strains carrying a *norB* mutation (Fig. 3). These assays were conducted in a sealed vessel, in which the remaining oxygen is consumed very rapidly, resulting in anaerobic conditions. These results indicate that the *M. catarrhalis norB* gene product is required for NO· consumption.

Analysis of AniA protein expression. In this study, nitrite was used as a source of NO· biologically generated by the action of the *M. catarrhalis* AniA protein. The expression of AniA protein by *M. catarrhalis* O35E strains was determined by Western blotting using a mouse polyclonal AniA antibody (72). Interestingly, expression of AniA protein in the *M. catarrhalis* O35E *norB* mutant (Fig. 4A, lane 2) was higher than in wild-type O35E (Fig. 4A, lane 1). As expected, the *M. catarrhalis* O35E *aniA norB* mutant (Fig. 4A, lane 3) did not express AniA, and the *M. catarrhalis* O35E *nsrR norB* mutant (Fig. 4A, lane 4) exhibited the highest expression of AniA, similar to that of an O35E *nsrR* mutant (72). Expression of the *M. catarrhalis* CopB protein was measured as a loading control (Fig. 4B).

AniA is required for NO_2^- consumption. To confirm that the AniA protein expressed by *M. catarrhalis norB* mutants is functional, NO_2^- consumption by mutant *M. catarrhalis* strains was determined as described in Materials and Methods. The three AniA-expressing *M. catarrhalis* strains, including wild-type O35E (Fig. 5, blue line), the *norB* mutant (Fig. 5, red line), and the *nsrR norB* double mutant (Fig. 5, green line) were able to consume NO_2^-. In contrast, the O35E *aniA norB* mutant (Fig. 5, yellow line) did not consume NO_2^-. This result confirmed that the AniA proteins expressed by wild-type O35E, the O35E *norB* mutant, and the O35E *nsrR norB* mu-

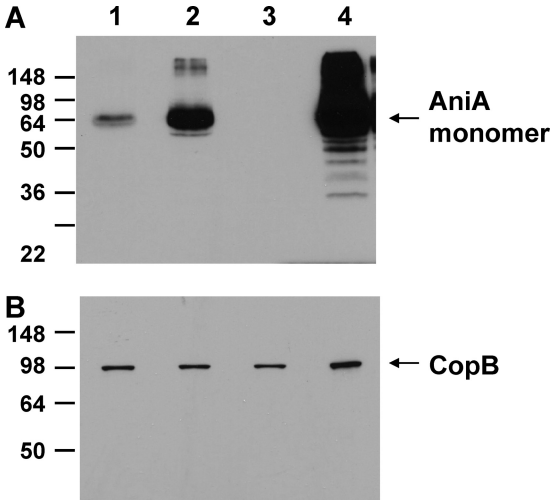

FIG. 4. Expression of AniA protein in wild-type and mutant strains of *M. catarrhalis* O35E. Whole-cell lysates were probed by Western blot analysis with polyclonal AniA antiserum (A) or with the CopB-specific MAb 10F3 (26) (B) as the primary antibody. Lane 1, wild-type O35E; lane 2, O35E *norB*; lane 3, O35E *norB aniA*; lane 4, O35E *norB nsrR*. The position of the putative AniA monomers is indicated by an arrow on the right side of panel A. The CopB outer membrane protein was used as a loading control, and its position is indicated by an arrow on the right side of panel B. Molecular weight position markers (in thousands) are present on the left side of each panel.

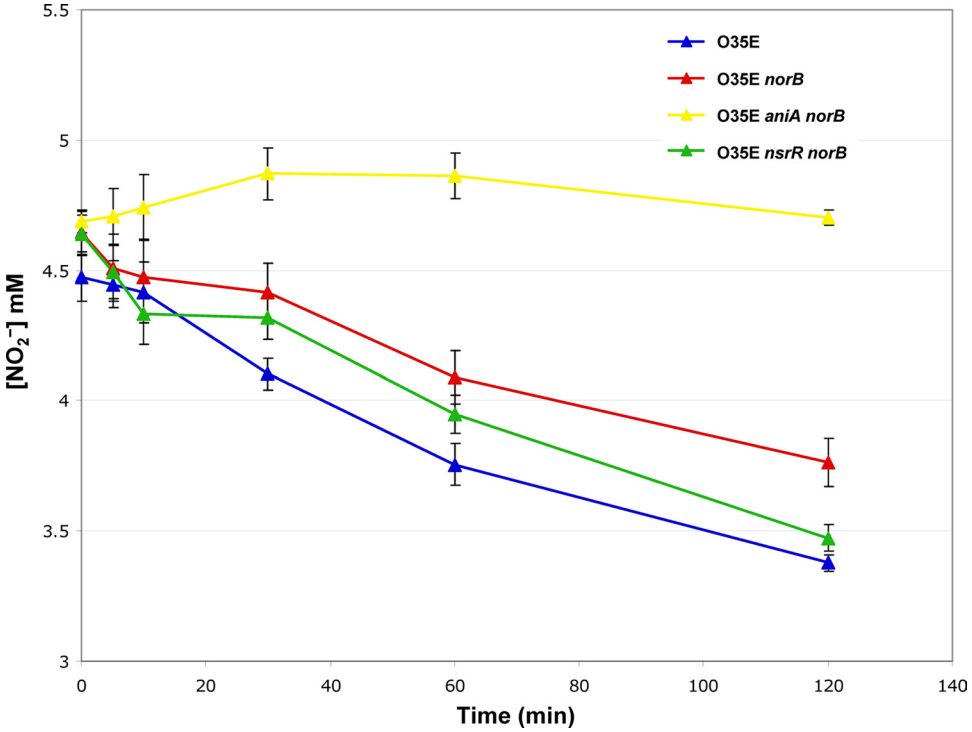

FIG. 5. NO_2^- consumption by wild-type and mutant strains of *M. catarrhalis* O35E. Cell suspensions of the wild-type strain, the *norB* mutant, the *nsrR norB* mutant, and the *aniA norB* mutant in BHI medium supplemented with 5 mM $NaNO_2$ were monitored for the presence of NO_2^- using the Griess reaction. The data shown are the averages of results from three independent experiments, with standard errors indicated.

tant are functional. Although both the O35E *norB* and O35E *nsrR norB* mutants expressed higher levels of AniA than the parental wild-type strain O35E (Fig. 4A), the two *norB* mutants did not consume NO_2^- significantly faster than wild-type O35E (Fig. 5).

Production of NO· and N_2O by wild-type and mutant *M. catarrhalis* O35E strains. Following the addition of 500 μM $NaNO_2$, wild-type *M. catarrhalis* simultaneously produced and consumed NO· to form the final product, N_2O (Fig. 6A). The *nsrR norB* and *norB* mutants consumed nitrite to form NO·, which accumulated to high levels in both cultures (Fig. 6B and D). A low level of erratic signal of the N_2O sensor observed in the experiments with the O35E *norB* and O35E *nsrR norB* mutants was due to chemical reduction of NO· to N_2O within the sensor compartment occurring at high concentrations of NO· As expected, the O35E *aniA norB* mutant failed to generate either NO· or N_2O from nitrite (Fig. 6C). This study confirms that AniA is required for nitrite reduction to NO· and that NorB reduces NO· to N_2O in the truncated *M. catarrhalis* denitrification pathway.

Effect of NO_2^- on growth of the wild-type, mutant, and repaired mutant strains of *M. catarrhalis*. To investigate the biological relevance of the *M. catarrhalis norB* gene product, the effect of nitrite at low concentrations on the growth of *M. catarrhalis* O35E strains was examined. The presence of 5 mM $NaNO_2$ had no effect on the aerobic growth of wild-type O35E (Fig. 7A), as reported previously (72). In contrast, the growth of the O35E *norB* mutant and the O35E *nsrR norB* mutant was completely inhibited by 5 mM NO_2^- (Fig. 7B and D). This NO_2^--dependent growth inhibition was completely relieved by disrupting the *aniA* gene in an O35E *norB* mutant background (Fig. 7C). The growth of the *M. catarrhalis* 7169 *norB* mutant (data not shown) and the ETSU-9 *norB* mutant (Fig. 7E) was also completely inhibited by NO_2^-, suggesting that the growth inhibition of a *norB* mutant by nitrite is not strain specific.

Gene repair was performed to restore a functional *norB* gene in the chromosome of the O35E *norB* mutant, resulting in the strain O35E *norB*(*norB*) as described in Materials and Methods. Growth of the repaired O35E *norB*(*norB*) strain was not affected by 5 mM NO_2^- (Fig. 7F). These experiments confirmed that a functional *norB* gene is essential for *M. catarrhalis* to grow in the presence of nitrite at low concentrations.

NO· produced from nitrite by the AniA protein is bactericidal for a *Moraxella catarrhalis* O35E *norB* mutant. The viability of *M. catarrhalis* O35E strains under aerobic growth at 37°C in BHI with or without the addition of nitrite at low concentrations was examined. Wild-type *M. catarrhalis* O35E, *norB* mutant, and *aniA norB* mutant cells were used to inoculate BHI with or without the addition of 5 mM $NaNO_2$ to a final concentration of approximately 10^8 CFU/ml (Fig. 8, 0 h). The growth of both the wild-type O35E and *aniA norB* mutant strains was not affected by nitrite, as viable counts for these strains increased to $\sim 9 \times 10^9$ CFU/ml in BHI either with or without $NaNO_2$ (Fig. 8, 6 h). In contrast, viable counts of the O35E *norB* mutant increased to $\sim 9 \times 10^9$ CFU/ml in BHI but decreased to 10^5 CFU/ml in BHI containing nitrite. NO· produced from nitrite is bactericidal for an *M. catarrhalis*

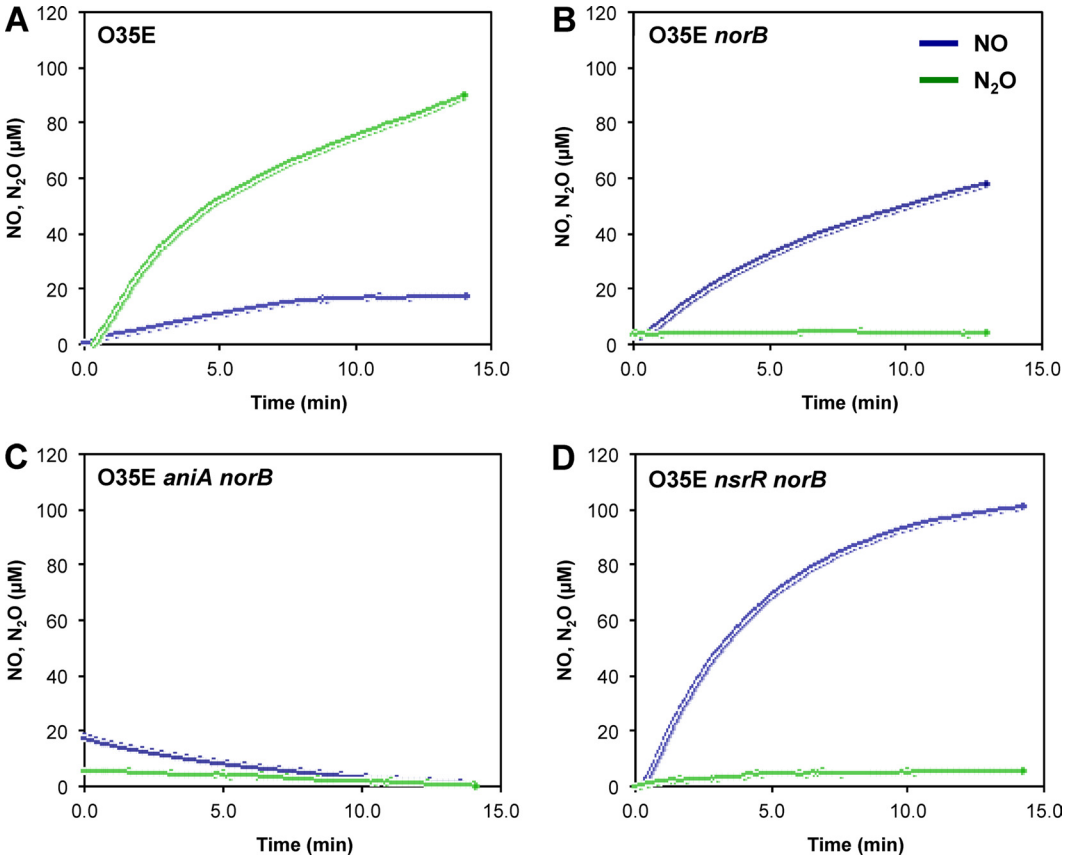

FIG. 6. Production of NO and N_2O from NO_2^- by wild-type and mutant strains of *M. catarrhalis* O35E. Cell suspensions of the wild-type strain (A), the *norB* mutant (B), the *aniA norB* mutant (C), and the *aniA norB* mutant (D) in BHI medium supplemented with 500 μM $NaNO_2$ were monitored for 15 min for the presence of NO· (blue lines) using an NO· -specific electrode. Probe specificity was affirmed by the ability of an NO· scavenger, Carboxy-PTIO, to quench measurable signal (not shown). The presence of N_2O (green lines) was measured simultaneously using an oxygen-insensitive, N_2O-specific probe as described in Materials and Methods. The data shown are a representative set of results from two independent experiments.

O35E *norB* mutant and the ETSU-9 *norB* mutant (data not shown) but not for wild-type *M. catarrhalis*, indicating that *M. catarrhalis* relies on NorB for NO· detoxification.

NO· produced from nitrite by the AniA protein increases bacterial protein *S*-nitrosylation. To investigate whether NO· produced from nitrite by the AniA protein is responsible for the nitrite-dependent growth inhibition of both the *M. catarrhalis* O35E *nsrR* mutant and the O35E *norB* mutant, bacterial protein *S*-nitrosylation profiles were measured as described in Materials and Methods. Studies have shown that nitrite/nitrate is present in various animal tissues at levels between 0.5 and 50 μM (2, 48), which can support NO generation (38). We have briefly determined that BHI contains approximately 50 μM nitrate (data not shown), which can be reduced to a trace amount of NO· Protein S nitrosylation in *M. catarrhalis* cells grown in medium without the addition of nitrite was measured as the steady-state endogenous *S*-nitrosylation level (Fig. 9A, − lanes). Increased *S*-nitrosylation was observed in all three AniA-expressing O35E strains in the presence of nitrite, especially a band slightly above 22 kDa that was present only in all three AniA-expressing O35E strains in the presence of nitrite (Fig. 9A, lanes wt +, *norB* +, and *nsrR* +). Increased *S*-nitrosylation levels, indicative of nitrosative stress, correlated with AniA expression levels in the presence of nitrite: highest in the O35E *nsrR* mutant (72), modest in wild-type O35E (Fig. 4, lane 1), and intermediate in the O35E *norB* mutant (Fig. 4, lane 2). As expected, the O35E *aniA norB* mutant did not express AniA protein (Fig. 4, lane 3), and its overall level of protein *S*-nitrosylation was not substantially affected by the presence of nitrite (Fig. 9, lane *aniA norB* +), except in a protein migrating at ~64 kDa. This might reflect the high sensitivity of the SNOB reagent for *S*-nitrosothiols and the nonenzymatic generation of NO· from the added nitrite. Fig. 9 shows representative data from two experiments. The identities of S-nitrosylated proteins are presently unknown. These observations suggest that high levels of nitrosative stress can inhibit the growth of *M. catarrhalis*.

DISCUSSION

Bacterial denitrification pathways, consisting of four sequential enzymatic steps that reduce nitrate to gaseous nitrogen, have been identified in bacteria living in various environments (reviewed in reference 75). Anaerobic bacteria use denitrification pathways as an alternative means of energy production, with nitrogen oxides functioning as electron acceptors (75).

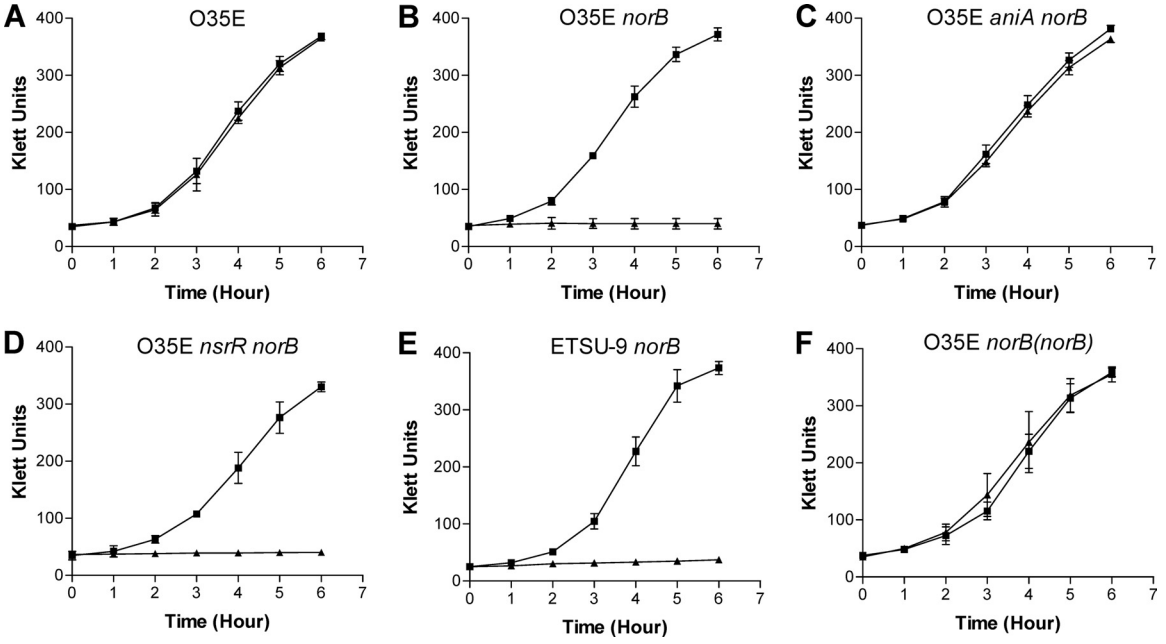

FIG. 7. Effect of NO_2^- on growth of the wild-type, mutant, and repaired mutant strains of *M. catarrhalis*. Cells of the wild-type strain (A), the O35E *norB* mutant (B), the O35E *aniA norB* mutant (C), the O35E *nsrR norB* mutant (D), the ETSU-9 *norB* mutant (E), and the repaired strain O35E *norB*(*norB*) (F) were grown in BHI medium (■) or BHI containing 5 mM $NaNO_2$ (▲). The data shown are the means of results from three independent growth experiments.

Recently, denitrification by bacterial pathogens has attracted increasing interest (51). The nitrate reductase complex NarGHJI was shown to be involved in *Mycobacterium bovis* BCG virulence in an animal model (73) and was implicated in *Pseudomonas aeruginosa* biofilm formation and virulence (65, 66). The *Brucella melitensis* denitrification pathway is required for virulence in mice (6, 23). Denitrification promotes the growth of *Neisseria meningitidis*, a strictly aerobic human pathogen, under oxygen-limited conditions (3, 55). Barth and colleagues (8) recently showed that, among all *Neisseria* strains tested, a nitrate reductase complex could be identified only in *Neisseria mucosa*. However, a nitrite reductase (AniA or NirK) and nitric oxide reductase (NorB) were present in all *Neisseria* species tested. Interestingly, a nitrous oxide reductase (Nos) is present in some commensal *Neisseria* species but absent from the pathogenic species *Neisseria gonorrhoeae* and *N. meningitidis*. The fumarate and nitrate reductase (FNR), NarQP, and NsrR transcriptional regulators are highly conserved in *Neisseria* species. The transcriptional regulator NsrR is conserved in several *M. catarrhalis* clinical isolates; however, the ATCC 43617 genome does not encode an FNR protein regulator (72). Instead, the *M. catarrhalis* genome encodes a two-component system NarXL and a homologue of the DnrD protein which lacks the [4Fe-4S] center and belongs to a new subgroup of the FNR regulator family (72). The nitric oxide reductase NorB is

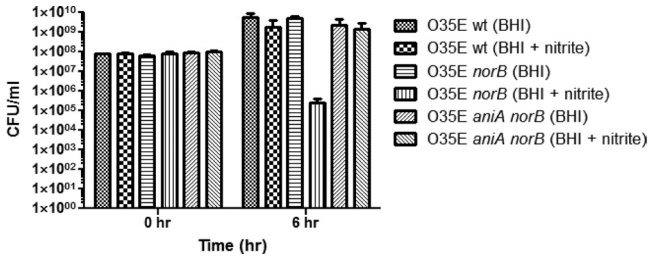

FIG. 8. NO· produced from nitrite by the AniA protein is bactericidal for an *M. catarrhalis* O35E *norB* mutant. Cells of the wild-type strain (wt), the *norB* mutant (*norB*), and the *aniA norB* mutant (*aniA norB*) were grown in BHI without or with the addition of 5 mM nitrite (+ nitrite). Aliquots from each culture were taken at the beginning (0 h) and end (6 h) of aerobic growth for the determination of viable cell counts (CFU/ml). The data shown are the means of results from three independent growth experiments.

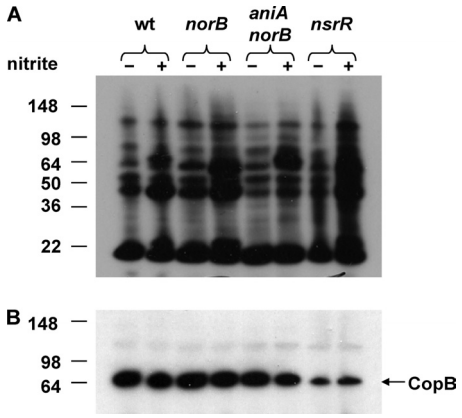

FIG. 9. Bacterial cellular protein *S*-nitrosylation profiles of wild-type and mutant strains of *M. catarrhalis* O35E. (A) Cell suspensions of the wild-type strain, the *norB* mutant, the *aniA norB* mutant, and the *nsrR* mutant were incubated with SNOB reagent in medium with (+) or without (−) added nitrite. S-nitrosylated proteins were visualized using streptavidin-HRP. (B) *M. catarrhalis* CopB was detected as a loading control.

responsible primarily for NO detoxification (61, 62) in *Neisseria* species, and a *cycP* gene product also functions in NO detoxification (3, 62). The *M. catarrhalis* ATCC 43617 genome has 18 open reading frames (ORFs) encoding cytochrome *c* proteins (71). Six of the *M. catarrhalis* cytochrome *c* proteins contain a heme-binding motif (CXXCH) (15). Four of these heme-binding cytochrome *c* proteins have a signal peptide sequence identified with SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). Only ORF 192 (152 amino acids; also known as Msp22 [56]) has both a signal peptide sequence and a single CXXCH motif that is located at its C terminus, which are features specific for cytochrome *c′* (*cycP*). However, a role for the ORF 192 protein in *M. catarrhalis* NO· detoxification has not been established.

It was recently reported that *M. catarrhalis* forms biofilms on the mucosal surface of the middle ear in children with chronic otitis media (24). Studies of *M. catarrhalis* gene expression have revealed that the expression of the enzymes comprising the truncated *M. catarrhalis* denitrification pathway was highly upregulated in biofilm-grown *M. catarrhalis* cells *in vitro* (71). Elevated expression of denitrification genes has been reported in other pathogens, including *N. gonorrhoeae* (17) and *P. aeruginosa* (65), during growth in biofilms *in vitro*. *M. catarrhalis* is known to reduce NO_3^- (reviewed in reference 12), although the genes that encode this activity (likely *narGHJI*) have not been fully described. An initial study showed that an *M. catarrhalis narGH* mutant grows as well in a continuous-flow biofilm system as its wild-type parent (71). The importance of denitrification for *M. catarrhalis* biofilm formation remains to be fully elucidated. Recent studies showed that a low level of NO· can promote biofilm dispersal in *P. aeruginosa* (65) and *N. gonorrhoeae* (18). In contrast, high chemically generated levels of NO· inhibit early-stage biofilm formation but enhance *N. gonorrhoeae* growth in established biofilms (18).

The present study shows that a functional *norB* gene is required for *M. catarrhalis* to reduce chemically generated NO· (Fig. 3, blue line), as NO· is not utilized by an O35E *norB* isogenic mutant (Fig. 3, red, green, and yellow lines). It is not apparent why *M. catarrhalis norB* mutant cells express higher levels of AniA (Fig. 4A, lane 2) than the wild type (Fig. 4A, lane 1), even when cells are grown on BHI agar without the addition of nitrite. The NsrR-repressed aerobic expression of *M. catarrhalis* AniA is insensitive to chemically generated NO· (from 50 μM spermine NONOate; half-life, about 39 min at 37°C) in wild-type O35E cells (72). Although both *norB* and *nsrR norB* mutants express significantly higher levels of AniA protein (Fig. 4, lanes 2 and 4), they do not reduce nitrite faster (Fig. 5, red and green lines) than the wild type (Fig. 5, blue line). In contrast, an *M. catarrhalis nsrR* mutant reduces nitrite more rapidly than the wild type (72). This is attributable to the accumulation of NO· in the absence of NorB, some of which undergoes auto-oxidation to nitrite (30), and the inability of the Griess reagent to distinguish NO· and nitrite.

Bacteria employ different mechanisms for NO· detoxification to withstand nitrosative stress. An NsrR-regulated and NO·-inducible flavohemoglobin protein (Hmp) is the major NO· detoxifier in *Salmonella enterica* serovar Typhimurium (7, 22) and *Escherichia coli* (19). The enteric pathogen *S.* Typhimurium requires Hmp for virulence in mice expressing inducible NO· synthase (7). In *E. coli*, Hmp is required for resistance to nitrosative stress (25) and for bacterial survival within macrophages (63). The *M. catarrhalis* ATCC 43617 genome does not contain a gene encoding a Hmp-like protein (72). Furthermore, a low level of nitrite completely inhibits the growth of an *M. catarrhalis* O35E *norB* mutant (Fig. 7B), strongly suggesting that this bacterium relies on NorB for NO· detoxification. It has not been immediately obvious why nitrite supplementation also completely inhibits the aerobic growth of an *M. catarrhalis nsrR* mutant, because this mutant rapidly reduces nitrite to nitrous oxide with little or no detectable steady-state NO· accumulation (72). To investigate whether an increased flux of NO· is responsible for nitrite-related inhibition of the aerobic growth of an *M. catarrhalis nsrR* mutant, we examined bacterial protein *S*-nitrosylation profiles.

In an enzyme-independent chemical reaction, NO· can bond covalently with the thiol groups of protein cysteine residues to form *S*-nitrosothiols (SNOs), a posttranslational modification of cellular proteins known as *S*-nitrosylation (59, 60). *S*-nitrosylation has been implicated in mammalian cell apoptosis (9, 33–35, 39, 53). A recent study reported that bacterial proteins involved in NO· detoxification, including NorB of *N. meningitidis* and the flavohemoglobins (Hmp) of *S. enterica* and *E. coli*, prevent host cell SNO formation (37).

S-nitrosylation of bacterial proteins was determined in wild-type O35E, *norB* mutant, *aniA norB* mutant, and *nsrR* mutant *M. catarrhalis* cells as described in Materials and Methods. The source of additional NO· was nitrite that was reduced by the activity of the *M. catarrhalis* AniA protein. The O35E *aniA norB* mutant does not express AniA (Fig. 4A, lane 3) and is unable to reduce nitrite (Fig. 5, yellow line). The O35E *norB* mutant and *nsrR* mutant strains express significantly higher levels of AniA (Fig. 4A, lane 2, and reference 72) than the parental strain, O35E (Fig. 4A, lane 1). By Western blot analysis, a band slightly above 22 kDa is present only in the three AniA-expressing O35E strains, and an overall increase in *S*-nitrosylation was observed in all AniA-expressing *M. catarrhalis* strains in the presence of nitrite (Fig. 9A, wt +, *norB* +, and *nsrR* + lanes). Increased *S*-nitrosylation correlated well with levels of AniA expression and was highest in an *nsrR* mutant (Fig. 9A, *nsrR* + lane) and lowest in wild-type O35E (Fig. 9A, wt + lane). These observations strongly suggest that NO· is the inhibitory factor responsible for the nitrite-dependent inhibition of the growth of the *norB* and *nsrR* mutant strains. Although the *nsrR* mutant exhibits low measurable steady-state NO· concentrations (72), the increased flux of NO· produced from nitrite reduction appears to cause nitrosative stress, which could account for growth inhibition by nitrite. The specific protein targets of *S*-nitrosylation in *M. catarrhalis* remain to be identified.

The AniA protein of *N. gonorrhoeae* is expressed *in vivo* during infection in humans (13). The *M. catarrhalis* AniA protein, also known as Msp78 (56), has recently been shown to be present in patients during COPD exacerbations associated with *M. catarrhalis* infection (56). The transcriptional regulatory network that controls the expression of the truncated denitrification pathway in *M. catarrhalis* under various *in vitro* growth conditions is under active investigation. It is hoped that such work will help to elucidate the importance of denitrification in *M. catarrhalis* pathogenesis.

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