INFECTION AND IMMUNITY, May 2009, p. 1945–1958 Vol. 77, No. 5
0019-9567/09/$08.00+0 doi:10.1128/IAI.01365-08

Resistance of *Haemophilus influenzae* to Reactive Nitrogen Donors and Gamma Interferon-Stimulated Macrophages Requires the Formate-Dependent Nitrite Reductase Regulator-Activated *ytfE* Gene▽

Jane C. Harrington,[1] Sandy M. S. Wong,[1] Charles V. Rosadini,[1] Oleg Garifulin,[2] Victor Boyartchuk,[2] and Brian J. Akerley[1]*

Department of Molecular Genetics and Microbiology[1] and Program in Gene Function and Expression,[2] University of Massachusetts Medical School, Worcester, Massachusetts 01655

Received 6 November 2008/Returned for modification 16 December 2008/Accepted 2 March 2009

Haemophilus influenzae **efficiently colonizes and persists at the human nasopharyngeal mucosa, causing disease when it spreads to other sites. Nitric oxide (NO) represents a major antimicrobial defense deployed by host cells in locations colonized by *H. influenzae* during pathogenesis that are likely to vary in oxygen levels. Formate-dependent nitrite reductase regulator (FNR) is an oxygen-sensitive regulator in several bacterial pathogens. We report that *fnr* of *H. influenzae* is required for anaerobic defense against exposure to NO donors and to resist NO-dependent effects of gamma interferon (IFN-γ)-activated murine bone marrow-derived macrophages. To understand the mechanism of resistance, we investigated the role of FNR-regulated genes in defense against NO sources. Expression analysis revealed FNR-dependent activation of *nrfA*, *dmsA*, *napA*, and *ytfE*. Nonpolar deletion mutants of *nrfA* and *ytfE* exhibited sensitivity to NO donors, and the *ytfE* gene was more critical for survival. Compared to the wild-type strain, the *ytfE* mutant exhibited decreased survival when exposed to macrophages, a defect that was more pronounced after prior stimulation of macrophages with IFN-γ or lipopolysaccharide. Complementation restored survival of the mutant to the level in the parental strain. Increased sensitivity of the *ytfE* mutant relative to that of the parent was abrogated by treatment of macrophages with a NO synthase inhibitor, implicating YtfE in resistance to a NO-dependent pathway. These results identify a requirement for FNR in positive control of *ytfE* and indicate a critical role for *ytfE* in resistance of *H. influenzae* to reactive nitrogen species and the antibacterial effects of macrophages.**

Haemophilus influenzae is a gram-negative bacterial pathogen of humans that colonizes the nasopharynx of healthy individuals, causing primarily asymptomatic infections. Disease occurs by extension of bacteria to privileged anatomical sites, leading to otitis media, meningitis, septicemia, or respiratory infections (59). Despite the availability in developed countries of an effective vaccine against type b strains, which cause meningitis, *H. influenzae* type b remains a significant cause of disease worldwide (13). The incidence of infection by nontypeable *H. influenzae* strains (NTHi) appears to be unaffected by the type b vaccine, and NTHi remain a frequent cause of otitis media (65). NTHi is also a significant medical concern in recurring bacterial exacerbations of chronic obstructive pulmonary disease and cystic fibrosis (33, 43, 58, 60). The mechanisms by which this bacterium survives and causes disease at diverse sites of infection are not well understood.

In different tissues or stages of pathogenesis, bacteria must recognize environmental signals as cues for appropriate regulation of gene expression. The results of recent studies suggest that levels of oxygen availability generate signals leading to modulation of virulence factor expression in *H. influenzae.* Modification of *H. influenzae*'s lipooligosaccharide with phosphorylcholine, a virulence-associated structure that is also targeted by host immunity (86), is upregulated when oxygen availability is decreased (89). Genes similar to *arcA* and *fnr*, global genetic regulatory genes critical to the response to anaerobiosis in other species, are present in *H. influenzae*. The *H. influenzae* ArcA/ArcB system is a two-component signal transduction system that is active under low-oxygen conditions and functions via a phosphorelay from a sensor kinase protein, ArcB, to activate the DNA binding response regulator, ArcA, analogous to the well-characterized signal-transduction mechanism of ArcA/ArcB of *Escherichia coli* (30, 45). Phosphorylated ArcA then controls a set of genes involved in adaptation to respiratory conditions of growth that is similar but not identical to the ArcA/ArcB regulon of *E. coli* (47, 62, 72, 91). In support of the hypothesis that *H. influenzae* encounters a low-oxygen environment in vivo in the host, *H. influenzae* mutants lacking ArcA are attenuated for survival in murine models of pathogenesis (19, 91).

In *E. coli* and other bacteria, transitions between aerobic and anaerobic environments also involve the global regulator FNR (formate-dependent nitrite reductase regulator), a cyclic AMP receptor protein/catabolite activator protein-like transcriptional regulatory protein that activates and represses the expression of multiple genes under anaerobic conditions (75). FNR's functional role in *H. influenzae* has not been characterized; however, its predicted amino acid sequence is 79% iden-

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, 55 Lake Ave., N., S6-242, Worcester, MA 01655. Phone: (508) 856-1442. Fax: (508) 856-1422. E-mail: Brian.Akerley@umassmed.edu.

▽ Published ahead of print on 16 March 2009.

tical to *E. coli* FNR, which has been studied intensively. Unlike ArcB, which indirectly senses oxygen via changes in the reduction/oxidation status of the quinone pool (52), FNR of *E. coli* is considered to be a direct oxygen sensor by virtue of its iron-sulfur center, which is required for maintaining an active conformation that promotes dimerization and DNA binding (46). Upon oxidation, the iron-sulfur center undergoes a transition leading to the conversion of FNR to its inactive monomeric form (16, 20). FNR and FNR-like anaerobic regulators have been implicated in the pathogenesis of *Salmonella enterica* serovar Typhimurium (28), *Neisseria meningitidis* (8), *Actinobacillus pleuropneumoniae* (5), and *Bordetella pertussis* (92). The mechanisms by which these regulators contribute to virulence are not fully understood; however, FNR-regulated genes that participate in virulence properties have been identified in several organisms. *A. pleuropneumoniae* HlyX is an FNR-like regulator that controls *dmsA* and *aspA*, genes involved in anaerobic respiration that are required for virulence (39). In addition, genes of nitrosative defense constitute an important class of FNR-regulated genes that have been identified in diverse bacterial species, including *Campylobacter jejuni* (25), *S. enterica* serovar Typhimurium (28, 32), and *E. coli* (14, 64, 67, 68).

During immune responses to bacteria, host cells produce the free radical gas nitric oxide (NO) via metabolism of L-arginine by inducible NO synthase (iNOS) (for reviews, see references 10 and 51). NO is highly reactive and unstable in biological systems, especially in the presence of oxygen at neutral pH, which results in its conversion to nitrite (87). Additional reactions with NO generate a collection of other reactive nitrogen species (RNS), such as *S*-nitrosothiols, which are thought to exceed the cytotoxicity of NO, leading to diverse mechanisms of antimicrobial activity (11, 26). Alcohol dehydrogenase (*adhC*) has been implicated in defense of *H. influenzae* against one type of RNS, *S*-nitrosoglutathione (GSNO), under normoxic conditions (44); however, information regarding how *H. influenzae* defends itself from host-derived RNS under oxygen-limiting conditions has not been reported. Genome analysis suggests that *H. influenzae* possesses a single nitrite reductase, encoded by the *nrfABCD* operon, comprised of nitrite reductase genes similar to those implicated in NO metabolism in other species (66, 76). Also present in the *H. influenzae* genome is the *ytfE* gene, predicted to encode a di-iron protein similar to a family of iron-sulfur repair proteins involved in RNS and reactive oxygen species resistance in *E. coli* and a diverse group of additional bacterial species (63).

In this study, we investigated the role of FNR in regulation of the defense of *H. influenzae* from RNS generated in vitro and by activated macrophages. A single-copy, chromosomal reporter fusion to *nrfA* was generated to monitor expression from its endogenous promoter. The fusion strain was used to identify a range of low-oxygen conditions that are required for FNR-mediated transcriptional activation in *H. influenzae*. Control of candidate FNR-regulated genes, including the *nrfA* and *ytfE* genes, which have not been investigated in this bacterium, was assessed by mRNA analysis. Mutants lacking FNR or genes that it regulates were evaluated for resistance to two chemical donors of NO in vitro, implicating each of these genes in RNS defense. To investigate the context in which anaerobic sensitivity to RNS could influence pathogenesis, the effect of macrophage activation with lipopolysaccharide (LPS) or gamma interferon (IFN-γ) on the survival of wild-type, *fnr*, and *ytfE H. influenzae* strains was determined, and the role of NO in this process was addressed by using an inhibitor of NOS. We report that FNR is required for anaerobic defense of *H. influenzae* against exposure to NO donors and IFN-γ-treated macrophages. Furthermore, the results demonstrate positive control by FNR of *H. influenzae ytfE* and reveal a critical role for *ytfE* in resistance of this bacterium to RNS and the antibacterial effects of activated macrophages.

(Parts of the manuscript have been included in a Ph.D. dissertation by Jane Harrington.)

MATERIALS AND METHODS

Strains and culture conditions. *H. influenzae* Rd BA042, a capsule-deficient derivative obtained from the serotype d source that was used to generate Rd KW20 (89), and NTHi clinical isolate NT127 (provided by Robert N. Husson), were grown in brain heart infusion broth (BHI) supplemented with 10 μg/ml hemin, 10 μg/ml NAD, 2 mM D-xylose (sBHI) or on sBHI agar plates at 35°C. For growth in semidefined medium lacking detectable nitrate or nitrite sources, bacteria were grown in MIc, a low-nutrient medium capable of supporting growth of *H. influenzae* (7). To generate a condition approximating anaerobiosis, strains were grown in anaerobic chambers with BBL GasPak Plus generators to deplete oxygen (Becton Dickenson and Company, Sparks, MD); for simplicity, these are termed low-oxygen conditions. Development of competence for transformation of *H. influenzae* was accomplished as previously described (7). For selection of Rd-derived strains, antibiotics at the following concentrations were used: 8 μg/ml tetracycline (Tc), 20 μg/ml kanamycin (Km), and 10 μg/ml gentamicin (Gm). Selection of NT127 strains was conducted at the same antibiotic concentrations, except that Gm was used at 20 μg/ml.

Plasmid and *H. influenzae* strain construction. Plasmids and PCR products were constructed using standard molecular biology techniques (3). For complementation of mutants, DNA fragments were amplified by PCR and cloned between adjacent SapI restriction sites of the chromosomal delivery vector pXT10, which does not replicate in *H. influenzae* (90). The pXT10-based plasmids contain upstream (*xylF*) and downstream (*xylB*) homologous regions flanking the SapI cloning sites that allow precise fusion of genes of interest to the xylose-inducible *xylA* promoter, as previously described (90). Recombination at the xylose catabolic locus replaces the endogenous *xylA* gene with the cloned fragment and the *tetAR* Tc resistance (Tc^r) cassette. Plasmids were linearized by digestion with ApaLI and introduced into competent *H. influenzae*, and tetracycline-resistant recombinants were selected on sBHI agar plates. Double-crossover homologous recombination between the *H. influenzae* chromosome and constructs for each mutation in this study was confirmed by PCR with primers specific for sequences outside the inserted recombinant region. All genes introduced for complementation of mutations were verified by DNA sequence analysis and were identical to corresponding genes in *H. influenzae* Rd KW20 (GenBank accession number L42023, updated 21 December 2005).

For gene replacement of *nrfA* with a hemagglutinin (HA)-tagged reporter fusion construct, a 1,360-bp fragment corresponding to the region immediately 5′ of the *nrfA* translational start site was amplified by PCR using primers rtnrfARI and 5nrfAexp, with Rd genomic DNA as template. Primer 5nrfAexp, which includes a BssHII restriction site at its 5′ end, binds to the *nrfA* translational start codon and to the sequence directly upstream of the start codon. A second fragment was generated by PCR using primers 3nrfArevexp and lt1068nrfARI, which amplify the 725-bp product of a corresponding region downstream of *nrfA* starting at the termination codon. Primer 3nrfArevexp carries a StuI restriction site at its 5′ end and binds the *nrfA* stop codon, the *nrfB* start codon, and an *nrfB* sequence immediately downstream of the start codon. Primers 5nrfAexp and 3nrfArevexp include 17 bp of overlapping sequence, allowing them to be joined by overlap extension PCR using primers rtnrfARI and lt1068nrfARI. The resulting product was cloned into the EcoRI site of vector pBR322, generating vector pBNA1. The overlap region added by primers 5nrfAexp and 3nrfArevexp introduced BssHII and StuI cloning sites, allowing the *hel* gene coding sequence, which was amplified by PCR from Rd genomic DNA with primers 5′helORF and 3′helORF, to generate an 863-bp product incorporating a C-terminal HA epitope tag to be precisely cloned from the translation initiation to the termination codon of *nrfA*, resulting in vector pBNheltag. Finally, a fragment containing the *nrfA* promoter upstream of the *hel*-HA fusion gene flanked by sequences

identical to those flanking *nrfA* was generated by PCR with primers rtnrfARI and lt1068nrfARI, using pBNheltag as a template. The coding sequence of the fusion protein was verified by DNA sequence analysis. The resulting 3-kb product was used to transform Rd, and recombinants were screened nonselectively for loss of the ability to reduce nitrite, generating strain RdHA. This strain contains an HA epitope-tagged reporter controlled by the 5′ *nrfA* transcriptional and translational signals. In addition, the *H. influenzae* lipoprotein P4, encoded by the *hel* gene, is reported to be accessible to antibodies at the cell surface (35), potentially allowing detection applications that do not require cell lysis.

To complement the *nrfA* mutation in strain RdHA with the wild-type *nrfA*, introduced at the *xyl* locus, the *nrfA* gene coding sequence and upstream promoter elements were amplified from Rd by PCR using primers 5′nrfAsapI and 3′nrfAsapI to generate a 1.8-kb fragment. SapI restriction sites at the 5′ ends of the primers were used to clone the resulting fragments into the adjacent SapI sites of pXT10, resulting in vector pXTnrfAC. To generate strain RnrfAC, pXTnrfAC was digested with ApaLI, gel purified, and transformed into RdHA, followed by selection on sBHI containing Tc.

Strain Rfnr containing a transposon insertion mutation in *fnr* was generated by targeted in vitro transposon mutagenesis of the *fnr* locus with the *Himar1* derivative *magellan1*, followed by identification of the desired mutant by genetic footprinting as previously described (1). Rfnr contains a *magellan1* insertion ~170 bp from the 5′ end of the 770-bp *fnr* protein-coding region. To generate strain RHAfnr, a fragment consisting of 1.4 kb upstream of the transposon insertion in *fnr* to 1.3 kb downstream of the insertion was amplified by colony PCR using Rfnr as a template with primers 5′FNRupstreamCK and 3′FNRdownstreamCK. The resulting product was used to transform strain RdHA, resulting in strain RHAfnr. Kanamycin-resistant recombinants were selected on sBHI agar, and the resulting mutation was verified by PCR. To complement *fnr* mutants, a 1,286-bp PCR product containing the HI1425 gene and its putative promoter region upstream (468 bp 5′ of initiation codon ATG of *fnr* coding region) was amplified from Rd with primers FNRMlu1FORWARD and FNRMlu1REV. This PCR product was digested with MluI and cloned into the AscI site of pXT10. This plasmid, pXTfnrC, was introduced into the *xyl* locus of strain Rfnr or RHAfnr by double-crossover homologous recombination, and transformants were selected on sBHI agar containing Tc to create strains RfnrC and RHAfnrC, respectively.

A nonpolar, in-frame deletion of HI677 (*ytfE*) was created by replacing the protein-coding sequences with the *aacC1* gentamicin resistance cassette to create strain Rytfe by overlap extension PCR as follows. A 1,042-bp PCR product containing the 5′ flanking region of HI1677 was amplified from Rd with primers 1kb5′1677 and HI1677-5′out. A 1,044-bp PCR product containing the 3′ flanking region of HI1677 was amplified from Rd with primers HI1677-3′out and 1kb3′1677. A 536-bp fragment containing the *aacC1* gentamicin resistance cassette was amplified with primers aacC1-5ORF and aacC1-3ORF from pBSL182 (2). The 1,042-bp, 1,044-bp, and 536-bp products were combined in a PCR with primers 1kb5′1677 and 1kb3′1677. The resultant 2.6-kb product was introduced into Rd, and Gm^r transformants were selected on sBHI agar containing Gm to create strain Rytfe.

To generate RytfeC, the *ytfE* gene, including its native promoter, was amplified by PCR using Rd as template with primers 1677upcomp and 1677-3′sap, which introduce SapI restriction sites flanking *ytfE* and its predicted promoter region. The resulting 1.4-kb product was digested with SapI and cloned into SapI-digested pXT10. The resulting plasmid, pXTPytfeC, was linearized with ApaLI and transformed into Rytfe with selection for Tc^r.

The NT127 *xyl* locus differs from that of Rd in that it contains *xylAB* but lacks the *xylHGF* genes. To facilitate complementation of mutants by introduction of genes at the NT127 *xyl* locus with our exchange vector, pXT10, the *xylHGF* genes were cloned into a Km^r-marked derivative of pXT10 and introduced into NT127 at the *xyl* locus as they are arranged in Rd, providing sequences for homologous recombination with pXT10 and its derivatives. The resulting strain was transformed with pXT10 or the *ytfE* mutation to generate strains NTV ("vector only" strain) and NytfE (*ytfE* mutant), respectively. To generate strain NytfeV containing a *ytfE* mutation and "empty vector" sequences, the *ytfE* mutation was amplified with primers 3′HI1676 and 3′ytfe.down using RytfE as a template. The resulting 3-kb PCR product was transformed into the parental strain NTV, and transformants selected for Gm^r under anaerobic conditions. Sequence analysis verified that the *ytfE*-flanking regions of Rd and NT127 strains are identical. For complementation of the *ytfE* mutation in the NT127 background, primers 5′ytfEc and 1677-3′sap were used to amplify the *ytfE* protein-coding sequence, followed by digestion with SapI and cloning into the SapI sites of pXT10, placing *ytfE* under the control of the *xylA* promoter. The resulting plasmid was transformed into Nytfe with selection for Tc^r. RfnrV, RHAfnrV, and RytfeV were generated by transforming an ApaLI-digested linear pXT10 fragment into Rfnr, RHAfnr, and RytfE, respectively, with selection for Tc^r.

Strains and plasmids are listed in Table 1, and primers are listed in Table 2.

Assay for nitrite reduction. To measure reduction of nitrite by *H. influenzae*, triplicate cultures of MI_c in 25-ml DeLong flasks (Bellco) were inoculated from standing overnight cultures to a starting optical density at 600 nm (OD_{600}) of 0.02. To minimize aeration, flasks were filled to the necks to a 30-ml total volume (25-ml markings denote the typical maximum culture volume, but flasks hold 30 ml when filled to the neck). The resulting cultures were incubated at 35°C with shaking at 250 rpm. When cultures reached mid-log phase (OD_{600} of 0.4 to 0.5), 7.5 OD_{600} equivalents of cells were harvested by centrifugation at 5,000 × *g* for 5 min and resuspended in 25 ml MI_c supplemented with $NaNO_2$ to a starting concentration of 0.25 mM in 25-ml Erlenmeyer flasks. These cultures were then incubated at 35°C with shaking at 200 rpm, and 200-μl aliquots were removed at selected time points and stored on ice in a 96-well flat-bottom dish until completion of the assay. The 96-well dish was centrifuged at 5,000 × *g* at 4°C for 5 min to pellet cells. The presence of nitrite in cell-free-supernatant medium was quantified by using the Griess reaction. Supernatants were incubated with 50 μl of a solution of 5% phosphoric acid, 1% sulfanilamide for 5 min, followed by the addition of 50 μl of aqueous 0.1% *N-N*-1-napthylethylenediamine dihydrochloride for 5 min. Subsequently, the OD_{540} was recorded with a $Versa_{Max}$ microplate reader (Molecular Devices, Sunnyvale, CA) and nitrite concentrations were calculated using a standard curve. For assays of nitrite reduction after a 24-hour incubation, cultures were inoculated in 200 μl sBHI supplemented with $NaNO_2$ at a concentration of 1.0 mM to a starting OD_{600} of 0.01 in a 96-well dish. Cultures were incubated for 24 h at 35°C, and remaining nitrite concentrations in the supernatants determined as described above.

Western blotting. To determine the role of FNR in reporter expression, strains were grown to mid-log phase in 30 ml of sBHI in 25-ml DeLong flasks (filled to the neck) and were lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled for 5 min, centrifuged for 1 min at 16,000 × *g*, and then resolved on 12% SDS–PAGE gels (0.4 OD_{600} equivalents per lane). Gels were electrotransferred to Immobilon-P membranes (Millipore Corporation, Billerica, MA). Aliquots of each sample were analyzed in parallel on a gel stained with Coomassie blue (3) to verify equal sample concentrations. Membranes were blocked with 1% Tris-buffered saline–0.1% Tween (TBS-T) supplemented with 1% instant milk, probed with a 1:1,000 dilution of anti-HA.11 (Covance, Berkeley, CA) for 60 min, washed with TBS-T, probed with a 1:5,000 dilution of secondary antibody goat anti-mouse immunoglobulin G-horseradish peroxidase conjugate (Upstate, Lake Placid, NY) for 30 min in TBS-T, and washed with TBS-T. The *Pnrf*-HA-encoded reporter protein was then detected with a West-One Western blot detection system (iNtRON Biotechnology).

To monitor the expression of the *Pnrf-hel*-HA fusion under a range of aeration conditions, volumes of 7.5, 10, 30, 45, or 60 ml (filled to the neck for 60 ml) of sBHI in 50-ml DeLong flasks (nominal volume) were inoculated in triplicate from overnight cultures at a starting OD_{600} of 0.005 and incubated at 35°C with shaking at 250 rpm. When cultures reached mid-log phase, 0.25 OD_{600} equivalents of each sample were centrifuged for 5 min at 16,000 × *g*, the supernatant was removed, and the resulting pellets were resuspended in Hank's balanced salt solution. Subsequently, samples were lysed in SDS-PAGE sample buffer and proteins were separated by 12% SDS–PAGE. The upper portion of the gel (~98 to 64 kDa) was removed and stained with Coomassie blue to verify equal loading. The lower portion of the gel was electrotransferred to Immobilon-P and analyzed via Western blotting as described above, except that blocking was conducted with TBS-T containing 2% instant milk and secondary probing with goat anti-mouse immunoglobulin G ReserveAP phosphatase-labeled antibody (KPL, Gaithersburg, MD) at a 1:30,000 dilution. The *Pnrf*-HA-encoded reporter protein was then detected with CDP-Star detection reagent (GE Healthcare). Densitometry was performed on the original scanned image using ImageJ (National Institutes of Health, Bethesda, MD), and local background was measured and subtracted for each individual lane. The contrast of the displayed blot was adjusted (applied to entire image) using Adobe Photoshop 10.0.1 (Adobe Systems, Incorporated). In parallel with sample collection, for each of the three cultures per condition, percent oxygen saturation levels were measured with a Clark-type oxygen probe (model DO-166; Lazar Research labs) calibrated according to the manufacturer's instructions.

RNS sensitivity assays. To determine the sensitivity of *fnr*, *nrfA*, and *ytfE* mutants to RNS, strains were cultured overnight in an anaerobic chamber with BBL GasPak Plus generators and the cultures were used to inoculate 5 ml of sBHI broth in 18- by 150-mm culture tubes to an initial OD_{600} of 0.02 and then incubated in an anaerobic chamber at 35°C with shaking at 120 rpm until they reached mid-log phase. Triplicate 200-μl aliquots of each culture were incubated in sBHI in 96-well flat-bottom microtiter plates overnight in an anaerobic cham-

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype, description, and/or relevant features	Reference or source
Strains		
Rd	BA042; *H. influenzae* capsule-deficient type d	89
RdHA	Rd Δ*nrfA*::*hel-ha*; *nrfA* deletion-replacement strain containing a C-terminal HA epitope fusion to the *hel* gene driven by the *nrfA* promoter, replacing *nrfA*	This study
RnrfAC	RdHA $xylA_{\Delta 4\text{-}804}$::*nrfA*; *nrfA* mutant complemented with *nrfA* expressed via the *nrfA* promoter from pXTnrfC, replacing *xylA*	This study
Rfnr	Rd *fnr′*::*nptII*; *fnr* mutant with Km^r transposon insertion in *fnr*	This study
RfnrV	Rfnr $xylA_{\Delta 4\text{-}804}$::*tetAR*; *fnr* mutant carrying empty Tet^r vector sequence from pXT10, replacing *xylA*	This study
RfnrC	Rfnr $xylA_{\Delta 4\text{-}804}$::*fnr*; *fnr* mutant complemented with *fnr* expressed via the *fnr* promoter from pXTfnrC, replacing *xylA*	This study
RHAfnr	RdHA *fnr′*::*nptII*; *Pnrf-hel*-HA reporter strain and *fnr* mutant with Km^r transposon insertion in *fnr*	This study
RHAfnrV	RHAfnr $xylA_{\Delta 4\text{-}804}$::*tetAR*; *Pnrf-hel*-HA reporter strain and *fnr* mutant carrying empty Tet^r vector sequence from pXT10, replacing *xylA*	This study
RHAfnrC	RHAfnr $xylA_{\Delta 4\text{-}804}$::*fnr*; *Pnrf-hel*-HA reporter strain and *fnr* mutant complemented with *fnr* expressed via the *fnr* promoter from pXTfnrC, replacing *xylA*	This study
RV	Rd $xylA_{\Delta 4\text{-}804}$::*tetAR*; parental Rd carrying a deletion of nonessential phase-variable repeats in *lic1A* and empty Tet^r vector sequence from pXT10, replacing *xylA*	89
RytfeV	Rd Δ*ytfE*::*aacCI* $xylA_{\Delta 4\text{-}804}$::*tetAR*; *ytfE* mutant carrying empty Tet^r vector sequence from pXT10, replacing *xylA*	This study
RytfeC	Rd Δ*ytfE*::*aacCI* $xylA_{\Delta 4\text{-}804}$::*ytfE*; *ytfE* mutant complemented with *ytfE* expressed via *ytfE* promoter from pXTPytfeC, replacing *xylA*	This study
NT127	Nontypeable *H. influenzae* clinical isolate	R. Husson
NTV	NT127 $xylA_{\Delta 4\text{-}804}$::*tetAR*; contains Tet^r vector sequence from pXT10, replacing *xylA*	This study
NytfeV	NT127 Δ*ytfE*::*aacCI* $xylA_{\Delta 4\text{-}804}$::*tetAR*; *ytfE* mutant carrying empty Tet^r vector sequence from pXT10 replacing *xylA*	This study
NytfeC	NT127 Δ*ytfE*::*aacCI*, $xylA_{\Delta 4\text{-}804}$::*ytfE*; *ytfE* mutant complemented with *ytfE* expressed via the *xylA* promoter from pXTytfeC replacing *xylA*	This study
Plasmids		
pXT10	Delivery vector for chromosomal expression at the *H. influenzae* xylose locus, containing *xylF*, *xylB*, $xylA_{\Delta 4\text{-}804}$, and the *tetAR* tetracycline resistance cassette	90
pXTnrfC	pXT10 carrying *nrfA* expressed from the *nrfA* promoter	This study
pXTfnrC	pXT10 carrying *fnr* expressed from the *fnr* promoter	This study
pXTPytfeC	pXT10 carrying *ytfE* expressed from the *ytfE* promoter	This study
pXTytfeC	pXT10 carrying *ytfE* expressed from the *xylA* promoter	This study
pBNheltag	pBR322 vector carrying the *Pnrf-hel*-HA reporter construct	This study

ber in the presence or absence of RNS donors and then diluted and plated on sBHI agar for enumeration of CFU. For acidified sodium nitrite (ASN) exposure, 5×10^5 cells were incubated in sBHI adjusted to pH 6.5 in the presence or absence of a final concentration of 10 mM $NaNO_2$. GSNO (Sigma-Aldrich St. Louis, MO) was used at a final concentration of 5 mM with 5×10^4 cells in sBHI at pH 7.5.

To evaluate the dynamics of killing by ASN, time-kill curves were generated. Anaerobic overnight cultures of strains were used to inoculate sBHI adjusted to pH 5.5 for an initial OD_{600} of 0.01. Cells were then inoculated in triplicate 200-μl cultures into 96-well flat-bottom microtiter plates and treated with the addition of $NaNO_2$ to a final concentration of 15 mM or not treated. For low-oxygen conditions, dishes were incubated at 35°C in individual BD GasPak EZ anaerobe gas-generating pouches. For higher-oxygen conditions, microtiter plates were incubated in ambient air (35°C). Bacteria were diluted and plated for enumeration of CFU at various time points throughout the assay.

Generation of FNR binding consensus sequence. Putative FNR binding sites were identified upstream of *nrfA*, *napA*, *dmsA*, and *ytfE* based on previously reported candidate FNR binding sequences for *E. coli* and *H. influenzae* (31, 69, 83). DNA sequencing verified that promoter regions in Rd BA03 were identical to those of Rd KW20 (GenBank accession number L42023, updated 21 December 2005). Putative binding sequences were used to generate a sequence logo diagram (http://weblogo.berkeley.edu/logo.cgi/).

Detection of mRNA by reverse-transcription quantitative PCR (RT-qPCR). To quantify mRNA of FNR-controlled genes, total RNA was isolated from 40-ml cultures of Rd, RfnrV, and RfnrC grown to mid-log phase in anaerobic chambers by using TRIzol reagent (Invitrogen). Isolation of intact RNA for each sample was verified by agarose gel electrophoresis with ethidium bromide staining. RNA was quantified by measuring UV absorption at 260 and 280 nm with a SmartSpec plus spectrophotometer (Bio-Rad, Hercules, CA). Samples were then treated with DNase I (Ambion, Austin, TX) and phenol extracted. To verify the absence of DNA contamination, 1 μg of the RNA sample was used as template for real-time PCR containing primers HI1069-5′ and HI1069-3′ (89) and iQ SYBR green supermix (Bio-Rad Laboratories, Hercules, CA) with fluorescence in real-time PCR measured with a DNA Engine Opticon II system (MJ Research, Waltham, MA). No DNA contamination was detected with a limit of detection equal to 0.001 ng, as determined via a genomic DNA standard curve. The RNA (5 μg total) samples served as a template for cDNA synthesis using random primers (New England Biolabs, Beverley, MA) and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). PCR was performed using iQ SYBR green Supermix, with fluorescence measured in real-time PCR by using the DNA Engine Opticon II system. Amounts of 1/10 of the cDNA reactions were used as a template for PCR with primers specific for *nrfA* (HI1069-5′ and HI1069-3′; 89), *dmsA* (HI1047-5′ and HI1047-3′; 89), *napA* (5′napA-RT and 3′napA-RT), *ytfE* (5′ytfe-RT and 3′ytfe-RT), and *rpoA* (HI0802-5′ and HI0802-3′; 91). Approximate transcript values were calculated from a standard curve generated with each primer set. Relative expression was standardized by determining the ratio of the transcript value of each gene to the *rpoA* transcript value. Control reactions were performed in parallel with mock cDNA reactions generated without reverse transcriptase to verify specific amplification, and the presence of single products of correct approximate sizes was verified by agarose gel electrophoresis and ethidium bromide staining. Real-time cycler conditions were as described previously (91).

Exposure of *H. influenzae* to macrophages. Bone marrow-derived macrophages (BMM) were generated by differentiating C57BL/6J bone marrow cells in a

TABLE 2. Oligonucleotides used in this study

Primer	Sequence[a]	Feature(s) and/or sequence amplified
rtnrfARI	5′-ggaattcAGTTGGTGATTGCGCTG-3′	EcoRI restriction site is underlined; primer binds bp −1357 to −1341 upstream of *nrfA* start codon
5nrfAexp	5′-ctagatctatcctggcgcgc**CAT**TCAAGCGTAAGATACCA-3′	BssHII restriction site is underlined, and *nrfA* start codon position is in boldface
3nrfArevexp	5′-cgccaggatagatctaggcct**TAA**ATGAAAAATGCACCG-3′	StuI restriction site is underlined, and *nrfA* stop codon is in boldface
lt1068nrfARI	5′-ggaattcATAGTTTATCCTTTTCTTCG-3′	EcoRI restriction site is underlined; primer binds bp +721 to +702 downstream of last base of *nrfA* stop codon
5′helORF	5′-aaaatggcgcgctaagaacataat**ATG**AAAACAACGTTAAAAATGAC-3′	BssHII restriction site is underlined, and start codon of *hel* is in boldface
3′helORF	5′-**tta**tgcataatctggcacatcatattgataTTTACCATCCCAAGCTTGTACTGC-3′	Sequence complementary to HA-epitope coding sequence is underlined, and position of stop codon of *hel*-HA fusion is in boldface
5′nrfAsapI	5′-ccctagctagctcttcT**ATG**ATTGAGGAATTGCGCGTAAG-3′	SapI restriction site is underlined, and *nrfA* start codon is in boldface
3′nrfAsapI	5′-ccacatcgtgctcttcC**TTA**TTTTTGTGCTGGTGGATCAACTAATAAGC-3′	SapI restriction site is underlined, and position of the *nrfA* stop codon is in boldface
5′FNRupstreamCK	5′-GCGGCAAGAATGGCAGCGTTATCC-3′	Primer binds bp −1663 to −1640 upstream of *fnr* start codon
3′FNRdownstreamCK	5′-ACCTGTGCGTCCCACTGTGCC-3′	Primer binds bp +954 to +934 downstream of last base of *fnr* stop codon
FNRMlu1FORWARD	5′-gcgacgcgtAGGGGTTTATGGCTGATGTGAAC-3′	MluI restriction site is underlined; primer binds bp −468 to −446 upstream of *fnr* start codon
FNRMlu1REVERSE	5′-cgcacgcgtAAAAAGGCGTGATATTCAC-3′	MluI restriction site is underlined; primer binds bp +27 to +9 downstream of last base of *fnr* stop codon
1kb5′1677	5′-ATTGGGCGATCTTTAACCCAAGCGA-3′	Primer binds bp −1025 to −1001 upstream of *ytfE* start codon
HI1677-5′out	5′-gctgctgcgtaa**CAT**AAATCACTCCTATATTGATTAAGT-3′	Complementary sequence directly upstream of *ytfE* start codon; *ytfE* start codon is in boldface
HI1677-3′out	5′-agtaccgccacc**TAA**TTTAATATTCGTCAAAAAATAACCG-3′	Sequence directly downstream of *ytfE* stop codon; *ytfE* stop codon is in boldface
1kb3′1677	5′-ATATCTAGAACATAATCTGCATGG-3′	Primer binds bp +1676 to +1699 upstream of *ytfE* start codon
aacC1-5ORF	5′-**ATG**TTACGCAGCAGCAACGATGTTACGCAGCAGG-3′	Start codon of *aacC1* is in boldface
aacC1-3ORF	5′-**TTA**GGTGGCGGTACTTGGGTCGAT-3′	Stop codon for *aacC1* is in boldface
1677upcomp	5′-aaagatctgctcttcAATGGGCTTGAGCAAGATGGGTAATTTC-3′	SapI site is underlined; primer binds bp −646 to −619 upstream of *ytfE* start codon
1677-3′sap	5′-aaagatctgctcttcT**TTA**ATTAAAGTGCGGTTATTTTTTGAC-3′	SapI site is underlined, and position of *ytfE* stop codon is in boldface
3′HI1676	5′-GCCACCAATAGAAGCTAAATG-3′	Primer binds bp −1455 to −1435 upstream of *ytfE* start codon
3′ytfe.down	5′-GCGCAAGATTATTTGGACAGA-3′	Primer binds bp +1065 to +1045 downstream of last base of *ytfE* stop codon
5′ytfEc	5′-tttgctcttcT**ATG**TCTTTTGCCCAACAAAAAC-3′	Sequence directly downstream of the *ytfE* start codon, which is in boldface; SapI site is underlined
5′ytfE-RT	5′-CATGGTGATCGTGATGACTGTCCT-3′	Within *ytfE* coding sequence
3′ytfE-RT	5′-CCAACTGAAACAAGCATCTGCTGG-3′	Within *ytfE* coding sequence
5′napA-RT	5′-GCACCCATTAGATGCACAAGCAC-3′	Within *napA* coding sequence
3′napA-RT	5′-ATTGGGTCTGTCGCATCTAGTGTTA-3′	Within *napA* coding sequence

[a] Uppercase letters indicate sequences identical to the *H. influenzae* genome. Lowercase letters indicate sequences added to allow generation of recombinant DNA constructs.

complete bone marrow medium (Dulbecco's modified Eagle's medium, 10% heat-inactivated fetal calf serum [Invitrogen], 100 U/ml penicillin [Invitrogen], 100 μg/ml streptomycin [Invitrogen], and 10% L929 fibroblast-conditioned medium as a source of macrophage colony-stimulating factor) for 6 days in 10-cm petri dishes (VWR). Prior to exposure to bacteria, BMM were washed in antibiotic-free bone marrow medium and incubated with or without 100 U/ml IFN-γ (Peprotech) for 24 h, 100 μM N_ω-nitro-L-arginine methyl ester HCl (L-NAME) for 24 h, or 100 ng/ml LPS for 2 h.

To evaluate the survival of *fnr* and *ytfE* mutants in the presence of BMM, strains were cultured anaerobically as described above for the RNS sensitivity assay. Cultures were then diluted in Dulbecco's modified Eagle's medium, and 2.5×10^5 bacterial cells were aliquoted into each well of a 96-well dish containing 5×10^4 adherent BMM per well. The 96-well dish was centrifuged for 5 min at $200 \times g$, sealed in a BD GasPak EZ anaerobe gas-generating pouch, and incubated for 0 or 30 min at 36°C, 5% CO_2. Following incubation, wells were treated with saponin to a final concentration of 0.1%, a concentration that does not influence bacterial viability, and samples were triturated vigorously by pipetting to lyse macrophages and release bacteria. Samples were diluted and plated for CFU enumeration. This assay measures the ability of BMM to limit survival of *H. influenzae* by killing or growth inhibition during a transient incubation. Rel-

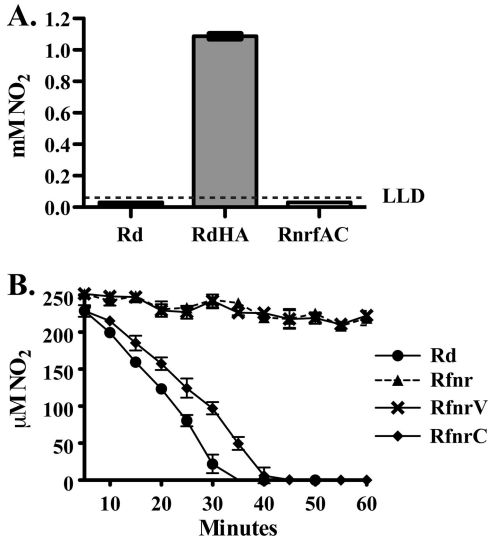

FIG. 1. Effects of *fnr* and *nrfA* mutations on nitrite reductase activity. (A) Strains Rd, RdHA, and RnrfAC were cultured in sBHI with 1 mM $NaNO_2$. Supernatants were assayed for remaining nitrite after 24 h. Lower level of detection (LLD) was 3 μM. Error bars indicate standard deviations of the mean values obtained in the three independent experiments. (B) Strains Rd, Rfnr, RfnrV, and RfnrC, grown microaerobically to mid-log phase, were normalized to a density of 0.3 OD_{600} in MI_C medium. After addition of $NaNO_2$ to an initial concentration of 250 μM, nitrite concentrations in supernatants were monitored at 5-min intervals. Mean nitrite levels for three independent cultures of each strain monitored in parallel are shown. Error bars represent standard deviation values, which were less than 5% of the means and are not visible for all points.

ative survival is calculated as the ratio of the number of bacteria recovered after treatment with macrophages compared to the number of bacteria recovered from parallel cultures incubated in medium alone.

RESULTS

FNR regulates anaerobic nitrite reductase activity in *H. influenzae*. As a starting point for investigating whether FNR of *H. influenzae* participates in the regulation of defense against RNS, including NO, we evaluated the role of FNR as an anaerobically active regulator of nitrite reductase in *H. influenzae*. The *H. influenzae* genome contains a single putative nitrite reductase locus that is comprised of the *nrfABCD* genes (57%, 50%, 64%, and 48% amino acid identity, respectively, to their putative homologs in *E. coli*) (18, 29, 37). To verify the predicted role of *nrfA* in reduction of nitrite by *H. influenzae*, we compared the parental strain Rd, a mutant containing a nonpolar deletion of *nrfA* (strain RdHA), and a complemented strain in which *nrfA* was provided in *trans* to the deletion (RnrfAC) for their ability to reduce nitrite within 24 h in the presence of 1 mM nitrite under microaerobic conditions (Fig. 1A). At 24 h, nitrite levels from cultures of Rd and the *nrfA*-complemented strains were below the limit of detection. In contrast, the *nrfA* mutant was incapable of reducing nitrite and the concentrations at the end of the assay remained equivalent to the initial values. These results indicate that *nrfA* is required for nitrite reduction by *H. influenzae* in this assay.

To determine whether FNR is required for nitrite reduction, we compared microaerobically grown cultures of the parental strain (Rd); an *fnr* disruption mutant (Rfnr); an *fnr* mutant carrying vector sequences in the *xyl* locus (RfnrV); and an *fnr* mutant complemented with a wild-type copy of the *fnr* gene, provided at the *xyl* locus (RfnrC), for the ability to deplete nitrite from culture supernatants during a 1-h assay (Fig. 1B). Nitrite was steadily depleted from supernatants of Rd and RfnrC cultures during the first 45 min, at which time residual nitrite became undetectable. In contrast, the concentrations of nitrite did not detectably decrease in culture supernatants of the *fnr* mutants (Rfnr and RfnrV), providing evidence that FNR is required for appreciable activity or expression of nitrite reductase under these conditions.

FNR controls *nrfA* expression in response to changes in culture aeration conditions. To facilitate studies of nitrite reductase gene expression, we utilized strain RdHA, a *nrfA* deletion mutant which also serves as a reporter strain, as it contains the *nrfA* promoter precisely fused to the *hel* gene encoding *H. influenzae* protein P4. A fusion of an influenza virus HA epitope to the C terminus of P4 allows immunological detection of the reporter fusion with monoclonal antibody HA.11. To preserve the native expression elements controlling *nrfA*, the reporter was designed to replace the native *nrfA* by homologous recombination at its endogenous locus. A markerless allelic exchange procedure (see Materials and Methods) was used to introduce this construct into *H. influenzae* Rd to create strain RdHA.

This reporter strain was used to assess whether FNR is required for expression from the *nrfA* promoter. RdHA derivatives were generated (Table 1) that contained the *fnr* mutation (RHAfnr); the *fnr* mutation and "empty vector" sequences at the *xyl* locus (RHAfnrV); or the *fnr* mutation and a wild-type copy of *fnr*, provided at the *xyl* locus (RHAfnrC). These strains were assayed by Western blotting for levels of the fusion protein after growth under low-oxygen conditions predicted to activate FNR (Fig. 2A). The fusion protein was readily detectable as a band migrating at ~29 kDa in lysates from strains RdHA and RHAfnrC that contain *fnr*, consistent with the predicted 29.5-kDa molecular mass of the mature fusion protein. As expected, no reactivity with monoclonal antibody HA.11 was detected in the parental strain Rd, which lacks the fusion construct. Lysates from *fnr* mutant strains RHAfnr and RHAfnrV contained no detectable fusion protein, indicating that FNR is required for expression of the fusion from the *nrfA* promoter.

The dependence of the *Pnrf-hel*-HA reporter fusion on FNR for its expression suggested that this system could be used to investigate the aeration conditions influencing FNR activity in *H. influenzae*. A range of oxygen conditions was generated by varying the volume of culture medium in a series of flasks of the same size. As the culture volume increases, the liquid/air interface decreases, thus yielding lower rates of oxygen supply (21). Genomic transcription-profiling experiments with *H. influenzae* have shown that this range of conditions modulates the expression of *nrfA* and other genes predicted to be controlled by FNR (89). The effect of these conditions on oxygen levels was verified by measuring dissolved oxygen levels in cultures at the time of sample harvesting (see Materials and Methods). In parallel, fusion protein levels in cells from the same cultures were assessed by Western blot analysis. Increased expression of *Pnrf-hel*-HA was observed in samples

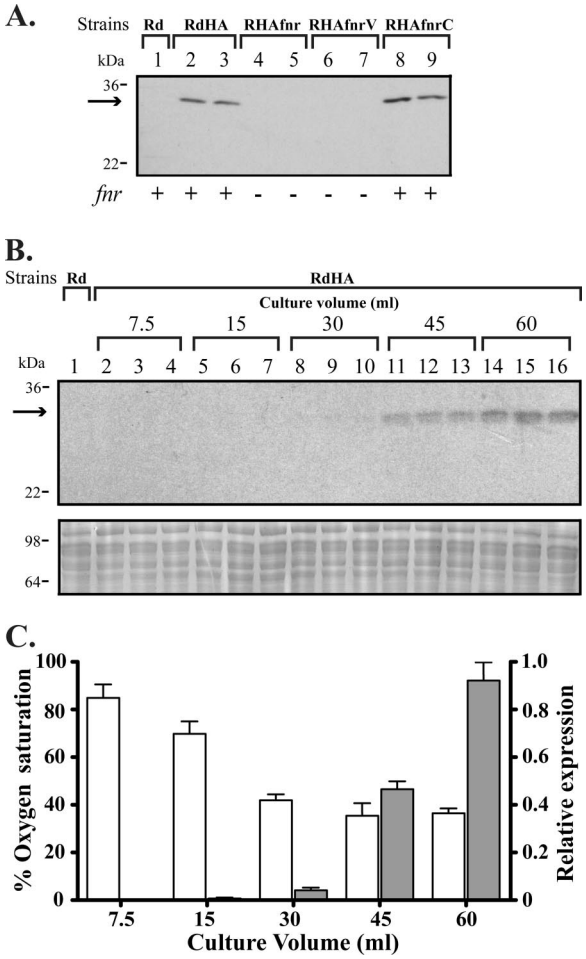

FIG. 2. Modulation of *nrfA* reporter fusion expression by FNR and oxygen availability. (A) Whole-cell lysates from duplicate independent microaerobic cultures of Rd, RdHA, RHAfnr, RHAfnrV, and RHAfnrC were resolved with SDS-PAGE and analyzed by anti-HA immunoblotting. Arrow indicates the band migrating at ~29 kDa, corresponding to the predicted size of the *Pnrf-hel*-HA-encoded reporter fusion protein. +, present; −, absent. (B) Whole-cell lysates of Rd and RdHA, as indicated above the panels, grown in triplicate to mid-log phase at the indicated range of culture medium volumes, were resolved via SDS-PAGE and analyzed by anti-HA immunoblotting (top). Arrow is as described for panel A. Equal sample loading was verified by Coomassie blue staining of the upper portion of the gel (bottom). (C) Percent oxygen saturation (white bars) determined in each of the triplicate cultures for each condition at the time of sampling is shown. Relative expression represents the intensity value of each band divided by the highest intensity value obtained in the experiment as determined by densitometry performed on the Western blot shown in panel B. Differences in mean expression levels between the 30-, 45-, and 60-ml cultures were statistically significant ($P < 0.001$) as determined by analysis of variance with Tukey's multiple comparison test. Error bars show standard deviations.

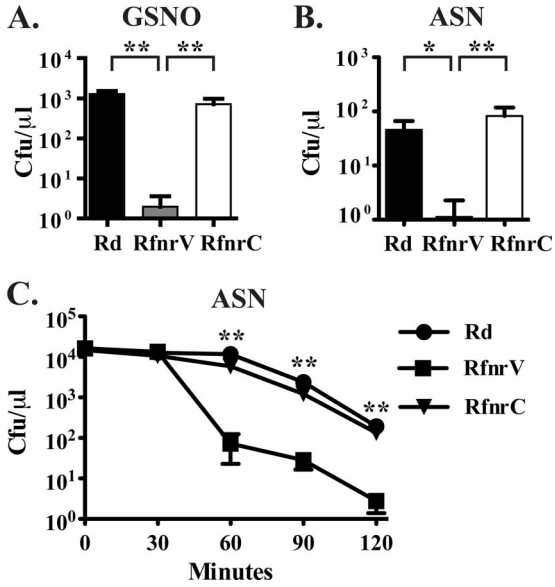

FIG. 3. Role of *fnr* in resistance of *H. influenzae* to NO donors GSNO and ASN. *H. influenzae* strains were incubated in sBHI containing either 5 mM GSNO at pH 7.5 for 14 h (A), 10 mM $NaNO_2$ at pH 6.5 for 14 h (B), or 15 mM $NaNO_2$ at pH 5.5 (C) for the times indicated, and viability was assessed by CFU determination. Values are the means ± standard deviations of the results of three independent experiments. Analysis of variance with Tukey's multiple comparison test detected statistically significant differences between results for Rd and RfnrV and between results for RfnrC and RfnrV (*, $P < 0.01$; **, $P < 0.001$).

with decreased oxygen levels (Fig. 2B and C) Although the intracellular oxygen concentration sensed by FNR is related to a complex function of the oxygen consumption rate and other factors not measured here, the results demonstrate that activation of the *nrfA* promoter occurs under low-oxygen conditions but does not require complete anaerobiosis. Together, these results indicate that FNR of *H. influenzae* increases *nrfA* expression in response to conditions of decreased culture aeration.

FNR is required for resistance to NO donors under low-oxygen conditions. In other bacteria, FNR has been implicated in both negative and positive control of genes for nitrosative stress defense. To investigate the potential role of FNR in NO resistance by *H. influenzae*, we evaluated the sensitivity of the *fnr* mutant to two NO donors, GSNO and nitrite. GSNO acts by slow release of NO, causing bacterial damage through protein transnitrosation, a covalent transfer of a NO group to free thiol groups (77). Under acidic conditions, nitrite is a NO donor, commonly called ASN (73). Strains Rd, RfnrV, and RfnrC were compared for survival after challenge with GSNO under anaerobic conditions. Recovery of CFU of the *fnr* mutant strain RfnrV was decreased by 600-fold relative to the rate of recovery of parental strain Rd, and complementation with *fnr* in strain RfnrC restored resistance to wild-type levels (Fig. 3A). Treatment of cultures with GSNO in the presence of oxygen resulted in no measurable growth inhibition for all strains (data not shown), consistent with the instability of NO in the presence of oxygen, particularly in biological systems (34). Exposure to ASN yielded results with the *fnr* mutant RfnrV that were comparable to those obtained with GSNO, exhibiting a 40-fold decrease in recoverable CFU compared to the number of CFU recovered for the parental strain, Rd; complementation with the wild-type *fnr* in strain RfnrC restored resistance to parental levels (Fig. 3B).

ASN releases NO rapidly, providing the opportunity to monitor decreases in CFU over a relatively short period of time

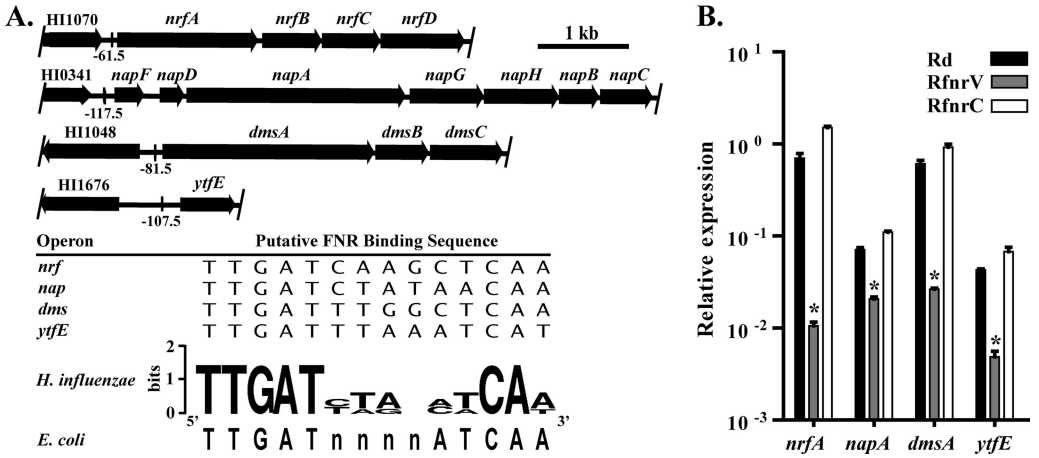

FIG. 4. FNR-mediated transcriptional regulation in *H. influenzae*. (A) Genetic organization of *nrf*, *nap*, *dms*, and *ytfE* loci showing positions of potential FNR binding sites relative to the predicted translational start sites of the designated genes. A sequence logo shows conserved residues within the putative FNR binding sites of *H. influenzae* in comparison to the *E. coli* FNR binding site consensus (24, 55). (B) Total RNA was extracted from mid-log-phase cultures of Rd, RfnrV, and RfnrC cultured in triplicate under low-oxygen conditions, and levels of expression of *nrfA*, *napA*, *dmsA*, and *ytfE* were examined with RT-qPCR. Transcript levels were normalized to *rpoA* expression level. Values are the means ± standard deviations of the results of three independent experiments. Statistically significant differences were determined by analysis of variance with Tukey's multiple comparison test (*, $P < 0.01$).

(73). Cultures of strains Rd, RfnrV, and RfnrC were incubated in acidified medium under oxygen-depleted conditions in the presence or absence of ASN and monitored for recoverable CFU for 120 min. The cultures incubated in this acidified medium in the absence of ASN exhibited negligible growth or loss of viability over the course of the assay (data not shown), indicating that decreases in recoverable CFU when ASN is present are not a direct result of pH sensitivity. In contrast, in the presence of ASN, the *fnr* mutant (RfnrV) exhibited a dramatic decrease in CFU by 60 min (Fig. 3C), with CFU dropping by 161- and 81-fold relative to CFU of the parental and complemented strains, respectively, which exhibited only 1.4- and 2.5-fold decreases in survival at this time point relative to their starting CFU. Rd and RfnrC were affected by ASN at later times, exhibiting ~100-fold reductions relative to their starting CFU at 120 min, yet the *fnr* mutant remained significantly more sensitive than parental and complemented strains, by 69- and 28-fold, respectively (Fig. 3C). As was observed with GSNO, cultures exposed to ASN under oxygen-rich conditions exhibited no appreciable decreases in CFU (data not shown), consistent with the rapid oxidation of NO to nitrite expected to occur under these conditions (87). Together, these results indicate that FNR is required to promote resistance of *H. influenzae* to killing by NO donors under low-oxygen conditions.

Transcriptional regulation of genes with potential roles in NO defense by FNR. To identify mechanisms by which FNR could participate in defense against RNS, we examined FNR-mediated transcriptional control of a selected set of genes with potential FNR binding sites in their putative promoters (Fig. 4A). Included in the analysis were genes with potential roles in NO resistance and, for comparison, several genes predicted to be members of the FNR regulon based on their regulation in other bacteria. Based on results obtained with the *Pnrf-hel*-HA reporter fusion in nitrite reductase assays and Western blotting (Fig. 1 and 2), FNR was expected to positively control transcription of the *nrfA* gene. The *dmsA* operon encodes a putative homolog of dimethyl sulfoxide reductase, which is also positively regulated by FNR in *E. coli* (15) and was included to further evaluate similarities between the FNR regulon of *H. influenzae* and those of other bacteria. The predicted *H. influenzae napA* gene product has 74% amino acid identity to the product of the FNR-regulated *napA* that is required for nitrate reductase activity in *E. coli* (80, 81), and a transposon insertion in *H. influenzae napA* abrogates nitrate reductase activity (J. C. Harrington, unpublished results). The *ytfE* of *E. coli* encodes a di-iron protein that repairs nitrosative damage (42). HI1677, the putative homolog of *ytfE* in *H. influenzae*, encodes a predicted protein with 57% amino acid identity to *E. coli* YtfE. The presence of a potential FNR binding site in the promoter region of *ytfE* in *H. influenzae* suggested that it could play a role in FNR-mediated RNS resistance.

To evaluate the potential regulation of these genes by FNR, total RNA was obtained from cultures of Rd, RfnrV, and RfnrC grown under low-oxygen conditions, and RT-qPCR was used to measure the transcript levels of each gene, normalized to the transcript level of the housekeeping gene *rpoA*. The transcript abundance of each of the candidate FNR-regulated genes was decreased in the *fnr* mutant and restored to wild-type levels in the complemented strain (Fig. 4B). Specifically, a 70-fold decrease in *nrfA* mRNA levels was observed in the *fnr* mutant relative to the level in the parent strain, consistent with the pronounced effect of the *fnr* mutation on the expression of the *Pnrf-hel*-HA reporter fusion (Fig. 2B). The *dmsA* transcript levels were similarly affected, exhibiting a decrease of 23-fold in the *fnr* mutant. The *napA* gene was moderately influenced, and its expression was decreased by threefold in the *fnr* mutant. This result with *napA* is consistent with a partial decrease in nitrate reductase activity in the *fnr* mutant (data not shown). The mRNA levels of *ytfE* decreased by 8.8-fold in the *fnr* mutant relative to the level in the wild type. Therefore, unlike *E. coli ytfE*, which is negatively regulated by FNR (41), *ytfE* is positively controlled by FNR in *H. influenzae*.

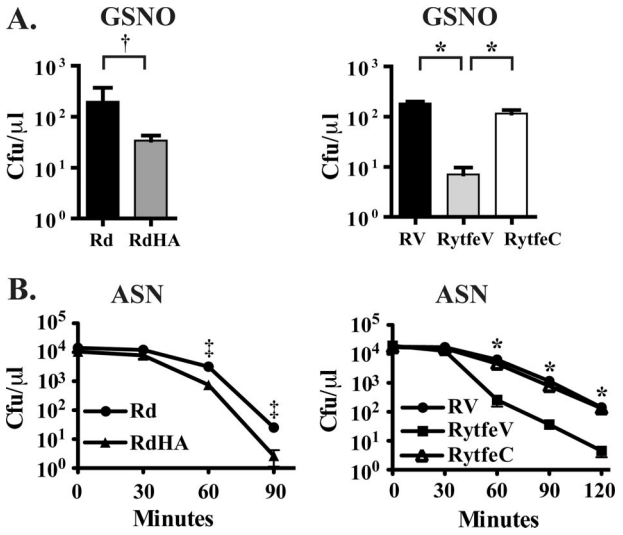

FIG. 5. Effect of *nrfA* and *ytfE* on susceptibility to NO donors. *H. influenzae* strains were cultured in triplicate under low-oxygen conditions in sBHI with either 5 mM GSNO at pH 7.5 for 24 h (A) or 15 mM $NaNO_2$ at pH 5.5 (B) for the times indicated, and viability was assessed by CFU determination. Values are the means ± standard deviations of the results of three independent experiments. Differences between strains under each condition or at each time were evaluated by Student's *t* test (†, $P < 0.05$; ‡, $P < 0.001$) or analysis of variance with Tukey's multiple comparison test (*, $P < 0.001$).

Roles in RNS resistance of genes subject to positive control by FNR. FNR-dependent transcription of *nrfA* and *ytfE* indicated a potential role for these genes in FNR-mediated resistance to RNS in *H. influenzae*. The *nrfA* mutant strain, RdHA, and strain RytfeV containing an in-frame, nonpolar deletion of *ytfE*, were compared to $NrfA^+$ $YtfE^+$ parental strains for resistance to RNS generated by ASN or GSNO. Recovery of CFU of the *nrfA* mutant strain relative to that of wild-type Rd was decreased by 5.8-fold after exposure to GSNO (Fig. 5A) and 4.2-fold after 90 min of exposure to ASN (Fig. 5B). The *ytfE* deletion mutant, RytfeV, exhibited a 25-fold decrease in CFU relative to its parental strain, RV, after exposure to GSNO, and complementation restored its resistance to parental levels (Fig. 5A). Similarly, after 60 min of exposure to ASN, 25-fold fewer CFU were recovered from cultures of RytfeV relative to the amount recovered for the wild-type strain, RV, and complementation restored resistance (Fig. 5B). These results indicate that the *nrfA* and *ytfE* deletion mutants exhibit hypersensitivity to RNS exposure. The sensitivity of the *nrfA* mutant, RdHA, to NO donors was less striking than that of RytfeV, indicating that although *nrfA* appears to contribute to RNS resistance, the requirement for *ytfE* is greater under these conditions.

FNR and YtfE participate in resistance to NO produced by macrophages. *H. influenzae* is likely to encounter low-oxygen conditions at sites of infection in which macrophages and other cells are sources of RNS. We investigated the potential role of FNR in defense against macrophages by comparing the rates of recovery of bacteria incubated under low-oxygen conditions in the presence and absence of BMM from C57BL/6 mice. The parent strain, RV, and the *fnr* mutant, RfnrV, both exhibited ~70% survival when incubated with BMM for 30 min, and no significant difference in survival was observed between these two strains under these conditions (Fig. 6A). The expression of iNOS and production of NO has previously been shown to increase in IFN-γ-stimulated murine macrophages exposed to bacterial LPS (48), an effect that is enhanced under low-oxygen conditions (53, 54). Therefore, the effect on the survival of *H. influenzae* of prestimulating BMM with IFN-γ or LPS was evaluated. Prestimulation resulted in decreased survival of both strains; however, the *fnr* mutant, RfnrV, exhibited a greater decrease than the parent, RV, after exposure to either type of stimulated macrophages, and the attenuation of the mutant was statistically significant in both cases (Fig. 6A). Based on these data, we concluded that *fnr* is required for optimal survival of *H. influenzae* after exposure to activated macrophages.

Because our data suggested that the *fnr*-regulated gene, *ytfE*, is essential for NO resistance under low-oxygen conditions, we investigated the potential role of *ytfE* in resistance to killing by macrophages under these conditions. To determine whether increased sensitivity of the *ytfE* mutant to activated macro-

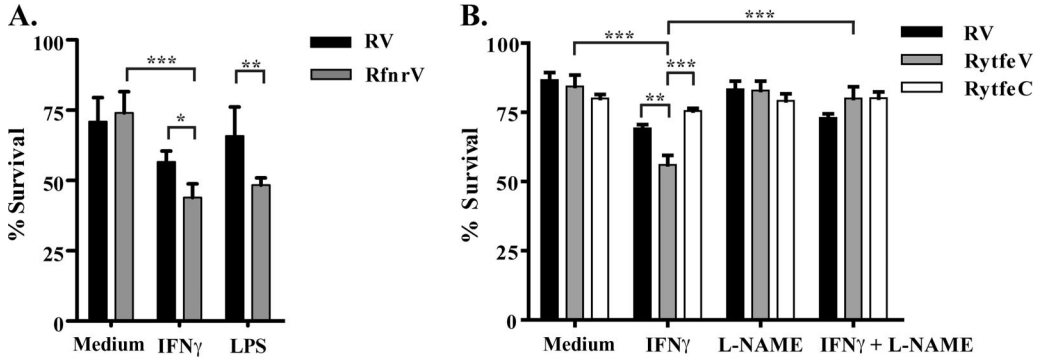

FIG. 6. Effects of *fnr* and *ytfE* mutations and NO inhibitor L-NAME on susceptibility of *H. influenzae* to activated macrophages. *H. influenzae* strains grown under low-oxygen conditions were added at a multiplicity of infection of 5:1 to primary C57BL/6 BMM pretreated with medium alone, IFN-γ for 24 h, or purified LPS for 2 h (A) or medium alone, IFN-γ for 24 h, L-NAME for 24 h, or IFN-γ plus L-NAME for 24 h (B). Percent survival represents the ratio of CFU recovered in the presence of macrophages versus CFU recovered after incubation in medium alone. Values are the means ± standard deviations of the results of four independent experiments. Statistically significant differences were determined by analysis of variance with Tukey's multiple comparison test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

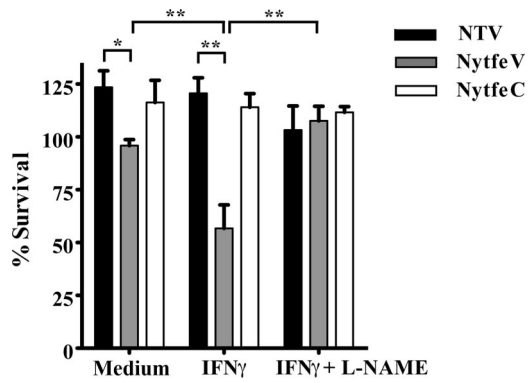

FIG. 7. Role of *ytfE* in resistance of NTHi to NOS-dependent antibacterial effects of IFN-γ-activated macrophages. *H. influenzae* strains grown under low-oxygen conditions were added at a multiplicity of infection of 2:1 to BMM pretreated for 24 h with IFN-γ, IFN-γ plus L-NAME, or medium alone. Percent survival represents the ratio of CFU recovered in the presence of macrophages versus CFU recovered after incubation in medium alone. Values are the means ± standard deviations of the results of five independent experiments. Statistically significant differences were determined by analysis of variance with Tukey's multiple comparison test (*, $P < 0.01$; **, $P < 0.001$).

phages could involve a NO-dependent mechanism, we pretreated IFN-γ-stimulated and unstimulated BMM with L-NAME, a competitive inhibitor of NOS. The parental strain (RV), the *ytfE* deletion strain (RytfeV), and the *ytfE* deletion strain complemented with *ytfE* at the *xyl* locus (RytfeC) were evaluated in this assay (Fig. 6B). Unstimulated BMM reduced the survival of each strain slightly relative to the survival of untreated controls. In contrast, after exposure to IFN-γ-stimulated macrophages, the percent survival of the *ytfE* mutant (RytfeV) was decreased by a greater extent than that of the parent (RV) or the complemented strain (RytfeC) (Fig. 6B). Unstimulated BMM treated with or without L-NAME reduced the recovery of all strains slightly and to an approximately equal extent. Treatment of BMM with L-NAME during IFN-γ stimulation eliminated the survival defect of the *ytfE* mutant relative to the survival of the parent and complemented strains in this assay. Together, these results indicate that the susceptibility of *H. influenzae* to IFN-γ-stimulated macrophages involves a mechanism dependent upon the ability of macrophages to produce NO.

Results with *H. influenzae* Rd-derived strains implicated *ytfE* in the defense of *H. influenzae* from activated macrophages. To investigate whether this mechanism is conserved in a pathogenic NTHi strain, the *ytfE* mutation was moved into a clinical NTHi strain, NT127. The resulting mutant, NytfeV, was tested for susceptibility to macrophages that had been preincubated with or without IFN-γ (Fig. 7). Unlike that of Rd-derived strains, the survival of the NTHi parental (NTV) and complemented (NytfeC) strains was unaffected by exposure to macrophages with or without prior stimulation, suggesting that this clinical isolate may evade killing by means of additional mechanisms that are absent in Rd. Nevertheless, the *ytfE* mutant, NytfeV, exhibited a 27% decrease in survival compared to that of the parent and a 20% decrease relative to that of the complemented strains when exposed to unstimulated BMM. Moreover, in the presence of BMM that had been stimulated with IFN-γ, the survival of the *ytfE* mutant was decreased by 64% relative to that of the parent and 57% compared to that of the complemented strain. Treatment of BMM with L-NAME concurrently with IFN-γ stimulation completely eliminated the survival defect of the *ytfE* mutant that was seen with BMM in the absence of inhibitor, implicating *ytfE* in defense against BMM-derived NO. The marked survival defect of the *ytfE* mutant relative to the survival of the parental and complemented strains after exposure to macrophages highlights the importance of this gene in the ability of NTHi to evade a major antimicrobial defense mechanism of innate immunity.

DISCUSSION

The concentration of oxygen present within the microenvironment dramatically affects the reactivity of NO by influencing its stability and the range of additional RNS generated. NO is oxidized to nitrite more rapidly as oxygen levels increase, and therefore, oxygen is considered to be one significant factor in limiting the half-life of NO in biological systems (12, 87). Bacteria growing in high- versus low-oxygen conditions exhibit different expression profiles of RNS-detoxifying enzymes, likely as an adaptation to coordinate the production of appropriate RNS detoxification mechanisms with other metabolic pathways active under these environmental conditions (14, 67, 68). Furthermore, inappropriate expression of enzymes involved in RNS defense can be detrimental to bacterial survival. For example, under aerobic conditions, *E. coli* and *S. enterica* serovar Typhimurium (56, 79) detoxify RNS via *hmp*, which catalyzes oxygen-dependent conversion of NO to nitrate and is repressed by FNR under low-oxygen conditions (17, 28, 56, 57, 79, 94). Deletion of *hmp* reduces the virulence of *S. enterica* serovar Typhimurium in mice; however, constitutive expression of *hmp* can lead to microbial-derived oxidative stress (6), implying that tight regulation of genes involved in nitrosative stress defense is essential for bacteria to withstand challenge from host immunity. Therefore, bacteria must tailor their defense strategies against these reactive species according to the conditions in which they are encountered.

In this study, we investigated the role of *H. influenzae* FNR in the regulation of resistance to RNS. FNR in other bacterial species plays complex roles in RNS defense. In addition to its well-characterized function in oxygen sensing, FNR of *E. coli* was also shown to respond directly to NO (68). In *Neisseria meningitidis*, the FNR response to NO mediates part of a homeostatic regulatory circuit by decreasing the expression of the FNR-activated nitrite reductase AniA (36), which is capable of generating a high level of endogenous NO (71). A denitrification pathway resembling that of *N. meningitidis* is not present in *H. influenzae*; however, the *nrfA* gene in *H. influenzae* is similar at the predicted amino acid level to the cytochrome *c* nitrite reductase *nrfA* of *E. coli.* Unlike AniA, *nrfA* has been implicated in NO detoxification (66, 85). We found that the *H. influenzae fnr* gene is required for appreciable levels of nitrite reductase activity (Fig. 1B), suggesting positive control by FNR of the *nrfABCD* operon, predicted to encode the sole nitrite reductase in this species. A nonpolar in-frame deletion of the *nrfA* gene abrogated the FNR-dependent dissimilatory nitrite reduction by *H. influenzae*, and complementation restored activity (Fig. 1A). Studies with a reporter fused to the

nrfA gene confirmed FNR-dependent activation of *nrfA* expression and demonstrated *nrfA* expression under a range of low-oxygen conditions, consistent with the predicted role of FNR in anaerobic transcriptional regulation (Fig. 2). Under low-oxygen conditions that promote *fnr* activity, as inferred by its ability to positively control *nrfA* expression, the *fnr* mutant was hypersusceptible to NO donors GSNO and ASN, implicating genes of the FNR regulon in RNS defense (Fig. 3). Consistent with this hypothesis, *nrfA* was also required for resistance to NO donors (Fig. 5). The sensitivity of the *nrfA* mutant was not as pronounced as that of the *fnr* mutant, suggesting that additional *fnr*-regulated genes play a role in RNS defense.

A previous study using a bioinformatic approach to identify potential FNR-regulated genes in *H. influenzae* identified potential FNR binding sites in the promoter for *ytfE* (83). YtfE is a member of a broadly conserved family of di-iron proteins that participate in the biogenesis and repair of iron-sulfur centers (41, 42, 63). Our transcriptional analysis revealed FNR-dependent positive control over transcript levels of *ytfE* in *H. influenzae* (Fig. 4B). Because *ytfE* participates in RNS resistance mechanisms in other bacteria, we examined its role in the resistance of *H. influenzae* to chemical NO donors GSNO and ASN. Deletion of *ytfE* led to marked attenuation for survival after exposure to these NO donors, and complementation with *ytfE*, provided in single copy at an ectopic chromosomal site, restored resistance (Fig. 5). Therefore, *ytfE* of *H. influenzae* is positively controlled by FNR and mediates resistance to donors of RNS, implicating the activation of *ytfE* expression as a primary mechanism of the observed *fnr*-dependent RNS resistance in *H. influenzae*.

These results indicate a different mode of control in *H. influenzae* than in a diverse set of pathogenic bacteria in which *ytfE* regulation has been examined. In *E. coli*, *ytfE* is negatively controlled by FNR and by an additional NO-sensitive DNA binding protein, NsrR, which is absent in *H. influenzae*, based on genome sequence analysis (9, 27, 41). The NsrR protein of *E. coli* has been implicated as a direct repressor (9, 22), whereas the absence of a recognizable FNR binding site in the *E. coli ytfE* promoter has suggested that the effect of FNR may be indirect (41). In the presence of NO, both negative regulators become inactive, leading to *ytfE* expression. Much of this regulatory circuitry and expression pattern appears to be conserved in bacterial species as diverse as *Staphylococcus aureus*, *S. enterica* serovar Typhimurium, and *N. gonorrhoeae*, each of which contains genes with functional and sequence similarity to *nsrR*, *fnr*, and *ytfE* (63). Moreover, the *N. gonorrhoeae* NsrR has been demonstrated to directly repress target promoters in a NO-sensitive manner (38). In contrast, *H. influenzae* lacks a gene with any significant similarity to *nsrR*, and FNR positively controls *ytfE*, potentially via interaction with the possible FNR binding site located in its promoter. The apparent conservation of the regulatory pathway controlling *ytfE* in phylogenetically diverse bacteria but not in *H. influenzae* may reflect unique aspects of the regulation of RNS defense pathways in *H. influenzae* pathogenesis. One possibility is that *H. influenzae* primarily encounters RNS in a relatively low-oxygen environment that it likely experiences during invasion of the epithelium or in biofilms that appear to form at the mucosal surface during infection (23). Entry into such stages of colonization could signal the requirement for induction of preemptive defenses against RNS generated when host cells respond to increased concentrations of bacterial products. In this model, an *nsrR*-mediated response to NO would not be required. Alternatively, the configuration of multiple systems to metabolize toxic reactive oxygen species and RNS in different bacteria may constrain the allowable expression patterns for certain enzymes, as exemplified by the toxic effect of ectopic expression of *hmp* in *Salmonella enterica* serovar Typhimurium (6). *H. influenzae* lacks many enzymes of RNS defense that are present in other bacteria and, therefore, may not require the same degree of complex control to prevent such aberrant interactions.

Control of *H. influenzae* by phagocytic cells has been shown to be critical during early phases of infection and during adaptive immunity (4, 61, 82, 84, 88). To investigate the potential role of *fnr* regulation in defense against host cells capable of RNS generation during the immune response, we evaluated the ability of *H. influenzae* to resist the antibacterial effects of BMM with or without prior stimulation with IFN-γ or LPS, which trigger iNOS production (49). The *fnr* mutant was attenuated for survival in the presence of activated macrophages (Fig. 6A). Resistance also required *ytfE*, both in the laboratory strain Rd and in a clinical NTHi background. Complementation of the *ytfE* mutants or inhibition of macrophages with L-NAME restored the mutants to wild-type survival levels (Fig. 6B and 7). It has been shown that NO can inhibit the growth of *E. coli* under fully anaerobic conditions by damaging the iron-sulfur centers of branched-chain amino acid synthesis enzymes (70). Such bacteriostatic effects could contribute to the decreased net survival of *H. influenzae* mutants that we observed in our assays. Nevertheless, it appears likely that RNS also kills *H. influenzae* under the conditions we used. Experiments measuring sensitivity to ASN during a short time course of exposure revealed a decrease in CFU (Fig. 3C), whereas control cultures of *H. influenzae* did not multiply in the same acidified medium in the absence of nitrite during this period, indicating that ASN exerted a bactericidal effect. In either case, a destructive effect of RNS on iron-sulfur centers in *H. influenzae* would be consistent with the role in RNS resistance that we have detected with mutants deficient in *ytfE*, whose product is a protein involved in repairing iron-sulfur centers. Therefore, the data indicate that FNR and the *ytfE* gene are probably required to resist inhibitory or antimicrobial effects of RNS produced by the immune response during infection.

The role of RNS resistance in *H. influenzae* pathogenesis is at the early stages of investigation. Because NO-producing cells, including macrophages and epithelial cells, are abundant in the human nasopharynx and high levels of NO are present in this primary site of asymptomatic colonization (50), it is likely that resistance to RNS is an important adaptation for this organism. *Neisseria meningitidis*, another nasopharyngeal colonizer, has been shown to require RNS defenses for viability during coincubation of bacteria with nasopharyngeal explants (78), implicating defense against RNS as an important factor in colonization of human respiratory mucosa. Disease states also expose *H. influenzae* to RNS derived from phagocytes and other cell types. Macrophages are abundant in the lung, and bacterial infection, implicated in exacerbations of chronic obstructive pulmonary disease, is associated with elevated num-

bers of neutrophils (74). Otitis media pathology involves an influx of polymorphonuclear leukocytes during acute infection and elevated numbers of macrophages during chronic disease (93), and human middle ear effusions contain NO metabolites, including nitrate and nitrite (40). Therefore, it will be informative to exploit knowledge of RNS resistance pathways in *H. influenzae* with models such as mucosal tissue explant systems, infections of mice deficient in RNS production, or otitis media models of middle ear infection. Our investigation of FNR-mediated environmental control of defense against RNS and phagocytic killing of *H. influenzae* suggests that this regulation is likely required for the survival of *H. influenzae* in an in vivo environment in which oxygen levels are low, conditions that may occur when bacterial density increases on mucosal surfaces or during invasion into submucosal sites. Identifying the specific stages of colonization or disease that involve these pathways will be important for understanding how this pathogen evades host immunity.

ACKNOWLEDGMENTS

This work was supported by a grant from the American Heart Association and by NIH NIAID grant number 1RO1-AI49437 to B.J.A.

We thank Jeffrey Gawronski for helpful comments.

REFERENCES

1. **Akerley, B. J., and D. J. Lampe.** 2002. Analysis of gene function in bacterial pathogens by GAMBIT. Methods Enzymol. **358:**100–108.
2. **Alexeyev, M. F., and I. N. Shokolenko.** 1995. Mini-Tn10 transposon derivatives for insertion mutagenesis and gene delivery into the chromosome of gram-negative bacteria. Gene **160:**59–62.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. E. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1995. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, NY.
4. **Bakaletz, L. O., T. F. DeMaria, and D. J. Lim.** 1987. Phagocytosis and killing of bacteria by middle ear macrophages. Arch. Otolaryngol. Head Neck Surg. **113:**138–144.
5. **Baltes, N., M. N′Diaye, I. D. Jacobsen, A. Maas, F. F. Buettner, and G. F. Gerlach.** 2005. Deletion of the anaerobic regulator HlyX causes reduced colonization and persistence of *Actinobacillus pleuropneumoniae* in the porcine respiratory tract. Infect. Immun. **73:**4614–4619.
6. **Bang, I. S., L. Liu, A. Vazquez-Torres, M. L. Crouch, J. S. Stamler, and F. C. Fang.** 2006. Maintenance of nitric oxide and redox homeostasis by the salmonella flavohemoglobin hmp. J. Biol. Chem. **281:**28039–28047.
7. **Barcak, G. J., M. S. Chandler, R. J. Redfield, and J. F. Tomb.** 1991. Genetic systems in *Haemophilus influenzae*. Methods Enzymol. **204:**321–342.
8. **Bartolini, E., E. Frigimelica, S. Giovinazzi, G. Galli, Y. Shaik, C. Genco, J. A. Welsch, D. M. Granoff, G. Grandi, and R. Grifantini.** 2006. Role of FNR and FNR-regulated, sugar fermentation genes in *Neisseria meningitidis* infection. Mol. Microbiol. **60:**963–972.
9. **Bodenmiller, D. M., and S. Spiro.** 2006. The *yjeB* (*nsrR*) gene of *Escherichia coli* encodes a nitric oxide-sensitive transcriptional regulator. J. Bacteriol. **188:**874–881.
10. **Bogdan, C., M. Rollinghoff, and A. Diefenbach.** 2000. The role of nitric oxide in innate immunity. Immunol. Rev. **173:**17–26.
11. **Brunelli, L., J. P. Crow, and J. S. Beckman.** 1995. The comparative toxicity of nitric oxide and peroxynitrite to *Escherichia coli*. Arch. Biochem. Biophys. **316:**327–334.
12. **Butler, A. R., and P. Rhodes.** 1997. Chemistry, analysis, and biological roles of S-nitrosothiols. Anal. Biochem. **249:**1–9.
13. **Centers for Disease Control and Prevention.** 2002. Progress toward elimination of *Haemophilus influenzae* type b invasive disease among infants and children: United States, 1998–2000. MMWR Morb. Mortal. Wkly. Rep. **51:**234–237.
14. **Constantinidou, C., J. L. Hobman, L. Griffiths, M. D. Patel, C. W. Penn, J. A. Cole, and T. W. Overton.** 2006. A reassessment of the FNR regulon and transcriptomic analysis of the effects of nitrate, nitrite, NarXL, and NarQP as *Escherichia coli* K12 adapts from aerobic to anaerobic growth. J. Biol. Chem. **281:**4802–4815.
15. **Cotter, P. A., and R. P. Gunsalus.** 1989. Oxygen, nitrate, and molybdenum regulation of *dmsABC* gene expression in *Escherichia coli*. J. Bacteriol. **171:**3817–3823.
16. **Crack, J., J. Green, and A. J. Thomson.** 2004. Mechanism of oxygen sensing by the bacterial transcription factor fumarate-nitrate reduction (FNR). J. Biol. Chem. **279:**9278–9286.
17. **Cruz-Ramos, H., J. Crack, G. Wu, M. N. Hughes, C. Scott, A. J. Thomson, J. Green, and R. K. Poole.** 2002. NO sensing by FNR: regulation of the *Escherichia coli* NO-detoxifying flavohaemoglobin, Hmp. EMBO J. **21:**3235–3244.
18. **Darwin, A., H. Hussain, L. Griffiths, J. Grove, Y. Sambongi, S. Busby, and J. Cole.** 1993. Regulation and sequence of the structural gene for cytochrome c552 from *Escherichia coli*: not a hexahaem but a 50 kDa tetrahaem nitrite reductase. Mol. Microbiol. **9:**1255–1265.
19. **De Souza-Hart, J. A., W. Blackstock, V. Di Modugno, I. B. Holland, and M. Kok.** 2003. Two-component systems in *Haemophilus influenzae*: a regulatory role for ArcA in serum resistance. Infect. Immun. **71:**163–172.
20. **Dibden, D. P., and J. Green.** 2005. In vivo cycling of the *Escherichia coli* transcription factor FNR between active and inactive states. Microbiology **151:**4063–4070.
21. **D'Mello, R. A., P. R. Langford, and J. S. Kroll.** 1997. Role of bacterial Mn-cofactored superoxide dismutase in oxidative stress responses, nasopharyngeal colonization, and sustained bacteremia caused by *Haemophilus influenzae* type b. Infect. Immun. **65:**2700–2706.
22. **Efromovich, S., D. Grainger, D. Bodenmiller, and S. Spiro.** 2008. Genome-wide identification of binding sites for the nitric oxide-sensitive transcriptional regulator NsrR. Methods Enzymol. **437:**211–233.
23. **Ehrlich, G. D., R. Veeh, X. Wang, J. W. Costerton, J. D. Hayes, F. Z. Hu, B. J. Daigle, M. D. Ehrlich, and J. C. Post.** 2002. Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. JAMA **287:**1710–1715.
24. **Eiglmeier, K., N. Honore, S. Iuchi, E. C. Lin, and S. T. Cole.** 1989. Molecular genetic analysis of FNR-dependent promoters. Mol. Microbiol. **3:**869–878.
25. **Elvers, K. T., S. M. Turner, L. M. Wainwright, G. Marsden, J. Hinds, J. A. Cole, R. K. Poole, C. W. Penn, and S. F. Park.** 2005. NssR, a member of the Crp-Fnr superfamily from *Campylobacter jejuni*, regulates a nitrosative stress-responsive regulon that includes both a single-domain and a truncated haemoglobin. Mol. Microbiol. **57:**735–750.
26. **Fang, F. C.** 2004. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat. Rev. **2:**820–832.
27. **Filenko, N., S. Spiro, D. F. Browning, D. Squire, T. W. Overton, J. Cole, and C. Constantinidou.** 2007. The NsrR regulon of *Escherichia coli* K-12 includes genes encoding the hybrid cluster protein and the periplasmic, respiratory nitrite reductase. J. Bacteriol. **189:**4410–4417.
28. **Fink, R. C., M. R. Evans, S. Porwollik, A. Vazquez-Torres, J. Jones-Carson, B. Troxell, S. J. Libby, M. McClelland, and H. M. Hassan.** 2007. FNR is a global regulator of virulence and anaerobic metabolism in *Salmonella enterica* serovar *Typhimurium* (ATCC 14028s). J. Bacteriol. **189:**2262–2273.
29. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, et al.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
30. **Georgellis, D., O. Kwon, E. C. Lin, S. M. Wong, and B. J. Akerley.** 2001. Redox signal transduction by the ArcB sensor kinase of *Haemophilus influenzae* lacking the PAS domain. J. Bacteriol. **183:**7206–7212.
31. **Gerasimova, A. V., D. A. Rodionov, A. A. Mironov, and M. S. Gel'fand.** 2001. Computer analysis of regulatory signals in bacterial genomes. Fnr binding segments. Mol. Biol. (Moscow) **35:**1001–1009. (In Russian.)
32. **Gilberthorpe, N. J., and R. K. Poole.** 2008. Nitric oxide homeostasis in *Salmonella typhimurium*: roles of respiratory nitrate reductase and flavohemoglobin. J. Biol. Chem. **283:**11146–11154.
33. **Gilligan, P. H.** 1991. Microbiology of airway disease in patients with cystic fibrosis. Clin. Microbiol. Rev. **4:**35–51.
34. **Goldstein, S., and G. Czapski.** 1995. The reaction of NO with O_2 and HO_2: a pulse radiolysis study. Free Radic. Biol. Med. **19:**505–510.
35. **Green, B. A., J. E. Farley, T. Quinn-Dey, R. A. Deich, and G. W. Zlotnick.** 1991. The *e* (P4) outer membrane protein of *Haemophilus influenzae*: biologic activity of anti-*e* serum and cloning and sequencing of the structural gene. Infect. Immun. **59:**3191–3198.
36. **Heurlier, K., M. J. Thomson, N. Aziz, and J. W. Moir.** 2008. The nitric oxide (NO)-sensing repressor NsrR of *Neisseria meningitidis* has a compact regulon of genes involved in NO synthesis and detoxification. J. Bacteriol. **190:**2488–2495.
37. **Hussain, H., J. Grove, L. Griffiths, S. Busby, and J. Cole.** 1994. A seven-gene operon essential for formate-dependent nitrite reduction to ammonia by enteric bacteria. Mol. Microbiol. **12:**153–163.
38. **Isabella, V. M., J. D. Lapek, Jr., E. M. Kennedy, and V. L. Clark.** 2009. Functional analysis of NsrR, a nitric oxide-sensing Rrf2 repressor in *Neisseria gonorrhoeae*. Mol. Microbiol. **71:**227–239.
39. **Jacobsen, I., I. Hennig-Pauka, N. Baltes, M. Trost, and G. F. Gerlach.** 2005. Enzymes involved in anaerobic respiration appear to play a role in *Actinobacillus pleuropneumoniae* virulence. Infect. Immun. **73:**226–234.
40. **John, E. O., P. T. Russell, B. H. Nam, T. H. Jinn, and T. T. Jung.** 2001. Concentration of nitric oxide metabolites in middle ear effusion. Int. J. Pediatr. Otorhinolaryngol. **60:**55–58.
41. **Justino, M. C., C. C. Almeida, V. L. Goncalves, M. Teixeira, and L. M.**

Saraiva. 2006. *Escherichia coli* YtfE is a di-iron protein with an important function in assembly of iron-sulphur clusters. FEMS Microbiol. Lett. **257:** 278–284.
42. **Justino, M. C., C. C. Almeida, M. Teixeira, and L. M. Saraiva.** 2007. *Escherichia coli* di-iron YtfE protein is necessary for the repair of stress-damaged iron-sulfur clusters. J. Biol. Chem. **282:**10352–10359.
43. **Kainulainen, L., J. Suonpaa, J. Nikoskelainen, E. Svedstrom, T. Vuorinen, O. Meurman, and O. Ruuskanen.** 2007. Bacteria and viruses in maxillary sinuses of patients with primary hypogammaglobulinemia. Arch. Otolaryngol. Head Neck Surg. **133:**597–602.
44. **Kidd, S. P., D. Jiang, M. P. Jennings, and A. G. McEwan.** 2007. Glutathione-dependent alcohol dehydrogenase AdhC is required for defense against nitrosative stress in *Haemophilus influenzae*. Infect. Immun. **75:** 4506–4513.
45. **Kwon, O., D. Georgellis, and E. C. Lin.** 2000. Phosphorelay as the sole physiological route of signal transmission by the *arc* two-component system of *Escherichia coli*. J. Bacteriol. **182:**3858–3862.
46. **Lazazzera, B. A., H. Beinert, N. Khoroshilova, M. C. Kennedy, and P. J. Kiley.** 1996. DNA binding and dimerization of the Fe-S-containing FNR protein from *Escherichia coli* are regulated by oxygen. J. Biol. Chem. **271:** 2762–2768.
47. **Liu, X., and P. De Wulf.** 2004. Probing the ArcA-P modulon of *Escherichia coli* by whole genome transcriptional analysis and sequence recognition profiling. J. Biol. Chem. **279:**12588–12597.
48. **Lorsbach, R. B., W. J. Murphy, C. J. Lowenstein, S. H. Snyder, and S. W. Russell.** 1993. Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. Molecular basis for the synergy between interferon-gamma and lipopolysaccharide. J. Biol. Chem. **268:**1908–1913.
49. **Lorsbach, R. B., and S. W. Russell.** 1992. A specific sequence of stimulation is required to induce synthesis of the antimicrobial molecule nitric oxide by mouse macrophages. Infect. Immun. **60:**2133–2135.
50. **Lundberg, J. O., T. Farkas-Szallasi, E. Weitzberg, J. Rinder, J. Lidholm, A. Anggaard, T. Hokfelt, J. M. Lundberg, and K. Alving.** 1995. High nitric oxide production in human paranasal sinuses. Nat. Med. **1:**370–373.
51. **MacMicking, J., Q. W. Xie, and C. Nathan.** 1997. Nitric oxide and macrophage function. Annu. Rev. Immunol. **15:**323–350.
52. **Malpica, R., B. Franco, C. Rodriguez, O. Kwon, and D. Georgellis.** 2004. Identification of a quinone-sensitive redox switch in the ArcB sensor kinase. Proc. Natl. Acad. Sci. USA **101:**13318–13323.
53. **Melillo, G., T. Musso, A. Sica, L. S. Taylor, G. W. Cox, and L. Varesio.** 1995. A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. J. Exp. Med. **182:**1683–1693.
54. **Melillo, G., L. S. Taylor, A. Brooks, G. W. Cox, and L. Varesio.** 1996. Regulation of inducible nitric oxide synthase expression in IFN-gamma-treated murine macrophages cultured under hypoxic conditions. J. Immunol. **157:**2638–2644.
55. **Melville, S. B., and R. P. Gunsalus.** 1996. Isolation of an oxygen-sensitive FNR protein of *Escherichia coli*: interaction at activator and repressor sites of FNR-controlled genes. Proc. Natl. Acad. Sci. USA **93:**1226–1231.
56. **Membrillo-Hernandez, J., M. D. Coopamah, M. F. Anjum, T. M. Stevanin, A. Kelly, M. N. Hughes, and R. K. Poole.** 1999. The flavohemoglobin of *Escherichia coli* confers resistance to a nitrosating agent, a "nitric oxide releaser," and paraquat and is essential for transcriptional responses to oxidative stress. J. Biol. Chem. **274:**748–754.
57. **Mills, C. E., S. Sedelnikova, B. Soballe, M. N. Hughes, and R. K. Poole.** 2001. *Escherichia coli* flavohaemoglobin (Hmp) with equistoichiometric FAD and haem contents has a low affinity for dioxygen in the absence or presence of nitric oxide. Biochem. J. **353:**207–213.
58. **Moller, L. V., A. G. Regelink, H. Grasselier, J. E. Dankert-Roelse, J. Dankert, and L. van Alphen.** 1995. Multiple *Haemophilus influenzae* strains and strain variants coexist in the respiratory tract of patients with cystic fibrosis. J. Infect. Dis. **172:**1388–1392.
59. **Moxon, E. R., and T. F. Murphy.** 2000. *Haemophilus influenzae*, p. 2369–2378. *In* G. L. Mandell, J. R. Bennett, and R. Dolin (ed.), Mandell, Douglas, and Bennett's principles and practices of infectious diseases, 5th ed., vol. 2. Churchill Livingstone, New York, NY.
60. **Murphy, T. F., A. L. Brauer, A. T. Schiffmacher, and S. Sethi.** 2004. Persistent colonization by *Haemophilus influenzae* in chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. **170:**266–272.
61. **Noel, G. J., S. K. Hoiseth, and P. J. Edelson.** 1992. Type b capsule inhibits ingestion of *Haemophilus influenzae* by murine macrophages: studies with isogenic encapsulated and unencapsulated strains. J. Infect. Dis. **166:**178–182.
62. **Oshima, T., H. Aiba, Y. Masuda, S. Kanaya, M. Sugiura, B. L. Wanner, H. Mori, and T. Mizuno.** 2002. Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. Mol. Microbiol. **46:**281–291.
63. **Overton, T. W., M. C. Justino, Y. Li, J. M. Baptista, A. M. Melo, J. A. Cole, and L. M. Saraiva.** 2008. Widespread distribution in pathogenic bacteria of di-iron proteins that repair oxidative and nitrosative damage to iron-sulfur centers. J. Bacteriol. **190:**2004–2013.
64. **Partridge, J. D., R. K. Poole, and J. Green.** 2007. The *Escherichia coli yhjA* gene, encoding a predicted cytochrome *c* peroxidase, is regulated by FNR and OxyR. Microbiology **153:**1499–1507.
65. **Pichichero, M. E., J. R. Casey, A. Hoberman, and R. Schwartz.** 2008. Pathogens causing recurrent and difficult-to-treat acute otitis media, 2003-2006. Clin. Pediatr. **47:**901–906.
66. **Poock, S. R., E. R. Leach, J. W. Moir, J. A. Cole, and D. J. Richardson.** 2002. Respiratory detoxification of nitric oxide by the cytochrome *c* nitrite reductase of *Escherichia coli*. J. Biol. Chem. **277:**23664–23669.
67. **Poole, R. K., M. F. Anjum, J. Membrillo-Hernandez, S. O. Kim, M. N. Hughes, and V. Stewart.** 1996. Nitric oxide, nitrite, and Fnr regulation of *hmp* (flavohemoglobin) gene expression in *Escherichia coli* K-12. J. Bacteriol. **178:**5487–5492.
68. **Pullan, S. T., M. D. Gidley, R. A. Jones, J. Barrett, T. M. Stevanin, R. C. Read, J. Green, and R. K. Poole.** 2007. Nitric oxide in chemostat-cultured *Escherichia coli* is sensed by Fnr and other global regulators: unaltered methionine biosynthesis indicates lack of S nitrosation. J. Bacteriol. **189:** 1845–1855.
69. **Ravcheev, D. A., A. V. Gerasimova, A. A. Mironov, and M. S. Gelfand.** 2007. Comparative genomic analysis of regulation of anaerobic respiration in ten genomes from three families of gamma-proteobacteria (Enterobacteriaceae, Pasteurellaceae, Vibrionaceae). BMC Genomics **8:**54.
70. **Ren, B., N. Zhang, J. Yang, and H. Ding.** 2008. Nitric oxide-induced bacteriostasis and modification of iron-sulphur proteins in *Escherichia coli*. Mol. Microbiol. **70:**953–964.
71. **Rock, J. D., M. R. Mahnane, M. F. Anjum, J. G. Shaw, R. C. Read, and J. W. Moir.** 2005. The pathogen *Neisseria meningitidis* requires oxygen, but supplements growth by denitrification. Nitrite, nitric oxide and oxygen control respiratory flux at genetic and metabolic levels. Mol. Microbiol. **58:**800–809.
72. **Salmon, K. A., S. P. Hung, N. R. Steffen, R. Krupp, P. Baldi, G. W. Hatfield, and R. P. Gunsalus.** 2005. Global gene expression profiling in *Escherichia coli* K12: effects of oxygen availability and ArcA. J. Biol. Chem. **280:**15084–15096.
73. **Samouilov, A., P. Kuppusamy, and J. L. Zweier.** 1998. Evaluation of the magnitude and rate of nitric oxide production from nitrite in biological systems. Arch. Biochem. Biophys. **357:**1–7.
74. **Sethi, S., J. Maloney, L. Grove, C. Wrona, and C. S. Berenson.** 2006. Airway inflammation and bronchial bacterial colonization in chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. **173:**991–998.
75. **Shaw, D. J., D. W. Rice, and J. R. Guest.** 1983. Homology between CAP and Fnr, a regulator of anaerobic respiration in *Escherichia coli*. J. Mol. Biol. **166:**241–247.
76. **Simon, J.** 2002. Enzymology and bioenergetics of respiratory nitrite ammonification. FEMS Microbiol. Rev. **26:**285–309.
77. **Singh, R. J., N. Hogg, J. Joseph, and B. Kalyanaraman.** 1996. Mechanism of nitric oxide release from S-nitrosothiols. J. Biol. Chem. **271:**18596–18603.
78. **Stevanin, T. M., J. W. Moir, and R. C. Read.** 2005. Nitric oxide detoxification systems enhance survival of *Neisseria meningitidis* in human macrophages and in nasopharyngeal mucosa. Infect. Immun. **73:**3322–3329.
79. **Stevanin, T. M., R. K. Poole, E. A. Demoncheaux, and R. C. Read.** 2002. Flavohemoglobin Hmp protects *Salmonella enterica* serovar *typhimurium* from nitric oxide-related killing by human macrophages. Infect. Immun. **70:**4399–4405.
80. **Stewart, V., P. J. Bledsoe, and S. B. Williams.** 2003. Dual overlapping promoters control *napF* (periplasmic nitrate reductase) operon expression in *Escherichia coli* K-12. J. Bacteriol. **185:**5862–5870.
81. **Stewart, V., Y. Lu, and A. J. Darwin.** 2002. Periplasmic nitrate reductase (NapABC enzyme) supports anaerobic respiration by *Escherichia coli* K-12. J. Bacteriol. **184:**1314–1323.
82. **Sun, J., J. Chen, Z. Cheng, J. B. Robbins, J. F. Battey, and X. X. Gu.** 2000. Biological activities of antibodies elicited by lipooligosaccharide-based conjugate vaccines of nontypeable *Haemophilus influenzae* in an otitis media model. Vaccine **18:**1264–1272.
83. **Tan, K., G. Moreno-Hagelsieb, J. Collado-Vides, and G. D. Stormo.** 2001. A comparative genomics approach to prediction of new members of regulons. Genome Res. **11:**566–584.
84. **Toews, G. B., W. C. Vial, and E. J. Hansen.** 1985. Role of C5 and recruited neutrophils in early clearance of nontypable *Haemophilus influenzae* from murine lungs. Infect. Immun. **50:**207–212.
85. **van Wonderen, J. H., B. Burlat, D. J. Richardson, M. R. Cheesman, and J. N. Butt.** 2008. The nitric oxide reductase activity of cytochrome *c* nitrite reductase from *Escherichia coli*. J. Biol. Chem. **283:**9587–9594.
86. **Weiser, J. N., N. Pan, K. L. McGowan, D. Musher, A. Martin, and J. Richards.** 1998. Phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* contributes to persistence in the respiratory tract and sensitivity to serum killing mediated by C-reactive protein. J. Exp. Med. **187:**631–640.
87. **Wink, D. A., J. F. Darbyshire, R. W. Nims, J. E. Saavedra, and P. C. Ford.** 1993. Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: determination of the kinetics for oxidation and nitrosa-

tion by intermediates generated in the NO/O2 reaction. Chem. Res. Toxicol. **6:**23–27.
88. **Winter, L. E., and S. J. Barenkamp.** 2006. Antibodies specific for the high-molecular-weight adhesion proteins of nontypeable *Haemophilus influenzae* are opsonophagocytic for both homologous and heterologous strains. Clin. Vaccine Immunol. **13:**1333–1342.
89. **Wong, S. M., and B. J. Akerley.** 2005. Environmental and genetic regulation of the phosphorylcholine epitope of *Haemophilus influenzae* lipooligosaccharide. Mol. Microbiol. **55:**724–738.
90. **Wong, S. M., and B. J. Akerley.** 2003. Inducible expression system and marker-linked mutagenesis approach for functional genomics of *Haemophilus influenzae*. Gene **316:**177–186.
91. **Wong, S. M., K. R. Alugupalli, S. Ram, and B. J. Akerley.** 2007. The ArcA regulon and oxidative stress resistance in *Haemophilus influenzae*. Mol. Microbiol. **64:**1375–1390.
92. **Wood, G. E., N. Khelef, N. Guiso, and R. L. Friedman.** 1998. Identification of Btr-regulated genes using a titration assay. Search for a role for this transcriptional regulator in the growth and virulence of *Bordetella pertussis.* Gene **209:**51–58.
93. **Wright, C. G., and W. L. Meyerhoff.** 1994. Pathology of otitis media. Ann. Otol. Rhinol. Laryngol. **163:**24–26.
94. **Wu, G., H. Corker, Y. Orii, and R. K. Poole.** 2004. *Escherichia coli* Hmp, an "oxygen-binding flavohaemoprotein," produces superoxide anion and self-destructs. Arch. Microbiol. **182:**193–203.

Editor: J. B. Bliska