INFECTION AND IMMUNITY, Sept. 2007, p. 4506–4513 Vol. 75, No. 9
0019-9567/07/$08.00+0 doi:10.1128/IAI.00487-07

Glutathione-Dependent Alcohol Dehydrogenase AdhC Is Required for Defense against Nitrosative Stress in *Haemophilus influenzae*[▽]

Stephen P. Kidd, Donald Jiang, Michael P. Jennings, and Alastair G. McEwan*

Australian Bacterial Pathogenesis Program and Centre for Metals in Biology, School of Molecular and Microbial Sciences, University of Queensland, Brisbane, Queensland 4072, Australia

Received 5 April 2007/Returned for modification 29 May 2007/Accepted 15 June 2007

In *Haemophilus influenzae* Rd KW20, we identified a gene, *adhC*, which encodes a class III alcohol dehydrogenase (AdhC) and has *S*-nitrosoglutathione reductase activity. *adhC* exists on an operon with *estD*, which encodes an esterase. Divergent to the *adhC-estD* operon is the *Haemophilus influenzae nmlR* gene ($nmlR_{HI}$), which encodes a MerR family regulator that is homologous to the *Neisseria* MerR-like regulator (NmlR). Analysis of an $nmlR_{HI}$ mutant indicated that expression of the *adhC-estD* operon is regulated by $NmlR_{HI}$ in strain Rd KW20. Chromosomal inactivation of either *adhC* or $nmlR_{HI}$ resulted in sensitivity to *S*-nitrosoglutathione and decreased *S*-nitrosoglutathione reductase activity. Examination of the $NmlR_{HI}$-AdhC system in the genome sequences of nontypeable *H. influenzae* strains R2846, R2866, and 86-028NP identified significant variations. The *adhC* gene of 86-028NP was predicted to be nonfunctional due to a premature stop codon. Polymorphisms in the operator/promoter region of R2866 resulted in reduced enzyme activity. This correlated with an increased sensitivity to *S*-nitrosoglutathione. The *adhC*-$nmlR_{HI}$ system was examined in thirty-three clinical isolates (both capsular and nontypeable strains). Nucleic acid sequence data showed that only strain 86-028NP contained a premature stop codon. There were some variations in the DNA sequence of the operator/promoter region which altered the $nmlR_{HI}$ promoter. However, the clinical isolates still possessed *S*-nitrosoglutathione reductase activity and showed at least the equivalent ability to grow in the presence of *S*-nitrosoglutathione as Rd KW20. These data suggest that the $nmlR_{HI}$-*adhC* system has a role in the defense against nitrosative stress in *Haemophilus influenzae*.

Haemophilus influenzae is an obligate human parasite (25). This bacterium is able to survive in the human nasopharynx as a harmless commensal and is present in the upper respiratory tracts of up to 80% of healthy individuals (40). *H. influenzae* does possess the ability to move to other sites within the body, resulting in a variety of diseases. Isolates of *H. influenzae* can be subdivided into encapsulated or nonencapsulated strains. The latter are defined by their failure to agglutinate with antisera against the recognized *H. influenzae* capsular polysaccharides and are referred to as nontypeable *H. influenzae* (NTHi). Encapsulated and nonencapsulated strains have similar morphological and metabolic properties, but they manifest differently in a clinical context (32). NTHi is a common cause of otitis media, sinusitis, and conjunctivitis (37), as well as chronic bronchitis, and is an important cause of community-acquired pneumonia. In the transition from colonization to cause of these pathologies, the organism must overcome innate immunity.

The innate immune response produces reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS, which include a superoxide anion ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2), and a hydroxyl radical (HO·), cause damage to proteins, nucleic acids, and cell membranes (24, 38).

Nitric oxide (NO), produced by inducible nitric oxide synthase (iNOS), is recognized as an important mediator of the innate immune response to infection (10). NO interacts with biomolecules within its immediate environment to form other RNS, such as *S*-nitrosoglutathione (GSNO) and nitrosothiols through interactions with glutathione (GSH) and thiols, respectively. RNS possess antimicrobial activity through their interaction with numerous targets within the bacterial cell. NO and *S*-nitrosothiols can inhibit bacterial DNA replication via the release of zinc from metalloproteins (35). NO also inhibits bacterial respiration (29, 36). By inhibiting ribonucleotide reductase, RNS limit the availability of precursors for synthesis and repair of DNA (21). There is also evidence that Fe-S clusters of some proteins are targets for RNS.

In humans and animal model systems, it has been shown that there are increased levels of iNOS expression and NO production upon microbial infection (12, 28). In the case of otitis media, it is the middle ear epithelium that responds to invading pathogens, such as *Streptococcus pneumoniae* and *H. influenzae*, by releasing proinflammatory cytokines and other mediators, a process that might facilitate invasion (27, 34, 39). In experiments with ear effusions from patients, it has been shown that middle ear infections result in the induction of expression of iNOS (23) and that the resulting NO production has a role in bacterial killing in this environment. If iNOS was inhibited, there was little NO present, and a decrease in bacterial killing was observed (28). RNS defense mechanisms have been described for the enteric pathogen *Salmonella enterica* serovar Typhimurium. This bacterium possesses a number of enzymes with the ability to metabolize RNS. The flavohemoglobin Hmp, the flavorubredoxin NorV, and the nitrite reductase Nrf all have the ability to reduce NO, and the class

* Corresponding author. Mailing address: Australian Bacterial Pathogenesis Program and Centre for Metals in Biology, School of Molecular and Microbial Sciences, University of Queensland, Brisbane, Queensland 4072, Australia. Phone: 61 7 3365 4622. Fax: 61 7 3365 4273. E-mail: mcewan@uq.edu.au.

▽ Published ahead of print on 25 June 2007.

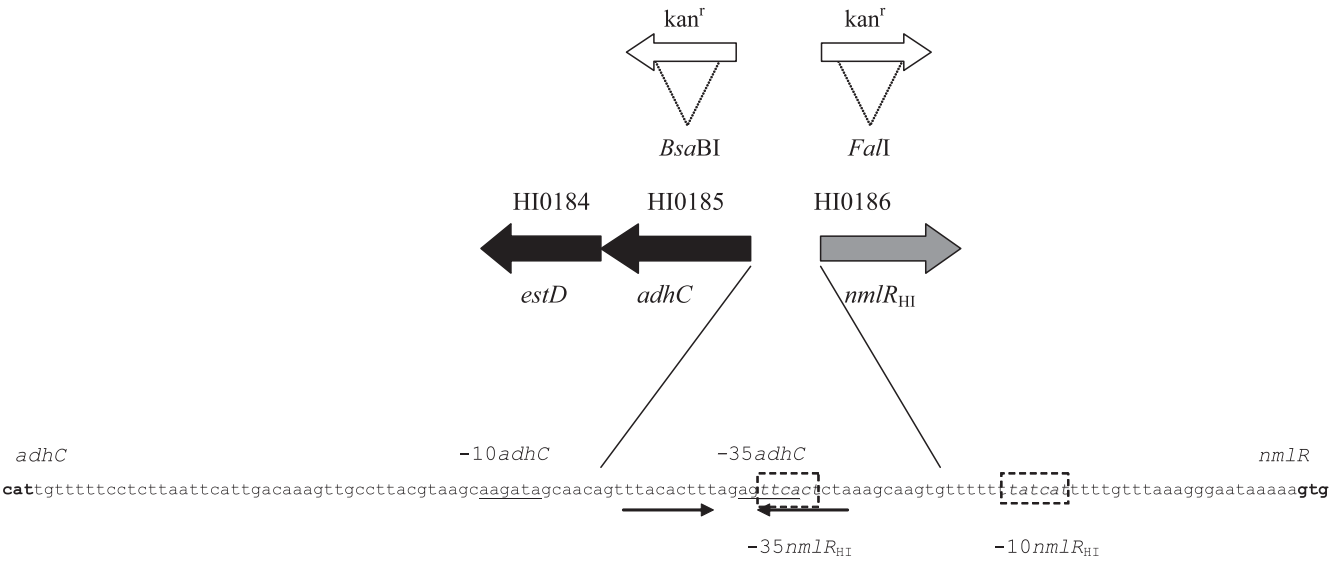


FIG. 1. Organization of the $nmlR_{HI}$ and *adhC* gene cluster of *H. influenzae*. The DNA sequence for the intergenic spacer region of *adhC-estD* (HI0185 and HI0184) to $nmlR_{HI}$ (HI0186) is shown. The −10 and −35 promoter elements for $P_{nmlR_{HI}}$ are boxed and annotated −10 $nmlR_{HI}$ and −35 $nmlR_{HI}$; for P_{adhC}, they are underlined and annotated −10 *adhC* and − 35 *adhC*. The dyad symmetry is indicated with arrows. The start of translation is in bold type.

III alcohol dehydrogenase (AdhC) has been shown to reduce GSNO (10, 22). In *S. enterica* serovar Typhimurium, it has been shown that Hmp plays the principle role in defense against NO, and the *hmp* mutant is avirulent in a mouse model system (1). AdhC from *Escherichia coli* has been shown to use GSNO as a substrate (22), but AdhC was not essential for virulence in *S. enterica* serovar Typhimurium (1).

Recently, we described a MerR-like regulator in *Neisseria gonorrhoeae* which we called *Neisseria M*erR-*l*ike *R*egulator (NmlR). We have established that NmlR regulates its own expression and that of a divergently transcribed gene, *adhC* (18). The *adhC* gene encodes a class III alcohol dehydrogenase that, in different systems, is known to possess NADH-GSNO oxidoreductase activity (22). A survey of bacterial genome sequences identified NmlR homologs in numerous bacteria, including several pathogens. NmlR forms a subfamily of the MerR family, which includes a member in *H. influenzae*, $NmlR_{HI}$. Here we describe experiments to investigate the regulation of *adhC* by $NmlR_{HI}$ and the function of AdhC in defense against nitrosative stress. The organization and function of this system were also compared in a collection of encapsulated and NTHi strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Thirty-three *H. influenzae* strains were used in this study. These strains included isolates from the Finnish otitis media collection as used in previous studies (3, 6, 16, 26) and encapsulated clinical isolates from the A. Smith laboratory (9). Brain heart infusion (BHI) medium was prepared with 3.7% (wt/vol) BHI Powder (Oxoid). For solid medium, 1.5% agar powder was added. Medium was sterilized by autoclaving at 121°C for 20 min. Levinthal blood (10% [wt/vol]) was added for solid medium. BHI broth required NAD^+ (2 μg/ml) and 10 μl/ml hemin solution (0.1% [wt/vol] hemin, 0.1% [wt/vol] L-histidine, 4% [vol/vol] triethanolamine). M-IV competence medium was prepared as described by Herriott et al. (14). Solutions for media were sterilized individually, either by filter sterilizing or by autoclaving. The solutions were mixed under sterile conditions. Chemically defined medium (CDM) was prepared as described by Coleman et al. (7). The final pH of CDM was adjusted to 7.56 by $NaHCO_3$. CDM was sterilized by filter sterilization through a 0.22-μm filter.

Chromosomal inactivation of *adhC* and $nmlR_{HI}$. A 1.3-kb DNA fragment containing $nmlR_{HI}$ and flanking DNA sequences was amplified by PCR from *H. influenzae* using the following primers: HI0186KOF (5′-ACTCACATTCGCCA CATTCA-3′) and HI0186KOR (5′-CACGGATTAAGCCTGTTCCT-3′). A 2.0-kb DNA fragment containing *adhC* and flanking DNA sequences was amplified by PCR from *H. influenzae* using the following primers: HI0185KOF (5′-CAGTG CCAATACCAATGCAC-3′) and HI0185KOR (5′-CGTTTTAATTCGGCAAT TTGA-3′). PCR was carried out at 96°C for 10 min, then 30 cycles of 96°C for 30 s, 55°C for 30 s, and 72°C for 3 min, followed by a final extension step of 72°C for 10 min. PCR products were purified (using a QIAquick PCR purification kit) and cloned into pGEM-T Easy Vector (Promega), creating the plasmids pGEM-T::$nmlR_{HI\text{-}ko}$ and pGEM-T::*adhC-ko*. These plasmids were digested with BsaBI and FalI (New England Biolabs), respectively (Fig. 1), and treated with calf intestinal alkaline phosphatase. The kanamycin-resistant gene cassette from pUC4Kan was isolated by digestion with HincII and extracted from a 1% agarose gel by using a QIAquick gel extraction kit (QIAGEN). This cassette has no promoter or terminator and does not effect transcription or create a polar effect. The kanamycin-resistant cassette was phosphorylated and cloned into the digested clones pGEM-T::$nmlR_{HI\text{-}ko}$ and pGEM-T::*adhC-ko*. The orientation of the insert was identified, and plasmids containing the kanamycin resistance open reading frame (ORF) in the same orientation as the genes were selected. The resulting plasmids, pGEM-T::$nmlR_{HI\text{-}ko}$::*kan* and pGEM-T::*adhC-ko*::*kan*, were linearized with NdeI and Sau96I, respectively, before being transformed into *H. influenzae* Rd KW20. Transformation into *H. influenzae* was performed as described by Herriott et al. (14). Competent *H. influenzae* was incubated with linearized DNA at 37°C for 30 min. Mutants were created by double crossover, and recombinant colonies were selected on BHI plates with 20 μg/ml kanamycin.

Site-specific recombination of *adhC*::Kan^r into the chromosome was confirmed by PCR amplification with HIadhCkOF-F (5′-AAAGGCTTTTTCTCCCC AAG-3′) and HIadhCkOF-R (5′-CAAACGTTTCGGGCCTAATA-3′). These primers are located outside the sites used for generating the original construct. Site-specific recombination of $nmlR_{HI}$::Kan^r was confirmed by PCR using a primer within the kanamycin resistance cassette Kan-F (5′-CGAGGCGGCGA TTAAATTCC-3′) and a primer located on the *H. influenzae* chromosome and outside the sites used to generate the original construct, HInmlRko-scr (5′-CA CGATCTTCCCCTAAATAG-3′).

A chromosomal preparation from the *H. influenzae adhC* gene was sheared by repeatedly passing it through a needle (23 gauge by 1.5 inches; Terumo Corp.). The sheared product was transformed into clinical isolates of *H. influenzae*. Site-specific recombination of *adhC*::Kan^r into the chromosome was confirmed

by PCR amplification with HIadhCkOF-F (5′-AAAGGCTTTTTCTCCCCAAG-3′) and HIadhCkOF-R (5′-CAAACGTTTCGGGCCTAATA-3′).

Reverse transcriptase PCR. RNA was extracted from *H. influenzae* Rd KW20 and *H. influenzae* $nmlR_{HI}$ strains by using a QIAGEN RNeasy minikit. RNA was quantified using an A_{260} reading. RNA was checked for DNA contamination by PCR, and no product was detected. Prior to the reverse transcriptase (RT) reaction, RNA was further treated to remove any residual DNA by using Promega DNase (Promega). The RT reaction was performed using a QIAGEN Omniscript reverse transcriptase kit. Following the RT reaction, a multiplex PCR was performed with primers for the 16S rRNA gene (16SFOR, 5′-AGTCCACGCCCTAAACGATGT-3′; 16SREV, 5′-TACTCCCCAGGCGGTCAAT-3′) and primers from *estD* (E1, 5′-CCCAAGGCTGCTCGGTC-3′) to *adhC* (A1, 5′-TTCAACGCGTCCGTTCCAA-3′). PCR was carried out with New England Biolabs *Taq* polymerase using an initial 96°C for 10 min followed by 30 cycles of 96°C for 45 s, 54°C for 45 s, and 72°C for 30 s and a final elongation step of 72°C for 10 min.

Growth assays. Growth assays were performed with *H. influenzae* strains using CDM. Cells were grown overnight in 5 ml of CDM and, prior to the assay, were measured at an optical density of 600 nm (OD_{600}; Hitachi U-3000 spectrophotometer) to standardize the amount of cells inoculated. From the overnight culture, 1 ml was inoculated into 50 ml of CDM in a 250-ml conical flask. Different specific concentrations of stress agent were added to the medium. Cultures were incubated aerobically at 37°C with shaking at 190 rpm. OD_{600} measurements were taken at different time points for 10 h. The assays were done in triplicate. Assay results were represented as growth curves over this period or, for clarity for the large set of clinical isolates, as percentages of survival at this time point.

GSNO reductase enzyme assays. NADH-dependent GSNO reductase activity was measured as previously described (22). Fresh overnight cultures of *H. influenzae* were inoculated into 100 ml of CDM in 500-ml conical flasks and grown aerobically at 37°C with shaking at 190 rpm until an OD_{600} measurement between 0.4 and 0.7 was obtained. The cells were harvested (5,000 × *g* at 4°C for 10 min) and washed twice with 0.1 M phosphate buffer (pH 7.0) before resuspension in 2 ml of phosphate buffer. The suspension was frozen at −80°C, thawed at room temperature, given a brief vortexing, and frozen again at −80°C. This freeze-thaw process was performed four more times before the cells were centrifuged at 13,000 × *g* at 4°C for 15 min. The final supernatant (cell extract) was used for assays. The total protein concentration of the supernatant was determined spectrophotometrically using the formula protein (mg/ml) = $1.55 \times A_{280} - 0.76 \times A_{260}$ (19). GSNO reductase activity was expressed as μmol of NADH oxidized per minute per mg of total protein. The assays were done in triplicate.

PCR-based sequence analysis of clinical isolates of *H. influenzae*. PCR using primers HIadhCko-scr and HInmlRko-scr was used to generate a DNA fragment covering the *adhC-nmlR*$_{HI}$ region of 33 clinical isolates of *H. influenzae*. PCR was carried out using Vent polymerase (New England Biolabs) and the following conditions: 96°C for 10 min and then 30 cycles of 96°C for 30 s, 55°C for 30 s, and 72°C for 3 min, followed by a final extension step of 72°C for 10 min. PCR products were sequenced using a BigDye Terminator v3.1 sequencing kit (Applied Biosystems).

Nucleotide sequence accession numbers. The nucleotide sequences have been submitted to GenBank under accession numbers EF523999 to EF524031.

RESULTS

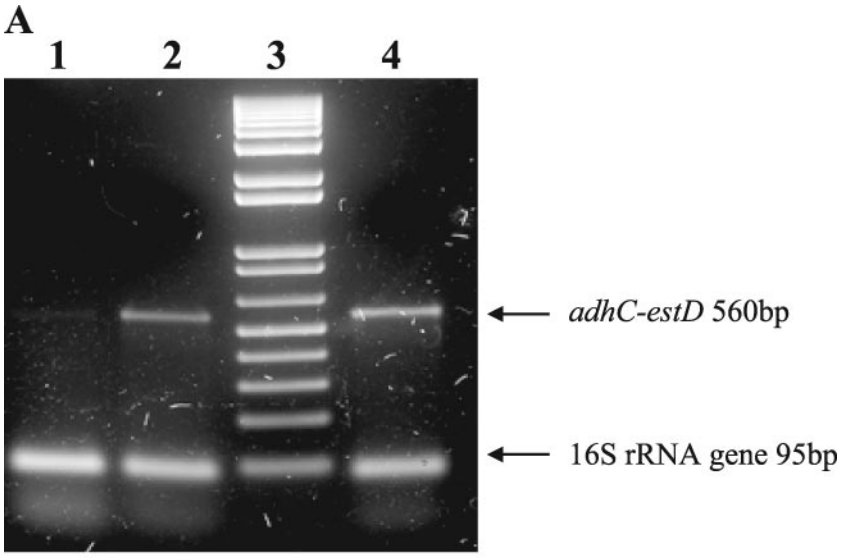
***H. influenzae* possesses an NmlR-like transcription factor.** A survey of the *H. influenzae* Rd KW20 genome sequence (11) showed that *H. influenzae* contained an NmlR homolog that we have named $NmlR_{HI}$ (HI0186; genome location, 200733 to 201140). $NmlR_{HI}$ shows high identity (93%) to the NmlR from *N. gonorrhoeae* (18). As in the *nmlR* system of *N. gonorrhoeae*, the $nmlR_{HI}$ gene is adjacent to a putative operon transcribed from the opposite DNA strand that is composed of two genes annotated as *adhC* and *estD*, which encode a class III alcohol dehydrogenase and an esterase, respectively. The promoters for the $nmlR_{HI}$ and *adhC-estD* cistrons are overlapping and divergent, with a dyad symmetry (the $NmlR_{HI}$ binding site), located between the promoter elements for *adhC-estD* (Fig. 1). This is consistent with the arrangement for the prototype for the MerR family, *merR-merTPAD*, and is present in other MerR family regulators (4). The predicted −10 (5/6 identity to the consensus sequence) and −35 (4/6) elements for the promoter for $nmlR_{HI}$ are separated by 18 bp. The operator/promoter for P_{adhC} fulfils the criteria for a MerR operator/promoter (4). The promoter elements for *adhC* (−10 with 4/6 and −35 with 3/6 identity to the consensus sequence) are separated by a nonoptimal spacer region of 19 bp. Overlapping with the −35 element and extending within the P_{adhC} spacer is a 9-2-9 dyad symmetry.

$NmlR_{HI}$ regulates the *adhC-estD* operon. We have already established that in *N. gonorrhoeae*, NmlR regulates the expression of the *adhC-estD* operon (18). Using primers from within the *estD* and *adhC* genes of *H. influenzae*, we first performed RT-PCR. This generated a 560-bp product, which was the expected size if the mRNA derived from *adhC* and *estD* was contiguous (Fig. 2A, lane 2). RT-PCR was then performed using RNA extracted from the *H. influenzae* wild type and the $nmlR_{HI}$ mutant and, using known RNA concentrations for the RT reaction and a multiplex PCR with a 16S rRNA gene as a standard, the yields of the 560-bp *adhC-estD* RT-PCR products were compared. Figure 2 shows that there was significantly more of the *adhC-estD* RT-PCR product from *H. influenzae* (Fig. 2A, lane 2) than from the $nmlR_{HI}$ mutant (Fig. 2A, lane 1). Thus, we concluded that the *adhC-estD* genes form an operon that requires $NmlR_{HI}$ for maximal expression.

In silico promoter analysis. Promoters within the *adhC-nmlR* intergenic spacer regions of the NmlR subfamily from a number of bacteria were identified using BPROM software available from Softberry (Mount Kisco, NY). The predicted binding sites for the $NmlR_{HI}$ subfamily were then identified and aligned (Fig. 2B). From this alignment, a consensus binding site for $NmlR_{HI}$ transcription factors was formulated using WebLogo (http://weblogo.berkeley.edu/logo.cgi) (Fig. 2C). This was used to search the whole-genome sequence of *H. influenzae* Rd KW20, using both PRODORIC, release 2, Virtual Fooprint Web-based facility (http://prodoric.tu-bs.de/), and Predict Regulon Server software (41). Both of these programs resulted in the sole identification of the promoter region for HI0185 (*adhC*). The next best scores were investigated as possible binding sites with lower identity to the consensus sequence, but none contained a dyad symmetry or was located in the promoter elements. Taken together, these data suggest that $NmlR_{HI}$ is a specific regulator of the *adhC-estD* operon and does not act as a global regulator of gene expression.

***H. influenzae* Rd KW20 AdhC exhibits NADH-dependent GSNO reductase activity.** Class III alcohol dehydrogenases have been shown to catalyze the reduction of GSNO using NADH as an electron donor (22). We tested whether this activity was also associated with AdhC in *H. influenzae* by determining NADH-GSNO oxidoreductase activity in cell extracts. Figure 3A shows that the rate of GSNO-dependent oxidation of NADH in the $nmlR_{HI}$ mutant was more than 10-fold lower than in wild-type cells. In the *adhC* mutant strain, most of the GSNO reductase activity was reduced compared to that of both the $nmlR_{HI}$ mutant and the wild type. This indicates that AdhC is responsible for the NADH-dependent GSNO reductase activity in *H. influenzae*.

***H. influenzae* Rd KW20 genes *adhC* and $nmlR_{HI}$ are required for defense against GSNO.** Following the confirmation that



B

```
Hi RdKW20     CTTTAGAGTTCACTCTAAAG
Hi 86-028NP   CTTTAGAGTTCACTCTAAAG
Hi R2866      CTTTAGAGCTCTCTCTAAAG
Hi R2846      CTTTAGAGTTCACTCTAAAG
Ng PadhC      CTTAGAGTGAACTCTAAA
Ng PcopA      TTTCGGATTCTACCCGAA
Lac.plant     CCTTAGACAACACTCTAAGG
En.faec      AACTTGACTTTAGGTCAAGT
Cl.aceto     ACTTGAGAGTGTACTCTAAGT
Oc.ihey      ACTTAGAGTTAACTATAACGT
Str.pn       ACTTGGAGTCAACTCAAAGT
Str.aga      TCTTGACTTGAAGTTAACTCTAAGA
NMA           CTTTAGAGTTAACTCTAAAG
```

C

FIG. 2. Regulation of the *adhC-estD* operon. (A) Analysis of transcription of *adhC-estD* using RT-PCR. Template RNA from *H. influenzae* Rd KW20 (lane 2) and *H. influenzae* $nmlR_{HI}$ (lane 1). RNA size marker (lane 3) and the PCR product using genomic DNA (lane 4). (B) Consensus NmlR binding site. The binding sites as determined by in silico analysis for *H. influenzae* strains are as follows: Rd KW20 (Hi RdKW20) (GenBank accession number L42023; nucleotide coordinates 200673–200692), R2846 (accession number AADO0000000; coordinates 86235–86258), R2866 (accession number AADP0000000; coordinates 46407–46387), 86-028NP (accession number CP000057; coordinates 261657–261638), *Lactobacillus plantarum* (Lac.plant) (accession number CR377164; coordinates 2681213–2681231), *Enterococcus faecium* (En.faec) (accession number AAAK00000000; coordinates 9495–9515), *Clostridium acetobutylicum* (Cl.aceto) (accession number AE001437.1; coordinates 113616–113636), *Oceanobacillus iheyensis* (Oc.ihey) (accession number BA000028; coordinates 841653–841673), *Streptococcus pneumoniae* (Str.pn) (accession number AE008532; coordinates 9226–9245), *Streptococcus agalactiae* (Str.aga) (accession number AE0014211; coordinates 10838–10861), and *Neisseria meningitidis* (NMA) (accession number AL162756.2; coordinates 80451–80471), as well as the two proven binding sites by genetic studies for NmlR in *N. gonorrhoeae* P_{adhC} (Ng P*adhC*) and P_{copA} (Ng P*copA*) (18). The dyad symmetries are underlined. (C) WebLogo consensus sequence for the binding site for NmlR.

AdhC catalyzed the removal of GSNO, we tested whether *adhC* and $nmlR_{HI}$ were of significance for defense against nitrosative stress in *H. influenzae*. Figure 3B shows a series of growth curves in which wild-type and mutant strains were challenged with GSNO. For the wild-type *H. influenzae* strain, the presence of 0.5 mM GSNO restricted growth during the first 4 h. However, after this time point, the growth rate increased, and cells reached a density that was close to that seen for

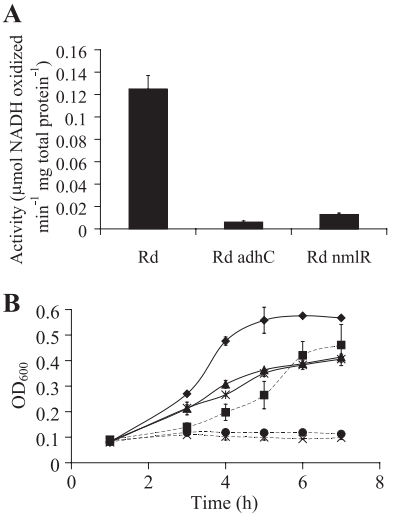


FIG. 3. Roles for *H. influenzae*, *H. influenzae adhC*, and *H. influenzae nmlR*$_{HI}$ in GSNO metabolism. (A) *S*-Nitrosoglutathione reductase activities of *H. influenzae* strains Rd KW20 wild type (Rd), Rd KW20 *adhC* (Rd adhC), and Rd KW20 *nmlR*$_{HI}$ (Rd nmlR). *Y*-axis error bars indicate ±1 standard deviation of the mean. Each assay experiment was conducted in triplicate and repeated with separated bacterial cultures for both strains. (B) Effect of GSNO on *H. influenzae*, *H. influenzae adhC*, and *H. influenzae nmlR*$_{HI}$. Wild-type strain Rd KW20 was grown in CDM (◆) as were Rd KW20 *adhC* (X) and Rd KW20 *nmlR*$_{HI}$ (▲). Results are shown alongside results for each strain grown in the presence of 0.5 mM GSNO, shown as dotted lines (Rd KW20 [■] Rd KW20 *adhC* [●] and Rd KW20 *nmlR*$_{HI}$ [X]. *Y*-error bars indicate ±1 standard deviation of the mean. Experiments were conducted in triplicate.

wild-type cells in the absence of the GSNO. In contrast, the presence of 0.5 mM GSNO abolished growth of the *adhC* and *nmlR*$_{HI}$ strains. We also tested an *estD* strain for GSNO reductase activity and its ability to grow in the presence of GSNO. It grew with the same profile as the wild type and possessed similar levels of GSNO reductase activity, indicating that the phenotype of the *adhC* strain is not due to effects on *estD* expression (S. P. Kidd, M. P. Jennings, and A. G. McEwan, unpublished data). Interestingly, even in the absence of nitrosative stress, the *adhC* and *nmlR*$_{HI}$ mutants grew slower than the wild-type *H. influenzae* strain (Fig. 3B.).

The genome sequences of NTHi harbor the *adhC-nmlR*$_{HI}$ system. Initial in silico analysis identified the *nmlR*$_{HI}$-*adhC* system within various strains of *H. influenzae*, including the NTHi strains R2866, R2846, and 86-028NP. When these strains of *H. influenzae* were analyzed, their systems showed considerable identity to the system in strain Rd KW20, with some exceptions. In strain 86-028NP, *adhC* has been annotated as a pseudogene. It contains a mutation that results in a premature stop codon terminating the protein at amino acid 109, suggesting that this strain might not make a functional AdhC. There were also polymorphisms observed in the *adhC-nmlR*$_{HI}$ intergenic spacer region, including an impaired dyad symmetry and −10 and −35 promoter elements in strain R2866. These differences between the nontypeable genome strains and our previous observation that *adhC* of *N. gonorrhoeae* is a pseudogene (31) suggested the possibility that the role in defense against GSNO demonstrated in strain Rd KW20 (as described above) may not be representative of *H. influenzae* clinical isolates.

Clinical isolates of *H. influenzae* have a functional *adhC* gene as part of their GSNO defense mechanisms. A collection of 33 clinical isolates of *H. influenzae* was used to measure the activity and genetic variation of the *adhC-nmlR*$_{HI}$ system. A region covering the *adhC* and *nmlR*$_{HI}$ genes and intergenic spacer region was sequenced and aligned. The *adhC* gene of strain 86-028NP has a premature stop codon. No other strain contained this mutation. There were some variations in the operator/promoter region of the different strains. Many strains (NTHi strains 1247, 1207, 1181, 1124, 723, and 667 and capsular strains R539, R540, R543, R546, R1831, R3368, R3230, R874, and RM7004 and the Eagan strain) included a mutation which resulted in an altered −10 element (less optimal, 4/6 consensus, and shortened spacer region) for the $P_{nmlR_{HI}}$ compared to that of the Rd KW20 strain. Strain R2866 had a shortened dyad symmetry, and strain 176 (an NTHi strain) had a mutation which interrupted the dyad symmetry.

Enzyme assays were performed using representative strains of each of the variations, as well as clinical isolates of both NTHi and capsular strains, which were identical to Rd KW20 (strains 981, 447, R541, and R3327). Strain 86-028NP had very low GSNO reductase activity, close to 10-fold less than that of Rd KW20, consistent with the prediction that *adhC* from 86-028NP is a nonfunctional gene (Fig. 4). Strain R2866 also exhibited very low GSNO reductase activity, presumably as a result of a nonoptimal operator/promoter region (as noted above). Although there was some variation in the activities of GSNO reductase in the other strains tested, the majority of strains had activity levels similar to that of Rd KW20. The exceptions were strain 176, which had twofold higher activity than Rd KW20, and strain 723, which had an activity level that was lower than that of Rd KW20 but higher than those of 86-028NP and R2866.

In order to assess whether there was any correlation between GSNO reductase activity and defense against nitrosative stress, we measured the ability of each strain to survive a GSNO challenge. Figure 4 shows that the three strains with the lowest GSNO reductase activities exhibited a low percentage of survival in comparison to that of strain Rd KW20 and the other strains with high GSNO reductase activity. The correlation between GSNO reductase activity and survival against nitrosative stress was not as clear in strain R541 or R3327 (Fig. 4). Chromosomal inactivation of *adhC* from representative strains of the clinical strains R539, R874, and 667 resulted in a significant reduction of GSNO reductase activity (data not shown).

DISCUSSION

Class III alcohol dehydrogenases are pyridine nucleotide-dependent enzymes that catalyze the transformation of adducts of glutathione such as hydroxymethylglutathione or GSNO. The GSNO-metabolizing activity of GSH-dependent alcohol dehydrogenases has been known for some time (17), but it was Liu and coworkers who recognized the widespread distribution of this enzyme in both the eukaryotic and the prokaryotic world and proposed that GSNO reductase activity of this enzyme is physiologically relevant (22). Although the phenotype of an *adhC* mutant in *S. enterica* serovar Typhi-

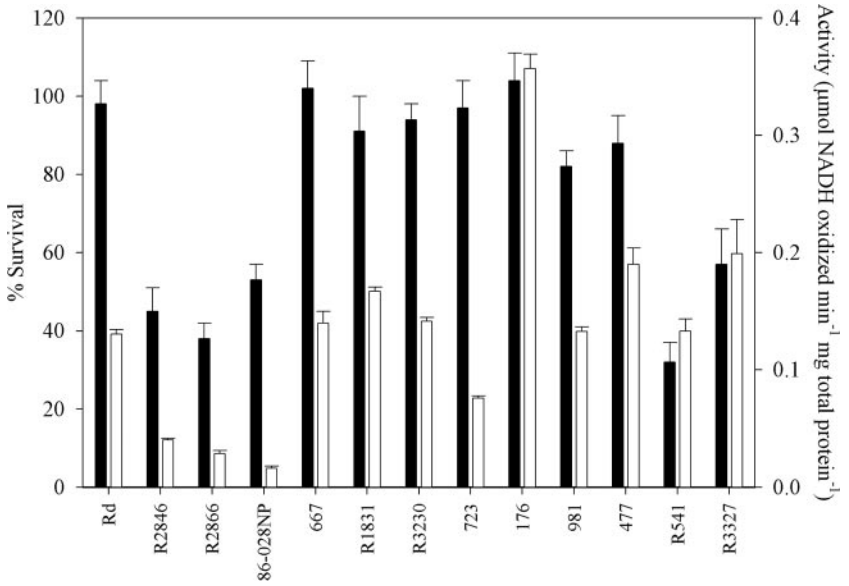


FIG. 4. Effect of GSNO on clinical isolates of *H. influenzae*. *S*-Nitrosoglutathione reductase activities of *H. influenzae* strains are shown with filled bars. *Y*-error bars indicate ±1 standard deviation of the mean. Each enzyme assay experiment was conducted in triplicate and repeated with separated bacterial cultures for both strains. The sensitivities of clinical isolates of *H. influenzae* to GSNO are shown with open bars. Separate cultures were grown in CDM and CDM with 0.5 mM GSNO. The growth was followed over 18 h, and the results are presented as the percentages of CFU/ml in 0.5 mM GSNO relative to that of CDM alone. *Y*-error bars indicate ±1 standard deviation of the mean. Each assay experiment was conducted in triplicate.

murium has not been described in detail, it is known that *adhC* is not required for systemic virulence of *S. enterica* serovar Typhimurium in a mouse model system (1). This may mean that *adhC* is not involved in defense against NO in *S. enterica* serovar Typhimurium or that there is redundancy in NO defense systems, consistent with the presence of a number of enzymes involved in protection against NO. While mechanisms for defense against ROS by *H. influenzae* have been described previously (40), *H. influenzae* does not possess all of the enzymes that have been shown to be involved in NO protection from *S. enterica* serovar Typhimurium. It does possess AdhC. Like AdhC from eukaryotic and bacterial sources, the *H. influenzae* enzyme is able to reduce GSNO. However, for the first time, the results presented in this report suggest that this activity is physiologically relevant, acting as a key enzyme in defense against RNS. Our observation that the *adhC* mutant was sensitive to nitrosative stress is strong evidence that AdhC has a role in defense against RNS killing in *H. influenzae*. The present result and our recent observation that *adhC-nmlR* is required for systemic virulence in *S. pneumoniae* (38a) suggest that this is an important system for defense against nitrosative stress in nonenteric mucosal pathogens.

Class III alcohol dehydrogenases have been characterized in a number of bacteria, including *Rhodobacter sphaeroides* and *Paracoccus denitrificans* (2, 33). Both of these bacteria are facultative methylotrophs; thus, it is essential that they are able to defend against toxic formaldehyde. In these bacteria, there is clear evidence that the AdhC functions as an NAD^+-dependent dehydrogenase that oxidizes hydroxymethylglutathione (formed from the reaction of formaldehyde and GSH). A mutant lacking *adhC* in *R. sphaeroides* was highly sensitive to killing by methanol, indicative of an inability to remove formaldehyde. In *E. coli*, the expression of *adhC* is controlled by the FrmR transcriptional repressor that has been shown to respond to formaldehyde but not to GSNO (13). Inspection of the genomic organization of the *adhC* gene cluster in *S. enterica* serovar Typhimurium suggests a similar pattern of regulation, although no analysis of gene expression has been reported. In the case of *R. sphaeroides*, the expression of *adhC* in response to formaldehyde is positively and negatively regulated by two sensor histidine kinase-response regulator two-component systems (15). These data support the conclusion that in enteric bacteria and in facultative methylotrophs, the expression of *adhC* is associated with defense against aldehyde stress. There were no differences in sensitivity to formaldehyde between the *H. influenzae* strain Rd KW20 and the *adhC* strain (data not shown).

A key difference between the *adhC* system in *H. influenzae* and that in *E. coli* appears to be in regulation of their expression. In *H. influenzae*, there is no FrmR repressor, and the complex sensor histidine kinase-response regulator system that regulates expression of *adhI* in response to methanol/formaldehyde in *R. sphaeroides* also appears to be absent. Instead, regulation is via $NmlR_{HI}$, and the *nmlR-adhC* gene pair is found in a number of bacteria, including *N. gonorrhoeae*, for which it was first described (18). In the pathogen *Neisseria*, NmlR is the sole MerR-like regulator, and there are three regulatory targets, *adhC*, *trxB*, and *copA* (18). In contrast, *H. influenzae* harbors three MerR-like regulators. Analysis of the binding sites for NmlR homologs from the bacteria which harbor an *nmlR-adhC* pair has identified a significant degree of identity between the dyad symmetries of these predicted binding sites, and a consensus sequence has been formulated (Fig. 2). Our analysis predicts $NmlR_{HI}$ has only one target within the

H. influenzae genome, the divergent *adhC* gene (Fig. 1). The $NmlR_{HI}$-AdhC system is distinctive from the previously published NmlR system of *N. gonorrhoeae*, not only in its regulon but also because, as we have recently reported for *N. gonorrhoeae*, the *adhC* is nonfunctional (31). Together with the difference in the genes that NmlR and $NmlR_{HI}$ regulate, these distinctive features may indicate differences in the signal to which these NmlR regulators respond. We have not yet identified the stress signal to which $NmlR_{HI}$ responds. The transcription factor contains two cysteine residues that are conserved in all proteins of the NmlR subfamily, except for those in streptococci (Stroeher et al., submitted). It is tempting to suggest that the activation of $NmlR_{HI}$ may involve oxidation of these cysteine residues, as observed for a number of other transcription factors (30). The trigger for this may be disulfide stress, although this needs to be tested in future experiments. It is also possible, considering the role for GSNO, that the cysteine residues are nitrosalated and that this is acting as the switch to an activator form for $NmlR_{HI}$.

Analysis of *adhC* from the genome sequences of nontypeable strains 86-028NP, R2846, and R2866 indicated variations that might result in nonfunctional AdhC (strain 86-028NP) or reduction of *adhC* expression (strain R2866). This casts doubt on the importance of *adhC* in GSNO defense as a general mechanism in *H. influenzae.* Assays of AdhC expression and GSNO defense in a set of 33 clinical isolates confirmed that these genome strains were atypical, with all other strains expressing high levels of AdhC activity that correlated well with GSNO defense. Chromosomal inactivation of *adhC* from a selection of these clinical strains resulted in a significant loss of GSNO reductase activity.

Our study suggests that the *adhC-nmlR*$_{HI}$ system may be important for virulence in *H. influenzae*. NO is recognized as a key component of the innate immune response to bacterial pathogens, particularly the niches inhabited by *H. influenzae*. Specifically with the ear, nose, and throat, different studies have shown that NO is produced in response to infection and that NO functions in restricting the spread of bacteria from this niche (20). Also, relevant studies have shown the survival of *H. influenzae* in macrophage lines and adenoid tissues and have identified NO production and the antimicrobial functions of NO in otitis media (8, 20, 23, 25, 27). A study which used *H. influenzae* to examine bacterial meningitis in rats has shown that NO was important in increasing the blood-brain barrier permeability, although it did not investigate the antimicrobial activity of NO (5). Implicit in these results but not elaborated is that while increasing NO levels have a role in bacterial passage from the blood to the brain, maybe through altering cerebral blood flow or brain edema or other consequences of subarachnoid space inflammation, the bacteria need to be able to defend against the levels of NO being produced. Our studies identify the *adhC-nmlR*$_{HI}$ system as the major mechanism in *H. influenzae* for defense against GSNO, a stress which is physiologically relevant in a host environment producing NO, and show that this system is functional in most clinical isolates.

ACKNOWLEDGMENT

This work was supported by program grant 284214 from the National Health and Medical Research Council of Australia to M.P.J. and A.G.M.

REFERENCES

1. **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.
2. **Barber, R. D., and T. J. Donohue.** 1998. Function of a glutathione-dependent formaldehyde dehydrogenase in *Rhodobacter sphaeroides* formaldehyde oxidation and assimilation. Biochemistry **37:**530–537.
3. **Bolduc, G. R., V. Bouchet, R.-Z. Jiang, J. Geisselsoder, Q. C. Truong-Bolduc, P. A. Rice, S. I. Pelton, and R. Goldstein.** 2000. Variability of outer membrane protein P1 and its evaluation as a vaccine candidate against experimental otitis media due to nontypeable *Haemophilus influenzae*: an unambiguous, multifaceted approach. Infect. Immun. **68:**4505–4517.
4. **Brown, N. L., J. V. Stoyanov, S. P. Kidd, and J. L. Hobman.** 2003. The MerR family of transcriptional regulators. FEMS Microbiol. Rev. **27:**145–163.
5. **Buster, B. L., A. C. Weintrob, G. C. Townsend, and W. M. Scheld.** 1995. Potential role of nitric oxide in the pathophysiology of experimental bacterial meningitis in rats. Infect. Immun. **63:**3835–3839.
6. **Cody, A. J., D. Field, E. J. Feil, S. Stringer, M. E. Deadman, A. G. Tsolaki, B. Gratz, V. Bouchet, R. Goldstein, D. W. Hood, and E. R. Moxon.** 2003. High rates of recombination in otitis media isolates of non-typeable *Haemophilus influenzae*. Infect. Gen. Evol. **3:**57–66.
7. **Coleman, H. N., D. A. Daines, J. Jarisch, and A. L. Smith.** 2003. Chemically defined media for growth of *Haemophilus influenzae* strains. J. Clin. Microbiol. **41:**4408–4410.
8. **Craig, J. E., A. Cliffe, K. Garnett, and N. J. High.** 2001. Survival of nontypeable *Haemophilus influenzae* in macrophages. FEMS Microbiol. Lett. **203:**55–61.
9. **Erwin, A. L., K. L. Nelson, T. Mhlanga-Mutangadura, P. J. Bonthuis, J. L. Geelhood, G. Morlin, W. C. T. Unrath, J. Campos, D. W. Crook, M. M. Farley, F. W. Henderson, R. F. Jacobs, K. Muhlemann, S. W. Satola, L. van Alphen, M. Golomb, and A. L. Smith.** 2005. Characterization of genetic and phenotypic diversity of invasive nontypeable *Haemophilus influenzae*. Infect. Immun. **73:**5853–5863.
10. **Fang, F. C.** 2004. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat. Rev. Microbiol. **2:**820–832.
11. **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, K. McKenney, G. Sutton, W. Fitzhugh, C. Fields, J. D. Gocayne, J. Scott, R. Shirley, L. I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
12. **Gantt, K. R., T. L. Goldman, M. L. McCormick, M. A. Miller, S. M. B. Jeronimo, E. T. Nascimento, B. E. Britigan, and M. E. Wilson.** 2001. Oxidative responses of human and murine macrophages during phagocytosis of *Leishmania chagasi*. J. Immunol. **167:**893–901.
13. **Herring, C. D., and F. R. Blattner.** 2004. Global transcriptional effects of a suppressor tRNA and the inactivation of the regulator *frmR*. J. Bacteriol. **186:**6714–6720.
14. **Herriott, R. M., E. M. Meyer, and M. Vogt.** 1970. Defined nongrowth media for stage II development of competence in *Haemophilus influenzae*. J. Bacteriol. **101:**517–524.
15. **Hickman, J. W., V. C. Witthuhn, Jr., M. Dominguez, and T. J. Donohue.** 2004. Positive and negative transcriptional regulators of glutathione-dependent formaldehyde metabolism. J. Bacteriol. **186:**7914–7925.
16. **Hood, D. W., K. Makepeace, M. E. Deadman, R. F. Rest, P. Thibault, A. Martin, J. C. Richards, and E. R. Moxon.** 1999. Sialic acid in the lipopolysaccharide of *Haemophilus influenzae*: strain distribution, influence on serum resistance and structural characterization. Mol. Microbiol. **33:**679–692.
17. **Jensen, D. E., G. K. Belka, and G. C. Du Bois.** 1998. S-Nitrosoglutathione is a substrate for rat alcohol dehydrogenase class III isoenzyme. Biochem. J. **331:**659–668.
18. **Kidd, S. P., A. J. Potter, M. A. Apicella, M. P. Jennings, and A. G. McEwan.** 2005. NmlR of *Neisseria gonorrhoeae:* a novel redox responsive transcription factor from the MerR family. Mol. Microbiol. **57:**1676–1689.
19. **Layne, E.** 1957. Spectrophotometric and turbidimetric methods for measuring proteins, vol. 3, p. 447–454. Academic Press, New York, NY.
20. **Lee, H., A. Andalibi, P. Webster, S.-K. Moon, K. Teufert, S.-H. Kang, J.-D. Li, M. Nagura, T. Ganz, and D. J. Lim.** 2004. Antimicrobial activity of innate immune molecules against *Streptococcus pneumoniae*, *Moraxella catarrhalis* and nontypeable *Haemophilus influenzae*. BMC Infect. Dis. **4:**12.
21. **Lepoivre, M., F. Fieschi, J. Coves, L. Thelander, and M. Fontecave.** 1991. Inactivation of ribonucleotide reductase by nitric oxide. Biochem. Biophys. Res. Commun. **179:**442–448.
22. **Liu, L., A. Hausladen, M. Zeng, L. Que, J. Heitman, and J. S. Stamler.** 2001. A metabolic enzyme for *S*-nitrosothiol conserved from bacteria to humans. Nature **410:**490–494.
23. **Long, J. P., H. H. Tong, P. A. Shannon, and T. F. DeMaria.** 2003. Differential

expression of cytokine genes and inducible nitric oxide synthetase induced by opacity phenotype variants of *Streptococcus pneumoniae* during acute otitis media in the rat. Infect. Immun. **71:**5531–5540.
24. **Lushchak, V. I.** 2001. Oxidative stress and mechanisms of protection against it in bacteria. Biochemistry **66:**476–489.
25. **Marrs, C. F., G. P. Krasan, K. W. McCrea, D. L. Clemans, and J. R. Gilsdorf.** 2001. *Haemophilus influenzae*: human specific bacteria. Front. Biosci. **6:**e41–e60.
26. **Meats, E., E. J. Feil, S. Stringer, A. J. Cody, R. Goldstein, J. S. Kroll, T. Popovic, and B. G. Spratt.** 2003. Characterization of encapsulated and noncapsulated *Haemophilus influenzae* and determination of phylogenetic relationships by multilocus sequence typing. J. Clin. Microbiol. **41:**1623–1636.
27. **Melhus, A., and A. F. Ryan.** 2000. Expression of cytokine genes during pneumococcal and nontypeable *Haemophilus influenzae* acute otitis media in the rat. Infect. Immun. **68:**4024–4031.
28. **Orman, K. L., J. L. Shenep, and B. K. English.** 1998. Pneumococci stimulate the production of the inducible nitric oxide synthase and nitric oxide by murine macrophages. J. Infect. Dis. **178:**1649–1657.
29. **Pacelli, R., D. A. Wink, J. A. Cook, M. C. Krishna, W. DeGraff, N. Friedman, M. Tsokos, A. Samuni, and J. B. Mitchell.** 1995. Nitric oxide potentiates hydrogen peroxide-induced killing of *Escherichia coli*. J. Exp. Med. **182:** 1469–1479.
30. **Paget, M. S. B., and M. J. Buttner.** 2003. Thiol-based regulatory switches. Annu. Rev. Genet. **37:**91–121.
31. **Potter, A. J., S. P. Kidd, M. P. Jennings, and A. G. McEwan.** 2007. Evidence for distinctive mechanisms of *S*-nitrosoglutathione metabolism by AdhC in two closely related species, *Neisseria gonorrhoeae* and *Neisseria meningitidis*. Infect. Immun. **75:**1534–1536.
32. **Rao, V. K., G. P. Krasan, D. R. Hendrixson, S. Dawid, and J. W. St. Geme.** 1999. Molecular determinants of the pathogenesis of disease due to nontypeable *Haemophilus influenzae*. FEMS Microbiol. Rev. **23:**99–129.
33. **Ras, J., P. Van Ophem, W. Reijnders, R. Van Spanning, J. Duine, A. Stouthamer, and N. Harms.** 1995. Isolation, sequencing, and mutagenesis of the gene encoding NAD- and glutathione-dependent formaldehyde dehydrogenase (GD-FALDH) from *Paracoccus denitrificans*, in which GD-FALDH is essential for methylotrophic growth. J. Bacteriol. **177:**247–251.
34. **Sato, K., C. L. Liebeler, M. K. Quartey, C. T. Le, and G. S. LGeibink.** 1999. Middle ear fluid cytokine and inflammatory cell kinetics in the chinchilla otitis media model. Infect. Immun. **67:**1943–1946.
35. **Schapiro, J. M., S. J. Libby, and F. C. Fang.** 2003. Inhibition of bacterial DNA replication by zinc mobilization during nitrosative stress. Proc. Natl. Acad. Sci. USA **100:**8496–8501.
36. **Stevanin, T. M., N. Ioannidis, C. E. Mills, S. O. Kim, M. N. Hughes, and R. K. Poole.** 2000. Flavohemoglobin Hmp affords inducible protection for *Escherichia coli* respiration, catalyzed by cytochromes bo′ or bd, from nitric oxide. J. Biol. Chem. **275:**35868–35875.
37. **St. Geme, J. W.** 2002. Molecular and cellular determinants of non-typeable *Haemophilus influenzae* adherence and invasion. Cell. Microbiol. **4:**191–200.
38. **Storz, G., and J. A. Imlay.** 1999. Oxidative stress. Curr. Opin. Microbiol. **2:**188–194.
38a. **Stroeher, U. H., S. P. Kidd, S. L. Stafford, M. P. Jennings, J. C. Paton, and A. G. McEwan.** A pneumococcal MerR-like regulator and *S*-nitrosoglutathione reductase are required for systemic virulence. J. Infect. Dis., in press.
39. **Tong, H. H., Y. Chen, M. James, J. Van Deusen, D. B. Welling, and T. F. DeMaria.** 2001. Expression of cytokine and chemokine genes by human middle ear epithelial cells induced by formalin-killed *Haemophilus influenzae* or its lipooligosaccharide *htrB* and *rfaD* mutants. Infect. Immun. **69:**3678–3684.
40. **Vergauwen, B., F. Pauwels, and J. J. Van Beeumen.** 2003. Glutathione and catalase provide overlapping defenses for protection against respiration-generated hydrogen peroxide in *Haemophilus influenzae*. J. Bacteriol. **185:** 5555–5562.
41. **Yelboina, S., J. Seshadri, M. S. Kumar, and A. Ranjan.** 2004. PredictRegulon: a web server for the prediction of the regulatory protein binding sites and operons in prokaryote genomes. Nucleic Acids Res. **32:**W318–W320.

Editor: F. C. Fang