MAJOR ARTICLE

A Pneumococcal MerR-Like Regulator and *S*-nitrosoglutathione Reductase Are Required for Systemic Virulence

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A transcriptional regulator, $NmlR_{sp}$, has been identified in *Streptococcus pneumoniae* that is required for defense against nitric oxide (NO) stress. The $nmlR_{sp}$ gene is cotranscribed with *adhC*, which encodes an alcohol dehydrogenase that is able to reduce *S*-nitrosoglutathione (GSNO) with NADH as reductant. $nmlR_{sp}$ and *adhC* mutants exhibited a reduced level of NADH-GSNO oxidoreductase activity and were more susceptible to killing by NO than were wild-type cells. Comparison of the virulence of wild-type and mutant strains by use of a mouse model system showed that $NmlR_{sp}$ and AdhC do not play a key role in the adherence of pneumococci to the nasopharynx in vivo. An intraperitoneal challenge experiment revealed that both $NmlR_{sp}$ and AdhC were required for survival in blood. These data identify novel components of a NO defense system in pneumococci that are required for systemic infection.

Although *Streptococcus pneumoniae* is a commensal bacterium of the nasopharynx, it is also an important cause of human disease. A key event in pneumococcal pathogenesis is migration from the nasopharynx to the lungs, where the bacterium causes pneumonia. Subsequently, the organism enters the blood, causing bacteremia. NO is recognized as an important mediator of the innate immune response to infection with many microorganisms [1]. Recently, it has been shown that, after phagocytosis of pneumococci, NO contributes to the decline in the viability of internalized bacteria [2] and that NO has direct antibacterial activity [3].

To date, no mechanism has been proposed to explain how the pneumococcus is able to withstand challenge with NO. This contrasts with other pathogens, such as *Salmonella enterica* ser. Typhimurium, for which defense mechanisms against NO have been described [4]. Enzymes involved in this defense include hemoproteins and respiratory enzymes with NO reductase activity; these are absent from *S. pneumoniae*, which does not possess a respiratory chain, as are key regulators of the response to NO in enteric bacteria, NorR [5] and NsrR [4].

We recently characterized a novel regulator of gene expression in *Neisseria gonorrhoeae*, NmlR (the *Neisseria merR*-like regulator), which was proposed to control a defense system to cope with disulfide stress caused by the action of electrophiles, including NO. This regulator controlled the expression of a class 3 alcohol dehydrogenase, AdhC, which is known to catalyse the reduction of *S*-nitrosoglutathione (GSNO) in microorganisms and humans [6]. Here, we report the first description of an NO defense system based on an NmlR-like regulator and AdhC in *S. pneumoniae* and assess its importance in systemic disease.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *S. pneumoniae* virulent type 2 strain D39 [7] was grown overnight on blood agar and inoculated into either serum broth (meat-extract broth supplemented with 10% horse serum)

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or Todd-Hewitt broth with 0.5% yeast extract (THY broth) for growth before inoculation or challenge. D39 was transformed as described elsewhere [8, 9], and transformants were selected on blood agar containing 0.2 μg/mL erythromycin.

DNA manipulations. Chromosomal DNA was extracted and purified using the Wizard Genomic DNA Purification Kit (Promega) in accordance with to the manufacturer's instructions, except that cell lysis was induced with 0.1% (wt/vol) deoxycholate and by incubation at 37°C for 10 min. Polymerase chain reaction (PCR) amplification was performed in a Hybaid Touchdown thermal cycler, and the 50-μL reaction volume contained PCR buffer (1.5 mmol/L $MgCl_2$, 10 mmol/L Tris [pH 8.4], 50 mmol/L KCl, and 100 μg/mL gelatine), 1.5 U of *Taq* polymerase, 1 μmol/L each primer, 100 ng of DNA template, and 200 μmol/L each of the 4 dNTPs. Amplification involved 25 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and elongation at 72°C for 1–4 min.

Construction of* nmlR$_{sp}$ *and* adhC *mutants. Deletion-replacement mutations in *nmlR*$_{sp}$ and *adhC* were constructed using overlap-extension PCR mutagenesis and direct transformation, essentially as described elsewhere [10]. The oligonucleotides used for first round PCR amplification of *nmlR*$_{sp}$ and *adhC* were designed with reference to the sequences in the genome of *S. pneumoniae* R6 (a derivative of D39) (accession number NC_003098). The primers used were as follows: upstream NmlR$_{sp}$, 5'-TCAGCTAGCACGGAATAGAC-3'; downstream NmlR$_{sp}$, 5'-CAAGGAGGATTTATGGATACC-3'; EryNmlR$_{sp}$I, 5'-TTGTTCATGTAATCACTCCTTCCTCTCATTTCATTCACTGAATTC-3'; EryNmlR$_{sp}$II, 5'-CGGGAGGAAATAATTCTATGAGGAAAAATTTTAAATGAATCAGCAGTATATAC-3'; EryAdhCI, 5'-TTGTTCATGTAATCACTCCTTCTGCCTTTGTATATACTGCTCATTCAT-3'; and EryAdhCII, 5'-CGGGAGGAAATAATTCTATGAGTCTATGATTGTAATCGAATAAAAAACG-3'. The primers used for the amplification of the erythromycin-resistance cartridge (*erm* from pVA891 [11]) were JM214, 5'-GAGGAGTGATTACATGAAACAA-3', and JM215, 5'-CTCATAGAATTATTTCCTCCC-3'. The individual PCR products were then amplified together to obtain a single overlap-extension PCR product that was transformed into D39.

Phylogenetic analysis. Protein sequences of MerR family proteins were aligned using the CLUSTAL W algorithm [12]. An unrooted tree was constructed using PHYLIP [13].

Extraction of RNA and reverse-transcriptase PCR (RT-PCR). RNA was isolated from bacterial pellets with acid-phenol:chloroform:isoamyl alcohol (125:24:1; pH 4.5), as described elsewhere [14]. The extract was precipitated at −80°C overnight and subsequently treated with 10 U of RNase-free DNase (Roche) at 37°C for 60 min, to remove DNA contamination. Relative levels of mRNA were determined using real-time RT-PCR. The levels of 16S rRNA were used as an internal control to standardize the amount of mRNA. Oligonucleotides for 16S rRNA were 16sFor, 5'-GGTGAGTAACGCGTAGGTAA-3', and 16sRev, 5'-ACGATCCGAAAACCTTCTTC-3'; oligonucleotides for the *adhC* transcript were AdhCFor, 5'-CGGATGATGTGATTATTCGTGT-3', and AdhCRev, 5'-CGGCTAGACATCTGCAAGAGTC-3'. Real-time RT-PCR was performed on a Rotorgene RG-2000 thermocycler using the Superscript III One-Step RT-PCR System (Invitrogen). Quantitative fold differences for each transcript were determined using the $2^{-\Delta\Delta C_T}$ method described by Livak and Schmittgen [15].

For induction of *adhC* with GSNO, pneumococci were grown to an absorbance at 600 nm (A_{600}) of 0.25 in THY broth and then stressed by addition of GSNO to a final concentration of 100 μmol/L. Cells were allowed to grow for an additional 20 min before harvesting and extraction of total cellular RNA.

GSNO killing assays. Triplicate samples of D39 and the *adhC* and *nmlR*$_{sp}$ mutants were grown to an A_{600} of 0.3–0.6 in THY broth. Cells were resuspended in 200 μL of PBS. Ten microliters of cell suspension was added to 490 μL of NaOH/SDS solution, A_{260} was measured, and colony-forming units per milliliter was calculated using a standard curve. The volumes of the cell suspensions were then adjusted to ensure that wild-type and mutants had equivalent densities of colony-forming units per milliliter. Ten microliters of each cell suspension was then added to 90 μL of PBS with or without 2.5 mmol/L GSNO in a microtiter plate. Ten-microliter samples were withdrawn at 10-min intervals, serially diluted, and plated.

Measurement of NADH-GSNO oxidoreductase activity. Cells were grown in THY broth to an A_{600} of 0.5–0.7. Cells were harvested, washed twice, resuspended in 5 mL of 150 mmol/L potassium phosphate (pH 7.0) and lysed by addition of 0.1% deoxycholate. After 30 min at 37°C, cell debris was removed by centrifugation at 13,000 *g* for 15 min, and the cell-free extract was passed through a 0.45-μm filter. GSNO-dependent oxidation of NADH by cell-free extracts was followed spectrophotometrically as described elsewhere [16]. Protein concentration was measured using the Sigma Protein Determination Kit.

Intranasal colonization model. The method used was a modification of that described elsewhere [17, 18]. Bacterial strains used for the intranasal challenge were grown in THY broth to an A_{600} of 0.25. Ten 5-week-old female CD1 mice were anaesthetised before challenge by intraperitoneal injection with pentobarbitone sodium (Nembutal; Rhone-Merieux); 25 μL of a mixture of *S. pneumoniae* D39 and the *nmlR*$_{sp}$ mutant, at concentrations of $\sim 1 \times 10^8$ cfu/mL, was pipetted into the nares and involuntarily inhaled. Five mice were killed at 24 and 48 h after challenge. The nasopharynx was washed with 1 mL of buffer (0.5% trypsin and 0.02% EDTA in sterile PBS). Blood (75 μL) was collected from the heart. The mouse was perfused with sterile saline, and both lungs were removed and placed in 2 mL sterile PBS. The nasopharyngeal cavity was excised and placed in 5 mL of sterile PBS. The lungs and nasopharyngeal tissues were then homogenized using a CAT X120 homogeniser. Samples

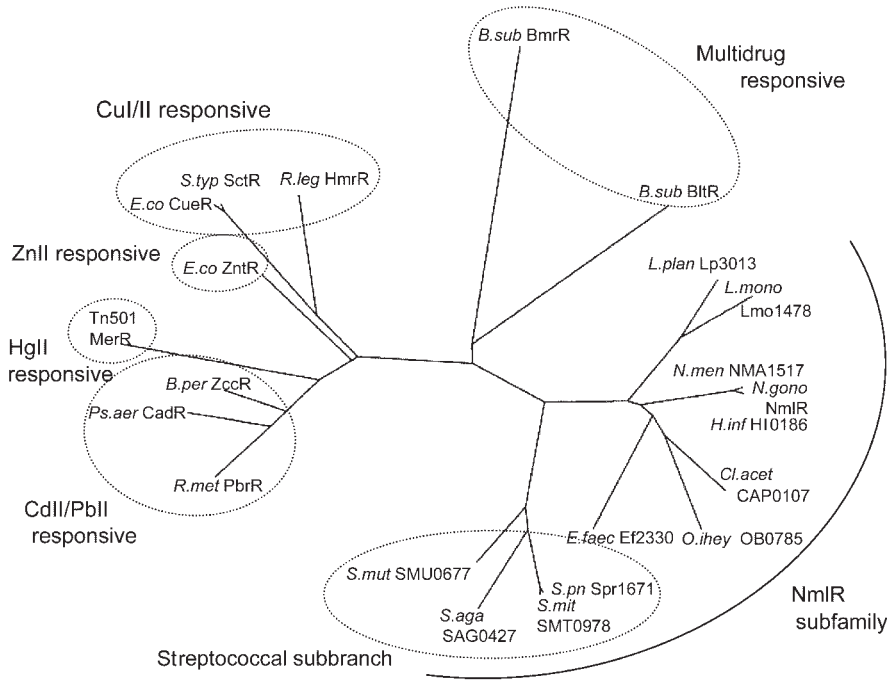

Figure 1. Unrooted phylogenetic tree of $NmlR_{sp}$ and selected MerR regulators. Sequences are indicated by name and accession no., as follows: ZntR (P36676), MerR (P06688), CueR (P77565), ZccR (CAE43000), SctR (AF409088), BmrR (P39075), BltR (L32599), PbrR (CAC28872.1), CadR (AAK48830), and HmrR (NP_435795). The NmlR clade is EF2330 (ZP_00037431), *Enterococcus faecium;* OB785 (BAC12741), *Oceanobacillus iheyensis;* CAP0107 (AKK76852), *Clostridium acetobutylicum;* Lmo1478 (CAC99556.1), *Listeria monocytogenes* EGD-e; Lp3013 (NP_786333.1), *Lactobacillus plantarum;* HI0186 (I64052), *Haemophilus influenzae;* and NMA1517 (CAB84745), *Neisseria meningitidis*. The *Streptococcus* subgroup is Spr1671 (NP_359263), *S. pneumoniae* R6; SAG0427 (NP_687461), *S. agalactiae* 2603 V/R; SMU0677 (NP_721105), *S. mutans* UA159; and SMT0978, *S. mitis* NCTC 12261.

were then serially diluted and plated on blood agar (supplemented with 0.2 μg/mL erythromycin in the case of the $nmlR_{sp}$ and *adhC* mutants) to determine the number of viable pneumococci. Differences in the level of colonization were analyzed using Student's paired *t* test (2-tailed).

Intraperitoneal competition and virulence models. Five mice were injected intraperitoneally with 0.1 mL of serum broth containing 1×10^3 cfu of *S. pneumoniae* comprising a 1:1 mixture of either D39:$nmlR_{sp}$ or $nmlR_{sp}$:*adhC*. Blood was collected from mice at 24 and 48 h, and serially diluted blood was plated on blood agar with or without erythromycin to enumerate wild-type and mutant *S. pneumoniae*. Differences in the level of colonization were analyzed using Student's paired *t* test (2-tailed). Alternatively, groups of 15 mice were challenged with 1×10^2 cfu of each strain and were observed for 10 days. Differences in the overall survival rates between groups were analyzed by Fisher's exact test.

Adherence assay. Adherence of pneumococci to A549 cells (human type 2 pneumocytes) and HEp-2 cells (human laryngeal carcinoma) was determined essentially as described elsewhere [19]. Pneumococci were suspended at a density of 1×10^6 cfu/mL. Washed A549 or HEp-2 monolayers in 24-well tissue-culture plates were infected with 1 mL of the bacterial suspension. After incubation at 37°C for 2 h, culture medium was removed, and the monolayers were washed 4 times with PBS. The cell monolayers were detached from the plate by treatment with 0.25% trypsin and 0.02% EDTA. Cells were lysed by the addition of 400 μL of 0.025% Triton X-100, and serial 10-fold dilutions thereof were plated on blood agar to determine the number of colony-forming units of adherent bacteria.

RESULTS

Identification of a MerR-like regulator in* S. pneumoniae *related to neisserial NmlR. The MerR family of regulators sense a variety of physiological changes (reviewed in [20]). Three *merR*-like genes were identified in the genome of *S. pneumoniae* D39, and the protein encoded by one of these, Spr1671, aligned most closely with *N. gonorrhoeae* NmlR. We have named Spr1671 "the NmlR-like regulator of pneumococcus," or $nmlR_{sp}$. In an earlier study, we observed that 2 cysteine residues (at positions 54 and 71 in NmlR) were conserved within the proteins of the NmlR subfamily, although it should be noted that NmlR itself possesses 4 cysteine residues [16]. NmlR-like regulators have been identified in several streptococcal species (figure 1), and these form a distinct clade within the MerR family. These streptococcal transcription factors possess only a single cysteine (C54), except for the NmlR from *Streptococcus agalactiae,* in which a tyrosine residue replaced the cysteine (data not shown). Available sequenced *S. pneumoniae* genomes and our in-house

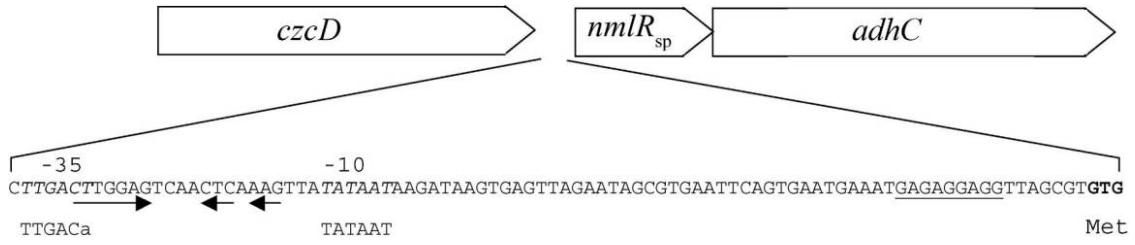

Figure 2. Organization of the intergenic region between the *czcD* gene and the upstream promoter region of $NmlR_{sp}$ (Spr1671). The translational start of $nmlR_{sp}$ is indicated by boldface type, and the proposed −10 and −35 promoter elements are marked and indicated by italics; below is the consensus sequence of these elements. The small arrows show the region of dyad symmetry that lies between the promoter elements. The putative ribosome-binding site (RBS) is underscored. The arrowheads on the open reading frames indicate the direction of transcription.

microarray data show that $nmlR_{sp}$ is present in all strains examined (including serotypes 1, 2, 3, 4, 6, 11, 14, and 23F).

***Transcriptional coupling of a gene encoding a class III alcohol dehydrogenase to* nmlR$_{sp}$.** The $nmlR_{sp}$ gene is flanked by genes predicted to encode a zinc-containing alcohol dehydrogenase (*adhC;* Spr1670) and a cation efflux system protein of the CDF family (*czcD;* Spr1672) (figure 2). All 3 genes are predicted to be transcribed from the same DNA strand. The spacing of 243 bp between the end of *czcD* and $nmlR_{sp}$ is sufficient for a MerR-regulated promoter. It also appears that *adhC* is transcriptionally coupled to $nmlR_{sp}$, given that there is no gap between the stop codon for $nmlR_{sp}$ and the start codon for *adhC* (figure 2). This was confirmed using RT-PCR (data not shown). The *N. gonorrhoeae adhC* is also adjacent to *nmlR* [16], but the genetic arrangement is different because the 2 genes are transcribed from opposite strands. MerR regulators are targeted to promoters with a characteristic elongated −35 to −10 spacer region, usually between 2 overlapping promoters of divergently transcribed genes, each with a sequence exhibiting dyad symmetry [20]. The $nmlR_{sp}$ promoter exhibits the characteristic 19-bp spacer containing a dyad symmetry between the −10 and −35 elements (figure 2).

Requirement of NmlR$_{sp}$ and AdhC for resistance to nitrosative stress. To assess the physiological role played by $nmlR_{sp}$ and *adhC,* we constructed a deletion-replacement mutation in the highly virulent type 2 *S. pneumoniae* strain D39. That portion of the $nmlR_{sp}$ open reading frame encoding aa 1–115 (of a total of 117 aa) was replaced in-frame with an erythromycin-resistance cartridge (*erm* from pVA891) by overlap-extension PCR and transformation of D39. The last 3 codons of $nmlR_{sp}$ (encoding aa 116–117 plus the stop codon) were left intact so as not to disturb the putative ribosome-binding site for the downstream gene. A similar approach was used to construct an *adhC* mutant.

Because it has been established that different AdhC enzymes are able to reduce GSNO, we tested the effect of mutations in $nmlR_{sp}$ and *adhC* on the ability of pneumococci to survive nitrosative stress. Figure 3*A* shows the effect of challenge with GSNO (2.5 mmol/L for 60 min) on the survival of *S. pneumoniae* D39 and its otherwise isogenic $nmlR_{sp}$ and *adhC* mutants. Both mutants exhibited hypersusceptibility to NO stress, with viability after a 60-min exposure significantly reduced to 4.17% and 0.39% of that of the wild-type strain for the $nmlR_{sp}$ mutant and the *adhC* mutant, respectively ($P < .0002$ and $P < .0003$, respectively).

NADH-dependent GSNO reductase activity was assayed in lysates of D39 as well as the *adhC* and $nmlR_{sp}$ mutants, as described in Materials and Methods (figure 3*B*). The results closely paralleled the GSNO survival data, with GSNO reductase activity

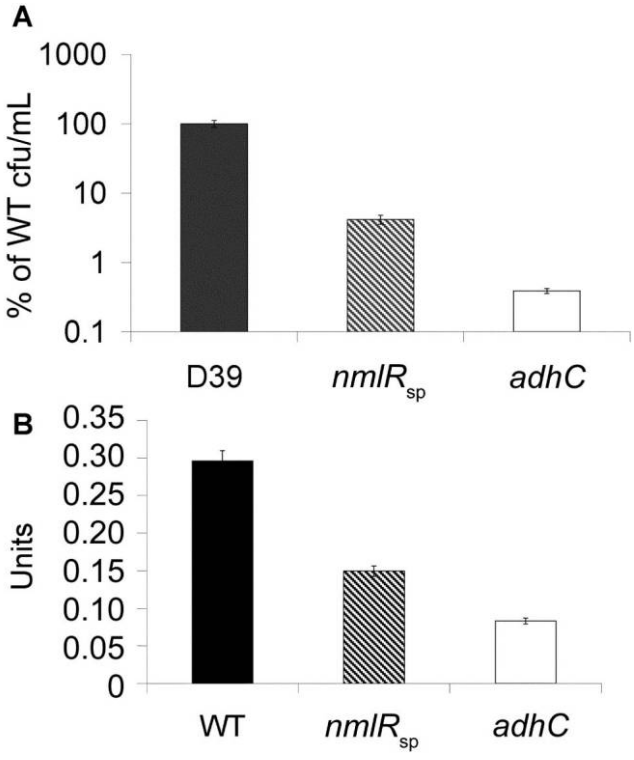

Figure 3. Phenotype of *adhC* and $nmlR_{sp}$ mutants. *A,* Percentage of survival (relative to that of wild-type [WT] D39 cfu/mL survival) after a 60-min exposure to 2.5 mmol/L *S*-nitrosoglutathione (GSNO). *B,* NADH-GSNO oxidoreductase activity in mutant and WT strains (μmol of NADH consumed/min/mg of protein).

in the *nmlR*$_{sp}$ and *adhC* cell-free extracts reduced to 50% (0.15 U) and 28% (0.08 U) of that for the wild type (0.30 U), respectively.

Induction of* adhC *expression by NO stress. We examined the level of *adhC* mRNA by real-time RT-PCR. The *adhC* mRNA level was 6.25-fold lower in the *nmlR*$_{sp}$ mutant compared with D39, suggesting that transcription of the *nmlR*$_{sp}$-*adhC* operon is under the direct control of NmlR$_{sp}$. However, transcription of *adhC* was not completely abolished, presumably because of residual promoter activity, since the −10 and −35 regions and the ribosome binding site are left intact in the *nmlR*$_{sp}$ mutant. To test whether NO stress could induce transcription of *adhC* in *S. pneumoniae,* we compared the level of *adhC* mRNA in wild-type D39 and *nmlR*$_{sp}$ mutant cells stressed for 20 min by the addition of GSNO at a final concentration of 100 μmol/L. The level of transcription for *adhC* in the D39 parent was 4-fold higher in the GSNO-treated bacteria than in the nonstressed control culture. In comparison, there was no induction of *adhC* mRNA by GSNO in the *nmlR*$_{sp}$ mutant. This clearly indicates that GSNO can induce *adhC* transcription and that induction requires the regulator NmlR$_{sp}$.

Contribution of NmlR$_{sp}$ and AdhC to systemic virulence of* S. pneumoniae *but not to nasopharyngeal colonization or adherence to respiratory epithelial cells. The hypersensitivity to NO caused by the mutation of either *nmlR*$_{sp}$ or *adhC* could potentially affect the virulence of *S. pneumoniae* D39. To examine this possibility, we measured the capacity of the mutants to compete with either D39 or each other in an intraperitoneal challenge model. In the first experiment, 5 mice were injected with ~1 × 10^3 cfu of a 1:1 mix of D39 and the *nmlR*$_{sp}$ mutant. Blood was collected at 24 and 48 h, and the numbers of each strain were determined after plating on blood agar with or without erythromycin. As shown in figure 4*A*, in each mouse, the number of wild-type D39 bacteria was several orders of magnitude greater than that of the *nmlR*$_{sp}$ mutant. The mean competitive index of the mutant with respect to the wild-type strain was 0.008 and 0.0036 at 24 and 48 h, respectively. These differences in the capacity to survive in the blood were highly significant ($P = .004$ and $P = .034$, respectively). In the second experiment (figure 4*B*), competitive intraperitoneal challenge of mice with 1 × 10^3 cfu of the *nmlR*$_{sp}$ and *adhC* mutants at a 1:1 ratio showed that the latter is less fit than the former (figure 4*B*). The mean competitive index of the *adh* mutant with respect to the *nmlR*$_{sp}$ mutant was 0.11 and 0.10 at 24 and 48 h, respectively. These differences were also significant ($P = .002$ and $P = .011$, respectively).

In view of the significant systemic attenuation of both the *nmlR*$_{sp}$ and *adhC* mutants with respect to D39, the fitness of the *nmlR*$_{sp}$ mutant and the wild-type strain were also compared in an intranasal challenge model of nasopharyngeal colonization and lung infection. After intranasal challenge, there was no significant difference in the capacity of the 2 strains to survive in the nasopharyngeal niche (competitive index of ~1.0). In the lungs,

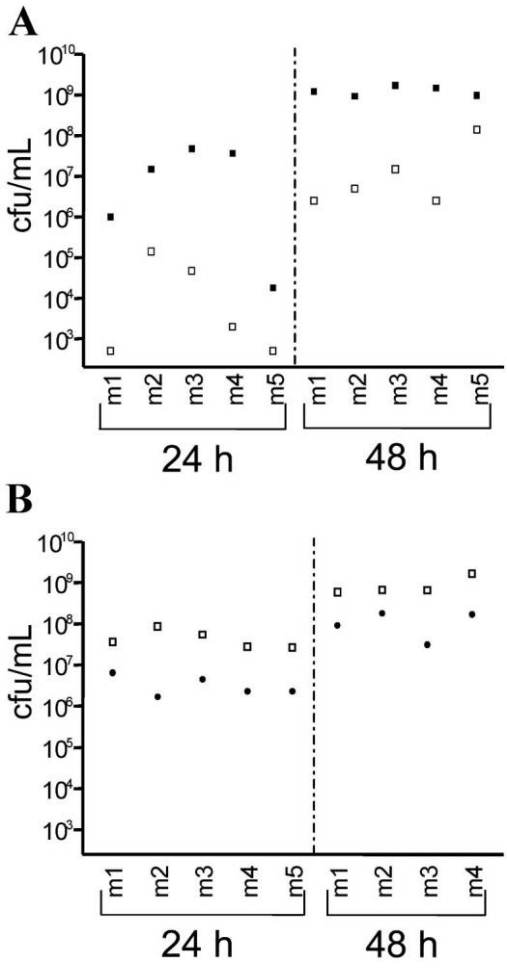

Figure 4. Systemic virulence competition assay. Equal nos. of D39 *(black squares)* and the *nmlR*$_{sp}$ mutant *(white squares)* were injected intraperitoneally, and the nos. of each strain were determined in blood collected at 24 and 48 h, as described in Materials and Methods *(A).* Data are shown for each of 5 mice (m1–m5). Panel B shows results for a similar competition experiment after coinjection of the *nmlR*$_{sp}$ mutant *(white squares)* and the *adhC* mutant *(black circles).*

the *nmlR*$_{sp}$ mutant showed a small decrease in fitness relative to D39 at 24 and 48 h (competitive indices of 0.5 and 0.1, respectively), but this trend did not reach statistical significance. We also directly compared the intraperitoneal virulence of D39 and the *nmlR*$_{sp}$ and *adhC* mutants. Mice were challenged with 1 × 10^2 cfu of either D39, the *nmlR*$_{sp}$ mutant, or the *adhC* mutant, and their survival was followed over 10 days. The survival rate for mice challenged with D39 was 4/15 (median survival time, 4.08 days), compared with 13/15 and 11/15 for the groups challenged with the *nmlR*$_{sp}$ and *adhC* mutants ($P = .0025$ and $P = .027$, respectively).

We also compared the ability of the *nmlR*$_{sp}$ mutant and D39 to adhere to Detroit 562 (human nasopharyngeal) or A549 (human type II pneumocytes) cells in vitro but were unable to show any significant difference in adherence to either cell line (data not shown). Collectively, these findings suggest that NmlR$_{sp}$ does

not play a role either directly or via regulatory effects in the adherence of *S. pneumoniae* to human respiratory epithelial cells in vitro or to the murine nasopharynx in vivo but that it is of considerable importance during invasive disease.

DISCUSSION

Little is known about the role played by NO in the clearance of pneumococcal infections and even less about the defense mechanisms that pneumococci use to protect against reactive nitrogen species. Our results show that defense against NO in pneumococcus includes at least 2 proteins, AdhC and $NmlR_{sp}$. Our data using wild-type D39 cells and the *adhC* mutant clearly show that AdhC catalyses the NADH-dependent reduction of GSNO. We therefore propose that this system is one mechanism whereby *S. pneumoniae* resists NO stress. Although this enzyme is present in *Salmonella* Typhimurium, it does not appear to be essential for its virulence in mice [4].

We initially considered the NmlR subfamily of MerR regulators to be a single clade [16], but clearly there is considerable diversity within this subfamily. $NmlR_{sp}$ is different from all of the other forms of NmlR that we have identified. The differences in the number and position of cysteine residues in NmlR regulators suggests that there may be differences in the way in which they sense stress induced by NO or other oxidants.

In a number of bacteria, an *adhC*-like gene is associated with an NmlR-like regulator [16], and this is the case for *S. pneumoniae*. However, there are some important differences between the organization of the pneumococcal and gonococcal gene clusters. First, the pneumococcal *adhC* and *nmlR* are cotranscribed, whereas, in the gonococcus, the 2 genes are transcribed from opposite DNA strands. It follows that the promoter regions differ and the gonococcal *adhC-nmlR* exhibits divergent overlapping promoters with NmlR-binding sites, a genetic organization that is typical of the majority of MerR-regulated systems. Second, unlike in *N. gonorrhoeae,* there is no esterase-encoding gene distal to *adhC* in *S. pneumoniae* [16]. Examination of the *adhC-*$nmlR_{sp}$ gene clusters in other *Streptococcus* species have revealed that they all have a similar genetic organization to that found in *S. pneumoniae*. We were able to show that *adhC* is induced by GSNO. This induction required $NmlR_{sp}$, because *adhC* in the $nmlR_{sp}$ mutant was no longer inducible. Furthermore, the $nmlR_{sp}$ mutant showed a reduced level of *adhC* mRNA, suggesting that $nmlR_{sp}$ acts as a transcription activator for *adhC*. Because the *erm* cassette lacks a promoter or transcription terminator, such insertion-replacement mutations are nonpolar, and all previous insertion mutants of this type have never shown any polar effects. Thus, it seems unlikely that the reduction of *adhC* transcription seen in this $nmlR_{sp}$ mutant is due to polarity.

During the normal course of *S. pneumoniae* infection, the organism translocates from the nasopharynx to the lungs and subsequently to the blood to cause bacteremia. In the lungs, the bacteria will encounter large numbers of resident macrophages that are part of the innate immune system. Once the pneumococci are phagocytosed the internalized bacteria are subject to killing by bactericidal agents, including NO. The pneumococcal toxin pneumolysin is known to contribute significantly to lung damage during pneumonia, and one mechanism involves the triggering of NO production in macrophages [2]. This leads to apoptosis of the macrophage but, in so doing, also places the bacteria under considerable NO stress. The $nmlR_{sp}$-*adhC* system appears to be an important mechanism whereby the bacteria could protect themselves from elevated NO levels. This does not appear to be important during the nasophayngeal colonization phase, and competitive-index data from the intranasal-challenge experiments suggests at best a modest effect in the lungs. Furthermore, mutation of *adhC* or $nmlR_{sp}$ does not affect the growth rate of *S. pneumonieae* in vitro (S.P.K., S.L.S., and A.G.M., unpublished data), and this indicates that neither gene is required during growth in the absence of nitrosative stress. However, once the bacteria cross into the blood, they encounter not only pulmonary macrophages but also neutrophils, which are also capable of killing via NO. Our data clearly show that, in the blood, there is a significant decrease in the fitness of the $nmlR_{sp}$ and *adhC* mutants compared with that of wild-type D39 *S. pneumoniae,* which correlated with in vitro AdhC activity and resistance to GSNO challenge. The $nmlR_{sp}$-*adhC* system is the first NO resistance mechanism to be described for *S. pneumoniae,* and our data underscore the importance of this system for countering innate host defences during invasive pneumococcal disease.

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