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NOTES

The MerR/NmlR Family Transcription Factor of *Streptococcus pneumoniae* Responds to Carbonyl Stress and Modulates Hydrogen Peroxide Production▽

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The $NmlR_{sp}$ transcription factor of *Streptococcus pneumoniae* is shown to induce *adhC* (alcohol dehydrogenase) expression in the presence of both formaldehyde and methylglyoxal. $nmlR_{sp}$ and *adhC* mutant strains display altered and opposite aerobic growth phenotypes. The $nmlR_{sp}$ strain exhibits increased resistance to high oxygen tension, attributable to decreased H_2O_2 production, which correlated with downregulation of carbamoyl phosphate synthase (*carB*). This indicates a possible role for AdhC in aldehyde metabolism and a broader role for $NmlR_{sp}$ in the regulation of carbon metabolism.

The Gram-positive bacterium *Streptococcus pneumoniae* is a human pathogen of major significance, causing approximately 1 million deaths in children under 5 years annually (11). The pneumococcus is carried asymptomatically in the nasopharynx of a large proportion of the human population but is capable of invading internal sites within the body, resulting in pathology such as otitis media, pneumonia, sepsis, and meningitis (3). Invasive pneumococcal disease is typically associated with an intense inflammatory response in host tissues and recruitment of cells involved in the innate immune response (2). It is clear that the pneumococcus must possess mechanisms to cope with the various stresses imposed by the host immune response in order to cause invasive disease. In a previous publication (17), we characterized two pneumococcal genes ($nmlR_{sp}$ and *adhC*) that are required for invasive disease but not colonization, using a mouse model of infection. $NmlR_{sp}$ was characterized as a transcription factor of the MerR/NmlR family that regulates the expression of a class III alcohol dehydrogenase (*adhC*) in the pneumococcus (17). Class III alcohol dehydrogenases are known to catalyze the metabolism of two electrophilic adducts of glutathione: the nitric oxide adduct (*S*-nitrosoglutathione [GSNO]) and the formaldehyde adduct (*S*-hydroxymethylglutathione) (16). Our earlier study led to the hypothesis that the *S*-nitrosoglutathione reductase activity of AdhC protects the pneumococcus against host-produced nitric oxide (NO) during systemic infection. In the current study we have explored an alternate/additional role for $NmlR_{sp}$ in the response to aldehyde stress and regulation of central carbon metabolism in the pneumococcus.

***adhC* expression is induced by $NmlR_{sp}$ in the presence of formaldehyde and methylglyoxal.** Given the known role of class III alcohol dehydrogenases in the detoxification of formaldehyde, a possible role for pneumococcal AdhC in formaldehyde metabolism was investigated. Real-time reverse transcription-PCR (RT-PCR) was used to measure the expression of *adhC* upon treatment of pneumococci with formaldehyde. *S. pneumoniae* D39 (virulent serotype 2 strain [1]) was grown to early exponential phase in Todd-Hewitt broth supplemented with 0.5% (wt/vol) yeast extract (THY) at 37°C before the culture was divided in half, and 0.5 mM formaldehyde (Sigma) was added to one sample. The cultures were incubated for a further 15 min before cells were collected by centrifugation, and total RNA was extracted using acid phenol-chloroform-isoamyl alcohol (125:24:1) as described elsewhere (12). The extract was precipitated at −80°C overnight in ethanol-sodium acetate and subsequently treated with RNase-free DNase (Promega), to remove DNA contamination. Relative levels of *adhC* mRNA transcripts were determined in relation to 16S rRNA levels using the SuperScript III Platinum SYBR green One-Step quantitative RT-PCR (qRT-PCR) kit (Invitrogen) and the LightCycler 480 II (Roche). The nucleotide sequences of primers used for real-time RT-PCR can be found in Table 1. *adhC* expression was found to be approximately 31-fold higher in the sample treated with formaldehyde than in the nontreated sample (Fig. 1A). To determine whether this formaldehyde-dependent induction is mediated by $NmlR_{sp}$, the experiment was repeated using an *S. pneumoniae* $nmlR_{sp}$ mutant strain (described in reference 17). *adhC* was found to be expressed in the sample treated with formaldehyde to a level similar to that in the nontreated sample (Fig. 1A). Thus, induction of *adhC* expression in the presence of formaldehyde is dependent on the cells expressing an active $NmlR_{sp}$ protein. The results presented in Fig. 1A also indicate that $NmlR_{sp}$ acts as a repressor of *adhC* expression under standard culture conditions (no formaldehyde), as indicated by the fact that *adhC*

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TABLE 1. Primers used for real-time RT-PCR

Primer name	Nucleotide sequence (5′–3′)
16S-F	GGTGAGTAACGCGTAGGTAA
16S-R	ACGATCCGAAAACCTTCTTC
adhC-RT-F	CGGATGATGTGATTATTCGTGT
adhC-RT-R	CCTGGTTTCACCGTCGTAATGGC
spxB-RT-F	CAACATGTGCTACCCAGACG
spxB-RT-R	CGAGCATCGATGACAACAGT
lox-RT-F	GCGCCTTTAACCACAAACTC
lox-RT-R	AACCAAACTCATGCACACCA
carB-RT-F	GTGGTATGTGTGCCAACGAG
carB-RT-R	AATGGAATCCCCTGTGTGAA

expression is almost 5-fold higher in the $nmlR_{sp}$ strain than in wild-type pneumococci. This is consistent with the unique mode of action of MerR family transcription factors acting as both activators and repressors of transcription (6). Despite the significant transcriptional response of *adhC* to formaldehyde, neither the *S. pneumoniae* $nmlR_{sp}$ strain nor the *adhC* strain (described in reference 17) exhibited an altered growth phenotype upon challenge with formaldehyde, compared with wild-type pneumococci (data not shown). It has recently been shown that the $NmlR_{sp}$ homologue in *Bacillus subtilis* upregulates the expression of its regulon (including an alcohol dehydrogenase) in response to both formaldehyde and methylglyoxal (10). To test if $NmlR_{sp}$ also induces *adhC* expression in the presence of methylglyoxal, the above experiment was repeated using 0.01% (vol/vol) methylglyoxal (Sigma). *adhC* expression was found to be induced approximately 100-fold in the wild-type strain upon treatment with methylglyoxal, while treatment of the $nmlR_{sp}$ strain did not result in differential expression (Fig. 1B). Thus, it seems likely that $NmlR_{sp}$ is a general aldehyde-responsive regulator, as opposed to selectively responding to a particular compound. $NmlR_{sp}$ shares a conserved cysteine residue with its *B. subtilis* homologue (17), which was shown to be essential for activation of transcription in response to formaldehyde and methylglyoxal (10). This suggests that thiol-(*S*)-alkylation of this residue by aldehyde compounds may be responsible for $NmlR_{sp}$-mediated activation of *adhC* in pneumococci.

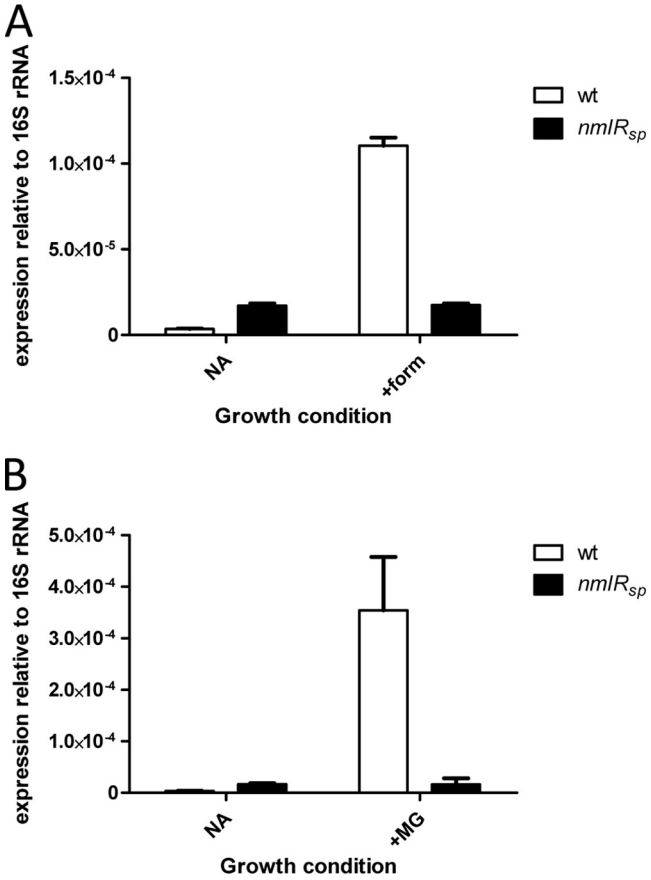

FIG. 1. Real-time quantification of *adhC* in *S. pneumoniae* D39 wild-type (wt) and $nmlR_{sp}$ strains grown in the presence or absence (NA) of formaldehyde (+form) (A) and methylglyoxal (+MG) (B). Error bars indicate standard deviations from the means. Experiments were conducted in triplicate.

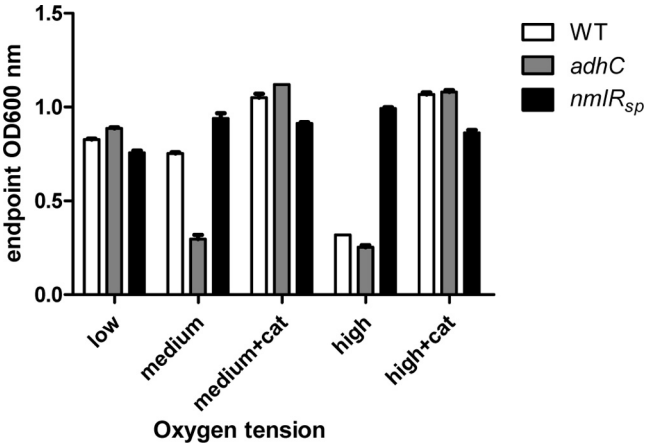

FIG. 2. Growth of *S. pneumoniae* wild-type (WT), *adhC*, and $nmlR_{sp}$ strains in THY broth under different oxygen tensions. +cat, exogenous catalase added to cultures. Error bars indicate standard deviations from the mean. Experiments were conducted in triplicate.

$NmlR_{sp}$ and AdhC have a role the in growth of the pneumococcus under different oxygen tensions. As was the case with formaldehyde, neither the *S. pneumoniae* $nmlR_{sp}$ strain nor the *adhC* strain exhibited an altered growth phenotype upon challenge with methylglyoxal (data not shown). However, an altered growth phenotype for these strains was observed in standard culture medium (THY) during growth at increased oxygen tension. Figure 2 shows endpoint growth measurements of wild-type, *adhC*, and $nmlR_{sp}$ pneumococcal cultures grown overnight in THY with 0.5% (wt/vol) choline chloride under different oxygen tensions at 37°C. Differential oxygenation was achieved through varying the culture volume-to-tube volume ratio as well as the shaking speed. Low-oxygen cultures were 5-ml cultures in 10-ml tubes incubated without shaking. Medium-oxygen cultures were 5-ml cultures in 50-ml tubes with shaking at 125 rpm. High-oxygen cultures were 5-ml cultures in 50-ml tubes with shaking at 150 rpm. While all strains exhibited similar growth characteristics under low oxygen tension, the *adhC* strain showed impaired growth under medium oxygen tension compared with the wild-type strain ($P < 0.0001$). However, growth could be restored completely by supplementing the medium with 6 μg/ml beef liver catalase

(Roche), indicating that hydrogen peroxide (H_2O_2) toxicity is responsible for growth inhibition. Conversely, the *nmlR*$_{sp}$ strain exhibited increased resistance to aerobic growth as evidenced by the fact that wild-type pneumococci were unable to grow at high oxygen tensions, while growth of the *nmlR*$_{sp}$ strain was not inhibited ($P < 0.0001$). This growth difference also appears to be due to H_2O_2 toxicity, as wild-type growth was restored by supplementing the medium with catalase. Pneumococci are known to produce high levels of H_2O_2 under aerobic conditions, primarily through the action of pyruvate oxidase (SpxB), which, together with acetate kinase, provides a pathway to increased ATP generation in the presence of oxygen (15). Despite the fact that pneumococci produce high concentrations of H_2O_2, little is known about the mechanisms employed by the cell to defend against this toxic compound. Pneumococci do not possess catalase or the well-described peroxide-responsive regulators OxyR and PerR (19). To investigate if the different aerobic growth phenotypes of the strains may be due to variation in the levels of H_2O_2 that accumulates during growth of each strain, H_2O_2 production assays were performed on each of the strains under low oxygen tension using the horseradish peroxidase/phenol red assay described elsewhere (14). *S. pneumoniae* wild-type and *adhC* strains were found to produce identical amounts of H_2O_2 under these conditions (146 ± 4 μM and 147 ± 10 μM, respectively [$P = 0.89$]). However, the *nmlR*$_{sp}$ strain was found to produce approximately 6-fold-less H_2O_2 (26 ± 1 μM [$P < 0.0001$]). Thus, the increased resistance of the *nmlR*$_{sp}$ mutant to growth at high oxygen tension appears to be due to reduced H_2O_2 production/accumulation in this strain.

Variation in peroxide production is due to downregulation of *carB*. To further investigate the low-H_2O_2-producing phenotype of the *nmlR*$_{sp}$ strain, the expression of 3 genes known to be associated with H_2O_2 production in *S. pneumoniae* was determined using real-time RT-PCR. Pyruvate oxidase (SpxB) is known to be responsible for the majority of the H_2O_2 produced by pneumococci (15). Lactate oxidase (Lox) has recently been described in the pneumococcus and produces H_2O_2 as a by-product of converting lactate to pyruvate (18). Carbamoyl phosphate synthase (CarB) also influences H_2O_2 production, as evidenced by the fact that a *carB* mutant produces amounts of H_2O_2 similar to those produced by an *spxB* mutant (8), although the mechanism by which this occurs is unknown. The expression of *spxB*, *lox*, and *carB* was measured in *S. pneumoniae* wild-type and *nmlR*$_{sp}$ strains grown with and without formaldehyde supplementation as described previously. The levels of *spxB* and *lox* transcripts were found to be essentially identical in the two strains and under the two growth conditions (Fig. 3A and B). However, the level of *carB* transcript was found to be approximately 5-fold lower in the *nmlR*$_{sp}$ strain than in the wild-type strain (Fig. 3C). Treatment of the cultures with formaldehyde resulted in a small (approximately 2-fold) increase in *carB* expression in both strains. Thus, the low-H_2O_2-producing phenotype of the *nmlR*$_{sp}$ strain appears to be due to reduced levels of CarB in this strain. Furthermore, the fact that *carB* expression was not decreased in wild-type pneumococci in the presence of formaldehyde (conditions that lead to activation of *adhC*) suggests that differential regulation of *carB* in the *nmlR*$_{sp}$ strain is not due to derepression of *adhC*. Carbamoyl phosphate synthase (composed of 2 subunits; CarAB) catalyzes the reaction of bicarbonate with ATP and glutamine to produce carbamoyl phosphate, glutamic acid, ADP, and inorganic phosphate (P_i) (9). The link between CarAB activity and hydrogen peroxide production in pneumococci is unclear; however, it should be noted that one of the products of the CarAB-catalyzed reaction (P_i) is a required substrate for SpxB. The major product of CarAB activity, car-

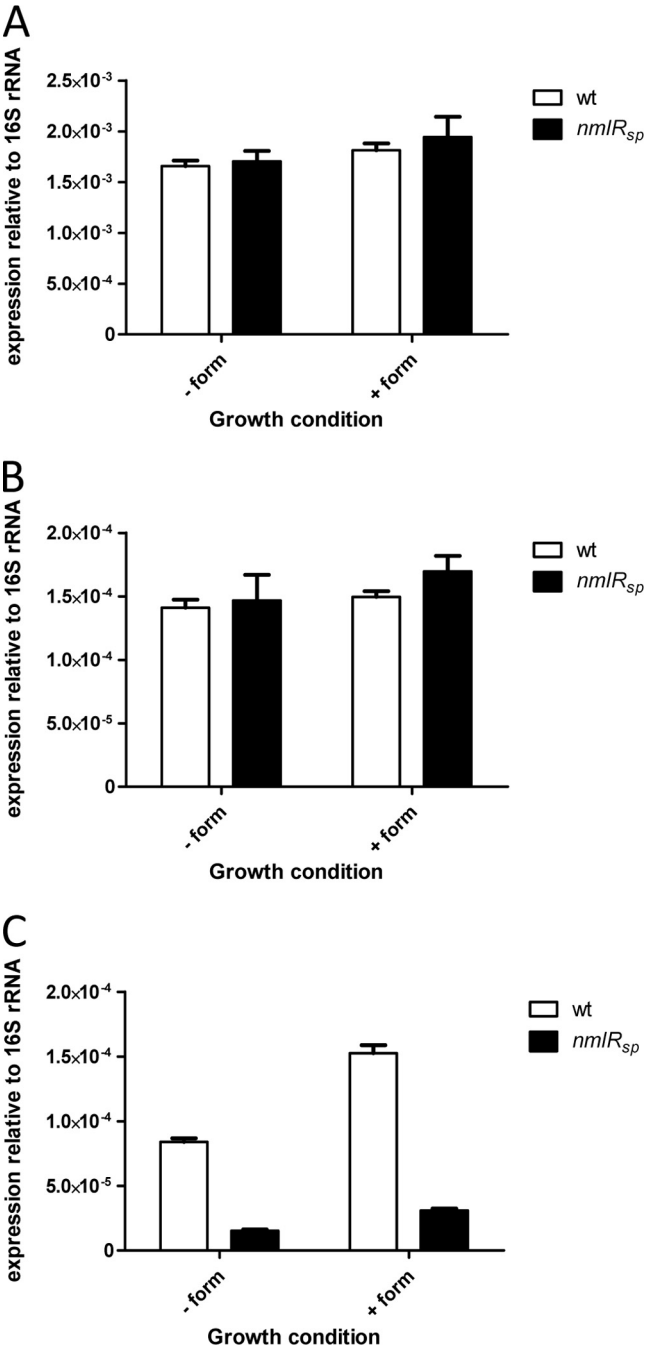

FIG. 3. Real-time quantification of *spxB* (A), *lox* (B), and *carB* (C) in *S. pneumoniae* D39 wild-type (wt) and *nmlR*$_{sp}$ strains grown with (+form) or without (−form) formaldehyde. Error bars indicate standard deviations from the mean. Experiments were conducted in triplicate.

bamoyl phosphate, is a central intermediate in both the pyrimidine and arginine biosynthetic pathways (4). Regulation of *carB* in *S. pneumoniae* has not been studied, but in other organisms *carB* expression has been shown to be influenced by both pyrimidine and arginine availability, as well as the intracellular inorganic carbon concentration (CO_2/bicarbonate) (4, 5). Analysis of the *carB* promoter sequence of *S. pneumoniae* D39 did not reveal the presence of a typical MerR family regulator binding site; thus, it is expected that $NmlR_{sp}$ mediates differential expression of *carB* via an indirect mechanism.

Conclusions. The results presented here indicate that the $NmlR_{sp}$ regulator has a broader function in *S. pneumoniae* than previously recognized. $NmlR_{sp}$ was shown to induce the expression of the class III alcohol dehydrogenase gene *adhC* in the presence of both formaldehyde and methylglyoxal, suggesting that it may function as a general aldehyde-responsive regulator. The physiological aldehydic compound for $NmlR_{sp}$ and AdhC in the pneumococcus remains to be elucidated. It seems unlikely that it will be formaldehyde, since pneumococcus does not oxidize methanol, the principal source of this aldehyde. Similarly, *S. pneumoniae* lacks the methylglyoxal synthase that is found in *Escherichia coli* and is associated with a response to phosphate limitation (7). However, the requirement for AdhC at medium and high oxygen tension suggests that this enzyme may be required to protect cells against reactive aldehydes and dicarbonyl compounds that can arise from C_3 sugars during carbon metabolism (13). In *S. pneumoniae*, carbon metabolism under aerobic conditions coincides with the production of H_2O_2 via the action of SpxB. Although pneumococcus lacks catalase, it can protect itself against this oxidant by using glutathione peroxidase (PsaD) (20). However, PsaD is dependent upon a supply of glutathione that is likely to be limiting in pneumococcus, since it cannot synthesize this tripeptide. It follows that the central role of AdhC may actually be to regenerate reduced glutathione following the formation of a glutathione-aldehyde adduct. Loss of the ability to perform this catalytic step results in glutathione limitation and a failure to tolerate hydrogen peroxide. The fact that $NmlR_{sp}$ influences carbamoyl phosphate synthase expression via a mechanism independent of *adhC* regulation suggests that there may be additional members of the $NmlR_{sp}$ regulon that are yet to be described. Our data are consistent with the view that $NmlR_{sp}$ activates AdhC and modulates gene expression either directly or indirectly to provide protection against hydrogen peroxide stress. We suggest that this functionality is essential for pneumococci to cause invasive disease.

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