A molecular link between cell wall biosynthesis, translation fidelity, and stringent response in *Streptococcus pneumoniae*

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Survival in the human host requires bacteria to respond to unfavorable conditions. In the important Gram-positive pathogen *Streptococcus pneumoniae*, cell wall biosynthesis proteins MurM and MurN are tRNA-dependent aminoacyl transferases which lead to the production of branched muropeptides. We demonstrate that wild-type cells experience optimal growth under mildly acidic stressed conditions, but ΔmurMN strain displays growth arrest and extensive lysis. Furthermore, these stress conditions compromise the efficiency with which alanyl-tRNAAla synthetase can avoid noncognate mischarging of tRNAAla with serine, which is toxic to cells. The observed growth defects are rescued by inhibition of the stringent response pathway or by overexpression of the editing domain of alanyl-tRNAAla synthetase that enables detoxification of tRNA misacylation. Furthermore, MurM can incorporate seryl groups from mischarged Seryl-tRNAAla into cell wall precursors with exquisite specificity. We conclude that MurM contributes to the fidelity of translation control and modulates the stress response by decreasing the pool of mischarged tRNAs. Finally, we show that enhanced lysis of ΔmurMN pneumococci is caused by LytA, and the murMN operon influences macrophage phagocytosis in a LytA-dependent manner. Thus, MurMN attenuates stress responses with consequences for host-pathogen interactions. Our data suggest a causal link between misaminoacylated tRNA accumulation and activation of the stringent response. In order to prevent potential corruption of translation, consumption of seryl-tRNAAla by MurM may represent a first line of defense. When this mechanism is overwhelmed or absent (ΔmurMN), the stringent response shuts down translation to avoid toxic generation of mistranslated/misfolded proteins.

**Streptococcus pneumoniae** | cell wall | translation quality control | stringent response | autolysis

Gram-positive bacteria have evolved a thick and sophisticated cell wall that ensures bacterial structural integrity and is critical for cellular viability. This dynamic structure includes peptidoglycan, surface anchored proteins, wall teichoic acids, lipoteichoic acids, lipoproteins, and capsular polysaccharides (1, 2). It is also a major target of immune defenses and antibiotics (3, 4). Bacteria with high pathogenic potential, such as *Streptococcus pneumoniae* (pneumococcus), encounter hostile environments within the human host, where the cell wall serves as a barrier and an interface between the bacterium and its host.

The pneumococcal peptidoglycan consists of glycan chains that are cross-linked directly or indirectly via peptide bridges (5). These glycan chains consist of alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) residues. Each NAM subunit is attached to a stem peptide which is cross-linked to an adjacent stem peptide from a nearby glycan chain. In the pneumococcus, this cross-link can be direct or indirect through a dipeptide branch that is assembled by MurM and MurN, two transfer RNA (tRNA)-dependent aminoacyl transferases (6, 7). Indirect cross-linking requires the synthesis of lipid II-linked branched peptidoglycan precursors on the cytoplasmic face of the pneumococcal cell membrane. MurM transfers alanyl or seryl residues from aminoacyl-tRNAs to the ε-amino group of the stem peptide lysine of the peptidoglycan precursor lipid II (6), and MurN appends an alanyl residue from alanyl-tRNAAla to the residue added by MurM (7). Lipid precursors are then flipped to the extracellular face of the cell membrane to be polymerized by transglycosylation. The nascent glycan strands are then cross-linked through the third residue (1-Lys, which can be branched or unbranched) of a stem peptide emanating from one glycan strand to the fourth residue (D-Ala) of the stem peptide of an adjacent glycan strand by penicillin binding proteins (8, 9). Thus, pneumococcal peptidoglycan synthesis links stem peptides with a dipeptide comprising a C-terminal 1-Ala or 1-Ser and an N-terminal 1-Ala.

**Significance**

During infection, microbes must survive the hostile environmental conditions of the human host. When exposed to stresses, bacteria activate an intracellular response, known as the stringent response pathway, to ensure their survival. This study connects two fundamental pathways important for cellular growth in a Gram-positive pathogen: it demonstrates that enzymes responsible for cell wall biosynthesis are connected to the stringent response pathway via their ability to ameliorate errors in protein translation. Our study was performed on *S. pneumoniae*, where the MurM cell wall biosynthesis enzyme, a tRNA-dependent aminoacyl transferase, is linked to penicillin resistance. We now demonstrate the importance of MurM in translation quality control and establish that it serves as a gatekeeper of the stringent response pathway.

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The authors declare no competing interest.

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Allelic variants of murM lead to diversity in the nature of stem peptide branching in the cell wall peptidoglycan (10–12). Similarly, allelic variation among pneumococcal penicillin binding protein genes is responsible for drastically increasing the minimum inhibition concentration for β-lactam antibiotics. However, such clinical high-level resistance to β-lactams additionally requires murMN as inactivation of this operon leads to a complete loss of penicillin resistance (8, 13).

The employment of aminoacyl-tRNAs as MurM and MurN substrates juxtaposes the fundamental cellular functions of peptidoglycan biosynthesis and protein translation with each other and antibiotic resistance. The ability of MurM to incorporate both seryl and alanine residues from tRNA into peptidoglycan branches is intriguing. Serine is an amino acid that is erroneously recognized by alanyl-tRNAAla synthetase (AlaRS), which itself possesses editing mechanisms to forestall noncognate seryl-tRNAAla synthesis and consequent misincorporation of serine at alanine codons (14, 15).

In vitro, MurM can deacylate seryl and alanyl-tRNAAla by hydrolysis (6, 16). This led to the hypothesis that MurM-catalyzed aminoacyl-tRNA deacylation provides an in trans mechanism for the correction of deficits in the editing activity and fidelity of AlaRS (16). However, several observations are inconsistent with the role of MurM in editing misacylation of tRNA via simple hydrolytic deacylation of a mischarged substrate: 1) initial reports were made with a vast excess of enzymes relative to the tRNA substrate which precluded demonstration of MurM catalysis (16), 2) MurM-supported aminoacyl-tRNA hydrolysis rates are negligible when compared with those of peptidoglycan precursor aminoacylation (6), and 3) MurM-mediated alanyl-tRNAAla deacylation is significantly more rapid than seryl-tRNAAla deacylation (16). Therefore, the tantalizing possibility that seryl-tRNAAla consumption by MurM-catalyzed lipid II serylation contributes to maintaining accuracy of alanine codon translation remains to be validated in the literature.

Under stress, many bacterial cellular processes including translation are regulated by the stringent response pathway. Deprivation of amino acids or carbon sources, elevated temperature, and acidic conditions can trigger the stringent response and reconfigure cellular metabolism to adapt to challenging conditions to ensure bacterial survival (17–20). In addition, the stringent response plays a role in regulation of bacterial virulence and susceptibility to antimicrobials (18, 21–23). The stringent response pathway is characterized by the accumulation of guanosine tetra- (ppGpp) and pentaphosphate (pppGpp), collectively referred to as alarmones [(p)ppGpp]. In the majority of Gram-negative bacteria, these alarmones are produced by the synthetase RelA and hydrolyzed by SpoT. In these bacteria, binding of deacylated-tRNA to ribosomes activates alarmon production (24–27). In Gram-positive bacteria, these molecules are produced by a bifunctional RSH (RelA/SpoT homolog) protein, which possesses both alarmon synthetase and hydrolase activity (19, 28). Much less is understood about activation of the stringent response in Gram-positive bacteria. However, recent work in Bacillus subtilis suggests that alarmon target initiation factor 2 (IF2) and, in doing so, inhibit translation (29). Together, these data suggest a clear link between alarmones, tRNA targeting, and translation.

In this study, we show that MurMN is a molecular link between cell wall biosynthesis and translation quality control through its preferential utilization of misacylated tRNA for the formation of indirect cross-links in peptidoglycan peptidoglycan. Our findings indicate that the absence of these proteins sensitizes pneumococcal cells to acidic stress. Additionally, we found that a murMN deletion strain presents growth defects when grown in mildly acidic conditions, similar to those in which the ability of AlaRS to edit serine misacylation of tRNAAla is reduced. The impairment of AlaRS editing activity likely resulted in the accumulation of mischarged tRNA and the subsequent activation of the stringent response pathway. Furthermore, our data suggest that activation of the stringent response is associated with the modulation of LytA activity, and this is reflected in changes in the initiation of stationary phase-induced autolysis and in the extent of phagocytosis of pneumococci. These findings provide insight into cell wall function by suggesting that cell wall biosynthesis enzymes can buffer the deleterious consequences of intracellular stress on protein synthesis and modulate entry into the stringent response pathway.

Results

The murMN Operon Protects Cells against Acid-Induced Growth Defects. When growing ΔmurMN cells in planktonic culture, we observed a pronounced growth defect in rich media in mildly acidic conditions. Growth was compared for wild-type and ΔmurMN strains grown in normal (●) and acidic conditions (▲). (A) Representative growth curves for R6D wild-type and ΔmurMN strains grown in normal (●) and acidic conditions depicted in A (▲). (B) Representative growth curves comparing R6D ΔmurMN→murMN+ growth with wild-type and ΔmurMN strains grown in acidic conditions depicted in A (▲). (C) Violin plot depicting maximum growth of wild-type, ΔmurMN, and ΔmurMN→murMN+ strains grown in acidic conditions. Mean ± SD of max ODs: wild type (0.89 ± 0.07), ΔmurMN (0.35 ± 0.04), and ΔmurMN→murMN+ (0.88 ± 0.1). ***P < 0.001 relative to wild-type strain by ANOVA followed by Tukey’s posttest. Growth curves were started at an OD600 of 0.05 (at least n = 3).
acridic conditions (pH 6.6) (Fig. 1A). The mutant exhibited increased susceptibility to stationary phase-induced autolysis and a decrease in the maximum optical density (max OD) reached during growth, consistent with growth arrest. This growth defect was rescued in a ΔmurMN strain with a wild-type murMN knock-in (ΔmurMN/+murMN) (Fig. 1B and C). Although minor differences in growth of wild-type and ΔmurMN strains were observed in rich media in normal conditions (pH 7.4), these differences were much more pronounced in mildly acidic conditions.

MurM and MurN contribute to cell wall branching by adding dipeptide crossbridges to the peptidoglycan stem peptide that leads to production of branched muropeptides (8). The loss of branching alters pneumococcal cell wall composition and increases bacterial sensitivity to lysis by cell wall inhibitors (30). Thus, one hypothesis for the difference in sensitivity of wild-type and ΔmurMN strains to acidic stress is that wild-type cells increase the number of cell wall bridges as a response to low pH and that the ΔmurMN strain cannot mount this defense. To test this hypothesis, we employed high-performance liquid chromatography to analyze the peptidoglycan composition of pneumococci grown in rich media at normal (pH 7.4) and mildly acidic (pH 6.6) conditions for both wild-type and ΔmurMN strains. We did not observe a significant difference in the extent of cross-links (direct or via cross-bridges) of the wild-type cells between normal and acidic conditions (Fig. 2A and B, i). As expected, we observed that deletion of murMN led to the loss of branched peptides from the peptidoglycan as compared to wild-type cells (Fig. 2A and B, i). Although the ΔmurMN strain had a substantially higher number of direct cross-links, the total number of cross-links was not significantly different when this strain was grown at pH 6.6 or pH 7.4 (listed as monomers in Fig. 2B, i). Similarly, the total number of cross-links was similar for the wild-type strain when grown at pH 6.6 or pH 7.4. Thus, the observation that the ΔmurMN strain displays a pronounced growth defect at pH 6.6, but not at pH 7.4, could not be attributed to pH-dependent alterations in the extent of peptidoglycan cross-linking.

A second hypothesis for the difference in sensitivity of wild-type and ΔmurMN strains to acidic stress is that the absence of cross-bridges in the ΔmurMN peptidoglycan sensitizes the cell to LytA-mediated autolysis during log phase growth. Thus, we tested whether there was any difference in the sensitivity of wild-type and ΔmurMN cells in log phase (when wild-type cells are refractory to LytA activity) grown in mildly acidic media to recombinant LytA (the major pneumococcal autolysin). Our data demonstrated that the absence of murMN did not increase the sensitivity of cells to autolysis by exogenously added LytA during log phase (Fig. 2C).

We conclude that, relative to the wild type, the ΔmurMN strain displays a growth defect in mildly acidic conditions and that this was not attributed to cell wall changes induced at the lower pH (as we did not detect changes in cell wall composition of wild-type cells between normal and mildly acidic pH) or to sensitivity to LytA-autolysis during log phase growth.

MurMN Attenuates Activation of the Stringent Response Pathway. The stringent response is a well-conserved pathway utilized by bacteria to ensure survival under stressed conditions. Mediated by the production of alarmones, the activation of this pathway reconfigures bacterial metabolism and coordinates cellular entry into stationary phase (17). We hypothesized that the differences in growth of the ΔmurMN strain, relative to the wild-type strain,

Fig. 2. Analysis of peptidoglycan structure of cells grown in normal and acidic conditions. (A) High-performance liquid chromatography profiles of the stem peptide compositions of peptidoglycan from strain R6D wild-type (WT) and R6D ΔmurMN strain, grown in rich media in normal (blue) and mildly acidic (red) pH and harvested during exponential growth. In addition, strain Pen6 (WT and ΔmurMN) is shown as a reference to identity key peaks (black) (B, i) Table presenting percentage and, in parentheses, the SD of each stem peptide structure in cell wall preparation from R6D strains grown in rich media at normal (blue) or mildly acidic (red) pH. Reference strain Pen6 (WT and ΔmurMN) are shown in black. Summary rows present the average ratio (and SD) of total Ser-Ala to Ala-Ala under normal and mildly acidic pH and the average numbers of monomers (inverse measure of the level of peptidoglycan cross-linking). NA: nonapplicable. *P < 0.05 relative to normal pH by unpaired Student’s t test (at least n = 3, described in detail in Materials and Methods). (**) Composition of the corresponding peptide may not be identical to that of peak five from other strains (B, ii) Structures of the cell wall stem peptides that comprise pneumococcal peptidoglycan based on Pen6 references. (C) Representative curve depicting OD₆₀₀ of R6D wild-type and ΔmurMN cells harvested during the exponential phase in the absence or presence of 2 μg/mL recombinant LytA (at least n = 3).

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was a consequence of activation of the stringent response pathway.

If activation of the stringent response pathway contributes to the low pH-induced early onset of stationary phase in ∆murMN pneumococci, this growth phenotype should be decreased or abrogated in a ∆murMN strain where the stringent response pathway cannot be activated. This pathway is activated by accumulation of the intracellular alarmones, ppGpp or pppGpp, which are hyperphosphorylated forms of guanosine 5′-diphosphate or guanosine 5′-triphosphate (GTP), respectively, synthesized by the addition of a pyrophosphate molecule obtained from adenosine 5′-tripophosphate (ATP) (Fig. 3A). In the pneumococcus, the primary source of alarmone production is RSH, a bifunctional synthetase and hydrolase of (p)ppGpp (31). Thus, we deleted rsh (spr_1487) to generate strains defective in the ability to activate the stringent response pathway.

We compared growth among wild-type, Δrsh, ∆murMN, and ΔmurMNΔrsh strains (Fig. 3B). The ∆murMNΔrsh double mutant did not exhibit the growth defects observed for the ΔmurMN strain in mildly acidic conditions. Thus, inhibition of the stringent response abrogates the growth defects in a ΔmurMN background. To measure the relative alarmone levels in the ΔmurMN strain relative to the wild-type strain in mildly acidic conditions, we employed thin-layer chromatography. The ΔmurMN strain displayed increased alarmone levels compared to the wild-type strain, as observed by 2.5-fold higher levels of alarmone relative to GTP, congruent with growth defects in the absence of murMN (Fig. 3C and SI Appendix, Fig. S1). Furthermore, knock-in of ΔmurMN pneumococci with wild-type murMN restored alarmone concentrations to those found in wild-type cells, while the Δrsh mutants displayed very low levels of alarmone. We conclude that acidic conditions promote entry into stringent response and that MurMN attenuates the activation of stringent response and consequently entry into stationary phase.

Disruption of the Translation Quality Control Function of MurM Leads to Pneumococcal Growth Defects.

MurMN plays a role in correcting misaminoacylation of noncognate tRNA. How can a cell wall synthesis protein influence activation of the stringent response? We hypothesized that MurMN could be involved in the modulation of the stringent response due to its proposed role in the elimination of misacylated tRNAs.

MurM is an aminocyl transferase that transfers L-serine or L-alanine from tRNAs to lipid II to ultimately form dipetidic cross-bridges in the peptidoglycan (8–10). The responsibility of ensuring fidelity of amino acid charging to their cognate tRNAs depends on the proofreading ability of aminoacyl-tRNA synthetases and, in many cases, on additional editing proteins that can reduce the number of misacylated tRNAs. For some aminoacyl-tRNA synthetases, errors resulting in aminoacylation of tRNAs depend on the proofreading ability of aminoacyl-tRNA synthetases, ensuring fidelity of amino acid charging to their cognate tRNAs (10). The responsibility of MurM is to generate strains defective in the ability to activate the stringent response pathway due to its proposed role in the elimination of misacylated tRNAs.

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Consistent with these observations, relative to cognate aminoacylation of tRNAAla with alanine, pneumococcal AlaRS generation of seryl-tRNAAla was easily detectable with high (8 μM) concentrations of AlaRS. However, we also observed that misaminoacylation of tRNAAla with glycine by this AlaRS was almost undetectable (SI Appendix, Fig. S2). These data suggested that the fidelity of pneumococcal protein synthesis was challenged by the aberrant amino acid specificity of AlaRS for serine. Therefore, if activation of the stringent response is sensitive to misacylated tRNAs, enzymes that decrease the number of misacylated tRNAs or that decrease errors in the aminoacylation of tRNAs would buffer entry into stringent response.

Accumulation of Ser-tRNAAla is toxic to cells (14, 34), such that many organisms across all the three kingdoms of life encode AlaXp. This protein is homologous to the editing domain of the AlaRS protein and functions in hydrolytic editing of misaminoacylated tRNAAla to ensure fidelity of tRNA aminoacylation (35–39). The pneumococcus does not encode an AlaXp, and MurM has been proposed to fulfill this role (16). However, the catalytic turnover of seryl-tRNAAla hydrolysis by MurM has not been demonstrated, and there is no evidence for discrimination by MurM against utilizing cognate alanyl-tRNAAla (which are hydrolyzed...
corporates seryl-tRNAAla (relative to both seryl-tRNASer and alanyl-tRNAAla) at normal pH (Fig. 2A). In order to use accumulating molecules of Ser-tRNAAla in a drainage system to use accumulating molecules of Ser-tRNAAla, in the context of peptidoglycan synthesis in translation quality control versus normal conditions emphasize the importance of MurM within the pneumococcal translation machinery. The high specificity with which MurM incorporates seryl-tRNAAla (relative to both seryl-tRNAAla and alanyl-tRNAAla), the absence of a gene encoding AlaXp in the pneumococcal genome, and the absence of serine in cell wall bridges in acidic versus normal conditions emphasize the importance of MurM in the pneumococcal synthesis in translation fidelity control under certain conditions that stress pneumococci.

**The efficiency of AlaRS editing is pH dependent.** Deletion of murMN clearly abrogates the ability of the pneumococcus to correct misaminoacylation of noncognate tRNA, yet why would levels of misaminoacylation of tRNAAla be higher at a lower pH? We hypothesized that the strategies used by pneumococci to correct the misaminoacylation of tRNAAla were impaired. AlaRS avoids misaminoacylation of tRNAAla by hydrolysis of misacylated noncognate amino acyl adenylate to form 5′-AMP and noncognate amino acid prior to transfer of the aminoacyl group to the tRNA (pretransfer editing) or by hydrolysis of the noncognate aminoacyl-tRNA once this transfer has occurred (posttransfer editing) (SI Appendix, Fig. S3 and B). Therefore, it was possible that amino acid lability of pre- or posttransfer editing by pneumococcal AlaRS could lead to an increase in misacylated tRNAs in the cell. To test this hypothesis, we devised a simple spectrophotometric method to follow the steady state proofreading rates of pre- (tRNA independent) and posttransfer (tRNA dependent) editing by AlaRS in acidic (pH 6.5) and normal (pH 7.6) conditions in the absence and presence of tRNA as a function of 5′-AMP release (SI Appendix, Fig. S3C). At either pH, addition of 5′-AMP to the assay led to an instantaneous consumption of all of the coupling enzyme system NADH substrate, indicating that pH does not impact the ability of the assay to follow AlaRS activity.

At pH 7.6, pretransfer editing in the presence of noncognate 100 mM L-serine was catalyzed by AlaRS at a rate of 2.15 ± 0.45 min⁻¹. Addition of 3.35 mg · mL⁻¹ Escherichia coli crude tRNA, containing 2.12 μM tRNAAla isoacceptors, caused cycling of the tRNA pool between tRNAAla and seryl-tRNAAla. This registered as a pronounced 3.47-fold increase of 5′-AMP production to a postransfer rate of seryl-tRNAAla editing of 5.32 ± 0.53 min⁻¹ (Fig. 5A, i and ii). Using a saturating concentration of the cognate amino acid L-alanine at 50 mM as a control, at pH 7.6, there was a much lower tRNA-independent turnover of alanine-adenylate of 0.71 ± 0.12 min⁻¹ and a negligible tRNA-dependent posttransfer rate (0.52 ± 0.09 min⁻¹) (Fig. 5B, i and ii). To confirm that this assay did indeed follow postransfer editing, we repeated these assays with AlaRS from which the postransfer editing domain had been removed (AlaRS1463). Pretransfer editing of serine was still detectable (0.70 ± 0.08 min⁻¹); however, tRNAAla supported a posttransfer editing rate (0.49 ± 0.06 min⁻¹) that was less than pretransfer editing (Fig. 5A, iii). These data were consistent with the ability of the tRNA-dependent 5′-AMP release assay to specifically follow AlaRS postransfer editing.

On transition of AlaRS from pH 7.6 to 6.5, there was no significant drop in the rate of pretransfer serine editing, which was now 1.80 ± 0.07 min⁻¹ (Fig. 5A, i and ii). In contrast, at this lower pH, postransfer serine editing was not stably maintained. Within 4 min of tRNA addition, this activity had decelerated to a steady state rate of 1.85 ± 0.49 min⁻¹ that was equal to the rate of pretransfer editing and at this point was threefold less than the corresponding rate at pH 7.6 (Fig. 5A, i and ii). When this experiment was repeated with alanine in place of serine at pH 6.5, there was a much lower

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**Fig. 4.** Impact of misaminoacylation of tRNA on MurM-catalyzed aminoacylation of lipid II-lys. Each MurM species was assayed at a constant concentration of 10 μM lipid II-lys with increasing concentrations of [3H]-seryl-tRNAAla, [3H]-alanyl-tRNAAla, and [3H]-seryl-tRNASer at a specific activity of 100 cpm · pmol⁻¹ in which pneumococcal tRNAAlaUGG and tRNAAlaUGA isoacceptors were employed. Respective MurM159, MurM10K/70 and MurM65 concentrations were 0.63, 2.00, and 1.00 nM in assays utilizing [3H]-seryl-tRNAAla; 13, 91, and 126 nM in assays utilizing [3H]-alanyl-tRNAAla; and 6, 18, and 11 nM in assays utilizing [3H]-seryl-tRNASer. Initial velocities of MurM activity (error bars represent variation between duplicate measurements) are plotted versus [aminoacyl-tRNA]. Data were fitted to the Michaelis–Menten equation using GraphPad Prism version 8.2.1.

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Table 1. Catalytic efficiency of MurM encoded by three alleles, expressed as $k_{cat}^{app}/K_m^{app}$, relative to seryl-tRNA$^{Ala}$, seryl-tRNA$^\text{Ser}$, and alanyl-tRNA$^{Ala}$.

| MurM: Pneumococcal origin | Allele type | Aminoacyl-tRNA | $K_m^{app}$ (μM) | $k_{cat}^{app}$ (min$^{-1}$) | $k_{cat}^{app}/K_m^{app}$ (min$^{-1}$ · μM$^{-1}$) |
|---------------------------|------------|----------------|-----------------|-----------------|-----------------------------|
| 159                       | murMB1     | Alanyl-tRNA$^{Ala}_{\text{UGC}}$ | 0.5 ± 0.02       | 11 ± 0.53       | 22 ± 1.40                   |
|                            |            | Seryl-tRNA$^{Ala}_{\text{UGA}}$ | 0.2 ± 0.01       | 7 ± 0.30        | 31 ± 1.95                   |
|                            |            | Seryl-tRNA$^{\text{Ser}}_{\text{UGC}}$ | 0.1 ± 0.01       | 42 ± 0.21       | 698 ± 116.44                |
| 86                        | murMA      | Alanyl-tRNA$^{Ala}_{\text{UGC}}$ | 0.3 ± 0.02       | 4 ± 0.02        | 12 ± 1.00                   |
|                            |            | Seryl-tRNA$^{\text{Ser}}_{\text{UGC}}$ | 0.2 ± 0.01       | 4 ± 0.22        | 20 ± 1.55                   |
| 110K/70                   | murMA(V101 → A101) | Alanyl-tRNA$^{Ala}_{\text{UGC}}$ | 1 ± 0.09         | 73 ± 7.34       | 77 ± 10.48                  |
|                            |            | Seryl-tRNA$^{Ala}_{\text{UGC}}$ | 1.3 ± 0.21       | 2 ± 0.19        | 2 ± 0.65                    |
|                            |            | Seryl-tRNA$^\text{Ser}_{\text{UGC}}$ | 0.3 ± 0.03       | 2 ± 0.06        | 9 ± 1.11                    |
|                            |            | Seryl-tRNA$^{\text{Ala}}_{\text{UGC}}$ | 0.4 ± 0.08       | 37 ± 2.60       | 98 ± 32.56                  |

Bold italicized data correspond to those obtained with noncognate misaminoacylated seryl-tRNA$^{Ala}_{\text{UGC}}$.

tRNA-independent pretransfer turnover of alanyl-adenylate of 0.91 ± 0.09 min$^{-1}$, and as at pH 7.6, there was a residual tRNA-dependent posttransfer editing rate of 0.34 ± 0.05 min$^{-1}$ (Fig. 5, i and ii). We therefore concluded that if intracellular pH is modified, the ability of pneumococcal AlaRS to support sustained elimination of seryl-tRNA$^{\text{Ala}}$ is compromised (we discuss this data further in relation to mechanisms by which pH might impact AlaRS posttransfer editing and the impact of pH on the turnover of mis-serylated tRNAs in SI Appendix, Supplemental Discussion of Figure 5).

Overexpression of the editing domain of AlaRS can suppress the growth defect of ΔmurMN. The increased preference of MurM for misacylated tRNAs over correctly acylated tRNA species, combined with the drop of AlaRS editing efficiency caused by lowering pH, led us to propose that the growth phenotype of wild-type pneumococcus in acidic conditions resulted from compensation of the loss of translation quality control by MurM-catalyzed elimination of seryl-tRNA$^{\text{Ala}}$.

We reasoned that an increased expression of a tRNA-editing protein in a ΔmurMN background could allow us to distinguish between the cell wall cross-linking and the translation quality control roles of MurM and therefore establish whether disruption of one or both of these roles was responsible for the growth defect of the ΔmurMN strain. To this end, we identified the posttransfer editing domain of AlaRS encoded by pneumococcal alanRS

![Fig. 5. Analysis of the impact of pH on serine editing by AlaRS. All assays were performed with AlaRS or its catalytic domain at an assay concentration of 0.98 μM. Absorbance data (340 nm) were converted to [S-AMP] generated by editing. tRNA$^{\text{Ala}}$ was added as constituent of a crude E. coli total tRNA preparation (Sigma) at a stock concentration 111.16 mg·mL$^{-1}$ based on absorbance at 260 nm, containing 70.34 μM tRNA$^{\text{Ala}}$ (all isoacceptors as assayed by aminoacylation with $^3$H]-alanyl, AlaRS, and trichloroacetic acid precipitation of the $^3$H]-alanyl-tRNA$^{\text{Ala}}$ product (6) in which the final [tRNA$^{\text{Total}}$] assay and [tRNA$^{\text{Ala}}$] assay were 0.09 min$^{-1}$ and 0.005 min$^{-1}$, respectively. All data are in triplicate with SDs. Posttransfer editing was calculated by subtraction of steady state rates in the presence of all components from rates without tRNA. Pretransfer editing rates were the difference between rates obtained in the absence of tRNA and the absence of AlaRS. (A) Pre- and posttransfer serine editing by AlaRS at pH 7.6 and 6.5: pH 6.5 data, red data points and purple error bars; pH 7.6 data, blue data points and purple error bars; (A, i) editing time course; (A, ii) AlaRS steady state serine editing rates where **p < 0.005 and ***p < 0.02 levels of significance in Student’s t test comparisons; and (A, iii) AlaRS$^{\text{Ala}-463}$ steady state serine editing rates. (B) Pre- and posttransfer alanine editing by AlaRS at pH 7.6 and 6.5: (B, i) time course; (B, ii) AlaRS steady-state alanine editing rates.]
The pneumococcal editing domain was therefore identified as residues 437 to 873 of the pneumococcal AlaRS, and this portion of the protein was used for functional complementation (AlaRS editing) (Fig. 6A).

Ectopic expression of AlaRS editing is predicted to deacylate mischarged tRNAAla species, the most abundant of which are Ser-tRNAAla and Gly-tRNAAla (16, 33, 34) (Fig. 6B). Since AlaRS editing only deacylates mischarged tRNAAla molecules, its expression would not be expected to facilitate the correction of otherwise erroneously alanoylated or serylated tRNA species such as Ser-tRNAThr, Ala-tRNAPro, Ala-tRNALys, or Ser-tRNALys synthetized by the class II synthetases (32, 42) (ThrRS, LysRS, or ProRS) (orange in Fig. 6B).

The expression of AlaRS editing in the ΔmurMN strain partially rescued the growth defect displayed by this strain in mildly acidic conditions (Fig. 6C). Specifically, the ΔmurMN/alaRS editing strain resembled the wild type in that it did not exhibit the dramatically increased stationary phase-induced lysis associated with the ΔmurMN strain, and the max OD achieved by this strain was intermediate between the ΔmurMN and wild-type strains. Finally, we tested whether the expression of AlaRS editing in ΔmurMN also influenced alarmone levels, and as predicted by our model, the levels of alarmone in the ΔmurMN/alaRS editing strain resembled that of the wild type (Fig. 3C). These findings illustrate the importance of MurM consumption of misacylated tRNA species in vivo and imply that MurM ensures normal growth in mildly acidic conditions by its ability to consume toxic and mischarged tRNAs through peptidoglycan precursor acylation. Moreover, we conclude that in the absence of MurM, the accumulation of misacylated tRNA species triggers the stringent response.

MurMN Dampens LytA-Mediated Lysis. The role of MurMN in preventing activation of the stringent response pathway via its ability to deacylate mischarged tRNAs explains the growth arrest described in Fig. 1. Next, we questioned the mechanism responsible for the increased lysis subsequent to growth arrest observed in the ΔmurMN strain when grown in mildly acidic conditions (Fig. 1). Having established that MurMN influences activation of the stringent response pathway, we searched for the molecule(s) responsible for the autolysis. Autolysis of pneumococcal cells is carried out by autolysins, peptidoglycan hydrolases containing choline-binding domains that enable their binding to phosphocholine residues present on the teichoic acids of the cell wall (43–45). The addition of exogenous choline inhibits this binding and the consequent autolysis (43, 46–48). The addition of choline chloride (final concentration of 2% [weight/volume]) to ΔmurMN grown in mildly acidic conditions abrogated lysis, suggesting lysis is triggered by choline-binding autolysins (Fig. 7A, i). Of the multiple choline-binding proteins encoded in the pneumococcal genomes, LytA is the major autolysin. To investigate whether the increased lysis of ΔmurMN is induced by LytA, we tested growth of ΔmurMN with a deletion in lytA (ΔmurMNΔlytA). The double mutant resembled the wild-type strain in that it did not display lysis (Fig. 7A, ii). Importantly, the lower maximal OD observed in the ΔmurMN strain was not restored to wild-type levels in the ΔmurMNΔlytA strain. This rescue of only the lysis phenotype suggests that in the absence of MurMN, activation of the stringent response may induce early onset of stationary phase and that LytA is responsible for the subsequent autolysis phenotype. The dramatically increased sensitivity of the ΔmurMN strain to LytA activity, relative to both the wild-type strain at mildly acidic pH and the ΔmurMN strain in normal conditions, suggest that MurMN is positively associated with the activation of LytA under mildly acidic conditions. The autolytic activity of LytA is linked to stationary phase onset (49, 50), and thus, we propose that early entry into the stationary phase may be a trigger for LytA.

MurMN Impacts Macrophage Phagocytosis by Influencing LytA Activity. Pneumococcal cells encounter numerous environmental stresses in an infection setting. Among these is acidic pH, encountered as a result of the inflammatory response mounted by the host against the invading bacteria (51, 52). Previous work suggests that LytA contributes to pneumococcal evasion from phagocytosis (53), and our data revealed that ΔmurMN cells display increased activation of LytA activity. Thus, we hypothesized that macrophages would be less efficient at uptake of ΔmurMN pneumococci relative to wild-type cells.

Since the capsule is a major contributor to the evasion of cells from phagocytosis, we moved to the encapsulated strain D39 (serotype 2 strain, which is the ancestor of Rd6 strain) to test whether MurMN influenced macrophage phagocytosis of pneumococci.

**Fig. 6.** Analysis of translation quality control function of MurM (A) Schematic representation of the domain architecture of full-length and ectopically expressed editing domains of AlaRS. (B) Schematic represents the various naturally mischarged species that MurM is likely to encounter (underlined). AlaRS can misacylate tRNAAla with Ser, ThrRS can misacylate tRNAThr with Ser, ProRS can misacylate tRNAPro with Ala, and LysRS can misacylate tRNAlys with Ala and Ser. Expression of AlaRS editing protein can deacylate mischarged tRNAAla (blue). Other mischarged tRNA moieties cannot be corrected by AlaRS editing protein (orange). Schematic based on findings from previous work (16, 32–34, 41). (C) Representative growth curve for Rd6 wild-type and isogenic ΔmurMN and ΔmurMN/alaRS editing strains grown in acidic conditions (▲). Growth curves were started at an OD600 of 0.05 (at least n = 3).
The number of macrophages positive for pneumococci was about twofold lower when infected with ΔmurMN relative to the wild-type strain (Fig. 7 B and C and SI Appendix, Fig. S4). Furthermore, this phenotype was reversed for the ΔmurMNΔlytA strain, demonstrating the importance of LytA to this phenotype (Fig. 7 B and C). Finally, in agreement with previous work (53), the number of macrophages positive for pneumococcal evasion was increased upon infection with a ΔlytA strain relative to the wild type (Fig. 7 B and C).

Our data suggest that the activation of LytA in the ΔmurMN strain requires activation of the stringent response pathway. Thus, if a ΔmurMN strain does not induce the stringent response, it should not display increased evasion from macrophages. In support of this contention, the number of macrophages that internalized the ΔmurMNΔrsh strain was higher than those that internalized the ΔmurMN strain. Finally, our model predicts that MurMN buffers the stringent response and LytA-mediated autolysis via its role in deacylating the misacylated tRNAs. Consistently, we observed a higher number of macrophages positive for the ΔmurMNΔalaRSΔclading strain relative to the ΔmurMN strain (Fig. 7 B and C). These data are consistent with a role for MurMN in activation of the stringent response and subsequent increase in LytA activity and reveal the consequence of MurMN-mediated stringent response suppression for phagocytosis.

The decreased percentage of macrophages positive for ΔmurMN pneumococci could be a consequence of either greater pneumococcal evasion of phagocytes or increased lysis of pneumococcal cells upon their internalization by macrophages (where the pH of phagolysosomes is much lower than the pH 6.6 used in our studies). If the acidic environment of macrophages promotes increased lysis of the ΔmurMN pneumococci, there should be a rapid decrease in internalized bacteria over time. In contrast, we observe a more gradual decline in internalized ΔmurMN pneumococci relative to wild-type cells (SI Appendix, Fig. S5). Thus, the ΔmurMN strain appears to evade phagocytosis, and, as previously reported, this process is LytA dependent (53).

Discussion

Elucidating the mechanisms that bacterial cells employ to compartmentalize their functions or organize cell-wide responses to environmental stresses is of fundamental interest to the field of microbiology. The staphylococci have evolved mechanisms to partition cognate aminoacylated tRNA molecules between translation and cell wall biosynthesis. For instance, Staphylococcus aureus encodes a set of isoacceptor tRNA<sup>gly</sup> species that display a greater propensity for biosynthesis of peptidoglycan than for protein synthesis via diminished binding to the EF-Tu elongation factor utilized in translation (54–56). Similarly, Staphylococcus epidermidis contains a tRNA<sup>ser</sup> and two tRNA<sup>gly</sup> species that are only competent in peptidoglycan synthesis (56–58). In contrast, in other bacterial species, the same pool of aminoacyl-tRNAs is utilized for both purposes of peptidoglycan modification and translation (54). The tRNA-dependent amino acyl transferases MurM and MurN are involved in pneumococcal cell wall biosynthesis: they build dipeptide cross bridges that serve as a major component of the bacterial cell wall and contribute to penicillin resistance. Here, we demonstrate that in doing so, one such enzyme, MurM, resolves the threat that tRNA misaminoacylation poses to protein synthesis by utilizing misaminoacylated

![Image](https://doi.org/10.1073/pnas.2018089118)
tRNA to aminoacylate peptidoglycan precursors with remarkable specificity.

We found that three enzymes encoded by different murM alleles use none cognate misaminoacylated seryl-tRNA to seryl the peptidoglycan precursor lipid II-Lys with up to 20-fold greater catalytic efficiency than either correctly aminoacylated cognate alanyl-tRNA or seryl-tRNA. This strongly suggests that MurM can attenuate intracellular stress through preferential sequestration of incorrectly aminoacylated tRNA into peptidoglycan synthesis. It has been proposed that MurM acts to preserve fidelity of protein synthesis through catalyzing the simple hydrolysis of misaminoacylated tRNA (16). However, this was based upon assays where enzyme to seryl- or alanyl-tRNA ratios were 10,000:1 (16) and could not therefore address the catalytic properties of MurM. In contrast, our MurM lipid II-Lys aminoacylation assays were performed with enzyme:seryl-tRNA
greater than the rate of seryl-tRNA deacylation, inconsistent with the proposed role of MurM in the control of the fidelity of protein biosynthesis (16). In contrast, and consistent with its proposed role, we found that MurM was considerably more specific for the misaminoacylated seryl-tRNA than the cognate alanyl-tRNA (37). Finally, hydrolytic deacylation of alanyl-tRNA by MurM was negligible compared to rates of aminoacylation of peptidoglycan precursors (6). These observations lead us to conclude that the most likely mechanism by which MurM corrects AlaRS editing deficiencies is by incorporation of misdirected seryl residues into the pneumococcal peptidoglycan.

In vivo, our analysis of cell wall composition is consistent with functional MurM leading to an increased amount of seryl residues in peptidoglycan dipeptide branches at pH 6.5 compared to 7.6. This fits a model where MurM acts to consume misaminoacylated tRNA generated by a diminution of the ability of AlaRS (and perhaps ThrRS) to carry out effective posttransfer editing at a reduced pH. Pneumococcal isolates display wide variation in the extent and composition of the branched muranopeptides within their peptidoglycan (11). We suggest that stresses such as, but not limited to, pH on editing by aminoacyl-tRNA synthetases contribute to this variability.

There are precedents from several lactic acid bacteria that low pH conditions trigger a drop in internal pH, allowing these cells to maintain a constant pH gradient across the membrane. For example, in Streptococcus thermophilus, reduction in external pH between 7 and 5 is mirrored by reduction in intracellular pH to as low as pH 5.5 (59). Similarly, in Streptococcus bovis, an external pH range of 5.4 to 6.5 translates to an internal pH of 5.5 to 6.7; this maintains a constant ΔpH across the cell membrane and forestalls toxic accumulations of fermentation product anions (60, 61). Furthermore, Streptococcus mutans with a glucose energy source maintains a pH gradient across the cell membrane, which dissipates on exhaustion of the carbon source and depletion of ATP (62). Therefore, for S. pneumoniae, which can generate up to 35 mM D-lactic acid from the fermentation of glucose (63), it is likely that this organism also meets the challenge of acidic growth conditions by optimizing the pH gradient across its cytoplasmic membrane through reduction of its intracellular pH. On exhaustion of the carbon source, and entry into stationary phase, it is likely that the pneumococcal intracellular pH will fall. We did not confirm the intracellular pneumococcal pH relative to the corresponding external pH. However, it seems likely that the drop in external pH may have induced a drop in intracellular pH (before or on consumption of the pneumococcal carbon source), which compromised translational fidelity via decreased editing by AlaRS (Fig. 5). During pneumococcal infection, this effect may be exacerbated in the phagolysosomal environment, where the pH in the phagosomal lumen can be as low as 4.5 (64). The heterogeneity of cell wall composition that results from these stresses may make peptidoglycan an environmentally sensitive readout of the response of MurM activity to variable levels of mischarged seryl-tRNAs.

A drop in intracellular pH and consequent decreased editing of AlaRS provides one hypothesis for the increased level of mischarged seryl-tRNAs. Another hypothesis is that the drop in extracellular pH impacts the function of an extracellular protein. For instance, if decreased pH impacts the activity of penicillin binding proteins (PBPs), this could induce a stress response. In support of this, there is an association between pH and pneumococcal cell shape (65), and in E. coli, PBP function is dependent on pH (66).

The posttransfer editing domain of AlaRS is functional in the absence of the N-terminal tRNA aminoacylation domain of the enzyme (37). We used this functionality to confirm that the increase in seryl-tRNAΔ due to the absence of MurMN was a cause of acid stress-induced early onset of stationary phase. We suggest that, in the ΔmurMN strain, this construct augmented the compromised editing capacity of AlaRS at this pH, allowing sufficient restoration of editing activity to consume seryl-tRNAΔ that would have been utilized by MurM in the wild-type pneumococcus.

However, overexpression of the posttransfer editing domain of pneumococcal AlaRS as a seryl-tRNAΔ deacylase in our ΔmurMN strain only partially reversed its growth deficit at acidic pH. This could be related to levels of expression or activity of the AlaRS posttransfer editing domain. Alternatively, the incomplete restoration of growth at the lower pH might also be the result of impaired ThrRS posttransfer editing in these conditions (67), which would not have been reversed by overexpression of the posttransfer editing domain of AlaRS. Additionally, if a component of the acid-sensitive ΔmurMN phenotype was contributed by an impediment to PBP-catalyzed peptidoglycan transpeptidation at low pH, this could similarly also account for the partial reversal of this phenotype by expression of the editing domain of AlaRS.

Our results further revealed that in addition to suppression of seryl-tRNAΔ accumulation through expression of MurM or the posttransfer editing domain of AlaRS, the acid-sensitive growth phenotype of ΔmurMN strains could be reversed by deletion of rsh, which reduced alarmon synthesis and therefore suppressed expression of the ΔmurMN phenotype. Furthermore, alarmon levels were inversely related to expression of murMN. This suggested that there is a causal link between misaminoacylated tRNA accumulation and activation of the pneumococcal stringent response. It therefore seems that in order to prevent potential corruption of translation, consumption of seryl-tRNAΔ by MurM may represent a first line of defense. When this mechanism is overwhelmed (or as here, absent in ΔmurMN), the stringent response shuts down translation to avoid further toxic generation of mistranslated/misfolded protein (SI Appendix, Fig. S6).

In summary, our studies in the pneumococcus revealed that in the absence of MurM, the accumulation of mischarged tRNAs (possibly via pH-induced diminution of posttransfer editing by AlaRS) initiates the stringent response, premature entry into stationary phase, and subsequent activation of the murein hydrolase LytA. In this context, the cell wall biosynthesis protein MurM, by virtue of its role in preferentially using mischarged seryl-tRNAΔ to aminoacylate peptidoglycan precursors, dampens activation of the stringent response and calibrates the cellular reaction to this stress.

An outstanding question in this model is how mischarged tRNAs accumulated because of the absence of MurM trigger the stringent response? Binding of aminoacyl-tRNA to the ribosomal A-site provides the required amino acid for polypeptide elongation (19). In Gram-negative bacteria, binding of decaylated tRNAs to the ribosome activates RelA and induces alarmon production (19). Activation of the stringent response is
less understood in Gram-positive bacteria, where the alarmon e synthetase and hydrolyse functions are fused in the bifunctional RSH enzyme (17, 68, 69). Our data, which suggests an accumulation of mischarged tRNAs concurrent with initiation of RSH-catalyzed alarmone production to trigger the stringent response in pneumococcus, could indicate that RSH may be activated by binding of mischarged tRNAs to the ribosome.

A second hypothesis is that the stringent response is activated by unfolded proteins. In the case of seryl-tRNA<sup>Ala</sup> accumulation through mutation of AlaRS posttransfer editing domains (34, 70), and in other examples of unrestrained tRNA mischarging (71), errors in protein synthesis accumulate and account for substantial losses of cellular viability. The production of mistranslated proteins leads to up-regulation of the heat shock response genes <i>rpoH</i>, <i>dnaK</i>, and <i>mapl</i> in <i>E. coli</i> (71, 72). In this context, it is interesting to note that (p)ppGpp accumulation has recently been shown to be an integral part of the Firmicute heat shock response of <i>Bacillus subtilis</i> (73). Therefore, we propose that a deficit in pneumococcal translational quality control caused by the absence of MurM activity and pH-driven attenuation of posttransfer editing by AlaRS, and possibly ThrRS, generates sufficient seryl-tRNA<sup>Ala</sup> to overwhelm the capacity of pretransfer editing to prevent access of these noncognate species into protein synthesis. The consequential generation of misfolded protein arising from substitution of serine for alanine at GGN codons triggers (p)ppGpp accumulation by RSH, which inhibits translation IF2 (29), thereby attenuating protein synthesis and transitioning the organism into a quiescent state and therefore triggering the stringent response to cause cessation of growth.

Consistent with its role in the response to stress, RSH is the main source of pneumococcal alamone and in the nutritional stringent response, provides increased fitness in cells subjected to mupirocin, an antibiotic that causes increasing cellular levels of decacylated tRNA<sup>Ala</sup>. Additionally, RSH is a virulence determinant in murine models of pneumococcal pneumonia and sepsis and promotes resistance to killing by neutrophils to provide a fitness advantage in a murine model of colonization (31, 74). Together, these studies strongly suggest that the ability of the pneumococcus to trigger the stringent response is critical for virulence. We therefore propose that MurM plays a role in delaying or fine-tuning entry into the stringent response and as such may contribute to the overall virulence of pneumococcus.

The ability of MurM to forestall acid-induced entrance into the stringent response on phagocytosis is likely to play a role in macrophage survival during pathogenesis.

In eukaryotes, prokaryotes, and the archaea, AlaXp proteins are conserved in their ability to decay seryl-tRNA<sup>Ala</sup> but not necessarily glycyl-tRNA<sup>Ala</sup>, which has recently been shown to be decacylated by D-aminoacyl-tRNA deaclyase (38, 80). Similarly, here we showed that pneumococcal AlaRS misaminoacylation of tRNA<sup>Ala</sup> with glycine is negligible compared to that with serine, a characteristic also shared by eukaryotic AlaRSs (34). This, in addition to the specificity of D-aminoacyl-tRNA deaclyase, suggests that unlike seryl-tRNA<sup>Ala</sup>, glycyl-tRNA<sup>Ala</sup> does not accumulate enough to threaten fidelity of protein synthesis that MurM is required to correct. Consistent with this hypothesis, pneumococcal peptidoglycan cell wall bridges do not contain glycine (11).

Decacylated mischarged tRNA<sup>Ala</sup> is crucial, wherein accumulation of seryl-tRNA<sup>Ala</sup> is toxic and where even partial diminution of posttransfer editing of this species has pathological consequences (33, 67). It has been proposed that AlaXp is an evolutionary solution to the ubiquitous challenge of Ser-tRNA<sup>Ala</sup> production (38). We suggest that MurM is an alternative evolutionary solution to this challenge during conditions of cellular stress.

Genomic analysis reveals that many Firmicutes encoding cell wall tRNA-dependent aminoacyl transferases that produce peptide bridges do not appear to encode for an AlaXp protein. These include the following: <i>Staphylococcus aureus</i>, <i>Staphylococcus epidermidis</i>, <i>Streptococcus thermophilus</i>, <i>Streptococcus agalactiae</i>, <i>Streptococcus salivarius</i>, <i>Enterococcus faecalis</i>, and <i>Weissella viridescens</i> (75). It is therefore tempting to speculate that cell wall bridges containing proteogenic amino acids may reflect the function of cell wall tRNA-dependent aminoacyl transferase in translation quality control. Consistent with this hypothesis, Firmicutes that encode AlaXp proteins include <i>Bacillus subtilis</i> and <i>Bacillus megaterium</i>, which have no cell wall bridges, and <i>Enterococcus faecium</i>, <i>Pediococcus pentosaceus</i>, and <i>Lactobacillus cellobiosus</i> (syn. <i>fermentans</i>), which do have cell wall bridges but those that are composed of nonproteogenic D-iso-asparagine.

We contend that these species encode AlaXp proteins to carry out the role performed by MurM and its homologs in other Firmicutes (75–77).

In conclusion, this study suggests that MurM provides a molecular link between cell wall biosynthesis and translation quality control in <i>S. pneumoniae</i> that produce short peptide bridges in their peptidoglycan layer. These bridges are structural components that contribute to antibiotic resistance (8–10, 13). This study provides in vivo evidence to suggest that the propensity of MurM to utilize mischarged tRNA allows it to serve as a translation quality control checkpoint and dampen entry into the stringent response. Through these mechanisms, MurM influences the activity of LytA and phagocytosis. These findings implicate MurM and therefore peptidoglycan synthesis in the survival of bacteria as they encounter unpredictable and hostile conditions in the host. The question of how the regulation of these fundamental biological processes by MurM influences the extent of pathogenesis still remains to be investigated. Our work provides functional insight into the role of peptidoglycan peptide bridge-generating enzymes in modulating intracellular stress and entry into the stringent response.

Materials and Methods

Growth Curves. Strains of interest were streaked on TSA II agar plates supplemented with 5% (volume/volume [vol/vol]) sheep blood. These streaked cells were then inoculated into fresh Columbia broth (normal or acidic) and incubated at 37 °C and 5% (vol/vol) CO<sub>2</sub> without shaking. Once the cultures reached an OD<sub>600</sub> of 0.05, the growth of the cultures was followed every 10 min and resuspended in acidic Columbia broth to an OD<sub>600</sub> of 0.05 by recording their optical density at a wavelength of 600 nm. Strains with a Δ<i>rsh</i> genetic background showed a much longer lag phase. To circumvent this, when comparing growth of cells with strains in Δ<i>rsh</i> background, we adapted our protocol from a previous study measuring growth of these cells (31). The cells were grown in acidified Columbia broth to an OD<sub>600</sub> of 0.1. The cells were collected by centrifugation at 3,000 rpm for 10 min and resuspended in acidic Columbia broth to an OD<sub>600</sub> of ~0.1. The growth of these cultures at 37 °C and 5% (vol/vol) CO<sub>2</sub> without shaking was followed every 30 min by recording their optical density at 600 nm.

All strains whose growth curves are represented in a given figure panel were grown in the same batch of media. The replicates were performed with fresh preparations of media on different days.

AlaRS Serine Editing Assays. AlaRS editing assays were performed to follow serine- and tRNA-dependent generation of 5′-AMP at 37 °C in a continuous coupled assay in a final volume containing 50 mM Hepes, pH 7.6, or 50 mM 3-(N-morpholino)propanesulfonic acid, pH 6.5, 10 mM Mg<sub>2+</sub>, 50 mM KC1, 1 mM dithiothreitol, 2 mM ATP, 2 mM phosphoenol pyruvate, 0.3 mM NADH, 40 mM · min<sup>−1</sup> rabbit muscle myokinase, 8 mM · min<sup>−1</sup> rabbit muscle pyruvate kinase, and 11.5 mM · min<sup>−1</sup> rabbit muscle pyruvate kinase, and 11.5 mM · min<sup>−1</sup> rabbit muscle pyruvate kinase, and 11.5 mM · min<sup>−1</sup> rabbit muscle pyruvate kinase, and 11.5 mM · min<sup>−1</sup> rabbit muscle pyruvate kinase, and 11.5 mM · min<sup>−1</sup> rabbit muscle pyruvate kinase, and 11.5 mM · min<sup>−1</sup> rabbit muscle pyruvate kinase, and 11.5 mM · min<sup>−1</sup> rabbit muscle pyruvate kinase, and 11.5 mM · min<sup>−1</sup> rabbit muscle pyruvate kinase, and 11.5 mM · min<sup>−1</sup> rabbit muscle pyruvate kinase, and 11.5 mM · min<sup>−1</sup> rabbit muscle pyruvate kinase, and 11.5 mM · min<sup>−1</sup> rabbit muscle pyruvate kinase, and 11.5 mM · min<sup>−1</sup> rabbit muscle pyruvate kinase. In this assay, 100 UV-Vis double beam spectrophotometer for 3 min, at which point assays were supplemented with 100 mM L-serine. To initiate pretransfer editing, 0.98 μM AlaRS was added to the cuvette, and 5′-AMP generated by seryl-adenylate formation was followed as a drop in OD<sub>600</sub>. To initiate posttransfer addition, 3 mg · mL<sup>−1</sup> crude <i>E. coli</i> tRNA comprising 2.12 μM tRNA<sup>Ala</sup> isoacceptors [assayed by [3H]-L-alanine aminoacylation of <i>S. pneumoniae</i> tRNA<sup>Ala</sup> according to Lloyd et al. (6)] were added to the cuvette. The 5′-AMP additionally generated from the continual formation and hydrolysis of seryl-tRNA<sup>Ala</sup> was then followed as a drop in OD<sub>600</sub>. Assays were additionally performed in which L-serine was replaced by
l-alanine and 5'-AMP concentration was determined assuming coupling of two equivalents of NADH-AMP in which for NADH, 1×10^-3 M = 6,220 M-1.cm^-1.

AlaRS reporter assays. AlaRS reporter assays were performed using the reporter system in E. coli to measure the activity of AlaRS. The assays were carried out in 50 μL of reaction mixture containing 100 mM Tris–HCl (pH 7.5), 50 mM MgCl2, 200 μM [methyl-3H]-L-alanine, 50 μM L-alanine, 30 mM ATP, 50 μM GTP, 10 μg of tRNA, and 50 μg of AlaRS. The reactions were incubated at 37 °C for 30 min, and the reactions were stopped by the addition of 1 mL of ice-cold ethanol. The samples were then filtered through millipore filters, and the radioactivity was measured.

MurM enzyme assays. MurM enzyme assays were performed as previously described (18). MurM was expressed in E. coli and purified using Ni-affinity chromatography. The enzyme was then used for the assay, and the activity was determined by measuring the incorporation of [3H]-L-alanine or [3H]-L-serine into lipid II.

MurM aminoacyl transferase assays. MurM aminoacyl transferase assays were performed using [3H]-L-alanine-lipid II-Lys or [3H]-L-serine-lipid II-Lys as substrates. The reactions were carried out in 50 μL of reaction mixture containing 100 mM Tris–HCl (pH 7.5), 50 mM MgCl2, 200 μM [methyl-3H]-L-alanine, 50 μM L-alanine, 100 μM L-serine, 100 μM L-lysine, 20 μM tRNA, and 50 μg of MurM. The reactions were incubated at 37 °C for 30 min, and the reactions were stopped by the addition of 1 mL of ice-cold ethanol. The samples were then filtered through millipore filters, and the radioactivity was measured.

Data availability. All data used in the article and its supporting information are available in the supplemental materials and methods.

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