Lipid II-Independent Trans Editing of Mischarged tRNAs by the Penicillin Resistance Factor MurM

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Lipid II-independent \textit{trans} Editing of Mischarged tRNAs by the Penicillin Resistance Factor MurM*

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\textbf{Background: }MurM utilizes aminoacyl-tRNAs and Lipid II for peptidoglycan biosynthesis in \textit{Streptococcus pneumoniae}. Results: MurM decaysates mischarged aminoacyl-tRNAs in the absence of Lipid II. Conclusion: The ability of MurM to function in quality control can compensate for the absence of AlaXp proteins in \textit{S. pneumoniae}. Significance: MurM can function in translation as a lipid-independent \textit{trans} editing factor.

\textit{Streptococcus pneumoniae} is a causative agent of nosocomial infections such as pneumonia, meningitis, and septicemia. Penicillin resistance in \textit{S. pneumoniae} depends in part upon MurM, an aminoacyl-tRNA ligase that attaches \textit{l}-serine or \textit{l}-alanine to the stem peptidase lysine of Lipid II in cell wall peptidoglycan. To investigate the exact substrates the translation machinery provides MurM, quality control by alanyl-tRNA synthetase (AlaRS) was investigated. AlaRS mischarged serine and glycine to tRNA\textit{Ala}, as observed in other bacteria, and also transferred alanine, serine, and glycine to tRNA\textit{Phe}. \textit{S. pneumoniae} tRNA\textit{Phe} has an unusual U4:C69 mismatch in its acceptor stem that prevents editing by phenylalanyl-tRNA synthetase (PheRS), leading to the accumulation of misacylated tRNAs that could serve as substrates for translation or for MurM. Although the peptidoglycan layer of \textit{S. pneumoniae} tolerates a combination of both branched and linear muropeptides, deletion of MurM results in a reversion to penicillin sensitivity in strains that were previously resistant. However, because MurM is not required for cell viability, the reason for its functional conservation across all strains of \textit{S. pneumoniae} has remained elusive. We now show that MurM can directly function in translation quality control by acting as a broad specificity lipid-independent \textit{trans} editing factor that deacylates tRNA. This activity of MurM does not require the presence of its second substrate, Lipid II, and can functionally substitute for the activity of widely conserved editing domain homologues of AlaRS, termed AlaXp proteins, which are themselves absent from \textit{S. pneumoniae}.

\textit{Streptococcus pneumoniae} is a Gram-positive diplococcus that is carried asymptotically in the nasopharynx of 5–10% of healthy adults and 20–40% of healthy children. Clinically, \textit{S. pneumoniae} is the common causative agent of several community and hospital acquired infections including pneumonia, otitis media, meningitis, and septicemia. According to the Centers for Disease Control (CDC), ~5 million fatal cases of pneumococcal pneumonia in children under the age of five are reported globally each year (37). Pneumococci have an unusual lifestyle because they produce high levels of hydrogen peroxide that provides a competitive advantage for the organism during colonization of the nasopharynx (1, 2). In other organisms, it has been reported that exposure to increased levels of hydrogen peroxide can enhance cellular mistranslation rates both \textit{in vivo} and \textit{in vitro} (3, 4). For example, in mammalian cells, ~1% of protein synthesis-directed methionine residues are aminoacylated onto noncognate tRNA molecules. This methionine misacylation is increased as much as 10-fold in the presence of reactive oxygen species, such as hydrogen peroxide. Substitution of coded amino acids with methionine is believed to protect proteins against oxidative damage under stress conditions (3). In \textit{Escherichia coli}, exposure to hydrogen peroxide causes reduction in the fidelity of translation. This effect has been directly attributed to oxidation of Cys-182 within threonyl-tRNA synthetase, which subsequently impairs the editing ability of the enzyme and results in the production of misacylated Ser-tRNA\textit{Thr} (4). How translation quality control is maintained in pneumococci, which are routinely exposed to elevated hydrogen peroxide levels, is unknown.

Aminoacyl-tRNA synthetases are the first step in quality control of protein synthesis because they are responsible for amino acid activation and transfer to cognate tRNA (5). Following this process, the aminoacyl-tRNA is released from the synthetase and bound by elongation factor Tu (EF-Tu)\textsuperscript{2} for delivery to the ribosome and use in protein synthesis (6, 7). Aminoacyl-tRNA synthetases are usually highly selective for their cognate tRNA due to the availability of a large surface area for recognition, identity elements within the tRNA molecule itself, and also kinetic proofreading during the aminoacylation reaction (8–10). In contrast, some amino acids are difficult for aminoacyl-tRNA synthetases to distinguish with high accuracy as they can differ by as little as a single methyl group (11). For example, isoleucyl-tRNA synthetase has difficulty distinguishing between the isosteric amino acids isoleucine and valine, whereas the active site of alanyl-tRNA synthetase (AlaRS) is able to accommodate alanine, glycine, and serine (12–16).

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\footnote{The abbreviations used are: EF-Tu, elongation factor Tu; AlaRS, alanyl-tRNA synthetase; PheRS, phenylalanyl-tRNA synthetase.}
Misactivation of noncognate serine and glycine by AlaRS occurs at frequencies of 1/500 and 1/250, respectively. This is higher than the overall error rate for translation, which is typically from 1/3000 to 1/10,000 (10). Amino acid activation errors can be corrected both by the synthetase itself at a distinct editing site, as is the case for AlaRS, and also by free-standing editing domain homologues, as exemplified by the widely conserved AlaXPs proteins that edit Ser-tRNAAla (17, 18).

S. pneumoniae encodes no known AlaXPs, suggesting that the genes encoding these AlaRS editing domain homologues may have been lost from the pneumococcal genome during gene shuffling, which occurs rapidly within the organism as a result of exposure to antibiotics (19). This loss would be feasible in the presence of another conserved protein able to perform the same function. One such candidate protein is MurM, an Ala/Ser-tRNA-dependent aminoacyl-tRNA ligase that is involved in the synthesis of branched-structured muropeptides in pneumococcal peptidoglycan (20). MurM catalyzes the transfer of either alanine or serine to the stem peptide lysine of Lipid II and, in combination with MurN, generates the substrate for indirect cross-linking of peptidoglycan. Until recently, the only aminoacyl-tRNA substrates recognized by MurM were thought to be Ser-tRNA<sup>Ser</sup>, provided by seryl-tRNA synthetase, and Ala-tRNA<sup>Ala</sup>, provided by AlaRS (38). However, MurM is also able to efficiently transfer serine to Lipid II from misaminoacylated Ser-tRNA<sup>Ala</sup>, which is also produced by pneumococcal AlaRS (21). The observed preference for misaminoacylated Ser-tRNA<sup>Ala</sup> suggests that MurM could function as a trans editing factor and influence translation quality control by channeling appropriate misaminoacylated tRNA species into the peptidoglycan biosynthesis pathway. Here we show that pneumococcal AlaRS also misaminoacylates an unusual tRNA<sup>Phe</sup> isoacceptor to generate substrates for MurM, which is able to catalyze deacylation in the absence of Lipid II.

### EXPERIMENTAL PROCEDURES

**Strains, Plasmids, and General Protein Expression and Purification—**S. pneumoniae strain D39 chromosomal DNA for use as a template in the cloning of AlaRS, PheRS, EF-Tu, and MurM was a gift from B. Lazazzera (University of California, Los Angeles). An expression construct for producing His<sub>6</sub>-tagged *E. coli* AlaRS and pUC19 containing the *E. coli* tRNA<sup>Ala</sup> gene for production by *in vitro* transcription were gifts from K. Musier-Forsyth (Ohio State University, Columbus, OH).

The genes encoding *S. pneumoniae* AlaRS full-length protein, AlaRS residues 1–460 (catalytic domain only), EF-Tu, and MurM were cloned into pET21b (Novagen) by virtue of the NdeI and XhoI restriction sites. Subsequent expression constructs allowed for the production of recombinant proteins tagged at the C termini with a hexahistidine tag. The genes encoding *S. pneumoniae* PheRS<sup>α</sup> and PheRS<sup>β</sup> subunits were cloned into pET Duet-1 (Novagen) multiple cloning sites one and two, respectively, such that the protein was produced with an N-terminal hexahistidine tag. All proteins were overexpressed in *E. coli* strain BL21 (DE3) by the addition of a final concentration of 1 mM isopropyl-β-D-1-thigalactopyranoside at an A<sub>600</sub> of 0.4–0.6 followed by a reduction in growth temperature from 37 °C to 28 °C for 3–5 h. Proteins were purified on BD TALON cobalt resin using equilibration/wash buffer (50 mM sodium phosphate, pH 7.2, 500 mM sodium chloride, and 20% glycerol) containing 250 mM imidazole. MurM was solubilized prior to purification as described (21, 38).

*S. pneumoniae* tRNA<sup>Phe</sup> and tRNA<sup>Phe</sup> (wild type and U4G) and the *E. coli* wild type equivalents were produced by *in vitro* T7 RNA polymerase runoff transcription as described (22).

**Site-directed Mutagenesis—**Site-directed mutagenesis of pneumococcal tRNA<sup>Phe</sup> was carried out by the polymerase chain reaction using *Pfu* Turbo polymerase. Methodology
was obtained from the Stratagene site-directed mutagenesis manual.

**Aminoacylation**—Aminoacylation time courses were carried out across a time period of 1 h at 37 °C in the presence of 0.1 M Na-HEPES, pH 7.2, 30 mM KCl, 10 mM MgCl₂, 2 mM ATP, 7 μM trNA²⁰⁺⁶;/³⁰⁻° transcript, 40 or 110 μM [³H]Ser/[¹⁴C]Ala/[¹⁴C]Gly (150–200 cpm/pmol), and 310 nM active AlaRS (as determined by active site titration). Reactions were repeated in the presence of 50 nM active *S. pneumoniae* PheRS, 300 nM *S. pneumoniae* MurM, or 3 μM activated *S. pneumoniae* EF-Tu. EF-Tu was activated in 50 mM Tris-HCl, 1 mM DTT, 68 mM KCl, 2.5 mM phosphoenolpyruvate, 0.5 mM GTP, and 30 mg/ml pyruvate kinase as described (23). 10-μl samples were taken for each time point and spotted onto 3-mm Whatman filter paper discs, which were immediately dropped into 5% TCA. Discs were subjected to further washes with 5% TCA and ethanol prior to drying and scintillation counting.

**Kinetics of Phenylalanylation of tRNAPhe Wild Type and U4G by PheRS**—To determine Michaelis-Menten kinetics for pneumococcal PheRS with either wild type or mutant U4G trNA²⁰⁺⁶;/³⁰⁻°, phenylalanylation time courses were carried out at 37 °C at both the lowest (0.05 μM) and the highest (10.0 μM) tRNA concentration in the presence of 0.1 M Na-HEPES, pH 7.2, 30 mM KCl, 10 mM MgCl₂, 2 mM ATP, 50 μM [¹⁴C]Phe (200 cpm/pmol), and 50 nM active PheRS (as determined by active site titration). Because the linear region was determined to be within the first 2 min, 10-μl samples were spotted onto 3-mm Whatman filter paper and dropped into 5% TCA at four time points (0.5, 1.0, 1.5, and 2 min) at each of the tRNA concentrations (0.05, 0.10, 0.20, 0.40, 0.60, 0.80, 1.00, 3.00, 5.00, 10.00 μM) for determination of gradients and key kinetic parameters from triplicate data sets.

**Decacylation Assays**—Aminoacylation reactions were set up in four 200-μl reactions each consisting of 30 mM HEPES, pH 7.6, 15 mM MgCl₂, 10 mM DTT, 2 mM ATP, 110 μM [³H]alanine (with a specific activity of ~300 cpm/pmol), 10 μM *S. pneumoniae* tRNA²⁰⁺⁶;/³⁰⁻° transcript (prior to use, stock was resuspended in 4 mM MgCl₂ and heated at 80 °C for 10 min followed by slow cooling to room temperature to allow refolding), 2 μmol min⁻¹ ml⁻¹ inorganic pyrophosphatase, and 3 μM
alanyl-tRNA synthetase catalytic domain. The reactions were incubated at 37 °C for 2 h and then quenched by the addition of 20 μl of 3 mM sodium acetate, pH 4.5. [3H]Aminoacyl-tRNA purification was achieved by the addition of 220 μl of phenol, pH 4.5, to each of the four reactions followed by mixing and centrifugation at 13,000 rpm for 5 min. After centrifugation, the aqueous phase was retained, and an equal volume of 24:1 chloroform:isoamyl alcohol was added. 550 μl of −20 °C RNase-free ethanol was added to the aqueous phase, which was subsequently incubated at −80 °C for 1 h. The precipitated aminoacyl-tRNA was pelleted by centrifugation at 13,000 rpm for 30 min and washed with 1 ml of 70% ethanol. After a final centrifugation step at 13,000 rpm for 30 min, the pellet was dried at room temperature for 5 min and resuspended in 50 μl of 3 mM sodium acetate, pH 4.5. At this point, all four reactions were pooled, yielding 200 μl of [3H]Ser or [3H]Ala-tRNAAla, the concentration of which was determined by 5% TCA precipitation of 2 μl of the stock and scintillation counting. Deacylation assays were carried out by incubation of 50 pm [3H]Ser or Ala-tRNAAla in buffer composed of 0.1 M Na-HEPES, pH 7.2, 30 mM KCl, and 10 mM MgCl2. In addition, 0.5 μM AlaRS, 0.5 μM MurM, or an equal volume of protein storage buffer was added to the reaction, which was monitored by TCA precipitation and scintillation counting.

Due to our inability to successfully isolate sufficient yields of misaminoacylated tRNAPh, an aminoacylation-coupled deacylation reaction was developed. 100-μl aminoacylation reactions were incubated at 37 °C in the presence of 0.1 mM Na-HEPES, pH 7.2, 30 mM KCl, 10 mM MgCl2, 2 mM ATP, 7 μM tRNAAlaPhe transcript, 100 μM [3H]Ser (PerkinElmer)/[3H]Ala (Moravek Biochemicals) at a specific activity of 200–300 cpm/μmol, and 0.5–1.0 μM active AlaRS catalytic domain (or full-length AlaRS in the case of cognate Ala-tRNAAla). After a 1-h incubation, sodium chloride (final concentration 100 mM) and adenosine 5’-triphosphatase from porcine cerebral cortex (final concentration of 0.5 units, Sigma-Aldrich) were added to the reaction. Incubation at 37 °C was continued for an additional 30 min to the plateau of aminoacylation prior to the addition of the potential deacylation factor (enzyme storage buffer in the case of the control, full-length AlaRS, PheRS, or MurM) in a 34-μl volume where 4 μl was composed entirely of 100 mM unlabeled serine or alanine as appropriate. After equilibration for 2 min, a 10-μl volume was taken from the reaction and spotted onto 3-mm Whatman filter paper, which was immediately dropped into 5% TCA. This was used as the zero time point, and the deacylation reaction was monitored for an additional 10 min with samples taken at 1, 2, 4, 8, and 10 min. After all time points had been taken, filter papers were washed a further two times in 5% TCA and then once in 100% ethanol prior to drying and liquid scintillation counting. All samples were repeated in triplicate. Full-length AlaRS, PheRS, and MurM were at final concentrations of 0.6, 1, and 1 μM, respectively.

RESULTS

Pneumococcal AlaRS Displays Relaxed Specificity for Amino Acid and tRNA Recognition—The ability of AlaRS to misacylate both serine and glycine is well documented, as is its high specificity for cognate tRNAAla, which results from recognition of the conserved G3:U70 base pair in the acceptor stem (14, 24). The potential ability of pneumococcal AlaRS to utilize noncognate tRNAPh as a substrate was investigated here due to the presence of an unusual U4:C69 mismatch within the acceptor stem of the molecule, which may affect the ability of AlaRS to accurately discriminate against it (Fig. 1). In vitro assays showed that pneumococcal AlaRS was able to aminoacylate both tRNAAla and tRNAPh with any of Ala, Gly, or Ser (Fig. 2). As expected, an equivalent active concentration of the isolated catalytic domain of AlaRS, which has no editing function, was more efficient in tRNA misaminoacylation than the full-length editing-proficient enzyme (Fig. 3).

To determine whether aminoacylation of tRNAPh by AlaRS is a unique feature of the pneumococcal system, alanylation time courses were carried out with an equivalent concentration of full-length E. coli AlaRS and either the cognate E. coli tRNAAla or the noncognate E. coli tRNAPh (data not shown). E. coli AlaRS does not use tRNAPh as a substrate, consistent with the absence of either the G3:U70 alanylation identity element or other atypical structures such as the U4:C69 mismatch present in S. pneumoniae tRNAPh (Fig. 1). Previous studies have indicated that mutation of E. coli tRNAPh to contain the G3:U70 identity element is required for aminoacylation by E. coli AlaRS (24).

Pneumococcal PheRS Is Unable to Edit Misaminoacylated tRNAPh—The ability of pneumococcal PheRS to aminoacylate both wild type pneumococcal tRNAPh and mutant tRNAPhUAG was tested. In the later species, the distorted region of the acceptor stem was replaced by a Watson-Crick base pair conformation,
making the structure more closely resemble that of E. coli tRNAPhe (Fig. 1). The tRNAPhe_U4G mutant was aminoacylated 5-fold more efficiently than the wild type species by PheRS (Fig. 4A), whereas full-length AlaRS was able to alanylate both tRNAs equally efficiently (Fig. 4B). Kinetic characterization of PheRS with both wild type and mutant tRNAPhe indicated that this effect was not caused by a change in $K_m$ but rather by an approximate 2.5-fold increase in $k_{cat}$ for tRNAPhe (Table 1).

To test whether pneumococcal PheRS could hydrolyze misaminoacylated Ala-, Ser-, and Gly-tRNAPhe, AlaRS-catalyzed misaminoacylation reactions were repeated in the presence of pneumococcal PheRS (Fig. 4C–E). PheRS addition caused a marked enhancement of Ala-tRNAPhe production by pneumococcal AlaRS, suggesting that PheRS binds and protects this misaminoacylated tRNA from spontaneous deacylation (Fig. 4C). Some protection was also demonstrated for Gly-tRNAPhe (Fig. 4E) but not for Ser-tRNAPhe (Fig. 4D). In the absence of a successful procedure for isolating adequate yields of misaminoacylated tRNA$^{Phe}$, a coupled aminoacylation/deacylation assay was developed to assess the ability of PheRS to edit both wild type and mutant Ala- and Ser-tRNAPhe. PheRS addition had no effect on the deacylation of either Ser-tRNAPhe species (Fig. 5, A and B, respectively). For Ala-tRNAPhe, PheRS addition led to deacylation of Ala-tRNAPhe$^{U4G}$ but not wild type Ala-tRNAPhe$^{Phe}$ (Fig. 5, D and C, respectively). This suggests that the U4:C69 mismatch in wild type pneumococcal tRNAPhe is an anti-determinant for PheRS-mediated editing of Ala-tRNAPhe.

The Effect of Pneumococcal EF-Tu on tRNA Misaminoacylation—The sequence of pneumococcal EF-Tu is diverged from that of other bacteria at four conserved positions: P129K, M140L, T230S, and E234D. Of particular interest is the substi-

TABLE 1

| tRNA$^{Phe}$ | $K_m$ | $V_{max}$ | $k_{cat}$ | $k_{cat}/K_m$ |
|--------------|------|----------|----------|---------------|
| Wild type tRNA$^{Phe}$ | 1.05 ± 0.19 | 0.15 ± 0.04 | 0.32 ± 0.09 | 0.36 |
| Mutant tRNA$^{Phe}_{U4G}$ | 1.09 ± 0.30 | 0.38 ± 0.06 | 0.78 ± 0.10 | 0.72 |
tution of Thr-230 with Ser in the third β strand of the second domain of the protein, which comprises the aminoacyl-tRNA binding pocket (25). The effects of pneumococcal EF-Tu addition on the generation of misaminoacylated tRNAAla and tRNAphe by AlaRS were investigated. EF-Tu addition resulted in modest enhancement of the production of Gly-tRNAAla by pneumococcal AlaRS (Fig. 6) but had no significant effect on other AlaRS-catalyzed reactions (data not shown).

MurM Functions as a trans Editing Factor in Pneumococci—Pneumococcal peptidoglycan is unusual in that it typically consists of a combination of both branched and linear muropeptides. Within the structure, branched muropeptides consist of either a serine-alanine or an alanine-alanine dipeptide bridge attached to the stem peptide lysine of Lipid II. This dipeptide bridge is synthesized by the action of the MurM and MurN proteins (26–28). The substrates for MurM include pneumococcal Ser-tRNAser and Ala-tRNAAla provided by seryl-tRNA synthetase and AlaRS, respectively. Previous work also demonstrated that the catalytic efficiency of MurM is greater when Ser-tRNAAla is provided as a substrate as opposed to Ala-tRNAAla (21). The ability of MurM to utilize Ser-tRNAAla and the absence of any genome-encoded AlaXPs proteins in S. pneumoniae prompted us to investigate the ability of MurM to trans edit mischarged tRNAs. In the case of AlaRS synthesis of Ser-tRNAAla, the addition of MurM suppressed mischarging for the first 15 min of the reaction (Fig. 7A). After this time, product formation increased, possibly due to a loss of MurM stability during incubation for prolonged time periods at 37 °C. The initial suppression of mischarging suggests that MurM may act as a trans editing factor capable of hydrolyzing Ser-tRNAAla in the absence of its second substrate, Lipid II. AlaRS generation of Ala-tRNAphe was also reduced by the addition of MurM to the mischarging time course, providing additional support for a trans editing function (Fig. 7E). The ability of MurM to hydrolyze Ser-tRNAAla and Ala-tRNAAla was investigated in the absence of Lipid II (Fig. 8, A and B, respectively). The addition of MurM led to rapid deacylation of both Ala-tRNAAla and Ser-tRNAAla, consistent with trans editing activity. Direct deacylation assays could not be performed with MurM and Ala-tRNAphe due to the comparative instability of this mischarged tRNA species (see above).

DISCUSSION

Pneumococcal AlaRS Is Error-prone during Aminoacylation—Extensive studies on E. coli AlaRS have indicated that the G3:U70 wobble base pair in the acceptor stem of tRNAAla is a critical identity element for alanylation by AlaRS (24, 29–32). In addition to this, it has been demonstrated that E. coli tRNAphe can only be alanylated by E. coli AlaRS if the acceptor stem is mutated so that it contains the G3:U70 base pair (24, 29). The predicted cloverleaf structure of pneumococcal tRNAphe indicates the presence of an unusual wobble base pair in its acceptor stem, U4:C69, which is not found in E. coli
tRNA^Phe (Fig. 1) (33, 34). In addition to misaminoacylating tRNA^Ala, pneumococcal AlaRS is also able to charge tRNA^Phe with alanine, serine, and glycine. Production of high levels of hydrogen peroxide during the life cycle of pneumococcus could potentially accelerate misaminoacylation of tRNA^Ala and tRNA^Phe by AlaRS. In support of this hypothesis, it has been shown that exposure of E. coli threonyl-tRNA synthetase to hydrogen peroxide results in exacerbated production of misaminoacylated Ser-tRNAThr. This is due to oxidation of an editing site cysteine residue and subsequent loss of zinc ion coordination (4). Therefore, in the absence of free-standing homologues of the editing domain of AlaRS (AlaXPs proteins), pneumococcus may require MurM to maintain translation quality control, particularly if tRNA mischarging is elevated under oxidative stress as has been previously observed in other organisms (3, 4).

Evolution of Pneumococcal tRNA for Dual Functions in Protein and Peptidoglycan Biosynthesis—The peptidoglycan structure of pneumococcus is particularly unusual in that it contains a combination of both branched and linear muropeptides. The MurMN proteins are responsible for the synthesis of branched muropeptides and are one of the requirements for high level penicillin resistance within this bacterium (27, 28). Therefore, the mechanisms used by pneumococcus to ensure sufficient division of aminoacylated tRNA species between peptidoglycan biosynthesis and protein synthesis are of great interest.

In Staphylococcus aureus, pentaglycine bridge formation is essential for cell viability and is catalyzed by the Gly-tRNAGly requiring FemXAB proteins (35). In S. aureus, four annotated tRNA^Gly isoacceptors have been identified in addition to a pseudogene encoding an unusual fifth tRNA^Gly isoacceptor. Although all five tRNA^Gly isoacceptors have been shown to be substrates for the S. aureus glycyl-tRNA synthetase, the pseudogene and two of the other tRNA^Gly isoacceptors with the same anticodon were shown to possess sequence identity elements favoring weak binding interaction with EF-Tu. This allows S. aureus to ensure proper division of Gly-

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**FIGURE 7.** MurM decreases production of serylated tRNA^Ala/Phe and alanylated tRNA^Phe by AlaRS. A–E, misaminoacylation time courses for generation of Ser-tRNA^Ala (A), Ser-tRNA^Phe (B), Gly-tRNA^Ala (C), Gly-tRNA^Phe (D), or Ala-tRNA^Phe (E) by 310 nM active full-length pneumococcal AlaRS in the presence of 300 nM MurM. The concentration of wild type pneumococcal tRNA^Ala or tRNA^Phe used was 7 μM. The concentration of [14C]alanine, [3H]serine, and [14C]glycine used was 110 μM. Data sets are the average of three independent experiments. Error bars indicate S.E.
Lipid II-independent Editing by MurM

![Graphs showing deacylation of mischarged pneumococcal tRNAAla by MurM.](image)

**FIGURE 8. Deacylation of mischarged pneumococcal tRNAAla by MurM.** A and B, deacylation time courses were carried out as described by incubation of 50 μM [3H]Ser-tRNAAla (A) or Ala-tRNAAla (B) with 0.5 μM pneumococcal AlaRS or 0.5 μM MurM. In the control reaction, an equal volume of protein storage buffer was added. Error bars indicate S.E. FL, full-length.

In pneumococcus, no unique tRNAAla or tRNASer isoacceptors have been identified that would enable the bacterium to achieve division of aminoacylated tRNA between protein synthesis and peptidoglycan biosynthesis. In the same way *S. aureus* does. The inability of pneumococcal PheRS to edit misaminoacylated Ala- and Ser-tRNApPhe may ensure that these species are potential cellular substrates for the peptidoglycan biosynthesis pathway. This is supported by the finding that the mismatch within the acceptor stem of pneumococcal tRNAPhe may allow AlaRS to compete with PheRS for this substrate by compromising the aminoacylization activity of the latter protein. Our data suggest that, once released from AlaRS, misaminoacylated tRNA species can be hydrolytically cleaved by MurM, but it remains unclear whether they are specifically diverted away from the translation machinery as is the case for some forms of Gly-tRNAGly in *S. aureus*. Further characterization of these and other adaptations pneumococci have made to ensure that the fidelity of protein synthesis and peptidoglycan biosynthesis are maintained during antibiotic and oxidative stress may enable new drug targets to be identified in the future. This has the potential to result in subsequent restoration of the potency of penicillin in the treatment of infections by this bacterium.

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