CHARACTERIZATION OF tRNA-DEPENDENT PEPTIDE BOND FORMATION BY MURM IN THE SYNTHESIS OF STREPTOCOCCUS PNEUMONIAE PEPTIDOGLYCAN

+Adrian J. Lloyd, Andrea M. Gilbey, Anne M. Blewett, *Gianfranco De Pascale, $Ahmed El Zoeiby, ^Roger C. Levesque, Anita C. Catherwood, & Alexander Tomasz, *Timothy D. H. Bugg, David I. Roper and Christopher G. Dowson

Department of Biological Sciences and *Department of Chemistry, University of Warwick, Gibbet Hill Road, Coventry, CV4 7AL, UK.

$Centre de Recherche sur la Fonction, Structure et Ingénierie des Protéines, Faculté de Médecine, Pavillon Charles-Eugène Marchand, Université Laval, Ste-Foy, Quebec, G1K 7P4, Canada.

&Laboratory of Microbiology, The Rockefeller University, 1230 York Avenue, New York, NY 10021. USA.

Running Title: Enzymology of the aminoacyl ligase MurM

+Address correspondence to: Adrian J. Lloyd, Department of Biological Sciences, University of Warwick, Gibbet Hill Road, Coventry, CV4 7AL, UK. Tel: +(44)-(0)2476-522568; FAX: +(44)-(0)2476-523701. Email: adrian.lloyd@warwick.ac.uk

MurM is an aminoacyl ligase that adds L-serine or L-alanine as the first amino acid of a dipeptide branch to the stem peptide lysine of the pneumococcal peptidoglycan. MurM activity is essential for clinical pneumococcal penicillin resistance. Analysis of peptidoglycan from the highly penicillin resistant Streptococcus pneumoniae strain 159 revealed that in vivo and in vitro, in the presence of the appropriate acyl-tRNA, MurM159 alanylated the peptidoglycan ε-amino group of the stem peptide lysine, in preference to its serylation. However, in contrast, identical analyses of the penicillin-susceptible strain Pn16 revealed that MurMPn16 activity supported serylation more than alanylation both in vivo and in vitro. Interestingly, both MurM Pn16 acylation activities were far lower than the alanylation activity of MurM159. The resulting differing stem peptide structures of 159 and Pn16 were caused by the profoundly greater catalytic efficiency of MurM159 compared to MurMPn16 bought about by sequence variation between these enzymes and to a lesser extent, differences in the in vivo tRNA^{A^4u}:tRNA^{Ser} ratio in 159 and Pn16. Kinetic analysis revealed that MurM159 acted during the lipid-linked stages of peptidoglycan synthesis, that the D-alanyl-D-alanine of the stem peptide and the lipid II N-acetyl-glucosaminyl group were not essential for substrate recognition, that ε-carboxylation of the lysine of the stem peptide was not tolerated and that lipid II-alanine was a substrate, suggesting an evolutionary link to staphylococcal homologues of MurM such as FemA. Kinetic analysis also revealed that MurM recognised the acceptor stem and/or the TΨC loop stem of the tRNA^{A^4u}. It is anticipated that definition of the minimal structural features of MurM substrates will allow development of novel resistance inhibitors that will restore the efficacy of β-lactams for treatment of pneumococcal infection.
(undecaprenyl-pyrophosphoryl-N-acetyl-muramyl-L-alanyl-\(\gamma\)-D-glutamyl-L-lysyl-D-alanyl-D-alanine) which is then glycosylated with UDP-N-acetyl-glucosamine by MurG to form lipid II (undecaprenyl-pyrophosphoryl-N-acetyl-muramyl (N-acetyl-glucosaminyl)-L-alanyl-\(\gamma\)-D-glutamyl-L-lysyl-D-alanyl-D-alanine) [1] (Figure 1). The dipeptide branch is added to the stem peptide lysine at some point after the stem peptide is constructed [5-9] (e.g. lipid II in Figure 1). After transport to the outer face of the cytoplasmic membrane, lipid II is polymerized by transglycosylation. This nascent peptidoglycan is given structural rigidity by transpeptidation between the position three lysine (with or without a dipeptide branch) and the fourth position D-alanine of adjacent stem peptides (Figure 1; [1]).

The pneumococcal genes encoding the enzymes that construct the dipeptide branch, MurM and MurN, add the first and second amino acids to the stem peptide lysine respectively [10,11]. \(S.\ pneumoniae\) has acquired related MurM sequences, within its genome by homologous recombination to create a family of mosaics of related \(murM\) genes [12,13]. This has endowed the resulting family of MurM variants with vastly differing levels of activity \(in vivo\) and differing amino acid specificities for incorporation of alanine and serine [2,12,14].

Although not absolutely essential for high level penicillin resistance in laboratory strains of \(S.\ pneumoniae\), clinical strains of this organism depend upon the activity of MurM for high level penicillin resistance [14-16]. Despite the medical importance of this protein, knowledge of the enzyme biochemistry of MurM is sketchy and inferred. Unavailability of the peptidoglycan precursor substrates of MurM has previously restricted analysis of this enzyme and inferred. Unavailability of the peptidoglycan precursor substrates, allowing delineation of what is required for substrate binding by this enzyme and (4) define those regions of the t\(RNA^{\text{Ala}}\) substrate of MurM that are required for binding and catalysis.

**Materials and Methods**

**Chemicals** \(RNA\) species corresponding to the sequence of the full length pneumococcal t\(RNA^{\text{Ala\_UGC}}\) isoacceptor GGG GCC UUA GCU CAG CUG GGA GAG CGC CUG CUA ACC A (corresponding to the \(3^\prime\)-aminoacylation site, acceptor stem, \(T\_\gamma C\) loop and stem of the full length pneumococcal t\(RNA^{\text{Ala\_UGC}}\): GGG GCC UAG CGG UUC GAU CCC GCU AGGCAC CA (RNA minihelix) were synthesised, purified and supplied with a \(5^\prime\)-phosphorylation by Dharmacom Inc. \(S.\ pneumoniae\) Pn16 MurE (Mur\(E_{\text{Pn16}}\)) and \(Pseudomonas\ aeruginosa\) MurA, MurB, MurC, MurD, MurE and MurF were over-expressed and purified [18,20]. Bacterial purine nucleoside phosphorylase (Sigma) was repurified [21]. Pig heart isocitrate dehydrogenase (NADP\(^+\); Sigma) was repurified by elution from Sepharose 4B-Procion Blue MX2G with 1 mM NADPH. Undecaprenyl-MurVac(Glc/ Vac)-L-alanyl-\(\gamma\)-D-glutamyl-L-lyssyl(\(\psi\)-L-alanine)-D-alanyl-D-alanine (Lipid II-Ala) was a generous gift from Dr. G. dePascale (Warwick University). Other chemicals are recorded in Supplementary Materials and Methods, were sourced as in [22] or were from Sigma or Melford Laboratories Ltd.

**Escherichia coli** strains and plasmids Details of \(E.\ coli\) strains and plasmids used in this study are recorded in Supplementary Materials and Methods.

**\(S.\ pneumoniae\) strains and isolation of pneumococcal DNA** Pn16 (110K/70) Serotype 42, was isolated in Papua New Guinea and was penicillin sensitive (minimum inhibitory concentration (MIC) < 0.016 \(\mu\)g.ml\(^{-1}\)) [23]. 159 serotype 19A was isolated in Hungary [15] and was penicillin resistant (MIC >16 \(\mu\)g.ml\(^{-1}\)). Strains were propagated on brain heart infusion (BHI) agar containing 5 % (\(v/v\)) sheep blood at 37\(^\circ\)C in 5 % (\(v/v\)) \(CO_2\) or in liquid medium in BHI broth at 37\(^\circ\)C in 5 % (\(v/v\)) \(CO_2\). DNA was extracted from lawns of pneumococci on BHI blood agar as described [23].
**Micrococcus flavus membranes** Details of preparation of *M. flavus* membranes are recorded in Supplementary Materials and Methods.

**Peptidoglycan analysis** Peptidoglycan was extracted from late exponential phase *S. pneumoniae*, purified and digested with muramidase and the resulting stem peptides were extracted and fractionated by reverse phase HPLC on a Vydac 218TP54 column [4,24]. Peptidoglycan fragment structural assignments were made according to [2-4,11].

**Protein analytical methods** Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS- PAGE), N-terminal protein sequencing, protein assays and Western blotting for histidine tags were performed according to [22] and references therein.

**Identification and sequencing of the murM alleles from Pn16 and 159** To sequence the Pn16 and 159 murM genes, primers were designed using the *S. pneumoniae* R6 genome sequence [25] at the J. Craig Venter Institute (formally The Institute for Genome Research) web site (www.tigr.org) to amplify the region between 232 nucleotides 5′ to the initiator ATG (primer 1; Table 1 Supplemental Data) of the *murM* gene to 162 nucleotides 3′ of the *murM* (primer 2; Table 1 Supplemental Data) by PCR. DNA sequence between these primers was amplified by PWO DNA polymerase according to the manufacturer’s instructions and a product of the correct size (1.6 kb) was purified using a Qiagen spin column and sequenced in both directions.

**Cloning, over-expression and purification of MurM from Pn16 and 159** To construct an expression vector carrying a *murM* allele with a 3′ sequence encoding a hexa-histidine (His), peptide, 1.3 kb fragments containing the *murM* allele from Pn16 and 159 were amplified by PCR from the appropriate pneumococcal DNA. Due to sequence divergence at the 5′ end of the open reading frame between the *murM* alleles, a 5′ primer for each gene was designed incorporating an Nde1 restriction site for amplification of *murMPn16* and *murM159* respectively (Primers 3 and 4, Table 1 Supplemental Data). A single 3′ primer for the amplification of both alleles was designed to incorporate a 3′ Xho1 site and eliminate the 3′ stop codon: (Primer 5; Table 1 Supplemental Data). On PCR with PWO polymerase, products of the correct mass for *murMPn16* and *murM159* were obtained, purified, restricted with Nde1 and Xho1 and ligated into similarly restricted pET21b as described in [22]. Clones carrying the recombinant *murMPn16* and *murM159* genes were verified by sequencing and one correct clone was retained for expression of each protein (pET21b::*murMPn16* and pET21b::*murM159*).

To over-express the MurM proteins, 650 ml cultures of *E. coli* C41 (DE3)/pRIL, harbouring either pET21b::*murMPn16* or pET21b::*murM159* in luria broth (LB) + 50 µg/ml carbenicillin + 30 µg/ml chloramphenicol were grown at 37°C to an A600 of 0.6-1.0, when MurM expression was induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside, concurrent with the growth temperature being reduced to 25°C. *E. coli* cells were harvested after 4 hours and washed at 4°C in 50mM N-(2-Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES), 1mM MgCl2 pH 7.5 and 2mM β-mercaptoethanol (β-ME).

Analysis of whole cells and sub cellular fractions thereof by SDS-PAGE and Western blotting suggested that MurM*pn16* and MurM*159* were mostly insoluble but could be solubulised by 1 M NaCl (Lloyd, Unpublished). To purify MurM*pn16* or MurM*159*, all steps were performed at <4°C: Cell pellets suspended/g in 3 ml of 50mM HEPES, 1mM ethylene glycol-bis-(2-aminoethylether)-N,N',N′,N″-tetra acetate (EGTA), 2mM β-ME, 0.2mM phenylmethanesulphonyl fluoride (PMSF), 1µM leupeptin, 1µM pepstatin pH 7.5 + 2.5mg ml⁻¹ chicken egg white lysozyme (lysis buffer) were shaken for 30 minutes, disrupted by sonication and centrifuged at 10,000 x g for 30 minutes. The 10,000 x g pellet was extracted in 50mM sodium phosphate, 1M NaCl, 0.5mM ethylene glycol-bis-(2-aminoethylether)-N,N',N″,N‴-tetra acetate (EGTA), 2mM β-ME, 0.2mM phenylmethanesulphonyl fluoride (PMSF), 1µM leupeptin, 1µM pepstatin and 0.2 mM PMSF pH 7.0 for 30 minutes and then centrifuged for 30 minutes at 100,000 x g. The supernatant was retained and the 100,000 x g pellet was re-extracted as above and the supernatants were combined. The supernatant was sequentially fractionated between 25 and 50 % saturation ammonium sulphate, and by gel exclusion chromatography on a 500 ml Sephacryl S-200 column in 50mM NaH₂PO₄, 0.5M NaCl, 0.2mM PMSF, 1µM leupeptin, 1µM pepstatin and 0.2 mM PMSF pH 7.0 for 30 minutes and then centrifuged for 30 minutes at 100,000 x g. The supernatant was retained and the 100,000 x g pellet was re-extracted as above and the supernatants were combined. The supernatant was sequentially fractionated between 25 and 50 % saturation ammonium sulphate, and by gel exclusion chromatography on a 500 ml Sephacryl S-200 column in 50mM NaH₂PO₄, 0.5M NaCl, 0.2mM PMSF, 1µM leupeptin, 1µM pepstatin pH 7.0 (phosphate buffer). Fractions containing MurM by Western blot were further purified by immobilised metal affinity chromatography (IMAC) on a 25 ml column of cobalt-talon resin (Clontech, USA) in phosphate buffer. Once unbound proteins were eluted, MurM was eluted by a 0-0.2M imidazole gradient. The purity and identity of the final products of these purifications were
assessed by SDS-PAGE, Western blotting and N-terminal sequencing.

Cloning, over-expression and purification of alanyl-tRNA\text{Ala} synthetase (AlaRS) and seryl-tRNA\text{Ser} synthetase (SerRS) from \textit{Pn16} and MurF from 159

Using the pneumococcal sequences in [26], the above genes were cloned, over-expressed and their products purified by IMAC and anion exchange chromatography. Details of these procedures are given in Supplementary Materials and Methods.

Synthesis of UDP-linked peptidoglycan precursors (i) \textit{UDP-N-acetyl-muramyl-L-alanyl-\textdagger{}D-glutamyl-L-lysine} (UDP-MurNac-AEK) Syntheses were conducted at 37°C overnight in 2 ml volumes in air-tight tubes with no head space, in 50 mM HEPES, 1 mM dithiothreitol (DTT), 50 mM KCl, 10 mM MgCl\textsubscript{2} adjusted to pH 7.5, 3.65 µM MurA, 7.24 µM MurB, 3.50 µM MurC, 9.91 µM MurD, 9.09 µM MurEPn16, 0.4 µmol.m\textsuperscript{-1}.ml\textsuperscript{-1} NADP\textsuperscript{-} linked isocitrate dehydrogenase, 6.7 µmol.m\textsuperscript{-1}.ml\textsuperscript{-1} rabbit muscle pyruvate kinase, 26.7 mM D,L-isocitrate, 79.8 mM phosphoenolpyruvate, 13.3 mM UDP-GlcN, 0.1 mM NADPH, 5 mM ATP, 20 mM L-alanine, 22.9 mM D-glutamate and 15 mM L-lysine.

(ii) \textit{UDP-MurNacAEKAA} Syntheses were conducted as for UDP-MurVacAEK, except that the phosphoenolpyruvate concentration was 99.8 mM and the incubations also contained 15 mM D-alanyl-D-alanine and 9.09 µM MurF\textsubscript{159}.

(iii) \textit{UDP-N-acetyl-muramyl-L-alanyl-\textdagger{}D-glutamyl-meso-diaminopimelyl-D-alanine} (UDP-MurNacAE(DAP)AA) Syntheses were conducted as described for UDP-MurVacAEKAA, except that lysine was replaced by 30 mM \textit{meso}-diaminopimelic acid (DAP) and MurEPn16 was replaced by \textit{P. aeruginosa} MurE.

In all cases, the UDP-MurVac peptide product was freed from protein by centrifugation through a 10,000 M\textsubscript{r} cut-off membrane, and the filtrate was fractionated on a 50 ml column of Source 30 Q anion exchange resin from which it was eluted using a 0-1 M ammonium acetate gradient at pH7.5. Fractions containing the UDP-MurVac product were identified enzymatically utilising MurF\textsubscript{159} and by negative ion electrospray mass spectrometry (ES-MS) for this and all other UDP-MurVac peptides. All products were lyophilised three times versus water and stored in solution at -20°C.

Synthesis of lipid-linked peptidoglycan precursors

All syntheses were conducted using \textit{M. flavus} membranes essentially as described in [19]. The lipid I or lipid II products were purified as described [19].

Analysis of lipid-linked peptidoglycan precursors

To assay lipid-linked precursors, 50 µl of Lipid I or II species suspended in 50 mM HEPES, 10 mM MgCl\textsubscript{2}, 30 mM KCl and 1.5 % (w/v) 3-(3-cholamidopropyl)dimethyl-ami-no-1-propanesulphonate (CHAPS), pH 7.6 were added to 50 µl 1 M HCl. Samples were boiled for 30 minutes and neutralized with 2 M NaOH. The phosphate released was assayed according to [21,27]. Synthesis of lipid precursors was confirmed by thin layer chromatography (TLC) on silica and by negative ion ES-MS as in [19].

Sequencing of tRNA\text{Ala} and tRNA\text{Ser} genes from \textit{Pn16} and 159

Four tRNA\textsuperscript{Ser} genes: tRNA\textsuperscript{Ser(1)}, tRNA\textsuperscript{Ser(2)}, tRNA\textsuperscript{Ser(3)}, and tRNA\textsuperscript{Ser(4)} corresponding to (anticodon/locus tag) GCU/SP2253, UGA/SP2258, UGA/SP2291 and GGA/SP2247 respectively and four UGC anticodon tRNA\textsuperscript{Ala} genes (1 to 4 with locus tags SP2270, SP2282, SP2295 SP2243 respectively) were identified in the \textit{S. pneumoniae} TIGR4 genome (www.tigr.org, [26]). Of these, tRNA\textsuperscript{Ala(2)}\textsuperscript{UGC} and tRNA\textsuperscript{Ala(3)}\textsuperscript{UGC} were located within blocks of sequence that were identical for 3.294 kb 5' and 1.995 kb 3' to the gene of interest, and were not amenable to PCR amplification. For the remaining genes, primers 12-23 were designed starting at 250 bp upstream and 250 bp downstream of the mature tRNA sequence (Table 1, Supplemental Data), and genes encoding \textit{Pn16} tRNA\textsuperscript{Ala(1)}, \textit{Pn16} tRNA\textsuperscript{Ala(4)}, \textit{Pn16} tRNA\textsuperscript{Ser(1-4)} and 159 tRNA\textsuperscript{Ala(1)} were amplified with Taq DNA polymerase, 159 tRNA\textsuperscript{Ala(4)} and 159 tRNA\textsuperscript{Ser(2)} were amplified by platinum pfx DNA polymerase and 159 tRNA\textsuperscript{Ser(4)} was amplified with PWO DNA polymerase. All products were of the expected size (0.6 kb) and were sequenced.

No conditions could be found for the amplification of 159 tRNA\textsuperscript{Ser(3)}. 159 tRNA\textsuperscript{Ser(1)} was amplified with platinum pfx DNA polymerase, however, unexpectedly, a clean 2 kb product was obtained, the 3' termini of which were tagged with ATP and TA cloned into a linearised pCR\textsuperscript{2.1} vector, according to the manufacturer’s instructions (Invitrogen). The 2 kb insert was then sequenced using the vector-specific m13 primer sequences (Table 1, supplemental Data) either side of the insert.

Total tRNA preparation, Aminoacyl-tRNA preparation and tRNA\textsuperscript{Ala} or tRNA\textsuperscript{Ser} determination

Techniques employed to isolate, preaptatively acylate and assay tRNA\textsuperscript{Ala} and
tRNA\textsuperscript{Ser} are described in Supplementary Materials and Methods.

**MurM enzyme assays:** (1) **Spectrophotometric.** This assay followed the cycling of tRNA\textsuperscript{Ser} between MurM and SerRS. To a 0.2 ml assay was added 50 mM HEPES, 30 mM KCl, 10 mM MgCl\textsubscript{2}, pH 7.6, 1 mM DTT, 1.5 % (w/v) CHAPS, 0.25 mM NADH, 2 mM phosphoenolpyruvate, 0.2 mM ATP, 6.2 mg.ml\textsuperscript{-1} total tRNA\textsuperscript{159} (1.5 µM in terms of tRNA\textsuperscript{Ser}), 10 mM L-serine, 4.89 µM SerRS, 52.5 µmol.min\textsuperscript{-1}.ml\textsuperscript{-1} myokinase, 6.60 µmol.min\textsuperscript{-1}.ml\textsuperscript{-1} pyruvate kinase, 10.50 µmol.min\textsuperscript{-1}.ml\textsuperscript{-1} lactate dehydrogenase and 0.14 µM MurM\textsubscript{159}. The ΔA\textsubscript{340} of NADH (ε\textsubscript{340 nm} = 6220 M\textsuperscript{-1}.cm\textsuperscript{-1}) was followed at 37ºC and MurM\textsubscript{159} activity was then initiated with 25 µM lipid II.

(2) **Radiochemical** These assays were designed to follow the transfer of label from \[^{3}H\]-acyl-tRNA\textsuperscript{Ala} and \[^{3}H\]-seryl-tRNA\textsuperscript{Ser} to the peptidoglycan precursor. Initial experiments examining the stability of the aminoacyl-linkage to the tRNA suggested that at 37ºC the pH employed in the spectrophotometric method (7.6) the half life of *M. flavus* \[^{3}H\]-acyl-tRNA\textsuperscript{Ala} was 9.8 minutes. However, this could be extended to 46 minutes by dropping the pH of the assay to 6.8 (A. Lloyd, unpublished). Therefore, to avoid interference by depletion of acyl-tRNA substrate in MurM assays, initial rate data were usually obtained at pH 6.8 within the first 10 minutes of reaction, where loss of acyl-tRNA\textsuperscript{Ala} through chemical deacylation was <5%.

(2.1) **Transfer of \[^{3}H\] amino acid between \[^{3}H\]-acyl-tRNA and lipid-linked peptidoglycan precursors** To follow generation of \[^{3}H\]-acyl-tRNA\textsuperscript{Ala} and \[^{3}H\]-seryl-tRNA\textsuperscript{Ser} to the peptidoglycan precursor. Peptidoglycan analysis reveals the relative activity and amino acid specificity of MurM in vivo. In order to gain insight into the activity of MurM in vivo, we examined the peptide structure of muramidase-digests of Pn16 and 159 peptidoglycan by reverse-phase HPLC (Figure 2). This revealed that the major cross-linked species in Pn16 peptidoglycan is dimeric, composed of two adjacent peptides, without any dipeptide branch between them (peak 4). Unbranched single peptide species (monomers, peaks 1, 2) also predominated in the first 15 minutes of the Pn16 chromatogram. Beyond 25 minutes, multimers of cross-linked peptidoglycan precursors (peaks 5-9) eluted (Figure 2). These formed the minority of the Pn16 material fractionated and are composed of cross-linked peptide dimers (peaks 5-7) and trimers (peaks 8,9) which are linked by dipeptide branches which mostly had serine attached to the lysine of the peptidoglycan peptide (Figure 2).
This suggested that MurM activity in Pn16 is predominantly serine-specific. In contrast after 20 minutes, the majority of material eluted on chromatography of muramidase digests of 159 peptidoglycan was composed of monomers (peaks I-III), dimers (peaks IV-VI) and trimers (peaks VII-IX), all of which were substituted by dipeptide branches (Figure 2). It was clear that in vivo, MurM activity was considerably greater in 159 than in Pn16. Further, in vivo MurM159 specificity was heavily biased in favour of addition of alanine onto the stem peptide in preference to serine, where the converse was true for MurMPn16 (Figure 2).

murM159 and murMPn16 genes display marked sequence divergence that underpins the variation in peptidoglycan structure between 159 and Pn16 murM genes display mosaic sequences resulting from homologous recombination amongst natural populations of streptococci [12,13]. To determine if this might underlie the marked differences between the stem peptide branching in Pn16 and 159, the murM159 and murMPn16 alleles were sequenced. Both genes encoded proteins of 406 amino acids. Comparison of the inferred amino acid sequences of MurMPn16 and MurM159 in Supplementary Figure 1(a) (Figure S1(a)) revealed considerable amino acid sequence divergence (18%) between the two enzymes overall, with the N-terminal 61 amino acids, residues 115-149 and residues 229-298 differing in sequence by as much as 31%, 42% and 44% respectively. Clearly, the sequence divergence and differing genetic lineages of murMPn16 and murM159 could contribute to the very differing activities of MurMPn16 and MurM159 in vivo. MurM species with a threonine or lysine in position 260 insert predominantly serine or alanine [14]. Consistent with this observation and the in vivo preferences of MurMPn16 and MurM159, these proteins possessed a threonine and lysine respectively at position 260 (Figure S1(a)).

Over-Expression and purification of MurM159 and MurMPn16 In order to characterize the enzymology of MurM159 and MurMPn16 and relate their in vitro behaviour to their activity in vivo, we cloned, over-expressed and purified MurM159 and MurMPn16 as their C-terminal [C-(His)6] fusions. Pilot experiments in E. coli C41(DE3)/pRIL demonstrated that MurMPn16 over-expression was in large excess over that of MurM159 as evidenced by SDS-PAGE and Western blot analysis of whole cells. Sub-cellular fractionation suggested that both MurM species were almost completely expressed as inclusion bodies. Attempts to obtain soluble MurM including varying growth temperature and co-expression with chaperones failed (Gilbey and Lloyd, unpublished). However, both MurM species could be solubilised by 1 M NaCl. Therefore, we developed a high ionic strength-tolerant purification protocol: high salt solubilization followed by ammonium sulphate precipitation followed by gel filtration followed by immobilized cobalt affinity chromatography to purify MurM.

The protocol yielded per litre of culture, approximately 3 mg and 0.5 mg MurMPn16 and MurM159 respectively at a purity in excess of 95% (Figure S2(a)). Confirmation of the identity of the purified products involved Western blot identification of the C-(His)6 tag on the purified proteins (Figure S2(b)), while N-terminal sequencing revealed N-terminal sequences of MurM159 and MurMPn16 were M.Y.R.Y.Q.I.G.I.P.T. and M.Y.R.Y.Q.L.G respectively, where the 6th residue (isoleucine) of MurMPn16 is substituted by a leucine in MurM159 (Figure S2(c)). These results exactly matched N-terminal sequences inferred from sequencing the murM159 and murMPn16 genes.

MurM substrate synthesis To provide MurM substrates for enzymological studies we synthesised UDP-MurNacAEK, UDP-MurNacAEKAA and UDP-MurNacAE(DAP)AA from UDP-GlcNac, the required amino acids and the appropriate combinations of MurA→F from P. aeruginosa and S. pneumoniae. The purity of the peptides after purification were ≥ 98% by analytical anion exchange on MonoQ™ FPLC. Syntheses were confirmed by negative ion ES-MS (observed m/z/ expected m/z): UDP-MurNacAEK (502.6326/502.6308); UDP-MurNacAEKAA (573.6773/573.6679); UDP-MurNacAE(DAP)-AA (595.6585/ 595.6629).

The UDP-MurNac peptides were converted to their lipid I or lipid II derivatives using the MraY and MurG activity associated with M. flavus membranes. Both UDP-MurNacAEKAA and UDP-MurNacAE(DAP)-AA were completely converted to their lipid II derivatives in the presence of UDP-GlcNac as judged by TLC. Likewise, UDP-MurNacAEKAA was completely converted to its lipid I derivative in the absence of UDP-GlcNac. However, even under regimes employing extended incubation times and doubling the quantity of M. flavus membranes and UDP-
determine if MurM159 depended on tRNA for m-2/2): Lipid II (936.4341/936.5222); Lipid I was observed (r_f = 0.27) that was absent if (ii) Transfer of serine collision-induced dissociation (Supplementary fragmentation analysis of this species by ES-MS analysis (Figure 3(c)) showed the product was dominated by ion ES-MS (observed m -2/2/ expected m/z of 980.5023, associated with triply and singly charged species with m/z values of 653.3488 and 1945.6179, associated with triply and doubly charged species with an m/z of 1961.0848, 1961.1393 which correspond precisely with the expected m/z values suggestive of lipid II-Ser. No ions with m/z values of lipid II were detected in this sample. The identification of the MurM159 product as lipid II-Ser was further confirmed by ES-MS fragmentation analysis of this species by collision-induced dissociation. (Supplementary Results; Figure S3). MurM regenerates unacylated tRNA on acylation of lipid II. To further confirm the coupling of SerRS activity to that of MurM159 implied above, and to demonstrate that unacylated tRNA was the other product of MurM159, a continuous spectrophotometric method for monitoring this process was devised. Where SerRS was not limiting for MurM159 activity, with catalytic concentrations of Ser-tRNA Ser, synthesis of lipid II-Ser from lipid II by MurM159 generated equivalent amounts of tRNA Ser. This could be reacylated by SerRS, generating an equivalent amount of adenosine 5'-monophosphate, which could be coupled to yield a continuous spectrophotometric signal (Assay 1, Materials and Methods; Figure S4(a)). This assay system generated a SerRS, tRNA Ser, ATP, serine, lipid II and MurM159 dependent decrease in NADH absorbance (Figure S4(b)). This confirmed that where MurM159 accumulated lipid II-Ser (above), tRNA Ser was generated by this enzyme and was recycled with SerRS. The NADH oxidation kinetics suggested consumption of 83% of the lipid II over the duration of the assay. This and the kinetics of the process in Figure S4(b) leads us to tentatively suggest that there is molar equivalence between lipid II-Ser and tRNA Ser generation.

The true amino acid substrates of MurM are aminocyl-tRNAs. To establish that the actual MurM tRNA substrate was an acyl-tRNA, using M. flavus total tRNA, we precharged [3H]-alanyl-tRNA Ala and tested the ability of MurM to transfer [3H]-alanine from the [3H]-alanyl-tRNA to lipid II, using the vastly enhanced solubility of lipid II in n-butanol relative to [3H]-alanyl-tRNA Ala to separate the [3H]-acyl-tRNA substrate from the [3H]-acylated lipid II product (assay 2.1 Materials and Methods). MurM159 catalysed the transfer of [3H]-alanyl groups from [3H]-alanyl-tRNA Ala to lipid II as evidenced by the incorporation of 78% of the [3H] added to the assay, into n-butanol-extractable material in the complete incubation (Table 1). Controls minus MurM159 or lipid II accumulated only 3.3% or 3.0% of the [3H] added to the incubation into n-butanol-extractable products, demonstrating the essential requirement of these components for

GlcVac, it proved impossible to completely convert UDP-MurVacAEK to its corresponding lipid II derivative as determined by TLC. Therefore, UDP-MurVacAEK was only converted to its lipid I derivative.

After purification of the lipid I and II species as in [19], they were judged to be at least 95% pure by TLC of the final purified products. Again, syntheses were confirmed by negative ion ES-MS (observed m/z/ expected m/z 2/2): Lipid II (936.4341/936.5222); Lipid I (834.8976/834.9828); Lipid II (DAP) (958.5496/958.5174) lipid I-AEK (763.9486/763.9457).

MurM depends upon tRNA for the transfer of alanine and serine to lipid II to generate lipid II-Ala or Lipid II-Ser in vitro. To determine if MurM159 depended on tRNA for the acylation of lipid II, we carried out a series of incubations designed to generate alanyl-tRNA Ala or seryl-tRNA Ser in situ to determine if MurM159 could then transfer the aminocyl group to lipid II:

(i) Transfer of alanine: On TLC analysis of the n-butanol soluble components of incubations involving alanyl-tRNA Ala, a product was observed (r_f = 0.27) that was absent if ATP, tRNA 159, AlaRS, lipid II, MurM or alanine was omitted (Figure 3(a)). This product was purified by anion exchange chromatography ([19]; Figure 3(b)). Negative ion ES-MS analysis (Figure 3(c)) showed the mass spectrum of the product was dominated by a doubly charged species with an (observed/expected for lipid II-Ala) m/z of 971.9925/972.0410, associated with triply and singly charged species with m/z values of 647.9958/647.6914 and 1945.6179/1945.0899 respectively, suggesting the product was lipid II-Ala. This was further confirmed by ES-MS fragmentation analysis of this species by collision-induced dissociation (Supplementary Results; Figure S3).

(ii) Transfer of serine: On TLC analysis of the n-butanol-soluble components of the incubations involving serine, tRNA Ser, SerRS, ATP, MurM159 and lipid II, no unique product was observed (Figure 3(d)). Purification of the components of this incubation ([19]; Figure 3(e)) yielded a product that on negative ion ES-MS (Figure 3(f)) was characterised by a major doubly charged species with an m/z of 980.5023, associated with triply and singly charged species with m/z values of 653.3488 and 1945.6179 which correspond precisely with the expected m/z values 980.0385, 653.0231 and 1961.0848 for the doubly, triply and...
MurM<sub>159</sub> activity (Table 1). To demonstrate the requirement of MurM<sub>159</sub> for the tRNA portion of the [3H]-alanyl-tRNA<sub>Ala</sub> substrate, complete reactions were treated with 0.1 mg.ml<sup>-1</sup> RNase A, which reduced incorporation of [3H] into lipid products to 2.4%, comparable to control values obtained without lipid II or MurM<sub>159</sub> (Table 1). Similar results were obtained with MurM<sub>Pn16</sub>, however the incorporation of radioactivity in the presence of all components was 8.8% of that of the MurM<sub>159</sub> incubations (Table 1).

These assays were conducted for 60 minutes. However, under initial rate conditions, accumulation of radiolabelled lipid-II-Ala product was linear for about 3 minutes and within this time frame, the relationship between the measured rate and protein concentration was linear.

MurM<sub>159</sub> and MurM<sub>Pn16</sub> activity with crude acyl-tRNAs reflect the branch composition of the stem of 159 and Pn16 peptidoglycans. <i>M. flavus</i> [3H]-seryl-tRNA<sub>Ser</sub> only barely supported the activity of MurM<sub>159</sub> (Table 1). Therefore, to assay the relative abilities of MurM<sub>Pn16</sub> and MurM<sub>159</sub> to serylate or alanylate lipid II in vitro, crude tRNA<sub>Pn16</sub> and tRNA<sub>159</sub> were charged with [3H]-serine or [3H]-alanine, and the Pn16 and 159 [3H]-acyl-tRNAs were tested as substrates for acylation of lipid II by MurM<sub>Pn16</sub> and MurM<sub>159</sub> respectively.

Initial velocity measurements (n = 5) demonstrated that when MurM<sub>159</sub> was challenged with lipid II and either [3H]-alanyl-tRNA<sub>Ala</sub> or [3H]-seryl-tRNA<sub>Ser</sub> from 159, the enzyme was 6.9-fold more active with [3H]-alanyl-tRNA<sub>Ala</sub> than with [3H]-seryl-tRNA<sub>Ser</sub> (Table 2; p<0.0005 by Students t-Test). This result was entirely consistent with the observed prevalence of insertion of alanine in preference to serine into peptidoglycan branches by MurM<sub>159</sub> in vivo. When these experiments were repeated with MurM<sub>Pn16</sub> and either [3H]-alanyl-tRNA<sub>Ala</sub> or [3H]-seryl-tRNA<sub>Ser</sub> from Pn16, it was apparent that there had been a switch in specificity in that unlike MurM<sub>159</sub>, MurM<sub>Pn16</sub> was 2.2-fold more active with [3H]-seryl-tRNA<sub>Ser</sub> than with [3H]-alanyl-tRNA<sub>Ala</sub> (Table 2; p<0.0005) which is entirely consistent with the preference of serine over alanine displayed by the MurM<sub>Pn16</sub> in vivo. The intrinsic alanylation activity of MurM<sub>159</sub> was also 11.3-fold and 5.2-fold greater than the lipid II-alanylation (<i>p</i><0.0005) and serylation (<i>p</i><0.0005) activities of MurM<sub>Pn16</sub> (Table 2). These data are consistent with the far higher levels of peptidoglycan branching in the 159 peptidoglycan compared to that of Pn16.

The competence of tRNA<sub>Ala</sub> and tRNA<sub>Ser</sub> from 159 and Pn16 as MurM substrates does not influence branch composition of the 159 and Pn16 peptidoglycan MurM<sub>159</sub> supported lipid-II alanylation to the greatest extent, however, it was not clear whether the tRNA pool in 159 was better suited to supporting MurM activity than the tRNA pool in Pn16. To address this issue, the sequencing of tRNA genes from 159 and Pn16 was undertaken to determine if there were sequence differences in the tRNA genes that could explain the enhanced levels of MurM activity in 159.

It was only possible to sequence tRNA<sub>Ala<i>(i)</i></sub> and tRNA<sub>Ala<i>(4)</i></sub> from Pn16 and 159 (see Materials and Methods) which showed that tRNA<sub>Ala<i>(i)</i></sub> and tRNA<sub>Ala<i>(4)</i></sub> were identical in sequence and identical from either organism. All four tRNA<sub>Ser</sub> genes from Pn16 were sequenced and were found to be identical in sequence and arrangement to the tRNA<sub>Ser</sub> genes found in penicillin sensitive pneumococci such as TIGR4 and R6 [25,26]. In the case of 159, tRNA<sub>Ser<i>(4)</i></sub> and tRNA<sub>Ser<i>(2)</i></sub> were identical to those in Pn16. The gene encoding 159 tRNA<sub>Ser<i>(1)</i></sub>, although identical in sequence to that of Pn16, had, as indicated by BLAST analysis [28] been subject to a IS1167 transposon insertion immediately 5' to its start increasing the size of the insert from 573 bp to 2043 bp. The remaining tRNA<sub>Ser<i>(3)</i></sub> gene could not be isolated by PCR suggesting sequence rearrangement local to this gene.

It was thus clear that no complete conclusion regarding tRNA sequence identity and MurM activity could be drawn. Thus, to determine if the in vitro activity of MurM<sub>Pn16</sub> and MurM<sub>159</sub> was either a function of their amino acid sequence divergence or of the relative ability of the tRNAs of 159 and Pn16 to serve as MurM substrates, MurM<sub>Pn16</sub> was assayed with 159 [3H]-alanyl-tRNA<sub>Ala</sub> and 159 [3H]-seryl-tRNA<sub>Ser</sub>, while MurM<sub>159</sub> was assayed with Pn16 [3H]-alanyl-tRNA<sub>Ala</sub> and Pn16 [3H]-seryl-tRNA<sub>Ser</sub> (n = 5 for all measurements).

MurM<sub>159</sub> was equally active with Pn16 [3H]-alanyl-tRNA<sub>Ala</sub> and 159 [3H]-alanyl-tRNA<sub>Ala</sub> (Table 2; <i>p</i><0.3). Similarly, the activity of MurM<sub>Pn16</sub> with 159 [3H]-alanyl-tRNA<sub>Ala</sub> was equal to that with Pn16 [3H]-alanyl-tRNA<sub>Ala</sub> (Table 2; <i>p</i><0.45). Evidently, both 159 and Pn16 [3H]-alanyl-tRNA<sub>Ala</sub> could support enhanced MurM<sub>159</sub> activity, but 159 [3H]-alanyl-tRNA<sub>Ala</sub> could not elevate the
specific activity of MurM$_{pn16}$ to that of the 159 enzyme. This strongly suggested that in vivo, in 159, the enhanced insertion of alanine into peptidoglycan branches was a function of MurM$_{159}$ but not of the presence of an intrinsically efficient alanyl-tRNA$_{Ala}$ substrate of MurM in 159.

MurM$_{159}$ was equally active with Pn16 [3H]-seryl-tRNA$_{Ser}$ and 159 [3H]-seryl-tRNA$_{Ser}$ (Table 2; p<0.05), and the activity of MurM$_{pn16}$ with Pn16 [3H]-seryl-tRNA$_{Ser}$ was only 1.5 times that with 159 [3H]-seryl-tRNA$_{Ser}$ (Table 2; p<0.0005). This suggested that enhanced insertion of serine over alanine in Pn16 peptidoglycan branches was a function of the acyl-tRNA specificity of MurM$_{pn16}$ but not caused by a particularly competent seryl-tRNA$_{Ser}$ substrate of this MurM in Pn16.

Changes in the ratio of tRNA$_{Ala}$ to tRNA$_{Ser}$ in 159 and Pn16 could influence the stem peptide branch composition of 159 and Pn16 peptidoglycans. Although the competence of alanyl-tRNA$_{Ala}$ and seryl-tRNA$_{Ser}$ from 159 and Pn16 to perform as MurM$_{159}$ and MurM$_{pn16}$ substrates was equivalent in vitro, in vivo, perturbations in the tRNA$_{Ala}$:tRNA$_{Ser}$ ratio possibly influenced by the transposon insertion S' to 159 tRNA$_{Ser}$ GCU could affect the substrate availability for MurM and so could impact on the peptidoglycan composition of 159 and Pn16. Therefore pneumococcal AlaRS and SerRS were used to assay the relative concentration of tRNA$_{Ala}$ and tRNA$_{Ser}$ in 159 and 159 total tRNA. This revealed that the 159 tRNA$_{Ala}$ pool [1.363 ± 0.102 pmol tRNA$_{Ala}$/µg total tRNA; n = 4 cultures] was 2.2-fold larger than the Pn16 tRNA$_{Ala}$ pool [0.5973 ± 0.107 pmol tRNA$_{Ala}$/µg total tRNA; n = 3 cultures; p < 0.001], whilst the tRNA$_{Ser}$ pool was the same size in both 159 and Pn16 [0.549 ± 0.096 pmol tRNA$_{Ser}$/µg total tRNA (n=5 cultures) and 0.519 ± 0.099 pmol tRNA$_{Ser}$/µg total tRNA (n=3 cultures) respectively; p < 0.3]. The tRNA$_{Ala}$:tRNA$_{Ser}$ ratio in 159 (2.3) relative to Pn16 (1.2), was raised two fold which could contribute to the enhanced levels of stem peptide branch alanylation in 159 relative to that in Pn16.

Selection of [3H]-acyl-tRNA substrates for kinetic analysis of the peptidoglycan precursor substrate specificity of MurM. To establish the position of MurM within peptidoglycan synthesis and to determine what portions of the peptidoglycan precursor substrate were crucial for catalysis required a considerable quantity of acyl-tRNA. Therefore, M. flavus [3H]-alanyl-tRNA$_{Ala}$ was chosen for these experiments. However, it was necessary to demonstrate that this choice was appropriate, especially as M. flavus [3H]-seryl-tRNA$_{Ser}$ had proven to be essentially catalytically inert. (Table 1). Therefore the initial velocity of MurM$_{159}$ was determined over a variety of lipid II concentrations between 0 and 50 µM in the presence of 0.45 µM M. flavus [3H]-alanyl-tRNA$_{Ala}$. The experiment was then repeated with 0.45 µM [3H]-alanyl-tRNA$_{Ala}$, obtained by alanylation of a synthetic RNA sequence corresponding to the 76 nucleotides of S. pneumoniae tRNA$_{Ala}$ (see Chemicals; Figure 4(ai) and (4(b)).

The velocities obtained at increasing lipid II concentrations in the presence of M. flavus [3H]-alanyl-tRNA$_{Ala}$ were related to those obtained in the presence of [3H]-alanyl-tRNA$_{Ala}$ by a straight line represented by a linear regression equation (r$^2 = 0.9358$) of

$$V_o(\text{Alanyl-tRNA}_{Ala}) = 0.9234 ± 0.0344(V_o(\text{M. flavus Alanyl-tRNA}_{Ala}))$$

(Figure 4(ai)) insert which demonstrated that MurM activities supported by M. flavus [3H]-alanyl-tRNA$_{Ala}$ accurately reflected those supported by pure pneumococcal [3H]-alanyl-tRNA$_{Ala}$$_{UGC}$. Indeed, both data sets delineated hyperbolic dependencies of $V_o$ on lipid II concentration which when fitted by non-linear regression to the Michaelis-Menten equation ([S] = [Lipid II]; Equation 1),

$$V_o = \frac{V_{max}}{K_m + [S]}$$

yielded identical values for the $k_{cat}^{app}$ and $K_m^{app}$ of MurM$_{159}$ for lipid II (Table 3).

Kinetic analysis of MurM$_{159}$ suggests that stem peptide branches are added exclusively during the lipid-linked stages of cell wall synthesis. In order to determine if there was any preference displayed by MurM$_{159}$ towards lipid I or lipid II, both peptidoglycan precursors were tested as MurM$_{159}$ substrates in the n-butanol extraction assay. MurM$_{159}$ displayed a hyperbolic dependence on lipid I (Figure 4(aii)). The similarity in $K_m^{app}$ and the modest diminution in the lipid I $k_{cat}^{app}$ when compared to lipid II (Table 3) indicated that lipid I would be as significant as lipid II as a MurM$_{159}$ substrate.

To determine if MurM$_{159}$ substrate specificity extended to the cytoplasmic peptidoglycan precursors, the MurM$_{159}$ and UDP-MurNac peptide-dependent consumption of [3H] alanyl-tRNA$_{Ala}$ was measured as a function of time-dependent loss of TCA-precipitable radioactivity. Despite the slight
loss of label due to chemical deacylation of M. flavus \(^{[\text{H}]\text{-alanyl-RNAA}}\), MurM\(_{159}\) activity was clearly detectable (Figure S5). Comparison of \(k_{\text{cat}}/K_m\) for both lipid I-AEK and lipid I - 0.46 s\(^{-1}\).mM\(^{-1}\) respectively (Table 3), suggests that the extra alanylation of the lipid II substrate supported catalysis 8-fold more rapidly than the crude 159 \(^{[\text{H}]\text{-alanyl-RNA}}\). These results indicated that secondary modifications were not important for the recognition by MurM\(_{159}\) of alanyl-RNAs.

(ii) The anticodon, variable and D-loops and stems of the \(^{[\text{H}]\text{-Alanyl-RNA}}\) are not required for substrate recognition by MurM\(_{159}\).

Pilot experiments showed \(^{[\text{H}]\text{-alanyl-RNA}}\) minihelix supported MurM\(_{159}\) activity. To further characterise these interactions we compared the kinetics of dependence of MurM\(_{159}\) activity on \(^{[\text{H}]\text{-alanyl-RNA}}\) with those on the \(^{[\text{H}]\text{-alanyl-RNA}}\) minihelix (Table 4). Both relationships were hyperbolic (Figures 5(aii) and (bi)). However, MurM\(_{159}\) concentrations in these assays were significant compared to that of the alanyl-RNAs (Table 4).
To obtain the kinetic constants that characterized the dependence of MurM\textsubscript{159} on its alanylated RNA substrates, the data was fitted by non-linear regression to equation 3 below [29,30]:

\begin{equation}
V_0 = \frac{V_{\text{max}}^{\text{app}}}{2[E]} \left\{ \frac{[E] + [S] + K_m^{\text{app}}}{\sqrt{([E] + [S] + K_m^{\text{app}})^2 - 4[E][S]}} \right\}
\end{equation}

The MurM\textsubscript{159} \( k_{\text{cat}}^{\text{app}}/K_m^{\text{app}} \) ratios for \([	ext{H}]\)-alanyl-tRNA\textsubscript{Ala}\textsubscript{UGC} and \([	ext{H}]\)-alanyl-RNA minihelix (Table 4) were identical (0.101 s\(^{-1}\).µM\(^{-1}\) and 0.099 s\(^{-1}\).µM\(^{-1}\) respectively), indicating that both RNA substrates were catalytically equivalent. Thus, MurM\textsubscript{159}:tRNA substrate recognition was likely to be limited to the alanyl-acceptor stem, the T\(\Psi\)C loop and the T\(\Psi\)C stem, and did not involve the anticodon, D-, or variable loops and stems.

**Comparison of the dependencies of MurM\textsubscript{159} and MurM\textsubscript{Pn16} on \([	ext{H}]\)-alanyl-tRNA\textsubscript{Ala}\textsubscript{UGC} and lipid II**

MurM\textsubscript{Pn16} was considerably less active than MurM\textsubscript{159} in vivo and in vitro. To characterize this discrepancy kinetically, the dependence of MurM\textsubscript{Pn16} on lipid II and \([	ext{H}]\)-alanyl-tRNA\textsubscript{Ala}\textsubscript{UGC} was analysed (Table 4). The catalytic efficiency (\(k_{\text{cat}}^{\text{app}}/K_m^{\text{app}}\)) of MurM\textsubscript{Pn16} for lipid II was 72.3-fold lower than that for the 159 enzyme (Table 4), while the \(k_{\text{cat}}^{\text{app}}/K_m^{\text{app}}\) of MurM\textsubscript{Pn16} for \([	ext{H}]\)-alanyl-tRNA\textsubscript{Ala}\textsubscript{UGC} was 46-fold lower than the corresponding value for the 159 enzyme (Table 4). These data are consistent with the performance of MurM\textsubscript{Pn16} and MurM\textsubscript{159} in vivo in Pn16 and 159.

**Discussion**

Peptide bond formation is crucial to the synthesis of the peptidoglycan which involves three of the four commonly used biochemical mechanisms of amino acid carboxyl activation: the stem peptide is constructed from amino acids that are activated by esterification to phosphate [31]; the stem peptides are cross-linked after activation by esterification to a highly reactive serine within a penicillin binding protein transpeptidase [32], or inter-stem peptide branches are built up by condensation of L-amino acids activated by esterification to their cognate tRNA [5-9,33-36]. It is with this latter class of reactions that this paper is concerned.

*S. pneumoniae*, probably because of the plasticity of its genome is unique because of the strain-dependent and highly non-uniform chemical nature and abundance of dipeptide branches in its peptidoglycan [12,14], as exemplified here with strains Pn16 and 159.

Sequence similarity to *S. aureus* f\(mhb\) (femX) led Filipe and Tomasz to insertional disrupt the pneumococcal murM sequence. The resulting peptidoglycan phenotype identified the murM gene as being responsible for the addition of the first amino acid of the pneumococcal stem peptide branch [10]. We therefore over-expressed and purified MurM\textsubscript{Pn16} and MurM\textsubscript{159} to correlate their enzymological properties with their contribution to the structure of pneumococcal peptidoglycan for the first time. The purification of both enzymes was complicated because their solubility depended upon maintenance of a high ionic strength. This feature is also shared with the MurM homologue *Enterococcus faecalis* BppA1 [33].

A further hurdle in the study of MurM was the generation of peptidoglycan precursors. Although the synthesis of these molecules in vitro had been previously described, NADPH oxidase activity of MurB, coupled with its sensitivity to substrate inhibition by NADPH required use of very high concentrations of NADPH and MurB for synthesis of UDP-MurNac peptides by these methods [17,18,37-40]. Here, however, by recycling the NADP\(^+\) co-factor with isocitrate dehydrogenase, we maintained sub-inhibitory NADPH concentrations that were ~1% of the final yield of UDP-MurNac peptide. Similarly, by recycling ADP produced by MurC to F (Figure 1), with pyruvate kinase to reform ATP, UDP-MurNac peptide purification was simplified because although ADP co-purified with many UDP-MurNac peptides, ATP eluted after all of them (Lloyd, unpublished).

Our results conclusively showed for the first time that MurM\textsubscript{159} and by inference, MurM\textsubscript{Pn16} supported dipeptide branch synthesis by transferring either alanyl- or seryl residues from alanyl-tRNA\textsubscript{Ala} or seryl-tRNA\textsubscript{Ser} to the \(\varepsilon\)-amino group of the lipid II stem peptide lysine (Figures 3, S3, and S4). This is consistent with inter-stem branch synthesis in other Gram positive organisms where L-amino acids or glycerine are activated by esterification to tRNA prior to insertion into the stem peptide [5-9,33-36]. The work here is an advance on previous analyses of the MurM homologues *E. faecalis* BppA1 and *S. aureus* FemX, A or B. In these cases, no demonstration of the transfer of
aminoacyl groups from a pre-acylated tRNA to a peptidoglycan precursor was achieved and so no kinetic analyses of acyl-tRNA usage could be performed [8,33], as was done here. Further, the BppA1 results were based upon utilization of UDP-MurNAc pentapeptide as the stem peptide substrate, where, as the authors of this work concede, it is more likely that the true BppA1 substrate was lipid I or lipid II [33]. Unlike the studies of the E. feacalis and S. aureus enzymes, our reconstruction of peptidoglycan intermediate synthesis in vitro allowed us to probe in detail the interaction of MurM with its lipid substrates (Figure 4; Table 3; [8,33]). Finally, the ES-MS-fragmentation results presented here are the first direct confirmation that the site of aminoacylation of a lipid peptidoglycan precursor by this class of enzymes is the ε-amino group of the lipid II lysine (Figure S3). This contrasts with previous studies of the S. aureus FemXAB [8], where the site of glycolation of lipid II was not identified.

The markedly different branching phenotype displayed by Pn16 and 159, corresponding to the in vivo activity of MurM (Figure 2) was reflected in vitro by MurM<sub>159</sub> activity with pneumococcal alanyl-tRNA<sub>Ala</sub> which was considerably greater than with seryl-tRNA<sub>Ser</sub> and greater than any acyl-tRNA supported MurM<sub>Ph16</sub> activity (Table 2). This was consistent with the almost entire alanylation of stem peptides in 159 (Figure 2). For MurM<sub>Ph16</sub>, seryl-tRNA<sub>Ser</sub> was a better substrate than alanyl-tRNA<sub>Ala</sub>, consistent with the residual amounts of serylation and alanylation (where the serylation was greater than alanylation) of stem peptides in Pn16 (Figure 2; Table 2). The markedly differing properties of MurM<sub>Ph16</sub> and MurM<sub>159</sub> were also reflected in their kinetics of substrate dependence, where consistent with their in vivo activity, both lipid II and alanyl-tRNA<sub>Ala</sub> were used considerably less efficiently by the Pn16 enzyme compared to its 159 counterpart (Table 4).

Involvement of both MurM and pneumococcal tRNA in the synthesis of dipeptide branches suggested that the tRNA or the MurM protein had been modified to reflect the differing nature of the peptidoglycan in Pn16 and 159. Analysis of pneumococcal genomes coupled with our own sequencing of tRNA genes of 159 and Pn16 failed to reveal any sequence differences that could account for the elevated levels of peptidoglycan branching in 159. Furthermore, equal amounts of [<sup>3</sup>H]-seryl-tRNA<sub>Ser</sub> and [<sup>3</sup>H]-alanyl-tRNA<sub>Ala</sub> from either 159 or Pn16 were equally competent at supporting MurM<sub>159</sub> and MurM<sub>Ph16</sub> activity, suggesting that tRNA<sub>Ala</sub> or tRNA<sub>Ser</sub> from 159 were as capable of supporting enhanced peptidoglycan branching as tRNA<sub>Ala</sub> or tRNA<sub>Ser</sub> from Pn16.

Nevertheless, one of the 159 tRNA<sub>Ser</sub><sup>GCU</sup> genes was directly adjacent to the 3′ end of an IS1167 transposon insertion that was absent in Pn16. tRNA gene rearrangement by transposon insertion is well known and it is conceivable that this interfered with transcription of this 159 tRNA<sub>Ser</sub><sup>GCU</sup> gene, elevating the tRNA<sub>Ala</sub>/tRNA<sub>Ser</sub> ratio in 159 relative to Pn16 [41,42]. This suggests that alterations in tRNA expression as opposed to the tRNA itself may contribute to the high levels of alanylation of peptidoglycan of 159 relative to those in the Pn16 peptidoglycan.

Homologous recombination of streptococcal genes generates a myriad of gene products of variable sequence that has allowed the Pneumococcus and other streptococci to develop resistance to major classes of antibiotic as evidenced by sequence differences that have generated β-lactam-resistant penicillin binding proteins and sulphonamide-resistant dihydropteroate synthase variants [43,44]. This type of genetic rearrangement has led to a series of families of <i>murM</i> alleles, of which <i>murM<sub>Ph16</sub></i> is 99% identical to the <i>murMA</i> class of <i>murM</i> alleles typified by <i>S. pneumoniae</i> R6 <i>murM</i> and of which <i>murM<sub>159</sub></i> is 100% identical to the <i>murMB1</i> allele typified by Hungarian isolate Hun663 (Figure S1(b); [12,25]). Such strain-dependent sequence variation of MurM is believed to underpin variations in MurM activity and peptidoglycan composition [12,14] similar to those observed here.

What does our data reveal about tRNA recognition by MurM? MurM<sub>Ph16</sub> and MurM<sub>159</sub> can utilise alanyl-tRNA<sub>Ala</sub> and seryl-tRNA<sub>Ser</sub> (Table 2). Therefore, anticodon recognition is not important, which is also so for AlaRS and SerRS [45,46]. This correlates with the [<sup>3</sup>H]-alanyl-minihelix data which suggested that no more than the acceptor stem and the TFY loop of tRNA<sub>Ala</sub> were required for interaction with MurM<sub>159</sub> (Table 4; Figure 5(bi-iii)). Similar conclusions have been drawn regarding the <i>Lactobacillus</i> FemX [47].

AlaRS recognition of tRNA<sub>Ala</sub> depends on the highly conserved mispaired G:U at position 3:70 of the acceptor stem of the tRNA (Figure 5(aii); [45]). Although is possible that this recognition element is targeted by pneumococcal MurM, very recent work by
Villet et al. [48] showed that Lactobacillus FemX does not depend upon the G3:U70 base pair for alanyl-tRNA$_{\text{Ala}}^{\text{UGC}}$ substrate recognition, but instead depends principally upon the first two base pairs in the acceptor stem of tRNA$_{\text{Ala}}^{\text{UGC}}$ (G2:C71 and G1:C72; Figure 5(aii)). All of the S. pneumoniae tRNA$_{\text{Ser}}$ and tRNA$_{\text{Ala}}^{\text{UGC}}$ species have acceptor stems whose first two base pairs are G2:C71 and G1:C72 [25,26 and data reported herein]. Additionally, this acceptor stem motif is shared by pneumococcal tRNA isoacceptors for (amino acid/anticodon) Y/GUA, R/GCG, I/GAU, F/GAA, M/CAU, D/GUC and V/UAC [25,26,49]. Thus, if MurM shares the Lactobacillus FemX mode of tRNA recognition [48], then discrimination between seryl and alanyl addition and limitation of MurM activity to just alanylation and serylation of the stem peptide largely results from recognition of the aminocyl moiety of the acyl-tRNA substrate by this enzyme.

Comparison of the rates of MurM$_{159}$ supported by crude 159 [3H]-alanyl-tRNA$_{\text{Ala}}$ and synthetic [3H]-alanyl-tRNA$_{\text{Ala}}^{\text{UGC}}$ suggested that the latter was an 8-fold better substrate. This result highlighted the redundancy of tRNA secondary modifications in MurM catalysis. Additionally, these data suggested that the crude pneumococcal tRNA pool contains components that inhibit utilization of alanyl-tRNA$_{\text{Ala}}$ by MurM$_{159}$. These components could well be unacylated tRNA species with the G2:C71 and G1:C72 motif (above) including the tRNA$_{\text{Ser}}$ isoacceptors which implies a subtlety in the interaction of MurM with the pneumococcal tRNA pool which might impact upon the final composition of the cell wall.

What do the MurM alanyl-tRNA$_{\text{UGC}}$ kinetics reveal about the relationship between peptidoglycan and protein synthesis? $K_{\text{app}}$ values of MurM$_{159}$ and MurM$_{\text{Pa}16}$ for [3H]-alanyl-tRNA$_{\text{Ala}}^{\text{UGC}}$ were considerably higher (Table 4) than the 6.2 nM $K_{\text{D}}$ of EF-TU for alanyl-tRNA$_{\text{Ala}}$ [50]. Thus there is potential for considerable competition between ribosomal protein and cell wall synthesis for alanyl-tRNA$_{\text{Ala}}$ particularly where amino acids and therefore aminocyl-tRNA species are in short supply and where, necessarily, alanyl-tRNA$_{\text{Ala}}$ will be drawn into protein synthesis. In the staphylococci, this issue is resolved by mutations in the TΨC and D-loops of two specialised UCC isoacceptor tRNA$_{\text{gly}}$ species that bar involvement of these tRNA$_{\text{UCC}}$ species in protein synthesis, thus reserving a pool of glycyl-tRNA$_{\text{gly}}$ for peptidoglycan synthesis [51-54]. In the case of the lactobacilli, maintenance of cell wall branching despite the demands of protein synthesis for alanyl-tRNA$_{\text{Ala}}$ probably depends upon the high $k_{\text{cat}}$ of FemX [47] relative to that of MurM.

S. pneumoniae is distinguishable from L. viridescens and S. aureus because it is not essential for pneumococcal peptidoglycan to be entirely composed of branched stem peptides, nor even to contain any at all [2,4,10,55]. It is likely therefore that the pneumococcus is more tolerant to loss of acyl-tRNA from peptidoglycan to protein synthesis than L. viridescens and S. aureus. Nevertheless, it should be noted that such diminution of pneumococcal stem peptide branching may well adversely impact upon the attachment of important cell surface protein adhesins and virulence determinants ligated to branched stem peptides via sortases prior to peptidoglycan crosslinking [56].

What can be concluded from the in vitro peptidoglycan intermediate substrate specificity of MurM? MurM probably utilises lipid I and/or II in vivo as suggested by Filipe et al. [14]. This is inconsistent with exclusive specificity of S. aureus FemX for lipid II [8]. Usage of UDP-MurVacAEKAA by MurM$_{159}$ was insignificant (Figure S5; Table 3) in comparison to that of L. viridescens FemX which utilises only this substrate [5].

The presentation of the preferred peptidoglycan precursor substrates to MurM and L. viridescens FemX is likely to be radically different. In the case of MurM, its lipid II substrate is tethered to a phospholipid bilayer by an undecaprenyl tail, which is therefore unlikely to interact with the enzyme, while the rest of the molecule including the stem peptide (pyrophosphoryl-(GlcNac)-Mur-Nac-AEKAA) extends from the membrane surface [57] into the active site of MurM. In contrast, the stem peptide of the soluble peptidoglycan substrate of the L. viridescens FemX is tethered to UDP which is available for and is indeed essential for interaction with this enzyme [58,59]. The vastly differing properties and availabilities of the groups appended to the MurNac-stem peptide of UDP-MurVacAEKAA and lipid I or II probably have precluded the evolution of a FemX or MurM active site that can bind both substrates and might dictate whether peptidoglycan stem peptide branching is a cytoplasmic or a membrane-bound process.

Usage by MurM of either lipid I or lipid II in vivo, suggests the N-acetyl-glucosaminyl group of lipid II was not essential for
recognition of the lipid precursor by MurM (Figure 4; Table 3). It also indicated that in vivo, MurG probably tolerates lipid I species with an acylation of the stem peptide lysine ε-NH₂ group to avoid wasteful accumulation of lipid I precursors. This is likely because M. flavus MurG can even process lipid I modified by the addition of bulky pyrene-based fluorophores to the stem peptide lysine [19].

To extend our substrate specificity studies, we examined the consequences of carboxylation of the ε-carbon atom of the stem peptide lysine on MurM159 catalysis. MurM159 was entirely intolerant of this modification as judged by the observation that lipid II (DAP) was not a substrate. This has also been observed for S. aureus FemA and L. viridescens FemX [8,9]. The lysine-containing stem peptide specific members of the FemXAB family contain a catalytic aspartate [47], which is similarly conserved in MurM (D107; Figure S1a). It is possible that a substrate (DAP) carboxyl adjacent to the ε–NH₂ undergoing acylation, would perturb the environment of the catalytic carboxyl of D107 of MurM sufficiently to prevent catalysis. In this context, it is interesting to note that Streptomyces coelicolor FemX and VanK which append glycine onto an LL-DAP containing stem peptide do not possess conserved active site aspartates analogous to L. viridescens FemX [58,61].

Our results indicated that the stem peptide C-terminal D-alanyl-D-alanine was not essential for MurM159 catalysis (Figure 4(aiii)). These results contrasted starkly with those of Malliard et al. [58] who found a stem peptide D-alanyl-D-alanine C-terminus was required in L. viridescens FemX substrates. In this context, the lipid I-AEK substrate inhibition of MurM159 might originate in a set of interactions such as those evident in the L. viridescens FemX:UDP-MurNacAEKAA crystal structure [58], which is not a bound conformation of the substrate that can be catalytically active.

Surprisingly, we found that MurM159 could append an alanyl-group from [3H]-alanyl-tRNAAla onto lipid II-Ala (Figure 4(aiiv)). Although this was not evident from the ES-MS analysis of the products of MurM (Figures 3, and S3), the kinetics of alanylation of lipid II-Ala were so poor in the absence of lipid II (Figure 4(aiiv); Table 3), that in its presence, it is unlikely that lipid II-Ala-Ala formation would have been detected by the experiments represented by Figure S3 and Figure 3.

The alanylation of lipid II-Ala by MurM is the activity of MurN in vivo [11]. This showed that MurM is an enzymatic homologue of S. aureus FemA [8]. A similar flexibility in the active site of S. aureus FemX also allows it under forcing conditions to add two glycine residues instead of one [8]. Although clearly of mechanistic and evolutionary relevance, in vivo, mutational inactivation of MurN caused the branches of the stem peptides to be substituted by a single alanine or serine in S. pneumoniae, and consequently, the alanylation by MurM159 of lipid II-Ala cannot be physiologically significant [11].

What relevance have our results for pneumococcal penicillin resistance? Clinically, MurM is essential for high level penicillin resistance [14-16]. Our data demonstrate the central role played by tRNA in streptococcal cell wall synthesis, and link tRNA metabolism with β-lactam resistance. Interestingly, competition of peptidoglycan branching and ribosomal protein synthesis for acyl tRNA might influence the response of pneumococcal infection to β-lactam therapy which in turn might be modified by ribosomally directed antibiotics such as erythromycin and chloroamphenicol. Our results have revealed many of the important features of the substrates of MurM involved in binding and catalysis. Pursuit of the structural basis of these interactions and methods for their disruption to furnish novel antibiotic therapies is currently underway in our laboratory.

Acknowledgments
We thank Dr. E. Breukink (University of Utrecht, The Netherlands) for assistance with lipid I and II synthesis, Dr. M. Mystry (MRC Unit, University of Leicester, UK) for N-terminal sequencing and Ms. S. Slade and Ms. H. Bird (Dept. of Biological Sciences, University of Warwick, UK) for mass spectrometric and DNA sequence analyses, and the Wellcome Trust (grant 066443) for support.

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Table Legends

Table 1: Demonstration of acyl-tRNA dependence of MurM from Pn16 and 159: Assays were for 60 minutes in 50 µl of 10 µM lipid II and 0.29 µM M. flavus [3H]-alanyl-tRNAAla (6669 cpm/assay; 457 cpm.pmol⁻¹) or 0.26 µM M. flavus [3H]-seryl-tRNASer (3971 cpm/assay; 303 cpm.pmol⁻¹). MurM Pn16 and MurM 159 were 5.81 and 2.91 µM respectively. n-Butanol extracted cpm are averages of duplicate incubations, that varied by ≤10%. Background was 8.5 cpm.

Table 2: Activity of MurM Pn16 and MurM159 with [3H]-alanyl and [3H]-seryl-tRNA species from 159 and Pn16: Assays were performed at 10 µM lipid II and 0.45 µM pneumococcal [3H]-acyl-tRNA from Pn16 or 159 as indicated in the Text. [MurM Pn16] and [MurM 159] were 4.2 µM and
0.03 µM respectively. Specific activities were calculated from initial rates of time courses, typically from the first three minutes of data.

Table 3: Kinetics of utilization of peptidoglycan precursor and peptidoglycan precursor derivatives by MurM<sub>159</sub>: All assays were performed for 1 minute at 0.45 µM [3H]-alanyl-tRNA<sub>Ala</sub>. All assays with lipid derivatives followed the n-butanol extraction method (2.1; Materials and Methods), all assays with UDP-MurNac substrates used the TCA precipitation procedure (2.2; Materials and Methods). Lipid I-AEK kinetic constants were determined according to equation 2 in the text, all other kinetic constants were determined according to equation 1 (see text). Fitting data to both equations was performed by non-linear regression.

Table 4: Comparison of the substrate dependencies of MurM<sub>Pn16</sub> and MurM<sub>159</sub>: All assays were performed for 1 minute. All assays followed the n-butanol extraction method. Lipid II kinetic constants were determined according to equation 1 in the text, all other kinetic constants were determined according to equation 3 to account for the relatively high [MurM]:[3H]-alanyl-(t)RNA substrate ratio (see text). Fitting data to both equations was performed by non-linear regression.

Figure Legends

Figure 1: The peptidoglycan synthetic pathway: Intracellular cytoplasmic and cell membrane bound steps are shown with the polymerization reactions that generate the nascent peptidoglycan on the extra cellular face of the cell membrane. L-I and L-II denote lipid I and lipid II respectively. The C-terminal D-alanine that is lost in transpeptidation is shown in yellow on a green background, the remaining stem peptide amino acids are in black. The GlcNac residues of the carbohydrate backbone of the peptidoglycan are violet, the serine or alanine added by MurM is in light green and the alanine added by MurN is brown. The undecaprenyl-phosphate carrier that is cyclised between the extra and intracellular faces of the cytoplasmic membrane is in red. For purposes of simplification, the Figure is drafted assuming that MurM and MurN act exclusively after the formation of lipid II, where formally, they could utilise any intermediate after the MurE step.

Figure 2: Finger prints of stem peptides fractionated by reverse phase HPLC from muramidase digests of peptidoglycans from Pn16 and 159: Stem peptides were isolated from pneumococcal peptidoglycans as in Materials and Methods and fractionated by reverse phase HPLC with a C-18 column eluted by an 80 minute gradient between 0 and 15% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid [4]. Peptide elution was monitored at 215 nm and peptides were identified by comparison with elution of known peptidoglycan fragments (see [2,4,11,12,14]). Stem peptide sequences assigned to each species are in black type, amino acids added by MurM are in red type, amino acids added by MurN are in purple type. Labels 1-9 on the Pn16 HPLC trace and 1,3 and IX on the 159 HPLC trace correspond to stem peptides categorised in [2,4,11,12,14].

Figure 3: Transfer of alanine and serine to lipid II as mediated by MurM<sub>159</sub> and tRNA<sub>Ser</sub>. 1.14 µM MurM<sub>159</sub> was incubated in 50 mM HEPES, 10 mM MgCl<sub>2</sub>, 30 mM KCl, pH 7.6 and 1.5% (v/v) CHAPS, with 1 mM DTT, 5.9 µM AlaRS (or 19.5 µM SerRS), 10 mM L-alanine (or 10 mM L-serine), 0.43 mM lipid II, 5 mM ATP and 1.72 mg.ml<sup>-1</sup> total tRNA<sub>159</sub> (2.34 µM tRNA<sub>Ala</sub> or 0.944 µM tRNA<sub>Ser</sub> total isoacceptors) at 37°C for 60 minutes. Incubations omitting MurM<sub>159</sub>, lipid II, ATP, tRNA<sub>159</sub>, aminoacyl-tRNA synthetase or L-amino acid were also carried out. Reactions were terminated and extracted with 1 volume of ice-cold 6 M pyridinium acetate and 2 volumes of ice-cold n-butanol. The n-butanol phases were dried. Figure 3(a): Incubations to demonstrate alanyl-transfer to lipid II. Samples were resuspended in 10 µl of 2:3:1 chloroform: methanol:water, and fractionated by TLC on silica according to [19]. The TLC plate was stained by iodine vapour. Lane (1) corresponds to the complete incubation. Lanes (2)-(7) are identical to lane (1) except that incubations omitted MurM, lipid II, ATP, tRNA<sub>159</sub>, AlaRS and alanine respectively. The product unique to the complete incubation is highlighted (→). Figure 3(b): Purification of lipid II-Ala: Incubations were resuspended in 10 ml of 2:3:1 chloroform:methanol:water, loaded onto a 3 ml column of diethylaminoethyl (DEAE) Sephacel (acetate counter ion) and isocratically eluted with increasing [NH<sub>4</sub>HCO<sub>3</sub>]. 0.5 ml of each wash was dried and analysed by TLC as for Figure 3(a). Samples loaded in lanes (1)-(5) are from eluates from washes containing 2 parts chloroform to 3 parts methanol to 1 part 0.15 M, 0.2 M, 0.25 M, 0.3 M and 1.0 M NH<sub>4</sub>HCO<sub>3</sub> respectively. Species labelled (i) and (ii) correlate in abundance with lipid II and lipid II-Ala as determined by ES-MS analysis (Not shown except for final wash – see Figure 3(c)). Figure 3(c): ES-MS analysis of lipid II-Ala: 0.5 ml of DEAE
Figure 4: Kinetics of dependence of MurM159 activity on lipid substrates. Initial velocity (V₀) is in units of nmol.lipid-[3H]-Ala.min⁻¹.mg⁻¹ (MurM159)⁻¹. Unless otherwise stated, all data were obtained with 0.45 μM M. flavus [3H]-alanyl-tRNA⁰⁰₆. Figure 4a(i): lipid II dependence in the presence of [3H]-alanyl-tRNA⁰⁰₆ from M. flavus or synthetic [3H]-alanyl-tRNA⁰⁰₆. Main figure: V₀ is plotted vs. [lipid II]. M. flavus [3H]-alanyl-tRNA⁰⁰₆ data is represented by filled triangles ▲. Data obtained with 0.45 μM synthetic [3H]-alanyl-tRNA⁰⁰₆ is shown by filled squares ■. [MurM159] was 21 nM. Data were fitted by non-linear regression to equation 1 in the text. Insert: V₀ at a given [lipid II] with [3H]-alanyl-tRNA⁰⁰₆ (▲) are plotted against V₀ at the same [lipid II] with M. flavus [3H]-alanyl-tRNA⁰⁰₆ (■). The data were fitted by linear regression yielding the equation V₀([Alanyl-tRNA⁰⁰₆]_{M. flavus}) = 0.9234 ± 0.0344(V₀([Alanyl-tRNA⁰⁰₆]_{M. flavus})). Figure 4a(ii): lipid I Dependence V₀ is plotted vs. [lipid I]. [MurM159] was 21 nM. Data were fitted to equation 1 in the text. Figure 4a(iii): lipid I-AEK Dependence V₀ is plotted vs. [lipid I-AEK]. [MurM159] was 33 nM. Data were fitted to equation 2 in the text. Figure 4a(iv): lipid II-Ala Dependence V₀ is plotted vs. [lipid II-Ala]. [MurM159] was 100 nM. Data were fitted to equation 1 in the text. Figure 4b(i-iv): Lipid substrates conversions: Panels show the lipid substrates and products that participate in and result from alanylation of lipid II {Figure 4b(i)}, lipid I {Figure 4b(ii)}, lipid I-AEK {Figure 4b(iii)} and lipid II-Ala {Figure 4b(iv)}. Figure 5: Kinetics of dependence of MurM159 activity on RNA substrates. V₀ is in nmol.lipid-II-[3H]-Ala.min⁻¹.mg⁻¹ (MurM159)⁻¹. Figure 5a(i-iii): Alanyl-tRNA⁰⁰₆ UGC: (i): Dependence of MurM159 on [3H]-alanyl-tRNA⁰⁰₆ UGC. V₀ is plotted vs. [3H]-alanyl-tRNA⁰⁰₆ UGC. The [lipid II] was 10 μM, [MurM159] was 67.2 nM. Data were fitted to equation 3 in the text. (ii): Secondary Structure of the Pneumococcal tRNA⁰⁰₆ UGC Isoacceptor: Sequence was determined from the gene sequence determined in Results and was synthesised as an oligonucleotide for use as a MurM substrate. The anticodon and all stems and loops are labelled, the G3:U70 base pair that is crucial for recognition by AlaRS is highlighted in white against grey (*). (iii): Tertiary Structure of the pneumococcal tRNA⁰⁰₆ UGC isoacceptor: A model of tRNA⁰⁰₆ based on that of tRNA⁰⁰₆ is shown, with the major structural elements labelled. Figure 5b(i-iii): Alanyl-minihelix: (i): Dependence of MurM159 on [3H]-alanyl-minihelix. V₀ is plotted vs. [3H]-alanyl-minihelix. The [lipid II] was 10 μM, [MurM159] was 16.6 nM. Data were fitted to equation 3 in the text. (ii): Secondary structure of the minihelix derivative of the pneumococcal tRNA⁰⁰₆ UGC isoacceptor: Sequence of the acceptor stem and T Loop, comprised the contiguous sequence of nucleotides 1-7 and 49-76 of the full length tRNA⁰⁰₆. The G3:U70 base pair in the acceptor stem crucial for recognition by AlaRS is highlighted in white against grey (*). (iii): Tertiary structure of the minihelix derivative of the pneumococcal tRNA⁰⁰₆ UGC isoacceptor: A model of the tertiary structure of the portion of tRNA⁰⁰₆ recapitulated by the minihelix based on the structure of tRNA⁰⁰₆ is shown, with the major structural elements labelled accordingly.
## Table 1

**Demonstration of acyl-tRNA dependence of MurM from Pn16 and 159**

| [\(^3\)H]-Acyl-tRNA | Incubation | MurM origin | Butanol soluble [\(^3\)H] - background (cpm) | % Label in butanol phase |
|----------------------|------------|-------------|---------------------------------|-------------------|
| \textit{M. flavus} [\(^3\)H]-alanyl-tRNA\textsuperscript{Ala} | Complete | 159 | 5217 | 78 |
| | | Pn16 | 462 | 6.9 |
| | No MurM | None | 221 | 3.3 |
| | No Lipid II | 159 | 203 | 3.0 |
| | | Pn16 | 170 | 2.5 |
| | Complete + 0.1 mg.ml\(^{-1}\) RNAse A | 159 | 162 | 2.4 |
| | | Pn16 | 208 | 3.1 |
| \textit{M. flavus} [\(^3\)H]-seryl-tRNA\textsuperscript{Ser} | Complete | 159 | 492 | 12.3 |
| | | Pn16 | 130 | 3.2 |
| | No MurM | None | 209 | 5.2 |
| | No Lipid II | 159 | 145 | 3.7 |
| | | Pn16 | 156 | 3.9 |
| | Complete + 0.1 mg.ml\(^{-1}\) RNAse A | 159 | 335 | 8.4 |
| | | Pn16 | 310 | 7.8 |
### Table 2

Activity of MurM<sub>Pn16</sub> and MurM<sub>159</sub> with [³H]-alanyl and [³H]-seryl-tRNA species from 159 and Pn16

| [³H]-Acyl-tRNA | MurM species | tRNA source | MurM specific activity ± S.E (nmol.min⁻¹.mg (MurM)⁻¹) |
|---------------|---------------|-------------|--------------------------------------------------|
|               | Pn16          | Pn16        | 0.172 ± 0.025                                    |
| [³H]-Alanyl-tRNA<sub>Ala</sub> | Pn16          | 159         | 0.175 ± 0.022                                    |
|               | 159           | Pn16        | 2.095 ± 0.285                                    |
|               |               | 159         | 1.924 ± 0.466                                    |
| [³H]-Seryl-tRNA<sub>Ser</sub> | Pn16          | Pn16        | 0.370 ± 0.038                                    |
|               | Pn16          | 159         | 0.247 ± 0.025                                    |
|               | 159           | Pn16        | 0.312 ± 0.055                                    |
|               |               | 159         | 0.281 ± 0.096                                    |
### Table 3

**Kinetics of utilization of peptidoglycan precursor and peptidoglycan precursor derivatives by MurM$_{159}$**

| Peptidoglycan precursor substrate | [Substrate] range | $[^3]$H-Alanyl-tRNA$^{\text{Ala}}$ source | [MurM$_{159}$]$_{\text{Assay}}$ µM | $k_{\text{cat}}^{\text{app}}$ (s$^{-1}$) | $K_{m}^{\text{app}}$ (µM) | $K_s$ (µM) | $k_{\text{cat}}^{\text{app}}/K_{m}^{\text{app}}$ (s$^{-1}$.mM$^{-1}$) |
|---------------------------------|------------------|----------------------------------------|-------------------------------|--------------------------|----------------|-----------|----------------------------------|
| Lipid II                        | 0 to 50 µM       | *M. flavus*                            | 0.021                         | 0.033 ± 0.0039           | 34.78 ± 7.29   | N/A       | 0.948                                 |
| Lipid II                        | 0 to 50 µM       | synthetic                              | 0.021                         | 0.032 ± 0.0042           | 37.45 ± 8.59   | N/A       | 0.854                                 |
| Lipid II-Ala                    | 0 to 200 µM      | *M. flavus*                            | 0.100                         | 0.0031 ± 0.002           | 236.8 ± 153.3  | N/A       | 0.0131                                |
| Lipid II (DAP)                  | 10 µM, 100 µM    | *M. flavus*                            | 0.028                         | No activity              | N/A            | N/A       | N/A                                  |
| Lipid I                         | 0 to 50 µM       | *M. flavus*                            | 0.021                         | 0.017±0.002              | 37.37 ± 6.99   | N/A       | 0.205                                 |
| Lipid I AEK                     | 0 to 80 µM       | *M. flavus*                            | 0.033                         | 0.0157 ± 0.0037          | 31.03 ± 6.33   | 51.98 ± 5.57 | 0.460                                 |
| UDP-MurNacAEKAA                 | 0 to 4.54 mM     | *M. flavus*                            | 1.130                         | 7.02.10$^{-4}$ ± 2.1.10$^{-5}$ | 2445 ± 1499   | N/A       | 2.87.10$^{-4}$                        |
| UDP-MurNacAEK                   | 100 µM           | *M. flavus*                            | 0.576                         | No activity              | N/A            | N/A       | N/A                                  |
Table 4

Comparison of the substrate dependencies of MurM<sub>Pn16</sub> and MurM<sub>159</sub>

| MurM  | Varied substrate       | [MurM] (nM) | [Substrate] Range (µM) | Co-substrate concentration | K<sub>m</sub><sub>app</sub> (µM) | k<sub>cat</sub><sub>app</sub> (s⁻¹) | k<sub>cat</sub><sub>app</sub>/K<sub>m</sub><sub>app</sub> (s⁻¹.mM⁻¹) |
|-------|------------------------|-------------|------------------------|-----------------------------|-------------------------------|-----------------------------|-----------------------------|
| Pn16  | Lipid II               | 450         | 0-50                   | 0.45 µM M. flavus [³H]-Alanyl-tRNA<sub>Ala</sub> | 236.8 ± 153.3                 | 0.0031 ± 0.0017             | 0.0131                      |
|       | [³H]-Alanyl-tRNA<sub>Ala</sub><sub>UGC</sub> | 743         | 0-0.5                  | 10 µM Lipid II              | 0.589 ± 0.038                 | 0.0013 ± 0.0003             | 0.0022                      |
| 159   | Lipid II               | 21          | 0-50                   | 0.45 µM M. flavus [³H]-Alanyl-tRNA<sub>Ala</sub> | 34.78 ± 7.29                  | 0.033 ± 0.0039              | 0.948                       |
|       | [³H]-Alanyl-tRNA<sub>Ala</sub><sub>UGC</sub> | 67.2        | 0-0.3                  | 10 µM Lipid II              | 0.209 ± 0.007                 | 0.021 ± 0.002              | 0.101                       |
|       | [³H]-Ala-RNA minihelix | 16.6        | 0-0.531                | 10 µM Lipid II              | 0.756 ± 0.048                 | 0.075 ± 0.023              | 0.099                       |
Fig. 4

(a): $V_o$ vs. [Lipid Substrate]

(b): Conversion

(i) D-Ala $\xrightarrow{\text{Ala-tRNA}^{\text{Ala}}_{\text{M}159}}$ D-Ala
L-Lys $\xrightarrow{\text{MurM}_{159}}$ L-Lys-Ala
D-Glu $\xrightarrow{\text{L-Ala}}$ D-Glu
L-Ala $\xrightarrow{\text{MurNAc-GlcNAc}}$ MurNAc-GlcNAc

(ii) Undecaprenyl tail

(iii) L-Lys $\xrightarrow{\text{Ala-tRNA}^{\text{Ala}}_{\text{M}159}}$ L-Lys-Ala
D-Glu $\xrightarrow{\text{L-Ala}}$ D-Glu
L-Ala $\xrightarrow{\text{MurNAc}}$ MurNAc

(iv) D-Ala $\xrightarrow{\text{Ala-tRNA}^{\text{Ala}}_{\text{M}159}}$ D-Ala
L-Lys-Ala $\xrightarrow{\text{M}159}$ L-Lys-Ala-Ala
D-Glu $\xrightarrow{\text{L-Ala}}$ D-Glu
L-Ala $\xrightarrow{\text{MurNAc-GlcNAc}}$ MurNAc-GlcNAc
Characterization of tRNA-dependent peptide bond formation by MurM in the synthesis of Streptococcus pneumoniae peptidoglycan
Adrian J. Lloyd, Andrea M. Gilbey, Anne M. Blewett, Gianfranco De Pascale, Ahmed El Zoeiby, Roger C. Levesque, Anita C. Catherwood, Alexander Tomasz, Timothy D.H. Bugg, David I. Roper and Christopher G. Dowson

J. Biol. Chem. published online December 12, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M708105200

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