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Direction of aminoacylated transfer RNAs into antibiotic synthesis and peptidoglycan-mediated antibiotic resistance

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Abstract

Prokaryotic aminoacylated-transfer RNAs often need to be efficiently segregated between translation and other cellular biosynthetic pathways. Many clinically relevant bacteria, including *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* direct some aminoacylated-tRNA species into peptidoglycan biosynthesis and/or membrane phospholipid modification. Subsequent indirect peptidoglycan cross-linkage or change in membrane permeability is often a prerequisite for high-level antibiotic resistance. In Streptomycetes, aminoacylated-tRNA species are used for antibiotic synthesis as well as antibiotic resistance. The direction of coding aminoacylated-tRNA molecules away from translation and into antibiotic resistance and synthesis pathways are discussed in this review.

Keywords

Antibiotic; antibiotic resistance; pathogenesis; peptidoglycan; protein synthesis; translation; tRNA

1. Introduction

Protein synthesis by ribosomes utilizes aminoacyl-tRNAs to translate the codons of mRNA into the corresponding polypeptide sequence. Within the cell, each aminoacyl-tRNA species is produced by a two-stage reaction that is catalyzed specifically by one of twenty aminoacyl-tRNA synthetase enzymes (aaRSs). In the first stage of the reaction, an amino acid is activated with adenosine triphosphate (ATP) to form an aminoacyl-adenylate with concurrent release of inorganic pyrophosphate (PPi). In the second stage of the reaction, the activated amino acid moiety is transferred either to the 2’ or the 3’ hydroxyl of adenine-76 at the 3’ end of the bound tRNA substrate with the subsequent formation of an aminoacyl-ester bond [1,2]. Following this process, the aminoacylated-tRNA product is released and sequestered by elongation factor Tu (EF-Tu) for delivery to the ribosome and use in protein synthesis (Fig. 1) [3,4]. Aminoacylated tRNA substrates are also required for cellular processes outside of translation including both peptidoglycan and antibiotic biosynthesis pathways in prokaryotes (Fig. 2). The processes that sequester aminoacyl-tRNAs away from translation so that they can, instead, be used in other specific biosynthetic pathways will be discussed in this review.

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2. Direction of aminoacyl-tRNA into peptidoglycan biosynthesis

Peptidoglycan is a key component of the bacterial cell wall responsible for providing protection against osmotic stress and turgor pressure [5]. It forms a thicker layer in Gram-positive bacteria, many of which also heavily use the structure for anchoring of important virulence factors, when compared to Gram-negative bacteria [6]. The carbohydrate backbone of peptidoglycan consists of an alternating chain of N-acetylglucosamine (GlucNAc) and N-acetylmuramic acid (MurNAc) residues with a pentapeptide side chain attached to the lactyl ether appendage of the latter component. A single GlucNAc-MurNAc pentapeptide subunit in combination with undecaprenyl-pyrophosphate is the building block for peptidoglycan formation and is commonly referred to as Lipid II. Pentapeptide side chains are comprised of alternating D- and L- amino acids with an overall common structure of L-Ala-γ-D-Glu-X-D-Ala-D-Ala. Gram-negative bacteria typically have *meso*-diaminopimelic acid at position X whereas in Gram-positive bacteria this is replaced most frequently by L-Lys [7]. An additional level of peptidoglycan structure is obtained by cross-linking between the pentapeptide side chains of adjacent strands. The rigidity of the final murein structure is dictated by the degree of cross-linking. Typically Gram-negative bacteria, such as *Escherichia coli*, have a low level of pentapeptide cross-linking (25–50%) in comparison to Gram-positive bacteria (70–90%). In addition to this, cross-links can either be direct, involving linkage of the amino acid at position X of the first pentapeptide side chain with the L-Ala at the fourth position of another, or indirect which first requires the formation of a connecting amino acid bridge. Indirect cross-linking is more common in bacteria that have L-Lys at position X and the structure of the required intervening amino acid bridge can vary between different genera and species [7,8]. The amino acids required for bridge formation are typically derived from appropriately aminoacylated-tRNA donor molecules. Utilization of these donor tRNA molecules in peptidoglycan biosynthesis and how this relates to antibiotic resistance, are discussed below.

2.1. MurM and MurN in Streptococcus pneumoniae

The peptidoglycan layer of *S. pneumoniae* is unusual in that it contains a combination of both linear and branched muropeptides within the structure. Branched muropeptides carry either an L-Ala-L-Ala or an L-Ser-L-Ala dipeptide that is attached to the third L-Lys of the pentapeptide side chain of Lipid II [9] (Fig. 3). Formation of this dipeptide substrate for indirect peptidoglycan cross-linking requires the activities of the MurM and MurN tRNA-dependent aminoacyl-ligases. MurM and MurN are encoded within an operon by the *fibA* and *fibB* (factors important in β-lactam resistance) genes, respectively. In addition, MurMN activity is functionally conserved across all strains of pneumococcus [10,11]. Selective inactivation of these two genes has shown that the protein products act within a specific order. MurM is responsible for the addition of either L-Ala or L-Ser as the first amino acid of the cross-link and then MurN invariably adds L-Ala as the second amino acid [12]. In both cases, appropriately aminoacylated-tRNA species serve as the amino acid donors for the reaction [13]. Provision of adequate substrates for MurMN is currently thought to be achieved solely by the activities of alanyl- and seryl-tRNA synthetases within this bacterium. No MurMN-specific tRNA isoacceptors have been identified to date that would explain how a balance between direction of these aminoacylated-tRNA species into the protein and peptidoglycan biosynthesis pathways is either established or maintained.

In 1990, prior to the identification of the *fibAB* operon, a link was established between levels of indirect cross-linking in the pneumococcal cell wall and penicillin resistance. At this time, it was observed that a penicillin-resistant isolate from South Africa had a highly branched peptidoglycan that could be co-transferred with penicillin resistance to susceptible pneumococci [14,15]. Since then, many highly penicillin-resistant strains of pneumococcus have also been shown to have an increased level of branched muropeptides within their cell

wall in comparison to penicillin-sensitive isolates. Further investigation by insertion duplication mutagenesis targeting the *fibAB* operon showed that inactivation of MurMN had no significant effect on cell viability. Notably, interruption of the *fibAB* operon in *S. pneumoniae* strain Pen6 caused a marked reduction in penicillin resistance from 6 μg mL^{-1} to 0.032 μg mL^{-1}. This effect was also seen in other penicillin-resistant strains of pneumococcus regardless of their genetic background, labeling MurM and MurN as the first major non-penicillin binding protein determinants of β-lactam resistance within this bacterium [10].

Further elucidation of the role of MurMN in β-lactam resistance has been complicated by the finding that transformation with a MurM allele from a penicillin-resistant strain enriches branched muropeptides but does not increase penicillin-resistance. This suggests that, whilst MurM is necessary for high-level penicillin resistance, it is not sufficient in the absence of low affinity forms of the penicillin binding proteins. It has also been demonstrated that inactivation of *murMN* results in increased susceptibility to cell lysis during exposure to low concentrations of other antibiotics that target peptidoglycan biosynthesis including fosfomycin, vancomycin, D-cycloserine and nisin. This is predictive of stress response mediated re-direction of aminoacylated-tRNA species into peptidoglycan biosynthesis via the MurM and MurN proteins [12,16,17].

2.2. FemXAB in Staphylococcus aureus

Indirect cross-linking within the peptidoglycan of *S. aureus* requires modification of the Lipid II intermediate with a pentaglycine bridge, which is subsequently used as a platform for anchoring many virulence factors within this bacterium [5,18]. Pentaglycine bridge formation is catalyzed in a three-step process by the tRNA-dependent aminoacyl ligases FemX (FhmB), FemA and FemB. The genes encoding FemXAB are transcribed from an operon and the protein products act in a specific order. FemX is responsible for addition of the first Gly residue of the bridge [19,20]. Following this, FemA sequentially adds two more Gly residues prior to pentaglycine bridge completion by FemB. All three of these proteins require Gly-$tRNA^{Gly}$ as the amino acid donor and adequate provision of this substrate within the cell is achieved by glycyl-tRNA synthetase [21]. This is, in part, aided by the fact that three out of the five $tRNA^{Gly}$ isoacceptors encoded in the *S. aureus* genome have sequence identity elements consistent with weak binding to EF-Tu [22]. These specific sequence elements include replacement of the strong EF-Tu binding pairs G49–U65 and G51–C63 [23–25] with A49–U65 and A51–U63, respectively, in the TψC loop. In addition the three non-proteinogenic $tRNA^{Gly}$ isoacceptors also exhibit replacement of GG at positions 18 and 19 with either UU or CU [22]. This allows *S. aureus* to maintain adequate provision of Gly-$tRNA^{Gly}$ for two essential processes: translation and cell wall modification by the FemXAB proteins, even though it only has one glycyl-tRNA synthetase encoded in its genome [22].

Determination of the X-ray crystal structure of FemA indicated that the protein likely has only one binding site for Gly-$tRNA^{Gly}$ [26]. This initially indicated that an additional aminoacyl-tRNA binding event would be required for transfer of the second Gly residue by this protein. Further studies resulted in the detection of protein-protein interactions between two monomers of FemA, two monomers of FemB and also between FemA and FemB directly. No such interactions were found for FemX [27]. This suggests that FemX carries out its function as a monomer whereas FemA and FemB carry out their functions as homodimers. Dimerization of two FemA monomers and then, in a separate event, two FemB monomers, would allow for transfer of one Gly residue to Lipid II by each of the proteins in the dimer without the need for an additional tRNA binding event [27].

In contrast to *S. pneumoniae*, indirect peptidoglycan cross-linking in *S. aureus* is essential for cell viability as determined by *femX* inactivation studies. Furthermore, selective *femA* or *femB* inactivation was determined to be lethal without the acquisition of complementary mutations due to a combination of phenotypic effects. These effects included reduction in the cell wall Gly content, reduction in cell wall turnover, disruption to cell septum formation, retarded separation of cells and loss of methicillin resistance with concurrent increase in sensitivity to β-lactam antibiotics [28–30]. These detrimental effects make sequestration of Gly-tRNA^{Gly} away from protein synthesis and into peptidoglycan synthesis essential in this bacterium.

2.3. Lif and Epr in Staphylococcus simulans and Staphylococcus capitis

Both *S. simulans* and *S. capitis* produce glycyl-glycine endopeptidases that specifically target and cleave the pentaglycine bridge that is formed during indirect cross-linking of staphylococcal peptidoglycan. Possession of such endopeptidases confers a competitive advantage on these particular bacteria during niche colonization in the presence of other staphylococcal species. In order to protect itself from its own endopeptidase (lysostaphin), *S. simulans* specifically produces lysostaphin immunity factor, otherwise known as Lif [31]. Similarly, *S. capitis* produces endopeptidase resistance factor, or Epr, to provide protection against its endopeptidase Ale-1 [32]. Lif and Epr have been shown to have up to 41% identity to FemAB and, therefore, it is no surprise that they require aminoacylated-tRNA species for their function. Lif and Epr have a high specificity for incorporation of Ser instead of Gly at the third and fifth position of the typical staphylococcal pentaglycine bridge. The resulting peptidoglycan structure, which has an interpeptide bridge comprised of alternating Ser and Gly, is protected from hydrolysis by the glycyl-glycine endopeptidases as they are unable to break the peptide bonds between these two amino acids [33]. Currently, it is not known how Lif and Epr hijack SertRNA^{Ser} away from protein synthesis, however, neither of these proteins are able to extend the monoglycine bridge found in *femAB* deletion strains. Therefore it is clear that they depend on FemA and FemB in some way for activity [33]. L-Ser has also been detected in the peptidoglycan cross-links of other staphylococci including *S. epidermidis* [34].

2.4. FemX in Weissella viridescens

In *W. viridescens*, indirect peptidoglycan cross-linking involves the formation of an L-Ala-L-Ser or an L-Ala-L-Ser-L-Ala peptide bridge. Within this bacterium, the Ala-tRNA-dependent enzyme, FemX, achieves initiation of peptide bridge formation by transfer of the first L-Ala residue to the epsilon amino group of L-Lys found at the third position of the pentapeptide side chain. The enzymes involved in the subsequent transfer of the second position Ser and third position Ala residues have not yet been formally identified [35]. In contrast to the substrate specificity of many of the other Fem enzymes, *W. viridescens* FemX exclusively utilizes the cytoplasmic peptidoglycan precursor, UDP-MurNAc pentapeptide, rather than membrane bound Lipid II [36]. Kinetic characterization of *W. viridescens* FemX has indicated that its catalytic mechanism can be divided into four highly ordered and sequential stages. In the first two stages, the enzyme binds to UDP-MurNAc and then to Ala-tRNA^{Ala}. In the third stage, L-Ala is transferred from the bound tRNA donor molecule to the accepting L-lys at the third position of the pentapeptide side chain. Finally the reaction is completed by release of the tRNA donor and the UDP-MurNAc hexapeptide product. Asp-108 has been identified as an essential residue within FemX since mutation to Asn is directly correlated with a 230-fold reduction in the catalytic efficiency of the enzyme [37]. Recent crystallization of FemX in complex with a peptidyl-tRNA conjugate has identified Lys-305 and Phe-304 as additional critical residues for optimal catalytic activity of the enzyme. Replacement of Lys-305 with Ala, Met or Arg decreases the turnover number of the enzyme by 1.3×10^4-, 1.9×10^2- and 9.9×10^2- fold,

respectively. The critical nature of this highly conserved Fem-ligase family residue is likely due to the amine stabilizing effect it has during the development of a negative charge on the carbonyl group of L-Ala upon nucleophilic attack of the Ala-tRNA^{Ala} ester bond by the amine group of L-Lysine in the pentapeptide side chain. In contrast, replacement of Phe-304 with Ala or Leu decreases the turnover number of the enzyme by 16- and 29-fold, respectively. This is due to the critical nature of this residue in establishing correct stacking interactions between the enzyme and its tRNA substrate [38].

The X-ray crystal structure of FemX shows that it has two domains, each with a distinct role. The first domain of the protein is involved in the binding of UDP-MurNAc, whereas the presence of a positively charged channel across domain II indicates a potential role in the accommodation and binding of the negatively charged backbone of the Ala-tRNA^{Ala} substrate [39]. Following on from this discovery, the interaction of FemX with its second substrate has been characterized by the introduction of specific modifications within both the tRNA and the amino acid moiety. These studies indicated that FemX has a preference for L-Ala addition to UDP-MurNAc pentapeptide because it reacts much more unfavorably with both L-Ser and the acceptor arm of tRNA^{Gly}. More specifically, *in vitro* activity assays show that FemX turns over Ser-tRNA^{Ser} and Gly-tRNA^{Gly} 17- and 38-fold less efficiently than Ala-tRNA^{Ala}, respectively. In the latter case, this can be explained by site-directed mutagenesis studies indicating the importance of the penultimate base pair of tRNA^{Ala}, G2–C71, as an essential identity element for FemX. This is typically replaced by C2–G71 in tRNA^{Gly} species [40]. Specific FemX residues involved in tRNA binding have been identified by crystallization of the enzyme with a peptidyl-RNA conjugate and include Ile-208, which forms a hydrophobic interaction with the CCA end of the aminoacyl-tRNA substrate. In addition to this, Arg-205, Ser-259 and His-206 form a hydrogen-bonding network that stabilizes the phosphates of the C75 and A76 nucleotides located at the 3' terminus of the tRNA substrate [38]. *W. viridescens* FemX has been shown to specifically transfer Ala from tRNA that is aminoacylated on the 2' hydroxyl of adenine-76 of the tRNA molecule [35,41]. This may account for successful substrate competition with the A site of the ribosome which transfers amino acids from the 3' hydroxyl of the tRNA using the 2' hydroxyl as a catalyst for peptide bond formation [42].

2.5. BppA1 and BppA2 in Enterococcus faecalis

The peptidoglycan of *E. faecalis* comprises a series of indirect cross-links that result from the production of an L-Ala-L-Ala dipeptide bridge. The dipeptide bridge is attached to the epsilon amino group of the third position L-Lys of the pentapeptide side chain in a process that requires alanyl-tRNA synthetase, Ala-tRNA^{Ala} and the two Fem ligases BppA1 and BppA2. BppA1 is responsible for transfer of the first Ala of the dipeptide to its cytoplasmic precursor, UDP-MurNAc pentapeptide. BppA2 is subsequently responsible for transfer of the second Ala residue. BppA1 and BppA2 are orthologues of the *S. pneumoniae* Fem proteins sharing 39% identity to MurM and 38% identity to MurN at the amino acid level, respectively. Deletion of BppA2 results in a peptidoglycan structure comprised of a mono-Ala bridge and simultaneous reduction in the intrinsic β-lactam resistance level of this bacterium. Resistance of *E. faecalis* to third generation cephalosporins results from production of a specific low affinity penicillin binding protein, PBP5, by nearly all members of the species. Optimal activity of PBP5 requires peptidoglycan precursor substrates to carry the completed di-Ala bridge [43]. Therefore, successful diversion of Ala-tRNA^{Ala} away from protein synthesis and into this alternative pathway has direct effects on β-lactam resistance. The mechanism by which Ala-tRNA^{Ala} partitioning is achieved in *E. faecalis* to ensure adequate provision of this substrate for both protein and peptidoglycan biosynthesis is currently unknown.

2.6. FemX and VanK in Streptomyces coelicolor

S. coelicolor peptidoglycan is indirectly cross-linked by virtue of a mono-Gly branch that is attached to meso-diaminopimelic acid at the third position of the cytoplasmic UDP-MurNAc pentapeptide side chain precursor. Mono-Gly branch formation is achieved by one of the Gly-tRNA-dependent aminoacyl ligases FemX or VanK. FemX and VanK are not active at the same time and control of their gene expression depends upon vancomycin levels in the immediate environment of the bacterium. In the absence of vancomycin, FemX, which recognizes UDP-MurNAc pentapeptide terminating in D-Ala-D-Ala, is active and responsible for the addition of mono-Gly to the third position of the side chain. Conversely, in the presence of this antibiotic, expression of the vancomycin gene cluster, *VanSRJKHAX*, is switched on by the two component regulatory system VanRS [44]. Expression from this gene cluster results in VanHAX mediated termination of the pentapeptide side chain of UDP-MurNAc in D-Ala-D-Lactate (Lac) instead of D-Ala-D-Ala. Since vancomycin is unable to bind pentapeptide that terminates in D-Ala-D-Lac, this minor change in the peptidoglycan precursor is enough to confer vancomycin resistance on *S. coelicolor*. FemX is unable to recognize UDP-MurNAc pentapeptide terminating in D-Ala-D-Lac and so cannot branch the precursor in the presence of vancomycin. Therefore, to maintain cell viability in the presence of this antibiotic, FemX activity is substituted for by VanK activity [45]. The factors involved in diversion of Gly-tRNA^{Gly} away from protein synthesis and into peptidoglycan biosynthesis in *S. coelicolor* are currently unknown.

3. Direction of aminoacylated-tRNA into antibiotic biosynthesis and resistance pathways

In addition to having essential roles in protein synthesis and Gram-positive peptidoglycan cross-linking, aminoacyl-tRNAs are also used in pathways directly implicated in antibiotic biosynthesis and resistance. These reactions involve different amino acid-tRNA pairs and a variety of acceptor molecules as discussed below.

3.1. MprF mediated antibiotic resistance

MprF proteins are broadly defined as virulence factors that control the permeability of the cell wall to cationic antimicrobials by catalyzing the aminoacylation of inner membrane lipids (reviewed in [46]. Aminoacylation of membrane lipids adjusts the net negative charge of the membrane bilayer in a manner that is dependent on the aminoacyl-tRNA substrate [47]. The most well characterised MprF proteins are the aminoacyl-phosphatidylglycerol synthases (aa-PGSs). The aa-PGSs are integral membrane proteins found in the cytoplasmic membrane of both Gram-positive and Gram-negative bacteria. These enzymes are directly responsible for the transfer of amino acids from aminoacyl-tRNA donors to the polar head group of the common membrane lipid, phosphatidylglycerol [48]. In each case, aa-PGSs have an N-terminal membrane-spanning domain that varies in length and is thought to be involved in flipping the aminoacylated-phospholipid across the membrane. At the C-terminus, there is a separate hydrophilic domain that catalyzes the aminoacylation process [49]. Some forms of aa- PGSs, including LysX in *Mycobacterium tuberculosis*, also have a bound tRNA synthetase enzyme that is dedicated to providing the MprF part of the protein with its charged tRNA substrate [50].

In some bacteria, including *Clostridium perfringens*, *Staphylococcus aureus* and *Mycobacterium tuberculosis*, Lys-tRNA^{Lys} is used both for translation and as a substrate for the aminoacylation of membrane lipids [48,50–52]. Within these organisms, aa-PGSs transfer the amino acid from LystRNA^{Lys} to phosphatidylglycerol. The overall effect of this process is an increase in the net positive charge of the cytoplasmic membrane. This subsequently lowers permeability to cationic molecules including defensins,

aminoglycosides, β-lactams and glycopeptides. Recently, aa-PGSs that are able to transfer amino acids other than Lys to phosphatidylglycerol have been discovered. *C. perfringens* and *Pseudomonas aeruginosa* both have Ala-PGSs which are able to aminoacylate phosphatidylglycerol with Ala and thus neutralize the overall charge of the membrane [48,53]. This modification has been shown to enhance the resistance of *P. aeruginosa* to a multitude of negatively charged β-lactam antibiotics such as oxacillin and methicillin as well as providing protection against acidic or osmotically stressful growth conditions. *Enterococcus faecium* has been shown to have an aa-PG with triple specificity for Ala, Arg and Lys [54]. This is indicative of the fact that these aa-PG enzymes confer antibiotic resistance by a combination of two mechanisms: lowering the net negative charge of the membrane and altering its general biophysical properties including fluidity and permeability. The latter of these two underexplored mechanisms could result in significant changes in the membrane proteome and a shift in the expression of virulence phenotypes.

The process of directing appropriately aminoacylated-tRNA donor molecules into membrane lipid modification and away from protein synthesis is not fully understood. Since MprF and EF-Tu have been demonstrated to have comparable affinities for Lys-tRNA^{Lys} under physiological conditions (Fig. 4, [53]), it is possible that different MprF proteins sample different aminoacylated-tRNA substrates under different environmental conditions [48]. This may be directly related to the availability of these donor molecules within the intracellular aminoacyl-tRNA pool at the time, which is likely to change based upon the growth conditions of the bacterium. Alternatively, MprF expression could be down regulated under such conditions to reduce the detrimental effect of competition with the translation machinery for the same aminoacylated-tRNA donor molecules.

3.2. VlmA mediated valanimycin synthesis in Streptomyces viridifaciens

Within the genome of *S. viridifaciens* there is a 14-gene cluster encoding for the biosynthesis of the naturally occurring azoxy-containing antibiotic, valanimycin. Valanimycin is derived from L-Val and L-Ser via isobutylamine and isobutylhydroxylamine intermediates. Encoded within the valanimycin biosynthesis gene cluster, there is a dedicated class II seryl-tRNA synthetase, VlmA. VlmA is only 39% homologous to the other housekeeping seryl-tRNA synthetase found within this actinomycete. This can be explained by the different substrate specificity of VlmA, which is responsible for the transfer of L-Ser from Ser-tRNA^{Ser} to the hydroxyl group of isobutylhydroxylamine. The product of the VlmA catalyzed reaction is *O*-(L-seryl)-isobutylhydroxylamine, which is subsequently able to rearrange spontaneously to form *N*-(L-seryl)-isobutylhydroxylamine [55,56]. The reaction catalysed by VlmL is essential for efficient production of valanimycin as demonstrated by gene disruption studies. Phenotypic effects seen in a *vlmA* mutant suggest that the Ser-tRNA^{Ser} produced by the *S. viridifaciens* housekeeping seryl-tRNA synthetase can be directed into valanimycin production but very inefficiently. Potential explanations for this have included the possible compartmentalization of the valanimycin pathway within this bacterium or inability of VlmA to access the full set of tRNA^{Ser} isoacceptors in the cell [57]. The mechanisms for directing tRNA^{Ser} into the valanimycin pathway rather than translation have not been fully characterized.

3.3. PacB mediated pacidamycin biosynthesis in Streptomyces coeruleorubidus

Genome mining within *S. coeruleorubidus* has resulted in the identification of a biosynthetic gene cluster that allows this bacterium to synthesize members of the anti-pseudomonal pacidamycin family of antibiotics [58]. Pacidamycins are broadly defined as a group of uridyl tetra- or pentapeptide antibiotics that target the MraY translocase in the prokaryotic peptidoglycan biosynthesis pathway [59]. The common scaffold for all pacidamycins is based upon a central N_{β}-methyl *2S*, *3S*-diaminoburytic acid (DABA) moiety. The DABA

moiety typically has an N-terminal amino acid (Ala/*m*-Tyr_2) and a bicyclic heterocycle or dipeptide (Ala/Gly_1-m-Tyr_2). In addition, there is a C-terminal ureido dipeptide (Ala_4-Phe/Trp/*m*-Tyr_5) and a carboxy-linkage to a 3'-deoxy-4', 5'-enaminouridine [60]. Production of the 5'aminouridyl-tetrapeptide pacidamycin framework requires a total of nine nonribosomal peptide synthetases [61]. Specifically, PacN generates the ureido dipeptide moiety of the scaffold and PacD subsequently ligates this to the α-amino region of DABA. DABA is then further modified at the carboxyl group by PacI-catalyzed addition of an amide-linked modified uridine moiety. Addition of the N-terminal Ala/Gly_1 has been shown to require PacB, a structural homologue of *W. viridescens* FemX (see section 2.4 of this review). PacB has functional similarity to the Fem ligases due to its role in the transfer of L-Ala from aminoacyl-tRNA donors to the m-Tyr_2 residue of the growing PacH-anchored antibiotic scaffold. No pacidamycin-dedicated aminoacyl-tRNA synthetases or tRNAs have been found within the gene cluster encoding the components for synthesis of this class of antibiotic. This suggests that PacB must sequester aminoacyl-tRNA donors in direct competition with the protein synthesis pathway [62]. The mechanism by which this is achieved and controlled is currently unknown.

3.4. Cyclodipeptide synthesis

Cyclodipeptides form a large class of natural secondary metabolites that belong to the diketopiperazine (DKP) family [63]. Many of these secondary metabolites are potentially clinically relevant exhibiting a broad range of biological activities [64,65] including those that are antibacterial [66–68], antifungal [69,70], antiviral [71], immunosuppressive [72], antitumor [73–76] or anti-inflammatory [77] in nature. Initial characterization of cyclodipeptide synthesis pathways resulted in the hypothesis that nonribosomal peptide synthetases are essential to the process due to their role in catalysis of DKP scaffold formation [63]. In 2002, a nonribosomal peptide synthetase-independent DKP biosynthesis pathway was discovered in *Streptomyces noursei* [78]. Characterization of this albonoursin synthesis pathway identified a unique tRNA-dependent cyclodipeptide synthase, AlbC. AlbC is catalytically responsible for the synthesis of the albonoursin precursor cyclo(L-Phe-L-Leu) from Phe- and Leu-tRNA substrates. This precursor is then converted to albonoursin by a cyclic dipeptide oxidase that is responsible for two sequential α,β-dehydrogenations [63,78]. Since the discovery of AlbC, two other prokaryotic cyclodipeptide synthase-requiring pathways have been characterized. Synthesis of the cyclo(L-Leu-L-Leu) precursor for the iron chelator, pulcherrimin, in *Bacillus subtilis* requires the Leu-tRNA-dependent cyclodipeptide synthase, YvmC. After its synthesis, cyclo(L-Leu-L-Leu) is then converted to the iron-binding unit, pulcherriminic acid, by CypX-catalyzed diketopiperazine ring oxidation [63,79]. In *Mycobacterium tuberculosis*, the cyclo(L-Tyr-L-Tyr) precursor for the potential cell viability factor mycocyclosin is produced by the Tyr-tRNA-dependent cyclodipeptide synthase Rv2275. Following its synthesis, the cyclo(L-Tyr-L-Tyr) precursor is subjected to C-C aryl coupling by CYP121 to form the final mycocyclosin product [63,80,81]. Following elucidation of the pathways employed by these three cyclodipeptide synthases, it has been determined that they form a unique family of enzymes detected across at least 50 different bacterial genomes. The enzyme family is most well represented across Actinobacteria, Proteobacteria and Firmicutes but they have also been found in Bacteroidetes, Chlamydiae, Cyanobacteriae and five eukaryotic genomes. Despite this fairly broad evolutionary distribution, cyclodipeptide synthases show very little to no significant structural and functional similarity to the non-ribosomal peptide synthetases or Fem ligases. Sequence alignment of nine members of this family has resulted in identification of conservation at only seven residues (Gly-35, Ser-37, Gly-79, Tyr-128, Tyr-178, Glu-182 and Tyr-202) and no apparent essential consensus sequence was found (reviewed in [82]). Therefore, it is currently unclear how the cyclodipeptide synthases hijack aminoacylated-tRNA species from primary metabolism (Fig. 5).

4. Diverting aminoacyl-tRNAs away from translation

In addition to their primary role in translation, aminoacyl-tRNAs are also critical substrates for other cellular processes including peptidoglycan biosynthesis, membrane lipid modification, protein tagging for N-end rule degradation (reviewed in [83]), synthesis of other tRNAs to compensate for an absent aminoacyl-tRNA synthetase (reviewed in [84]), tetrapyrrole synthesis (reviewed in [84]), antibiotic biosynthesis and antibiotic resistance pathways. With recent advances in post-genomic techniques ensuring the frequent discovery of novel aminoacyl-tRNA-dependent pathways, there is renewed interest in both the proteins that are involved in hijacking these substrates from primary metabolism and in the associated mechanisms that ensure this does not become detrimental to the cell.

The mechanism for ensuring adequate diversion of aminoacylated-tRNA donor molecules into peptidoglycan biosynthesis, thereby surpassing direct competition from the translation machinery, depends upon the bacterium and, in some cases, is still not fully understood. In *S. aureus*, three of the five encoded $tRNA^{Gly}$ isoacceptors escape protein synthesis and are, instead, directed into pentaglycine peptidoglycan cross-bridge formations by virtue of them containing sequence specific identity elements that are associated with weak binding to EF-Tu. In *S. epidermidis*, both Ser and Gly are found within the penta-amino acid peptidoglycan cross-bridge, and at least two of the identified $tRNA^{Gly}$isoacceptors (named IA and IB) participate in peptidoglycan synthesis but not in translation [46,85]. Both of these tRNA molecules differ from others in that their dihydrouridine (D) loops contain an unusually low number of modified uridine nucelotides as well as lacking guanine at positions 18 and 19. In addition to this, the D-loop of IB has two opposing uridine residues that prevent the normal formation of the first base pair of this stem structure. In both species, the typical $GT\psi C$ sequence found in many tRNAs is replaced by GUGC and there is an additional base pairing event in the anticodon loop, between C32 and G38, which may distort this region enough to prevent binding to the ribosome, thus accounting for efficient escape into peptidoglycan biosynthesis [86,87]. In contrast, no single species of $tRNA^{Ser}$ or mutations within this molecule have been identified that would allow it to act as an exclusive Ser donor for peptidoglycan biosynthesis. In fact, it has been demonstrated that Ser is integrated into the penta-amino acid bridge of this bacterium in a random manner and approximately 20% of the cross-links within *S. epidermidis* strain Texas 26 consist solely of Gly [88]. This suggests that Ser integration could be regulated by growth conditions or environmental factors that would make it beneficial for Ser-$tRNA^{Ser}$ to be hijacked away from protein synthesis. For example, it would be advantageous for *S. epidermidis* to increase levels of Ser in its peptidoglycan cross-bridges during colonization of a niche inhabited by other staphylococcal species that are able to produce and secrete glycyl-glycine endopeptidases [31,32]. This is plausible when it is taken into consideration that the two well characterized glycl-glycine endopeptidase secreting staphylococcal species, *S. simulans* and *S. capitis* (reviewed in section 2.3), have both been isolated from human skin, which is also the common niche of *S. epidermidis* [89].

Since indirect peptidoglycan cross-linking has only been directly correlated with cell viability in members of the genus Staphylococcus, it is of no surprise that the Fem ligases within this bacterium are provided with designated non-proteinogenic $tRNA^{Gly}$ isoacceptors to perform this task. No such designated tRNA isoacceptors have been identified in *S. pneumoniae* or *W. viridescens*, the two other well characterized bacterial systems that exhibit Fem ligase mediated indirect peptidoglycan cross-linking. MurMN from *S. pneumoniae* require Ser-$tRNA^{Ser}$ and Ala-$tRNA^{Ala}$ as amino acid donors whereas *W. viridescens* FemX requires only the latter species. In both cases, attempts to characterize the substrate specificities of *W. viridescens* FemX and *S. pneumoniae* MurM have indicated that they only accept the amino acid moiety from the 2’ hydroxyl of adenine-76 of the tRNA

donor molecule [41,90]. This contrasts with the described specificity of the ribosome, which utilizes tRNA species that are aminoacylated on the 3' hydroxyl group of adenine-76 [42]. Spontaneous transesterification of the amino acid between the 3' and the 2' hydroxyl group of the terminal adenine of any tRNA molecule ensures that these species exist in a 2:1 ratio at equilibrium [91]. Therefore this differential specificity of MurM and FemX in comparison to the ribosome may be one mechanism that is exploited by the cell to ensure adequate provision of aminoacyl-tRNA species for translation and peptidoglycan biosynthesis.

In addition to mechanisms based on dedicated tRNAs, some non-ribosomal biosynthetic pathways depend on dedicated aminoacyl-tRNA synthetases. Some MprF proteins, most notably LysX in *Mycobacterium tuberculosis*, have a bound tRNA synthetase enzyme that is dedicated to providing the MprF part of the protein with its charged tRNA substrate [50]. The valanimycin antibiotic biosynthesis pathway in *S. viridifaciens* has a dedicated seryl-tRNA synthetase that is responsible for production of the Ser-tRNA^{Ser} intermediate [55]. Within these systems, presence of a dedicated synthetase could directly address the problem of substrate competition with the translation machinery by ensuring the required aminoacyl-tRNA species are directly transferred to the proteins involved in these pathways without being subjected to sampling by EF-Tu. The pacidamycin and characterized cyclodipeptide biosynthesis pathways do not encode dedicated aminoacyl-tRNA synthetase enzymes and are likely to hijack aminoacylated-tRNA donors directly from primary metabolism [58,82]. Therefore, the balance between translation and these pathways may shift in favour of the later during stages in the growth cycle when production of these compounds is advantageous to the bacterium. How this is achieved is currently unknown and further studies are required to fully understand the interactions of these proteins with their tRNA substrates [62,82].

5. Future perspectives

Aminoacyl-tRNA species are the prevalent amino acid donors in the cell due to their critical role as substrates for ribosomal protein biosynthesis. It has become increasingly clear that aminoacyl-tRNAs are also utilized in an ever-growing number of other non-ribosomal biosynthetic pathways particularly in bacteria, although some pathways also exist in eukaryotes [92]. To date over half of the 20 canonical aminoacyl-tRNAs required to translate the genetic code have been shown to have one or more alternative functions in bacteria, and it is likely that many more will be found to have roles in other pathways in all domains of life. As discussed here, there is a clear link between many of the non-ribosomal aminoacyl-tRNA dependent activities and antibiotic resistance or production, and other stress responses. As a result of this, further characterization of these pathways may enable development of methodologies for increasing the yield of natural antibiotic biosynthesis. More importantly, research into these pathways will also provide the potential for identification of new drug targets, which could restore the potency of antibiotics already used in the clinical setting. Of particular interest here may be the discovery of "missing links" in some of these non-ribosomal aminoacyl-tRNA dependent pathways, which might be expected to fall into two broad categories. Firstly, it is possible that non-canonical aminoacylation events may provide new and useful substrates to diversify non-ribosomal biosynthetic pathways just as they do for protein synthesis [93]. Secondly, the partitioning of substrates away from protein synthesis may require the presence of dedicated complexes either between know factors [94], which in some cases may be fused [50], or the presence of as yet uncharacterized factors that can sequester substrates for non-ribosomal pathways. Overall, it seems clear that our current understanding of the role of aminoacyl-tRNAs outside of protein synthesis will continue to expand and add to our broader understanding of how translation and other cellular pathways are linked.

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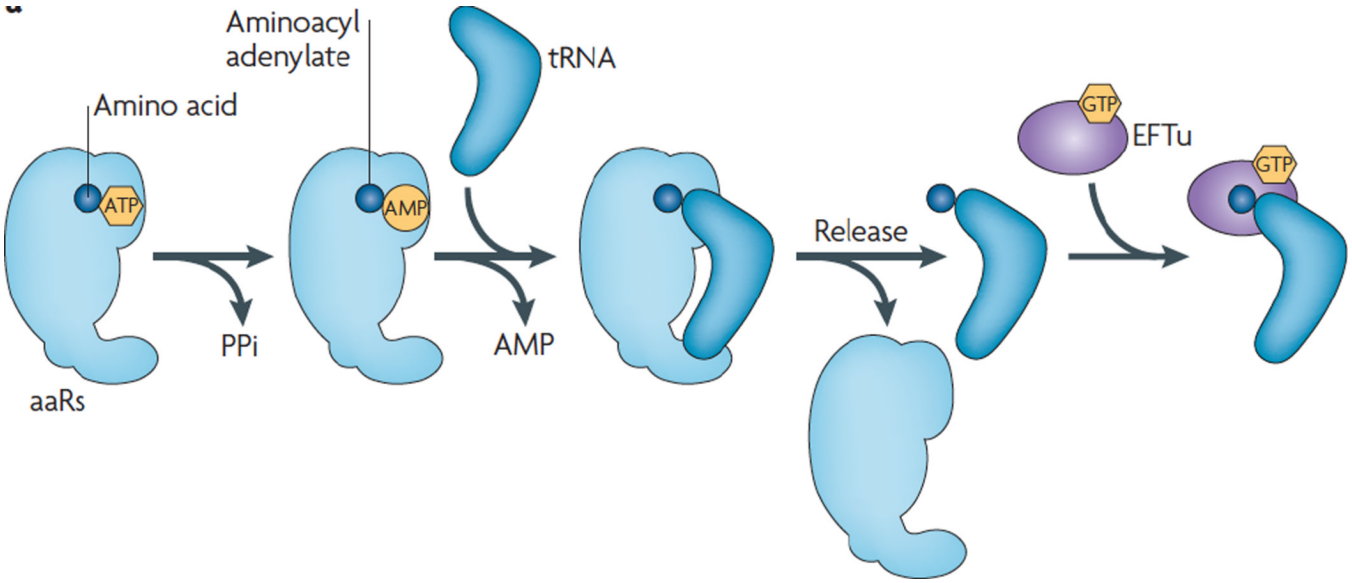

Figure 1. Formation of an amino acid-tRNA pair by an aminoacyl-tRNA synthetase (aaRS)

An amino acid (blue) is activated by an aaRS to form an aminoacyl-adenylate. This process requires adenosine triphosphate (ATP) and results in the release of pyrophosphate (PPi). Following tRNA binding to the aaRS, the activated amino acid is transferred to the 3' end of the tRNA molecule forming aminoacyl-tRNA with concurrent release of adenosine monophosphate (AMP). The aminoacyl-tRNA product is released from the aaRS and is either subject to binding by elongation factor Tu (EF-Tu) for delivery to the ribosome or hijacking by other factors for diversion into biosynthetic pathways outside of translation. Figure reproduced with permission from [2].

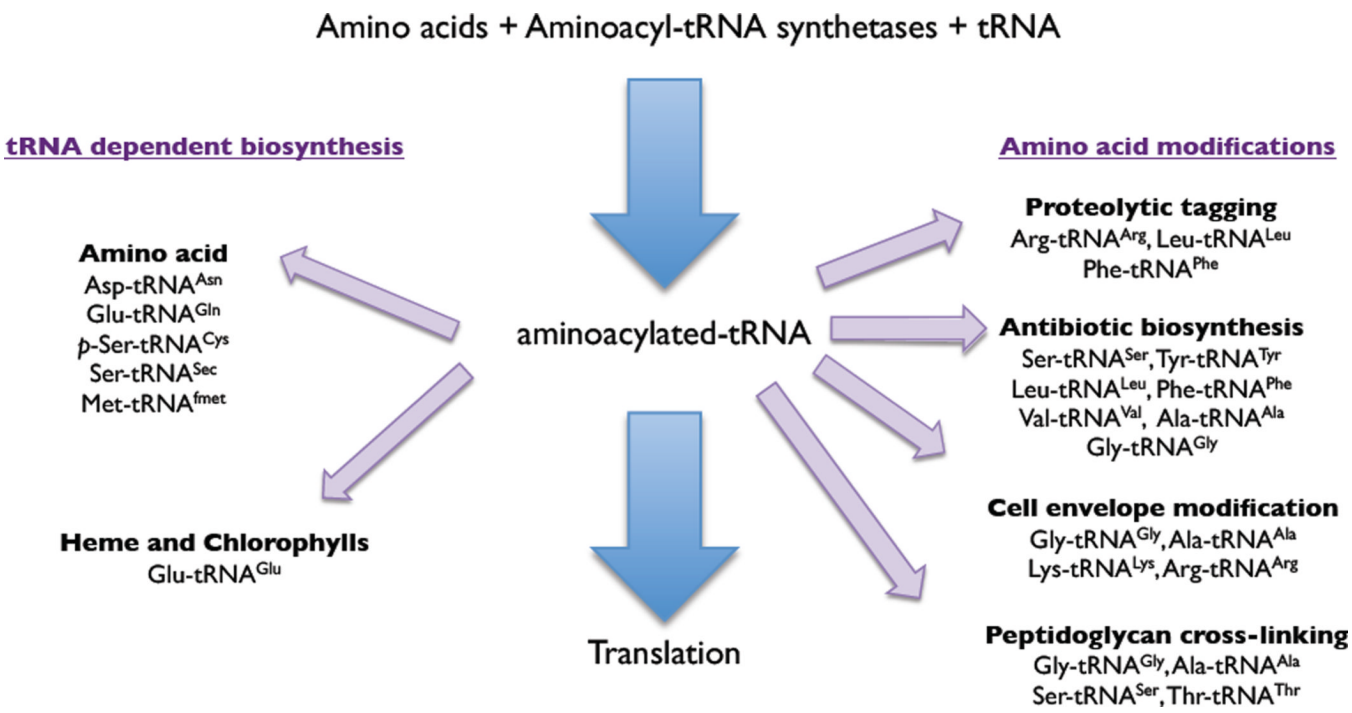

Figure 2. Cellular biosynthetic pathways that utilize aminoacyl-tRNAs as substrates

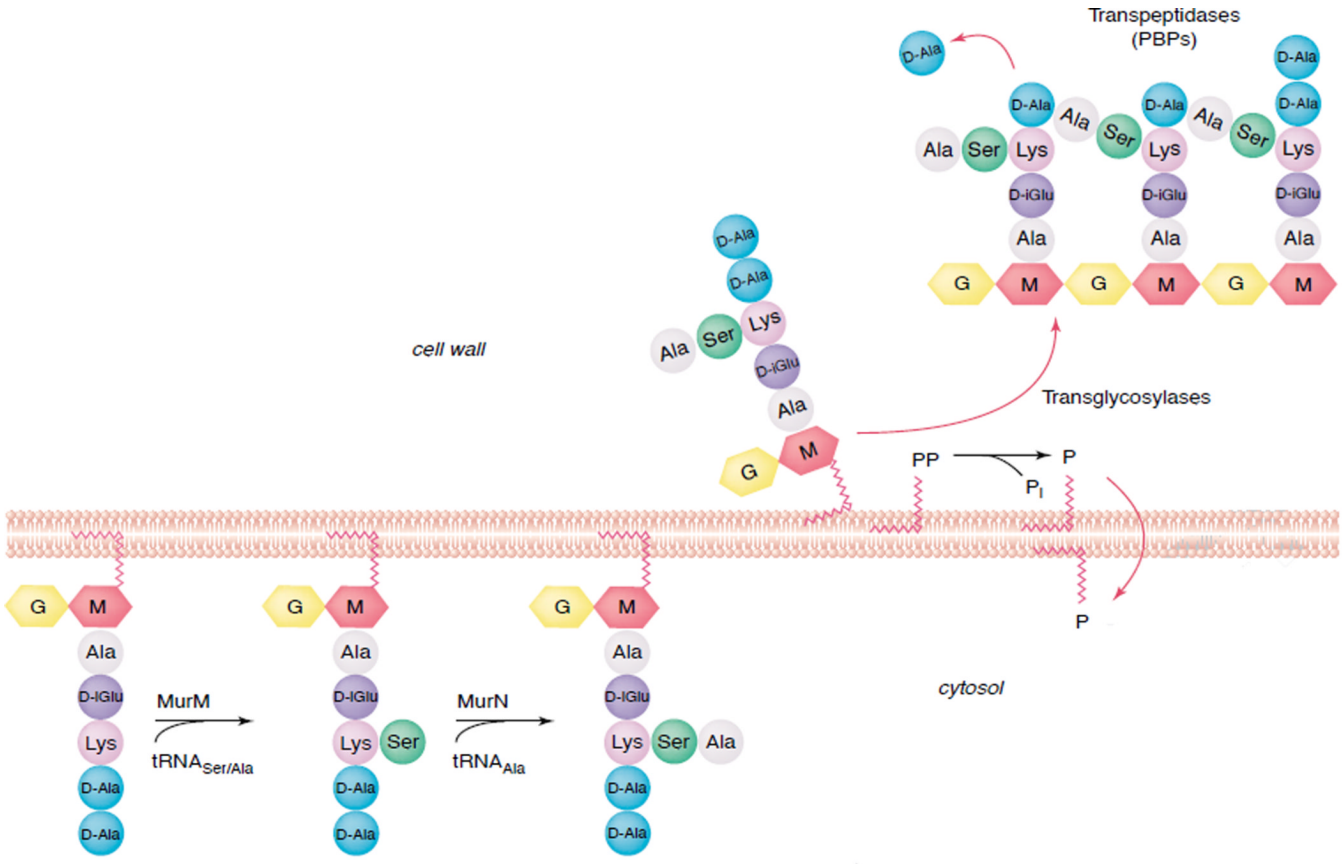

Figure 3. The role of MurM and MurN in cell wall branching using Lipid II as substrate

Lipid II is comprised of N-acetylated disaccharide units of glucosamine (yellow hexagon labeled G) and muramic acid (pink hexagon labeled M). The pentapeptide side chain is enzymatically attached to the muramic acid residues. Lipid II itself is anchored via bactoprenyl pyrophosphate (pink zig-zag line) to the plasma membrane. MurM is an aminoacyl ligase responsible for the addition of either L-Ser or L-Ala to the cell-wall precursor L-Lys residue. MurN is responsible for the invariable addition of L-Ala as the second amino acid of the cross-link. Reproduced with permission from [9].

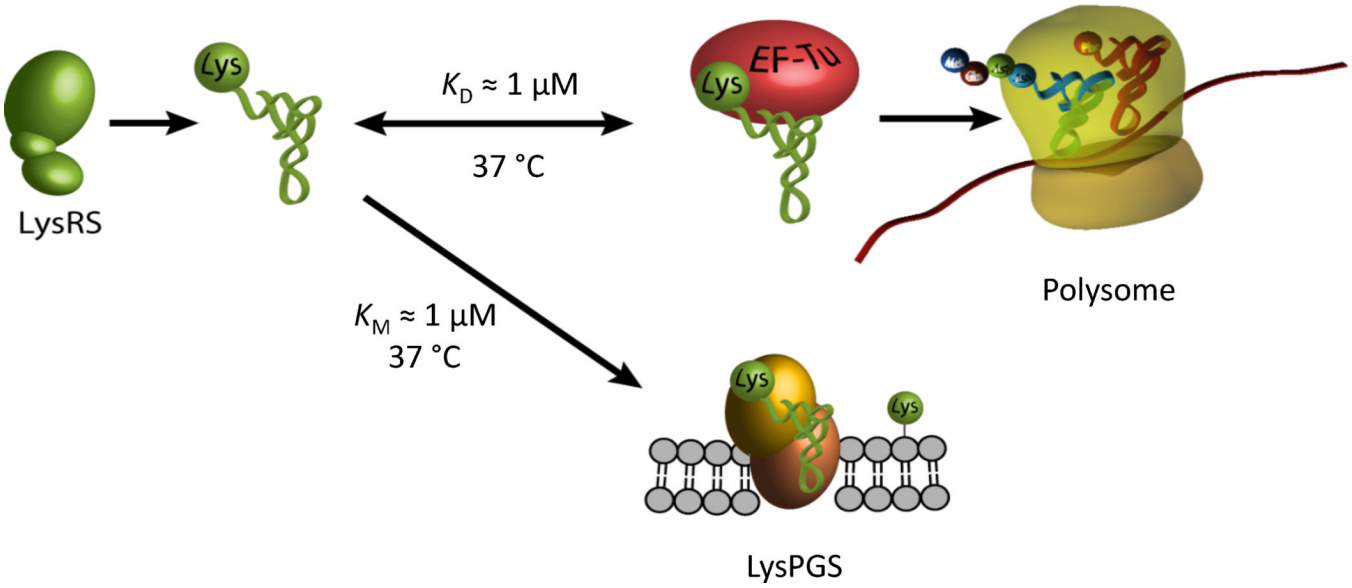

Figure 4. Partitioning of Lys-tRNA^{Lys} between protein synthesis and membrane lipid modification by MprF proteins

Lys-specific MprF (LysPGS) and Elongation factor Tu (EF-Tu) show comparable affinities for Lys-tRNA^{Lys} potentially allowing this aminoacylated tRNA species to enter two different biosynthetic pathways simultaneously. Reproduced with permission from [95].

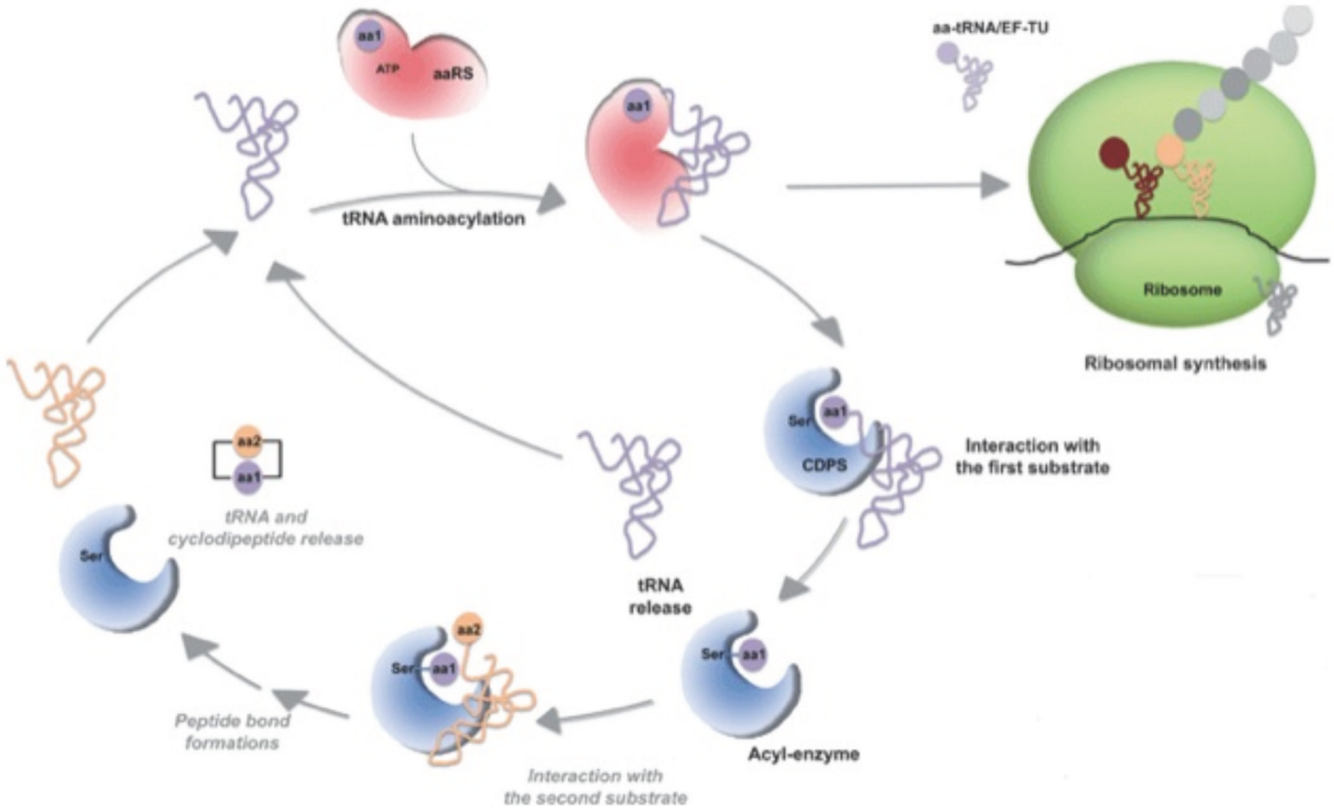

Figure 5. Hijacking of aminoacylated-tRNA donor molecules by cyclodipeptide synthases for use in biosynthesis of the natural secondary metabolites, cyclodipeptides

Aminoacylated-tRNA donor molecules are generated by aminoacyl-tRNA synthetases (aaRS, shown in red). Typically the aminoacylated-tRNA product of the aaRS catalyzed reaction is bound by elongation factor Tu (EF-Tu) and delivered to the ribosome (shown in green) for use in protein synthesis. Cyclodipeptide synthases (blue) must hijack certain aminoacylated tRNA species and divert them into the cyclodipeptide biosynthesis pathway. Putative steps in the pathway are shown in italics. Reproduced and modified with permission from [82].