Published online 16 January 2009 *Nucleic Acids Research, 2009, Vol. 37, No. 5* ***1589–1601***
doi:10.1093/nar/gkn1039

Aminoacyl-tRNA recognition by the $FemX_{Wv}$ transferase for bacterial cell wall synthesis

Matthieu Fonvielle[1,2,3], Maryline Chemama[4,5], Régis Villet[1,2,3], Maxime Lecerf[1,2,3], Ahmed Bouhss[6,7], Jean-Marc Valéry[4,5], Mélanie Ethève-Quelquejeu[4,5] and Michel Arthur[1,2,3,*]

[1]Centre de Recherche des Cordeliers, LRMA, Equipe 12, INSERM, [2]Université Pierre et Marie Curie – Paris 6, [3]Université Paris Descartes, UMR S 872, [4]Synthèse, Structure et Fonction de Molécules Bioactives, CNRS UMR 7613, [5]Université Pierre et Marie Curie – Paris 6, UMR 7613, F-75006, Paris, [6]Laboratoire des Enveloppes Bactériennes et Antibiotiques, Institut de Biochimie et Biophysique Moléculaire et Cellulaire, UMR 8619 CNRS and [7]Université Paris-Sud, F-91405, Orsay, France

Received October 8, 2008; Revised December 11, 2008; Accepted December 12, 2008

ABSTRACT

Transferases of the Fem family catalyse peptide-bond formation by using aminoacyl-tRNAs and peptidoglycan precursors as donor and acceptor substrates, respectively. The specificity of Fem transferases is essential since mis-incorporated amino acids could act as chain terminators thereby preventing formation of a functional stress-bearing peptidoglycan network. Here we have developed chemical acylation of RNA helices with natural and non-proteinogenic amino acids to gain insight into the specificity of the model transferase $FemX_{Wv}$. Combining modifications in the RNA and aminoacyl moieties of the donor substrate revealed that unfavourable interactions of $FemX_{Wv}$ with the acceptor arm of $tRNA^{Gly}$ and with L-Ser or larger residues quantitatively accounts for the preferential transfer of L-Ala observed with complete aminoacyl-tRNAs. The main $FemX_{Wv}$ identity determinant was identified as the penultimate base pair (G^2-C^{71}) of the acceptor arm instead of $G^3 \bullet U^{70}$ for the alanyl-tRNA synthetase. $FemX_{Wv}$ tolerated a configuration inversion of the Cα of L-Ala but not the introduction of a second methyl on this atom. These results indicate that aminoacyl-tRNA recognition by $FemX_{Wv}$ is distinct from other components of the translation machinery and relies on the exclusion of bulky amino acids and of the sequence of $tRNA^{Gly}$ from the active site.

INTRODUCTION

Peptidoglycan is a giant macromolecule, in the order of 3×10^9 to 30×10^9 Da, that completely surrounds the cytoplasmic membrane and thereby provides a mechanical protection against the turgor pressure of the cytoplasm. Since the osmoprotective function is required in continuity throughout the cell cycle, peptidoglycan metabolism is intimately involved in cell division (1). Peptidoglycan also provides a scaffold to anchor various surface polymers that interact with host cells and the immune system (2,3). These multiple functions are fulfilled by polymerization of a relatively simple subunit, a disaccharide peptide, that was recently shown to display little conformational heterogeneity by solid-state nuclear magnetic resonance of the intact polymer (4). Formation of the peptidoglycan network involves two main enzyme activities, glycosyltransferase and D,D-transpeptidase, that are often combined in multifunctional proteins belonging to the penicillin-binding protein family (PBP). The glycosyltransferases polymerize glycan strands made of alternating β,1→4-linked *N*-acetyl-glucosaminyl (GlcNAc) and *N*-acetyl-muramyl (MurNAc) residues. The 3D nature of the peptidoglycan network is provided by the cross-linking of short stem peptides born by MurNAc residues from adjacent glycan strands (5).

Formation of the peptidoglycan network (Figure 1) involves different types of peptide and amide bond synthesizing enzymes. The Mur synthetases form cytoplasmic UDP-MurNAc-pentapeptide by sequential addition of amino acids with alternating L and D configurations except for the terminal D-Ala-D-Ala dipeptide (6).

*To whom correspondence should be addressed. Tel: +33 1 43 25 00 33; Fax: +33 1 43 25 68 12; Email: michel.arthur@crc.jussieu.fr

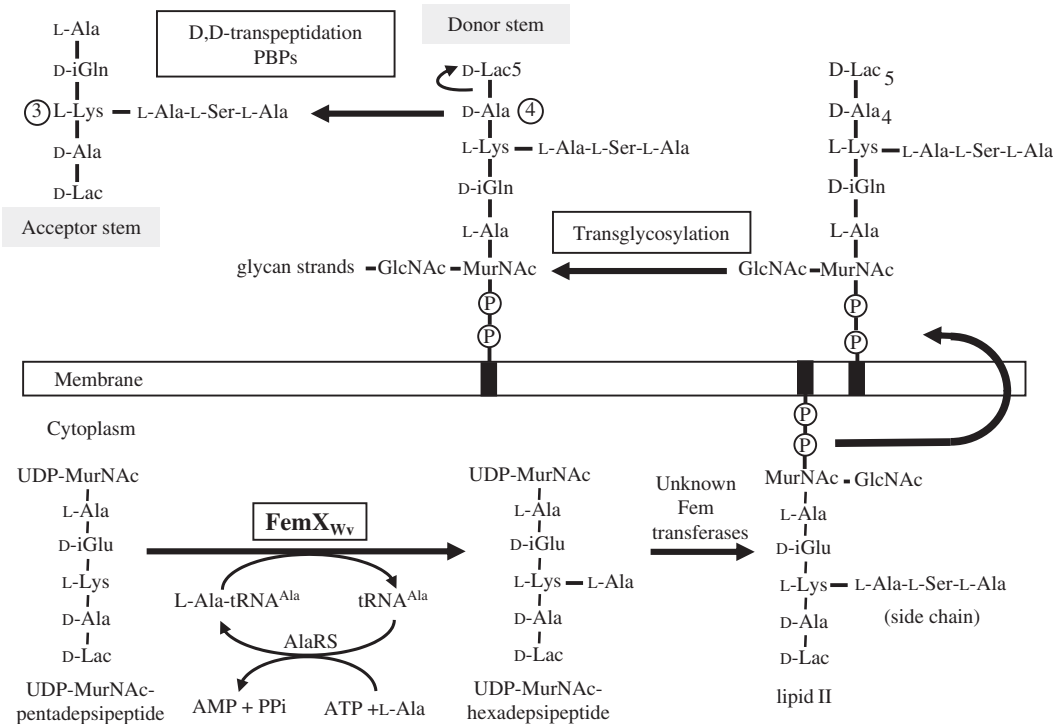


Figure 1. Peptidoglycan synthesis pathway in *W. viridescens*. The $FemX_{Wv}$ transferase adds the first residue of the L-Ala-L-Ser-L-Ala side chain onto the nucleotide precursor UDP-MurNAc-pentadepsipeptide. The Ala-$tRNA^{Ala}$ substrate of $FemX_{Wv}$ is produced by the alanyl-tRNA-synthetase (AlaRS). Additional unknown Fem transferases add the second (L-Ser) and third (L-Ala) residues of the side chain onto the precursors linked to the undecaprenyl lipid carrier (30). Black box, undecaprenyl; D-Lac, D-lactate; lipid II, undecaprenyl-diphospho-MurNAc(pentadepsipeptide)-GlcNAc. D,D-transpeptidases belonging to the penicillin-binding protein (PBP) family catalyse formation of a peptide bound between the carbonyl of D-Ala^4 of an acyl donor stem and the amine at the side chain extremity of an acceptor stem.

These enzymes use ATP as a cofactor and activate the carboxyl of the acyl donor by formation of an acyl phosphate. The precursors of many Gram-positive bacteria contain an additional side chain linked to the third amino acid of the stem pentapeptide. The side chain comprises from one to seven amino acids belonging both to the L and D series (7). Glycine and L-amino acids are activated as aminoacyl-tRNAs by the aminoacyl-tRNA synthetases involved in protein synthesis and are transferred to the peptidoglycan precursors by Fem transferases that belong to the GCN5-related *N*-acetyltransferase (GNAT) protein superfamily (8,9). The side chain carboxyl of D-Asp and D-Glu is activated as an acyl-phosphate and subsequently ligated to the precursors by members of the ATP-Grasp protein superfamily (10). The final cross-linking step is performed by active-site serine peptidases that cleave the D-Ala^4-D-Ala^5 peptide bond of an acyl donor (transpeptidase of the D,D specificity) and link the carbonyl of D-Ala^4 to the amine located at the extremity of the side chain of an acyl acceptor (11). In enterococci resistant to β-lactams and in *Mycobacterium tuberculosis*, these members of the PBP family can be replaced by active-site cysteine peptidases that cleave the L-Lys^3-D-Ala^4 peptide bond of an acyl donor (transpeptidase of the L,D specificity) and link the carbonyl of L-Lys^3 to the amine of the acyl acceptor (12,13).

The specificity of peptide and amide forming enzymes is essential for bacteria since mis-incorporated amino acids can act as chain terminators (6,14) and block the final cross-linking step of peptidoglycan polymerization (15,16). The sequence of the peptide network has a critical impact on the activity of amidases and peptidases that fulfil a wide variety of physiological functions including peptidoglycan recycling, separation of daughter cells after division and partial hydrolysis of the peptidoglycan network thought to be required both for insertion of novel subunits into the pre-existing material and for crossing of macromolecular structures such as pili and flagella through the peptidoglycan layer (17). Finally, the sequence of the peptide network determines the susceptibility of the peptidoglycan to hydrolases produced by competing bacteria (18).

Fem transferases are considered as attractive targets for the development of novel antibiotics active against multiresistant bacteria (14). These enzymes have a unique catalytic mechanism (19,20) and are essential either for viability (21) or for expression of β-lactam resistance mediated by low-affinity PBPs (22–24). Characterized members of this family include FemABX from *Staphylococcus aureus* that sequentially add one (FemX) or two (FemA and FemB) glycines (25) and homologues from *Streptococcus pneumoniae* (MurMN) (22,26) and *Enterococcus faecalis* (BppA1A2) (27,28) for incorporation of single residues into L-Ala (or L-Ser)-L-Ala side chains. In addition, $FemX_{Wv}$ from *Weissella viridescens* has been widely used as a model transferase since the UDP-MurNAc-pentapeptide substrate of this enzyme

(Figure 1) is more easily obtained than the lipid intermediates used by other members of the family (20,27). $FemX_{Wv}$ catalysis proceeds by an ordered bi-bi mechanism with sequential fixation of the UDP-MurNAc-pentapeptide and Ala-$tRNA^{Ala}$ substrates and sequential release of the $tRNA^{Ala}$ and UDP-MurNAc-hexapeptide products (19). Structure-based site-directed mutagenesis of the UDP-MurNAc-pentapeptide-binding cavity of $FemX_{Wv}$ revealed that a complex hydrogen bond network connects two residues of the enzyme (Lys^{36} and Arg^{211}) with two regions of UDP-MurNAc-pentapeptide (both phosphate groups and both D-Ala residues) and constrains the substrate in a bent conformation essential for the aminoacyl transferase activity (8,29). Analysis of the interaction of $FemX_{Wv}$ with the second substrate (Ala-$tRNA^{Ala}$) showed that the acceptor stem of $tRNA^{Ala}$ is sufficient for aminoacyl transfer (30). Saturation mutagenesis of this region of the substrate and modelling of the acceptor stem in the $FemX_{Wv}$ catalytic cavity suggested that the enzyme only interacts with the two distal base pairs (G^2-C^{71} and G^1-C^{72}) and the single-stranded 3′-end ($^{73}ACCA^{76}$) (30). We have analysed the specificity of $FemX_{Wv}$ in the aminoacyl transfer reaction by systematically exploring the impact of modifications in the aminoacyl residue and RNA sequence on the catalytic efficiency of $FemX_{Wv}$.

MATERIALS AND METHODS

Enzyme purification

$FemX_{Wv}$ (29), alanyl-tRNA synthetase (AlaRS) (27), T4 RNA ligase (30) and T7 RNA polymerase (30) were purified according to previously published procedures.

Substrates

Full-length $tRNA^{Ala}$ (5′-GGGGCCUUAGCUCAGCUG GGAGAGCGCCUGCUUUGCACG CAGGAGGUCA GCGGUUCGAUCCCGCUAGGCUCCACCA-3′) corresponds to the three identical sequences annotated as $tRNA^{Ala}$ in the genome sequence of *E. faecalis* strain V583 (http://www.tigr.org/). This 76-nucleotide RNA was obtained by *in vitro* transcription using T7 RNA polymerase (30). The choice of the *E. faecalis* rather than a *W. viridescens* $tRNA^{Ala}$ sequence was dictated by the fact that the sequence of the genome of the latter bacteria is unknown.

The peptidoglycan precursor UDP-MurNAc-L-Ala^1-D-$iGlu^2$-L-Lys^3-D-Ala^4-D-Ala^5 (UDP-MurNAc-pentapeptide) was synthesized as previously described (31). Labelled UDP-MurNAc-L-[^{14}C]Ala^1-D-$iGlu^2$-L-Lys^3-D-Ala^4-D-Ala^5 was prepared by sequential addition of L-[^{14}C]Ala (6.3 GBq.mmol^{-1}; Perkin Elmer), D-Glu, L-Lys and D-Ala-D-Ala by the purified MurC, D, E, F synthetases (32).

Reagents and materials for organic synthesis

Solvents were dried using standard methods and distilled before use. Unless otherwise specified, materials were purchased from commercial suppliers and used without further purification. TLC: precoated silica gel thin layer sheets 60 F_{254} (Merck). Flash chromatography: silica gel 60 Å, 180–240 mesh from Merck. ^{1}H (250.13 MHz), ^{13}C (62.90 MHz) spectra were recorded on Brüker ARX 250 spectrometer in $CDCl_3$. Chemicals shifts (δ) are expressed in ppm relative to residual $CDCl_3$ (δ 7.26) for ^{1}H, $CDCl_3$ (δ 77.16) for ^{13}C as internal references. Signals were attributed based on COSY and DEPT 135 (^{13}C). High resolution mass spectroscopy (HRMS) spectra were carried out on a LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific Inc.) in the positive or negative electrospray ionization modes (ESI) at the Mass Spectrometry Centre of the University Pierre & Marie Curie (Paris). High-performance liquid chromatography (HPLC) was performed with reverse phase C-18 columns (analytic column: 250 × 4.6 mm, HYPERSIL-100 C18; semipreparative column: 250 × 21.2 mm, HYPERSIL HS C18; Thermoelectron Corporation). Compounds were eluted at flow rates of 1 and 17 ml.min^{-1} (for the analytic and semipreparative columns, respectively) with a linear gradient of CH_3CN (0–33% in 45 min) in 50 mM aqueous NH_4OAc (pH 4.5).

Synthesis of [*N*-(4-pentenoyl)-aminoacyl]-pdCpAs (compounds 2a-d)

The dinucleotides acylated by protected Gly, L-Ala, D-Ala and 2-aminobutyrate (Abu) (compounds **2a–d** in Figure 2A) were synthesized according to a previously described method (33,34). A solution of freshly distilled DMF (100 µl) containing 10 mg of pdCpA tetrabutylammonium salt (7.35 µmol) was added to 39.7 µmol of *N*-(4-pentenoyl)-amino-acid cyanomethyl esters (**1a–d**). The reaction mixtures were stirred at room temperature and monitored by rp-HPLC. Purification on the semipreparative C18 reversed phase column led to compounds **2a–d** (retention times: **2a**, 13.8 min; **2b**, 14.7 min; **2c**, 14.9 min; **2d**, 16.3 min) that were recovered as colourless solids after lyophilization and analysed by ESI-HRMS. **2a**: 44% yield (2.5 mg); observed and calculated *m*/*z* of 774.1655 and 774.1650 for the $[M - H]^-$ ion, respectively; **2b**: 48% yield (2.8 mg); *m*/*z* 790.1959 $[M + H]^+$ (calculated 790.1963); **2c**: 52% yield (3.0 mg); *m*/*z* 788.1785 $[M - H]^-$ (calculated 788.1806); **2d**: 60% yield (3.6 mg); *m*/*z* 802.1959 $[M - H]^-$ (calculated 802.1963).

Synthesis of *N*-(4-pentenoyl)-L-serine methyl ester (compound 4)

$NaHCO_3$ (135 mg; 1.61 mmol) was added to an aqueous solution (4 ml) of L-serine methylester hydrochloride **3** (250 mg; 1.61 mmol) (Figure 2B). A solution of 4-pentenoic acid succinimide ester (35) (177 mg in 4 ml of dioxane) was added under stirring at room temperature. After overnight stirring at room temperature, the reaction mixture was diluted with 8 ml of EtOAc and 8 ml of 1 M $NaHSO_4$, and then extracted with EtOAc. The combined organic extracts were dried over Na_2SO_4 and concentrated under diminished pressure. The crude product was purified by flash chromatography on a silica gel column. Elution with 9:1 CH_2Cl_2/MeOH gave **4** as a colourless oil: 95% yield (307 mg); ^{1}H NMR

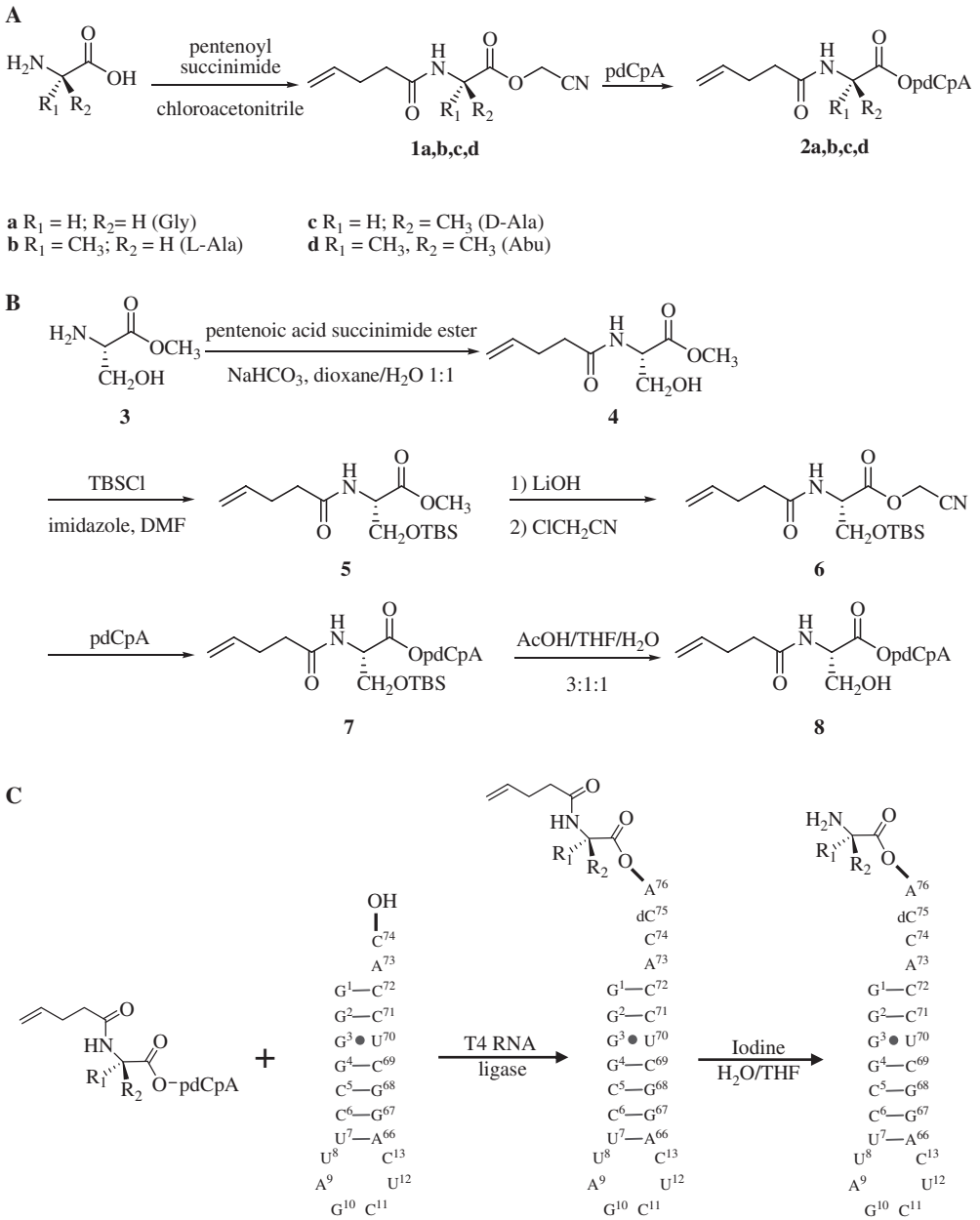


Figure 2. Semi-synthesis of Ala-tRNA^{Ala} analogues. (**A**) Organic synthesis of dinucleotides acylated by Gly, L-Ala, D-Ala and Abu protected with a pentenoyl group. pdCpA, (5′)phospho(2′)deoxycytidine-(5′)phosphoadenine. (**B**) Organic synthesis of pdCpA acylated by L-Ser protected with a pentenoyl group. (**C**) Ligation of acylated dinucleotides to RNA helix^{Ala}. The product of the reaction catalysed by the T4 RNA ligase was deprotected with iodine.

(250 MHz, $CDCl_3$) δ 7.05 (br d, J = 7.9, 1H), 5.72 (dd, J = 10.1, 17.0, 1H), 5.05 – 4.82 (m, 2H), 4.60 – 4.42 (m, 1H), 3.80 (ddd, J = 3.6, 11.3, 34.1, 2H), 3.65 (s, 3H), 2.28 (m, 4H). ^{13}C NMR (63 MHz, $CDCl_3$) δ 173.6, 171.0, 136.6, 115.5, 62.5, 54.4, 52.5, 35.1, 29.3 ppm.

N-(4-pentenoyl)-O-*tert*-butyldimethylsilyl-L-serine methyl ester (compound 5)

Compound **4** (286 mg; 1.42 mmol) was dissolved in 5 ml of anhydrous DMF. After sequential addition of TBSCl (321 mg; 2.13 mmol) and imidazole (160 mg; 2.34 mmol), the reaction mixture was stirred under argon overnight at room temperature. The solution was diluted with 50 ml of brine and extracted with EtOAc. The combined organic phase was washed with water, dried over Na_2SO_4 and concentrated under diminished pressure. Purification by flash chromatography on a silica gel column (95:5 CH_2Cl_2/MeOH) gave **5** as a colourless oil: 88% yield (395 mg); ^{1}H NMR (250 MHz, $CDCl_3$) δ 6.35 (br d, J = 7.9, 1H), 5.92–5.67 (m, 1H), 5.12–4.90 (m, 2H), 4.70–4.57 (m, 1H), 3.88 (ddd, J = 2.9, 10.1, 60.3, 2H), 3.69 (s, 3H), 2.45–2.24 (m, 4H), 0.81 (s, 9H), –0.02 (s, 3H), –0.03 (s, 3H). ^{13}C NMR (63 MHz, $CDCl_3$)

δ 172.0, 171.0, 136.9, 115.7, 63.6, 54.2, 52.4, 35.6, 29.4, 25.7, 18.2, −5.5, –5.7 ppm.

N-(4-pentenoyl)-O-*tert*-butyldimethylsilyl-L-serine cyanomethyl ester (compound 6)

LiOH (139 mg; 3.31 mmol) was added to a suspension containing 348 mg (1.10 mmol) of **5** in 4 ml of 1:1 THF/water. The reaction mixture was stirred at room temperature for 1 h 30 min, diluted with 40 ml of EtOAc, washed with 20 ml of 1 M $NaHSO_4$, dried over Na_2SO_4 and concentrated under diminished pressure. The crude residue was dissolved in 2.4 ml of anhydrous CH_3CN and chloroacetonitrile (349 µl; 5.5 mmol) and triethylamine (766 µl; 5.5 mmol) were sequentially added. The reaction mixture was stirred at room temperature overnight, diluted with 40 ml of EtOAc, washed with 20 ml of 1 N $NaHSO_4$, dried over Na_2SO_4 and concentrated under diminished pressure. Purification by flash chromatography on a silica gel column (4:6 cycohexane/EtOAc) gave **6** as a colourless oil: 65% yield (243 mg); ^{1}H NMR (250 MHz, $CDCl_3$) δ 6.87 (br d, $J = 8.3$, 1H), 6.47−6.26 (m, 1H), 5.69−5.50 (m, 2H), 5.42−5.20 (m, 3H), 4.47 (ddd, $J = 2.9$, 10.2, 68.1, 2H), 3.00−2.85 (m, 4H), 1.39 (s, 9H), 0.57 (s, 6H). ^{13}C NMR (63 MHz, $CDCl_3$) δ 172.3, 169.3, 136.7, 115.9,113.9, 63.3, 54.0, 49.1, 35.4, 29.3, 25.7, 18.2, −5.6 ppm.

N-(4-pentenoyl)-(S)- seryl-pdCpA (compound 8)

A solution of freshly distilled DMF (200 µl) containing 20 mg of pdCpA tetrabutylammonium salt (14.7 µmol) was added to *N*-(4-pentenoyl)-O-*tert*-butyldimethylsilyl-L-serine cyanomethyl ester **6** (27 mg; 79 µmol). The reaction mixture was stirred at room temperature and monitored by rp-HPLC. *N*-(4-pentenoyl)-O-*tert*-butyldimethylsilyl-(S)-seryl-pdCpA (compound **7**) was purified by preparative rp-HPLC (retention time 27.9 min) and recovered by lyophilization as a white solid: 36% yield (5.0 mg). Final deprotection was performed by treating protected compound **7** (5 mg, 5.15 µmol) with 680 µl of 3:1:1 AcOH/THF/H_2O (36) for 24 h at room temperature under stirring. Compound **8** was purified by rp-HPLC (retention time 15.9 min) and recovered as a colourless solid after lyophilization with a yield of 72% (3.2 mg). HRMS-ESI analysis revealed an *m*/*z* of 804.1756 $[M - H]^-$ that matched the calculated value of 804.1755.

Synthesis of the aminoacyl-tRNA analogues containing an oxadiazole ring

Synthesis of the modified nucleotide containing a 3-(*S*)-1-aminoethyl-1,2,4-oxadiazole ring as a mime of the ester bond at the 3′-end of the RNA helices was performed as previously described (37).

Ligation of modified dinucleotides to RNA helices

Modified dinucleotides [pdCpA-aminoacyl-pentenoyl and pdCpA substituted by a 3-(*S*)-1-aminoethyl-1,2,4-oxadiazole ring] were ligated to RNA helices with purified T4 RNA ligase (37) (Figure 2C). The RNA helices, which did not contain the terminal pCpA, were synthesized by the phosphoramidite method and purified by polyacrylamide gel electrophoresis (Eurogentec). The inhibitors containing the oxadiazole ring were purified by anion exchange chromatography (DEAE column, DNAPac-100, Dionex) with a linear gradient of ammonium acetate pH 8.0 (25–2500 mM) containing 0.5% acetonitrile. Fractions containing the ligation product were identified by denaturing polyacrylamide gel electrophoresis (37), lyophilized, resuspended in RNAse free water (Sigma) and stored at –20°C. The concentration of the inhibitors was determined spectrophotometrically ($\varepsilon = 2.26\ 10^5\ M^{-1}\ cm^{-1}$ at 260 nm). The substrates containing the aminoacyl-pentenoyl groups were purified by ethanol precipitation (34). The pentenoyl group was removed with iodine prior to enzyme assay (35).

Determination of the relative activity of $FemX_{Wv}$ with aminoacylated RNA helices

$FemX_{Wv}$ activity was determined in 50 mM ammonium acetate (pH 6.5) containing 12.5 mM $mgCl_2$, 0.1 mg/ml bovine serum albumin, enzyme (0.75 nM to 10 µM), UDP-MurNAc-[^{14}C]pentapeptide (78 µM) and acylated helices (3 –10 µM). The reaction was allowed to proceed for 30 min at 37°C and an incubation of 10 min at 96°C was used to stop the reaction. UDP-MurNAc-[^{14}C]hexapeptides produced by $FemX_{Wv}$ were determined by rp-HPLC coupled to a radioflow detector using a C18 column (Nucleosil; 25 cm, 3 µM; Machery–Nagel). Elution was performed at a flow rate of 0.5 ml/min with a linear gradient of acetonitrile (0–4%) in 50 mM ammonium acetate pH 5.0, which was applied between 15 and 55 min. Reproducibility was assessed with two to three independent preparations of aminoacyltated RNA helices and standard deviations were calculated by linear regression using a set of data generated with the same batch of substrate.

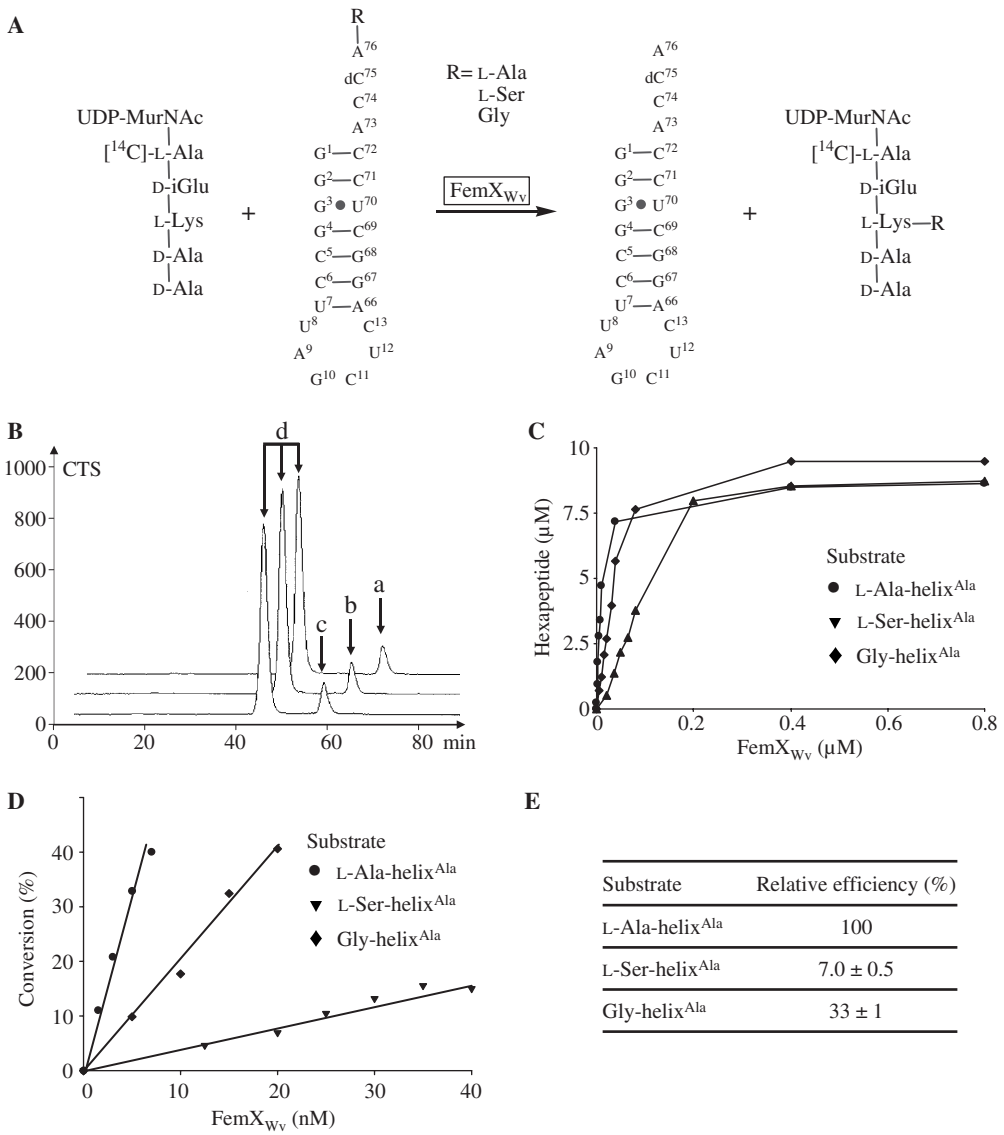
Inhibition of $FemX_{Wv}$ by analogues of Ala-$tRNA^{Ala}$

The assay contained Tris–HCl (50 mM, pH 7.5), alanyl-tRNA synthetase of *E. faecalis* (800 nM), ATP (7.5 mM), $MgCl_2$ (12.5 mM), L-[^{14}C]Ala (50 µM, 3700 Bq/nmol; ICN, Orsay, France), $FemX_{Wv}$ (2 nM), UDP-MurNAc-pentapeptide (5 µM), $tRNA^{Ala}$ (0.4 µM) and inhibitors (0–200 µM). The reaction was performed at 37°C for 10 min with a preincubation of 2 min in the absence of $FemX_{Wv}$ for synthesis of Ala-$tRNA^{Ala}$ by the auxiliary system. The reaction was stopped at 96°C for 10 min and analysed by descending paper chromatography (Whatman 4 mm, Elancourt) with isobutyric acid-ammonia, 1 M (5:3 per vol). Radioactive spots were identified by autoradiography, cut out and counted by liquid scintillation.

RESULTS

Synthesis of aminoacyl-tRNA analogues and design of a $FemX_{Wv}$ assay for the resulting substrates

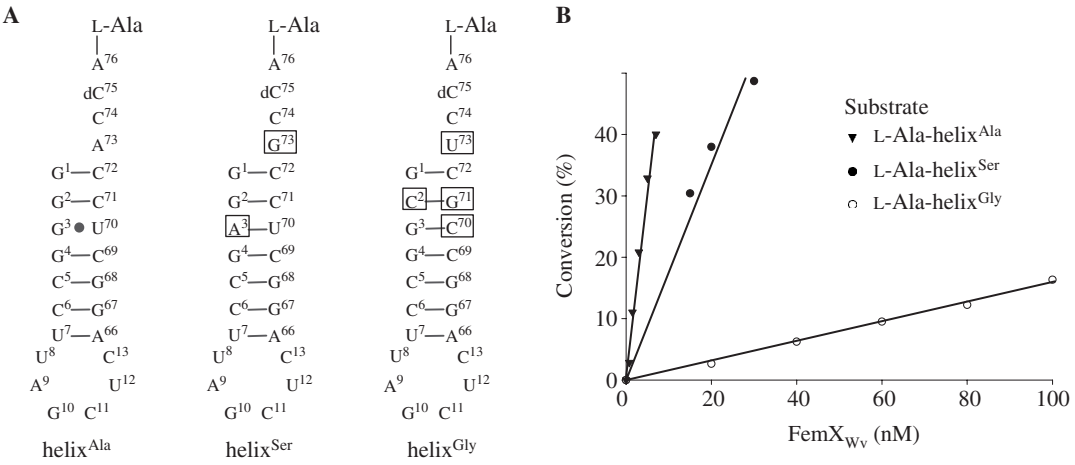
Acylated RNA helices were obtained by semi-synthesis (Figure 2). The main steps involved organic synthesis of



Substrate	Relative efficiency (%)
L-Ala-helix^{Ala}	100
L-Ser-helix^{Ala}	7.0 ± 0.5
Gly-helix^{Ala}	33 ± 1

Figure 3. Specificity of $FemX_{Wv}$ for the aminoacyl residue. (**A**) The relative efficiency of $FemX_{Wv}$ was estimated for the transfer of L-Ala, L-Ser and Gly from acylated RNA helix^{Ala} to UDP-MurNAc-[^{14}C]pentapeptide. (**B**) The radiolabelled substrate and product of the reaction catalysed by $FemX_{Wv}$ were determined by rp-HPLC coupled to a radioflow detector. The superimposed chromatograms provide examples of the separation of the UDP-MurNAc-[^{14}C]hexapeptide product containing L-Ala (a), L-Ser (b) or Gly (c) from the UDP-MurNAc-[^{14}C]pentapeptide substrate (d) common to the three reactions. CTS, counts per s. (**C**) The initial concentration of acylated RNA helix^{Ala} in each reaction was estimated following complete transfer of the residue at high concentrations of $FemX_{Wv}$ (after completion of the aminoacyl transfer reaction, the concentration of UDP-MurNAc-[^{14}C]hexapeptide was equal to the initial concentration of the Ala-helix^{Ala}). (**D**) At lower concentrations of $FemX_{Wv}$, the extent of the transfer was proportional to the concentration of $FemX_{Wv}$. (**E**) The slopes were used to estimate the relative efficiency of the enzyme for the different acylated helices.

dinucleotides acylated by protected amino acids (Figure 2A and B). The acylated dinucleotides were ligated with T4 RNA ligase to 22-nt RNA helices that mimic the acceptor arm of tRNAs (Figure 2C). The extent of the transfer of amino-acid residues from acylated helices to UDP-MurNAc-[^{14}C]pentapeptide (reaction depicted in Figure 3A) was determined by rp-HPLC coupled to a radioflow detector (Figure 3B). Since the ester link connecting the amino-acid residues to the RNA helices was unstable, the deprotection step (Figure 2C) was performed immediately prior to the assay and the initial concentration of the aminoacylated helices was determined in each experiment. For this purpose, high concentrations of $FemX_{Wv}$ and an excess of UDP-MurNAc-pentapeptide were used to obtain full transfer of the residue (Figure 3C). The concentration of UDP-MurNAc-[^{14}C]hexapeptide obtained after full transfer was used to evaluate the initial concentrations of the acylated RNA helices that varied between experiments according to the yield (30–80%) of the ligation and deprotection steps. For each substrate, a range of $FemX_{Wv}$ concentrations was identified in which formation of the product was proportional to the time of reaction (data not shown) and to the enzyme concentration (Figure 3D). The slopes of the



C

Substrate	Relative efficiency (%)
L-Ala-helix^{Ala}	100
L-Ala-helix^{Ser}	31 ± 2
L-Ala-helix^{Gly}	2.9 ± 0.2

Figure 4. Specificity of $FemX_{Wv}$ for the sequence of the acceptor arm of $tRNA^{Ala}$, $tRNA^{Ser}$ and $tRNA^{Gly}$. (**A**) RNA sequence of the helices. Nucleotide substitutions introduced into $helix^{Ala}$ in order to obtain $helix^{Ser}$ and $helix^{Gly}$ are boxed. The sequence of the distal portion of the $helix^{Ser}$ and $helix^{Gly}$ (base pairs 1–72, 2–71, 3–70 and unpaired bases 73–76) were design according to the sequence of the acceptor arm of $tRNA^{Ser}$ and $tRNA^{Gly}$. (**B** and **C**) The relative efficiency of $FemX_{Wv}$ was estimated for the transfer of L-Ala from the RNA helices to UDP-MurNAc-[^{14}C]pentapeptide.

linear portion of the curves were used to determine the relative efficiency of the transfer reaction with the different acylated donor substrates (Figure 3E).

Relative activity of $FemX_{Wv}$ for the transfer of L-Ala, L-Ser and Gly from $helix^{Ala}$ to UDP-MurNAc-pentapeptide

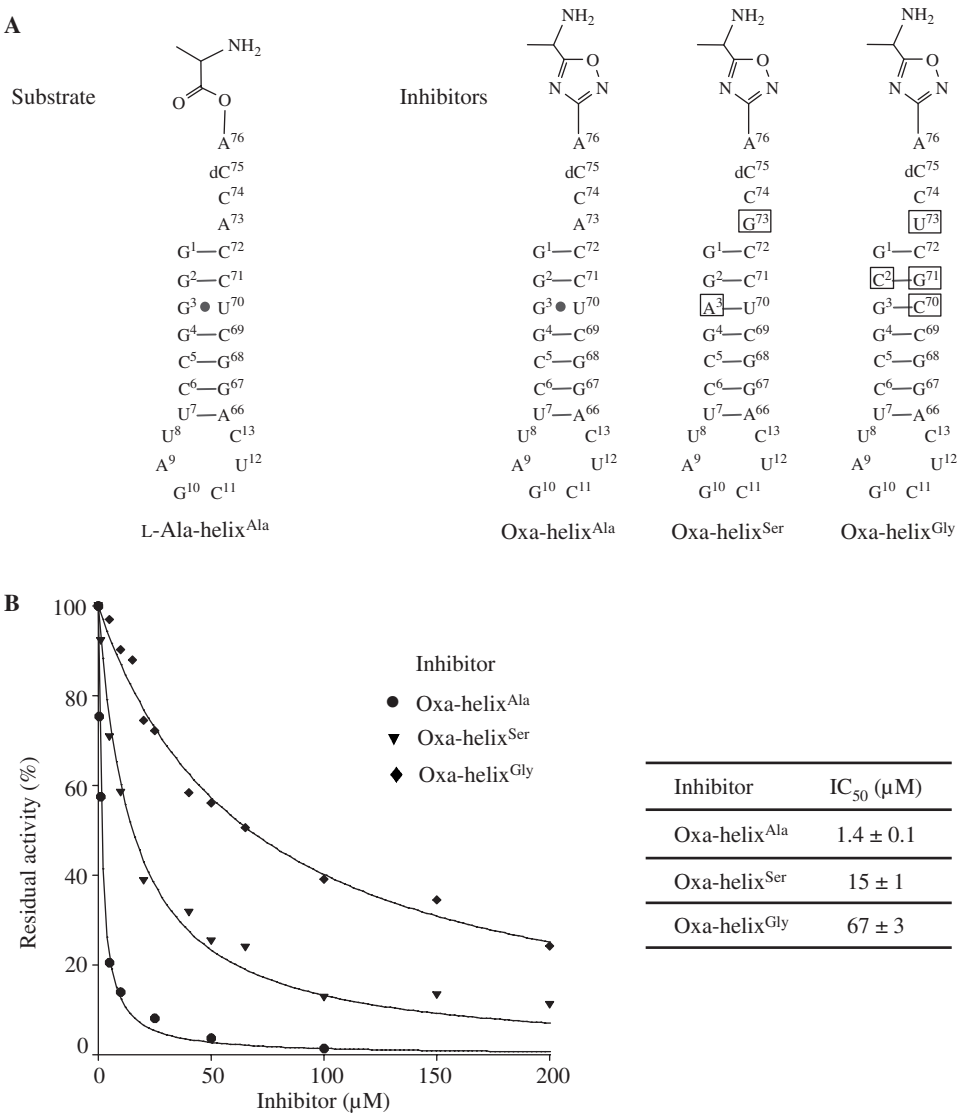
Comparison of the efficiency of transfer of L-Ala and L-Ser from $helix^{Ala}$ to UDP-MurNAc-[^{14}C]pentapeptide indicated that $FemX_{Wv}$ efficiently discriminated between the methyl and hydroxymethyl side chains of the residues (7.0% relative efficiency). A similar relative efficiency (6.0%) was previously reported for the complete aminoacyl-tRNAs (Ala-$tRNA^{Ala}$ versus Ser-$tRNA^{Ser}$) (30). Thus, exclusion of Ser from the active site of $FemX_{Wv}$ was sufficient to account for enzyme specificity since the Ala-$helix^{Ala}$ and Ser-$helix^{Ala}$ only differed by the aminoacyl residue. In contrast, Gly-$helix^{Ala}$ was efficiently used by $FemX_{Wv}$ (33% relative efficiency) whereas Gly-$tRNA^{Gly}$ was a poor substrate (2.6%). Thus, discrimination between Ala-$tRNA^{Ala}$ and Gly-$tRNA^{Gly}$ involved different interactions between the $FemX_{Wv}$ and the RNA moiety of the substrate.

Transfer of L-Ala from helices mimicking $tRNA^{Ala}$, $tRNA^{Ser}$ and $tRNA^{Gly}$

The sequence of $tRNA^{Ala}$, $tRNA^{Ser}$ and $tRNA^{Gly}$ differs at four positions in the distal portion of the acceptor arm (boxed in Figure 4A). Nucleotide substitutions were introduced in these positions of $helix^{Ala}$ to generate helices that mimic the acceptor arm of $tRNA^{Ser}$ and $tRNA^{Gly}$ (designated $helix^{Ser}$ and $helix^{Gly}$, respectively). Introduction of the combination of the $G^3 \rightarrow A$ and $A^{73} \rightarrow G$ substitutions, required to generate $helix^{Ser}$ from $helix^{Ala}$, had a moderate impact on $FemX_{Wv}$ activity (relative efficiency of 31%; Figure 4B and C). Thus, modification of the acceptor arm of $helix^{Ala}$ confirmed that $FemX_{Wv}$ discriminates between Ala-$tRNA^{Ala}$ and Ser-$tRNA^{Ser}$ mainly at the level of the aminoacyl residue. The opposite result was observed for Ala-$helix^{Gly}$. In this case, the relative efficiency of transfer of L-Ala from $helix^{Gly}$ and $helix^{Ala}$ (2.9%) was similar to the relative efficiency observed for the complete aminoacyl-tRNAs (2.6% for comparison of Ala-$tRNA^{Ala}$ and Gly-$tRNA^{Gly}$) (30). Together, these results indicate that the specificity of $FemX_{Wv}$ depends both on the aminoacyl residue (discrimination between L-Ser and L-Ala) and the nucleotide sequence of the RNA moiety of the substrate (discrimination between $tRNA^{Ala}$ and $tRNA^{Gly}$).

Inhibition of $FemX_{Wv}$ by stable analogues of aminoacyl-tRNAs

To gain insight into the interaction of $FemX_{Wv}$ with $helix^{Ala}$, $helix^{Ser}$ and $helix^{Gly}$, stable analogues of the aminoacylated helices were synthesized by introducing an oxadiaxole-containing substituant at the 3′-end of the helices (Figure 5A). The oxadiazole ring was previously shown to act as an isoster of the ester link connecting



Inhibitor	IC_{50} (μM)
Oxa-helix^{Ala}	1.4 ± 0.1
Oxa-helix^{Ser}	15 ± 1
Oxa-helix^{Gly}	67 ± 3

Figure 5. Inhibition of $FemX_{Wv}$ by substrate analogues. (**A**) Structure of the Ala-helix^{Ala} and analogues containing an oxadiazole moiety linked to helix^{Ala}, helix^{Ser} and helix^{Gly}. (**B**) IC_{50} values were determined in the AlaRS-$FemX_{Wv}$ coupled assay.

the amino-acid residue to the tRNA (37). The oxadiazole ring was substituted by a 2-amino-ethyl group mimicking L-Ala (oxa). The IC_{50}s for the oxa-helix^{Ser} and oxa-helix^{Gly} were 11- and 48-fold higher than the IC_{50} of the oxa-helix^{Ala}, respectively (Figure 5B). These results show that differences in the sequence of the acceptor arm of $tRNA^{Ala}$, $tRNA^{Ser}$ and $tRNA^{Gly}$ affect binding of $FemX_{Wv}$ to the RNA substrate. The largest effect was observed between helix^{Ala} and helix^{Gly} underscoring again the key role of differences in the mode of recognition of the acceptor arm of the $tRNA^{Ala}$ and $tRNA^{Gly}$ for the specificity of $FemX_{Wv}$.

$FemX_{Wv}$ identity determinants in the acceptor arm of $tRNA^{Ala}$

Substitutions were introduced in the distal portion of the acceptor arm of Ala-helix^{Ala} to evaluate the role of specific bases on $FemX_{Wv}$ activity (Figure 6). The discriminator base (position 73) was not a key element for $FemX_{Wv}$ specificity since a residual activity of 62% was observed for the modified Ala-helix^{Ala} containing the $A^{73}\rightarrow G$ substitution. The $C^{72}\rightarrow G$ substitution in the first base pair of the acceptor stem led to a 100-fold decrease in the catalytic activity of $FemX_{Wv}$. The impact of the substitution involved at least in part the destabilization of the RNA helix since introduction of a second substitution $G^1\rightarrow C$, which restored a Watson–Crick base pair, increased the residual activity 6.5-fold. In contrast, a similar analysis of the 2-71 base pair led to the opposite conclusion since the replacement of G^2-C^{71} by $G^2\bullet G^{71}$ and C^2-G^{71} led to residual activity of 38 and 1.8%, respectively. This result indicates that the C^2-G^{71} base pair of $tRNA^{Gly}$ acts as a major anti-determinant to prevent incorporation of Gly into peptidoglycan precursors by $FemX_{Wv}$. Conversely, the moderate impact of the $G^3\rightarrow A$ (45% residual activity) and $A^{73}\rightarrow G$ (62%) substitutions are in agreement with the comparison of misacylated tRNAs (Figure 4) that

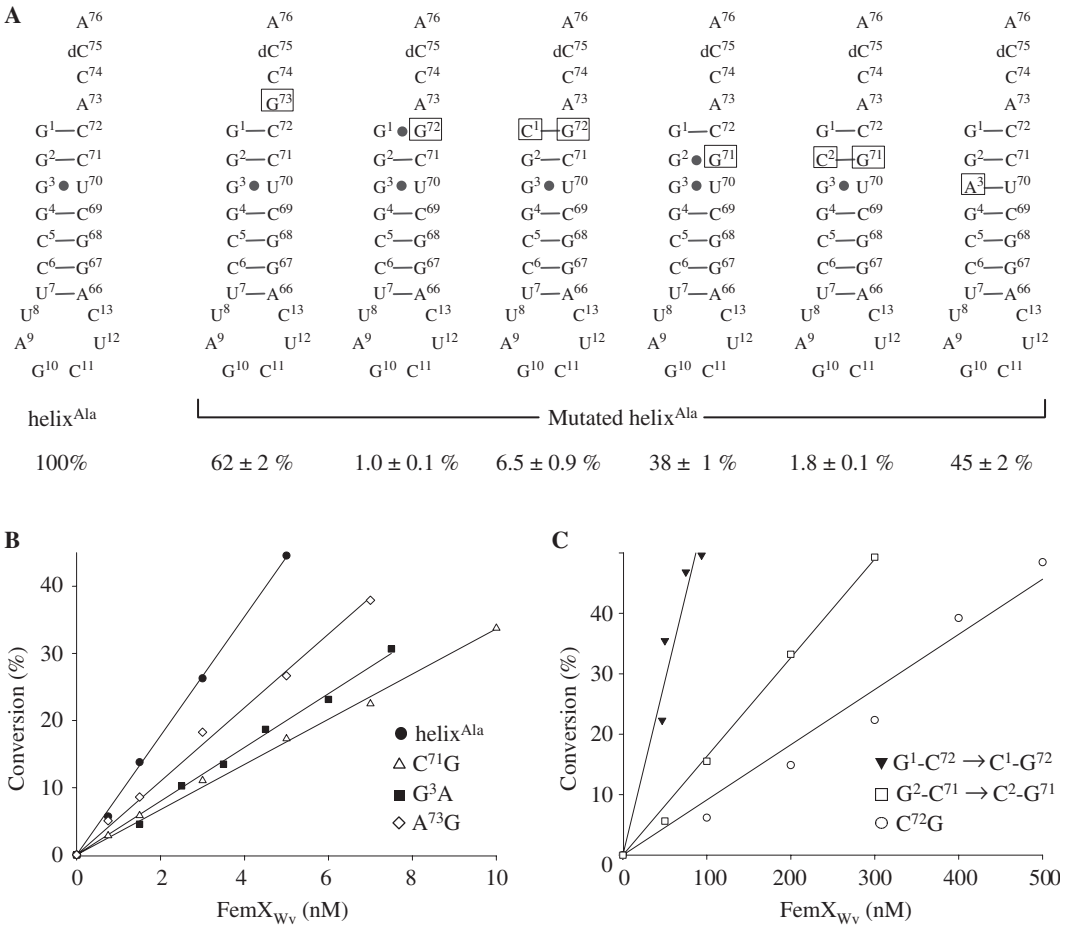


Figure 6. Specificity of $FemX_{Wv}$ for the discriminator base and the first three base pairs of the acceptor arm. (**A**) Nucleotide substitutions were introduced into the distal portion of the RNA helix^{Ala} (boxed). (**B** and **C**) The relative efficiency of $FemX_{Wv}$ was estimated for the transfer of L-Ala from the acylated helices to UDP-MurNAc-[^{14}C]pentapeptide. Data are presented in two graphs according to the range of $FemX_{Wv}$ concentrations required to estimate enzyme activity.

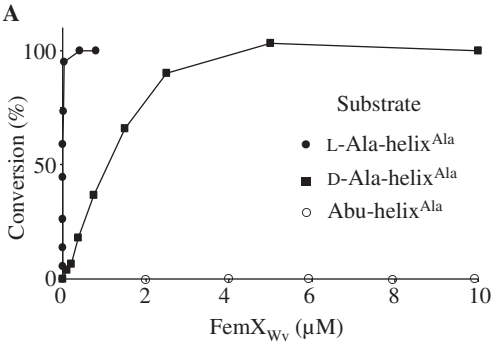
led to the conclusion that Ser-tRNA^{Ser} is mainly excluded from the $FemX_{Wv}$ active site due to unfavourable interaction with the aminoacyl residue.

Stereospecificity of $FemX_{Wv}$

The helix^{Ala} substituted by D-Ala instead of L-Ala was used as a substrate by $FemX_{Wv}$ albeit with a 110-fold reduced catalytic efficiency (Figure 7). Thus, the configuration of the Cα of the L-alanyl residue was preferred but not essential for $FemX_{Wv}$ activity. 2-Amino-isobutyrate, which combines two methyl groups at this position, was not used as a substrate.

DISCUSSION

The main function of aminoacyl-tRNAs is the interpretation of the genetic code by providing the interface between nucleic acid triplets in mRNA and the corresponding amino acids in proteins. Aminoacyl-tRNAs also participate in the synthesis of proteinogenic amino acids such as glutamine and asparagine by amidation of Glu-tRNA^{Gln} and Asp-tRNA^{Asn} or selenocysteine by conversion of Ser-tRNA^{Sec} into Sec-tRNA^{Sec} (38,39). The misacylated tRNA intermediates of the tRNA-dependent amino-acid biosynthesis pathways are not recognized by EF-Tu or by the corresponding specific elongation factors (38,39). The fidelity of translation is preserved by this mechanism and by the assembly of the participating biosynthetic enzymes in stable ribonucleoprotein complexes that channel the misacylated tRNAs through the different catalytic steps and prevent their interaction with the translation machinery (40). Independently from their role as translation substrates, certain aminoacyl-tRNAs act as aminoacyl donors in several biosynthesis pathways (41) including the assembly of the side chain of peptidoglycan precursors (42) (Figure 1). In addition, Glu-tRNA^{Glu} participates in the synthesis of porphyrins (43), Lys-tRNA^{Lys} and Ala-tRNA^{Ala} are used for aminoacylation of phosphatidylglycerol (44) and Phe-tRNA^{Phe} and Leu-tRNA^{Leu} act as aminoacyl donors for labelling the *N*-terminus of proteins targeted for degradation by the proteasome-like protease ClpAP (45). The aminoacyl-tRNA substrates of all these reactions are correctly acylated and have therefore the dual capacity to participate in two pathways. Partial uncoupling mediated by



Substrate	Relative efficiency (%)
L-Ala-helix^{Ala}	100
D-Ala-helix^{Ala}	0.9 ± 0.1
Abu-helix^{Ala}	< 0.01

Figure 7. Stereospecificity of FemX$_{Wv}$. The relative efficiency of FemX$_{Wv}$ was estimated for the transfer of L-Ala, D-Ala and 2-amino-isobutyric acid (Abu) from helix^{Ala} to UDP-MurNAc-[^{14}C]pentapeptide. A modified acetonitrile gradient (0–20%) followed by a step elution at 100% was used to confirm the absence of the transfer of Abu since the corresponding UDP-MurNAc-hexapeptide is expected to be hydrophobic and might have been retained in the column under the classical conditions.

dedicated tRNAs have been reported in rare instances. In *Acidithiobacillus ferrooxidans* for example, one of the three Glu-tRNA^{Glu} that act as translation substrates does not participate in porphyrin synthesis probably ensuring an adequate supply of Glu-tRNA^{Glu} upon high heme demand (46). In *Staphylococcus epidermidis*, an adequate supply of Gly-tRNA^{Gly} to the synthesis of the side chain of peptidoglycan precursors could be provided by two closely related tRNA^{Gly} that do not participate in protein synthesis (47). The remaining tRNA^{Gly} are functional in both pathways as are a wide variety of tRNAs that have been investigated in other organisms (19,20,25,26,30). This implies that the tRNA-dependent transferases have developed their own interpretation of the genetic code to achieve specificity, a question typically investigated with substrates obtained by enzymatic acylation of tRNAs (19,30,48). Since the aminoacyl-tRNA synthetases are highly specific, the latter experimental approach is severely limited by the substrates that can be analysed. Here we have developed chemical acylation of RNA helices that allowed for the first time combining modifications in the aminoacyl residue with any base substitution in the RNA moiety of the substrate (Figure 2). Since the complex organic syntheses were not feasible with radiolabelled material, determination of FemX$_{Wv}$ activity with these substrates required to develop a novel assay in which the radioisotope is introduced in the peptidoglycan precursor rather than in the aminoacyl residue (Figure 3).

In a first set of experiments, the contribution of the aminoacyl residue to the FemX$_{Wv}$ specificity was investigated by comparing substrates obtained by misacylation of an RNA helix that mimics the acceptor arm of tRNA^{Ala} (helix^{Ala}). L-Ser and L-Ala were found to be transferred from acyl donors containing an identical helix with a relative efficiency of 7.0% (Figure 3). Since a relative efficiency of 6.0% was previously observed for comparison of the entire substrate (Ala-tRNA^{Ala} versus Ser-tRNA^{Ser}) (30), this observation indicates that exclusion of L-Ser from the FemX$_{Wv}$ active site is in itself sufficient to quantitatively account for discrimination between these two aminoacyl-tRNAs (Figure 8). In agreement, transfer of L-Ala from helices mimicking the acceptor arm of tRNA^{Ala} and tRNA^{Ser} occurred at relative efficiencies of 31% reflecting the moderate impact of the A^{73}G (62%) and G^{3}A (45%) substitutions that were also tested individually (Figure 6 and 8). Cys is likely to be similarly excluded from the FemX$_{Wv}$ active site although this was not directly tested.

Several lines of evidence indicate that discrimination against L-Ser, and presumably L-Cys, is due to steric hindrance. Helix^{Ala} charged with L-Ala and Gly were used with relative efficiencies of 33% indicating that recognition of the methyl group of L-Ala has a marginal role in the catalytic efficiency of FemX$_{Wv}$ (Figure 3). In addition, 2-aminobutyrate was not transferred by FemX$_{Wv}$ (<0.01%) although D-Ala-helix^{Ala} was a substrate (0.9%) (Figure 7). *N*-acetylation of L-Ala and extension of the side chain from a methyl (L-Ala) to a CH_2-CH_2-S-CH_3 group (L-Met) both abolished FemX$_{Wv}$ activity (M. Lecerf, unpublished results). Together these results indicate that the specificity of FemX$_{Wv}$ involves steric hindrance rather than the recognition of the methyl group of L-Ala. This mode of substrate recognition implies that FemX$_{Wv}$ can discriminate between L-Ala and all other proteinogenic amino acids except Gly.

Since recognition of the L-Ala and Gly residues cannot account for the specificity of FemX$_{Wv}$, we searched for anti-determinants in the tRNA^{Gly} sequence (Figure 6). Similar residual activities were observed for the transplantation of the C^{2}-G^{71} base pair of tRNA^{Gly} into the Ala-Helix^{Ala} (1.8%) and the comparison of Ala-tRNA^{Ala} and Gly-tRNA^{Gly} (2.6%) (Figure 8). Thus, the C^{2}-G^{71} base pair of tRNA^{Gly} acts as a major anti-determinant that quantitatively accounts for the specificity of FemX$_{Wv}$ independently from the discrimination between the Ala and Gly residues. The role of the recognition of the RNA moiety of the substrate is also supported by the inhibition of FemX$_{Wv}$ by stable Ala-tRNA^{Ala} analogues containing a mimetic of the acceptor stem of tRNA^{Gly} since substitution of helix^{Ala} by helix^{Gly} in the RNA moiety of the inhibitors led to a 48-fold increase in the IC$_{50}$s (Figure 5). Thus, the specificity of FemX$_{Wv}$ for the aminoacyl-tRNA donor substrate is based on excluding aminoacyl residues larger than L-Ala from its active site

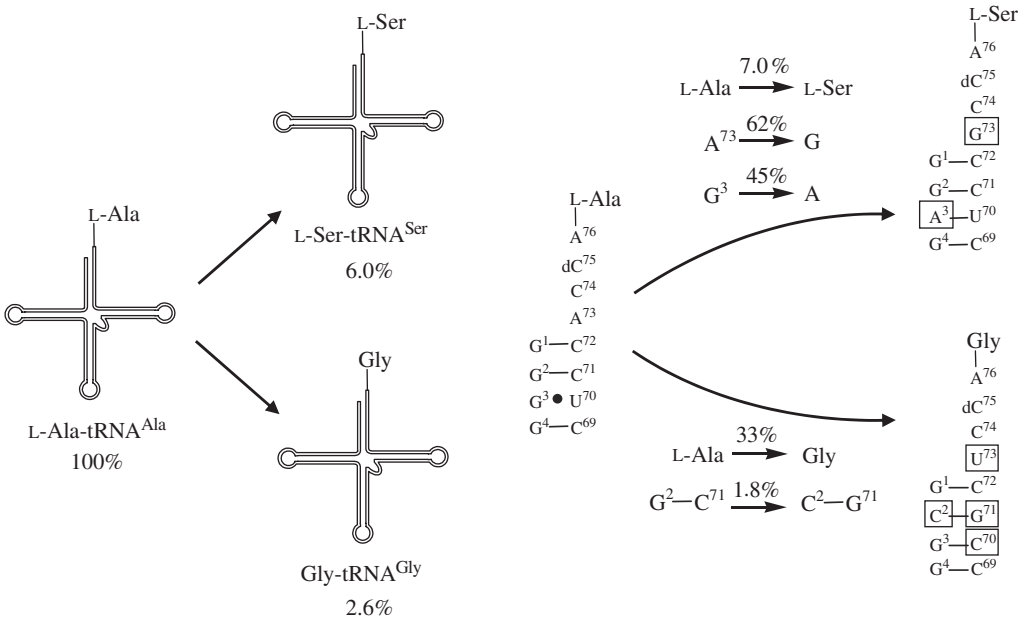


Figure 8. Relative contribution of the aminoacyl residue and of the nucleotide sequence to the specificity of $FemX_{Wv}$. Previous comparisons of full tRNAs obtained by *in vitro* transcription indicated that Ser-$tRNA^{Ser}$ and Gly-$tRNA^{Gly}$ are used 17- and 38-fold less efficiently than Ala-$tRNA^{Ala}$ by $FemX_{Wv}$ (6.0% and 2.6%, respectively) (30). In this study, the main identity determinants of $FemX_{Wv}$ were identified based on independently testing the impact of substitutions of the aminoacyl residue and of nucleotides on $FemX_{Wv}$ activity. Substitutions were chosen according to the differences found in the distal portion of the acceptor arm of $tRNA^{Ala}$, $tRNA^{Ser}$ and $tRNA^{Gly}$. The discrimination between L-Ala and L-Ser is the main factor that prevents incorporation of L-Ser by $FemX_{Wv}$ whereas the C^2-G^{71} base pair in the acceptor arm of $tRNA^{Gly}$ is the major anti-determinant that prevents incorporation of Gly.

and exploiting the first divergent base pair in the acceptor stems of $tRNA^{Ala}$ and $tRNA^{Gly}$ as an antideterminant to prevent mis-incorporation of Gly (G^2-C^{71} in $tRNA^{Ala}$ versus C^2-G^{71} in $tRNA^{Gly}$). In agreement, modelling of the donor substrate in the catalytic cavity of $FemX_{Wv}$ indicated that the enzyme can interact with the first two base pairs (1-72 and 2-71) of the acceptor stem of Ala-$tRNA^{Ala}$, the single stranded ACCA extremity and the aminoacyl residue (30). Efficient binding of elongation factor EF-Tu to aminoacyl-tRNAs also requires the correct combination of amino acid and tRNA body that have independent and compensatory thermodynamic contributions to the overall affinity thereby ensuring uniform binding to the 20 aminoacyl-tRNA types (49).

Aminoacyl-tRNA recognition by $FemX_{Wv}$ and the leucyl/phenyl-tRNA protein transferase (L/F-transferase) involved in the control of protein degradation are very different although the two enzymes share a similar fold in the absence of primary amino-acid sequence conservation (50,51). The catalytic cavity of the L/F-transferase contains a hydrophobic pocket that accommodates the side chain of Leu and Phe and efficiently excludes the β-branched side chains of Ile and Val (48,50). The size of the Ala and Pro side chains is not large enough to fit within the hydrophobic pocket whereas low activity was detected with Met-$tRNA^{Met}$ (50). Steric hindrance accounts for the low activity observed with Trp-$tRNA^{Trp}$ (50). In contrast to $FemX_{Wv}$, biochemical studies led to the conclusion that the L/F-transferase recognizes the aminoacyl-tRNA substrates in a sequence-independent manner (48,51). A double-stranded acceptor stem is fully dispensable for the L/F-transferase and replacement of G-C by weaker base pairs in the acceptor stem reduces the K_m of the aminoacyl-tRNA substrate (48). The opposite result was obtained for $FemX_{Wv}$ (Figure 6) since replacement of G^1-C^{72} by unpaired $G^1 \bullet G^{72}$ had a greater impact on $FemX_{Wv}$ activity than a double substitution that restored a Watson–Crick base pairing (C^1-G^{72}) at this position (1.0% versus 6.5% residual activity, respectively).

Comparison of the L/F-transferase and $FemX_{Wv}$ indicates that the mode of recognition of the aminoacyl-tRNA substrate is not conserved among enzymes that use this type of activated substrate in non-translational processes. Aminoacyl-tRNA recognition by component of the translation machinery is also different, as exemplified by the analysis of the $G^3 \bullet U^{70}$ base pair, which is essential for the activity of the alanyl-tRNA synthetase, but not of $FemX_{Wv}$ (45% relative efficiency; Figure 6). These observations suggest that Fem transferases are parasitic on the translation machinery that provides a ubiquitous and permanent supply of amino acids activated in the form of aminoacyl-tRNAs. Fem transferases may have therefore evolved from a translation-independent pathway involving aminoacyl activation by adenylation, although the enzymes may have lost the capacity to use a mono-nucleotide as the substrate. The L/F-transferase binds phenylalanyl-adeosine (rA-Phe) (45) and its analogue puromycine (51). Transfer of Phe from rA-Phe to the *N*-terminal Arg residue of a hexapeptide fragment of α casein has been observed in co-crystals (51) and in solution at a rate sufficient for detection of the product by mass

spectrometry (51), although a larger portion of the aminoacyl-tRNA substrate is required for efficient aminoacylation (48). Recognition of the acceptor stem can be selectively advantageous, not only to provide specificity determinants (as shown for the 2–71 base pair in this work), but also to ensure sufficient affinity for the aminoacyl-tRNA substrates in order to compete with EF-Tu. The predominant recognition of the aminoacyl moiety of the substrates by the L/F-transferase and $FemX_{Wv}$ as well as the different modes of recognition of the acceptor stems suggest multiple origins for these aminoacyl transferases that are unrelated to any tRNA-interacting protein of the translation machinery. $FemX_{Wv}$ and the L/F-transferase should therefore be considered as enzymes that have developed their own interpretation of the genetic code but did not participate in its elaboration.

ACKNOWLEDGEMENTS

We thank Antoine P. Maillard for helpful discussion.

FUNDING

This work was supported by the European Community (EUR-INTAFAR, Project N° LSHM-CT-2004-512138, 6th PCRD) and by the Fondation Recherche Médicale (fin de thèse to Régis Villet). Funding for open access charge: INSERM.

Conflict of interest statement. None declared.

REFERENCES

1. den Blaauwen,T., de Pedro,M.A., Nguyen-Distèche,M. and Ayala,J.A. (2008) Morphogenesis of rod-shaped sacculi. *FEMS Microbiol. Rev.*, **32**, 321–344.
2. Dramsi,S., Magnet,S., Davison,S. and Arthur,M. (2008) Covalent attachment of proteins to peptidoglycan. *FEMS Microbiol. Rev.*, **32**, 307–320.
3. Vollmer,W. (2008) Structural variation in the glycan strands of bacterial peptidoglycan. *FEMS Microbiol. Rev.*, **32**, 287–306.
4. Kern,T., Hediger,S., Muller,P., Giustini,C., Joris,B., Bougault,C., Vollmer,W. and Simorre,J.P. (2008) Toward the characterization of peptidoglycan structure and protein-peptidoglycan interactions by solid-state NMR spectroscopy. *J. Am. Chem. Soc.*, **130**, 5618–5619.
5. Vollmer,W., Blanot,D. and de Pedro,M.A. (2008) Peptidoglycan structure and architecture. *FEMS Microbiol. Rev.*, **32**, 149–167.
6. Barreteau,H., Kovac,A., Boniface,A., Sova,M., Gobec,S. and Blanot,D. (2008) Cytoplasmic steps of peptidoglycan biosynthesis. *FEMS Microbiol. Rev.*, **32**, 168–207.
7. Schleifer,K.H. and Kandler,O. (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.*, **36**, 407–477.
8. Biarrotte-Sorin,S., Maillard,A.P., Delettre,J., Sougakoff,W., Arthur,M. and Mayer,C. (2004) Crystal structures of *Weissella viridescens* FemX and its complex with UDP-MurNAc-pentapeptide: insights into FemABX family substrates recognition. *Structure*, **12**, 257–267.
9. Benson,T.E., Prince,D.B., Mutchler,V.T., Curry,K.A., Ho,A.M., Sarver,R.W., Hagadorn,J.C., Choi,G.H. and Garlick,R.L. (2002) X-ray crystal structure of *Staphylococcus aureus* FemA. *Structure*, **10**, 1107–1115.
10. Bellais,S., Arthur,M., Dubost,L., Hugonnet,J.E., Gutmann,L., van Heijenoort,J., Legrand,R., Brouard,J.P., Rice,L. and Mainardi,J.L. (2006) Aslfm, the D-aspartate ligase responsible for the addition of D-aspartic acid onto the peptidoglycan precursor of *Enterococcus faecium*. *J. Biol. Chem.*, **281**, 11586–11594.
11. Sauvage,E., Kerff,F., Terrak,M., Ayala,J.A. and Charlier,P. (2008) The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiol. Rev.*, **32**, 234–258.
12. Mainardi,J.L., Fourgeaud,M., Hugonnet,J.E., Dubost,L., Brouard,J.P., Ouazzani,J., Rice,L.B., Gutmann,L. and Arthur,M. (2005) A novel peptidoglycan cross-linking enzyme for a beta-lactam-resistant transpeptidation pathway. *J. Biol. Chem.*, **280**, 38146–38152.
13. Lavollay,M., Arthur,M., Fourgeaud,M., Dubost,L., Marie,A., Veziris,N., Blanot,D., Gutmann,L. and Mainardi,J.L. (2008) The peptidoglycan of stationary-phase *Mycobacterium tuberculosis* predominantly contains cross-links generated by L,D-transpeptidation. *J. Bacteriol.*, **190**, 4360–4366.
14. Kopp,U., Roos,M., Wecke,J. and Labischinski,H. (1996) Staphylococcal peptidoglycan interpeptide bridge biosynthesis: a novel antistaphylococcal target? *Microb. Drug. Resist.*, **2**, 29–41.
15. Arbeloa,A., Hugonnet,J.E., Sentilhes,A.C., Josseaume,N., Dubost,L., Monsempes,C., Blanot,D., Brouard,J.P. and Arthur,M. (2004) Synthesis of mosaic peptidoglycan cross-bridges by hybrid peptidoglycan assembly pathways in gram-positive bacteria. *J. Biol. Chem.*, **279**, 41546–41556.
16. Magnet,S., Arbeloa,A., Mainardi,J.L., Hugonnet,J.E., Fourgeaud,M., Dubost,L., Marie,A., Delfosse,V., Mayer,C., Rice,L.B. *et al.* (2007) Specificity of L,D-transpeptidases from gram-positive bacteria producing different peptidoglycan chemotypes. *J. Biol. Chem.*, **282**, 13151–13159.
17. Vollmer,W., Joris,B., Charlier,P. and Foster,S. (2008) Bacterial peptidoglycan (murein) hydrolases. *FEMS Microbiol. Rev.*, **32**, 259–286.
18. Ehlert,K., Tschierske,M., Mori,C., Schroder,W. and Berger-Bachi,B. (2000) Site-specific serine incorporation by Lif and Epr into positions 3 and 5 of the Staphylococcal peptidoglycan interpeptide bridge. *J. Bacteriol.*, **182**, 2635–2638.
19. Hegde,S.S. and Blanchard,J.S. (2003) Kinetic and mechanistic characterization of recombinant *Lactobacillus viridescens* FemX (UDP-N-acetylmuramoyl pentapeptide-lysine N6-alanyltransferase). *J. Biol. Chem.*, **278**, 22861–22867.
20. Hegde,S.S. and Shrader,T.E. (2001) FemABX family members are novel nonribosomal peptidyltransferases and important pathogen-specific drug targets. *J. Biol. Chem.*, **276**, 6998–7003.
21. Rohrer,S., Ehlert,K., Tschierske,M., Labischinski,H. and Berger-Bachi,B. (1999) The essential *Staphylococcus aureus* gene *fmhB* is involved in the first step of peptidoglycan pentaglycine interpeptide formation. *Proc. Natl Acad. Sci. USA*, **96**, 9351–9356.
22. Filipe,S.R., Severina,E. and Tomasz,A. (2002) The *murMN* operon: a functional link between antibiotic resistance and antibiotic tolerance in *Streptococcus pneumoniae*. *Proc. Natl Acad. Sci. USA*, **99**, 1550–1555.
23. Filipe,S.R., Severina,E. and Tomasz,A. (2001) The role of *murMN* operon in penicillin resistance and antibiotic tolerance of *Streptococcus pneumoniae*. *Microb. Drug Resist.*, **7**, 303–316.
24. Berger-Bachi,B., Barberis-Maino,L., Strassle,A. and Kayser,F.H. (1989) FemA, a host-mediated factor essential for methicillin resistance in *Staphylococcus aureus*: molecular cloning and characterization. *Mol. Gen. Genet.*, **219**, 263–269.
25. Schneider,T., Senn,M.M., Berger-Bachi,B., Tossi,A., Sahl,H.G. and Wiedemann,I. (2004) *In vitro* assembly of a complete, pentaglycine interpeptide bridge containing cell wall precursor (lipid II-Gly5) of *Staphylococcus aureus*. *Mol. Microbiol.*, **53**, 675–685.
26. Lloyd,A.J., Gilbey,A.M., Blewett,A.M., De Pascale,G., El Zoeiby,A., Levesque,R.C., Catherwood,A.C., Tomasz,A., Bugg,T.D., Roper,D.I. *et al.* (2008) Characterization of tRNA-dependent peptide bond formation by MurM in the synthesis of *Streptococcus pneumoniae* peptidoglycan. *J. Biol. Chem.*, **283**, 6402–6417.
27. Bouhss,A., Josseaume,N., Allanic,D., Crouvoisier,M., Gutmann,L., Mainardi,J.L., Mengin-Lecreulx,D., van Heijenoort,J. and Arthur,M. (2001) Identification of the UDP-MurNAc-pentapeptide:L-alanine ligase for synthesis of branched

peptidoglycan precursors in *Enterococcus faecalis*. *J. Bacteriol.*, **183**, 5122–5127.
28. Bouhss,A., Josseaume,N., Severin,A., Tabei,K., Hugonnet,J.E., Shlaes,D., Mengin-Lecreulx,D., Van Heijenoort,J. and Arthur,M. (2002) Synthesis of the L-alanyl-L-alanine cross-bridge of *Enterococcus faecalis* peptidoglycan. *J. Biol. Chem.*, **277**, 45935–45941.
29. Maillard,A.P., Biarrotte-Sorin,S., Villet,R., Mesnage,S., Bouhss,A., Sougakoff,W., Mayer,C. and Arthur,M. (2005) Structure-based site-directed mutagenesis of the UDP-MurNAc-pentapeptide-binding cavity of the FemX alanyl transferase from *Weissella viridescens*. *J. Bacteriol.*, **187**, 3833–3838.
30. Villet,R., Fonvielle,M., Busca,P., Chemama,M., Maillard,A.P., Hugonnet,J.E., Dubost,L., Marie,A., Josseaume,N., Mesnage,S. *et al.* (2007) Idiosyncratic features in tRNAs participating in bacterial cell wall synthesis. *Nucleic Acids Res.*, **35**, 6870–6883.
31. Babic,A., Patin,D., Boniface,A., Hervé,M., Mengin-Lecreulx,D., Pecar,S., Gobec,S. and Blanot,D. (2007) Chemoenzymatic synthesis of the nucleotide substrates of the Mur ligases. In *Proceedings of the 5th Joint Meeting on Medicinal Chemistry, June 17-21, Portoroz, Slovenia, Medimond Srl, Bologna, Italy*, Medimond Srl., Bologna, Italy, pp. 1–4.
32. Bouhss,A., Crouvoisier,M., Blanot,D. and Mengin-Lecreulx,D. (2004) Purification and characterization of the bacterial MraY translocase catalyzing the first membrane step of peptidoglycan biosynthesis. *J. Biol. Chem.*, **279**, 29974–29980.
33. Robertson,S.A., Noren,C.J., Anthony-Cahill,S.J., Griffith,M.C. and Schultz,P.G. (1989) The use of 5'-phospho-2 deoxyribocytidylylriboadenosine as a facile route to chemical aminoacylation of tRNA. *Nucleic Acids Res.*, **17**, 9649–9660.
34. Lodder,M., Golovine,S., Laikhter,A.L., Karginov,V.A. and Hecht,S.M. (1998) Misacylated Transfer RNAs Having a Chemically Removable Protecting Group. *J. Org. Chem.*, **63**, 794–803.
35. Lodder,M., Wang,B. and Hecht,S.M. (2005) The N-pentenoyl protecting group for aminoacyl-tRNAs. *Methods*, **36**, 245–251.
36. Chung,H.H., Benson,D.R., Cornish,V.W. and Schultz,P.G. (1993) Probing the role of loop 2 in Ras function with unnatural amino acids. *Proc. Natl Acad Sci. USA*, **90**, 10145–10149.
37. Chemama,M., Fonvielle,M., Villet,R., Arthur,M., Valery,J.M. and Etheve-Quelquejeu,M. (2007) Stable analogues of aminoacyl-tRNA for inhibition of an essential step of bacterial cell-wall synthesis. *J. Am. Chem. Soc.*, **129**, 12642–12643.
38. Ibba,M. and Soll,D. (2004) Aminoacyl-tRNAs: setting the limits of the genetic code. *Genes Dev.*, **18**, 731–738.
39. Sheppard,K., Yuan,J., Hohn,M.J., Jester,B., Devine,K.M. and Soll,D. (2008) From one amino acid to another: tRNA-dependent amino acid biosynthesis. *Nucleic Acids Res.*, **36**, 1813–1825.
40. Bailly,M., Blaise,M., Roy,H., Deniziak,M., Lorber,B., Birck,C., Becker,H.D. and Kern,D. (2008) tRNA-dependent asparagine formation in prokaryotes: characterization, isolation and structural and functional analysis of a ribonucleoprotein particle generating Asn-tRNA(Asn). *Methods*, **44**, 146–163.
41. RajBhandary,U.L. and Soll,D. (2008) Aminoacyl-tRNAs, the bacterial cell envelope, and antibiotics. *Proc. Natl Acad. Sci. USA*, **105**, 5285–5286.
42. Mainardi,J.L., Villet,R., Bugg,T.D., Mayer,C. and Arthur,M. (2008) Evolution of peptidoglycan biosynthesis under the selective pressure of antibiotics in Gram-positive bacteria. *FEMS Microbiol. Rev.*, **32**, 386–408.
43. Randau,L., Schauer,S., Ambrogelly,A., Salazar,J.C., Moser,J., Sekine,S., Yokoyama,S., Soll,D. and Jahn,D. (2004) tRNA recognition by glutamyl-tRNA reductase. *J. Biol. Chem.*, **279**, 34931–34937.
44. Roy,H. and Ibba,M. (2008) RNA-dependent lipid remodeling by bacterial multiple peptide resistance factors. *Proc. Natl Acad. Sci. USA*, **105**, 4667–4672.
45. Watanabe,K., Toh,Y., Suto,K., Shimizu,Y., Oka,N., Wada,T. and Tomita,K. (2007) Protein-based peptide-bond formation by aminoacyl-tRNA protein transferase. *Nature*, **449**, 867–871.
46. Levican,G., Katz,A., Valenzuela,P., Soll,D. and Orellana,O. (2005) A tRNA(Glu) that uncouples protein and tetrapyrrole biosynthesis. *FEBS Lett.*, **579**, 6383–6387.
47. Roberts,R.J. (1974) Staphylococcal transfer ribonucleic acids. II. Sequence analysis of isoaccepting glycine transfer ribonucleic acids IA and IB from *Staphylococcus epidermidis* Texas 26. *J. Biol. Chem.*, **249**, 4787–4796.
48. Abramochkin,G. and Shrader,T.E. (1996) Aminoacyl-tRNA recognition by the leucyl/phenylalanyl-tRNA-protein transferase. *J. Biol. Chem.*, **271**, 22901–22907.
49. LaRiviere,FJ., Wolfson,AD. and Uhlenbeck,OC. (2001) Uniform binding of aminoacyl-tRNAs to elongation factor Tu by thermodynamic compensation. *Science*, **294**, 165–168.
50. Dong,X., Kato-Murayama,M., Muramatsu,T., Mori,H., Shirouzu,M., Bessho,Y. and Yokoyama,S. (2007) The crystal structure of leucyl/phenylalanyl-tRNA-protein transferase from *Escherichia coli*. *Protein Sci.*, **16**, 528–534.
51. Suto,K., Shimizu,Y., Watanabe,K., Ueda,T., Fukai,S., Nureki,O. and Tomita,K. (2006) Crystal structures of leucyl/phenylalanyl-tRNA-protein transferase and its complex with an aminoacyl-tRNA analog. *EMBO J.*, **25**, 5942–5950.