Structural basis of resistance to lincosamide, streptogramin A, and pleuromutilin antibiotics by ABCF ATPases in Gram-positive pathogens

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Abstract

Target protection proteins bind to antibiotic targets and confer resistance to the host organism. One class of such proteins, termed antibiotic resistance (ARE) ATP binding cassette (ABC) proteins of the F-subtype (ARE ABCFs), are widely distributed throughout Gram-positive bacteria and bind the ribosome to alleviate translational inhibition by antibiotics that target the large ribosomal subunit. Using single-particle cryo-EM, we have solved the structure of ARE ABCF–ribosome complexes from three Gram-positive pathogens: Enterococcus faecalis LsaA, Staphylococcus haemolyticus VgaA_{LC} and Listeria monocytogenes VgaL. Supported by extensive mutagenesis analysis, these structures enable a comparative approach to understanding how these proteins mediate antibiotic resistance on the ribosome. We present evidence of mechanistically diverse allosteric relays converging on a few peptidyltransferase center (PTC) nucleotides, and propose a general model of antibiotic resistance mediated by these ARE ABCFs.
Introduction

The bacterial ribosome is a major antibiotic target (Wilson, 2014). Despite the large size of the ribosome, and the chemical diversity of ribosome-targeting small compounds, only a few sites on the ribosome are known to be bound by clinically-used antibiotics. On the 50S large ribosomal subunit, two of the major antibiotic binding sites are the peptidyltransferase center (PTC) and the nascent peptide exit tunnel. The PTC is targeted by pleuromutilin, streptogramin A, and lincosamide (PSAL) antibiotics, as well as phenicols and oxazolidinones (Dunkle et al., 2010; Matzov et al., 2017; Schlünzen et al., 2004; Tu et al., 2005; Wilson et al., 2008). Representatives of macrolide and streptogramin B classes bind at adjacent sites at the beginning of the nascent peptide exit tunnel (Dunkle et al., 2010; Tu et al., 2005).

Many mechanisms have evolved to overcome growth inhibition by such antibiotics in bacteria, among them target protection mediated by a subset of ABC family of proteins (Wilson et al., 2020). ATP-binding cassette (ABC) ATPases are a ubiquitous superfamily of proteins found in all domains of life, best-known as components of membrane transporters (Krishnan et al., 2020; Rees et al., 2009). A typical ABC transporter contains two nucleotide-binding domains (NBDs), each of which contribute one of two faces to an ATP-binding pocket, as well as transmembrane domains (Thomas & Tampé, 2020). Some sub-groups of ABC proteins, however, lack membrane-spanning regions and have alternative cytoplasmic functions, such as being involved in translation (Davidson et al., 2008; Fostier et al., 2020; Gerovac & Tampé, 2019). For example, in eukaryotes Rli1/ABCE1 is a ribosome splitting factor involved in recycling after translation termination, and the fungal eEF3 proteins bind the ribosome to facilitate late steps of translocation and E-site tRNA release (Andersen et al., 2006; Ranjan et al., 2020). The F-type subfamily of ABC proteins, which are present in bacteria and eukaryotes, contain at least two NBDs separated by an α-helical interdomain linker and notably lack transmembrane regions (Murina et al., 2019; Ousalem et al., 2019).

One group of bacterial ABCFs, which are termed antibiotic resistance (ARE) ABCFs (Dorrian & Kerr, 2009), confer resistance to antibiotics that bind to the 50S subunit of the bacterial ribosome (Ero et al., 2019; Ousalem et al., 2019; Sharkey & O’Neill, 2018; Wilson et al., 2020). Characterized ARE ABCFs are found predominantly in Gram-positive bacteria, including human and animal pathogens, typically have a restricted host specificity, and can be further divided into eight subfamilies (Allignet et al., 1992; Murina et al., 2019; Wilson et al., 2020). Although initially thought to act as part of efflux systems (Ross et al., 1990; Ross et al., 1989), these proteins were subsequently shown instead to bind the ribosome, oppose antibiotic binding, and to reverse antibiotic-mediated translation inhibition of translation in vitro (Sharkey et al., 2016).
Phylogenetic analyses indicate that ARE ABCFs may have arisen multiple times through convergent evolution, and that antibiotic specificity can be divergent within a related subgroup (Murina et al., 2019). Classified by the spectrum of conferred antibiotic resistance, ARE ABCFs can be categorized into three groups (Murina et al., 2019; Sharkey & O’Neill, 2018):

1. A highly polyphyletic group of ARE ABCFs that confer resistance to the PTC-binding PS\textsubscript{L}L antibiotics (ARE1, ARE2, ARE3, ARE5 and ARE6 subfamilies). The most well-studied representatives are VmlR, VgaA, SalA, LmrC and LsaA (Allignet et al., 1992; Hot et al., 2014; Koberska et al., 2020; Ohki et al., 2005; Singh et al., 2002). Additionally, a lincomycin-resistance ABCF that belongs to this group, termed Lmo0919, has been reported in Listeria monocytogenes (Chesneau et al., 2005; Dar et al., 2016; Duval et al., 2018).

2. ARE ABCFs that confer resistance to antibiotics that bind within the nascent peptide exit channel (a subset of the ARE1 subfamily, and ARE4). The most well-studied representatives are Macrolide and streptogramin B resistance (Msr) proteins (Reynolds & Cove, 2005; Ross et al., 1990; Su et al., 2018).

3. Poorly experimentally characterized ARE ABCF belonging to subfamilies ARE7 (such as OptrA) and ARE8 (PoxT4). These resistance factors confer resistance to phenicols and oxazolidinones that bind in the PTC overlapping with the PS\textsubscript{L}L binding site (Antonelli et al., 2018; Wang et al., 2015; Wilson et al., 2020) and are spreading rapidly throughout bacteria in humans and livestock by horizontal gene transfer (Freitas et al., 2017; Limura et al., 2020; Sadowy, 2018; Zhang et al., 2020).

Additionally, several largely unexplored groups of predicted novel ARE ABCFs are found in high-GC Gram-positive bacteria associated with antibiotic production (Murina et al., 2019).

So far, two structures of ARE ABCFs bound to the 70S ribosome have been determined (Crowe-McAuliffe et al., 2018; Ero et al., 2019; Su et al., 2018). In each instance, the ARE ABCF interdomain linker extended from the E-site-bound NBDs into the relevant antibiotic-binding site in the ribosome, distorting the P-site tRNA into an unusual state in the process. The tip of the interdomain linker—termed the antibiotic resistance determinant (ARD)—is not well conserved among (or sometimes even within) subfamilies, and mutations in this region can abolish activity as well as change antibiotic specificity. Mutagenesis indicates that both steric overlap between the ARD and the antibiotic, as well as allosteric reconfiguration of the rRNA and the antibiotic-binding site, may contribute to antibiotic resistance (Crowe-McAuliffe et al., 2018; Ero et al., 2019; Lenart et al., 2015; Su et al., 2018). Non-ARE ribosome-associated ABCFs that do not confer resistance to antibiotics—such as EttA—tend to have...
relatively short interdomain linkers that contact and stabilize the P-site tRNA (Chen et al., 2014). ARE ABCFs that confer resistance to PS₅₅L antibiotics (such as VmlR) have extensions in the interdomain linker that allow them to reach into the antibiotic-binding site in the PTC (Chen et al., 2014; Crowe-McAuliffe et al., 2018; Lenart et al., 2015). The longest interdomain linkers belong to ARE ABCFs that confer resistance to macrolides and streptogramin Bs (e.g. MsrE), and such linkers can extend past the PTC into the nascent peptide exit tunnel (Su et al., 2018). The length of the bacterial ABCF ARD generally correlates with the spectrum of conferred antibiotic resistance. Notable exceptions to this pattern are OptrA and PoxA ARE ABCF which have short interdomain linkers, yet still confer resistance to some PTC-binding antibiotics (Antonelli et al., 2018; Wang et al., 2015), while typically PTC-protecting ARE ABCFs such as VmlR, LsaA and VgaA, typically have comparatively long interdomain linkers (Lenart et al., 2015; Singh et al., 2001).

The available ARE ABCF-ribosome structures were generated by in vitro reconstitution. *Pseudomonas aeruginosa* MsrE, which confers resistance to tunnel-binding macrolides and streptogramin Bs (that inhibit translation elongation) was analyzed bound to a heterologous *Thermus thermophilus* initiation complex (Su et al., 2018). *Bacillus subtilis* VmlR, which confers resistance to PS₅₅L antibiotics that bind in the PTC (which stall translation at initiation) was analyzed in complex with an *B. subtilis* 70S ribosome arrested during elongation by the presence of a macrolide antibiotic (Crowe-McAuliffe et al., 2018; Dornhelm & Högenauer, 1978; Meydan et al., 2019; Ohki et al., 2005; Orelle et al, 2013). Structures of native physiological complexes (such as those generated using pull-down approaches from the native host) are currently lacking.

Here we have thoroughly characterized the antibiotic resistance specificity and determined the structure of three native ARE ABCF-70S ribosome complexes using affinity chromatography and cryo-electron microscopy (cryo-EM). We selected ARE ABCFs that confer resistance to PS₅₅L antibiotics in clinically-relevant Gram-positive pathogens: ARE3 representative *Enterococcus faecalis* LsaA (Singh et al., 2002), and ARE1 representatives *Listeria monocytogenes* Lmo0919 (Chesneau et al., 2005; Dar et al., 2016; Duval et al., 2018)—which we have termed VgaL—as well as the well-characterized VgaA₇₀C protein, initially isolated from *Staphylococcus haemolyticus* (Allignet et al., 1992; Chesneau et al., 2005; Jacquet et al, 2008; Lenart et al., 2015; Novotna & Janata, 2006). *Staphylococcus* and *Enterococcus* are commensal organisms that are prevalent in diverse healthcare-associated infections, and antibiotic resistance is spreading through these species (Magill et al, 2014; Mantora et al, 2019; Mendes et al, 2019; Pfaller et al, 2019). *L. monocytogenes* is a foodborne pathogen that poses particular risk to pregnant women and immunocompromised patients (Camargo et al, 2016). Our structures, supported by extensive mutagenesis
experiments, provide much needed insight into the mechanism by which these distinct ARE ABCFs displace antibiotics from their binding site on the ribosome to confer antibiotic resistance.
Results

Cryo-EM structures of native ARE ABCF-70S complexes

To obtain native ARE ABCF-70S complexes, we expressed C-terminally FLAG$_3$-tagged ATPase-deficient EQ$_2$ variants of *E. faecalis* LsaA, *S. aureus* VgaA$_{LC}$, and *L. monocytogenes* VgaL in their corresponding native host bacterial species. The FLAG$_3$ tag was used for affinity purification of each protein locked on the ribosomal target. The ARE ABCFs co-migrated with the 70S fraction through sucrose gradients—with the complex further stabilized in the presence of ATP in the case of LsaA and VgaA$_{LC}$—and co-eluted with ribosomal proteins after affinity purification (Figures S1–3).

The resulting native complexes were characterized by single-particle cryo-EM (see Methods), yielding ARE–70S complexes with average resolutions of 2.9 Å for *E. faecalis* LsaA, 3.1 Å for *S. aureus* VgaA$_{LC}$, and 2.9 Å for *L. monocytogenes* VgaL (Figure 1A–C, Table S4, Figures S4–S6). In each instance, the globular nucleotide-binding domains (NBDs) of the ARE ABCF bound in the E-site, and the α-helical interdomain linker extended towards the peptidyl-transferase center (PTC, Figure 1A–C). Additionally, a distorted tRNA occupied the P-site (Figure 1A–C), similarly to what was observed previously for *P. aeruginosa* MsrE and *B. subtilis* VmlR (Crowe-McAuliffe *et al*., 2018; Su *et al*., 2018). For the LsaA and VgaL samples, occupancy of the factor on the ribosome was high, with >95% or ~70% of picked ribosomal particles containing LsaA or VgaL, respectively (Figures S4 and S6). By contrast, VgaA$_{LC}$ had lower occupancy (~60%), implying that the factor dissociated after purification and/or during grid preparation (Figure S5). *In silico* 3D classification revealed that the major class not containing VgaA$_{LC}$ in the dataset was a 70S ribosome with P-tRNA, which could also be refined to an average resolution of 3.1 Å (Figure S5). Generally, the 50S ribosomal subunit and ARE ABCF interdomain linkers were well-resolved (Figures 1D–F and S4–S6).

While ARE ABCF NBDs, occupying the E site, had a lower resolution—especially in the regions that contact the ribosomal L1 stalk and the 30S subunit—the density was nonetheless sufficient to dock and adjust homology models in each instance (Figures 1D–F and S4–S6). Densities corresponding to the 30S subunits were less clear, indicating flexibility in this region, but nonetheless sufficient to build near-complete models of each ribosome.

Density corresponding to ATP and a coordinated magnesium ion was observed in both nucleotide-binding sites for each ARE ABCF (Figure 1D–F and S7). Density for the ATP bound in the peripheral nucleotide-binding site was relatively poor, with little density corresponding to the nucleobase moiety, consistent with the relaxed nucleotide specificity of these proteins (Figure S7) (Murina *et al*., 2018).
By comparison to structures of other ABC proteins, the NBDs adopted a closed conformation bound tightly to each nucleotide (Figure S8). In each ARE ABCF–70S map, the acceptor stem of the P-site tRNA was distorted, as observed previously for MsrE and VmlR (Crowe-McAuliffe et al., 2018; Su et al., 2018). The CCA 3′ end was particularly disordered, precluding any additional density corresponding to an amino acid or nascent chain from being visualized (Figures 1A–C and S4–S6). To our knowledge, this is the first model of the ribosome from the Gram-positive pathogen L. monocytogenes that have been described. Additionally, we have used our high-resolution map to create an updated model of the S. aureus ribosome (Khusainov et al., 2016). Our models of the E. faecalis and S. aureus ribosomes are generally in agreement with those recently described (Golubev et al., 2020; Murphy et al., 2020).

**LsaA, VgaLc and VgaL bind to translation initiation states**

In each cryo-EM map, the P-site tRNA body was sufficiently well-resolved so as to unambiguously assign the density to initiator tRNAfMet, on the basis of (i) general fit between sequence and density, (ii) the well-resolved codon-anticodon interaction, and (iii) a characteristic stretch of G:C base pairs found in the anticodon stem loop of tRNAfMet (Figure 2A–C). Additionally, in the small subunit mRNA exit tunnel, density corresponding to a putative Shine-Dalgarno–anti-Shine-Dalgarno helix was observed, consistent with the ARE ABCF binding to an initiation complex containing tRNAfMet (Figure 2D). LsaA–E. faecalis 70S samples were further analyzed with a custom tRNA microarray, which confirmed tRNAfMet was the dominant species found in the sample (Figure 2E). Collectively, these observations indicate that in our structures the majority of the ARE ABCFs are bound to 70S translation initiation complexes.

Further examination of the LsaA–70S volume revealed weak density in the ribosomal A site (Figure S4F), suggesting that some complexes had entered into the first elongation cycle. This was unexpected, as the distorted P-site tRNA is predicted to overlap with an accommodated A-site tRNA, although as noted would be compatible with a pre-accommodated A/T-tRNA (Crowe-McAuliffe et al., 2018). A mask around the A site was used for partial signal subtraction, and focused 3D classification was used to further sub-sort the LsaA–70S volume. One class, containing approximately one third of the particles, was shown to indeed contain a tRNA in the A site (Figures S4, S9A). This tRNA was poorly resolved, suggesting flexibility, and was slightly rotated compared to a canonical, fully accommodated A-site tRNA, and, as for the P-site tRNA, the acceptor stem was significantly disordered and displaced (Figure S9B,C). This state likely reflects an incomplete or late-intermediate
accommodation event, as observed previously when translation is inhibited by PTC binding antibiotics hygromycin A or A201A, both of which were shown to sterically exclude the acceptor stem of a canonical A-site tRNA (Polikanov et al., 2015). A very weak density corresponding to an A-site tRNA was also observed in VgaA<sub>LC</sub> and VgaL volumes, but sub-classification was unsuccessful for these datasets.

VgaA<sub>LC</sub> and VgaL, both of which belong to the ARE1 subfamily—although not LsaA, which belongs to the ARE3 subfamily—contain a short C-terminal extension predicted to form two α-helices (Crowe-McAuliffe et al., 2018; Murina et al., 2019). Although not conserved among all AREs, deletion of the CTE abolished antibiotic resistance in VmlR and reduces antibiotic resistance in VgaA, implying that this extension is necessary for function in some ARE ABCFs (Crowe-McAuliffe et al., 2018; Jacquet et al., 2008). Density for this region, which emanates from NBD2 and was located between ribosomal proteins uS7 and uS11, was present in the VgaA<sub>LC</sub>–70S and VgaL–70S maps and was essentially consistent with the position of the VmlR C-terminal extension, although was not sufficiently resolved to create a model for this region. Although bound close to the mRNA exit channel, the CTEs of VgaA<sub>LC</sub> and VgaL did not contact the Shine-Dalgarno–anti-Sine-Dalgarno helix of the initiation complexes, indicating they are not critical for substrate recognition in these ARE ABCFs (Figure S10).

The location and conformation of short and long ARDs on the ribosome

The ARD loop, positioned between the two long α-helices that link the NBDs, is a critical determinant of antibiotic resistance (Crowe-McAuliffe et al., 2018; Lenart et al., 2015; Murina et al., 2018; Sharkey et al., 2016; Su et al., 2018). Despite sharing a similar antibiotic specificity profile, the ARDs of LsaA, VgaA<sub>LC</sub>, VgaL, and VmlR are divergent in both amino acid composition and length, which is consistent with the polyphyletic nature of this group but precludes confident sequence alignment of this region (Figure 3A). Despite such sequence divergence, the position of the ARDs on the ribosome is broadly similar in each instance (Figure 3B–G). By comparison to tiamulin, which overlaps with the aminoacyl moieties of A- and P-tRNAs in the PTC, VmlR, LsaA, VgaA<sub>LC</sub>, and VgaL are all positioned similarly on the ribosome, with the ARD backbone adjacent to the antibiotic binding site (Figure 3B–F) (Polikanov et al., 2015; Schlünzen et al., 2004). Compared to VmlR, the additional residues in the ARDs of LsaA, VgaA<sub>LC</sub>, and VgaL extend away from the antibiotic binding site, towards the CCA 3’ end of the distorted P-tRNA (Figure 3C–F). By contrast, MsrE, which confers resistance to tunnel-binding antibiotics deeper in the ribosome, has a longer ARD that extends both past the PTC to approach the macrolide/streptogramin A binding site, as well as towards the distorted P-tRNA (Figure 3A, G) (Dunkle et al., 2010; Su et al., 2018). Thus,
the length of the ARD does not necessarily provide insights into the extent to which the ARD will extend into the ribosomal tunnel and thus one cannot easily predict whether long ARDs will confer resistance to macrolide antibiotics.

Position of the ARDs with respect to PSₐL antibiotic binding site

We next made a careful comparison of the LsaA, VgaAₐC, and VgaL ARDs with the binding sites of relevant antibiotics within the PTC (Figure 4A, B) (Dunkle et al., 2010; Matzov et al., 2017; Schlünzen et al., 2004; Tu et al., 2005). For LsaA, the side chain of Phe257 overlapped with the binding sites of tiamulin, virginiamycin M, and lincomycin, but was not close to erythromycin (Figure 4A–C), consistent with the spectrum of antibiotic resistance conferred by this protein (Table S1). In the VgaAₐC ARD, Val219 was situated close to tiamulin and virginiamycin M, and had a modest predicted overlap with lincomycin (Figure 4D). Notably, in the closely related variant VgaA, which has a similar specificity with modestly higher resistance to tiamulin and virginiamycin M, residue 219 is a glycine, which we predict would not overlap with the PSₐL binding site (Lenart et al., 2015). Thus, VgaAₐC confers resistance to virginiamycin M and tiamulin despite the lack of overlap between the ARE ABCF and the antibiotic binding site (Table S2). For VgaL, the closest residue to the PSₐL binding site was Ala216, which had no predicted overlap with tiamulin, virginiamycin M, or lincomycin (Figure 4E). Strikingly, VgaL therefore confers resistance to lincomycin, virginiamycin M, and tiamulin without directly overlapping the binding sites of these antibiotics. In summary, there was no general pattern of overlap or non-overlap with the PSₐL binding sites among LsaA, VgaAₐC, and VgaL.

Mutational analysis of LsaA and VgaAₐC ARDs

Our models of the ARD loops allowed us to design and test mutants for capacity to confer antibiotic resistance. When LsaA Phe257, which directly overlaps the PSₐL binding site (Figure 4C), was mutated to alanine, no change in resistance was observed (Figure S11). By contrast, mutation of Lys244, which is not situated close to the PSₐL binding sites but forms a hydrogen bond with 23S rRNA G2251 and G2252 of the P-loop (Escherichia coli numbering is used for 23S rRNA nucleotides), nearly abolished antibiotic resistance activity (Figure S11 and S12A–C). Combined, these observations indicate that LsaA does not confer resistance via simple steric occlusion, and that interactions with the P-loop may be required for positioning the LsaA ARD. For VgaAₐC, extensive alanine mutations within the ARD were
explored (Table S2). As expected from the above analyses and natural variants, mutating Val219—the only residue in VgaA_LC that sterically overlaps the LS_A binding site—did not affect the antibiotic resistance profile. Three residues at the beginning of α2, directly after the ARD loop, were required for resistance: Tyr223, which stacks with U2585 (part of the pleuromutilin and lincomycin binding sites); Phe224, which stacks with A2602 held in the center of the ARD; and Lys227, which forms a hydrogen bond with the 5’ phosphate of C2601 (Table S2). These residues do not overlap with the PS_AL binding site, but may be required to position the ARD in the PTC to impede antibiotic binding, or for the folding of the ARD itself (Figure S12D–F). In the naturally variable VgaA_LC ARD, mutation of Ser213, which sits adjacent to U2506 and C2507 (Figure S12E), to alanine similarly reduced antibiotic resistance (Table S2). Of note, mutating the most conserved residue among VgaA variants in this region, Lys218, did not substantially affect resistance (Table S2) (Vimberg et al., 2020).

Extensive alanine substitutions in the surrounding residues that contact the 23S rRNA (Figure S12D–F) either did not affect, or had only a mild influence on, the antibiotic resistance conferred by this protein (Table S2). In summary, mutation of VgaA_LC residues that interact with 23S rRNA nucleotides that form part of the LS_AP binding pocket affected antibiotic-resistance activity.

**Modulation of the ribosomal antibiotic binding site by ARE ABCFs**

We next sought to explore how the ARDs of LsaA, VgaA_LC, and VgaL affect the conformation of the ribosomal PTC. The 23S rRNA A2602, which is flexible in the absence of tRNAs and positioned between the P- and A-tRNAs during peptidyl transfer, is bound and stabilized by all structurally characterized ARE ABCFs. In LsaA and VmlR, a tryptophan stacks and stabilizes A2602 in a flipped position (Figure S13) (Crowe-McAuliffe et al., 2018). In VgaA_LC, VgaL, and MsrE, A2602 is instead positioned within the ARD loop, interacting with multiple residues from the ARE (Figure S13) (Su et al., 2018). We have labelled five regions of domain V of the 23S rRNA, which form the PTC, PTC loops (PLs) 1–5 (Figure 5A) (Polacek & Mankin, 2005). There was a significant overlap between nucleotides that form the PS_AL binding pockets, nucleotides that were shifted when LsaA, VgaA_LC, or VgaL bound the 70S, and nucleotides known to be mutated or modified in antibiotic-resistant strains of bacteria (summarized in Figure 5A). Broadly, changes to the PTC were similar between the VgaA_LC- and VgaL-bound 70S structures, consistent with the grouping of these proteins together in the ARE1 subfamily (Figures 5E–G, S14, S15) (Murina et al., 2019). Loop PL3, which contains nucleotides A2503 to U2506, was shifted upon binding of each ARE ABCF (Figure 5B–G). However, no residues from VgaA_LC or VgaL directly contact PL3 (Figures 5E–G, S14
and S1). Rather, these ARE ABCFs directly displace PL2, which ordinarily positions PL3, perhaps thereby facilitating the distorted conformation of PL3 that is incompatible with antibiotic binding. In the VgaA_LC-bound state, U2585, which was poorly ordered in the LsaA- and VgaL-bound 70S, stacks with Tyr223 and would not be available to interact with tiamulin or virginiamycin M (Figure S14D–F). Substituting VgaA_LC Tyr223 to alanine diminished antibiotic resistance, indicating that the reposition of U2585 contributes to antibiotic resistance conferred by this ARE ABCF (Table S2 and Figure S12F). 23S rRNA U2506 is additionally displaced in the VgaA_LC- and VgaL-bound 70S compared to the tiamulin- or lincomycin-bound 70S, potentially disrupting the binding site of these antibiotics (Figures S14A–C, S15A–C). By contrast, LsaA induced the most dramatic rearrangements in the PTC, with U2504 and G2505 in the LsaA-bound state predicted to strongly clash with each relevant antibiotic bound to the ribosome (Figure S5E–G and S16A–C). In the LsaA-bound ribosome, A2453 is shifted slightly away from the PTC and pairs with G2499 instead of U2500. This allows C2452, which normally pairs with U2504 to form part of the PS_AL binding pocket, to instead hydrogen-bond with U2500, thereby freeing U2504, and PL3 more generally, to reposition when LsaA is bound (Figure S16D, E).
Discussion

Model of antibiotic resistance mediated by LsaA, VgaA\textsubscript{LC}, and VgaL

These observations allow us to propose a model for how ARE ABCFs confer antibiotic resistance to the host organism (Figure 6). PS\textsubscript{A\textsubscript{L}} antibiotics have binding sites overlapping with the nascent polypeptide chain, and inhibit translation at, or soon after, initiation (Figure 6A) (Dornhelm & Högenauer, 1978; Meydan \textit{et al.}, 2019; Orelle \textit{et al.}, 2013). The incoming ARE ABCF binds in the E-site, triggering closure of the L1 stalk and inducing a distorted conformation of the P-tRNA. The ARD disrupts the antibiotic binding pocket in the PTC, causing drug release (Figure 6B). An incoming ternary complex delivers a tRNA to the A-site, which upon ARE ABCF egress and successful accommodation ‘sweeps’ the 3’ end of the P-tRNA into the PTC (Figure 6C, D). The trigger for nucleotide hydrolysis and exit of the ARE ABCF from the E site is unknown. We propose that rapid peptidyl transfer then creates a short nascent chain that overlaps with the antibiotic binding site, preventing re-binding of the PS\textsubscript{A\textsubscript{L}} drug until the next round of translation (Figure 6D). Alternatively, an A-tRNA may partially accommodate on the stalled initiation complex prior to ARE ABCF binding, and become distorted as part of a ‘knock-on’ effect of P-tRNA disruption, consistent with the ability of ARE ABCFs to ‘reset’ the P-tRNA independently of additional accommodation events (Murina \textit{et al.}, 2018). In this model, potentially only one round of ATP hydrolysis per translation cycle is necessary to confer resistance.

ARE ABCFs such as LsaA, VgaA\textsubscript{LC}, VgaL, and VmlR confer resistance to PS\textsubscript{A\textsubscript{L}} antibiotics but not phenicols or oxazolidinones (Sharkey & O’Neill, 2018). This observation has been puzzling, as both groups of antibiotics have overlapping binding sites (Dunkle \textit{et al.}, 2010; Matzov \textit{et al.}, 2017; Schlünzen \textit{et al.}, 2004; Tu \textit{et al.}, 2005; Wilson \textit{et al.}, 2008). However, phenicols and oxazolidinones inhibit translation during elongation at specific motifs (Marks \textit{et al.}, 2016; Orelle \textit{et al.}, 2013), while PS\textsubscript{A\textsubscript{L}} antibiotics instead inhibit translation at the initiation stage (Dornhelm & Högenauer, 1978; Meydan \textit{et al.}, 2019; Orelle \textit{et al.}, 2013). The apparent specificity of LsaA, VgaA\textsubscript{LC}, and VgaL for initiation complexes in our immunoprecipitations (Figure 2) matches the specificity of the antibiotics to which they confer resistance.

Do EQ\textsubscript{2}-substituted ATPase-deficient variants of ARE ABCF, like the ones used in this study, bind the ribosome in the pre- or post-antibiotic-dissociation state (Figure 6B)? Although direct evidence is lacking, three reasons lead us to propose that these proteins are bound in the post-antibiotic-release state:

1) In the case of VgaA\textsubscript{LC} and VmlR the position of the ARD directly overlaps with the antibiotic binding site. Although the side chain of the overlapping amino acid is not
critical for antibiotic resistance in most instances, the overlap nonetheless implies mutually exclusive binding.

2) MsrE-EQ stimulates dissociation of azithromycin from the ribosome (Su et al., 2018).

3) Our attempts to form complexes containing both antibiotic and ARE ABCF have been unsuccessful, resulting in exclusive binding of either the ARE ABCF or the antibiotic, similarly to what we observed for TetM, a tetracycline-resistance ribosome protection protein (Arenz et al., 2015).

How does the ARE ABCF ARD mediate antibiotic resistance (Figure 6B, C)? In one model, by analogy to the TetM tetracycline resistance protein (Arenz et al., 2015; Wilson et al., 2020), the ARD may induce antibiotic dissociation by a direct steric overlap with the antibiotic. In the case of VmlR, substitutions of the Phe237 residue that overlaps the binding site of PS₄₅L antibiotics affect resistance to one of three relevant antibiotics, indicating that both direct steric overlap and an indirect, allosteric mechanism can contribute to resistance (Crowe-Mcauliffe et al., 2018). In the case of MsrE substitution of Leu242, which overlaps with the erythromycin binding site, as well as adjacent residues abolished or severely reduced the antibiotic resistance activity of this protein (Su et al., 2018). In both cases, a mixture of direct steric overlap and allostery is consistent with the available data (Ero et al., 2019). The ARDs of LsaA, VgaA₉, and VgaL either do not directly overlap with the PS₄₅L binding site, or where there is an overlap, as with LsaA Phe257 and VgaA₉ Val219, the side chains are not essential for resistance, implicating an allosteric mechanism for these proteins (Figures 4–5, S11, S12, Table S2). Alanine mutagenesis instead indicates that the side chains of residues surrounding the amino acid closest to the antibiotic-binding pocket, as well as those that contact the 23S rRNA, are necessary for resistance (Figures S11, S12 and Table S2). These residues may position the ARD in the PTC. No single set of 23S rRNA rearrangements was identical among LsaA, VgaA₉, and VgaL, although displacement of PTC loop PL3, especially residue U2504, was ultimately observed in each ARE ABCF–70S structure (Figure 5).

In summary, we present three new structures of ARE ABCFs bound to 70S ribosomes from relevant Gram-positive pathogenic bacteria and present the first model of the ribosome from Listeria monocytogenes. Our structures and mutagenesis experiments support an allosteric mechanism of ARE ABCF action, and hint at a rationalization for the specificity of LsaA, VgaA₉, and VgaL for PS₄₅L antibiotics. Each ARE ABCF binds the 70S similarly as observed for other bacterial ABCF proteins, but alters the geometry of the PTC distinctively, consistent with the convergent evolution—and divergent sequences—of this class of ABCF proteins.
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Fig. 1 Cryo-EM structures of ARE ABCF–ribosome complexes. (A–C) Cryo-EM maps with isolated densities for (A) *E. faecalis* LsaA (green), (B) *S. aureus* VgaA<sub>LC</sub> (magenta), (C) *L. monocytogenes* VgaL (yellow) as well as P-site tRNA (cyan), small subunit (SSU, yellow) and large subunit (LSU, grey). (D–F) Density (grey mesh) with molecular model for (D) LsaA, (E) VgaA<sub>LC</sub>, and (F) VgaL, coloured according to domain as represented in the associated schematics: nucleotide binding domain 1 (NBD1, red), antibiotic-resistance domain (ARD, cyan), nucleotide binding domain 2 (NBD2, blue) and C-terminal extension (CTE, grey, not modelled). In (D–F), the ATP nucleotides are coloured green.
Figure 2. The LsaA–70S complex contains an initiator tRNA and SD-helix. (A–D) Isolated density (grey mesh) with molecular models (sticks) for (A) initiator tRNA$^{fMet}$ (cyan), (B) interaction between AUG start codon of the mRNA (magenta) and anticodon of initiator tRNA$^{fMet}$ (cyan) in the P-site, (C) three G-C base pairs specific to the initiator tRNA$^{fMet}$ (cyan), and (D) helix formed between Shine-Dalgarno (SD) sequence of the mRNA (magenta) and anti-SD of the 16S rRNA (yellow). (E) Replicate tRNA microarray analysis of the LsaA–70S complex, illustrating the enrichment of initiator tRNA$^{fMet}$ in the LsaA–70S complex over the lysate. Confidence intervals between replicates were 92%.
Fig. 3. Comparison of the ARD loops of different ARE ABCFs. (A) The sequence length of the ARD loops differs significantly for VmlR, VgaL, VgaA_{LC}, LsaA and MsrE. Although the lack of sequence homology precludes accurate sequence alignment of the ARD loops, the red highlighted residues can be aligned structurally. (B–G) Comparison of the positions of (B) A-site tRNA (grey) and P-site tRNA (cyan) from pre-attack state (PDB 1VY4) (Polikanov et al., 2014), with shifted P-site tRNA (cyan) and ABCF ARD from ribosome complexes containing (C) VmlR (orange, PDB 6HA8) (Crowe-McAuliffe et al., 2018), (D) LsaA (green), (E) VgaA_{LC} (magenta), (F) VgaL (yellow), and (G) MsrE (blue, PDB 5ZLU) (Su et al., 2018). In (B–G), the relative position of either tiamulin (Tia, magenta, PDB 1XBP) (Schlünzen et al., 2004) or erythromycin (Ery, red, PDB 4V7U) (Dunkle et al., 2010) has been superimposed.
**Fig. 4 Interaction of LsaA, VgaA<sub>LC</sub> and VgaL at the peptidyltransferase centre. (A–B)**

LsaA and distorted P-site tRNA superimposed on a transverse section of the large subunit (LSU, grey) to reveal (A) the ARD of LsaA extending into the nascent polypeptide exit tunnel (NPET) and (B) the relative position of Phe257 of LsaA to tiamulin (Tia, purple, PDB 1XBP) (Schlünzen et al., 2004) and erythromycin (Ery, red, PDB 4V7U) (Dunkle et al., 2010). (C–E) Relative position of LsaA (green, row C), VgaA<sub>LC</sub> (pink, row D) and VgaL (yellow, row E) to tiamulin (Tia, purple, PDB 1XBP), virginiamycin M (VgM, lime, PDB 1YIT) (Tu et al., 2005), lincomycin (Lnc, tan, PDB 5HKV) (Matzov et al., 2017). Clashes in C–E are shown with red outlines.
Fig. 5 ARE ABCF binding induces allosteric conformational changes at the PTC. (A) Secondary structure of peptidyltransferase ring within domain V of the 23S rRNA, highlighting residues within PTC loops 1–4 (PL1–4) that (i) comprise the binding site of PS$_{AL}$ antibiotics (blue), (ii) undergo conformational changes upon ARE ABCF binding (grey) and (iii) confer resistance to PS$_{AL}$ antibiotics (red circles). (B–D) Binding site of (B) tiamulin (Tia, magenta, PDB 1XBP; (Schlünzen et al., 2004), (C) virginiamycin M (VgM, lime, PDB YIT; (Tu et al., 2005) and (D) lincomycin (Lnc, tan, PDB 5HKV) (Matzov et al., 2017) on the ribosome. (E–G) Comparison of conformations of rRNA nucleotides comprising the (E) Tia, (F) VgM and (G) Lnc binding site (shown as grey cartoon ladder representation), with rRNA conformations when LsaA (green), Vga$_{LC}$ (magenta) or VgaL (yellow) are bound.
Fig. 6 Model for ribosome protection by ARE ABCFs VmlR, LsaA, VgaA_{LC} and VgaL.

(A) PS_{A}L-stalled ribosomes containing an initiator-tRNA in the P-site are recognized by the ARE ABCFs such as VmlR, LsaA, VgaA_{LC} and VgaL, which bind to the E-site of the ribosome with a closed ATP-bound conformation. (B) Binding of the ARE ABCF induces a shifted P-site tRNA conformation in the ribosome allowing the ARD of the ARE ABCF to access the peptidyl-transferase center (PTC). The ARD induces conformational changes within the 23S rRNA at the PTC that promotes dissociation of the drug from its binding site (shown as dashed lines). (C) Aminoacyl-tRNAs can still bind to the ARE ABCF-bound ribosomal complex, but cannot accommodate at the PTC due to the presence of the ABCF and shifted P-site tRNA conformation. (D) Hydrolysis of ATP to ADP leads to dissociation of ARE ABCF from the ribosome, which may allow the peptidyl-tRNA as well as the incoming aminoacyl-tRNA to simultaneously accommodate at the PTC. Peptide bond formation can then ensue, converting the ribosome from an initiation to an elongation (pre-translocation) state, which is resistant to the action of initiation inhibitors, such as PS_{A}L antibiotics.
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Tu D, Blaha G, Moore PB, Steitz TA (2005) Structures of MLSB antibiotics bound to mutated large ribosomal subunits provide a structural explanation for resistance. *Cell* 121: 257-270


**Materials and methods**

### Strains and plasmids

All strains and plasmids used in this work are listed in Table S5.

**E. faecalis** OG1RF and TX5332, a LsaA disruption mutant of OG1RF (Singh et al., 2002), were kindly provided by Dr. Barbara E. Murray (Health Science Center, University of Texas). All cloning was performed by Protein Expertise Platform at Umeå University. **E. faecalis** LsaA ORF was PCR amplified from pTEX5333 plasmid and cloned into either pCIEcam (Weaver et al., 2017) (used for preparation of LsaA-70S complexes) or pCIEcam derivative with the Cm' gene swapped to the spectinomycin resistance Sc' gene; used for MIC testing) vector for cCF10-inducible expression. To allow detection by immunoblotting and preparation of LsaA-70S complexes, the LsaA ORF was supplemented with C-terminal His6-TEV-FLAG3 tag (HTF tag) and the ribosome binding site was optimized for high expression yield. Point mutations E142Q and E452Q were introduced to LsaA resulting in pCIE_LsaA-EQ2-HTF.

**S. haemolyticus** vga(A)LC gene was PCR-amplified from a S. haemolyticus isolate held in the O'Neill strain collection at the University of Leeds, using oligonucleotide primers vgaALC-F (5′-GGTGGTGTTACCAGGATGAGAAATATGAAAA-3′) and vgaALC-R (5′-GGTGGTGAATTCGGTAATTTATTTATCTAATTTTCT-3′) (engineered restriction sites shown underlined). The protein encoded by this gene is identical to that previously reported (Novotna & Janata, 2006) (accession number DQ823382). The fragment was digested with KpnI and EcoRI and ligated into the tetracycline-inducible expression vector pRMC2 (Corrigan & Foster, 2009). Constructs encoding the VgaALC protein fused with a C-terminal FLAG3 tag were obtained by synthesis (Genewiz), with E105Q, E410Q and EQ2 mutants subsequently created by site-directed mutagenesis. Generation of other point mutants of untagged Vga(A)LC was performed by NBS Biologicals, again using chemical synthesis to generate the original vga(A)LC template, followed by site-directed mutagenesis.

**L. monocytogenes** VgaL (Lmo0919). In order to construct L. monocytogenes EDGe::∆lmo0919, regions corresponding to the upstream and downstream flanking regions of lmo0919, present on the EDGe genome were amplified with primer pairs VKT35 (5′-GGGGGGATCCATCACTAGCCGAATCCAAAC-3′) and VgALC-R (5′-GGTGGTGAATTCGGTAATTTATTTATCTAATTTTCT-3′) (engineered restriction sites shown underlined). The protein encoded by this gene is identical to that previously reported (Novotna & Janata, 2006) (accession number DQ823382). The fragment was digested with KpnI and EcoRI and ligated into the tetracycline-inducible expression vector pRMC2 (Corrigan & Foster, 2009). Constructs encoding the VgaALC protein fused with a C-terminal FLAG3 tag were obtained by synthesis (Genewiz), with E105Q, E410Q and EQ2 mutants subsequently created by site-directed mutagenesis. Generation of other point mutants of untagged Vga(A)LC was performed by NBS Biologicals, again using chemical synthesis to generate the original vga(A)LC template, followed by site-directed mutagenesis.

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lm00919 was amplified from EDGe genomic DNA using primers VHKT12 (5'-CCCCCATGGCATTACAATCGAATAAATC-3') and VHKT39 (5'-GGGGCTGCAGTTAAATTGCTGTCTTTTG-3'), and cloned into pIMK3 using Ncol and PstI restriction sites, resulting in plasmid VHp690.

Overlap extension PCR was used in order to introduce a HTF tag at the C-terminus of lm00919 (Ho et al., 1989). The lm00919 locus and HTF tag were amplified with primer pairs VHKT12 and VHKT15 (5'-ATGATGATGGCCGCCACTAAATTGCTGTCTTTTG-3') and VHKT14 (5'-AGACAGCAATTAGTGCGGCATTACTCATCATC-3'), VHKT13 (5'-GGGGCTGCAGTTAGCCTTTGTCATCGTC-3'), the lmo0919 locus and HTF tag were amplified with primer pairs VHKT12 and VHKT14 (5'-TGGTTGGTTGATCAAGAATCAAGAAATTGGCGT-3') using EDGe genomic DNA and VHp100 template DNA respectively, producing fragments with overlapping ends. VHKT12 and VHKT13 were then used to fuse the fragments and the resulting PCR product was cloned into pIMK3 using Ncol and PstI sites resulting in VHp692.

To introduce EQ2 mutations (E104Q and E408Q) simultaneously into the VHp692 plasmid, primers VHT266 (5'-TCTTGATCAACCAACATTTGAGTTATGTCGCAATGGAA-3') and VHT267 (5'-CTTGTTGGTTGGTCTGCTAGGAGAACACTTGGATTTTGGCG-3') containing both mutations were used to extend out from lm00919 HTF to amplify the VHp692 backbone. Primers VHT264 (5'-AGCAGACCAACCAAGCAATCTTGATGTCG-3') and VHT265 (5'-TTGTTGGTTGATCAAGAATCAAGAAATTGGCGT-3') also containing lmo0919EQ2 mutations were used to amplify a fragment with overlapping sequence to the backbone fragment. Both PCR products were then assembled using NEBuilder® HiFi DNA Assembly Master Mix (NEB), resulting in VHp693.

B. subtilis: To construct the VHB109 [trpC2 ΔvmlR thrC::Phy-spank-lsaA kmR] strain untagged LsaA under the control of an IPTG-inducible Pphy-spank promotor, a PCR product encoding lsa(A) was PCR-amplified from pTEX5333 using the primers VHT127 (5'-CGACAGGAAGAGAGCGATAATGTCGAAATTGAAATTGCAATGGG-3') and VHT128 (5'-CACCCGAATTAGTTGCAAGAATCATTGCAATGGG-3') containing both mutations were used to extend out from lm00919HTF to amplify the VHp692 backbone. Primers VHT264 (5'-AGCAGACCAACCAAGCAATCTTGATGTCG-3') and VHT265 (5'-TTGTTGGTTGATCAAGAATCAAGAAATTGGCGT-3') also containing lmo0919EQ2 mutations were used to amplify a fragment with overlapping sequence to the backbone fragment. Both PCR products were then assembled using NEBuilder® HiFi DNA Assembly Master Mix (NEB), resulting in VHp693.

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GTACGGCAACGCTAAGGAAAAAGGGAGCGGGGCGA-3′), according to directions of Phusion Site-Directed Mutagenesis Kit (Thermo Fisher Scientific), yielding, yielding VHplug6 (pHT009-IsaAK244A) plasmid which was used to transform the VHB5 [trpC2 ΔvmlR] strain. Selection for kanamycin resistance yielded the desired VHB168 strain. To construct the VHB169 [trpC2 ΔvmlR thrC::P_{hy}-spnak-IsaAF257A kmR] strain, VHp369 plasmid was subjected to site-directed mutagenesis using primer VHP305 (5′-CAATCGCCCCGCTCCCTTTTTCCTAGCGT-3′) and VHP306 (5′-CGGATACAGGAGCCATTGGTGCCCGGGCA-3′), according to directions of Phusion Site-Directed Mutagenesis Kit (Thermo Fisher Scientific), yielding, yielding VHplug7 (pHT009-IsaAF257A) plasmid which was used to transform the VHB5 [trpC2 ΔvmlR] strain. Selection for kanamycin resistance yielded the desired VHB169 strain.

## Bacterial transformation

### E. faecalis: Electrocompetent cells were prepared as per Bhardwaj and colleagues (Bhardwaj et al, 2016). Shortly, an over-night culture grown in the presence of appropriate antibiotics was diluted to OD_{600} of 0.05 in 50 mL of BHI media (supplemented with 2 mg/mL kanamycin in case of TX5332), grown to OD_{600} of 0.6-0.7 at 37 °C with moderate shaking (160 rpm). Cells were collected by centrifugation at 4,000 rpm at 4 °C for 10 min. Cells were resuspended in 0.5 mL of sterile lysozyme buffer (10 mM Tris-HCl pH 8; 50 mM NaCl, 10 mM EDTA, 35 µg/mL lysozyme), transferred to 1.5 mL Eppendorf tube and incubated at 37 °C for 30 minutes. Cells were pelleted at 10,000 rpm at 4 °C for 10 min and washed three times with 1.5 mL of ice-cold electroporation buffer (0.5M sucrose, 10% glycerol(w/v)). After last wash the cells were resuspended in 500 µL of ice-cold electroporation buffer and aliquoted and stored at –80°C. For electroporation 35 µL of electrocompetent cells were supplemented with 1 µg of plasmid DNA, transferred to ice-cold 1 mm electroporation cuvette and electroporated at 1.8 keV. Immediately after electroporation 1 mL of ice-cold BHI was added to the cells, the content of the cuvette was transferred to 1.5 mL Eppendorf tubes and the cells were recovered at 37 °C for 2.5 hours and plated to BHI plates containing appropriate antibiotics (10 µg/mL chloramphenicol and 2 mg/mL kanamycin).

### S. aureus: the preparation and transformation of S. aureus electrocompetent cells followed the method of Schenk & Laddaga (Schenk & Laddaga, 1992), though used Tryptone soya broth (Oxoid) containing 2.5% yeast extract in place of B2 medium. Sequence-verified constructs established in E. coli were transferred into the restriction deficient S. aureus RN4220 strain (Fairweather et al, 1983), before recovery and introduction into S. aureus SH1000 (Horsburgh et al, 2002; O’Neill, 2010).
**L. monocytogenes**: pIMK3 integrative plasmids were transformed into *L. monocytogenes* via conjugation. *E. coli* S17.1 harbouring pIMK3 and its derivatives, was grown at 37 °C overnight in LB media supplemented with 50 µg/mL Kanamycin, 1 mL of culture was washed three times with sterile BHI media to remove antibiotics. 200 µL of washed *E. coli* culture was mixed with an equal volume of *L. monocytogenes* overnight culture grown at 37 °C in BHI media. 200 µL of mixed bacterial suspension was then dropped onto a conjugation filter (Millipore #HAEP047S0) placed onto a BHI agar plate containing 0.2 µg/mL penicillin-G. After overnight incubation at 37 °C, bacterial growth from the filter was re-suspended in 1 ml of BHI and 100-300 µL plated onto BHI-agar plates supplemented with 50 µg/mL Kanamycin (to select for pIMK3), 50 µg/mL Nalidixic acid and 10 µg/mL Colistin sulfate (Sigma-Aldrich C4461-100MG). Resulting colonies were checked for correct integration via PCR and subsequent sequencing using primers VHKT42 and VHKT43.

**Antibiotic susceptibility testing**

Minimum Inhibitory Concentrations (MIC) were determined based on guidelines from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/ast_of_bacteria/mic_determination).

**E. faecalis**: bacteria were grown in BHI media supplemented with 2 mg/mL kanamycin (to prevent *lsa* revertants), either 0.1 mg/mL spectinomycin (to maintain the pCIEspec plasmid), or 20 µg/mL of chloramphenicol (to maintain the pCIEcam plasmid used to validate the functionality of the HTF-tagged LsaA variant), 100 ng/mL of cCF10 peptide (to induce expression of LsaA protein) as well as increasing concentrations of antibiotics was inoculated with 5 × 10^5 CFU/mL (OD_600 of approximately 0.0005) of *E. faecalis* ∆lsaA (lsa::Kan) strain TX5332 transformed either with empty pCIEspec plasmid, or with pCIEspec encoding LsaA. After 16-20 hours at 37 °C without shaking, the presence or absence of bacterial growth was scored by eye.

**S. aureus**: bacteria were grown in cation-adjusted Mueller-Hinton Broth (MHB) at 37 °C with vigorous aeration, supplemented with 10 mg/L chloramphenicol to maintain the pRMC2 plasmid. Upon reaching an absorbance of OD_625 of 0.6, anhydrotetracycline (ATC) (Sigma-Aldrich, UK) was added at a final concentration of 100 ng/mL to induce expression from pRMC2, and incubated for a further 3 hours. Cultures were then diluted to 5 × 10^5 CFU/mL using MHB supplemented with ATC (100 ng/mL) and used in MIC determinations essentially as described above (though cultures were shaken).

**L. monocytogenes**: bacteria were grown in BHI media supplemented with 50 µg/mL kanamycin (to prevent loss of the integrated pIMK3 plasmid), 1 mM of IPTG (to induce expression of VgaL protein) as well as increasing concentrations of antibiotics was inoculated
with 5 x 10^5 CFU/mL (OD_{600} of approximately 0.0003) of *L. monocytogenes* EDG-e wildtype strain or EDG-e::Δlmo0919 strain transformed either with empty pIMK3 plasmid, or with pIMK3 encoding VgaL variants. After 16–20 hours at 37 °C without shaking, the presence or absence of bacterial growth was scored by eye.

**B. subtilis** (for LsaA mutants): *B. subtilis* strains were pre-grown on LB plates either supplemented with 1 mM IPTG overnight at 30 °C. Fresh individual colonies were used to inoculate filtered LB medium in the presence of 1 mM IPTG, and OD_{600} adjusted to 0.01. The cultures were seeded on a 100-well honeycomb plate (Oy Growth Curves AB Ltd, Helsinki, Finland), and plates incubated in a Bioscreen C (Labsystems, Helsinki, Finland) at 37°C with continuous medium shaking. After 90 min (OD_{600} ~ 0.1), antibiotics were added and growth was followed for an additional 6 hours.

### Preparation of bacterial lysates

#### Preparation of bacterial biomass

**E. faecalis**: *E. faecalis* TX5332 transformed with pCIE plasmids (either empty vector and expressing either wild-type or EQ_2 variants of C-terminally HTF-tagged LsaA) were grown overnight from single colony in BHI supplemented with 2 mg/mL kanamycin and 10 µg/mL of chloramphenicol. Next day overnight cultures were diluted to starting OD_{600} of 0.05 in 160 mL BHI supplemented with 0.5 mg/mL kanamycin and 10 µg/mL of chloramphenicol. Cells were grown with intensive shaking at 37 °C till OD_{600} of 0.6 and were induced with 300 ng/mL of cCF10 peptide for 30 minutes prior harvesting by centrifugation at 10,000 × g for 15 minutes at 4 °C.

**S. aureus**: *S. aureus* SH1000 transformed with pRMC2 plasmids (empty vector, wild-type and EQ_2 VgaA_{LC}-FLAG_3) were grown in LB supplemented with 25 µg/mL of chloramphenicol. Saturated cultures were diluted to an OD_{600} of 0.1 in 400 mL LB supplemented with 20 µg/mL of chloramphenicol and grown at 37 °C with vigorous aeration to an OD_{600} of 0.6. Protein expression was induced with 100 ng/mL of anhydro-tetracycline for 30 minutes prior to harvesting by centrifugation at 10,000 × g for 15 minutes at 4 °C.

**L. monocytogenes**: *L. monocytogenes* EDG-e was transformed with pIMK3 plasmids (empty vector, wild-type and EQ_2 VgaL-HTF) were grown overnight from single colony in LB supplemented with 50 µg/mL of kanamycin. Next day overnight cultures were diluted till starting OD_{600} of 0.005 in 200 mL BHI supplemented with 50 µg/mL of Kanamycin. Cells were grown at 37 °C with shaking at 160 rpm till OD_{600} of 0.6 and were induced with 1 mM IPTG for 60 minutes prior harvesting by centrifugation at 10,000 × g for 15 minutes at 4 °C.
Preparation of clarified lysates

Cell pellets were resuspended in 1.5 mL of cell lysis buffer (95 mM KCl, 5 mM NH₄Cl, 20 mM HEPES pH 7.5, 1 mM DTT, 5 mM Mg(OAc)₂, 0.5 mM CaCl₂, 8 mM putrescine, 1 mM spermidine, 1 tablet of cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche) per 10 mL of buffer and in the absence or presence of either 0.5 or 0.75 mM ATP), resuspended cells were opened by FastPrep homogeniser (MP Biomedicals) with 0.1 mm Zirconium beads (Techtum) in 4 cycles by 20 seconds with 1 minute chill on ice. Cell debris was removed after centrifugation at 14,800 × g for 15 minutes at 4 °C. Total protein concentration in supernatant was measured by Bradford assay (BioRad), supernatant was aliquoted and frozen in liquid nitrogen.

Polysome fractionation and immunoblotting

Sucrose density gradient centrifugation

After melting the frozen lysates on ice, 2 A₂₆₀ units of each extract was aliquoted into three tubes and supplemented with or without 0.5-0.75 mM ATP and was loaded onto 5–25% or 7–35% (w/v) sucrose density gradients in HEPES:Polymix buffer (Takada et al, 2020), 5 mM Mg(OAc)₂ and supplemented or not with 0.5–0.75 mM ATP. Gradients were resolved at 35,000 rpm for 2.5 hours at 4 °C in SW41 rotor (Beckman) and analysed and fractionated using Biocomp Gradient Station (BioComp Instruments) with A₂₈₀ as a readout.

Immunoblotting

LsaA and VgaA LC: Schleicher & Schuell Minifold II Slot Blot System SRC072/0 44-27570

Manifold was used for transferring samples from sucrose gradient fractions to PVDF membranes (Immobilon PSQ, Merk Millipore). Shortly, 15-100 μL of each sucrose gradient fraction was added to 200 μL of Slot-blotting Buffer (20 mM HEPES:KOH pH 7.5, 95 mM KCl, 5 mM NH₄Cl, 5 mM Mg(OAc)₂) in slots and blotted onto PVDF membrane that had been activated with methanol for one minute, wetted in MilliQ water and equilibrated with Slot-blotting Buffer (1x PM 5 mM Mg²⁺ without putrescine and spermidine) for 10 minutes. After blotting of the samples each slot was washed twice with 200 μL of Slot-blotting Buffer. The membrane was removed from the blotter, transferred to hybridization bottle, equilibrated for 10 minutes in PBS-T (1x PBS supplemented with 0.05% Tween-20) and blocked in PBS-T supplemented with 5% w/v nonfat dry milk for one hour. Antibody incubations were performed for one hour in 1% nonfat dry milk in PBS-T with five 5-minute washes in fresh PBS-T between and after antibody incubations. HTF-tagged LsaA and FLAG₃-tagged VgaA LC proteins were detected using anti-Flag M2 primary (Sigma-Aldrich, F1804; 1:10,000 dilution) antibodies.
combined with anti-mouse-HRP secondary (Rockland; 610-103-040; 1:10,000 dilution) antibodies. An ECL detection was performed on ImageQuant LAS 4000 (GE Healthcare) imaging system using Pierce® ECL Western blotting substrate (Thermo Scientific). The blotting and all incubations were performed at room temperature in hybridization oven.

VgaL (Lmo0919): Western blotting of lysates on sucrose gradient fractionation was performed as previously described (Takada et al., 2020). VgaL-HTF was detected using anti-Flag M2 primary (Sigma-Aldrich, F1804; 1:10,000 dilution) antibodies combined with anti-mouse-HRP secondary (Rockland; 610-103-040; 1:10,000 dilution) antibodies.

**Affinity purification on anti-FLAG M2 affinity gel**

100 µL of well mixed anti-FLAG M2 Affinity Gel aliquots were loaded on columns (Micro Bio-Spin Columns, Bio-Rad) and washed two times with 1 mL of cell lysis buffer by gravity flow. All incubations, washings and elutions were done at 4 °C. The total protein concentration of each lysate was adjusted to 2 mg/mL with cell lysis buffer and 1 mL of each lysate was loaded on columns and incubated for two hours with end-over-end mixing for binding. The columns were washed 5 times by 1 mL of cell lysis buffer by gravity flow. For elution of FLAG-tagged proteins and their complexes 100-300 µL of 0.1 mg/mL FLAG3 peptide (Sigma) was added to samples, the solutions were incubated at 4 °C for 20 minutes with end-over-end mixing. Elutions were collected by centrifugation at 2,000 ×g for 2 minutes at 4 °C.

20 µL aliquots of collected samples (flow-through, washes and elutions) were mixed with 5 µL of 5x SDS loading buffer and heated up at 95 °C for 15 minutes. The beads remaining in the column were washed twice with 1 mL of cell lysis buffer and resuspended in 100 µL of 1x SDS loading buffer. Denatured samples were resolved on 12-15% SDS-PAGE. SDS-gels were stained by “Blue-Silver” Coomassie Staining (Candiano et al., 2004) and washed with water for 6 hours or overnight before imaging with LAS4000 (GE Healthcare).

**tRNA microarrays**

To fully deacylate tRNAs, eluates and input lysate samples from two biological replicates were mixed with 80 µL 250 mM Tris-HCl, pH 9.0, 10 µL 0.2 M EDTA, 10 µL 1% SDS, and incubated for 45 min, and neutralised with 200 µL 1 M NaOAc, pH 5.5, before mixing 1:1 with acidic phenol:chloroform alcohol 5:1. The supernatant was precipitated with ethanol and dissolved in ddH2O.
tRNA microarrays were performed as described (Kirchner et al., 2017). Briefly, using the unique invariant single stranded 3′-NCCA-ends of intact tRNA a Cy3-labelled or Atto647-labeled RNA/DNA hybrid oligonucleotide was ligated to the tRNA extracted from the RqcH-50S samples and total *E. faecalis* tRNA (from the lysate), respectively. Labeled tRNA was purified by phenol:chloroform extraction and loaded on a microarray containing 24 replicates of full-length tDNA probes recognizing *E. faecalis* tRNA isoacceptors. Flourescence signals were normalized to three *in vitro* transcribed human tRNAs, spiked in to each sample. Microarrays were statistically analysed with in-house scripts written in Python 3.7.0.

**Grid preparation, cryo-electron microscopy and single-particle reconstruction**

**Preparation of cryo-EM grids and data collection**

Elutions from LsaA and VgaL pull-downs were loaded on grids within two hours after obtaining them without freezing, samples were kept on ice. The VgaAC sample was frozen in liquid nitrogen after pull-down, defrosted and loaded later. After glow-discharging of grids, 3.5 mL of sample was loaded on grids in Vitrobot (FEI) in conditions of 100% humidity at 4 °C, blotted for 5 seconds and vitrified by plunge-freezing in liquid ethane. Samples were imaged on a Titan Krios (FEI) operated at 300 kV at a nominal magnification of 165 k× (0.86 Å/pixel, later estimated to be 0.82 Å/pixel by comparing refined maps to structures with known magnification) with a Gatan K2 Summit camera at an exposure rate of 5.80 electrons/pixel/s with a 4 seconds exposure and 20 frames using the EPU software. Quantifoil 1.2/1.3 Cu200 grids were used for LsaA and VgaAC and Quantifoil 2/2 Cu200 grids were used for VgaL.

**Single-particle reconstruction**

Motion correction was performed with MotionCor2 with 5×5 patches (Zheng et al., 2017). Relion 3.0 or 3.1 was used for further processing unless otherwise stated and resolutions are reported according to the so-called ‘gold standard’ criteria (Henderson et al., 2012; Scheres & Chen, 2012; Zivanov et al., 2018). CTFFIND4 (LsaA dataset) or Gctf v1.06 (VgaAC and VgaL datasets) was used for CTF estimation (Rohou & Grigorieff, 2015; Zhang, 2016). Particles were picked with Gautomatch (https://www2.mrc-lmb.cam.ac.uk/research/locally-developed-software/zhang-software/gauto, developed by K. Zhang) without supplying a reference, and in the case of LsaA, re-picked using RELION autopicker after templates were generated by 2D classification. Particles were initially extracted at 2.46 Å/pixel and subjected to 2D classification. Classes that resembled ribosomes were used for 3D refinement, with a 60 Å low-pass filter applied to initial references. For 3D refinement of LsaA–70S, the initial reference was EMDB-0176, a *B. subtilis* 70S ribosome with no factor bound in the E-site (Crowe-McAuliffe et al., 2018); for VgaAC–70S and VgaL–70S 3D refinements the RELION initial
A model job type was used to create a reference from particles selected after 2D classification. 3D classification was performed without angular sampling, and classes of interest were re-extracted at 0.82 Å/pixel for further refinement.

In the case of LsaA, after initial 3D classification, a soft mask around the A-site was used for partial signal subtraction followed by focused classification. The classes with the strongest and weakest A-site density were selected for signal restoration and refinement. In the case of the VgaA\textsubscript{LC} dataset, initial 3D classification yielded a class with apparent sub-stoichiometric density in the E-site corresponding to VgaA\textsubscript{LC}. Micrographs with poor values from CTF estimation were discarded, particles were re-extracted, subjected to an additional 2D classification and 3D refinement, followed by Bayesian polishing and CTF refinement. An additional 3D classification yielded a class with strong E-site density corresponding to the factor. Refer to Figures S4–S6 for details.

For multibody refinements, soft masks around the small subunit body, small subunit head, and large subunit/ARD were applied. In the case of the VgaA\textsubscript{LC} dataset, particles were first re-extracted in a smaller box (360×360 pixels) and subjected to 3D refinement prior to multibody refinement.

ResMap was used to estimate local resolution (Kucukelbir \textit{et al.}, 2014). Maps were locally filtered using SPHIRE (Moriya \textit{et al.}, 2017).

\textit{Molecular modelling}

For the \textit{E. faecalis} and \textit{L. monocytogenes} ribosomes, homology models were generated with SWISS-MODEL (Waterhouse \textit{et al.}, 2018), mostly from PDB 6HA1/6HA8 (Crowe-McAuliffe \textit{et al.}, 2018). PDBs 4YBB (Noeske \textit{et al.}, 2015) 5MDV (James \textit{et al.}, 2016) were used as additional templates and references where necessary, 4V9O (Pulk & Cate, 2013) was used for bS21, 5ML7 (Gabdulkhakov \textit{et al.}, 2017) and 3U4M (Tischchenko \textit{et al.}, 2012) were used for the L1 stalk region, 5AFI (Fischer \textit{et al.}, 2015) and 5UYQ (Loveland \textit{et al.}, 2017) were used for tRNAs, and 6QNQ was used to help placing metal ions (Rozov \textit{et al.}, 2019). PDB 5L10 (Khusainov \textit{et al.}, 2016) was used as a starting model for the \textit{S. aureus} ribosome. Where appropriate, individual components of multibody refinements were fitted into density from the corresponding locally filtered map to help modelling. Models were adjusted with Coot (Casañal \textit{et al.}, 2020) and refined using locally filtered maps in Phenix version 1.14 3260 (Liebschner \textit{et al.}, 2019).

Figures were created with PyMOL 2.0 (Schrödinger, LLC), UCSF Chimera (Pettersen \textit{et al.}, 2004), RELION (Zivanov \textit{et al.}, 2018), and Igor Pro (WaveMetrics, Inc.). Structures were aligned in PyMOL using the 23S rRNA unless otherwise noted.
Figures were assembled with Adobe Illustrator (Adobe Inc.).

Supplementary Material

Figure S1. Characterization of *E. faecalis* LsaA interactions with ribosomes and preparation of samples for cryo-EM. (A) Polysome profiles and immunoblot analyses of C-terminally His$_6$-TEV-FLAG$_3$-tagged (HTF) ATPase-deficient (EQ$_2$) LsaA-EQ$_2$ ectopically expressed in ΔlsaA *E. faecalis* TX5332. Experiments were performed both in the presence or absence of 0.75 mM ATP in gradients. (B, C) Affinity purification of wild-type and EQ$_2$ *E. faecalis* LsaA-HTF ectopically expressed in TX5332 *E. faecalis*. Pull-down experiments were performed either in the presence (B) or absence (C) of 0.75 mM ATP using clarified lysates of *E. faecalis* either transformed with empty pCIE vector (background control), expressing *E. faecalis* LsaA-HTF (VHp100) or expressing *E. faecalis* LsaA-EQ$_2$-HTF (VHp149). Samples: M: molecular weight marker; Lys: 2 μL of clarified lysate, FT: 2 μL of...
flow-through; W5: 10 μL of last wash before specific elution; E1: 10 μL of the first elution with FLAG$_3$ peptide; E2: 10 μL of the second elution with FLAG$_3$ peptide; B: 10 μL of SDS-treated post-elution anti-FLAG beads; 70S: purified *E. faecalis* 70S ribosomes. The samples were resolved on 15% SDS-PAGE gel. The 0.75 mM ATP *E. faecalis* LsaA-EQ$_2$-HTF puldown sample was used for further cryo-EM and tRNA array analysis.
Figure S2. Characterization of *S. haemolyticus* VgaA<sub>LC</sub> interactions with ribosomes and reparation of samples for cryo-EM reconstructions. (A) Polysome profiles and immunoblot analyses of FLAG<sub>3</sub>-tagged *S. haemolyticus* VgaA<sub>LC</sub>-EQ<sub>2</sub> ectopically expressed in wild-type SH-1000 *S. aureus*. Experiments were performed both in the presence or absence of 0.5 mM ATP in gradients. (B) Affinity purification of wild-type and EQ<sub>2</sub> *S. haemolyticus* VgaA<sub>LC</sub>-FLAG<sub>3</sub> ectopically expressed in SH-1000 *S. aureus*. Immunoprecipitations were performed in the presence of 0.5 mM ATP and the samples were resolved on a 15% polyacrylamide gel by SDS-PAGE. Samples: M: 2 μL of molecular weight marker; FT: 2 μL of flow-through, W: 10 μL of last wash before specific elution; E: 10 μL of elution with FLAG<sub>3</sub> peptide; B: 2 μL of SDS-treated post-elution anti-FLAG beads; 70S: 1 pmol of purified *S. aureus* 70S ribosomes. The 0.5 mM ATP *S. haemolyticus* VgaA<sub>LC</sub>-EQ<sub>2</sub>-HTF pulldown sample was used for cryo-EM reconstructions.
**Figure S3.** Characterization of *L. monocytogenes* VgaL (Lmo0919) interactions with ribosomes and reparation of samples for cryo-EM reconstructions. (A) Polysome profiles and immunoblot analyses of HTF-tagged *L. monocytogenes* VgaL-EQ2 (Lmo0919-EQ2) ectopically expressed in wild-type EDG-e *L. monocytogenes*. Experiments were performed both in the presence or absence of 0.5 mM ATP in gradients. (B) Affinity purification of *L. monocytogenes* VgaL-EQ2 ectopically expressed in EDG-e *L. monocytogenes*. Pull-down experiments were performed in the presence of 0.5 mM ATP using clarified lysates of *L. monocytogenes* transformed with empty integrative pIMK3 vector (background control), expressing VgaL-HTF (VHp692) or expressing VgaL-EQ2-HTF (VHp149). Samples: M: 2 μL of molecular weight marker; FT: 2 μL of flow-through; W: 10 μL of last wash before specific elution; E: 10 μL of elution with FLAG3 peptide; B: 2 μL of SDS-treated post-elution anti-FLAG beads; 70S: purified *B. subtilis* 70S ribosomes, the samples were resolved on 15% SDS-PAGE gel. The 0.5 mM ATP *L. monocytogenes* VgaL-EQ2-HTF pulldown sample was used for cryo-EM reconstructions.
Figure S4. Processing of the cryo-EM data of LsaA-70S complex. (A) Processing scheme for the LsaA-70S complex, yielding two subpopulations of LsaA-70S complexes with and without A-site tRNA. (B, C) Fourier Shell Correlation (FSC) curves of the LsaA-70S (B) with A-tRNA and (C) without A-tRNA with a dashed line at 0.143 indicating average resolutions of 3.1 Å and 2.9 Å, respectively. (D) Overview (left) and transverse section (right) of the cryo-EM map of the LsaA-70S (without A-tRNA) coloured according to local resolution. (E) Isolated density of LsaA (left) and P-site tRNA (right) from (D). (F) Isolated density of LsaA, P-site and A-site tRNA from the LsaA-70S map (with A-tRNA) coloured according to local resolution.
Figure S5. Processing of the cryo-EM data of VgaA_{LC}–70S complex. (A) Processing scheme for the LsaA–70S complex, yielding a VgaA_{LC}–70S and 70S–P-tRNA complex without VgaA_{LC}. (B) Fourier Shell Correlation (FSC) curves of the VgaA_{LC}–70S and 70S–P-tRNA complexes with a dashed line at 0.143 indicating average resolutions of 3.1 Å. (C, D) Overview (left) and transverse section (right) of the cryo-EM map of the (C) VgaA_{LC}–70S and (D) 70S–P-tRNA complexes coloured according to local resolution. (E) Isolated density of VgaA_{LC} (left) and P-site tRNA (right) from the VgaA_{LC}–70S complex, and the P-site-tRNA from the 70S–P-tRNA complex, coloured according to local resolution.
Figure S6. Processing of the cryo-EM data of VgaL–70S complex. (A) Processing scheme for the VgaL–70S complex. (B) Fourier Shell Correlation (FSC) curves of the VgaL-70S complex with a dashed line at 0.143 indicating average resolutions of 2.9 Å. (C) Overview (left) and transverse section (right) of the cryo-EM map of the VgaL–70S complex coloured according to local resolution. (D) Isolated density of VgaL (left) and P-site tRNA (right) from the VgaL–70S complex coloured according to local resolution.
Figure S7. ATP in the ARE-bound 70S structures. Model and density surrounding the innermost ATP bound by LsaA (A), VgaA\textsubscript{LC} (B), and VgaL (C) viewed from the direction of the signature sequence of NBD2 (model and density not shown). A black outline highlights a magnesium ion. D–F, as for A–C except for the peripheral nucleotide-binding site viewed from the direction of the signature sequence of NBD1 (model and density not shown). Density from post-processed maps is shown.
**Figure S8 LsaA, Vgaₐₐ and VgaL NBDs exhibit a closed conformation.** (A) The closed conformation of the multidrug transporter MRP1 (grey) with bound ADP molecules (blue, PDB 6BHU) (Johnson & Chen, 2018). (B, C) Alignment (based on NBD1) and superimposition of the closed conformation of MRP1 from (A) with the ABC domains of (B) Rli1p (cyan) in closed conformation with bound ADPNP (red, PDB 5LL6) (Heuer et al, 2017), (C) ABCE1 (brown) in open conformation with bound ADP (red, PDB 3J15) (Becker et al, 2012), and with (D–F) closed ARE ABCF NBD conformations with bound ATP (red) for (D) LsaA (green), (E) Vgaₐₐ (magenta) and (F) VgaL (yellow).
Figure S9. Presence of A-site tRNA in the LsaA-70S complex. (A) Cryo-EM map density for LsaA (green), P-site tRNA (cyan) and A-site tRNA (brown) in the LsaA–70S complex with A-site tRNA. Density for small subunit (yellow) is shown for reference. Density for the large subunit is not shown. (B) The same view as A, except with molecular models. The brown dashed line indicates a likely path for the 3’ CCA end of the distorted A-tRNA. A pre-accommodation A/T tRNA (pink, PDB 4V5L) (Voorhees et al., 2010) is superimposed. The position of the lincomycin binding site (red dotted circle) is shown for comparison (PDB 5HKV) (Matzov et al., 2017). (C) Similar to (B) except with classical accommodated A- and P-site tRNAs from pre-attack state superimposed (both grey, PDB 1VY4) (Polikanov et al., 2014).
**Figure S10. C-terminal extensions of VgaA<sub>LC</sub> and VgaL on the small subunit. (A-C)**

Cryo-EM map (grey) with molecular model for (B–C) VgaA<sub>LC</sub>–70S complex, and (C) VgaL–70S complex, showing density for L1 stalk (grey) on the large subunit, and ribosomal proteins S7 (blue), S11 (green) as well as the SD–anti-SD helix on the small subunit (yellow).

In (A) and (B), density for the C-terminal extension (CTE) of VgaA<sub>LC</sub> (magenta mesh) is fragmented, and in (B) fitted with the model of the CTE from VmlR (orange, PDB 6HA8) (Crowe-McAuliffe et al., 2018) based on alignment of the NBDs. In (C), density for the C-terminal extension (CTE) of VgaL (yellow mesh) also reaches between the S7–S11 cleft and is consistent with an α-helical conformation, but appears to be distinct from VmlR and VgaA<sub>LC</sub> and could not be modelled at this resolution.
Figure S11. Effect of amino acid substitutions in ARD on antibiotic resistance in LsaA.

Growth of *B. subtilis ΔvmlR* expressing the indicated LsaA variants over time in the presence of lincomycin (A), tiamulin (B), and virginiamycin M (C). *B. subtilis* strains (VHB109, 168 and 169) were grown in LB media with 1 mM IPTG at 37 °C with medium shaking. At the 90 minutes time point (OD$_{600}$ ≈ 0.1) antibiotics were added to the final concentrations as indicated on the figure. The SD of three biological replicates is indicated with pale shading.
Figure S12. Visualisation of tested mutations in VgaA_{LC} and LsaA. Residues in blue did not affect antibiotic resistance when mutated to alanine, and residues in yellow reduced antibiotic resistance when mutated to alanine. A–C, three views of the LsaA ARD with selected E. faecalis 23S 23S rRNA nucleotides shown. D–F, three views of the VgaA_{LC} ARD with selected S. aureus 23S 23S rRNA nucleotides shown. See also Tables S1 and S2.
Figure S13. Comparison of A2602 position between ribosomes with and without bound AREs. (A) A2602 with accommodated A- and P-site tRNAs in the ‘pre-attack’ state (PDB 1VY4) (Polikanov et al., 2014) (B) Conformation of A2602 with bound LsaA with 23S rRNA from (A). (C–F) Similar to (B), except for VmlR, VgaA\textsubscript{LC}, VgaL, and MsrE (Crowe-McAuliffe \textit{et al.}, 2018; Su \textit{et al.}, 2018).
Figure S14. Conformation of the PTC in the presence of VgaA\textsubscript{LC} and antibiotics. (A–C)
The conformation of selected 23S rRNA nucleotides at the PTC in the presence of either (A) tiamulin (Tia, purple, PDB 1XBP) (Schlünzen \textit{et al.}, 2004), (B) virginiamycin M (VgM, green, PDB 1YIT) (Tu \textit{et al.}, 2005), or (C) lincomycin (Lnc, tan, PDB 5HKV) (Matzov \textit{et al.}, 2017). Left panels show the antibiotic-bound structures only, right panels have superimposed nucleotides and protein from the VgaA\textsubscript{LC}-bound ribosome (pink). (D–F) As for A–C, except with focus on U2585. Red arrows indicate significant shifts in nucleotide positions from antibiotic-bound to VgaA\textsubscript{LC}-bound ribosomes.
**Figure S15. Conformation of the PTC in the presence of VgaL and antibiotics.** The conformation of selected 23S rRNA nucleotides at the PTC in the presence of either (A) tiamulin (Tia, purple, PDB 1XBP) (Schlünzen et al., 2004), (B) virginiamycin M (VgM, green, PDB 1YIT) (Tu et al., 2005), or (C) lincomycin (Lnc, tan, PDB 5HKV) (Matzov et al., 2017). Left panels show the antibiotic-bound structures only, right panels have superimposed nucleotides and protein from the VgaL-bound ribosome (yellow). (D–F) As for A–C, except with focus on U2585. Red arrows indicate significant shifts in nucleotide positions from the antibiotic-bound to VgaL-bound ribosome. An asterisk indicates low confidence in the position of U2585 due to weak density.
**Figure S16. Conformation of the PTC in the presence of LsaA and antibiotics. (A–C)**

The conformation of selected 23S rRNA nucleotides at the PTC in the presence of either (A) tiamulin (Tia, purple, PDB 1XBP) (Schlünzen et al., 2004), (B) virginiamycin M (VgM, green, PDB 1YIT) (Tu et al., 2005), or (C) lincomycin (Lnc, tan, PDB 5HKV) (Matzov et al., 2017).

Left panels show the antibiotic-bound structures only, right panels have superimposed nucleotides and protein from the LsaA-bound ribosome (green). (D–F) As for A–C, except with focus on U2585 (D, E) or U2504 (F). Red arrows indicate significant shifts in nucleotide positions from antibiotic-bound to LsaA-bound ribosomes, and red crosses indicate significant overlap between the lincomycin-binding site and U2504 in the LsaA-bound ribosome. An asterisk indicates low confidence in the position of U2585 due to weak density.
Table S1. Minimum inhibitory concentrations (MICs) of ribosome-targeting antibiotics against *E. faecalis* expressing LsaA. 5 x 10^5 CFU/mL (OD$_{600}$ approximately 0.0005) of either *E. faecalis* OG1RF, ΔlsaA (*lsa::Kan*) strain TX5332 transformed with empty pCIE$_{spec}$ plasmid, or with pCIE$_{spec}$ derivative for expression of LsaA was used to inoculate BHI media supplemented with 2 mg/mL kanamycin to prevent *lsa* revertants, 0.1 mg/mL spectinomycin to maintain the pCIE$_{spec}$ plasmid, 100 ng/mL of cCF10 peptide to induce expression of LsaA as well as increasing concentrations of antibiotics. After 16-20 hours at 37 °C without shaking, the presence or absence of bacterial growth was scored by eye. The MIC values that exceed the empty vector control are shown in bold.

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<th>E. faecalis TX5332 pCIE$_{spec}$ <em>lsaA</em> (VHp431)</th>
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Table S2. Minimum inhibitory concentrations (MICs) of ribosome-targeting antibiotics against *S. aureus* expressing V\(g\)a\(_{LC}\) *S. aureus* strain SH1000, harbouring empty vector pRMC2 or pRMC2 expressing wild-type vga\(_{LC}\) or its mutants.

<table>
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<th>Construct (mutation)</th>
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<th>tiamulin</th>
<th>retapamulin</th>
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<td>0.25</td>
<td>0.06</td>
<td>1</td>
</tr>
<tr>
<td>pRMC2: vga(_{LC}) (S226A)</td>
<td>16</td>
<td>2</td>
<td>16</td>
<td>8</td>
<td>4</td>
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<tr>
<td>pRMC2: vga(_{LC}) (K227A)</td>
<td>4</td>
<td>0.25</td>
<td>1</td>
<td>0.125</td>
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<tr>
<td>pRMC2: vga(_{LC}) (G228A)</td>
<td>8</td>
<td>2</td>
<td>8</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>pRMC2: vga(_{LC}) (K229A)</td>
<td>16</td>
<td>2</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>pRMC2: vga(_{LC}) (K230A)</td>
<td>16</td>
<td>2</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>pRMC2: vga(_{LC}) (R232A)</td>
<td>16</td>
<td>2</td>
<td>8</td>
<td>2</td>
<td>4</td>
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**Table S3. Minimum inhibitory concentrations (MICs) of ribosome-targeting antibiotics against *L. monocytogenes* EDG-e expressing VgaL (Lmo0919).** 5 x 10^5 CFU/mL (approximately OD<sub>600</sub> 0.0003) of *L. monocytogenes* EDGe, Δlmo0919 (markerless) strain with integrated empty pIMK3 plasmid, or with pIMK3 encoding VgaL or VgaL-HTF was used to inoculate BHI media supplemented with 50 µg/mL kanamycin to maintain the integrative pIMK3 plasmid, 1 mM IPTG to induce expression of VgaL as well as increasing concentrations of antibiotics. After 16-20 hours at 37 °C without shaking, the presence or absence of bacterial growth was scored by eye. The MIC values that exceed the empty vector control lacking chromosomal *lmo0919* are shown in bold.

<table>
<thead>
<tr>
<th>Antibiotic class</th>
<th>Antibiotic</th>
<th>L. monocytogenes EDGe::pIMK3</th>
<th>L. monocytogenes EDGe::Δlmo0919 pIMK3</th>
<th>L. monocytogenes EDGe::Δlmo0919 pIMK3 vgaL</th>
<th>L. monocytogenes EDGe::Δlmo0919 pIMK3 vgaL&lt;sub&gt;HTF&lt;/sub&gt;</th>
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</thead>
<tbody>
<tr>
<td>Phenicols</td>
<td>Chloramphenicol</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
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<tr>
<td>Oxazolidinones</td>
<td>Linezolid</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Macrolides</td>
<td>Erythromycin</td>
<td>&lt; 0.125</td>
<td>&lt; 0.125</td>
<td>&lt; 0.125</td>
<td>&lt; 0.125</td>
</tr>
<tr>
<td>Lincosamides</td>
<td>Lincomycin</td>
<td>2</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
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<tr>
<td>Pleuromutilins</td>
<td>Tiamulin</td>
<td>16-32</td>
<td>0.125</td>
<td>32</td>
<td>16-32</td>
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<tr>
<td>Streptogramins</td>
<td>Virginiamycin M1</td>
<td>32</td>
<td>4-8</td>
<td>64</td>
<td>32</td>
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<tr>
<td></td>
<td>Virginiamycin S1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Tetracycline</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
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Table S4. Cryo-EM data collection, modelling and refinement statistics.

<table>
<thead>
<tr>
<th></th>
<th><em>E. faecalis</em> 70S–LsaA</th>
<th><em>S. aureus</em> 70S–P-tRNA</th>
<th><em>S. aureus</em> 70S–VgaA&lt;sub&gt;e&lt;/sub&gt;</th>
<th><em>L. monocytogenes</em> 70S–VgaL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data collection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Magnification (×)</td>
<td>130 000</td>
<td>165 000</td>
<td>165 000</td>
<td>165 000</td>
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<tr>
<td>Electron dose (e&lt;sup&gt;−&lt;/sup&gt;/Å&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>38.0</td>
<td>26.3</td>
<td>26.3</td>
<td>28.28</td>
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<tr>
<td>Defocus range (µm)</td>
<td>−0.7–2.2</td>
<td>−0.7–1.9</td>
<td>−0.7–1.9</td>
<td>−0.8–2.0</td>
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<tr>
<td>Pixel size (Å)</td>
<td>1.041</td>
<td>0.82</td>
<td>0.82</td>
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<tr>
<td>Initial particles</td>
<td>61 009</td>
<td>165 827</td>
<td>165 827</td>
<td>83 340</td>
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<tr>
<td>Final particles</td>
<td>59 262</td>
<td>61 910</td>
<td>35 129</td>
<td>45 548</td>
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<tr>
<td>Average resolution (Å)</td>
<td>2.9</td>
<td>3.1</td>
<td>3.1</td>
<td>2.9</td>
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<tr>
<td><strong>Model composition</strong></td>
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<tr>
<td>Atoms</td>
<td>144 982</td>
<td>139 909</td>
<td>145 651</td>
<td>144 492</td>
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<tr>
<td>Protein residues</td>
<td>5 753</td>
<td>5 330</td>
<td>5 783</td>
<td>5 715</td>
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<tr>
<td>RNA bases</td>
<td>4 627</td>
<td>4554&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4 647</td>
<td>4617</td>
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<tr>
<td><strong>Refinement</strong></td>
<td></td>
<td></td>
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<tr>
<td>Map CC around atoms</td>
<td>0.85</td>
<td>0.89</td>
<td>0.88</td>
<td>0.86</td>
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<tr>
<td>Map CC whole unit cell</td>
<td>0.85</td>
<td>0.88</td>
<td>0.87</td>
<td>0.85</td>
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<tr>
<td>Map sharpening B</td>
<td>−35.42</td>
<td>−56.43</td>
<td>−62.31</td>
<td>−68.16</td>
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<td>R.M.S. deviations</td>
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<tr>
<td>Bond lengths (Å)</td>
<td>0.008</td>
<td>0.010</td>
<td>0.009</td>
<td>0.014</td>
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<td>Bond angles (°)</td>
<td>0.906</td>
<td>0.947</td>
<td>0.933</td>
<td>1.082</td>
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<td><strong>Validation</strong></td>
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<tr>
<td>MolProbity score</td>
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<td>1.73</td>
<td>1.76</td>
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<tr>
<td>Clash score</td>
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<td>3.87</td>
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<td>Poor rotamers (%)</td>
<td>0.02</td>
<td>0.07</td>
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<td>Ramachandran</td>
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<td>Favoured (%)</td>
<td>90.48</td>
<td>89.88</td>
<td>89.64</td>
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<tr>
<td>Outlier (%)</td>
<td>0.16</td>
<td>0.02</td>
<td>0.05</td>
<td>0.02</td>
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*23S rRNA helices H76–H78 of the L1 stalk were flexible and not modelled.
**Table S5. Strains and Plasmids used in this study.** Plasmid and strain construction is described in detail in supplemental text. *Denotes a plasmid constructed by the PEP facility at Umeå University.

<table>
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<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
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</thead>
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<tr>
<td><em>L. monocytogenes</em> EDGE</td>
<td>Wild-type serotype 1/2a strain</td>
<td>(Glaser et al., 2001)</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> EGDe::pIMK3</td>
<td>EGDe with empty pIMK3 plasmid containing P_{help} promoter integrated at tRNA^{Arg} locus</td>
<td>This work</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> EGDe::pIMK3{lmo0919}HTF</td>
<td>EGDe with VgaL-HTF overexpressed from the P_{help} promoter integrated at tRNA^{Arg} locus</td>
<td>This work</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> EGDe::pIMK3{lmo0919}EQ2-HTF</td>
<td>EGDe with VgaL EQ2-HTF overexpressed from the P_{help} promoter integrated at tRNA^{Arg} locus</td>
<td>This work</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> EDGe::Δlmo0919</td>
<td>EGDe harboring a <em>lmo0919</em> marker less deletion lacking VgaL</td>
<td>This work</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> EDGe::Δlmo0919::pIMK3</td>
<td>EGDe::Δlmo0919 with empty pIMK3 plasmid containing P_{help} promoter integrated at tRNA^{Arg} locus</td>
<td>This work</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> EDGe::Δlmo0919::pIMK3{lmo0919}</td>
<td>EGDe::Δlmo0919 with VgaL overexpressed from the P_{help} promoter integrated at tRNA^{Arg} locus</td>
<td>This work</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> EDGe::Δlmo0919::pIMK3{lmo0919}HTF</td>
<td>EGDe::Δlmo0919 with VgaL-HTF overexpressed from the P_{help} promoter integrated at tRNA^{Arg} locus</td>
<td>This work</td>
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<td><em>L. monocytogenes</em> EDGe::Δlmo0919::pIMK3{lmo0919}HTF-EQ2</td>
<td>EGDe::Δlmo0919 with VgaL-EQ2-HTF overexpressed from the P_{help} promoter integrated at tRNA^{Arg} locus</td>
<td>This work</td>
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<tr>
<td><em>E. faecalis</em> OG1RF</td>
<td>Rif(^r); Fus(^r); WT <em>E. faecalis</em></td>
<td>(Singh et al., 2002)</td>
</tr>
<tr>
<td><em>E. faecalis</em> TX5332</td>
<td>Rif(^r); Fus(^r); Kan(^r); <em>lsa</em> gene disruption mutant (OG1RF <em>lsa</em>::pTEX4577)</td>
<td>(Davis et al., 2001)</td>
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<td><em>S. aureus</em> SH1000</td>
<td>Functional <em>rsbU</em>(^r) derivative of <em>S. aureus</em> 8325-4</td>
<td>(Horsburgh et al., 2002; O’Neill, 2010)</td>
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<td><em>E. coli</em> S17.1</td>
<td><em>E. coli</em> strain used for conjugative plasmid transfer to <em>L. monocytogenes</em></td>
<td>(Simon et al., 1983)</td>
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<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Reference</td>
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<tr>
<td>-------------</td>
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<td>pIMK3</td>
<td>Kan’; Listerial tRNA&lt;sup&gt;419&lt;/sup&gt; locus specific integrative vector for high-level IPTG-induced protein expression from the P&lt;sub&gt;help&lt;/sub&gt; promoter</td>
<td>(Monk et al, 2008)</td>
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<tr>
<td>pMAD</td>
<td>Amp’, Ery’; lacZ, thermosensitive shuttle vector used for allelic exchange in &lt;i&gt;L. monocytogenes&lt;/i&gt;</td>
<td>(Arnaud et al., 2004)</td>
</tr>
<tr>
<td>pHT009</td>
<td>Amp’, Km’; thrC locus specific integrative vector for high-level IPTG-induced protein expression from the P&lt;sub&gt;hy-snak&lt;/sub&gt; promoter</td>
<td>(Crowe-McAuliffe et al., 2018)</td>
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<td>VHp689</td>
<td>pMAD Δ&lt;i&gt;imo0919&lt;/i&gt;</td>
<td>This work</td>
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<tr>
<td>V Hp690</td>
<td>pIMK3;&lt;i&gt;imo0919&lt;/i&gt;</td>
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</tr>
<tr>
<td>V Hp692</td>
<td>pIMK3;&lt;i&gt;imo0919-HTF&lt;/i&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>V Hp693</td>
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<td>pTX5333</td>
<td>Cm’; &lt;i&gt;E. faecalis&lt;/i&gt;-&lt;i&gt;E. coli&lt;/i&gt; shuttle plasmid expressing LsaA from native promoter</td>
<td>(Singh et al., 2002)</td>
</tr>
<tr>
<td>pCIE</td>
<td>Cm’; &lt;i&gt;E. faecalis&lt;/i&gt;-&lt;i&gt;E. coli&lt;/i&gt; shuttle plasmid for cCF10 induced expression of proteins</td>
<td>(Weaver et al., 2017)</td>
</tr>
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<td>VHp100</td>
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<td>V Hp369</td>
<td>pHT009;&lt;i&gt;lsaA&lt;/i&gt;</td>
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<td>V Hp426</td>
<td>pCIE, Sc’; Cm’ gene swapped to spectinomycin resistance (Sc’) gene</td>
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<td>V Hp431</td>
<td>V Hp426;&lt;i&gt;lsa&lt;/i&gt;</td>
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<td>V Hp526</td>
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<td>pRMC2</td>
<td>Amp’, Cm’, &lt;i&gt;E. coli&lt;/i&gt; - &lt;i&gt;S. aureus&lt;/i&gt; shuttle plasmid for tetracycline-regulable expression of proteins in the latter host.</td>
<td>(Corrigan &amp; Foster, 2009)</td>
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<td>pRMC2::&lt;i&gt;vgaA-FLAG&lt;/i&gt;&lt;sub&gt;3&lt;/sub&gt;</td>
<td>pRMC2 expressing C-terminally FLAG&lt;sub&gt;3&lt;/sub&gt; tagged Vga&lt;sub&gt;ALC&lt;/sub&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pRMC2::&lt;i&gt;vgaA-EQ2&lt;/i&gt;-&lt;i&gt;FLAG&lt;/i&gt;&lt;sub&gt;3&lt;/sub&gt;</td>
<td>pRMC2 expressing C-terminally FLAG&lt;sub&gt;3&lt;/sub&gt; tagged Vga&lt;sub&gt;ALC&lt;/sub&gt;-&lt;i&gt;E&lt;/i&gt;&lt;sub&gt;160Q&lt;/sub&gt;, &lt;i&gt;E&lt;/i&gt;&lt;sub&gt;410Q&lt;/sub&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pRMC2::&lt;i&gt;vgaALC&lt;/i&gt;</td>
<td>pRMC2 expressing wild-type Vga&lt;sub&gt;ALC&lt;/sub&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pRMC2::&lt;i&gt;vgaALC&lt;/i&gt;(&lt;i&gt;K&lt;/i&gt;&lt;sub&gt;208A&lt;/sub&gt;)</td>
<td>pRMC2 expressing Vga&lt;sub&gt;ALC&lt;/sub&gt;&lt;i&gt;K&lt;/i&gt;&lt;sub&gt;208A&lt;/sub&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pRMC2::&lt;i&gt;vgaALC&lt;/i&gt;(&lt;i&gt;S&lt;/i&gt;&lt;sub&gt;211A&lt;/sub&gt;)</td>
<td>pRMC2 expressing Vga&lt;sub&gt;ALC&lt;/sub&gt;&lt;i&gt;S&lt;/i&gt;&lt;sub&gt;211A&lt;/sub&gt;</td>
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<td>pRMC2::&lt;i&gt;vgaALC&lt;/i&gt;(&lt;i&gt;S&lt;/i&gt;&lt;sub&gt;212A&lt;/sub&gt;)</td>
<td>pRMC2 expressing Vga&lt;sub&gt;ALC&lt;/sub&gt;&lt;i&gt;S&lt;/i&gt;&lt;sub&gt;212A&lt;/sub&gt;</td>
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<td>pRMC2::&lt;i&gt;vgaALC&lt;/i&gt;(&lt;i&gt;S&lt;/i&gt;&lt;sub&gt;213A&lt;/sub&gt;)</td>
<td>pRMC2 expressing Vga&lt;sub&gt;ALC&lt;/sub&gt;&lt;i&gt;S&lt;/i&gt;&lt;sub&gt;213A&lt;/sub&gt;</td>
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<td>pRMC2::&lt;i&gt;vgaALC&lt;/i&gt;(&lt;i&gt;K&lt;/i&gt;&lt;sub&gt;216A&lt;/sub&gt;)</td>
<td>pRMC2 expressing Vga&lt;sub&gt;ALC&lt;/sub&gt;&lt;i&gt;K&lt;/i&gt;&lt;sub&gt;216A&lt;/sub&gt;</td>
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<td>pRMC2::&lt;i&gt;vgaALC&lt;/i&gt;(&lt;i&gt;K&lt;/i&gt;&lt;sub&gt;218A&lt;/sub&gt;)</td>
<td>pRMC2 expressing Vga&lt;sub&gt;ALC&lt;/sub&gt;&lt;i&gt;K&lt;/i&gt;&lt;sub&gt;218A&lt;/sub&gt;</td>
<td>This work</td>
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<tr>
<td>pRMC2::&lt;i&gt;vgaALC&lt;/i&gt;(&lt;i&gt;V&lt;/i&gt;&lt;sub&gt;219A&lt;/sub&gt;)</td>
<td>pRMC2 expressing Vga&lt;sub&gt;ALC&lt;/sub&gt;&lt;i&gt;V&lt;/i&gt;&lt;sub&gt;219A&lt;/sub&gt;</td>
<td>This work</td>
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<td>pRMC2::&lt;i&gt;vgaALC&lt;/i&gt;(&lt;i&gt;W&lt;/i&gt;&lt;sub&gt;223A&lt;/sub&gt;)</td>
<td>pRMC2 expressing Vga&lt;sub&gt;ALC&lt;/sub&gt;&lt;i&gt;W&lt;/i&gt;&lt;sub&gt;223A&lt;/sub&gt;</td>
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<td>pRMC2 expressing Vga&lt;sub&gt;ALC&lt;/sub&gt;&lt;i&gt;F&lt;/i&gt;&lt;sub&gt;224A&lt;/sub&gt;</td>
<td>This work</td>
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<td>pRMC2::&lt;i&gt;vgaALC&lt;/i&gt;(&lt;i&gt;S&lt;/i&gt;&lt;sub&gt;226A&lt;/sub&gt;)</td>
<td>pRMC2 expressing Vga&lt;sub&gt;ALC&lt;/sub&gt;&lt;i&gt;S&lt;/i&gt;&lt;sub&gt;226A&lt;/sub&gt;</td>
<td>This work</td>
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<td>pRMC2::&lt;i&gt;vgaALC&lt;/i&gt;(&lt;i&gt;K&lt;/i&gt;&lt;sub&gt;227A&lt;/sub&gt;)</td>
<td>pRMC2 expressing Vga&lt;sub&gt;ALC&lt;/sub&gt;&lt;i&gt;K&lt;/i&gt;&lt;sub&gt;227A&lt;/sub&gt;</td>
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<tr>
<td>pRMC2::&lt;i&gt;vgaALC&lt;/i&gt;(&lt;i&gt;G&lt;/i&gt;&lt;sub&gt;228A&lt;/sub&gt;)</td>
<td>pRMC2 expressing Vga&lt;sub&gt;ALC&lt;/sub&gt;&lt;i&gt;G&lt;/i&gt;&lt;sub&gt;228A&lt;/sub&gt;</td>
<td>This work</td>
</tr>
<tr>
<td><strong>pRMC2:vgaALC(K229A)</strong></td>
<td>pRMC2 expressing VgaALC K229A</td>
<td>This work</td>
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<tr>
<td><strong>pRMC2:vgaALC(K230A)</strong></td>
<td>pRMC2 expressing VgaALC K230A</td>
<td>This work</td>
</tr>
<tr>
<td><strong>pRMC2:vgaALC(A232A)</strong></td>
<td>pRMC2 expressing VgaALC A232A</td>
<td>This work</td>
</tr>
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