Antibiotic resistance ABCF proteins reset the peptidyl transferase centre of the ribosome to counter translational arrest

Victorii Murina1,2, Marje Kasari1, Vasili Hauryliuk1,2,3,4 and Gemma C. Atkinson1,*

1Department of Molecular Biology, Umeå University, 901 87 Umeå, Sweden, 2Laboratory for Molecular Infection Medicine Sweden (MIMS), Umeå University, 901 87 Umeå, Sweden and 3University of Tartu, Institute of Technology, 50411 Tartu, Estonia

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ABSTRACT

Several ATPases in the ATP-binding cassette F (ABCF) family confer resistance to macrolides, lincomycines and streptogramins (MLS) antibiotics. MLS are structurally distinct classes, but inhibit a common target: the peptidyl transferase (PTC) active site of the ribosome. Antibiotic resistance (ARE) ABCFs have recently been shown to operate through direct ribosomal protection, but the mechanistic details of this resistance mechanism are lacking. Using a reconstituted translational system, we dissect the molecular mechanism of Staphylococcus haemolyticus VgaALC and Enterococcus faecalis LsaA on the ribosome. We demonstrate that VgaALC is an NTPase that operates as a molecular machine strictly requiring NTP hydrolysis (not just NTP binding) for antibiotic protection. Moreover, when bound to the ribosome in the NTP-bound form, hydrolytically inactive EQ2 ABCF ARE mutants inhibit peptidyl transferase activity, suggesting a direct interaction between the ABCF ARE and the PTC. The likely structural candidate responsible for antibiotic displacement by wild type ABCF AREs, and PTC inhibition by the EQ2 mutant, is the extended inter-ABC domain linker region. Deletion of the linker region renders wild type VgaALC inactive in antibiotic protection and the EQ2 mutant inactive in PTC inhibition.

INTRODUCTION

Approximately half of the antibiotics currently in use for treating bacterial infections inhibit protein synthesis, predominantly by targeting key functional sites of the ribosome (1). To counteract antibiotics, bacteria have developed an array of resistance mechanisms (2). These mechanisms can be broadly classified into two categories. The first strategy is to decrease the intracellular concentration of the active antibiotic. This can be achieved by preventing antibiotic uptake (3), by actively excreting the antibiotic (4) or by inactivating the drug via chemical modification or degradation (5). The second strategy is to render the pathway that is targeted by the antibiotic immune to the drug. This can be achieved by mutation or modification of the antibiotic target (6). Alternatively, a resistance factor can directly interact with the antibiotic target to displace the antibiotic or prevent its binding in the first place, as exemplified by the ribosome protection factors TetO and TetM (7,8). These members of the EF2 family of translational GTPases share the domain structure of elongation factor EF-G (9). Like EF-G, Tet proteins bind to the A-site of the ribosome, where they sterically clash with tetracycline bound to the ribosome, thus effectuating antibiotic dissociation from its target (10,11).

Antibiotic resistance proteins in the ABC (ATP-binding cassette) superfamily of ATPases are well known for conferring resistance through the first of the two strategies, i.e. by reducing the concentration of the drug in the cell by pumping it out (4). However, the crucial component of the classical ABC pump, its transmembrane domain (12,13) is lacking in antibiotic resistance factors that belong to the ABCF subfamily of ABC ATPases (14) (BioRxiv: https://www.biorxiv.org/content/early/2017/11/16/220046). This raises the question of whether resistance is achieved instead via the second strategy described above i.e. via direct protection of the inhibited pathway.

Two archetypal antibiotic resistance (ARE) ABCF factors are VgaA and LsaA, which carry two nucleotide binding domains, separated by a linker region (Figure 1). Staphylococcal VgaA (standing for ‘virginiamycin A-like antibiotic resistance’) is a plasmid-encoded resistance factor described in Staphylococcus aureus (15), Staphylococcus epidermidis (16) and Staphylococcus haemolyticus (17). LsaA (standing for ‘lincomycin and streptogramin A-re...
Figure 1. Maximum Likelihood phylogenetic analysis and domain structure of confirmed bacterial ABCF AREs. The tree shows phylogenetic relationships between ABCF AREs from bacterial pathogens (pale yellow), such as \textit{S. haemolyticus} VgaALC and \textit{E. faecalis} LsaA (highlighted in bold) and bacterial antibiotic producers (pale green). All ABCFs contain two universal ABC domains (blue) separated by an inter-ABC domain linker (green). The first ABC can contain an additional sequence element corresponding to the ‘arm’ subdomain of EttA (32,33) (yellow), and a conserved C-terminal extension can also be present (turquoise). Protein sequences of ABCF AREs presented on the tree were retrieved from the CARD database (37) or from UniProt (36). Numbers on branches show bootstrap support from 100 replicates.

Resistance’ is encoded on the chromosome of \textit{Enterococcus faecalis} (18,19). The two terms, ‘virginiamycin A-like antibiotics’ and ‘streptogramin A’ are synonymous and refer to the same chemical group of compounds (20). VgaA and LsaA confer protection against the same antibiotic classes, streptogramin A and lincosamides (so-called \textit{LS} \textit{A} phenotype) and pleuromutulins, such as tiamulin, although the relative activity against different antibiotics varies (15–19,21,22). An extreme example is \textit{S. haemolyticus} VgaALC, which has a substrate specificity that is strongly shifted towards lincosamides, such as lincomycin and its derivative, clindamycin (17).

While chemically unrelated, pleuromutilin, streptogramin A and lincosamide antibiotics bind to a common target, the peptidyl transferase centre (PTC) of the large (50S) ribosomal subunit (23–29). The PTC is the catalytic centre of the ribosome, where during each iteration of the elongation cycle the amino acid moiety of an incoming aminoacyl-tRNA to the ribosomal A-site forms a peptide bond with the peptidyl-tRNA in the P-site (30). As the ARE ABCFs confer resistance to antibiotics that are chemically unrelated but share a binding target, it was hypothesized that AREs directly interact with the ribosome to displace the drug (14). A decade later this hypothesis has been experimentally substantiated both for \textit{S. aureus} VgaA and \textit{E. faecalis} LsaA (31). While the molecular mechanism of resistance is still unknown, an inter-ATPase domain linker region that protrudes towards the PTC in the ribosome-binding translation factor ABCF EttA (32,33) is extended in AREs (Supplementary Figure S1). Therefore, it was hypothesized that the linker could directly displace the antibiotic from the PTC (21,34). Indeed, mutations in
this region affect the antibiotic specificity of the ARE (21). These initial discoveries have set the stage for dissecting the system biochemically using a reconstituted translational system.

Here, we use antibiotic inhibition of transpeptidation of S. aureus 70S ribosomal initiation complexes and rescue by S. haemolyticus VgaALC to probe three aspects of the molecular mechanism of ARE ABCFs. First, we addressed the question of nucleotide substrate specificity. Not all the ABC enzymes are strict ATPases, and certain representatives are, essentially, NTPases that in addition to ATP can utilize GTP, CTP and UTP (35). Second, we clarified the functional role of NTP hydrolysis in the functional cycle of VgaALC. NTPases can operate either as a ‘molecular switch’ where NTP versus NDP binding alters the structural conformation of the enzyme thereby exerting its function, or as a ‘molecular machine’, which uses NTP to NDP hydrolysis to drive a ‘powerstroke’ fueling the performed mechanical work. Both versions are present in the ABC family (12). Finally, we probed structure-function relationships, focusing on the extended alpha-helical linker region connecting the two ATP cassette domains of antibiotic resistance ABCFs (Figure 1).

MATERIALS AND METHODS

Phylogenetic analysis

Sequences of previously documented ABCF AREs were retrieved from UniProt (36) and the Comprehensive Antibiotic Resistance Database (CARD) (37). Sequences were aligned with Mafft v7.164b with the l-ins-i and maximum likelihood phylogenetic analysis carried out with RAxML-HPC v.8 (38) on the CIPRES Science Gateway v3 (39) with 100 bootstrap replicates and the LG model of substitution. Alignment positions with > 50% gaps were excluded from the analysis.

Protein cloning, expression and purification

All cloning was performed by the Protein Expertise Platform at Umeå University. S. haemolyticus VgaALC ORF was PCR-amplified from pRB374 VgaALC plasmid (17) and sub-cloned to the pET24d expression vector with a C-terminal 6His tag preceded by a single glycine linker. E. faecalis LsaA ORF was PCR amplified from pTEX533 plasmid (18) and sub-cloned into pCA528 vector for 6His-SUMO-tagging (40). ATPase deficient (EQ3) mutants were generated by introducing E105Q and E410Q (VgaALC) or E142Q and E452Q (LsaA) point mutations. The linker deletion (∆L) VgaALC mutants were generated by preplacing K199-A226 with GSG in ether wt or EQ2 VgaALC.

Both LsaA and VgaALC (wt and mutants) were overexpressed in freshly transformed Escherichia coli BL21 DE3 Rosetta (Novagen). An overnight culture in LB supplemented with 50 mg/ml kanamycin and 25 mg/ml chloramphenicol was diluted to 0.06 in the same media, grown at 37°C until an OD600 of 0.6–0.7, induced with 1 mM IPTG (final concentration) and grown for 2 h at 30°C. The cells were harvested by centrifugation and resuspended in lysis buffer (VgaALC: 1 M NaCl, 100 mM Tris:HCl pH 7.5, 5 mM imidazole, 10 mM MgCl2, 2 mM β-mercaptoethanol, 10% glycerol; LsaA: 0.7 M KCl, 50 mM HEPES pH 7.5, 5 mM MgCl2, 10 mM imidazole, 2 mM β-mercaptoethanol). The column was washed with high salt buffer (buffer B) (VgaALC: 2 M NaCl, 100 mM Tris:HCl pH 7.5, 25 mM imidazole, 10 mM MgCl2, 2 mM β-mercaptoethanol; LsaA: 2 M KCl, 50 mM HEPES pH 7.5, 20 mM imidazole, 5 mM MgCl2, 2 mM β-mercaptoethanol), and the proteins were eluted with a gradient of 0.5 M imidazole buffer (buffer C) (0.7 M KCl, 0.5 M imidazole, 50 mM Tris:HCl pH 7.5, 10 mM MgCl2, 2 mM β-mercaptoethanol). The following polishing steps were different for 6His-tagged VgaALC and 6His-SUMO-tagged LsaA.

In the case of VgaALC, after HisTRAP chromatography the protein was buffer-exchanged on 10 MWCO centrificon (Amicon) into low salt buffer (buffer A) (50 mM Tris:HCl pH 7.5, 5 mM MgCl2, 2 mM β-mercaptoethanol) and 50% μg of Ulp1 per 1 mg of protein was added. After the 6His-SUMO tag was cut off during buffer exchange to loading buffer (buffer A2) (500 mM KCl, 50 mM HEPES pH 7.5, 5 mM MgCl2, 10% glycerol, 2 mM β-mercaptoethanol) and 35 μg of Ulp1 per 1 mg of protein was added. The 6His-SUMO-tag was cut off during buffer exchange to loading buffer (buffer A) (500 mM KCl, 50 mM HEPES pH 7.5, 5 mM MgCl2, 10% glycerol, 2 mM β-mercaptoethanol) on 3 MWCO centrificons (Amicon) (1 h at 19°C), the protein was passed through 1 ml HisTRAP HP column (GE Healthcare) pre-equilibrated with LsaA buffer A (see above). Flow-through fractions were collected, diluted to 300 mM KCl (final concentration) in dilution buffer and passed through an anion exchange column (HiPrep Q XL 16/10 20 ml, GE Healthcare) pre-equilibrated with mid-salt buffer (300 mM KCl, 50 mM HEPES pH 7.5, 5 mM MgCl2, 2 mM β-mercaptoethanol) on 10 MWCO centrificons (Amicon).

The purity of protein preparations was assessed by SDS-PAGE and spectrophotometrically (OD280/OD260 ratio of ≈1.8 for VgaALC and 1.6–1.8 for LsaA). The proteins were aliquoted and stored at −20°C.
Preparation of *S. aureus* and *E. faecalis* 70S ribosomes

LB (SH-1000 *S. aureus*) or BHI (OG1RF *E. faecalis*) liquid cultures (12 × 400 ml) were inoculated with an overnight culture to OD$_{600}$ of 0.05–0.06 and grown at 37°C with vigorous shaking. At OD$_{600}$ 2–2.5 (*S. aureus*) or 1.5 (*E. faecalis*), cells were pelleted at 4°C (TLA10.500 rotor, Beckman, 15 min at 5000–8000 rcf), resuspended with either LB (*S. aureus*) or cell opening buffer (20 mM Tris·HCl, 100 mM NH$_4$Cl, 20 mM Tris·HCl, pH 7.4), pelleted again in falcon tubes, frozen with liquid nitrogen and stored at −80°C.

*S. aureus* frozen cells (15 g) were opened by cryomilling (Spex Freezer Mill, 8 cycles at 14 fps frequency interspersed with 2 min rest-work intervals). The powder was melted on ice during 3 h before opening the tube in a class II biosafety cabinet, followed by addition of 50 ml of cell opening buffer (100 mM NH$_4$Cl, 15 mM Mg(OAc)$_2$, 0.5 mM EDTA, 3 mM β-mercaptoethanol, 20 mM Tris·HCl pH 7.5) supplemented with 0.4 mM Turbo DNase (Thermo Fisher Scientific), 0.1 mM PMSF and 35 μg/ml lysozyme and additional incubation on ice for 1 h. *Enterococcus faecalis* frozen cells (15 g) were directly resuspended in cell opening buffer and opened by high-pressure cell disrupter (Stansted Fluid Power) (350 MPa, three passages).

Lysed cells were clarified by centrifugation for 40 min at 40,000 rpm (Ti 45 rotor, Beckman), the supernatant loaded onto sucrose cushions (1.1 M sucrose, 500 mM NH$_4$Cl, 15 mM Mg(OAc)$_2$, 0.5 mM EDTA, 3 mM β-mercaptoethanol, 20 mM Tris·HCl pH 7.5) and centrifuged for 18–19 h at 30,000 rpm. Ribosomal pellets were dissolved in high salt buffer (500 mM NH$_4$Cl, 15 mM Mg(OAc)$_2$, 0.5 mM EDTA, 3 mM β-mercaptoethanol, 20 mM Tris·HCl pH 7.5 supplemented with 0.5–1 mM puromycin), incubated for 1 h at 4°C with gentle mixing and pelleted again (8 h at 35,000 rpm or 19 h at 28,000 rpm) through 40 ml sucrose cushions. Resultant ribosomal pellets were combined in 15 ml of overlay buffer (60 mM NH$_4$Cl, 15 mM Mg(OAc)$_2$, 0.25 mM EDTA, 3 mM β-mercaptoethanol, 20 mM Tris·HCl pH 7.5) and resolved on a 10–40% sucrose gradient in overlay buffer in a zonal rotor (Ti 15, Beckman, 17 h at 21,000 rpm). The peak containing 70S ribosomes was pelleted by centrifugation (20 h at 35,000 rpm), pure 70S dissolved in 1 ml of HEPES:Polymix buffer (20 mM HEPES:Polymix pH 7.5, 2 mM DTT, 5 mM Mg(OAc)$_2$, 95 mM KCl, 5 mM NH$_4$Cl, 0.5 mM CaCl$_2$, 8 mM putrescine, 1 mM spermidine) and 70S concentration measured spectrophotometrically (1 OD$_{260}$ = 23 nM of 70S). Ribosomes were aliquoted, frozen in liquid nitrogen and stored at −80°C.

Preparation of 70S initiation complexes (70S IC)

Initiation complexes were prepared by combining 70S ribosomes (final concentration of 4 μM) with IF2 (2 μM), IF1 (1.5 μM), IF3 (1.5 μM), 35S-fMet-tRNA$_{iMet}$ (6 μM), mRNA MF (6 μM), 5'-GGCAAGGGAGGUAAGGUCAA-3', 1 mM GTP and 2 mM DTT in 1× HEPES:Polymix buffer. The reaction mix was incubated at 37°C for 30 min, the ICs were pelleted through a sucrose cushion (1.1 M sucrose, HEPES:Polymix buffer 15 mM Mg$^{2+}$ final concentration) at 50,000 rpm during 2 h (TLS-55, Beckman), the pellet was dissolved in 100 μl of HEPES:Polymix buffer (5 mM Mg(OAc)$_2$), aliquoted, frozen in liquid nitrogen and stored at −80°C.

Puromycin reaction

The puromycin reaction was carried out at 37°C in HEPES:Polymix pH 7.5 2+ buffer (41). The biochemical system from purified *E. coli* components has been described earlier (42, 43). 35S-Met-puromycin release was followed using 10% TCA precipitation and centrifugation with scintillation counting of the supernatant (35S-Met-puromycin) and pellet (intact 35S-fMet-tRNA$_{iMet}$). The percentage of 35S-Methionine released from the 70S IC was calculated by dividing the signal from the supernatant by the sum of signals from supernatant and pellet. The data are presented as geometric means, and error bars represent the range of experimental values.

Detailed experimental protocols, buffer preparations, expression and preparation of initiation factors and Ulp1 protease are found in SI Materials and Methods.

RESULTS

*S. haemolyticus* Vga$_{1,2}$C ATP-dependently rescues the puromycin reactivity of *S. aureus* initiation complexes inhibited by lincomycin, clindamycin, virginiamycin M1 or tiamulin

We used the so-called puromycin reaction to assay transpeptidation functionality of the ribosome. The aminonucleoside antibiotic puromycin is a structural mimic of the 3′ end of aminoacyl-tRNA that prematurely terminates translation by substituting for the incoming aminoacyl-tRNA and acting as an acceptor either for the growing peptide chain donated by the P-site peptidyl-tRNA, or, during initiation, the fMet donated by P-site initiator tRNA (fMet-tRNA$_{iMet}$) (Figure 2A) (44). Using the puromycin reactivity of 35S-methionine-labeled 70S initiation complexes (70S IC) as a biochemical system, we followed the inhibition of PTC reaction by antibiotics and PTC rescue by ABCF ARE factors. The 70S IC was formed from purified 70S ribosomes isolated from either *E. coli*, *S. aureus* or *E. faecalis*, a synthetic mRNA encoding the Met-Phe dipeptide (mRNA(MF)), and *E. coli* initiator tRNA (fMet-tRNA$_{iMet}$) that is aminoaoylated with 35S-methionine and subsequently formylated (35S-fMet-tRNA$_{iMet}$) (42). Formation of the 70S ICs was catalyzed by *E. coli* initiation factors that were subsequently removed by ultracentrifugation through the sucrose cushion.

Since *E. coli* 70S ribosomes are intrinsically resistant to lincomycin (27, 45), we opted for *S. aureus* 70S purified from the SH1000 model laboratory strain (46). Unlike *E. coli*, *S. aureus* ribosomes are highly sensitive to lincomamide antibiotics which are used to treat Staphylococcal infections in clinical practice (47), and several AREs have been identified as resistance factors in these bacteria. Already in moderate excess over ribosomes (0.7 μM antibiotic versus 0.5 μM 70S) both lincomycin (Figure 2B) and clindamycin (Figure 2C) completely abrogate the puromycin reactivity of *S. aureus* 70S IC. In the presence of saturating concentrations of lincomycin (0.7 μM and above) around 20% of
Figure 2. *S. haemolyticus* VgaALC ATP-dependently counteracts inhibition of puromycin reactivity of *S. aureus* 70S initiation complexes by lincosamide antibiotics. Puromycin reactivity of *S. aureus* 70S initiation complexes (IC) is used as a test reaction for biochemical studies of *S. haemolyticus* VgaALC resistance factor protecting against LSₐ antibiotics targeting the ribosomal peptidyl transferase centre (PTC) (A). Increasing concentrations of lincosamides lincomycin (B) and its 7(S)-chloro-7-deoxy derivative clindamycin (C) abrogate the $^{35}$S-Methionine release from P-site $^{35}$S-fMet-tRNA$^{Met}$ by 1 mM puromycin. At 1 µM both antibiotics saturate the ribosomal complexes and the observed slow puromycin reactivity reflects the kinetics of antibiotic dissociation. In the presence of 1 mM ATP (filled red circles) 1 µM *S. haemolyticus* VgaALC rescues the puromycin reactivity inhibited by either lincomycin (D) or clindamycin (E). In the absence of ATP (empty red circles) addition of VgaALC has no effect. All experiments were performed at 37°C in HEPES:Polymix pH 7.5 buffer, 5 mM Mg$^{2+}$. 0.5 µM *S. aureus* IC(MF) were programmed with synthetic mRNA(MF) encoding Met-Phe dipeptide and *E. coli* $^{35}$S-fMet-tRNA$^{Met}$ (occupancy 60–80%). ICs were incubated with lincomycin for 5 min to allow equilibrium binding to be achieved prior the addition of puromycin. The data are presented as geometric means, and error bars represent the range of experimental values.
\[ ^{35}\text{S}-\text{methionine} \text{ is released after 1000 s, reflecting slow disso-} \]
\[ \text{ociation of the drug followed by the puromycin reaction. Clindamycin} \]
\[ \text{near-completely inhibits puromycin reactivity even after a 1000-s incubation} \]
\[ \text{with puromycin, indicating even slower dissociation. Conversely, in the case} \]
\[ \text{of intrinsically} \]
\[ \text{lincomycin resistant} \text{ E. coli 70S IC, puromycin releases} \]
\[ ^{35}\text{S}-\text{methionine} \text{ even in the presence of 5 or 15 } \mu\text{M} \]
\[ \text{lincomycin, resulting in a near-complete deacylation of the} \]
\[ \text{35S-methionine is released after 1000 s, reflecting slow dis-} \]
\[ \text{ociation. Even in these conditions, puromycin reactivity is not} \]
\[ \text{completely restored. In the following puromycin reaction} \]
\[ \text{assays we used excess concentrations of VgaALC and nucleotides: 1 } \mu\text{M}\]
\[ \text{and 1 mM, respectively.} \]
\[ \text{Next, we characterized the nucleoside triphosphate specifici-} \]
\[ \text{ty of VgaALC-mediated lincomycin resistance. The} \]
\[ \text{puromycin release kinetics is identical when ATP is substi-} \]
\[ \text{tuted for GTP, CTP or UTP, suggesting that VgaALC is} \]
\[ \text{an NTPase (Figure 3C). In the absence of VgaALC none} \]
\[ \text{of the NTPs alone have an effect on the system (Supple-} \]
\[ \text{mental Figure S4A). To characterize the NTP specificity} \]
\[ \text{of VgaALC, we titrated GTP, the second most-abundant} \]
\[ \text{NTP species in bacteria after ATP (49) (Supplementary} \]
\[ \text{Figure S4B). Similarly to ATP, the full activity of} \]
\[ \text{VgaALC} \text{ is achieved at 0.5 mM GTP, suggesting that in the} \]
\[ \text{cell the enzyme operates at saturating concentrations of both} \]
\[ \text{NTP species. Finally, to probe the role of NTP hydrolysis} \]
\[ \text{by VgaALC, we compared ATP, its non-hydrolysable} \]
\[ \text{analogue ADPNP (adenosine 5'-\text{\(\beta\text{-}\gamma\text{-imidoo} \}} \]
\[ \text{triphosphate, App(NH)pp}, \text{a slow-hydrolysable analogue ATPyS (ado-} \]
\[ \text{nosine 5'-O-(3-thio) triphosphate) and the product of ATP hyd-} \]
\[ \text{rolysis, ADP (Figure 3D). Neither ADPNP, nor ATPyS, nor ADP} \]
\[ \text{support VgaALC activity, suggesting driving the} \]
\[ \text{conformational switch by ATP analogues is not sufficient to} \]
\[ \text{rescue the PTC activity.} \]
\[ \text{Since VgaALC can utilize GTP as a substrate, we tested} \]
\[ \text{if the alarmone nucleotide ppGpp could suppress the} \]
\[ \text{VgaALC-mediated resistance acting as an orthosteric inhibi-} \]
\[ \text{tor the same way as it inhibits GTPases and nucleotide} \]
\[ \text{metabolism enzymes (50). During acute amino acid starva-} \]
\[ \text{tion, ppGpp becomes the dominant guanosine nucleotide,} \]
\[ \text{reaching sub-mM concentration (49). However, even when} \]
\[ \text{added at 0.5 mM, ppGpp does not have a pronounced inhi-} \]
\[ \text{bitory effect on VgaALC (Supplementary Figure S5).} \]
\[ \text{The hydrolytically inactive S. haemolyticus VgaALC EQ2 and} \]
\[ E. faecalis LsaAEQ2} \text{ mutants inhibit ribosomal peptidyl} \]
\[ \text{transferase in the presence of NTP} \]
\[ \text{Use of non- or slowly-hydrolysable ATP analogues is one} \]
\[ \text{approach to generate an ATP-bound state of VgaALC.} \]
\[ \text{An alternative is to mutate conserved catalytic glutamate} \]
\[ \text{residues following the Walker B motif in the two ABC} \]
\[ \text{cassettes and use the native ATP substrate. These glutamates} \]
\[ \text{form strong hydrogen bond interactions with the attacking} \]
\[ \text{water molecule, polarizing it as a nucleophile (51). Simul-} \]
\[ \text{taneous mutation of the residues in both cassettes for glu-} \]
\[ \text{tamidine (E105Q and E410Q; EQ2) results in a deficiency in} \]
\[ \text{ATP hydrolysis by ABC ATPases (52) and locks the enzyme} \]
\[ \text{in an ATP-bound active conformation (33,53).} \]
\[ \text{The hydrolytically deficient VgaALC EQ2:ATP does not} \]
\[ \text{rescue inhibition of transpeptidation by lincomycin and} \]
\[ \text{compromises protection by the wild type VgaALC (Fig-} \]
\[ \text{ure 4A). A possible explanation is that NTPaseinactive} \]
\[ \text{VgaALC EQ2 competes with the wild type for binding to the} \]
\[ \text{ribosome, thus preventing the wild type from carrying out} \]
\[ \text{its protective function. A more non-trivial scenario is that} \]
\[ \text{VgaALC is an NTPase that operates as a molecular ma-} \]
\[ \text{chine, not as a molecular switch.} \]
\[ \text{In the following experiments we focused on ABCF-} \]
\[ \text{mediated protection from lincomycin. The addition of 1 } \mu\text{M} \]
\[ \text{VgaALC and 1 mM ATP rescues the puromycin reactivity} \]
\[ \text{inhibited by saturating concentration of the antibi-} \]
\[ \text{otic (1 } \mu\text{M). However, the protection is not complete; the} \]
\[ \text{transpeptidation reaction is still significantly slower than in} \]
\[ \text{the absence of the antibiotic. One possible reason could be} \]
\[ \text{that the system is not saturated with either the nucleotide}
Figure 3. VgaALC activity requires mM-range concentrations of NTP nucleotides (ATP, GTP, CTP or UTP) and is not sustained either by the non-hydrolysable ATP analogue ADPNP, the slow-hydrolysable analogue ATPγS nor the product of ATP hydrolysis, ADP. Puromycin reactivity is progressively rescued by 1 µM VgaALC and increasing concentrations ATP (A) or by increasing concentrations VgaALC in the presence of constant 1 mM ATP (B). The resistance effect saturates at 0.5 mM ATP and 0.5 µM VgaALC, respectively. VgaALC-mediated rescue of puromycin reactivity is supported by either ATP, GTP, CTP or UTP nucleotides added at 1 mM, suggesting that VgaALC is an NTPase, rather than a strict ATPase (C). Neither ADP, nor the non-hydrolysable ATP analogue ADPNP nor the slow-hydrolysable analogue ATPγS can support VgaALC activity, indicating that NTP hydrolysis, not just the NTP binding, are necessary (D).

Deletion of the linker region renders wild type VgaALC inactive in antibiotic protection and the EQ2 mutant inactive in PTC inhibition

The likely structural candidate responsible for antibiotic displacement by wild type VgaALC and PTC inhibition by the EQ2 mutant is the extended inter-ABC linker region (21) (Figure 1). This hypothesis, however, has never been tested experimentally. In the absence of structural information on VgaALC it is impossible to design a ‘clean’ deletion of the linker region. We designed a deletion that reduces the length of the linker to that seen for EttA. In this truncated VgaALCΔL mutant, amino acids K199-S226 are substituted for a ‘stump’ formed by a flexible GSG linker. Additionally, the ΔL deletion was combined with the EQ2 double point mutation (E105Q and E410Q) to inactive hydrolytic activity. Deletion of the linker region has a profound effect on the functionality of VgaALC: neither does VgaALCΔL protect from lincomycin nor does VgaALCΔL/EQ2ΔL compromise the protective effect of the wild type VgaALC (Figure 4D). This suggests that the truncated protein can no longer reach and interact with the PTC region (Supplementary Figure S1). However, it is also possible that the deletion alters the overall structural integrity of VgaALC thus inhibiting the protein’s activity indirectly.
Figure 4. The linker region is essential for NTP-dependent inhibition of ribosomal peptidyl transferase activity by the NTP hydrolysis-incompetent VgaALC-EQ2 mutant. The NTPase-incompetent VgaALC-EQ2 mutant does not rescue the puromycin reactivity of S. aureus 70S IC(MF) inhibited by 1 µM lincomycin (dark blue filled circles), and compromises protection by the wild type protein (dark blue circles, red fill) (A). The VgaALC-EQ2 mutant inhibits IC(MF) puromycin reactivity in the absence of antibiotics in the presence of 1 mM ATP (B and C) or GTP (C), but not 1 mM ADP, ATPγS or ADPNP (C). The VgaALCΔL mutant in which inter-ABC linker region (amino acids K199-S226) is substituted for a GSG linker neither protects the 70S IC(MF) inhibited by 1 µM lincomycin (filled green circles), and its EQ2 variant, VgaALC-EQ2ΔL, does not abolish the protective effect of the wild type protein (black circles with red fill) (D).

DISCUSSION

We suggest a model of ARE-mediated ribosome protection from PTC inhibition by LSₐ antibiotics (Figure 6). Powered by NTP-hydrolysis, ARE factors dislodge LSₐ antibiotics from the ribosome. In the cell, AREs can use any NTP species, the most abundant being GTP and ATP. The association of the NTP-bound ARE with the ribosome causes transient arrest of transpeptidation, conceivably via a direct contact of the linker region with the PTC.

The relaxed nucleotide substrate specificity seen with VgaALC is not unusual for ABC enzymes, e.g. E. coli transporter CvaB uses both GTP and ATP (54), and ribosome-associated ABCF EttA was mentioned by Böel and colleagues to interact with both ATP and GTP (no data shown in the original publication) (32). To achieve its maximum activity, VgaALC requires ≥500 µM of the NTP substrate. While this is a relatively high concentration, it follows the general trend of enzyme affinity to substrate correlating with the concentration of substrate (55): there is no evolutionary pressure selecting for high affinity to abundant substrates. Since two NTP species—GTP and ATP—are present in bacterial cell in the mM concentration range (49), it is not surprising that in the test tube VgaALC requires high NTP concentration.

Our biochemical analyses raise several pertinent questions that can be addressed by structural and microbiological approaches. First, high resolution structural information is necessary to unequivocally establish the role of the ‘linker’ region in ARE’s molecular mechanism. Second, the low efficiency of protection by VgaALC in the reconstituted system indicates that the biochemical experiments do not reveal the full story. One possible explanation could be that in the test tube, the dislodged antibiotic rapidly rebinds to the ribosome, thus compromising VgaALC-mediated protection. However, this is unlikely, since in direct competition experiments the puromycin test reaction is sufficiently fast to kinetically outcompete the slowly binding lincomycin (Supplementary Figure S2B). A second explanation is that the reconstituted system is missing one or more components or co-factors crucial for the full activity of VgaALC. It is tempting to speculate that in order to achieve efficient removal of the dislodged antibiotic, the activity of the ABCF ARE is coordinated with a direct interaction of the ARE with an efflux pump. Experiments with Streptococcus pneumoniae macrolide resistance ARE MsrD indicate a possible function for ABCF efflux systems.
A BBBBBB

Figure 5. Wild type E. faecalis LsaA ATP-dependently protects E. faecalis 70S initiation complexes from lincomycin antibiotics, and the NTP hydrolysis-incompetent LsaAEQ2 mutant inhibits the peptidyl transferase activity. In the presence of 1 mM ATP (filled red circles) 1 µM E. faecalis LsaA rescues the puromycin reactivity inhibited by 1 µM lincomycin; in the absence of ATP LsaA has no effect (empty red circles) (A). Increasing concentrations of LsaAEQ2 progressively inhibit the transpeptidase activity of E. faecalis 70S initiation complexes (B).

Figure 6. Model of ARE-mediated ribosome protection from antibiotics targeting the PTC. LSA antibiotics directly bind to the PTC and inhibit its catalytic activity. ARE factors dislodge the antibiotics in an NTP-dependent manner. Association of the ARE with the ribosome causes transient arrest of transpeptidation, conceivably via a direct contact of the linker region with the PTC.

candidate. The msrD gene is co-transcribed with another ORF encoding MefE (macrolide efflux E) efflux pump, a member of major facilitator superfamily (56). While acting alone in the absence of MefE, the MsrD ABCF confers only low-level macrolide resistance (57), but the two proteins synergize when acting together (58). Although they are Streptococcal resistance factors, MefE and MsrD confer macrolide resistance in E. coli, suggesting that the proteins are functional when expressed heterologously (59). While GFP-labelled Msrd localized in the E. coli cytoplasm, GFP-labelled MefE is localized at cell poles—but only when co-expressed with wild type MsrD. This indicates a physical interaction between the two proteins. However, just how general this kind of interaction might be among ABCF AREs is unknown. Genetic, microbiological and molecular biology experiments with Staphylococcal VgaA are the next step for identification of its potential interactive partners, paving the way for more refined biochemical and structural studies.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR online.

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