Regulation and mechanistic basis of macrolide resistance by the ABC-F ATPase MsrD

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SUMMARY

Antibiotic resistance ABC-Fs (ARE ABC-Fs) are translation factors currently proliferating among human pathogens that provide resistance against clinically important ribosome-targeting antibiotics. Here, we combine genetic and structural approaches to determine the activity of the *streptococcal* ARE ABC-F protein MsrD on the ribosome and its regulation in response to macrolide exposure. We show that cladinose-containing macrolides lead to insertion of MsrDL leader peptide into a conserved crevice of the ribosomal exit tunnel, which remained thus far undocumented, concomitantly with 23S rRNA rearrangements that preclude proper accommodation of release factors and inhibits termination. The stalled ribosome obstructs formation of a Rho-independent terminator which prevents *msrD* transcriptional attenuation. This stalled ribosome is rescued by MsrD powered by its two functionally asymmetric ATPase sites, but not by MsrD mutants which do not provide antibiotic resistance, showing evidence of equivalence between MsrD function in antibiotic resistance and its action on this complex.

KEYWORDS

Ribosome, protein synthesis, antibiotic resistance, ARE ABC-F protein family, leader peptide, cryo-EM.

INTRODUCTION

ABC-F ATPases belonging to the ATP-Binding Cassette (ABC) superfamily are translation factors and some of them, termed antibiotic resistance ABC-Fs (ARE ABC-Fs), confer resistance to clinically important antibiotics that bind to the ribosomal peptidyl transferase center (PTC) and/or the nascent peptide exit tunnel (NPET) (Crowe-McAuliffe et al., 2018, 2021; Sharkey et al., 2016; Su et al., 2018). ABC-F proteins are composed of two ABC domains (or Nucleotide Binding Domains, NBDs) joined by a linker region called P-site tRNA-interaction motif (PtIM) (Boël et al., 2014; Chen et al., 2014), also named antibiotic resistance determinant (ARD) for ARE ABC-Fs (Crowe-McAuliffe et al., 2018, 2021). The two ABC domains dimerize after binding of two ATP molecules and in this conformation the factor can bind the ribosomal E-site (Chen et al., 2014), where the PtIM adopts an α -helical hairpin conformation that interacts directly with the peptidyl-tRNA and extends toward the PTC/NPET. Three antibiotic resistance phenotypes are associated with ARE ABC-Fs: (i) MKS_B (Macrolides, Ketolides, Streptogramins group B); (ii) PLS_A (Pleuromutilins, Lincosamides, Streptogramins group A); (iii) PhO (Phenicols, Oxazolidinones) (Fostier et al., 2021; Ousalem et al., 2019). However, despite structural investigations (Crowe-McAuliffe et al., 2018, 2021; Su et al., 2018), the exact molecular mechanism of action of ARE ABC-Fs remains unclear, so is their genetic regulation.

Over the last forty years, biochemical and structural investigations demonstrated the ability of elongating nascent chains (NC) into the ribosomal tunnel to interact with metabolites or antibiotics, thus adapting protein synthesis to environmental cues (Arenz et al., 2014a, 2016; Beckert et al., 2021; Herrero del Valle et al., 2020; van der Stel et al., 2021). In human pathogens, antibiotic-dependent formation of stalled ribosome complexes (SRCs) on regulatory ORFs, named leader peptides, can subsequently allow regulation of downstream resistance genes in response to antibiotic exposure (Horinouchi and Weisblum, 1980; Ito and Chiba, 2013; Narayanan and Dubnau, 1985; Vazquez-Laslop et al., 2008).

ARE ABC-F gene *msrD* is a member of the *msr* (macrolide and streptogramin B resistant) gene group previously referred as *mel* (Gay and Stephens, 2001; Roberts et al.,

1999). This gene is generally found in operon with a macrolide efflux facilitator (*mefA* or *mefE*)
gene, the two corresponding proteins acting synergistically to confer macrolide resistance
(Daly et al., 2004; Nunez-Samudio and Chesneau, 2013; Tatsuno et al., 2018; Zhang et al.,
2016). This operon is part of the Macrolide Efflux Genetic Assembly (MEGA) (Del Grosso et
al., 2004, 2006) that disseminates in human pathogens on mobile genetic elements:
conjugative transposons like the tn916-type (Chancey et al., 2015a) and conjugative
prophages like the Φ 1207.3 (Iannelli et al., 2014) among streptococci, conjugative plasmids
like the pMUR050 (González-Zorn et al., 2005) among enterobacteria. In streptococci, the
operon is transcribed in presence of erythromycin (ERY) as a polycistronic mRNA from a single
promoter located ~350 bp upstream *mefA* (Ambrose et al., 2005; Chancey et al., 2015b). It is
also regulated by ribosome-mediated transcription attenuation via MefAL leader peptide
(MTASMLRLR), which is closely related to ErmDL leader peptide (MTHSMRLRFPTLNQ), both
peptides harboring the characteristic macrolide-arrest motif “RLR” (Beckert et al., 2021;
Chancey et al., 2015b; Ramu et al., 2009; Sothiselvam et al., 2014).

Here, we report the presence of a second transcriptional attenuator on the *mefA/msrD*
operon regulating exclusively *msrD* expression upon macrolide exposure. Presence of ERY
induces ribosome stalling on the previously identified *msrDL* leader peptide (encoding
MYLIFM) (Ramu et al., 2009), allowing RNA polymerase (RNAP) to bypass an intrinsic
terminator. Our findings demonstrate stalling occurs due to hampered translation termination
on UAA stop codon, action of both release factors 1 and 2 (RF1 and RF2) being inhibited. Our
results are supported by the cryo-EM structure of MsrDL-SRC that provides molecular insight
on how the PTC precludes productive accommodation of RF1/RF2 and how the NC
discriminates tunnel-bound cladinose-containing macrolides. The path of the NC within the
tunnel greatly differs from previously described leader peptides (Arenz et al., 2014a, 2016;
Beckert et al., 2021; Herrero del Valle et al., 2020; van der Stel et al., 2021) with the NC
latching into a ribosomal crevice, conserved from prokaryotes to eukaryotes, delimited by 23S
rRNA nucleotides U2584, U2586, G2608 and C2610 that may form a novel ribosomal
functional site. Finally, our results demonstrate that the two ATPase sites of MsrD perform

different functions and the protein can negatively self-regulate its synthesis in presence of ERY by direct action on MsrDL-stalled ribosomes.

RESULTS

MsrD provides macrolide and ketolide resistance by direct interaction with ribosome

The *mefA/msrD* macrolide resistance operon (**Figure 1A**) is part of the MEGA element currently spreading among clinical isolates and livestock (de Block et al., 2021; Chancey et al., 2015b; Lu et al., 2010; Sharma et al., 2020). A phylogenetical analysis (**Figure S1A**) shows that the operon disseminates predominantly in Gram-positive firmicutes (mostly streptococci) and in some Gram-negative proteobacteria (such as *Haemophilus influenzae*, *Neisseria gonorrhoeae* or *Escherichia coli*). Gene *msrD* shares ~62 % identity with its closest homolog *msrE* (Su et al., 2018), both factors exhibiting the canonical ABC-F organization (**Figure 1B**).

Model bacteria *E. coli* presents undeniable advantages for genetic and molecular biology, but presence of multidrug efflux systems greatly limits its use in antibiotic research (Nikaido, 2009; Nikaido and Pagès, 2012). To circumvent this limitation, we took advantage of *E. coli* DB10 strain which exhibits exacerbated sensitivity to macrolide antibiotics (Datta et al., 1974). Heterologous expression of *msrD* from an arabinose-inducible promoter conferred macrolide and ketolide resistance phenotype to *E. coli* DB10 strain (**Table 1**), demonstrating the functionality of the factor in this organism. Expression of *msrD* resulted in 8-fold minimal inhibitory concentration (MIC) increase for 14-membered macrolide ERY, 8-fold for 15-membered macrolide azithromycin (AZI) and 2-fold for telithromycin (TEL, ketolide antibiotic derived from 14-membered macrolides). However, no change in MIC was observed for 16-membered macrolides tylosin (TYL) and spiramycin (SPI), neither for non-macrolide antibiotics lincomycin (LNC), linezolid (LNZ) and retapamulin (RTP) (**Table 1**).

Polysome profile from bacteria exposed to ERY show a stabilization of ribosome disomes (**Figure 1C**) as expected for an antibiotic that inhibits ribosome elongation. The disomes accumulation likely results from elongating ribosomes that collide on ERY-stalled ribosomes. Induction of *msrD* alleviates the accumulation of disomes and restores a profile

identical to that without ERY (**Figure 1D**). Western blots of the polysome fractions show an MsrD association with the 70S ribosome and the 50S subunit with a higher affinity for the latter one. These results agree with a model in which MsrD directly rescues ERY-stalled ribosomes and that MsrD can restore normal translation in presence of the antibiotic.

To understand the function of the ATPase activity of MsrD, we constructed ATPase-deficient variants MsrD_{E125Q}, MsrD_{E434Q} and MsrD_{E125Q/E434Q} (hereafter referred as MsrD_{EQ2}) by replacing the catalytic glutamic acid residue (E) in Walker B motif of each or both ABC domain by a glutamine (Q) (**Figures 1B** and **S1B**). This mutation strongly reduces the ATP hydrolysis while preserving the local stereochemistry of active site and has been extensively used to trap ABC-F factors on the ribosome in ATP-bound conformation (Chen et al., 2014; Crowe-McAuliffe et al., 2018, 2021). Expression of *msrD*_{EQ2} in absence of ERY shows an accumulation of disomes and western-blotting reveals its association with the 30S subunit, 70S ribosome and polyribosomes. This result shows that MsrD_{EQ2} binds to the ribosome and inhibits its elongation (**Figure 1E**) as observed for all reported ABC-F carrying the E-to-Q mutation (Boël et al., 2014; Murina et al., 2019), with the exception of MsrE (Su et al., 2018). Expression of *msrD*_{EQ2} also alters the 30S peak, an alteration often observed during translation inhibition and presumably due to pre-30S maturation defects (Siibak et al., 2009). In presence of ERY, the disome and trisome accumulation increases and western blots show an increase of MsrD_{EQ2} in the disome fraction (**Figure 1E**, fraction 13). Still, the ATPase activity of MsrD impacts its binding affinity, MsrD having a higher affinity for the 50S subunit in contrast to MsrD_{EQ2} having a higher affinity for the 70S ribosome (**Figures 1D** and **E**). A model consistent with these observations would imply that MsrD binds to ERY-stalled ribosomes in an ATP-bound close conformation without the need of its ATPase activity, then ATPase activity triggers 70S dissociation, MsrD staying associated with the 50S subunit. Similar results were observed by expressing *msrD* and *msrD*_{EQ2} in *E. coli* K12 MG1655 (**Figure S1C**) without the presence of ERY. They also show a decrease of the 70S peak correlated with an increase of 30S and 50S peaks for *msrD* condition when compared to the control, supporting the proposed dissociation model. Western blots show more MsrD_{EQ2} than MsrD in total lysates (**Figures 1D**

and **E**), MsrD_{EQ2} inhibiting protein synthesis and therefore its own synthesis, we hypothesized that MsrD_{EQ2} is less expressed but more soluble, which has been confirmed with a solubility test (**Figure S1D**).

We evaluated the influence of E-to-Q mutations of MsrD on bacterial growth and the antibiotic resistance phenotype. All the mutations impair bacterial growth and antibiotic resistance phenotype (**Figure 1F**), while MsrD_{E125Q} and MsrD_{EQ2} have a similar effect on bacterial growth in absence or at low ERY concentration, MsrD_{E434Q} is more toxic. However, MsrD_{E125Q} variant maintains some resistance (MIC = 4 μ M) compared to control (MIC = 2 μ M) which is also supported by the fact that MsrD_{E125Q} IC₅₀ is ~8 times higher than Control IC₅₀ (**Figure 1F, Supplemental Table 1**). The two ATP hydrolysis sites (**Figure S1B**) have therefore different tasks and are functionally asymmetric, ATP site II achieving in part the mechanism of ERY-stalled ribosome rescue. We also evaluated the importance of other residues of MsrD. Truncation of the PtlM (replaced by three glycine) or just the Loop (Δ PtlM, Δ Loop respectively) completely abolished the resistance phenotype *in vivo* as reported *in vitro* for MsrE (Su et al., 2018) (**Figure 1G**). Punctual mutations in the Loop (**Figure 1B**) affect the resistance to a different extent, mutation of R241 into alanine has almost no effect on the phenotype, but equivalent mutation for L242 or H244 reduces IC₅₀ by ~3 fold (**Figure 1H and Supplemental Table 1**), demonstrating that these residues are important for MsrD function, but not essential even if they are predicted at the vicinity of the antibiotic (**Figure S1E**) in the MsrE-ribosome complex (Su et al., 2018). Interestingly, this finding contrasts with mutagenesis assays for MsrE showing that mutation R241A reduces the resistance by more than 50%. Replacement of H244 by the larger residue tryptophan should displace the drug by steric occlusion, but the variant lost most of its antibiotic resistance phenotype (**Figure 1H**). Similarly, MsrD does not provide resistance to TYL nor SPI (**Table 1**), while both antibiotics should clash with MsrD according to MsrE structure (**Figure S1E**). Same observation can be made with LNC, LNZ or RTP (**Table 1 and Figure S1E**). Therefore, MsrD action on the antibiotic is indirect and direct steric occlusion to displace the antibiotic seems excluded.

Ribosome-mediated transcriptional attenuation regulates *msrD* expression

Analysis of the *mefA/msrD* operon sequence exposes a stringent ribosome binding site (RBS, whose sequence is 5'-GGAGGA-3') only 8 bp downstream *mefA* stop codon and possibly required for translation of the downstream putative small ORF *msrDL* (**Figure S2A**). In order to test the influence of *msrDL* on *msrD* expression, a fluorescent reporter, which contains the sequence spanning from the first nucleotide downstream *mefA* stop codon to *msrD* three first codons fused to the Yellow Fluorescent Protein gene (YFP), was cloned in the low copy pMMB plasmid (Fürste et al., 1986) under the control of an IPTG-inducible promoter (**Figure 2A**). The resulting plasmid, pMMB-*msrDL*-*msrD*₍₁₋₃₎:*yfp*, was used to directly follow the fluorescence reflecting *yfp* expression.

Basal fluorescence level was found stable in *E. coli* DB10 strain containing pMMB-*Control* independently of ERY concentration (**Figure 2A**) while a fluorescence increase correlated with ERY concentration was observed in cells containing pMMB-*msrDL*-*msrD*₍₁₋₃₎:*yfp*, indicating an ERY-dependent induction (**Figure 2A**). Optimal induction was found at 100 nM ERY while only 6.25 nM was sufficient to significantly induce *msrD*₍₁₋₃₎:*yfp* expression, demonstrating the high sensitivity of the system toward its inducer. Moreover, a reduced but significative fluorescence signal was detected at 0 nM ERY indicating an imperfect repression. These results are consistent with previous description of *ermC* induction by nanomolar ERY concentrations in *S. aureus* and *B. subtilis* (Mayford and Weisblum, 1989; Weisblum et al., 1971), contrary to *E. coli* strains JM109 and RR1 where micromolar concentrations are required (Bailey et al., 2008; Kirsch and Lai, 1984). Inactivation of *msrDL* ORF by replacement of the start codon by an amber stop codon (pMMB-*msrDL*_(no_ORF)-*msrD*₍₁₋₃₎:*yfp*) resulted in the elimination of the fluorescence induction (**Figure 2A**), demonstrating that *msrDL* translation is an important and necessary cis-acting feature to regulate *msrD*₍₁₋₃₎:*yfp* expression as reported for *ermC* (Dubnau, 1985).

In bacteria, antibiotic-dependent gene induction relies mainly on translational or transcriptional attenuation (Dersch et al., 2017). To determine if MsrDL prompts a transcriptional or translational attenuation, northern blots analysis with a probe located in

msrD₍₁₋₃₎:*yfp* 3' UTR were performed on total RNA extracted from cell exposed or not to ERY and/or IPTG. A 1.2-kb band corresponding to *msrDL*-*msrD*₍₁₋₃₎:*yfp* mRNA is transcribed in presence of IPTG and starts to increase steadily 5 min after exposure to 100 nM ERY (**Figure 2B**). Consistent with this ERY-dependent transcript accumulation, we concluded that *msrD* regulation by *msrDL* occurs at the transcription level.

Transcription attenuation employs premature transcription termination via: (i) Rho-dependent terminator (RDT) by binding of Rho factor, a RecA-type ATPase, to a C-rich and G-poor sequence known as Rho utilization site (Banerjee et al., 2006); (ii) Rho-independent terminator (RIT) where a stable GC-rich stem-loop followed by a poly-uridine sequence causes RNAP to drop off (Ray-Soni et al., 2016). Treatment with bicyclomycin, a Rho inhibitor, did not result in constitutive expression of the reporter, thus excluding the possibility of RDT attenuation (**Figure S2B**). Analysis of the intergenic region between *msrDL* and *msrD* using ARNold server (Naville et al., 2011) revealed the presence of a RIT with a ΔG of -7.9 kcal.mol⁻¹ (**Figure 2C**). In order to test this possible transcription termination activity, we generated a mutant by removing the putative terminator (referred as pMMB-*msrDL*_(no_term)-*msrD*₍₁₋₃₎:*yfp*). Deletion of the terminator resulted in a constitutive expression of the fluorescent reporter in absence of ERY, indicating that the terminator is a necessary cis-acting regulatory feature that can terminate transcription (**Figure 2C**). These results validate the presence of a second transcriptional attenuator in the *mefA/msrD* operon that regulates only *msrD*.

Selective drug-sensing by MsrDL prevents its translation termination

To determine how this regulation occurs via ribosome-mediated mechanism, translation of *msrDL* mRNA with the PURE system (Shimizu et al., 2001) was subjected to various antibiotics and its propensity to form SRC was analyzed by toeprinting (Hartz et al., 1988) (**Figure 3** and **Figure S3**). A strong toeprint signal was observed in presence of ERY and AZI, while it was absent in presence of TEL, TYL and SPI (**Figure 3A**). This *in vitro* result is correlated to *in vivo* observations made with the *msrD*₍₁₋₃₎:*yfp* reporter gene which was also

only induced by ERY and AZI, but not by TEL, TYL, SPI and non-macrolide antibiotics (**Figure 3B**).

We further characterized the translation of *msrDL* mRNA and showed that in presence of 50 μ M RTP, an inhibitor of the first peptide bond formation which allows to identify ORFs start codon (Meydan et al., 2019), a clear toeprint occurred (**Figure 3C**). This toeprint corresponds to a ribosome stalled with the initiating codon in the ribosomal P site and confirms that ribosomes can initiate *msrDL* translation. The toeprint observed at positions +31, +32 and +33 relative to *msrDL* 5' end (**Figures 3A and C**) in presence of ERY and AZI indicates an SRC located at the termination step of MsrDL synthesis with the C-terminal methionine (M6) codon in the P site and UAA stop codon in the A site. In accordance with this, reactions depleted of release factors (RF1, RF2, RF3) (**Figure 3C**) show the same toeprint result. Moreover, addition of puromycin led to the loss of this toeprint signal, while preserving it in presence of ERY (**Figure 3C**). Puromycin is an aminonucleoside antibiotic mimicking A site substrate tyrosyl-tRNA and causing premature chain termination. While actively translating ribosomes are sensitive to this antibiotic, stalled ones tend to be refractory (Muto et al., 2006; Seip and Innis, 2016; Vazquez-Laslop et al., 2008). The resistance of MsrDL-SRC to puromycin is consistent with an ERY-sensing NC that leads to PTC silencing and stalling. The stalled ribosome on *msrDL* covers the first half of the hairpin of the RIT and therefore prevents its formation and transcription termination (**Figure 2C and S3**).

The main dissimilarity between tested macrolides resides mainly in the presence or absence of a cladinose moiety on C3 of the macrocyclic lactone ring which appears to be critical for MsrDL-SRC formation (**Figure 3A**) similarly to ErmCL (Vazquez-Laslop et al., 2008). Absence of significant toeprint in presence of TEL (whose C3 cladinose sugar is replaced by a keto group) suggests that *msrDL* may be translated in this condition. TYL and SPI differ from the other macrolides by the presence of disaccharide mycaminose-mycarose in place of the C5 monosaccharide desosamine. When bound to the ribosome, this extended sugar moiety protrudes into the PTC of the ribosome (Hansen et al., 2002). Absence of intermediate toeprint and increase of the toeprint corresponding to initiation complex in presence of these two drugs

imply that these antibiotics stabilize the initiation complex. Previous results (Hansen et al., 2002; Mao and Robishaw, 1971; Poulsen et al., 2000) have shown that they inhibit the formation of the peptide bond, our result suggests that it occurs mainly for the first peptide bond formation (**Figure 3A**).

We submitted the sequence of MsrDL to an alanine-scanning to investigate the importance of each residue and their ability to induce *msrD*₍₁₋₃₎:*yfp* reporter, which indirectly reports the formation of an ERY-dependent SRC. The scan shows a strong reduction of inducibility for all the mutated residues with the exception Y2 (**Figure 3D**). Since MsrDL is a hexapeptide, it is evident that most of the amino acids would have their importance to maintain a conformation able to sense ERY. A complete loss of inducibility has been observed for residues L3 and I4 suggesting that these two residues were directly involved in drug-sensing, which was structurally confirmed and detailed in the next section (**Figure 4**). Moreover, considering that MsrDL inhibits the action of both RF1/RF2 in presence of ERY, we mutated UAA stop codon into RF1-specific UAG and RF2-specific UGA stop codons to investigate a putative preferential inhibition as previously shown for TnaC (Emmanuel et al., 2019). Mutation of UAA into UAG or UGA reduces the level of inducibility of the reporter gene (**Figure 3E**), demonstrating a better expression of the reporter gene when both RF1 and RF2 are inhibited compared to situations where RF1 or RF2 are inhibited separately. This observation is corroborated by our phylogenetic analysis which revealed a large prevalence of UAA stop codon among the different *msrDL* variants (**Figure S2A**). Most likely the UAA stop codon has been selected by evolution to maximize *msrD* expression in presence of an inducer.

Molecular mechanism of ribosome stalling by MsrDL

To understand how MsrDL inhibits translation termination in presence of ERY, a synthetic mRNA containing a single *msrDL* copy was translated *in vitro* with purified *E. coli* DB10 ribosomes in presence of ERY as described in **Figure S4A** and the resulting sample was subjected to cryo-EM. Single-particle reconstruction of MsrDL-SRC was done by refining two subclasses, one containing only P-tRNAs and one containing P/E-tRNAs (respectively

11% and 28.1% of total particles), as described in methods and on **Figure S4B**. It resulted an initial map of 70S ribosome (EMD-13805) with an average resolution of 3.0 Å that was subjected to multibody refinement, generating reconstructions for the body and the head of the small subunit (EMD-13807 and EMD-13808) and the large subunit (EMD-13806), presenting average resolutions of 3.08, 3.3 and 2.97 Å, respectively (**Figures S4B and C**). The resolutions of the derived reconstructions were consistent with clear and unambiguous assignment of ribosomal proteins side chains, rRNA nucleotides and some post-transcriptional modifications (**Figures S4D, E and F**). Local resolution of the codon-anticodon allowed unambiguous identification of the P tRNA as an elongator $_{Met}tRNA^{Met}$ (**Figure S4E**), which is also confirmed by the presence of distinctive elements such as N4-acetylcytidine at position 34 (ac^4C34) and N6-threonylcarbamoyladenosine at position 37 (t^6A37) (**Figures S4E and F**) (Ohashi et al., 1972; Varshney et al., 1993), consistently with the toeprint results which assigned the M6 codon in the P site and UAA stop codon in the A site (**Figure 3C**).

A clear density corresponding to ERY bound in its canonical position was found at the entrance of the NPET (**Figures 4A, 4B and S4D**), allowing unambiguous attribution of macrocyclic lactone ring, cladinose and desosamine sugars. A continuous density at the 3' end of P tRNA extending within the entrance of ribosome tunnel was identified as MsrDL-NC and local resolution of ~3 Å allowed to model the peptide *de novo* (**Figure 4B and S4D**). It adopts a hook-like shape with its C-terminal extremity protruding into a cavity at the entrance of the tunnel delimited by 23S rRNA nucleotides U2584, U2586, G2608 and C2610 (**Figure 4B**). One noticeable feature of MsrDL interaction with the ribosome is its residue Y2 that budge out the base U2584 to form a π -stacking with the base G2583 (**Figures 4C and D**) in place of the U2584. This unique interaction should stabilize the peptide conformation, while it appears structurally significant, it is not strictly essential since replacement of the Y2 by an alanine reduces by less than 50% the induction of the reporter gene (**Figure 3D**). In addition, the Y2 is substituted in some MsrDL variants (**Figure S2A**). MsrDL does not show numerous significant electrostatic interactions with the ribosome since it is mostly composed of hydrophobic amino acids. It is likely stabilized by hydrophobic interactions.

Our biochemical investigations showed that MsrDL-SRC were formed due to hampered translation termination on UAA stop codon. This stop codon is recognized by RF1 and RF2 which subsequently catalyze peptide hydrolysis via their conserved GGQ motif protruding within the PTC. Structural alignment of our model to RF1- and RF2-containing ribosome structures (Pierson et al., 2016) revealed that proper accommodation of the RFs is prevented by steric clashes of the methylated glutamine and the following glycines of the GGQ motif with the residue F5 of MsrDL and 23S rRNA bases U2584 and U2585 respectively (**Figure 4E**). The conformation of MsrDL peptide stabilizes the PTC in an uninduced conformation rather than an induced one (Martin Schmeing et al., 2005) which catalyzes the peptide bond formation (**Figure 4F**) as 23S rRNA base U2585 would clash with MsrDL. Structural alignment of MsrDL-SRC with ErmBL- and ErmCL-SRC demonstrates that bases U2506 and 2585 are not in the same conformation (**Figures S5A and B**), these bases in MsrDL-SRC being in a conformation more similar to ErmDL-, SpeFL- and TnaC(R23F)-SRC (**Figures S5C to E**) (Arenz et al., 2014a, 2016; Beckert et al., 2021; Herrero del Valle et al., 2020; van der Stel et al., 2021). However, unusual position of base U2584 seems to be a unique feature of MsrDL-SRC.

MsrDL may monitor the presence of cladinose-containing drugs as our *in vivo* and *in vitro* experiments suggested. Mutation of residues L3 and I4 abolished induction of the reporter gene suggesting that they were critical for drug recognition and/or conformation of the peptide (**Figure 3D**). Consistently, in our model we identified residue L3 as the closest amino acid from cladinose moiety and residue I4 as the closest from desosamine moiety (**Figure 4B and 4G**). However, similarly to ErmBL, no close contact between the nascent chain and the drug was observed (Arenz et al., 2014b, 2016), residue L3 being ~3.8 Å away from cladinose sugar and residue I4 being ~3.9 Å away from desosamine sugar, consistently with a hydrophobic interaction between MsrDL and the drug (**Figures 4G to I**). This observation is also corroborated by previous descriptions of pentapeptides conferring ERY resistance in which the presence of a leucine or isoleucine at position 3 was critical for drug recognition (Lovmar et al., 2006; Tenson et al., 1997). Moreover, the importance of 23S rRNA residue A2062 in acting

as an ERY-sensor and in contributing to silence the PTC, has been demonstrated for some regulatory leader peptides (Koch et al., 2017; Vázquez-Laslop et al., 2010). In the case of ErmCL, the critical feature for macrolide-dependent stalling is the rotation of the base A2062 that forms an Hoogsteen base pairing with m²A2503 (Arenz et al., 2014a; Koch et al., 2017; Vázquez-Laslop et al., 2010). The structure of MsrDL-SRC shows that residue I4 restrains rotation of base A2062 (**Figure 4J**). Therefore, MsrDL induces an A2062/m²A2503-independent ribosome stalling (Vázquez-Laslop et al., 2010).

The path of MsrDL-NC within the ribosomal tunnel contrasts from all previous descriptions of elongating polypeptides (**Figures 5A to 5C**). Indeed, ERY-sensing ErmBL, ErmCL and ErmDL leader peptides (Arenz et al., 2014a, 2016; Beckert et al., 2021) engage in the NPET and go partially through ERY, while MsrDL curves before encountering the ligand then engages into a dead-end crevice at the entrance of NPET (**Figures 4B and 5B**). The same observation (**Figure 5C**) can be made with metabolite-sensing SpeFL/TnaC(R23F) leader peptides (Herrero del Valle et al., 2020; van der Stel et al., 2021). The first methionine of MsrDL-NC latches into the dead-end crevice which is delimited by 23S rRNA nucleotides U2584, U2586, G2608 and C2610 (**Figures 5A, 5B and 5C**). This crevice, named proximal crevice, located at the base of helix 93 (h93) is part of domain V of 23S rRNA and is conserved from bacteria to human mitoribosome and cytosolic ribosome (**Figures 5D, S5F and S5G**).

MsrD negatively regulates its own expression

MsrD protein provides antibiotic resistance by rescuing ERY-stalled ribosome while *msrDL* translation in presence of ERY leads to the formation of a SRC. If MsrD is also able to rescue this SRC, it will repress its own expression and form a feedback loop similarly to Vga(A) (Vimberg et al., 2020). We tested this hypothesis using *E. coli* DB10 strains double transformed with pMMB-*msrDL-msrD*₍₁₋₃₎:*yfp* and the compatible pBAD plasmid expressing various *msrD* variants. Strains were grown in presence of ERY at 300 nM with their optical density and fluorescence monitored (**Figure 6A**). Strain carrying the pBAD-*control* showed high fluorescence and low OD₆₀₀ as observed in **Figure 2A**, reflecting an induction of the reporter

and a susceptibility to ERY. On the contrary, expression of *msrD* led to higher cell growth and a strong reduction of fluorescence which correspond respectively to antibiotic resistance and repression of the expression of the reporter. These results support that MsrD can rescue MsrDL-SRC and therefore releases the RIT repressing *msrD* transcription.

The strain expressing *msrD*_{E125Q} has a low ERY resistance (**Figure 1F**). When it is co-transformed with the pMMB-*msrDL-msrD*_{(1-3):yfp} plasmid, no resistance is observed, possibly because the presence of the two plasmid creates some toxicity. Overall, the results show that MsrD variants that lost the ability to provide antibiotic resistance (Δ PtIM, Δ Loop and ATPase deficient mutants) also lost the ability to rescue MsrDL-SRC (**Figure 6A**). Point mutations in the Loop of the PtIM indicated some discrepancy between antibiotic resistance phenotype and MsrDL-SRC rescue. All the mutants show an intermediary phenotype with less antibiotic resistance and less repression of reporter compared to MsrD. The mutant MsrD_{H244W} has the same repression effect on the reporter than MsrD_{H244A} for an equivalent OD₆₀₀ but it provides more than 3 times less antibiotic resistance (**Figures 1H and 6A**). The mutant MsrD_{R241A} which provides a resistance close to MsrD does not rescue the MsrDL-SRC as efficiently as the latter one (**Figures 1H and 6A**). Mutants MsrD_{L242A} and MsrD_{H244A} provide similar resistance, but MsrD_{H244A} confers a stronger repression of the reporter (**Figures 1H and 6A**). Together these observations demonstrate that the determinants of the dual function of MsrD in conferring antibiotic resistance and rescuing MsrDL-SRC are overall similar.

DISCUSSION

We used genetic, molecular biology and structural approaches to dissect the regulation of the ARE ABC-F MsrD, its effect on ERY-stalled ribosomes and to provide information on ARE ABC-F mechanisms of action that were lacking to date despite structural investigations (Crowe-McAuliffe et al., 2018, 2021; Su et al., 2018). We characterized the *msrDL* regulatory ORF which inhibits its own translation termination in presence of ribosome-bound cladinose-containing macrolides (**Figures 3, 4 and 6B**). Our structural data show that this inhibition results from the conjoint effects of: (i) MsrDL maintaining 23S rRNA bases U2506 and U2585

in an uninduced conformation (**Figure 4F**), (ii) the steric occlusion by U2584, U2585 and MsrDL residue F5 that precludes the productive accommodation of RF1/RF2 (**Figure 3C** and **4E**). MsrDL-stalled ribosome then hampers formation of a RIT allowing transcription of *msrD* (**Figures 2C, 3A, 3C** and **6B**).

MsrD can bind with some specificity to ERY-stalled ribosome in an ATP-bound conformation (**Figures 1D** and **1E**) and then its ATPase activity drives mechanical rearrangements to rescue stalled ribosome. The ATPase mechanism that allows the rescue is still unknown for ARE ABC-Fs and all the current structures of an ARE ABC-F in complex with the ribosome were generated with an ATPase-dead mutant (Crowe-McAuliffe et al., 2018, 2021) or use of non-hydrolysable ATP analogues (Su et al., 2018). These structures do not capture an antibiotic stalled complex and embodies a reaction intermediary before or after antibiotic release of an ARE ABC-F trapped on the ribosome. We provide here some evidence about possible mechanisms (**Figure 6B**). We show that only one active ATPase site (Site II) can provide some antibiotic resistance (**Figures 1F**) while the mutation inactivating Site I generates a strong toxicity for the cell independently of the antibiotic (growth reduced by 70 %, **Figures 1F**). This suggests that even if Site II is used for antibiotic resistance mechanism, it is also necessary for MsrD dissociation from the ribosome. The mutant of Site II shows a stronger toxicity (growth reduced by 80 %, **Figures 1F**) and no antibiotic resistance, effects related to less dissociation from the ribosome, Site I is therefore key for the release of MsrD. Unexpectedly, mutation of the 2 sites leads to the same toxicity as for Site I (**Figures 1F**), suggesting that having only Site I mutated induces a new MsrD conformation that inhibits the ribosome further.

Results of polyribosomes experiments (**Figure 1C to E, Figure S1C**) suggest that MsrD rescues stalled ribosomes by disassembling the two subunits. However, without an *in vitro* assay that has been impeded by the low solubility of purified MsrD, we were not able to confirm this observation. We envision two putative non-exclusive scenarios that involve for the first one, a disassembling of ERY-stalled ribosomes, and for the other one, a displacement of the drug followed by a restart of the translation process (**Figure 6B**). In one case or the other,

direct drug displacement by the factor seems excluded (**Figure S1E**). A key point for future studies will be to explore the ATPase activity of MsrD in a stalled-ribosome context to determine how many rounds of ATP hydrolysis are necessary to rescue the latter one. The dissociation model requires the hydrolysis of only two ATP molecules, while antibiotic displacement may require multiple hydrolysis rounds in Site II to displace the drug and to position back the elongating peptide in a correct conformation in the NPET. So far, those experiments have been challenging due to the low solubility of all the ARE ABC-F proteins (Crowe-McAuliffe et al., 2021; Sharkey et al., 2016).

Alongside with ErmBL, ErmCL and ErmDL, MsrDL constitutes the fourth ERY-sensing NC structurally described, however mechanisms of drug-sensing and PTC silencing greatly differ. First, similarly to ErmCL, the presence of C3 cladinose sugar is required for stalling (**Figures 3A and 3B**) contrary to ErmBL and ErmDL that senses drugs lacking this moiety such as oleandomycin, solithromycin or telithromycin (Arenz et al., 2014b; Beckert et al., 2021). Second, similarly to ErmBL, no close contact between the drug and MsrDL was observed (**Figures 4H and 4I**) (Arenz et al., 2014b). Third, MsrDL is the only described leader peptide that employs inhibition of its hydrolysis by RFs to induce stalling in presence of antibiotic, similarly to the metabolite sensors TnaC and SpeFL (Herrero del Valle et al., 2020; van der Stel et al., 2021). On the contrary, ErmBL (Arenz et al., 2016), ErmCL (Arenz et al., 2014a) and ErmDL (Beckert et al., 2021) employ elongation inhibition. Fourth, ERY-dependent stalling on *msrDL* regulates downstream *msrD* via transcriptional attenuation (**Figure 2B and 2C**) while it relies on translational attenuation for ErmBL, ErmCL and ErmDL (Arenz et al., 2014b; Beckert et al., 2021; Mayford and Weisblum, 1989). Finally, MsrDL-NC conformation and path within the ribosome greatly differ from previously described ligand-sensing leader peptides that elongate within the tunnel (**Figures 5B and 5C**) (Arenz et al., 2014a, 2016; Beckert et al., 2021; Herrero del Valle et al., 2020; van der Stel et al., 2021). It adopts a hook-like shape possibly due to hydrophobic interactions with the drug (**Figures 4B, 4G to 4H**), while its initiating methionine engages in a dead-end crevice at the NPET entrance (**Figure 5**). To our knowledge, this is the first description of this crevice, conserved in prokaryotes and eukaryotes

(**Figures S5F and S5G**), as a functional site within the ribosome. We propose to name it proximal crevice and believe it may contribute to the design of new antimicrobial drugs.

Some pentapeptides are known to mediate ERY resistance (Tenson et al., 1997; Vimberg et al., 2004) by a mechanism that allows the exclusion of the ribosomal bond ERY (Lovmar et al., 2006) upon peptidyl-tRNA hydrolysis by release factors. Exclusion and hydrolysis are reduced for equivalent hexapeptides and the precise molecular mechanism is still unknown, though it has been proposed that they engage in a putative dead end (Lovmar et al., 2006). Those peptides share common features with the MsrDL hexapeptide: the third amino acid is Leu/Ile, the forth is a hydrophobic one and they modulate termination in presence of ERY. Thus, we propose that they could use the same crevice as MsrDL to catalyze their action on ERY.

ARE ABC-F genes tend to be transcriptionally regulated as illustrated by *msrD*, *vmIR*, *vgaA* and *Imo0919* (Dar et al., 2016; Ohki et al., 2005; Vimberg et al., 2020). However, as described in the introduction, *msrD* forms an operon with the efflux pump *mefA* (**Figure 1A**) which is also regulated *via* ribosome-mediated transcriptional attenuation, translational stalling occurring on *mefAL* (Chancey et al., 2015b). Therefore, *msrD* is under the dual-regulation of two ERY-sensing leader peptides. One explanation for this redundancy may reside in the need for the bacteria to tightly regulate *msrD* because of its toxicity. Expression of *msrD* in *E. coli* DB10 led to a 20 % growth defect compared to control condition (**Figure 1F**) suggesting that *msrD* expression has a fitness cost for the bacteria and can be beneficial only in presence of antibiotic. The presence of two attenuators creates a double-lock system to avoid any basal expression in absence of inducer; and the feedback a loop, a mechanism to express only a minimal amount of MsrD. Thus, as long as MefA can export enough antibiotic to provide resistance, *msrD* should be kept repressed. The fitness cost of the expression of ARE ABC-F genes is possibly the Achilles heel of bacteria containing those genes and may be exploited to fight antibiotic resistance. MsrD provides resistance to ERY, AZY and TEL but only the first two induce its expression, indicating that MsrD can provide some resistance for an antibiotic

which does not induce its expression. Thus, MsrDL facilitates the use of MsrD by the bacteria
and maintains its evolution under its control.

ACKNOWLEDGMENTS

This work has benefited from the facilities and expertise of the Biophysical and Structural Chemistry platform (BPCS) at IECB, CNRS UMS3033, Inserm US001, University of Bordeaux. C.R.F. is funded by a doctoral grant from the french Ministère de l'Enseignement supérieur, de la Recherche et de l'Innovation, F.O. and G.B. have received support from the LABEX program (DYNAMO ANR-11-LABX-0011), and the ANR grants EZOtrad (ANR-14-ACHN-0027) for G.B. and ABC-F_AB (ANR-18-CE35-0010) for G.B. and Y.H., ERC-2017-STG #759120 "TransTryp" to Y.H. E.C.L. and C.A.I. have received funding for this project from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (Grant Agreement No. 724040). Authors would like to thank Dr. Olivier Chesneau (Département de Microbiologie, Institut Pasteur, Paris - France) for providing *E. coli* DB10 strain, plasmids pBAD33 and pVN50; Dr. Sylvain Durand and Dr. Maud Guillier (UMR 8261, CNRS, Université de Paris, Institut de Biologie Physico-Chimique, Paris - France) that kindly provided respectively bicyclomycin and equipment for toeprinting experiments. Authors would like also to thank Laura Monlezun and Tina Wang for proofreading the manuscript.

AUTHOR CONTRIBUTIONS

F.O. performed the first experiments that initiated the project. C.R.F. performed most of the experiments. S.N. performed northern blotting experiments. C.R.F. prepared the cryo-EM sample. H.S. prepared cryo-EM grids and imaged them. C.A.I. and Y.H. processed cryo-EM data. C.R.F. and E.C.L. reconstructed the atomic model. G.B. initiated the project and designed the research program with Y.H. C.R.F. and G.B. wrote the paper with input from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests. Requests for materials should be addressed to G.B. (boel@ibpc.fr).

MAIN FIGURE TITLES AND LEGENDS

Figure 1. Translation factor MsrD alleviates erythromycin effects upon translation *in vivo*. (A) Organization of the *mefA/msrD* macrolide resistance operon. In presence of ERY, ribosomes stall during translation of *mefA* leading to transcription anti-attenuation, both *mefA* and *msrD* being then transcribed. Similar mechanism occurs during translation of *msrDL*, which regulates transcription of *msrD* only. (B) Sequence alignment of various *msr* homologs visualized with Jalview according to percentage of identity. Positions of tested mutations are highlighted in red and indicated by arrows. Main features of ABC-F proteins are indicated on the schematic. Genebank accession numbers are indicated between brackets. See also **Figure S1B**. (C, D and E) Polyribosomes analysis and western blotting of *E. coli* DB10 expressing *msrD* (pBAD-*msrD*_{WT}), *msrD*_{EQ2} (pBAD-*msrD*_{EQ2}) or a control (pBAD-*Control*) in presence or absence of ERY. “L” stands for lysate. See also **Figure S1C** and **S1D**. (F, G and H) Relative growth of *E. coli* DB10 expressing *msrD* variants in presence of ERY after 24 h. Optical densities were normalized relative to optical densities of *E. coli* DB10 pBAD-*Control* grown in the absence of ERY. Error bars represent mean $\pm$ s.d. for triplicate experiments. See also **Table S1**.

Figure 2. Erythromycin-dependent transcriptional termination regulates *msrD* expression. (A) ERY-dependent induction of *msrD*₍₁₋₃₎:*yfp*. Fluorescent reporters shown on schematics have been introduced in *E. coli* DB10 and grew in presence of 1 mM IPTG and increasing sublethal ERY concentrations during 17 h. Fluorescence has been plotted against OD₆₀₀, error bars for both axes represent mean $\pm$ s.d. for triplicate experiments. (B) Northern analysis of *msrD* transcript. RNAs were extracted before adding or not 1 mM IPTG (T₀), 10 min after adding IPTG (T₁₀), and 5, 15, 30, 60, 120 min after adding or not 100 nM ERY. Location of probe in *msrD*₍₁₋₃₎:*yfp* 3' UTR is shown on **Figure 2A**. The presence of a second band was also observed but we hypothesized that it was abortive transcript resulting from the construct insofar as its presence correlates with induction by IPTG and ERY, and is no longer detectable using other probes (data not shown). (C) Deletion of the intrinsic terminator between *msrDL* and *msrD*₍₁₋₃₎:*yfp* leads to a constitutive induction in absence of ERY. Error bars for both axes represent mean $\pm$ s.d. for triplicate experiments.

Figure 3. MsrDL is a macrolide-sensing nascent chain inhibiting its translation termination. (A) Toeprinting assay of *msrDL* in the absence (-) or in the presence of 50 μ M of various macrolide antibiotics. Empty arrow indicates initiation codon. Plain arrows indicate ribosome stalling. Chemical structure of antibiotics is shown, C3 cladinose sugar of ERY and AZI being highlighted. See also **Figure S3**. (B) *In vivo* induction of *msrD*₍₁₋₃₎:*yfp* by various PTC/NPET targeting antibiotics. Bacteria containing pMMB-*msrDL*-*msrD*₍₁₋₃₎:*yfp* were grown

during 17 h in presence of 1 mM IPTG, in the absence (grey histograms) or in the presence of 50 nM antibiotics (red histograms). Error bars represent mean $\pm$ s.d. for triplicate experiments. (C) Toeprinting assay depleted of release factors was performed in the absence of ribosome (line 1), in presence of 50 μ M RTP to assess start codon (line 2), without or with 50 μ M ERY (line 3 and 4), without or with 50 μ M ERY then supplemented with 100 μ M puromycin (line 5 and 6), without or with 50 μ M ERY in presence of RF1/RF2/RF3 (line 7 and 8). The schematic indicates position of toe-printing signal on the synthetic mRNA, P site codon of MsrDL-SRC is underlined. See also **Figure S3**. (D and E) Effects of *msrDL* variants on the expression of *msrD*_{(1:3);yfp}. Bacteria were grown during 17 h in presence of 1 mM IPTG, in the absence (grey histograms) or in the presence of 100 nM ERY (red histograms). Error bars represent mean $\pm$ s.d. for triplicate experiments.

Figure 4. Structure of MsrDL-SRC. (A) Transverse section of the cryo-EM map showing the 30S (grey) and 50S (white) ribosomal subunits, mRNA (purple), ERY (red) and MsrDL-NC (gold) bound to P-site tRNA^{Met} (beige). (B) Close-up of MsrDL-NC within ribosomal tunnel showing experimental density and modelled structure, colored as **Figure 4A** while 23S rRNA nucleotides are shown in light grey. (C-D) Presence of MsrDL residue Y2 displace nucleotide U2584 out of its position when compared to RF1- (PDB 5J30, light blue) and RF2-containing (PDB 5CZP, blue) termination complex, thus forming a π -stacking with G2583 (Pierson et al., 2016). (E) Conformation of U2584 and U2585 prevent productive accommodation of RF1 (PDB 5J30, light blue) and RF2 (PDB 5CZP, blue) while catalytic methylated glutamine would clash with MsrDL residue F5 (Pierson et al., 2016). (F) PTC in MsrDL-SRC (light grey) is stabilized in an uninduced state (PDB 1VQ6, pink) rather than in an induced state (PDB 1VQN, green) as U2585 is pushed back by MsrDL residue Y2 (Martin Schmeing et al., 2005). (G-I) Molecular basis for C3 cladinose sugar recognition by MsrDL. Residue L3 is at proximity of cladinose sugar while residue I4 is at proximity of desosamine sugar. TEL lacking cladinose sugar and failing to form MsrDL-SRC has been aligned (PDB 4V7S) (Dunkle et al., 2010). (J) Presence of residue I4 avoids rotation of A2062 to form an Hoogsteen base pairing with m²A2503 as it is the case for ErmCL-SRC (PDB 3J7Z, green) (Arenz et al., 2014a). Light blue dashed lines indicate hydrogen bonds formed by Hoogsteen base pairing. For the whole figure, structures were aligned on domain V of 23S rRNA. Spheres represent van der Waals radii.

Figure 5. MsrDL engages in a conserved crevice at the NPET entrance. (A) Secondary structure of the *E. coli* 23S rRNA domain V showing location of the proximal crevice at the base of h93. For the whole figure, nucleotides delimitating proximal crevice are shown in red. (B-C) Comparison of MsrDL path (gold) with ERY-dependent leader peptides ErmBL (PDB 5JTE, blue), ErmCL (PDB 3J7Z, green), ErmDL (PDB 7NSO, teal) as well as L-ornithine-

sensing SpeFL (PDB 6TC3, pink) and L-tryptophan-sensing TnaC(R23F) (PDB 7O1A, purple) (Arenz et al., 2014a, 2016; Beckert et al., 2021; Herrero del Valle et al., 2020; van der Stel et al., 2021). See also **Figure S5A to S5E**. (D) Proximal crevice in domain V is conserved from bacteria to human (MsrDL-SRC, grey; *S. aureus* PDB 6YEF, light green; *H. sapiens* 55S mitoribosome PDB 7A5F, light blue; *H. sapiens* 80S ribosome PDB 6OLI, marine blue) (Desai et al., 2020; Golubev et al., 2020; Li et al., 2019). See also **Figure S5G and S5F**. For the whole figure, atomic model of MsrDL is shown with its experimental density. Structures were aligned on domain V of 23S rRNA.

Figure 6. MsrD negatively regulates its own synthesis upon erythromycin exposure. (A) Effects of MsrD variants on MsrDL. *E. coli* DB10 containing pMMB-*msrDL-msrD*(1-3):*yfp* and expressing various *msrD* mutants were grown in presence of 0.2 % L-Arabinose, 1 mM IPTG and 300 nM ERY, both OD₆₀₀ and fluorescence being recorded over 24 h. Fluorescence has been plotted against OD₆₀₀, error bars for both axes represent mean $\pm$ s.d. for triplicate experiments. Color code is same as **Figures 1F to 1H**. Light green rectangle indicates bacterial growth over control plasmid. (B) Model of MsrD regulating its own expression and providing antibiotic resistance. In absence of ERY, RNAP drops off at Rho-independent transcription terminator. In presence of ERY, the ribosome following the RNAP stalls and unwinds the terminator leading to *msrD* transcription. Once translated, MsrD negatively regulates its own expression on one side, and provides antibiotic resistance on the other side. ATP-bound MsrD recognizes ERY-stalled ribosome and may either expel the antibiotic or dissociate the ribosome, ATP site II being active. Activity in ATP site I leads to MsrD dissociation and recycling.

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Table 1. Minimum inhibitory concentration (MIC) and half maximal inhibitory concentration (IC₅₀) of *E. coli* DB10 expressing *msrD* in presence of ribosome-targeting antibiotics. See methods for experimental details.

	pBAD-Control		pBAD-<i>msrD</i>_{WT}	
Antibiotic	MIC (μM)	IC₅₀ (μM)	MIC (μM)	IC₅₀ (μM)
Erythromycin	2	0,179 ± 0,007	16	4,592 ± 0,582
Azithromycin	0.25	0,046 ± 0,002	2	0,953 ± 0,053
Telithromycin	1	0,086 ± 0,008	2	0,714 ± 0,066
Tylosin	4	1,649 ± 0,151	4	1,672 ± 0,103
Spiramycin	2	0,758 ± 0,063	2	0,81 ± 0,078
Lincomycin	32	12,61 ± 2,154	32	12,62 ± 1,27
Linezolid	32	13,62 ± 2,064	32	13,41 ± 1,137
Retapamulin	1	0,071 ± 0,004	1	0,075 ± 0,007

STAR METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-6xHis-tag (mouse - monoclonal)	Covalab	Cat# mab90001-P
Anti-mouse-HRP IgG (goat – polyclonal)	Covalab	Cat# Lab0252
Bacterial strains		
<i>E. coli</i> DB10 (Derived from <i>E. coli</i> PR7)	(Datta et al., 1974)	N/A
<i>E. coli</i> K12 MG1655	N/A	N/A
Chemicals, Peptides, and Recombinant Proteins		
[γ ³² P]ATP 3000 Ci/mmol, 50 mCi/ml	Hartmann Analytic	Cat# SRP-301
Ampicillin	Euromedex	Cat# EU0400-D
Avian Myeloblastosis Virus Reverse Transcriptase	Promega	Cat# M5101
Azithromycin	SYNkinase	Cat# SYN-3010
Chloramphenicol	Euromedex	Cat# 3886-B
CloneAmp HiFi PCR Premix	Takara	Cat# 639298
ddNTPs	Sigma Aldrich	Cat# GE27-2045-01
dNTPs	New England Biolabs	Cat# N0447S
Erythromycin	Toku-E	Cat# E002-5G
Formamide Ultra High Purity	Electron Microscopy Sciences	Cat# 15745
Isopropyl β -D-1-thiogalactopyranoside	Euromedex	Cat# EU0008-C
K-Glutamate	Sigma Aldrich	Cat# 49601-100G
Lincomycin	Sigma Aldrich	Cat# 62143-1G
Linezolid	Sigma Aldrich	Cat# PZ0014-5MG
Mineral oil	Sigma Aldrich	Cat# M8410-1L
Mueller Hinton Broth	Sigma Aldrich	Cat# 70192-500G
NEBuilder HiFi DNA Assembly Master Mix	New England Biolabs	Cat# E2621L

Protease Inhibitor Cocktail	Sigma Aldrich	Cat# P8340-1ML
Puromycin	Sigma Aldrich	Cat# 540222-25MG
Putrescine	Sigma Aldrich	Cat# D13208-25G
Retapamulin	Sigma Aldrich	Cat# CDS023386-25MG
Spermidine	Sigma Aldrich	Cat# S2626-1G
Spiramycin	Sigma Aldrich	Cat# 46745-100MG
T4 Polynucleotide kinase	New England Biolabs	Cat# M0201L
T7 RiboMAX Large Scale RNA Production System	Promega	Cat# P1300
Taq DNA polymerase with ThermoPol Buffer	New England Biolabs	Cat# M0267S
Telithromycin	Sigma Aldrich	Cat# SML2162-5MG
THE Ambion RNA Storage Solution	Thermo Fisher Scientific	Cat# AM7000
TRIzol Reagent	Thermo Fisher Scientific	Cat# 15596026
Tylosin tartrate	Sigma Aldrich	Cat# 33847-250MG
ULTRAhyb-Oligo hybridization buffer	Thermo Fisher Scientific	Cat# AM8663
Critical commercial assays		
PUREfrex 2.0	GeneFrontier	Cat# PF201-0.25-EX
PURExpress ΔRF123 kit	New England Biolabs	Cat# E6850S
PURExpress ΔRibosome kit	New England Biolabs	Cat# E3313S
Deposited data		
Atomic coordinates of erythromycin-stalled <i>Escherichia coli</i> 70S ribosome with streptococcal MsrDL nascent chain	This study	PDB 7Q4K
Cryo-EM maps of erythromycin-stalled <i>Escherichia coli</i> 70S ribosome with streptococcal MsrDL nascent chain	This study	EMD-13805, EMD-13806, EMD-13807, EMD-13808
Oligonucleotides		
See supplementary table 3	Eurofins Genomics	N/A

Recombinant DNA		
pBAD-Control (pBAD33)	(Nunez-Samudio and Chesneau, 2013)	N/A
pBAD- <i>msrD</i> _{WT} (pVN50)	(Nunez-Samudio and Chesneau, 2013)	N/A
pBAD- <i>msrD</i> _{EQ2}	This study	N/A
pBAD- <i>msrD</i> _{E125Q}	This study	N/A
pBAD- <i>msrD</i> _{E434Q}	This study	N/A
pBAD- <i>msrD</i> _{ΔLoop}	This study	N/A
pBAD- <i>msrD</i> _{ΔPILM}	This study	N/A
pBAD- <i>msrD</i> _{R241A}	This study	N/A
pBAD- <i>msrD</i> _{L242A}	This study	N/A
pBAD- <i>msrD</i> _{H244A}	This study	N/A
pBAD- <i>msrD</i> _{H244W}	This study	N/A
pMMB-67EH	(Fürste et al., 1986)	N/A
pMMB-67EH- <i>yfp</i>	This study	N/A
pMMBpLacO-67EH- <i>yfp</i>	This study	N/A
pMMB- <i>msrDL</i> - <i>msrD</i> ₍₁₋₃₎ : <i>yfp</i>	This study	N/A
pMMB- <i>msrDL</i> _(no_ORF) - <i>msrD</i> ₍₁₋₃₎ : <i>yfp</i>	This study	N/A
pMMB- <i>msrDL</i> _(no_term) - <i>msrD</i> ₍₁₋₃₎ : <i>yfp</i>	This study	N/A
pMMB- <i>msrDL</i> _(Y2A) - <i>msrD</i> ₍₁₋₃₎ : <i>yfp</i>	This study	N/A
pMMB- <i>msrDL</i> _(L3A) - <i>msrD</i> ₍₁₋₃₎ : <i>yfp</i>	This study	N/A
pMMB- <i>msrDL</i> _(I4A) - <i>msrD</i> ₍₁₋₃₎ : <i>yfp</i>	This study	N/A
pMMB- <i>msrDL</i> _(F5A) - <i>msrD</i> ₍₁₋₃₎ : <i>yfp</i>	This study	N/A
pMMB- <i>msrDL</i> _(M6A) - <i>msrD</i> ₍₁₋₃₎ : <i>yfp</i>	This study	N/A
pMMB- <i>msrDL</i> _(UAA>UGA) - <i>msrD</i> ₍₁₋₃₎ : <i>yfp</i>	This study	N/A
pMMB- <i>msrDL</i> _(UAA>UAG) - <i>msrD</i> ₍₁₋₃₎ : <i>yfp</i>	This study	N/A
Software and Algorithms		
ARNold	(Naville et al., 2011)	N/A

Clustal W	(Larkin et al., 2007)	N/A
Coot	(Emsley and Cowtan, 2004)	N/A
Fiji	(Schindelin et al., 2012)	N/A
Gctf	(Zhang, 2016)	N/A
Jalview	(Waterhouse et al., 2009)	N/A
MotionCor2	(Zheng et al., 2017)	N/A
Phenix	(Liebschner et al., 2019)	N/A
PRISM 7	GraphPad	N/A
PyMOL Molecular Graphics System	Schrödinger, Inc.	N/A
RELION 3	(Zivanov et al., 2018)	N/A
ResMap	(Kucukelbir et al., 2014)	N/A
SWISS-MODEL	(Waterhouse et al., 2018)	N/A
UCSF Chimera	(Pettersen et al., 2004)	N/A
UCSF ChimeraX	(Goddard et al., 2018)	N/A
Other		
Amersham Hybond-N+ Membrane	GE Healthcare	Cat# RPN203B
Biocomp Gradient Master	Biocomp Instruments	N/A
Biocomp Piston Gradient Fractionator	Biocomp Instruments	N/A
BMG ClarioStar	BMG Labtech	N/A
Clarity Western ECL Substrate	Bio-Rad	Cat# 170-5061
Direct-zol RNA Miniprep kit	Zymo Research	Cat# R2051
Immun-Blot PVDF Membranes for Protein Blotting	Bio-Rad	Cat# 1620177
Li-Cor Odyssey FC imaging system	Li-cor	N/A

QIAquick Nucleotide Removal kit	Qiagen	Cat# 28304
Storage Phosphor Screen BAS-IP MS 2040	Fujifilm	Cat# 28956474
Typhoon FLA 9500	GE Healthcare	N/A

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METHOD DETAILS

Construction of plasmids

All strains and plasmids used in this study are listed in **key resources table**. Oligonucleotides are listed in **supplementary table 3**. For the whole study, the considered reference sequence for *mefA/msrD* macrolide resistance operon was a Tn916-type transposon inserted in *Streptococcus pneumoniae* strain 23771 genome (Genbank accession number FR671415.1) (Croucher et al., 2011). Plasmids pBAD-*Control* and pBAD-*msrD*_{WT} containing a C-terminal hexahistidine tag (originally referred as pBAD33 and pVN50) were kindly retrieved from Olivier Chesneau at Institut Pasteur (Paris, France) (Nunez-Samudio and Chesneau, 2013). Both *mefA/msrD* macrolide resistance operon and pBAD-*msrD*_{WT} encode the same protein MsrD (Uniprot accession number A0A496M710).

pBAD plasmids. Those plasmids allow a tight and stringent expression control via catabolite repression, interest gene being repressed in presence of 0.4 % (w/v) β-D-Glucose and induced in presence of 0.2 % (w/v) L-Arabinose. Transformed clones were selected with 20 µg.ml⁻¹ chloramphenicol. In this study, all *msrD* mutants contain a C-terminal hexahistidine tag. Catalytic mutant *msrD*_{EQ2} was generated by amplifying pBAD-*msrD*_{WT} with primer pairs 5/6 and 7/8 (**Table S3**), both fragments being assembled with NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs). Catalytic mutants *msrD*_{E125Q}, *msrD*_{E434Q} were generated via quickchange mutagenesis with primers pairs 9/10 and 11/12. Mutants *msrD*_{ΔLoop} and *msrD*_{ΔPTM} were generated based on phylogenetic alignments by deleting residues between K216/K254 and E189/A274 respectively, a three-glycine linker being added to allow flexibility, via quickchange mutagenesis using primer pairs 13/14 and 15/16 (**Table S3**). Variants *msrD*_{R241A}, *msrD*_{L242A}, *msrD*_{H244A}, *msrD*_{H244W} were generated via fusion PCR by amplifying *msrD*_{WT} with primers pairs 1/18, 1/20, 1/22, 1/24 and 2/17, 2/19, 2/21, 2/23 respectively (**Table S3**). Backbone was amplified with primer pair 3/4 and fragments were assembled with NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs).

pMMB plasmids. All pMMB constructs originated from a low copy IPTG-inducible plasmid pMMB67EH-*yfp*, consisting in original pMMB67EH (Fürste et al., 1986) containing optimized *venus-yfp* enhancing its translation under the control of P_{tac} promoter (manuscript in preparation). Transformed clones were selected with 100 µg.ml⁻¹ ampicillin. To test *msrDL* functions *in vivo*, several fluorescent reporter genes have been designed. First, pMMB67EH-*yfp* native P_{tac} promoter was replaced by P_{LacO} promoter as previously described (Lutz and Bujard, 1997):

5'- ATAAATGTGAGCGGATAACATTTGACATTGTGAGCGGATAACAAGATACTGAGCACA -
3' (lac operators, shaded grey; transcription start, bold). This IPTG-inducible promoter allows a tight and stringent transcription regulation compared to P_{tac} promoter, insofar as no regulatory

element is found in the 5' UTR. Promoter was replaced by amplifying pMMB67EH-*yfp* with primer pairs 25/26 (**Table S3**), PCR fragment being then re-circularized via NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs). Plasmid pMMB-*msrDL-msrD*₍₁₋₃₎:*yfp* has been designed by introducing the sequence spanning from the first nucleotide downstream *mefA* stop codon to *msrD* three first codons fused to *yfp*. The introduced sequence is as follows:

5'-
ACAATATT**GGAGGA**AATATTTATGTATCTTATTTTCATGTAACTCTTCCTGCTAAAATCGCA
GGGTTTTCCCTGCATACAAGCAAATGAAAGCATGCGATTATAGACAG**GGAGGAAATGTTA**
TGGAATTA-3' (RBS, bold; *msrDL*, shaded grey, *msrD* three first codons are underlined). To clone such construct, *yfp* gene was amplified from pMMB67EH-*yfp* with primer pairs 27/29 then with 28/29 to generate the insert, backbone was amplified with primer pair 30/31 (**Table S3**), both fragments being then assembled with NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs). Plasmid pMMB-*control* was built by removing the *msrDL-msrD*₍₁₋₃₎:*yfp* cassette via quickchange mutagenesis using primer pair 32/33, the resulting construction containing only the P_{LacO} promoter followed by the plasmid endogenous *rrnB* transcription terminator. Plasmid pMMB-*msrDL*_(no_term)-*msrD*₍₁₋₃₎:*yfp* (where the RIT between *msrDL* and *msrD* is deleted) was generated by amplifying pMMB-*msrDL-msrD*₍₁₋₃₎:*yfp* with primer pair 34/35, PCR fragment being then re-circularized via NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs). The various *msrDL* mutants were cloned via quickchange mutagenesis by amplifying pMMB-*msrDL-msrD*₍₁₋₃₎:*yfp* with primers 36 to 51.

Antibiotic susceptibility testing, MIC and IC50 determination

A saturated preculture of *E. coli* DB10 transformed with pBAD plasmid was grown overnight at 37 °C under vigorous shaking in Luria-Bertani Miller broth (LB), 20 µg.ml⁻¹ chloramphenicol and supplemented with 0.4 % (w/v) β-D-Glucose. Antibiotic susceptibility testing assay was performed in Mueller-Hinton broth (MH, Sigma Aldrich), antibiotics being diluted via serial dilutions. A 96-wells flat-bottom plate (Flacon) was filled with a final volume per well of 200 µl, containing 20 µg.ml⁻¹ chloramphenicol and 0.2 % (w/v) L-Arabinose and antibiotic to test. Wells were inoculated at OD₆₀₀ ~0.03-0.04 prior to addition of 60 µl mineral oil (Sigma Aldrich) avoiding evaporation but not oxygen diffusion. Plates were therefore incubated for 24 h in CLARIOstar Plus plate reader (BMG Labtech) at 37 °C with 600 rpm double-orbital shaking, OD₆₀₀ being measured each 30 min. Optical densities at 24 h were then normalized relative to optical densities of *E. coli* DB10 pBAD-*Control* grown in the absence of antibiotic in Prism 7 (GraphPad). MIC was determined as absence of growth compared to blank, IC50 was calculated in Prism using equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + ((X^{\text{HillSlope}}) / (\text{IC50}^{\text{HillSlope}})))$ and the standard deviation was calculated by multiplying standard error by square root of n (n being at least 3 replicates). To generate

curves, for bacteria grown in absence of antibiotic (0 μM), since coordinates are plotted as logarithms and since $\log(0)$ is undefined, this point has been approximated 2 log units below the lowest tested value (*i.e.* 0.0625 μM) consistently with Prism user guide (Miller, 2003). Curve fitting was performed with non-linear fitting function “log(inhibitor) vs. response -- Variable slope” using $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogIC}_{50} - X) * \text{HillSlope}))}$. Both equations gave strictly the same result for IC_{50} .

***In vivo* induction assay**

A saturated preculture of *E. coli* DB10 transformed with pMMB plasmid was grown overnight at 37 °C under vigorous shaking in LB with 100 $\mu\text{g} \cdot \text{ml}^{-1}$ ampicillin. For bacteria double-transformed with pMMB and pBAD plasmids, media was also supplemented with 20 $\mu\text{g} \cdot \text{ml}^{-1}$ chloramphenicol and supplemented with 0.4 % (w/v) β -D-Glucose. *In vivo* induction assay was performed in Mueller-Hinton broth (MH, Sigma Aldrich). A 96-wells flat-bottom plate (Falcon) was filled with a final volume per well of 200 μl , containing 100 $\mu\text{g} \cdot \text{ml}^{-1}$ ampicillin, 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG). Growth medium was supplemented with 20 $\mu\text{g} \cdot \text{ml}^{-1}$ chloramphenicol and 0.2 % (w/v) L-Arabinose if pBAD plasmid is present. Induction of fluorescent reporters being antibiotic-dependent, growth media were supplemented with required antibiotic accordingly. Plates were therefore incubated in CLARIOstar Plus plate reader (BMG labtech) at 37 °C with 600 rpm double-orbital shaking up to 24 h, OD_{600} and fluorescence (excitation: 497-15 nm, emission: 540-20 nm, gain 1600) being measured each 30 min.

Polyribosomes fractionation

Saturated precultures of *E. coli* DB10 transformed with pBAD-control, pBAD-*msrD*_{WT} or pBAD-*msrD*_{EQ2} were grown overnight at 37 °C under vigorous shaking in LB, 20 $\mu\text{g} \cdot \text{ml}^{-1}$ chloramphenicol, 0.4 % (w/v) β -D-Glucose. Growth media (MH, 20 $\mu\text{g} \cdot \text{ml}^{-1}$ chloramphenicol, 0.2 % (w/v) L-Arabinose) was inoculated at $\text{OD}_{600}=0.05$ with normalized precultures, and cells were grown at 37 °C and 180 rpm. When $\text{OD}_{600}=0.5$ was reached, cultures were splitted in two and one half was treated with a 25 μM erythromycin. Cultures were then incubated 1 hour at 37 °C and 180 rpm before harvesting. Cultures were chilled and cells were harvested by centrifuging 10 min at 8 000 rpm, 4 °C. Supernatant was discarded and cells were resuspended in 350 μl of lysis buffer (20 mM Tris pH 8, 20 mM MgCl_2 , 100 mM $\text{NH}_4(\text{OAc})$, 2 mM β -mercaptoethanol, 10 $\text{mg} \cdot \text{ml}^{-1}$ lysozyme) and transferred in microtubes. Then, 30 μl of sodium deoxycholate 10 % (w/v) was added and cells were mechanically lysed by 3 rounds of freezing-thawing. Lysates were clarified by centrifuging 20 min at 18 000 xg, 4 °C and total RNAs were quantified. Finally, 500 μg of total RNAs were loaded onto 10-40 % (w/v) sucrose gradients (20 mM Tris pH 8, 20 mM MgCl_2 , 100 mM $\text{NH}_4(\text{OAc})$, 2 mM β -mercaptoethanol) and

centrifuged 2 h at 40 000 rpm, 4 °C in SW41 rotor (Beckman Coulter). Samples were fractionated and collected using Biocomp Piston Gradient Fractionator (BioComp Instruments), absorbance being measured at 254 nm.

Immunoblotting

Fractions of 300 µl were precipitated overnight in 150 µg.ml⁻¹ sodium deoxycholate and 15 % trichloroacetic acid. Pellets were washed twice in cold acetone, dried and resuspended in 25 µl 1X Laemmli buffer, 10 µl being loaded on 12.5 % acrylamide SDS-PAGE gels. Concerning total lysates, 10 µg of normalized total RNAs were loaded as control. Gels were resolved by migrating at 0.04 A and then applied on Immun-Blot PVDF membrane (Bio-Rad) that had been activated in absolute ethanol then washed in electro-transfer buffer (25 mM Tris base, 192 mM glycine, 0.1 % (w/v) SDS, 10 % (v/v) absolute ethanol). Proteins were electro-transferred at 100 V during 1 h. Membranes were blocked by incubating 1 h in 1X PBS supplemented with 0.5 % nonfat dry milk then washed with 1X PBS. C-term His-tagged MsrD_{WT} and MsrD_{EQ2} were detected using anti-6xHis-tag primary antibody (Covalab, 1:2 000 dilution in 1X PBS, 0.1 % Tween-20) combined with anti-mouse-HRP secondary antibody (Covalab, 1:20 000 dilution in 1X PBS, 0.1 % Tween-20). Immunoblots were revealed by performing an ECL detection using Clarity Western ECL Substrate (Bio-Rad) and imaged with Li-Cor Odyssey FC imaging system (Li-Cor).

Preparation of *E. coli* DB10 ribosomes

An overnight saturated *E. coli* DB10 preculture was used to inoculate growth medium (LB, dilution 1:100) and cells were grown at 37 °C and 180 rpm. When OD₆₀₀=0.5, cells were washed and harvested by two centrifugations during 20 min, 5 000 xg at 4°C followed by resuspensions in Buffer A (20 mM Tris pH 7.4, 10 mM Mg(OAc), 100 mM NH₄(OAc), 0.5 mM EDTA). Pellets were resuspended in Buffer A supplemented with 6 mM β-mercaptoethanol, 10 mg.ml⁻¹ lysozyme, 0.001 % (v/v) protease inhibitor cocktail (Sigma Aldrich) and lysed three times at 2.5 kBar using a cell disrupter (Constant Systems Limited). Lysate was clarified by two centrifugations during 15 min, 22 000 xg at 4°C then spun for 20 h, 34 700 rpm at 4 °C in a Type 70 Ti rotor (Beckmann Coulter) through a 37.7 % (w/v) sucrose cushion in Buffer B (20 mM Tris pH 7.4, 10 mM Mg(OAc), 500 mM NH₄(OAc), 0.5 mM EDTA, 6 mM β-mercaptoethanol). Sucrose cushions were decanted and ribosome pellets resuspended in Buffer C (20 mM Tris pH 7.4, 7.5 mM Mg(OAc), 60 mM NH₄(OAc), 0.5 mM EDTA, 6 mM β-mercaptoethanol). Finally, 12 mg of ribosomes were loaded onto 10-40 % (w/v) sucrose gradients in Buffer C and centrifuged 18 h at 20 000 rpm, 4 °C in SW28 rotor (Beckman Coulter). Gradients were fractionated using Biocomp Piston Gradient Fractionator (BioComp Instruments), absorbance being measured at 254 nm. Fractions containing 70S ribosomes

were pooled, washed and concentrated in Amicon 50k (Merckmillipore) using Buffer C. Ribosomes concentration was adjusted to 18-20 μM , then aliquoted and flash frozen in liquid nitrogen.

RNA extraction and northern blotting

Total RNA extraction was realized using the RNAsnap method as previously described (Stead et al., 2012). In brief, a preculture of *E. coli* DB10 containing pMMB-*msrDL-msrD*₍₁₋₃₎:*yfp* was grown overnight at 37 °C under vigorous shaking in LB supplemented with 100 $\mu\text{g}.\text{ml}^{-1}$ ampicillin. Growth medium (MH, 100 $\mu\text{g}.\text{ml}^{-1}$ ampicillin) was inoculated at OD₆₀₀=0.05, and cells were grown at 37 °C and 180 rpm. When OD₆₀₀=0.5, cells were treated (or not) with 1 mM IPTG during 10 min, then treated (or not) with 100 nM erythromycin. Samples of 2 ml were collected (T_0 = before IPTG was added, T_{10} = 10 min after IPTG was added, and 5 min, 15 min, 30 min, 60 min and 120 min after erythromycin was added), then spun during 1 min at 15 000 rpm. Growth media was removed and cells were resuspended in 100 μl RNAsnap buffer (95 % (v/v) formamide, 18 mM EDTA, 0.025 % (v/v) SDS, 1 % (v/v) β -mercaptoethanol). Samples were heated for 10 min at 95 °C then clarified by centrifuging 10 min, at 16 000 rpm at 16 °C. For northern blotting analysis, 6 μg of total RNAs were resolved on a 1 % (w/v) agarose gel then transferred onto Amersham Hybond-N+ Membrane (GE Healthcare) by capillary transfer. Radioactive probe was prepared using 40 pmol of primer 55 and 5'-labelled with 10 U of T4 Polynucleotide Kinase (New England Biolabs) and [$\gamma^{32}\text{P}$]ATP (150 μCi). Probe was hybridized overnight at 42 °C using ULTRAhyb-Oligo hybridization buffer (Thermo Fisher Scientific). Membrane was washed three times at 42 °C during 15 min (once in 2x SSC + 0.1 % (v/v) SDS, once in 1x SSC + 0.1 % (v/v) SDS and finally in 0.1x SSC + 0.1 % (v/v) SDS). Radioactive signal was visualized by exposing 4 h a Storage Phosphor Screen BAS-IP MS 2040 (Fujifilm) then imaged with a Typhoon FLA 9500 (GE Healthcare).

In vitro transcription

In vitro transcription was carried out in T7 RiboMAX Large Scale RNA Production System kit (Promega) according to manufacturer instructions. Briefly, to generate DNA matrix (Table S4), T7 promoter (5'-GCGAATTAATACGACTCACTATAGGG-3') was added by PCR using primers pair 52/53 (Table S3) and pMMB plasmids as templates. Transcription reactions were incubated 4 h at 37 °C and transcripts were purified with TRIzol Reagent (Thermo Fisher Scientific) and Direct-zol RNA Miniprep kit (Zymo Research), samples being eluted in THE Ambion RNA Storage Solution (Thermo Fisher Scientific). Final concentration was adjusted to 5 pmol. μl^{-1} .

Toeprinting assay

Position of stalled ribosomes on mRNA was determined by toe-printing assay, slightly adapted from previously described methods (Herrero del Valle et al., 2020; Seefeldt et al., 2015). *In vitro* translation reactions were performed using PURExpress Δ RF123 kit and PURExpress Δ Ribosome (New England Biolabs) according to manufacturer instructions. Briefly, prior to *in vitro* translation reactions, 0.5 μ l of 500 μ M ligand (e.g. erythromycin etc) was dried in a micro-centrifuge tube, using a SpeedVac vacuum concentrator (Thermo Fisher Scientific), final concentration being 50 μ M once resuspended in a 5 μ l reaction. Reactions were incubated during 15 min at 37 °C using 5 pmol of RNA templates (generated as described above) and 3.3 μ M of purified *E. coli* DB10 ribosomes. When needed, samples were treated with 100 μ M final puromycin and incubated 3 min more. Immediately, 2 pmol of CY5-labelled primer 54 complementary to NV1 sequence (Vazquez-Laslop et al., 2008) was added and incubated for 5 min at 37 °C. For each reaction, reverse transcription was performed with 0.5 μ l (corresponding to 5 U) of Avian Myeloblastosis Virus Reverse Transcriptase (Promega), 0.1 μ l dNTP mix (10 mM), 0.4 μ l Pure System Buffer (5 mM K-phosphate pH 7.3, 9 mM Mg(OAc), 95 mM K-glutamate, 5 mM NH₄Cl, 0.5 mM CaCl₂, 1 mM spermidine, 8 mM putrescine, 1 mM DTT) and incubated 20 min at 37 °C. Reactions were quenched with 1 μ l NaOH 5 M and incubated 15 min at 37 °C. Alkali were therefore neutralized by adding 0.7 μ l HCl 25% immediately supplemented with 20 μ l toe-printing resuspension buffer (300 mM Na(OAc) pH 5.5, 5 mM EDTA, 0.5% (v/v) SDS). Finally, cDNAs were purified using QIAquick Nucleotide Removal kit (Qiagen), vacuum dried and resuspended in 6 μ l formamide dye (95% (v/v) formamide, 20 mM EDTA, 0.25% (w/v) bromophenol blue). Sanger sequencing was performed on DNA matrix used for *in vitro* transcription. Briefly, each 20 μ l reaction contained 7.5 nM DNA matrix, 75 nM CY5-labelled primer 58, 40 μ M of each dNTPs, 0.025 U Taq Pol (New England Biolabs), 1x Thermo Pol Buffer (New England Biolabs) and corresponding ddNTPs (625 μ M ddCTP/ddTTP/ddATP or 50 μ M ddGTP). After PCR, 20 μ l formamide dye was added to each sequencing reaction. Samples were denatured 3 min at 80 °C, then 5 μ l of sequencing reaction and 1.5 μ l of toe-printing reaction were loaded on a 6% acrylamide/bis-acrylamide (19:1) sequencing gel containing 8 M urea. Gel was resolved by migrating 90 min at 50 W, then imaged using a Typhoon FLA 9500 (GE Healthcare Life Sciences) using CY5 mode, LPR Ch.2 filter and 635 nm laser.

Preparation of MsrDL-SRC

A DNA matrix (**Table S4**) was prepared with CloneAmp HiFi PCR Premix (Takara) using primer pair 52/56 (**Table S3**) and plasmid pMMB-*msrDL-msrD*₍₁₋₃₎:*yfp* as matrix. The corresponding mRNA was generated as described above. The complete sequence of MsrDL-SRC mRNA is:

5'- GGAGCGGAUAACAAGAUACUGAGCACAACAAUAUUGGAGGAUAUUUAUGUAU
CUUAUUUUCAUGUAAACUCUCCUGCUAAAAUCGCAGGGUUUUGCCUGC -3' (RBS, bold;
msrDL, shaded grey; the ATG codon in the P site of stalled ribosomes is underlined). Prior to
in vitro translation reaction, 1 µl of 500 µM erythromycin (50 µM final concentration once
resuspended in 10 µl) has been dried in a micro-centrifuge tube, using a SpeedVac vacuum
concentrator (Thermo Fisher Scientific). Dried erythromycin has been resuspended in a 10 µl
in vitro translation reaction carried out in PUREflex 2.0 kit (Genescript), containing 1.83 µM
of purified *E. coli* DB10 ribosomes and 3.6 µM mRNA (molar ratio 1:2). The reaction has been
incubated for 10 minutes at 37 °C, before adding 100 µM final puromycin, and incubated 3 min
more. Reaction was therefore diluted in ribosome purification Buffer C (20 mM Tris pH 7.4, 7.5
mM Mg(OAc), 60 mM NH₄(OAc), 0.5 mM EDTA, 6 mM β-mercaptoethanol) to reach a
concentration of 150 nM ribosomes. Cryo-EM grids were immediately prepared.

Cryo-EM grids preparation

Safematic ccu-010 HV carbon coater was used to coat Quantifoil carbon grids (QF-R2/2-Cu)
with a thin carbon layer of approximate thickness of 2nm. Grids were therefore glow discharged
for 30 sec at 2 mA. Then, 4 µl of *in vitro* translation reaction diluted to 150 nM were applied,
and after a 2 sec blotting (force 5) and 30 sec waiting time, grids were vitrified in liquid ethane
using a Vitrobot Mark IV (FEI) set to 4 °C and 100 % humidity.

Image acquisition and processing

Data collection was performed on a Talos Arctica instrument (FEI Company) at 200 kV using
the EPU software (Thermo Fisher Company) for automated data acquisition. Data were
collected at a nominal under focus of -0.5 to -2.7 µm at a magnification of 120,000 X yielding
a calibrated pixel size of 1.2 Å. Micrographs were recorded as movie stack on a K2 Summit
direct electron detector (Gatan), each movie stack were fractionated into 65 frames for a total
exposure of 6.5 sec corresponding to an electron dose of 64 e⁻/Å². MotionCor2 (Zheng et al.,
2017) was used for dose weighting, drift and whole-frame motion correction. A dose weighted
average image of the whole stack was used to determine the contrast transfer function with
the software Gctf (Zhang, 2016). Particles were picked using a Laplacian of gaussian function
(min diameter 260 Å, max diameter 320 Å). A total of ~254k particles were extracted from a
subset of motion-corrected images (1523 micrographs) presenting a resolution equal or better
than 4 Å with a box size of 360 pixels. The particles were binned three folds for 2D and
subsequent 3D classification. After 2 rounds of 2D classification in RELION 3 (Zivanov et al.,
2018), ~158k particles were selected and submitted to Relion 3D classification (Zivanov et al.,
2018). A classe of non-rotated 70S depicting high-resolution features and bearing a tRNA with
various occupancies was selected, representing ~104k particles. This class was further 3D-

classified with a spherical mask engulfing the tRNAs binding sites (**Figure S4B**) into 4 classes, thus yielding P/E-tRNAs, P-tRNA, A/P-tRNAs and E-tRNA -containing 70S reconstructions. Only P-site containing reconstructions were used for further processing, which represents ~62k particles displaying the same global conformation of the 70S. These particles were re-extracted at the full pixel size (1.2 Å) and refined through the 3D auto-refinement performed in RELION 3 (Zivanov et al., 2018) resulting in a 3Å reconstruction (**Figure S4C**), after CTF-refinement, Bayesian particle polishing and post-processing in RELION 3. In spite of the sufficient local resolution at the vicinity of MsrDL peptide at the PTC (~3Å), residual movements of the 30S around the 50S can be deduced by the lower local resolutions of the head and the body of the 30S. In order to improve their resolution so to derive a complete atomic model of the entire 70S complex, we applied RELION 3 multi-body refinement by defining three bodies; 50S, 30S-head and 30S-body. After completion of the refinement, the reconstructions of the 30S-head, 30S-body and 50S reached average resolutions of 3.3, 3.08 and 2.97 Å, respectively. The local resolution (**Figure S4B** and **D**) was estimated using ResMap (Kucukelbir et al., 2014).

Model building and refinement

The atomic model of erythromycin-stalled *Escherichia coli* 70S ribosome with streptococcal MsrDL nascent chain was built into cryo-EM maps using Coot and Phenix (Emsley and Cowtan, 2004; Liebschner et al., 2019). Insofar as *E. coli* DB10 hasn't been sequenced yet, we assumed that ribosomal proteins and rRNAs were strictly identical to *E. coli* K12. Furthermore, we did not notice any significant features in the map. Structure of SpeFL-SRC in response to L-ornithine was used as initial model (PDB 6TC3) (Herrero del Valle et al., 2020) and has been fitted in 70S ribosome map EMD-13805. Then, each part of the ribosome (50S, 30S Body and 30S Head) has been individually inspected in the corresponding map (respectively EMD-13806, EMD-13807 and EMD-13808), modified if necessary in Coot and refined with Phenix. P-site elongator tRNA_{Met}tRNA^{Met} was modeled *de novo* based on *E. coli* K12 MG1655 *metT* gene, posttranscriptional modifications were added consistently with Modomics database (Boccalletto et al., 2018) (<http://genesilico.pl/modomics/>) and refined in map. E-site tRNA_{Phe}tRNA^{Phe} was derived from crystal structure of phenylalanine tRNA from *E. coli* (PDB 6Y3G) with minor adjustments (Bourgeois et al., 2020). Both tRNAs were refined in Phenix using map EMD-13805. ERY, MsrDL leader peptide and mRNA were modeled *de novo* and refined in the corresponding map.

Figure preparation

Growth curves, histograms and polyribosomes profiles were generated using GraphPad Prism 7 (GraphPad). Sequence alignments were visualized with JalView (Waterhouse et al., 2009).

Western blotting, northern blotting and toe-printing gels were analyzed using Fiji (Schindelin et al., 2012). Figures depicting molecular structures or electronic density maps were prepared using PyMOL Molecular Graphics System, Chimera and ChimeraX (Goddard et al., 2018; Pettersen et al., 2004).

Quantification and statistical analysis

Statistical details can be found in the figure legends. Statistical significance was assessed using unpaired t-Test function in Prism 7 (GraphPad).

Data and software availability

Cryo-EM map of erythromycin-stalled *Escherichia coli* 70S ribosome with streptococcal MsrDL nascent chain has been deposited at the Electron Microscopy Data Bank (EMDB) with accession code EMD-13805, as well as 50S, 30S Body and 30S Head maps obtained after multibody refinement with accession code EMD-13806, EMD-13807, EMD-13808 respectively. Corresponding atomic model has been deposited in the Protein Data Bank (PDB) with accession code 7Q4K.

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FIGURE 1

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FIGURE 6