Safe and easy in vitro evaluation of tmRNA-SmpB-mediated *trans*-translation from ESKAPE pathogenic bacteria

MARION THÉPAUT,[1,2,5] RODRIGO CAMPOS-SILVA,[1,3,5] EVA RENARD,[4] FRÉDÉRIQUE BARLOY-HUBLER,[1] ERIC ENNIFAR,[4] DANIEL BOUJARD,[1] and REYNALD GILLET[1]

[1]Université Rennes, CNRS, Institut de Génétique et Développement de Rennes (IGDR) UMR 6290, 35043 Rennes, France

[2]SATT Ouest-Valorisation, 35750 Rennes, France

[3]Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre 90610-000, Brazil

[4]Architecture et Réactivité de l'ARN—CNRS UPR 9002, Institut de Biologie Moléculaire et Cellulaire, Université de Strasbourg, 67084 Strasbourg, France

ABSTRACT

In bacteria, *trans*-translation is the major quality control system for rescuing stalled ribosomes. It is mediated by tmRNA, a hybrid RNA with properties of both a tRNA and a mRNA, and the small protein SmpB. Because *trans*-translation is absent in eukaryotes but necessary for bacterial fitness or survival, it is a promising target for the development of novel antibiotics. To facilitate screening of chemical libraries, various reliable in vitro and in vivo systems have been created for assessing *trans*-translational activity. However, the aim of the current work was to permit the safe and easy in vitro evaluation of *trans*-translation from pathogenic bacteria, which are obviously the ones we should be targeting. Based on green fluorescent protein (GFP) reassembly during active *trans*-translation, we have created a cell-free assay adapted to the rapid evaluation of *trans*-translation in ESKAPE bacteria, with 24 different possible combinations. It can be used for easy high-throughput screening of chemical compounds as well as for exploring the mechanism of *trans*-translation in these pathogens.

Keywords: *trans*-translation; tmRNA; ESKAPE; antibiotics; HTS; ribosome

INTRODUCTION

The World Health Organization (WHO) designated six "ESKAPE" pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*) as critical targets for drug discovery (Rice 2008; Tacconelli and Magrini 2017). Indeed, these bacteria are the leading cause of nosocomial infections throughout the world, and most are multidrug-resistant isolates (Santajit and Indrawattana 2016). The WHO recommendation is to focus specifically on the discovery and development of new antibiotics that are active against multidrug- and extensively drug-resistant ESKAPE bacteria. However, the hazardous nature of these pathogens makes it highly challenging to develop high-throughput screening methods for identifying and evaluating any new antimicrobial agents for future clinical use. To aid in this, the molecular process to be targeted must first be identified, and ideally this process should be: (i) conserved among all pathogenic ESKAPE bacteria; (ii) indispensable to bacterial survival or at least its fitness; (iii) sufficiently variable that different species can be distinguished from each other; (iv) absent in eukaryotes; (v) not targeted by current antibiotics; (vi) unrelated to existing resistance mechanisms; and finally (vii) reproducible in nonhazardous in vitro experiments.

In fact, *trans*-translation appears to be the perfect candidate. This mechanism is the primary bacterial rescue system, allowing for the release of ribosomes stalled on faulty mRNAs that lack stop codons as well as the elimination of these mRNAs and mistranslated peptides. The *trans*-translation process is performed by hybrid transfer-messenger RNA (tmRNA) and its protein partner SmpB (Giudice et al. 2014). Briefly, the tmRNA–SmpB complex recognizes the stalled ribosome and associates with it. In a finely orchestrated ballet, translation then resumes on

[5]These authors contributed equally to this work.

Corresponding author: reynald.gillet@univ-rennes1.fr

Article is online at http://www.rnajournal.org/cgi/doi/10.1261/rna.078773.121.

tmRNA's internal mRNA-like domain (MLD), which encodes a specific sequence that is recognized by proteases. This process permits the stalled ribosomes to be recycled, the degradation of the incomplete peptide after its release, and elimination of the problematic nonstop mRNA. Remarkably, genes coding for tmRNA and SmpB have been found in nearly all bacterial genomes, yet not in eukaryotes, with the exception of a very few rare organelles (Hudson and Williams 2015). Despite high sequence conservation at both the 5′- and 3′-ends of tmRNA genes, the internal sequences of tmRNA are considerably divergent among different species (Supplemental Fig. 1), and this property makes tmRNA a good tool for species identification (Schönhuber et al. 2001). In the same way, despite global structural conservation, variations in *smpB* sequences are also considerably divergent among different species (Supplemental Fig. 1).

While resolving stalled ribosomal complexes is undoubtedly a matter of life or death (Keiler and Feaga 2014), *trans*-translation itself is not always indispensable to bacterial survival. This irregularity was the subject of a long debate until the discovery of backup systems, mechanisms which take over if *trans*-translation is deficient or overwhelmed. However, even when they are present, these systems are not enough to ensure a steady and prolonged fitness to the cell, as impaired *trans*-translation is known to result in various phenotypes varying from mild (such as loss of tolerance to multiple antibiotics and stresses) to severe (including lethality or loss of virulence) (Li et al. 2013; Keiler and Feaga 2014). To date, *trans*-translation has not been yet exploited for clinical use. In the search for inhibitors specific to the process, initial assays led to the discovery of 1,3,4 oxadiazole molecules (Ramadoss et al. 2013), but their specificity for *trans*-translation in vivo is still in question (Macé et al. 2017; Brunel et al. 2018). It has been suggested that *trans*-translation is inhibited by pyrazinamide (PZA), a first-line anti-tuberculosis drug (Shi et al. 2011), but it was finally recently shown the action of PZA is entirely independent of *trans*-translation in *M. tuberculosis* (Dillon et al. 2017).

Because of its biological properties, transfer of *trans*-translation into a nonhazardous system that could allow for rapid and easy evaluation of its activity would greatly help in the search for new antibiotics which target this system. While there are routine methods for screening the antimicrobial activity of compounds from chemical libraries, a combination of this primary screening with the specification of a molecular target is much harder to implement (Osterman et al. 2016). An ideal method would allow not just the identification of the targeted cellular process, but also its level of specificity toward a bacterial genus or species. Furthermore, an easy quantitative and rapid analysis of the process should be possible even in small volumes. Reporter assays are the best candidates for efficient initial high-throughput screening (HTS) methods, as they can be quick and automated, as well as quite useful for screening unpurified mixtures of natural extracts (Osterman et al. 2016). Accordingly, we recently used a commercial reconstituted in vitro translation system (PURExpress) to create a reliable in vitro reporter system that detects the *E. coli trans*-translation activity (Guyomar et al. 2020). This assay, based on reassembling an active "superfolder" Green Fluorescent Protein (sfGFP) after tmRNA tagging (Fig. 1), was designed and validated for the specific in vitro quantification of *trans*-translation in ESKAPE pathogenic bacteria, and we report on that here.

RESULTS

Distribution of ArfA, ArfB, and RQC in ESKAPE bacterial genomes

While some bacteria can survive without *trans*-translation, this is only because of the existence of backup systems, such as the two alternative release factors, ArfA and ArfB or the bacterial ribosome-associated quality control (RQC) mediated by RqcH and RqcP. The Arf mechanisms can be divided in release factor (RF)-dependent and RF-independent mechanisms. ArfA recruits RF2 to hydrolyze the nascent polypeptide chain from the P-site tRNA, while ArfB, a class I release factor homolog, performs hydrolysis itself (Himeno et al. 2015; Müller et al. 2021)

On the other hand, RqcH and RqcP act in concert to mediate the ribosome-associated quality control (RQC) pathway, triggering carboxy-terminal tailing of stalled peptides in the large ribosomal subunit. RqcH belongs to the NEMF family proteins (homolog of the eukaryotic RQC factor Rqc2/NEMF, while RqcP (ribosome quality control P-tRNA, formerly YabO), belongs to the widely distributed S4 RNA-binding family, and is homologous to *E. coli* heat shock protein 15 (Hsp15) (Lytvynenko et al. 2019; Müller et al. 2021). Depending on backup system status, therefore, the effects of specific inhibitors of *trans*-translation will vary, from increasing the activity of currently used antibiotics to outright cell death. It was therefore important for us to begin by pinpointing the phylogenetic distribution of ArfA, ArfB, and RQC in ESKAPE pathogens. To do this, we investigated the sequences of those rescue factors using a combination of in silico methods including keyword searches, similarity detection, protein domain prediction, ortholog clustering, and synteny analysis. This pipeline was applied to the complete genomic sequences of 1670 species: 147 *E. faecium*, 473 *S. aureus*, 465 *K. pneumoniae*, 188 *A. baumannii*, 259 *P. aeruginosa*, and 151 *Enterobacter* spp. Interestingly, among these ESKAPE pathogens, none of the back-up systems were found in *A. baumannii* while the two Gram-positive bacteria *E. faecium* and *S. aureus* displayed RQC only (Table 1). While we cannot categorically state that no other backup systems exist in these bacteria—see for instance the recent reports on ArfT

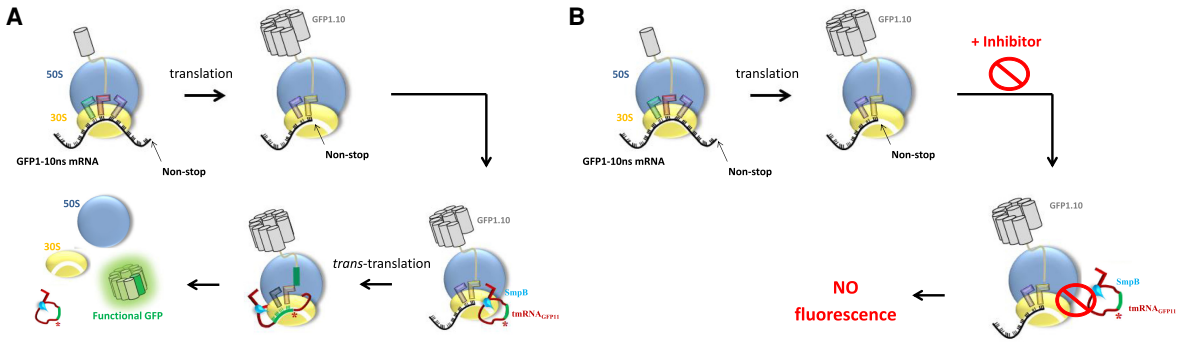

FIGURE 1. (*A*) *Trans*-translation of sfGFP1-10 mRNA lacking a stop codon. The tmRNA$_{GFP11}$-SmpB complex binds to the stalled ribosome, and translation resumes thanks to the tmRNAGFP11 mRNA-like domain (MLD). The MLD encodes the missing eleventh beta-strand of the sfGFP, and the complete sfGFP is released and becomes fluorescent. (*B*) Impairment of the process in the presence of *trans*-translation inhibitors. The ribosomes stay stalled on the problematic mRNA and fluorescence is impaired.

in *Francisella tularensis* and BrfA in *Bacillus subtilis* (Goralski et al. 2018; Shimokawa-Chiba et al. 2019)—we can however suppose that their viability highly depends on *trans*-translation impairment. On the other hand, we found genes encoding ArfA and/or ArfB in most if not all of the *K. pneumonia, P. aeruginosa*, and *Enterobacter* spp. studied. The impairment of *trans*-translation in these bacteria is probably less severe, therefore, even if it still detrimental to bacterial fitness.

ESKAPE tmRNA and SmpB production

To allow for independent monitoring of *trans*-translation in the six ESKAPE pathogens, we engineered their tmRNAs by replacing their internal MLD with a sequence of 16 amino acids that encodes GFP's eleventh beta-strand (Supplemental Table 1). To conserve the tmRNA H5 helix that is instrumental during *trans*-translation, we also engineered compensatory mutations (Supplemental Fig. 2A, B; Guyomar et al. 2020). Unlike those of the other bacteria, the natural tmRNA 3′-ends in *E. faecium* and *S. aureus* are not CCA but UUG and UAU, respectively, so these were replaced by CCA to ensure correct aminoacylation by *E. coli* AlaRS (Barends et al. 2000), and these variants were named tmRNA$_{GFP11}$. Urea-PAGE analysis indicated that the six tmRNA variants were successfully produced at the expected size, without any noticeable degradation or unexpected bands (Supplemental Fig. 2D). We started with 10 µg plasmidic DNA, and the final yields were about 4 nmol of transcribed RNAs for each reaction. The six corresponding SmpB proteins were cloned and produced in vivo in *E. coli* (see Materials and Methods). After purification, polyacrylamide gel analysis confirmed the correct size of each protein (Supplemental Fig. 2E). The final yields for each ESKAPE SmpB were about half the amount of the *E. coli* SmpB produced.

ESKAPE trans-translation reactions

In order to obtain nonproductive translation complexes (NTCs) to be targeted by *trans*-translation, we used a reconstituted cell-free protein synthesis (NEB PURExpress) system from *E. coli* (Shimizu et al. 2001; Shimizu and Ueda 2002). By adding a nonstop DNA template, we accumulated stalled ribosomes with the ten first beta-strands of sfGFP stuck in the ribosome exit tunnel (Fig. 1A). When tmRNA$_{GFP11}$ and *E. coli* SmpB are added, the ribosomes are freed and the intensity of the fluorescent signal

TABLE 1. Distribution of *arfA, arfB, rqcH*, and *rqcP* in ESKAPE bacteria

ESKAPE pathogen	# of screened genomes	*arfA*	*arfB*	*rqcH*	*rqcP*
Enterococcus faecium	147	0	0	147	147
Staphylococcus aureus	473	0	0	473	473
Klebsiella pneumoniae	465	464 + 1 Δ	459 + 6 Δ	0	0
Acinetobacter baumannii	188	0	0	0	0
Pseudomonas aeruginosa	259	259	259	0	0
Enterobacter spp.	151	151	150 + 3 Δ	0	0

(Δ) Pseudogenes with frameshift or "in-frame" stop codon.

increases over time while the complete sfGFP protein is produced. A plateau is reached at ~4 h of incubation, and the fluorescence remains stable for at least 710 min (Fig. 2A, black curve).

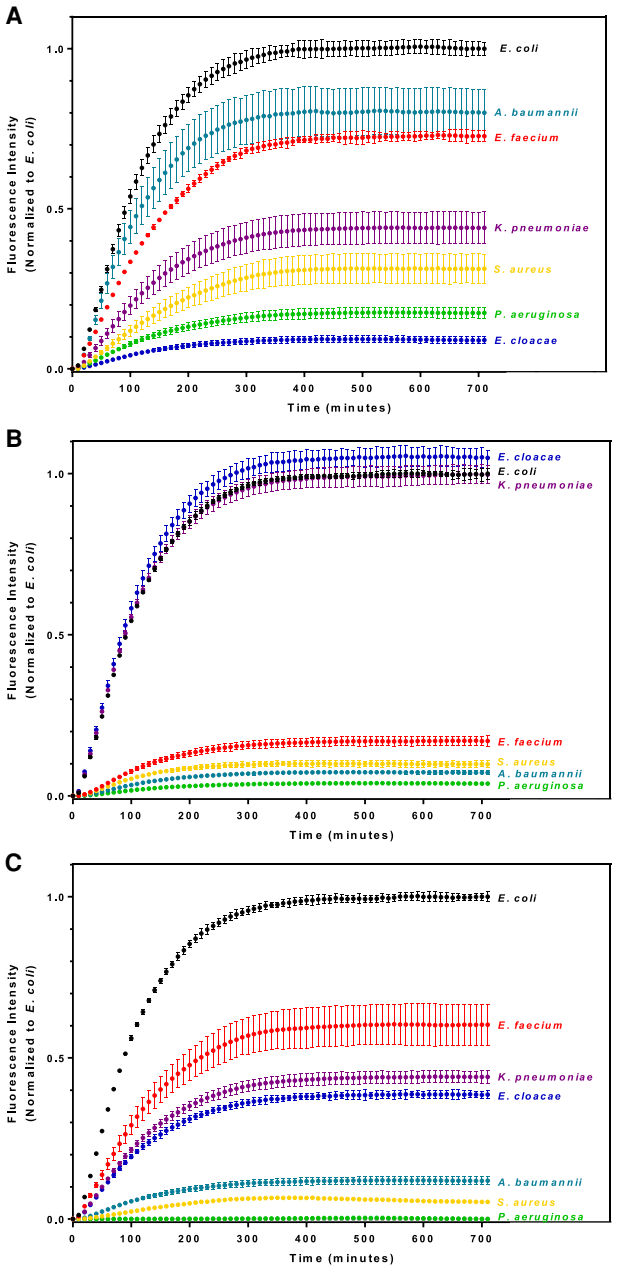

FIGURE 2. *Trans*-translation kinetics over time using *Escherichia coli* ribosomes. Fluorescence increases are directly linked to *trans*-translation activity. (*A*) *Trans*-translation assays were done on *E. coli* $tmRNA_{GFP11}$ using the SmpBs from each ESKAPE pathogen, with the *E. coli* SmpB as a control. (*B*) *Trans*-translation assays keeping the *E. coli* SmpB but using the $tmRNA_{GFP11}$ variants of each ESKAPE pathogen, with an *E. coli* $tmRNA_{GFP11}$ as the control. (*C*) Both SmpB and $tmRNA_{GFP11}$ are from each ESKAPE pathogen, with the *E. coli* SmpB-$tmRNA_{GFP11}$ as a control. The results are shown as means ± standard deviation and normalized to the *E. coli* conditions.

In a first set of heterologous experiments, we kept the *E. coli* $tmRNA_{GFP11}$, but replaced its SmpB by one from an ESKAPE pathogen. A fluorescent signal was still recovered with each one of the hetero-complexes, albeit at different levels (Fig. 2A). The *E. cloacae*, *S. aureus*, and *P. aeruginosa* SmpBs displayed the lowest signals, less than 30% of the *E. coli* control, while the *K. pneumoniae* SmpB signal was about half the control, and *E. faecium* and *A. baumannii* at 80%. This demonstrates that all of the ESKAPE SmpBs are functional and sufficiently conserved to be interchangeable in the presence of *E. coli* tmRNA. While it confirms that SmpB is highly conserved (Supplemental Fig. 1), it also supports the use of this simple system for screening molecules that target SmpB but not tmRNA. Indeed, since SmpB is essential for tmRNA's peptide-tagging activity (Karzai et al. 1999), disrupting SmpB is one of the most promising ways to impair *trans*-translation. In fact, aptamers that inhibit SmpB functioning were recently shown to trigger strong growth defects in *Aeromonas veronii* C4 (Liu et al. 2016).

We then performed the experiments the other way around, using the *E. coli* SmpB but the tmRNAs from the ESKAPE pathogens. Contrary to the previous experiments, only the heteroduplexes combining *E. coli* SmpB and the tmRNAs from *K. pneumoniae* and *E. cloacae* gave out strong signals, about the same levels as those recovered in the *E. coli* tmRNA control (Fig. 2B). This is not a surprise since, like *E. coli*, both *K. pneumoniae* and *E. cloacae* are *Enterobacteriaceae* with very similar tmRNA sequences (≥95% identity with *E. coli*, see Supplemental Fig. 1). The four other bacterial species all produced signals, but at lower levels (about 5% to 20% of the reference).

We continued by performing homologous experiments, using SmpB and $tmRNA_{GFP11}$ from the same ESKAPE pathogen, but still with *E. coli* ribosomes (Fig. 2C). Five of the six complexes yielded positive results. Three of these were at high levels (~50% compared to the *E. coli* reference): *K. pneumonia*; *E. cloacae* and *E. faecium*. The other two were at lower levels (about 5%–10% of the reference): *A. baumannii*, another Gammaproteobacteria that is relatively close to *Enterobacteriaceae*; and the Gram-positive *S. aureus*. The Gammaproteobacteria *P. aeruginosa* did not work at all.

In a final set of experiments, we used homologous tmRNA–SmpB complexes in the presence of their corresponding ESKAPE ribosomes. The use of the PURExpress Δ Ribosome Kit allowed us to substitute commercial *E. coli* ribosomes with ESKAPE variants we had prepared in-house. We first confirmed the effectiveness of translation using these ribosomes by synthesizing full-size sfGFP. For all ribosomes used, a fluorescent signal was recovered, indicating that the ESKAPE ribosomes translate well even if at lower levels (Fig. 3A). The *P. aeruginosa* and *E. cloacae* *Enterobacteriaceae* ribosomes gave the strongest signals,

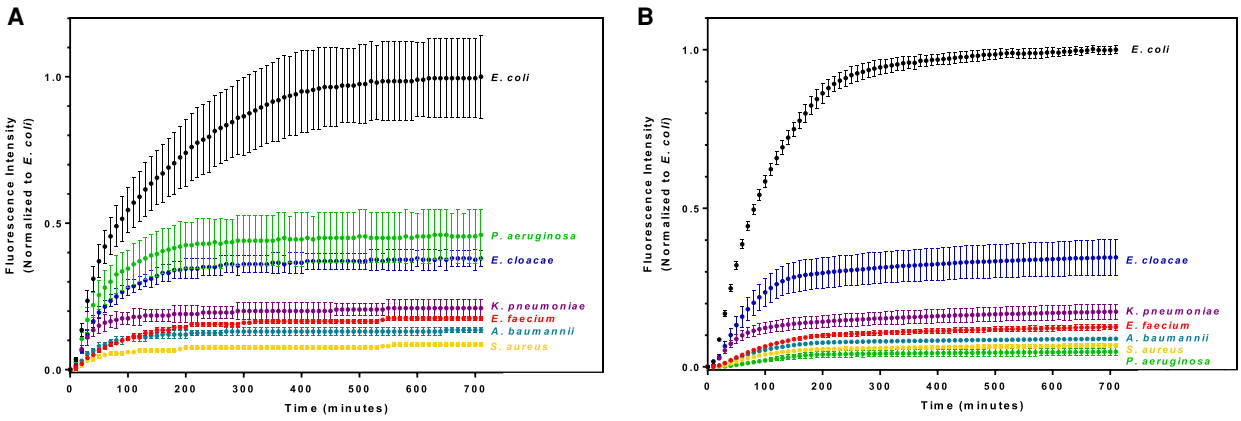

FIGURE 3. Translation and *trans*-translation kinetics over time. (*A*) Translation kinetics over time: the increase in fluorescence of full-length GFP (i.e., encompassing the 12 beta-strands) is directly linked to translation. (*B*) *Trans*-translation kinetics over time using ESKAPE ribosomes. All results are shown as means ± standard deviation and normalized to *E. coli*.

~45% and 35%, respectively, as compared to that of *E. coli*. All of the other signals were below 20%, even dropping under 10% in the case of *S. aureus*.

Despite these rather poor translation rates, fluorescence was easily detected, so we also performed *trans*-translation experiments using ESKAPE ribosomes (Fig. 3B). The goal was to improve the levels of the *trans*-translation signals previously recovered, but more importantly to obtain a positive result for *P. aeruginosa*. The results were finally conclusive for that bacteria, which gave a fluorescent signal of ~10% compared to the control. This positive result could be linked to the quite efficient translation obtained with these ribosomes (Fig. 3A). On the other hand, the *trans*-translation levels of the other bacteria did not improve, and were even lower in *S. aureus*. This could be due to the fact that the PURExpress system is based on only *E. coli* translation factors, and their low count limits their handling of canonical translation (see Fig. 3A) or specific tmRNAs (e.g., tmRNA aminoacylation by *E. coli* AlaRS or tmRNA–SmpB transport by *E. coli* EF-Tu-GTP). However, and since our goal was to detect variations of fluorescence after drug treatment within each of the ESKAPE species, rather than comparing the strains between each other, it was important to get a correct and satisfactory signal for each one of the strains individually. Toward this aim, we decided to use a more sensitive spectrophotometer, that is, Synergy HTX from BioTek. We adjusted the spectrophotometer gain function in order to ensure optimal detection of GFP fluorescence without saturation and applied the technique to the homologous systems (tmRNA–SmpB and ribosomes from the same ESKAPE), the ones that are the most interesting for developing new inhibitors. The data obtained were finally conclusive, within a range of 20,000–90,000AU for translation as well as for *trans*-translation, allowing for an accurate internal control in case of inhibition (Supplemental Fig. 3).

DISCUSSION

Here we describe the use of GFP as a reporter for safe measurement of the *trans*-translation activity of the six ESKAPE systems in a cell-free protein synthesis system. The various combinations we evaluated (four for each ESKAPE pathogen) have yielded different interesting strategies for the disruption of *trans*-translation (Fig. 4).

The molecules being investigated for the development of new anti-*trans*-translation antibiotics will have different ways of interfering with tmRNA–SmpB binding to stalled ribosomes. They could disrupt tmRNA–SmpB interactions, or they could prevent interactions between the complex and the ribosome, such as by blocking the entrance of SmpB entirely or by preventing the passage of the complex through the bridges which have to be open during the process. Therefore, it is of great interest to have the ability to evaluate the targeting of the three main actors (tmRNA, SmpB, and the ribosome) independently as well as in each ESKAPE system. Of the 24 combinations we tested, 23 exhibited a signal strong enough for evaluating the possible activity of inhibitors. The only one that did not was the *P. aeruginosa* tmRNA–SmpB complex when used with *E. coli* ribosomes. We first suspected that the tmRNA H5 helix, inspired from the *E. coli* helix (Supplemental Fig. 2B), might somehow have altered its activity. Therefore, to avoid any possible effects of the helical rearrangement, we constructed and tested new $tmRNA_{GFP11}$ variants for *P. aeruginosa* but also *E. coli*, *S. aureus*, and *E. faecium*. These $tmRNA_{GFP11}$V2 constructs all have the full sequence that encodes the eleventh beta-strand of GFP upstream of the natural H5 helix (Supplemental Fig. 2C). However, these variants did not have improved fluorescence, and *P. aeruginosa* still did not emit signals. We also performed new experiments by increasing twofold the amounts of SmpB and two- to fourfold the amounts of tmRNA, but without further success (not shown). We can thus exclude

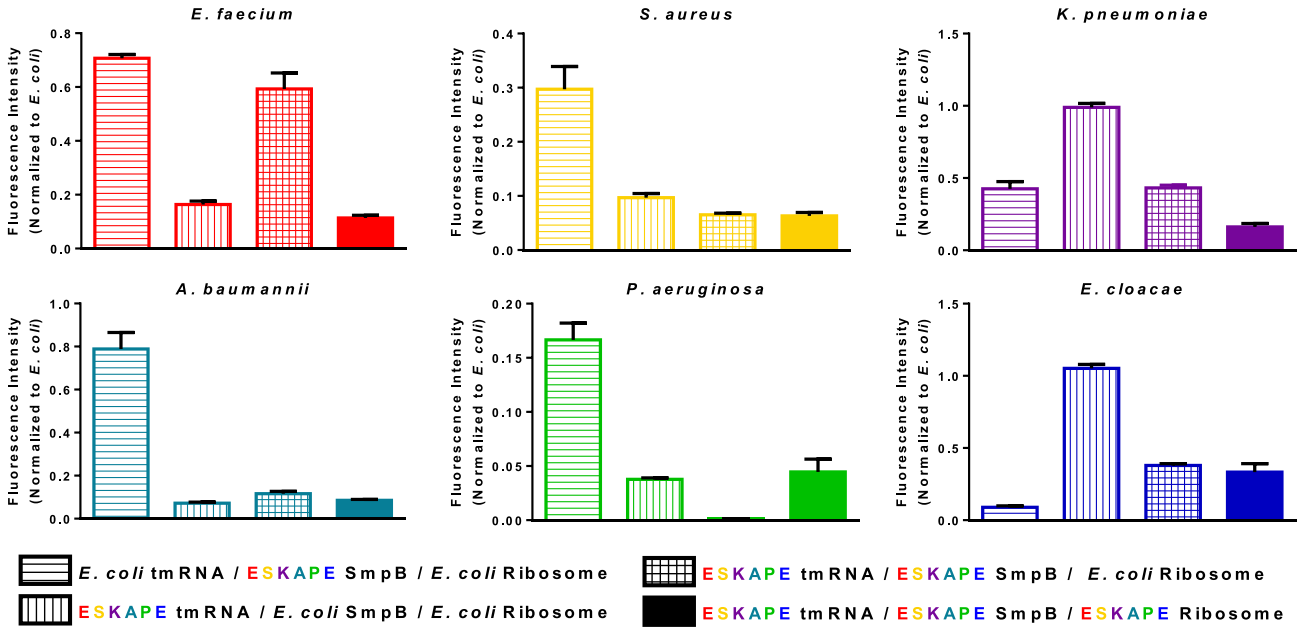

FIGURE 4. Quantification of in vitro *trans*-translation. Normalized fluorescence obtained in heterologous and homologous systems are shown at 310 min of incubation and reassembled by species. The results were normalized to the *E. coli* conditions and are shown as means ± standard deviations.

the idea that the different structural features between the *P. aeruginosa* and *E. coli* ribosomes (Halfon et al. 2019) are important enough to prevent the correct process from occurring.

To permit the high-throughput screening of chemical compounds in multiwell microplates it was important to lower average screening costs of the current assay. To enable this, we decreased the reaction scale of the assays by reducing the final reaction volumes down to a microliter scale. Proof-of-concept experiments were performed with *E. coli* or ESKAPE homologous systems in final volumes of 2 µL using the MANTIS liquid-handler instrument (Formulatrix) or simply by using an electronic micropipette. The resulting signals were strong enough to allow for the easy detection of *trans*-translational activity. Indeed, the objective of this study was to create a nonhazardous in vitro screening system for evaluating *trans*-translation in ESKAPE pathogens, and to miniaturize it for HTS applications, and the assays we performed were convincing. We then decided to perform an experiment demonstrating proof of principle by using an oligonucleotide that interferes with the mRNA-like domain (MLD) of tmRNA as well as CT1-83 and KKL-35, two 1,3,4-oxadiazole derivatives that were recently shown to display a low in vitro activity against *E. coli trans*-translation (Guyomar et al. 2020). Toward this aim we used again the homologous system including tmRNA, SmpB and ribosomes from the same ESKAPE. The results on *trans*-translation show a total inhibition of the process when using the anti-sense, whatever the pathogen. On the other hand, and despite a very slight and dose-dependent effect of CT1-83 on *P. aeruginosa* and *E. cloacae* and KKL-35 on *S. aureus*, none of the compounds displayed any noticeable activity on the six ESKAPE systems (Fig. 5A). Of course, to avoid compounds that inhibit any necessary step for fluorescence (e.g., transcription, translation, or GFP folding) to be scored as positive and result in false positive hits, transcription-translation assays were also performed using full GFP in the absence of tmRNA–SmpB. This set of results confirmed the absence of noticeable effect of the two oxadiazole compounds on transcription-translation, while the signal was completely abolished after chloramphenicol treatment (Fig. 5B). While this is interesting, it especially confirms that new classes of more efficient molecules are needed to target *trans*-translation in ESKAPE pathogens.

Therefore, the system will clearly be very effective for benchmarking the effects of new antibiotic compounds that target *trans*-translation in highly pathogenic bacteria, as well as aiding us to better understand the *trans*-translation process in these bacteria. Its flexibility in the choice of target bacterial species and the possibility for varying the combinations of tmRNA, SmpB, and ribosomes are advantageous, making the identification of new specific antimicrobial inhibitors easier. Ongoing experiments in our laboratory are using this to screen large chemical and natural product libraries for drug discovery.

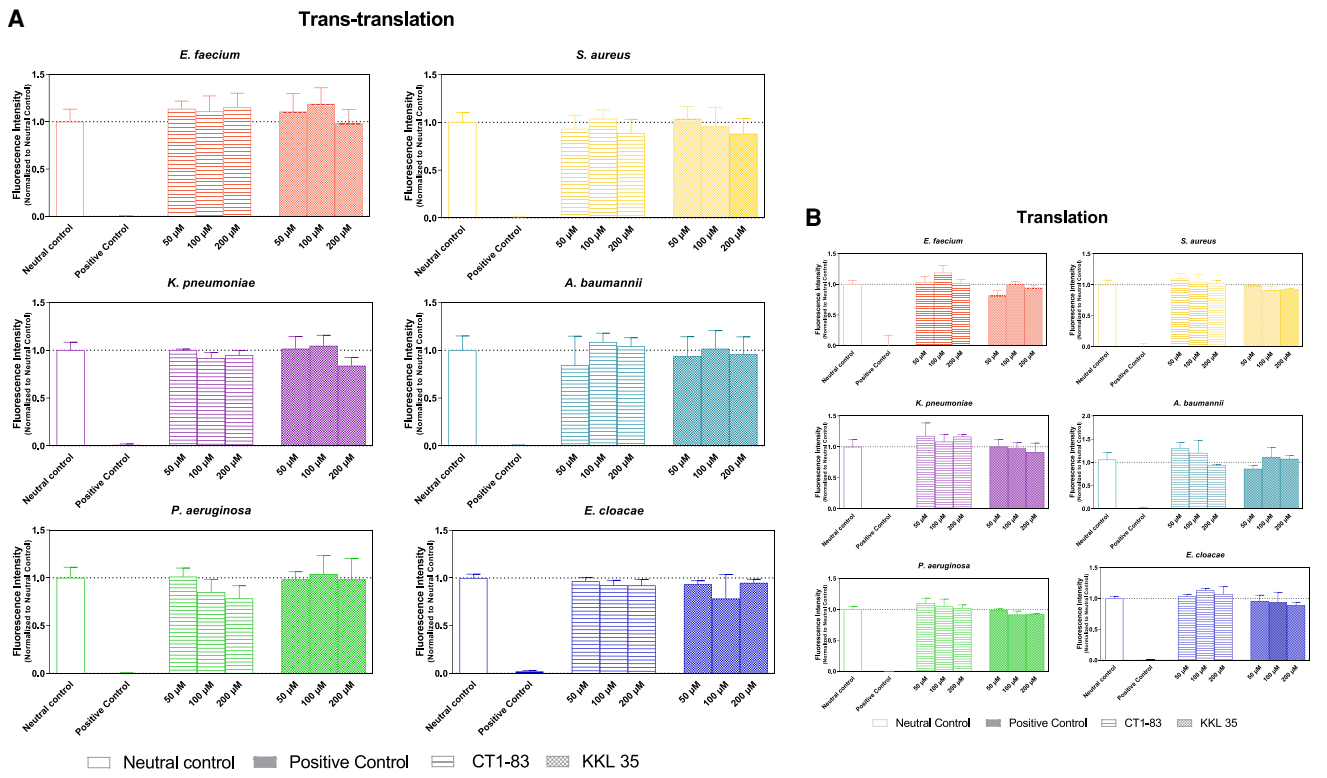

FIGURE 5. Quantification of in vitro ESKAPE *trans*-translation and translation assays after CT1-83 and KKL-35 treatment. Experiments were conducted in microplates, using ESKAPE homologous systems and increasing concentrations (50, 100, and 200 µM) of CT1-83 and KKL-35 oxadiazole compounds. Normalized fluorescence intensities obtained are presented after 310 min of incubation and sorted by species. The results were normalized to the neutral control conditions and are shown as means ± standard deviations. (*A*) *Trans*-translation assay with the oxadiazole compounds CT1-83 and KKL-35. The positive control was obtained by using 10 µM Antisense B in 10% DMSO. (*B*) Translation assay with the oxadiazole compounds CT1-83 and KKL-35. The positive control was obtained by using Chloramphenicol at 100 µM in 10% DMSO. Using the Anova test, results were considered statistically significant when $P \leq 0.01$. Only positive controls presented a difference of $P \leq 0.0001$ for translation and *trans*-translation (stars were not represented to facilitate graph reading).

MATERIALS AND METHODS

In silico analysis

Complete genomes were retrieved from the NCBI database (March 2020). Chromosomes and plasmids (when present) were studied separately. GenBank files were first searched based on their textual annotation entries, using the keywords "ArfA," "yhdL," and "alternative ribosome-rescue factor" (for ArfA), or "ArfB," "yaeJ, " "ribosome-associated protein," and "peptidyl-tRNA hydrolase" (for ArfB), or "RqcH," "Rqc2 homolog" (for RqcH), or "RqcP," "YabO" (for RqcP). Missing loci were checked using BlastN, BlastP, and tBlastN similarity-detection strategies (Altschul et al. 1990) as well as comparative genomics, with synteny analysis done using progressiveMauve (Darling et al. 2010). All retrieved loci were compared using the Reciprocal Best Hits method, and InterProScan (Jones et al. 2014) was used on the corresponding proteins to check for the presence of the IPR005589/PF03889 (ArfA) and IPR000352/PF00472 (ArfB) domains. Frameshifted loci were indicated as annotated in the GenBank files. Finally, the presence and absence of K09890 (ArfA) and K15034 (ArfB) were checked in the KEGG ORTHOLOGY database (Kanehisa et al. 2016).

Plasmid construction and preparation

For each ESKAPE tmRNA, the internal open reading frame was replaced by the eleventh beta-strand of the superfolder GFP (sfGFP) preceded by the first conserved alanine of native tmRNA. In order to preserve the H5 helix, compensatory mutations were added (Fig. 2B). Additionally, the sequences were designed to carry a T7 promotor sequence in the 5′-end in order to realize transcription in vitro. Note that the tmRNA 3′-end natural sequences from *E. faecium* (UUG) and *S. aureus* (UAU) were replaced by CCA so that the *E. coli* AlaRS could correctly aminoacylate them.

We also produced $tmRNA_{GFP11}$V2 variants for *E. coli*, *P. aeruginosa*, *S. aureus*, and *E. faecium* species. This $tmRNA_{GFP11}$ series carries the full sequence encoding the eleventh beta-strand of GFP upstream of the *E. coli*

H5 helix (Supplemental Fig. 2C). In order to obtain mature tmRNA$_{GFP11}$ by in vitro transcription, the tmRNA$_{GFP11}$ and tmRNA$_{GFP11}$V2 ESKAPE sequences were synthesized and cloned into the pUC19 vector between the HindIII and BamHI restriction sites (Supplemental Table 1). For each ESKAPE SmpB, GenScript synthesized the sequences with codon optimization for *E. coli*, cloning them into the pET22b(+) vector between the NdeI and XhoI restriction sites to add a 6His histidine tag (Supplemental Table 2). The generated plasmids, pUC19ESKAPEtmRNA$_{GFP11}$ and pET22b + ESKAPESmpB (Supplemental Table 5), were amplified in *E. coli* NM522 cells, then extracted using a NucleoBond Xtra Midi Kit (Macherey-Nagel). Quantification was performed using a SimpliNano Spectrophotometer (Biochrom).

SmpB purification

The bacterial cultures and SmpB purification were all done as previously described (Guyomar et al. 2020). His-tagged *E. coli* and ESKAPE SmpB proteins (Supplemental Table 2) were expressed from the pF1275 and the pET22b + ESKAPE SmpB vectors under the control of a T7 promoter in BL21(DE3)Δ*ssrA* cells (Cougot et al. 2014). Briefly, BL21 (DE3)Δ*ssrA* cells were grown in lysogeny broth (LB) at 30°C supplemented with ampicillin (100 µg/ml) and kanamycin (50 µg/mL). Protein expression was induced in the exponential phase (OD_{600} = 0.6) with 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) overnight at 16°C. Cells were harvested and washed, then resuspended in lysis buffer (50 mM HEPES-KOH, 200 mM KCl, 20 mM imidazole, and 1 mM DTT pH 7.5). Cell lysis was performed using a French press, and the lysate was centrifuged at 15,000 rpm for 45 min at 4°C in a Beckman J2-MC with a JA-17 rotor. The supernatant was then filtrated (0.2 µm) and injected onto a Ni-NTA sepharose column (HisTrap FF, GE Healthcare) previously equilibrated with the lysing buffer. The column was washed with 100 mL lysis buffer and 50 mL washing buffer (50 mM HEPES-KOH, 200 mM KCl, 1 M NH_4Cl, imidazole 20 mM, and 1 mM DTT pH 7.5) before elution with 500 mM imidazole. Finally, a 10 kDa Amicon Ultra Centrifugal Filter (Merck Millipore) was used to concentrate the fractions containing pure SmpB, changing the buffer to a concentration buffer (50 mM HEPES-KOH, 100 mM KCl, 10% glycerol, and 1 mM DTT pH 7.5). In order to visualize SmpB, 50 pmol of denatured proteins was analyzed on 15% SDS-PAGE gels. Proteins were detected using InstantBlue protein stain (Expedeon) according to the supplier's instructions.

tmRNA$_{GFP11}$ production

E. coli and ESKAPE tmRNA$_{GFP11}$ were produced as previously described (Guyomar et al. 2020). Each ESKAPE tmRNA$_{GFP11}$ was transcribed in vitro from the pUC19ESKAPEtmRNA$_{GFP11}$ plasmids. To generate the 3′ end needed for aminoacylation by AlaRS, the plasmid (10 µg) was completely digested by NEB BsmBI or EarI restriction enzymes (Supplemental Table 5). After phenol/chloroform extraction, the purified digested plasmid was precipitated, and the resulting pellets resuspended in 40 µL nuclease-free water. A MEGAscript T7 Transcription Kit (Thermo Fisher Scientific) was used to produce each ESKAPE tmRNA$_{GFP11}$ before its purification using the corresponding MEGAclear Kit. Denatured tmRNA$_{GFP11}$ was checked by electrophoresis on 8% Urea-PAGE gels, stained with ethidium bromide, and visualized under ultraviolet light.

DNA templates and oligonucleotide production

For *trans*-translation assays, the nonstop GFP1-10 sequence was produced by PCR using primers #1 and #2 and Q5 High-Fidelity DNA Polymerase (NEB) with pETGFP 1–10 vector as a template (Cabantous and Waldo 2006; Supplemental Tables 3, 4). For translation assays, primers #1 and #3 from the same template were used to amplify sfalaGFP, the superfolder GFP having an additional conserved alanine between the sfGFP1–10 and sfGFP11 beta-strands (Supplemental Tables 3, 4). The resulting PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and checked by agarose electrophoresis. Both PCR products have a T7 promoter upstream of their coding sequences. Antisense oligonucleotide "A" was supplied by Eurofins (Supplemental Table 3).

ESKAPE ribosome purification

Ribosomes were purified from *Acinetobacter baumannii* (clinical isolate); *Staphylococcus aureus* (clinical isolate); *Pseudomonas aeruginosa* (ATCC 27853); *Enterobacter cloacae* (clinical isolate); *Klebsiella pneumoniae* (clinical isolate); and *Enterococcus faecium* (HM1070). From an overnight starter culture, 6–9 L of LB medium were inoculated to reach an OD_{600} of 0.05, then stirred at 150 rpm at 37°C. Bacterial growth was stopped when the OD_{600} reached 0.8 to 1.0. The cells were then centrifuged at 4000 rpm for 20 min at 4°C. Pellets (~2 g/L of culture) were washed in a lysis buffer (20 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 200 mM NH_4Cl, 0.1 mM EDTA, and 6 mM β-mercaptoethanol), centrifuged at 4000 rpm for 15 min at 4°C, and kept overnight at −80°C. Pellets were then suspended in a Potter homogenizer in another lysis buffer complemented with 1 mM $CaCl_2$. Cells were lysed in a French press at 1.0 kbar. To remove cellular debris, the lysates were centrifuged using a type 50.2 Ti rotor at 18,200 rpm for 30 min at 4°C. The superficial pellet layer was then discarded, and the pellet resuspended in lysis buffer. Ribosomes were isolated by centrifuging lysates on a

30% sucrose cushion at 31,500 rpm for 19 h at 4°C. The superficial layer of pellets was again discarded, leaving only the transparent pellets which were then resuspended in conservation buffer (20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NH_4Cl, 0.1 mM EDTA, and 6 mM β-mercaptoethanol). Any remaining contaminants were removed by a final centrifugation at 18,200 rpm for 1 h at 4°C. Ribosomes were concentrated using a Centricon (Merck Millipore) with a cut-off of 100K, flash-frozen in nitrogen, and conserved at −80°C.

Trans-translation assays

In vitro *trans*-translation assays were performed using the PURExpress In Vitro Protein Synthesis and Δ Ribosome Kits (New England Biolabs). For *trans*-translation assays, PURExpress was supplemented by 62.5 ng purified PCR product encoding for nonstop sfGFP1-10, 12.5 pmol $tmRNA_{GFP11}$, 25 pmol SmpB, and 50 pmol antisense A. Where necessary (Δ Ribosome), 6.725 pmol ribosomes were also added. These reactions were performed in a final reaction volume of 10 µL, with PURExpress diluted by a final factor of 1.6 with Buffer III (HEPES-KOH 5mM pH7.5, MgOAc 9mM, NH4Cl 10mM, KCl 50mM, and DTT 1mM). A Step One Plus PCR system (Applied Biosystems) was used for incubation at 37°C as well as for fluorescence measurements over 710 min.

Translation assays

In vitro translation assays were performed using a PURExpress Δ Ribosome Kit. To produce the sfalaGFP, the PURExpress Δ Ribosome was diluted to a final factor of 1.6 with Buffer III, to which was added 62.5 ng purified PCR product and 6.725 pmol of the appropriate ribosomes in a final reaction volume of 10 µL. The translation reactions were incubated at 37°C, and fluorescence was measured over 710 min using a Step One Plus.

Miniaturization of the *trans*-translation assays for HTS

In vitro miniaturization of the *trans*-translation assays was performed using the PURExpress In Vitro Protein Synthesis Δ Ribosome Kit (New England Biolabs). The mix was diluted by a factor of 1.6 after addition of 2.5 µM SmpB, 1.25 µM tmRNAGFP11, 672.5 nM ribosomes, 6.25 ng/µL of purified PCR product encoding for nonstop sfGFP1-10 and 5 µM antisense A. A total of 2 µL of neutral control (10% DMSO), 2 µL of positive control (10 µM Antisense B in 10% DMSO) and compounds in 10% DMSO were mixed together in a qPCR 96-well plate. CT1-83 oxadiazole compound was provided by Dr. Mickael Jean (Univ. Rennes) and KKL35 by Sigma Chemicals, respectively. Compounds and controls were then dried in a SpeedVac Concentrator before being resolubilized by adding 2 µL of PURExpress Mix in the same plate. Incubation at 37°C and fluorescence measurements over 310 min were simultaneously performed thanks to Synergy HTX from BioTek. The intensities of GFP were measured with the excitation filter at 485/20 and the emission filter at 528/20. The gain used was 116 for *E. faecium, S. aureus, A. baumannii* and *P. aeruginosa* and 100 for *K. pneumoniae* and *E. cloacae*.

The transcription-translation control assays were performed in the same way, except that nonstop sfGFP1-10 was replaced by full sfGFP, in the absence of tmRNA and SmpB. The positive control was then Chloramphenicol at 100 µM in 10% DMSO.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

COMPETING INTEREST STATEMENT

Reynald Gillet is co-inventor of the system described here (patent application #EP/2018/063780).

ACKNOWLEDGMENTS

The authors are particularly grateful to Axel Innis and Anne-Xander van der Stel for sharing their Mantis experiment expertise and to Charlotte Guyomar and Emmanuel Giudice for insightful comments on the manuscript. We thank Sylvie Georgeault and Fanny Demay for their help in purifying *E. faecium, S. aureus*, and *A. baumanni* ribosomes and to Mickael Jean for providing CT1-83 oxadiazole compound. This work was supported by the Agence Nationale pour la Recherche as part of the EU's Joint Programming Initiative on Antimicrobial Resistance project (JPIAMR) project "Ribotarget—Development of novel ribosome-targeting antibiotics" as well as by SATT Ouest-Valorisation (DV 2552 and DV 3506).

Author contributions: M.T. cloned and purified the SmpB and tmRNA variants and performed and analyzed the *trans*-translation in vitro assays. R.C.D.S. performed translation and *trans*-translation in vitro assays. E.R. purified ribosomes from *A. baumannii, E. cloacae, K. pneumoniae, P. aeruginosa*, and *S. aureus*. F.B.H. performed in silico analysis of ArfA and ArfB in bacterial genomes. R.G. designed the study. E.E., D.B., and R.G. supervised the project. M.T. and R.G. wrote the manuscript, and all authors approved its final version.

Received April 14, 2021; accepted July 27, 2021.

REFERENCES

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J Mol Biol* **215:** 403–410. doi:10.1016/S0022-2836(05)80360-2

Barends S, Wower J, Kraal B. 2000. Kinetic parameters for tmRNA binding to alanyl-tRNA synthetase and elongation factor Tu from

Escherichia coli. *Biochemistry* **39:** 2652–2658. doi:10.1021/bi992439d

Brunel R, Descours G, Durieux I, Doublet P, Jarraud S, Charpentier X. 2018. KKL-35 exhibits potent antibiotic activity against *Legionella* species independently of *trans*-translation inhibition. *Antimicrob Agents Chemother* **62:** e01459-17. doi:10.1128/AAC.01459-17

Cabantous S, Waldo GS. 2006. In vivo and in vitro protein solubility assays using split GFP. *Nat Methods* **3:** 845–885. doi:10.1038/nmeth932

Cougot N, Molza AE, Delesques J, Giudice E, Cavalier A, Rolland JP, Ermel G, Blanco C, Thomas D, Gillet R. 2014. Visualizing compaction of polysomes in bacteria. *J Mol Biol* **426:** 377–388. doi:10.1016/j.jmb.2013.09.035

Darling AE, Mau B, Perna NT. 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS ONE* **5:** e11147. doi:10.1371/journal.pone.0011147

Dillon NA, Peterson ND, Feaga HA, Keiler KC, Baughn AD. 2017. Anti-tubercular activity of pyrazinamide is independent of *trans*-translation and RpsA. *Sci Rep* **7:** 6135. doi:10.1038/s41598-017-06415-5

Giudice E, Macé K, Gillet R. 2014. *Trans*-translation exposed: understanding the structures and functions of tmRNA-SmpB. *Front Microbiol* **5:** 113. doi:10.3389/fmicb.2014.00113

Goralski TDP, Kirimanjeswara GS, Keiler KC. 2018. A new mechanism for ribosome rescue can recruit RF1 or RF2 to nonstop ribosomes. *MBio* **9:** e02436-18. doi:10.1128/mBio.02436-18

Guyomar C, Thépaut M, Nonin-Lecomte S, Méreau A, Goude R, Gillet R. 2020. Reassembling green fluorescent protein for *in vitro* evaluation of *trans*-translation. *Nucleic Acids Res* **48:** e22. doi:10.1093/nar/gkz1204

Halfon Y, Jimenez-Fernandez A, La Rosa R, Espinosa Portero R, Krogh Johansen H, Matzov D, Eyal Z, Bashan A, Zimmerman E, Belousoff M, et al. 2019. Structure of *Pseudomonas aeruginosa* ribosomes from an aminoglycoside-resistant clinical isolate. *Proc Natl Acad Sci* **116:** 22275–22281. doi:10.1073/pnas.1909831116

Himeno H, Nameki N, Kurita D, Muto A, Abo T. 2015. Ribosome rescue systems in bacteria. *Biochimie* **114:** 102–112. doi:10.1016/j.biochi.2014.11.014

Hudson CM, Williams KP. 2015. The tmRNA website. *Nucleic Acids Res* **43:** D138–D140. doi:10.1093/nar/gku1109

Jones P, Binns D, Chang HY, Fraser M, Li W, McAnulla C, McWilliam H, Maslen J, Mitchell A, Nuka G, et al. 2014. InterProScan 5: genome-scale protein function classification. *Bioinformatics* **30:** 1236–1240. doi:10.1093/bioinformatics/btu031

Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M. 2016. KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res* **44:** D457–D462. doi:10.1093/nar/gkv1070

Karzai AW, Susskind MM, Sauer RT. 1999. SmpB, a unique RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA). *EMBO J* **18:** 3793–3799. doi:10.1093/emboj/18.13.3793

Keiler KC, Feaga HA. 2014. Resolving nonstop translation complexes is a matter of life or death. *J Bacteriol* **196:** 2123–2130. doi:10.1128/JB.01490-14

Li J, Ji L, Shi W, Xie J, Zhang Y. 2013. *Trans*-translation mediates tolerance to multiple antibiotics and stresses in *Escherichia coli*. *J Antimicrob Chemother* **68:** 2477–2481. doi:10.1093/jac/dkt231

Liu P, Chen Y, Wang D, Tang Y, Tang H, Song H, Sun Q, Zhang Y, Liu Z. 2016. Genetic selection of peptide aptamers that interact and inhibit both small protein B and alternative ribosome-rescue factor A of *Aeromonas veronii* C4. *Front Microbiol* **7:** 1228. doi:10.3389/fmicb.2016.01228

Lytvynenko I, Paternoga H, Thrun A, Balke A, Müller TA, Chiang CH, Nagler K, Tsaprailis G, Anders S, Bischofs I, et al. 2019. Alanine tails signal proteolysis in bacterial ribosome-associated quality control. *Cell* **178:** 76–90. doi:10.1016/j.cell.2019.05.002

Macé K, Demay F, Guyomar C, Georgeault S, Giudice E, Goude R, Trautwetter A, Er-mel G, Blanco C, Gillet R. 2017. A genetic tool to quantify *trans*-translation activity in vivo. *J Mol Biol* **429:** 3617–3625. doi:10.1016/j.jmb.2017.10.007

Müller C, Crowe-McAuliffe C, Wilson DN. 2021. Ribosome rescue pathways in bacteria. *Front Microbiol* **12:** 652980. doi:10.3389/fmicb.2021.652980

Osterman IA, Bogdanov AA, Dontsova OA, Sergiev PV. 2016. Techniques for screening translation inhibitors. *Antibiotics (Basel)* **5:** 22. doi:10.3390/antibiotics5030022

Ramadoss NS, Alumasa JN, Cheng L, Wang Y, Li S, Chambers BS, Chang H, Chatter-jee AK, Brinker A, Engels IH, et al. 2013. Small molecule inhibitors of *trans*-translation have broad-spectrum antibiotic activity. *Proc Natl Acad Sci* **110:** 10282–10287. doi:10.1073/pnas.1302816110

Rice LB. 2008. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *J Infect Dis* **197:** 1079–1081. doi:10.1086/533452

Santajit S, Indrawattana N. 2016. Mechanisms of antimicrobial resistance in ESKAPE pathogens. *Biomed Res Int* **2016:** 2475067. doi:10.1155/2016/2475067

Schönhuber W, Le Bourhis G, Tremblay J, Amann R, Kulakauskas S. 2001. Utilization of tmRNA sequences for bacterial identification. *BMC Microbiol* **1:** 20. doi:10.1186/1471-2180-1-20

Shi W, Zhang X, Jiang X, Yuan H, Lee JS, Barry CE III, Wang H, Zhang W, Zhang Y. 2011. Pyrazinamide inhibits *trans*-translation in *Mycobacterium tuberculosis*. *Science* **333:** 1630–1632. doi:10.1126/science.1208813

Shimizu Y, Ueda T. 2002. The role of SmpB protein in *trans*-translation. *FEBS Lett* **514:** 74–77. doi:10.1016/S0014-5793(02)02333-5

Shimizu Y, Inoue A, Tomari Y, Suzuki T, Yokogawa T, Nishikawa K, Ueda T. 2001. Cell-free translation reconstituted with purified components. *Nat Biotechnol* **19:** 751–755. doi:10.1038/90802

Shimokawa-Chiba N, Müller C, Fujiwara K, Beckert B, Ito K, Wilson DN, Chiba S. 2019. Release factor-dependent ribosome rescue by BrfA in the Gram-positive bacterium *Bacillus subtilis*. *Nat Commun* **10:** 5397. doi:10.1038/s41467-019-13408-7

Tacconelli E, Magrini N. 2017. *Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics*. World Health Organization, Geneva.