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

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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-translation 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
Evaluation of trans-translation in ESKAPE

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 into 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.
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<sub>GFP11</sub>. 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<sub>GFP11</sub> 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 |

(A) 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).

In a first set of heterologous experiments, we kept the *E. coli* tmRNAGFP11, 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. cloaeca*, *S. aureus*, and *P. aeruginosa* SmpB signals were the lowest, 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 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. cloaeca* 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. cloaeca* 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. pneumoniae; E. cloaeca* 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. cloaeca* Enterobacteriaceae ribosomes gave the strongest signals.

**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.
∼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 GFP11V2 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
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.
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. The generated plasmids, pUC19 ESKAPE tmRNA GFP11 and pET22b + ESKAPESmpB (Supplemental Table 5), were amplified in E. coli NM522 cells, then extracted using a NucleoBond Xtra Mdi 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 control of a T7 promoter in BL21 (DE3) ΔssrA cells (Cougot et al. 2014). Briefly, BL21 (DE3) ΔssrA 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 (OD600 = 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 lysis buffer. The column was washed with 100 mL lysis buffer and 50 mL washing buffer (50 mM HEPES-KOH, 200 mM KCl, 1 M NH2Cl, 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 pUC19 ESKAPE tmRNA 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 (ATCC 27853). From an overnight starter culture, 6–9 L of LB medium were inoculated to reach an OD600 of 0.05, then stirred at 150 rpm at 37°C. Bacterial growth was stopped when the OD600 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 MgCl2, 200 mM NH4Cl, 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 CaCl2. 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 then discarded, leaving only the transparent pellets which were then resuspended in conservation buffer (20 mM Tris-Cl pH 7.5, 10 mM MgCl₂, 50 mM NH₄Cl, 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) 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 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 tmRNA_GFP11, 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).

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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.

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