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

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19

20 **Running title:** Evaluation of *trans*-translation in ESKAPE bacteria

21 **Keywords:** *Trans*-translation, tmRNA, ESKAPE, antibiotics, HTS, ribosome

22

23 **Abstract**

24 In bacteria, *trans*-translation is the major quality control system for rescuing stalled ribosomes.

25 It is mediated by tmRNA, a hybrid RNA with properties of both a tRNA and a mRNA, and the

26 small protein SmpB. Because *trans*-translation is absent in eukaryotes but necessary for

27 bacterial fitness or survival, it is a promising target for the development of novel antibiotics. To

28 facilitate screening of chemical libraries, various reliable *in vitro* and *in vivo* systems have been

29 created for assessing *trans*-translational activity. However, the aim of the current work was to

30 permit the safe and easy *in vitro* evaluation of *trans*-translation from pathogenic bacteria, which

31 are obviously the ones we should be targeting. Based on green fluorescent protein (GFP)  
32 reassembly during active *trans*-translation, we have created a cell-free assay adapted to the  
33 rapid evaluation of *trans*-translation in ESKAPE bacteria, with 24 different possible  
34 combinations. It can be used for easy high-throughput screening of chemical compounds as  
35 well as for exploring the mechanism of *trans*-translation in these pathogens.

36

## 37 **Introduction**

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

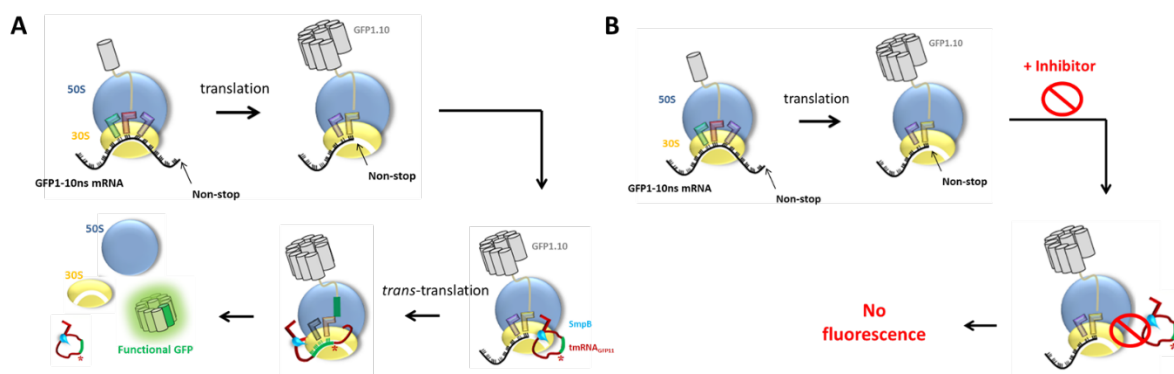
53 In fact, *trans*-translation appears to be the perfect candidate. This mechanism is the  
54 primary bacterial rescue system, allowing for the release of ribosomes stalled on faulty mRNAs  
55 that lack stop codons as well as the elimination of these mRNAs and mistranslated peptides.

56 The *trans*-translation process is performed by hybrid transfer-messenger RNA (tmRNA) and its  
57 protein partner SmpB (Giudice *et al.*, 2014). Briefly, the tmRNA-SmpB complex recognizes  
58 the stalled ribosome and associates with it. In a finely orchestrated ballet, translation then  
59 resumes on tmRNA's internal mRNA-like domain (MLD), which encodes a specific sequence  
60 that is recognized by proteases. This process permits the stalled ribosomes to be recycled, the  
61 degradation of the incomplete peptide after its release, and elimination of the problematic non-  
62 stop mRNA. Remarkably, genes coding for tmRNA and SmpB have been found in nearly all  
63 bacterial genomes, yet not in eukaryotes, with the exception of a very few rare organelles  
64 (Hudson and Williams, 2015). Despite high sequence conservation at both the 5'- and 3'-ends  
65 of tmRNA genes, the internal sequences of tmRNA are considerably divergent among different  
66 species (Supp. Fig. 1), and this property makes tmRNA a good tool for species identification  
67 (Schonhuber *et al.*, 2001). In the same way, despite global structural conservation, variations in  
68 *smpB* sequences are also considerably divergent among different species (Supp. Fig. 1).

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

81 by pyrazinamide (PZA), a first-line anti-tuberculosis drug (Shi *et al.*, 2011), but it was finally  
82 recently shown the action of PZA is entirely independent of *trans*-translation in *M. tuberculosis*  
83 (Dillon *et al.*, 2017).

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



100  
101 **Figure 1. A. *Trans*-translation of sfGFP1-10 mRNA lacking a stop codon.** The tmRNA<sub>GFP11</sub>-SmpB

102 complex binds to the stalled ribosome, and translation resumes thanks to the tmRNAGFP11 mRNA-like  
103 domain (MLD). The MLD encodes the missing eleventh beta-strand of the sfGFP, and the complete  
104 sfGFP is released and becomes fluorescent. **B. Impairment of the process in the presence of *trans*-**  
105 **translation inhibitors.** The ribosomes stay stalled on the problematic mRNA and fluorescence is  
106 impaired.

107

## 108 **Results**

### 109 *Distribution of ArfA, ArfB and RQC in ESKAPE bacterial genomes*

110 While some bacteria can survive without *trans*-translation, this is only because of the existence  
111 of backup systems, such as the two alternative release factors, ArfA and ArfB or the bacterial  
112 ribosome-associated quality control (RQC) mediated by RqcH and RqcP. The Arf mechanisms  
113 can be divided in release factor (RF)-dependent and RF-independent mechanisms. ArfA recruits  
114 RF2 to hydrolyze the nascent polypeptide chain from the P-site tRNA, while ArfB, a class I  
115 release factor homologue, performs hydrolysis itself (Himeno *et al.*, 2015; Müller *et al.*, 2021)  
116 On the other hand, RqcH and RqcP act in concert to mediate the Ribosome-associated quality-  
117 control (RQC) pathway, triggering C-terminal tailing of stalled peptides in the large ribosomal  
118 subunit. RqcH belongs to the NEMF family proteins (homolog of the eukaryotic RQC factor  
119 Rqc2/NEMF, while RqcP (ribosome quality control P-tRNA, formerly YabO), belongs to the  
120 widely distributed S4 RNA-binding family, and is homologous to *E. coli* heat shock protein 15  
121 (Hsp15) (Lytvynenko *et al.*, 2019; Müller *et al.*, 2021). Depending on backup system status,  
122 therefore, the effects of specific inhibitors of *trans*-translation will vary, from increasing the  
123 activity of currently used antibiotics to outright cell death. It was therefore important for us to  
124 begin by pinpointing the phylogenetic distribution of ArfA, ArfB and RQC in ESKAPE  
125 pathogens. To do this, we investigated the sequences of those rescue factors using a  
126 combination of *in silico* methods including keyword searches, similarity detection, protein  
127 domain prediction, ortholog clustering, and synteny analysis. This pipeline was applied to the  
128 complete genomic sequences of 1,670 species: 147 *E. faecium*, 473 *S. aureus*, 465 *K.*  
129 *pneumoniae*, 188 *A. baumannii*, 259 *P. aeruginosa*, and 151 *Enterobacter* spp. Interestingly,

130 among these ESKAPE pathogens, none of the back-up systems were found in *A. baumannii*  
 131 while the two Gram-positive bacteria *E. faecium* and *S. aureus* displayed RQC only (Table 1).  
 132 While we cannot categorically state that no other backup systems exist in these bacteria – see  
 133 for instance the recent reports on ArfT in *Francisella tularensis* and BrfA in *Bacillus subtilis*  
 134 (Goralski *et al.*, 2018; Shimokawa-Chiba *et al.*, 2019) – we can however suppose that their  
 135 viability highly depends on *trans*-translation impairment. On the other hand, we found genes  
 136 encoding ArfA and/or ArfB in most if not all of the *K. pneumonia*, *P. aeruginosa*, and  
 137 *Enterobacter* spp. studied. The impairment of *trans*-translation in these bacteria is probably less  
 138 severe, therefore, even if it still detrimental to bacterial fitness.

139

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

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

141  $\Delta$  *pseudogenes* with frameshift or « in-frame » stop codon.

#### 142 **ESKAPE tmRNA and SmpB production**

143 To allow for independent monitoring of *trans*-translation in these six ESKAPE pathogens, we  
 144 engineered their tmRNAs by replacing their internal MLD with a sequence of 16 amino acids  
 145 that encodes GFP's eleventh beta-strand (Supp. Table 1). To conserve the tmRNA H5 helix that  
 146 is instrumental during *trans*-translation, we also engineered compensatory mutations (Guyomar  
 147 *et al.*, 2020, and Supp. Fig. 2A, B). Unlike those of the other bacteria, the natural tmRNA 3'-  
 148 ends in *E. faecium* and *S. aureus* are not CCA but UUG and UAU, respectively, so these were

149 replaced by CCA to ensure correct aminoacylation by *E. coli* AlaRS (Barends *et al.*, 2000), and  
150 these variants were named tmRNA<sub>GFP11</sub>. Urea-PAGE analysis indicated that the six tmRNA  
151 variants were successfully produced at the expected size, without any noticeable degradation or  
152 unexpected bands (Supp. Fig. 2D). We started with 10 µg plasmidic DNA, and the final yields  
153 were about 4 nmol of transcribed RNAs for each reaction. The six corresponding SmpB proteins  
154 were cloned and produced *in vivo* in *E. coli* (see Experimental Procedures). After purification,  
155 polyacrylamide gel analysis confirmed the correct size of each protein (Supp. Fig. 2E). The  
156 final yields for each ESKAPE SmpB were about half the amount of the *E. coli* SmpB produced.

157

### 158 ***ESKAPE trans-translation reactions***

159 In order to obtain non-productive translation complexes (NTCs) to be targeted by *trans-*  
160 translation, we used a reconstituted cell-free protein synthesis (NEB PURExpress®) system  
161 from *E. coli* (Shimizu, *et al.*, 2001, Shimizu and Ueda, 2002). By adding a non-stop DNA  
162 template, we accumulated stalled ribosomes with the ten first beta-strands of sfGFP stuck in the  
163 ribosome exit tunnel (Fig. 1A). When tmRNA<sub>GFP11</sub> and *E. coli* SmpB are added, the ribosomes  
164 are freed and the intensity of the fluorescent signal increases over time while the complete  
165 sfGFP protein is produced. A plateau is reached at ~4 hours of incubation, and the fluorescence  
166 remains stable for at least 710 minutes (Fig. 2A, black curve).

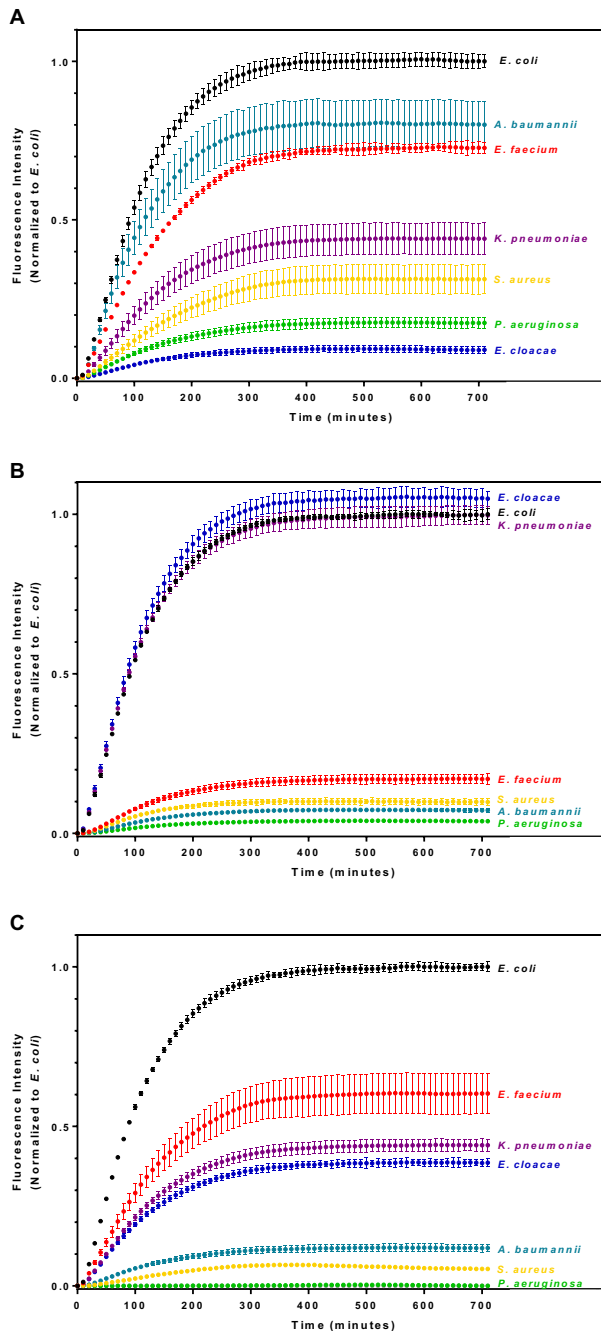
167 In a first set of heterologous experiments, we kept the *E. coli* tmRNA<sub>GFP11</sub>, but replaced  
168 its SmpB by one from an ESKAPE pathogen. A fluorescent signal was still recovered with each  
169 one of the hetero-complexes, albeit at different levels (Fig. 2A). The *E. cloacae*, *S. aureus*, and  
170 *P. aeruginosa* SmpBs displayed the lowest signals, less than 30% of the *E. coli* control, while  
171 the *K. pneumoniae* SmpB signal was about half the control, and *E. faecium* and *A. baumannii*  
172 at 80%. This demonstrates that all of the ESKAPE SmpBs are functional and sufficiently  
173 conserved to be interchangeable in the presence of *E. coli* tmRNA. While it confirms that SmpB



174 is highly conserved (Supp. Fig. 1), it also supports the use of this simple system for screening  
175 molecules that target SmpB but not tmRNA. Indeed, since SmpB is essential for tmRNA's  
176 peptide-tagging activity (Karzai *et al.*, 1999), disrupting SmpB is one of the most promising  
177 ways to impair *trans*-translation. In fact, aptamers that inhibit SmpB functioning were recently  
178 shown to trigger strong growth defects in *Aeromonas veronii* C4 (Liu *et al.*, 2016).

179 We then performed the experiments the other way around, using the *E. coli* SmpB but  
180 the tmRNAs from the ESKAPE pathogens. Contrary to the previous experiments, only the  
181 heteroduplexes combining *E. coli* SmpB and the tmRNAs from *K. pneumoniae* and *E. cloacae*  
182 gave out strong signals, about the same levels as those recovered in the *E. coli* tmRNA control  
183 (Fig. 2B). This is not a surprise since, like *E. coli*, both *K. pneumoniae* and *E. cloacae* are  
184 *Enterobacteriaceae* with very similar tmRNA sequences ( $\geq 95\%$  identity with *E. coli*, see Supp.  
185 Fig. 1). The four other bacterial species all produced signals, but at lower levels (about 5 to  
186 20% of the reference).

187 We continued by performing homologous experiments, using SmpB and tmRNA<sub>GFP11</sub>  
188 from the same ESKAPE pathogen, but this time with *E. coli* ribosomes (Fig. 2C). Five of the  
189 six complexes yielded positive results. Three of these were at high levels ( $\sim 50\%$  compared to  
190 the *E. coli* reference): *K. pneumoniae*; *E. cloacae* and *E. faecium*. The other two were at lower  
191 levels (about 5-10% of the reference): *A. baumannii*, another Gammaproteobacteria that is  
192 relatively close to *Enterobacteriaceae*; and the Gram-positive *S. aureus*. The  
193 Gammaproteobacteria *P. aeruginosa* did not work at all.

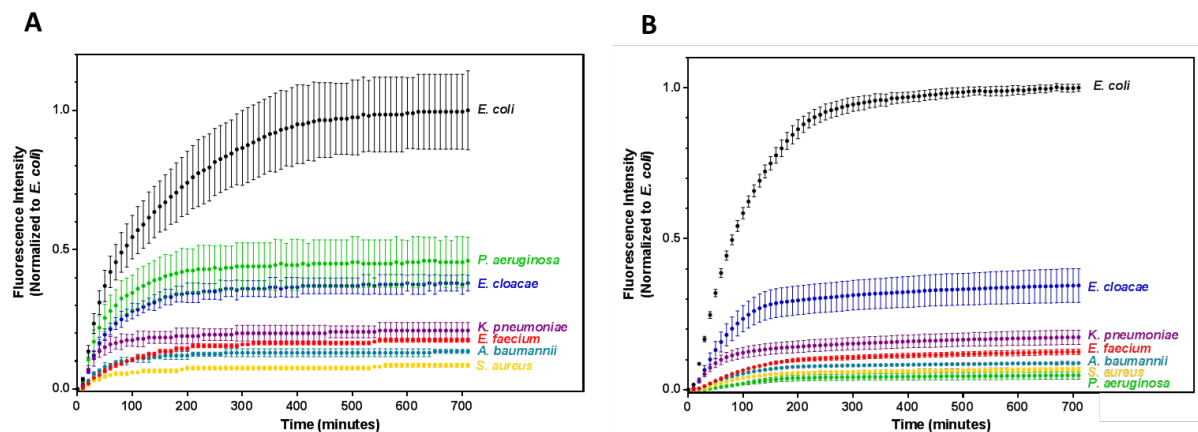


194

195 **Figure 2: *Trans*-translation kinetics over time using *Escherichia coli* ribosomes.** Fluorescence  
 196 increases are directly linked to *trans*-translation activity. **A)** *Trans*-translation assays were done on *E.*  
 197 *coli* tmRNA<sub>GFP11</sub> using the SmpBs from each ESKAPE pathogen, with the *E. coli* SmpB as a control.  
 198 **B)** *Trans*-translation assays keeping the *E. coli* SmpB but using the tmRNA<sub>GFP11</sub> variants of each  
 199 ESKAPE pathogen, with an *E. coli* tmRNA<sub>GFP11</sub> as the control. **C)** Both SmpB and tmRNA<sub>GFP11</sub> are from  
 200 each ESKAPE pathogen, with the *E. coli* SmpB-tmRNA<sub>GFP11</sub> as a control. The results are shown as  
 201 means ± standard deviation and normalized to the *E. coli* conditions.

202

203 In a final set of experiments, we used homologous tmRNA-SmpB complexes in the  
 204 presence of their corresponding ESKAPE ribosomes. The use of the PURExpress  $\Delta$  Ribosome  
 205 kit allowed us to substitute commercial *E. coli* ribosomes with ESKAPE variants we had  
 206 prepared in-house. We first confirmed the effectiveness of translation using these ribosomes by  
 207 synthesizing full-size sfGFP. For all ribosomes used, a fluorescent signal was recovered,  
 208 indicating that the ESKAPE ribosomes translate well even if at lower levels (Fig. 3A). The *P.*  
 209 *aeruginosa* and *E. cloacae* *Enterobacteriaceae* ribosomes gave the strongest signals, around 45  
 210 and 35% respectively as compared to that of *E. coli*. All of the other signals were below 20%,  
 211 even dropping under 10% in the case of *S. aureus*.



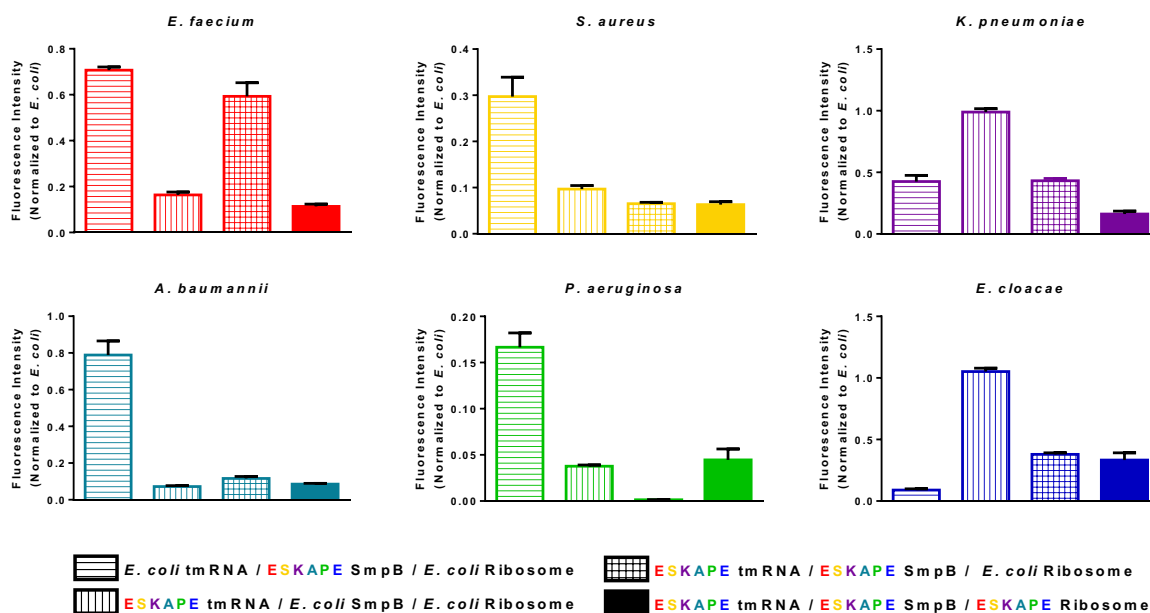
212 **Figure 3. Translation and *trans*-translation kinetics over time. A) Translation kinetics over time:**  
 213 **the increase in fluorescence of full-length GFP (*i.e.* encompassing the 12 beta-strands) is directly linked**  
 214 **to translation. B) *Trans*-translation kinetics over time using ESKAPE ribosomes.** All results are  
 215 shown as means  $\pm$  standard deviation and normalized to *E. coli*.  
 216

217  
 218 Despite these rather poor translation rates, fluorescence was easily detected, so we also  
 219 performed *trans*-translation experiments using ESKAPE ribosomes (Fig. 3B). The goal was to  
 220 improve the levels of the *trans*-translation signals previously recovered, but more importantly  
 221 to obtain a positive result for *P. aeruginosa*. The results were finally conclusive for that bacteria,  
 222 which gave a fluorescent signal of  $\sim$ 10% compared to the control. This positive result could be  
 223 linked to the quite efficient translation obtained with these ribosomes (Figure 3A). On the other  
 224 hand, the *trans*-translation levels of the other bacteria did not improve, and were even lower in

225 *S. aureus*. This could be due to the fact that the PURExpress system is based on only *E. coli*  
226 translation factors, and their low count limits their handling of canonical translation (see Fig.  
227 3A) or specific tmRNAs (e.g. tmRNA aminoacylation by *E. coli* AlaRS or tmRNA-SmpB  
228 transport by *E. coli* EF-Tu-GTP). However, and since our goal was to detect variations of  
229 fluorescence after drug treatment within each of the ESKAPE species, rather than comparing  
230 the strains between each other, it was important to get a correct and satisfactory signal for each  
231 one of the strains individually. Towards this aim, we decided to use a more sensitive  
232 spectrophotometer, *ie* Synergy HTX from Biotek©. We adjusted the spectrophotometer gain  
233 function in order to ensure optimal detection of GFP fluorescence without saturation and  
234 applied the technique to the homologous systems (tmRNA-SmpB and ribosomes from the same  
235 ESKAPE), the ones that are the most interesting for developing new inhibitors. The data  
236 obtained were finally conclusive, within a range of 20000-90000AU for translation as well as  
237 for *trans*-translation, allowing for an accurate internal control in case of inhibition (Supp. Fig.  
238 3).

## 239 **Discussion**

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



244

245 **Figure 4: Quantification of *in vitro* trans-translation.** Normalized fluorescence obtained in  
 246 heterologous and homologous systems are shown at 310 minutes of incubation and reassembled by  
 247 species. The results were normalized to the *E. coli* conditions and are shown as means  $\pm$  standard  
 248 deviations.

249

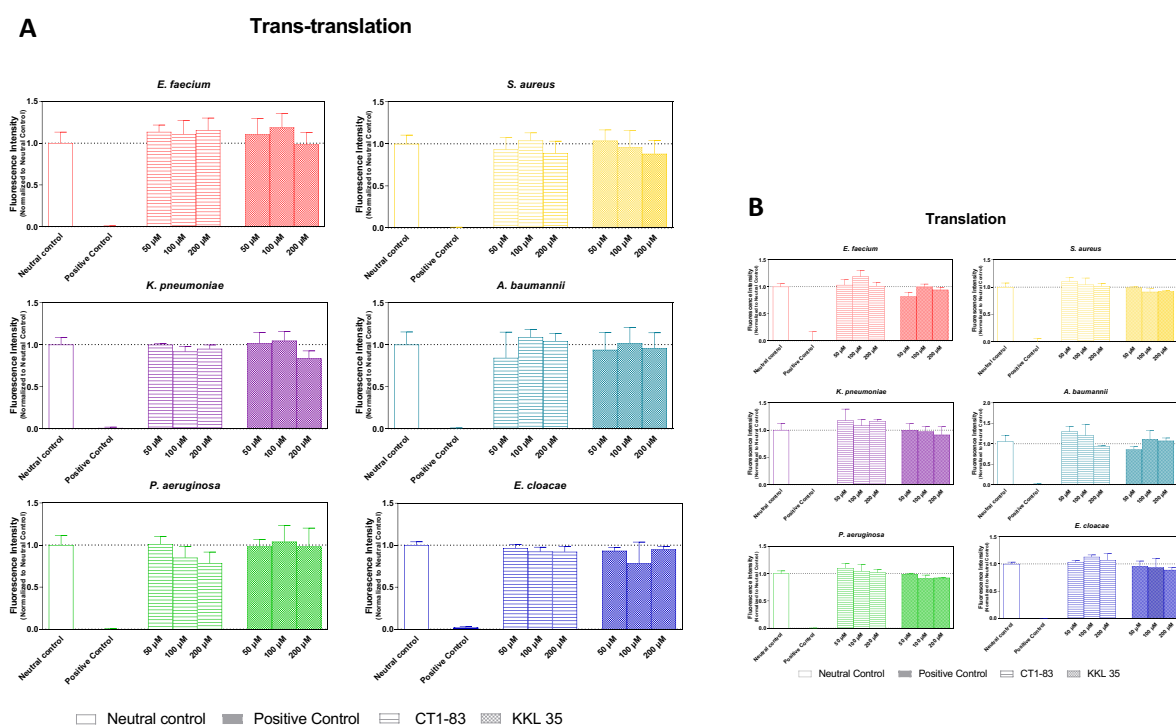
250 The molecules being investigated for the development of new anti-*trans*-translation antibiotics  
 251 will have different ways of interfering with tmRNA-SmpB binding to stalled ribosomes. They  
 252 could disrupt tmRNA-SmpB interactions, or they could prevent interactions between the  
 253 complex and the ribosome, such as by blocking the entrance of SmpB entirely or by preventing  
 254 the passage of the complex through the bridges which have to be open during the process.  
 255 Therefore, it is of great interest to have the ability to evaluate the targeting of the three main  
 256 actors (tmRNA, SmpB, and the ribosome) independently as well as in each ESKAPE system.  
 257 Of the 24 combinations we tested, 23 exhibited a signal strong enough for evaluating the  
 258 possible activity of inhibitors. The only one that did not was the *P. aeruginosa* tmRNA-SmpB  
 259 complex when used with *E. coli* ribosomes. We first suspected that the tmRNA H5 helix,  
 260 inspired from the *E. coli* helix (Supp. Fig. 2B), might somehow have altered its activity.  
 261 Therefore, to avoid any possible effects of the helical rearrangement, we constructed and tested  
 262 new tmRNA<sub>GFP11</sub> variants for *P. aeruginosa* but also *E. coli*, *S. aureus* and *E. faecium*. These

263 tmRNA<sub>GFP11V2</sub> constructs all have the full sequence that encodes the eleventh beta-strand of  
264 GFP upstream from the natural H5 helix (Supp. Fig. 2C). However, these variants did not have  
265 improved fluorescence, and *P. aeruginosa* still did not emit signals. We also performed new  
266 experiments by increasing two fold the amounts of SmpB and two to four fold the amounts of  
267 tmRNA, but without further success (not shown). We can thus exclude the idea that the different  
268 structural features between the *P. aeruginosa* and *E. coli* ribosomes (Halfon *et al.*, 2019) are  
269 important enough to prevent the correct process from occurring.

270 To permit the high-throughput screening of chemical compounds in multi-well microplates it  
271 was important to lower average screening costs of the current assay. To enable this, we  
272 decreased the reaction scale of the assays by reducing the final reaction volumes down to a  
273 microliter scale. Proof-of-concept experiments were performed with *E. coli* or ESKAPE  
274 homologous systems in final volumes of 2  $\mu$ l using the MANTIS<sup>®</sup> liquid-handler instrument  
275 (Formulatrix) or simply by using an electronic micropipette. The resulting signals were strong  
276 enough to allow for the easy detection of *trans*-translational activity. Indeed, the objective of  
277 this study was to create a non-hazardous *in vitro* screening system for evaluating *trans*-  
278 translation in ESKAPE pathogens, and to miniaturize it for HTS applications, and the assays  
279 we performed were convincing. We then decided to perform an experiment demonstrating proof  
280 of principle by using an oligonucleotide that interferes with the mRNA-like domain (MLD) of  
281 tmRNA as well as CT1-83 and KKL-35, two 1,3,4-oxadiazole derivatives that were recently  
282 shown to display a low *in vitro* activity against *E. coli* *trans*-translation (Guyomar *et al.*, 2020).

283 Towards this aim we used again the homologous system including tmRNA, SmpB and  
284 ribosomes from the same ESKAPE. The results on *trans*-translation show a total inhibition of  
285 the process when using the anti-sense, whatever the pathogen. On the other hand, and despite a  
286 very slight and dose-dependent effect of CT1-83 on *P. aeruginosa* and *E. cloacae* and KKL-35  
287 on *S. aureus*, none of the compounds displayed any noticeable activity on the six ESKAPE

288 systems (Figure 5A). Of course, to avoid compounds that inhibit any necessary step for  
 289 fluorescence (*e.g.* transcription, translation, or GFP folding) to be scored as positive and result  
 290 in false positive hits, transcription-translation assays were also performed using full GFP in t  
 291 the absence of tmRNA-SmpB. This set of results confirmed the absence of noticeable effect of  
 292 the two oxadiazole compounds on transcription-translation, while the signal was completely  
 293 abolished after chloramphenicol treatment (Figure 5B). While this is interesting, it especially  
 294 confirms that new classes of more efficient molecules are needed to target *trans*-translation in  
 295 ESKAPE pathogens.



296  
 297 **Figure 5: Quantification of *in vitro* ESKAPE *trans*-translation and translation assays after CT1-**  
 298 **83 and KKL-35 treatment.** Experiments were conducted in microplates, using ESKAPE homologous  
 299 systems and increasing concentrations (50, 100 and 200  $\mu$ M) of CT1-83 and KKL-35 oxadiazole  
 300 compounds. Normalized fluorescence intensities obtained are presented after 310 minutes of incubation  
 301 and sorted by species. The results were normalized to the Neutral control conditions and are shown as  
 302 means  $\pm$  standard deviations. **A) *Trans*-translation assay with the oxadiazole compounds CT1-83**  
 303 **and KKL-35.** The positive control was obtained by using 10 $\mu$ M Antisense B in 10% DMSO. **B)**

304 **Translation assay with the oxadiazole compounds CT1-83 and KKL-35.** The positive control was  
305 obtained by using Chloramphenicol at 100  $\mu$ M in 10% DMSO. Using the Anova test, results were  
306 considered statistically significant when  $P \leq 0.01$ . Only positive controls presented a difference of  $P \leq$   
307 0.0001 for translation and *trans*-translation (stars were not represented to facilitate graph reading).

308  
309 Therefore, the system will clearly be very effective for benchmarking the effects of new  
310 antibiotic compounds that target *trans*-translation in highly pathogenic bacteria, as well as  
311 aiding us to better understand the *trans*-translation process in these bacteria. Its flexibility in  
312 the choice of target bacterial species and the possibility for varying the combinations of tmRNA,  
313 SmpB, and ribosomes are advantageous, making the identification of new specific antimicrobial  
314 inhibitors easier. When hits are recovered it is important to perform a translation assay to  
315 confirm that the compounds do not inhibit any other step necessary for fluorescence (*e.g.*  
316 transcription, amino-acylation, translation, or GFP folding). Ongoing experiments in our  
317 laboratory are using this to screen large chemical and natural product libraries for drug  
318 discovery.

319

## 320 **Experimental Procedures**

### 321 ***In silico* analysis**

322 Complete genomes were retrieved from the NCBI database (March 2020). Chromosomes and  
323 plasmids (when present) were studied separately. GenBank files were first searched based on  
324 their textual annotation entries, using the keywords ‘ArfA,’ ‘yhdL,’ and ‘alternative ribosome-  
325 rescue factor’ (for ArfA), or ‘ArfB,’ ‘yaeJ,’ ‘ribosome-associated protein,’ and ‘peptidyl-tRNA  
326 hydrolase’ (for ArfB). Missing loci were checked using BlastN, BlastP, and tBlastN similarity-  
327 detection strategies (Altschul *et al.*, 1990) as well as comparative genomics, with synteny  
328 analysis done using progressiveMauve (Darling *et al.*, 2010). All retrieved loci were compared  
329 using the Reciprocal Best Hits method, and InterProScan (Jones *et al.*, 2014) was used on the



330 corresponding proteins to check for the presence of the IPR005589/ PF03889 (ArfA) and  
331 IPR000352/PF00472 (ArfB) domains. Frameshifted loci were indicated as annotated in the  
332 GenBank files. Finally, the presence and absence of K09890 (ArfA) and K15034 (ArfB) were  
333 checked in the KEGG ORTHOLOGY database (Kanehisa *et al.*, 2016).

### 334 ***Plasmid construction and preparation***

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

342 We also produced tmRNA<sub>GFP11</sub>V2 variants for *E. coli*, *P.aeruginosa*, *S.aureus* and *E. faecium*  
343 species. This tmRNA<sub>GFP11</sub> series carries the full sequence encoding the eleventh beta-strand of  
344 GFP upstream from the *E. coli* H5 helix (Supp. Fig. 2C). In order to obtain mature tmRNA<sub>GFP11</sub>  
345 by *in vitro* transcription, the tmRNA<sub>GFP11</sub> and tmRNA<sub>GFP11</sub>V2 ESKAPE sequences were  
346 synthesized and cloned into the pUC19 vector between the HindIII and BamHI restriction sites  
347 (Supp. Table 1). For each ESKAPE SmpB, GenScript synthesized the sequences with codon  
348 optimization for *E. coli*, cloning them into the pET22b(+) vector between the NdeI and XhoI  
349 restriction sites to add a 6His histidine tag (Supp. Table 2). The generated plasmids,  
350 pUC19ESKAPetmRNA<sub>GFP11</sub> and pET22b+ESKAPESmpB (Supp. Table 5), were amplified in  
351 *E. coli* NM522 cells then extracted using a NucleoBond Xtra Midi kit (Macherey-Nagel).  
352 Quantification was performed using a SimpliNano spectrophotometer (Biochrom).

### 353 ***SmpB purification***

354 The bacterial cultures and SmpB purification were all done as previously described (Guyomar  
355 *et al.*, 2020). His-tagged *E. coli* and ESKAPE SmpB proteins (Supp. Table 2) were expressed  
356 from the pF1275 and the pET22b+ESKAPE SmpB vectors under the control of a T7 promoter  
357 in BL21(DE3) $\Delta$ *ssrA* cells (Cougot *et al.*, 2014). Briefly, BL21(DE3) $\Delta$ *ssrA* cells were grown in  
358 lysogeny broth (LB) at 30 °C supplemented with ampicillin (100 µg/ml) and kanamycin (50  
359 µg/ml). Protein expression was induced in the exponential phase ( $OD_{600} = 0.6$ ) with 0.1 mM  
360 isopropyl- $\beta$ -D-1-thiogalactopyranoside (IPTG) overnight at 16 °C. Cells were harvested and  
361 washed, then resuspended in lysis buffer (50 mM HEPES-KOH, 200 mM KCl, 20 mM  
362 imidazole, and 1 mM DTT pH 7.5). Cell lysis was performed using a French press, and the  
363 lysate was centrifuged at 15,000 rpm for 45 minutes at 4 °C in a Beckman J2-MC with a JA-17  
364 rotor. The supernatant was then filtrated (0.2 µm) and injected onto a Ni-NTA sepharose  
365 column (HisTrap FF, GE Healthcare) previously equilibrated with the lysing buffer. The  
366 column was washed with 100 ml lysis buffer and 50 ml washing buffer (50 mM HEPES-KOH,  
367 200 mM KCl, 1 M NH<sub>4</sub>Cl, imidazole 20 mM, and 1 mM DTT pH 7.5) before elution with 500  
368 mM imidazole. Finally, a 10kDa Amicon Ultra centrifugal filter (Merck Millipore) was used to  
369 concentrate the fractions containing pure SmpB, changing the buffer to a concentration buffer  
370 (50 mM HEPES-KOH, 100 mM KCl, 10% glycerol, and 1 mM DTT pH 7.5). In order to  
371 visualize SmpB, 50 pmol of denatured proteins was analyzed on 15% SDS-PAGE gels. Proteins  
372 were detected using InstantBlue protein stain (Expedeon) according to the supplier's  
373 instructions.

#### 374 ***tmRNA<sub>GFP11</sub> production***

375 *E. coli* and ESKAPE tmRNA<sub>GFP11</sub> were produced as previously described (Guyomar *et al.*,  
376 2020). Each ESKAPE tmRNA<sub>GFP11</sub> was transcribed *in vitro* from the  
377 pUC19ESKAPEtmRNA<sub>GFP11</sub> plasmids. To generate the 3' end needed for aminoacylation by  
378 AlaRS, the plasmid (10 µg) was completely digested by NEB BsmBI or EarI restriction

379 enzymes (Supp. Table 5). After phenol/chloroform extraction, the purified digested plasmid  
380 was precipitated, and the resulting pellets resuspended in 40  $\mu$ l nuclease-free water. A  
381 MEGAscript T7 transcription kit (Thermo Fisher Scientific) was used to produce each ESKAPE  
382 tmRNA<sub>GFP11</sub> before its purification using the corresponding MEGAclear kit. Denatured  
383 tmRNA<sub>GFP11</sub> was checked by electrophoresis on 8% Urea-PAGE gels, stained with ethidium  
384 bromide, and visualized under ultraviolet light.

### 385 ***DNA templates and oligonucleotide production***

386 For *trans*-translation assays, the nonstop GFP1-10 sequence was produced by PCR using  
387 primers #1 and 2 and Q5 High-Fidelity DNA Polymerase (NEB) with pETGFP 1–10 vector as  
388 a template (Cabantous *et al.*, 2006; Supp. Table 3 and 4). For translation assays, primers #1 and  
389 #3 from the same template were used to amplify sfalaGFP, the superfolder GFP having an  
390 additional conserved alanine between the sfGFP1–10 and sfGFP11 beta-strands (Supp. Table 3  
391 and 4). The resulting PCR products were purified using a QIAquick PCR Purification Kit  
392 (Qiagen) and checked by agarose electrophoresis. Both PCR products have a T7 promoter  
393 upstream from their coding sequences. Antisense oligonucleotide “A” was supplied by Eurofins  
394 (Supp. Table 3).

### 395 ***ESKAPE ribosome purification***

396 Ribosomes were purified from *Acinetobacter baumannii* (clinical isolate); *Staphylococcus*  
397 *aureus* (clinical isolate); *Pseudomonas aeruginosa* (ATCC 27853); *Enterobacter cloacae*  
398 (clinical isolate); *Klebsiella pneumoniae* (clinical isolate); and *Enterococcus faecium*  
399 (HM1070). From an overnight starter culture, 6-9 L of LB medium were inoculated to reach an  
400 OD<sub>600</sub> of 0.05, then stirred at 150 rpm at 37 °C. Bacterial growth was stopped when the OD<sub>600</sub>  
401 reached 0.8 to 1.0. The cells were then centrifuged at 4,000 rpm for 20 minutes at 4 °C. Pellets  
402 (around 2 g/L of culture) were washed in a lysis buffer (20 mM Tris-HCl pH 7.5, 20 mM MgCl<sub>2</sub>,  
403 200 mM NH<sub>4</sub>Cl, 0.1 mM EDTA, and 6 mM  $\beta$ -mercaptoethanol), centrifuged at 4,000 rpm for

404 15 minutes at 4 °C, and kept overnight at -80 °C. Pellets were then suspended in a Potter  
405 homogenizer in another lysis buffer complemented with 1 mM CaCl<sub>2</sub>. Cells were lysed in a  
406 French press at 1.0 kbar. To remove cellular debris, the lysates were centrifuged using a type  
407 50.2 Ti rotor at 18,200 rpm for 30 minutes at 4 °C. The superficial pellet layer was then  
408 discarded, and the pellet resuspended in lysis buffer. Ribosomes were isolated by centrifuging  
409 lysates on a 30% sucrose cushion at 31,500 rpm for 19 hours at 4 °C. The superficial layer of  
410 pellets was again discarded, leaving only the transparent pellets which were then resuspended  
411 in conservation buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 50 mM NH<sub>4</sub>Cl, 0.1 mM EDTA,  
412 and 6 mM β-mercaptoethanol). Any remaining contaminants were removed by a final  
413 centrifugation at 18,200 rpm for 1 hour at 4 °C. Ribosomes were concentrated using a Centricon  
414 (Merck Millipore) with a cut-off of 100K, flash-frozen in nitrogen, and conserved at -80 °C.

#### 415 ***Trans-translation assays***

416 *In vitro trans-translation* assays were performed using the PURExpress *In Vitro* Protein  
417 Synthesis and Δ Ribosome kits (New England Biolabs). For *trans-translation* assays,  
418 PURExpress was supplemented by 62.5 ng purified PCR product encoding for nonstop sfGFP1-  
419 10, 12.5 pmol tmRNA<sub>GFP11</sub>, 25 pmol SmpB, and 50 pmol antisense A. Where necessary (Δ  
420 Ribosome), 6.725 pmol ribosomes were also added. These reactions were performed in a final  
421 reaction volume of 10 μL, with PURExpress diluted by a final factor of 1.6 with Buffer III  
422 (HEPES-KOH 5mM pH7.5, MgOAc 9mM, NH<sub>4</sub>Cl 10mM, KCl 50mM, and DTT 1mM). A  
423 Step One Plus PCR system (Applied Biosystems) was used for incubation at 37 °C as well as  
424 for fluorescence measurements over 710 minutes.

#### 425 ***Translation assays***

426 *In vitro* translation assays were performed using a PURExpress Δ Ribosome kit. To produce  
427 the sfalaGFP, the PURExpress Δ Ribosome was diluted to a final factor of 1.6 with Buffer III,  
428 to which was added 62.5 ng purified PCR product and 6.725 pmol of the appropriate ribosomes

429 in a final reaction volume of 10  $\mu$ L. The translation reactions were incubated at 37 °C, and  
430 fluorescence was measured over 710 minutes using a Step One Plus.

#### 431 ***Miniaturization of the trans-translation assays for HTS***

432 *In vitro* miniaturization of the *trans*-translation assays was performed using the PURExpress *In*  
433 *Vitro* Protein Synthesis  $\Delta$  Ribosome kit (New England Biolabs). The mix was diluted by a factor  
434 of 1,6 after addition of 2,5 $\mu$ M SmpB, 1,25 $\mu$ M tmRNAGFP11, 672,5nM ribosomes, 6,25ng/ $\mu$ L  
435 of purified PCR product encoding for nonstop sfGFP1-10 and 5  $\mu$ M antisense A. 2 $\mu$ L of Neutral  
436 control (10% DMSO), 2 $\mu$ L of Positive Control (10 $\mu$ M Antisense B in 10% DMSO) and  
437 compounds in 10% DMSO were mixed together in a qPCR 96-well plate. CT1-83 oxadiazole  
438 compound was provided by Dr. Mickael JEAN (Univ. Rennes) and KKL35 by Sigma  
439 Chemicals, respectively. Compounds and controls were then dried in SpeedVac Concentrator  
440 before being resolubilized by adding 2 $\mu$ L of PURExpress Mix in the same plate. Incubation at  
441 37°C and fluorescence measurements over 310 minutes were simultaneous performed thanks  
442 to Synergy HTX from Biotek. The intensities of GFP were measured with excitation filter at  
443 485/20 and emission filter at 528/20. The gain used was 116 for *E. faecium*, *S. aureus*, *A.*  
444 *baumannii* and *P. aeruginosa* and 100 for *K. pneumoniae* and *E. cloacae*.

445 The transcription-translation control assays were performed in the same way, except that  
446 nonstop sfGFP1-10 was replaced by full sfGFP, in the absence of tmRNA and SmpB. The  
447 Positive Control was then Chloramphenicol at 100  $\mu$ M in 10% DMSO.

448

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455

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461

462 Author contributions

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

469

470 **Conflict of interest**

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

473

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