UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Trans-translation inhibition in ESKAPE bacteria and its perspectives as an antibiotic adjuvant

Rodrigo Campos da Silva

Porto Alegre, 2023

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Tese apresentada por Rodrigo Campos da Silva para obtenção do TÍTULO DE DOUTOR em Ciências Farmacêuticas

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“Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world.”

"A ciência não conhece país, porque o conhecimento pertence à humanidade e é a tocha que ilumina o mundo."

Louis Pasteur

RESUMO

Hoje em dia estamos ameaçados sob o risco de voltar à era pré-antibiótica devido à disseminação da resistência antimicrobiana. O ressurgimento da morte por infecções, que eram consideradas facilmente tratáveis, exige a descoberta de novos antibióticos e, mais importante, de novas classes de antibióticos com mecanismos diferentes dos atuais. Nesse contexto, podemos citar o processo de trans-tradução. A trans-tradução é o principal sistema de resgate ribossomal bacteriano que libera o ribossomo parado no final de 3' do RNA e há algum tempo o processo de trans-tradução tem sido considerado como um grande alvo antibiótico, possuindo várias características que o tornam uma boa promessa: i) só está presente em células procarióticas, ii) é onipresente em todos os gêneros de bactérias, iii) não visado pelos antibióticos atuais e iv) é essencial para o *fitness* bacteriano, pois foi demonstrado que, mesmo que sua ausência não mate as bactérias, cria vários fenótipos diferentes, o que inclui perda de tolerância ao antibiótico ou perda de virulência. Nosso objetivo foi rastrear uma grande biblioteca química em busca de moléculas que visem a trans-tradução e combiná-las com antibióticos utilizados na prática médica. Examinamos a Biblioteca Química Francesa (CN - coleção essencial de 1080 compostos) para encontrar moléculas capazes de inibir a trans-tradução. Os experimentos foram realizados conforme previamente publicado por Macé *et al* e Guyomar *et al* onde a trans-tradução foi avaliada *in vitro* e *in vivo* (modelo celular). O modelo celular foi realizado utilizando um mutante de *Escherichia coli* contendo um plasmídeo constituído por genes repórteres que indicavam quando a trans-tradução era inibida. O modelo molecular envolvia a produção de um marcador fluorescente quando a trans-tradução estava ativa, portanto, se a molécula estivesse inibindo a trans-tradução, não haveria fluorescência. Para isso, utilizamos o kit de síntese de proteínas in vitro PURExpress® da NEB. Após a triagem inicial, foi identificado um potencial hit ("molécula 404") inibindo aproximadamente 50% da trans-tradução in vitro. A partir desse acerto, 28 derivados foram sintetizados e testados: 6 outros compostos inibiram pelo menos 20% da trans-tradução. A molécula 404

apresentou forte atividade de inibição *in vivo* com dois de seus derivados mais ativos, PD5 e PD24. Combinações e ensaios de inibição foram realizados contra patógenos ESKAPE com uma variedade de antibióticos. Dentre as 90 combinações testadas, 16 apresentaram redução positiva na concentração inibitória mínima (CIM) do antibiótico em relação ao seu uso isolado. Considerando que todas as bactérias utilizadas estão na lista prioritária da OMS para serem alvo do desenvolvimento de antibióticos e que os antibióticos utilizados estavam na Lista de Medicamentos Essenciais da OMS, a possibilidade de reduzir os perfis de resistência inibindo a trans-tradução merece atenção especial. Este trabalho é a primeira prova de conceito mostrando que os inibidores da trans-tradução podem atuar como adjuvantes de antibióticos para o tratamento de bactérias clinicamente importantes e podem servir como um guia para as possibilidades desse alvo.

Palavras-Chave: Ribossomo; tmRNA; Trans-tradução; Bacteriologia; Resistência aos Antibióticos.

ABSTRACT

Nowadays we are threatened under the risk of going back to the pre-antibiotic era due to the spreading of antimicrobial resistance. The resurgence of death to infections, which were considered easily treatable calls for the discovery of new antibiotics and most importantly new antibiotic classes with mechanism that are different from the current ones. In this context we can cite the trans-translation process. The trans-translation is the main ribosomal rescue system that frees ribosome stalled at 3' end of nonstop RNA and for some time now the trans-translation process has been considered as a great antibiotic target, possessing several traits that makes it a good promise: i) it is only present in procaryotic cells, ii) it is ubiquitous to all bacteria genera, iii) not targeted by current antibiotics and iv) it is essential to the bacterial fitness as it was demonstrated that even if its absence does not kill the bacteria, it creates several different phenotypes which includes loss of tolerance to antibiotic or loss of virulence. Our objective was to screen a large chemical library in search of molecules that target trans-translation and combine them with antibiotics used in medical practice. We screened the French Chemical Library (CN - essential collection 1080 compounds) to find molecules capable of inhibiting *trans*-translation. The experiments were performed as previously published by Macé *et al* and Guyomar *et al* where trans-translation was evaluated i*n vitro* and *in vivo* (cell model). The cell model was performed using a mutant of *Escherichia coli* containing a plasmid consisting of reporter genes that indicated when the trans-translation was inhibited. The molecular model involved in producing a fluorescent marker when trans-translation was active so if the molecule was inhibiting trans-translation there would not be fluorescence. To do this we utilized the PURExpress® in vitro protein Synthesis kit by NEB. After the initial screening, a potential hit was identified ("molecule 404") inhibiting approximately 50% of trans-translation in vitro. From this hit, 28 derivatives were synthesized and tested: 6 other compounds inhibited at least 20% of trans-translation. Molecule 404 showed strong trans-translation inhibition activity in vivo with two of its most active derivatives, PD5 and PD24. Combinations and

inhibition assays have been performed against ESKAPE pathogens with a range of antibiotics. Among 90 combinations tested, 16 showed a positive reduction in the minimum inhibitory concentration (MIC) of the antibiotic compared to its use alone. Considering that all the bacteria used are on the WHO priority list to be targeted by antibiotic development and that the antibiotics used were on the WHO Essential Medicines List, the possibility of reducing resistance profiles by inhibiting trans-translation deserves special attention. This work is the first proof of concept showing that trans-translation inhibitors can act as antibiotic adjuvants to treat clinically important bacteria and may serve as a guide to the possibilities of this target.

Keywords: Ribosome; SmpB; tmRNA; *trans*-translation; Bacteriology; Antibiotic Resistance.

SUMMARY

INTRODUCTION

In the year of 1928, Sir Alexander Fleming discovered that the fungus *Penicillium notatum,* accidentally grown, inhibited *Staphylococcus aureus* growth. This was the beginning of what would soon become the golden age of antibiotics. This period began after the commercialization of Benzylpenicillin, or Penicillin G, isolated by Ernst Chain and Howard Florey in 1942(DURAND; RAOULT; DUBOURG, 2019). Penicillin G is an antibiotic possessing high effectiveness against many bacterial infections, notably due to Gram positive pathogens. Life expectancy at the time was as low as 47 years average due to the many infection diseases, like pneumonia and cholera, that ravaged the population. With antibiotic commercialization, average life expectancy in developed countries increased to incredible 78 years (ADEDEJI, 2016). Currently, this scenario is being threatened by the rise of antibiotic resistance (AR) which is now a worldwide issue (WHO, 2020).

Intrinsic AR is the capability of a microorganism to survive and reproduce under the presence of antimicrobials to which it was previously sensitive. This leads to the use of higher and possibly toxic concentrations to try to achieve the desired result (ADEDEJI, 2016). AR is not strictly related to antibiotic discovery by humans but has been around for about the same time as microbes started to produce antibiotics to fight against each other for nutrients and space. In fact, this was demonstrated by the finding of resistance genes in permafrost ice that dated 30.000 years (D'COSTA *et al.*, 2011). However, AR has been accelerated due to modern socioeconomic practices like the indiscriminate use of antibiotics. It is estimated that, by 2050, 10 million people could die due to resistant pathogens, approximately 3 person each second, with trillions of dollars in treatment expenses (O´NEILL, 2016). It is no surprise that World Health Organization (WHO) appointed AR as one of the main global health issues to track (WHO, 2020).

It has been 30 years since the discovery of the last antibiotic class and now all of them possess described resistances (W.C REYGAERT, 2018). Therefore, new efforts in the discovery of antibiotics are of extreme importance. In this quest, the discovery of new targets for which no resistance mechanisms were already reported, should be prioritized (TYERS; WRIGHT, 2019). To be of interest, a

bacterial new target should be conserved among all bacteria; indispensable to bacteria survival or fitness; sufficiently variable so that different species can be distinguished; present in prokaryotes only. Finally, it should not be targeted by current antibiotics or subjected to known resistance mechanisms (THEPAUT *et al.*, 2021). When considering all the above criteria, it looks like an impossible task

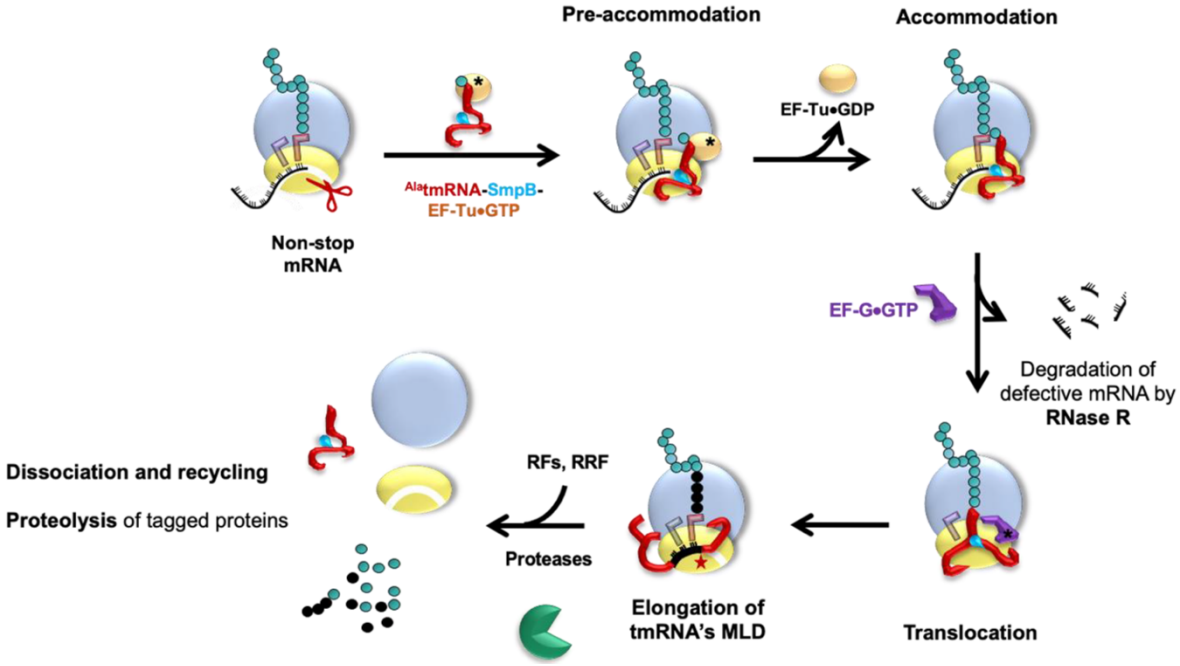

Figure 1 Trans-translations mechanism adapted from CAMPOS-SILVA et al., 2021. https://doi.org/10.3390/microorganisms10010003.

to achieve. Though, there is one target that fits all of this: *trans*-translation (CAMPOS-SILVA *et al.*, 2021).

Trans-translation is the main quality control system allowing for the release of stalled ribosomes during bacterial protein synthesis. Its leading actors are a hybrid RNA named transfer-messenger RNA (tmRNA) and its protein partner SmpB. When the ribosome reaches the 3'-end of a non-stop RNA, it gets stuck due to the lack of stop-codon. This situation leaves the stalled ribosome with an incomplete peptide bound to the P-site tRNA and a vacant decoding site (A site) (Figure 1). During *trans*-translation, the tmRNA-SmpB complex enters this vacant A site to resume translation (GIUDICE; MACÉ; GILLET, 2014). This complex system will be discussed in further depth in the next chapter.

Trans-translation possesses all the qualities above mentioned that are needed for an antibiotic candidate. In fact, recent works have demonstrated the efficacity of inhibiting *trans*-translation to kill several bacteria. And even if the complete inhibition of *trans*-translation does not kill the bacteria, due to back up rescuing systems, it leads to the appearance of antibiotic sensitive phenotypes (KEILER; FEAGA, 2014). To date there are no antibiotics on the market that target *trans*-translation and only a few molecules have appeared as possible candidates but even them had their activity put into question (CAMPOS-SILVA *et al.*, 2021). This all makes the research of anti-*trans*-translation compounds a promising and possible safe alternative to cope with the resistance that is spreading through the bacterial world.

OBJECTIVES

2.1 General Objective

Considering the above-mentioned considerations, the objective of this work was to discover new compounds that inhibit *trans*-translation in bacteria of clinical importance.

2.2 Specific Objectives

- To describe the importance of *trans*-translation as a target for the development of antibiotic alternatives.
- To screen for *trans*-translation inhibitors.
- To test the use of these compounds as antibiotics or antibiotic adjuvants against bacteria of clinical importance belonging to the ESKAPE group.
- To evaluate the toxicity of these compounds in cellular models and in *Galleria mellonella* animal model.
- To study the mechanisms by which *trans*-translation is inhibited.

CHAPTER 1: TRANS-TRANSLATION ISAN APPEALING TARGET FOR THE DEVELOPMENT OF NEW ANTIMICROBIAL COMPOUNDS.

In this section we present a review article about *tran*s-translation as a potent target for new antimicrobial compounds. We approach the *trans*-translation system in more depth, talking about the mechanism at the molecular level, its particularities and the current research that is being done to use it as a target for antibiotic development. By the end we can see that despite the existence of a few molecules that were proposed as inhibitors, a lot of controversy surrounding their actual mechanism of action persists. One of the most promising candidates, the oxadiazole KKL-35, was questioned by us and other researchers and indeed, it was recently proved that oxadiazoles are multitarget antibiotics (NACLERIO *et al.*, 2022). In this review article we also discuss the possibility of using anti-*trans*-translation compounds not as proper antibiotics but also as adjuvants to commercial antibiotics with the intention of lowering their concentration and eventually even rescuing antibiotics that normally wouldn't work due to resistance.

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Review

Trans-Translation Is an Appealing Target for the Development of New Antimicrobial Compounds

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Abstract: Because of the ever-increasing multidrug resistance in microorganisms, it is crucial that we find and develop new antibiotics, especially molecules with different targets and mechanisms of action than those of the antibiotics in use today. Translation is a fundamental process that uses a large portion of the cell's energy, and the ribosome is already the target of more than half of the antibiotics in clinical use. However, this process is highly regulated, and its quality control machinery is actively studied as a possible target for new inhibitors. In bacteria, ribosomal stalling is a frequent event that jeopardizes bacterial wellness, and the most severe form occurs when ribosomes stall at the 3′-end of mRNA molecules devoid of a stop codon. *Trans*-translation is the principal and most sophisticated quality control mechanism for solving this problem, which would otherwise result in inefficient or even toxic protein synthesis. It is based on the complex made by tmRNA and SmpB, and because *trans*-translation is absent in eukaryotes, but necessary for bacterial fitness or survival, it is an exciting and realistic target for new antibiotics. Here, we describe the current and future prospects for developing what we hope will be a novel generation of *trans*-translation inhibitors.

Keywords: antibiotics; ribosome; SmpB; tmRNA; *trans*-translation

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1. Introduction

Protein synthesis, or translation, is a fundamental biological process that occurs on ribonucleoprotein nanomachines named ribosomes. The bacterial ribosome is, therefore, a major antibiotic target, and many types of inhibitors can stop bacterial growth by binding its functional centers and interfering with the ribosome's ability to synthesize proteins [1]. However, bacteria have evolved a wide set of mechanisms to resist the inhibitory effect of antibiotics, including those that target the ribosome. Indeed, resistance mechanisms have been identified for nearly every antibiotic currently in clinical use. Combined with the fact that pharmaceutical companies have not developed more than a few antibiotics recently, infections that are treatable now will probably, once again, become life threatening [2].

It is generally accepted that among the most important bacteria to target, those in the ESKAPE pathogens group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) are of enormous interest when it comes to drug discovery [3,4]. They are the leading cause of nosocomial infections throughout the world, and most are multidrug-resistant isolates [5]. The World Health Organization (WHO) regularly issues global reports on antimicrobial resistance (AMR) surveillance [6], and the topic has ranked in the top 10 global health issues over the past few years [4,7].

To combat this crisis, we need new antibiotics, and, most importantly, we need new classes of antibiotics with novel mechanisms of action [8]. To do this, we must first identify

new molecular processes that can be targeted. Ideally, these should be conserved among pathogenic bacteria; indispensable to the survival, or at least to the fitness, of the bacteria; sufficiently specific, so that they can distinguish between bacterial species and minimize microbiotal damage; not targeted by current antibiotics; absent in eukaryotes to limit toxicity. In fact, *trans*-translation, the primary quality control mechanism for rescuing stalled ribosomes in bacteria, appears to be a perfect candidate, allowing us to target this key cellular process in a totally new way. Here, we discuss the potential of targeting this pathway with novel antimicrobial compounds.

2. Ribosomal Stalling: From No-Go to Non-Stop

Several phenomena can cause the production of aberrant mRNA molecules that lead to the accumulation of stalled ribosomes in bacteria. The most frequently observed are spontaneous mutations in their corresponding genes, as well as transcription defects after the RNA polymerase prematurely terminates transcription, or does not correctly transcribe the stop codon [9]. Other phenomena include mRNA degradation, caused by either endo- or 3′–5′ exo-ribonucleases, or by environmental stresses that result in chemical and physical damage [10]. "Non-stop" situations (readthrough) can also occur when a canonical stop codon is translated in the presence of non-sense suppressor tRNA [11,12], aberrant frameshifts [13], or translational error-inducing drugs [14]. In bacteria, translation initiation mainly relies on the binding of the ribosomal binding site, the Shine–Dalgarno (SD) sequence to the 3′-end of 16S ribosomal RNA. This, therefore, means that translation can start before transcription is actually complete, and that non-stop events, such as degradation, can occur both before translation starts or while the ribosome advances along the mRNA [15]. It must be noted that another type of defective translation event can also appear during certain stressful conditions (e.g., starvation), which, during translation, slow or stop ribosomes upstream from the stop codon. Due to the presence of a stop codon, this situation is called "no-go" instead of "non-stop." Even though this process could eventually be reversed, it is problematic if it occurs for too long, as endonucleases, such as RelE (the toxin component of the type II RelE–RelB toxin–antitoxin system), will cut the mRNA within the ribosomal A site to facilitate tmRNA-mediated rescue, and conserve the energy and nutrients being used to combat stress [16]. The "no-go" then becomes "non-stop," and triggers the same quality control mechanisms for ribosomal release. In all of these cases, the rescue of non-stop ribosomes is essential in most or all bacteria [17], suggesting that interference with non-stop quality control mechanisms is surely a promising antibiotic development path.

3. *Trans*-Translation Components Are Major Targets for Interference

Despite the recent discovery of several back-up systems (see [18], for a complete review), *trans*-translation is the principal and most sophisticated quality control mechanism for avoiding inefficient protein synthesis on stalled non-stop bacterial ribosomes. It mainly relies on the complex between tmRNA and SmpB, the two main actors in the process.

3.1. Transfer-Messenger RNA (tmRNA)

Having both transfer and messenger RNA functions, tmRNAs are chimeric RNA molecules that are typically 260 to 420 nucleotides in length (363 nts in *Escherichia coli*). The *ssrA* gene, which encodes tmRNA, has been found in nearly all bacterial genomes [19]. tmRNA is always first transcribed as a precursor, and it is subsequently processed at its CCA 5′- and 3′-ends [20–23]. The number of tmRNA molecules per cell has been estimated to be 500–700, roughly 5% of the total number of ribosomes, as estimated from the ratio of tmRNA-to-5S ribosomal RNA [24,25]. As with classical tRNA, the T-loop undergoes some base modifications, with the TrmA enzyme catalyzing 5-methyluridine and TruB enabling pseudouridine production [26,27]. The classic mature tmRNA is composed of a tRNA-like domain (TLD), a messenger-like domain (MLD), and a large, halo-shaped pseudoknot (PK) ring (Figure 1).

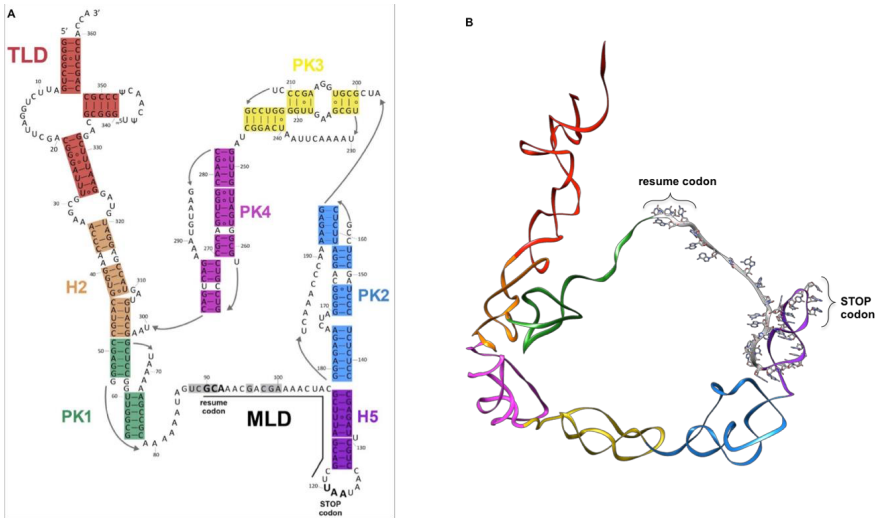

Figure 1. tmRNA. (**A**) Organization of the secondary structure of *Escherichia coli* tmRNA. The internal open reading frame is underlined. (**B**) 3D structure of the *E. coli* tmRNA molecule. In both panels, the tRNA-like domain (TLD, red) is followed by helix H2 (orange). The pseudoknot ring is composed of PK1 (dark green), PK2 (steel blue), PK3 (yellow), PK4 (magenta), helix H5 (purple), and the mRNA-like domain (MLD, grey). The resume and stop codons are indicated.

The TLD portion plays the same role as in classical tRNA; the acceptor stem is always recognized and aminoacylated by alanine tRNA synthetase (AlaRS) after recognition of a G3:U base pair, a motif also present in canonical $tRNA^{Ala}$ [28–30]. The domain displays a classical T-loop, and a small D-loop without a stem. It is also devoid of an anticodon loop, since no codon will need to be recognized within the vacant decoding site of a stalled ribosome [20,26,31]. In fact, this ostensible problem is overcome by SmpB; when interacting with the TLD region, it mimics codon–anticodon recognition and allows tmRNA to accommodate into the ribosomal A site (see below). The MLD is the RNA portion that contains the internal open reading frame (ORF) of tmRNA, which encodes the aminoacidic sequence A*ANDENYALAA in *E. coli*, with the first A* being carried by the TLD. This sequence is added to the stalled protein during *trans*-translation. The tag sequence displays strong phylogenetic conservation, with the consensus sequence A*AN----ALAA. The final three alanines (AxAA) are crucial, allowing for specific recognition of the tagged protein by proteases. The nature of the RNA sequence upstream from the resume codon allows for the correct placement of the codon into the decoding center. Accordingly, mutations in this region can lead to reading frameshifts or a loss of tmRNA function [32–34]. In fact, the structural elements that precede the resume codon, rather than the sequence itself, are important for the reinitiation of translation [35–39]. Once the ORF is completely translated, the tagged peptide is specifically degraded by several proteases. In addition to this classical single-chain conformation, tmRNA also exists (in alpha-proteobacteria, cyanobacteria, and some beta-proteobacteria lineages) as a two-piece molecule, a formation caused by a circular gene permutation that splits it into two molecules [40,41]. In this case, the TLD, MLD, and PK1 are similar to those of "one-piece" tmRNA, but the loop containing the tag reading frame is broken, and there are fewer pseudoknots [42].

3.2. SmpB

SmpB is a small, basic protein of ~160 amino acids, encoded by the *smpB* gene. In the *E. coli* genome, it is located just upstream from the *ssrA* gene that codes for tmRNA [43]. SmpB binds to tmRNA with high affinity, and is its most important partner during ribosome rescue. In fact, in its absence, tmRNA can no longer accommodate its TLD portion into the vacant A site [44–47]. A comparison between the SmpB proteins in the various ESKAPE bacteria reveals that the six proteins conserve the same fold, but the sequence and length of the C-terminal tail differs, especially in *S. aureus*, *A. baumannii*, and *E. cloacae* (Figure 2).

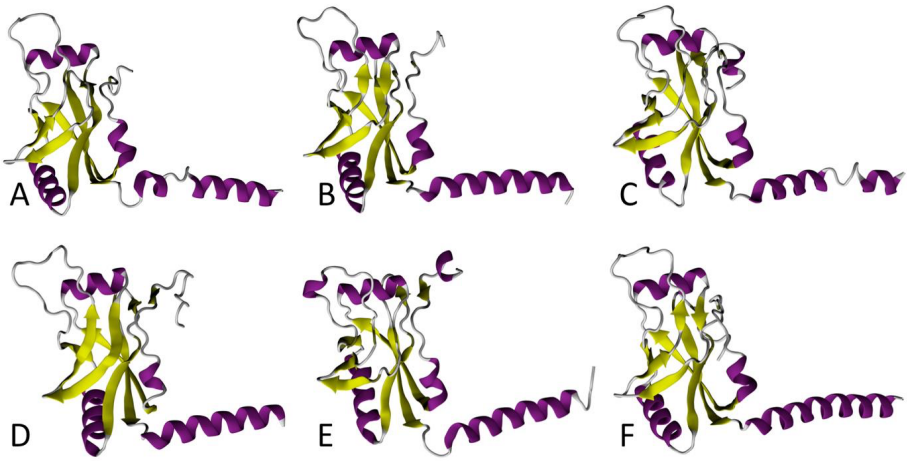

Figure 2. Comparison between 3D models of SmpB in ESKAPE bacteria. In each conformation, the tmRNA TLD contact region is on the left side, and the helix-shaped C-terminal end is shown to the right. The α-helices and β-strands are purple and yellow, respectively. These models of *Enterococcus faecium* (**A**), *Staphylococcus aureus* (**B**), *Klebsiella pneumoniae* (**C**), *Acinetobacter baumannii* (**D**), *Pseudomonas aeruginosa* (**E**), and *Enterobacter cloacae* (**F**) were all computed with the I-TASSER program using *E. coli* SmpB as the structural template (PDB 7ACJ).

The SmpB body is arranged in an oligonucleotide/oligosaccharide binding (OB) domain that folds in a classical fashion into a β-barrel made up of six antiparallel β-strands, which is also typical of other RNA-binding proteins, such as IF1 or bS1 [48–50]. By interacting with the tmRNA TLD, SmpB mimics the missing D-loop and anticodon stem–loop present in classical tRNA [51,52]. The interesting shape assumed by the tmRNA–SmpB complex is important for its entry into the ribosome, as it simulates the codon–anticodon pairing, which then promotes the reactivity of a cognate tRNA. Of the ~160 amino acids, the last 30 C-terminal residues form a tail, which is unstructured in solution, but folds into an α-helix during *trans*-translation. This C-terminal tail is rich in positively charged side-chain residues, essential for contacts with the tmRNA helix H5, as well as for interactions with the negatively charged nucleotides within the decoding site of the 30S ribosomal subunit [53–57]. Indeed, by inserting into the mRNA entry channel, the C-terminal tail is instrumental in selecting the stalled ribosomes with empty mRNA entry channels. The recent cryo-electron microscopy (cryo-EM) structures of *E. coli* tmRNA–SmpB bound to a stalled ribosome [56,57], and the previous crystallographic study of *trans*-translation in *Thermus thermophilus* [58], both show that, just as in canonical translation, the presence of the protein in the decoding center induces reorientation of nucleotides A1492 and A1493 in helix 44. Besides its main RNA-binding site on the TLD, SmpB also has a secondary RNA-binding site, which later binds the MLD to ensure that the resume codon is correctly

positioned in the ribosomal A site [56]. These results confirm the long-predicted importance of SmpB in the *trans*-translation partnership [53,55,59].

4. The Molecular Process of *Trans*-Translation

During *trans*-translation (Figure 3), the tmRNA–SmpB complex is first brought to the ribosome with EF-Tu•GTP. Stalled ribosomes are selected by SmpB, whose C-terminal tail probes the mRNA entrance channel [58]. In this pre-accommodation state, GTP hydrolysis in EF-Tu is favored, as are conformational changes in the ribosomal subunits, and this induces the accommodation of tmRNA–SmpB into the vacant ribosomal A site. After transpeptidation occurs between the stalled incomplete peptide and the tmRNA alanine, a swap between the tmRNA MLD and the non-stop mRNA allows translation to resume. The C-terminal tail of SmpB, which was involved in ribosomal vacant A site recognition, then rotates by 60° to allow the MLD to move into the mRNA channel, as well as to allow ejection of the problematic mRNA. The ribosome translates the MLD until it reaches the stop codon, after which the stalled ribosomes are recycled (see [60], for the structural details of the process). The incomplete peptides are tagged with a signal sequence that results in quick proteolysis. Two AAA+ proteolytic enzymes (ATPases associated with various cellular activities), ClpXP and ClpAP, are able to degrade the tagged proteins by converting ATP hydrolysis energy into mechanical work [61]. FtsH, a hexameric protease anchored to the internal side of the cytoplasmic membrane, is also involved in degrading a small subset of tagged proteins present in the inner membrane [43]. On the other hand, the energy-independent protease Tsp takes over the tmRNA-tagged substrates in the periplasm [62]. The problematic mRNAs are degraded by RNase R [63]. This enzyme, of 92 kDa, belongs to the RNase II superfamily, a group of exoribonucleases able to degrade the RNA molecules in the 3′ → 5′ direction [64], as well as digest various RNA substrates [65,66]. However, the details of how the ribonuclease works with the complex to promptly recognize and handle problematic mRNA is still unclear.

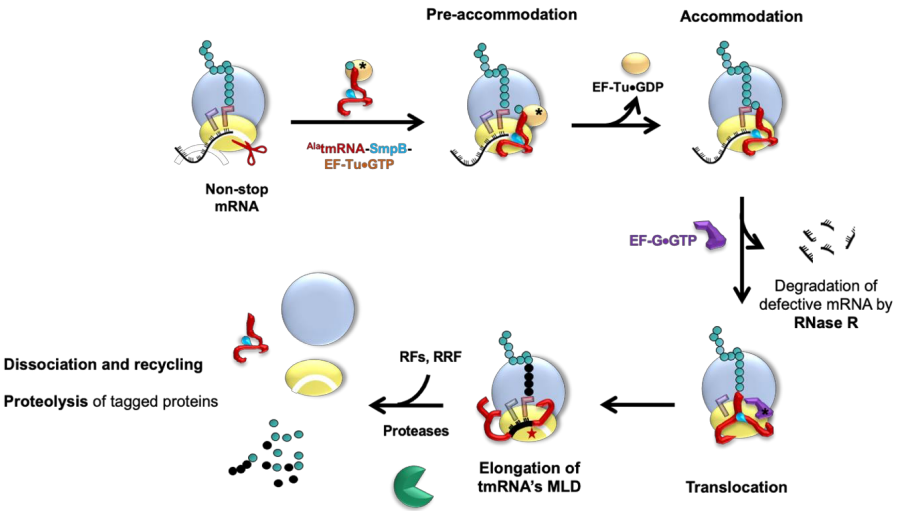

Figure 3. The complete *trans*-translation quality control cycle. **Pre-accommodation state:** tmRNA associates with its partner SmpB to form a complex. Elongation factor EF-Tu•GTP binds to ^{Ala}tmRNA–SmpB, thereby forming the quaternary complex needed to rescue the ribosome stalled on a non-stop mRNA. To recognize these ribosomes, this quaternary complex enters the vacant ribosomal A site. There, SmpB mimics a codon–anticodon pairing while its C-terminal tail inserts into the mRNA channel.

The EF-Tu•GDP is then released after GTP hydrolysis. **Accommodation**: The ^{Ala}tmRNA–SmpB complex is accommodated into the A site, triggering the peptidyl transfer reaction. **Translocation:** Thanks to GTP hydrolysis, EF-G•GTP helps shift the tmRNA–SmpB into the P site. EF-G•GDP is released, and the non-stop mRNA is ejected then degraded by RNase R. **Elongation**: The tmRNA open reading frame is placed into the A site, and new tRNAs arrive at the ribosome to resume translation. **Termination**: The tmRNA–SmpB complex moves towards the E site, and the TLD and SmpB are promptly ejected. Translation of the MLD continues until translation of the tmRNA-encoded tag is terminated at the stop codon with the help of the release factors (RFs). The ribosomal subunits are then dissociated by the ribosome recycling factor (RRF), and the nascent peptide is degraded by ClpXP/ClpAP/FtsH/Tsp proteases. All of the components are now recycled and ready for a new round.

5. *Trans*-Translation as a Target for New Antimicrobial Compounds

Considering that *trans*-translation is absent in eukaryotes, tmRNA–SmpB is an especially promising target for novel antibiotics. Obviously, when it is essential to the survival of pathogenic bacteria, the *trans*-translation machinery is an excellent specific target for use in developing molecules to kill bacteria directly [35,67,68]. When non-lethal, because alternative rescue factors can take over the rescue process, deletion of tmRNA and/or SmpB induces various phenotypes, including loss of virulence or loss of antibiotic tolerance [69–72]. These hypersensitive mutants are not viable in the presence of low doses of some protein synthesis inhibitors (chloramphenicol, lincomycin, spiramycin, tylosin, erythromycin, and spectinomycin) that do not otherwise significantly affect the growth of wild-type cells [69,70,73]. Strikingly, mutants deleted for tmRNA are also more sensitive to antibiotics that do not target translation than wild-type cells, such as inhibitors of cell wall synthesis. This is probably because these drugs stress the bacteria, and this is handled more efficiently when *trans*-translation is active [74]. In all of these cases, it is possible that *trans*-translation inhibitors could be used in combination with already commercialized antibiotics, in order to diminish their minimal inhibitory concentration (MIC) in pathogens, or even to reenable the use of antibiotics no longer used because of resistance. Finally, *trans*-translation is also important for persister survival, as well as tolerance to a variety of antibiotics and stresses [75]. Despite the enormous potential and extensive research into how it works and how this pathway can be targeted for treatments against bacterial infection, there are currently no drugs on the market that use this mechanism. Since this review focuses on *trans*-translation, we will only discuss the possible strategies for specifically impairing that process via targeting tmRNA, SmpB, and/or the ribosome itself. However, we must mention that a global strategy should not overlook the possibility of altering the activity of supporting actors, such as the highly conserved aminoacyl-tRNA synthetase (AlaRS) enzyme, serine protease ClpP, or ribonuclease RNase R.

6. Antibiotics Targeting *Trans*-Translation: Are We There Yet?

6.1. Oxadiazole Compounds

In 2013, based on a luciferase assay, Keiler's group performed a high-throughput screening assay on a library of 663,000 candidate compounds. This led to the identification of 1,3,4-oxadiazole and tetrazol-based compounds as broad-spectrum antibiotics that specifically inhibited the pathway [67]. The most promising compound was the oxadiazole KKL-35 (Figure 4), which displays an antibiotic effect against very distantly related bacteria, suggesting that it may have antibiotic activity against a broad spectrum of species, thus paving the way for the development of the first class of small molecules inhibiting *trans*-translation. How KKL-35 targets *trans*-translation could not be easily identified. KKL-35 binds poorly to tmRNA and SmpB, suggesting that the compound probably affects a later step in the quality control process. Indeed, later biochemical experiments, using *Mycobacterium smegmatis* and *Staphylococcus aureus* cells, highlighted KKL-2098, an analog of KKL-35 that incorporates a photoreactive azide group and a terminal alkyne moiety. KKL-2098 targets helix 89 of 23S rRNA, but in a region not targeted by conventional antibiotics. It

binds to a pocket adjacent to the peptidyl transfer center (PTC), without inhibiting canonical translation [68,76]. More recently, this result was confirmed by cryo-EM (EMDB with the accession code EMD-20121). Despite a rather low occupancy, KKL-2098, cross-linked to a non-stop ribosome, binds near the PTC and significantly alters the conformation of the ribosomal protein bL27. This suggests that 1,3,4-oxadiazoles may, at least in part, inhibit *trans*-translation by preventing tmRNA–SmpB binding at the A site, or by interfering with the translocation of the complex from the A to the P site [77]. In another oxadiazole example, a *Bacillus subtilis* proteomic response library was used to show that KKL-35 and other oxadiazole derivatives induce responses that are similar to those of ionophores, which disturb metal homeostasis, and to other agents, causing oxidative stress responses. This activity could be linked to the importance of *trans*-translation in cells undergoing oxidative stress [78].

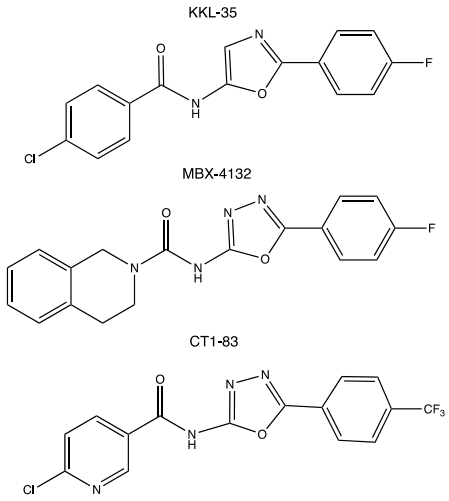

Figure 4. Chemical structures of the experimental oxadiazole compounds KKL-35, MBX-4132, and CT1-83.

In 2017, our group developed a new double-fluorescence reporter system for the simultaneous and specific quantification of bacterial *trans*-translation, as well as proteolysis, in *E. coli* [79]. However, when we tested KKL-35, we did not observe any significant changes in fluorescence levels, despite its strong antibiotic activity, suggesting that *trans*-translation is not its only target, or that the molecule is rapidly metabolized (certainly due to amide bond fragility, see below) and the resulting products of degradation act on another target in *E. coli*. These data were supported by the fact that the inhibitory activity of KKL-35 is similar in both a Δ*arfA* strain (in which *trans*-translation is essential) and in a Δ*ssrA* strain deprived of *trans*-translation. Furthermore, in the human pathogen *Legionella pneumophila* (which causes Legionnaires' disease), the antibiotic activity of KKL-35 is not related to the specific inhibition of *trans*-translation, as it remained active against *L. pneumophila* mutants expressing an alternate ribosome-rescue system and lacking tmRNA [80].

Because the characterization of a new antibiotic target in living cells can be slow, difficult, and treacherous (as shown with KKL-35), we recently constructed a system to detect *trans*-translation in vitro [81]. It is based on an engineered tmRNA variant that reassembles green fluorescent protein (GFP) when *trans*-translation is active. This system is, thus, adapted for the high-throughput screening of chemical compounds by fluorescence, and the limited number of reaction components allows for the direct detection of the relevant targets of *trans*-translation, which are as follows: tmRNA, SmpB, and the ribosome itself.

Based on this simple system, we demonstrated that several 1,3,4-oxadiazole compounds do, indeed, inhibit *trans*-translation in vitro, though only moderately [81,82]. In KKL-35, replacing the benzene of the chloro-aryl moiety with a pyridine group (compound CT1-83, see Figure 4) results in much stronger inhibition of *trans*-translation.

However, because of the rapid hydrolysis of the amide bond of KKL-35 in liver microsomes, it cannot be used in animals. A recent structure–activity relationship (SAR) program thus led to the development of a new uriedo-oxadiazole derivate, MBX-4132 (Figure 4). This compound is much more stable and not significantly less potent, able to inhibit *trans*-translation both in vitro and in vivo, and clears multidrug-resistant *Neisseria gonorrhoeae* in infected mice [77]. While the oxadiazole strategy has been deeply studied, its cellular targets and mode of action remain uncertain, which justifies further investigation, as well as the continued search for other molecules.

6.2. Pyrazinamide

In 2011, it was proposed that pyrazinamide (PZA), a mainstay of anti-tuberculosis combination therapy [83], inhibits *trans*-translation [84]. Using proteomic studies, pyrazinoic acid (POA), the hydrolyzed and active form of PZA, was shown to bind to the ribosomal protein S1, encoded by the *rpsA* gene [84]. Interestingly, POA only inhibits *trans*-translation and not canonical translation, and this inhibition depends strictly on wild-type *M. tuberculosis* S1. Crystal structures of the S1–POA complex revealed that the residues Lys303, Phe307, Phe310, and Arg357 in the S1 domain directly interact with POA, and that mutations on these locations blocked the interaction with the drug, and diminished the binding between S1 and tmRNA [85]. However, the action of PZA on S1 and *trans*-translation in *M. tuberculosis* was called into question, and experiments suggest that this drug directly targets a critical player in the metabolism of coenzyme A instead [86]. A recent study seems to confirm this hypothesis, since no measurable binding between POA and S1 could be recovered, despite the use of a wide panel of biophysical methods, including nuclear magnetic resonance (NMR) spectroscopy, isothermal titration calorimetry (ITC), microscale thermophoresis (MST), and electrophoretic mobility shift assays (EMSA) [87].

6.3. Peptides and Oligonucleotides

Peptide aptamers (PA) are combinatorial proteins that consist of a stable scaffold protein and random amino acids designed to bind to specific targets, in order to disrupt their activity [88]. In a recent study into the ways to vaccinate and protect zebrafish against infection, PAs were developed to target SmpB in *Aeromonas veronii* [89]. These opportunistic bacteria depend on *trans*-translation for virulence, and they are commonly found in aquaculture, where they cause wound infection, diarrhea, and septicemia [90]. The aptamers directed against SmpB were selected from a PA library, and the leading aptamer PA-1 (sequence: GGVTFLVNTYPNGVQSRAGG) was shown to specifically target SmpB, and to knockdown its functioning. When PA-1 was introduced into *A. veronii*, the engineered strain was much less virulent and could be used as a potential attenuated live vaccine, thereby providing a novel strategy for preventing *A. veronii* infection [89]. A second aptamer PA-2 (sequence: IGQEWGLGVRGPLSAK) was demonstrated to interact not only with SmpB, but also with the alternative rescue factor ArfA, resulting in the dysfunction of both rescue factors [91]. Considering the expected conservation of the fold in SmpB (see Figure 2), PA-1 and PA-2 could theoretically target a wide range of different bacteria.

Another peptide strategy involves using a peptide that mimics the SmpB C-terminal tail to compete with endogenous SmpB for binding in the vacant ribosomal A site, thus preventing tmRNA recruitment and, in turn, inhibiting *trans*-translation. We showed that the peptide that corresponds to the C-terminal extremity of *E. coli* SmpB (sequence: GKKQHDKRSDIKEREWQVDKARIMKNAHR) acts as a potent *trans*-translation inhibitor in vivo [79].

Finally, the most obvious strategy is to use antisense oligonucleotides directed towards the genes encoding SmpB or tmRNA (*ssrA* gene), or towards the mature tmRNA itself.

This approach is already in use in vitro by various laboratories, often as an internal control, with an antisense oligonucleotide targeting the tmRNA MLD and, thereby, very efficiently inhibiting *trans*-translation (for an example, see [92]).

7. Conclusions

Although *trans*-translation was discovered more than 25 years ago, and has been studied carefully ever since, with several attempts made to develop molecules to target it, the only chemical family that has displayed potential activity derives from 1,3,4-oxadiazole compounds. The recent development of sensitive and selective high-throughput screening assays that target ESKAPE pathogenic bacteria will undoubtedly help us to find new scaffolds that specifically target ribosome rescue [92]. Current studies work from scratch, by screening pharmacologically active small molecules from large chemical or natural product libraries [93], or are based on rational drug design, attempting to target the interactions between tmRNA, SmpB, and the ribosome (Figure 5), as recently described in cryo-EM structural studies [56,57]. Among the interactions discussed, the most promising targets may be the TLD–SmpB interface (in order to inhibit the tmRNA–SmpB interaction before the complex enters the ribosome); the mechanism of stalled ribosome recognition (to block or compete with SmpB C-terminal tail insertion into the empty mRNA channel); the SmpB–MLD binding site, which allows resume codon registration during translocation (to impede protein tagging). The possible specific integration of such molecules within pathogenic bacteria would be an extraordinary tool in the fight against multiresistance. There is no doubt that the groundwork already laid will soon respond to this increasingly urgent antibiotic resistance emergency.

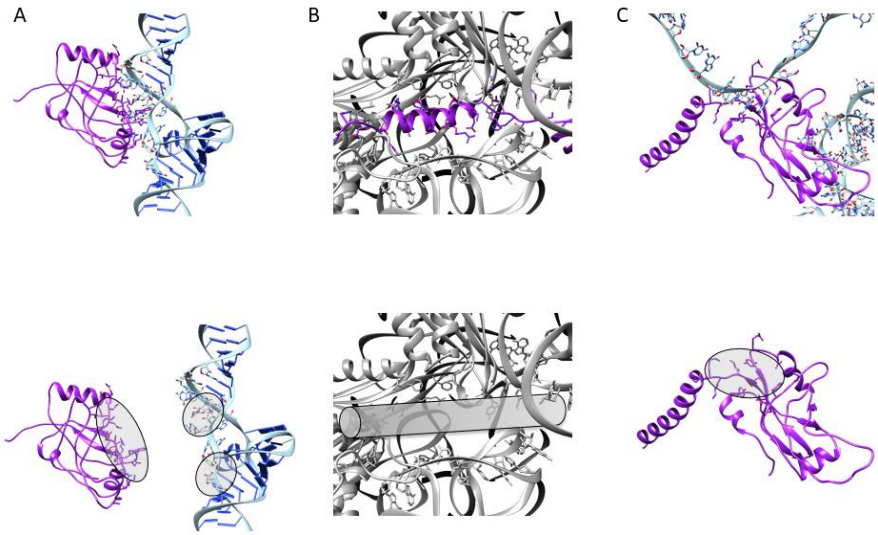

Figure 5. Potential anti-*trans*-translation targets. To interfere with *trans*-translation, one can (**A**) inhibit the tmRNA–SmpB interaction by targeting the binding sites of either partner; (**B**) compete with the SmpB C-terminal tail for stalled ribosome recognition; (**C**) alter the tagging process by targeting the binding site between SmpB and the MLD.

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CHAPTER 2: SAFE AND EASY IN VITRO EVALUATION OF TMRNA-SMPB-MEDIATED *TRANS*-TRANSLATION FROM ESKAPE PATHOGENIC BACTERIA

The methods that we used to screen for anti-*trans*-translation compounds involved two already established, previously published and patented assays developed by Reynald Gillet's team. One has been developed *in vitro*, the other one *in vivo* (*i.e.* in *E. coli*). Since the reagents needed for the *in vitro* method are expensive, our first goal has been the miniaturization of the reaction. This way, we could sharply diminish the costs of large screening assays. At the same time, as we were miniaturizing our method, we also adapted it to the ESKAPE *trans*-translation machineries so we could safely test our candidates against it. This generated an article that was published in ***RNA***

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METHOD

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*-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

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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

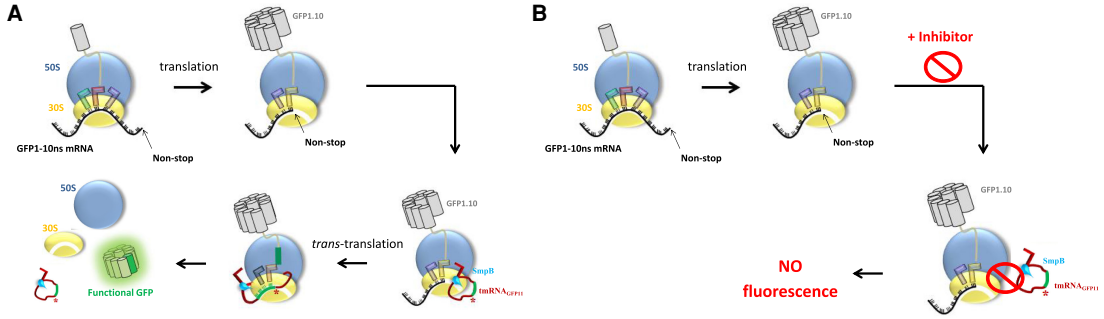

FIGURE 1. (*A*) *Trans*-translation of sfGFP1-10 mRNA lacking a stop codon. The tmRNA$_{GFP11}$-SmpB complex binds to the stalled ribosome, and translation resumes thanks to the tmRNAGFP11 mRNA-like domain (MLD). The MLD encodes the missing eleventh beta-strand of the sfGFP, and the complete sfGFP is 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).

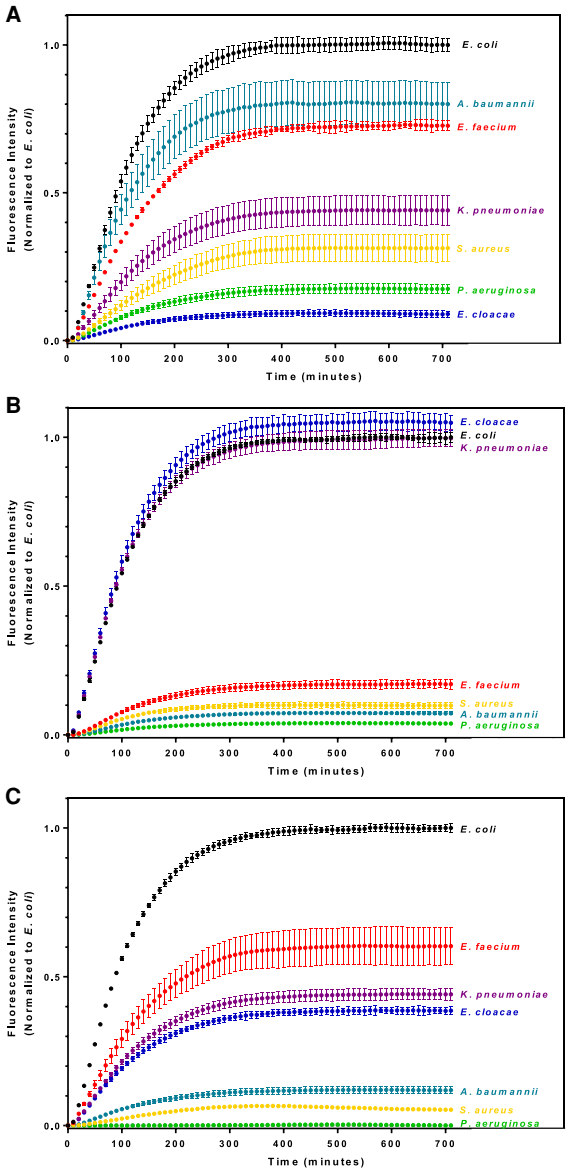

FIGURE 2. *Trans*-translation kinetics over time using *Escherichia coli* ribosomes. Fluorescence increases are directly linked to *trans*-translation activity. (*A*) *Trans*-translation assays were done on *E. coli* $tmRNA_{GFP11}$ using the SmpBs from each ESKAPE pathogen, with the *E. coli* SmpB as 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,

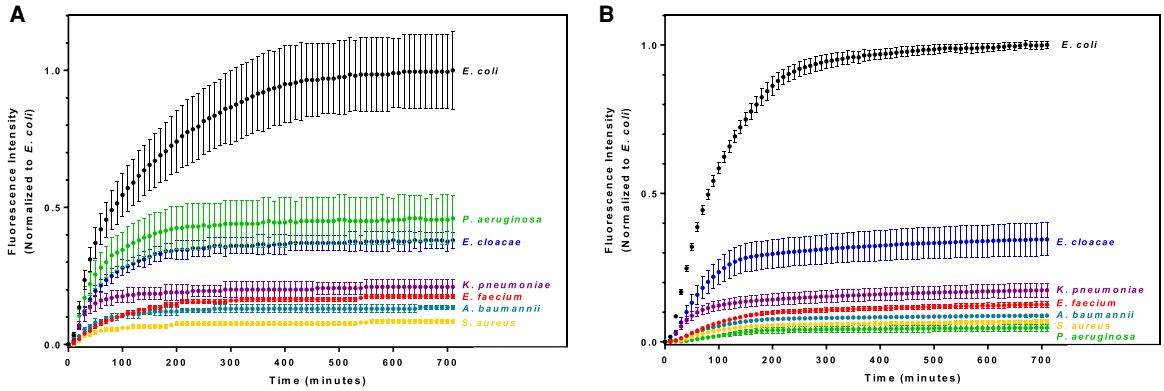

FIGURE 3. Translation and *trans*-translation kinetics over time. (*A*) Translation kinetics over time: the increase in fluorescence of full-length GFP (i.e., encompassing the 12 beta-strands) is directly linked to translation. (*B*) *Trans*-translation kinetics over time using ESKAPE ribosomes. All 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

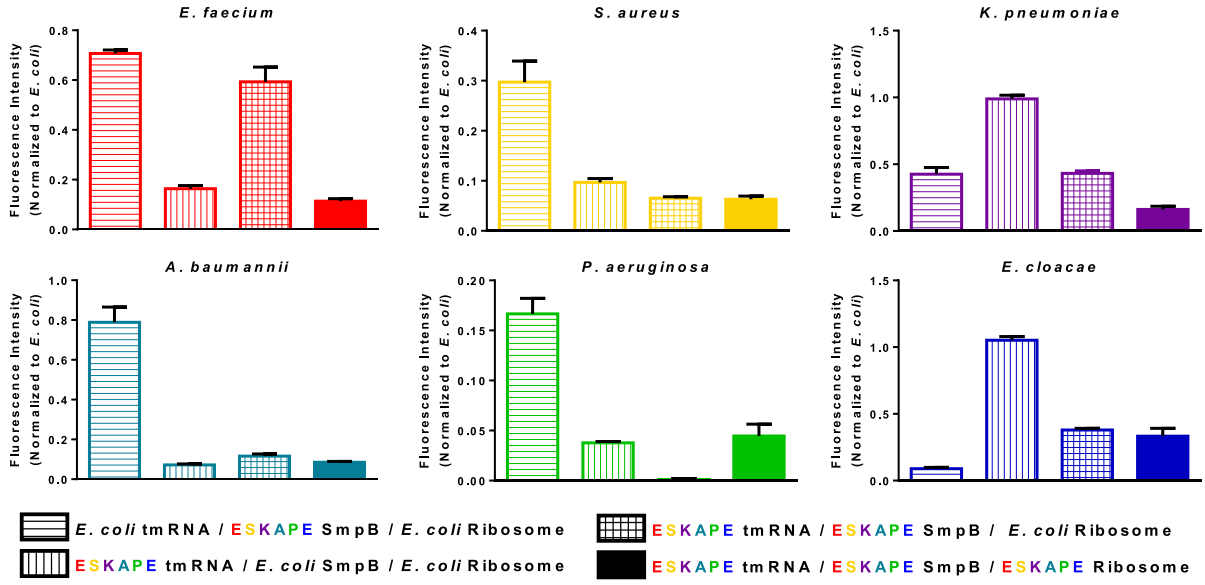

FIGURE 4. Quantification of in vitro *trans*-translation. Normalized fluorescence obtained in heterologous and homologous systems are shown at 310 min of incubation and reassembled by species. The results were normalized to the *E. coli* conditions and are shown as means ± standard deviations.

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.

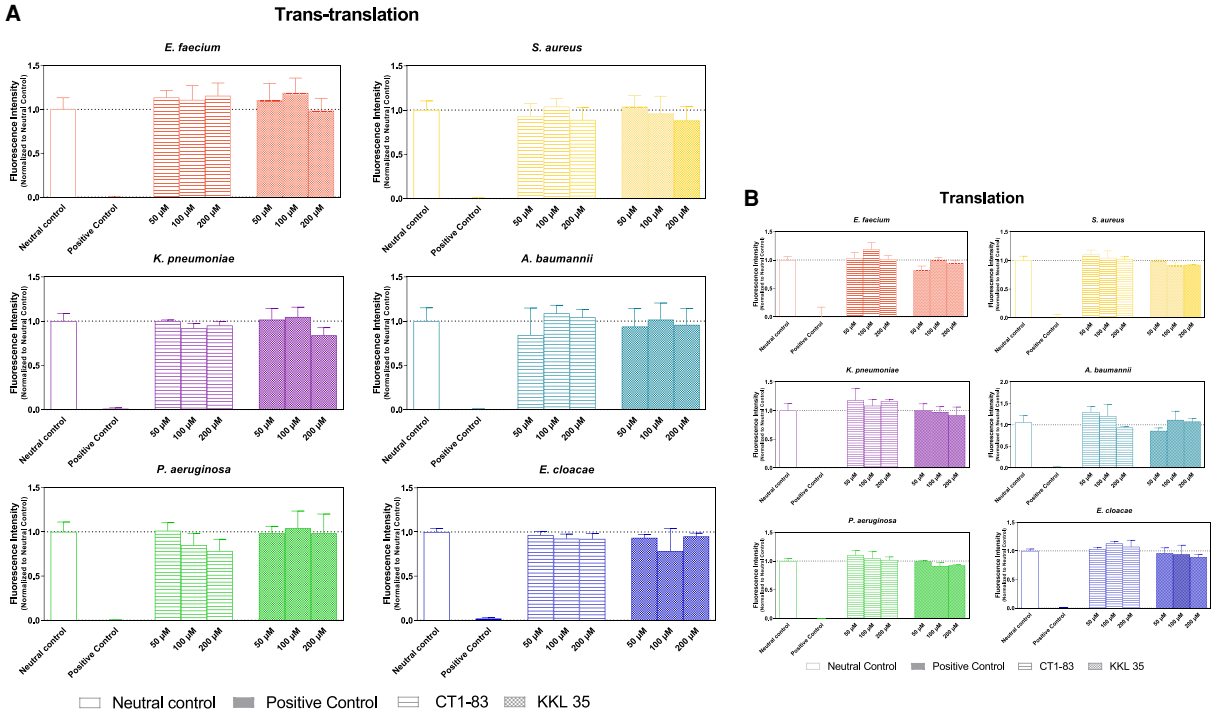

FIGURE 5. Quantification of in vitro ESKAPE *trans*-translation and translation assays after CT1-83 and KKL-35 treatment. Experiments were conducted in microplates, using ESKAPE homologous systems and increasing concentrations (50, 100, and 200 µM) of CT1-83 and KKL-35 oxadiazole compounds. Normalized 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).

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

Marion Thépaut, Rodrigo Campos-Silva, Eva Renard, et al.

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1.1. Supplementary data

Supporting Information

A – Phylogenetic comparison of tmRNA sequences

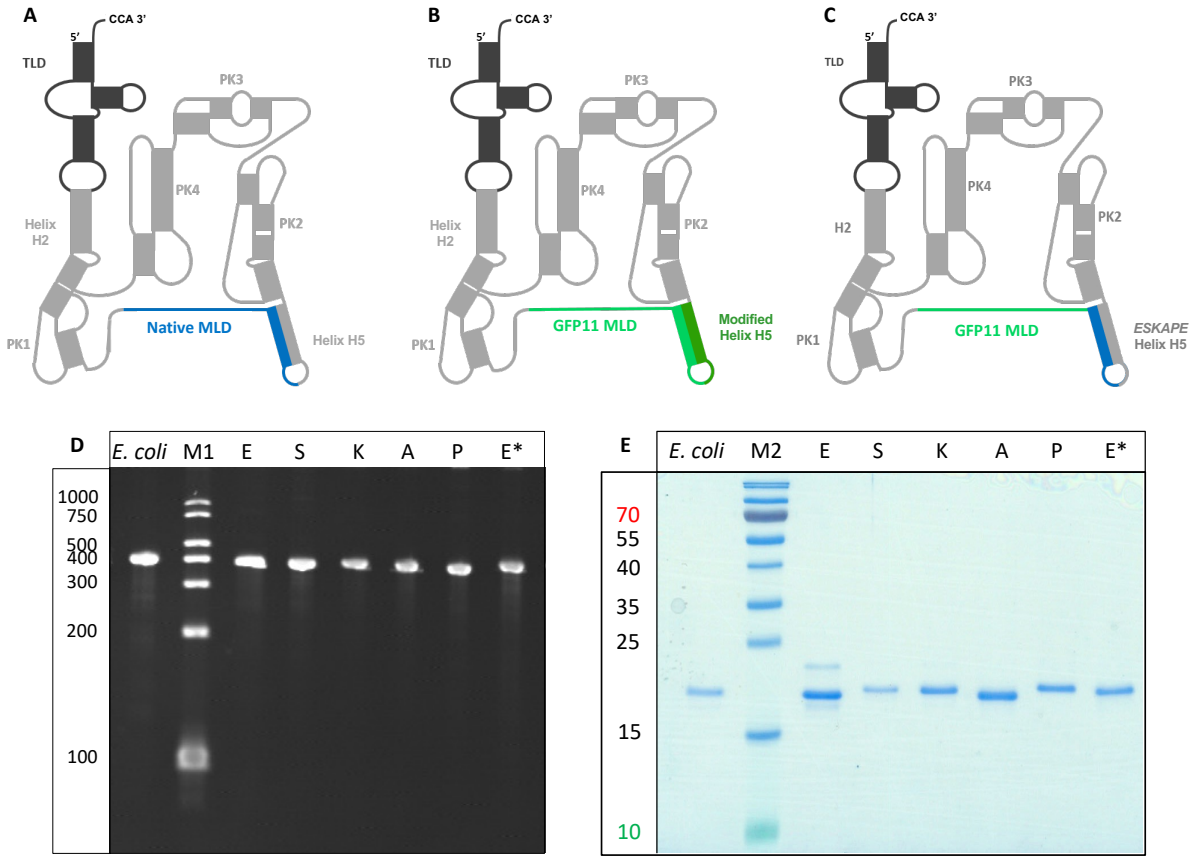
Adapted from the tmRNA Website by Kelly Williams and Corey Hudson (All tmRNA Alignment 08-JUL-2014), with base-pairing color-coded (P1, P2, P3, P4, MLD, P5, P6, P7, P8, P9, P10, P11, and P12). The predicted reading frame for the proteolysis-inducing peptide tag is underlined, and the portions of the expected 3′ CCA tail that are not encoded in the genome are lowercase.

B – Phylogenetic comparison of SmpB amino-acid sequences

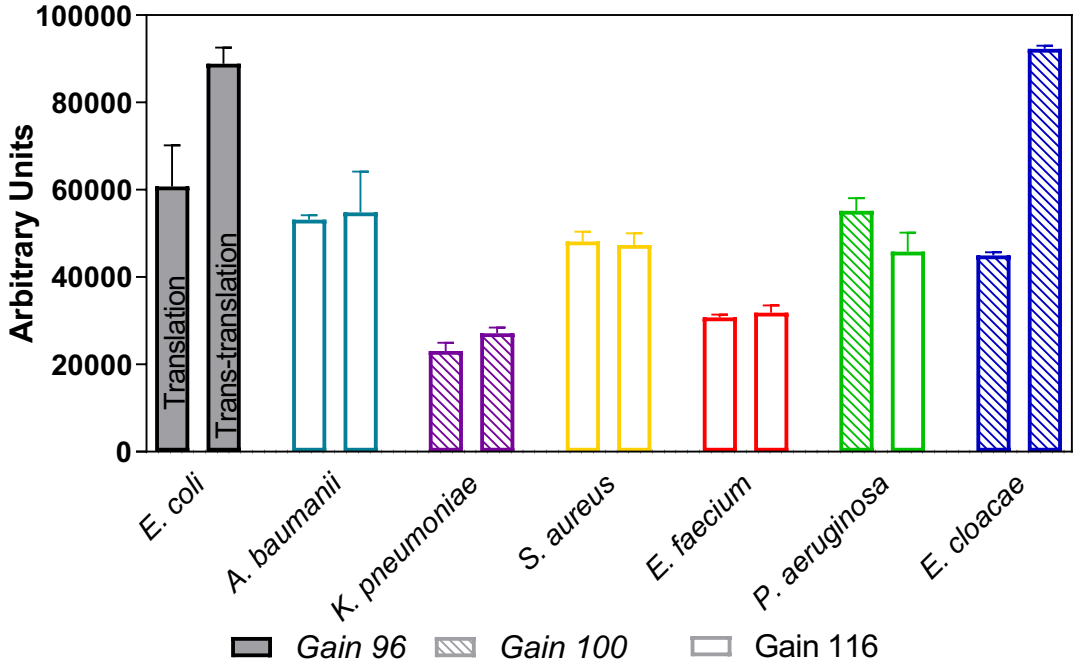
```
E. coli             -MTKKKAHKPGSATIALNKRARHEYFIEEEFEAGLALQGWEVKSLRAGKANISDSYVLLR     59
K. pneumoniae       -MTKKKAHKPGSATIALNKRARHEYFIEDEYEAGLALQGWEVKSLRAGKANIGDSYVILK     59
E. cloacae          -MTKKKAHKPGSATIALNKRARHEYFIEEEFEAGLALQGWEVKSLRAGKANIGDSYVILK     59
A. baumannii        MAKATVVKKHNGGTIAQNKRARHDYFIEEKFEAGMSLLGWEVKSLRAGRMSLTESYVIFK     60
P. aeruginosa       ---MAKQKKHPSGTIAQNKKALHDYFIEQRFEAGVALAGWEVKSLRAGKAQLVDSYVLLK     57
E. faecium          ------MPKGEGKLIAQNKKARHDYSIIDTMEAGMVLQGTEIKSIRNSRINLKDGFIRVR     54
S. aureus           -----MAKKKSPGTLAENRKARHDYNIEDTIEAGIVLQGTEIKSIRRGSANLKDSYAQVK     55
                          *     :* *::* *:* * :  ***: * * *:**:* .  .: :.:  .:

E. coli             DGEAFLFGANITPMAVASTHVVCDPTRTRKLLLNQRELDSLYGRVNREGYTVVALSLYWK     119
K. pneumoniae       DGEAFLFGANFTPMAVASTHYVCDPTRTRKLLLNQRELDTLYGRINREGYTVVALSLYWK     119
E. cloacae          DGEAFLFGANFTPLTVASSHYVCDPTRTRKLLLNKRELESLYGRINREGFTVVALSLYWK     119
A. baumannii        NGEAFLFGAQIQPLLSASTHIVPEATRTRKLLLSRRELEKLMGAVNQKGYSCVPLACYWK     120
P. aeruginosa       DGEAWLLGSHITPLTTASTHVIADPVRTRKLLLHKRELGKLFGAVQQKGYACVALSMYWK     117
E. faecium          NGEAFLHNVHISPYEQG-NIFNHDPLRTRKLLLHKKQIIRLENELKNTGITVVPLKVYIR     113
S. aureus           NGEMYLNNMHIAPYEEG-NRFNHDPLRSRKLLLHKREIIKLGDQTREIGYSIVPLKLYLK     114
                    :** :* . :: *   . .    :  *:***** ::::  * .  .. * : * *  * :

E. coli             NAWCKVKIGVAKGKKQHDKRSDIKEREWQVDKARIMKNAHR-       160
K. pneumoniae       NAWCKVKIGVAKGKKQHDKRTDLKDREWALDKARIMKHAGR-       160
E. cloacae          NAWCKVKVGVAKGKKQHDKRTDLKEREWQLDKARIMKNAGR-       160
A. baumannii        GHLVKLEIALVKGKQLHDKRATEKERDWQRDKARIFHK----       158
P. aeruginosa       KHLVKCEIALAKGKKDFDKRHTEKERDSDREIQRAMRHGKDD       159
E. faecium          NGYAKVLIGLAKGKKSYDKREDLKRKDIDRQIDRTLKNFSR-       154
S. aureus           HGHCKVLLGVARGKKKYDKRQALKEKAVKRDVARDMKARY--       154
                        *  :.:.:**: .***   * :    :  * ::
```

Residue properties are color-coded: small [small+ hydrophobic (incl.aromatic -Y)]; acidic; basic – H; Hydroxyl + sulfhydryl + amine + G, unusual amino or imino acids, etc.
Key: *, positions having a single and fully conserved residue; . , weakly similar properties conserved between groups; :, strongly similar
properties conserved between groups.

Supplementary Figure 1. Phylogenetic comparison of SmpBs and tmRNAs from ESKAPE pathogens and *E. coli*. A) Shown here are the RNA sequences of tmRNAs and B) the SmpB amino acid sequences (Clustal Omega) from ESKAPE and *E. coli* bacteria.



Supplementary Figure 2. Schematic illustrations of tmRNA secondary structures as well as production patterns of tmRNA and SmpB variants. (A) Wild-type tmRNA, with a black transfer-like domain (TLD) and blue messenger-like domain (MLD). (B) Mutated tmRNA$_{GFP11}$ has an engineered MLD (light green) that encodes the eleventh GFP beta-strand. Compensatory mutations (dark green) maintain the base-pairing interactions of the H5 helix, and the 3′-ends for all species are CCA. (C) Version 2 mutated tmRNA$_{GFP11}$. An engineered MLD encoding the eleventh GFP beta-strand is green, the WT 3′-end MLD is blue, and the WT H5 helix is gray. (D) Visualization of ESKAPE tmRNA$_{GFP11}$ variants (5 pmol) on 8% urea-PAGE, with *E. coli* tmRNA$_{GFP11}$ used as a control. (E) As C, but showing 50 pmol *E. coli* and ESKAPE SmpB on 15% SDS-PAGE. Key: M1, RNA Century™-Plus Markers (ThermoFisher Scientific), M2, PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (ThermoFisher Scientific), E, *Enterococcus faecium*; S, *Staphylococcus aureus*; K, *Klebsiella pneumoniae*; A, *Acinetobacter baumannii*; P, *Pseudomonas aeruginosa*; E*, *Enterobacter cloacae*.



Supplementary Figure 3: Arbitrary units of fluorescence for the controls of the *E. coli* and ESKAPE homologous systems translation assays. The smaller the gain unit is, the higher is the fluorescence detected by the equipment.

Supplementary Table 1: *E. coli* and ESKAPE tmRNA$_{GFP11}$ sequences for *trans*-translation *in vitro*. The eleventh beta-strand is light green and the compensatory mutations forming the helix H5 are darker green.

Species and Accession	tmRNA$_{GFP11}$ sequence
E. faecium NC_017960.1/1726235-1726601	GGGGACGUUACGGAUUCGACAGGCACAGUCGAGCUUGAAUUGCGUUUCGUAGGUUACGUCUAC GUAAAAACGUUACAGUUAAAUAUAACU**GCACGUGACCACAUGGUCCUUCAUGAGUACGUAAAU GCUGCUGGGAUUACAUAA**UAACCUAAUCCUAGCGAUCCUCUCGGCAUCGCCCAUGUGCUCGAG UAAGGGUCCUAACUUUAGUGGGAUACGUUUCAACUUUCCGUCUGUAAGUUGAAAAAGAGAACA UCAGACUAGCGAUACAGAAUGCCUGUCACUCGGCAAGCUGUAAAGUGAAUCCUUAAAUGAGUU GACUAUGAACGUAGAUUUUUAAGUGGCGAUGUGUUUGGACGCGGGUUCGACUCCCGCCGUCUC CACCA
S. aureus NZ_GG774480.1/1107214-1106853	GGGGACGUUCAUGGAUUCGACAGGGGUCCCCCGAGCUCAUUAAGCGUGUCGGAGGGUUGUCUU CGUCAUCAACACACACAGUUUAUAAUAACU**GCACGUGACCACAUGGUCCUUCAUGAGUACGUA AAUGCUGCUGGGAUUACAUAA**UAACCUAAUCCUAGCAUCGCCUAACAGCAUUUCCUAUAUGCU GUUAACGCGAUUCAACCUUAAUAGGAUAUGCUAAACACUGCCGUUUGAAGUCUGUUUAGAAGA AACUUAAUCAAGCUAGCAUCAUGUUGGUUGUUUAUCACUUUUCAUGAUGCGAAACCUUUCGAU AAACUACACACGUAGAAAGAUGUGUAUCAGGACCUCUGGACGCGGGUUCAAAUCCCGCCGUCU CCACCA
K. pneumoniae NC_017540.1/3743434-3743796	GGGGCUGAUUCUGGAUUCGACGGGAUUUGCGAAACCCAAGGUGCAUGCCGAGGGGCGGUUGGC CUCGUAAAAAGCCGCAAAAAAUAGUC**GCACGUGACCACAUGGUCCUUCAUGAGUACGUAAAUG CUGCUGGGAUUACAUAA**UAACCUAAUCCUAGCCCUCUCUCCCUAGCUUCCGCUCUUAAGACGG GGAUCAAAGAGAGGUCAAACCCAAAAGAGAUCGCGUGGAUGCCCUGCCUGGGGUUGAAGCGUU AAAUCUCAUCAGGCUAGUUUGUUAGUGGCGUGUCUGUCCGCAGCUGGCAAGCGAAUGUAAAGA CUGACUAAGCAUGUAGUGCCGAGGAUGUAGGAAUUUCGGACGCGGGUUCAACUCCCGCCAGCU CCACCA
A. baumannii NZ_KB849843.1/1574036-1573677	GGGGAUGUUAUUGGCUUCGACGCCGGUGAUGAAACUCAUAGAUGCAUGCCGAGAGCGCAUUUU CUCUCGUAAAUAAAAUUUGCAUUUAAAUAGUC**GCACGUGACCACAUGGUCCUUCAUGAGUACG UAAAUGCUGCUGGGAUUACAUAA**UAACCUAAUCCUAGCGUCCGCUUCCUAGAAUACUUGUGGU CUGGGAACCCGACUGAAGCGCACGCACACAAGUCCGUAUAGAGUCAAGCCUCGGGGCUUUAUA CCAAACUUAGAGGAUCGCACUUUGUACCCUGUUCGUCGGGUCACUUGGUGUUAAAACAAUAGA CGAUAUCUAAGCAUGUAGUAUUCUCGAGCGUAGUGCUGGCGGACGCGGGUUCAACUCCCGCCA UCUCCACCA
P. aeruginosa NC_002516.2/901872-901520	GGGGCCGAUUAGGAUUCGACGCCGGUAACAAAACUUGAGGGGCAUGCCGAGCUGGUAGCAGAA CUCGUAAAUUCGCUGCUGCAAACUUAUAGUU**GCACGUGACCACAUGGUCCUUCAUGAGUACGU AAAUGCUGCUGGGAUUACAUAA**UAACCUAAUCCUAGCCAGUCGCUAGGGGAUGCCUGUAAACC CGAAACGACUGUCAGAUAGAACAGGAUCGCCGCCAAGUUCGCUGUAGACGUAACGGCUAAAAC UCAUACAGCUCGCUCCAAGCACCCUGCCACUCGGGCGGCGCGGAGUUAACUCAGUAGAGCUGG CUAAGCAUGUAGAACCGAUAGCGGAGAGCUGGCGGACGGGGGUUCAAAUCCCCCCGGCUCCAC CA
E. cloacae NC_016514.1/3580054-3580417	GGGGCUGAUUCUGGAUUCGACGGGAUUUGCGAAACCCAAGGUGCAUGCCGAGGGGCGGUUUGC CUCGUAAAAAGCCGCAAAAAAAUAGUC**GCACGUGACCACAUGGUCCUUCAUGAGUACGUAAAU GCUGCUGGGAUUACAUAA**UAACCUAAUCCUAGCCCUCUCUCCCUAGCUUCCGCUCUUAAGACG GGGAUUCAAGAGAGGUCAAACCCAAAAGAGAUCGCGUGGAAGCCCUGCCUGGGGUUGAAGCGU UAAAACUAAUCAGGCUAGUACGUUAGUGGCGUGUUUGUUCGCAGCUGGCGUGCGAAUGUAAAG ACAAACUAAGCAUGUAGUACCGAGGAUGUAGAAAUUUCGGACGCGGGUUCAACUCCCGCCAGC UCCACCA
E. coli (Guyomar et al ; 2020) NC_000913.2/2753615-2753977	GGGGCUGAUUCUGGAUUCGACGGGAUUUGCGAAACCCAAGGUGCAUGCCGAGGGGCGGUUGGC CUCGUAAAAAGCCGCAAAAAAUAGUC**GCACGUGACCACAUGGUCCUUCAUGAGUACGUAAAUG CUGCUGGGAUUACAUAA**UAACCUAAUCCUAGCCCUCUCUCCCUAGCCUCCGCUCUUAGGACGG GGAUCAAGAGAGGUCAAACCCAAAAGAGAUCGCGUGGAAGCCCUGCCUGGGGUUGAAGCGUUA AAACUUAAUCAGGCUAGUUUGUUAGUGGCGUGUCCGUCCGCAGCUGGCAAGCGAAUGUAAAGA CUGACUAAGCAUGUAGUACCGAGGAUGUAGGAAUUUCGGACGCGGGUUCAACUCCCGCCAGCU CCACCA
	tmRNA$_{GFP11}$V2 sequence
P. aeruginosa	GGGGCCGAUUAGGAUUCGACGCCGGUAACAAAACUUGAGGGGCAUGCCGAGCUGGUAGCAGAA CUCGUAAAUUCGCUGCUGCAAACUUAUAGUU**GCACGUGACCACAUGGUCCUUCAUGAGUACGU AAAUGCUGCUGGGAUUACAUAA**CUAGCUGCUUAAUGCGGCUAGCAGUCGCUAGGGGAUGCCUG UAAACCCGAAACGACUGUCAGAUAGAACAGGAUCGCCGCCAAGUUCGCUGUAGACGUAACGGC UAAAACUCAUACAGCUCGCUCCAAGCACCCUGCCACUCGGGCGGCGCGGAGUUAACUCAGUAG AGCUGGCUAAGCAUGUAGAACCGAUAGCGGAGAGCUGGCGGACGGGGGUUCAAAUCCCCCCGG CUCCACCA

Supplementary Table 2: *E. coli* and ESKAPE SmpB amino acid sequences

Species	Accession numbers	SmpB sequence
E. faecium	NC_017960.1/2002719-2002255	MPKGEGKLIAQNKKARHDYSIIDTMEAGMVLQGTEIKSIR NSRINLKDGFIRVRNGEAFLHNVHISPYEQGNIFNHDPLR TRKLLLHKKQIIRLENELKNTGITVVPLKVYIRNGYAKVL IGLAKGKKSYDKREDLKRKDIDRQIDRTLKNFSR
S. aureus	NZ_GG774480.1/1107769-1107305	MAKKKSPGTLAENRKARHDYNIEDTIEAGIVLQGTEIKSI RRGSANLKDSYAQVKNGEMYLNNMHIAPYEEGNRFNHDPL RSRKLLLHKREIIKLGDQTREIGYSIVPLKLYLKHGHCKV LLGVARGKKKYDKRQALKEKAVKRDVARDMKARY
K. pneumoniae	NC_017540.1/3742807-3743289	MTKKKAHKPGSATIALNKRARHEYFIEDEYEAGLALQGWE VKSLRAGKANIGDSYVILKDGEAFLFGANFTPMAVASTHY VCDPTRTRKLLLNQRELDTLYGRINREGYTVVALSLYWKN AWCKVKIGVAKGKKQHDKRTDLKDREWALDKARIMKHAGR
A. baumannii	NZ_KB849843.1/1643614-1644090	MAKATVVKKHNGGTIAQNKRARHDYFIEEKFEAGMSLLGW EVKSLRAGRMSLTESYVIFKNGEAFLFGAQIQPLLSASTH IVPEATRTRKLLLSRRELEKLMGAVNQKGYSCVPLACYWK GHLVKLEIALVKGKQLHDKRATEKERDWQRDKARIFHK
P. aeruginosa	NC_002516.2/5353783-5354262	MAKQKKHPSGTIAQNKKALHDYFIEQRFEAGVALAGWEVK SLRAGKAQLVDSYVLLKDGEAWLLGSHITPLTTASTHVIA DPVRTRKLLLHKRELGKLFGAVQQKGYACVALSMYWKKHL VKCEIALAKGKKDFDKRHTEKERDSDREIQRAMRHGKDD
E. cloacae	NC_016514.1/3579482-3579964	MTKKKAHKPGSATIALNKRARHEYFIEEEFEAGLALQGWE VKSLRAGKANIGDSYVILKDGEAFLFGANFTPLTVASSHY VCDPTRTRKLLLNKRELESLYGRINREGFTVVALSLYWKN AWCKVKVGVAKGKKQHDKRTDLKEREWQLDKARIMKNAGR
E. coli	NC_000913.2/2752918-2753400	MTKKKAHKPGSATIALNKRARHEYFIEEEFEAGLALQGWE VKSLRAGKANISDSYVLLRDGEAFLFGANITPMAVASTHV VCDPTRTRKLLLNQRELDSLYGRVNREGYTVVALSLYWKN AWCKVKIGVAKGKKQHDKRSDIKEREWQVDKARIMKNAHR

Supplementary Table 3: Primers and antisense oligonucleotide sequences.

	Name	Letter/ Number	Sequence	Reference
Primers	GFPfold_for	#1	**5'** CTCGATCCCGCGAAATTAATACG **3'**	Guyomar *et al.*, 2020
	GFP1-10nonSTOP_rev	#2	**5'** CTTTTCGTTGGGATCTTTCG **3'**	
	alaGFPfold_rev	#3	**5'** CCGGCCTAGGTTATGTAATCCCAGCAGCATTT ACGTACTCATGAAGGACCATGTGGTCACG**TGC** CTTTTCGTTGGGATCTTTCGAAAG **3'**	
Antisense oligonucleotide	Antisense tmRNA	A	**5'** GCTGCTAAAGCGTAGTTTTCGTCGTT **3'**	

Supplementary Table 4: PCR product sequences for *in vitro trans*-translation. The T7 promoter is underlined, the RBS sequence is green, the start and stop codons are red, and the tmRNA alanine resume codon is orange.

Name	Sequence	Reference
sfGFP1-10	**5'**CTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATT CCCCTCTAGAAATAATTTTGTTTAACTTTAAGA**AGGAGA**TATACAT**ATG**GGTGGCACTAG TAGCAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGA TGTTAATGGGCACAAATTTTCTGTCAGAGGAGAGGGTGAAGGTGATGCTACAATCGGAAA ACTCACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGT CACTACTCTGACCTATGGTGTTCAATGCTTTTCCCGTTATCCGGATCACATGAAAAGGCA TGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAACGCACTATATCTTTCAA AGATGACGGGAAATACAAGACGCGTGCTGTAGTCAAGTTTGAAGGTGATACCCTTGTTAA TCGTATCGAGTTAAAGGGTACTGATTTTAAAGAAGATGGAAACATTCTCGGACACAAACT CGAGTACAACTTTAACTCACACAATGTATACATCACGGCAGACAAACAAAAGAATGGAAT CAAAGCTAACTTCACAGTTCGCCACAACGTTGAAGATGGTTCCGTTCAACTAGCAGACCA TTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCT GTCGACACAAACTGTCCTTTCGAAAGATCCCAACGAAAAG**3'**	Guyomar *et al.*, 2020
sfalaGFP	**5'**CTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATT CCCCTCTAGAAATAATTTTGTTTAACTTTAAGA**AGGAGA**TATACAT**ATG**GGTGGCACTAG TAGCAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGA TGTTAATGGGCACAAATTTTCTGTCAGAGGAGAGGGTGAAGGTGATGCTACAATCGGAAA ACTCACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGT CACTACTCTGACCTATGGTGTTCAATGCTTTTCCCGTTATCCGGATCACATGAAAAGGCA TGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAACGCACTATATCTTTCAA AGATGACGGGAAATACAAGACGCGTGCTGTAGTCAAGTTTGAAGGTGATACCCTTGTTAA TCGTATCGAGTTAAAGGGTACTGATTTTAAAGAAGATGGAAACATTCTCGGACACAAACT CGAGTACAACTTTAACTCACACAATGTATACATCACGGCAGACAAACAAAAGAATGGAAT CAAAGCTAACTTCACAGTTCGCCACAACGTTGAAGATGGTTCCGTTCAACTAGCAGACCA TTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCT GTCGACACAAACTGTCCTTTCGAAAGATCCCAACGAAAAG**GCA**CGTGACCACATGGTCCT TCATGAGTACGTAAATGCTGCTGGGATTACA**TAA**CCTAGGCCGG**3'**	Guyomar *et al.*, 2020

Supplementary Table 5: Plasmid list. These synthesized sequences were cloned by GenScript in pUC19 between the HindIII and BamHI restriction sites or in pET22b+ between the NdeI and XhoI restriction sites. The T7 promoter is grey and underlined, the eleventh beta-strand is light green, and the mutations making up for the formation of the H5 helix are dark green. BsmB1 or EarI allow generation of the 3 'end of tmRNA. SmpB sequences are codon optimized for *E. coli.*

pUC19*ESKAPE*tmRNA$_{GFP11}$	pUC19*E.faecium*tmRNA$_{GFP11}$	aagctTAATACGACTCACTATAGGGGACGTTACGGATTCGACAGGCACAGTCGAG CTTGAATTGCGTTTCGTAGGTTACGTCTACGTAAAAACGTTACAGTTAAATATAA CTGCACGTGACCACATGGTCCTTCATGAGTACGTAAATGCTGCTGGGATTACATA ATAACCTAATCCTAGCGATCCTCTCGGCATCGCCCATGTGCTCGAGTAAGGGTCC TAACTTTAGTGGGATACGTTTCAACTTTCCGTCTGTAAGTTGAAAAAGAGAACAT CAGACTAGCGATACAGAATGCCTGTCACTCGGCAAGCTGTAAAGTGAATCCTTAA ATGAGTTGACTATGAACGTAGATTTTTAAGTGGCGATGTGTTTGGACGCGGGTTC GACTCCCGCCGTCTCCAccaCGAAGAGgatcc
	pUC19*S.aureus*tmRNA$_{GFP11}$	aagctTAATACGACTCACTATAGGGGACGTTCATGGATTCGACAGGGGTCCCCCG AGCTCATTAAGCGTGTCGGAGGGTTGTCTTCGTCATCAACACACACAGTTTATAA TAACTGCACGTGACCACATGGTCCTTCATGAGTACGTAAATGCTGCTGGGATTAC ATAATAACCTAATCCTAGCATCGCCTAACAGCATTTCCTATATGCTGTTAACGCG ATTCAACCTTAATAGGATATGCTAAACACTGCCGTTTGAAGTCTGTTTAGAAGAA ACTTAATCAAGCTAGCATCATGTTGGTTGTTTATCACTTTTCATGATGCGAAACC TTTCGATAAACTACACACGTAGAAAGATGTGTATCAGGACCTCTGGACGCGGGTT CAAATCCCGCCGTCTCCAccatCGAAGAGgatcc
	pUC19*K.pneumoniae*tmRNA$_{GFP11}$	aagctTAATACGACTCACTATAGGGGCTGATTCTGGATTCGACGGGATTTGCGAA ACCCAAGGTGCATGCCGAGGGGCGGTTGGCCTCGTAAAAAGCCGCAAAAAATAGT CGCACGTGACCACATGGTCCTTCATGAGTACGTAAATGCTGCTGGGATTACATAA TAACCTAATCCTAGCCCTCTCTCCCTAGCTTCCGCTCTTAAGACGGGGATCAAAG AGAGGTCAAACCCAAAAGAGATCGCGTGGATGCCCTGCCTGGGGTTGAAGCGTTA AATCTCATCAGGCTAGTTTGTTAGTGGCGTGTCTGTCCGCAGCTGGCAAGCGAAT GTAAAGACTGACTAAGCATGTAGTGCCGAGGATGTAGGAATTTCGGACGCGGGTT CAACTCCCGCCAGCTCCAccaGGAGACGgatcc
	pUC19*A.baumannii*tmRNA$_{GFP11}$	aagctTAATACGACTCACTATAGGGGATGTTATTGGCTTCGACGCCGGTGATGAA ACTCATAGATGCATGCCGAGAGCGCATTTTCTCTCGTAAATAAAATTTGCATTTA AATAGTCGCACGTGACCACATGGTCCTTCATGAGTACGTAAATGCTGCTGGGATT ACATAATAACCTAATCCTAGCGTCCGCTTCCTAGAATACTTGTGGTCTGGGAACC CGACTGAAGCGCACGCACACAAGTCCGTATAGAGTCAAGCCTCGGGGCTTTATAC CAAACTTAGAGGATCGCACTTTGTACCCTGTTCGTCGGGTCACTTGGTGTTAAAA CAATAGACGATATCTAAGCATGTAGTATTCTCGAGCGTAGTGCTGGCGGACGCGG GTTCAACTCCCGCCATCTCCAccaGGAGACGgatcc
	pUC19*P.aeruginosa*tmRNA$_{GFP11}$	aagctTAATACGACTCACTATAGGGGCCGATTAGGATTCGACGCCGGTAACAAAA CTTGAGGGGCATGCCGAGCTGGTAGCAGAACTCGTAAATTCGCTGCTGCAAACTT ATAGTTGCACGTGACCACATGGTCCTTCATGAGTACGTAAATGCTGCTGGGATTA CATAATAACCTAATCCTAGCCAGTCGCTAGGGGATGCCTGTAAACCCGAAACGAC TGTCAGATAGAACAGGATCGCCGCCAAGTTCGCTGTAGACGTAACGGCTAAAACT CATACAGCTCGCTCCAAGCACCCTGCCACTCGGGCGGCGCGGAGTTAACTCAGTA GAGCTGGCTAAGCATGTAGAACCGATAGCGGAGAGCTGGCGGACGGGGGTTCAAA TCCCCCCGGCTCCACCAGGAGACGgatcc
	pUC19*E.cloacae*tmRNA$_{GFP11}$	aagctTAATACGACTCACTATAGGGGCTGATTCTGGATTCGACGGGATTTGCGAA ACCCAAGGTGCATGCCGAGGGGCGGTTTGCCTCGTAAAAAGCCGCAAAAAAATAG TCGCACGTGACCACATGGTCCTTCATGAGTACGTAAATGCTGCTGGGATTACATA ATAACCTAATCCTAGCCCTCTCTCCCTAGCTTCCGCTCTTAAGACGGGGATTCAA GAGAGGTCAAACCCAAAAGAGATCGCGTGGAAGCCCTGCCTGGGGTTGAAGCGTT AAAACTAATCAGGCTAGTACGTTAGTGGCGTGTTTGTTCGCAGCTGGCGTGCGAA TGTAAAGACAAACTAAGCATGTAGTACCGAGGATGTAGAAATTTCGGACGCGGGT TCAACTCCCGCCAGCTCCAccaGGAGACGgatcc
	pUC19*P.aeruginosa*tmRNA$_{GFP11}$V2	aagctTAATACGACTCACTATAGGGGCCGATTAGGATTCGACGCCGGTAACAAAA CTTGAGGGGCATGCCGAGCTGGTAGCAGAACTCGTAAATTCGCTGCTGCAAACTT ATAGTTGCACGTGACCACATGGTCCTTCATGAGTACGTAAATGCTGCTGGGATTA CATAActagctgcttaaTGCGGCTAGCAGTCGCTAGGGGATGCCTGTAAACCCGA AACGACTGTCAGATAGAACAGGATCGCCGCCAAGTTCGCTGTAGACGTAACGGCT AAAACTCATACAGCTCGCTCCAAGCACCCTGCCACTCGGGCGGCGCGGAGTTAAC TCAGTAGAGCTGGCTAAGCATGTAGAACCGATAGCGGAGAGCTGGCGGACGGGGG TTCAAATCCCCCCGGCTCCACCAGGAGACGgatcc

pET22b+*ESKAPE*SmpB	pET22b+*E.faecium*SmpB	CAT**ATG**CCGAAGGGCGAGGGCAAACTGATTGCGCAGAACAAGAAAGCGCGTCACG ACTACAGCATCATTGATACCATGGAGGCGGGTATGGTGCTGCAAGGCACCGAAAT CAAAAGCATTCGTAACAGCCGTATCAACCTGAAGGACGGTTTCATTCGTGTGCGT AACGGCGAGGCGTTTCTGCACAACGTTCACATCAGCCCGTATGAACAGGGTAACA TTTTCAACCACGATCCGCTGCGTACCCGTAAACTGCTGCTGCACAAGAAACAAAT CATTCGTCTGGAGAACGAACTGAAAAACACCGGTATCACCGTGGTTCCGCTGAAG GTGTACATTCGTAACGGCTATGCGAAGGTTCTGATCGGTCTGGCGAAAGGCAAGA AAAGCTACGACAAGCGTGAAGATCTGAAGCGTAAAGACATCGATCGTCAGATTGA CCGTACCCTGAAGAACTTTAGCCGTCTCGAG
	pET22b+*S.aureus*SmpB	CAT**ATG**GCGAAGAAAAAGAGCCCGGGTACCCTGGCGGAGAACCGTAAAGCGCGTC ACGACTATAACATCGAGGATACCATTGAAGCGGGTATCGTGCTGCAGGGCACCGA GATCAAGAGCATTCGTCGTGGTAGCGCGAACCTGAAAGACAGCTACGCGCAAGTT AAGAACGGCGAAATGTATCTGAACAACATGCACATTGCGCCGTACGAGGAAGGTA ACCGTTTCAACCACGACCCGCTGCGTAGCCGTAAGCTGCTGCTGCACAAACGTGA GATCATTAAGCTGGGTGATCAGACCCGTGAAATTGGCTACAGCATCGTGCCGCTG AAGCTGTATCTGAAACACGGCCACTGCAAAGTGCTGCTGGGTGTTGCGCGTGGCA AAAAGAAATATGATAAGCGTCAAGCGCTGAAGGAAAAAGCGGTGAAACGTGACGT TGCGCGTGATATGAAGGCGCGTTACCTCGAG
	pET22b+*K.pneumoniae*SmpB	CAT**ATG**ACCAAGAAAAAGGCGCACAAACCGGGTAGCGCGACCATCGCGCTGAACA AGCGTGCGCGTCACGAATACTTCATTGAGGACGAATATGAGGCGGGTCTGGCGCT GCAGGGTTGGGAAGTGAAAAGCCTGCGTGCGGGCAAGGCGAACATCGGCGACAGC TATGTTATTCTGAAAGATGGTGAAGCGTTCCTGTTTGGCGCGAACTTTACCCCGA TGGCGGTGGCGAGCACCCACTACGTTTGCGACCCGACCCGTACCCGTAAGCTGCT GCTGAACCAGCGTGAACTGGATACCCTGTACGGTCGTATCAACCGTGAGGGCTAT ACCGTGGTTGCGCTGAGCCTGTACTGGAAAAACGCGTGGTGCAAAGTGAAGATTG GTGTTGCGAAGGGCAAAAAGCAACACGACAAACGTACCGACCTGAAGGATCGTGA GTGGGCGCTGGATAAAGCGCGTATCATGAAGCACGCGGGCCGTCTCGAG
	pET22b+*A.baumannii*SmpB	CAT**ATG**GCGAAGGCGACCGTGGTTAAGAAACACAACGGTGGCACCATCGCGCAGA ACAAGCGTGCGCGTCACGACTACTTCATTGAGGAAAAATTTGAGGCGGGTATGAG CCTGCTGGGCTGGGAAGTGAAGAGCCTGCGTGCGGGTCGTATGAGCCTGACCGAG AGCTATGTTATCTTCAAAAACGGTGAAGCGTTCCTGTTTGGTGCGCAGATCCAAC CGCTGCTGAGCGCGAGCACCCACATTGTGCCGGAGGCGACCCGTACCCGTAAACT GCTGCTGAGCCGTCGTGAGCTGGAAAAGCTGATGGGTGCGGTGAACCAAAAAGGC TACAGCTGCGTTCCGCTGGCGTGCTATTGGAAGGGTCACCTGGTGAAACTGGAAA TCGCGCTGGTTAAGGGCAAACAGCTGCACGATAAGCGTGCGACCGAGAAAGAACG TGACTGGCAACGTGATAAGGCGCGTATTTTTCACAAACTCGAG
	pET22b+*P.aeruginosa*SmpB	CAT**ATG**GCGAAACAGAAGAAACACCCGAGCGGTACCATCGCGCAAAACAAGAAAG CGCTGCACGACTACTTCATTGAGCAGCGTTTTGAAGCGGGTGTGGCGCTGGCGGG TTGGGAAGTGAAAAGCCTGCGTGCGGGCAAGGCGCAACTGGTGGACAGCTATGTT CTGCTGAAAGATGGTGAAGCGTGGCTGCTGGGTAGCCACATTACCCCGCTGACCA CCGCGAGCACCCACGTGATTGCGGATCCGGTTCGTACCCGTAAGCTGCTGCTGCA CAAACGTGAGCTGGGCAAGCTGTTCGGCGCGGTGCAGCAAAAGGGTTACGCGTGC GTTGCGCTGAGCATGTATTGGAAGAAACACCTGGTGAAATGCGAGATCGCGCTGG CGAAGGGCAAGAAAGACTTTGATAAACGTCACACCGAGAAGGAACGTGACAGCGA TCGTGAAATTCAGCGTGCGATGCGTCACGGCAAGGACGATCTCGAG
	pET22b+*E.cloacae*SmpB	CAT**ATG**ACCAAGAAAAAGGCGCACAAACCGGGTAGCGCGACCATCGCGCTGAACA AGCGTGCGCGTCACGAATACTTCATTGAGGAAGAGTTTGAGGCGGGTCTGGCGCT GCAGGGTTGGGAAGTTAAAAGCCTGCGTGCGGGCAAGGCGAACATCGGCGACAGC TACGTGATTCTGAAAGATGGCGAGGCGTTCCTGTTTGGCGCGAACTTCACCCCGC TGACCGTTGCGAGCAGCCACTATGTGTGCGACCCGACCCGTACCCGTAAACTGCT GCTGAACAAGCGTGAACTGGAGAGCCTGTACGGTCGTATCAACCGTGAAGGCTTT ACCGTGGTTGCGCTGAGCCTGTATTGGAAAAACGCGTGGTGCAAAGTGAAGGTTG GTGTGGCGAAGGGCAAAAAGCAGCACGACAAACGTACCGATCTGAAGGAGCGTGA GTGGCAACTGGATAAAGCGCGTATTATGAAGAACGCGGGCCGTCTCGAGCTCGAG

CHAPTER 3: *TRANS*-TRANSLATION INHIBITORS ENHANCE ANTIBIOTIC ACTIVITY AND RESCUE THEIR ACTIVITY UPON ESKAPE STRAINS.

This manuscript will be submitted to the International Journal of Antimicrobial Agents and is referent to pages 63 to 85 and, since it is a still work-in progress, the pages were excluded

GENERAL DISCUSSION

As we have seen during the first two segments of this work, the search of new weapons to fight in the never-ending war against bacteria is of upmost importance since our current arsenal is threatened by the bacterial resistance. Since all current targets already present at least one resistance mechanism it stands to reason that we should start searching for new ones. In fact, one of the best candidates that could give us the upper hand in this war is the discovery of new targets that are not currently used and for which no resistance was yet reported. This is not a simple task since it is necessary to find a target that is present only in bacteria, preferentially widespread in all genres and then search for a molecule that targets it specifically. In this sense, we explored herein a target candidate that has been gaining a lot of attention, the *trans*-translation rescue system.

In the first segment of this work (*Trans*-Translation Is an Appealing Target for the Development of New Antimicrobial Compounds) we present a review that discusses the *trans*-translation mechanism, its peculiarities as a reason to why this is an appealing target and the current research that has been done to find its inhibitors. It was possible to see that even though *trans*-translation has great potential as a new target, more research is needed to have a definitive inhibitor since the molecules that we currently have as candidates are still in an experimental phase and some other have already been heavily contested for their activity and specificity.

We have screened 1080 molecules using two previously well stablished assays. From these molecules, one presented a high activity and specificity in inhibiting *trans*-translation. The 404 molecule inhibited approximately 50% of *in vitro trans*-translation without affecting translation and had the best activity in a bacterial cell (*E. coli in vivo* model). When tested against ESKAPE strains only the derivates PD5 and PD24 presented a MIC value and only against one strain (*E. faecium*). We then decided to combine the compounds with commercial antibiotics since it has already been stablished that an antibiotic acting alongside a molecule that interferes with bacterial fitness helps diminish the emergence of resistance. It

is also possible to rescue antibiotics that have already been disregarded since no bacteria dies from it (TYERS; WRIGHT, 2019).

Combinatory assays confirmed that the compounds indeed lowered the MIC concentrations of some of the tested ESKAPE bacteria, which is great evidence that inhibitors of *trans*-translation can be used as antibiotic adjuvants and minimize the impact of antibiotic resistance. Despite cytotoxicity presenting low IC50, no toxicity was observed in concentrations of 25 mg/Kg for neither the 404 molecule nor its two derivates in *G. mellonella* toxicity model.

CONCLUSION

In conclusion, *trans*-translation inhibitors may be used as alternatives to slow down emergence of resistance when combined with current antibiotics since it has the capability of lowering antibiotic concentrations needed to treat resistant strains. This lower bacterial fitness making them more sensitive to antibiotics and it also lowers the selective pressure. Considering that antibiotic resistance is a never-ending threat, new alternatives will always be needed. This is currently a proof-of-concept research and further investigation is needed to fully comprehend the mechanism in which this activity takes place. This way in the future we may be able to rationally design trans-translation inhibitors.

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