Published in final edited form as:
Biochemistry. 2019 January 15; 58(2): 75–84. doi:10.1021/acs.biochem.8b00927.

Using the bacterial ribosome as a discovery platform for peptide-based antibiotics

Justine Charon[†,‡], **Aitor Manteca**[†], **C. Axel Innis**[*,†]

[†]Institut Européen de Chimie et Biologie, Univ. Bordeaux, Institut National de la Santé et de la Recherche Médicale (U1212) and Centre National de la Recherche Scientifique (UMR 5320), Pessac 33607, France

Abstract

The threat of bacteria resistant to multiple antibiotics poses a major public health problem requiring immediate and coordinated action worldwide. While infectious pathogens have become increasingly resistant to commercially available drugs, antibiotic discovery programs in major pharmaceutical companies have produced no new antibiotic scaffolds in forty years. As a result, new strategies must be sought to obtain a steady supply of novel scaffolds capable of countering the spread of resistance. The bacterial ribosome is a major target for antimicrobials and is inhibited by more than half of the antibiotics used today. Recent studies showing that the ribosome is a target for several classes of ribosomally synthesized antimicrobial peptides point to ribosome-targeting peptides as a promising source of antibiotic scaffolds. In this perspective, we revisit the current paradigm of antibiotic discovery by proposing that the bacterial ribosome can be used both as a target and as a tool for the production and selection of peptide-based antimicrobials. Turning the ribosome into a high-throughput platform for the directed evolution of peptide-based antibiotics could be achieved in different ways. One possibility would be to use a combination of state-of-the-art microfluidics and genetic reprogramming techniques, which we will review briefly. If successful, this strategy has the potential to produce new classes of antibiotics for treating multidrug-resistant pathogens.

Antibiotics are the workhorses of modern medicine, but their misuse has favored the emergence of resistant bacterial strains that reduce the effectiveness of our drug arsenal over time. Strains resistant to multiple antibiotics are particularly worrying, as they can cause infections that are difficult to treat and seriously compromise clinical outcomes. The fight against antibiotic resistance is a complex problem that requires coordinated action on many levels, from the way antibiotics are prescribed to the development of new drugs. This last point is essential because a common tactic used by physicians to treat resistant pathogens is to switch a patient to a class of antibiotics with an unrelated molecular scaffold, a different mode of action and, importantly, a distinct mechanism of resistance. Without an

[*] **Corresponding author** Address: Institut Européen de Chimie et Biologie, 2 rue Robert Escarpit, 33607 Pessac Cedex, France. axel.innis@inserm.fr. Phone: +33 540006149.
[‡]Present address: School of Life & Environmental Sciences, The University of Sydney, Camperdown campus, Sydney, NSW 2006, Australia.

The authors declare no competing financial interest.

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effective discovery platform to deliver a steady supply of new antibiotic scaffolds, it will be increasingly difficult to contain the spread of resistance [1–3].

This perspective focuses on antimicrobials that inhibit the bacterial ribosome, the complex molecular machine responsible for producing the cellular proteome. Given its central role, the bacterial ribosome is a target for more than half of the antibiotics available today [4, 5]. The majority of these are based on molecular scaffolds that date back to the golden era of antibiotic discovery (1950-1960), when soil-dwelling bacteria were mined extensively for antimicrobial compounds. As resistance against these early antibiotics began to spread in the 1960s, new scaffolds also became more difficult to find and the focus shifted to replicating and expanding antibiotic scaffolds from the golden era through synthetic chemistry. Derivatives produced during this period showed greater potency and improved pharmacokinetic parameters, were more effective against resistant strains and had fewer side effects than their predecessors. Unfortunately, efforts since the 1990s to identify new antibiotic scaffolds by high-throughput screening of large compound libraries or by rational drug design have failed to deliver inhibitors with the necessary physical and chemical properties to either target a broad spectrum of pathogens or to effectively penetrate Gram-negative bacteria [2]. As a result, drug discovery programs have not yielded a new class of ribosome targeting antibiotic in the past forty years and novel strategies are needed to find molecules that can successfully reach and block their cellular target.

Recent studies showing that ribosomally synthesized antimicrobial peptides are potent inhibitors of the bacterial ribosome point to peptide-based antibiotics as a possible way forward [6–13]. Long dismissed by the pharmaceutical industry, peptides are increasingly considered as drug candidates[14, 15]. They are easy to synthesize chemically, tend to show greater target selectivity and fewer side effects compared to conventional small molecules (MW < 500 Da) and, once they have been suitably modified, offer the potential for better bioavailability, membrane permeability and metabolic stability relative to protein therapeutics (MW > 5000 Da). In addition, peptides can be synthesized biologically by the ribosome, simply by encoding the desired amino acid sequence in a DNA template. This property makes peptides stand out relative to other antimicrobials and offers us a unique opportunity to revisit the antibiotic discovery paradigm that has prevailed for more than half a century.

Our basic premise is simple: the ribosome should not only be considered a target for antibiotics, but also a discovery platform for peptide-based antibiotics. Instead of screening large libraries of synthetic compounds or natural products for antimicrobial activity, we could use the ribosome to produce complex peptide libraries *in vitro* and select those peptides that are most effective at reaching and blocking ribosomes within bacterial cells. This could be achieved through a directed evolution approach, a well-established strategy that mimics the natural selection process to evolve proteins or nucleic acids with a set of desired properties. Directed evolution relies on similar principles as Darwinian selection: genetic diversity giving rise to varied phenotypes, a strong phenotype-genotype linkage to ensure the recovery of desired genotypes and amplification of the selected genotypes. Although the implementation of these basic principles could vary significantly, considerations such as the quality and complexity of the input library and the sensitivity

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and specificity of the selection procedure are the key to ensuring success. Directed evolution has been used to discover or improve therapeutic peptides [16], proteins [17] and aptamers [18]. Its remarkable success was rewarded with the 2018 Nobel Prize in Chemistry. Yet, with one possible exception[19], it has never been applied to the search for novel antimicrobials to block the ribosome. Here, we will review recent biochemical advances that uphold the validity of developing ribosome-targeting peptides as antibiotic scaffolds and that suggest how this could actually be achieved using directed evolution. The general approach outlined in this perspective could be implemented in a number of different ways, but we hope that its underlying principle – that the ribosome can be used both as a production and a selection tool to discover new peptide-based antibiotics – will drive further efforts to replenish our dwindling collection of antimicrobials.

Ribosomally-Synthesized Peptides are Promising Antibiotic Scaffolds

The ribosome is a validated antibiotic target

The ribosome is an extremely complex and sophisticated macromolecular machine, composed of multiple functional sites and moving parts. In bacteria, the ribosome consists of two subunits composed of about two-thirds of ribosomal RNA (rRNA) and one-third of protein. The small 30S subunit contains the decoding center, where the anticodon of an aminoacylated transfer RNA (tRNA) recognizes its cognate codon on the messenger RNA (mRNA), effectively translating the 4-nucleotide language used to store genetic information into the 20-amino acid language of proteins. The large 50S subunit includes the peptidyl transferase center (PTC), where amino acids are added one at a time to the elongating polypeptide, and the nascent polypeptide exit tunnel, a long cavity through which newly synthesized proteins transit on their way out of the ribosome. Protein synthesis is a dynamic process that requires additional protein factors to facilitate the initiation, elongation, release and recycling steps that make up the translation cycle [5]. During the course of translation, factors come in and out of the ribosome, subunits move relative to each other and the ribosome inches along the mRNA, one codon at a time, in a process known as translocation.

Just like the machines we use in every day life, the ribosome has weak points that can be targeted to block the activity of the entire complex. Small molecules that efficiently block the bacterial ribosome, but fail to inhibit or reach our cytoplasmic and mitochondrial ribosomes, account for a large portion of commonly used antibiotics. Different antibiotics target different steps of the translation cycle, either by binding to various functional sites on the ribosome or by preventing factors from binding to it. The mechanisms of action of these antibiotics and the means by which bacteria become resistant to them have been reviewed extensively [5] and will not be addressed here. Instead, we will focus on peptides that block the ribosome when they are added extraneously (*in trans*) or that inhibit the ribosome that is translating them while they are still attached to a ribosome-bound tRNA (*in cis*). While the former are obvious candidates for further development as antimicrobials, the latter share enough common points with certain ribosome-targeting antibiotics to deserve a mention here.

Antimicrobial peptides block the ribosome *in trans*

Several classes of naturally occurring ribosome-targeting antimicrobial peptides exist, based on either linear or cyclic peptide backbones (Figure 1a) [20]. These peptides can be classified into three groups: (i) peptides that are produced by the ribosome, such as the recently characterized proline-rich antimicrobial peptides (PrAMPs) [21]; (ii) ribosomally synthesized and post-translationally modified peptides (RiPPs) [22] like klebsazolicin or thiostrepton; or (iii) peptides that are synthesized by non-ribosomal peptide synthetases (NRPSs) [23], such as edeine or GE81112. Peptides that affect the 30S subunit target various steps of the translation cycle, including initiation (edeine [24], GE81112 [25]), mRNA decoding (odilorhabdins [26]) or tRNA translocation (GE82832/dityromycin [27], viomycin and capreomycin [28, 29]) (Figure 1b). Peptide-based antibiotics that affect the large subunit do so by targeting the PTC or the nascent polypeptide exit tunnel in order to block different processes, namely the transition from the initiation to the elongation phase of translation (Type-I PrAMPs like oncocin[8, 11, 13] or Bac-7 [7, 9, 12]), the elongation phase (streptogramins A and B [30–33], klebsazolicin [34]) or the termination phase (Type-II PrAMPs like apidaecin [6]) (Figure 1b). Some peptide antibiotics, like thiostrepton, block translation by preventing the binding of key factors to the ribosome [35]. With the exception of streptogramins and the anti-tuberculosis drugs capreomycin and the now-retired viomycin, peptide-based ribosomal antibiotics have yet to be used clinically as antibiotics [20].

Ribosomally synthesized antimicrobial peptides in particular are a promising source of antibiotic scaffolds, as it should be possible to produce them in an *in vitro* translation system at a high enough concentration to measure the inhibitory effect that they exert upon their own synthesis. Using a ribosome to produce a peptide that has the capacity to inhibit it may seem counter-intuitive at first. However, it is important to remember that this peptide cannot act on the same ribosome that is translating it and that it will only become inhibitory once a sufficient peptide concentration is reached in the *in vitro* translation reaction. For example, the insect-derived PrAMP Onc112 blocks luciferase production in an *Escherichia coli* (*E. coli*) *in vitro* translation system with a half-inhibitory concentration (IC_{50}) [13]. This value is one order of magnitude smaller than the ~10 μM of peptide that can theoretically be obtained using a commercial PURExpress system, if we assume that each ribosome in the reaction translates a minimum of 5 mRNA molecules and the concentration of ribosomes is 2 μM [36]. Significantly, recent data from our group show that recombinant Onc112 can inhibit its own synthesis once a sufficient amount of peptide has been produced by this type of *in vitro* translation system (*to be published*). This means that a directed evolution strategy could be devised in which a collection of peptides is translated *in vitro* and a selection procedure ensures that only those peptides that inhibit translation with a given IC_{50} are retained. Similarly, peptides produced *in vitro* could accumulate at a sufficient concentration to block bacterial cell growth, as suggested by the minimum inhibitory concentration (MIC) of 10 μM measured for Onc112 [13]. This means that assays could be devised that test the ability of peptides produced by a cell-free translation system to reach their ribosomal target inside growing bacterial cells.

For a directed evolution strategy to succeed, the genetic diversity of the input DNA or mRNA library is critical. On the one hand, a library that is too focused on a particular region

of sequence space might compromise our ability to discover truly novel inhibitory peptides. On the other hand, the selection method could fail to yield any hits if the library is too complex and variants with the desired phenotype only occur at a very low frequency. The availability of known antimicrobial peptide sequences makes it possible to prepare focused libraries comprised of millions of peptide variants differing by only a few amino acids, in addition to using truly random peptide-encoding libraries. Focused libraries based on insect or mammalian-derived PrAMPs like Onc112 [8, 11, 13] or Bac-7 [7, 9, 12], respectively, could help identify shortened variants with enhanced activity against the ribosomes of many or perhaps even just a single bacterial species, including resistant strains. Indeed, the high degree of conservation of the bacterial ribosome makes it a prime target for broad-spectrum antibiotics, but small sequence variations among ribosomes from different species could be exploited to develop species-specific translation inhibitors. The choice of selection scheme is important as well and care must be taken that the variants identified not only display high inhibitory activity against the ribosome *in vitro* (inhibitory peptides), but also retain their ability to cross the bacterial membrane and block cell growth (antimicrobial peptides). Additional selection schemes to isolate protease-resistant variants could also be established in order to produce antibiotic scaffolds that are suitable for further development. Establishing reliable protocols in future directed evolution strategies will be the key to ensuring success.

Arrest peptides inhibit translation *in cis*

An intriguing but so far unexploited source of translation inhibition is that of ribosome-arresting peptides, or arrest peptides for short (Figure 1a) [37–39]. During their synthesis by the ribosome, arrest peptides make extensive interactions with the nascent polypeptide exit tunnel that force them to remain trapped inside the large ribosomal subunit (Figure 1b). This in turn results in the inactivation and stalling of the ribosome that was translating the arrest peptide on the mRNA. Translation inhibition mediated by arrest peptides is dependent on the nascent amino acid sequence and sometimes requires the presence of a low molecular weight ligand, with the ribosome and arrest peptide effectively acting as a metabolite sensor [37, 39, 40]. As a result, ribosome stalling induced by arrest peptides can regulate the expression of other genes on the same mRNA through diverse mechanisms, both in bacteria and in eukaryotes. Biological phenomena that rely on arrest peptides in bacteria include the induction of drug-resistance genes by antibiotics of the macrolide family (e.g. Erm leader peptides) [41–45], the sensing of soluble tryptophan by a ribosome-associated TnaC peptide [46, 47], targeting of the expression of the SecA pre-protein translocase to the cell membrane by the nascent SecM polypeptide [48–50], the expression of the YidC2 membrane insertase by the MifM peptide [51–53] and the regulation of SecDF2 in low-salinity environments by the arrest peptide VemP [54]. Arrest can occur during the elongation phase of translation by inhibiting peptide bond formation or aminoacyl-tRNA accommodation, or during termination by preventing peptide release from the ribosome [39, 46]. It may also take place at a clearly defined location on the mRNA or at multiple sites within the same sequence [51].

The nascent polypeptide exit tunnel of the ribosome measures ~80 Å in length and has a diameter of 10-20 Å in bacteria. Several classes of antibiotics [5], natural antimicrobial

peptides [20] and all arrest peptides characterized to date [37, 38] exert their effects in the upper and central sections of this tunnel, where the PTC and the exit tunnel constriction formed by the extensions of ribosomal proteins uL22 and uL4 are located. As a result, the significant structural and functional overlap between these three classes of molecules suggests that arrest peptides could help inspire new peptide-based translation inhibitors that target the exit tunnel of bacterial ribosomes. Like ribosome-targeting antimicrobial peptides, known arrest peptides are short. Only a few conserved amino acids within a stretch of 10-30 residues appear to be needed to induce translational arrest. However, one significant difference exists between antimicrobial peptides that target the ribosomal exit tunnel and arrest peptides. The latter exert their inhibitory effects *in cis*, while they are still covalently linked to a tRNA molecule bound to the P-site of the ribosome. The additional affinity for the ribosome provided by the tRNA moiety is likely to be partly responsible for the ability of arrest peptides to stably interact with the ribosomal exit tunnel and no ribosomal inhibition has been reported to date for an arrest peptide provided *in trans*. Nevertheless, focused libraries encoding variants of arrest peptides (in particular ligand-independent ones, such as enhanced SecM variants [55]) could provide a suitable input for a directed evolution scheme in which peptides that gain the ability to inhibit the ribosome *in trans* are selected. Although there is no evidence at present that arrest peptides can successfully be converted into *trans*-inhibitors of translation, this option is certainly worth exploring.

Establishing an Effective Selection Procedure for Ribosome-Targeting Peptides

A strong phenotype-genotype linkage is the cornerstone of directed evolution

Known peptides that target translation interact with the ribosome using 4-20 amino acids [20]. Given the set of 20 standard amino acids, the number of possible 10-amino acid peptides produced by the ribosome (and hence starting with formyl-methionine) is ~$5x10^{11}$, a number greatly exceeding, for example, the >450,000 compounds made available for screening by the seven established pharmaceutical partners of the European Lead Factory [56, 57]. As a result, the limiting factor in accessing this molecular diversity will be our ability to develop suitable assays to select peptides with the highest inhibitory activity against the ribosome and the best cell-penetrating ability and intracellular stability. While choosing a suitable peptide-encoding library is important, the most critical requirement to ensure a successful directed evolution experiment is to achieve a strong linkage between the desired phenotype and the genotype that has given rise to it. Possible selection strategies based on existing technologies are discussed below, both for *trans* and *cis* peptide inhibitors of translation.

Compartmentalization can be used for high-throughput selection of *trans*-acting peptides

Directed evolution techniques such as ribosome display [58] or mRNA display [59] have been used successfully to identify proteins or peptides with a high binding affinity for a molecular target of interest. In both cases, the link between phenotype and genotype is established directly at the molecular level, be it through the formation of a stable ribosome nascent chain complex carrying both the peptide of interest (phenotype) and the mRNA that encodes it (genotype) [58] or through the covalent linkage of the nascent peptide and its mRNA [59]. While

these approaches are effective at selecting proteins or peptides that bind to the exposed surface of a macromolecular target, ribosome-targeting inhibitory peptides present additional challenges. Indeed, the peptides under selection in a ribosome display or mRNA display experiment are attached to a 2.5 MDa ribosome or to a long mRNA molecule, respectively. This could lead to non-specific interactions between these molecules and the selection target (the bacterial ribosome) or, conversely, to the loss of potential binders that are unable to access buried active sites on the target. How then, might one proceed in the absence of a direct physical linkage between the peptide and the mRNA that encodes it?

An elegant way to circumvent this limitation would be to compartmentalize the *in vitro* translation reaction [60] using droplet-based microfluidics [61, 62] (Figure 2a). Indeed, several recent studies have shown that picoliter-sized droplets can be used as individual reaction chambers for high-throughput analyses of libraries with therapeutic uses [63, 64]. Unlike conventional screening that relies on microplates, droplet-based microfluidics offer a relatively inexpensive way of ensuring high-throughput, making it possible to study $>10^7$ mutants per experiment. In our proposed scheme, each droplet would contain multiple copies of a unique peptide-encoding sequence, an expression cassette for a reporter protein and all the molecular components necessary to perform *in vitro* translation. The reporter protein would provide a means to monitor expression levels within each droplet and would thus reflect the inhibitory activity of the peptides produced. The most promising peptides could later be specifically selected by means of microfluidic sorting systems [65]. In this way, templates encoding the peptides of interest would be pooled and analyzed by next-generation sequencing, leading to the large-scale identification of *trans*-acting protein synthesis inhibitors, including peptides that directly block the ribosome. An efficient counter-selection procedure would be needed to eliminate transcription inhibitors and peptides that target components of the translational machinery other than the ribosome, such as translation factors or aminoacyl-tRNA synthetases. An additional selection system could then be devised to isolate ribosome-targeting peptides with antimicrobial activity, by placing the expression reporter cassette inside bacteria [66–68] contained within the droplets or within liposomes that mimic the bacterial cell wall [69]. This would be effective as a secondary strategy to eliminate peptides that display inhibitory activity *in vitro* but fail to inhibit bacterial cell growth.

High-throughput inverse toeprinting enables selection of *cis*-acting peptides

As reported above, arrest peptides are a promising albeit immature source of translation inhibitors. A method to identify new ligand-independent arrest peptides would yield *cis*-acting molecules with the potential to be evolved into *trans*-inhibitory peptides. To this end, our group has developed inverse toeprinting, a method to map the position of a stalled ribosome on the mRNA with codon resolution, while protecting the entire peptide-encoding sequence up to the point of stalling [70] (Figure 2b). This contrasts with ribosome profiling [71], which only generates a short ribosome-protected footprint and therefore loses sequence information for a majority of the coding region. Like ribosome profiling, however, inverse toeprinting is characterized by a strong phenotype-genotype linkage, made possible by the inherent stability of stalled ribosome nascent chain complexes. The use of next-generation sequencing as readout and the specificity of inverse toeprinting make it suitable for

incorporation into directed evolution schemes, as evidenced by the ability of a single cycle of inverse toeprinting to identify variants of existing drug-dependent arrest peptides with specificities for different antibiotics [70]. Inverse toeprinting allows $>10^{12}$ sequences to be analyzed in parallel in a single experiment and thus might have a reasonable chance of finding inhibitory peptides within a random pool of sequences. This could be beneficial by providing additional templates as input for the microfluidics-based approach, provided that the resulting *cis*-acting peptides could eventually be made to work *in trans*.

Expanding the Chemical Diversity of Ribosome-Targeting Peptides

Non-proteinogenic groups make peptides more drug-like

Peptides that inhibit the bacterial ribosome *in trans* must also possess drug-like properties to become viable antibiotic scaffolds, such as the capacity to cross biological membranes, good oral bioavailability, low toxicity against eukaryotic cells and sufficient stability in a physiological environment. Despite their huge potential as pharmaceutical compounds, most peptides are not considered good drug candidates in their natural, unmodified state, due to their limited stability and short half-life *in vivo*, a very low membrane permeability and negligible oral bioavailability [72, 73]. Drawing inspiration from small natural macrocyclic peptides produced via ribosome-independent pathways (e.g. cyclosporin A, vancomycin, actinomycin D…), a strategy aimed at overcoming these limitations consists in adding non-canonical chemical groups to the peptides of interest [74, 75]. Indeed, the incorporation of residues capable of inducing the formation of macrocycles, complemented with N-methylation, D-amino acids or residues harboring bulky side chains have shown great promise for the design of new antibiotics, as their chemical properties can confer rigidity and protease resistance, help cross biological membranes and increase the affinity for their target [76]. Significant efforts have been made over the past decade to introduce these non-canonical groups into peptide backbones of interest in a site-specific manner. Within the context of this perspective, we have chosen to focus on the co-translational incorporation of non-proteinogenic side chains and backbones into a ribosomally synthetized peptide by *in vitro* genetic code reprogramming.

In vitro genetic code reprogramming

Genetic code reprogramming is the artificial reassignment of codons to non-canonical residues in order to allow the ribosome-based synthesis of polypeptides that incorporate these unnatural moieties (Figure 3). Various exotic groups have been incorporated at the N-terminus of proteins using a standard bacterial *in vitro* translation system, including a large selection of unnatural L-amino acids, residues to induce peptide cyclization[77], N-methyl amino acids[78], β-amino acids[79], D-amino acids[80, 81] and even helical aromatic foldamers[82]. Incorporation at internal positions of ribosomally synthesized proteins has also been achieved for many of these groups, with efforts focused over the past decade on increasing the efficiency of this process for ‘difficult’ or bulky non-canonical groups. However, the incorporation of a single or consecutive residues with a backbone chemistry differing from that of standard L-amino acids remains a significant challenge in the field [83–86], though the recent development of Ribo-T [87] and splinted orthogonal ribosome systems [88] suggest that this aim is now within reach. Genetic reprogramming can be achieved *in vitro*

or *in vivo*, but in the context of discovering new antibiotics, we will focus exclusively on one of the most promising *in vitro* approaches: the flexizyme technology. Complementary information about *in vivo* and other *in vitro* approaches can be found in several excellent reviews [76, 89, 90].

The first step of genetic reprogramming is the mis-acylation of a tRNA molecule with an unusual amino acid or group of interest (Figure 3a). This can be accomplished in a relatively easy and flexible way using small RNA enzymes called flexizymes [91] or, traditionally, using mutated amino acyl-tRNA synthetases (aaRSs) with more permissive active sites and/or altered editing sites[90]. A distinct advantage of the flexizyme technology over aaRSs-based approaches is its applicability to virtually any non-canonical amino acid. Three types of flexizyme are typically used – eFx, aFx and dFx – that act upon amino acid substrates previously activated with specific ester-based leaving groups [91, 92]. The only sequence element in the tRNA needed for recognition by flexizymes is an intact 3' CCA end. This flexibility ensures that a wide range of substrates can be acylated onto any tRNA molecule, irrespective of its anticodon. Thus, more than 300 non-canonical amino acids, exotic monomers or short polymers (N-alkyl, N-acyl, D-amino acids, β-amino acids, γ-amino acids, macrocyclic peptides, aromatic-helix folding foldamers hybrids) have been attached to the 3' end of tRNAs since flexizymes were first introduced [77, 81, 82, 92].

The second step of genetic reprogramming is the pairing of the mis-acylated tRNA to its cognate codon in the mRNA and the subsequent incorporation of the unusual moiety it carries into the nascent polypeptide chain. This can be achieved thanks to a fully reconstituted and tunable bacterial *in vitro* translation system comprising mRNA, mis-acylated-tRNA, ribosomes, translation factors and all other necessary substrates and components of the translational machinery[93]. While the flexizyme technology has considerably extended the array of non-canonical groups that can be charged onto tRNAs, recent efforts have sought to expand the number of codons that can be reassigned in a single translation reaction. Owing to the redundancy of the genetic code, the reallocation of a given codon to an unusual amino acid limits the sequence space available, as the amino acid that is encoded naturally by this codon must be omitted from the reaction. To overcome this, codons must artificially be reassigned to increase the total number of codons available for genetic reprogramming.

Two strategies have been developed to address the issue of codon reassignment (Figure 3b). The 'artificial division of codon boxes' strategy reduces the redundancy of codon assignment by replacing all wild-type tRNAs with 32 *in vitro* transcribed tRNAs bearing SNN anticodons (S=G or C; N = U, C, A or G) [94]. Among these, tRNAs with GNN anticodons can decode NNY (Y=C or T) codons independently from tRNAs with CNN anticodons from the same codon box, which recognize NNG codons. After the 20 standard amino acids have been accounted for, the redundancy of the genetic code leaves 11 codons vacant that could theoretically be reprogrammed. Three such codons have successfully been reallocated to date, enabling the synthesis of a linear peptide containing 20 natural amino acids and 3 reassigned N-methyl-amino acids, as well as a macrocyclic N-methyl peptide. The 'codon table duplication' strategy makes use of orthogonal ribosome/tRNA pairs that do not cross-react with the wild-type translational machinery. This is made possible by

introducing mutations in the A- and P-sites of the orthogonal ribosome that allow it to interact with orthogonal tRNAs bearing a C75G mutation [95]. Introducing the orthogonal tRNA/ribosome pair into an *in vitro* translation system containing a single mRNA yields two distinct peptides in similar ratios: one translated by the wild-type ribosome and one translated by the orthogonal ribosome, which uses the parallel genetic code. Used in combination with the flexizyme technology, these approaches make *in vitro* genetic reprogramming a powerful tool to introduce chemical and functional diversity into directed evolution experiments aimed at identifying linear or cyclic peptide-based antibiotic scaffolds to target the ribosome.

Turning the Ribosome Into an Antibiotic Discovery Platform

A possible way forward

Various strategies could foreseeably be adopted to use the ribosome as a production and selection platform for peptides with the potential to become new ribosome-targeting antibiotic scaffolds. The strategy that we favor is outlined in Figure 4. In brief, peptide-encoding template libraries will be used to drive the expression of short peptides in a compartmentalized *in vitro* translation system, such that no more than a single peptide variant is expressed in each compartment. The choice of methodology for compartmentalization will be important, with droplet-based microfluidics providing an attractive solution in terms of consistency, reproducibility and ability to sort different phenotypes. Different readouts could be used to determine whether the peptides produced in each drop inhibit translation, an obvious solution being the use of a fluorescent reporter protein. Sorting of the droplets would yield a pool of templates encoding potential inhibitory peptides, which could be enriched through further cycles of selection and amplification. An additional selection strategy could be used to retain peptides that display antimicrobial properties against live bacterial cells, which will again require the use of compartmentalization to link phenotype to genotype. It is important to note that this approach could foreseeably lead to the identification of peptides that inhibit transcription, components of the translation machinery other than the ribosome (translation factors, aminoacyl-tRNA synthetases) or enzymes tasked with replenishing the *in vitro* translation system with energy (ATP, GTP). Different counter-selection schemes may thus need to be developed to identify and remove these unwanted peptides. Eventually, repeated cycles of selection and counter-selection would ensure that the most active peptide variants are retained. In parallel, *cis*-acting arrest peptides could be identified by inverse toeprinting and further mutated to act *in trans* using the directed evolution scheme described above. Directed evolution experiments could either make use of focused libraries of known antimicrobial peptides or arrest peptides variants as input or could be initiated with truly random peptide-encoding libraries. Finally, the use of genetic reprogramming to introduce chemical and molecular diversity into the peptides produced could yield modified linear or macrocyclic molecules with the desired properties for cellular uptake, oral bioavailability and *in vivo* stability. Implementing this or any other strategy will require new methods to be developed and many hurdles to be overcome, but ensuring a steady supply of novel antibiotic scaffolds for the generations to come is certainly worth the effort.

Acknowledgments

The authors thank A.-X. van der Stel for productive discussions and reading of the manuscript.

Funding

J.C. was supported by a grant form the Aquitaine Regional Council (2015-1R30104 – 00005037). A.M. is the recipient of an EMBO Long-Term Fellowship. C.A.I. is an EMBO Young Investigator and received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (Grant Agreement No. 724040).

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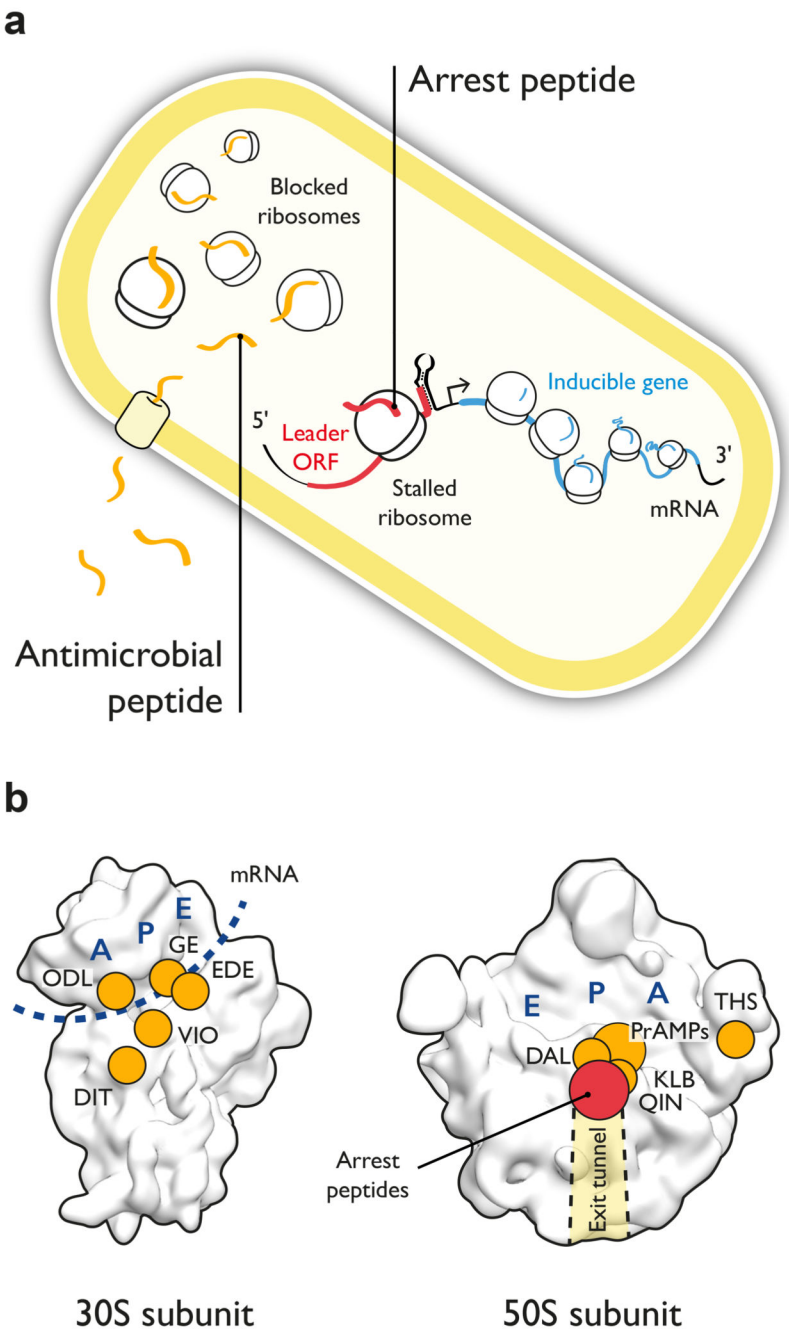

Figure 1. Antimicrobial peptides and arrest peptides are *trans*- and *cis*-acting inhibitors of bacterial translation, respectively.

(a) Antimicrobials peptides (left part, orange) are produced by the host immune response of eukaryotes and must cross the bacterial membrane to inhibit the translational machinery *in trans*. Arrest peptides (center, red) are *cis*-acting inhibitors of the translational machinery that regulate the expression of inducible genes in bacteria and in eukaryotes. Inhibition *in cis* results from interactions between an arrest peptide in its nascent state and components of the large ribosomal subunit. (b) Surface representation of the *E. coli* 30S and 50S ribosomal subunits (PDB: 4ybb [96]) showing the sites of action of various ribosome-targeting

antimicrobial peptides (orange) and of arrest peptides (red). Abbreviations for antimicrobial peptides are DAL (Dalfopristin), DIT (Dityromycin), EDE (Edeine), GE (GE81112), KLB (Klebsazolicin), ODL (Odilorhabdin), PrAMPs (Proline-rich antimicrobial peptides), QIN (Quinupristin), THS (Thiostrepton), VIO (Viomycin). The A, P and E tRNA binding sites are in dark blue and the path of the mRNA on the 30S subunit is indicated with a dark blue dotted line. The nascent polypeptide exit tunnel is indicated with a shaded area.

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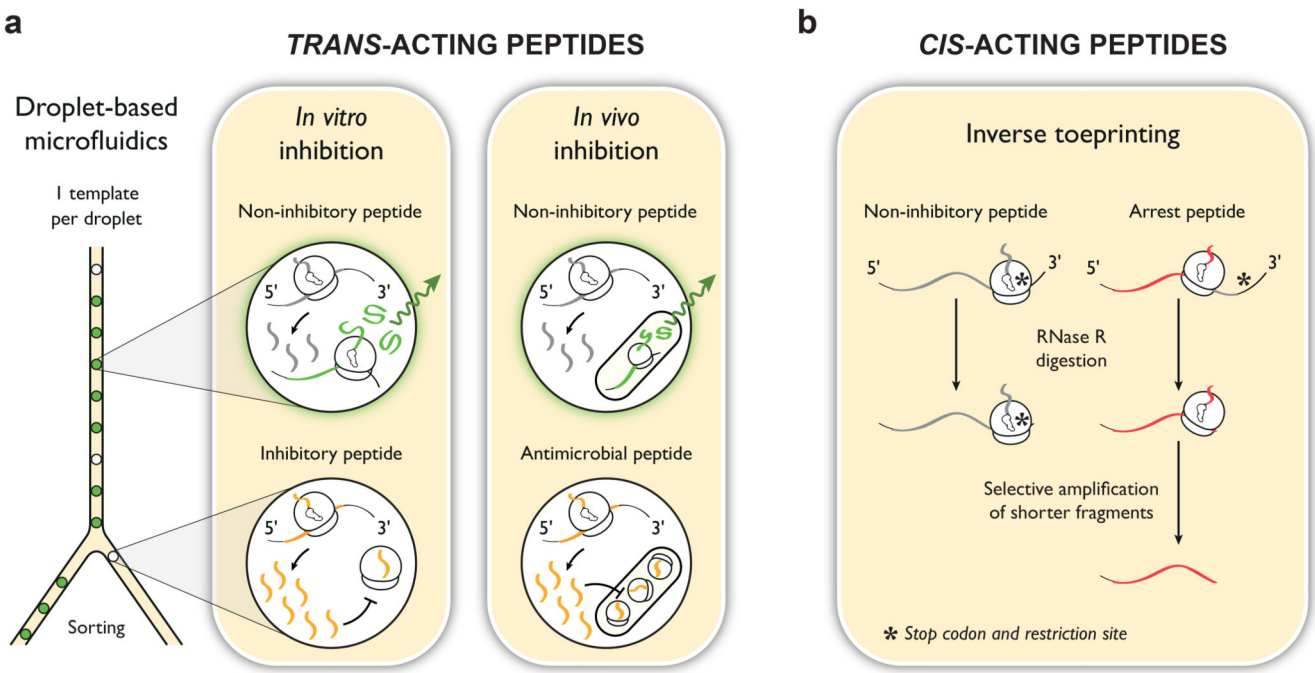

Figure 2. Possible selection schemes for identifying *trans*- or *cis*-acting peptides that inhibit bacterial translation.

(a) Approaches for the identification of *trans*-acting peptides capable of *in vitro* translation inhibition or *in vivo* cell growth inhibition (orange). The proposed microfluidic strategy allows the sorting of picoliter-sized droplets based on the level of expression of a fluorescent reporter protein (green). Each droplet contains multiple copies of a unique peptide variant. For the *in vitro* inhibition approach, the expression level of the reporter protein will be reduced inside droplets that contain inhibitory concentrations of a *trans*-acting inhibitory peptide, leading to low fluorescence. In contrast, drops that contain non-inhibitory peptides will show strong fluorescence. For the *in vivo* inhibition approach, the same principle applies, but the reporter protein is expressed inside bacterial cells contained within the droplets. (b) Identification of *cis*-acting peptides (red) by inverse toeprinting [70]. On the left, ribosomes translating a non-inhibitory peptide reach the stop codon and remain stalled on the mRNA due to the omission of release factor-2 from the *in vitro* translation reaction. On the right, translation is inhibited during the elongation step due to the presence of an arrest sequence. This yields shorter RNA fragments after RNase R digestion, which can be specifically amplified and sequenced.

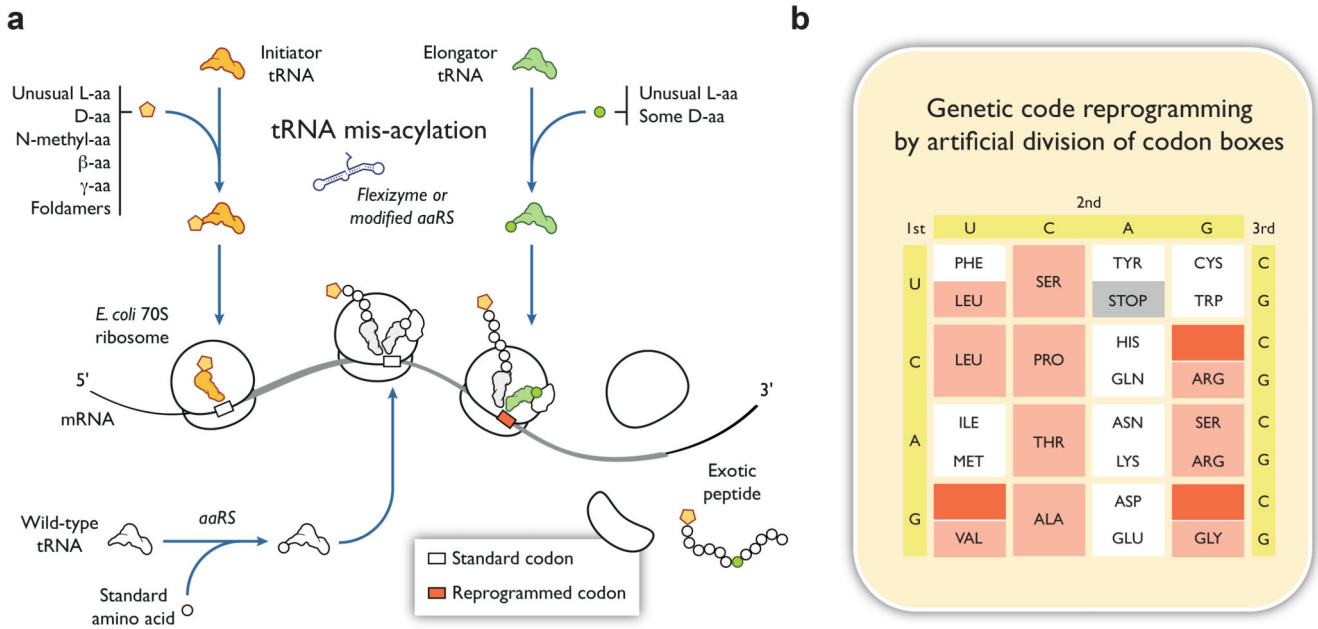

Figure 3. *In vitro* genetic code reprogramming to extend the chemical diversity of ribosomally synthetized peptides.

(a) Flexizyme-based mis-acylation of initiator and elongator tRNAs (yellow and green respectively) with diverse non-proteinogenic groups (top). Addition of the acyl-tRNA to an *in vitro* bacterial translation system allows the synthesis of peptides with both proteinogenic (white) and non-proteinogenic groups at their N-terminus (orange) or at internal positions (green). (b) Genetic code expansion strategies [94, 95] permit the reallocation of some codons to non-proteinogenic groups without compromising the presence of natural amino acids in the final genetic code. For the 'artificial division of codon boxes' approach shown here, codons that have been successfully reprogrammed are shown in red, whereas redundant codons that could theoretically be reprogrammed are shown in salmon.

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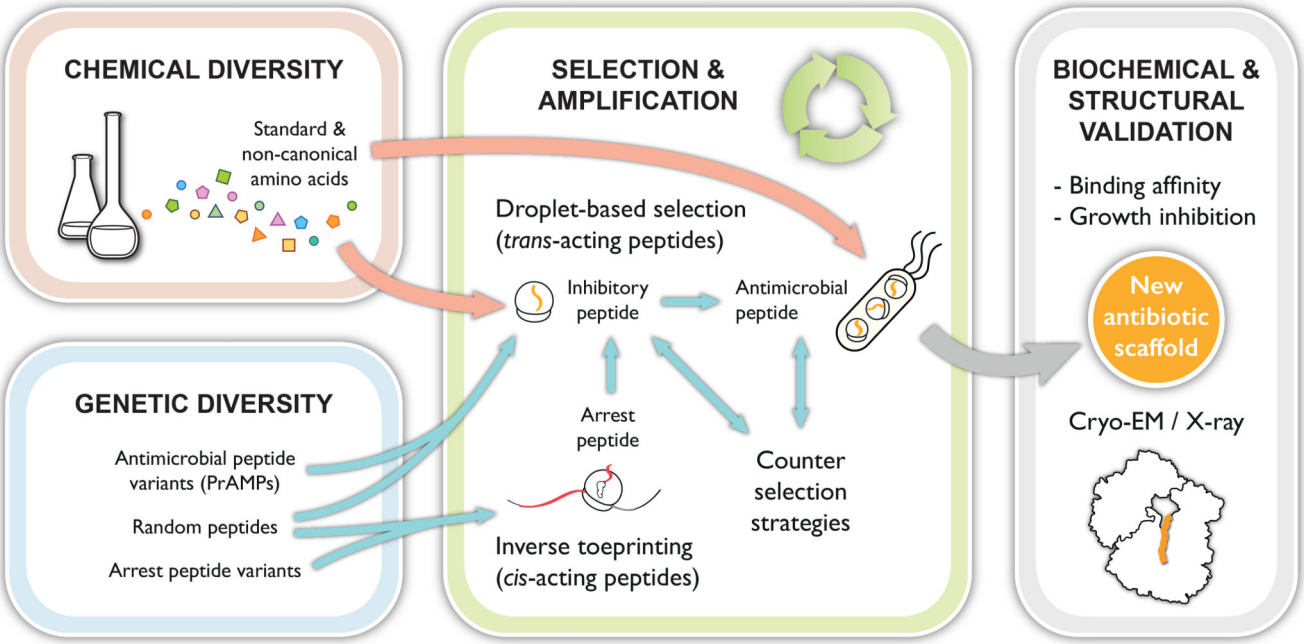

Figure 4. Proposed strategy for the discovery of new peptide-based antibiotic scaffolds that target the ribosome.

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