



Review

Multiple ways to regulate translation initiation in bacteria: Mechanisms, regulatory circuits, dynamics

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ABSTRACT

To adapt their metabolism rapidly and constantly in response to environmental variations, bacteria often target the translation initiation process, during which the ribosome assembles on the mRNA. Here, we review different mechanisms of regulation mediated by *cis*-acting elements, sRNAs and proteins, showing, when possible, their intimate connection with the translational apparatus. Indeed the ribosome itself could play a direct role in several regulatory mechanisms. Different features of the regulatory signals (sequences, structures and their positions on the mRNA) are contributing to the large variety of regulatory mechanisms. Ribosome heterogeneity, variation of individual cells responses and the spatial and temporal organization of the translation process add more layers of complexity. This hampers to define manageable set of rules for bacterial translation initiation control.

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1. Introduction

1.1. Bacterial translational control: fast and reversible adaptation

Bacteria are versatile organisms able to live in a wide range of conditions and to colonize various ecological niches. Their high capacity of adaptation is the result of a robust metabolism [1], which is yet controlled by a network of regulatory circuits linked together to face a large range of conditions [2]. To accustom to environmental changes, bacteria fine-tune their proteome via multi-step regulations at transcriptional and post-transcriptional levels which respond to several extracellular and intracellular stress signals. Regulation of translation has an immediate impact on the proteome and is largely used in stress response mechanisms, as recently demonstrated using a combination of quantitative proteomics and RNA-Seq on different stress conditions in various bacteria [3–7]. These studies have highlighted the poor correlation between the mRNA levels and protein abundance with greater

variations observed for the latter. Indeed, mRNA concentration alone can only explain a fraction of the total variation of protein quantity (50% at most in *Escherichia coli* [5,6], less in other bacteria [8] while protein turnover has only a marginal role [9]. Protein synthesis control can therefore ensure a rapid and transient response to a variety of stimuli or environmental changes, allowing a fast adaptation of cell growth.

1.2. Impact of the transcription–translation–degradation coupling on regulation

Changes in translational efficiency could be correlated with several features on the mRNAs, and some of them affect the translation initiation steps, others the elongation process. Even if codon usage in relation to variation of the tRNAs pool has a strong impact in some stress conditions (i.e. altered O₂ levels [10]) and arrest peptides can stop translation elongation in a manner regulated by environmental cues [11], the majority of the known mechanisms of translation regulation are targeting the translation initiation process [12] during which the ribosome assembles on the mRNA in the order of seconds [13,14]. This slow, rate-limiting step of protein synthesis provides the time window necessary for regulation between mRNA transcription and active translation or mRNA degradation. Indeed, in bacteria, transcription, translation and mRNA degradation are interconnected processes [15–19] with strong impact on regulation and quality control.

Abbreviations: 30SIC, 30S initiation complex; 70SIC, 70S initiation complex; IF, initiation factors; PRE, pH responsive element; r-proteins, ribosomal proteins; RBP, ribosome binding protein; RBS, ribosome binding site; RNA-Seq, sequencing of RNA; RNAP, RNA polymerase; sRNA, small non coding RNA; SD, Shine Dalgarno; aSD, anti SD; ThrRS, threonyl tRNA synthetase; TPP, thiamine pyrophosphate; UTR, untranslated regions.

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Recent single-particle tracking methods and time-resolved quantitative single cell imaging of *E. coli* chromosome and ribosome spatial distributions revealed a possible compartmentalization of the translation machinery. These studies suggest that the ribosomal subunits can initiate translation on the nascent mRNA in the nucleoid dense environment while actively translating polysomes form at the periphery and in close proximity of the membrane [20–22]. In line with the localization of the ribosome at the nucleoid, a direct link between the RNA polymerase and the small ribosomal subunit has been proposed to involve the universally conserved transcription factor NusG and ribosomal protein S10 [17]. The pioneer round of translation is thought to tightly control transcription rate [18] by preventing spontaneous backtracking of RNA polymerase [23,24] to facilitate readthrough of roadblocks *in vivo*. This might imply that transcription is slower in the 5' untranslated regions (5' UTR) and at the translation start sites. Furthermore, the elongation speed of RNA polymerase and specific pauses of the enzyme influence the dynamics of mRNA folding [25–28] and as a consequence the formation of alternative mRNA structures and the access of *trans*-acting regulators may be favoured. It has been hypothesized that this is why the 5' UTRs of numerous mRNAs are structured and have freely evolved dedicated regulatory sites [29,30]. Indeed, mRNA structures at 5' regions strongly impact translation initiation and are therefore target of several mechanisms of regulations. Besides, recent observations also revealed that bacterial mRNAs carry large 3' UTRs, which impact the translation efficiency [31].

1.3. Translation initiation as target for regulation

In contrast to eukaryotes and archaea (for reviews see Refs. [32,33]), the initiation process in bacteria involves a rather low number of *trans*-acting factors. The ribosome together with the aminoacylated and formylated initiator tRNA (fMet-tRNA^{fMet}), the mRNA, and the three initiation factors (IF1, IF2, IF3) assemble in a multi-step process which leads to the consecutive formation of two successive initiation complexes. The “30S initiation complex” (30SIC) is formed in the very early steps at the Ribosome Binding Site (RBS) of the mRNA. The mRNA stably binds the 30S *via* its Shine-Dalgarno sequence (SD) complementary to the anti SD (aSD) sequence at the 3' end of the 16S rRNA [34–36]. For many bacterial mRNAs, the selection of the appropriate initiation codon (the canonical AUG, GUG or UUG) depends largely on the formation of this short SD–aSD helix. The three initiation factors (IF1, IF2, and IF3) kinetically assist the formation of the first specific codon–anticodon interactions between the fMet-tRNA^{fMet} and the mRNA [37]. They cooperate to ensure the correct location of the initiator tRNA on the 30S, proofread the first codon–anticodon interaction and stimulate the transition to the elongation phase (for a review see Ref. [38]). IF1 binds to 30S and promotes a conformational rearrangement [39] which favours the binding of IF3 and IF2 [40]. The GTPase IF2 maintains the initiator tRNA in the correct P/I position which allows fast subunit association [37,41]. IF3 discriminates against non-canonical initiation codon (AUU and AUC) [42–46] exerting this proofreading activity during the joining of the subunits [47]. Once the 30SIC complex is formed, the translational reading frame is set and protein synthesis can start. During the following steps of translation initiation, the joining of the large ribosomal subunit (50S) to the 30SIC leads to the formation of the “70S initiation complex” (70SIC; made by the small and the large ribosomal subunits, the fMet-tRNA^{fMet} and the mRNA), ready for the first peptide bond formation. During this transition, the adjustment of fMet-tRNA^{fMet} in the ribosomal P-site and the release of all factors are coupled with the hydrolysis of the GTP molecule

bound to IF2 [37,48–50]. The formation of the correct 70SIC marks the irreversible transition to the elongation phase.

The binding of the mRNA to the 30S subunit is one of the most critical steps of translation initiation and several aspects of this process are not yet fully understood. For some mRNAs, a first binding with the 30S might occur at the stand-by site (Fig. 1), a site distinct and sometime distant from the RBS on the mRNA [51–54]. Differently from the SD/aSD interaction, this contact is thought to be weak but essential to relocate the 30S at the RBS. Only a few examples of this binding site have been described so far [51–53] and the structural or sequence features are not yet fully understood. It has been proposed that folded regions of the 5' UTRs could interact with a specific positively charged environment of the 30S, its platform. Located between the body and the head of the 30S, r-proteins S2, S7, S11, S18 and S21 form a nest around the aSD of the 16S rRNA [55]. Increasing the local concentration of mRNAs on the 30S platform could kinetically help the formation of more specific interactions, i.e. as soon as the SD will be available [55–57] (Fig. 1). The strength of the SD–aSD interaction obviously depends on the extent of base pairing interactions and on the mRNA SD sequence. This varies largely in mRNAs and the proportion of strong SD containing mRNAs is highly diverse in bacteria [58].

The location of the SD–aSD helix and the path of an unstructured mRNA were solved on the ribosome by X-ray crystallography [56,57]. These studies show that about 30 unstructured nucleotides encompassing the SD and the AUG codon are lying in a groove that encircles the head of the 30S, resulting in the correct positioning of the initiation codon in the decoding centre. The accommodation of the mRNA in the 30S decoding channel (Fig. 1) in *E. coli* is stimulated by a specific ribosomal protein of the platform, S1 [59]. This protein is also important for the docking of weak SD containing mRNAs to the 30S [59,60]. In addition to the SD sequence, several mRNA elements have been shown to affect the kinetics of the initiation complex formation: the nature of the initiation codon, the spacing between the initiation codon and the SD, the non-random distribution of nucleotides upstream and downstream of the SD, and structure or sequence elements present in the 5' UTRs [50,51,61–63]. These elements *per se* are able to fine tune translation interplaying directly with the ribosome. In fact, the IFs seem to have no role in the binding and accommodation of several mRNAs into the 30S decoding channel [40]. Nevertheless, it has been reported that IF2 selectively stimulates the recruitment of leaderless mRNAs [64], a process which take place directly on the 70S [65], while IF3 antagonizes their authentic start codon selection [66], indicating that the translational efficiency of this mRNA class can be modulated depending on the availability of components of the translational machinery. The three factors are expressed in equivalent amounts balanced to the amount of the ribosome in the cell. The genetic mechanism which ensures this concerted expression is still unknown, but cold shock was found to cause a strong (two-to threefold) stoichiometric imbalance of the ratio between initiation factors (IF1, IF2, IF3) and ribosomes without altering the stoichiometric ratio between the factors themselves [67]. In this condition, IF3 has been reported to selectively favour translation of specific cold-shock mRNAs, while IF1 stimulates general translation in the cold. Finally, only for IF3 a feedback mechanism of translational control has been proposed [68].

Structural works [55,69] suggested how the ribosome acts as an active player in translational control. The docking and unfolding of these 5' structured mRNAs on the 30S subunit are critical steps that modulate translation [29]. These mRNA structures, also called translational operators, can directly sense the environmental cues, and/or can be recognized by *trans*-acting factors, which could range from metabolites to *trans*-acting small non coding RNA (sRNA) and

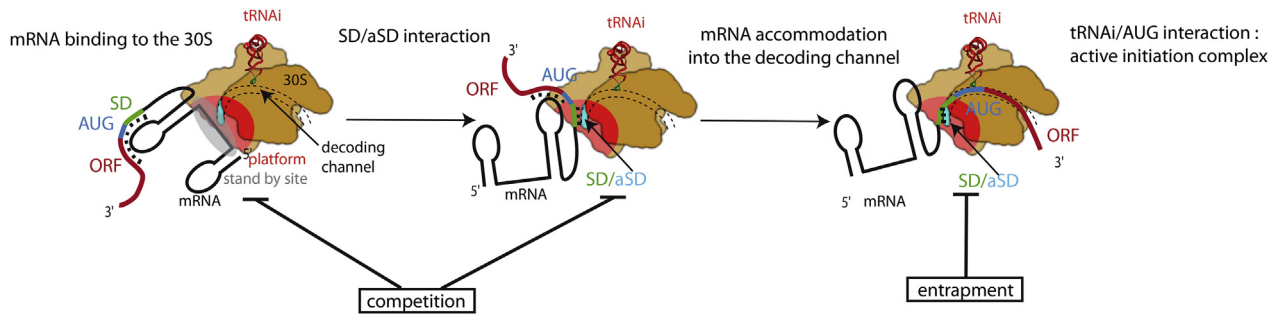


Fig. 1. Schematic view of bacterial translation initiation and regulation. During translation initiation, the mRNA binds the 30S ribosomal subunit (brown) in a process that depends on its sequence/structural features and that can include up to three distinct steps. In chronological order, a first 30S–mRNA interaction could take place at the mRNA stand-by site (grey oval, left part), usually located at its 5' untranslated region (UTR). Then the Ribosome Binding Site (RBS), composed of the SD (SD, green) and the AUG start codon (blue), locates onto the 30S platform (light red) in order to establish SD/antiSD interactions (SD/aSD, in green/cyan, middle part). Several mRNAs contain a SD sequence and a short 5' UTR and can bind the ribosome directly at this step [58]. Finally, ribosomal protein S1 (not shown in the figure) promotes mRNA accommodation in the 30S decoding channel (dotted lines) allowing the interaction between the AUG start codon and the initiator tRNA (tRNA_i). This active 30S Initiation Complex (30SIC) will recruit the 50S ribosomal subunit and elongation will start. Regulation of the initiation of translation includes two major mechanisms: (1) the competition mechanism, in which ribosome–mRNA interactions are inhibited at either the stand-by site or at the RBS and (2) the entrapment mechanism, in which the accommodation step is prevented. Initiation Factors are omitted in the figure for simplicity.

proteins. Regulation is often based on mRNA conformational rearrangements that lead to either repression or activation of translation. Repression of translation involves two fundamentally different mechanisms: (1) the competition model, i.e. a *trans*- or *cis*-acting element prevents binding of the ribosome to the mRNA acting either at the stand-by site or at the RBS, and (2) the entrapment model, i.e. the regulatory factor stalls the ribosome in a dead-end complex (Fig. 1).

Here we will illustrate several examples representative of protein-mediated and sRNA-mediated regulations, and of *cis*-acting regulations, solely relying on mRNA structures.

2. Proteins regulating translation initiation

A large number of translational regulators are RNA-binding proteins (RBPs), which regulate initiation of translation by a variety of mechanisms depending on the location of their binding sites on the mRNA [12]. These proteins can prevent the formation of the 30SIC, and/or induce conformational rearrangements of the mRNA that permit or prevent translation initiation. RBPs have RNA binding domains that can recognize specific RNA sequences, structures or both, and their targets can be restricted to a single mRNA or to a class of mRNAs. The modes of recognition and the stabilities of the complexes are highly diverse; for instance, they can involve stacking and hydrophobic interactions between RNA bases and aromatic amino acids, and/or electrostatic interactions between positively charged amino acids and the phosphate backbone of the RNA.

Here, we provide examples of three mechanisms of negative regulation (Fig. 2) mediated by ribosomal protein S15 (entrapment and direct competition), by the general regulator *E. coli* CsrA (direct competition) and by *E. coli* threonyl-tRNA synthetase (ThrRS; indirect competition).

2.1. Ribosomal protein S15, direct competition or entrapment

All of ribosomal proteins (r-proteins), except L7/L12, have the stoichiometry of one per ribosome and their synthesis is strictly proportional [70] to the abundance of 16S or 23S rRNAs [71] which is under growth rate control [72,73]. In *E. coli*, only a few r-protein genes are individually transcribed whereas most of them are synthesized from large translationally regulated operons. In *E. coli*, combination of auto-regulation and translational coupling by single repressor r-protein allows their coordinated synthesis [71,74]. The

feedback mechanism relies on the ability of the repressor r-protein to recognize specific structures on their own mRNAs (at the 5' UTRs of their mRNA or of other mRNAs of the same operon) which mimic their rRNA binding sites [29,75–79]. Many of the regulatory r-proteins are primary rRNA binding proteins, which interact with the rRNA early during the ribosome assembly (namely, S4, S7, S8, S15, S20, L1, L4, L10–L7/L12, L20) [80,81]. Regulatory activity has been reported also for tertiary rRNA binding proteins such as the r-proteins S1 and S2 [82,83].

Among these regulatory r-proteins, S15 (*rpsO*) auto-regulation has been extensively studied *in vivo* and *in vitro* in *E. coli* and some *in vitro* analyses has been also done with *Geobacillus stearothermophilus* and *Thermus thermophilus* protein. Although S15 is highly conserved [30] a diversity of regulatory mechanisms has been described (Fig. 2A and B). Early during the 30S assembly process, S15 binds the central domain of the 16S rRNA recognizing two adjacent motifs, a three-way junction and a G–U/G–C motif [84,85]. The same surface of S15 is used to interact with *rpsO* mRNAs and to regulate its translation in *E. coli*, *G. stearothermophilus* and *T. thermophilus* [76,86,87]. However, the mRNA determinants for S15 recognition and the position relative to the SD are different [30].

The binding of *T. thermophilus* S15 (TtS15; Fig. 2A) to *rpsO* mRNA triggers the formation of an RNA three-way junction that partially mimics the 16S rRNA binding site. In the stable complex TtS15–*rpsO* mRNA ($K_d \sim 5$ nM) the RBS is no more accessible, thus preventing the formation of the SD–aSD helix on 30S [86] (Fig. 2A). Such a competition mechanism between S15 and 30S has also been proposed for *G. stearothermophilus* S15 [87].

In contrast, the operator region of *E. coli* *rpsO* mRNA adopts a different topology, involving a pseudoknot that sequesters 10 nucleotides of the coding region [55,88]. *E. coli* S15 (EcS15; Fig. 2B) binds to the pseudoknot recognizing a G–U/G–C motif similar to the 16S rRNA and other elements of the pseudoknot that are not present in the rRNA [89]. The complex is less stable ($K_d \sim 200$ nM) than in *T. thermophilus* case. Part of the *rpsO* RBS, including the SD sequence, is located in a large loop accessible for 30S interaction [88,89]. As a result, the SD–aSD helix anchors the EcS15–*rpsO* mRNA complex on the platform of the 30S but translation is inhibited (Fig. 1). This complex was proposed to be a stalled 30SIC where EcS15 stabilizes the pseudoknot blocking the transition to the productive initiation complex [55]. Recent observations suggested that EcS15 counteracts the action of r-protein S1, which is essential to melt the pseudoknot structure to relocate the AUG

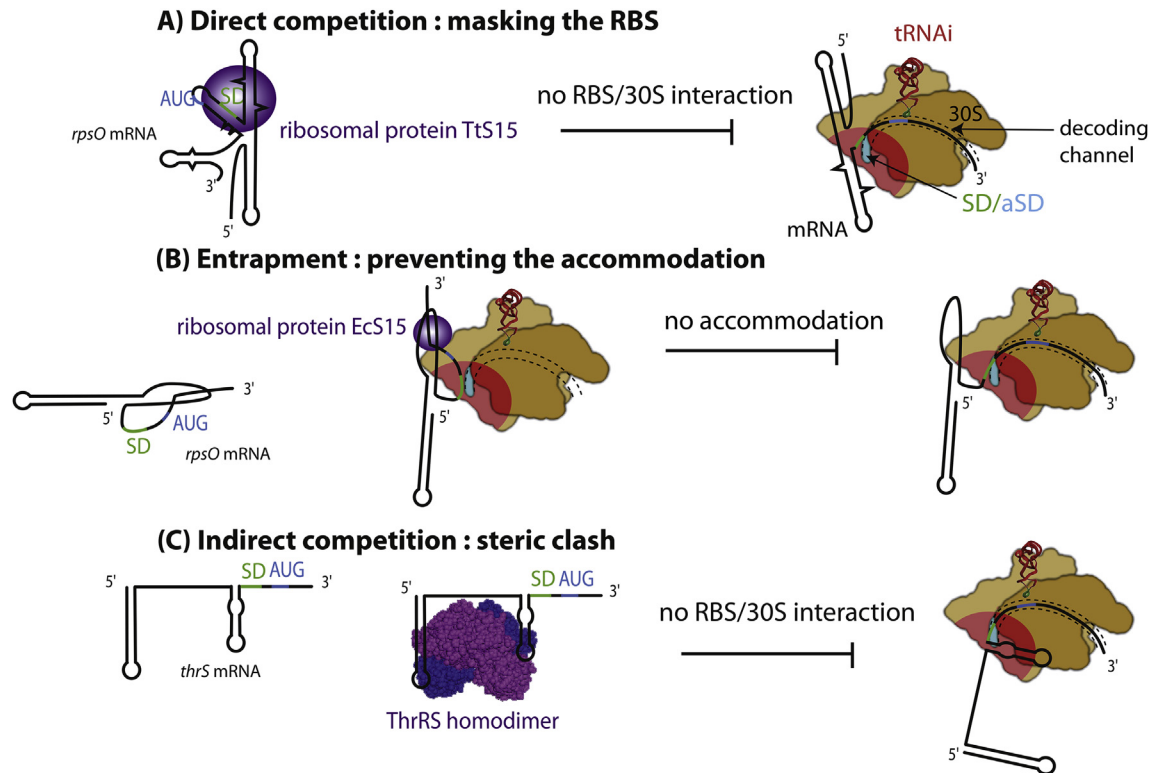


Fig. 2. Feedback regulations by proteins. Autogenous feedback mechanisms rely on the ability of the repressor regulators to recognize specific structures on their own mRNAs and prevent different steps of translation initiation. (A) Ribosomal protein S15 from *T. thermophilus* (in purple) autoregulates its expression by masking the RBS of its own mRNA in a direct competition mechanism with the ribosome (brown). (B) Ribosomal protein S15 from *E. coli* (in purple) prevents accommodation of its own mRNA in the decoding channel (dashed lines) using an entrapment mechanism. (C) *E. coli* Threonyl tRNA synthetase (ThrRS) homodimer (violet/dark blue) binds the mRNA close to its RBS and inhibits ribosome interaction via steric hindrance with its N-terminal region. This is a case of an “indirect” competition mechanism. Colour codes are as in Fig. 1.

codon into the decoding channel [59]. The pseudoknot structure in *rpsO* gene is only conserved in a subset of the γ -proteobacteria suggesting that the entrapment mechanism is conserved in closely related bacteria [81].

The difference between the two translation regulation mechanisms of S15 highlights the importance of the position of the regulatory signals on the mRNAs relatively to the coding region and ribosome binding site. Comparative genomics further identified other potential RNA structure motifs recognized by S15 for regulation in various bacteria, in which are located at different position in *rpsO* mRNA including the coding region [90]. In these cases, new strategy of regulations could be used, demonstrating how regulatory mechanisms can evolve along with mRNA features independently from the strong conservative pressure imposed by the ribosome architecture.

2.2. Global regulators by direct competition or translation activation: the case of *E. coli* CsrA

Differently from the auto-regulatory examples described above, bacteria have also evolved global protein-mediated translation regulation mechanisms which allow contemporaneous modulation of the translation of a large variety of mRNAs in response to environmental changes. A well described example is the *E. coli* major carbon storage coordinator CsrA and its orthologs in *Erwinia carotovora* and *Pseudomonas* RsmA/RsmE (repressors of secondary metabolites). CsrA affects the simultaneous expression of several proteins involved in carbon metabolism, peptide transport, biofilm formation, motility, quorum sensing and virulence (for a recent review see Ref. [91]). The mechanism of translation repression has

been detailed by the NMR structure of CsrA–mRNA complex [92]. CsrA is a homodimer which recognizes two distinct regions on the target mRNAs, a stem-loop containing a GGA motif generally located in the 5' UTRs and the SD sequence also present in an apical loop [93]. The stability of CsrA–mRNA complex varies greatly (~10 nM to ~3 mM, K_d) depending on the sequence and structural context of the GGA binding motifs [94]. It is generally assumed that by binding to the SDs, CsrA would compete against 30S binding [95]. CsrA has been also reported to positively regulate translation of some target mRNAs [96,97], but the mechanism of this activation is not yet clear. In the γ -proteobacteria, the activity of CsrA is antagonized by sRNAs which sequester multiple CsrA dimers away from mRNA via a molecular mimicry mechanism (for a review see Ref. [98]). The *E. coli* CsrB and CsrC sRNAs contain 22 and 13 GGA repeats, respectively, which are predicted to bind several CsrA dimers. The expression of CsrB/CsrC sRNAs is stimulated by CsrA through a two-component system BarA–UvrY, thereby producing an autoregulatory circuit [99,100]. Interestingly, also in *Bacillus subtilis* the activity of CsrA is modulated, but in this case is the protein FlhW that binds to CsrA and inactivates it [101].

2.3. “Indirect” competition with 30S: the case of *E. coli* ThrRS

The expression of aminoacyl-tRNA synthetases (aaRSs) is adjusted to the levels of tRNAs and amino acids in a growth dependent fashion. A well-characterized example is the *thrS* gene encoding the threonyl-tRNA synthetase (ThrRS). In *B. subtilis*, the expression of *thrS* and of most aminoacyl-tRNA synthetase genes is controlled by a T-Box and an anti-termination mechanism at the level of transcription [102] while *E. coli* *thrS* regulated its own

translation at the initiation step [63,103]. This regulatory mechanism has been solved at the atomic level [69], revealing a variation on the theme of translation repression by competition.

E. coli *thrS* mRNA contains a large unstructured RBS and two adjacent hairpin structures mimicking two tRNA^{Thr} anticodon stem-loop [104,105]. When the level of uncharged tRNA^{Thr} decreases, ThrRS homodimer recognizes the two hairpins to hinder the 30S anchoring step (Fig. 2C) [106–111]. ThrRS and the 30S bind to distinct but interspersed domains of the mRNA. In this case, repression of translation resulted from a steric clash between the 30S and the N-terminal domain of ThrRS [69].

Alignment of γ -proteobacterial 5' UTR sequences of the *thrS* gene [107] indicated that a similar feedback mechanism is conserved in bacterial species closely related to *E. coli*. Interestingly, the rather weak SD sequence is also highly conserved. In *E. coli*, it was recently shown that the docking of *thrS* mRNA on the 30S requires r-protein S1 [59]. The replacement of its weak SD with a stronger one alleviates the requirement of S1 for the formation of 30SIC but at the same time weakens the translation repression [59,104]. As for the EcS15, the repressor activity of *E. coli* ThrRS targets one of the functions of r-protein S1 [59].

3. Translation regulation by sRNAs

Bacterial sRNAs vary in size and structure among species, but functionally participate to environmental response pathways (metabolism, virulence, iron homeostasis, etc...). Genes encoding sRNAs are either *cis*-encoded, i.e., synthesised from the opposite strand or *trans*-encoded at a locus different from that encoding the

target(s). *Cis*-encoding antisense RNAs are fully complementary to targets, whereas *trans*-encoding RNAs show only partial complementarity. These *trans*-acting sRNAs have the potential to regulate multiple targets and one mRNA can be regulated by more than one sRNA [112,113]. A key helper protein that is required for the activity of the *trans*-encoded sRNAs, is the RNA chaperone protein Hfq (for review see Ref. [114]). Indeed, Hfq intervenes with sRNA to activate or repress translation, protects sRNA from degradation, or recruits RNase E to cleave sRNA and its target mRNAs leading to an irreversible outcome.

Three different examples of translational regulation by sRNAs in *E. coli* will be described below: two repression mechanisms targeting different steps of the translation initiation process (RyhB/*sodB* affecting 30S docking and IstR/*tisAB* involving repression at the stand-by binding step) and one example of translation activation (DsrA/*rpsO*).

3.1. RyhB/*sodB*, translation inhibition by targeting the RBS and mRNA degradation

Most *bona fide* sRNAs act at the translational level by base-pairing with the 5'-UTR of mRNA thus preventing 30S binding at the SD, which results in translation arrest. In several examples, duplexes formed by sRNA and their targets are often cleaved by ribonucleases (mainly RNase E or RNase III). Repression of *E. coli* *sodB* mRNA by the iron-regulated sRNA RhyB has been extensively studied [115] (Fig. 3A). *sodB* encodes the superoxide dismutase enzyme, responsible of cell protection from oxygen damage and is synthesized only in presence of abundant iron. In contrast, when

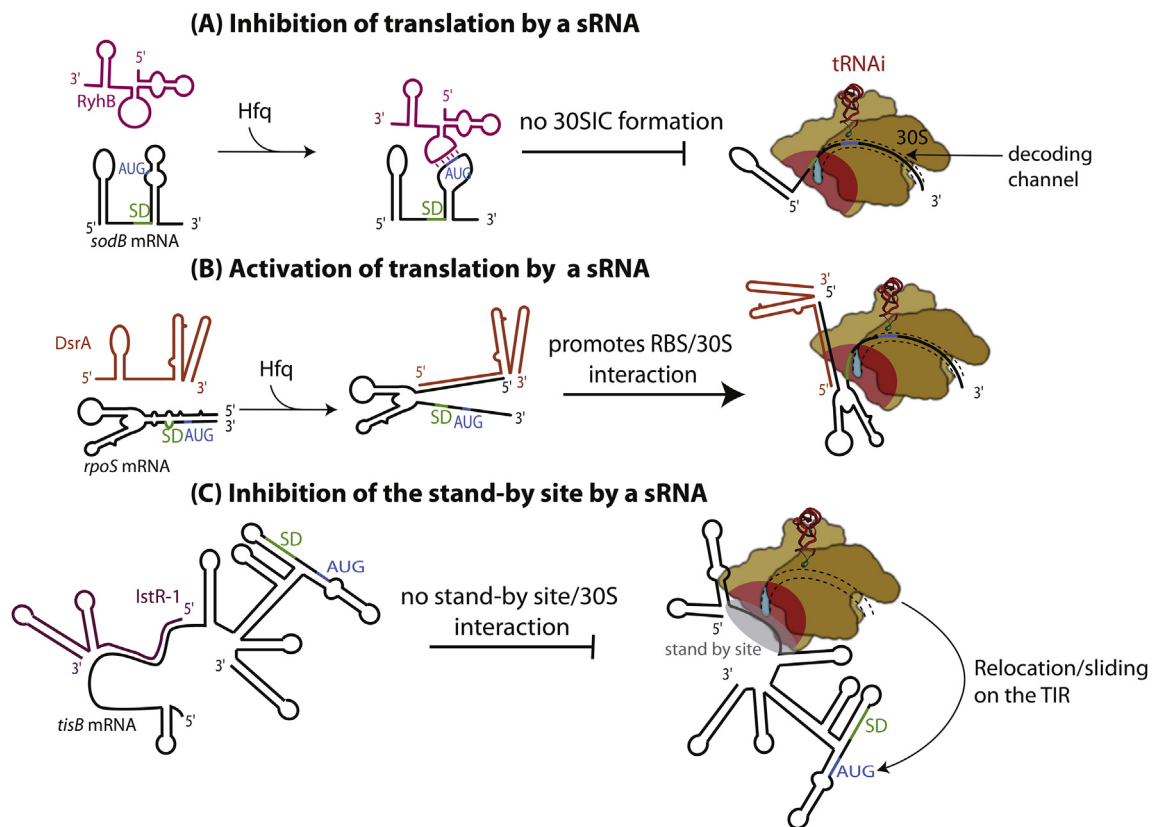


Fig. 3. sRNA mediated regulatory mechanisms. Most *bona fide* sRNAs act at the translational level, by base-pairing with the 5'-UTR of mRNAs thus altering ribosome binding at the SD sequence (RyhB and DsrA), or at the stand-by site (IstR-1). (A) RyhB sRNA (pink) prevents *sodB* mRNA translation with Hfq by targeting *sodB* RBS thus preventing ribosome (brown) recruitment and inducing mRNA degradation. (B) DsrA sRNA (orange) promotes *rpsO* mRNA translation with Hfq by liberating *rpsO* SD from a trapping structure. (C) IstR-1 sRNA (magenta) prevents *tisB* mRNA translation by targeting its stand-by site (grey oval). Colour codes are as in Fig. 1.

iron is low, RhyB is highly transcribed and prevents *sodB* translation, with a mechanism requiring Hfq and RNase E, which is part of the degradosome composed of PNPase, RNA helicase RhlB and enolase [116]. Under iron limitation, RhyB was shown to be essential for normal growth in *E. coli* [117]. Structural analysis of *sodB* mRNA revealed that its RBS is unstructured with both a strong SD sequence and AUG codon directly accessible to the ribosome [59]. Indeed, *sodB* is one typical example of *E. coli* mRNA whose translation does not necessitate the action of S1 [59]. When Hfq and RhyB target the RBS, the ribosomes cannot bind [118]. RNase E is then recruited to a far downstream site in the coding region of *sodB* to initiate cleavage and irreversible repression, which means that mRNA is cleared off translating ribosomes before degradation [119,120]. The model could be generalized to two other mRNAs (*fumA* and *iscRSUA*), where RhyB induces distal mRNA degradation by RNase E.

3.2. *DsrA/rpoS*, translation activation

The most predominant mechanism of sRNA-dependent activation of translation involves melting of a secondary structure sequestering the RBS of mRNA. The mechanism is well exemplified by the regulation of *rpoS* mRNA by DsrA (Fig. 3B) and RprA. *RpoS* encodes the stress induced transcription factor sigma B (σ B), which is translationally repressed under normal condition of growth due to trapping of the SD sequence in a stable structure targeted by RNase III for degradation [121]. However, under cold shock and in the presence of Hfq, base-pairing of DsrA with the 5' leader of *rpoS* liberates the RBS and thus promotes translation of σ B [122]. Based on structure probing, a model of Hfq bound to *rpoS* was recently proposed [123]. This model adopts a compact tertiary structure where the distal face and the lateral rim of Hfq interact with three sites in the *rpoS* 5' UTR. This distorted *rpoS* structure enables its correct positioning adjacent to DsrA binding region present on the proximal face of Hfq. A similar mechanism exists between RprA and *rpoS* under envelope stress and in response to osmotic shock [124,125]. In both cases, *rpoS* mRNA is stabilized in the presence of sRNAs and protected from RNase E degradation [126]. A recent *in vivo* analysis [127] suggested the following mechanistic model: Hfq associates with the helicase CsdA and binds to an A-rich sequence in the leader of *rpoS*, allowing the annealing of DsrA to *rpoS* and efficient translation. Nevertheless, Hfq binding does not seem to be necessary for the interaction of *rpoS* and DsrA with 30S [128]. The same group showed using photochemical crosslinking that the r-protein S1 interacts with both RNAs on the 30S [129] and suggested that S1 might play a role in facilitating translation of sRNA regulated mRNAs.

3.3. *IstR-1/tisB*, translation inhibition by targeting the stand-by site

A very unusual mechanism involves a sRNA, which inhibits the binding of the ribosome at a distant site from the RBS, also called the stand-by site (Fig. 1). The toxin/antitoxin system IstR-1/*tisB* encodes a *tisB* mRNA, a toxin whose transcription is induced by SOS response, which is maintained repressed by the sRNA IstR-1. During the SOS response *tisB* transcription is promoted and the basal level of IstR-1 would not anymore be sufficient for efficient repression. Under these conditions, the accumulation of the toxin would slow down growth, allowing DNA repair. However, under normal conditions, *tisB* is poorly transcribed and further repressed by IstR-1. This sRNA binds to a 23-nucleotide region located 100 nts upstream the RBS in the 5' UTR of *tisB* (Fig. 3C). As a consequence, the mRNA could not be loaded on the 30S [53]. Translation inhibition eventually leads to RNase III dependent degradation [130]. Translation of *tisB* requires accessibility of the stand-by site, which is

made by a stretch of unpaired AU-rich nucleotides bordered by an unstable upstream structure, where the 30S would load to further slide to the RBS and start translation.

The IstR-1/*tisB* system is not the only example of sRNA targeting a region outside the RBS and affecting translation initiation. Among others, the sRNA GcvB regulating more than 40 genes in *Salmonella enterica*, has been found to target a translational enhancer C/A rich motif located at various distances from the RBS [131,132].

4. Translation regulation by *cis*-acting mRNA elements

Cis-acting regulatory elements are special mRNA structures present in 5' UTR or 3' UTR that can affect ribosome binding or prevent the formation of an active translation initiation complex. These structural elements of the mRNAs are able to sense physicochemical signals, like metabolite concentration, temperature and pH. Noteworthy, bacterial transcription–translation coupling is crucial in this fine-tuning mechanism since transcription speed and RNA polymerase pauses kinetically link the RNA polymerisation, RNA folding and translation initiation [133].

The rules governing RNA polymerase pausing are not yet fully understood. However, there are evidences showing how pausing of RNA polymerase at specific locations provides a time-window to modify the RNA folding pathway. This phenomenon prevents the formation of competitive structure in RNAs [134,135]. In this subsection, we describe how bacteria can regulate gene expression to face various environmental stresses using *cis*-acting regulatory mechanism like riboswitches, thermoswitches, thermosensors and pH sensors. We will also illustrate how the 3' UTR can influence translation initiation efficiency in *cis*.

4.1. Riboswitches elements

Present mainly in the 5' UTR of the mRNA, these regulatory elements are widespread in bacteria [136,137], and have been also found in eukaryotes [138–140]. Riboswitches are defined as RNA structure sensors for metabolites, cofactors and ions (reviewed in Refs. [141,142]). They are composed of two regions: the «aptamer domain» where the metabolite binds once a threshold concentration is reached, and the «expression platform» which triggers the regulatory response. By forming a Rho-independent terminator or an anti-termination stem-loop, they can modulate transcription (especially in Gram-positive) [137,143]. Alternatively, riboswitches can act on translation initiation efficiency by trapping or liberating the RBS. This is the major mechanism existing in Gram-negative bacteria [144].

A typical example of riboswitch is the thiamine pyrophosphate binding riboswitch called «thi-box» (TPP, coenzyme B1, [145–147]). Genes containing this riboswitch element in the 5' UTRs are able to sense the intracellular level of TPP and control the downstream gene at either transcriptional or translational levels [148]. The *E. coli* *thiM* mRNA encoding for hydroxyethylthiazol kinase, an enzyme implicated in TPP production and transport [147], is regulated at the translational level. Indeed, the binding of the TPP metabolite to the aptamer region induces a structural refolding of the expression platform, that in turn traps its RBS into an inhibitory stable hairpin (Fig. 4A; [148–150]).

For some riboswitches, metabolite binding likely occurs during the transcription of the riboswitch, thus dictating the structure of the nascent mRNA [151]. On a dynamic point of view, the comparison of the ligand affinity, the transcription speed and the time needed by RNA to refold after ligand binding implicate that transcriptional pauses are necessary to increase the switch efficiency [151].

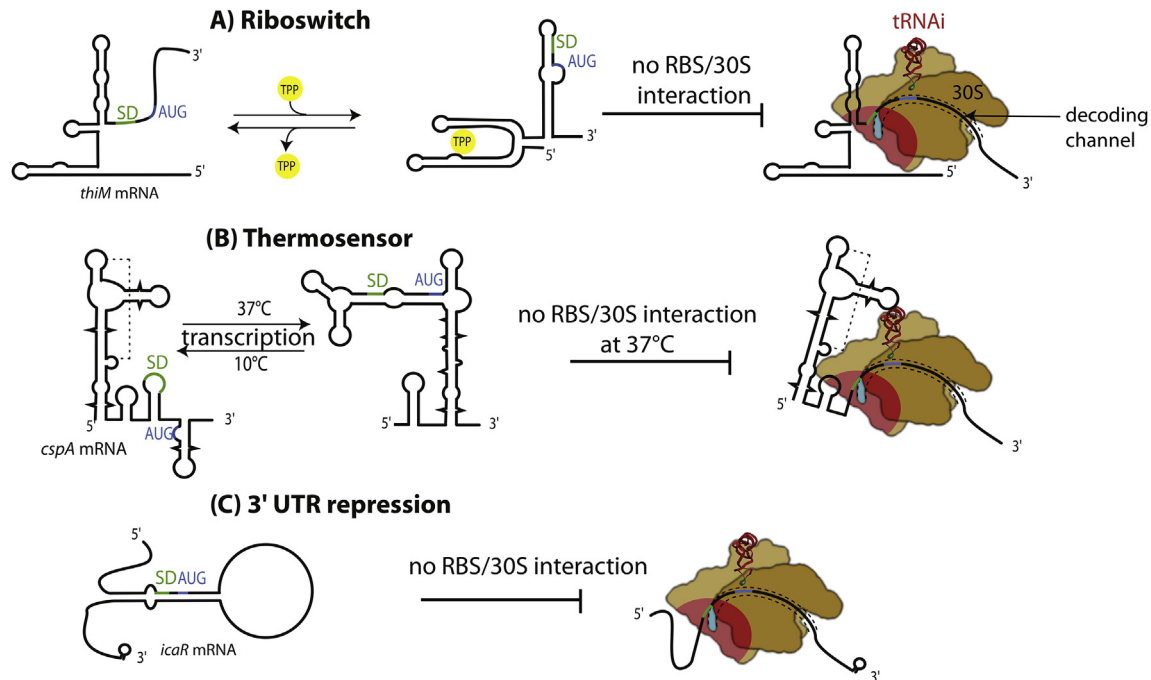


Fig. 4. Regulation by *cis*-elements. Translation can be regulated by RNA structures borne by the mRNA itself, which respond to physicochemical variations like metabolites concentration, pH, or temperature. The mRNA structure modulates RBS accessibility, influencing translation efficiency. (A) Binding of thiamine pyrophosphate (TPP, yellow) to the aptamer region of the riboswitch located in the 5' UTR of *thiM* mRNA turns off its translation. The RBS accessibility on the expression platform is thus reduced via the formation of an alternative structure. (B) *CspA* translation is activated at low temperature by a mechanism which involves a thermosensor element. Transcription in the cold of *cspA* mRNA stabilizes a pseudoknot intermediate which favours the open conformation of the nearby RBS. At 37 °C *cspA* mRNA folds differently. Its SD is buried in a stable structure which prevents ribosome binding. (C) The long 3' UTR of *icaR* mRNA represses its own translation by long range interactions with its 5' UTR and RBS. All mRNA are depicted in black. Colour codes are as in Fig. 1.

4.2. Thermosensors and pH sensitive elements

As bacteria are able to live in a wide range of conditions, adaptation to temperature changes is crucial to face their environment [152]. It is particularly true for pathogenic bacteria, which must respond to new conditions, when they enter their host [153,154]. Thermosensors or RNA thermometers (RNAT) are riboregulators, often situated at the 5' UTR of heat or cold shock proteins, or virulence factors [153], which modulate the accessibility of downstream RBSs in response to temperature changes. Temperature variations have different impacts on the dynamics of RNA folding and several mechanisms respond to heat or cold shock conditions (reviewed in Ref. [155]).

Thermosensors, like the 5' UTR-RBS region of *Listeria monocytogenes prfA* [154], are hairpin structures, which contain a proper balance of stabilizing G–C base pairs and destabilizing mismatches and bulges. This structure gradually melts with increasing temperatures in a zipper-like manner initiated by the melting of the non canonical base pairs [155–157]. These motifs have been first demonstrated in the 5' UTRs of heat shock genes and have been named ROSE (repressor of heat-shock gene expression) elements. ROSE are hairpins composed of non-canonical base pairs (G–U), which are highly sensitive to heat, and melt when temperature rises thus exposing the RBS [158,159]. It is not known whether a 30S-stand-by site is present in the vicinity of the RBS that would increase the efficacy of translation at higher temperature [29].

Other thermosensors operate through a switch-like mechanism that is dependent on the transcription. These are RNA elements, which adopt distinct structures when transcribed at different temperatures. The best characterized example is *E. coli cspA*, the major cold shock protein. This RNA chaperone accumulates in the cold to favour several RNA related processes at low temperature

[160,161]. The structure of several *cspA* mRNA fragments mimicking the transcriptional process has been probed using chemicals and enzymes at different temperatures [162]. When transcribed at 10 °C, the mRNA adopts a pseudoknot structure, which exposes the SD and the start codon and enhances translation of *CspA* in the cold (Fig. 4B). Conversely, transcription at 37 °C favours a conformation in which the SD is masked within a double stranded region, and this structure was shown to be poorly translated. RNA transcription speed is highly influenced by the temperature. Indeed, a single-molecule kinetic study of *E. coli* RNA polymerase (RNAP) at different temperatures showed a five times higher pause density at cold temperatures (7–20 °C) than that measured above 20 °C [163]. Such pauses could modify the folding dynamics of the RNA [27,164]. It is noteworthy to mention that *cspA* pseudoknot folded in the cold could easily adopt the 37 °C conformation, when temperature increases [162]. Therefore, it was postulated that *cspA* mRNA undergoes a temperature-dependent structural rearrangement, which result from stabilization in the cold of an otherwise thermodynamically unstable folding intermediate, the pseudoknot structure.

A very similar study revealed the importance of RNA polymerase pauses on the dynamics of RNA folding in response to changes of pH. This “pH responsive element” (PRE) was first demonstrated in *E. coli alx* gene [28,165,166]. Under normal growth conditions, *alx* mRNA is transcribed into a translationally inactive structure. Conversely, when transcribed under alkaline conditions, PRE forms an active structure producing the Alx protein. RNA polymerase transcriptional pauses were modified at high pH and as the results folding of the active structure was facilitated. Specific sequence elements seem to be responsible of the pH-dependent pauses [28]. These sequences have also been found in the riboswitch *yybP–ykoY* located at the 5' UTR of other bacteria such as

Staphylococcus aureus SA0878 gene, encoding a transporter protein [137,167]. However, whether this riboswitch responds to pH variations, remains to be addressed.

4.3. 3' UTR regulatory elements affecting translation

In bacteria, the importance of 5' UTR for the regulation of gene expression has been extensively described, but the role of the 3' UTR is less well understood. These regions are known to be important for mRNA stability, as intrinsic Rho-independent terminator stem-loop stabilizes the transcript [168,169]. At least in three cases, it was shown that the 3' UTR has an impact on translation efficiency: *S. aureus* *icaR* [31]; *S. aureus* RNAIII [170]; *E. coli* *hok/sok* [171]. Despite the notion that large 3' UTRs are mostly found in eukaryotes [172], recent genome-wide studies showed that they are also frequently found in bacteria like *S. aureus* [31], *L. monocytogenes* [143], and *B. subtilis* [173].

IcaR is the repressor of the *icaADBC* operon involved in the synthesis of the main biofilm matrix exopolysaccharide. Its mRNA exhibits a 390 nt-long 3' UTR with regulatory functions [31,174]. Autogenous regulation of *icaR* is possible since is a poorly translated mRNA. It is possible that, as in the case of the similar *hok/sok* system [175], a transient hairpin involving the SD or more in general the RBS, could form during RNA synthesis of *icaR*, thus preventing premature ribosome binding until the 3' UTR is synthesized. When completely transcribed, a C-rich motif of its 3' UTR recognises and binds the SD reducing ribosome loading (Fig. 4C), and inducing mRNA degradation by RNase III [31]. Whether base pairings between the 3' and 5' UTRs occur on the same *icaR* mRNA or between two mRNA molecules is not known. It is also not yet clear how activation of translation would work. It is possible that induction of *trans*-acting factors could relieve the 3'-UTR away from the 5'-UTR to change the RBS accessibility. Although

neither Hfq nor RNA helicase (YqfR) are involved [31], other RNA binding proteins or unknown sRNAs might participate in the activation process in response to environmental signals. Because 35% of *S. aureus* mRNAs contain 3' UTR longer than 100 nts and 68% have a longer 3' UTR compared to the 5' UTR, novel regulatory mechanisms are expected to be discovered [31].

5. Perspectives: the ribosome as a regulatory machine and the deciphering of the translation regulation code

The rules governing mRNA–ribosome interactions are not yet fully understood and the presence of specific signals close to RBSs leaves open the possibility that the ribosome itself could play a direct role in several regulatory mechanisms. Indeed, in some cases its involvement in the regulatory complex has been clearly proven. The best example is the entrapment mechanism of EcS15 (Fig. 2B) which takes physically place on the 30S subunit [55]. The direct visualization of this ribosome–mRNA–repressor regulatory complex underlined the importance of the 30S platform as dedicated binding site for translation initiation (Fig. 1) and regulation. This region between the head and the body of the 30S (Fig. 5) is formed by several r-proteins (S1, S2, S7, S11, S18 and S21) together with two rRNA helices (h26 and h40) that are surrounding the aSD close to one extremity of the decoding channel. Since both r-protein S1 and the aSD are on the platform, we can imagine that several mRNAs would transit on this site before getting into the mRNA decoding channel. A systematic structure and sequence analysis revealed that conserved residues of the r-proteins of the platform form patches of positive charges on its surface that could localize folded mRNA regardless of their specific structure or sequence [55]. Interestingly, it has been observed that even sequences that have low propensity to form structures, like poly(A) or poly(U), get folded into compact structures similar to hairpins close to r-protein S2

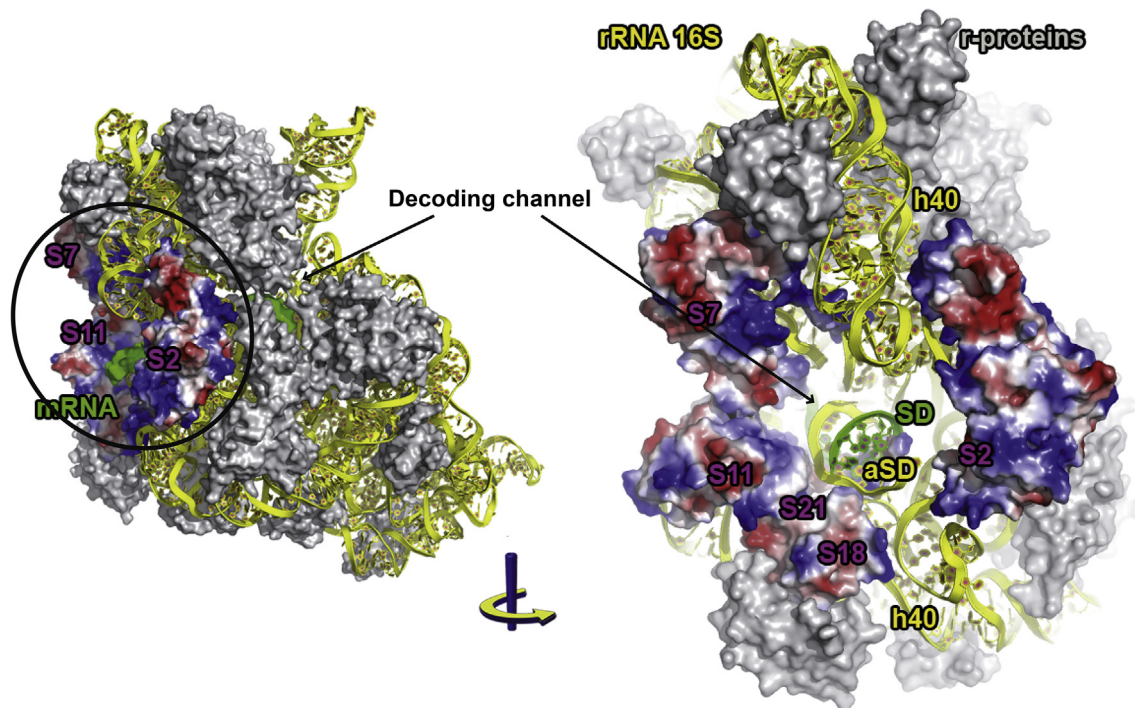


Fig. 5. The platform of the 30S. On the left, view of the 30S subunit showing the localization of the platform. On the right, enlarged and rotated view of the platform showing the charge distribution of the r-proteins composing it. S2, S7, S11, S18 and S21 are forming a nest of positive charges (blue surfaces) around the aSD. Negatively charged amino acids are represented in red. The mRNA (green) is shown accommodated into the decoding channel. The model in the figure has been obtained using the coordinates of *E. coli* ribosome (pdb file 4TP8 [188]) which contains all the r-proteins of the platform including S21 and the mRNA pathway described in the *T. thermophilus* translation initiation complex (pdb file 4HGR [56]).

[56]. Furthermore, at least in *E. coli*, r-protein S1 is known to have RNA chaperone activities which also contribute to remodelling of mRNA structures in different ways [59,176,177]. Are these features of the platform exploited by regulatory mechanisms distinct from the EcS15 entrapment?

Recent observations have suggested a possible role of the ribosome in sRNA-mediated regulation. In *E. coli*, it was demonstrated that RhyB-dependent repression of *sodB* translation is enhanced if the translation rate of the mRNA target is increased [178]. In other words, the repressor sRNA appears to be more efficient in the presence of actively translating polysomes. This phenomenon is observed only if the SD and the sRNA binding site are in close proximity. Based on these facts, it was proposed that the ribosome might recruit RhyB for targeting the mRNA. However, another possibility might be that the sRNA traps the ribosome into an inactive complex similarly to EcS15. Indeed, because the SD of *sodB* mRNA is still accessible in the complex formed with RyhB (Fig. 3A), it could potentially anchor the regulatory complex through the formation of the SD–aSD helix on the 30S platform. In this case, RyhB binding would inhibit translation by preventing *sodB* accommodation into the decoding channel. Hence, the platform is a well appropriate site where *trans*-acting regulators (sRNA or proteins) could interfere with the ribosome functioning at the initiation step.

Another important feature is the fact that the translation speed has been reported to affect regulation. *E. coli* SgrS sRNA is produced during glucose-phosphate stress, which is characterized by the accumulation of phosphosugars like glucose 6-phosphate (reviewed in Ref. [179]). SgrS negatively regulates the translation of *ptsG* mRNA via base pairing interactions with the RBS in an Hfq-dependent manner and stimulates the rapid degradation of the repressed mRNA by an RNase E-dependent mechanism [180,181]. It was also reported that the ability of SgrS to pair and to induce rapid mRNA degradation required the mRNA target to encode an integral membrane protein. The requirement for membrane targeting of the *ptsG* mRNA to allow SgrS to function as a repressor might be linked to the slow translation during targeting.

Protein levels are also modulated by the interplay between ribosome and mRNA features, even when no regulators or switches are involved. Growth conditions exert a passive control over translation of mRNAs with different SDs or start codons [182]. This non-specific regulation superimposes upon the specific, regulatory loops. Even in the absence of any stress, the abundance of functionally related proteins, which are working in complexes or are part of the same metabolic pathway, should be carefully balanced. These genes are often clustered into transcription operon units [183] and the differential expression of individual cistron is obtained at translational level. Genes on the same operon may exhibit large differences in their expression and their levels can vary independently (i.e. the *dusB*–*fis* operon [184]). More often, they encode for multi-subunit complexes with uneven stoichiometry and their relative levels are the results of very accurate differential translation [185]. A classic example is the *rplJ*–*rplL* operon encoding the single copy ribosomal protein L10 (*rplJ*) and the multicopy ribosomal protein L12 (*rplL*) composing the L7/L12 stalk of the large ribosomal subunit, respectively [186]. The measure of absolute protein synthesis rates, based on ribosome profiling, confirmed a four time higher translation for *rplL* than for *rplJ*. This study revealed that this phenomenon is not an exception, because other members of multi-protein complexes are made in precise proportion to their stoichiometry, whereas components of functional modules are produced differentially according to their hierarchical roles [70]. In these cases, no induction of regulators is involved and the different levels of translation seem to be dictated simply by the interplay between ribosome and mRNA features.

Furthermore, it has been recently proposed that a limited set of distinct ribosome–mRNA interactions can modulate translation initiation rates by over 100-fold [54]. Protein level variations have been predicted via biophysical modelling that takes into account only mRNA–ribosome interactions [54]. These models have been used to build synthetic biology circuits [187] although we need to accumulate more knowledge to get a full appreciation of the functional importance of these variations. The definition of a manageable set of rules to decipher bacterial translation initiation regulation is hampered by the large variety of mechanisms of regulations and by the different nature (sequence/structure) of the mRNA regulatory signals. The list of mechanisms that control protein synthesis levels continues to grow and more mechanistic studies will be necessary to determine a possible code for translation control. Ribosome heterogeneity (discussed elsewhere in this special issue) and variation of individual cells responses are also adding other levels of complexity in bacterial translation regulation. Moreover, the recent advancement in single-particle tracking super-resolution microscopy methods has evidenced a spatial and temporal organization of the translation process in bacteria [19–22], and more specific investigation would be necessary to understand the impact of such organization on translation regulation.

Conflict of interest

The authors declare no conflict of interest.

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