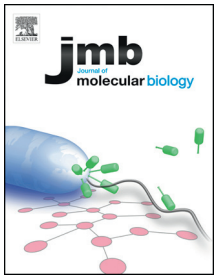

Ribosome Shut-Down by 16S rRNA Fragmentation in Stationary-Phase *Escherichia coli*

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Abstract

Stationary-phase bacterial cells are characterized by vastly reduced metabolic activities yielding a dormant-like phenotype. Several hibernation programs ensure the establishment and maintenance of this resting growth state. Some of the stationary phase-specific modulations affect the ribosome and its translational activity directly. In stationary-phase *Escherichia coli*, we observed the appearance of a 16S rRNA fragmentation event at the tip of helix 6 within the small ribosomal subunit (30S). Stationary-phase 30S subunits showed markedly reduced activities in protein biosynthesis. On the other hand, the functional performance of stationary-phase large ribosomal subunits (50S) was indistinguishable from particles isolated from exponentially growing cells. Introduction of the 16S rRNA cut *in vitro* at helix 6 of exponential phase 30S subunits renders them less efficient in protein biosynthesis. This indicates that the helix 6 fragmentation is necessary and sufficient to attenuate translational activities of 30S ribosomal subunits. These results suggest that stationary phase-specific cleavage of 16S rRNA within the 30S subunit is an efficient means to reduce global translation activities under non-proliferating growth conditions.

Introduction

Stationary-phase cells of bacterial cultures have often been regarded as rather static and homogenous organisms possessing little or no metabolic activity. However, recent years have led to an increasing interest and appreciation of the possibility of single-cell heterogeneity in various biological processes, including stationary-phase cells [1,2]. One of the examples of phenotypic heterogeneity in bacterial populations is persistence [3]. Persisters are a subpopulation of dormant cells that can survive antibiotic treatment due to their resting metabolic state and are capable of resuming growth once the inhibitory drug concentration has declined. During the last decade, several studies have been carried out to describe the physiology and molecular mechanisms causing metabolic shut-down in non-dividing, dormant bacteria (Refs. 3, 4 and references therein). However, so far no clear-cut picture emerged and it remains largely unknown how bacteria manage to establish the dormant or persister phenotype on the molecular level [5–7]. In a stationary-phase bacterial population, nutrients are limited and thus bacteria respond to these environmental changes by a controlled downregulation of metabolic activities. In general, bacterial stress response systems are often, but not exclusively, executed at the transcriptional level. In *Escherichia coli*, a very prominent player during stationary-phase entry is the sigma factor RpoS which guides the RNA polymerase to genes crucial for stress adaptation and has been shown to regulate the expression of about 10% of genes [8]

More recently, regulatory small non-protein-coding RNAs (ncRNAs or sRNAs) appeared on the scene of stress response in prokaryotes (reviewed in Ref. [9]). These ~70- to 500-nt-long sRNAs have been shown to regulate the access of the translation machinery to and the stability of stress response-relevant mRNAs including *rpoS*, *ompA*, *ompC* and *ompW* (reviewed in Refs. 10, 11). The main advantage of sRNA regulators

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compared to regulatory proteins is their immediate availability since these molecules function at the RNA level and thus do not have to be translated into proteins prior to becoming effective. Thus, sRNAs are believed to allow a swift response to changing environmental conditions and are therefore considered pivotal for the first wave of stress adaptation.

Very recently, we have discovered a so far largely unknown class of small ncRNAs that are capable of directly binding to the ribosome and regulating protein biosynthesis on the global level [12]. These so-called ribosome-associated ncRNAs (rancRNAs) have been found in organisms spanning all three domains of life and some of them have been shown to function as rapid modulators of gene expression on the translational level upon changing environmental inputs [13,14]. The ribosome, the central enzyme of protein biosynthesis in all cells, appears to be an ideally suited target for regulatory input. The so far characterized rancRNAs are functional only under particular environmental conditions and can, upon ribosome association, downregulate global protein biosynthesis. Thus, we reasoned that such ribo-regulators might also be at work in stationary-phase *E. coli* cultures to adjust metabolic activity under these non-proliferative conditions. To address this issue, we have deep-sequenced the small RNA population that co-purified with *E. coli* ribosomes during exponential growth as well as during early and late stationary phase.

Here we describe the processing of 16S ribosomal RNA (rRNA) close to the 5′ end, thus generating an ~80-nt-long fragment. This rRNA fragment is specifically generated during stationary phase and remains bound as integral part of the small 30S ribosomal subunit. Ribosomes carrying 30S particles with split 16S rRNA showed markedly reduced translational activity. Thus, 16S rRNA fragmentation within 30S ribosomal subunits might represent a means of adaptation to stationary-phase conditions.

Results

16S rRNA fragmentation in helix 6 of 30S ribosomal subunits

Bioinformatics analyses of the high-throughput sequencing reads of rancRNAs in the size range between 20 and 300 nt were performed using a custom-made version of the automated computational pipeline APART [15]. Particular emphasis was given to RNA reads overrepresented in stationary-phase *E. coli*. The most abundant RNA evident in stationary phase compared to exponentially growing cells was an ~80-nt-long RNA fragment mapping to the 5′ end of 16S rRNA (Fig. 1a and b). The most prominent cleavage sites are situated at positions 78 and 79 located immediately upstream of the terminal loop of 16S rRNA helix 6, a secondary structure element also referred to as the spur. The obtained sequence reads further indicate minor processing sites at 16S rRNA residues 89 and 90. Only very few sequencing reads of this 16S rRNA fragment were evident during exponential growth (Fig. 1a). A very similar 16S rRNA fragmentation pattern was observed when the growth experiment was performed in 3-(*N*-morpholino)propanesulfonic acid (MOPS) minimal medium instead of LB medium (Fig. S1), suggesting a physiologically relevant processing response rather than a growth medium-dependent degradation event. *E. coli* 16S rRNA contains five variable nucleobase positions at the cleavage sites in helix 6 that can be grouped into three allelic types, thus representing a region of 16S rRNA microheterogeneity (Fig. 1c). The putative 16S rRNA processing site within the context of the available bacterial 70S crystal structure [16] is located at the surface of the 30S particle and is thus easily accessible from the solvent side (Fig. S2).

16S rRNA fragmentation is specific for stationary phase

To corroborate the deep-sequencing results and to verify the existence of the ~80-nt-long RNA fragment in *E. coli* cells, Northern blot analysis was performed. While the signal for the 16S rRNA fragment was weak in RNA preparations from exponential phase ribosomes, a band of approximately 80 nt was easily detectable when RNA was extracted from stationary-phase 70S ribosomes (Fig. 2a) or 30S subunits (Fig. 2b). It can be argued that the 16S rRNA cleavage might occur only during the rather lengthy ribosome preparation including several centrifugation steps and thus might represent a non-physiological fragmentation event. However, a very similar 16S rRNA processing pattern was observed when the northern blot was repeated with a total RNA preparation of stationary *E. coli* cells, a procedure that is much faster and less challenging for the integrity of RNA molecules in general (Fig. S3). In addition, analysis of total RNA and ribosome-derived libraries showed also very similar 16S fragmentation patterns (Fig. 1a). These data allowed us to conclude that the 16S rRNA fragmentation occurs specifically in stationary phase and the produced ~80-nt-long rRNA fragment remains integral to the 30S and 70S ribosomal particles (Figs. 1 and 2a and b). To gain insight into the extent of 16S rRNA cleavage, a quantitative reverse transcription approach (also known as poisoned primer extension analysis) was utilized (see Materials and Methods). It turned out that in accordance with the northern blot analysis, 16S rRNA cleavage was dependent on the length of the stationary phase and reached a maximum cut efficiency of about 80% 16S rRNA cleavage after 40–48 h post-inoculation in LB medium (Fig. 2c). In late stationary phase, the 16S

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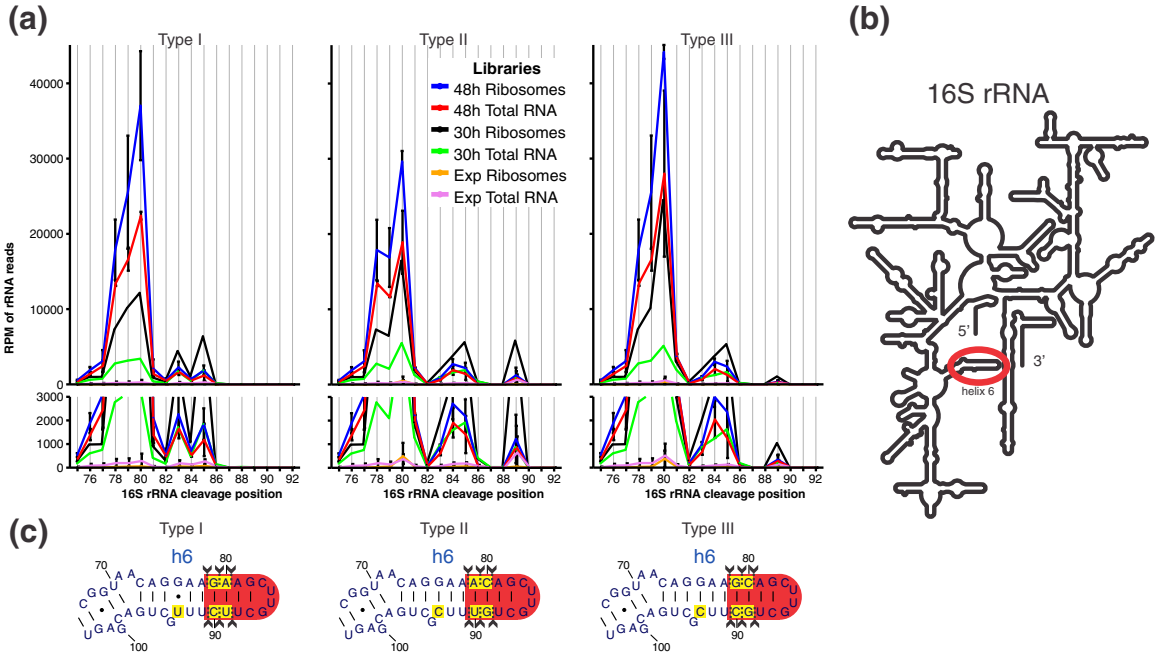

Fig. 1. Bioinformatics analysis of 16S rRNA-derived sequencing reads. (a) Reads from LB media culture-derived libraries which mapped exactly to the 5′ end of the *E. coli* 16S rRNA were counted according to their 3′ end position allele-wise. Reads mapping to several 16S rRNA operons were given fractional values resulting in a total value of 1 per read. To compare different libraries, the read counts were normalized to all reads mapped to the genomic regions of rRNAs and given as read per million (RPM) per allele. The mean and standard deviation of two biological replicates are given (except 30-h libraries with one sample). Libraries were derived from ribosome-associated RNA or from total RNA isolated from the exponential phase (Exp; orange and violet) or stationary-phase cultures at the indicated time points post-inoculation (30h, black and green; 48h, blue and red). (b) Secondary structure *of E. coli* 16S rRNA with the spur (helix 6) highlighted in red. (c) *E. coli* 16S rRNA alleles are combined into allelic types of helix 6. Variable regions in helix 6 are given as yellow, the cleaved off fragment as red and potential cleavage sites are marked with arrows. Type I (rrsA, rrsB, rrsE, rrsH), Type II (rrsC,rrsD), Type III (rrsG).

rRNA spur cleavage is so efficient that the shortened 16S rRNA lacking the 5′-terminal ~80 residues can be separated from full-length 16S rRNA and visualized in denaturing gels (Fig. 2d).

30S subunits carrying fragmented 16S rRNA impair efficient translation

To gain insight into the functional consequence of spur (helix 6) cleavage for ribosome functioning, we isolated 30S subunits from stationary-phase cultures *via* sucrose density gradient centrifugation (Fig. S4). From the gradients, it became furthermore evident that stationary-phase 30S subunits remain intact and sediment as a single sharp peak indistinguishable from exponential phase particles. The purified 30S subunits were subsequently joined with large 50S ribosomal subunits isolated from exponentially growing cells to yield reassociated 70S ribosomes for *in vitro* translation reactions [17]. To initiate *in vitro* translation, the ribosome-free S100 cell extract from exponentially growing *E. coli* cells was used [18,19]. This experimental set-up thus guarantees that solely the used 30S particles originate from the stationary phase. Using these ribosomes in the poly(U)-directed poly(Phe) synthesis approach [20] revealed markedly reduced product yields that correlated with the length of the stationary phase (Fig. 3a). Reversing the experimental set-up by combining stationary-phase 50S subunits and exponential phase 30S particles did not disclose deficits in poly(Phe) synthesis (Fig. 3b). This demonstrates that certain 30S alterations occurring during stationary phase, possible 16S rRNA fragmentation, are responsible for the reduced *in vitro* translation activities. A very similar activity profile was apparent when a physiologically more relevant genuine mRNA, coding for ribosomal protein L12, was used for *in vitro* translation (Fig. 3c and d). These data highlight that 30S subunits isolated from different time points of stationary phase correlate with reduced protein synthesis activities. Even though we did not observe any other 16S rRNA fragments with comparable abundance and specificity in our deep-sequenced cDNA library, we cannot exclude the possibility that additional 16S rRNA cuts might occur during stationary phase that could also contribute to the reduced activities. In addition, stationary phase-specific modification of rRNA [21] or ribosomal proteins

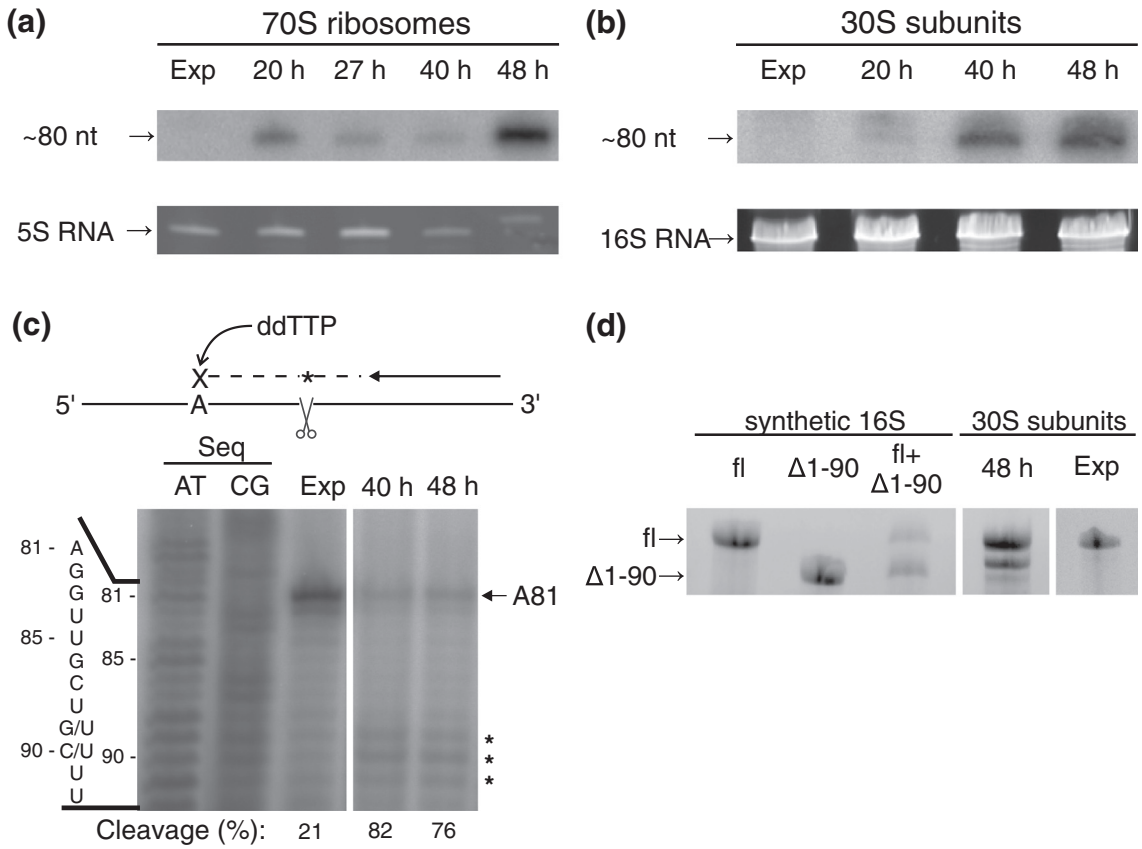

Fig. 2. 16S rRNA fragmentation occurs in stationary-phase 30S subunits. (a) Northern blot analysis on RNA isolated from 70S ribosomes from exponentially growing cells (Exp) or stationary bacteria after 20-48 h post-inoculation in LB medium. The presence of the ~80-nt 5′ 16S rRNA fragment is indicated by an arrow. The ethidium bromide-stained 5S rRNA serves as loading control. Note that the slight upward shifted 5S rRNA signal in the 48-h lane results from a gel artifact (ripped gel). (b) Northern blot analysis on RNA isolated from density gradient purified 30S subunits. The ~80-nt 5′ 16S rRNA fragment is indicated by an arrow. The ethidium bromide-stained 16S rRNA serves as loading control. (c) Cleavage efficiencies in helix 6 of 16S rRNA were quantified using a modified primer extension approach (see scheme included above the gel and Materials and Methods) in the presence of ddTTP which causes a reverse transcription stop at the first encountered adenosine of the 16S rRNA template (A81; X in the scheme). Primer extension products were separated on 12% denaturing polyacrylamide gels. Cleavage efficiencies at the three positions indicated by asterisks (residues 89, 90, 91) were quantified relative to the band intensity of A81. The quantified cleavage efficiencies are given in % below the respective lanes. Seq denotes dideoxy sequencing lanes, and the 16S rRNA sequence of the relevant region is shown on the left. (d) Separation of fragmented 16S rRNA isolated from gradient purified 30S subunits on a 5% denaturing polyacrylamide gel. Ethidium bromide-stained 16S rRNAs from exponentially growing cells (Exp) or stationary-phase *E. coli* (48 h) are shown. As size markers, *in vitro* transcribed full-length (fl) 16S rRNA (1–1542 nt) and fragmented (Δ1–90) 16S rRNA (90–1542 nt) are used.

[22] could additionally affect the functional performance of 30S subunits for protein production. To clarify if the 16S rRNA fragmentation at the spur is sufficient to hamper 30S subunit activities, we isolated ribosomes from exponentially growing cultures and subjected them to specific *in vitro* conditions (see Materials and Methods) that resulted in significant 16S rRNA fragmentation at helix 6 (Fig 4a) without affecting overall 30S integrity (Fig. S5). These 30S subunits were subsequently combined with 50S particles purified from exponentially growing *E. coli* and subjected to *in vitro* translation reactions. In the poly(Phe) assay, these *in vitro* fragmented 30S particles showed a clearly reduced activity compared to 30S subunits without spur cleavage (Fig 4b). These data indicate a causal link between 16S rRNA fragmentation at the 16S rRNA spur and the observed diminished activities during protein synthesis.

Stationary-phase 30S subunits show compromised initiation potential

In order to understand the molecular reasons for the compromised translation activities of ribosomes carrying stationary-phase 30S subunits, we performed toeprinting assays on initiation-like complexes. The

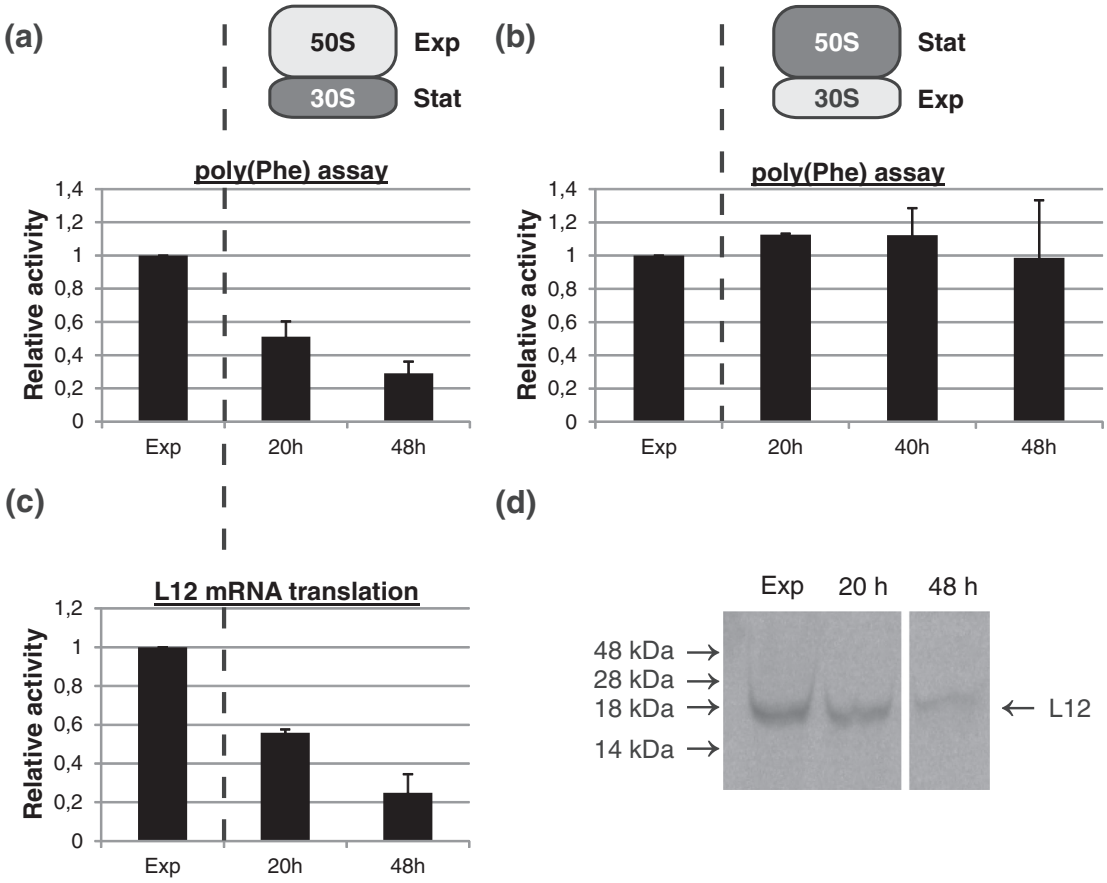

Fig. 3. *In vitro* translation activities of ribosomes containing stationary-phase subunits. (a) Poly(U)-directed poly(Phe) synthesis with ribosomes composed of stationary-phase 30S (Stat; dark gray) and exponential phase 50S (Exp; light gray) subunits. The product yields obtained with ribosomes carrying stationary-phase 30S subunits (from 20-h or 48-h cultures) were compared to ribosomes carrying solely exponential phase 50S and 30S (Exp). The mean and standard deviation are shown from three independent experiments. On average, 34 pmol Phe/pmol 70S was incorporated with ribosomes carrying both subunits from exponential phase cultures. (b) Same as in (a) but with stationary-phase 50S and exponential phase 30S subunits. The mean and the standard deviation are shown for two independent experiments. (c) Ribosomal protein L12 mRNA translation activities of ribosomes with stationary-phase 30S and exponential phase 50S subunits were compared to ribosomes with both subunits prepared from exponential phase cultures (Exp). The mean and standard deviations of two independent *in vitro* translation reactions are shown. (d) A representative SDS polyacrylamide gel is shown and the position of the ^{35}S-Met-labeled L12 protein is indicated by an arrow.

purified 30S subunits were incubated with *E. coli* $tRNA^{fMet}$ and an mRNA analog carrying a canonical Shine–Dalgarno sequence as well as a single AUG codon in an appropriate distance downstream thereof [23]. The formation of this 30S/mRNA/$tRNA^{fMet}$ complex, which mimics a translation initiation complex, was monitored by toeprinting [23,24]. Compared to 30S subunits isolated from exponentially growing cells, 30S subunits from late stationary phase, and thus mostly harboring the 16S rRNA cut at helix 6, showed a reduced toeprinting signal (Fig. 5a and b). It follows that the 30S/mRNA/$tRNA^{fMet}$ complex is less efficiently established with 30S subunits purified from late stationary-phase cultures. On the other hand, peptide bond formation utilizing *N*-acetyl-[^{3}H]Phe-$tRNA^{Phe}$ as P-site substrate and puromycin as A-site substrate was not affected when late stationary-phase 30S subunits and exponential phase 50S subunits were used (Fig. 5c). The transpeptidation data therefore demonstrate that P-site tRNA binding and association with the large ribosomal subunit are not seriously affected by stationary phase-specific alterations within the 30S subunit.

Discussion

Almost two-thirds of the microbiome's biomass has been predicted to be in a non-proliferating and thus resting growth state [25]. These microorganisms are thought to remain in the dormant status due to insufficient nutrient concentrations in the environment

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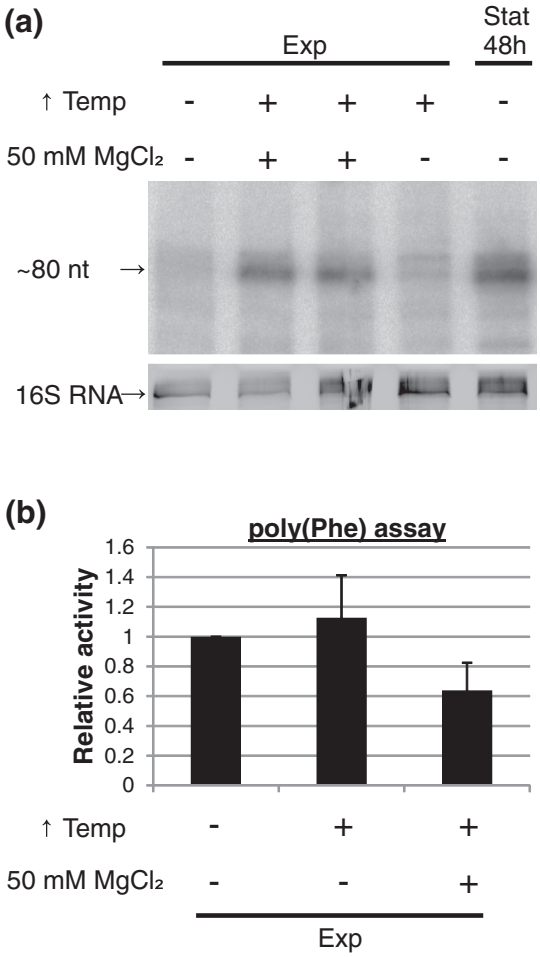

Fig. 4. *In vitro* fragmentation of 16S rRNA in 30S subunits from exponential phase cells. (a) Northern blot analysis on RNA isolated from 30S subunits obtained by density gradient purification from crude ribosomes treated at elevated temperatures (↑ Temp; 1.5 min 65 °C followed by 1 h 37 °C) at lower (10 mM) or elevated (50 mM) $MgCl_2$ concentrations. These incubation conditions at elevated Mg^{2+} concentration (see Materials and Methods) promote helix 6 fragmentation to a similar extent as observed in late stationary-phase 30S particles (48 h). The obtained ~80-nt 5′ 16S rRNA fragment is indicated by an arrow. Two biological replicates of *in vitro* fragmented 16S rRNA are shown. The ethidium bromide-stained 16S rRNA serves as loading control. (b) Poly(U)-directed poly(Phe) synthesis with ribosomes composed of exponential phase 30S and 50S subunits. When indicated, reactions were performed with *in vitro* fragmented 16S rRNA at helix 6 (same as in (a)). The product yields obtained with ribosomes carrying *in vitro* fragmented 30S subunits were compared to ribosomes carrying untreated 30S particles. The mean and standard deviation are shown from three independent experiments. On average, 27 pmol Phe/pmol 70S was incorporated with ribosomes carrying subunits from exponential phase cultures.

until more favorable growth conditions are reestablished. Therefore, entry into and escape from the stationary phase is crucial for long-term survival of a bacterial population and is therefore a tightly regulated process (reviewed in Ref. [11]). Bacteria have evolved many different mechanisms to cope with nutrient depletion and subsequent metabolic shut-down, and some of them directly affect or involve the ribosome, the central enzyme of protein biosynthesis. Among the best-characterized ribosome-dependent shut-down mechanisms are the stringent response, which involves the ribosome-bound RelA protein to produce the alarmone (p)ppGpp [26], the expression of specific ribosome-targeted regulatory proteins (e.g., hibernation promoting factor, HPF; stationary-phase-induced ribosome-associated protein, SRA; ribosomal silencing factor, RsfS; YqjD, a hypothetical protein) or the formation of translationally inactive 100S ribosome dimers (Refs. 27, 28 and references therein).

Recent insights from transcriptome-wide analyses suggest post-transcriptional processing events as being pivotal for enlarging the functional and regulatory repertoire of numerous RNA species [29]. Here we report on stationary phase-specific fragmentation of 16S rRNA in the small ribosomal subunit at the tip of helix 6 near the 5′-end (Figs. 1 and 2). These late stationary-phase 30S subunits showed reduced activities in several protein biosynthesis assays (Figs. 3a, c, and d, and 5a and b). In contrast, large ribosomal subunits isolated from stationary-phase populations did not show hampered translation activities *in vitro* (Fig. 3b), thus hinting at a 30S subunit-specific alteration during stationary phase. The cleavage site is located on the tip of helix 6, also referred to as the spur, which is solvent accessible (Fig. S2). Thus, endonucleases, putatively activated at the entry into the stationary phase, are considered to have access to this region of the small ribosomal subunit. Since 30S carrying fragmented 16S rRNA showed reduced activities in protein synthesis assays, it is conceivable that such a cleavage mechanism represents another layer of stationary phase-specific alteration of the translation apparatus involved in dimming metabolic activities in non-proliferating bacteria. If 16S helix 6 cleavage is cause or consequence of the stationary-phase hibernation program and whether or not the yet-to-be determined endonuclease cooperates with the already known ribosome-targeted modulation factors, such as RMF, HPF, SRA, YqjD or RsfA (recently renamed to RsfS) awaits further investigations. As first step toward addressing this issue, we have converted fully active exponential phase 30S subunits under particular *in vitro* conditions that promote helix 6 fragmentation (see Materials and Methods), into attenuated 30S mimicking late stationary-phase particles (Figs. 4 and S5). These findings suggest that 16S rRNA fragmentation at the spur (helix 6) is necessary and sufficient to shut-down translational activity of 30S ribosomal subunits.

16S rRNA cleavage upon particular environmental stimuli to modulate ribosome functions has already been observed before. In recent studies in *E. coli*, it

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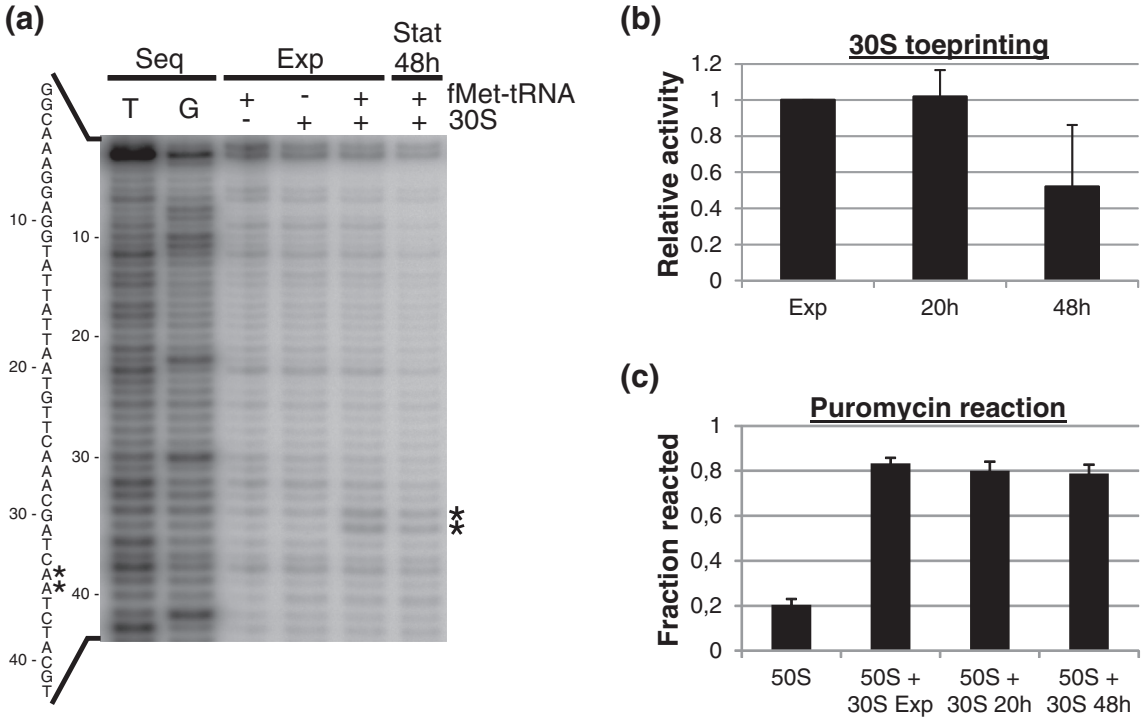

Fig. 5. Performance of stationary-phase 30S in initiation complex formation and peptide bond synthesis. (a) The efficiency of 30S/mRNA/tRNA^{fMet} complex formation was monitored by toeprinting and the primer extension products separated on an 8% denaturing polyacrylamide gel. The toeprints (bands labeled with asterisks) observed in the presence of 30S subunits from exponential cells (Exp) were compared to toeprints seen in the presence of stationary-phase 30S particles (isolated 48 h post-inoculation). T and G denote dideoxy sequencing lanes. (b) The quantification of three toeprinting experiments is shown. The toeprinting signal was normalized relative to the full-length mRNA band and a primer extension stop located 3′ of the observed toeprinting bands. (c) Peptide bond formation was assessed using the puromycin reaction with exponential phase 50S subunits and 30S subunits isolated either from exponential cultures (Exp) or from stationary-phase populations (20 or 48 h after inoculation). The background counts measured in the presence of 50S subunit alone represent putatively minor contaminations of exponential 30S subunits in the 50S subunit preparation. Radioactive counts measured in the absence of any ribosomal particles were subtracted from every experimental point. Under the applied single turnover conditions [43], the P-site substrate *N*-acetyl-[^{3}H]Phe-tRNA^{Phe} reacted almost quantitative with puromycin (fraction of reacted P-site substrate is given on the *y*-axis). The mean and standard deviation of two independent experiments are shown.

was shown that the RNA endonuclease MazF from the toxin–antitoxin pair MazE–MazF is capable of cleaving off a 43-residue-long fragment from the very 3′-end [30]. This MazF-dependent fragmentation is apparent upon stress induction such as oxidative stress, UV irradiation, heat, DNA damage or antibiotic treatment [31]. These 30S subunits carrying truncated 16S rRNA are subsequently recruiting leaderless mRNAs in order to synthesize a selective proteome thought to be needed to cope with the stress situation, thus promoting cell survival. Our deep sequencing data aiming at identifying the small RNome (sized between 20 and 300 nt) of *E. coli* during all growth phases failed to identify this particular 43-residue-long 16S rRNA piece (our unpublished data). This means that either the 43-nt-long 3′-end 16S rRNA fragment is not produced in stationary phase or our cDNA library preparation approach, which relies on the presence of a 5′-phosphate group as well as on efficient reverse transcription of rRNA (the 43-nt-long 3′-end fragment contains four post-transcriptional modifications), was unable to pick up this MazF-dependent processing product. In our hands, the most prevalent 16S rRNA-derived fragment turned out to be the ~80-nt-long piece originating from the 5′-end (Fig. 1). These two 16S rRNA fragmentation events might represent an analogous basic principle of rapidly altering the functional properties of the *E. coli* 30S subunit during particular environmental stimuli in a straightforward manner. Biogenesis of ribosomal subunits is resource-intensive, and thus, from an energetic point of view, it would be most reasonable to assume that the observed 16S rRNA fragmentation events would be reversible once the environmental stress ceases to exist. Future work needs to be dedicated toward this direction to uncover if such a 16S

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rRNA healing mechanism exists to re-activate the hibernating translation apparatus once growth conditions improve.

Materials and Methods

Strains and media

E. coli strain MG1655 (F–λ–ilvG rfb-50 rph-1) [32] carrying a plasmid pETgfp–mut2–AGGAGG(3) [33] was used to collect cells for experiments where exponential and stationary-phase time points were compared. To increase the reproducibility of those experiments, the used Lennox LB medium was prepared by filter sterilization rather than autoclaving as described [5]. MOPS medium was supplemented with 0.1% glucose (MOPS Glc) [5]. All media for this strain were supplemented with kanamycin (25 μg/ml). *E. coli* strain CAN20-12E [34] was grown in Lennox LB media supplemented with tetracycline (20 μg/ml) and used to isolate exponential phase ribosomal subunits. These subunits were used to generate 70S ribosomes for experiments shown in Figs. 3, 4b and 5c.

Cell growth and cell isolation

Ten microliters of an MG1655 DMSO stock (prepared as described in Ref. 5) was used to inoculate 2 ml of MOPS Glc, which was incubated for 24 h at 37°C. Next, 200 μl (for LB) or 1 ml (for MOPS Glc) thereof was used to inoculate 200 ml media (containing 250 μM IPTG to induce GFP production) in 1-L Erlenmeyer flasks. The cultures were incubated at 37 °C and cells were collected at desired time points (exponential phase: OD600 0.4, stationary phase: after 20, 28, 30, 40 or 48 h for LB, 2 or 5 days for MOPS by pelleting for 5 min at 20 °C (for volumes ≤200 ml) or for 10 min (for volumes >200 ml) at 11,000*g* at 4 °C. Cells were washed with 1–2 ml 1× PBS and pelleted again by centrifugation for 5 min at 8000*g* at 4 °C. The pelleted cells were frozen in liquid nitrogen and stored at −80 °C.

Preparation of crude ribosomes and ribosomal subunits

The crude ribosomes were prepared according to standard protocols [18,35] with the following modification. Briefly, the cells were opened using the FastPrep®-24 Instrument (MP Biomedicals) according to the manufacturer's protocol using 0.1-mm glass beads in buffer containing 20 mM Tris/Cl (pH 7.5), 100 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 mM EDTA and 6 mM β-mercaptoethanol. Purification of ribosomal subunits *via* sucrose density gradient centrifugation was performed as described previously [19].

RNA isolation

Total RNA was isolated as described [36] with the following modifications: Frozen cells were resuspended in 1× TEN buffer or in 1× buffer A [20 mM Tris/Cl (pH 7.5), 100 mM NH_4Cl, 10 mM $MgCl_2$, 0.5 mM EDTA, 6 mM β-mercaptoethanol]. Ribosome-associated RNA was isolated from crude, 70S ribosomes or isolated subunits in the presence of 0.5% SDS by extraction with acidic phenol.

cDNA library preparation and deep sequencing

Small RNAs sized between 18 and 300 nt from the total RNA or ribosome-associated RNA pool (extracted from crude ribosomes) isolated from exponential phase or stationary-phase cultures of *E. coli* MG1655 were size selected and purified as described [37]. RNA of 500 ng was treated with 10 U of Tobacco Acid Pyrophosphatase (Epicentre) for 1 h at 37 °C to convert 5′-triphosphate RNA (primary transcripts) into 5′-monophosphate RNA. cDNA libraries encoding these small RNAs were performed using the TruSeq Small RNA Library Prep TruSeq (Illumina) kit according to the manufacturer. For each RNA preparation, a unique index primer was used to allow for subsequent assignment of the sequencing reads. For every time point, two independent cDNA libraries (only one for the 30-h LB sample) were generated that were subjected to paired-end deep sequencing analyses on an Illumina HiSeq platform.

Bioinformatics analyses

Paired-end reads were quality trimmed (cut-off phred score of 30) and adapter trimmed (−O 1) from 3′ end while setting 18 bases as minimum length for processed reads by the program cutadapt (v1.5) [38]. Remaining reads were mapped to the *E. coli* MG1655 genome (NCBI Acc.-No: NC_000913.2; June 24, 2004) using the program Bowtie 2 [39] with the following parameters (to avoid indels: –rdg 100,3, –rfg 100,3; to report all alignments: –a; to make it more sensitive: −N 1). Using home-made python scripts, the best alignments (according to the alignment score) for each read pair were sorted and further processed by combing them to create pseudo single-end reads. The last step included also mismatch check allowing up to 2 mismatches. Generated pseudo single-end reads were further analyzed by the APART pipeline [15]. Quantification of the mapped reads was conducted by homemade python and R scripts.

Northern blot analysis

Northern blot analysis with 5 μg RNA and using DNA oligonucleotide 5′-CGCCAGCGTTCAATCTGAGCCATGATCAAAC-3′ was performed as previously described [13] with the following modifications: Hybridization was carried out at 50 °C and the second wash was performed at 42 °C.

Primer extension analysis (poisoned primer)

To assess the 16S rRNA cleavage efficiency, the primer 5′-ACCCGTCCGCCACTCGT-3′ was 5′-^{32}P-labeled and used together with 0.5–1 μg purified RNA in a modified primer extension protocol [40] using 5 μM ddTTP, 62.5 μM dATP, 62.5 μM dCTP and 62.5 μM dGTP as described [41]. Primer extension products were separated on 12% denaturing polyacrylamide gels and visualized by using a PhosphorImager (FLA-3000; Fuji Photo Film) and quantified with the densitometric program Aida Image Analyzer.

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In vitro translation

Poly(U)-directed poly(Phe) synthesis was performed with 2 pmol sucrose gradient purified 30S isolated from exponential or stationary-phase *E. coli* together with 2 pmol 50S subunits from exponentially growing cells and 5 μl *E. coli* S100 extracts as previously described [19]. The reaction was incubated for 120 min at 37 °C. To monitor the activities of ribosomes carrying stationary-phase or exponential-phase 30S subunits under more physiological conditions, a genuine mRNA *in vitro* transcript coding for ribosomal protein L12 was used. Translation was performed at 37 °C for 60 min as previously described [19].

In vitro transcription

All *in vitro* transcription reactions were performed as described [19,35]. The DNA template for 16S rRNA *in vitro* transcription (full length and fragments) was generated by PCR using the plasmid pAM552 as template. The following PCR primer pairs were used: 5′-GGATCCTAATACGACTCACTATAGGGAAATTGAAGAGTTTGATCATGGCTC-3′ and 5′-TAAGGAGGTGATCCAACCGCAGG-3′ for full-length 16S rRNA; 5′-GGATCCTAATACGACTCACTATAGGCTTTGCTGACGAGTGGCG-3′ and 5′-TAAGGAGGTGATCCAACCGCAGG-3′ for the 16S rRNA fragment 90-1542 lacking the first 90 residues on the 5′-end. Forward primers contain the T7 promoter (underlined) followed by two G residues for efficient transcription initiation.

Toeprinting

5′-^{32}P-labeled DNA oligonucleotide (6.2 pmol) 5′-CGTTAATCTGTGATG-3′ complementary to the mRNA was annealed to 25 pmol *in vitro* transcribed mRNA analog [23]. Annealing was carried out in 10 μl hybridization buffer containing 20 mM Hepes/KOH (pH 7.6), 150 mM NH_4Cl, 4 mM β-mercaptoethanol, 2 mM spermidine and 0.05 mM spermine by incubating 1 min at 95 °C and followed by 15-min incubation on ice. The formation of the initiation-like complex of 30S/mRNA/tRNA^{fMet} was initiated by mixing 5 pmol of 30S (pre-warmed at 37 °C for 15 min in 7 μl buffer containing 1 μl 10× hybridization buffer supplemented by 60 mM $Mg(OAc)_2$, 2 μl of annealed mRNA/primer mix (containing 2 pmol of mRNA and 1.24 pmol primer) and 1 μl deacylated tRNA^{fMet} (10 pmol/μl) by incubating 15 min at 37 °C. Reverse transcription was carried out by adding 2.5 μl reverse transcription mix containing 20 mM Tris/HCl (pH 7.4), 20 mM NH_4Cl, 100 mM $MgCl_2$, dNTP (each 14.5 mM) and 2.5 U AMV reverse transcriptase (*Promega*) and performed for 15 min at 37 °C. cDNA was precipitated, dissolved in loading dye and separated on 8% denaturing polyacrylamide gels as described [42]. The signals were monitored with a phosphor imager (FLA-3000; Fuji Photo Film) and analyzed quantitatively with the densitometric program Aida Image Analyzer.

Peptide bond formation

30S (5.5 pmol) isolated from stationary-phase cultures and 5.5 pmol 50S subunits isolated from exponential cells were reassociated for 20 min at 40 °C in 6.2 μl buffer containing 20 mM Hepes/KOH (pH 7.6), 20 mM Mg Ac_2, 30 mM KCl and 4 mM β-mercaptoethanol. Subsequently, the reassociated ribosomes were combined with 1 pmol *N*-acetyl-[^{3}H]Phe-tRNA^{Phe} (15,000 cpm/pmol) and 50 μg poly(U) in 20 μl buffer containing 20 mM Hepes/KOH (pH) 7.6, 6 mM Mg Ac_2, 150 mM NH_4Cl, 4 mM β-mercaptoethanol, 2 mM spermidine and 0.05 mM spermine for 15 min at 37 °C. The reaction was initiated by the addition of 5 μl puromycin (f.c. 2.1 mM) and incubated for 20 min at 37 °C. The reaction was stopped and the product extracted and quantified as described [19].

In vitro fragmentation of 16S rRNA

Crude ribosomes (1890 pmol) from *E. coli* strain MG1655 were incubated 90 s at 65 °C and followed by 1-h incubation at 37 °C in 200 μl buffer containing 50 mM Tris/HCl (pH 7.6), 100 mM NH_4Cl, 10 mM (or 50 mM when indicated) $MgCl_2$ and 6 mM β-mercaptoethanol. Under these incubation conditions and in the presence of 50 mM $MgCl_2$, efficient spur cleavage was observed. The overall integrity of the 30S particle and the 16S rRNA was not affected (Fig. S5). Subsequent purification of ribosomal subunits *via* sucrose density gradient centrifugation was performed as described previously [19].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2016.01.033.

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Abbreviations used:
30S, small ribosomal subunits; 50S, large ribosomal subunits; ncRNA, non-protein-coding RNA; sRNA, small non-coding RNA; rancRNA, ribosome-associated non-coding RNA; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DMSO, dimethyl sulfoxide.

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