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Published in:
Molecular Microbiology

Link to article, DOI:
[10.1111/mmi.14462](https://doi.org/10.1111/mmi.14462)

Publication date:
2020

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Fessler, M., Gummesson, B., Charbon, G., Lo Svenningsen, S., & Sørensen, M. A. (2020). Short-term kinetics of rRNA degradation in *Escherichia coli* upon starvation for carbon, amino acid, or phosphate. *Molecular Microbiology*, 113(5), 951-963. <https://doi.org/10.1111/mmi.14462>

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Article type : Research Article

Short-term kinetics of rRNA degradation in *Escherichia coli* upon starvation for carbon, amino acid, or phosphate.

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Running title: Kinetics of rRNA degradation in *E. coli*.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/MMI.14462](https://doi.org/10.1111/MMI.14462)

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SUMMARY

Ribosomes are absolutely essential for growth but are, on the other hand, energetically costly to produce. Therefore, it is important to adjust the cellular ribosome levels according to the environmental conditions in order to obtain the highest possible growth rate while avoiding energy wastage on excess ribosome biosynthesis. Here we show, by three different methods, that the ribosomal RNA content of *Escherichia coli* is downregulated within minutes of the removal of an essential nutrient from the growth medium, or after transcription initiation is inhibited. The kinetics of the ribosomal RNA reduction vary depending on which nutrient the cells are starved for. The number of ribosomes per OD unit of cells is roughly halved after 80 minutes of starvation for isoleucine or phosphate, whilst the ribosome reduction is less extensive when the cells are starved for glucose. Collectively, the results presented here support the simple model proposed previously, which identifies inactive ribosomal subunits as the substrates for degradation, since the most substantial rRNA degradation is observed under the starvation conditions that most directly affect protein synthesis.

Keywords: *Escherichia coli*; stable RNA degradation; bacterial stress response; nutrient starvation; ribosomal RNA

INTRODUCTION

The molecular basis for the growth physiology of *Enterobacteria*, mainly *Escherichia coli*, has been studied for more than half a century. Early on it became evident that the growth kinetics of bacterial cultures are tightly linked to the regulation of bacterial ribosome content (see e.g. (Schaechter *et al.*, 1958, Maaløe, 1979)). Since this fact was acknowledged, regulation of expression of the ribosomal components and the entire translational apparatus was studied intensively (see e. g. (Jinks-Robertson *et al.*, 1983, Davis *et al.*, 1986, Paul *et al.*, 2004, Bremer & Dennis, 2008)). A main conclusion resulting from such studies is that transcription of the ribosomal RNA (rRNA) operons is the pivotal regulatory point that controls ribosome content, because ribosomal protein (r-protein) content is adjusted to nascent rRNA availability. This adjustment occurs via feedback mechanisms rooted in competition between binding sites for some of the ribosomal proteins on the rRNA and regulatory binding sites for the ribosomal proteins on the mRNA encoding them (Keener & Nomura, 1996). Furthermore, since rRNA was found to be stable during growth (e.g. (Gausung, 1977)), degradation of ribosomal components is considered a negligible factor in the control of cellular ribosome content. The genes encoding the three rRNAs; 5S, 16S, and 23S, are present in seven operons controlled by conserved, strong promoters with special features. The most important player in the transcriptional regulation of rRNA synthesis, and thereby growth rate, is the small molecule guanosine pentaphosphate or tetraphosphate (collectively herein (p)ppGpp) (Potrykus *et al.*, 2011). Together with the protein DksA, (p)ppGpp binds to RNA polymerase, which affects promoter selectivity and reduces the rRNA promoter firing rates (Gummesson *et al.*, 2013, Ross *et al.*, 2016). In accordance with this role, there is an inverse correlation between the nutrient-dependent growth rate and the cellular content of (p)ppGpp during balanced growth (Lazzarini *et al.*, 1971).

The established way to obtain reproducible results in bacterial growth physiology is to perform experiments on cultures in balanced exponential growth (Ingraham *et al.*, 1983, Fishov *et al.*, 1995), and the vast majority of studies on ribosome content were carried out under these conditions. Since both rRNA and transfer RNA (tRNA) are stable during exponential growth (Neidhardt, 1964, Gausung, 1977, Piir *et al.*, 2011), the term stable RNA was adopted for these cellular components over the years.

In contrast to balanced growth, rRNA is degraded after prolonged starvation for various essential metabolites ((Mandelstam & Halvorson, 1960, Ben-Hamida & Schlessinger, 1966, Jacobson & Gillespie, 1968b)). Ribosome degradation under starvation conditions can serve to enhance survival

by making the released ribosomal building blocks available for other biosynthetic reactions. In addition to this role, the very reduction in the number of active ribosomes can allow the remaining ribosomes to maintain a functional translation elongation rate despite low substrate levels, as shown for *E. coli* growing at very slow rates in minimal medium supplemented with poor carbon and nitrogen sources (Dai *et al.*, 2016). Similarly, during conditions of Mg^{2+} limitation, a stark reduction in the number of ribosomes frees up cytosolic Mg^{2+} which supports the Mg^{2+} -assisted assembly and functionality of the remaining ribosomes, thereby allowing protein synthesis to proceed (Pontes *et al.*, 2016). It is thus clear that up- and down-regulation of ribosome content is central to both the growth and stress survival of bacterial cells.

In a previous study, we used a spike-in normalization strategy to show that tRNAs become unstable at the onset of amino acid starvation (Svenningsen *et al.*, 2017), a situation comparable to a nutritional downshift or entry into stationary phase. Here we apply similar methodology to show that rRNA levels are also quickly downregulated by degradation upon starvation, and we back up our findings by rRNA quantification with fluorescence *in situ* hybridization and by quantification of acid-soluble RNA degradation products. Further, we show that the rate and extent of rRNA degradation depends on which macronutrient the cells are starved for. Our findings imply that during nutritional downshifts and other growth conditions that reduce protein synthesis activity, rRNA degradation rates may be as important as rRNA synthesis rates for achieving the optimal number of ribosomes at the new growth condition.

RESULTS

Macromolecular changes upon valine-induced isoleucine limitation

We sought to measure rRNA degradation at the onset of amino acid starvation. To induce amino acid starvation in *E. coli* K-12 strains, it is common (e.g. (Traxler *et al.*, 2008, Laffler & Gallant, 1974)) to take advantage of a metabolic anomaly in these strains, which is caused by a frameshift mutation in *ilvGM* that inactivates one of three isozymes common to the valine and isoleucine biosynthetic pathways. Since the other two isozymes, *ilvBN* and *ilvIH*, are subject to feedback inhibition by valine (Leavitt & Umbarger, 1961), the consequence of the frameshift mutation is that high valine concentrations inhibit not only valine biosynthesis, but also isoleucine biosynthesis (Leavitt & Umbarger, 1962). Thus, addition of valine to *E. coli* K-12 cultures grown in isoleucine-free medium will result in isoleucine limitation.

We first quantified how severely valine addition affected the growth and macromolecular composition of our *E. coli* strain. Isoleucine starvation was induced by the addition of excess L-valine (400µg/ml) to balanced cultures of an *E. coli* strain auxotrophic for pyrimidines and arginine, grown in a MOPS-buffered minimal glucose medium supplemented with uracil and arginine. By adding [¹⁴C]-uracil and [³H]-arginine we could monitor the accumulation of radioactivity in DNA, RNA and protein prior to and during isoleucine starvation (Fig. 1). Consistent with previous reports (Cohen, 1958, Temple *et al.*, 1965), valine addition resulted in a reduced but nonzero growth rate, as measured by the optical density of the culture (Fig. 1A). We observed an immediate halt in the net protein synthesis upon valine addition (Fig. 1B), as well as a slight, but reproducible, reduction in the accumulation of ¹⁴C into RNA, indicative of net RNA degradation (Fig. 1C). After a transition period of approximately 80 minutes, the radioactivity incorporated into RNA and protein began to increase at a new constant rate similar to that of the other measured parameters, indicating *de novo* RNA and protein synthesis, and bacterial growth. The adverse effects of valine addition were less notable with regards to DNA synthesis (Fig. 1D) and counts of viable cells (Fig. 1E), which is in line with our expectations since *E. coli* is known to complete ongoing chromosomal replication events and reduce cell size upon amino acid starvation (Schreiber *et al.*, 1995, Ferullo & Lovett, 2008, Maciag-Dorszynska *et al.*, 2013). We have not been able to find an explanation for the continuation of macromolecular synthesis and growth in the presence of valine after the transition period, but remark that identical results were obtained regardless of whether we only used a single dose of valine to initiate isoleucine starvation or supplemented the culture with additional valine every hour for the duration of the starvation period. This observation shows that the resumption of growth after the transition period is not due to a reduction in the extracellular valine concentration over time. Based on these measurements, we focused our study of rRNA degradation kinetics upon isoleucine starvation on the 80-minute transition period where no net synthesis of RNA or protein was observed.

Rapid turnover of ribosomal RNA upon amino acid starvation

To determine the changes in rRNA levels during amino acid starvation, we quantified the amounts of full-length 5S, 16S, and 23S rRNA species by northern blot, using spike-in cells for normalization, as described previously (Svenningsen *et al.*, 2017). Briefly, an unstarved culture of spike-in cells, which are induced to express excessive amounts of the rarely used tRNA for

selenocysteine (tRNA^{Sec}) upon addition of IPTG, was grown in parallel with the valine-treated wildtype culture and harvested after IPTG induction. Prior to purification of RNA, defined volumes of the spike-in culture were mixed with each wildtype sample based on the OD units of the wildtype culture. This procedure allowed us to quantify rRNA/OD by using the tRNA^{Sec} band intensity in each lane of the northern blot as an internal standard. The use of spike-in cells allowed us to compare rRNA levels before and after starvation without making any assumptions about the levels of total RNA or any reference RNAs in the experimental samples.

A typical northern blot is shown in Fig. 2A. The rRNA levels are plotted relative to the average of three samples harvested during balanced growth before the addition of valine. After only 10 minutes of starvation, the levels of rRNA drop to ~85% of the pre-starvation levels, and after 80 minutes rRNA levels have decreased to 55% for 16S and 23S RNAs, and to 75% for the 5S RNA (Fig. 2B). Note that rRNA from the spike-in cells did not contribute significantly to the 16S and 23S band intensities (Fig. 2A, lane 14). At the 80 min timepoint, isoleucine was replenished, leading to immediate resumption of rRNA synthesis (Fig. 2B) and growth soon resumed at the pre-starvation rate (Fig. 2C).

Different types of starvation and stress result in different degrees of rRNA reduction.

It is well established that rRNA eventually becomes unstable when bacteria are deprived of a carbon source (Jacobson & Gillespie, 1968a, Zundel *et al.*, 2009), and extensive RNA degradation has also been reported for starvation for other nutrients, such as nitrogen (Ben-Hamida & Schlessinger, 1966, Kaplan & Apirion, 1975) and phosphate (Maruyama & Mizuno, 1970). However, it is not clear to what extent the kinetics of rRNA reduction differ between different types of starvation (Deutscher, 2003). We first focused on glucose starvation, which was the type of starvation employed when the rRNA degradation pathway was determined (Sulthana *et al.*, 2016). Despite the differences in metabolic function, glucose starvation and amino acid starvation provoke some of the same cellular responses. Both amino acid and glucose starvation eventually lead to stabilization of the stress response sigma factor RpoS (Traxler *et al.* 2008, Mandel and Silhavy 2004) and, thus, activation of the general stress response. For this reason, the two stress conditions might be expected to have comparable impacts on rRNA levels. To starve for glucose exponentially growing cells were filtered and resuspended in minimal medium lacking glucose. As shown in Fig. 3A, glucose starvation led to a reduction in rRNA content, but the rate of reduction in rRNA levels was

much slower than under amino acid limitation. Specifically, 80 minutes of glucose starvation led to a 10% reduction of the 16S and 23S RNAs (Fig 3A).

Next, we starved the cells for phosphate to assess how rRNA levels are affected when the supply of an essential component of the RNA backbone is removed. To do this, cultures in balanced growth were filtered and resuspended in medium without phosphate. This type of starvation led to the most severe rRNA reduction observed, amounting to a 60% reduction over 80 minutes for the three rRNAs (Fig 3B). Finally, to further investigate how protein synthesis activity affects rRNA instability we inhibited RNA synthesis with rifampicin, which binds RNA polymerase and inhibits transcription initiation (Campbell *et al.*, 2001). Ribosomes are affected by rifampicin in two ways. First, transcription of rRNA is blocked and second, the demand for translational activity diminishes as transcription of mRNA is inhibited as well. To assess the rRNA degradation upon transcription inhibition, rifampicin was added to cultures in balanced growth and samples harvested before and up to 80 minutes after rifampicin addition were treated as above. It was not possible to measure optical density after addition of rifampicin, since rifampicin affects the absorbance of light at the relevant wavelengths. Therefore, normalization was done under the assumption that the OD₄₃₆ of the culture did not change after rifampicin addition. Fig. 3C shows that all three rRNAs were degraded rapidly in response to rifampicin. Within the first 10 minutes, the amounts of 16S and 23S rRNA had been reduced to approximately 50% of the level found during balanced growth. After the initial rapid drop, rRNA levels stabilized for the remainder of the experiment. Thus, for both amino acid starvation and rifampicin treatment, the amount of rRNA is roughly halved at the end of the experiment. However, the kinetics are different, as the majority of the full-length rRNA disappears within the first 10 minutes in rifampicin-treated cells, whereas the isoleucine-limited cells lose their rRNA at a slower rate for an extended time.

E. coli* RNA degradation *in situ

To validate the rRNA levels observed in the Northern blots, we conducted a series of fluorescent *in situ* hybridization (FISH) experiments. FISH is a powerful alternative method for evaluating rRNA breakdown because no purification steps are required. Instead of purifying the RNA, the relative rRNA levels inside single cells are measured. There is a direct correlation between the cellular

rRNA content of cultures growing at different rates and the intensity of the fluorescent signal from an rRNA-targeted oligonucleotide probe, meaning that the method allows for rRNA quantitation (DeLong *et al.*, 1989). This method excludes any artifacts of the RNA extraction process that in theory could affect growing and starving cells differently, like differences in cell size (compare light scatter in Fig. 4A and 4B). Cells harvested from balanced growth and following 80 minutes of isoleucine limitation, glucose starvation, phosphate starvation or rifampicin treatment were fixed in formaldehyde. After cell permeabilization, a fluorescent probe recognizing a sequence in the 3'-end of the 16S subunit was allowed to hybridize overnight, excess probe was washed away, and the fluorescence intensity was measured by flow cytometry. The relative 16S fluorescence is shown in Fig. 4. The measured fluorescence values of individual cells were divided by the cell size measurement to more accurately reflect the rRNA concentration.

The results from the FISH experiments strongly agree with the northern blot data, confirming that rRNA is indeed degraded extensively during short-term starvation for all the tested nutrients. In both the northern and the FISH experiments, phosphate starvation causes the most drastic drop in rRNA levels, followed by isoleucine starvation, and finally glucose starvation, which has the smallest short-term impact on rRNA stability. In the case of rifampicin treatment, the results of the FISH experiment did only qualitatively agree with the other experiments, since the FISH quantification only indicates approximately 20% degradation of 16S rRNA after 80 min. We cannot explain the elevated fluorescence after rifampicin treatment but suggest that rifampicin may interfere with the fluorescence reading or create some other artifact in the FISH experiments. To more directly compare the FISH data with our northern blots, we used a northern probe with the exact same sequence as the FISH probe (Suppl. Fig. S1). The choice of probe did not change the outcome of the northern blot experiments in any of the four stress conditions.

Finally, we verified that the FISH probe binds to cellular RNA, as no fluorescent signal was observed in cells treated with RNase A after fixation, regardless of whether or not the cells had been subject to isoleucine limitation (Fig. 5C).

The pool of RNA degradation products increases during carbon-, amino acid-, and phosphate-starvation as well as rifampicin treatment

To further validate the fast disappearance of rRNA during starvation, a different approach based on the differential precipitation of short oligonucleotides and longer RNA molecules in acid (Cohen & Kaplan, 1977), was adopted. In short, bacterial cultures prelabelled with [^{14}C]-uracil were filtered into medium containing rifampicin, starvation media, or control medium allowing continued exponential growth. At each sampling point, two aliquots of the same culture were harvested into 5% TCA and 4M formic acid, respectively. The ^{14}C found in the TCA-precipitable material is a measure of pyrimidines found in RNA and DNA polymers longer than ~16 nt (Cleaver & Boyer, 1972), while counts found in the supernatant of the formic-acid-treated cells represents mononucleotides and short oligonucleotides because the formic acid treatment permits these short mono-, di- and oligomers to leak out of the cells (Cohen & Kaplan, 1977).

Since rRNA accounts for approximately 85% of total cellular RNA in exponential growth (Bremer & Dennis, 1996, Maaløe, 1979), and because rRNA (and tRNA) stability greatly exceeds mRNA stability during growth, the majority of the [^{14}C]-uracil would be incorporated into rRNA at the time the cells were exposed to rifampicin or nutrient starvation. Therefore, an increase in the free mono- and oligonucleotides primarily reflects nucleotides or short fragments released from ribosomes, and to a lesser extent possibly tRNA. This method was used previously to demonstrate degradation of rRNA during glucose starvation (Zundel *et al.*, 2009). Our experiment confirmed the reported accumulation of ^{14}C in the formic-acid-soluble fraction upon glucose starvation. In our measurements, the percentage of formic-acid-soluble radioactivity increased steadily until reaching a plateau at ~7% after 2.5 hours of glucose starvation (Fig. 5, triangles). Very similar results were obtained after isoleucine limitation (Fig. 5, squares). By contrast, a plateau was not reached for the duration of the phosphate starvation experiment, and the amount of formic-acid-soluble ^{14}C -labeled material was much higher, reaching 28% after 3 hours of starvation. Similarly, formic-acid-soluble counts from cells treated with rifampicin did not reach a plateau. Instead, the acid-soluble fraction increased at an almost constant rate over the three-hour period, reaching 14%. In comparison, acid-soluble counts from the exponentially growing control culture stayed at a basal level very close to 0% for the duration of the experiment. Replenishing the missing nutrient restored growth and led to a rapid decrease in the acid-soluble fraction in all cases (isoleucine, glucose or phosphate addition to the starved cells), in accordance with resumed net RNA synthesis.

As the formic acid ^{14}C -release assay reports on the free mono- and oligo-nucleotides and derivatives, a pool whose size depends on RNA degradation but also on the rates of reuse of the

nucleotides or their constituents for other metabolic reactions, it cannot be used to determine the kinetics of rRNA degradation directly. Nevertheless, the increase of the free nucleotide pool strongly supports the observed induction of rRNA degradation shown in Figs. 2-5 upon nutrient starvation or rifampicin treatment.

DISCUSSION

With the present set of experiments, we show that the levels of *E. coli* rRNA are dynamically regulated upon nutrient downshifts and starvation, and that regulation of rRNA levels does not only occur at the level of rRNA promoter activity but must also occur by degradation of existing rRNA in response to different types of starvation. rRNA breakdown has been reported before (Ben-Hamida & Schlessinger, 1966) but often under aberrant conditions (Okamura *et al.*, 1973, Kaplan & Apirion, 1974, Kaplan & Apirion, 1975) or with only a few time-points in studies that were focused on the mechanism rather than the kinetics of rRNA degradation (Basturea *et al.*, 2011, Zundel *et al.*, 2009, Sulthana *et al.*, 2016). Here, we used a method relying on the addition of spike-in cells expressing high amounts of a reference RNA to accurately quantify bacterial RNA content. The advantage of the spike-in method is that it allows for normalization of sample signals, which is required to reduce noise caused by variations in RNA recovery and blotting, without making assumptions about the existence of an invariable endogenous reference RNA or invariable total RNA contents of the cells, before and after starvation (Svenningsen *et al.*, 2017, Stenum *et al.*, 2017, Sorensen *et al.*, 2018). Although RNA was extracted with hot phenol (see Experimental Procedures), which efficiently disrupts gram-negative bacterial membranes (Ares, 2012), recovery of RNA from starved cells could be reduced compared to growing cells due to physiological changes of the cell envelope and size of the cells. Such an artifact could have caused the drop in RNA content we see in our northern blot analysis during starvation, since the spike-in cells to which we normalize, were not starved. We present several additional lines of evidence that rRNA degradation does in fact occur during starvation. First, a net loss of total RNA during the first 1-2 hours of isoleucine starvation was measured by TCA-precipitation of total cellular contents (Fig. 1 C). Next, we also performed FISH experiments (Fig. 4) and formic acid solubility experiments (Fig. 5), which do not involve RNA purification, to support our findings and to rule out that variations in RNA recovery could have biased our results. In particular, treatment with RNase A caused the loss of hybridization signals in both starved and unstarved cells in the FISH experiments (Fig. 4C)

which strongly suggests that there is no problem with permeability for the probe. The single stranded DNA probe we used has a molecular weight of about 6 kDa and is therefore expected to have access to cellular RNA that can be reached by RNase A (molecular weight ≈ 14 kDa).

Also, during the four different starvation conditions including rifampicin treatment, our northern blots showed that 5S RNA is the most stable of the three rRNAs. Assuming an extraction problem for starved cells, this could indicate that small RNAs are extracted easier than large RNAs and that we report on an extraction artifact. However, 23S RNA was consistently more stable than 16S RNA, and this contradicts extraction problems, since 23S is twice the size of 16S. We assume that our data reflects proper differences in the half-lives of the rRNAs and can only suggest that different binding affinities between ribosomal proteins and their RNA partners and different structural features may cause these differences in terms of accessibility for the degrading RNases. The consistent difference in half lives of the three RNAs at all four stress situations further suggest that it may be the same degradation mechanism that acts during the four stress conditions.

A key question is how degradation of rRNA is triggered. The Deutscher group has elegantly elucidated the molecular degradation pathway of rRNA in cells starved for glucose (Zundel *et al.*, 2009, Sulthana *et al.*, 2016) and shown that the initial sites of ribonuclease cleavage of the rRNA occur at the interface of the 30S and 50S subunits, suggesting that rRNA is primarily degraded when the two subunits are apart. These findings lend strong support to a model proposed previously by the same group, in which ribosomal inactivity is the trigger for degradation (Deutscher, 2003). This appealingly simple model predicts a dynamic scenario where the cellular ribosome content is continually adjusted to the protein synthesis activity because the degradation machinery specifically eliminates unengaged ribosomes. The model is also supported by the finding that isotope-labelled tagged rRNAs were stable during exponential growth, and somewhat stable after establishment of the stationary phase, but quite unstable during the transition from exponential growth into stationary phase where the protein synthesis activity would be declining (Piir *et al.*, 2011).

M. Deutscher's model fits very well with our northern blot data which are summarized in Figure 6 for the case of 16S rRNA.

First, we found that glucose starvation caused the weakest and slowest reduction of rRNA compared to any of the other treatments (Fig. 6). Glucose starvation does not directly affect the translation process, and both substrate (aminoacylated tRNA) and template (mRNA) are expected to be present in relatively high amounts while the cells prepare for stationary phase survival utilizing the

glycogen storage for energy supplies. Second, the translation process is directly affected by the downshift caused by valine addition, due to the lack of isoleucyl-tRNA substrate, and net protein synthesis was zero immediately after valine addition (Fig. 1). Consistent with reduced protein synthesis, our measurements reproducibly showed a net loss of RNA for the first 80 minutes after valine addition (Fig. 1). Indeed, during the same time span, northern blot analysis showed that rRNA levels were reduced to 50-60 % of their pre-starvation levels (Fig. 2). Our interpretation is that a fraction of the ribosomes was available for degradation in the transition period until a new steady state was reached, due to the lack of isoleucyl-tRNA substrate for translation. Lastly, the removal of phosphate from the medium resulted in a faster and more extensive reduction in the rRNA content than the other starvation types, and the only treatment that caused an even faster reduction of rRNA than phosphate starvation was the addition of rifampicin (Fig. 3 and Fig. 6). Using rifampicin, transcription initiation was prevented and since the average mRNA half-life is only a few minutes (Liang *et al.*, 2000), rifampicin causes a rapid decline in mRNA concentrations and therefore a fast decline in protein synthesis (Pedersen *et al.*, 1978), which would leave ribosomes inactive and available for degradation. Similarly, by removal of all phosphate from the medium, RNA synthesis decreases to low levels due to lack of substrate (St John & Goldberg, 1980), although cells have some residual transcriptional activity due to the phosphate stored in poly-P_i (Rao *et al.*, 1998) and presumably recycled nucleotides from rRNA as well. Rapid degradation of rRNA during phosphate starvation, and even faster degradation after rifampicin addition therefore fits very well with the inactivity model proposed by M. Deutscher's group. We observed the greatest release of the radioactive label in the cells under phosphate starvation (Fig. 5). Up to 70% of total cellular phosphate is found in RNA during rapid growth, while the remainder is mainly incorporated in the phospholipid membrane and to a lesser extent in DNA. Presumably, the phosphate released from the breakdown of rRNA during phosphate starvation is recycled for use elsewhere in the cells, so that the formic-acid-soluble [¹⁴C] is mainly in the form of the [¹⁴C]-uracil nucleobase. Indeed, extra- and intracellular accumulation of the uracil nucleobase has been reported at the entry to stationary phase (Rinas *et al.*, 1995). The ribosome content of *E. coli* growing balanced in chemostats on limiting carbon, nitrogen or phosphate concentrations has been measured recently (Li *et al.*, 2018) and it was confirmed that ribosome content decreases with a decrease in the quality of the growth medium. Furthermore, cultures growing at the same low rate but limited for glucose or nitrogen had equally low ribosome levels while, surprisingly, phosphate-limited cells growing at the same rate contained down to 50%

fewer ribosomes (Li *et al.*, 2018). This result indicates that phosphate starvation may cause very limited ribosome concentrations. The ribosome content of these steady-state growing chemostat cultures could have been reached solely by the ppGpp-mediated reduced synthesis rates of rRNA, compared to cultures growing in saturating levels of nutrients. Thus, no role for rRNA degradation can be evoked for these chemostat experiments. By contrast, we studied rRNA degradation in response to an abrupt change where the nutrient went from being in excess to exhaustion in a matter of seconds. The response to this abrupt change was a substantial drop in rRNA/OD both for amino-acid-starved and phosphate-starved cells, and a more modest drop for glucose-starved cells. The drop in rRNA levels cannot be explained solely by reduced rRNA synthesis and growth dilution in our experiments, but must also result from rRNA degradation, as is most clearly shown by an early net reduction of rRNA per culture volume (Fig. 1C, S2 & Supplementary Discussion) and the accumulation of RNA degradation products (Fig. 5). Based on the steady-state measurements made by Li *et al.* (Li *et al.*, 2018) we assume that starvation beyond 80 min in our experiments would have eventually led to a situation where phosphate-starved cells had the lowest rRNA content while glucose- and amino-acid-starved cells would have reached similar rRNA levels at times where the stored glycogen had been exhausted in the glucose-starved cells.

To summarize, our results show that rRNA breakdown can be an important factor when bacteria adapt to new growth conditions where the optimal steady-state ribosome content is lower than before the shift. While these results unequivocally demonstrate that a fraction of rRNA is being degraded upon rifampicin treatment and the three types of starvation, the differences in the magnitude of the net loss of rRNA are proportional to the expected differences in rRNA synthesis rates under the four conditions. Specifically, rRNA synthesis rates are initially limited only by the effect of ppGpp on transcription from the P1 promoter of rRNA operons in the cases of glucose-starvation and isoleucine-starvation (Maaløe, 1966, Sarmientos & Cashel, 1983), while rRNA synthesis would be inhibited both by ppGpp (Spira *et al.*, 1995) and lack of nucleotide substrates in the case of phosphate starvation (St John & Goldberg, 1980), and rRNA synthesis would be completely eliminated shortly after rifampicin treatment (Pato & Von Meyenburg, 1970). Thus, the up-regulation of rRNA degradation upon the different treatments can be understood as an additional layer of regulation acting additively with the down regulation of rRNA synthesis to rapidly adjust rRNA levels to the new condition. We have illustrated the interplay between rRNA synthesis and breakdown in Fig. 7. In this figure, the blue arrows show the well-described pathways

for regulation of the production of translational RNA (rRNA and tRNA) where an improvement of the growth medium leads to a higher saturation of translating ribosomes, followed by a reduction of the ppGpp production, an increase in the transcription rate of translational RNA genes and an increase in the growth rate. However, an abrupt nutrient downshift may lead to degradation of unengaged ribosomal subunits (Zundel *et al.*, 2009, Sulthana *et al.*, 2016), nascent rRNA (Jain, 2018) and vacant tRNA (Svenningsen *et al.*, 2017), here illustrated by red arrows pointing to the RNA degradation function in Fig. 7. RNA degradation probably serves to replenish important pools of building blocks thereby enabling the cell to reorganize gene expression patterns to cope with the nutritional down shift. In addition, RNA degradation may also serve to reduce the translational RNA pools to the appropriate size under circumstances where dilution of stable molecules by growth is either a very slow process or not an option at all, like at entrance into stationary phase. In conclusion, we suggest that degradation of translational RNA upon starvation is an important regulatory phenomenon that help cells cope with stresses that decrease translational activity. This stress-related rRNA breakdown is not unique to *E. coli*. For example, the ribosomal content of *Pseudomonas fluorescens* strain Ag1 dropped to ~45% of the pre-starvation level after 2 hours of carbon starvation (Boye *et al.*, 1995), and in the gram-positive bacterium *Lactococcus lactis*, rRNA levels dropped to 30% after 1 hour of sublethal heat treatment (43°C) (Hansen *et al.*, 2001). Lastly, in their seminal paper from 1958 on *Salmonella* transitions between different physiological states by Kjeldgaard, Maaløe and Schaechter (Kjeldgaard *et al.*, 1958), a net drop in RNA levels is also evident upon the downshift of a *Salmonella* culture from a defined complete medium to the same medium lacking amino acids.

Despite these examples, we think the labile nature of stable RNA under stress has received less attention than the regulation of stable RNA synthesis because the amount of total RNA harvested per cell is difficult to measure quantitatively, but this challenge is surmounted by the addition of spike-in cells for normalization of recovery during all steps of RNA purification.

EXPERIMENTAL PROCEDURES

Strains, media and growth conditions

Two derivatives of the *E. coli* K-12 MG1655 strain were used. For all experiments resulting in northern blots or FISH experiments the strain MAS1081 (MG1655 *rph*⁺ *gatC*⁺ *glpR*⁺) was used.

The cells were grown at 37°C in MOPS minimal medium (Neidhardt et al., 1974) supplemented with 0.2% glucose. Cultures were grown for at least 10 generations in exponential phase before every experiment unless otherwise stated. Isoleucine starvation was induced by adding valine to a final concentration of 400 µg/ml (Leavitt and Umbarger, 1962), glucose and phosphate starvation was induced by filtering the cells, washing in medium without glucose or phosphate, and subsequently resuspending them in MOPS media lacking either glucose or phosphate. These operations were performed at 37° C in less than two min. Rifampicin was added to a final concentration of 100 µg/ml. Cultures that came close to reaching an OD₄₃₆ of 1 during any experiment, were diluted in identical, preheated medium to ensure that no nutrients were exhausted from the medium besides those actively removed to induce starvation. Data reported after dilution were multiplied with the dilution factor.

For the internal standard in the Northern blots MAS1074 (Svenningsen *et al.*, 2017) a BL21(DE3) + pET11a(*sec*) strain was used, which was also grown in MOPS media with 0.2% glucose at 37°C. tRNA^{Sec} expression was induced by adding IPTG to a final concentration of 1mM.

The DNA, RNA and protein synthesis experiments were done in a MAS1081 background made auxotrophic for pyrimidines and arginine by removing the *pyrE* and *argG* genes from the WT background i.e. in MAS1083: *rph*⁺ *gatC*⁺ *glpR*⁺ Δ *pyrE*::*tet* Δ *argG*::(*cat sacB*).

RNA extraction, blotting, hybridization and quantification

1.5 ml samples were harvested by transferring into 300 µl stop-solution consisting of 95% ethanol and 5% phenol at 0°C. Samples were kept at 0°C until the final sample had been harvested. At this point the spike-in cells were added to each WT sample (5% spike-in culture was added based on OD) and subsequently total RNA was extracted with hot phenol. A detailed description of the northern blot procedure is present in Supplementary methods.

Each membrane was probed for 5S rRNA, 16S rRNA, 23S rRNA and tRNA^{Sec} (Sequences in Table S1). Normalization was done by calculating the ratio between the counts of a given rRNA and tRNA^{Sec} in the same lane of the blot, and this value was then plotted relative to the three samples harvested immediately before inducing starvation.

Macromolecular synthesis measurements

The MG1655 *rph*⁺ *gatC*⁺ *glpR*⁺ *ΔpyrE* *ΔargG* strain was grown overnight at 37°C in MOPS media supplemented with 0.2% glucose, 20 µg/ml uracil and 80 µg/ml arginine. The next morning, once the O/N culture reached OD₄₃₆ = 0.1, 0.33 µCi/ml [³H]-arginine (54.5 Ci/mmol) and 0.03 µCi/ml [¹⁴C]-uracil (58 mCi/mmol) was added to the culture. Every 20 minutes 0.5 ml samples were harvested into 5 ml 5% TCA and 0.5 ml 0.5 M NaOH both kept at 0°C. Further details for treatment of samples for measuring macromolecular synthesis and CFU determinations, see Supplementary Experimental Procedures.

Monitoring rRNA degradation products *in vivo*

MAS1081 (WT) cells were left to grow overnight in MOPS media with 0.2% glucose. The following day the culture was diluted 500x and 0.05 µCi/ml of [¹⁴C]-uracil (58 mCi/mmol) was added to label total RNA in the cells. Incorporation of the labeled uracil was considered to be fast and finished several generations before the actual experiment. After 5 generations, the culture was filtered to induce starvation and remove any remaining unincorporated [¹⁴C]-uracil from the medium. The cells were resuspended in media with rifampicin or media inducing either isoleucine starvation, glucose starvation, phosphate starvation or exponential growth. The release of radioactive degradation products were measured essentially as described by (Zundel *et al.*, 2009) but see Supplementary Experimental Procedures for details.

Fluorescence *in situ* hybridization

Four samples of 1 ml were harvested from an overnight culture of WT cells immediately before inducing starvation (or adding rifampicin) and another 4 samples after 80 minutes of starvation. Samples were treated as described by Parsley *et al.* (2010). Fluorescence was measured with an Apogee A10 Bryte flow cytometer equipped with a mercury arc lamp. A G1 filter cube (520-560 nm for excitation and emission at > 590 nm) was used. Two parameters were measured in the flow cytometer – forward light scatter (measure of size) and fluorescence. For each sample, the fluorescence/size ratio was calculated, and the appropriate background was subtracted. The formula is shown below, where “x” denotes the condition (exponential growth, isoleucine starvation, glucose starvation, phosphate starvation, rifampicin treatment or RNase treatment):

$$\text{value}_{\text{sample}(x)} = (\text{fluorescence}_{\text{sample}(x)} / \text{size}_{\text{sample}(x)}) - (\text{fluorescence}_{\text{background}(x)} / \text{size}_{\text{background}(x)})$$

Finally, the mean of the 4 starvation values was normalized to the mean of the 4 exponential phase values, which was set to 1.

Statistical testing

To test for significance a two-sided paired t-test was applied. The cut-off for significance was a p-value below 0.05. To use the t-test, the data must follow a normal distribution, which was tested for with a Shapiro-Wilk test. A p-value above 0.1 from this test suggests that the data is normally distributed.

Acknowledgements

The authors thank Marit Warrer for excellent technical assistance. Danish Council for Independent Research, Natural Sciences [1323-00343B to S.L.S.]; Novo Scholarship to M.F.; Danish National Research Foundation [DNRF120 to S.L.S. and M.A.S.]. Funding for open access charge: The Danish National Research Foundation.

Conflict of interest statement. None declared.

Author contributions

M. F., S.L.S and M.A.S. wrote the manuscript. S.L.S., M.A.S. and M. F. designed experiments; M. F., B. G., G. C. and M.A.S. performed experiments; M.A.S. and S.L.S. conceived and designed research.

Graphical Abstract

Abbreviated Summary

Escherichia coli ribosomal RNA is highly unstable right after nutritional deprivation. The kinetics differ depending on the type of starvation, in a manner consistent with active degradation of the unengaged ribosomes.

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Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Figure Legends

Figure 1. Growth parameters for a culture starved for isoleucine by addition of valine. A culture in balanced growth received 400 $\mu\text{g/ml}$ valine to induce starvation at time zero (vertical line at time 0) and additionally 100 $\mu\text{g/ml}$ extra valine every hour. After 8 hours the culture received isoleucine to a final concentration equal to the valine concentration to end the starvation (vertical line at 8h).

Optical density at 436 nm (A), accumulation of radioactivity in protein (B), accumulation of radioactivity in RNA (C) and accumulation of radioactivity in DNA (D) were measured during 80 minutes of exponential growth and 8 hours of isoleucine starvation in a pyrimidine- and arginine-auxotroph *E. coli* strain. CFU (colony forming units) were measured for 8 hours of starvation (E). All y-axes are $\log_{10}$ transformed.

Figure 2. Northern blot showing degradation kinetics for rRNA during valine-induced isoleucine limitation. **A:** A 1 % agarose gel was used for electrophoresis of total RNA from samples harvested immediately before starvation (lanes 1-3), during starvation at the indicated times (lanes 4-8), after starvation was ended by a 1:1 dilution into preheated medium containing isoleucine to a final concentration of 400 $\mu\text{g/ml}$ (lanes 9-13) and from spike-in cells only (lane 14) and was blotted. The resulting membrane was probed for tRNA^{Sec} and 5S, 16S and 23S rRNA as indicated on the left. **B:** The levels of 5S, 16S and 23S rRNAs were quantified by normalizing to tRNA^{Sec} originating from the spike-in cells (Svenningsen *et al.*, 2017). Spike-in-normalized RNA levels are shown relative to the average of the three RNA samples harvested prior to starvation. Error bars indicate SEM (n=3). **C:** Growth of the culture before, during and after starvation.

Figure 3. Quantification of rRNA reduction during starvation. **A:** Glucose starvation (n=5) **B:** phosphate starvation (n=3) **C:** rifampicin treatment (n=3). **D:** Growth curves for cultures starved for glucose, phosphate or treated with 100 $\mu\text{g/ml}$ of rifampicin. Vertical line indicates time of filtration and resuspension in starvation medium (red or blue curve), or rifampicin treatment (orange curve). To ease evaluation of the growth curves, all OD measurements obtained after filtration were corrected for the loss of cells (10-20%) that occurred during filtration into glucose-free or

phosphate-free medium. It was assumed that there is no change in OD after rifampicin treatment. Samples were harvested and treated as in Fig. 2.

Figure 4. Detection of 16S rRNA levels with FISH during glucose starvation (n=3), isoleucine starvation (n=3), phosphate starvation (n=3), and 80 min. of rifampicin treatment (n=3). Cells were fixed prior to or after 80 minutes of starvation and hybridized overnight to a fluorescent probe complementary to part of the 16S rRNA (1482-1499). The fluorescent signal was measured by flow cytometry. In addition, starved (n=2) and unstarved (n=2) cells were fixed and incubated overnight with RNase A, before the fluorescent probe was added. Representative scatter plots from exponential phase cells (A) and isoleucine starved cells (B) are shown. 16S rRNA levels were quantified (C). To test for significance a t-test was applied (* = p-value < 0.05, ** = p-value < 0.01) Normal distribution was assumed for samples only repeated two times. Error bars indicate SEM. a.u. = arbitrary unit.

Figure 5. *In vivo* assay of RNA degradation products during isoleucine starvation, glucose starvation, phosphate starvation and rifampicin treatment. Wildtype MAS1081 cells incorporated [¹⁴C]-uracil for 5 generations before filtration and resuspension in media inducing starvation or containing rifampicin. Samples were taken every 30 minutes until refeeding, at which point sampling was briefly intensified. The vertical line indicates when the three starved cultures were replenished with the missing nutrient (glucose, isoleucine or phosphate). Degradation was determined by plotting the formic-acid-soluble radioactivity as a percentage of the sum of radioactivity from the formic-acid-soluble and TCA-precipitable fractions, from three independent experiments. Error bars indicate SEM (n=3).

Figure 6: Data on 16S rRNA levels from Fig. 2 and 3 plotted in the same graph to ease comparison between the different treatments.

Figure 7. Major regulatory pathways for the homeostasis of translational RNA components as a function of nutrient influx. Blue connectors indicate pathways dominant during growth while

red connectors indicate pathways active upon a downshift in nutrient availability. Arrowheads indicate an increase in the component it points to while T-bars represent a decrease. Squared brackets mean "concentration of". Numbers in circles indicate the following representative references. 1: (Maaløe, 1979) 2: (Bremer & Dennis, 2008) 3: (Svenningsen *et al.*, 2017) ; 4:(Cashel & Gallant, 1969, Ross *et al.*, 2016) 5: (Jain, 2018, Gausing, 1977) 6: (Zundel *et al.*, 2009) and present data 7: (Hazeltine & Block, 1973, Winther *et al.*, 2018) 8: (Dong *et al.*, 1996, Sørensen *et al.*, 1989).

A **B** **C** **D** **E**

A

B

C

A

Glucose starvation

B

Phosphate starvation

C

Rifampicin

D

A

B

C