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Ribosome inactivation for preservation: concepts and reservations

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The role of the bacterial ribosome in the cellular response to environmental stress has been widely considered over last decade. Certain ribosome-associated proteins have been shown to induce conformational changes that lead to the formation of inactive forms of ribosomes that are presumed to be more stable during stationary phase. This was found to aid the survival of bacteria in this phase. Such proteins include ribosome modulation factor (RMF), YfiA and YhbH. Examining the influence of RMF on the survival of E. coli under heat, acid and osmotic stress showed that it was important for bacterial viability under these environmental pressures. However, the mechanism by which this protein exerts its effect has not been fully elucidated. The present work reviews the involvement of ribosomes in determining cell behaviour during stress. It focuses on the action of the ribosome-associated proteins and their role in inactivating ribosomes for preserving their integrity and aiding cell survival under stress.

Keywords: *bacterial ribosome, cellular response to environmental stress, RMF, YfiA, YhbH*

Ribosomes and stress

The ability of bacteria to express adaptive mechanisms to cope with environmental stress has received a lot of research and speculation over last few decades. These mechanisms involve various cell structures and networks of orchestrated behaviour. The ribosome is one cellular component whose reaction to stress and whose effect on cell survival have been extensively studied. An important reason for this interest is the involvement of ribosomes in protein synthesis, being the stage at which genetic codons are translated into proteins. The importance of ribosomes also arises from their representing

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approximately 40% of the mass of rapidly-growing cells and from that most of cellular energy is devoted for their synthesis and assembly[1]. The functional ribosomal unit in bacteria is designated 70S (S refers to Svedberg units for sedimentation rate) which consists of two subunits: 30S and 50S (Figure 1). Proteins and RNA constitute the major components of ribosomes; there are more than 50 proteins and three RNA species (23S, 16S and 5S) in *E. coli* ribosomes[2].

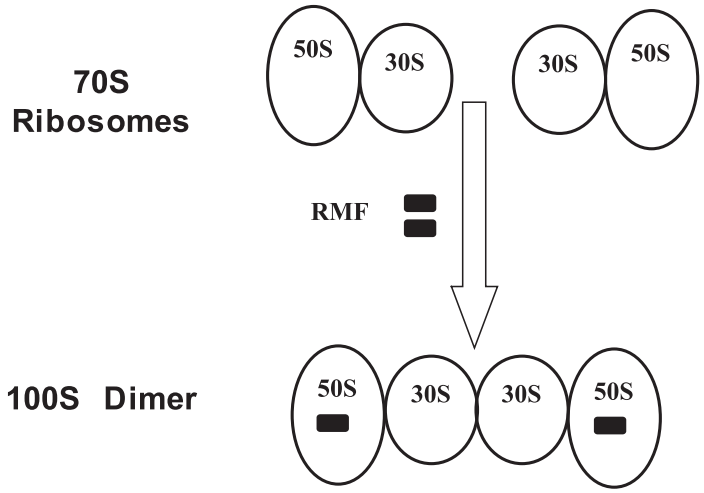

Fig. 1. Modulation of ribosome structure by RMF (after Ishihama[33]).

Early studies of the involvement of ribosomes in environmental stress showed that the degradation of ribosomal components, particularly RNA, correlated well with cell death during heat, cold and starvation[3-5]. This suggested an involvement of ribosome damage in cell death. This concept was supported by observations that the presence of factors stabilising ribosomes, such as Mg^{2+}, aided bacterial survival. Conversely, ribosome-destabilising agents, such as EDTA, increased cell death under stress[3-4].

With advances in molecular cell analysis, it was possible to provide closer visualisation of cell damage and to observe that rRNA species and ribosomal subunits are not equally vulnerable to stress. It was shown that 16S rRNA and 30S subunits are more sensitive to heat stress than 23S rRNA and 50S subunits, respectively[6]. Depriving *Salmonella* ser. Typhimurium of a metabolisable carbon source and growing *Listeria monocytogenes* under sub-lethal salt stress were observed to increase their tolerance to heat[7]. This phenomenon, termed

"cross protection", was found to be associated with increased stability of 16S rRNA and reduced ribosomal damage following exposure to heat. It has also been suggested that ribosomes were involved in the increased cell sensitivity to heat shock after being subjected to abrupt temperature downshift. Under these conditions, cold shock caused a decrease in thermal stability of 50S and 70S units that was proposed to be reflected in cell vulnerability to heat[8].

However, ribosome stability cannot be considered as the sole factor determining bacterial behaviour during stress. For example, although cell death correlated to ribosome damage under heat and cold stress, there was an initial stage during exposure to stress where there was rapid ribosome disintegration associated with no decline in cell viability[3,4]. It was suggested that ribosome damage was not a direct cause of cell death, which might have been the result of a rapid increase in the endogenous pool of components caused by RNA degradation. On the other hand, Niven *et al*.[9] showed that there was a reduction in ribosomal numbers and a decline in their stability on exposing *E. coli* to high pressure. While these were improved on eliminating pressure and incubating cultures at optimum growth conditions, bacterial viability continued to be lost.

Following interesting observations by VanBogelen and Neidhardt in 1990[10], ribosomes were suggested to be sensors to heat and cold shocks in *Escherichia coli*. It was found that the addition of antibiotics targeting ribosomes induced the expression of heat and cold shock proteins. These proteins are synthesized in bacteria under normal growth conditions, but their expression is induced significantly on expose to rapid temperature shift. They are presumed to aid cell survival by acting as chaperones interacting with other molecules to prevent or overcome temperature-induced damage[11]. It was interesting to observe that the patterns of heat or cold shock proteins induced by antibiotics simulated those induced by temperature shifts that the induction extent in the former case depended on drug dose in a similar way to the induction dependency on the severity of temperature shock. Zhang *et al*.[12] have revisited this phenomenon in *Bacillus subtilis*. They observed the involvement of the L11 ribo somal protein in the activation of sigma factor B, the general stress sigma factor in *B. subtilis*, following exposure to stress and concluded that ribosomes could serve as a sensor for most stresses encountered by this organism.

Not only can heat shock proteins aid cell survival by functioning as chaperones, but they were also found to be involved in facilitating

ribosomal translation during stress. For example, it was observed that the heat shock protein HrpA in *Mycobacterium bovis* was involved in the initiation step of translation under heat stress[13]. In *E. coli*, the heat shock protein Hsp15 was found to bind with very high affinity to free 50S subunits attached to a nascent polypeptide chain. It was thus proposed that it took part in recycling 50S subunits after completion of the polypeptide[14]. This function of a heat shock protein was also suggested by Teixeira-Gomes *et al*.[15] who identified the ribosome releasing factor as a heat shock protein in *Brucella melitensis*. Ribosome releasing (recycling) factor, is involved in the dissociation of ribosomes from mRNA after the termination of translation[16]. It is thus conceivable that the involvement of ribosomes in sensing stress and generating subsequent signals actually stems from their direct requirement of the action of the "response" molecules, e.g. heat and cold shock proteins for their function under stress[17].

Degrade or preserve!

A very frequent stress situation encountered by bacteria is their entrance into the stationary phase where starvation, accumulation of waste products, lack of proper aeration and other adverse conditions induce cells to show morphological, structural and metabolic adaptive changes (for review on these changes, see Huisman *et al*.[18]). One important change during this phase is the decline in protein synthetic capacity of the cells, which gives rise to the surplus ribosomes that are not involved in translation. Cells respond to this situation in one of two identified ways. They may degrade extra ribosomes, as in many *Salmonella* serovars, or preserve them as in *Escherichia coli*.

Ribosome degradation was observed in salmonellae, which showed a high degree of 23S rRNA fragmentation during the stationary phase[1,19]. This process was reported to be an active mechanism involving *de novo* protein synthesis as it was suppressed by the addition of chloramphenicol[19]. However, it is not clear whether this was the case since ribosome degradation may have been reduced by the inhibition of the synthesis of RNases and proteinases. So, were the ribosomes actively degraded or did the cells lack a mechanism to protect them from degradative enzymes during stationary phase? Although it causes loss of functional ribosomes, rRNA degradation could be viewed as a bacterial strategy to aid survival during stationary phase. When *E. coli* cells were starved for carbon, they showed dissociation of ribosomes followed by rRNA hydrolysis[20]. Interestingly, the ability of starved cells to recover their viability on

exposure to normal conditions was found to correlate with their capacity to degrade rRNA. This was explained as a consequence of RNA degradation providing cells with nucleotides, amino acids and energy. However, it is detrimental for cell when this process proceeds beyond its adaptive limits[20].

On the other hand, during stationary phase *E. coli* was found to increase the synthesis of certain ribosome-associated proteins (RAPs) that are thought to inactive but protect the ribosome. This therefore represents the sacrifice of ribosome functionality for survival during stress, which is perhaps analogous to the approach that sporeforming bacteria adopt for the whole cell functionality under adverse environmental conditions. To date, there are three RAPs proposed to serve this function: ribosome modulation factor (RMF), YfiA and YhbH. Characteristics of these proteins are shown in table 1.

Table 1 Characteristics of ribosome-associated proteins

RAP	*RMF*	*YfiA*	*YhbH*
Mr (kDa)	6.507	12.644	10.612
pI	11.3	6.2	6.6
Number of amino acid residues	55	113	95
Ribosomal binding subunit	50S	30S	30S

Ribosome-associated proteins (RAPs)

It is important to appreciate the difference between a ribosomal protein (RP) and a ribosome-associated protein (RAP). Ribosomal protein is an inherent part of ribosome, which can not be released by even extensive salt wash and is thus exclusively detected in ribosome fraction, whereas RAP is an accessory component that can be dissociated from ribosomes by washing with highly concentrated salt solution (usually 1M ammonium chloride or ammonium acetate or 0.4 M sodium chloride) or in the presence of low Mg^{2+} concentration (1 mM). RAP can therefore be detected associated with ribosomes and/or in the cytoplasmic fraction[2,21].

Ribosome modulation factor (RMF)

The discovery of RMF was a consequence of developing a novel technique of two-dimensional (2-D) gel electrophoresis. This method provided protein separation under highly-reducing conditions and the absence of free radicals and it was thus termed radical-free and highly reducing (RFHR) 2-D gel electrophoresis[22,23]. These modified conditions were presumed to prevent chemical

modification of RPs and RAPs caused by free radical and reactive oxygen molecules[2]. This resulted in the characterization of hitherto-unknown four RPs and one RAP. The latter was RMF which was found to be a small basic protein (Table 1) that exclusively associated with a non-functional ribosomal form named 100S ribosome or ribosomal dimer (two 70S units) (Figure 1). It is suggested that on entry to the stationary phase, one RMF molecule binds to 50S subunit generating conformational changes that stimulate the associated 30S subunit to bind to another one in another RMF-bound 70S ribosomes[2] (Figure 1). Within this context, the protein was presented as a modulator of ribosome conformation and was named ribosome modulation factor (RMF).

While ribosomal dimers were observed in early studies of ribosomes as particles formed *in vitro* in the presence of excessive magnesium ions[24-26], their physiological relevance to *E. coli* was only realised following the identification of RMF by Wada *et al*.[27] and Yamagishi *et al*. [28]. It was found that *rmf*-deficient *E. coli* mutant strains did not produce ribosomal dimers[28], and that RMF induced the dimerisation of 70S ribosomes *in vitro*[29]. A strong link between the expression of RMF and ribosome dimerisation was therefore suggested. An exception is the *E. coli* Q13 strain that is *rmf* $^{+}$ but does not form ribosomal dimers[28]. The RMF protein could not be detected in this strain[30], yet it synthesized *rmf* mRNA[28]. Further studies on this strain showed that it could not form ribosomal dimers even in the presence of RMF and it was concluded that it had structural ribosomal defects that inhibited RMF binding to the 70S ribosomes[30]. In addition to laboratory non-pathogenic *E. coli* strains expressing RMF, recent genome sequencing data show the presence of the *rmf* gene in various pathogens including *Salmonella* serovar Typhimurium, *Salmonella* serovar Typhi, *Yersinia pestia*, *Shigella flexneri* and *E. coli* O157: H7. Protein homologues to RMF and 100S dimers were also detected in G^{-}bacteria other than *E. coli*[31].

Wada *et al*.[29] found that protein synthesis was inhibited *in vitro* by the presence of RMF on the formation of 100S dimers. This provided evidence that ribosomal dimers are inactive in protein synthesis. An observation that supported this conclusion was that on inoculating stationary phase cells into fresh medium, 100S dimers dissociated into 70S, 30S and 50S units that to form functional 70S ribosomes and polysomes for cell growth during the exponential phase[2,27,32].

It was proposed that on entry into the stationary phase, protein synthetic capacity declines causing the emergence of extra "unused" ribosomes that become dimerised by increased expression of *rmf* [2,30,33]. We have found that when *E. coli* cells were grown at acid pH, their ability to form ribosomal dimers in the stationary phase was less than that of cultures grown in unacidified conditions (pH 7.3)[34]. This was consistent with the above hypothesis that growing cells under acid stress could reduce the number of ribosomes. And this made cell need most, if not, all of its ribosomes for survival in the stressful conditions of the stationary phase augmented by lower pH. Accordingly, less dimers were formed with the decrease in pH.
Ribosomal dimers are speculated to be more resistant than 70S ribosomes against ribonuclease and proteinase activities that are elevated during the stationary phase and they would thus provide more protection to ribosomes in this phase[28]. In a comparative assessment of ribosome stability on heating *in vitro*, it was observed that 70S particles isolated from an *rmf*-deficient mutant strain during stationary phase were less stable than 100S dimers isolated from the parent strain (Niven, *personal communication*).

YfiA and YhbH proteins

The significance of YfiA to ribosome stability was first demonstrated by Agafonov *et al*.[35] who reported the ability of this protein to stabilise 70S ribosomes of *E. coli* against dissociation. YhbH was later shown to be involved with inducing conformational changes in bacterial ribosomes during the stationary phase[32] (see below). Both YfiA and YhbH are weekly acidic proteins with molecular weight and number of amino acid residues higher than RMF (Table 1). They bind ribosomes more-tightly than RMF as they are not released by salt wash with 1 M ammonium acetate, but they dissociate in the presence of low Mg^{2+} concentration (1 mM) or by wash with 0.4 M sodium chloride solution[32,35]. YfiA was found to bind 30S units at their interface with 50S subunits[35]. Because of the similarity of the amino acid sequence of YfiA and YhbH, the latter was suggested to also bind to the 30S subunit[32]. However, YfiA could be detected in both 70S ribosomes and 100S particles, while YhbH was exclusively associated with 100S dimers[32]. Based on a series of experiments, Agafonov *et al*.[36] concluded that the binding of YfiA at the interface between 30S and 50S subunits may block the aminoacyl-tRNA site (A site). It was thus suggested that YfiA was a potent inhibitor of translation in *E. coli* under stress conditions. As a

result, the authors proposed the protein to be named "ribosome associated inhibitor A" (RaiA).
As with RMF, the expression of YfiA increases significantly during stationary phase[28,36]. Under optimal growth conditions, both proteins are detectable in the stationary phase but not in the exponential phase.

Ribosome inactivation by RAPs

Maki *et al*.[32] suggested an interesting model based on various hypothesises and data on RAPs generated by Wada *et al*.[27,29,30], Yamagishi *et al*.[28] and Agafonov *et al*.[35]. According to this model, the increased synthesis of RAP on entry to stationary phase causes the formation of non-translating but more protected ribosomes (Figure 2). One such ribosome form is the 100S dimer whose formation is induced by RMF. The model shows that proteins YfiA and YhbH bind 70S ribosomes decreasing their translation capacity. Most YhbH-bound ribosomes and some of the YfiA-associated ribosomes are dimerised into the 100S particles by RMF. This gives rise of the emergence of three relaxed ribosomal forms in the stationary phase: YfiA-associated 70S, YfiA-associated 100S and YhbH-associated 100S ribosomes. On inoculating cells into fresh medium, most RAPs disassociate from ribosomes, thus restoring their protein synthetic activity, which would be necessary for initiating growth.

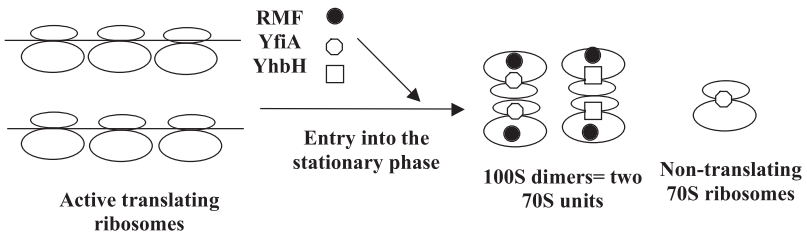

Fig. 2. Inactivation of ribosomes by RAPs on entry into the stationary phase (adapted after Maki *et al*. [32]).

RAPs and cell behaviour during environmental stress

The emergence of data suggesting increased expression of RAPs during stressful conditions of stationary phase beside observations indicating potential adaptive value of these proteins provoked interest as to whether RAPs aid cells to grow or survive under stress. Thus far, RMF and YfiA have captured most interest in this concern. It was observed that disruption of the *rmf* gene resulted in *E. coli*

mutant strains that were unable to form 100S dimers and less able to survive during the stationary phase[28,30]. This raised the possibility that the synthesis of RMF could be a survival mechanism under conditions unfavourable to growth. When this was examined against diverse physical and chemical stresses, it was found that *rmf*- deficient *E. coli* strains were less able to survive hyperosmotic shock[37], heat stress[38] and low pH[34] compared to their RMF$^+$ parent organisms.

It was further shown that the expression of *rmf* was inversely proportional to bacterial growth rate; slow growth induced the gene expression. It was interesting to find that under acid stress this relationship was independent of the acidifying agent with both mineral and organic acids[34]. These observations suggest that cells should take advantage of the increased *rmf* expression for adaptation during growth under stress.

We have been interested in investigating the mechanism(s) through which RMF aids survival or adaptation to stress. Surprisingly, the formation of 100S was found to be of little effect on the ability of *E. coli* to survive heat[38] and low pH[34]. In these experiments, stationary phase cultures were challenged against elevated temperature (50˚C) and acid pH (pH 3). While ribosomal dimers were detected in *rmf*$^+$ cells but not in an *rmf*-deficient mutant strain before challenge, they disappeared quickly on exposure to these stresses. It was found that the aggregation of the 70S units in the dimer form served to initially alleviate the effect of acid stress on these ribosomal units[34]. However, this effect did not persist that after few hours of acid challenge there was almost no difference in the ribosomal profile of RMF$^+$ and *rmf*-deleted strains, while there was a significant difference in viability between strains. This contradicted the concept presented by Wada[2], Ishihama[33], and Wada *et al*.[30], in which the deletion of *rmf* and thus the absence of 100S dimers were suggested to accelerate ribosome breakdown and viability decline during the stationary phase. They based this on the observation that the dissociation of dimers accompanied the loss of viability at latter stages in the stationary phase. Although cells are prone to stress in the stationary phase, this remains less severe than that imposed by acidification or heating. This is evidenced by more rapid loss of viability under the latter conditions compared to the stationary phase. The rapid dissociation of 100S dimers on exposure to low pH or high temperature compared with their stability for several days during the stationary phase could be due to the increased stressful effect of these environmental pressures. It might be possible that the degradation of the ribosomal dimers at latter stages

of the stationary phase coincided a state where the stress was great enough to induce the de-dimerisation and cell death. Wada *et al*.[30] similarly suggested that a "common factor" might be behind the simultaneous events of ribosome breakdown and viability decline during the late stationary phase.

We considered another route through which RMF might exert its effect that led to increased survivability of parental *E. coli* strains compared to *rmf*-deficient cells. This was to examine the influence of *rmf* deletion on the stability of rRNA during stress. We observed that *rmf*-deficiency was associated with higher extent of rRNA degradation in the mutant strain than the parent organism on exposure to heat[38] or low pH[34]. This correlated with the difference in viability between both strains during the stress challenge; in cases where parent strain had higher survival, it showed less rRNA degradation than the mutant strain. Recent data generated by Yoshida *et al*.[31] suggested that RMF bound 23S rRNA in *E. coli* and protected certain bases from chemical modification by dimethyl sulphate. Since 23S rRNA is the core, if not the unique, component of the peptidyl transferase centre on ribosomes[39], this RMF–rRNA binding was porposed to be a mechanism by which RMF inhibits translation. However, this may also serve a protective role decreasing rRNA disintegration under stress.

Taking these observations together, it could be concluded that RMF supported the survival of *E. coli* under various environmental stresses as it was reported during the stationary phase. However, this influence could not be attributed to the formation of ribosome dimers, but was rather associated with increased integrity of rRNA against dissociation on exposure to stress.

As demonstrated above, the expression of *rmf* was found to increase under conditions of slow growth. Considering data produced by Wada *et al*.[29] suggesting that RMF inhibited protein synthesis *in vitro*, the increased expression of *rmf* could be presumed to slow the rate of growth. So, if cells are grown in an environment that favours slow growth, it would not be clear whether this causes a decline in the growth rate that induces the expression of *rmf* or that *rmf* expression is induced by these suboptimal growth conditions and would cause a decrease in growth rate. This "chicken or egg" puzzle was resolved by showing that *rmf* expression was induced by the synthesis of guanosine-3', 5'-(bis) pyrophosphate (ppGpp), which is a cellular regulator stimulated by conditions of low growth rates[40]. When two genes encoding enzymes involved in the synthesis of ppGpp

were deleted (*relA* and *spot*), *rmf* expression remained low, even at low growth rates. However, this relationship does not exist under non-growing conditions as in the late stationary phase where the accumulation of spermidine caused an increase in ppGpp associated with a decline in RMF[41]. In addition, we observed that growth rates of an *rmf*-deficient strain were not higher than those of the parent strain under conditions of acid stress[34], which may have been expected if RMF reduced growth rate. These observations lead to the realisation that slow growth induces the expression of *rmf* but RMF does not slow the growth.

In spite of the increased expression of *rmf* during growth under environmental stress, 100S dimers could not be detected in ribosomal extracts of exponential phase cells[28,41,34]. It was observed that the expression of *rmf* was induced by acid pH during the exponential phase to levels approximating those expressed by stationary phase cells under low pH. However, ribosomal dimers were only detected in the stationary phase cultures[34]. *In vitro* studies using 70S ribosomes extracted from *E. coli* mixed with excess RMF showed that the levels of ribosome dimerisation were higher using ribosomes prepared from stationary phase cells than with those prepared from exponential phase organisms[29]. This suggested that the formation of 100S dimers may not be determined only by the expression of *rmf*, but other factors might also contribute to the process. Data generated by Niven[38] using differential scanning calorimetry, which is an *in situ* technique, suggested stationary phase conformational changes in *E. coli* ribosomes. These changes may initiate 70S ribosomes for dimerisation by RMF.

If RMF does not induce ribosome dimerisation during the exponential phase, what effect can it exert on cell growth under stress? Why do cells increase the expression of *rmf* when growth is slowed down? We have done *in vivo* experiments on the effect of RMF on the translation efficiency of *E. coli* under acid stress and found that it had positive influence on this process (El-Sharoud and Niven, unpublished results). It is possible that this effect of RMF might allow cells to compensate for the decrease in ribosome number caused by growth under stress. Cells may exploit this in the synthesis of chaperones that reduce or overcome damage in cellular components.

Agafonov *et al*.[36] demonstrated that YfiA was detected in the ribosome fraction of *E. coli* on exposure to cold shock, but not in cells grown under normal conditions. It was thus suggested that YfiA was a cold shock protein whose function was related to translation.

Further experiments in the same work showed YfiA inhibiting the elongation step in the translation process. Unlike RMF, YfiA was proposed to exert this effect by binding and blocking the aminoacyl-tRNA site (A site). However, the significance of the role of YfiA to cell's survival and growth under chilling stress was not elucidated.

Concluding remarks and future prospects

The described effect of RAPs, inducing the formation of non-functional ribosomes and thus inhibiting protein synthesis during environmental stress, appears to be a form of global bacterial survival behaviour. That is "maintaining function during stress may lead to cell death, while dormancy could aid survival or adaptation". In addition to above example of bacterial spore formation stimulated by adverse environmental conditions, vegetative cells also express the similar behaviour during their different growth phases. It is now widely-appreciated that cells in the exponential phase grow and increase in numbers, but are more vulnerable to stress than stationary phase organisms that show no net increase in numbers and show inhibition in protein synthesis.

Various survival-genes are expressed in the stationary phase to help cells overcome starvation and other adverse conditions in this phase. Most of these genes are recognised by the sigma factor S (σ^S or RpoS), the stationary phase specific sigma factor of RNA polymerase that is also induced under environmental stress. It is interesting to note that *rmf* is RpoS-independent which represents its survival-aiding role as a distinctive strategy during the stationary phase and under more severe environmental stress.

It could be realised from current literature on RAPs that they bind ribosomes to form "relaxed" ribosomes such as 100S dimers that are inactivate in translation but provide more protection to ribosomes during the stationary phase. This appears to be a temporary measure to protect ribosomes from moderate stress and, when environmental conditions improve, RAPs disassociate from ribosomes to allow the formation of functional 70S ribosomal units. Under more severe stress such as heat and acid pH, we found that RMF aided the survival of *E. coli*, but observed 100S dimers dissociating rapidly on exposures to these stresses. We thus could not attribute the protective effect of RMF against stress to its ability to induce ribosome dimerisation. More controversially is our observation that the deletion of *rmf* improved the efficiency of translation during exponential growth under acid stress. In those experiments, we used *in vivo*

assessment of the efficiency of protein synthesis compared with previous *in vitro* measurements suggesting RMF as an effective inhibitor of translation. However, we are in the process of developing a theory reconciling these contradictory conclusions.

An area of interest in the present topic is to examine the nature of relationship, if any, between RAPs. They have relatively contrasting characters that while RMF is a very basic protein, binds to 50S subunits and is presumed to inactivate the P-site on ribosomes, YfiA and YhbH are weakly-acid proteins binding to 30S subunits and proposed to inactivate the A-site. Is this diversity meant for providing the "inactivation for preservation" response under diverse stress conditions? That is, for example, pH conditions inappropriate for the activity of RMF could be suitable for the activity of YfiA and YhbH, and *vice-versa*. Or, does this diversity allow RAPs to work synergistically under the same conditions? In other words, binding the A-site by YfiA and YhbH may complement translation inhibition by RMF blocking the P-site. It is also reasonable to suggest that bacteria may use RAPs in either these ways depending on the environment.

Another aspect is the observations that the binding site of RMF was found to be close to that of the translation initiation factor-3 (IF-3)[31], and that RAPs and IF-3 having opposing functions, IF-3 causing subunit dissociation at the end of one protein synthesis cycle prior to initiation of the next and RAPs stabilising the intact ribosome. It is not determined yet whether RAPs and IF-3 perform simultaneously to compete on binding ribosomes or act in a coordinated sequential mode. Either way could be reasonable within certain context. The first approach may explain the inactivation of translation by RAPs during the stationary phase that the increase in these proteins and the decrease in IF-3 allow more RAPs binding ribosome and preventing the initiation of translation. On the other hand, during growth under stress, RAPs may bind ribosomes after the release of the initiation factors and thus stabilise them during subsequent translation steps. Further research is really needed to elucidate this point.

There is also a methodological issue that also deserves future consideration. That is, most knowledge on RAPs and their effect on the ribosome conformation and protein synthesis has been produced using *in vitro* measurements. It is thus difficult to determine whether the obtained results reflect real cellular events, or whether they involve artifacts generated by *in vitro* analysis conditions. Differential scanning calorimetry (DSC) is an *in situ* technique that can be adapted to study the effect of environmental stress on bacterial ribosomes[8,9]. However,

it produces ribosomal profile of two components: one pertaining to 30S subunit and the other representing 50S+70S units[8,42]. It therefore still requires further development to give more detailed description of the distribution of the ribosomal units.

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