

Gordon W. Niven

Ribosome modulation factor protects *Escherichia coli* during heat stress, but this may not be dependent on ribosome dimerisation

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Abstract The role of ribosome modulation factor (RMF) in protecting heat-stressed *Escherichia coli* cells was identified by the observation that cultures of a mutant strain lacking functional RMF (HMY15) were highly heat sensitive in stationary phase compared to those of the parent strain (W3110). No difference in heat sensitivity was observed between these strains in exponential phase, during which RMF is not synthesised. Studies by differential scanning calorimetry demonstrated that the ribosomes of stationary-phase cultures of the mutant strain had lower thermal stability than those of the parent strain in stationary phase, or exponential-phase ribosomes. More rapid breakdown of ribosomes in the mutant strain during heating was confirmed by rRNA analysis and sucrose density gradient centrifugation. Analyses of ribosome composition showed that the 100S dimers dissociated more rapidly during heating than 70S particles. While ribosome dimerisation is a consequence of the conformational changes caused by RMF binding, it may not therefore be essential for RMF-mediated ribosome stabilisation.

Keywords Ribosome stability · Ribosome modulation factor · Heat stress · Ribosome dimerisation

Abbreviations DSC Differential scanning calorimetry · MRD Maximum recovery diluent · RMF Ribosome modulation factor

Introduction

Although 100S ribosomes particles have been known in bacteria for many years (Tissières and Watson 1958; Tissières

et al. 1959; Macarthy 1960), their biological significance has only recently been investigated. Wada et al. (1990) isolated a ribosome-associated protein from stationary phase cultures, designated ribosome modulation factor (RMF). A mutant strain in which *rmf* had been inactivated failed to form 100S ribosomes in stationary phase (Wada et al. 1990; Yamagishi et al. 1993; Yoshida et al. 2002), and the isolated protein was shown to dimerise 70S ribosomes in vitro (Wada et al. 1995). Synthesis of RMF protein is induced in stationary phase cultures independently of the *rpoS*-encoded stationary-phase sigma factor, σ^S (Yamagishi et al. 1993; Izutsu et al. 2001), and strains lacking functional RMF showed reduced viability in stationary phase (Wada et al. 1990, 2000; Yamagishi et al. 1993). It was therefore suggested that ribosome dimers are a storage form of ribosome and that RMF plays a role in the stationary-phase survival strategy of *Escherichia coli*.

Fukuchi et al. (1995) observed increased ribosome breakdown in an RMF-deficient mutant strain and therefore suggested that it may function as an “anti-degradation factor”. However, the mechanism by which RMF may reduce ribosome degradation remains unclear. The death of the RMF-deficient mutant strain in stationary phase is exacerbated in mutants that also lack the cation-specific porin OmpC, and this can be relieved by addition of Mg^{2+} (Apirakamwong et al. 1998). It is well accepted that Mg^{2+} influences the stability of RNA and ribosomes (Gesteland 1966; Hapke and Noll 1976; Bonincontro et al. 1993; Laing et al. 1994). Since deletion of the OmpC gene alone did not increase cell death, these findings tend to support a link between RMF and ribosome stability.

The ribosome has been suggested as a potential site of lethal cell damage during heat stress (Hurst and Hughes 1978; Mohacsi-Farkas et al. 1994; Tolker-Nielsen and Molin 1996; Teixeira et al. 1997). It is therefore possible that any function of RMF in stabilising ribosome structure may also confer protection against such stress. Some support for a role of RMF in stress adaptation was provided by previous observations of increased *rmf* expression in exponential-phase cultures under conditions of nutrient limitation (Yamagishi et al. 1993; Izutsu et al. 2001). In

G. W. Niven (✉)
Food Microbial Sciences Unit, School of Food Biosciences,
The University of Reading,
P.O. Box 226, Whiteknights, Reading, RG6 6AP, UK
Tel.: +44-118-9357214, Fax: +44-118-9357222,
e-mail: g.w.niven@reading.ac.uk

addition, Garay-Arroyo et al. (2000) demonstrated that a mutant strain lacking RMF had increased sensitivity to hyperosmotic shock.

The aims of the current study were to determine if RMF plays a role in protecting *E. coli* cells against heat stress and to investigate the means by which such protection may be conferred. This was carried out by comparing the behaviour of a mutant strain lacking functional RMF (Yamagishi et al. 1993) with the parent strain (W3110).

Materials and methods

Organisms and culture conditions

E. coli strains W3110 and HMY15 were grown in tryptone soya broth (Oxoid) at 37°C. Cultures were inoculated (0.1%, v/v) with stationary-phase cells and incubated with shaking for 24 h to produce stationary-phase cultures, or to an OD of 0.2–0.3 at 600 nm to produce exponential-phase cultures. Strain HMY15 is a derivative of strain W3110 in which *rmf* had been disrupted by insertion of a chloramphenicol resistance gene (Yamagishi et al. 1993). Stocks of this strain were therefore maintained in medium supplemented with 10 µg chloramphenicol ml⁻¹, although this was not present in cultures during experimentation. HMY15 was the generous gift of Professor Akira Ishihama (National Institute of Genetics, Mishima, Shizuoka, Japan).

The effect of heat treatment on culture viability

Cultures were subjected to heat treatment by immersion of 0.5-ml aliquots in a waterbath at 50°C. After incubation for various time intervals, samples were cooled to room temperature by removal from the waterbath and immediate tenfold dilution in maximum recovery diluent (MRD) (Oxoid). Viable counts of heat-treated cultures were made after further tenfold serial dilution and plating 40-µl samples on tryptone soya agar (Oxoid). Colonies were counted after 24 h of incubation at 37°C, although plates were incubated for a further 24 h to ensure that no additional slow-growing colonies had appeared. All reported values are the means of three replicate counts.

Differential scanning calorimetry

Volumes of whole-cell culture (1.5–9 ml) were concentrated by centrifugation for 4 min at 10,000×g, resuspended in 20 µl culture supernatant and sealed in pre-weighed volatile-sample differential scanning calorimetry (DSC) pans. These were analysed using a Perkin-Elmer DSC7 differential scanning calorimeter and a temperature gradient of 30–90°C at 10°C min⁻¹. The result of a blank run containing only growth medium was subtracted from each thermogram. These were then normalised to sample weight (approximately 1 mg), determined after piercing sample pans and

drying at 80°C for 48 h. The reported peak temperature maxima and total enthalpy values are the means of 8–12 replicate analyses. Enthalpies were determined from the area under the thermogram after baseline subtraction. Due to the change in heat capacity during the DSC run, the baseline was estimated as consisting of three linear segments marking the pre-transition baseline, the transition baseline, and the post-transition baseline.

Ribosome analysis by sucrose density centrifugation

Cultures were subjected to heat treatment by growing 100-ml batch cultures to exponential or stationary phase at 37°C and then transferring them to an incubator at 50°C for the required time. All further actions during the isolation and analysis of ribosomes were carried out at 5°C. Unless stated otherwise, the buffer used contained 20 mM Tris-HCl, 10 mM magnesium acetate, 100 mM ammonium acetate and 6 mM 2-mercaptoethanol, pH 7.6. Volumes of cell culture (60 ml) were harvested by centrifugation at 10,000×g for 15 min and the pellets were stored frozen at -70°C. The pellet was homogenised for 10 min with 0.5 g alumina and 200 µl of buffer supplemented with DNase I (2 µg ml⁻¹). The homogeniser was washed using 800 µl of the same buffer and the washings were added to the homogenate. This was centrifuged for 3 min at 10,000×g to remove the alumina and for 30 min at 20,000×g to remove the cell debris. The resulting cell extracts were diluted in buffer to give an absorbance at 260 nm of 20 units using a 1-cm path length. Linear gradients of 5–20% (w/v) sucrose were prepared in buffer to a volume of 8 ml in 13-mm diameter centrifuge tubes. These were loaded with 200 µl cell extract and centrifuged for 3 h at 25,000 rpm in a Beckman SW40Ti ultracentrifuge rotor (maximum relative centrifugal force approximately 75,000×g). Ribosome profiles were determined by pumping the gradients through a UV monitor and continuously measuring the absorbance at 254 nm. This was carried out by positive displacement using 50% (w/v) sucrose. Ribosome profile traces were normalised by division by the absorbance value at 280 nm of the loaded cell extract, yielding data expressed in units of 254 A/280A. Individual peak areas were quantified using the PeakFit for Windows Version 4 computer program (SPSS Software).

Analysis of ribosomal RNA

Ribosomal RNA was analysed by agarose gel electrophoresis following lysozyme-mediated cell lysis by a variation of the method of Kornblum et al. (1988). Aliquots of cell culture (1 ml) were harvested by centrifugation at 10,000×g for 4 min and the pellets were resuspended in 100 µl of lysis buffer containing 20 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, 20% (w/v) sucrose and 1 mg lysozyme ml⁻¹, pH 7.6. Samples were left on ice for 15 min before addition of 100 µl SDS (2%, w/v) and 10 µl proteinase K (5 mg ml⁻¹). After shaking for 15 min at room temperature, samples

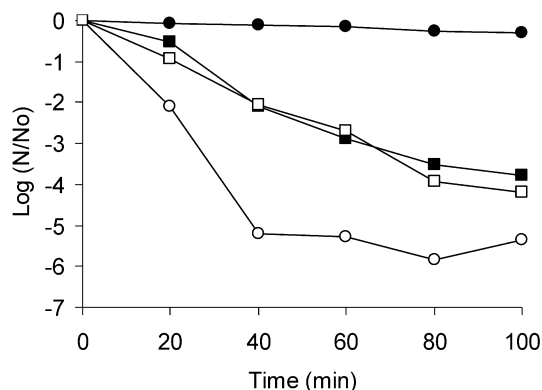


Fig. 1 The effect of heating at 50°C on survival of *Escherichia coli* W3110 (closed symbols) and the RMF-deficient mutant strain HMY15 (open symbols) in exponential-phase (squares) and stationary-phase (circles) cultures

were twice frozen at -70°C and thawed at 45°C . Loading solution (50 μl) containing 50% glycerol, 200 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol was added and 20- μl samples were loaded onto a 1.2% agarose gel containing ethidium bromide (1 $\mu\text{g ml}^{-1}$). The gel was run at 80 V in MOPS-acetate running buffer (Sigma). Ribosomal RNA bands were then viewed under UV light.

Results

Survival of *E. coli* during heating at 50°C

The influence of RMF on cell survival after heat treatment was investigated by comparing the viable cell counts in cultures of a mutant strain lacking RMF (HMY15) with those of the parent strain (W3110) on exposure to 50°C. As shown in Fig. 1, there was little decrease in viability of stationary-phase cultures of the parent strain after 100 min at 50°C, while the viability in exponential phase decreased gradually by approximately 4 log units after the same heat treatment. A similar decrease was observed in exponential-phase cultures of the mutant strain. Cultures of HMY15 in stationary phase were significantly more vulnerable to heat stress than either stationary-phase cells of the parent strain or exponential-phase cells, as viability decreased by over 5 log units within 40 min. It therefore appeared that the lack of RMF compromised the survival of *E. coli* during heat stress, and that this effect was limited to cultures in stationary phase. These observations are consistent with previous observations of the expression of *rmf* in stationary phase (Yamagishi et al. 1993; Izutsu et al. 2001), and with a role of RMF in cellular protection against heat stress.

Thermal stability of ribosomes in vivo by DSC

DSC of whole cell cultures was used to compare the heat stabilities of ribosomes of strains W3110 and HMY15 in situ in the cell cytoplasm. Using this method, samples were

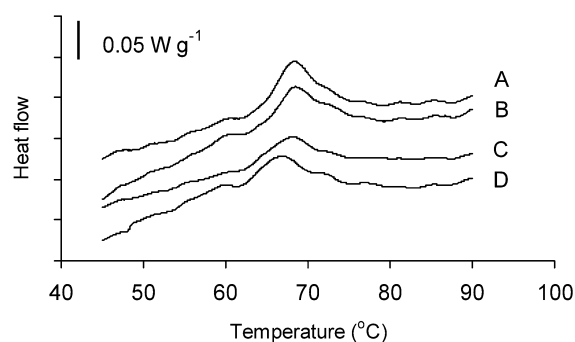


Fig. 2 Representative differential scanning calorimetry thermograms of whole cells of *E. coli* showing strains W3110 (A, C) and HMY15 (B, D) in exponential phase (A, B) and stationary phase (C, D)

heated according to a pre-defined temperature gradient and the amount of energy required to maintain that gradient was measured. Endothermic events associated with the heat denaturation of cell components are observed as peaks in the temperature versus energy-flow thermogram. The major endothermic reactions in the range of 50–90°C are associated with ribosome denaturation, the signals produced by the denaturation of individual cell proteins being negligible by comparison (Mackey et al. 1991; Teixeira et al. 1997). The relative temperatures at which the thermogram signals occur during analysis of whole bacterial cells therefore gives a broad indication of the overall thermal stability of the ribosomes in vivo.

Typical thermograms of exponential- and stationary-phase cultures of *E. coli* W3110 and HMY15 are shown in Fig. 2. The thermogram of the parent strain in exponential phase showed a main endothermic maximum located at 68.1°C ($\pm 0.3^{\circ}\text{C}$). There was also a shoulder to this peak at approximately 73°C and an area of increased heat flow at approximately 61°C . These features were similar to those identified in *E. coli* by Mackey et al. (1991) as m_1 , m_2 and m_3 , and identified as ribosome-associated transitions. The thermogram of the mutant strain in exponential phase was similar, with the main peak maximum at 68.4°C ($\pm 0.2^{\circ}\text{C}$). The similar thermal stabilities of the ribosomes of the parent and mutant strains in exponential phase were therefore consistent with the similar degrees of heat sensitivity of these cultures. The main peak maximum in stationary-phase cultures of the parent strain was located at 68.0°C ($\pm 0.3^{\circ}\text{C}$) and was therefore in a similar position to that of exponential-phase cultures. In contrast, the main peak maximum for stationary-phase HMY15 was located at 66.7°C ($\pm 0.2^{\circ}\text{C}$) and was therefore shifted to a lower temperature. These data indicate that the ribosome conformation of the RMF-deficient mutant strain in vivo was significantly less stable during heating in stationary phase than that of the parent strain.

The relative amounts of ribosomes can be compared using the total enthalpies, estimated from the area under the thermogram. For stationary-phase cultures, these were $3.1 \pm 0.4 \text{ J g}^{-1}$ for the parent strain and $3.1 \pm 0.5 \text{ J g}^{-1}$ for the mutant strain. In exponential-phase cultures, the total en-

thalpies were $5.0 \pm 0.5 \text{ J g}^{-1}$ and $4.3 \pm 0.3 \text{ J g}^{-1}$ for the parent and mutant strains, respectively. Although the amount of ribosomes was therefore greater in exponential phase than in stationary phase, there was no evidence of large differences between the parent and mutant strains.

The implications of these data are that the thermal stabilities of *E. coli* ribosomes were broadly similar in exponential and stationary phases of growth. However, the ribosome thermal stability was significantly reduced in the absence of functional RMF during stationary phase. This suggests that inactive ribosomes in stationary phase are more vulnerable to heat stress than active ribosomes during exponential growth, and that RMF is required to counter this instability.

Analysis of rRNA

Although the viability and DSC data suggested that RMF protects ribosomes during heat stress in stationary phase, further experiments were carried out to determine whether the RMF-inactivated strain and the parent strain showed any differences in ribosome damage during heating. An analysis of ribosomal RNA in heated stationary-phase cultures was therefore carried out. As shown in Fig. 3, two bands of rRNA were visualised in unheated stationary-phase cultures by agarose gel electrophoresis; these corresponded to 23S and 16S molecules. It was observed that

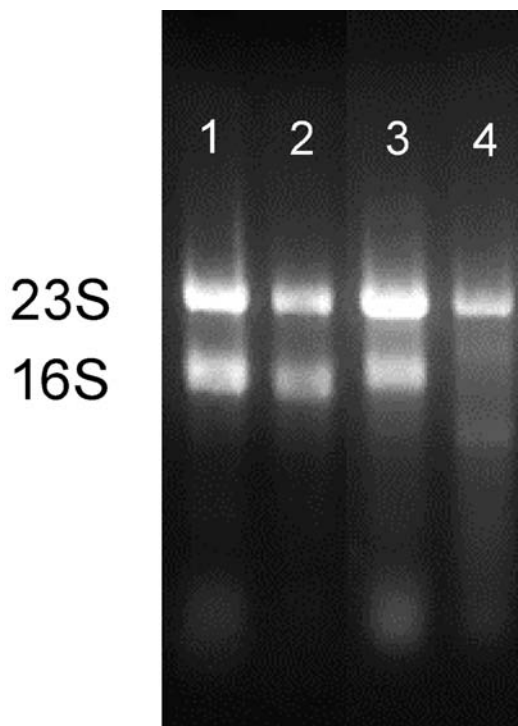


Fig. 3 Analyses of rRNA from stationary-phase cultures of *E. coli* strains W3110 (lanes 1, 3) and HMY15 (lanes 2, 4) before heating (lanes 1, 2) and after heating for 15 min at 50°C (lanes 3, 4). The bands for 23S and 16S rRNA are marked

the reduction in band intensities on heating cultures for 30 min at 50°C was greater for the RMF-deficient mutant strain than for the parent strain. This was particularly the case for the 16S rRNA molecule. Higher levels of ribosome damage in the mutant strain on heating were therefore apparent from the higher levels of rRNA degradation.

Ribosome composition by sucrose density centrifugation

The composition of ribosomes in stationary-phase cells during heat treatment was investigated by sucrose density centrifugation of cell-free extracts prepared from cultures that had been grown at 37°C and then incubated for various times at 50°C . In unheated cultures of the parent strain, 100S dimers accounted for approximately 35% of the total ribosomes (Fig. 4a). Approximately 45% of the total was 70S particles, with 30S and 50S subunits present in equal proportions and accounting for the remaining 20%. After exposure to 50°C for 20 min, the amount of dimers began to decrease, and none were detected after 60 min. There was a concomitant and equivalent increase in the amount of 70S particles with little increase in the amounts of 30S and 50S subunits. This indicates that a conversion of 100S particles to 70S units took place during this period with little loss of 70S ribosomes. Therefore, 100S dimers appeared to be more vulnerable to heat stress than 70S monomers. The amount of 70S particles began to decline after heating for 60 min, the amount remaining at 100 min being approximately 40% that existing at 60 min.

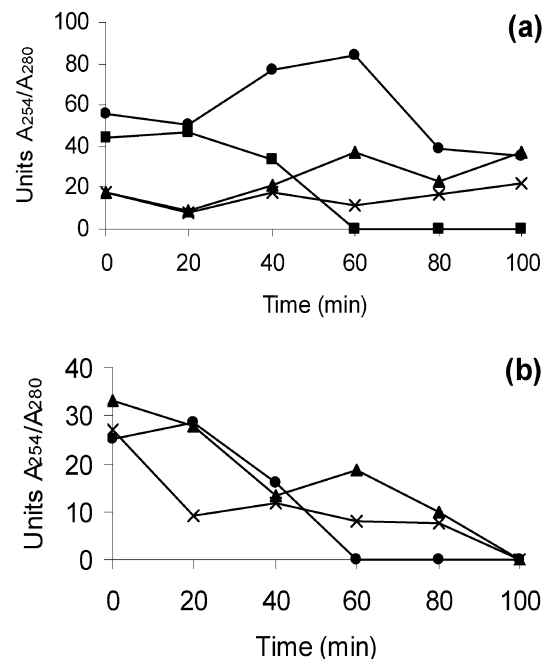


Fig. 4 The ribosome composition of stationary-phase cultures of **a** W3110 and **b** HMY15 during heating at 50°C . The ribosome particles quantified by sucrose density gradient centrifugation were 100S (open circles), 70S (filled circles), 50S (filled squares) and 30S (open squares)

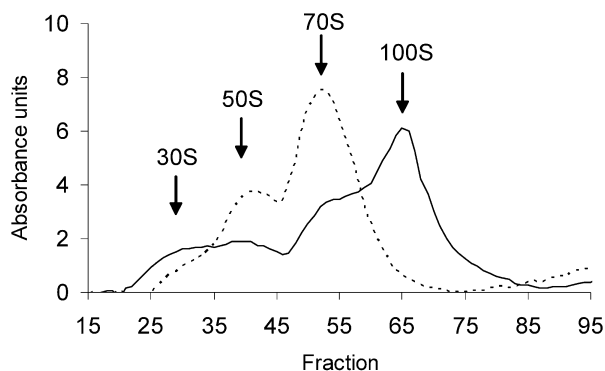


Fig. 5 Ribosome profiles by sucrose density gradient centrifugation of cell extracts of stationary-phase cultures of *E. coli* W3110 before heating (solid line) and after heating at 50°C for 60 min (broken line). The positions of the ribosome subunits are marked. Absorbance units are absorbance of each fraction at 254 nm divided by the absorbance of the cell extract at 280 nm

In the course of the experiment, the total amount of ribosomal particles recovered during analysis fell by approximately 30%, all of this loss occurring between 60 and 100 min. These observations suggested that the initial event on exposure of whole cells to elevated temperature was dissociation of 100S dimers to form 70S particles. On further heating, and after dissociation of all dimers, loss of 70S particles occurred, accompanied by the breakdown of a proportion of the resulting subunits. The initial loss of 100S dimers is also illustrated in Fig. 5, which shows the ribosome profiles of stationary-phase *E. coli* W3110 before heating and after heating for 60 min.

As expected from an RMF-deficient mutant strain and in accordance with previous work using this strain (Yamagishi et al. 1993), no 100S dimers were observed by sucrose density gradient centrifugation of stationary-phase cultures of strain HMY15. The ribosome composition consisted of similar amounts of 70S, 50S and 30S particles (Fig. 4b). The ribosome profiles obtained for heated stationary-phase cultures of the mutant strain contrasted strongly with the above findings for the parent strain. Ribosome loss was detected within 20 min of the start of heating, with 30S subunits being most vulnerable. In the period between 20 min and 60 min of exposure to 50°C, the amount of 70S particles declined to zero and no intact ribosomes particles were detected after 100 min. It was therefore clear that the overall rate of dissociation of ribosome particles on heating was significantly higher in the mutant strain than in the parent strain, which is consistent with the DSC and rRNA analyses described above.

Discussion

It is already well established that stationary-phase cells of *E. coli* are more resistant than exponential-phase cells to a range of environmental stresses, including heat shock. This may be attributed to a variety of structural and physiological changes that take place in the cell, many of which

are co-ordinated and mediated by expression of the *rpoS*-encoded sigma factor, σ^S (Lange and Hengge-Aronis 1991; Loewen et al. 1998). The observation of increased heat resistance in stationary-phase cells compared to those in exponential phase was therefore unsurprising. However, the extreme heat-sensitivity of the RMF-deficient mutant strain in stationary phase indicated that RMF-mediated modulation of ribosome conformation, which is σ^S -independent (Yamagishi et al. 1993; Izutsu et al. 2001), is an important element involved in the heat resistance of *E. coli* cells in stationary phase. That no difference in heat sensitivity was observed between the mutant and parent strains in exponential phase was consistent with the findings of Yamagishi et al. (1993) and Izutsu et al. (2001) that *rmf* is not expressed in rapidly growing exponential-phase cultures.

In order to investigate the influence of RMF on ribosome properties, DSC was used to give a broad indication of the relative thermal stabilities of ribosomes from the parent and the RMF-deficient mutant strains. It has previously been demonstrated that the irreversible endothermic events detected in the temperature range of 50–90°C in DSC thermograms of whole-cell bacterial cultures are associated with ribosome denaturation (Mackey et al. 1991; Teixeira et al. 1997). This type of analysis has been used to correlate ribosome thermal denaturation in vivo with the loss of viability in heated bacterial cultures (Miles et al. 1986; Lepock et al. 1990; Anderson et al. 1991; Mackey et al. 1993). DSC thermogram data therefore give a valid indication of the comparative levels of ribosome thermal stability in vivo. The significant advantage of this technique was that the ribosomes were analysed in situ in the cell cytoplasm, which overcame the effects of buffer composition that could influence studies of isolated ribosomes.

The DSC data indicated that ribosomes in stationary-phase cultures of the RMF-inactivated mutant strain were significantly less stable than either those in the parent strain during stationary phase, or than exponential-phase ribosomes. This suggests that inactive ribosomes may be particularly vulnerable to heat stress in the absence of RMF and that the function of RMF may therefore involve the stabilisation of inactive ribosomes. This idea is supported by analyses of rRNA breakdown and ribosome conformation by sucrose density gradient centrifugation, both of which confirmed more rapid breakdown of ribosomes in heated cultures of strain HMY15 than in strain W3110. This provides strong evidence that is consistent with previous suggestions of the role of RMF in forming a storage form of inactive ribosome that is more resistant to degradation (Wada et al. 1990; Fukuchi et al. 1995; Wada 1998). If the function of RMF relates specifically to inactive ribosomes, then this is also consistent with observations of increased *rmf* expression in slow-growing cultures since these would be expected to contain larger numbers of inactive ribosomes (Yamagishi et al. 1993; Izutsu et al. 2001).

There can be little doubt that RMF is a direct cause of the formation of the ribosome dimers observed in cell extracts of *E. coli* by sucrose density centrifugation. The 100S particles are not found in extracts of strains lacking

RMF (Wada et al. 1990; Yamagishi et al. 1993; Yoshida et al. 2002), and RMF has been shown to induce dimerisation of 70S ribosomes in vitro (Wada et al. 1995). It has therefore been assumed that the function of RMF is directly related to dimer formation (Wada 1998; Yoshida et al. 2002). Yet, the data presented here gave no evidence that 100S dimers were directly involved in protecting ribosomes in heated cultures. The analysis of the ribosome composition of stationary-phase cells during heating revealed complete dissociation of the 100S dimers before any degradation of 70S ribosomes was apparent. Similar results were obtained on exposure of *E. coli* to acid stress, in which immediate dissociation of ribosome dimers was observed despite higher survival of the parent strain than of the RMF-deficient mutant strain (El-Sharoud and Niven, unpublished data).

It has been demonstrated that ribosome dimers have one RMF molecule associated with each 70S particle (Wada 1998; Wada et al. 2000). It is likely that the interaction between RMF and the 70S particle causes a conformational change resulting in a more stable structure that also predisposes the ribosomes to dimerise. The observations reported here suggest that it is the initial conformational change associated with RMF binding that makes the ribosomes more stable rather than any inherent property of the dimers per se. On dissociation of the dimers following heating (or acid shock), the resulting 70S monomers therefore retain the bound RMF and the more robust conformation. Spontaneous ribosome dimerisation in vitro has been shown to be dependent on the chemical environment (Tissieres et al. 1959; Sabo and Spirin 1971; Bernabeu and Lake 1982). It is therefore conceivable that this may also be true of RMF-mediated dimerisation. This would offer an explanation of why higher *rmf* expression is observed in exponential-phase cells at low growth rates, but this does not result in the formation of 100S particles (Yamagishi et al. 1993; Izutsu et al. 2001). Again, we have made similar observations in acid-stressed cells (El-Sharoud and Niven, unpublished data). Although RMF is involved in ribosome dimerisation, the data presented here suggest that dimerisation is not essential to RMF function. However, a full understanding this phenomenon is currently limited by the fact that the method used to observe dimers is only applicable to isolated ribosomes and they have not yet been observed in living cells.

Two important facets of RMF activity have been demonstrated; firstly, that it has a role in the protection of *E. coli* against heat stress, and secondly, that this role is not dependent on the maintenance of ribosome dimers. Our data are consistent with the theory that RMF produces a storage form of inactive ribosomes (Wada et al. 1990, 2000; Yamagishi et al. 1993), suggesting that interaction between RMF and inactive ribosomes increases ribosome thermal stability, making them more resistant to subunit dissociation and rRNA degradation. It is clear that RMF is an important factor in the protection of *E. coli* during heat stress. Work by Garay-Arroyo et al. (2000) on osmotic stress and our own unpublished work on acid stress suggest that RMF may have a general role in stress protection in *E. coli*.

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