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Role of *Escherichia coli* YbeY, a highly conserved protein, in rRNA processing

Bryan W. Davies[1], **Caroline Köhrer**[1], **Asha I. Jacob**[1,4], **Lyle A. Simmons**[1,2,4], **Jianyu Zhu**[3], **Lourdes M. Aleman**[1], **Uttam L. RajBhandary**[1], and **Graham C. Walker**[1,5]

[1] Department of Biology, Massachusetts Institute of Technology, Cambridge, MA

[2] Department of Molecular, Cellular, and Development Biology, University of Michigan Ann Arbor, MI

[3] Department of Molecular, Cell and Developmental Biology and Center for Molecular Biology of RNA, University of California at Santa Cruz, Santa Cruz, CA

Abstract

The UPF0054 protein family is highly conserved with homologs present in nearly every sequenced bacterium. In some bacteria, the respective gene is essential, while in others its loss results in a highly pleiotropic phenotype. Despite detailed structural studies, a cellular role for this protein family has remained unknown. We report here that deletion of the *Escherichia coli* homolog, YbeY, causes striking defects that affect ribosome activity, translational fidelity and ribosome assembly. Mapping of 16S, 23S and 5S rRNA termini reveals that YbeY influences the maturation of all three rRNAs, with a particularly strong effect on maturation at both the 5′- and 3′-ends of 16S rRNA as well as maturation of the 5′-termini of 23S and 5S rRNAs. Furthermore, we demonstrate strong genetic interactions between *ybeY* and *rnc* (encoding RNase III), *ybeY* and *rnr* (encoding RNase R), and *ybeY* and *pnp* (encoding PNPase), further suggesting a role for YbeY in rRNA maturation. Mutation of highly conserved amino acids in YbeY, allowed the identification of two residues (H114, R59) that were found to have a significant effect *in vivo*. We discuss the implications of these findings for rRNA maturation and ribosome assembly in bacteria.

Keywords

rRNA maturation; ribosome

INTRODUCTION

Ribosome maturation and assembly occur in a cooperative and ordered fashion (Kaczanowska and Ryden-Aulin, 2007; Noller and Nomura, 1987). In bacteria, a 50S ribosomal subunit associates with a 30S ribosomal subunit to form an active 70S ribosome. The 50S ribosomal subunit is comprised of 23S and 5S rRNAs and 33 ribosomal proteins while the 30S ribosomal subunit is comprised of 16S rRNA and 21 ribosomal proteins (Wilson and Nierhaus, 2007). 16S, 23S and 5S rRNAs are cotranscribed as part of a large rRNA precursor. Before transcription is complete, ribosomal proteins associate with rRNA to form ribonucleoprotein complexes that are acted on by RNase III (Robertson *et al.*, 1967). RNase III cleaves the initial transcript into 17S, 25S and 9S rRNA precursors that undergo

[5]Corresponding author. Mailing address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139. Phone (617) 253 6711. Fax: (617) 253 2643. gwalker@mit.edu.
[4]These authors contributed equally to this work

further processing at their 5′- and 3′-termini to yield mature 16S, 23S and 5S rRNAs respectively (Gegenheimer *et al.*, 1977; Nierhaus, 1991).

Our understanding of the steps by which rRNA precursors are processed to their mature forms is still incomplete. In *Escherichia coli*, RNase G and RNase E are required for the maturation of the 5′-terminus of 16S rRNA (Li *et al.*, 1999b). RNase T is required for maturation of the 3′-terminus of 23S rRNA (Li *et al.*, 1999a). RNase E is required for partial maturation of the 5′-terminus of 5S rRNA (Misra and Apirion, 1979), and RNase E and RNase T are required for the maturation of the 3′-terminus of 5S rRNA (Li and Deutscher, 1995; Misra and Apirion, 1979). RNases responsible for the final step(s) in maturation of the 3′-terminus of 16S rRNA, 5′-terminus of 23S rRNA and 5′-terminus 5S rRNA remain unknown (Deutscher, 2009) (Fig. 1).

Reconstitution of active 30S and 50S ribosomal subunits has been performed *in vitro* using mature rRNAs and ribosomal proteins (Green and Noller, 1999; Nierhaus, 1980; Nomura and Erdmann, 1970; Wireman and Sypherd, 1974). However, the conditions required for *in vitro* reconstitution are far from physiological and it is well recognized that many additional proteins, that include processing, modification and assembly factors, are required for rRNA maturation and eventual 50S, 30S, and 70S ribosome formation *in vivo*. Several of these factors, such as Era and ObgE, are essential for cell viability (Wilson and Nierhaus, 2007). While these accessory factors are clearly important, in many cases we lack a good understanding of their role in ribosome biogenesis (Bunner *et al.*, 2010).

The UPF0054 protein family is highly conserved among prokaryotes. A gene encoding a member of this protein family is found in almost all sequenced bacterial genomes and it is one of the 206 genes that comprise the predicted minimal bacterial genome set (Gil *et al.*, 2004). In some bacteria including *Bacillus subtilis* (Kobayashi *et al.*, 2003), *Haemophilus influenzae* (Akerley *et al.*, 2002) and *Vibrio cholerae* (manuscript in preparation), the respective homolog is essential. In the plant symbiont, *Sinorhizobium meliloti*, the respective homolog is not essential, but loss of its activity results in extreme pleiotropy and an inability of *S. meliloti* to form a symbiosis with plant hosts (Davies and Walker, 2008).

Here, we show that loss of the *Escherichia coli* homolog, YbeY, results in viable cells with a pleiotropic phenotype and striking defects in ribosome function including decreased ribosome activity, reduced translational fidelity, and altered translation initiation factor binding. Furthermore, we demonstrate that deletion of *ybeY* strongly affects the maturation of all three rRNAs causing a particularly strong defect in maturation of the 16S rRNA 5′- and 3′-termini, as well as maturation of the 5′-termini of 23S and 5S rRNAs. Strong genetic interactions between *ybeY* and *rnc* (encoding RNase III), *ybeY* and *rnr* (encoding RNase R), and *ybeY* and *pnp* (encoding PNPase) additionally suggest a requirement for YbeY activity in rRNA maturation. We discuss the implications of these findings for rRNA maturation and ribosome assembly in bacteria.

RESULTS

UPF0054 protein family members in bacteria are functionally equivalent

Disruption of the gene encoding the *S. meliloti* UPF0054 protein family member, *SMc01113*, results in extreme pleiotropy (Davies and Walker, 2008). Deletion of the *E. coli* UPF0054 family member gene, *ybeY*, from the reference strain MC4100 similarly results in an extremely pleiotropic phenotype that includes a modest decrease in growth rate in rich medium (Fig. 2A), but a significant sensitivity to numerous physiologically diverse stresses including β-lactam antibiotics, temperature, detergents, and oxidative stress (Figs. 2B and C; Supplemental Fig. S1). Deletion of *ybeY* from a second reference *E. coli* strain, MG1655,

resulted in the same phenotypes (data not shown), indicating that the effects are not strain specific. All *ΔybeY* mutant phenotypes were rescued by ectopic expression of *ybeY* (Figs. 2A–C; Supplemental Fig. S1). Expression of either the *S. meliloti* homolog *SMc01113*, or the *B. subtilis* homolog *yqfG* rescues the *ΔybeY* mutant phenotypes as effectively as *ybeY* itself (Figs. 2A–C; Supplemental Fig. S1). Conversely, 6 expression of *ybeY* in the *S. meliloti SMc01113* mutant background rescues all free-living and symbiotic phenotypes of the *SMc01113* mutant (Supplemental Figs. S2A–C and data not shown) (Davies and Walker, 2008; Jones *et al*., 2007). Taken together, these results demonstrate a universally conserved function for UPF0054 members in bacteria.

Deletion of *ybeY* causes defects in polysome formation, ribosome activity, translational fidelity and translation initiation factor binding

Compared to the polysome profile of the parental strain, the *ΔybeY* mutant has a decrease in polysomes and a large increase in both free 50S and free 30S ribosomal subunits relative to 70S ribosomes (Fig. 2D). Ectopic expression of *ybeY* in the *ΔybeY* mutant restored the normal polysome profile (Supplemental Figs. S3A–C). After we had completed much of this work, a study investigating the role of YbeY in the *E. coli* heat shock response also reported that deletion of *ybeY* caused loss of polysomes (Rasouly *et al.*, 2009), however, there was no mention of a potential function for YbeY in ribosome maturation.

The decreased polysome population led us to examine the functional state of the intact ribosomes in the *ΔybeY* mutant. We reconstituted an *in vitro* translation system with an S100 extract from the wild type strain MC4100 and 70S ribosomes from either the *ΔybeY* mutant or its parent MC4100. Under saturating substrate conditions, ribosomes from the *ΔybeY* mutant had reduced translational activity compared to 70S ribosomes from MC4100 (Fig. 2E). Furthermore, both frameshifting and readthrough of nonsense codons were increased *in vivo* in the ***ΔybeY*** mutant, with the largest effect being for +1 frameshifts (Figs. 2F and G). The accumulation of defects in ribosomal function and polysome assembly, could account for the pleiotropic nature of the *ΔybeY* mutant since many stress response programs rely on the upregulation of defense proteins to ward off stress or to repair damage (Friedberg, 2005;Imlay and Linn, 1987).

The increased pools of free 30S and 50S ribosomal subunits found in the ***ΔybeY*** mutant suggest that a portion of these ribosomes may be defective in assembly of the 70S ribosome. In bacteria, translation initiation requires three protein initiation factors; IF1, IF2 and IF3. IF2 correctly positions the initiator tRNA in the ribosomal P-site and promotes association of 30S and 50S ribosomal subunits. While IF1 is also known to stabilize the binding of the initiator tRNA, IF3 acts as a proofreading and anti-association factor (Kaczanowska and Ryden-Aulin, 2007). Analysis of total cell lysates showed only a slight but reproducible decrease in IF2 and an increase in IF3 in the ***ΔybeY*** mutant (Fig. 2H). However, we found a substantial decrease in IF2 present in the fraction containing 30S subunits isolated from the ***ΔybeY*** mutant, suggesting that many of the mutant 30S ribosomal subunits are not available for 70S ribosome assembly. We also observed a slight increase in IF3 associated with ***ΔybeY*** mutant 30S subunits and 70S ribosomes, but the change was modest compared to IF2. This suggests that the 30S ribosomal subunits in a ***ΔybeY*** mutant have a defect that prevents ribosome assembly and/or promotes disassembly of 70S ribosomes when assembly is attempted.

The *ΔybeY* mutant is defective in rRNA maturation

The ribosome is primarily composed of rRNA (Wilson and Nierhaus, 2007) and incomplete processing of rRNA can lead to defects in ribosome function (Wireman and Sypherd, 1974). Comparison of total rRNA profiles by agarose gel electrophoresis from MC4100 and the

ΔybeY mutant showed the presence of a substantial amount of 17S rRNA in the *ΔybeY* mutant, as well as a faster migrating species, which we annotate as 16S*, indicating a defect in 16S rRNA maturation (Fig. 3A). 17S rRNA is the precursor of 16S rRNA (Fig. 1) and is generated from the initial rRNA transcript by RNase III cleavage (Gegenheimer *et al.*, 1977). The aberrant 16S* rRNA species may be a product of misprocessing and/or degradation of the 17S or 16S rRNA. Ectopic expression of *ybeY* in the *ΔybeY* mutant restored normal 16S rRNA maturation (Supplemental Fig. S3D). We were unable to assess the state of 5S and 23S rRNA processing in these total rRNA profile experiments (see below, however) because the difference between mature and immature 5S and 23S rRNAs is only a few nucleotides, respectively, and thus does not cause a detectable shift on agarose gels.

In strain MC4100, 30S ribosomes contained mostly 16S rRNA along with some 17S rRNA, whereas 70S ribosomes contained only 16S rRNA (Fig. 3A). In contrast, 30S ribosomal subunits from the *ΔybeY* mutant contained much more 17S rRNA than 16S rRNA as well as a substantial amount of 16S* rRNA (Fig. 3A). Furthermore, there was also a substantial amount of 17S rRNA in 70S ribosomes isolated from the *ΔybeY* mutant (Fig. 3A). The accumulation of 17S rRNA was not due to the slower growth rate of the *ΔybeY* mutant. When the strains were grown in minimal medium where MC4100 and the *ΔybeY* mutant show similar doubling times, we still observed similar levels of 17S rRNA accumulation (data not shown). 17S rRNA is thought not to be competent for translation *in vitro* (Wireman and Sypherd, 1974). The increased abundance of 17S and 16S* rRNA in the 30S ribosomal subunits could account for altered translation initiation factor binding and the other translational defects described above for the *ΔybeY* mutant. Similarly, the increased abundance of 17S rRNA in 70S ribosomes could explain, at least in part, the reduced translation activity and decreased translational accuracy of the *ΔybeY* 70S ribosomes. Interestingly, 16S* rRNA was not found in *ΔybeY* 70S ribosomes suggesting that it is not competent for assembly into the 70S ribosome and may thus be a non-functional product of rRNA processing in the *ΔybeY* mutant.

In agreement with the observed increase in 17S rRNA in the *ΔybeY* mutant, Northern blot analysis showed that both 5′- and 3′-termini of 17S rRNA were present at much higher levels in total RNA extracted from the *ΔybeY* mutant than in total RNA from the parental strain MC4100 (Fig. 3B). Use of primer extension to map the 5′-terminus (Fig. 3C) and site-specific RNase H cleavage followed by the Northern hybridization (Li *et al.*, 1999a) to map the 3′-terminus of 16S rRNA (Fig. 3D) showed that the termini of the mature and immature 16S rRNA in total RNA extracted from the *ΔybeY* mutant and the parental strain MC4100 were identical. However, consistent with the above results, both immature 5′- and 3′-termini of 16S rRNA were present at higher levels in total RNA extracted from the *ΔybeY* mutant (Figs. 3C and D). We did not observe either 5′- or 3′-termini shorter than that of mature 16S rRNA, although, if the 5′- or 3′-terminus of the 16S* species were extensively degraded, the assays used would not have detected them.

Primer extension and site-specific RNase H cleavage were also used to determine the maturation state of 23S and 5S rRNAs. Strikingly, along with 16S rRNA, the maturation of the 5′- and 3′-termini of both 23S and 5S rRNA were also affected, with all four termini showing increased amounts of the immature form in the *ΔybeY* mutant (Figs. 3C and D), indicating that *ybeY* function is required for normal 5′ and 3′ processing of 16S, 23S and 5S rRNAs. Nevertheless, the most significant defects in the *ΔybeY* mutant appeared to be in the complete maturation of the 5′- and 3′-termini of 16S rRNA.

Rasouly *et al.* demonstrated a defect in the polysome profile of a Δ*ybeY* mutant after heat shock (Rasouly *et al.*, 2009). After shifting the cultures to 45 °C for 1 h, we observed little

mature 16S rRNA in the Δ*ybeY* mutant by agarose gel electrophoresis; instead we saw two bands that migrate near the 17S and 16S* rRNA positions (Supplementary Fig. S3E). In contrast, there were no significant changes in the rRNA profile of MC4100 samples shifted to 45 °C compared to those at 37 °C. Since 17S rRNA is not competent for translation (Wireman and Sypherd, 1974), and 16S* rRNA appears to be excluded from 70S ribosomes (Fig. 3A), the increase in 17S and 16S* rRNA-like species and the decrease in 16S rRNA in the *ΔybeY* mutant following heat shock could explain the defect in polysome formation at elevated temperatures (Rasouly *et al.*, 2009) and the overall temperature sensitivity of the *ΔybeY* mutant described above (Fig. 2C).

ybeY shows strong genetic interactions with *rnc* (RNase III), *rnr* (RNase R) and *pnp* (PNPase)

The serious defects in translation and rRNA maturation in the *ΔybeY* mutant suggested that YbeY might be involved in rRNA metabolism. To explore the cellular role of YbeY, we compared the rRNA profile of the *ΔybeY* mutant to that of several well characterized *E. coli* RNase mutants disrupted in the following genes: *cafA* (RNase G), *rnc* (RNase III), *rnr* (RNase R), *pnp* (PNPase), *rnd* (RNase D), *rnt* (RNase T), and *rph* (RNase PH) (Fig. 4A). We also transduced each of these RNase mutations into the *ΔybeY* mutant background and examined the rRNA profiles of these double mutants (Fig. 4B). We then mapped the 5′- and 3′-termini of 16S, 23S and 5S rRNAs for each strain using primer extension and site-specific RNase H cleavage assays (Figs. 4C–H) as described above.

Profiles of total rRNA showed that, of all the mutants tested, the *ΔybeY* mutant had the greatest defect in maturation of 17S rRNA to 16S rRNA (Fig. 4A). Primer extension and site-specific RNase H cleavage assays confirmed this result showing high levels of both immature 5′ and 3′ 16S rRNA termini in the *ΔybeY* mutant compared to the other RNase mutants (Figs. 4C and D). As previously described (Li *et al.*, 1999b), the Δ*cafA* mutant also showed severe defects in maturation of the 16S rRNA 5′-terminus. However, in contrast to the *ΔybeY* mutant, which accumulates the full 17S rRNA 5′ precursor (+115 nucleotides), the Δ*cafA* mutant accumulates the shorter +66-nucleotides 5′ precursor (Fig. 4C). Also, of all the mutants tested, only the *ΔybeY* mutant showed accumulation of high levels of immature 16S rRNA 3′-terminus (Fig. 4D).

The introduction of either the *rnc* or *rnr* mutation into the *ΔybeY* mutant resulted in major alterations in the gross rRNA profile of the *ΔybeY* mutant (Fig. 4B). Strikingly, 17S rRNA was no longer present in the Δ*ybeY* Δ*rnc* mutant, instead 16S* rRNA and a new rRNA species migrating more slowly than 17S rRNA accumulated. Primer extension and site-specific RNase H cleavage assays confirmed this new precursor, which we designate 18S* rRNA, as a 16S rRNA derivative with additional nucleotides on both the 5′ and 3′-termini of 17S rRNA (Figs. 4C and D). Gegenheimer *et al*. reported a transient 18S rRNA species in a *Δrnc* mutant (Gegenheimer *et al.*, 1977) that is matured to 16S rRNA and has a similar mobility as the new precursor species we observe. We have not mapped the termini of 18S* rRNA or the 18S species found by Gegenheimer *et al.* to the nucleotide level, but based on their relative mobility, we suggest that the 18S* rRNA observed in the Δ*ybeY* Δ*rnc* mutant may be 18S rRNA. If 18S* and 18S rRNA are identical, our data suggest that the absence of YbeY influences the processing of 18S rRNA to 16S rRNA in a Δ*rnc* mutant. Further characterization of the 18S* rRNA species in the Δ*ybeY* Δ*rnc* mutant could be of much interest.

The Δ*ybeY* Δ*rnr* mutant showed a substantial decrease in 16S rRNA and reciprocal increase in both 17S and 16S* rRNA (Fig. 4B). This result was confirmed by mapping of the 5′- and 3′-termini (Figs. 4C and D). Furthermore, primer extension of 16S rRNA 5′-termini showed an increase in a set of new rRNA species of minor intensity that migrated between 17S and

16S rRNAs. These new species may represent partially mature or non-functional 16S-like rRNA species. RNase R has been shown to function as a scavenging RNase that removes non-functional rRNA from the cell (Cheng and Deutscher, 2003). Loss of this function is consistent with the appearance of 16S rRNA intermediates observed by primer extension in the Δ*ybeY* Δ*rnr* mutant. It also suggests that, in the absence of *ybeY*, the cell misprocesses large amounts of 17S rRNA generating abnormal species that RNase R normally helps to degrade.

Strikingly, we observed very little of the mature 16S rRNA 3′-terminus in either the Δ*ybeY* Δ*rnr* mutant or the Δ*ybeY* Δ*pnp* mutant rRNA (Fig. 4D). Deletion of either *rnr* or *pnp* alone does not significantly affect the maturation of the 16S rRNA 3′-terminus (Fig. 4D). The RNase(s) required for maturation of the 16S rRNA 3′-terminus has not been identified (Deutscher, 2009). Our results suggest that PNPase and RNase R may act in maturation of the 16S rRNA 3′-terminus and their role may be modulated by YbeY. Interestingly, while both the Δ*ybeY* Δ*rnr* mutant and the Δ*ybeY* Δ*pnp* mutant affect maturation of the 16S rRNA 3′-terminus, only the Δ*ybeY* Δ*rnr* mutant exhibits additional defects in 16S rRNA metabolism as shown by the appearance of extra aberrant rRNA species (Figs. 4B and C). This suggests that RNase R and YbeY interact in aspects of rRNA metabolism beyond 16S rRNA 3′-terminus maturation, such as quality control of rRNA.

Mapping of the 5′- and 3′-termini showed that deletion of *ybeY* also affected 5′ maturation of 23S and 5S rRNAs (Figs. 4E and H). The Δ*ybeY* mutant had a greater effect in 23S and 5S rRNA maturation than nearly all of the RNase mutants tested. As expected, deletion of *rnc* abolishes formation of mature 23S rRNA 5′ and 3′-termini (King *et al.*, 1984). Also as expected, deletion of *rnt* greatly inhibits maturation of the 23S and 5S rRNA 3′-termini (Li *et al.*, 1999a). Interestingly, we have also found that in the Δ*ybeY* Δ*rnt* mutant the 17S rRNA precursor was extended by 1 or 2 nucleotides at the 3′-terminus (Fig. 4D), suggesting the possible involvement of RNase T in trimming the 17S precursor at the 3′-terminus prior to complete 3′ maturation of 16S rRNA. Deletion of *pnp* and *rph* also caused accumulation of 5′-immature 5S rRNA precursor implicating a role of these two RNases in this step of rRNA maturation (Fig. 4G).

RNase E is known to function in the maturation of the 5′-terminus of 16S rRNA (Li *et al.*, 1999b), and the 5′ and 3′-termini of 5S rRNA (Li and Deutscher, 1995; Misra and Apirion, 1979). RNase E is essential in *E. coli* and temperature sensitive mutants have been used to explore its potential roles in rRNA maturation (Li *et al.*, 1999b). Using the *rne-1* temperature sensitive RNase E allele, we constructed a *rne-1* Δ*ybeY*::cat^{R} double mutant and attempted to explore their genetic interaction in rRNA processing at the non-permissive temperature (45 °C). The rRNA profile is shown (Supplementary Fig. S3F); mapping of the 16S rRNA termini showed that RNase E contributes to the maturation of the 5′-terminus of 16S rRNA as expected (Li *et al.*, 1999b), but does not contribute significantly to the maturation of the 3′-terminus under the conditions used (data not shown). Unfortunately, it is difficult to interpret the results from the *rne-1* Δ*ybeY*::cat^{R} double mutant grown at the non-permissive temperature because of the strong temperature-sensitivity of the Δ*ybeY* mutant by itself (Rasouly *et al.*, 2009; see also Fig. 2C and Supplementary Fig. S3E). Because of this, effects of the *rne-1* mutation at 45 °C are overshadowed by effects of the Δ*ybeY* deletion. A temperature-independent approach for conditional *rne* inactivation will be necessary for such experiments.

Growth rate analysis of the Δ*ybeY* double mutants

We next examined the growth behavior in rich medium of single and double mutants described above. Further strengthening a connection between YbeY and RNase R, we observed the greatest effect in the Δ*ybeY* Δ*rnr* mutant (Fig. 5). This mutant shows reduced

growth rate in addition to an extended lag phase. Interestingly, while both the Δ*ybeY* Δ*rnr* and the Δ*ybeY* Δ*pnp* mutants affect 16S rRNA 3′-terminus maturation strongly, the Δ*ybeY* Δ*pnp* grows identically to the Δ*ybeY* mutant, showing only a modest growth rate reduction. The change in growth behavior of the Δ*ybeY* Δ*rnr* mutant compared to the Δ*ybeY* mutant could be due to defects other than the incomplete maturation of the 16S rRNA, e.g. quality control of rRNA. Also, the significant accumulation of 16S* rRNA, a species that can be assembled in 30S ribosomal subunits, but not in functional 70S ribosomes (Fig. 3A) may be detrimental to the cell and be responsible for the growth behavior of the Δ*ybeY* Δ*rnr* mutant.

We also observed a decrease in growth rate in the Δ*ybeY* Δ*rnc* mutant in rich medium (Fig. 5) compared to the respective single mutants, although this was less pronounced than for the Δ*ybeY* Δ*rnr* mutant. Thus, the strong genetic interaction observed in the Δ*ybeY* Δ*rnr* and Δ*ybeY* Δ*rnc* mutants is manifested at both the rRNA processing and physiological levels.

Highly conserved amino acid residues required for YbeY activity *in vivo*

All UPF0054 family members contain a conserved H3XH5XH motif (Fig. 6A). The presence of this motif has led to the categorization of UPF0054 family members as putative metal-dependent hydrolases and, more specifically, by some as putative metal-dependent proteases due to the presence of a similar motif found in certain mammalian proteases (Koonin, 2003;Tatusov *et al.*, 2001;Verma *et al.*, 2002). The recently determined structures of *E. coli* YbeY and two additional UPF0054 family members (Oganesyan *et al.*, 2003;Yeh *et al.*, 2005;Zhan *et al.*, 2005) support a putative metal-dependent hydrolytic function, however, no substrate has been identified for this protein family despite extensive screening (Oganesyan *et al.*, 2003).

To study the structure-function relationship of the conserved UPF0054 motif and other highly conserved amino acids in this protein family, we mutated several conserved amino acid residues in *ybeY* on our complementation plasmid to Ala (Fig. 6A). We then determined the ability of these mutant *ybeY* alleles to complement the growth and rRNA processing defects of the Δ*ybeY* mutant (Figs. 6B and C). The first His residue (H114) in the conserved H3XH5XH motif was required for partial recovery of growth of the Δ*ybeY* mutant at 45 °C and to rescue rRNA processing defects of the Δ*ybeY* mutant, supporting the importance of this motif for YbeY function (Figs. 6B and C). Interestingly, changing the second and third His residues from this motif (H118, H124) to Ala did not have much of an effect on complementation. A highly conserved Arg residue (R59) was also found to be necessary for growth complementation. The loss of complementation by H114A and R59A alleles was not due to insufficient protein expression as plasmid-driven expression of all *ybeY* mutant alleles exceeded *ybeY* expression from the endogenous locus (Supplementary Fig. S3G). It should be noted that some of the *ybeY* mutant alleles that did not show an effect on complementation when expressed from a multicopy plasmid, may do so if they are expressed at levels of YbeY normally present in wild type cells.

DISCUSSION

We have shown that the functionally conserved UPF0054 protein family member, YbeY, influences rRNA maturation and ribosome formation in *E. coli*. Deletion of *ybeY* from *E. coli* results in defects in ribosome assembly and activity as well as in attenuation of 16S, 23S and 5S rRNA processing.

rRNA processing, ribosome maturation and translation are intertwined. Several reports have implicated partial ribosome assembly as a requirement for full rRNA maturation (Mangiarotti *et al.*, 1974; Mangiarotti *et al.*, 1975; Srivastava and Schlessinger, 1988) and, while deletion of specific RNases can lead to specific defects in rRNA maturation (Li and

Deutscher, 1995; Li *et al.*, 1999a, , 1999b), loss of non-RNase ribosome maturation factors can also lead to rRNA maturation defects. For example, both Era and ObgE are GTPases required for ribosome assembly. Era and ObgE are essential for viability in *E. coli* and loss of either activity causes accumulation of 17S rRNA (Inoue *et al.*, 2003; Sato *et al.*, 2005). Similarly, deletion of ribosome maturation factors RimB and RimP results in slower growth rates, reduced number of polysomes and accumulation of 16S rRNA precursor (Lövgren *et al.*, 2004). Furthermore, loss of RsgA, a GTPase activated by the 30S ribosomal subunit, results in the accumulation of both 17S rRNA and another species shorter than 16S rRNA (Himeno *et al.*, 2004). Our results could also mean that loss of YbeY affects a critical step in ribosome biogenesis and thereby slows rRNA maturation. It will be interesting to explore physical interactions of YbeY with the ribosome and rRNA directly as well as possible effects of YbeY on ribosomal proteins, e.g. protein modifications. Supporting a potential association of YbeY with the ribosome, recent work has identified interactions between YbeY and both 30S and 50S ribosomal proteins in a large scale pull-down experiment (Arifuzzaman *et al.*, 2006).

Active translation has also been suggested to be required for certain steps in rRNA maturation (Hayes and Vasseur, 1976; Sirdeshmukh and Schlessinger, 1985; Srivastava and Schlessinger, 1988). The dramatic decrease in IF2 binding to the 30S ribosomal subunit in the Δ*ybeY* mutant (Fig. 2H) could suggest that YbeY is needed, directly or indirectly, to form the translation initiation complex. A decrease in efficiency of initiation complex formation could slow translation to a point that negatively impacts maturation of rRNA.

The strongest evidence of a role for YbeY in rRNA and/or ribosome maturation comes from the analysis of rRNA in strains carrying the Δ*ybeY* mutation alone or in combination with other mutations in known RNases (Figs. 3 and 4). RNases required for the processing of the 16S rRNA 3′-terminus, 23S rRNA 5′-terminus and 5S rRNA 5′-terminus have not been identified. Interestingly, we observe significant defects in processing of all of these termini in the Δ*ybeY* mutant (Figs. 3C and D). The Δ*ybeY* mutant shows a much stronger overall effect on rRNA maturation than any of the RNases tested, even those with a known role in rRNA processing (Figs. 4C–H). Only RNase III, which is required for the initial cleavages of the rRNA precursor, also affects the maturation of multiple rRNA termini. Deletion of both *ybeY* and *rnc* stabilizes the accumulation of an 18S-like rRNA precursor with similar mobility to that of a transient 18S rRNA species previously observed in an *rnc* mutant (Gegenheimer *et al.*, 1977). Loss of YbeY activity may, therefore, exacerbate a specific rRNA maturation defect that is only observed in the absence of the RNase III required for the initial cleavage events in rRNA maturation.

Deletion of *ybeY* along with either *rnr* or *pnp* prevents, almost completely, the maturation of the 16S rRNA 3′-terminus (Fig. 4D), while maturation of all other rRNA termini proceeds to some degree in both of these mutants. Furthermore, large amounts of 16S rRNA are improperly processed in the Δ*ybeY* Δ*rnr* double mutant, as shown by the accumulation of additional aberrant 16S rRNA maturation intermediates. These results suggest that YbeY may function along with RNase R and PNPase in the maturation of the 16S rRNA 3′-terminus and potentially in other steps of rRNA metabolism such as rRNA quality control.

Site-directed mutagenesis of YbeY supports the requirement for one of the His residues (H114) in the highly conserved putative hydrolase domain and a highly conserved Arg residue (R59) for YbeY function *in vivo* (Fig. 6). For example, ectopic expression from a plasmid of *ybeY* mutants H114A and R59A in the Δ*ybeY* mutant failed to complement the growth defect of the mutant strain at elevated temperatures. The H3XH5XH motif is postulated to coordinate a Zn^{2+} ion that could act catalytically in a hydrolysis reaction (Zhan *et al.*, 2005). The crystal structure of YbeY also shows the highly conserved R59 pointing

inward towards the H3XH5XH motif (Zhan *et al.*, 2005). The location of R59 along with its positive charge could imply that R59 helps position a substrate near the H3XH5XH motif. Given the strong genetic interactions of *ybeY* with *rnc*, *rnr* and *pnp* (Figs. 4C–H) established here, along with the requirement of the putative hydrolase domain for *ybeY* activity, YbeY may also have RNase activity in the cell.

It is currently unclear why only one (H114) out of three His residues within the highly conserved H3XH5XH motif appears to be required for *ybeY* activity. It is possible that contributions of the other two His residues (H118, H124) towards YbeY activity may also be seen, when YbeY mutants are expressed at wildtype-like levels. It is interesting to note the H114A and R59A mutants show similar sensitivity to growth at elevated temperature, but the rRNA processing defects are markedly different (Figs. 6B and C). While the rRNA profile of the H114A mutant resembles that of the *ybeY* mutant, the rRNA profile of the R59A mutant more closely resembles that of the MC4100, suggesting that the different mutations affect different functions of YbeY or affect the same rRNA processing function to different degrees.

Finally, although deletion of *ybeY* results in severe rRNA maturation defects suggesting a role in rRNA and/or ribosome maturation it remains possible that YbeY actually has an alternative function in the cell that impacts these processes. Although this remains a formal possibility, the simplest explanation for the striking effects of deletion of *ybeY* on rRNA processing and ribosome assembly is that YbeY functions directly in rRNA and/or ribosome maturation and assembly.

METHODS

Strains, plasmids, growth conditions and DNA manipulations. Strains and plasmids are shown in Supplementary Table S1. Strains were grown aerobically in Luria-Bertani (LB) medium at 37 °C except for heat shock experiments where strains were grown at 45 °C. Antibiotics for strain selection were used at the following concentrations, ampicillin 100 μg/ml, chloramphenicol 20 μg/ml, and kanamycin 25 μg/ml. Deletion of *ybeY* and the addition of a C-terminal FLAG tag to the genomic copy of *ybeY* (strain BWD55) was performed using the methods of Wanner (Datsenko and Wanner, 2000). For deletion, *ybeY* was first replaced with a cat^R cassette creating the Δ*ybeY*::cat^R mutant. The cat^R cassette was then excised to create a clean Δ*ybeY* deletion mutant in MC4100. Deletion of *ybeY* maintains the ATG start of *ybeY* and the overlapping stop codon of the upstream gene *ybeZ*. Translation from the ATG start of *ybeY* terminates 8 amino acids into the flip recombinase site of the λ-red gene targeting recombinase system (Datsenko and Wanner, 2000). DNA primers used for deletion of *ybeY* and addition of the C-terminal FLAG tag are listed in Supplementary Table S2. Allele transfers were done by P1 transduction. DNA manipulations were performed according to the methods of Sambrook (Sambrook and Russell, 2001). Site-directed mutagenesis was performed using the Quikchange II kit from Stratagene. DNA primers used for site-directed mutagenesis are listed in Supplementary Table S2. For complementation studies, *ybeY*, *SMc01113* and *yqfG* were cloned downstream of the tetracycline promoter in pBR322 or the tryptophan promoter in pMS03. The DNA primers used for complementation cloning are shown in Supplementary Table S2.

Phenotypic analysis. Stress and plant assays were performed as previously described (Davies and Walker, 2008).

Polysome and rRNA analysis. rRNA and polysomes were extracted from cells growing exponentially at 37 °C, unless otherwise stated. Cells were pelleted, resuspended in buffer A (20 mM HEPES pH 7.5, 5 mM β-mercaptoethanol, 10 mM $MgCl_2$, 50 mM NH_4Cl and 0.1

mM PMSF) and lysed by repeated freeze/thaw cycles. After clarification by centrifugation, lysates were loaded on a 5–20% sucrose gradient. Polysomes and ribosomes were separated by ultracentrifugation followed by fractionation using a peristaltic pump. The A_{260} value was determined for each fraction.

rRNA was extracted from logarithmically growing cultures or separated ribosome fractions using Qiagen RNeasy Mini Kit. For gross analysis of 16S and 23S rRNA, 800 ng of rRNA were separated by synergel/agarose gel electrophoresis as described (Wachi *et al.*, 1999) using 0.9% synergel and 0.7% agarose.

Primer extension assays were performed using Superscript II reverse transcriptase (Invitrogen) as per manufacturer's instructions with primers annealing to the 5′ terminal region of mature 16S, 23S, and 5S rRNA, respectively. The site-specific RNase H cleavage assay was performed as described (Li and Deutscher, 1995; Li *et al.*, 1999a, , 1999b) followed by Northern hybridization using probes specific for the mature 3′-termini of 16S, 23S, and 5S rRNAs, respectively. 100 ng of total RNA were used for each assay. Reaction products were separated on denaturing polyacrylamide gels; 10 % polyacrylamide/7M urea for primer extensions and 6 % polyacrylamide/7 M urea for sitespecific RNase H cleavage assays. DNA oligonuleotides for primer extension and RNase H mapping are shown in Supplementary Table S2.

For the Northern blot in Fig. 2B, 250 ng of total MC4100 and ΔybeY RNA were separated on a 1 % agarose gel containing 2 % formamide. Probes used to identify 17S rRNA 5′ and 3′-termini are shown in Supplementary Table S2. The probes were detected with the AlkPhos Direct Labeling Kit (Amersham).

For temperature shift assays, cultures were grown to exponential phase at 37 °C. Samples were taken for rRNA analysis and the cultures were then shifted to 45 °C. After 1 h samples were taken for rRNA analysis.

In vitro translation and *lacZ* assays

In vitro translation assays were performed essentially as described (Matthaei and Nirenberg, 1961). Exponentially growing cells were pelleted, resuspended in buffer A and lysed by French press. Lysates were centrifuged twice at 30 000g and once at 100 000g. The resulting supernatant was kept as the S100 fraction, and the pelleted ribosomes were washed and resuspended in buffer A. Ribosomes were further purified by pelleting at 200 000g through a 20% sucrose cushion. Pelleted ribosomes were resuspended in buffer A and quantitated by UVabsorbance at 260 nm, and stored at −80 °C.

Mix I (25 μg of polyU mRNA and 10 pmoles of ribosomes) was pre-incubated at 37 °C for 15 min in polyU-mix (20 mM HEPES pH 7.5, 2 mM DTT, 10 mM $MgCl_2$, 50 mM NH_4Cl, 1 mM spermidine, 2 mM ATP, 0.5 mM GTP, 8 mM phosphoenolpyruvate and 2 μM pyruvate kinase). Mix II (50 μg of bulk tRNA, 100 μg S100 extact and 200 pmoles of C^{14}-Phe) was incubated at 37 °C for 15 min in polyU-mix. Mix I and II were mixed and incubated at 37 °C for 15 min. ^{14}C-Phe incorporation into protein was determined by TCA precipitation and scintillation counting. All reactions were performed in triplicate.

For LacZ assays, strains carrying wild type or mutant *lacZ* alleles on a plasmid were harvested in exponential phase. The assay for LacZ activity was then performed as described (Miller, 1972). The percent LacZ activity (in Miller units) of each mutant *lacZ* allele relative to the wild type *lacZ* allele for MC4100 or the *ΔybeY* mutant is reported.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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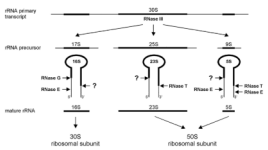

Figure 1.

Steps in the processing of rRNA in *E. coli*. All enzymes indicated are endonucleases with the exception of RNase T that generates mature 3' termini by trimming the rRNA precursors.

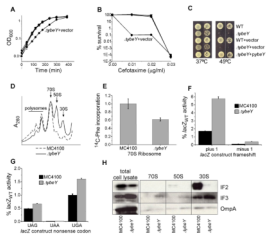

Figure 2.

Phenotypic analysis of the *E. coli* Δ*ybeY* mutant. (**A**) Growth curves of MC4100 (WT) and the Δ*ybeY* mutant complemented strains in LB at 37 °C. Doubling times: 40 ± 2 min (*ΔybeY* mutant) vs. 28 ± 3 min (MC4100). Sensitivity of the Δ*ybeY* mutant to stresses (**B**) cefotaxim and (**C**) temperature. The *ΔybeY* mutant with empty vector only (*ΔybeY*+vector) is shown on each plot for clarity. UPF0054 homologs: *ybeY* (*E. coli*), *yqfG* (*B. subtilis*) and *SMc01113* (*S. meliloti*). WT+vector (□), *ΔybeY*+vector (●), Δ*ybeY*+p*ybeY* (▲), Δ*ybeY*+p*yqfG* (▼) and Δ*ybeY*+p*SMc01113* (◀). "p" indicates that the gene indicated is expressed from a plasmid. In panel (C), the pairs of samples show a ten-fold dilution each. (D) Polysome profiles for MC4100 and the *ΔybeY* mutant. The positions of polysomes, 70S ribosomes and 50S and 30S ribosomal subunits are indicated. (E) *In vitro* translation assay under saturating substrate conditions. MC4100 S100 fractions were mixed with a polyU template and equal amounts of MC4100 or ***Δ****ybeY* mutant 70S ribosomes in an *in vitro* translation reaction as described in the Materials and Methods section. Translational activity is normalized to MC4100 70S ribosome reactions. MC4100 and the ***Δ****ybeY* mutant were transformed with plasmids expressing *lacZ* containing (**F**) frameshift mutations (+1 or −1) or (**G**) nonsense codons. LacZ activity was assayed as described in the Materials and Methods section. The 33 percent LacZ activity (in Miller units) of each mutant *lacZ* allele relative to the wild type *lacZ* allele for MC4100 or the *ΔybeY* mutant is reported. The value of LacZ activity from the wild type allele (18921 Miller units in the *ΔybeY* mutant and 12247 Miller units in MC4100; respectively) was set to as 100% activity. For clarity, wild type *lacZ* activity has been omitted from the plots. Each assay was performed in triplicate. (**H**) Immunoblots identifying IF2 and IF3 in whole cell lysates, 30S, 50S and 70S ribosome fractions from MC4100 and the *ΔybeY* mutant. Immunoblotting for OmpA is used as a loading control. Equal A_{260} amounts were loaded for the 30S, 50S and 70S ribosome fractions. The experiment was repeated 3 times and a representative result is shown.

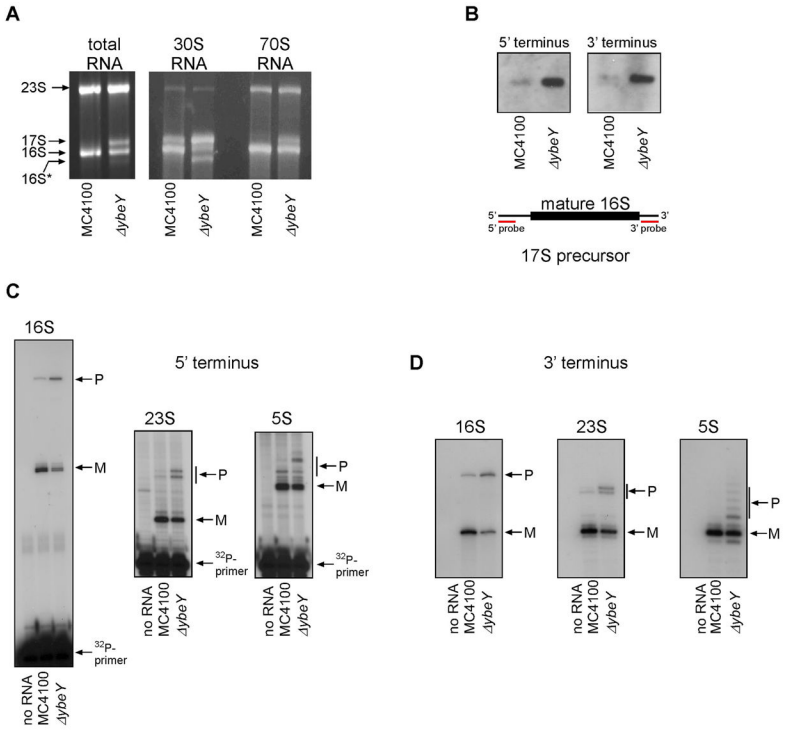

Figure 3.

Analysis of rRNA from *E. coli* MC4100 and the *ΔybeY* mutant. (**A**) Total RNA isolated from whole cells, 30S ribosomal subunits and 70S ribosomes from MC4100 and the *ΔybeY* mutant. The positions of 23S, 17S, 16S and 16S* rRNAs are indicated based on their mobility. (**B**) Northern blot analysis using probes directed against the 5′- and 3′-termini of 17S rRNA. Equal amounts of total RNA from MC4100 and the *ΔybeY* mutant were used. The locations of the probes are shown in the diagram below the blots. (**C, D**) Primer extension and site-specific RNase H cleavage assays to map the 5′- and 3′-termini of 16S, 23S and 5S rRNAs from MC4100 and the *ΔybeY* mutant. “P” and “M” indicate the location of bands corresponding to precursor and mature forms of the rRNA, respectively. Annotation of the positions of the 5′ and 3′-termini of 16S, 23S and 5S rRNAs, mature and precursor species, were based on previous observations (Li *et al.*, 1999b). Total RNA was prepared from MC4100 and the *ΔybeY* mutant strains as described in the Methods section. 34

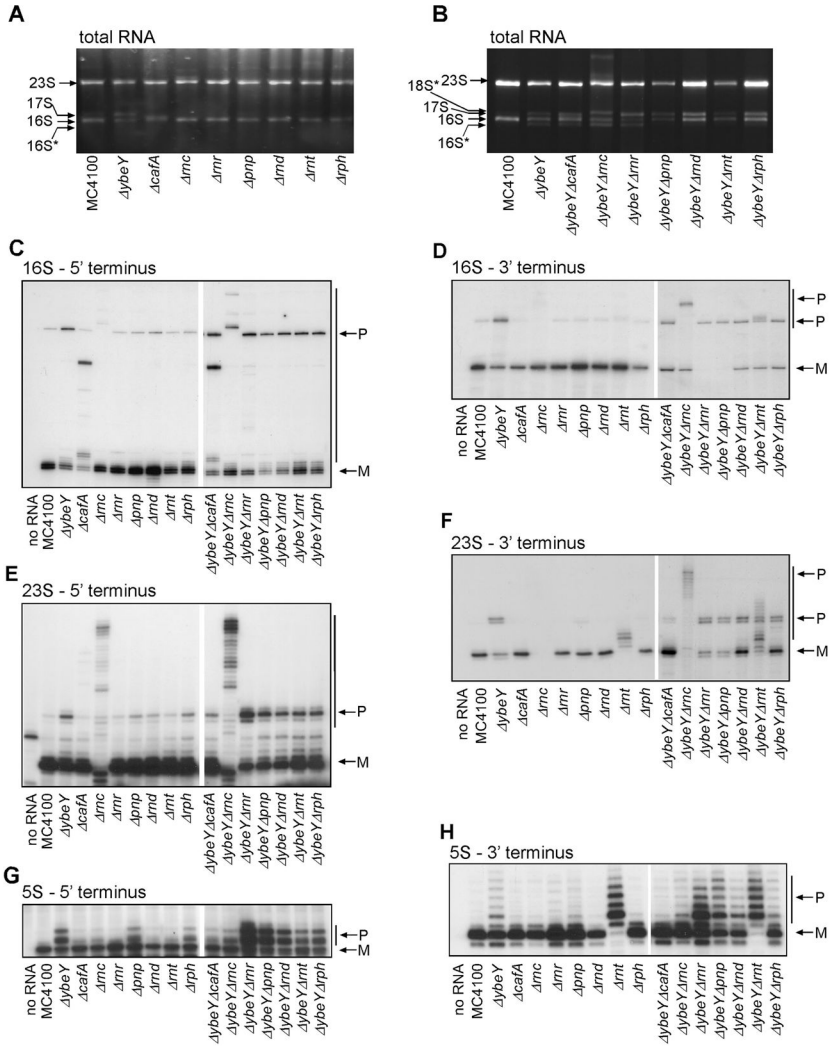

Figure 4.

Analysis of rRNA from the *ΔybeY* mutant and seven well characterized *E. coli* RNase mutant strains. The relevant genotype from which the rRNA was extracted is indicated under each lane. The parental strain MC4100 rRNA is shown in each case as a control. (**A–B**) Agarose gel electrophoresis of total rRNA from single and double RNase mutant strains. The positions of 23S, 18S, 17S, 16S and 16S* rRNAs are indicated. (**CH**) Primer extension and site-specific RNase H cleavage assays to map the 5′- and 3′-termini of 16S, 23S and 5S rRNAs from single and double RNase mutant strains. “P” and “M” indicate the position of bands corresponding to the precursor and mature form for each rRNA. Annotation of the positions of the 5′ and 3′-termini of 16S, 23S and 5S rRNAs, mature and precursor species, were based on previous observations (Li *et al.*, 1999b).

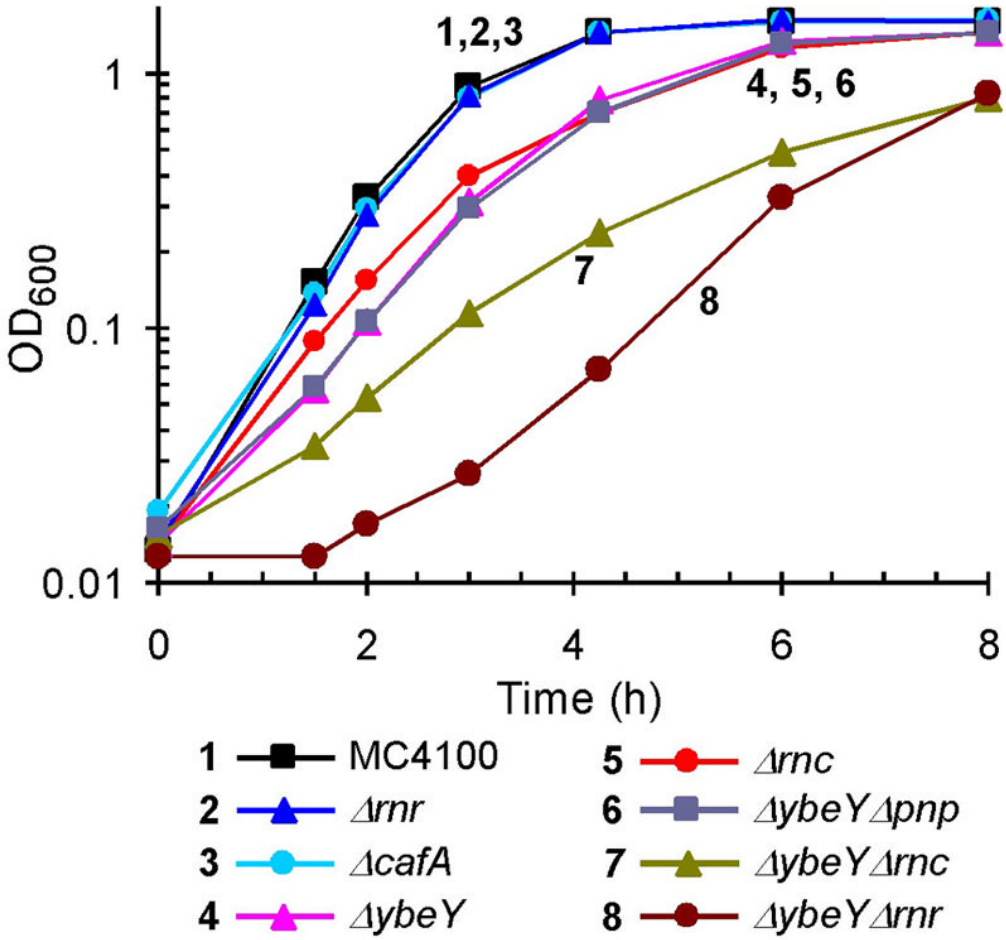

Figure 5.

Growth of MC4100, *ΔybeY* mutant and several *ΔybeY* double mutants in rich medium at 37 °C. Most *ΔybeY* double mutants did not show a growth defect (data not shown); the Δ*ybeY Δpnp* double mutant is shown as an example. In contrast, the Δ*ybeY* Δ*rnc* and Δ*ybeY* Δ*rnr* mutants showed a significant decrease in growth rate.

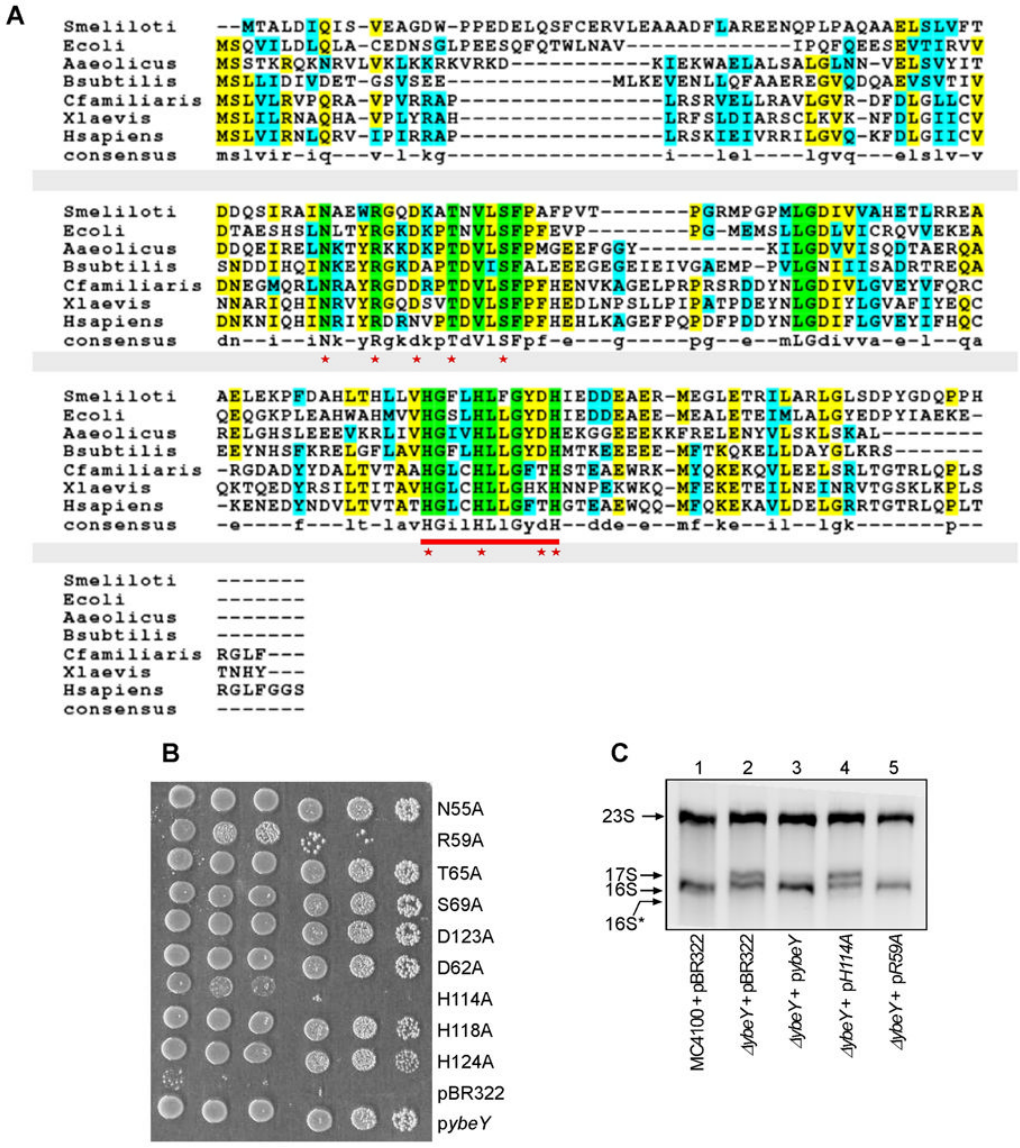

Figure 6.

(**A**) Sequence alignment of UPF0054 homologs from bacteria and eukaryotes. Alignments were performed using T-coffee (Poirot *et al.*, 2003). The red bar underlines the conserved H3XH5XH motif that is used to classify members of this family. Red asterisks indicate amino acid residues that were analyzed in this study by Ala 35 mutagenesis. (**B**) Sensitivity of the Δ*ybeY* mutant strain expressing mutant *ybeY* alleles to high temperature. Strains were serially diluted (1:10), plated on LB plates and incubated at 45 °C. (**C**) rRNA profiles of the Δ*ybeY* mutant expressing the mutant *ybeY* alleles H114A and R59A. The positions of 23S, 17S, 16S and 16S* rRNAs are indicated.