The essential nature of YqfG, a YbeY homologue required for 3′ maturation of *Bacillus subtilis* 16S ribosomal RNA is suppressed by deletion of RNase R

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ABSTRACT

Ribosomal RNAs are processed from primary transcripts containing 16S, 23S and 5S rRNAs in most bacteria. Maturation generally occurs in a two-step process, consisting of a first crude separation of the major species by RNase III during transcription, followed by precise trimming of 5′ and 3′ extensions on each species upon accurate completion of subunit assembly. The various endo- and exoribonucleases involved in the final processing reactions are strikingly different in *Escherichia coli* and *Bacillus subtilis,* the two best studied representatives of Gram-negative and Gram-positive bacteria, respectively. Here, we show that the one exception to this rule is the protein involved in the maturation of the 3′ end of 16S rRNA. Cells depleted for the essential *B. subtilis* YqfG protein, a homologue of *E. coli* YbeY, specifically accumulate 16S rRNA precursors bearing 3′ extensions. Remarkably, the essential nature of YqfG can be suppressed by deleting the ribosomal RNA degrading enzyme RNase R, i.e. a *ΔyqfG Δrnr* mutant is viable. Our data suggest that 70S ribosomes containing 30S subunits with 3′ extensions of 16S rRNA are functional to a degree, but become substrates for degradation by RNase R and are eliminated.

INTRODUCTION

Bacterial ribosomal RNAs are generally synthesized as long 30S precursor transcripts containing 16S, 23S and 5S rRNAs, sometimes interspersed with tRNAs. Processing of these transcripts into the final mature rRNA species typically takes place in two steps. In the two best studied bacteria *Escherichia coli* and *Bacillus subtilis*, the 30S transcript is first processed by the double-strand-specific ribonuclease RNase III at long processing stalks that form through base-pairing between the 5′ and 3′ extensions on either side of the 16S and 23S rRNAs (Figure 1) (1). This processing reaction occurs co-transcriptionally, as soon as the 3′ extension is transcribed and becomes available to hybridize to its complementary 5′ extension (2). Following RNase III cleavage, these leader and trailer sequences are removed in a second step that occurs once ribosome assembly is complete. Removal of the 5′ and 3′ extensions constitutes an important quality control step in ribosome biogenesis, because they can serve as on-ramps for exoribonucleases that can degrade incorrectly assembled particles (3).

The final processing steps for each of the rRNAs have been relatively well described in both *E. coli* and *B. subtilis*, but in both cases are incomplete (Figure 1). The 5′ end of 16S rRNA maturation in *E. coli* is catalysed by the joint action of the endoribonucleases RNase E and RNase G (4), whilst in *B. subtilis*, this reaction is performed by the 5′-3′ exoribonuclease activity of RNase J1 (5,6). For 23S rRNA, the enzyme performing the 5′ maturation step in *E. coli* is still unknown, whilst the 3′ side is matured by the 3′-5′ exoribonuclease RNase T (7). In *B. subtilis*, on the other hand, both sides of mature 23S rRNA are produced simultaneously through cleavage by the double-strand-specific endoribonuclease Mini-RNase III (8). This enzyme requires ribosomal protein L3 bound to its correct site on 23S rRNA to be able to perform the processing reaction efficiently (9). The final steps of *E. coli* 5S rRNA maturation occur in a two-step reaction involving endoribonucleolytic cleavage by RNase E (10), followed by trimming of 3 nt by an unknown enzyme on the 5′ side and by RNase T on the 3′ side (11). In *B. subtilis,* processing of 5S rRNA is performed by a specialist endoribonuclease RNase M5, which cleaves both sides of a double-stranded precursor helix in a single step (12–14). Like the 23S processing reaction, this step also requires a co-factor, in this case ribosomal protein L18 bound to its natural site on 5S rRNA (15). Up to now, none of the known enzymes involved in the final steps of rRNA maturation in *E. coli* existed in *B. subtilis* and *vice versa*. This is remarkable considering the conservation of ribosomal RNA, ribosomal proteins and the translational apparatus in general and im-

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Figure 1. Ribosomal RNA processing reactions in *Escherichia coli* (**A**) and *Bacillus subtilis* (**B**). Mature 16S, 23S and 5S rRNAs are represented by thick green, purple and blue lines, respectively. The cleavage sites of the major processing enzymes are indicated by arrows and their distances from the mature 5′ and 3′ ends are shown. Unknown enzymes are indicated in red. Ribosomal proteins that serve as co-factors in the *B. subtilis* processing reactions are shown as coloured spheres.
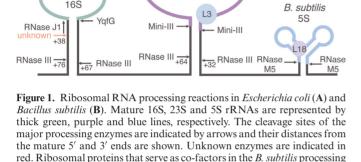
plies that nature has invented the processing machinery at least twice in bacteria, and multiple times if one considers that it is different again in the archaea and in eukaryotes.

Two pathways for the maturation of the 3′ end of 16S rRNA have recently been proposed in *E. coli*. The first involves the new endoribonuclease YbeY (16,17) and the second, a number of redundant 3′-5′ exoribonucleases, PNPase, RNase PH, RNase R and RNase II (18). This processing reaction is critical for making available the anti-Shine-Dalgarno (SD) sequence at the 3′ end of 16S rRNA and thus presumably for efficient translation initiation. The *ybeY* gene is ubiquitous in bacteria and is one of about 200 genes included in the minimal gene set considered to be essential for life in bacteria (19). In addition to its role in 16S rRNA processing, YbeY is also thought to play a role in degradation of defective 70S ribosome subunits in cooperation with RNase R in both *E. coli* and *Vibrio cholerae* (17,20).

Here we show that the essential enzyme YqfG, an orthologue of YbeY, is involved in the 16S 3′ processing reaction in *B. subtilis*, making it the only shared enzyme of the six major rRNA processing reactions. We characterize YqfG in some detail and show that cells depleted for this enzyme fail to accumulate 70S ribosomes. We also show that its essential nature is suppressed in cells lacking RNase R, a major 3′ exoribonuclease involved in ribosomal RNA turnover (21).

MATERIALS AND METHODS

Strains and constructs

Strains and oligonucleotides used are given in Supplementary Tables S1 and 2, respectively.

Strain CCB751 was constructed by Campbell integration of plasmid pMUTIN-YqfG in strain SSB1002 (W168) and subsequent transformation with pMAP65 (22). Plasmid pMUTIN-YqfG was constructed by amplifying an N-terminal portion of the *yqfG* gene by polymerase chain reaction (PCR) using oligos CC1564/1565, digestion with EcoRI and BamHI and cloning in pMUTIN-4M (23) cleaved with the same enzymes. Complementing strains CCB1051, CCB1033, CCB1034 and CCB1035 were made by transforming strain CC751 with wild-type (wt) and mutant derivatives of the integrative plasmid pX-YqfGHis. Plasmid pX-YqfGHis was constructed by amplifying a PCR fragment from pET28-YqfGHis with oligos CC1501/1949, cleaving with SpeI/BamHI and cloning into the vector pX (24) cleaved with the same enzymes. Plasmid pET28-YqfGHis was made by amplifying the *B. subtilis yqfG* gene with oligos CC1709/1710, cleaving the resulting PCR fragment with BamHI/HindIII and cloning in pET28a (Novagen) cut with the same enzymes. Mutant derivatives (R59A, H122A, R59A/H122A) of pET28-YqfGHis were made by site directed mutagenesis using oligo pairs CC1925/1926 and CC1927/1928 (in two steps for R59A/H122A) and then amplified and cloned into pX similarly to wt. pX-YqfGHis and mutant derivatives were linearized with KpnI before transformation in strain CCB751 for integration into the *amyE* locus. CCB795 was constructed by first cloning the native *yqfG* gene into pX to create plasmid pX-YqfG. The *yqfG* gene was amplified by PCR using oligos CC1501/1502 and cleaved with SpeI/BamHI before ligation into pX cut with the same enzymes. pX-YqfG was linearized by KpnI and integrated into the *amyE* gene to create intermediate strain CCB785. The *yqfG* gene was inactivated with a kanamycin resistance cassette in this strain by transformation with a DNA fragment assembled by 3-fragment overlapping PCR using oligo pairs CC1253/1237, CC1236/1239, CC1238/1254, reamplified with oligos CC1253/1254 in a second step. The final integrated construct was amplified from the genome by PCR and sequenced.

The double *Δrnr ΔyqfG* mutant (CBB1140) was constructed by transforming strain CCB327 (*rnr::tc*) with chromosomal DNA from CCB795 (*yqfG::kan amyE::pXyl-yqfG Cm*) with selection for kanamycin resistance. Kan-resistant colonies were screened for sensitivity to chloramphenicol to ensure non-transfer of the *amyE::pXyl-yqfG* construct. Deletion of the *yqfG* gene was confirmed by PCR.

RNA isolation and northern blots

RNA was isolated from mid-log phase *B. subtilis* cells growing in 2xYT medium by the glass beads/phenol method described in (25). Northern blots were performed as described previously (26).

Primer extension

Primer extension assays on total *B. subtilis* RNA have been described previously (5). Oligos CC058, CC257, HP246 were used to assay 5′ processing of 16S, 23S and 5S rRNAs, respectively.

Sucrose gradients

Bacillus subtilis 30S, 50S and 70S ribosomal particles were separated from 50 ml of log phase *B. subtilis* cells at OD_{600} = 0.5. Cells were centrifuged and resuspended in 2 ml ice cold Buffer A (10 mM Tris–HCl pH 7.5, 100 mM NH_4Cl, 10 mM $MgCl_2$, 6 mM β-Mercaptoethanol) plus 10 μg/ml DNase I and lysed by two passages in a French Press (20 000 psi). The lysate was cleared at 13 200 rpm for 30 min at 4°C in a bench top centrifuge. A total of 1 ml of lysate was loaded on a 10–30% sucrose gradient in Buffer A and centrifuged at 18 600 rpm for 16 h at 4°C in an SW41 rotor (Beckmann). A total of 1 ml fractions were collected using a Piston Gradient Fractionator (Biocomp) for analysis on agarose gels.

Labelled RT-PCR

Different exoribonuclease mutants were grown in 2xYT to mid-log phase (doubling times were as follows wt 26 min; *Δrnr* 27 min, *Δrph* 35 min; *Δpnp* 33 min; *Δrph pnp*, 36 min, *Δ rph rnr yhaM pnp* 73 min). A total of 5 μg of total RNA was ligated with RNA ligase (Biolabs). The 5′-P³²-labelled oligo CC1166 was used for first strand cDNA synthesis using MuLV RNase H⁻ reverse transcriptase (Finnzyme) and this cDNA was then used in a PCR reaction containing oligos CC1166 and CC1172. Products were run on a denaturing 5% polyacrylamide gel for size estimation.

RESULTS

The YbeY homolog, YqfG, is essential in *B. subtilis*

We previously showed that processing of the 3′ end of 16S rRNA in *B. subtilis* is catalysed by endonucleolytic cleavage 2 nt downstream of the mature 3′ end followed by trimming back to the fully processed species found in functional 30S ribosomal subunits [27] (Figure 2A). Neither the endoribonuclease, nor the trimming enzyme has so far been identified. Indeed, it is possible that both reactions are performed by the same enzyme in a two-step process or through multiple cleavages over the 2-nt interval. Cleavage by the endonuclease liberates a 65 nt fragment extending from the endonucleolytic cleavage site to the RNase III processing site further downstream. This fragment is degraded by the 5′-3′ activity of RNase J1, as evidenced by its substantial accumulation in *ΔrnjA* strains lacking this activity [27].

YbeY has been shown to be involved in 16S 3′ processing in both *E. coli,* where it is not necessary for viability and in *Vibrio cholerae,* where it is essential [17,20]. The *B. subtilis* homologue of YbeY is encoded by the *yqfG* gene, which is also thought to be essential. The *E. coli* and *B. subtilis* enzymes are 23% identical, 72% similar (Figure 2B) and the *B. subtilis yqfG* gene can complement *E. coli ybeY* deletion mutants for 16S rRNA processing *in vivo* [16]. To ask whether the product of the *yqfG* gene is involved in the 16S 3′ processing reaction in *B. subtilis*, we first made a construct, *Pspac-yqfG,* that allowed us to deplete this enzyme from *B. subtilis* in the absence of isopropyl β-D-1-thiogalactopyranoside (IPTG). CCB751 cells, which have the *Pspac-yqfG* construct integrated at the native locus and extra copies of LacI repressor expressed from plasmid pMAP65 to prevent leaky expression, showed strong growth in the presence of IPTG in spot dilution assays (Figure 2C). In contrast, this strain showed practically no growth in the absence of IPTG, consistent with the idea that the *yqfG* gene is essential in *B. subtilis*.

Two ribonucleases RNase Y and RNase J1 were erroneously deemed to be essential in *B. subtilis* based on similar plate assays of depletion strains grown in the absence of inducer. We subsequently showed that we could successfully knock out each of these genes by transforming wt *B. subtilis* with chromosomal DNA isolated from a strain bearing a deletion of the native gene (*rnjA::spc* or *rny::spc*) and surviving thanks to an ectopically expressed copy of the gene at the *amyE* locus (*amyE::Pxyl-rnjA or amyE::Pspac-rny*) [28]. To be sure that the *yqfG* gene was essential, we therefore constructed a strain (CCB795) in which the native *yqfG* gene was replaced by a kanamycin resistance cassette (*yqfG::kan*) and a copy of the *yqfG* gene was expressed from a xylose-inducible promoter at the *amyE* locus (*amyE::Pxyl-yqfG*). Three independent attempts to transform wt *B. subtilis* with chromosomal DNA from this strain failed to produce kanamycin resistant colonies bearing the *yqfG::kan* construct alone. We also failed to replace the chloramphenicol resistant *Pxyl-yqfG* construct in the *amyE* locus by transformation and selection for a spectinomycin resistant plasmid pDR111 [29] that integrates at the same site. We are thus confident that the *yqfG* gene is indeed essential in *B. subtilis*.

Role of YqfG in 16S rRNA 3′ processing in *B. subtilis*

To examine the role of YqfG in 16S processing, total RNA was isolated from strain CCB751 (*Pspac-yqfG* + pMAP65) grown in the presence and absence of IPTG and assayed by northern blot using an oligonucleotide probe (CC172) complementary to the 65 nt 3′ extension of 16S rRNA. In cells depleted for YqfG, the 65 nt processed species was no longer visible and there was a small but noticeable increase in the amount of intact 16S precursor (Figure 2D), suggesting this enzyme is indeed involved in 16S 3′ processing in *B. subtilis*.

In *E. coli, ΔybeY* mutants show defects in the maturation of both the 5′ and 3′ ends of all three ribosomal RNAs, leaving some questions about the specificity of the enzyme. We assayed for defects in 5′ processing of 16S and 23S rRNA in cells depleted for YqfG by primer extension assay. The 5′ processing of 16S rRNAs (assayed using oligo CC058) was unaffected in the YqfG depletion strain (Figure 3A). In contrast, deletion of the *rnjA* gene, encoding the 5′ processing enzyme, showed no mature 16S rRNA whatsoever and a strong accumulation of precursor species, as described previously for a depletion strain [5]. Primer extension assays using oligonucleotide (CC257), which hybridizes close to 5′ end of the mature 23S rRNA, also produced similar 23S precursor profiles for wt and YqfG depleted cells (Figure 3A). Since Mini-III produces the mature 5′ and 3′ ends of 23S by simultaneous cleavage on either side of a double-stranded helix [8], we consider 5′ processing as a proxy for both reactions. Lastly, we assayed for defects in 5S rRNA processing by northern blot, using oligo HP246, which hybridizes within 5S rRNA. No significant accumulation of longer species was observed in cells depleted for YqfG compared to wt. These experiments demonstrate that, unlike *E.*
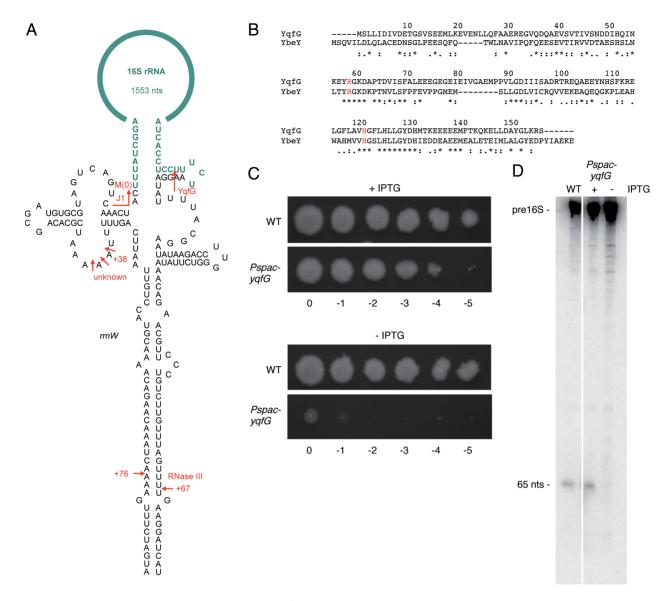
Figure 2. *Bacillus subtilis* YqfG is essential and is necessary for 3′ processing of 16S rRNA. (**A**) Schematic of 16S rRNA and precursor sequences. The mature 16S rRNA sequence is in green and precursor sequences in black. Processing reactions are shown in red. (**B**) Sequence alignment of *Escherichia coli* YbeY and *B. subtilis* YqfG. Identical amino acids are indicated by an asterisk; strongly similar amino acids by a colon; weakly similar amino acids by a full stop. Mutations were made in amino acids labelled in red (see below). (**C**) Spot dilution assays (log scale) of wt strain and CCB751 (*Pspac-yqfG* + pMAP65) in the presence and absence of IPTG. Mid-log phase cells were diluted to the same OD, then diluted in a 10-fold dilution series and 2 μl was spotted on plates. Note that the CCB751 strain was grown in the presence of antibiotics MLS and kanamycin to maintain the Campbell integrative and replicative plasmids. (**D**) Depletion of YqfG results in a lack of 16S 3′ processing and release of the 65 nt downstream fragment. Northern blot probed with an oligo (CC172) against the 3′ extension.

coli ΔybeY mutants, the effect of YqfG depletion is specific for the 16S 3′ processing reaction in *B. subtilis*.

YqfG depletion results in a lack of 70S ribosomes

Escherichia coli ΔybeY mutants have fewer polysomes and a significant increase in 30S and 50S subunits relative to 70S ribosomes compared to wt cells (16). To ask whether YqfG depletion impacts 70S ribosome levels in *B. subtilis*, we performed sucrose gradient analysis of whole cell extracts of wt and YqfG depleted cells. Remarkably, the 70S peak in YqfG depleted cells was only barely detectable, whilst the 30S and 50S subunits accumulated to similar levels to YqfG replete cells (Figure 4). This suggests that a lack of 16S 3′ processing has an even more dramatic effect on 70S particle levels in *B. subtilis* than in *E. coli*.

His122 is critical for both YqfG stability and activity

A conserved histidine and an arginine residue involved in metal ion coordination (either Ni or Zn (30,31)) were shown to be important for *E. coli* YbeY ribonuclease activity *in vitro* (17). We made mutations in the equivalent residues in YqfG, Arg59 and His122 (Figure 2B), to ask whether they could complement *B. subtilis* cells depleted for wt YqfG *in vivo*. For complementation assays, we used
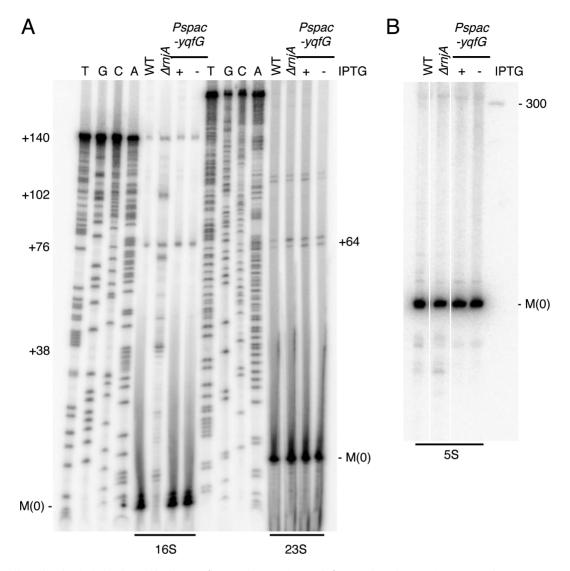
Figure 3. The effect of YqfG depletion is specific for 16S 3′ processing. (**A**) Assay of 5′ processing of 16S and 23S rRNA in wt, *ΔrnjA,* and *Pspac-yqfG* cells (CCB751) grown in the presence and absence of IPTG by primer extension using oligos CC058, CC257, respectively. The mature 5′ processing site is indicated by M(0) and precursor species according to the number of extra nucleotides on the 5′ end. A sequence reaction using the same labelled oligos is provided to the left of each primer extension assay. (**B**) Assay of processing of 5S rRNA by northern blot using oligo HP246. The mature 5′ processing site is indicated by M(0).

derivatives of strain CCB751, which contained the *Pspac-yqfG* construct at the native locus and an ectopic copy of wt or mutant variants of the *yqfG* gene under control of a xylose-inducible promoter integrated at the *amyE* locus (*amyE::Pxyl-yqfGHis*). The ectopically expressed YqfG variants bore C-terminal His-tags to allow us to do western blots to test protein stability *in vivo* (see below). Growing cells in the absence of IPTG allowed us to turn off expression of the wt *yqfG* gene at the native locus, whilst growing cells in the presence of xylose or glucose permitted us to turn on or off the complementing variants, respectively. In spot dilution assays, induction of expression of the wt *Pxyl-yqfGHis* construct in the presence of xylose allowed full growth of *B. subtilis* in the absence of IPTG (Figure 5A). Complementation also occurred in the presence of glucose, suggesting that even leaky expression of YqfG from the *Pxyl* promoter is sufficient for viability. In this assay, xylose-

induced expression of the R59A variant of YqfG complemented quite well, whereas both the single H122A and the double R59A/H122A mutant failed to complement. This result was confirmed in liquid cultures (Figure 5B). Note that leaky expression of the R59A variant in the presence of glucose in liquid culture did not complement as well as the wt, suggesting that it does have a small defect. However, of the two important residues identified in *E. coli* YbeY, His122 clearly plays the more important role in maintaining YqfG function and *B. subtilis* cell viability.

To correlate the growth defects observed in strains expressing the YqfG mutant variants with its roles in 16S rRNA 3′ processing and 70S ribosome assembly, we examined these two processes in the different complementation strains grown in the presence of glucose/xylose or ±IPTG. In cells depleted for a functional YqfG, we observed an accumulation of both a precursor 16S species and
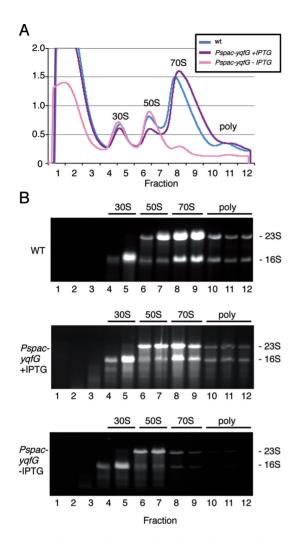
Figure 4. Depletion of YqfG results in a dramatic decrease in 70S ribosomes. (**A**) Sucrose gradients of the wt strain (blue) and the *Pspac-yqfG* strain (CCB751) grown in the presence (purple) and absence of IPTG (pink). (**B**) Agarose gels of sucrose gradient fractions from wt and strain CCB751 grown in the presence and absence of IPTG. Polysomal fractions (poly) are indicated.

smaller degradation product in total RNA (Figure 5C, upper panel). The accumulation of 16S precursor and lack of the 65-nt cleavage product in the H122A mutants was confirmed by northern blot (Figure 5C, middle and lower panels). Finally, the ribosome profiles showing a lack of 70S ribosome particles in strain expressing only the H122A variants were consistent with these results (Figure 5D). In conclusion, the 16S processing and 70S assembly patterns perfectly matched the growth phenotypes for the different complemented strains, with residue H122 of YqfG being critical for these processes.

To test whether R59A or H122A affected catalytic activity or protein stability of YqfG, we measured expression levels of the wt and mutant proteins by Western blot using antibodies against the C-terminal His-tags of the YqfG variants expressed under xylose control. Whilst the R59A mutant showed similar expression levels to wt in the presence of xylose, the H122A variants were expressed at a much lower level suggesting a problem with protein stability (Fig-

ure 5E). It should be noted, however, that expression of the H122A mutants in the presence of xylose was significantly higher than the leaky expression of the wt protein in the presence of glucose. Although this leaky expression of the wt protein is too low to be detected by western blot (Figure 5E, top left panel), it is sufficient to complement all growth and ribosome related phenotypes caused by YqfG depletion (Figure 5A–D). Thus, both mutations affect the catalytic activity of YqfG, with the H122A mutation having an additional impact on protein stability *in vivo*.

YqfG depletion results in degradation of 70S ribosomes by RNase R

To determine whether the lack of 70S ribosomes in cells depleted for YqfG was primarily the result of a defect in association between the 30S and 50S particles or an accelerated rate of 70S degradation, we examined the effect of depleting YqfG in cells lacking RNase R (strain CCB1107). This enzyme is known to be a key enzyme responsible for ribosomal RNA degradation in *E. coli* (21). Although both cultures grew quite slowly, depletion of YqfG in a *Δrnr* background did not appear to have a major growth defect compared to the same strain expressing the 16S 3′ processing enzyme in liquid medium (Figure 6A). Furthermore, ribosome profiles of *Δrnr Pspac-yqfG* cells grown in the absence of IPTG showed elevated levels of 70S particles on sucrose gradients (Figure 6B). In a spot dilution assay, an effect of YqfG depletion in a *Δrnr* background was much more strongly visible than in liquid medium (Figure 6C). However, YqfG depleted cells grew at a >10-fold higher dilution (10^{-1} versus undiluted) if RNase R was also lacking. These results suggest that the lack of 70S ribosomes in *B. subtilis* in cells depleted for YqfG is principally the result of degradation of these defective particles by RNase R.

A logical extension of this observation was that it might be possible to completely delete the *yqfG* gene in strains lacking RNase R. We therefore made competent cells of a *Δrnr* strain and transformed with chromosomal DNA from strain CCB795 (*yqfG::kan amyE::pX-yqfG Cm*). We readily obtained a slow-growing *Δrnr ΔyqfG* double mutant that was Cm sensitive i.e. that lacked the complementing copy of *yqfG* in *amyE*. Thus, a lack of RNase R allows suppression of the lethal phenotopye of YqfG depletion sufficient to form small slow-growing colonies (Figure 6D).

None of the known 3′ exoribonucleases make a significant contribution to the production of the mature 3′ end of 16S rRNA

The *in vivo* cleavage reaction occurs 2 nt downstream of the mature 3′ end of 16S rRNA (27). One possibility is that the removal of the remaining 2 nt to generate the final mature 16S rRNA occurs in a second step either by YqfG itself or by another enzyme, such as a 3′-exoribonuclease. We screened the four known 3′ exoribonuclease mutants of *B. subtilis* (PNPase, RNase R, RNase PH and YhaM) for this activity by RT-PCR. We first added RNA ligase to total RNA isolated from strains lacking one or more of the known 3′-exoribonucleases with the goal of ligating a portion of 16S rRNAs (or rRNA precursors) to the 5′ end of
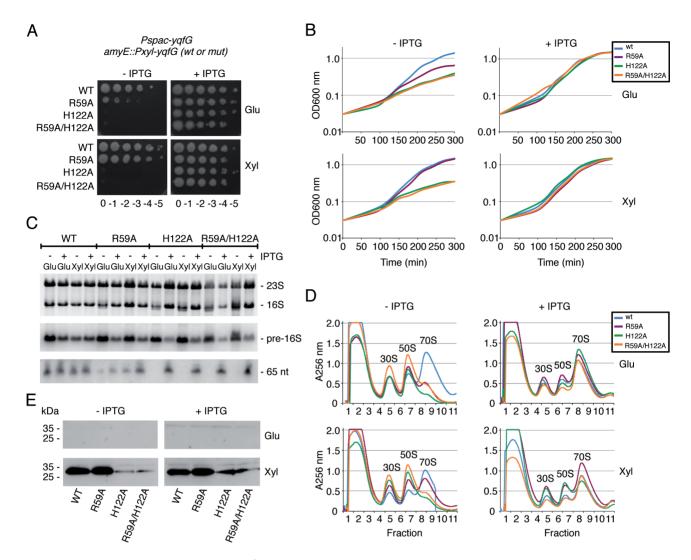
Figure 5. Residue H122 of YqfG is critical for 16S 3′ processing activity and cell growth. (**A**) Spot dilution assay (log scale) of YqfG-depleted cells complemented with wt and various mutants (R59, H122 and R59/H122) of YqfG (strains CCB1051, CCB1033, CCB1034 and CCB1035, respectively). Mid-log phase cells were diluted to the same OD, then diluted in a 10-fold dilution series and 2 μl was spotted on plates with or without IPTG and containing either glucose or xylose. (**B**) Growth curves of the same strains in liquid 2xYT medium. Complementation with the wt strain is shown in blue, the R59A mutant in green, the H122A mutant in red and the R59A/H122A double mutant in orange. (**C**) Upper panel: total RNA isolated from the different complemented strains showing 16S and 23S rRNAs. Middle panel: northern blot of the different complemented strains probed with oligo CC172 showing accumulation of 16SS precursor species. Lower panel: northern blot of the different complemented strains probed with oligo CC172 showing release of the 65 nt processed fragment. (**D**) Sucrose gradients showing 30S, 50S and 70S peaks in the different complemented strains. The traces are colour coded as in panel B. (**E**) Western blot, probed with anti-His antibody, showing the levels of YqfG in the different complemented strains grown ±IPTG and in the presence of glucose or xylose. The migration positions of a molecular weight marker (in kDa) are shown to the left of the blot.

5S rRNA (Figure 7A). We then primed a reverse transcriptase reaction with P^{32}-labelled oligo complementary to 5S rRNA (CC1166) and amplified the resulting cDNAs with a forward facing oligo (CC1172) close to the end of 16S rRNA. Ligation of 5S rRNA to mature 16S rRNA is expected to give a product 64 nt in length, whilst 16S rRNA where the second step has not occurred is expected to yield a 66 nt RT-PCR product. In wt cells we observed a band of the expected 64 nt size (Figure 7B). No single or multiple 3′-exoribonuclease mutant yielded a significant amount of larger RT-PCR product. A tiny amount of a larger species (∼67 nt) was visible in some lanes, but this was far lower than expected if this were the major pathway for the second step. We also observed a small accumulation of species

about 130, 105 and 85 nt in length in some mutants. The longest species seen in the quadruple mutant is consistent with the length expected for 16S rRNA extending as far as the RNase III cleavage site 67 nt downstream (131 nt). However, even in this strain, the vast majority of 16S rRNA is in the mature form. We conclude that whilst none of the known 3′-exoribonucleases makes a significant contribution towards removal of the two extra nucleotides after the cleavage involving YqfG, they can begin removing nucleotides from the downstream RNase III site to a certain extent. However, the fact that YqfG is essential *B. subtilis* suggests that any mature 16S product that might be produced via this pathway is insufficient for growth. Furthermore, our observation that deletion of RNase R can restore viability to cells
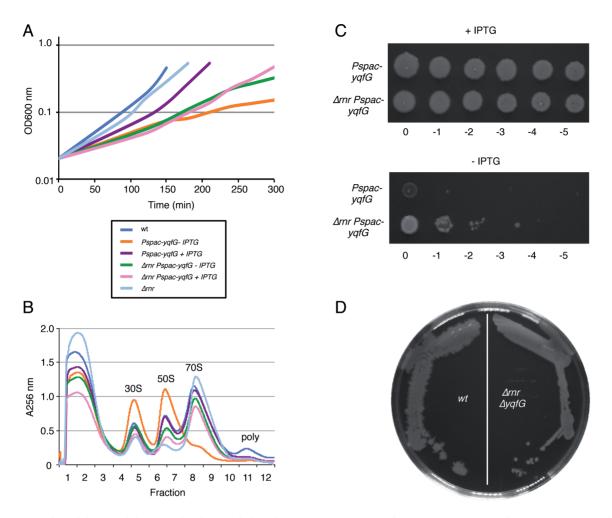
Figure 6. Suppression of the essential nature of YqfG by deletion of RNase R. (**A**) Growth of wt, *Δrnr*, and *Pspac-yqfG* and *Δrnr Pspac-yqfG* strains grown in the presence and absence of IPTG in 2xYT medium. (**B**) Sucrose gradients showing 30S, 50S and 70S peaks in the same strains as panel A. (**C**) Spot dilution assays of *Pspac-yqfG* and *Δrnr Pspac-yqfG* strains grown in the presence and absence of IPTG. (**D**) Comparison of growth of a wt and double *Δrnr ΔyqfG* mutant.

lacking YqfG suggests that, unlike in *E. coli* where the 3′ exoribonucleases including RNase R can stop at the mature 3′ end of 16S rRNA to yield a functional product ([18]), in *B. subtilis* RNase R at least principally functions in quality control mode to promote rRNA decay.

DISCUSSION

In this paper we identify a key enzyme involved in 16S rRNA 3′ processing, encoded by the essential *yqfG* gene. Cleavage occurs 2 nt downstream of the mature 3′ end of 16S rRNA *in vivo* ([27]), suggesting that the removal of the remaining 2 nt to generate the final mature 16S rRNA occurs in a second step. The known 3′ exoribonucleases do not appear to play a significant role in this process. Without an alternative candidate, our working hypothesis is that YqfG itself may be able to remove these 2 nt *in vivo* either through multiple endonucleolytic cleavages within this 2-nt interval or in a two-step endo/exo reaction. We have previously seen evidence for endonucleolytic cleavage and exonucleolytic removal of one or two extra nucleotides by a single enzyme in the maturation of certain tRNAs by RNase Z ([32]).

Recent evidence from Shetty and Varshney ([33]) has highlighted the importance of the initiator tRNA (i-tRNA) in 16S 3′ processing in *E. coli*, suggesting that initiation complex formation may be important for maturation in this organism. The same may be true in *B. subtilis,* but for another reason. Although we do not yet have experimental evidence to support its occurrence, we noticed that the first 5 nt of the 3′ extension (AAGGA) in *B. subtilis* can potentially fold back on complementary nucleotides of the anti-SD sequence (UCCUU) putting the processing site in a double-stranded configuration (Figure [2]). This would be predicted to be inhibitory for *E. coli* YbeY that prefers single stranded RNA ([17]). Indeed, this potentially inhibitory stem loop is not found in *E. coli*. The binding of the SD sequence of mRNA to the anti-SD sequence during initiation complex formation in *B. subtilis* would potentially free up the AAGGA sequence for YqfG promoted cleavage in a single-stranded conformation and serve a similar purpose to the i-tRNA which stabilizes the generally weaker SD:anti-SD pairings found in *E. coli*.

Escherichia coli and *Sinorhizobium meliloti ΔybeY* mutants are viable ([16,34]), whereas in *B. subtilis* and *V. cholerae*
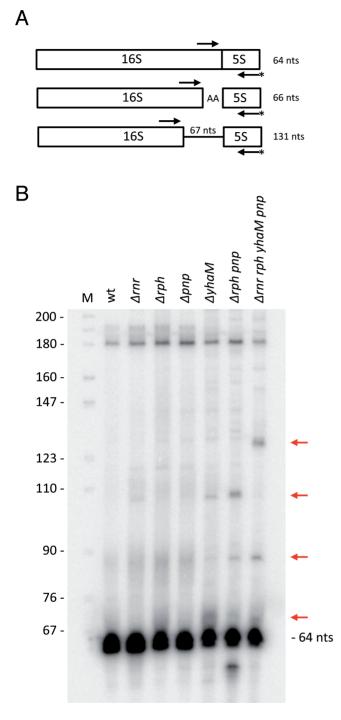
Figure 7. None of the four known 3′-exoribonucleases plays a significant role in the removal of the last 2 nt following YqfG cleavage. (**A**) Schematic of RT-PCR assays with anticipated products. (**B**) RT-PCR assays of total RNA isolated from different 3′-exoribonuclease mutants. Red arrows show migration positions of different length 3′ 16S precursors observed. Oligos used were CC1166 (5S; P[32]-labelled) and CC1172 (16S).

is the only enzyme that can perform 16S 3′ processing in *E. coli* at 45°C (17). Since the 3′ exoribonucleases PNPase and RNase R are expressed at their highest levels at cold temperatures (35,36), it is likely that their weaker levels of expression and/or activity at 45°C reduces the overall efficiency of the redundant 16S 3′ maturation pathway, providing a potential explanation for why YbeY becomes critical at high temperatures. Although the 3′ exoribonucleases do not play a significant role in removal of the last 2 nt after cleavage by YqfG in *B. subtilis*, we saw some evidence of 3′ exoribonucleolytic removal of nucleotides initiating at the downstream RNase III cleavage site in the RT-PCR assays (Figure 7). However, the fact that YqfG is essential would suggest that 16S 3′ maturation by this pathway, if it occurs, is extremely inefficient in *B. subtilis*. In *E. coli*, a strain lacking both YbeY and RNase R grows more slowly than a single ΔybeY mutant supporting RNase R's role as an alternative 3′ maturation enzyme. In *B. subtilis*, the opposite is true, i.e. the double YqfG RNase R mutant survives, whilst a ΔyqfG mutant alone is lethal. Our data are consistent with the idea that RNase R is primarily used as a quality control mechanism for turnover of defective particles rather than as a processing enzyme in *Bacillus*.

In *E. coli* and *V. cholerae*, it has also been suggested that RNase R plays a role in 70S ribosome quality control in conjunction with YbeY (17,20). *In vitro*, these two RNases can cooperate to degrade 70S ribosomes isolated from strains lacking YbeY, but leave wt 70S ribosomes intact. Indeed 70S ribosomes reconstituted from mutant 30S subunits and wt 50S subunits are also subject to *in vitro* degradation by YbeY and RNase R. Whilst this specific situation obviously cannot occur *in vivo* (i.e. the ribosome precursors that accumulate in a ΔybeY strain cannot be degraded in a pathway that also requires YbeY), the degradation of 70S particles by RNase R in a YqfG-depleted *B. subtilis* strain, may be an *in vivo* manifestation of a related phenomenon. It is intriguing that defective 30S particles bearing 3′ extensions do not seem to be a substrate for RNase R mediated quality control (Figure 6), suggesting that RNase R requires an association of the two subunits for this function.

Jacob *et al.* showed multiple cleavages by YbeY on a short RNA oligonucleotide overlapping the 3′ end of *E. coli* 16S rRNA, one of which corresponded to the expected processing site (17). This supports the idea that YbeY has ribonuclease activity, but leaves open the question of whether it is *the* endoribonuclease responsible for 16S 3′ processing *in vivo*. We made multiple attempts to cleave a similar (22-nt) oligoribonucleotide substrate corresponding to the 3′ end of *B. subtilis* 16S rRNA with YqfG purified from *E. coli* or translated *in vitro* using the PURExpress system, but failed to see specific YqfG-dependent cleavages (data not shown). In *E. coli*, YbeY has been shown to associate with a number of proteins including the ribosome assembly GTPases Der and Era, ribosomal protein S11, the ppGpp synthetase/hydrolase SpoT and a protein of unknown function YbeZ/PhoH, encoded in the same operon as YbeY (37). A three-way interaction between Era, YbeY and S11 is predicted to be the basis for the joining of YbeY to the ribosome. Consistent with this, the phenotype of Era de-

(20), the orthologous enzymes are essential. One possible explanation for this may be the relative strengths of alternative 3′ exoribonucleolytic processing pathways in these organisms and whether the exoribonucleases can be stopped efficiently at the mature 16S 3′ end (18). Interestingly, YbeY

pletion mutants (decreased 16S maturation and an increase in free 30S and 50S subunits compared to 70S ribosomes) is very similar to that of strains lacking YbeY (38) and, in some species of *Clostridium*, Era and YbeY are actually fused together as a single polypeptide (17). We therefore also attempted to perform the processing reaction *in vitro* on more physiologically relevant 16S rRNA molecules. These included pre-30S subunits isolated from cells depleted for YqfG, *in vitro*-transcribed full-length 16S precursors and an *in vitro*-transcribed fusion of the 5′ central domain to the 3′ min or domain of 16S rRNA containing binding sites for some key potential accessory proteins, e.g. S11. Adding various combinations of Era (± guanosine triphosphate (GTP) or non-hydrolysable GTP analogues), ribosomal proteins S2, S7, S11, S18, S21, mRNA and initiator tRNA to these substrates failed to yield specific YqfG cleavages (data not shown). We suspect therefore that *in vivo* the processing reaction by YqfG occurs on a very transient late intermediate of the 30S biogenesis pathway that remains to be identified. A similar conclusion was recently reached by the Culver group for *E. coli* YbeY (39). Although we consider it unlikely, we cannot at this point formally rule out the possibility that YqfG is only an accessory factor in the 16S 3′ processing reaction and that some other enzyme provides the catalytic function.

Cleavage by *E. coli* YbeY *in vitro* is thought to yield down-stream products bearing 5′ hydroxyl groups (17), which is unusual for a metal-dependent catalytic mechanism. In our previously reported experiment to map the 5′ end of the downstream cleavage product generated by YqfG following 16S 3′ processing in *B. subtilis*, we performed 5′ RACE PCR where we ligated the 65 nt species to the 3′ end of 5S rRNA and amplified the ligation product without a prior kinase step (27). This shows that the 65 nt downstream species has a 5′ monophosphate group in *B. subtilis in vivo*. The reasons for this apparent discrepancy with YbeY *in vitro* activity are unknown at this point.

A lack of YbeY leads to a plethora of phenotypes in different organisms, ranging from increased temperature sensitivity, to decreased virulence, to effects on sRNA regulation (17,20,34,40). Although it is possible that YbeY plays specific roles in the degradation of key mRNAs for these processes, or as an Hfq-like RNA chaperone as proposed in (34), it is also possible that these pleiotropic effects can be the result of a global problem with translation due either to the reduced 70S ribosome population or inefficient recognition of the SD sequence by 30S ribosomes bearing long 3′ extensions. Even the levels of non-translated sRNAs can be indirectly influenced by the levels of the target mRNAs, whose stability in turn depends heavily on translation levels. It remains to be seen whether YqfG has other specific substrates in *B. subtilis*, but it will be very difficult to untangle these from indirect translational effects that are due to the role of this enzyme in 16S 3′ processing and/or 70S ribosome deficiency.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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