

# Transfer RNA Maturation Defects Lead to Inhibition of ribosomal RNA Processing via Synthesis of pppGpp

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3 **Transfer RNA maturation defects lead**  
4 **to inhibition of ribosomal RNA processing via synthesis of pppGpp**  
5

6 **Aude Trinquier, Jonathan E. Ulmer<sup>1</sup>, Laetitia Gilet, Sabine Figaro, Philippe Hammann<sup>2</sup>,**  
7 **Lauriane Kuhn<sup>2</sup>, Frédérique Braun\* and Ciarán Condon\***

8  
9 \*Co-corresponding senior authors

10 UMR8261 (CNRS-Université Paris Diderot),

11 Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France

12 Lead contact : Ciarán Condon

13  
14 <sup>1</sup>Current address : UMR3664, Institut Curie-PSL-CNRS, 26 rue d'Ulm, 75248 Paris Cedex 05,  
15 France

16  
17 <sup>2</sup>Plateforme Proteomique Strasbourg – Esplanade, Institut de Biologie Moléculaire et Cellulaire,  
18 CNRS FR1589, 15 rue Descartes, 67084 Strasbourg cedex, France  
19

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25

26 Correspondence:

27 Tel: (33)-1-58 41 51 23

28 Fax: (33)-1-58 41 50 20

29 E-mail: [condon@ibpc.fr](mailto:condon@ibpc.fr); [frederique.braun@ibpc.fr](mailto:frederique.braun@ibpc.fr)  
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1 **Summary**

2 Ribosomal RNAs and transfer RNAs universally require processing from longer primary  
3 transcripts to become functional for translation. Here we describe an unsuspected link between  
4 tRNA maturation and the 3' processing of 16S rRNA, a key step in preparing the small ribosomal  
5 subunit for interaction with the Shine-Dalgarno sequence in prokaryotic translation initiation. We  
6 show that an accumulation of either 5' or 3' immature tRNAs triggers RelA-dependent  
7 production of the stringent response alarmone (p)ppGpp in the Gram-positive model organism  
8 *Bacillus subtilis*. The accumulation of (p)ppGpp and accompanying decrease in GTP levels  
9 specifically inhibit 16S rRNA 3' maturation. We suggest that cells can exploit this mechanism to  
10 sense potential slow-downs in tRNA maturation and adjust rRNA processing accordingly to  
11 maintain the appropriate functional balance between these two major components of the  
12 translation apparatus.

13

## 1 **Introduction**

2 Ribosomes are the platform for protein synthesis in all cells. Remarkably, the peptidyl  
3 transfer activity of this large ribonucleoprotein complex is provided by its RNA component, and  
4 the discovery of this property of ribosomes and those of other catalytic RNAs (ribozymes) has  
5 fueled the notion of an ancient RNA world in which all the major cellular functions,  
6 transcription, translation and replication were once RNA-based. Bacterial ribosomes contain  
7 three ribosomal RNAs (16S, 23S and 5S rRNA) that are generally transcribed as part of a large  
8 30S precursor molecule and that assemble with >50 ribosomal proteins to form this translation  
9 center (Noller and Nomura, 1987). In *E. coli* and in most other bacteria studied, transcription,  
10 initial separation of the individual rRNAs and r-protein assembly all occur concomitantly, and  
11 require additional co-factors and quality control check points along the way to ensure the correct  
12 order of events and a stable functional ribosome at the end of this intricate process (Shajani et al.,  
13 2011).

14 By far the greatest proportion of a bacterial cell's biosynthetic capacity and energy  
15 consumption is devoted to ribosome biogenesis (Bremer and Dennis, 1996). Because of this  
16 energy cost, rRNA transcription is tightly regulated to match the growth rate afforded by the  
17 culture medium, a phenomenon known as metabolic control (Pao and Gallant, 1978; Stent and  
18 Brenner, 1961). One of the key effectors of this process is guanosine penta- or tetra-phosphate,  
19 collectively referred to as (p)ppGpp, and historically known as magic spot (Cashel and Rudd,  
20 1987). In *E. coli*, (p)ppGpp binds to RNA polymerase with the help of the DksA protein to down-  
21 regulate initiation at rRNA promoters at slower growth rates, or during amino acid starvation (the  
22 stringent response) when rRNA transcription is essentially halted (Ross et al., 2016). The  
23 stringent response permits a global readjustment of the cell's metabolism, including inhibition of  
24 fatty acid biosynthesis, DNA replication, induction of amino acid biosynthesis and in the  
25 establishment of the persister cell state upon exposure to antibiotics or other severe stress  
26 conditions (Amato et al., 2013; Chowdhury et al., 2016; My et al., 2015; Polakis et al., 1973;  
27 Traxler et al., 2008; Wang et al., 2007). (p)ppGpp has also been implicated in the inhibition of  
28 translation by blocking the activity of translation factors EF-Tu, EF-G and IF2, and the  
29 association of ribosomal subunits through its interaction with ObgE (Feng et al., 2014; Miller et  
30 al., 1973; Milon et al., 2006; Mitkevich et al., 2010). In *B. subtilis*, (p)ppGpp is similarly an  
31 effector of the stringent response, but rather than binding to RNA polymerase, it inhibits the

1 synthesis of GTP by binding to two enzymes of the *de novo* and salvage pathways of GTP  
2 synthesis, Gmk and HprT, respectively (Kriel et al., 2012; Liu et al., 2015). Since ribosomal  
3 RNA promoters in both *E. coli* and *B. subtilis* are exquisitely sensitive to the concentration of the  
4 initiating nucleotide (iNTP), the decrease in GTP pools (the iNTP of all 10 rRNA operons in *B.*  
5 *subtilis*) leads to strong inhibition of rRNA transcription (Gaal et al., 1997; Krasny and Gourse,  
6 2004).

7         The maturation of rRNA is also remarkably different between *E. coli* and *B. subtilis*, with  
8 only two processing reactions being shared out of at least ten known intermediary and final  
9 processing steps. The first is the co-transcriptional cleavage of the primary transcript by RNase  
10 III that occurs in the long double-stranded processing stalks formed by hybridization of  
11 complementary precursor sequences at the 5' and 3' ends of both 16S (Fig. 1A) and 23S rRNA.  
12 Cleavage by RNase III separates the three rRNA molecules, which subsequently undergo further  
13 processing reactions to yield the mature functional rRNAs (Dunn and Studier, 1973; Herskovitz  
14 and Bechhofer, 2000). The enzymes responsible for most of the final maturation steps in *E. coli*,  
15 RNase E, RNase G and RNase T, are not found in *B. subtilis* and *vice versa*, where the enzymes  
16 RNase J1, Mini-III and RNase M5 play the key roles (Condon, 2014). The only exception is the  
17 enzyme involved in the maturation of the 3' end 16S rRNA, called YbeY in *E. coli* (Jacob et al.,  
18 2013) and YqfG in *B. subtilis* (Baumgardt et al., 2018).

19         The final rRNA trimming steps serve to protect rRNAs from degradation by limiting  
20 access to exoribonucleases and are thought to occur at the end of the assembly of each subunit to  
21 rubber stamp the assembly process (Baumgardt et al., 2018; Li et al., 1998). Thus mutations that  
22 perturb 30S or 50S subunit assembly generally block the final processing of 16S and 23S rRNA  
23 (Bylund et al., 1998; Charollais et al., 2003; Hase et al., 2009; Hwang and Inouye, 2006; Nord et  
24 al., 2009; Sayed et al., 1999). In *E. coli* a number of maturation factors, including GTPases  
25 (RsgA, Era), RNA chaperones (RimM, RimP, RbfA) and modification enzymes (RimJ, KsgA  
26 etc.) are known to be involved in 30S ribosomal subunit assembly (Shajani et al., 2011). In  
27 addition to having homologs for each of these factors, *B. subtilis* has at least one additional  
28 GTPase, called YqeH, involved in the 30S assembly process (Loh et al., 2007; Uicker et al.,  
29 2007).

30         In this paper, we describe the discovery of a link between transfer RNA (tRNA)  
31 maturation by RNase P and RNase Z in *B. subtilis*, and processing of the 3' end of 16S rRNA by

1 YqfG. RNase P is historically one of the first enzymes whose catalytic moiety was shown to be  
2 an RNA, encoded by the *rnpB* gene (Guerrier-Takada et al., 1983). Its primary function is the  
3 maturation of the 5' end of tRNAs. The enzyme also contains a small basic protein subunit,  
4 encoded by the *rnpA* gene, that plays a role in substrate recognition and binding (Crary et al.,  
5 1998; Reich et al., 1988). Although the RNA component of RNase P is sufficient for catalysis *in*  
6 *vitro*, both the RNA and protein moieties are essential for cell viability *in vivo* (Waugh and Pace,  
7 1990; Wegscheid et al., 2006). There are two major pathways for the maturation of the 3' end of  
8 tRNAs in *B. subtilis*. About two-thirds of *B. subtilis* tRNAs (59 tRNAs whose CCA motif is  
9 encoded by their genes) are matured by a 3'-5' exoribonucleolytic pathway involving the  
10 redundant activities of RNase PH, PNPase, RNase R and YhaM (Wen et al., 2005). RNase Z is  
11 required for the 3' maturation of 17 *B. subtilis* tRNAs lacking a CCA motif encoded in their  
12 genes (Pellegrini et al., 2012; Pellegrini et al., 2003), while 10 non-CCA encoding tRNAs can be  
13 matured by either pathway (Wen et al., 2005). Here, we propose a model that couples tRNA  
14 maturation by RNases P and Z to 16S 3' maturation *via* the production of (p)ppGpp, a decrease in  
15 GTP levels and a defect in 30S ribosomal subunit assembly by GTPases.

16

## 17 **Results**

18

### 19 **RNase P depletion inhibits maturation of the 3' end of 16S rRNA**

20 In an experiment originally designed to identify enzymes involved in the maturation of  
21 16S rRNA in *B. subtilis*, we screened a number of mutant strains lacking known ribonucleases  
22 for defects in 16S rRNA 3' processing, by Northern blotting of total RNA. In wild-type (wt)  
23 cells, a probe specific for 16S rRNA 3' precursors detects both full-length precursors (~1620 nts)  
24 and a 65-nt species extending from the proposed YqfG cleavage site to the downstream RNase III  
25 site (Fig. 1A) (Baumgardt et al., 2018 ; DiChiara et al., 2016). To our surprise, depletion of either  
26 the protein (*rnpA*) or RNA (*rnpB*) subunit of the tRNA 5' processing enzyme RNase P resulted in  
27 a strong reduction in 16S rRNA 3' processing as indicated by the absence of the 65-nt species  
28 (Fig. 1B). Depletion was achieved using integrative vectors that placed the native copy of the  
29 *rnpA* and *rnpB* genes under control of the xylose-inducible *Pxyl* promoter or the IPTG-inducible  
30 *Pspac* promoter, respectively, and growing cells in the presence of glucose to shut off expression  
31 of the *Pxyl-rnpA* construct or in the absence of IPTG to shut off expression of *Pspac-rnpB*.

1 Processing efficiency, quantified as the ratio of the 65-nt species to that of the full-length 16S  
2 rRNA precursor (65-nt/pre-16S), was reduced by 6.2 and 5.8-fold under conditions of *rnpB* and  
3 *rnpA* depletion, respectively, compared to those in the presence of inducer. Since we had already  
4 identified a candidate for the 16S rRNA 3' cleavage reaction, the essential enzyme YqfG  
5 (Baumgardt et al., 2018), we suspected the effect of RNase P depletion on 16S rRNA processing  
6 was indirect and was the consequence of a defect in tRNA maturation. Depletion of the RNA  
7 subunit of RNase P had a stronger effect on tRNA maturation than depletion of the protein  
8 subunit (Fig. S1), presumably reflecting the relative stabilities of the two components of the  
9 enzyme, and this more than likely accounts for the stronger effect of *rnpB* depletion on 3'  
10 processing of 16S rRNA.

11

### 12 **The effect of RNase P depletion is specific for 3' end of 16S rRNA**

13 We asked whether the effect of RNase P depletion was specific to the 3' end of 16S rRNA  
14 or whether other rRNA processing reactions were affected. The 5' end of 16S rRNA is matured  
15 by the 5'-exoribonuclease RNase J1 in *B. subtilis* (Britton et al., 2007; Mathy et al., 2007), while  
16 the 5' and 3' ends of 23S rRNA are simultaneously processed by the double-strand specific  
17 enzyme Mini-RNase III (Fig. S2A, B) (Redko et al., 2008). We examined the 5' processing of  
18 16S and 23S rRNA by primer extension using an oligonucleotide complementary to the early  
19 mature sequences to detect 5' precursors extending as far as the upstream RNase III cleavage  
20 sites. By proxy, assay of 23S rRNA 5' processing also determines the efficiency of 3' processing,  
21 since both strands of the processing stalk are cleaved together (Redko et al., 2008). Depletion of  
22 *rnpA* did not have a major effect on 5' maturation of either 16S or 23S rRNA (Fig. S2C, D),  
23 indicating that the coupling with tRNA maturation is specific to the 16S rRNA 3' processing  
24 reaction.

25

### 26 **Depletion of RNase Z has a similar effect on 16S 3' processing to depletion of RNase P**

27 We next asked whether the inhibition of 16S 3' processing was restricted to RNase P or  
28 whether it would similarly occur in cells depleted for the tRNA 3' processing enzyme RNase Z,  
29 involved in the maturation of about one-third of *B. subtilis* tRNAs (Wen et al., 2005). In cells  
30 depleted for RNase Z, under the control of the *Pspac* promoter, the levels of the 65-nt species  
31 were strongly reduced, but not completely absent. This corresponded to a 2.2-fold decrease in

1 processing efficiency (Fig. 1C). Thus, while the effect of RNase Z depletion on 16S 3' processing  
2 is less severe than depletion of RNase P, presumably because it has fewer tRNA substrates than  
3 RNase P, it is nonetheless evident. This result suggests that the 16S rRNA 3' processing defect is  
4 the result of a general deficiency in tRNA maturation.

### 6 **3' processing of 16S rRNA is affected in mutants of the 30S subunit assembly pathway**

7 The effect of the tRNA maturation defects on 16S rRNA processing could occur through  
8 an effect on the expression or activity of the 3' processing enzyme itself, YqfG, or on any of the  
9 major 30S ribosomal subunit assembly factors, since final processing is considered to occur post-  
10 assembly. We first screened a number of mutants lacking or depleted for specific 30S ribosomal  
11 proteins and assembly factors to determine which, if any, were affected in 16S rRNA 3'  
12 processing in *B. subtilis*. No major defects were observed in cells lacking the r-protein S5  
13 acetylase RimJ orthologs YdaF or YjcK, the 16S rRNA methylase KsgA, the RNA chaperones  
14 RimP (YlxS in *B. subtilis*) or RbfA, or the r-protein S21 ( $\Delta rpsU$ ) (Fig. 2A, B). However, strains  
15 lacking the RNA chaperone RimM, the GTPases CpgA (equivalent to *E. coli* RsgA) or the *B.*  
16 *subtilis*-specific YqeH, all showed greater degrees of 16S rRNA 3' processing deficiency, as did  
17 cells depleted for the essential GTPase Era or the 3' processing enzyme YqfG, as seen previously  
18 (Baumgardt et al., 2018). Thus, as in *E. coli*, a number of different proteins involved in *B. subtilis*  
19 30S subunit biogenesis have an impact on 16S rRNA processing and are potential intermediates  
20 in the mechanism coupling 16S rRNA 3' processing to tRNA maturation.

### 22 **Depletion of RNase P or RNase Z results in altered mRNA levels of several key 30S 23 assembly factors**

24 We performed Northern blots to determine whether the expression of any of the genes  
25 encoding different 30S assembly factors with a major impact on 16S rRNA 3' processing was  
26 altered in cells depleted for RNase P or RNase Z. Remarkably, mRNA levels coding for two 30S  
27 assembly proteins, the GTPases Era and YqeH, increased under depletion conditions for either  
28 tRNA maturase (Fig. 2C), while the expression of two mRNAs, encoding the RNA chaperone  
29 RimM and the GTPase CpgA, decreased (Fig. 2D). Some mRNAs were relatively unchanged  
30 (e.g. *rimP/ylxS*, *rbfA*), while others (e.g. *yqfG*) were too low to be detected by Northern blot (data  
31 not shown). Thus, under conditions of RNase P or Z depletion, the transcript levels of a number



1 of key assembly factors are perturbed and could account for the 16S 3' processing defect because  
2 their levels are insufficient for 30S assembly, or because they poison the assembly process when  
3 over-expressed.

#### 4 5 **Depletion of RNase P or RNase Z results in a defect in 30S subunit assembly**

6 To determine whether a decrease in tRNA maturation levels leads to a defect in 30S  
7 subunit assembly in *B. subtilis*, we subjected ribosomes isolated from cells depleted for RNase P  
8 (*rnpA*) or RNase Z to sucrose gradient analysis under low magnesium (Mg) conditions (3 mM) to  
9 dissociate ribosomal subunits. Under RNase P and RNase Z depletion conditions, the 30S peak  
10 was slightly broader than that seen in wt cells, with a small shoulder corresponding to the early  
11 30S fractions (Fig. 3A). The 16S ribosomal RNA present in these early fractions (fraction 10) is  
12 aberrant and shows two additional species, one slightly larger and one slightly smaller than  
13 mature 16S rRNA, corresponding to precursor and partially degraded 16S rRNA species (Fig.  
14 3B). We have seen this pattern previously with the depletion of the 16S 3' processing enzyme  
15 YqfG (Baumgardt et al., 2018). We measured the levels of individual ribosomal proteins present  
16 in fraction 10 by mass spectrometry (LC-MS/MS) and compared them to the content of the  
17 mature 30S peak (fraction 12) of wt cells. In cells depleted for RNase P (*rnpA*), a number of late  
18 assembly ribosomal proteins were significantly reduced, including S2, S3, S14 and S21, which  
19 were present at  $\leq 10\%$  of wt, and S5, S9 and S13 which were  $\leq 50\%$  of wt (Fig. 3C). A milder but  
20 overlapping defect was observed with the RNase Z depletion strain, with the late proteins S2, S3  
21 and S14 showing defective levels at  $\leq 50\%$  of wt (Fig. 3D). Thus, depletion of RNase P and  
22 RNase Z indeed results in a late 30S subunit assembly defect that could account for the defect in  
23 16S rRNA 3' processing. The observation that only specific (late) r-proteins were affected in this  
24 experiment confirms that at this point in the depletion curve, we have not yet reached the point of  
25 a global shut-down in r-protein synthesis.

26 The late assembly defect is very reminiscent of that seen in *E. coli* cells lacking RimM  
27 (Bunner et al., 2010; Guo et al., 2013; Leong et al., 2013), which along with *cpgA* was one of the  
28 two assembly factor mRNAs down-regulated by depletion of the tRNA maturation enzymes in *B.*  
29 *subtilis* (Fig. 2D). We therefore asked whether RimM performed a similar function in *B. subtilis*  
30 by performing sucrose gradient and mass spectrometry analysis similar to those described for the  
31 depletion of RNase P and RNase Z. In the  $\Delta rimM$  strain, the 30S peak was shifted significantly

1 towards a precursor form (Fig. S3A), confirmed by the analysis of 16S rRNA, which showed  
2 primarily the precursor and degraded 16S rRNA species, and very little mature 16S rRNA (Fig.  
3 S3B). We analysed the r-protein content of fractions 10 and 11 from this gradient by mass-  
4 spectrometry and saw very similar defects to those observed with the RNase P and the milder  
5 RNase Z depletion strains, respectively. Fraction 10 showed reduced levels of the late assembly  
6 proteins S2, S3, S9, S10, S14, S19, S21 ( $\leq 50\%$  of wt; Fig. S3C), whereas fraction 11 showed a  
7 milder defect with lower levels of S2, S3 and S14 (Fig. S3D). Thus, reduced levels of *rimM*  
8 expression in RNase P or RNase Z depletion strains could potentially account for the defect  
9 observed in 30S subunit assembly. In *E. coli*, RbgA (CpgA in *B. subtilis*) has been shown to have  
10 a similar role to RimM in late 30S assembly (Leong et al., 2013). Therefore, we also considered  
11 the possibility that the reduced *cpgA* mRNA levels in tRNA maturase depletion strains might  
12 equally contribute to the 30S assembly defect.

13  
14 **Perturbation of assembly factor mRNA levels upon RNase P depletion is not sufficient to**  
15 **account for the 16S rRNA processing defect.**

16 To directly test the hypothesis that the decreased *rimM* and *cpgA* mRNA levels could  
17 account for the defect in 16S rRNA 3' maturation in strains depleted for tRNA processing  
18 enzymes, we asked whether we could complement the processing deficiency by ectopic  
19 expression of these two mRNAs. We constructed a single integrative vector expressing both  
20 *rimM* under control of the arabinose-dependent *P<sub>xsA</sub>* promoter (Franco et al., 2007) and *cpgA*  
21 under control of the bacitracin-dependent *P<sub>lia</sub>* promoter (Toymentseva et al., 2012). Even leaky  
22 expression of *rimM* or *cpgA* from this construct was sufficient to complement the respective 16S  
23 3' processing defects in *rimM* and *cpgA* mutants, showing that the construct is functional (Fig.  
24 S4A, compare lanes 3 and 4, and lanes 6 and 8). However, ectopic expression of *rimM* alone, or  
25 *rimM* with *cpgA*, failed to rescue the 16S rRNA 3' processing defect in cells depleted for *rnpB*  
26 (Fig. S4B, compare lane 6 and 4). Similarly, ectopic expression of the 3' processing enzyme  
27 YqfG (Baumgardt et al., 2018), whose native mRNA we had failed to detect by Northern blot  
28 (above), did not restore 16S rRNA 3' processing under RNase P depletion conditions (Fig. S4C).  
29 Lastly, overexpression of the GTPase genes *era* or *yqeH* from a plasmid in a wt background had  
30 no impact on 16S rRNA 3' maturation (Fig. S4D, E), ruling out the possibility that the increase in  
31 expression of these mRNAs observed in RNase P and RNase Z depletion strains could poison

1 30S subunit assembly. Although we haven't formally ruled out the possibility that multiple  
2 cumulative effects are responsible, these experiments suggest that the impact of tRNA processing  
3 defects on 16S rRNA 3' maturation is unlikely to be due to the perturbation of the expression of  
4 30S assembly factors or processing enzymes alone.

5

## 6 **Defects in tRNA processing lead to the induction of the stringent response via RelA**

7       Having discounted the possibility that altered expression levels of 30S assembly factors  
8 were solely responsible for the 16S rRNA processing defect, we speculated that the activity of  
9 these proteins might be impacted by perturbations in tRNA processing. It was recently shown  
10 that the alarmone (p)ppGpp was a competitive inhibitor of GTPases involved in 30S ribosome  
11 biogenesis in *S. aureus*, notably Era and RbgA (CpgA in *B. subtilis*) (Corrigan et al., 2016). We  
12 therefore considered the possibility that the synthesis of (p)ppGpp might be induced by immature  
13 tRNA in a manner similar to the mechanism involving RelA and uncharged tRNA, *i.e. via* the  
14 stringent response. We first asked whether depletion of RNase P or RNase Z led to an  
15 accumulation of (p)ppGpp in *B. subtilis* by adding <sup>32</sup>P-labelled inorganic phosphate to cultures,  
16 extracting total nucleotides with formic acid and analyzing them by thin-layer chromatography  
17 (TLC). The migration position of (p)ppGpp was determined by adding the stringent response  
18 inducer arginine hydroxamate (RHX) to wt cells and to a ppGpp<sup>0</sup> strain unable to synthesize  
19 (p)ppGpp because it lacks the three known synthetases RelA, YwaC and YjbM (Kriel et al.,  
20 2012). Strains depleted for RNase P (*rnpA* or *rnpB*) showed strongly increased synthesis of  
21 pppGpp, while depletion of RNase Z showed a much weaker effect (Fig. 4A), coherent with their  
22 relative impacts on 16S 3' processing and 30S ribosome assembly. That the main form of the  
23 alarmone synthesized was the penta-phosphate derivative (pppGpp) was previously observed for  
24 *B. subtilis* (Wendrich and Marahiel, 1997).

25       Given the high back-ground signal in the TLC assay, we wished to confirm that the levels  
26 of (p)ppGpp observed upon depletion of RNase P or RNase Z were physiologically relevant. To  
27 do this, we assayed the expression of the *ywaA* and *ilvA* genes, two members of the *B. subtilis*  
28 CodY regulon, whose expression is known to increase during the stringent response due to  
29 resulting decrease in GTP pools (Kriel et al., 2012). The expression of both *ywaA* and *ilvA* was  
30 strongly derepressed in strains depleted for either the RNA or protein subunits of RNase P, and  
31 more weakly derepressed in response to depletion for RNase Z (Fig. 4B), consistent with the

1 direct assay of (p)ppGpp levels in these strains (Fig. 4A). We will use derepression of *ywaA* as an  
2 indirect measure of *in vivo* guanosine nucleotide pools for the rest of this paper.

3 To ask whether the synthesis of (p)ppGpp was dependent on the two synthetases YwaC  
4 and YjbM, or on the synthetase/hydrolase RelA, we examined the expression of *ywaA* in cells  
5 depleted for RNase P (*rnpA*) in a *ywaC yjbM* background or a strain lacking all three (p)ppGpp  
6 synthesizing enzymes. The experiment was done in this way because *relA* single mutants rapidly  
7 accumulate suppressor mutations in the two synthetase genes (Natori et al., 2009). Expression of  
8 *ywaA* was still strongly derepressed in the double *ywaC yjbM* mutant but no longer occurred in  
9 the triple *ywaC yjbM relA* mutant (ppGpp<sup>0</sup> strain; Fig. 4C), showing that the primary sensor of  
10 the tRNA maturation defect is the stringent response effector RelA.

### 11 12 **16S 3' processing is partially restored in ppGpp<sup>0</sup> strains depleted for RNase P and RNase Z**

13 If (p)ppGpp is an effector in the tRNA/16S rRNA maturation coupling mechanism, we  
14 would predict that 16S 3' processing should be impacted to a lesser degree by depletion of RNase  
15 P and RNase Z in ppGpp<sup>0</sup> strains. This was indeed the case. In RNase P depletion strains, 16S  
16 processing efficiency was partially restored in the ppGpp<sup>0</sup> background (Fig. 5). The processing  
17 efficiency improved from a 5.8-fold deficiency to only a 2.6-fold defect upon depletion of *rnpA*  
18 in a ppGpp<sup>+</sup> vs. ppGpp<sup>0</sup> background, and from 6.2-fold to 2.4-fold deficiency upon depletion of  
19 *rnpB*. In RNase Z depletion strains, which still show some 16S 3' processing in ppGpp<sup>+</sup> strains,  
20 there was no improvement in maturation efficiency the ppGpp<sup>0</sup> background (2.2-fold defect  
21 compared to 2.6-fold in ppGpp<sup>+</sup> vs. ppGpp<sup>0</sup> backgrounds; data not shown), consistent with the  
22 lower level of (p)ppGpp synthesis upon depletion of RNase Z and the generally milder effect of  
23 RNase Z depletion on 16S rRNA 3' processing. Since 16S rRNA maturation efficiency is not  
24 completely restored in a ppGpp<sup>0</sup> background, this suggests that (p)ppGpp is not the only effector  
25 of the coupling mechanism between tRNA and 16S rRNA 3' processing.

### 26 27 **Increased (p)ppGpp levels and decreased GTP pools inhibit 16S 3' processing in the 28 absence of tRNA processing defects**

29 We next asked whether the synthesis of (p)ppGpp or a decrease in intracellular GTP  
30 levels would inhibit 16S rRNA 3' processing in the absence of a defect in tRNA maturation. We  
31 did this in three ways, each time using the CodY-regulated *ywaA* gene as a sensitive gauge of the

1 changes in nucleotide pools. We first asked whether classical induction of the stringent response  
2 by amino acid starvation would also lead to a defect in 16S 3' rRNA processing. Addition of  
3 RHX to wt cells led to a decrease in the production of the 65-nt species over time that  
4 significantly out-paced the rate of inhibition of 16S rRNA transcription, as measured by levels of  
5 remaining full-length precursor (Fig. 6A). Thus, induction of the stringent response not only  
6 inhibits rRNA transcription as previously observed, but also impedes 16S rRNA 3' processing.  
7 Second, we constructed a strain that allowed us to induce (p)ppGpp synthesis in the absence of  
8 either defects in tRNA processing or amino acid starvation. In this strain, the three (p)ppGpp  
9 synthetase genes (*ywaC*, *yjbM*, *relA*) were inactivated in their native loci and an ectopic copy of  
10 the *ywaC* synthetase gene was placed under control of the *P<sub>xyl</sub>* promoter in the *amyE* locus.  
11 Addition of xylose to this strain led to a strong induction of ppGpp synthesis as measured by  
12 TLC (Fig. 6B) and this was confirmed by showing derepression of *ywaA* gene expression by  
13 Northern blot at different times after addition of xylose to the growth medium (Fig. 6C). Reduced  
14 levels of the 65-nt 16S 3' rRNA processed species were observed only 2 minutes after addition to  
15 xylose to the culture, well before synthesis of the full-length 16S rRNA precursor began to  
16 decrease, 15 minutes after xylose addition. In agreement, calculation of the processing efficiency  
17 (65-nt/full-length), showed a steady decrease over the full time-course of the experiment, even  
18 after the point where inhibition of 16S rRNA transcription became evident (Fig. 6C). Thus,  
19 induction of (p)ppGpp synthesis in the absence of amino acid starvation also results in a  
20 deficiency in 16S 3' processing. Lastly, we investigated whether a decrease in GTP synthesis  
21 would have an impact on 16S 3' rRNA processing independently of (p)ppGpp production, by  
22 adding the fungal GMP synthetase inhibitor decoyinine to cultures. Addition of decoyinine to wt  
23 cells led to a rapid decrease in 16S rRNA 3' processing efficiency, suggesting that a decrease in  
24 GTP levels is sufficient to inhibit the different GTPases that play a role in 30S ribosomal subunit  
25 assembly and ultimately cause the 16S rRNA 3' maturation defect (Fig. 6D). However, it does  
26 not rule out an additional contribution from direct inhibition of the ribosome biogenesis GTPases  
27 by (p)ppGpp, as proposed in *S. aureus* (Corrigan et al., 2016).

28

## 29 **Discussion**

30 This paper describes an intriguing observation that tRNA processing mutants completely  
31 abolish 16S 3' processing. We worked backwards from an original hypothesis that the expression

1 or activity of certain 30S ribosome assembly factors was affected to show that there was indeed a  
2 specific late 30S biogenesis defect. Finally, we discovered the missing link: that unprocessed  
3 tRNAs can induce the stringent response and the synthesis of (p)ppGpp. We propose a model in  
4 which unprocessed tRNAs enter the ribosome A-site similar to uncharged tRNA and trigger  
5 RelA-dependent (p)ppGpp synthesis (Fig. 7). The synthesis of (p)ppGpp can have two effects,  
6 either through direct competitive inhibition of assembly factor GTPase activity as proposed by  
7 Grundling and coworkers in *S. aureus* (Corrigan et al., 2016), or indirectly through the decrease  
8 in GTP pools necessary for their activity. Our data suggest that in *B. subtilis* at least, the decrease  
9 in GTP pools is sufficient to lead to problems in late 30S ribosome assembly and, consequently,  
10 to the deficiency in the maturation of 16S rRNA.

11 With only minor accommodations, the structure of RelA on the ribosome (PDB 5iqr)  
12 could accept both 5' and 3' tRNA precursors (Fig. S5). RelA recognizes C74 and C75 of the  
13 CCA motif of uncharged tRNA through a stacking interaction with His432 and hydrogen bond  
14 interactions with Arg438 (Arenz et al., 2016; Brown et al., 2016; Loveland et al., 2016). It is  
15 possible that similar interactions could occur with non-cytosine bases in the equivalent positions  
16 of non-CCA containing tRNA 3' precursors that accumulate in RNase Z-depleted cells. Since a  
17 free 3'-hydroxyl group of the terminal A-residue was proposed to be necessary for (p)ppGpp  
18 synthesis by RelA *in vitro* (Sprinzl and Richter, 1976), a different accommodation process would  
19 be necessary to account for the ability of 3' extended tRNA precursors to stimulate (p)ppGpp  
20 synthesis in RNase Z-depleted cells. It will be interesting to determine the molecular details of  
21 this recognition mechanism. Shetty and Varshney recently showed that three consecutive GC  
22 base pairs in acceptor stem of the initiator tRNA played an important role in licensing the final  
23 rRNA processing reactions during the first round of initiation complex formation in *E. coli*  
24 (Shetty and Varshney, 2016). Although these experiments did not directly implicate the stringent  
25 response, they showed that correct tRNA structure in the A-site is important for 16S rRNA  
26 processing. From a physiological standpoint, both these observations and ours indicate that other  
27 tRNA forms besides uncharged tRNA in the A-site may be able to activate RelA.

28 We have previously shown that the 16S 3' processing step is a quality control event that  
29 rubber stamps the correct completion of the 30S assembly process and that small subunits that are  
30 not processed correctly are rapidly degraded by RNase R (Baumgardt et al., 2018). Here, we  
31 show that inhibition of 16S 3' processing during the stringent response is much more rapid than

1 the inhibition of rRNA transcription. Hence, upon encountering translational stress, bacterial cells  
2 possess a mechanism not only to shut down transcription of rRNAs, but also to block the  
3 assembly of existing precursors into functional ribosomal subunits and to rapidly degrade the  
4 partially assembled pre-rRNAs. This suggests that the effect of (p)ppGpp production on *de novo*  
5 ribosome production is more rapid and extensive than previously understood. Our experiments  
6 show that cells can exploit this mechanism to sense potential slow-downs in tRNA maturation  
7 and adjust ribosome production accordingly. This would maintain the appropriate functional  
8 balance between these two major components of the translation apparatus. Indeed, it makes  
9 physiological sense to slow down ribosome assembly and processing under conditions where the  
10 levels of mature tRNAs available for translation are even transiently diminished. Expression  
11 levels of the *rnpA*, *rnpB* and *rnz* RNAs are relatively constant over about a hundred growth  
12 conditions tested (Nicolas et al., 2012), suggesting that the synthesis levels of these enzymes does  
13 not vary much in cells. We suspect therefore that the coupling mechanism may play a role in  
14 fine-tuning ribosome biogenesis and rRNA processing to minor perturbations in tRNA  
15 maturation that occur when the tRNA processing enzymes are transiently out-titrated by tRNA  
16 synthesis levels during the cell cycle.

17 Processing of the 3' end of 16S rRNA is restored to about half of its normal levels when  
18 tRNA maturation enzymes are depleted in a ppGpp<sup>0</sup> background, suggesting that (p)ppGpp is not  
19 the only effector of this coupling phenomenon. One possibility is that the perturbation in mRNA  
20 levels of the 30S assembly factors, in particular the decrease in levels of the mRNA encoding the  
21 GTPase CpgA contributes to this phenomenon. Although ectopic expression of *cpgA* failed to  
22 complement the 16S rRNA processing defect in cells depleted for RNase P, the ectopically  
23 produced enzyme would also be predicted to be inhibited by the ambient levels of (p)ppGpp and  
24 GTP in cells accumulating tRNA precursors. Second, although ectopic expression of the RNA  
25 chaperone RimM or the 16S 3' processing enzyme YqfG also failed to complement 16S rRNA  
26 processing in cells depleted for RNase P, we have not formally eliminated the possibility that the  
27 activity of these proteins is somehow impacted by (p)ppGpp or GTP levels. The mechanism  
28 through which tRNA depletion results in increased or decreased mRNA levels of several 30S  
29 mRNA assembly factors is currently unknown. Our preliminary data suggest that this is a mixture  
30 between transcriptional and post-transcriptional effects (data not shown) and this will be  
31 developed in detail in a later study.

1           The effect of RNase P and RNase Z depletion on rRNA processing is specific for the 3'  
2 end of 16S rRNA. Given the current models that all final processing steps occur post-assembly in  
3 *E. coli*, it is surprising that 5' maturation was not also affected. This may suggest some  
4 differences in the order of events between Gram-positive and negative bacteria. An earlier study  
5 showed that tRNA functional defects in *E. coli* could lead to non-specific problems with both 16S  
6 and 23S rRNA maturation, but the mechanism involved was not addressed (Slagter-Jager et al.,  
7 2007). In another study that may be related to the coupling mechanism described here, the  
8 Deutscher group showed in the 1970's that the strong growth defects of *E. coli* strains lacking  
9 nucleotidyl-transferase activity, required to repair the terminal CCA motif of tRNAs, could be  
10 suppressed by inactivation of the *relA* gene (Deutscher et al., 1977). This observation is coherent  
11 with our data that tRNA maturation defects promote the synthesis of (p)ppGpp, and potentially  
12 extends this phenomenon to Gram-negative bacteria. Coordination between tRNA processing and  
13 ribosome biogenesis has also been proposed in yeast, based on the observation that the transport  
14 factor Sxm1p ferries both the tRNA processing co-factor Lhp1p and the ribosomal proteins  
15 Rpl16p, Rpl21p and Rpl34p from the cytoplasm to the nucleoplasm (Rosenblum et al., 1997).  
16 The link between tRNA maturation and ribosome biogenesis may therefore be universally  
17 conserved, but with distinct mechanisms from one group of organisms to the next.

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## 27 28 **Author Contributions:**

29 AT, JU, LG and SF performed the experiments; PH and LK performed mass spectrometry  
30 analysis. All authors participated in analysing data. CC, FB and AT wrote the paper. FB and CC  
31 are co-supervisors of PhD student AT.

32



1 **Declaration of interests:**

2 The authors declare no competing interests.

3

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11



## 1 **Figure Legends**

2

3 **Figure 1.** Depletion of tRNA processing enzymes results in a defect in 3' processing of 16S  
4 rRNA. (A) Schematic of 16S rRNA (*rrnW*) precursor showing mature sequence in green,  
5 precursor sequences in black and key processing reactions in red. (B) and (C) Northern blots  
6 showing the effect of depleting RNase P (*rnpB* or *rnpA*) and RNase Z (*rnz*), respectively, on  
7 accumulation of the 65 nt 3' processing product. 5 µg of total RNA was probed with oligo CC172  
8 (Table S1), specific for the 16S rRNA 3' precursor, on agarose gels (upper panel) and  
9 polyacrylamide gels (lower panel) for optimal transfer of the ~1620 nt and 65 nt species,  
10 respectively. The histograms show the calculation of processing efficiency (65-nt/pre-16S) for  
11 each strain, normalized to wt (n=4 *rnpB* and *rnpA*; n=3 *rnz*; n=7 wt). The fold differences in  
12 processing efficiencies between depleted and non-depleted strains are indicated on each  
13 histogram.

14

15 **Figure 2.** Depletion of tRNA processing enzymes results in perturbed expression of genes  
16 involved in 30S ribosome biogenesis. (A) Northern blots showing the effects of  $\Delta ksgA$ ,  $\Delta ylxS$   
17 (*rimP*),  $\Delta rbfA$ ,  $\Delta ydaF$  (*rimJ1*),  $\Delta yjcK$  (*rimJ2*) deletions and Era depletion (in 168 *trpC2*  
18 background) on 16S rRNA 3' processing efficiency. (B) Northern blot showing the effects of  
19 YqfG depletion and  $\Delta rpsU$  (S21)  $\Delta rimM$ ,  $\Delta cpgA$ ,  $\Delta yqeH$  deletions (in W168 background) on 16S  
20 rRNA 3' processing efficiency. The histograms show the calculation of processing efficiency  
21 (65-nt/pre-16S) for each strain, normalized to wt (n=2). (C) Northern blots showing up-regulation  
22 of *era* (probe CC1846) and *yqeH* (probe CC1847) expression in strains depleted for RNase P  
23 (*Pxyl-rnpA* and *Pspac-rnpB*) and RNase Z (*Pspac-rnz*). (D) Northern blots showing down-  
24 regulation of *rimM* (probe CC1845) and *cpgA* (riboprobe) expression in the same depletion  
25 strains.

26

27 **Figure 3.** Effect of RNase P and Z depletions on ribosome assembly (A) Sucrose gradients in 3  
28 mM Mg of wt, *Pxyl-rnpA* and *Pspac-rnz* depletion strains grown in the absence of inducer. (B)  
29 16S rRNA profile in sucrose gradients from panel (A). (C) LC-MS/MS analysis (n=3) of pre-30S  
30 fractions in wt vs *Pxyl-rnpA* and *Pspac-rnz* depletion strains grown in the absence of inducer.  
31 The number of spectra for each protein were first normalized to the total spectra observed in each

1 fraction and then normalized to the equivalent number in wt. The percent fill of each box  
2 represents the amount of each ribosomal protein compared to wt. Proteins shown in red are  
3 represented at  $\leq 10\%$ ; orange  $>10\%$  but  $\leq 50\%$  of wt; green  $> 50\%$  of wt. Assembly map is from  
4 (Chen and Williamson, 2013).

5  
6 **Figure 4.** Depletion of tRNA processing enzymes leads to induction of the stringent response.

7 (A) Depletion of tRNA processing enzymes leads to the production of (p)ppGpp. Thin layer  
8 chromatography (TLC) analysis of  $^{32}\text{P}$ -labelled nucleotides extracted from *Pspac-rnpB*, *Pxyl-*  
9 *rnpA* and *Pspac-rnz* depletion strains grown in the absence of inducer. Arginine hydroxamate  
10 (RHX, 250  $\mu\text{g}/\text{mL}$ ) was added to wt cultures and strains unable to make (p)ppGpp ( $\text{ppGpp}^0$ ) as  
11 positive and negative controls. Note that the top and bottom halves of the chromatogram are  
12 exposed for different times. (B) Defects in tRNA processing cause derepression of the CodY  
13 regulon. Northern blot showing derepression of *ilvA* and *ywaA* gene expression in *Pspac-rnpB*,  
14 *Pxyl-rnpA* and *Pspac-rnz* depletion strains growing in the presence and absence of inducer. (C)  
15 Derepression of the CodY-regulated *ywaA* mRNA in *rnpA*-depleted strains is RelA-dependent.  
16 Northern blot showing *ywaA* gene expression in the *Pxyl-rnpA* depletion strains growing in the  
17 presence and absence of xylose in wt, *yjbM ywaC* double mutant and *yjbM ywaC relA* triple  
18 mutant ( $\text{ppGpp}^0$ ) genetic backgrounds.

19  
20 **Figure 5.** 16S rRNA 3' processing is partially restored in RNase P-depleted cells in a  $\text{ppGpp}^0$   
21 background. Northern blot comparing the effect of depleting RNase P (*rnpA* or *rnpB*) in a  
22  $\text{ppGpp}^+$  and  $\text{ppGpp}^0$  background on the accumulation of the 65 nt 3' processing product. The  
23 fold differences in processing efficiencies, normalized to wt, between depleted and non-depleted  
24 strains are indicated underneath the Northern blots (n=4 *rnpB* and *rnpA*; n=3 *rnz*; n=2 *rnpB*  
25  $\text{ppGpp}^0$  and *rnpA*  $\text{ppGpp}^0$ , n=7 wt).

26  
27 **Figure 6.** Increased (p)ppGpp synthesis and decreased GTP levels inhibit 16S rRNA 3'

28 processing in the absence of tRNA processing defects. (A) Induction of the stringent response by  
29 amino acid starvation results in defects in 16S rRNA 3' processing. Northern blots performed on  
30 total RNA after addition of arginine hydroxamate (RHX; 250  $\mu\text{g}/\text{mL}$ ) to wt cultures at times  
31 indicated. Quantification of processing efficiency (65-nt/full-length) is calculated underneath the

1 Northern blots, normalized to the untreated sample (n=2). The blot in the top panel was reprobred  
2 with oligo CC2213 specific for the CodY-regulated *ywaA* gene. (B)+(C) Induction of (p)ppGpp  
3 synthesis is sufficient to cause defects in 16S rRNA 3' processing. (B) TLC showing production  
4 of (p)ppGpp in *ywaC yjbM relA* strains expressing *ywaC* under control of the xylose promoter in  
5 the *amyE* locus 30 mins after addition of RHX (250  $\mu\text{g}/\text{mL}$ ) to cell cultures. (C) Northern blot  
6 performed on total RNA after addition of xylose (2%) to *ywaC yjbM relA* strains expressing  
7 *ywaC* under control of the xylose promoter at times indicated (n=2). (D) Inhibition of GTP  
8 synthesis results in defects in 16S rRNA 3' processing. Northern blot performed on total RNA  
9 after addition of decoyinine (dec; 500  $\mu\text{g}/\text{mL}$ ) to wt cultures at times indicated (n=2).

10

11 **Figure 7.** Model for coupling of tRNA and 16S rRNA 3' processing. Precursor tRNAs (green)  
12 and RelA occupy the A-site of the ribosome and provoke synthesis of (p)ppGpp. Upward  
13 pointing green arrows show increased synthesis or activity, downward pointing red arrows show  
14 decreases. Increased (p)ppGpp levels inhibit the synthesis of GTP by binding to Gmk and HprT  
15 (Kriel et al., 2012), leading to derepression of the CodY regulon, and potentially inhibit the  
16 GTPase activity of Era and CpgA (Corrigan et al., 2016). The decreased GTP pools may also  
17 affect the GTPase activity of Era, CpgA and YqeH, resulting in late 30S assembly defects,  
18 represented by the absence of ribosomal proteins S2, S3, S5, S14, S19 and S21. The assembly  
19 defect in turn leads to a defect in 16S rRNA 3' processing. It is also possible that perturbation of  
20 expression of 30S assembly factors contributes to the assembly defect upon accumulation of  
21 tRNA precursors.

22

23

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27

## 1 STAR METHODS

### 2 CONTACT FOR REAGENT AND RESOURCE SHARING

3 Further information and requests for resources and reagents should be directed to and will be  
4 fulfilled by the Lead Contact Ciarán Condon ([condon@ibpc.fr](mailto:condon@ibpc.fr)).

### 6 EXPERIMENTAL MODEL AND SUBJECT DETAILS

7 *B. subtilis* strain W168 and *B. subtilis* 168 *trpC*. All mutant strains used were derived from these  
8 two parental strains, by transformation with PCR products, plasmids or chromosomal DNA from  
9 both previously published strains or new constructs. A list of all strains used is provided in Table  
10 S1. Details of new strains and plasmid constructs are provided in Tables S2 and S4, respectively.

### 12 METHOD DETAILS

#### 14 **Bacterial cultures**

15 All cultures were grown in 2xYT medium. Overnight cultures were grown in the presence of  
16 appropriate antibiotics. Experimental cultures did not contain antibiotics, except when necessary  
17 for maintaining plasmids. For depletion studies, overnight cultures grown in the presence of  
18 inducer (1 mM IPTG or 2% xylose) were washed three times in an equal volume of pre-warmed  
19 medium and inoculated into fresh medium with or without inducer at  $OD_{600} = 0.05$  (*rnpA* and  
20 *rnz*) or 0.2 (*rnpB*), the empirically determined optimal depletion conditions for the respective  
21 strains. For the depletion of RnpA, cells were inoculated into fresh medium containing glucose  
22 (2%) to tighten repression of the *P<sub>xyI</sub>* promoter. Cultures were followed until they reached a  
23 plateau, typically around  $OD_{600}=0.6$  for *rnpA* and *rnz*, and 0.3 for *rnpB*, and harvested for RNA  
24 preparation.

25 For amino acid starvation, arginine hydroxamate (RHX) was added at 250  $\mu\text{g}/\text{mL}$  at  $OD_{600}$   
26 = 0.3. For inhibition of GTP synthesis, decoyinine was dissolved at 1  $\text{mg}/\text{mL}$  in 2xYT pre-  
27 warmed to 37°C and an equal volume added to 1 mL cultures at  $OD_{600} = 0.6$  (final concentration  
28 500  $\mu\text{g}/\text{mL}$ ). For the CRISPRi strain targeting *era* expression, cells were grown overnight in  
29 2xYT and diluted in the presence or absence of 1% xylose. Cells were grown to  $OD_{600} = 0.5$   
30 before harvesting.

1

## 2 **RNA isolation and Northern blots**

3 RNA was typically isolated from 1 mL mid-log phase *B. subtilis* cells growing in 2xYT medium  
4 by the glass beads/phenol, a modification of the method described in (Bechhofer et al., 2008). For  
5 strains that plateau at very low OD<sub>600</sub>, e.g. the *mprB* depletion strain, greater volumes of cell  
6 culture (up to 8 mL) were harvested. Frozen cell pellets were resuspended in 200 µL ice-cold  
7 TE-Buffer (10 mM Tris, pH 7.5, 1 mM EDTA) and transferred to a tube containing 25 µL  
8 chloroform, 6.25 µL 20% SDS and 100 µL glass beads for lysis by three 1 min vortexing steps at  
9 max speed on a Disruptor Genie (Scientific Industries) separated with 1 min intervals on ice.  
10 After centrifugation, the supernatant was transferred to 200 µL water-saturated phenol on ice and  
11 vortexed again (with the same vortexing protocol as above) before being centrifuged for 10 min  
12 at 16,000 x g at 4°C. The supernatant was then mixed with 200 µL water-saturated phenol and  
13 100 µL chloroform, vortexed for 1 min at full speed and centrifuged again for 10 min at 16,000 x  
14 g at 4°C. RNA was precipitated at -20°C by adding 3 volumes of 95% ethanol stored at -20°C  
15 and 0.1 volumes of 10M LiCl before being washed, dried and resuspended in 50 µL water. For  
16 Northern blots, 5 µg total RNA was run on 1% agarose or 5% acrylamide gels and transferred to  
17 hybond-N membranes (GE-Healthcare), by capillary transfer or electrotransfer, respectively.  
18 Hybridization was performed using 5'-labeled oligonucleotides using Ultra-Hyb (Life  
19 Technologies) or Roti-Hybri-Quick (Roth) hybridization buffers at 42°C for a minimum of 4  
20 hours. Membranes were washed twice in 2×SSC 0.1% SDS (once rapidly at room temperature  
21 (RT), once for 5 min at 42°C) and then twice for 5 mins in 0.2×SSC 0.1% SDS at 42°C, as  
22 described in (Durand et al., 2012).

23

## 24 **Primer extension**

25 Primer extension assays were done using a modified version of the protocol described in (Britton  
26 et al., 2007) on total *B. subtilis* RNA extracted as described above, but with an additional  
27 treatment with DNase I to remove chromosomal DNA. 0.5 pmol of 5'-labeled (<sup>32</sup>P)  
28 oligonucleotides was added to 5 µg of RNA in 5 µl final volume RT buffer (50 mM Tris-HCl, pH  
29 8.3, 10 mM MgCl<sub>2</sub>, 80 mM KCl) and denatured at 75°C for 4 min. The denatured mixture was  
30 frozen on dry ice for 2 mins and then transferred to ice to thaw. Oligonucleotides CC058 and  
31 CC257 were used to assay 5' processing of 16S and 23S rRNAs, respectively. A 5.2 µL mix

1 containing 2 mM each dNTP, 8 mM dithiothreitol (DTT), 4 units AMV reverse transcriptase  
2 (NEB) in RT buffer was then added. Reaction mixtures were incubated for 30 min at 45°C,  
3 stopped with 5 µL of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene  
4 cyanol and loaded on 5% sequencing gels.

5

## 6 **Sucrose gradients**

7 *B. subtilis* 30S and 50S ribosomal particles were separated from 50 mL of log phase *B. subtilis*  
8 cells (OD<sub>600</sub> = 0.5). Cells were centrifuged and resuspended in 1 mL ice cold Buffer A (20 mM  
9 Tris-HCl pH 7.5, 200 mM NH<sub>4</sub>Cl, 6 mM β-Mercaptoethanol) containing 3 mM MgCl<sub>2</sub> and 4  
10 µg/mL DNase I and lysed by two passages in an ice-cold French Press (Glen Mills) at 20,000 psi.  
11 The lysate was cleared at 13,200 rpm for 30 mins at 4°C in a bench top centrifuge. A maximum  
12 of 500 µL of lysate was loaded on a 10-30% sucrose gradient in Buffer A containing 3 mM  
13 MgCl<sub>2</sub> and centrifuged at 23,000 rpm for 16h at 4°C in an SW41 rotor (Beckmann). 500 µL  
14 fractions were collected using a Piston Gradient Fractionator (Biocomp) for analysis on agarose  
15 gels or mass spectrometry. 70S ribosomes were prepared similarly but with Buffer A containing  
16 10 mM MgCl<sub>2</sub> and centrifugation at 18,600 rpm for 16h.

17

## 18 **Mass spectrometry analysis and data processing**

19 Mass-spectrometry analysis was performed in triplicate. Proteins in sucrose gradient fractions  
20 were digested with sequencing-grade trypsin (Promega, Fitchburg, MA, USA). Each fraction  
21 (500 ng of digested peptides) was further analyzed by nanoLC-MS/MS on a QExactive+ mass  
22 spectrometer coupled to an EASY-nanoLC-1000 (Thermo-Fisher Scientific, USA) as described  
23 previously (Chicher et al., 2015). Data were searched against the *Bacillus subtilis* SwissProt sub-  
24 database with a decoy strategy (SwissProt release 2017\_09, taxon 224308, 4294 forward protein  
25 sequences). Peptides and proteins were identified with Mascot algorithm (version 2.5, Matrix  
26 Science, London, UK) and data were further imported into Proline v1.4 software  
27 (<http://proline.profi-proteomics.fr/>). Proteins were validated on Mascot pretty rank equal to 1,  
28 Mascot score above 25, and 1% FDR on both peptide spectrum matches (PSM score) and protein  
29 sets (Protein Set score). Proline package was further used to align proteins across all samples and  
30 to compute the Spectrum Counting values. The total number of MS/MS fragmentation spectra  
31 was used to relatively quantify each protein across all samples. The number of spectra for each

1 30S ribosomal protein was first normalized to the total number of spectra identified in each  
2 mutant sample and then normalized to the equivalent value obtained for the wt. Raw and  
3 processed data are given in Table S5. The % occurrence of each ribosomal protein compared to  
4 wt is reported as a % fill of the relevant box on the assembly maps shown in Figs. 3 and S3.

5

#### 6 **(p)ppGpp measurement**

7 Synthesis of (p)ppGpp was measured with a protocol adapted from (Wang et al., 2007).  
8  $\text{KH}_2^{32}\text{PO}_4$  (1mCi/ml; Perkin Elmer) was added to depletion cultures growing in 2xYT a final  
9 concentration of 100  $\mu\text{Ci/ml}$  at  $\text{OD}_{600} \approx 0.1$  (or from the beginning in the case of *mpB*). At  $\text{OD}_{600}$   
10  $= 0.5$  (or  $\approx 0.3$  for the *mpB* depletion strain) 250  $\mu\text{L}$  of culture was mixed with 55  $\mu\text{L}$  2M ice-cold  
11 formic acid and frozen on dry ice. Samples were left on ice for 20 mins and centrifuged at  $4^\circ\text{C}$   
12 for 15 mins to collect the supernatant. PEI-cellulose TLC plates (J.T.Baker) were prepared by  
13 sequential immersion in distilled  $\text{H}_2\text{O}$ , air drying, immersion in methanol and a second air-drying  
14 step. Then, 20  $\mu\text{L}$  of extracts were spotted progressively (2  $\mu\text{L}$  at a time, dried by hairdryer on  
15 cold setting) on the plate and plates were developed in 1.5 M  $\text{KH}_2\text{PO}_4$  (pH=3.4) as described in  
16 (Schneider et al., 2003). The region of the plate containing the unincorporated label was cut off  
17 before overnight exposure to PhosphorImager screens and scanning using a GE Typhoon  
18 scanner.

19

#### 20 QUANTIFICATION AND STATISTICAL ANALYSIS

21 Northern blots were scanned using a GE Typhoon scanner. The resulting (.gel) image was  
22 quantified by drawing rectangles around individual bands using Fiji software. Processing  
23 efficiency was calculated from the ratio of the 65-nt species to the pre-16S species and  
24 normalized to the ratios calculated for the wt samples present on each gel. Experiments were  
25 performed at least in duplicate (the actual number of repetitions is given in the legend to each  
26 figure). Standard errors were calculated in Microsoft Excel.

27

#### 28 DATA AND SOFTWARE AVAILABILITY

29 Raw and processed data for the mass-spectrometry experiment are given in Table S5. Raw  
30 imaging data (e.g. uncropped, unannotated agarose gels, Northern blots and thin layer  
31 chromatography autoradiograms) corresponding to individual figure panels have been deposited

1 in Mendeley Data ([https://data.mendeley.com/datasets/d4wrkvdtp/draft?a=ebd603ac-8c8f-4a7e-](https://data.mendeley.com/datasets/d4wrkvdtp/draft?a=ebd603ac-8c8f-4a7e-93e7-91668fe87f5c)  
2 [93e7-91668fe87f5c](https://data.mendeley.com/datasets/d4wrkvdtp/draft?a=ebd603ac-8c8f-4a7e-93e7-91668fe87f5c)).

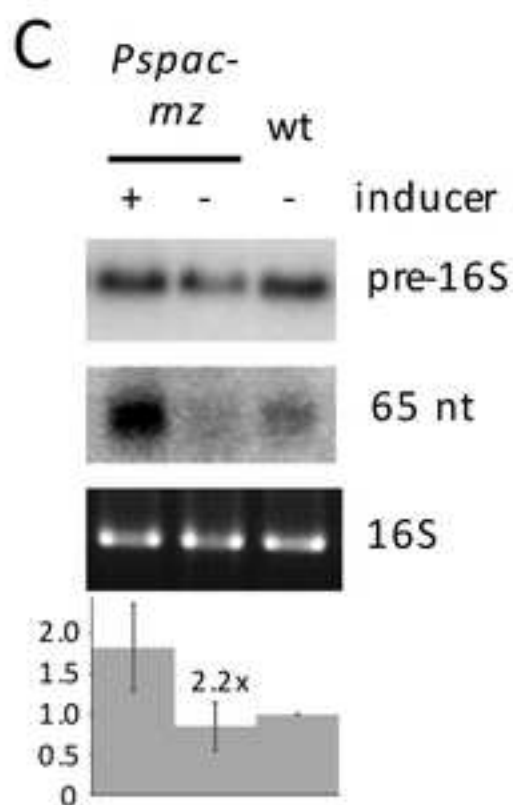
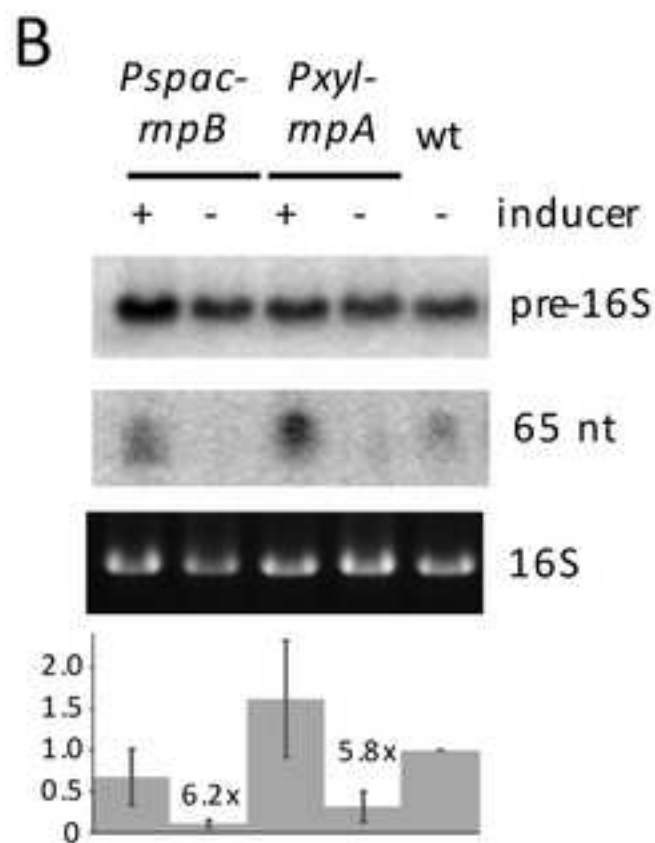
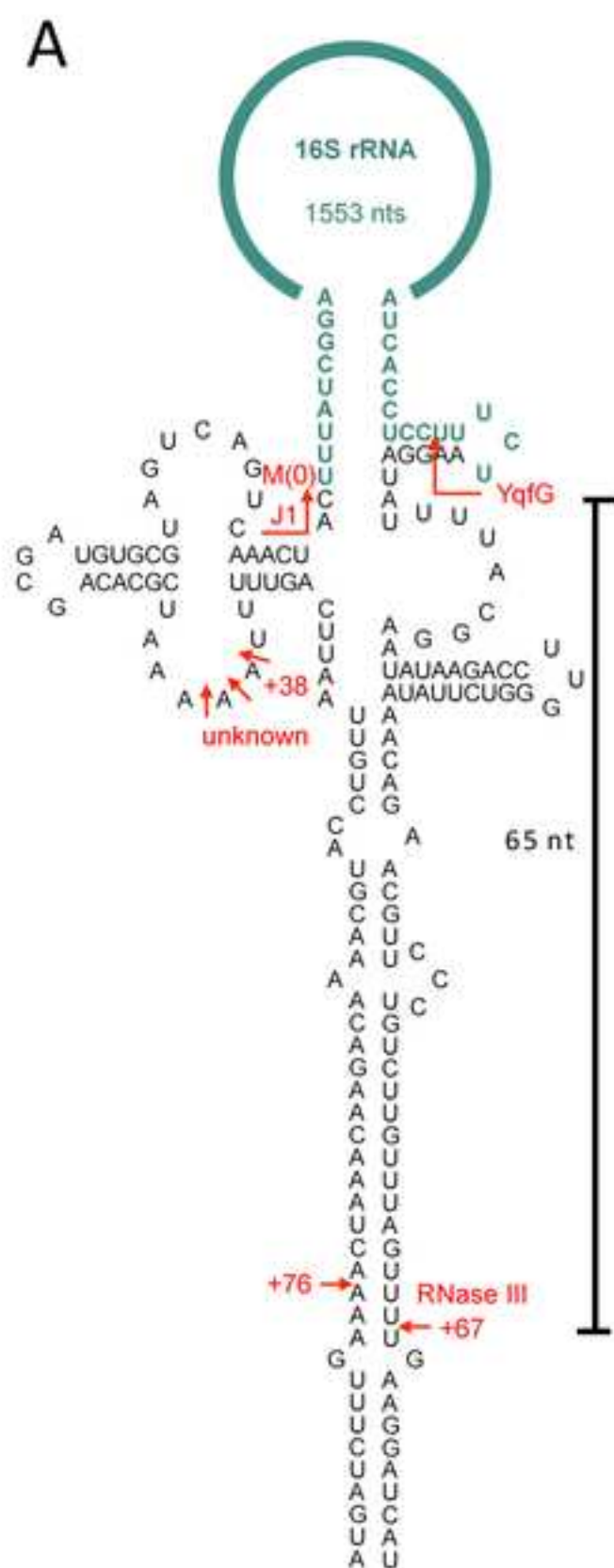
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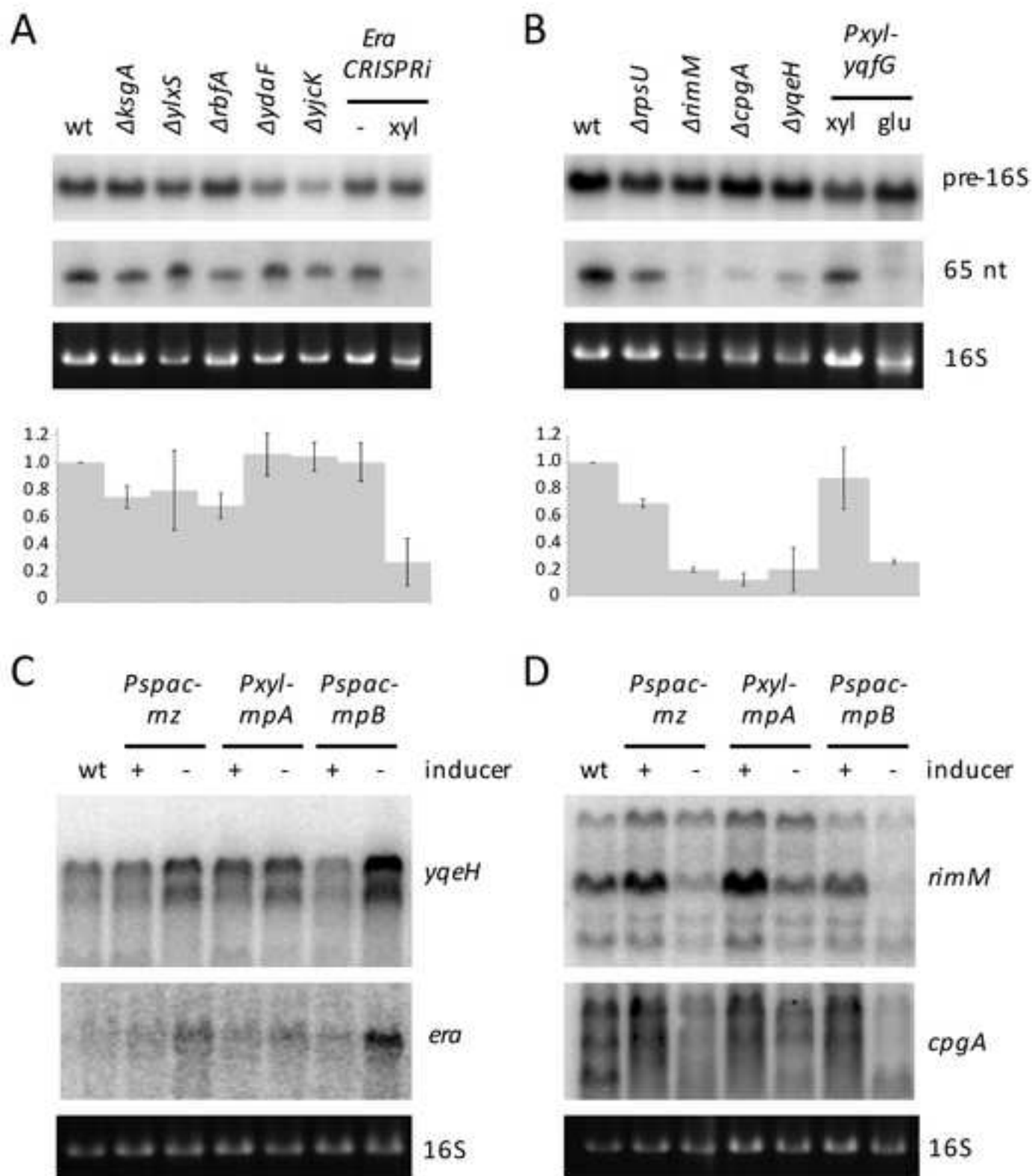


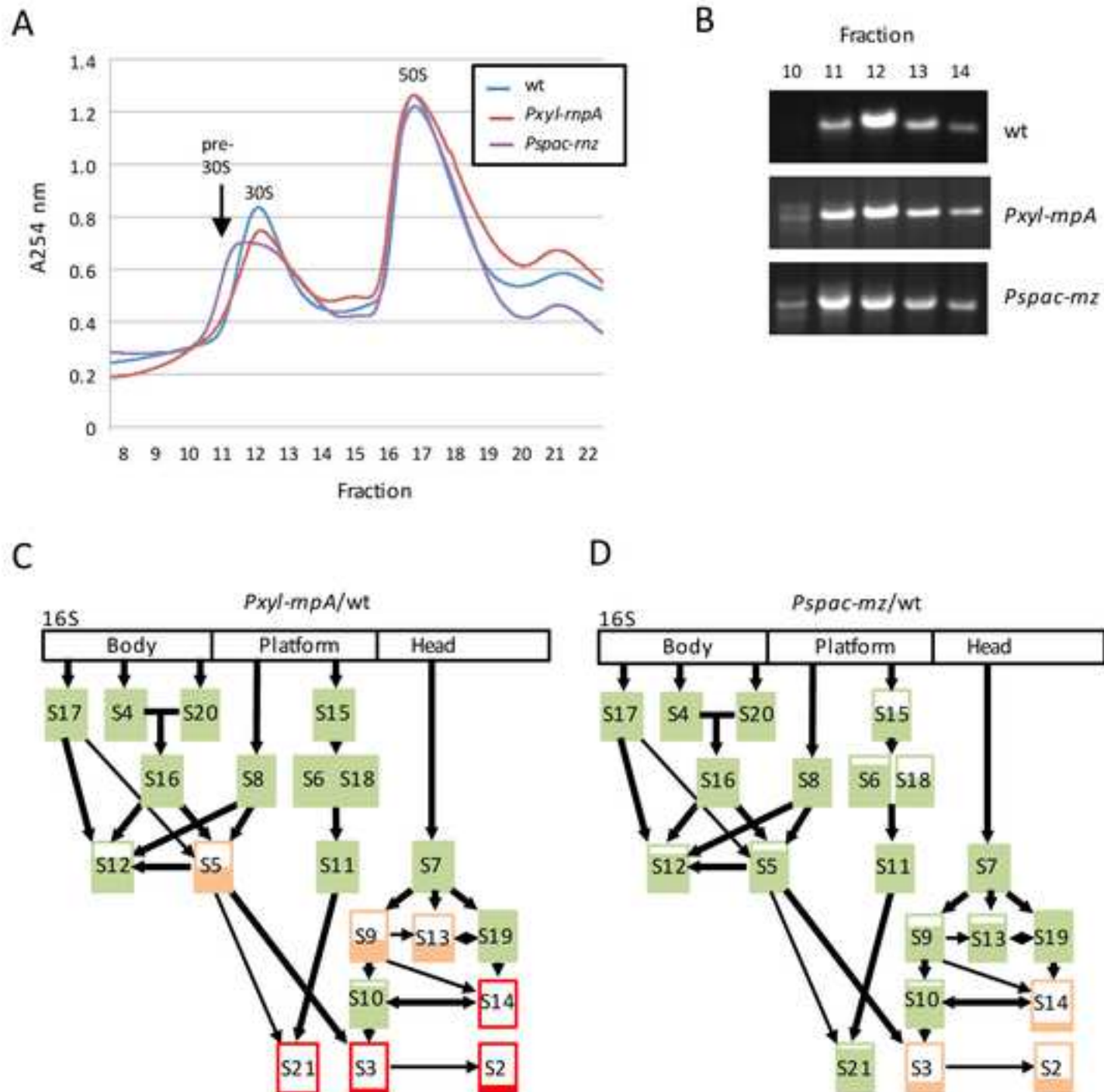
## KEY RESOURCES TABLE

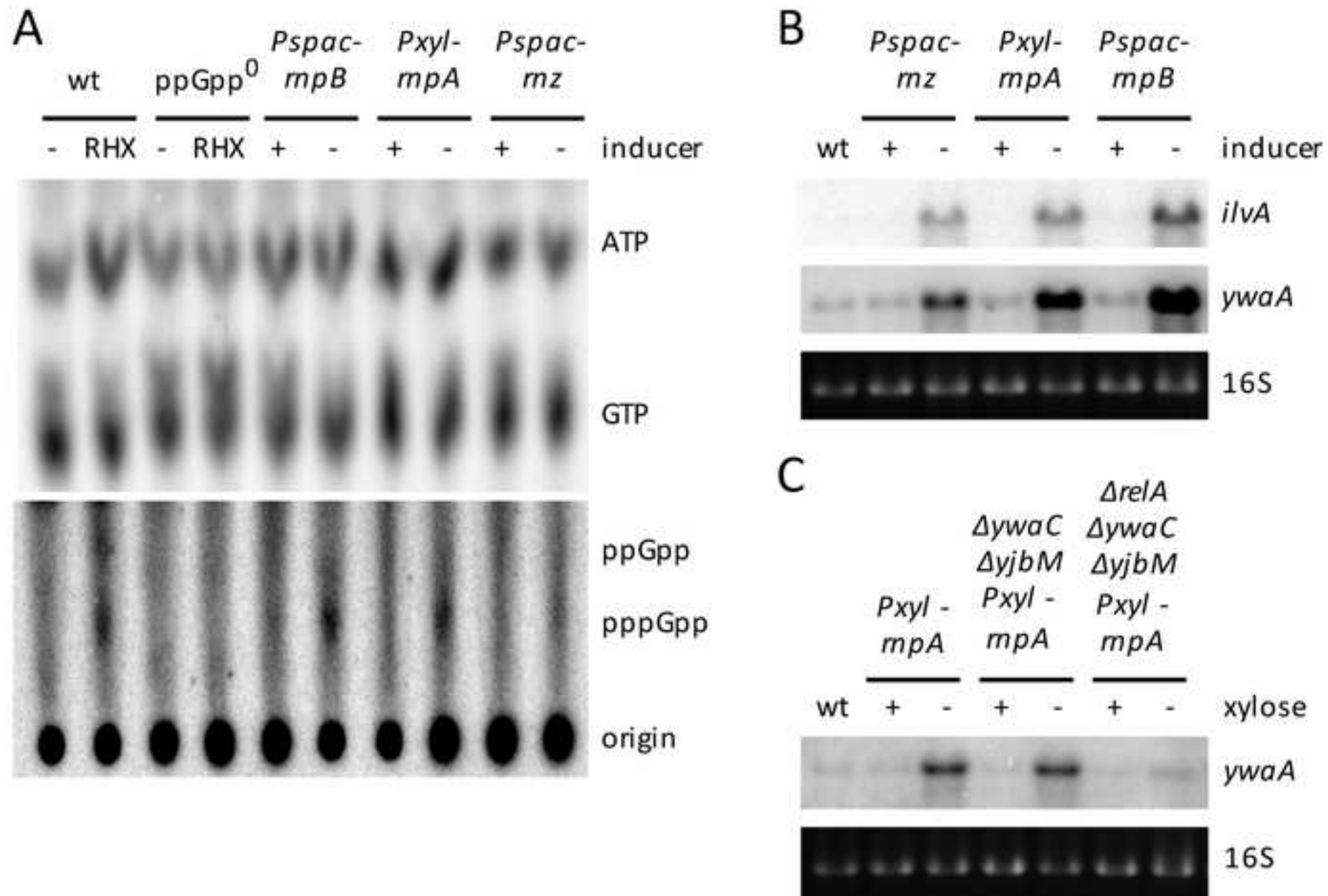
REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Bacterial and Virus Strains</b>		
See Table S1 for <i>Bacillus subtilis</i> strains used in this study and Table S2 for details on new constructs.	N/A	N/A
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
[P32] KH <sub>2</sub> <sup>32</sup> PO <sub>4</sub> (1mCi/ml)	Perkin-Elmer	Cat#NEX063001MC
ATP, [ $\gamma$ -32P]- 3000Ci/mmol 10mCi/ml EasyTide	Perkin-Elmer	Cat#BLU502A500UC
UTP, [ $\alpha$ -32P]- 3000Ci/mmol 10mCi/ml EasyTide	Perkin-Elmer	Cat#BLU507H500UC
Decoyinine (Augustmycin A)	Abcam	Cat#ab144238
L-Arginine hydroxamate hydrochloride	Sigma-Aldrich	Cat#A7380
<b>Bacitracin from <i>Bacillus licheniformis</i>, <math>\geq 60000</math> U/g (Potency)</b>	Sigma-Aldrich	Cat#11702
Formic acid ( $\geq 97\%$ )	MP biomedical	Cat#0215116290
Sequencing grade modified trypsin	Promega	Cat#V5111
DNase I	Sigma-Aldrich	Cat#DN25
RNase A	Sigma-Aldrich	Cat#R6513
Set of dATP, dCTP, dGTP and dTTP	Promega	Cat#U1240
rATP, rCTP, rGTP and rUTP	Promega	Cat#E6011, E6041, E6031, E6021
Roti Hybri Quick	Roth	Cat#A981.1
Ultrahyb	Life technologies	Cat#AM8669
Aquaphenol water-saturated	MP biomedical	Cat#AQUAPH01
AMV Reverse Transcriptase	New England Biolabs	Cat#M0277
<b>Deposited Data</b>		
Mendeley raw imaging data (e.g. uncropped and unannotated agarose gels, Northern blots and thin layer chromatography autoradiograms).	This work	<a href="https://data.mendeley.com/datasets/d4wrkvd tjp/draft?a=ebd603ac-8c8f-4a7e-93e7-91668fe87f5c">https://data.mendeley.com/datasets/d4wrkvd tjp/draft?a=ebd603ac-8c8f-4a7e-93e7-91668fe87f5c</a>
<b>Oligonucleotides</b>		
See Table S3 for list of primers used in this study	N/A	N/A
<b>Recombinant DNA</b>		
See Table S4 for list of plasmids used in this study.	N/A	N/A
<b>Software and Algorithms</b>		
Pymol 2.3.0	Schrödinger	RRID: SCR_000305
Fiji	<a href="https://fiji.sc/">https://fiji.sc/</a>	RRID: SCR_002285
Proline v1.4	<a href="http://proline.profipteomics.fr/">http://proline.profipteomics.fr/</a>	N/A
Mascot v2.5	Matrix Science, London, UK	N/A
<b>Other</b>		
QExactive+ mass spectrometer	Thermo-Fisher Scientific, USA	N/A
EASY-nanoLC-1000	Thermo-Fisher Scientific, USA	N/A
French Press	Glen Mills, USA	N/A

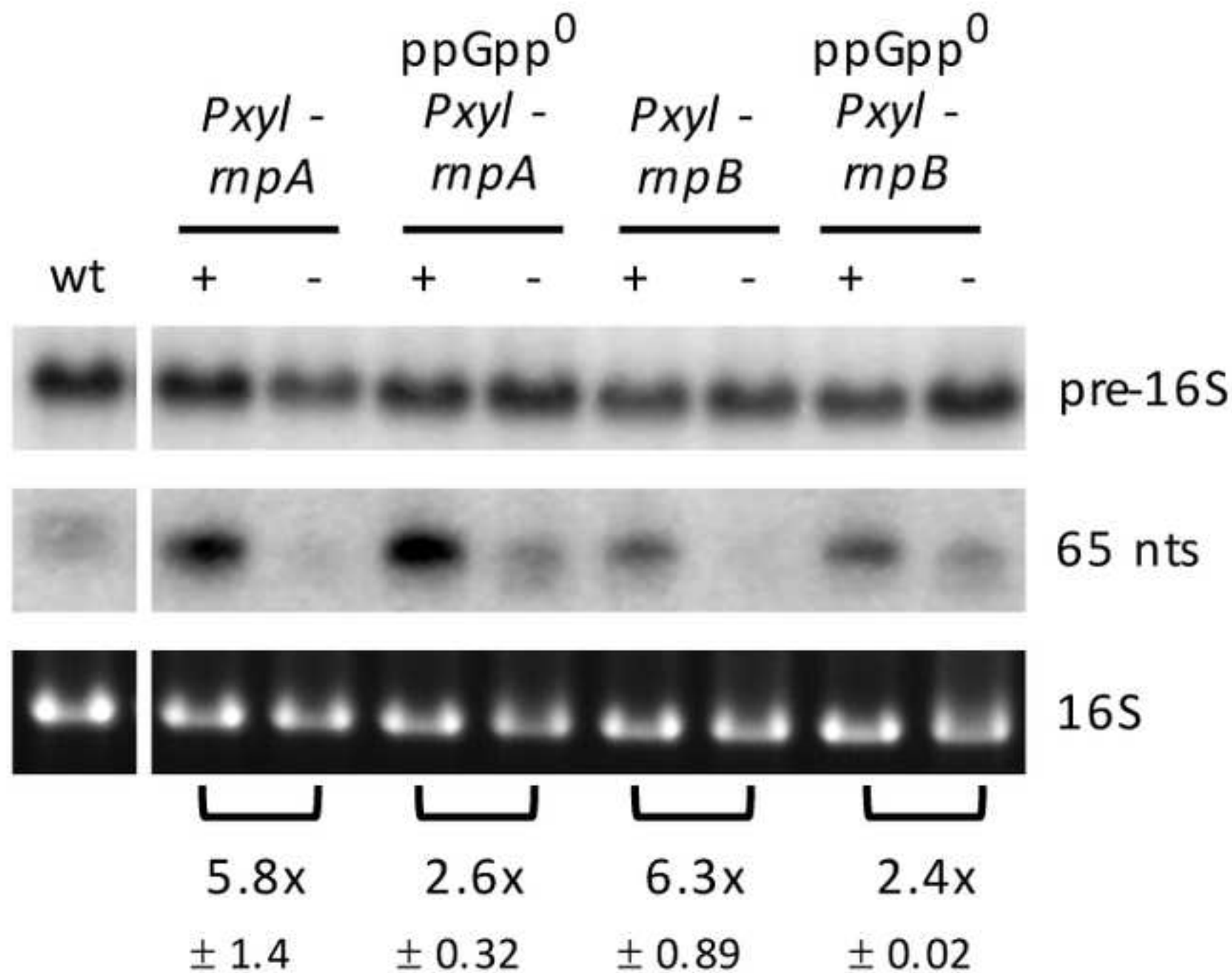
PhosphorImager screens	GE HealthCare Life Sciences	N/A
Typhoon scanner	GE HealthCare Life Sciences	N/A
Glass beads acid-washed	Sigma-Aldrich	Cat#G1145
Piston Gradient Fractionator	Biocomp	N/A
Gradient Master	Biocomp	N/A
Membrane Amersham Hybond-N+	GE HealthCare Life Sciences	Cat#RPN203B
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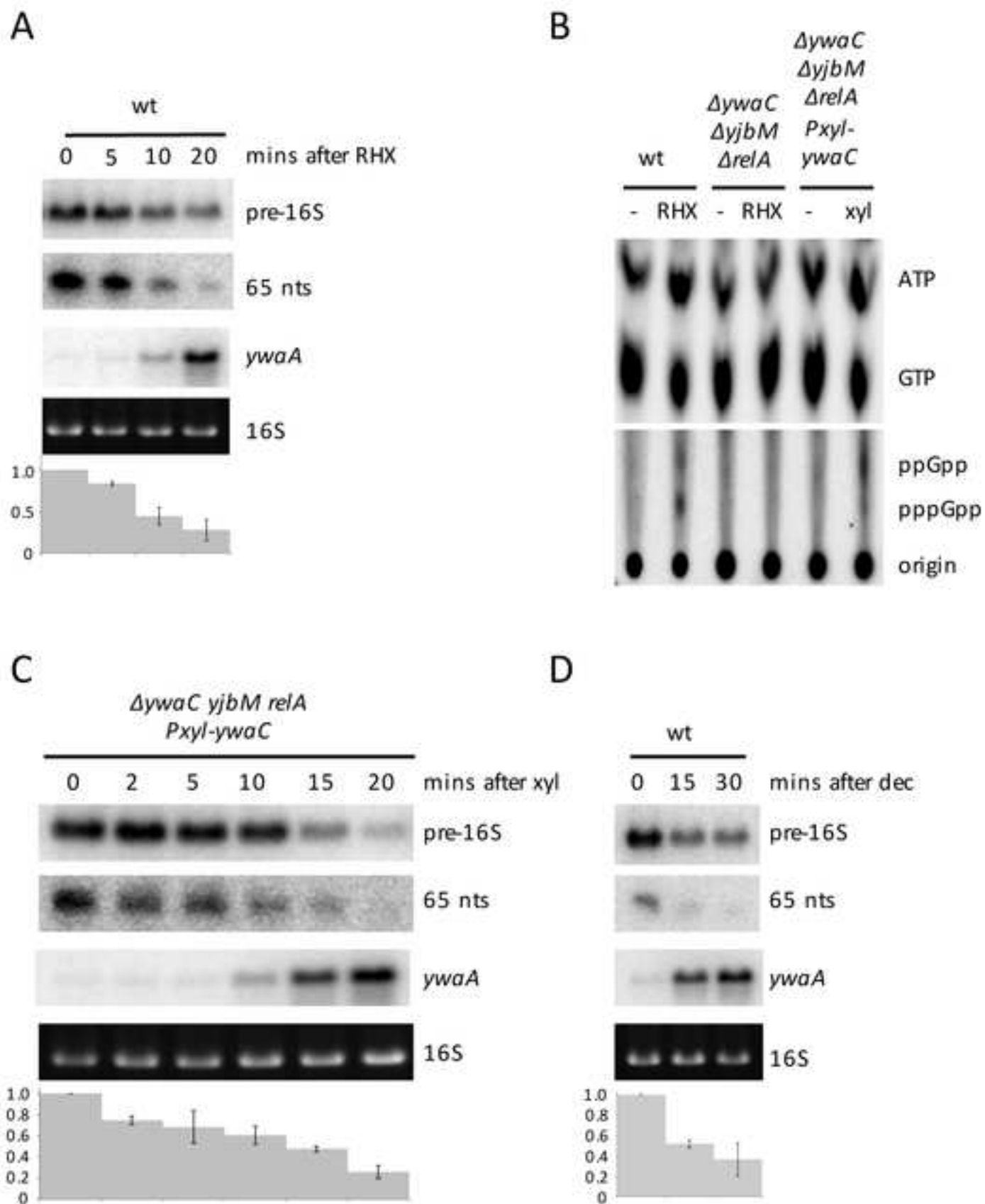




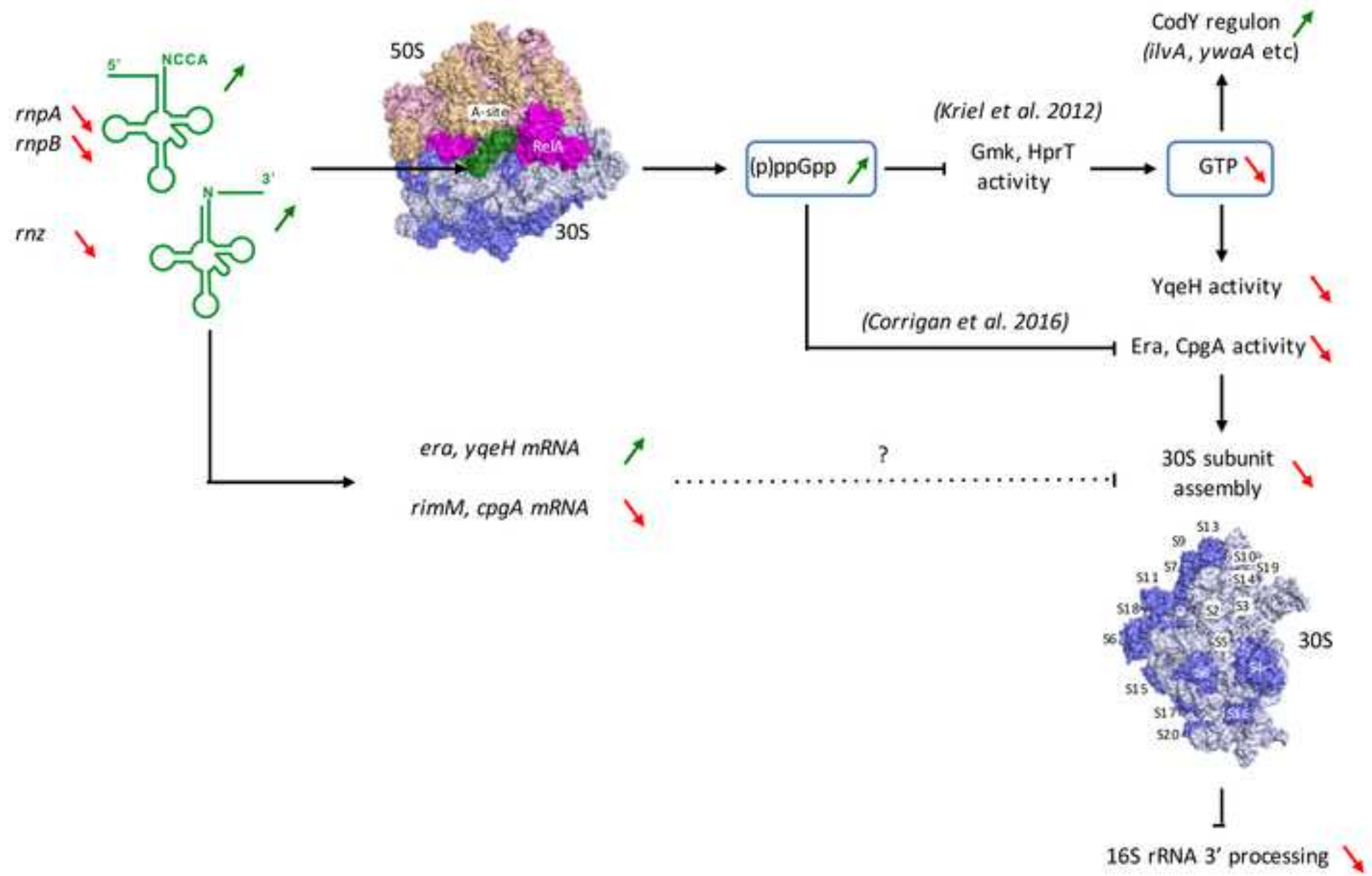












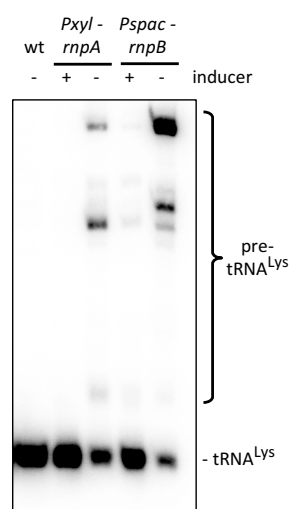


Figure S1, related to Figure 1: Effects of *rnpA* and *rnpB* depletion on processing of tRNA<sup>Lys</sup>. The Northern blot was probed with oligo CC1915, complementary to the mature portion of the *trnJ-lys* tRNA.

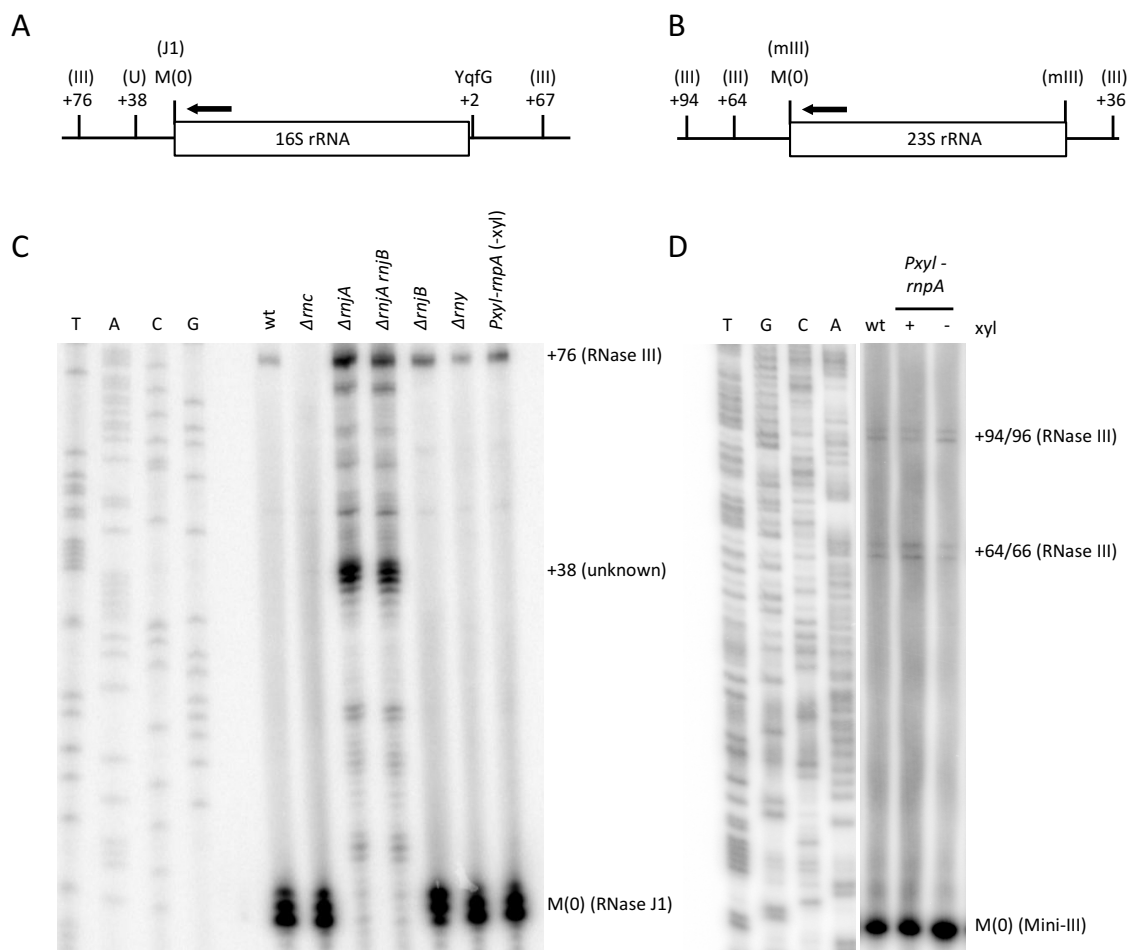


Figure S2, related to Figure 1: RNase P depletion is specific to the 3' processing of 16S rRNA. (A) Schematic of primer extension assay of 16S rRNA 5' processing, showing cleavage sites for RNase III (III), the unknown RNase (U), RNase J1 (J1) and YqfG. The primer used (CC058) is schematized by a black arrow. (B) Schematic of primer extension assay of 23S rRNA 5' processing, showing cleavage sites for RNase III (III) and Mini-III (mIII). The primer used (CC257) is schematized by a black arrow. (C) Primer extension assay of 16S rRNA 5' processing using oligo CC058 performed on total RNA isolated from wild-type (WT) or strains lacking RNase III ( $\Delta rnc$ ), RNase J1 ( $\Delta rnjA$ ), RNase J2 ( $\Delta rnjB$ ), RNase Y ( $\Delta rny$ ), or depleted for the protein subunit of RNase P (*rnpA*). (D) Primer extension assay of 23S rRNA 5' processing using oligo CC257, performed on total RNA isolated from WT and the *rnpA*-depletion strain in the absence of xylose (xyl).

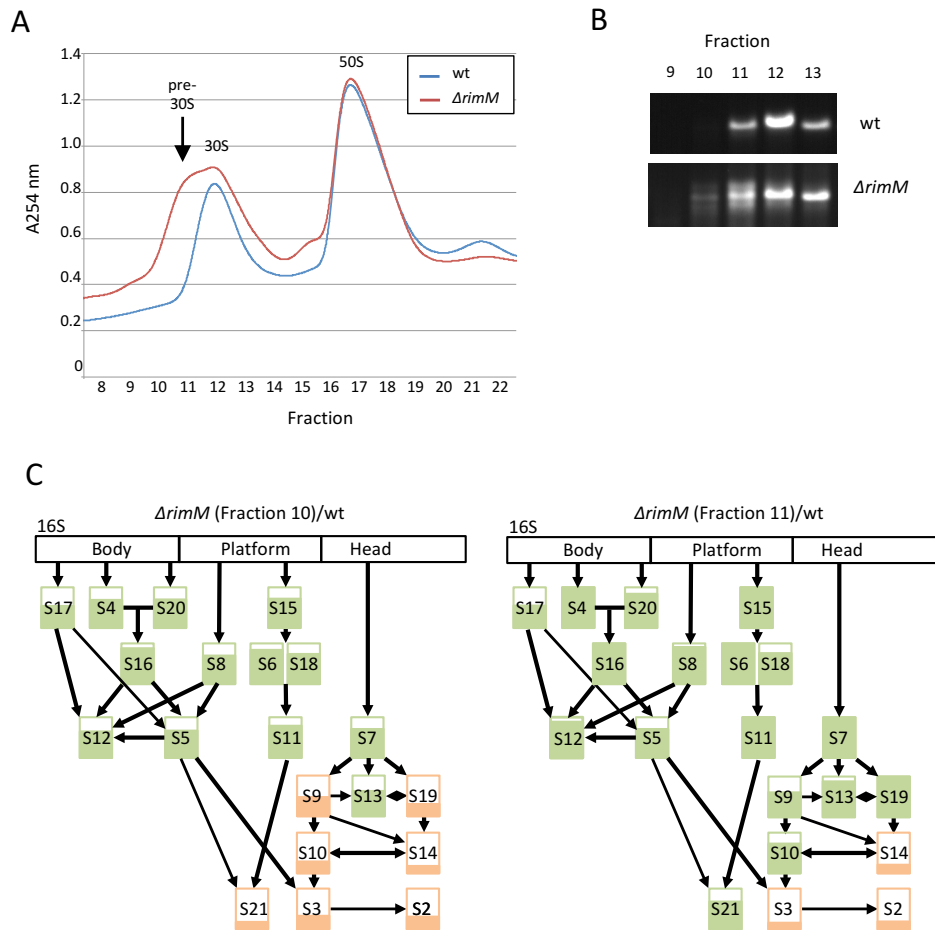


Figure S3, related to Figure 3: The effect of RNase P and Z depletions on ribosome assembly is similar to that of cells lacking the late assembly factor RimM. (A) Sucrose gradients of wt vs  $\Delta rimM$  mutants (B) 16S rRNA profile in sucrose gradients (C) LC-MS/MS analysis ( $n=2$ ) of pre-30S fractions in wt vs  $\Delta rimM$  mutants. The number of peptides for each protein were first normalized to the total peptides observed in each fraction and then normalized to the equivalent number in wt. Early fraction 10 in the  $\Delta rimM$  mutant was compared to early fraction 11 in wt; late fraction 11 in the  $\Delta rimM$  mutant was compared to mature fraction 12 in wt. The percent fill of each box represents the amount of each ribosomal protein compared to wt. Proteins shown in orange are represented at  $>10\%$  but  $\leq 50\%$  of wt; green  $> 50\%$  of wt.

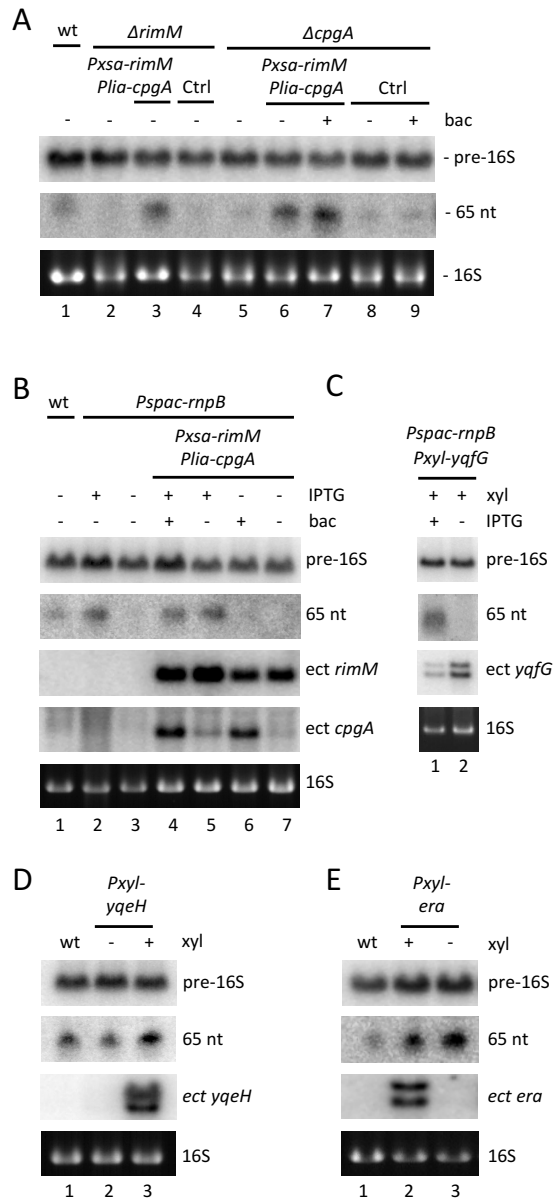


Figure S4, related to Figure 2: Perturbation of individual assembly factor mRNA levels is unlikely to explain defects in 16S 3' processing. (A) The *Pxsa-rimM* + *Plia-cpgA* vector is functional. Control experiment showing complementation of 16S rRNA 3' processing defects of  $\Delta rimM$  and  $\Delta cpgA$  strains by ectopic expression of *rimM* and *cpgA*, respectively. Note that expression of *Pxsa-rimM* is leaky and yields about 2-fold excess of *rimM* mRNA in the absence of arabinose, compared to expression from the native locus (not shown). Expression of *Plia-cpgA* is also leaky; addition of bacitracin (bac) yields similar levels of *cpgA* mRNA to expression from the native locus (not shown). Ctrl is the empty vector control. (B) 16S 3' processing is not restored in *rnpA*-depleted cells ectopically expressing *rimM* alone or together with *cpgA*. (C) 16S 3' processing is not restored in *rnpA*-depleted cells ectopically expressing *yqfG*. (D)+(E) 16S 3' processing is not inhibited upon over-expression of either *era* or *yqeH* in a wt background. 5  $\mu$ g of total RNA was probed with oligo CC172, specific for the 16S rRNA 3' precursor, on agarose gels (upper panel) and polyacrylamide gels (lower panel) in each case for optimal transfer of the  $\sim$  1620 nt and 65 nt species.

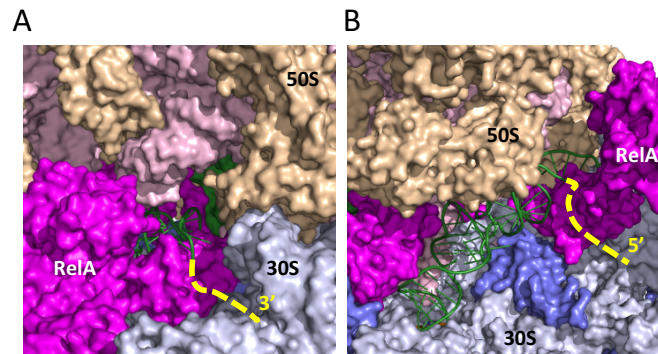


Figure S5, related to Figure 7: RelA bound to the A-site of the ribosome can accommodate tRNA precursors. (A) Possible pathway for tRNA 3' extensions (yellow dotted line). RelA is shown in space filling mode in pink, the 30S subunit in light blue (16S rRNA) and dark blue (30S proteins), the 50S subunit in pink (23S rRNA) and wheat (50S proteins), the A-site tRNA in cartoon mode and the P-site tRNA in space filling mode are shown in green (B) Possible pathway for tRNA 5' extensions. Color scheme as in panel (A).