The precursor tRNA 3'-CCA interaction with *Escherichia coli* RNase P RNA is essential for catalysis by RNase P in vivo

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ABSTRACT

The L15 region of *Escherichia coli* RNase P RNA forms two Watson–Crick base pairs with precursor tRNA 3'-CCA termini (G292-C₇₅ and G293-C₇₄). Here, we analyzed the phenotypes associated with disruption of the G292-C₇₅ or G293-C₇₄ pair in vivo. Mutant RNase P RNA alleles (*rnpBC292* and *rnpBC293*) caused severe growth defects in the *E. coli* *rnpB* mutant strain DW2 and abolished growth in the newly constructed mutant strain BW, in which chromosomal *rnpB* expression strictly depended on the presence of arabinose. An isosteric C293-G₇₄ base pair, but not a C292-G₇₅ pair, fully restored catalytic performance in vivo, as shown for processing of precursor 4.5S RNA. This demonstrates that the base identity of G292, but not G293, contributes to the catalytic process in vivo. Activity assays with mutant RNase P holoenzymes assembled in vivo or in vitro revealed that the C292/293 mutations cause a severe functional defect at low Mg²⁺ concentrations (2 mM), which we infer to be on the level of catalytically important Mg²⁺ recruitment. At 4.5 mM Mg²⁺, activity of mutant relative to the wild-type holoenzyme, was decreased only about twofold, but 13- to 24-fold at 2 mM Mg²⁺. Moreover, our findings make it unlikely that the C292/293 phenotypes include significant contributions from defects in protein binding, substrate affinity, or RNA degradation. However, native PAGE experiments revealed nonidentical RNA folding equilibria for the wild-type versus mutant RNase P RNAs, in a buffer- and preincubation-dependent manner. Thus, we cannot exclude that altered folding of the mutant RNAs may have also contributed to their in vivo defect.

Keywords: *E. coli* ribonuclease P; CCA-RNase P RNA interaction; in vivo complementation studies; point mutations

INTRODUCTION

Ribonuclease P (RNase P) is an essential ribonucleoprotein enzyme responsible for the 5'-end maturation of tRNAs (Altman and Kirsebom 1999; Schön 1999; Hartmann and Hartmann 2003; Evans et al. 2006). The bacterial RNase P holoenzyme consists of an RNA subunit of ~380 nucleotides (nt) and a small basic protein (~13 kDa), and in vitro, RNA subunits of bacterial RNase P enzymes are catalytically active in the absence of the protein subunit (Guerrier-Takada et al. 1983). Bacterial RNase P enzymes recognize the stacked acceptor stem-T arm module of

precursor tRNA (ptRNA) substrates, including specific elements at the cleavage site, such as the residue at nucleotide -1 and the 3'-₇₃NCCA₇₆ end (Harris and Christian 2003). The two cytosines of tRNA 3'-CCA (C₇₄ and C₇₅) termini were shown to form Watson–Crick (WC) base pairs with two conserved G residues (G292 and G293) in the loop L15 of *Escherichia coli* RNase P RNA (Fig. 1; Kirsebom and Svärd 1994). This interaction provides a substantial contribution to the free energy of ptRNA binding in vitro (Hardt et al. 1995; Oh et al. 1998; Busch et al. 2000), supports selection of the correct cleavage site (Kirsebom and Svärd 1994), and was suggested to play a role in the binding of catalytic Mg²⁺ (Oh et al. 1998; Busch et al. 2000). Another in vitro study provided evidence that base-pairing with 3'-CCA is also relevant to cleavage site selection in the holoenzyme reaction (Svärd et al. 1996).

We previously reported a kinetic and thermodynamic in vitro analysis of the G292-C₇₅ and G293-C₇₄ base-pairing

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FIGURE 1. Interaction of *Thermus thermophilus* ptRNA^{Gly} with the L15 loop of *E. coli* RNase P RNA. Highlighted nucleotides mark the sites of mutation investigated in this study. The arrow indicates the canonical RNase P cleavage site (between nucleotides -1 and $+1$). Nucleotides 291–295 of *E. coli* RNase P RNA are also termed J16/15.

interaction in the reaction catalyzed by *E. coli* RNase P RNA (Busch et al. 2000). The results were based on the study of wild-type RNase P RNA along with two variants carrying a single G-to-C exchange (C292 or C293), either acting on wild-type ptRNA^{Gly} or derivatives with a compensatory mutation in the tRNA 3'-end (3'-G₇₄CA or 3'-CG₇₅A) (Fig. 1). Disruption of either of the two intermolecular base pairs largely reduced ground state binding to *E. coli* RNase P RNA. Disruption of the G293-C₇₄ pair had a less detrimental effect on the single turnover kinetic parameters than did disruption of the G292-C₇₅ pair (Busch et al. 2000). This was consistent with previous findings (Oh et al. 1998), suggesting that the reduction in the efficiency of cleavage of a mutant ptRNA-CU₇₅A substrate resulted from impaired binding of Mg²⁺ important for catalysis. Restoration of base-pairing by compensatory base exchanges in the substrate (C293-G₇₄ and C292-G₇₅) restored catalytic performance in vitro, almost fully for the C293-G₇₄, but clearly less for the C292-G₇₅ combination (Busch et al. 2000).

Here, we investigated the role of the CCA interaction in vivo in *E. coli* to clarify how far previous in vitro findings, observed under unphysiologically high Mg²⁺ concentrations, are relevant to the cellular situation. This included the construction of a novel *E. coli* *rnpB* mutant strain (named BW), in which chromosomal *rnpB* expression is efficiently knocked down when glucose is substituted for arabinose in growth media. We demonstrate that the *rnpBC292* and *rnpBC293* mutant alleles are lethal in strain BW. In the case of the C293, but not the C292 mutant, a compensatory mutation in the substrate 3' end fully restored processing capacity in vivo and in vitro. Processing assays with partially purified and in vitro assembled holoenzymes further revealed that the defect of the C292/293 enzymes is most evident at free Mg²⁺ concentrations

assumed to be close to those in vivo. Since our results indicate that the phenotypes of the C292 and C293 mutations do not include substantial contributions from defects in protein binding, substrate affinity, or RNA degradation, we propose that the defect is primarily on the level of recruitment of catalytically important Mg²⁺. Native PAGE experiments revealed nonidentical RNA folding equilibria for the wild-type versus mutant RNase P RNAs, in a buffer- and preincubation-dependent manner. Thus, a contribution from nonnative folding to the defect of the mutant RNAs in vivo cannot be ruled out at present.

RESULTS

In vivo complementation in *E. coli* DW2

To investigate the ptRNA 3'-CCA interaction with RNase P RNA under in vivo conditions, plasmids carrying the wild-type RNase P RNA gene (*rnpB*^{wt}) or the C292 or C293 mutant alleles (*rnpBC292*, *rnpBC293*) were introduced into the temperature-sensitive *E. coli* RNase P mutant strain DW2/pDW160. In strain DW2/pDW160, the chromosomal gene encoding the RNase P RNA subunit (*rnpB*) is replaced with a chloramphenicol resistance cassette and a complementing *rnpB* gene is provided on a plasmid (pDW160) temperature sensitive for replication (Waugh and Pace, 1990). Suppression of the conditionally lethal phenotype at 43°C is achieved by expression of functional RNase P RNA from a second compatible plasmid.

As expected, *E. coli* *rnpB*^{wt} provided on the multicopy plasmid pSP64 rescued growth of the mutant strain at the nonpermissive temperature of 43°C (Table 1). The *rnpBC292* mutant allele caused only a partial rescue, and *rnpBC293* showed very weak complementation. For bacteria transformed with *rnpBC292* and *rnpBC293* mutant alleles and grown at 43°C, the corresponding mutant RNAs were verified to be the dominant RNase P RNA species in the cell by primer extension analyses (data not shown; see Materials and Methods). These control experiments excluded reversion of mutant alleles to the wild-type sequence.

Construction of a novel *E. coli* *rnpB* knockdown strain

The impaired phenotype of the *rnpBC293* allele relative to *rnpBC292* in *E. coli* DW2 cells (Table 1) was the reverse of what we had expected based on our previous in vitro results (Busch et al. 2000). Moreover, the DW2 strain has the drawback that complementation analyses are performed under stress conditions at 43°C. The heat-shock response has a global effect on gene expression (Richmond et al. 1999; Li et al. 2003), and induced metabolic changes could alter the mutant phenotype. Also, even cells expressing the

TABLE 1. Complementation analyses of *E. coli* RNase P mutant strain DW2 by *rnpB* alleles

	DW2/pDW160	
RNase P RNA variant	30°C	43°C
pSP64 <i>E. coli rnpB</i> wt	+++	+++ ^a
pSP64 <i>E. coli rnpBC292</i>	+++	++
pSP64 <i>E. coli rnpBC293</i>	+++	(+)
pSP64	+++	—

Growth of mutant strain DW2 transformed with different *rnpB* alleles (*E. coli rnpB*wt, *rnpBC292*, and *rnpBC293*) was analyzed on LB plates at the permissive (30°C) and nonpermissive (43°C) temperature. +++ > ++ > (+) > —, decreasing order of complementation efficiency based on number and size of colonies.

^aGrowth phenotype of cells harboring pSP64 *E. coli rnpB*wt at 43°C was set as standard, although colonies at 43°C were smaller than at 30°C.

wild-type allele (pSP64 *E. coli rnpB*wt) (Table 1) grew slower at 43°C compared with 30°C. We thus constructed a novel *E. coli* mutant strain in which the chromosomal *rnpB* gene was put under control of the arabinose promoter and repressor system (Fig. 2A). In the absence of arabinose and presence of glucose, *rnpB* expression is switched off, and cell survival depends on a second functional *rnpB* gene provided on a plasmid. The *E. coli* mutant strain (termed BW) exactly displayed the anticipated phenotype (Fig. 2B). In the BW strain, only the *rnpB*wt allele, but not *rnpBC292* and *rnpBC293*, restored growth in the absence of arabinose (Fig. 2C; Table 2A).

Defect in maturation exemplified for 4.5S RNA

We then analyzed if the *rnpBC292* and *rnpBC293* mutant alleles are associated with an RNase P processing defect in BW bacteria. For this purpose, BW cells expressing *rnpB*wt, *rnpBC292*, or *rnpBC293* from plasmid pACYC177 were initially grown in medium containing arabinose, followed by transfer into medium without arabinose, further incubation for 3 h at 37°C, and preparation of total cellular RNA. We then analyzed processing of 4.5S RNA, a prominent non-tRNA substrate of *E. coli* RNase P (Bothwell et al. 1976; Peck-Miller and Altman 1991), by primer extension (Fig. 3). Exclusively mature 4.5S RNA was detected in RNA extracts from cells expressing plasmid-encoded *rnpB*wt (Fig. 3, lane 2), whereas cells harboring *rnpBC292* and *rnpBC293* accumulated a 4.5S RNA precursor extended by 3 nt at the 5' end. Since the native 4.5S RNA primary transcript carries a 5' leader of 24 nt (see Fig. 4), nucleolytic activities other than RNase P apparently trimmed the leader to 3 nt. Likewise, accumulation of immature RNA with an extra 2–5 nt at the 5' end were observed for the bacteriophage P1/P7 regulatory C4 RNA, another non-tRNA substrate of *E. coli* RNase P, when processing by RNase P was defective (Hartmann et al. 1995). The ratio of

5'-extended to mature 4.5S RNA was reproducibly higher in the *rnpBC292* than *rnpBC293* background (Fig. 3, lanes 3,4), providing evidence that the processing defect of the C292 mutant RNA is more severe than that of the C293 variant, in line with the kinetic *in vitro* data (Busch et al. 2000).

FIGURE 2. Construction and analysis of the *E. coli rnpB* mutant strain BW. (A) Schematic presentation of the strain construction strategy. The native *rnpB* promoter region was replaced with a cassette (*cat*RExBAD, 2136 bp) carrying the chloramphenicol resistance gene (*cat*), a gene coding for an arabinose operon regulatory protein (*araC*), and the arabinose-inducible *pBAD* promoter. The cassette for homologous recombination (2669 bp) additionally contained flanking regions identical in sequence to *E. coli rnpB* and to a segment upstream of *rnpB*. (B) Cell growth rescue of strain BW in the presence of glucose (Glu) with a plasmid-encoded copy of *E. coli rnpB* (pACYC177 Ecwt). Note that growth curves in the presence of glucose versus arabinose differ in the same manner for strain BW and its progenitor MG1655. (C) Growth analyses of BW bacteria expressing plasmid-encoded *rnpB*wt, *rnpBC292*, or *rnpBC293* alleles (pACYC177 Ecwt, Ec292, or Ec293) in the presence of arabinose (Ara) or glucose (Glu).

TABLE 2. Complementation analyses in *E. coli* RNase P mutant strain BW

(A) Complementation of <i>E. coli</i> RNase P mutant strain BW by <i>rnpB</i> alleles			
RNase P RNA variant		10 mM arabinose	0.5% glucose
pACYC177 <i>E. coli rnpB</i> wt		+	+
pACYC177 <i>E. coli rnpBC292</i>		+	—
pACYC177 <i>E. coli rnpBC293</i>		+	—
pACYC177		+	—
(B) Complementation of <i>E. coli</i> RNase P mutant strain BW by <i>rnpB</i> alleles and simultaneous overexpression of <i>E. coli rnpA</i>			
RNase P RNA variant	<i>rnpA</i> ^a	10 mM arabinose,1 mM IPTG	0.5% glucose,1 mM IPTG
pACYC177 <i>E. coli rnpB</i> wt	+ <i>E. coli rnpA</i>	+	+
pACYC177 <i>E. coli rnpBC292</i>	+ <i>E. coli rnpA</i>	+	— ^b
pACYC177 <i>E. coli rnpBC293</i>	+ <i>E. coli rnpA</i>	+	+ ^c
pACYC177	+ <i>E. coli rnpA</i>	+	—

Growth of BW bacteria transformed with different *rnpB* alleles (*E. coli rnpB*wt, *rnpBC292* and *rnpBC293*, part A) and simultaneously overexpressing *E. coli rnpA* (part B); growth was analyzed on LB plates in the presence of 10 mM arabinose or 0.5% (w/v) glucose; for bacteria expressing *rnpA*, 1 mM IPTG was added to the medium. + indicates growth with equal numbers of colonies on arabinose and glucose plates; —, no growth;

^aCloned in plasmid pBR322 under control of the inducible pTrc promoter.

^bRetarded growth and less colonies on glucose plates (~10% of that of arabinose plates), likely revertants.

^cCells showed retarded growth.

Restoration of 4.5S RNA maturation in the *rnpBC293* background by a compensatory substrate mutation

From our previous results obtained in the RNA-alone reaction (Busch et al. 2000), it seemed possible that an isosteric C293-G₇₄ pair in enzyme–substrate complexes may fully restore activity to that of wild-type complexes. To test if this also holds for the cellular context, we constructed a plasmid-borne 4.5S RNA gene, in which we switched the identity of four consecutive base pairs in the terminal stem region. Expression of the mutated 4.5S RNA was not detrimental to cell viability. The sequence alteration in 4.5S RNA permitted primer annealing exclusively to this 4.5S RNA variant, but not the chromosomally encoded wild-type 4.5S RNA. In addition, two variants with C-to-G exchanges (G₇₄ or G₇₅) in the dangling 3'-CCC terminus were constructed (Fig. 4A). Primer extension analysis, performed essentially as in Figure 3, indeed revealed full restoration of 4.5S RNA maturation efficacy for the C293 holoenzyme acting on the precursor 4.5S RNA with a 3'-G₇₄ CC terminus (Fig. 4, cf. lanes 1,6 and 2–5,7–9). In contrast, the combination of C292 P RNA and p4.5S RNA 3'-CG₇₅C (Fig. 4, lane 8), although restoring base-pairing, did not reestablish maturation capacity to wild-type levels.

Overexpression of *rnpA*

We speculated that overexpression of the *E. coli* RNase P protein might increase the steady-state level of the RNase P holoenzyme and thereby alleviate the processing defects

FIGURE 3. Primer extension analysis using a primer specific to *E. coli* 4.5S RNA and total RNA from strain BW expressing pACYC177-encoded *E. coli rnpB*wt (lane 2), *rnpBC292* (lane 3), and *rnpBC293* (lane 4); Con (lane 1) indicates products of reverse transcription in the presence of ddGTP and using in vitro transcribed precursor 4.5S RNA as the template; prominent bands resulted from premature termination of reverse transcription at template C residues (marked by asterisks); the precursor segment of the in vitro transcribed p4.5S RNA had the natural sequence up to residue –23, but was different beyond this position and carried additional nucleotides for cloning reasons (5'-ggg aga ccg gaa uuc ccG GUU GGU UCU CAA CGC UCU CAA U_{–1}, deviations from the wild-type sequence in small letters). Samples were analyzed by 20% PAGE in the presence of 8 M urea. For growth of BW cells harboring the different plasmids (lanes 2–4) and preparation of total cellular RNA, see Materials and Methods.

FIGURE 4. Primer extension experiments to analyze processing of pBR322-encoded mutant p4.5S RNA in strain BW expressing *E. coli* *rnpBwt*, *rnpBC292*, or *rnpBC293*. (A) Schematic of the mutant p4.5S RNA: The gray-shaded box indicates base-pair exchanges relative to wild-type 4.5S RNA, which were introduced to permit specific reverse transcription of mutant (p)4.5S RNA; two variants carried 3'-terminal C to G exchanges (highlighted) at positions equivalent to nucleotides 74 and 75 of tRNA 3'-CCA ends; the arrow aligned to the stem depicts the reverse transcription primer, with the terminal asterisk illustrating the 5'-³²P-endlabel. (B) Analysis of primer extension products by 20% PAGE in the presence of 8 M urea. (Lanes C1,C2) Primer extension using total RNA from BW cells grown in the presence of glucose and expressing pACYC177-encoded *E. coli* *rnpBwt*, either utilizing the primer for wild-type 4.5S RNA (C1) or mutant 4.5S RNA (C2); the C2 control documents that the primer specific for mutant 4.5S RNA (panel A) did not prime on wild-type 4.5S RNA. (Lanes 1–9) Primer extension using the primer specific for mutant 4.5S RNA; total RNA was from BW cells grown in the presence of glucose and expressing pACYC177-encoded *E. coli* *rnpBwt* (lanes 1,4,7), *rnpBC292* (lanes 2,5,8), or *rnpBC293* (lanes 3,6,9); BW bacteria either expressed the pBR322-encoded mutant p4.5S RNA with the natural 3'-CCC (lanes 1–3), a mutated 3'-GCC (lanes 4–6), or a 3'-CGC (lanes 7–9) terminus. The natural 4.5S RNA primary transcript has a 5' leader of 24 nt (Bothwell et al. 1976; Hsu et al. 1984) as indicated on the left; longer transcripts in lanes 2–5 and 7–9 are attributable to transcriptional initiation from the β -lactamase promoter region still present in our pBR322 plasmid constructs.

caused by the *rnpBC292* and *rnpBC293* mutant alleles. Indeed, for BW cells expressing *rnpBC293*, concomitant overexpression of *rnpA* partially restored viability, although growth was retarded relative to cells harboring the *rnpBwt* allele (Table 2B). In contrast, the defect caused by the C292 mutation could not be cured by overexpressing *rnpA*. Overexpression of the *E. coli* RNase P protein (C5) was verified by Western blotting using an antiserum against the *E. coli* C5 protein (data not shown).

Processing of ptRNA by partially purified holoenzymes assembled in vivo

We partially purified in vivo assembled holoenzymes by anion exchange chromatography from DW2 cells grown at 43°C and either expressing *rnpBwt*, *rnpBC292*, or *rnpBC293* from plasmid pSP64. Holoenzymes were analyzed by primer extension in the presence of ddGTP (see Materials and Methods) and confirmed to contain $\geq 95\%$ of the respective mutant RNase P RNA (data not shown). We then tested these holoenzymes for processing of ptRNA^{Gly} (Fig. 1, termed ptRNA^{wt} in the following) and the two variant substrates thereof, ptRNA^{G74} and ptRNA^{G75}, with point mutations in 3'-CCA (3'-G₇₄CA and 3'-CG₇₅A) to compensate the C292/293 mutations in RNase P RNA. This enabled us to investigate rescue effects in terms of efficiency and cleavage site selection in the RNase P holoenzyme reaction. Processing assays contained 0.004 A₂₆₀ units of the partially purified holoenzyme fraction and trace amounts (<1 nM) of radiolabeled substrate. As illustrated in Figure 5, the wild-type holoenzyme substantially miscleaved the two mutant substrates (Fig. 5, panel A). As the wild-type holoenzyme, the C292 and C293 mutant holoenzymes cleaved ptRNA^{wt} to >95% at the canonical site. However, the C292 mutation suppressed miscleavage of the ptRNA^{G75} substrate (Fig. 5, panel B), as did the C293 mutant holoenzyme when acting on the ptRNA^{G74} substrate (Fig. 5, panel C). These findings were expected based on restoration of the intermolecular base pairs and are in line with results from previous in vitro studies (Kirsebom and Svärd 1994; Svärd et al. 1996; Busch et al. 2000). However, the mutant holoenzymes cleaved ptRNA^{wt}, the only substrate relevant to the in vivo situation, with good efficiency. The purification scheme was identical for DW2 cells expressing the *rnpBwt*, *rnpBC292*, or *rnpBC293* alleles, and equal amounts of extract based on absorption at 260 nm were used in these processing assays. Although we are aware that exact holoenzyme concentrations may not have been identical for all three extracts, good processing efficiencies of the two mutant holoenzymes appeared not fully consistent with their severe in vivo defect. We thus decided to increase the stringency of our processing assay by lowering the Mg²⁺ concentration to 2 mM, assuming that this better mimics the free Mg²⁺ concentration (1–2 mM) in *E. coli* cells (Alatossava et al. 1985). For wild-type RNase P, the cleavage rate at 10 mM Mg²⁺ relative to 2 mM Mg²⁺ was 25-fold increased ($k_{rel} = 25$), but this difference increased to ≥ 650 -fold for the C292 and C293 mutant enzymes. Thus, the defect of the mutant enzymes was exacerbated at low [Mg²⁺]. However, the C293 mutant holoenzyme cleaved the ptRNA^{G74} with the same efficiencies at 2 and 10 mM [Mg²⁺] as the wild-type holoenzyme acting on ptRNA^{wt} (Table 3), demonstrating that the natural G293-C₇₄ and the isosteric C293-G₇₄ pair are fully exchangeable. In contrast, the C292-G₇₅ pair only

FIGURE 5. Processing assays using RNase P holoenzymes (0.004 A₂₆₀ units) partially purified from strain DW2 grown at 43°C and complemented with *E. coli* (A) *rnpBwt*, (B) *rnpBC292*, and (C) *rnpBC293*; trace amounts (<1 nM) of 5'-end-labeled ptRNA^{Gly} (Fig. 1) and variants ptRNA_{G74} and ptRNA_{G75} with single point mutations were used as substrates in buffer A containing 10 mM Mg²⁺; con indicates trace amounts (<1 nM) of 5'-end-labeled ptRNA^{Gly} incubated with 5 μM *E. coli* RNase P RNA for 5 sec at 37°C in 50 mM PIPES, 0.1 M Mg(OAc)₂, and 1 M NH₄OAc (pH 7.0 at 37°C). 5'-cleavage products are indicated on the left (canonical cleavage site at +1/-1, miscleavage at -1/-2).

showed a partial rescue ($k_{rel} = 97$) of the defect, emphasizing the importance of base identity at position 292. Full restoration of function in the presence of the C293-G₇₄ pair, but not the C292-G₇₅ pair, exactly corresponds with what we have seen for 4.5S RNA maturation in vivo (Fig. 4).

Processing of ptRNA by holoenzymes reconstituted in vitro

The findings of Table 3 suggested that conditions of 2 mM Mg²⁺ may better mimic the intracellular conditions than

10 mM. Since enzyme concentrations can be better defined and equalized for in vitro reconstituted enzymes relative to those purified from cells, we further tested in vitro assembled *E. coli* RNase P holoenzymes under conditions optimized for ribosome function (buffer KN; Materials and Methods) at two different Mg²⁺ concentrations, 2 and 4.5 mM, and applying multiple turnover kinetics. The wild-type holoenzyme cleaved ptRNAwt with identical rates at 2 and 4.5 mM Mg²⁺ (Table 4). The mutant enzymes showed cleavage rates reduced by a factor of ~2 at 4.5 mM Mg²⁺, but this reduction amounted to 13- and 24-fold at 2 mM Mg²⁺. The pronounced activity drop for the mutant holoenzymes when [Mg²⁺] was only slightly lowered from 4.5 to 2 mM, which made it unlikely but did not exclude that a defect in substrate affinity or holoenzyme stability owing to the disruption of the CCA contact might be responsible for this effect. To address the latter possibilities, we tested processing activity under the same conditions but at a fivefold higher holoenzyme concentration (Table 4, lower part). If substrate binding or holoenzyme stability were substantially impaired in reactions catalyzed by the C292/3 mutant enzymes, one would have expected at least a partial activity rescue for the mutant enzymes at this higher enzyme concentration, which, however, was not observed (Table 4).

We then considered the possibility that the C292/3 mutant RNAs have an altered affinity for the protein subunit. This possibility was addressed by testing the activity of holoenzymes reconstituted at increasing excess amounts of RNase P protein over RNase P RNA. However, the dependence of cleavage rate on RNase P protein concentration showed a very similar saturation behavior for the wild-type and mutant RNase P RNAs, since in all cases activity leveled off above a protein:RNA ratio of 5:1 (Fig. 6). Likewise, the combination of RNase P RNA C293 and substrate ptRNA_{G74} resulted in essentially the same activity profile as for wild-type RNase P RNA acting on ptRNAwt (Fig. 6).

Analysis of RNase P RNA folding equilibria

At low Mg²⁺ concentrations, such as 2 mM, RNase P RNA folding may be incomplete (Zarrinkar et al. 1996) and particularly sensitive to structural alterations. To test the possibility that differences in RNA folding might have contributed to the in vivo and in vitro phenotypes of the C292 and C293 mutant RNase P RNAs, we performed a native PAGE analysis using the buffer systems applied in our kinetic analyses. To our surprise, the folding equilibria of wild-type, C292, and C293 RNase P RNA displayed clear differences. Without a preincubation step (Fig. 7, lanes 1-3), two major bands appeared, which are interpreted as folding intermediates (I₁ and I₂). For the wild-type RNA, formation of the faster migrating intermediate was favored relative to the two mutant RNAs.

TABLE 3. Activity of mutant *E. coli* RNase P holoenzymes purified from strain DW2

RNase P RNA	substrate	[Mg ²⁺] (mM)	<i>k</i> _{obs} (min ⁻¹)	<i>k</i> _{rel}
Wild type	ptRNA wild type	2	(15 ± 0.03) × 10 ⁻³	25
		10	0.37 ± 0.03	
Wild type	ptRNA G74	2	n.d.	
		10	0.21 ± 0.02	
Wild type	ptRNA G75	2	n.d.	
		10	0.047 ± 0.007	
C292	ptRNA wild type	2	(17 ± 3) × 10 ⁻⁵	647
		10	0.11 ± 0.02	
C292	ptRNA G74	2	n.d.	
		10	0.31 ± 0.02	
C292	ptRNA G75	2	(14.5 ± 1.5) × 10 ⁻⁴	97
		10	0.14 ± 0.004	
C293	ptRNA wild type	2	(47 ± 1) × 10 ⁻⁵	809
		10	0.38 ± 0.04	
C293	ptRNA G74	2	(17 ± 1) × 10 ⁻³	25
		10	0.42 ± 0.04	
C293	ptRNA G75	2	n.d.	
		10	0.13 ± 0.01	

Processing was assayed in buffer A: 50 mM Tris-HCl, 100 mM NH₄Cl, 6 mM DTT, 2 or 10 mM Mg(OAc)₂ (pH 7.5), 37°C; assays contained 0.004 A₂₆₀ units of partially purified holoenzymes and trace amounts (<1 nM) of 5'-end-labeled substrates. n.d. indicates no cleavage product detectable after 5 h of incubation; for further details, see Materials and Methods; *k*_{rel} is the ratio of *k*_{obs} values at 10 versus 2 mM Mg²⁺; *k*_{obs} values are based on at least three independent experiments.

In buffers A10 and KN4.5 combined with electrophoresis at 10 mM Mg²⁺, a preincubation step at 37°C (Fig. 7, lanes 4–9, A10 and KN4.5) led to formation of a more compact species assumed to represent a native-like conformation (termed N₁), in accordance with a previous study (Buck et al. 2005). This conformer was in equilibrium with conformer I₂, and the protein subunit did not influence this folding equilibrium (Fig. 7, lanes 4–6 versus 7–9). Here, the pattern was essentially identical for the wild-type and C293 mutant RNA, whereas the C292 RNA was still in equilibrium with the I₁ conformer (Fig. 7, lanes 5,8). An additional temperature step at 55°C led to almost complete disappearance of the I₁ conformer, thus largely eliminating the difference between the C292 and wild-type/C293 RNAs (Fig. 7, lanes 10–12).

In buffer KN2 combined with electrophoresis at 2 mM Mg²⁺, a band migrating slightly faster than the I₂ conformer appeared upon preincubation at 37°C or 55°C/37°C (Fig. 7, best visible in lane 1 versus lane 4, KN2), which we have termed N₂. This band migrated closer to conformer I₂ than did conformer N₁, but N₂ appeared on gels containing 2 mM Mg²⁺ and N₁ on

those supplemented with 10 mM Mg²⁺. Thus, it remains unclear if N₁ and N₂ represent identical or different conformers. Under KN2 conditions, all three RNAs mainly populated the N₂ fold, although gel migration of the C293 RNA was more smeary than that of wild-type and C292 RNA, and some I₁ conformer was still visible for the C292 RNA (Fig. 7, lanes 5,8). Upon additional preincubation at 55°C (Fig. 7, lanes 10–12), the differences between the wild-type and mutant RNAs were essentially eliminated.

In buffer A2 combined with electrophoresis at 2 mM Mg²⁺, the N₂ conformer formed only partially with wild-type RNA (Fig. 7, lanes 7,10, A2), and apparently not at all with the mutant RNAs. This suggests that the KN2 buffer containing spermine and spermidine favors formation of a native fold relative to the A2 buffer. When the native PAGE analysis was conducted with 5'- instead of 3'-end-labeled RNase P RNA, results were identical to those shown in Figure

7 (data not shown).

Although the structural differences between the conformers termed I₁, I₂, N₁, and N₂ remain to be characterized, the data in Figure 7 illustrate that the folding equilibria of wild-type and C292/293 mutant RNase P RNAs may differ depending on the particular buffer and

TABLE 4. Cleavage rates for *E. coli* wild-type and mutant RNase P holoenzymes at low Mg²⁺ concentrations

RNase P RNA	Enzyme concentration	[Mg ²⁺]	<i>k</i> _{obs}	activity decrease
Wild type	10 nM P RNA/50 nM RnpA	4.5	11.2 ± 0.7	—
C292	10 nM P RNA/50 nM RnpA	4.5	4.8 ± 0.1	2.33
C293	10 nM P RNA/50 nM RnpA	4.5	6.0 ± 0.6	1.87
Wild type	10 nM P RNA/50 nM RnpA	2.0	12.0 ± 1.4	—
C292	10 nM P RNA/50 nM RnpA	2.0	0.5 ± 0.06	24.0
C293	10 nM P RNA/50 nM RnpA	2.0	0.9 ± 0.04	13.3
Wild type	50 nM P RNA/250 nM RnpA	2.0	21.9 ± 2.5	—
C292	50 nM P RNA/250 nM RnpA	2.0	0.6 ± 0.06	36.5
C293	50 nM P RNA/250 nM RnpA	2.0	0.6 ± 0.07	36.5

Processing was assayed in buffer KN: 20 mM Hepes (pH 7.4, 37°C), 2 or 4.5 mM Mg(OAc)₂, 150 mM NH₄OAc, 2 mM spermidine, 0.05 mM spermine, 4 mM β-mercaptoethanol, and RNase P RNA (P RNA) and *E. coli* RNase P protein (RnpA) concentrations as indicated; the ptRNAwt concentration was 100 nM; *k*_{obs} is given as nmol substrate converted per nmol of RNase P RNA per min; values are based on at least four independent experiments; activity decrease is defined as the ratio of *k*_{obs} obtained with wild-type versus mutant holoenzyme under the respective conditions.

FIGURE 6. Activity of wild-type and mutant holoenzymes reconstituted at different RNase P protein/RNA ratios and acting on ptRNAwt; in addition, the activity profile for cleavage of ptRNA_{G74} by the C293 mutant holoenzyme is shown. Assay conditions were 20 mM Hepes (pH 7.4, 37°C), 2 mM Mg(OAc)₂, 150 mM NH₄OAc, 2 mM spermidine, 0.05 mM spermine, and 4 mM β-mercaptoethanol. RNase P RNA concentration was kept constant at 10 nM, and substrate concentrations were 100 nM; k_{obs} is given as nmol substrate converted per nmol of RNase P RNA per min; values are based on at least four independent experiments. The inset is a magnification of the lowest activity curves. P RNA, *E. coli* RNase P RNA.

preincubation conditions. It thus remains a possibility that the folding properties of the two mutant RNase P RNAs contributed to their defect in vivo. Unlike a recent study (Buck et al. 2005), we did not observe major effects of the protein component on RNase P RNA folding equilibria. This may be related to the fact that stabilization of the RNA fold by the protein was analyzed at <1 mM Mg²⁺ (Buck et al. 2005).

DISCUSSION

We have assessed the role of the CCA interaction in vivo by complementation analyses in two RNase P mutant strains. In both strains (DW2 and BW), *rnpBC292* and *rnpBC293* alleles encoding P RNA variants with a disrupted CCA interaction impaired or abrogated cell vitality (Tables 1, 2). Particularly, the phenotypes in strain BW (Table 2) demonstrate that base-pairing of G292 and G293 with C₇₄ and C₇₅ of RNase P substrates is essential in vivo, and its disruption is not compensated by the presence of the RNase P protein subunit in holoenzyme complexes. The BW strain also reconciles in vivo phenotype with kinetic data obtained in vitro: RNase P RNA C292 was more defective than the C293 variant in RNA alone (Busch et al. 2000) as well as in holoenzyme reactions using ptRNAwt as the substrate (Table 4; Fig. 6). In agreement, concomitant *rnpA* overexpression slightly improved viability of BW cells expressing *rnpBC293*, but not *rnpBC292* (Table 2B).

The molecular basis of increased viability owing to *rnpA* overexpression in BW bacteria expressing *rnpBC293* is not

fully clear yet. We think it likely that higher levels of RNase P protein lead to increased steady-state levels of holoenzyme, either by shifting the equilibrium between free RNase P subunits and holoenzyme toward holoenzyme or by a mechanism that couples RNase P RNA and protein expression, according to which increased RNase P protein levels would entail a corresponding increase in the level of the RNA subunit. The latter possibility has been discussed in the context of a study on RNase P RNA metabolism in *E. coli* (Kim et al. 2005). *E. coli rnpB* primary transcripts can undergo either 3'-end maturation or oligoadenylation resulting in degradation. A knockout of poly(A) polymerase (*pcnB*), which blocks the degradation pathway, thus increased the level of 3'-precursor RNase P RNA. However, this was not associated with a concomitant increase in the level of mature RNase P RNA, leading to the proposal that mature RNase P RNA may only stably accumulate when complexed with its protein subunit (Kim et al. 2005).

Previous in vitro studies, focusing on the *E. coli* RNA-alone reaction, have indicated that the CCA contact increases substrate affinity (Hardt et al. 1995; Oh et al. 1998;

FIGURE 7. Analysis of folding equilibria for wild-type and C292/C293 mutant *E. coli* RNase P RNAs by native PAGE. RNAs (50 fmol, 3' end-labeled) were preincubated either in buffer A containing 2 mM (A2) or 10 mM (A10) Mg²⁺, or in buffer KN containing 2 mM (KN2) or 4.5 mM (KN4.5) Mg²⁺ in a volume of 4–5 μL (for buffer A and KN compositions, see Materials and Methods). (Lanes 1–3) No preincubation (kept at 4°C); (lanes 4–6) preincubation of RNase P RNAs for 70 min at 37°C; (lanes 7–9) preincubation of RNase P RNAs for 55 min at 37°C, addition of 1 μL *E. coli* C5 protein (final concentration 50 nM) and further incubation for 15 min at 37°C; (lanes 10–12) as in lanes 7–9, but preincubation of RNase P RNAs for 5 min at 55°C and 50 min 37°C. Samples were run on 11.25% polyacrylamide gels in 1× THE buffer supplemented with 100 mM NH₄OAc and either 2 mM (A2, KN2) or 10 mM (A10, KN4.5) MgCl₂. The dashed lines confining lanes 4–6 in panel A2 indicate that these lanes were taken from a different gel.

Busch et al. 2000) and supports selection of the correct cleavage site (Kirsebom and Svärd 1994) as well as catalysis (Oh et al. 1998; Busch et al. 2000; Brännvall et al. 2003). Based on the data presented here, we infer that disruption of the CCA interaction primarily causes a defect in the binding of catalytically important Mg^{2+} under in vivo conditions, based on the following argumentation: The activity of mutant holoenzymes, either assembled in vivo (Table 3) or in vitro (Table 4), was reduced only about two- to three-fold relative to the wild-type holoenzyme at Mg^{2+} concentrations of 4.5–10 mM. However, a shift from 10 to 2 mM Mg^{2+} reduced the rate of ptRNA^{wt} cleavage 25-fold for the wild-type holoenzyme, but 650- and 810-fold for the C292 and C293 mutant enzymes (Table 3). We are aware that these rate differences may include contributions from deviating folding properties of the mutant RNAs in buffer A containing 2 mM Mg^{2+} (Fig. 7, panel A2). Since folding equilibria of the wild-type and mutant RNase P RNAs were much more similar in the KN buffer system (Fig. 7), the results shown in Table 4 are thus likely to be most meaningful. Nevertheless, a similar trend was observed: The shift from 4.5 to 2.0 mM Mg^{2+} had a negligible effect on cleavage by the wild-type enzyme but reduced the cleavage rate 13- and 24-fold for the C293 and C292 mutant enzymes, respectively (Table 4). Such pronounced effects on cleavage rate in response to a shift from 4.5 to 2 mM Mg^{2+} are hard to explain by reasons other than a catalytic defect, although contributions from decreased substrate affinity or reduced affinity of the protein subunit cannot be excluded. The latter possibility might be suggested by the finding that the RNase P protein–RNA complex is stabilized by addition of substrate in the *Bacillus subtilis* system (Day-Storms et al. 2004). To test the possibility of decreased substrate affinity as the cause of low mutant enzyme activity at 2 mM Mg^{2+} , we increased the holoenzyme concentration by a factor of 5 (Table 4). However, this only increased the cleavage rate for the wild-type enzyme but not for the mutant holoenzymes, suggesting that decreased substrate affinity owing to disruption of the CCA contact is not a major cause for the defect of the mutant holoenzymes. Likewise, in the RNase P protein titration experiment (Fig. 6), activity essentially saturated at a protein/RNA ratio of 5:1 for the wild-type and mutant holoenzymes acting on ptRNA^{wt}, thus providing no evidence for a major protein binding defect of the mutant RNase P RNAs. Finally, this is in line with the fact that we enriched the wild-type and mutant holoenzymes with comparable yields from cell extracts by anion exchange chromatography (Table 3).

Another potential defect of the C292 and C293 mutant RNAs in vivo could be aberrant folding leading to increased degradation. However, the mutant enzymes partially purified from strain DW2 had similar activities as the wild-type enzyme at 10 mM Mg^{2+} (Table 3). The same amounts of extract based on absorption at 260 nm were used in these processing assays, providing no evidence for altered RNase

P levels in bacteria expressing *rnpBC292* and *rnpBC293*. Likewise, wild-type-like maturation of plasmid-encoded 4.5S RNA by the C293 holoenzyme when base-pairing is restored by a compensatory mutation in the 3' end of this substrate (Fig. 4) argues against substantial degradation of the mutant RNA.

On the basis of the data shown in Figure 7, it remains a possibility that differences in the folding equilibria of the wild-type versus mutant RNase P RNAs may have contributed to the mutant defects in vivo. The sensitivity of RNA folding toward point mutations in loop L15 might also provide a clue as to why the relative phenotypes of the C292 and C293 mutant RNAs deviated in strain DW2 grown at 43°C compared with strain BW grown at 37°C. In any case, the results of Figure 7 point to the sensitivity of RNase P RNA folding to structural changes as small as point mutations, particularly in low Mg^{2+} buffers. This seems surprising in view of the highly dynamic nature of the loop L15 region, as inferred from biochemical experiments (Ciesiolka et al. 1994; Brännvall et al. 2003) and evident in the crystal structure of *Thermotoga maritima* RNase P RNA (Torres-Larios et al. 2005). On the other hand, the dynamic nature of this region might increase its propensity to interfere with folding of other structural elements in the molecule. It will be interesting to see if mutations in other RNase P RNA elements may be able to exert similarly pronounced effects on folding at low Mg^{2+} concentrations.

The mutant holoenzymes cleaved the ptRNA^{wt} substrate predominantly at the canonical site (Fig. 5), which also pertained to conditions of 2 mM Mg^{2+} (data not shown). Only the C292 enzyme showed some increase in miscleavage (<5%) (Fig. 5). A low extent of miscleavage is consistent with a previous study (Oh et al. 1998) analyzing processing of *B. subtilis* ptRNA^{Asp} with CCA mutations by the *E. coli* wild-type holoenzyme. Oh et al. (1998) did not observe aberrant cleavage under any set of conditions. The ptRNA^{Asp}, as the ptRNA^{Gly} used here, forms a canonical 7-bp acceptor stem and lacks any potential for extended acceptor stem base-pairing involving nucleotide –1/+73. Miscleavage in vivo may yet be increased for some ptRNAs, such as *E. coli* pSu1, which can form a C-1:G73 base pair (Svärd et al. 1996). In vitro processing of the latter substrate by reconstituted *E. coli* mutant holoenzymes gave substantial miscleavage (27% for the C293, 55% for the C292 variant) (Svärd et al. 1996). Thus, substantial aberrant cleavage of some cellular ptRNAs cannot be excluded and might contribute to the mutant phenotypes, particularly to that of *rnpB292* in strain BW (Table 2).

Our results rationalize some previous in vitro data. For example, *E. coli* RNase P-catalyzed cleavage of ptRNA substrates with single nucleotide substitutions in CCA or a CCA deletion suggested that the protein subunit suppresses the binding defect for catalytic Mg^{2+} (Oh et al. 1998). However, these experiments were done at 100 mM

Mg²⁺, conditions that mask the defect, as illustrated by the sharp activity differences we obtained at 2 versus 4.5 mM Mg²⁺ (Table 4).

The P15/L15/P16 region is a central Me²⁺ binding module in the active site of RNase P RNA (Kufel and Kirsebom, 1998; Altman and Kirsebom 1999; Brännvall et al. 2003). Binding of tRNA CCA ends suppresses, in vitro and in vivo, prominent Pb²⁺ hydrolysis at two locations in the L15 loop (sites III and V) and creates a new prominent Pb²⁺ hydrolysis site (IVb) nearby (Ciesiolka et al. 1994; Lindell et al. 2005). This indicates a structural rearrangement of the P15/L15/P16 region upon formation of the G292-C₇₅ and G293-C₇₄ intermolecular base pairs. The P15/L15/P16 module is also predicted to undergo a substantial conformational rearrangement upon substrate docking to the active site based on the recent crystal structure of *T. maritima* RNase P RNA (Torres-Larios et al. 2005). These observations combined with our findings presented here accentuate the need to include enzyme-substrate complexes in future structural analyses of RNase P in order to understand the catalytic mechanism of this ribozyme. Finally, our study suggests that activity tests of bacterial RNase P holoenzymes are better performed at Mg²⁺ concentrations <10 mM in order to be able to draw conclusions that are biologically relevant.

MATERIALS AND METHODS

Bacteria

E. coli strains DW2/pDW160 (Waugh and Pace 1990) and BW (this study) were used for complementation studies.

Complementation studies in strain DW2

For strain DW2/pDW160 (named DW2 in the following), complementing *rnpB* genes were under control of the native *E. coli rnpB* promoter and cloned in plasmid pSP64 as described (Hardt and Hartmann 1996). The pSP64 derivative carrying *E. coli rnpB* (pSP64 *E. coli rnpB*) served as template to introduce the point mutations C292 or C293 by the site-directed DpnI method according to the manual provided with the QuikChange XL Site-Directed mutagenesis Kit (Stratagene), using the primer pair 5'-GTA CTG AAC CCG CGT AGG CTG CTT GAGC and 5'-GCT CAA GCA GCC TAC GCG GGT TCA GTA C for the C292 mutation, and 5'-GTA CTG AAC CCG GCT AGG CTG CTT GAG C and 5'-GCT CAA GCA GCC TAG CCG GGT TCA GTA C for the C293 mutation (sites of mutation underlined). Recombinant *rnpB*-coding pSP64 derivatives (10 ng) were introduced into DW2 by electroporation, using a Biorad GenePulser (1.8 kV; 5 msec, 50 μ F, 100 Ω). After the pulse, 1 mL SOC medium (per liter: 20 g peptone, 5 g yeast extract, 0.6 g NaCl, 0.17 g KCl, 20 mM glucose, 10 mM MgCl₂, 10 mM MgSO₄ at pH 7.5) was added, and cells were shaken for 1 h at 30°C. Appropriate dilutions of cell suspensions were plated on two LB agar plates, containing 100 μ g/mL ampicillin and 30 μ g/mL chloramphenicol. The two sets of plates were incubated in parallel at 30°C or 43°C, respectively, for 20–40 h.

Construction of *rnpB* mutant strain BW

Construction of strain BW followed the strategy described by Roux et al. (2005). The so-called catRExBAD cassette (Fig. 2A) was amplified from strain TG1 cat-AraC (Roux et al. 2005) using primers 135 (5'-CTT CAG CGT ATT GAC CAG CAT AGG TAC GTT GGA CGA AGC ATT CCG CGG GCT TGC ATA ATG TGC CTG TC; the underlined sequence is identical to the region upstream of the *E. coli rnpB* promoter) and 134 (5'-GTC TCC CCC GAA GAG GAC GAC GAC GAA GCG GCG ACT GTC TGG TCA GCT TCT ATG GAG AAA CAG TAG AGA GTT GC; the underlined sequence is complementary to *E. coli rnpB*, corresponding to nucleotides 1–50 of mature RNase P RNA). Initially, we could isolate a recombinant strain, which had the 5' end of the described catRExBAD cassette successfully recombined within the 5'-*rnpB* promoter upstream region, but the 3' boundary was altered. This strain was used to amplify a 326-bp fragment, containing the 5' boundary, with primers 5'-*rnpB* ext (5'-CGA TTG GTG TCG CAA ACG TGG) and 3'-catRExBADveri (5'-CGC AAG GCG ACA AGG TGC TG). The *E. coli rnpB* gene was amplified from *E. coli* K12 with primers 3'-*rnpB* (5'-AGG TGA AAC TGA CCG ATA AGC C) and 5'-*rnpB* (5'-GAA GCT GAC CAG ACA GTC GC). This *rnpB* fragment and the 326-bp fragment with *E. coli rnpB* upstream sequence were combined with the catRExBAD cassette by overlapping PCR. The resulting PCR product was purified by agarose gel electrophoresis and transformed into *E. coli* MG1655 bacteria carrying pKD46 (temperature-sensitive helper plasmid expressing the λ red recombinase system) (Datsenko and Wanner 2000). For this purpose, MG1655/pKD46 cells were grown at 28°C to an OD₅₇₈ of 0.15, followed by adjusting cultures to 10 mM arabinose to induce the λ red genes. Induced bacteria were then further grown to an OD₅₇₈ of 0.6 and made electro-competent by 100-fold concentration and three washing steps with ice-cold 10% glycerol. Electroporation was performed with a Biorad Gene Pulser in 1-mm cuvettes (1.8 kV, 200 μ F, 25 Ω) using 40 μ L competent cells and 100–200 ng purified PCR fragment. Pulsed cells were added to 1 mL SOC medium supplemented with 10 mM arabinose, shaken for 1 h at 37°C and 900 rpm (Eppendorf Thermomixer comfort), and then plated and incubated at 37°C on LB agar plates containing 25 μ g/mL chloramphenicol and 10 mM arabinose. Clones were analyzed by PCR for correct 5' and 3' boundaries of the integrated catRExBAD cassette and screened for ampicillin sensitivity to test for loss of the temperature-sensitive helper plasmid pKD46. The following primers were used to scrutinize correct insertion and sequence of the integrated cassette:

primer 195 (3' AraC): 5'-GCA TGG AAG CGA TTA ACG AGT CGC

primer 194 (5' AraC): 5'-GCG ACT CGT TAA TCG CTT CCA TGC

primer 193 (5' *E. coli rnpB* promoter -58): 5'-CAC GTA ATA TCG CCG CGA CAC TGG

primer 158 (ext veri 3' *rnpB*): 5'-GTG AAA CTG ACC GAT AAG CCG C

primer 157 (5' ext veri *rnpB*): 5'-TGC AGG AGC TGC GGG TGG

primer 136 (RExBADveri 5'): 5'-CGG CGT CAC ACT TTG CTA TGC C

primer 138 (catBADveri3'): 5'-CGC AAG GCG ACA AGG TGC TG

primer 49 (ecRevT3): 5'-CTC ACT GGC TCA AGC AGC CT
 primer 186 (Ecm1 up +350): 5'-CAT TCG CGT CGC TTG
 TGA TGT C

primer 183 (Ecm1+350 3'): 5'-CGC CGG AAC GGT TTA TTA
 CGT AC

Integrity of the 5' boundary was checked by primer combinations 157/138 and 138/186, and that of the 3' boundary by the combinations 136/158 and 136/183; the absence of the native *rnpB* promoter region was verified with the primer pair 193/49, which produced a PCR fragment with genomic DNA from strain MG1655 (the original strain), but not with DNA from correct recombinants. Primer pairs 194/49 and 195/157 were used to amplify the inserted cassette in roughly two halves (upstream and downstream from *araC*) (see Fig. 2A) for verification by sequencing. Verified recombinant bacteria were named strain BW.

Construction of the low copy plasmid pACYC177 *E. coli rnpB*

To construct the derivatives of low copy plasmid pACYC177 encoding *E. coli rnpBwt*, *rnpBC292*, and *rnpBC293*, plasmids pSP64 *E. coli rnpBwt*, *rnpBC292*, or *rnpBC293* were digested with *NheI* and *HindIII* and the fragments carrying the different *E. coli rnpB* alleles were inserted into pACYC177 cut with *HindIII* and *NheI*.

Complementation studies in strain BW

Derivatives of pACYC177 or pBR322 were introduced into strain BW by electroporation as described for DW2 bacteria. However, after the pulse, 1 mL SOC medium supplemented with 10 mM arabinose was added and cells were incubated for 1 h at 37°C; cells were twice pelleted by centrifugation and resuspended in 1 mL LB medium. After the second washing step, cells were plated in appropriate dilutions on LB agar plates containing 100 µg/mL ampicillin, 25 µg/mL chloramphenicol, and either 10 mM arabinose or 0.5% (w/v) glucose; 10 µg/mL tetracycline was added if BW bacteria additionally harbored pBR322 derivatives expressing *E. coli rnpA* or mutant 4.5S RNA; 1 mM IPTG was further added for induction of plasmid-encoded *rnpA*. For growth curve measurements, an overnight culture grown at 37°C in LB medium containing 10 mM arabinose and respective antibiotics (see above) was washed twice as described above, and then 50 mL volumes of LB were inoculated to an OD₅₇₈ of 0.05–0.1, either supplemented with 10 mM arabinose or 0.5% glucose, followed by growth curve monitoring at 37°C and 180 rpm (Incubator GFL 3033).

Construction of pBR322 derivatives for expression of *E. coli rnpA*

To put *E. coli rnpA* under the control of the inducible pTrc-promoter (Amann et al. 1988), the *rnpA* gene was amplified with primers 5'-AGA CCA TGG TTA AGC TCG CAT TTC C (introducing an *NcoI* site, underlined, at the 5' end of *rnpA*) and 5'-ATC TCT AGA GTC AGG ACC CGC GAG CCA (introducing an *XbaI* site, underlined, at the 3' end of *rnpA*). The *E. coli rnpA* fragment was digested with *NcoI* and *XbaI*, and ligated into pTrc99a (Amann et al. 1988) also linearized with *NcoI* and *XbaI*, resulting in plasmid pTrc99a *E. coli rnpA*. This plasmid was unsuitable for our complementation studies, because it encodes an

ampicillin resistance as plasmid pACYC177; also, the high copy number of pTrc99a *E. coli rnpA* would have potentially increased an imbalance between *rnpA* expression and that of *rnpB* genes encoded on the low copy plasmid pACYC177. Therefore, we amplified the *rnpA* gene including the pTrc promoter and the *rrnBt1t2* terminator from pTrc99a *E. coli rnpA*, using the primers 5'-CAC ATG CAT GCA AAA AGG CCA TCC GTC AGG (introducing an *Mph1103 I* site, underlined) and 5'-ACA ATC GAT TCA ATT GCG CCG ACA TCA TAA CGG (introducing a *MunI* site, underlined) and ligated the fragment into pBR322 cut with *PstI* and *EcoRI*. Digestion of pBR322 with *PstI* and *EcoRI* (ends compatible with those of *Mph1103 I* and *MunI*, respectively) destroyed the ampicillin resistance, only leaving the tetracycline resistance.

Construction of pBR322 encoding mutated 4.5S RNAs

The chromosomal region of *E. coli* K12 harboring the bicistronic transcription unit of *ffs* (encoding 4.5S RNA) and *ybaA*, including the native promoter upstream of *ffs* as well as 100 bp downstream from *ybaA*, was amplified and cloned into pBR322. During PCR amplification, we already altered the terminal stem region by inversion of 4 bp within the stem structure (Fig. 4A) for specific reverse transcriptase priming in order to be able to distinguish native from plasmid-encoded 4.5S RNA. The promoter region and the 5' end of *ffs* were amplified using primers 5'-ACA GAA TTC AGC ATA ATC TGG AAA AAC GCC C (introducing an *EcoRI* site, underlined) and 5'-CCA ACA GAC **GGG** CCA TTG AGA GCG TTG AGA ACC (5'-terminal 4.5S RNA stem mutations in bold); the 5' portion of the *ffs* gene was amplified with the primer pair 5'-CTC AAT GGC CCG TCT GTT GGT TCT CCC GCA AC and 5'-TCT CTG CAG TAA CGC GTC ATC TGC CTT GGC (introducing *MluI* and *PstI* sites, underlined). These two fragments were combined by overlap extension PCR, cut with *EcoRI* and *PstI*, followed by ligation into pBR322 cut with the same enzymes. The 3' half of *ffs* was amplified with primer pair 5'-CTC AAT GGC CCG TCT GTT GGT TCT CCC GCA AC and 5'-TGG **CCC** GCC TGC CAG CTA CAT **CCC** GG (3'-terminal stem mutations in bold). The chromosomal region spanning the 3'-terminal 19 bp of *ffs* and the *ybaA* cistron was amplified using forward primer 5'-GCT GGC AGG CGG GCC ACC CAT TTC TGC CTC CCA CCG TTT C and reverse primer 5'-CAC CTG CAG CAA GGC ATT ACC GAA AGA AG. To further introduce point mutations into the 3'-CCC end of 4.5S RNA, the same PCR reaction was performed except that 5'-GCT GGC AGG CGG GCC AGC CAT TTC TGC CTC CCA CCG TTT C (3'-GCC₇₆) or 5'-GCT GGC AGG CGG GCC ACG CAT TTC TGC CTC CCA CCG TTT C (3'-CGC₇₆) was used as the forward primer. The latter three amplification products were combined with the PCR fragment harboring the 3' half of *ffs* (see above) by overlap extension PCR. The resulting product was then cut with *PstI*/*MluI* and ligated into the pBR322 derivative containing the promoter and 5' half of *ffs* and cut with the same restriction enzymes.

In vitro transcription and 5' and 3' endlabeling

Runoff transcription with bacteriophage T7 RNA polymerase as well as 5' and 3' endlabeling were performed essentially as described (Heide et al. 1999; Busch et al. 2000). The p^{trnA}^{Gly} substrate (Fig. 1) was transcribed from plasmid pSBpt3^{HH} (Busch et al. 2000). Mutations G74 and G75 were introduced by

the site-directed DpnI mutagenesis method according to the manual provided with the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene); the primer pair for introduction of the G₇₄ mutation was 5'-CCG TCT CCC GCT GCA GTC ACC GGA TGT GC and 5'-GCA CAT CCG GTG ACT GCA GCG GGA GAC GG; for introduction of the G₇₅ mutation, 5'-CCG TCT CCC GCT CGA GTC ACC GGA TGT GC and 5'-GCA CAT CCG GTG ACT CGA GCG GGA GAC GG were used (sites of mutation underlined). Precursor 4.5S RNA was transcribed from a PCR template under control of the T7 promoter; the PCR template was amplified from plasmid pT7-4.5S (constructed and kindly provided by Norman R. Pace, University of Colorado, Boulder, CO), a derivative of plasmid pT71 (Tabor and Richardson 1985) encoding *E. coli* 4.5S RNA with 23 nt of its natural 5'-flanking sequence and 17 additional 5'-flanking nucleotides; the 4.5S RNA precursor transcript carried an additional AUA at the 3' end in comparison with natural mature 4.5S RNA (Peck-Miller and Altman 1991); PCR primers were 5'-CCA GAA TTC GAA ATT AAT ACG ACT CAC TAT A (T7 promoter underlined) and 5'-TAT GGG TGG GGG CCC TGC CAG CTA. *E. coli* RNase P RNA and C292/3 mutant variants were synthesized as described previously (Busch et al. 2000).

RNA isolation for primer extension analysis

DW2 bacteria harboring derivatives of plasmid pSP64 were grown at 30°C overnight in LB containing 100 µg/mL ampicillin and 30 µg/mL chloramphenicol. On the next morning, the same culture was incubated for 2 h at 43°C, followed by inoculation of fresh medium (100 or 250 mL) to a starting OD₅₇₈ of 0.05–0.1, and further cultivation at 43°C until cell density reached an OD₅₇₈ of ~0.6. BW cells harboring pACYC177 and pBR322 derivatives were grown overnight at 37°C in 3 mL LB medium supplemented with 100 µg/mL ampicillin, 25 µg/mL chloramphenicol, 10 mM arabinose, and 10 µg/mL tetracycline in the case of cells harboring pBR322 derivatives. Cells were then used to inoculate 50 mL of the same medium to a starting OD₅₇₈ of 0.05–0.1, followed by incubation at 37°C. At an OD₅₇₈ of ~0.5, cells were centrifuged for 5 min at 5000 rpm and washed once with 20 mL LB to remove residual arabinose. Cells were resuspended in 100 mL LB supplemented with antibiotics and 0.5% glucose and incubated for another 3 h at 37°C and 180 rpm. Total cellular RNA from strains DW2 and BW was isolated by the TRIzol method (Invitrogen) according to the protocol provided by the manufacturer. After concentration by ethanol precipitation, RNA was further digested with DNase I (Turbo DNase, Ambion) followed by phenol/chloroform (1:1) and chloroform extractions (Busch et al. 2000) and ethanol precipitation in the presence of 0.12 M NaOAc (pH 5.0). RNA was dissolved in double-distilled H₂O, and concentrations were measured in H₂O at 260 nm (1 A₂₆₀ unit = 40 µg).

Primer extension assays

Primer extension assays were performed with total cellular RNA and primers specific to *E. coli* RNase P RNA (5'-CTC ACT GGC TCA AGC AGC CT), *E. coli* wild-type 4.5S RNA (5'-GGA GAA CCA ACA GAG CCC), or *E. coli* mutant 4.5S RNA (5'-GGA GAA CCA ACA GAC GGG). Primer annealing was conducted in 75 mM KCl and 68 mM Tris-HCl (pH 8.3) for ~60 min applying a linear temperature gradient from 90°C to 55°C. Reverse trans-

cription was then performed at 55°C using ThermoScript reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Nucleotide (dNTP) concentrations in primer extension assays were 0.5 mM each. For primer extension assays to verify expression of *E. coli* *rnpBC*292/293 mutant alleles, dGTP was replaced with ddGTP. For the size marker in Figure 3, lane 1, 0.5 mM dGTP and 0.5 mM ddGTP were used.

Partial purification of RNase P from *E. coli* cells

DW2 cells grown at 43°C as described (see RNA Isolation for Primer Extension Analysis) and resuspended in ~4 mL buffer A (60 mM NH₄Cl, 10 mM Mg[OAc]₂, 6 mM DTT, 50 mM Tris-HCl at pH 7.2–7.5) per 600 mg cell pellet were lysed by sonication (Branson Sonifier 250, output 20, duty cycle 30%–40%, 10 min on ice), followed by centrifugation for 45 min at 4°C and 10,000g. For the preparation of DEAE fractions, the supernatant was loaded onto a DEAE fast flow Sepharose column (Aekta basic, Pharmacia). Elution was performed by applying a continuous NH₄Cl gradient from 60–550 mM NH₄Cl. Active fractions eluted at ~300–400 mM NH₄Cl.

Preparation of recombinant RNase P protein

E. coli RNase P protein carrying an N-terminal His-tag (His-tagged peptide leader: MRGSHHHHHHGS, encoded in plasmid pQE-30 in *E. coli* JM109) was expressed essentially as described (Rivera-León et al. 1995). Cell cultures (LB broth containing 100 µg/mL ampicillin) were grown to an OD₅₇₈ of 0.6, and IPTG was added to a final concentration 1 mM. Cells were harvested after 3 h (OD₅₇₈ ~2.5) by centrifugation for 10 min at 5000 rpm at 4°C. The following steps were performed at 4°C or on ice, and all buffers contained 40 µg/mL of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Cells were then resuspended in 10–15 mL sonication buffer SB (50 mM Tris-HCl at pH 8.0, 0.3 M NaCl, 0.1% triton X-100, 1 M NH₄Cl). After sonication (Branson Sonifier 250, output 20, duty cycle 50%, 15 min on ice), the sample was centrifuged for 30 min (4°C, 14,500g), and the supernatant was mixed with Ni-NTA agarose (400 µL for 2 L of cell culture), which had been prewashed twice with 10 mL SB buffer. The sample was incubated for 1–2 h at 4°C under gentle mixing or rotating. The Ni-NTA agarose slurry was washed three times (centrifugation–resuspension cycles; centrifugation at 4°C and 8500 rpm in a desktop centrifuge) with ice-cold washing buffer (30 mM imidazol, 50 mM Tris-HCl at pH 8.0, 8 M urea, 0.1% triton X-100). The RNase P protein started to precipitate during this procedure; therefore, the supernatant after each washing step was removed carefully to avoid release of protein aggregates from the matrix. The protein was then eluted with 500 µL elution buffer (50 mM Tris-HCl at pH 7.0, 10% glycerol, 7 M urea, 20 mM EDTA, 0.3 M imidazol) for 45 min at 4°C under gentle shaking. Eluates were dialyzed twice for 1 h and subsequently overnight against volumes of 500 mL dialysis buffer (50 mM Tris-HCl at pH 7.0, 0.1 M NaCl, 10% glycerol; dialysis bags: Roth, molecular weight cut off 12–14 kDa); during dialysis, a white precipitate forms. The content of the dialysis bags was transferred to a 2-mL Eppendorf tube and centrifuged for 20 min at 4°C and 12,000 rpm in a desktop centrifuge. The supernatant contained RNase P protein devoid of any RNase P RNA contamination, whereas the pellet included traces of RNase P

RNA and was therefore discarded. All purification steps were monitored by 17% SDS-PAGE to assess the purity and concentration of RNase P proteins.

Processing assays

For RNase P-catalyzed processing of *T. thermophilus* ptRNA^{Gly} (ptRNAwt), or G₇₄ or G₇₅ variants thereof, active DEAE fractions (0.25–1.0 μ L; equal amounts [0.004 A₂₆₀ units] based on A₂₆₀ measurements) were incubated in buffer A (2 or 10 mM Mg[OAc]₂, 6 mM DTT, 50 mM Tris-HCl at pH 7.5) containing 100 mM NH₄Cl for 10 min at 37°C in a volume of 16 μ L. Trace amounts (<1 nM) of the 5'-end-labeled ptRNA^{Gly} substrate were preincubated under the same conditions for 5 min at 55°C and 25 min at 37°C. Processing reactions were started by combining enzyme and substrate solutions (final volume, 20 μ L) and assayed at 37°C. The Mg(OAc)₂ concentration was raised to 100 mM in control reactions with *E. coli* RNase P RNA alone. Buffer KN [20 mM Hepes-KOH at pH 7.4, 2 or 4.5 mM Mg(OAc)₂, 150 mM NH₄OAc, 2 mM spermidine, 0.05 mM spermine, and 4 mM β -mercaptoethanol] (Dinos et al. 2004), optimized for functional analyses of *E. coli* ribosomes, was used to closely mimic physiological conditions. In vitro reconstitution of RNase P holoenzymes was performed as follows: RNase P RNAs were incubated in buffer KN for 5 min at 55°C and 50 min at 37°C, after which RNase P protein was added, followed by another 5 min at 37°C before addition of substrate.

Folding analysis by native PAGE

We incubated 50 fmol of *E. coli* wild-type or C292/C293 mutant RNase P RNA, including trace amounts of 5'- or 3'-end-labeled material of the same RNA, in buffer A (100 mM NH₄Cl, 6 mM DTT, 50 mM Tris-HCl at pH 7.2–7.5, and 2 or 10 mM Mg[OAc]₂) or buffer KN (20 mM Hepes-KOH at pH 7.4, 150 mM NH₄OAc, 2 mM spermidine, 0.05 mM spermine, 4 mM β -mercaptoethanol, and 2 or 4.5 mM Mg[OAc]₂) in a volume of 5 μ L or 4 μ L if protein was added thereafter. Buffer conditions were the same as those used for the kinetic experiments. RNase P RNAs were either kept at 4°C (no preincubation) or preincubated for 55 min at 37°C, or 5 min at 55°C followed by 50 min at 37°C. We also tested a preincubation protocol starting with 2 min at 85°C, followed by 5 min at 55°C and 50 min at 37°C. The results were, however, identical to those obtained with 5 min at 55°C plus 50 min at 37°C. For samples including the protein subunit, 1 μ L of *E. coli* C5 protein was added (final concentration, 50 nM) after the preincubation step. All samples, except for those kept at 4°C, were then incubated for another 15 min at 37°C and finally adjusted to room temperature. Immediately before gel loading, 1 volume of loading buffer (10% [v/v] glycerol, 2 mM MgCl₂, xylene cyanol, bromphenol blue) was added. The nondenaturing 11.25% (v/v) polyacrylamide gels (thickness <1 mm) contained 66 mM Hepes, 33 mM Tris (pH 7.4), and 0.1 mM EDTA (1 $\times$ THE), supplemented with 100 mM NH₄OAc and 2 or 10 mM MgCl₂. Gels were run at 180–250 V until xylene cyanol had migrated 11.5 cm from the top. The gel temperature was controlled not to exceed 15°C. The RNA bands were visualized with a Bio-Imaging Analyzer (FLA 3000–2R, Fujifilm). Results were the same for 5'- and 3'-end-labeled RNase P RNAs. End homogeneity of RNase P RNAs was checked by denaturing PAGE. Results for RNase P

RNAs preincubated in buffer KN containing 4.5 mM MgCl₂ were the same if gels were run in the presence of 4.5 or 10 mM MgCl₂ (data not shown).

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