In vivo and in vitro investigation of bacterial type B RNase P interaction with tRNA 3'-CCA

Barbara Wegscheid and Roland K. Hartmann*

Institut für Pharmazeutische Chemie, Philipps-Universität Marburg, Marbacher Weg 6, D-35037 Marburg, Germany

Received November 6, 2006; Revised December 22, 2006; Accepted December 22, 2006

ABSTRACT

For catalysis by bacterial type B RNase P, the importance of a specific interaction with p(recursor)tRNA 3'-CCA termini is yet unclear. We show that mutation of one of the two G residues assumed to interact with 3'-CCA in type B RNase P RNAs inhibits cell growth, but cell viability is at least partially restored at increased RNase P levels due to RNase P protein overexpression. The in vivo defects of the mutant enzymes correlated with an enzyme defect at low Mg$^{2+}$ in vitro. For Bacillus subtilis RNase P, an isosteric C259-G74 bp fully and a C258-G75 bp slightly rescued catalytic proficiency, demonstrating Watson–Crick base pairing to tRNA 3'-CCA but also emphasizing the importance of the base identity of the 5'-proximal G residue (G258). We infer the defect of the mutant enzymes to primarily lie in the recruitment of catalytically relevant Mg$^{2+}$, with a possible contribution from altered RNA folding. Although with reduced efficiency, B. subtilis RNase P is able to cleave CCA-less ptRNAs in vitro and in vivo. We conclude that the observed in vivo defects upon disruption of the CCA interaction are either due to a global deceleration in ptRNA maturation or severe inhibition of 5'-maturation for a ptRNA subset.

INTRODUCTION

Ribonuclease P (RNase P), an essential ribonucleoprotein enzyme, catalyzes the 5'-end maturation of tRNAs in all Kingdoms of life (1,2). The bacterial RNase P holoenzyme consists of an RNA subunit (P RNA) of approximately 400 nt and a small basic protein (P protein) of approximately 13 kDa. In vitro, RNA subunits of bacterial RNase P enzymes are catalytically active in the absence of the protein subunit (3), but the protein is essential in vivo (4,5). Two major architectural subtypes of bacterial P RNA are known, type A (for ancestral, prototype Escherichia coli) and type B (represented by Bacillus subtilis) (6), each containing several secondary structural elements not present in the other. A major difference between type A and B structures is the L15 loop of the catalytic domain, which is an internal one in type A but apical in type B RNAs (Figure 1A–C). For E. coli P RNA, the model system of type A structures, it has been shown that two conserved G residues (G293 and G292) in L15 form Watson–Crick base pairs with the two cytosines of tRNA 3'-CCA (C74 and C75) termini (8). Several in vitro studies focussing on the E. coli RNA-alone reaction have indicated that the CCA contact increases substrate affinity (9–11) and supports selection of the correct cleavage site (8,12) as well as catalysis (10,11,13). Recently, disruption of this interaction was demonstrated to be lethal for E. coli cells (14). Analysis of processing of 4.5S RNA, a non-tRNA substrate of RNase P in E. coli, revealed that restoration of base pairing by an isosteric C293-G74 base pair, but not a C292-G75 pair, fully rescued catalytic performance in vivo. In vitro activity assays of E. coli wild type (wt) and C292/C293 mutant RNase P holoenzymes, at magnesium concentrations as low as 2 mM Mg$^{2+}$ suggested a defect in the recruitment of catalytically active Mg$^{2+}$ ions as the major cause for the lethal phenotype of the mutant P RNAs (14).

Type B RNase P RNAs, represented by those from B. subtilis and Staphylococcus aureus, also encode a conserved GG dinucleotide in their L15 loop (Figure 1A and B, G258/G259 in B. subtilis and G238/G239 in S. aureus P RNA). Photo-crosslinking of E. coli and B. subtilis P RNAs to tRNAs with photoreactive groups at the 3'-end has indicated that in enzyme–substrate complexes of both P RNA types the tRNA 3'-CCA terminus is positioned close to the L15 loop region (15). G258 and G259 of B. subtilis P RNA were further shown to be protected from kethoxal modification upon complexation with mature tRNA or precursor tRNA (ptRNA), and this protection was reduced with ptRNA lacking 3'-CCA (16). Potential base pairing to 3'-CCA was addressed by testing cleavage-site selection in L15 mutants of two type A RNAs (from E. coli and Mycobacterium tuberculosis) and
Figure 1. Secondary structure illustrations of (A) Bacillus subtilis (type B), (B) Staphylococcus aureus (type B) and (C) Escherichia coli (type A) RNase P RNA according to (7). The two G residues in L15, known (E. coli) or suspected (B. subtilis, S. aureus) to be involved in the interaction with tRNA 3'-CCA, are highlighted by gray ovals. (D) Proposed interaction of a canonical bacterial ptRNA (Thermus thermophilus ptRNAGly) with the L15 loop of B. subtilis RNase P RNA. Highlighted nucleotides mark the sites of mutation investigated in this study. The arrow indicates the canonical RNase P cleavage site (between nucleotide –1 and +1).
of the type B RNA from Mycoplasma hyopneumoniae. With M. hyopneumoniae P RNA, some of the observed mutational effects were consistent with base pairing to 3'-CCA, but the results were not as conclusive as for the type A RNAs (12).

The physiological role of a CCA contact has remained unclear in view of the observation that B. subtilis P RNA, in contrast to its E. coli counterpart, is rather insensitive to 3'-CCA mutations in terms of cleavage-site selection (17). Furthermore, the P protein of B. subtilis RNase P increases the affinity for the ptRNA more than 10^4-fold (18,19), raising the question whether a contribution of the CCA interaction to substrate affinity of type B enzymes would be significant. Also, the Michaelis constant K_m in multiple turnover reactions of the B. subtilis RNase P holoenzyme remained unaffected by the absence of CCA (15).

Furthermore, one-third of tRNAs (27 out of 86) in B. subtilis do not have the CCA motif encoded by their genes. RNase Z removes the 3'-trailers of such CCA-less tRNA primary transcripts by endonucleolytic cleavage 3' of the discriminator nucleotide as a prerequisite for CCA addition by tRNA nucleotidyl-transferase (CCase); since RNase Z is blocked by the presence of 3'-CCA, it acts specifically on tRNA transcripts lacking the 3'-CCA motif (20). However, B. subtilis RNase Z activity inversely correlates with the length of tRNA 5' extensions, which led to the proposal that processing by RNase P precedes that of RNase Z in the case of tRNA transcripts with larger 5' extensions (>30 nt) (20). This would suggest that in vivo B. subtilis RNase P might be able to process certain substrates in the absence of a 3'-CCA moiety.

Here we have analyzed the role of the CCA interaction by mutational analysis in vitro and in vivo in type B bacteria. We demonstrate that B. subtilis rnpBC258 and rnpBC259 mutant alleles carrying a G to C258 or G to C259 mutation in L15 are lethal in the B. subtilis rnpB mutant strain SSB318. We also included a second type B RNA from S. aureus in our complementation study. Corresponding S. aureus rnpBC238 and rnpBC239 mutant alleles also decreased cell viability of B. subtilis SSB318 cells, although not being lethal. Even though the growth defects could be (partially) rescued by parallel overexpression of the B. subtilis P protein, we found no evidence for defects of the mutant P RNAs in P protein or substrate binding. Rather, processing assays with in vitro assembled holoenzymes revealed a defect of the C258/C259 mutant enzymes at free Mg^{2+} concentrations assumed to be close to those in vivo. We infer that the defect lies primarily in the recruitment of catalytically important Mg^{2+}. An isosteric C259-G74 base pair fully and a C258-G75 pair slightly restored catalytic performance in vitro, which supports the notion that G258/G259 form Watson–Crick base pairs with ptRNA 3'-CCA, but also indicates that the base identity of G258 is important for enzyme function. This is remarkably similar to what has been found for E. coli RNase P (8,11,12,14). Native PAGE experiments further unveiled some differences in RNA-folding equilibria between the wt and mutant P RNAs at low [Mg^{2+}], but folding remained unaffected by the presence of the P protein. This raises the possibility that a folding defect of the C258/C259 mutant RNAs in vivo contributed to their phenotype. We finally addressed the question if RNase P can act on CCA-less ptRNAs in B. subtilis by 5'-RACE experiments in B. subtilis SSB320, an RNase Z-deficient strain that accumulates ptRNA transcripts with CCA-less 3'-extensions. We could demonstrate 5'-end maturation of such ptRNAs by RNase P in vivo, documenting that the presence of 3'-CCA is not an absolute requirement for catalysis by B. subtilis RNase P. We conclude that the in vivo defects of type B rnpB mutant alleles result from a global deterioration of tRNA 5'-end maturation; alternatively, a disruption of the CCA interaction may affect a subset of tRNAs more severely than the majority of tRNAs.

MATERIALS AND METHODS

Bacteria

Bacillus subtilis strain SSB318 (17) was used for complementation studies and SSB320 (20) for 5'-RACE experiments.

Complementation studies in strain SSB318

Recombinant rnpB-coding pHY300 derivatives were introduced into SSB318 using the LS/HS-medium protocol as described (17). Residual IPTG was removed by centrifugation (1 min, 7000 g) and resuspension of the cells in LB medium. Cells were plated in parallel on LB agar plates with or without 1 mM IPTG and containing 0.5 μg/ml erythromycin, 12.5 μg/ml lincomycin and 30 μg/ml tetracycline (to select for the presence of pHY300). In cases of poor transformation efficiency, cells were first plated exclusively in the presence of IPTG. For plasmids encoding rnpB or rnpA genes under control of the P_xyl promoter, the medium was additionally supplemented with 2% (w/v) glucose or 2% (w/v) xylose. For the construction of complementation plasmids, see Supplementary Data.

Growth curve monitoring

Bacillus subtilis SSB318 cells were grown overnight at 37°C in the presence of the appropriate antibiotics and 1 mM IPTG. IPTG was then washed out by repeated centrifugation and resuspension of the cell pellet in LB without IPTG; the final cell pellet was resuspended in LB, adjusted to a starting OD578 of 0.05–0.1 in 50 ml LB supplemented with the respective antibiotics and grown at 37°C under shaking.

In vitro transcription and 5'-endlabeling

T7 runoff transcription and 5'-endlabeling was performed as described (11). For transcription of ptRNA_Gly and G74 and G75 variants thereof as well as E. coli P RNAs, see (14). For transcription of B. subtilis wt and mutant P RNAs, as well as other mutant substrates (Table 5), see Supplementary Data.
Preparation of recombinant RNase P protein

Bacillus subtilis RNase P protein carrying an N-terminal His-tag (His-tagged peptide leader: MRRGSHHHHHHGS, encoded in plasmid pQE-30 in E. coli JM109) was prepared as described for the E. coli RNase P protein (14).

Processing assays

RNase P holoenzyme kinetics were performed in buffer F (2 or 10 mM MgCl₂, 50 mM Mes pH 6.1, 100 mM KCl) or buffer KN (20 mM HEPES-KOH, 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) (21). In vitro reconstitution of RNase P holoenzymes was performed as follows: P RNAs were incubated in the respective buffer 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. Processing reactions were started by combining enzyme and substrate solutions (final volume 10 µl) and assayed at 37°C.

Folding analysis by native PAGE

Folding analyses were conducted exactly as described (14).

5'-RACE

SSB320 cells were grown in parallel either in the presence or absence of IPTG to an OD₅₇₈ of 0.6. Total RNA was isolated using the TRIzol (Invitrogen) method (here we did not use the RNeasy Mini/Midi Kit from Qiagen employed below, because its cutoff of about 200 nt would have resulted in a substantial loss of smaller RNAs, such as the tRNA transcripts of interest in this experiment), followed by DNase I treatment (Turbo DNase, Ambion). In order to include primary transcripts, one half of total RNA was treated with tobacco acid pyrophosphatase (TAP, Epicentre). Afterwards, the adapter oligonucleotide A1 (5'-GTC AGC AAT CCC TAA GGA G; the three 5'-terminal residues were RNA, the remainder DNA) was ligated to the 5'-monophosphates of RNA molecules. Specific primers were designed to map 3'-unprocessed transcripts of trnSL-Ala1 (primer 180, 5'-TGG GTA TTA AAG GTA TGG AG) and trnSL-Val2 (primer 178, 5'-CTG CGC GTG GTA TGG AG) (5'-endlabeled in Figure S3). These primers were used in cDNA synthesis and in combination with primer 5'-GTC AGC AAT CCC TAA GGA G (matches the sequence introduced by the adapter oligonucleotide) in the following PCR reaction. PCR products were identified by native 8% PAGE, gel-purified and cloned into pCR2.1-TOPO for sequencing. Five clones were sequenced for each tRNA. For the ptRNA²⁺⁻ control (Figure 5, lanes 4-6), the reverse transcriptase primer was 5'-GAG TGG AGC GGG AGA CGG, yielding an RT-PCR product with an expected length of 112 bp.

Overexpression of rnpA—effect on P RNA levels

To determine rnpB expression levels in strain SSB318 complemented with S. aureus or B. subtilis rnpBwt or mutant alleles as a function of B. subtilis rnpA overexpression, cells were grown overnight at 37°C in 3 ml LB medium supplemented with 1 mM IPTG, 12.5 µg/ml lincomycin, 0.5 µg/ml erythromycin and 30 µg/ml tetracycline. IPTG was then washed out by repeated centrifugation/resuspension of the cell pellet in LB without IPTG; the final cell pellet was then resuspended in LB, and adjusted to a starting OD₅₇₈ of 0.5–0.1 in 50 ml LB; antibiotics (see above) and 2% xylose (w/v) were added and cells were grown at 37°C under aeration to an OD₅₇₈ of 0.5–0.6. Aliquots of the cell suspensions were then diluted and plated in parallel on four plates (+xylose and +IPTG; +xylose and −IPTG; +glucose and +IPTG; +glucose and −IPTG) supplemented with antibiotics (all concentrations as in liquid media) to verify the original growth phenotype (retarded growth with mutant rnpB alleles in the absence of Pₓyl rnpA, Table 2). Total RNA from the cultures was prepared using the RNeasy Mini/Midi Kit from Qiagen according to the protocol provided by the manufacturer, followed by DNase I treatment (Turbo DNase, Ambion). RT-PCR was performed with the Access RT-PCR System (Promega). PCR was stopped after 12 cycles within the exponential phase of amplification. Primers specific for S. aureus rnpB were 5'-GGG TAA TCG CTA TAT TAT ATA GAG G and combined with either 5'-ATT TGG ATT GCT CAC TCG AGG G (5'-endlabeled in Figure 3) or 5'-CTA GTA GTG ATA TTT CTA TAA GCC ATG (5'-endlabeled in Figure S1); primers specific for B. subtilis rnpB were 5'-CTT AAC GTT CGG GTA ATC GC and 5'-AAAG TGG TTG AAC GTT CCG TAA GCC (5'-endlabeled in Figure S2); primers specific for B. subtilis ribosomal protein S18 were 5'-GCA GAG GCC GTC GTG CGA AA and 5'-ACG TCG GCC TTT GAT CGC TGC A (5'-endlabeled in Figures 3, S1 and S2); RT-PCR reactions contained the normal amounts of unlabeled primers, and, in addition, trace amounts of the respective 5'-endlabeled primer.

RESULTS

Complementation studies in B. subtilis

The B. subtilis rnpB mutant strain SSB318 was employed to investigate the in vivo role of the tRNA 3'CCA interaction with type B RNase P RNA. In strain SSB318, the chromosomal rnpB gene is under the control of an IPTG-inducible Pₛₚₛₚₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛₚₛ₢
Table 1. Homologous complementation of B. subtilis RNase P mutant strain SSB318 by B. subtilis rnpB wild type and mutant alleles

| rnpB variants in pHY300 | IPTG | IPTG Aldose |
|------------------------|------|-------------|
| B. subtilis P_Bs rnpB rnpBwt | + + + + + | None |
| B. subtilis P_Bs rnpB rnpBC258 | a | None |
| B. subtilis P_Sa rnpB rnpBC259 | a | None |
| B. subtilis P_Sa rnpB rnpBC258 + P_Sa rnpA | + + + + + b Xylose | glucose |
| B. subtilis P_Sa rnpBC258 | a | None |
| B. subtilis P_Sa rnpBC259 | + + + + Xylose | |
| B. subtilis P_Sa rnpBC258 + P_Sa rnpA | + + + + + + Xylose | |
| B. subtilis P_Sa rnpBC258 + P_Sa rnpA-stop | + + + + + Xylose | |
| B. subtilis P_Sa rnpBC258 + P_Sa rnpA-stop | + + + + + Xylose | |
| B. subtilis P_Sa rnpBC258 + P_Sa rnpA-stop | + + + + + Xylose | |
| B. subtilis P_Sa rnpBC258 | + + + + Xylose | |
| pHY300 | + + + + Xylose | |
| pHY300 + P_Sa rnpA | + + + + Xylose | |
| pHY300 + P_Sa rnpA-stop | + + + + Xylose | |

Growth of mutant strain SSB318 transformed with wild-type B. subtilis rnpB (rnpBwt) and mutant (rnpBC258, rnpBC259) alleles on plasmid pHY300; promoter types: P_Bs rnpB, native B. subtilis rnpB promoter; P_Sa rnpBC258, inducible xylose promoter; B. subtilis rnpBC259 was overexpressed in parallel from the same plasmid; rnpA-stop designates the B. subtilis rnpA gene with two stop codons in the 5'-coding region. Cell growth was analyzed on LB plates (with appropriate antibiotics) in the presence (1 mM) or absence of IPTG; an aldose (xylose or glucose) at 2% (w/v) was added where indicated.

a Unable to clone.
b Basal expression of xylose promoter (in the presence of 2% glucose) is sufficient for cell growth in the absence of IPTG.
c Basal expression of xylose promoter (in the presence of 2% glucose) is not sufficient for cell growth in the absence of IPTG.

Growth of mutant strain SSB318 transformed with wild-type B. subtilis rnpB (rnpBwt) and respective mutant alleles on plasmid pHY300; promoter types: P_Bs rnpB, native B. subtilis rnpB promoter; P_Sa rnpBC258, inducible xylose promoter; B. subtilis rnpA was overexpressed in parallel from the same plasmid; rnpA-stop designates the B. subtilis rnpA gene with two stop codons in the 5'-coding region. Cell growth was analyzed on LB plates (with appropriate antibiotics) in the presence (1 mM) or absence of IPTG; an aldose (xylose or glucose) at 2% (w/v) was added where indicated.

a Unable to clone.
b Basal expression of xylose promoter (in the presence of 2% glucose) is sufficient for cell growth in the absence of IPTG.
c Basal expression of xylose promoter (in the presence of 2% glucose) is not sufficient for cell growth in the absence of IPTG.

Heterologous type B RNA from S. aureus was included in our complementation analysis to broaden the general significance of our findings on the CCA interaction of type B RNase P RNAs. Residues G238 and G239 in S. aureus P RNA correspond to G258 and G259, respectively.

Simultaneous overexpression of B. subtilis rnpA

We recently reported increased viability of E. coli cells expressing the E. coli rnpBC293 mutant gene when simultaneously overexpressing the homologous P protein (14). We thus transformed B. subtilis SSB318 cells with plasmids encoding the B. subtilis rnpA gene and either rnpBwt, rnpBC258 or rnpBC259, with both rnpA and rnpB alleles under control of the xylose promoter. In the presence of xylose, conditions which fully induced rnpA expression, cell viability in the absence of IPTG could indeed be substantially improved for cells expressing rnpBC258 or rnpBC259. For rnpBC259, basal expression of the P_Sa promoter (in the absence of xylose) supported retarded growth of SSB318 cells in the absence of IPTG. In contrast, for rnpBC258 full induction of the xylose promoter was required, with growth still somewhat retarded relative to rnpBwt and rnpBC259 (Table 1). In conclusion, P protein overexpression restored growth of bacteria expressing the rnpB mutant alleles, but fitness did not fully reach that of bacteria expressing the rnpBwt allele. To demonstrate that rnpA-related rescue effects were due to higher expression levels of the B. subtilis P protein and not to unspecific effects caused by the presence of additional copies of the rnpA gene, we also tested complementation with an rnpA gene with two stop codons in its 5'-portion (rnpA-stop), thus directing expression of a truncated inactive protein (5). As expected, rnpA-stop expression was unable to restore growth of SSB318 bacteria expressing rnpBC258 or rnpBC259 (Table 1). Our findings document a lethal phenotype associated with point mutations at G258 and G239 in L15 of B. subtilis P RNA. However, concomitant overexpression of the P protein (also verified by Western-blot analysis, data not shown) largely rescued cell viability. Furthermore, the phenotype was more severe for the C258 than C259 mutant RNA.

Table 2. Heterologous complementation of B. subtilis RNase P mutant strain SSB318 by S. aureus (type B) rnpB alleles.

| rnpB variants in pHY300 | IPTG | IPTG Aldose |
|------------------------|------|-------------|
| S. aureus P_Bs rnpB rnpBwt | + + + + | None |
| S. aureus P_Bs rnpB rnpBC258 | + + + + | None |
| S. aureus P_Bs rnpB rnpBC259 | + + + + | None |
| S. aureus P_Bs rnpB rnpBC258 + P_Sa rnpA | + + + + Xylose | |
| S. aureus P_Bs rnpB rnpBC259 + P_Sa rnpA | + + + + Xylose | |

Growth of mutant strain SSB318 transformed with wild-type S. aureus (rnpBwt) and respective mutant alleles on plasmid pHY300; promoter types: P_Bs rnpB, native B. subtilis rnpB promoter; P_Sa rnpBC258, inducible xylose promoter; B. subtilis rnpBC259 was overexpressed in parallel from the same plasmid. Cell growth was analyzed on LB plates (with appropriate antibiotics) in the presence (1 mM) or absence of IPTG; the aldose xylose was added to 2% (w/v) for pHY300 derivatives encoding B. subtilis rnpA. + + +: growth with equal numbers of colonies on the corresponding + and − IPTG plates; +: retarded cell growth, but equal numbers of colonies on + and − IPTG plates.
of *B. subtilis* P RNA (Figure 1A and B). Whereas *S. aureus rnpB*wt was fully functional in *B. subtilis* SS318, the mutant alleles *rnpBC238/C239* resulted in a significant growth defect, though not being lethal (Table 2). This was also confirmed by monitoring growth curves in liquid culture (Figure 2). Here, the phenotypes of *rnpBC238* and *rnpBC239* were very similar, thus deviating from the observation that the *B. subtilis rnpBC258* allele was more deleterious than *rnpBC259* (see above). The growth defect caused by the *S. aureus rnpBC238/C239* mutant alleles could be completely rescued by overexpression of *B. subtilis rnpA* (Table 2).

**Complementation with *E. coli* P RNA**

We have recently shown that *E. coli* P RNA (type A) can functionally replace *B. subtilis* P RNA (type B) in vivo, no matter if *E. coli rnpB*wt was expressed from its own native or from the *B. subtilis rnpB* promoter, and even a single copy of *E. coli rnpB*wt integrated into the *amyE* site of the *B. subtilis* chromosome was sufficient for cell viability (17). Since the *S. aureus rnpBC238/C239* mutant alleles displayed a somewhat relaxed phenotype relative to *B. subtilis rnpBC258/C259*, we also tested function of the corresponding *E. coli* mutant alleles (*rnpB*C292/C293) in the *B. subtilis* background of strain SS318. The *E. coli* mutant alleles were unable to restore growth under non-permissive conditions (without IPTG, Table S1). Even when co-expressed with *B. subtilis rnpB* (in the presence of IPTG), the *E. coli rnpBC292/C293* mutant alleles impaired cell growth; overexpression of *B. subtilis rnpA* did not rescue this growth defect (Table S1).

**Overexpression of rnpA—effect on P RNA levels**

The complementation results shown in Tables 1 and 2 revealed a rescue of the *B. subtilis rnpBC258/C259* and *S. aureus rnpBC238/C239* mutant phenotypes when the *B. subtilis* P protein was overexpressed. One explanation may be that higher levels of P protein increase the steady-state level of RNase P holoenzyme, thereby alleviating the processing defects caused by the mutant alleles. To test this possibility, we initially used SS318 bacteria expressing the *S. aureus rnpB* mutant alleles, because here the rescue by *rnpA* overexpression was complete, reducing the risk that spontaneous mutant bacteria might have dominated the liquid cultures. PCR reactions were limited to 12 cycles, because under these conditions product yields were essentially linearly dependent on RNA template amounts (Figure 3, lanes 25–30). The mRNA for ribosomal protein S18 served as the control to be able to assess experimental fluctuations. Overexpression of *rnpA* caused, on average, a 3- to 5-fold increase in the yields for the P RNA-specific product (based on five individual experiments), independent of whether bacteria expressed the *rnpB*wt or one of the mutant alleles (Figure 3, IPTG, Table S1).
lanes 1–12). No increase in the S18-specific RT-PCR product was observed upon overexpression of rnpA (lanes 13–24). Essentially the same results were obtained when the RT-PCR analysis was performed with a primer pair covering the 5'- and 3'-terminal regions of S. aureus P RNA in order to detect full-length P RNAs (Figure S1). This finding ruled out the possibility that partially degraded P RNA molecules might have contributed to the RT-PCR products in Figure 3. We also analyzed the levels of full-length B. subtilis P RNA as a function of rnpA overexpression. For the B. subtilis rnpBC258/C259 alleles, we could not include the control experiment (without rnpA overexpression), as SSB318 bacteria expressing these alleles did not grow under non-permissive conditions (−IPTG; Table 1); also, growth was impaired for rnpBC258, despite rnpA overexpression (Table 1), and this defect was even more pronounced in liquid culture (data not shown). For the above reasons, we compared P RNA levels for B. subtilis wt P RNA in the absence of a plasmid-borne rnpA gene with those for wt and C259 P RNAs in the presence of overexpressed P protein. As for S. aureus P RNA, we detected increased B. subtilis P RNA levels due to rnpA overexpression (Figure S2); intensity of the RT-PCR product for the C259 mutant RNA was reproducibly found to be somewhat lower than the signal for the wt P RNA, suggesting that the C259 mutation (and, by inference, the C258 mutation) may result in somewhat decreased P RNA levels. Overall, our RT-PCR results demonstrate that overexpression of the P protein causes an increase in the steady-state levels of P RNA, and by inference of the RNase P holoenzyme, which explains the observed rescue effects (Tables 1 and 2).

**In vitro processing of ptRNA by B. subtilis RNase P holoenzymes**

To shed light on the nature of the in vivo defect of the C258/C259 mutant P RNAs, we analyzed the kinetics of B. subtilis RNase P holoenzymes assembled in vitro. The rescue effect observed when the B. subtilis P protein was overexpressed (Table 1) pointed, among several possibilities, to a protein affinity defect of the mutant P RNAs. We thus tested the activity of B. subtilis wt and mutant holoenzymes as a function of P protein concentration. We applied a buffer system which has been used for B. subtilis holoenzyme kinetics, containing 10 mM MgCl₂, 50 mM Mes pH 6.1 and 100 mM KCl (referred to as buffer F1; adapted from (22)). Surprisingly, activities were indistinguishable for the wt and mutant holoenzymes at all P protein concentrations (Figure S3). Now the two mutant holoenzymes were more than 100-fold less active than wt RNase P (Table 3) when acting on ptRNA with a canonical 3'-CCA terminus (termed ptRNAwt in the following). However, combining the C259 enzyme with the mutant substrate ptRNAG74, restoring base pairing to the C259 P RNA, resulted in cleavage efficiencies

![Figure 3](https://www.nature.com/nar/article-assets/35/6/2060/fig3a.png)

**Figure 3.** Radioactive reverse transcription PCR (RT-PCR) analysis of strain SSB318 complemented with S. aureus rnpB wt or rnpBC238/C239. PCR products were analyzed on a 10% polyacrylamide/8 M urea gel. Lanes 1–30: total RNA from SSB318 complemented with S. aureus rnpBwt (lanes 1–12), or rnpBC238 (lanes 5–8, 17–20 and 25–30) grown at 37°C in the absence of IPTG and in the presence of 2% xylose (w/v); amounts of total RNA were 200 ng in lanes 1–24, 26 and 29, 100 ng in lanes 25 and 28, and 400 ng in lanes 27 and 30. P xyl rnpA: presence (+) or absence (−) of a xylose-inducible plasmid-encoded B. subtilis rnpA gene. Lanes 1–12 and 25–27: primers specific for S. aureus rnpB (rnpB); lanes 13–24 and 28–30: primers specific for the mRNA encoding B. subtilis ribosomal protein S18 (S18). AMV: presence (+) or absence (−) of reverse transcriptase. For details on RT-PCR, see the Material and Methods section. Lanes 25–30 document that the amount of RT-PCR product was sensitive to RNA template concentration. The figure illustrates a representative experiment, but the results shown here were reproduced in five individual experiments using three independent total RNA preparations.
Table 3. Cleavage activity of *B. subtilis* wt and mutant RNase P holoenzymes acting on ptRNA<sup> Gly</sup> wt and G74 or G75 mutant substrates at 2 mM Mg<sup>2+</sup> and two protein concentrations

| P RNA | ptRNA<sup>Gly</sup> | B. subtilis | k<sub>obs</sub> | k<sub>rel</sub> |
|-------|----------------|-------------|----------------|-------------|
| B. subtilis wt | wt | 37.5 | 0.18 ± 0.03 | 1.0 |
| B. subtilis wt | wt | 62.5 | 0.20 ± 0.04 | 1.1 |
| B. subtilis C258 wt | wt | 37.5 | (4.0 ± 0.2) x 10<sup>-4</sup> | 2.2 x 10<sup>-3</sup> |
| B. subtilis C258 wt | wt | 62.5 | (1.3 ± 0.5) x 10<sup>-3</sup> | 7.2 x 10<sup>-3</sup> |
| B. subtilis wt | C259 | 37.5 | (5.0 ± 1.0) x 10<sup>-4</sup> | 2.8 x 10<sup>-3</sup> |
| B. subtilis wt | C259 | 62.5 | (1.6 ± 0.9) x 10<sup>-3</sup> | 8.9 x 10<sup>-3</sup> |
| B. subtilis wt | G74 | 37.5 | (7.0 ± 1.0) x 10<sup>-4</sup> | 3.9 x 10<sup>-3</sup> |
| B. subtilis wt | G74 | 62.5 | (1.3 ± 0.3) x 10<sup>-3</sup> | 7.2 x 10<sup>-3</sup> |
| B. subtilis C258 wt | G74 | 37.5 | (2.0 ± 1.0) x 10<sup>-4</sup> | 1.1 x 10<sup>-3</sup> |
| B. subtilis C258 wt | G74 | 62.5 | (3.0 ± 2.0) x 10<sup>-4</sup> | 1.7 x 10<sup>-3</sup> |
| B. subtilis wt | C259 G74 | 37.5 | 0.15 ± 0.006 | 0.8 |
| B. subtilis wt | C259 G74 | 62.5 | 0.30 ± 0.04 | 1.7 |
| B. subtilis wt | C259 G75 | 37.5 | (3.0 ± 1.0) x 10<sup>-4</sup> | 7.2 x 10<sup>-3</sup> |
| B. subtilis wt | C259 G75 | 62.5 | (4.0 ± 3.0) x 10<sup>-4</sup> | 2.2 x 10<sup>-3</sup> |
| B. subtilis C258 wt | C259 G75 | 37.5 | (6.0 ± 4.0) x 10<sup>-4</sup> | 3.3 x 10<sup>-3</sup> |
| B. subtilis C258 wt | C259 G75 | 62.5 | (1.8 ± 0.2) x 10<sup>-3</sup> | 1.0 x 10<sup>-2</sup> |
| B. subtilis C259 wt | C259 G75 | 37.5 | (1.4 ± 0.8) x 10<sup>-4</sup> | 6.0 x 10<sup>-3</sup> |
| B. subtilis C259 wt | C259 G75 | 62.5 | (5.0 ± 1.1) x 10<sup>-4</sup> | 2.8 x 10<sup>-3</sup> |

Assay conditions: 50 mM Mes pH 6.1, 100 mM KCl, 2 mM MgCl<sub>2</sub>; P RNA concentration was 20 nM; protein concentration as indicated and substrate concentration was 200 nM; 5'-endlabeled substrate was added in trace amounts (<1 nM). k<sub>obs</sub> is given in pmol substrate converted per pmol of P RNA per min; for k<sub>rel</sub>, individual k<sub>obs</sub> values were divided by k<sub>obs</sub> for the wt P RNA at 37.5 nM P protein and acting on ptRNA wt (first line); values are based on at least three independent experiments.

In *vitro* processing of ptRNA by *B. subtilis* holoenzymes in a buffer assumed to closely mimic intracellular conditions

We recently showed (14) for *E. coli* P RNA that folding of the catalytic RNA may be incomplete in low Mg<sup>2+</sup> buffers, such as buffer F2 (Table 3). However, a buffer originally optimized for ribosome function (buffer KN, see Material and Methods) and containing spermine and spermidine appears to favor formation of a native fold (14). We therefore tested activity of the wt and mutant *B. subtilis* holoenzymes in buffer KN at two different Mg<sup>2+</sup> concentrations (2 and 4.5 mM, KN2 and KN4.5). At 4.5 mM Mg<sup>2+</sup>, the mutant enzymes cleaved ptRNA wt at almost identical (C259) or 2-fold reduced (C258) rates. However, at 2 mM Mg<sup>2+</sup>, the rate dropped 2-fold for the wt enzyme, but about 6-fold for the mutant enzymes (Table 4). Thus, the minor shift from 4.5 to 2 mM Mg<sup>2+</sup> exacerbated the defect of the mutant enzymes. To analyze if the defect was on the level of substrate affinity or holoenzyme stability, we tested processing activity under the same conditions but at a 5-fold higher holoenzyme concentration (Table 4). If substrate binding or holoenzyme stability were the major defect in reactions catalyzed by the C258/C259 mutant enzymes at 2 mM Mg<sup>2+</sup>, one would have expected that the ratio of cleavage rates for the mutant enzyme versus wt enzymes (k<sub>rel</sub>, Table 4) improves at the higher enzyme concentration. However, k<sub>rel</sub> values rather showed a tendency to further decrease (0.12 and 0.13 versus 0.17 and 0.30, Table 4).

We also analyzed cleavage of ptRNA<sup>Gly</sup> by the C259 mutant enzyme in buffer KN2 (Table 4). This fully restored the cleavage rate to that of the wt enzyme, in line with the results of Table 3.

Activity assays performed with chimeric holoenzymes consisting of *E. coli* P RNA (wt and the corresponding C292/C293 mutant variants) and the *B. subtilis* P protein (Table S2) resulted in essentially the same findings as with the *B. subtilis* holoenzymes.

Analysis of *B. subtilis* P RNA folding equilibria

Incomplete (24) or aberrant folding might have contributed to the *in vivo* and *in vitro* phenotypes of the
C258/C259 mutant P RNAs. To address this possibility, we performed a native PAGE analysis using the same buffer systems as for the kinetic experiments. When 5′-endlabeled wt, C258 or C259 P RNAs were directly loaded (after gel purification and ethanol precipitation) onto the native gel without a preincubation step, under all four tested conditions two major bands appeared, which we termed I1 and I2 (Figure 4, lanes 1–3). We are aware that I1 and I2 observed in the presence of 2 mM Mg²⁺ during electrophoresis may not necessarily be identical to those seen at 10 mM Mg²⁺. Upon P RNA preincubation for 55 min at 37°C (37°C) or for 5 min at 55°C followed by 50 min at 37°C (55/37°C), a faster migrating conformer appeared, which we assign to the native fold (termed ‘N’), in line with a previous study (19). Remarkably, in buffer F2 (2 mM Mg²⁺) the N conformer was much less populated with the two mutant than with the wt P RNA (Figure 4, lanes 4–12). This difference was largely reduced in buffer KN2. When preincubation was performed in buffers F10 and KN4.5 (10 and 4.5 mM Mg²⁺, respectively) and native PAGE at 10 mM Mg²⁺, differences between wt and mutant P RNAs were further mitigated (lanes 4–9) and essentially abolished after preincubation at 55/37°C (lanes 10–12). No substantial changes were seen when the P protein was added to the preincubation mix (lanes 4–6 versus 7–9). In summary, our native PAGE analysis indicates that (i) the KN2 buffer containing spermine and spermidine favors formation of the native fold relative to the F2 buffer and (ii) folding differences can be considered insignificant in buffer KN4.5 combined with our standard preincubation (5 min at 55°C, 50 min at 37°C). The data in Figure 4 illustrate that the folding equilibria of wt and C258/C259 mutant RNase P RNAs are sensitive to the buffer conditions. As we do not know the exact ionic milieu in vivo, a contribution from impaired folding of the mutant P RNAs to their growth defect in vivo cannot be excluded.

Processing of CCA-less ptRNAs

Since in B. subtilis only two-thirds of the tRNA genes encode 3′-CCA, the question arose whether tRNA transcripts not encoding 3′-CCA have to undergo 3′-end processing and CCA addition before 5′-end maturation by RNase P can occur. To address this question, we constructed ptRNA Gly 3′-variants, either encoding 3′-U73CCAUA, 3′-U73UAAUA or ending with the discriminator nucleotide (3′-U73¼/C1CCA); UAA instead of CCA downstream of the discriminator base is found in the native B. subtilis trnI-Thr precursor (20). Processing of these substrates by wt RNase P was tested in buffer KN, either at 4.5 or 2 mM Mg²⁺, and for 2 mM Mg²⁺ also at a 5-fold higher P RNA and P protein concentration (Table 5). The obtained pattern of relative cleavage rates roughly mirror-imaged what we had seen with the C258/C259 mutant enzymes in Table 4: (i) the cleavage rate was reduced for the UAA and ΔCCA variant, and the ratio of rates for the mutant versus canonical substrate worsened when the Mg²⁺ concentration was reduced from 4.5 to 2 mM; (ii) a 5-fold increase in P RNA and protein concentration did not improve performance of the mutant...
substrates relative to the canonical ptRNA (Table 5), suggesting that substrate affinity is not the major cause of the defect.

5'-RACE experiment of trnSL-Ala1 and trnSL-Val2

In vitro processing of the CCA-less substrates was impaired but not abolished, raising the question whether this residual activity would suffice for RNase P cleavage of CCA-less tRNA transcripts in vivo. This was addressed by 5'-RACE experiments. For this purpose, we used strain SSB320, in which the chromosomal rnz gene (encoding RNase Z) is put under control of the IPTG-inducible Pspac promoter. In B. subtilis, RNase Z is involved in the 3'-maturation of tRNA primary transcripts lacking the CCA motif. We chose two native tRNAs (trnSL-Ala1 and trnSL-Val2), which were shown to accumulate as 3'-precursors in strain SSB320 when rnz expression is decreased (20). Both tRNAs are transcribed from monocistronic genes with expected 5'-precursor segments of 8 nt (trnSL-Ala1) and 38 nt (trnSL-Val2), and both are 3'-extended by their transcription terminator stem-loop (Figure 5A) (20). To ensure that only tRNAs with unprocessed 3'-ends were reverse transcribed during 5'-RACE, we designed a primer for reverse transcription whose 5'-proximal and central portion targeted the 3'-precursor sequences (Figure 5A). A control experiment performed with RNA from SSB320 cells grown in the presence of IPTG (normal RNase Z expression) did not produce RT-PCR products (Figure 5B, lane 3), demonstrating that our primer was specific for the 3'-extended tRNA. Using total RNA isolated from SSB320 cells grown in the absence of IPTG we obtained a prominent 5'-RACE product, roughly corresponding to the size of 5'-matured 3'-precursor tRNA (ca. 110 nt; Figure 5B, lane 1). An additional treatment with tobacco acid pyrophosphatase (TAP; to permit RT-PCR of primary transcripts with 5'-triphosphates) did not give rise to an additional RT-PCR product (Figure 5B, lane 2). We further controlled for TAP activity by adding a T7 primary transcript of ptRNA^{Gly} (Figure 1D, carrying a 5'-triphosphate) to the total cellular RNA pool (Figure 5B, lanes 5 and 6). In the case of inefficient 5'-end processing we would have expected a product extended by 8 nt for tRNA^{Ala1} (20). To ensure that RT-PCR products corresponded to 5'-matured transcripts, we cloned the main band of Figure 5B (lanes 1 and 2, indicated by the arrow) into the pCR2.1-TOPO vector (Invitrogen) and sequenced 5 clones (the same was done for trnSL-Val2; data not shown). Sequencing analysis confirmed mature 5'-ends for both tRNAs. This was surprising as we would have anticipated, based on our kinetic in vitro results (Table 5), to detect at least some 3'-extended tRNA with immature 5'-ends. In any case, these results clearly demonstrated that RNase P processing can proceed in vivo in the absence of the CCA interaction.

DISCUSSION

In vivo phenotypes of P RNAs with disrupted CCA interaction

We have investigated the tRNA 3'-CCA interaction with type B RNase P under in vivo conditions, using B. subtilis mutant strain SSB318 which has the chromosomal rnpB gene under control of the IPTG-inducible Pspac promoter (17). For this purpose, complementation efficiencies of B. subtilis rnpB mutants relative to rnpBC258/C259 mutant alleles were analyzed in strain SSB318 under non-permissive conditions (−IPTG). The latter two rnpB alleles encode P RNAs with point mutations of the nucleotides thought to be involved in the CCA interaction. We also tested the functionality of S. aureus rnpBwt and its mutant rnpBC238/C239 mutant alleles (as representatives of a heterologous type B P RNA) and that of the corresponding E. coli rnpBC292/C293 alleles representing type A RNase P RNA architecture. All rnpBwt alleles (B. subtilis, S. aureus, E. coli) were functional in SSB318 bacteria (Tables 1, 2 and S1). However, the mutant P RNA alleles resulted either in a severe growth defect (S. aureus rnpBC238/C239; Table 2) or were even lethal (B. subtilis rnpBC258/C259 and E. coli rnpBC292/C293; Tables 1 and S1). Overexpression of the B. subtilis P protein partially (for B. subtilis rnpB alleles) or fully (for S. aureus rnpB alleles) restored cell viability of SSB318 bacteria expressing the mutant rnpB alleles (Tables 1 and 2). The rescue effect was also observed for the E. coli C293 mutant RNA in its natural host (14), but was not scrutinized there. Here we could attribute the rescue to increases in P RNA steady-state levels owing to P protein overexpression (Figures 3, S1 and S2). These findings demonstrate, for the first time, that the CCA interaction is important for the vitality of bacteria encoding a type B RNase P RNA (B. subtilis in this study), but not necessarily essential, as inferred from the complementation results with the mutant rnpB alleles from S. aureus (Table 2). RNase P is
able to cleave CCA-less ptRNAs in vivo, as inferred from 5'-RACE in the RNase Z mutant strain (Figure 5). Since RNase Z, which acts on CCA-less tRNA 3'-precursors, has largely impaired activity on ptRNAs with long 5'-leaders, RNase P cleavage was proposed to precede RNase Z cleavage on CCA-less ptRNAs carrying expanded 5'- in addition to 3'-extensions (20). Our results verify this hypothesis. The finding that B. subtilis RNase P is able to process CCA-less ptRNAs then raises the question why the C258/C259 (or C238/C239) mutations caused a lethal (or severe) phenotype in the presence of normal amounts of P protein (Tables 1 and 2). Most likely, the general deceleration in ptRNA maturation kinetics adds up to a collective defect caused by the incapability of the tRNA biosynthesis machinery to meet the demands of the protein synthesis apparatus. This defect is mitigated when the steady-state amounts of the RNase P holoenzyme increase severalfold as a result of P protein overproduction. It also cannot be ruled out that disruption of the CCA interaction may impair 5'-maturation of a subset of ptRNAs more severely than that of the bulk of ptRNAs.

P protein overexpression leads to increases in P RNA steady-state levels (Figures 3, S1 and S2), and, by inference, to increases in cellular RNase P holoenzyme concentrations. This is remarkable, taking into account that the P protein only covers a small surface area of the RNA subunit (2). In a study on P RNA metabolism in E. coli (25), it was found that rnpB primary transcripts can undergo either 3'-end maturation or oligoadenylation resulting in degradation. A knockout of poly(A) polymerase (pcnB) blocked the degradation pathway and consequently increased the level of 3'-precursor P RNA. Since these higher levels of 3'-precursor P RNA did not entail an increase in the level of mature P RNA, the authors proposed that mature RNase P RNA may only stably accumulate when complexed with its protein subunit (25). Our results (Figures 3, S1 and S2) are consistent with this model, suggesting similar mechanisms to regulate cellular RNase P levels in E. coli and B. subtilis despite fundamentally different P RNA maturation pathways in the two organisms. Whereas the mature 5'- and 3'-ends of B. subtilis P RNA appear to be generated by autolytic processing of precursor P RNA (26), E. coli P RNA primary transcripts are mainly initiated at the mature 5'-end, and 3'-precursor sequences are removed by RNase E followed by exonucleolytic removal of 1 or 2 remaining nucleotides (25,27).

In vitro results

The importance of the CCA interaction in reactions catalyzed by type B RNase P holoenzymes has been
underestimated in previous in vitro analyses (15). We could reconcile these earlier findings when we tested activity upon disruption of the CCA interaction at 10 mM Mg$^{2+}$ (Figure S3), which has been the ‘gold standard’ magnesium concentration for the testing of bacterial holoenzymes. However, the CCA interaction becomes critical in vitro at low Mg$^{2+}$ concentrations, such as 2 mM (Tables 3 to 5), assumed to better mimic the cellular milieu than 10 mM Mg$^{2+}$.

Our kinetic data clearly demonstrate that tRNA 3′-CCA ends form Watson–Crick base pairs with two guanosines in the L15 loop of type B RNase P RNAs. Evidence for the base pairing with C$_{74}$ was first suggested by a mutational study analyzing cleavage fidelity in the reaction catalyzed by the type B RNA from $M$. hyopneumoniae (12). As found for $E$. coli (11,14), an isosteric C259-G$_{74}$ base pair, but not a C258-G$_{75}$ pair, fully restored catalytic performance of type B RNase P to that of the wt holoenzyme acting on ptRNA with a canonical 3′-CCA end (Tables 3 and 4). This indicates that the base identity of the 5′-proximal G residue (G258 in $B$. subtilis) is critical for catalytic performance, as observed for G292 of $E$. coli P RNA (11,14). For $E$. coli, the importance of G292 identity can be attributed to the finding that the N7 of G292 is sensitive to a c7-deaza modification, which was interpreted to indicate hydrogen bonding with the 6-amino group of A258 in the 5′-portion of L15 (28). By inference, G258 might interact in an analogous manner with one of the adenines (A251–A253, Figure 1D) in L15 of $B$. subtilis P RNA.

Toward a better understanding of the defect of holoenzymes with a disrupted CCA interaction at low Mg$^{2+}$, it is instructive to briefly discuss what is known about the metal ion binding properties of the L15 loop. In $E$. coli, the P15/L15/P16 region has been identified as a central Me$^{2+}$ binding module in the active site of P RNA (13,29). There are sites of prominent Pb$^{2+}$ hydrolysis at two locations in the internal L15 loop (sites III and V). Binding of tRNA 3′-CCA suppresses, in vitro and in vivo, Pb$^{2+}$ hydrolysis at sites III and V and creates a new prominent Pb$^{2+}$ hydrolysis site (IVb) nearby (30,31). This indicates a structural rearrangement of the P15/L15/P16 region and a concomitant re-coordination of metal ions upon formation of the G292-C$_{74}$ and G293-C$_{74}$ intermolecular base pairs. For $B$. subtilis P RNA, four out of nine prominent Pb$^{2+}$ hydrolysis sites could also be assigned to its apical L15 loop (32). Combined with the phenotypic similarities of C258/C259 (C292/C293 in $E$. coli) mutations in the $E$. coli and $B$. subtilis systems, this supports the notion that the L15 loop is a central Me$^{2+}$ binding module in the active site of type B RNAs as well.

Based on the results shown in Figure 4, the low activity of mutant holoenzymes at 2 mM Mg$^{2+}$ might have been caused by a P RNA folding defect. However, the results of Table 5 argue against this view. When we deleted or mutated 3′-CCA in the substrate and assayed processing by wt RNase P, results were essentially a mirror image of what we had seen with the mutant enzymes acting on ptRNAwt (cf. Tables 4 and 5): catalytic performance for the mutant substrates relative to ptRNAwt deteriorated upon a reduction of [Mg$^{2+}$] from 4.5 to 2 mM, and the relative catalytic performance for the mutant substrates did not improve at 5-fold higher P RNA and protein concentrations.

The slight reduction in the fraction of correctly folded C258/C259 mutant P RNA molecules relative to the wt P RNA upon a decrease in Mg$^{2+}$ concentration from 4.5 to 2 mM (Figure 4) is nonetheless remarkable. Though unlikely to be the major cause of the catalytic defect of the mutant P RNAs at 2 mM Mg$^{2+}$, this finding is surprising as the L15 loop is thought to be quite unstructured in free P RNA based on its low resolution in the X-ray structure of $B$. stearothermophilus P RNA (33). Although the effect of the two point mutations in L15 on P RNA folding is not understood, this underscores the high sensitivity of P RNA folding to minimal structural changes at low Mg$^{2+}$ concentrations.

Formally, the defect of the mutant P RNAs may also include contributions from impaired P protein and/or substrate affinity. However, at 10 mM Mg$^{2+}$ (buffer F10) and increasing P protein concentrations, the reconstituted $B$. subtilis C258 and C259 mutant holoenzymes had activities indistinguishable from the wt enzyme (Figure S3). Likewise, in buffer KN supplemented with 4.5 mM Mg$^{2+}$ activity reductions for the mutant holoenzymes were marginal. Only at 2 mM Mg$^{2+}$, a substantial loss of activity was seen for the mutant enzymes, which correlated with the severe in vivo defects. Considering that a reduction of the Mg$^{2+}$ concentration from 10 to 2 mM and particularly from 4.5 to 2.0 mM Mg$^{2+}$ means only a minor decrease in ionic strength, we think it unlikely that the major defect of the mutant P RNAs is related to impaired P protein and/or substrate affinity. We rather infer that the L15 loop mutations lower the affinity for catalytically relevant Mg$^{2+}$ ions that either directly contribute to the catalytic process or help to fold the L15–CCA interaction module as part of the active site in E–S complexes.

**Differences between individual RNase P enzymes**

Despite the striking similarities in the CCA interaction of type A and B RNase P RNAs, some details are different. For $E$. coli RNase P, a 5-fold increase in P RNA and protein concentrations did not increase the cleavage rate for the C293 and C292 mutant enzymes at 2 mM Mg$^{2+}$ (14). In contrast, the $B$. subtilis C258 and C259 mutant enzymes cleaved ptRNAwt at a 4-to 7-fold higher rate when P RNA and protein concentrations were increased 5-fold (Table 4). Activity of the chimeric holoenzymes consisting of $E$. coli P RNA and $B$. subtilis P protein also improved 2- to 3-fold at the higher RNase P subunit levels (Table S2). This finding, if not due to differences in the quality of the recombinant $E$. coli and $B$. subtilis P proteins, may point to a specific effect exerted by the $B$. subtilis P protein, possibly related to the observation that binding of P protein and substrate to P RNA are cooperative in the $B$. subtilis system (34).

Also, the in vivo phenotypes of the C258 ($B$. subtilis) and C238 ($S$. aureus) type B mutant P RNAs could be partially ($B$. subtilis; Table 1) or fully ($S$. aureus; Table 2) rescued
by overexpression of \textit{B. subtilis} \textit{rnpA}. However, in the \textit{E. coli} system essentially no rescue was observed for the corresponding \textit{E. coli} C292 mutant P RNA upon overexpression of \textit{E. coli rnpA} (14), suggesting that the guanine identity at this position is more critical in type A versus B enzymes. In this context, the better complementation efficiency of mutant \textit{S. aureus} versus \textit{B. subtilis} \textit{rnpB} alleles was surprising. The \textit{S. aureus rnpB}C238/C239 alleles even enabled some cell growth at normal P protein levels (Table 2). This might be related to the general problem associated with studies of homologous genes especially in \textit{B. subtilis} which is known to possess an efficient recombination machinery. The presence of homologous \textit{rnpB} genes carrying point mutations may activate genetic repair systems, especially if the mutant genes cause stress to the cells. This would explain our severe problems to clone the homologous \textit{B. subtilis} \textit{rnpB}C258/C259 alleles in \textit{SSB318} combined with the high number of wild-type revertants. Finally, the severeness of phenotypes for \textit{B. subtilis} \textit{rnpB} versus \textit{B. subtilis} \textit{rnpB} was exceeded that of \textit{B. subtilis rnpB}C259 and \textit{E. coli rnpB}C293, respectively (Table 1) (14). In contrast, the \textit{in vivo} phenotypes of \textit{S. aureus rnpB}C238 and \textit{rnpB}C239 were indistinguishable (Table 2; Figure 2), which we do not understand at present.

Despite some context dependence of L15 mutations, we can conclude from our findings that the Watson–Crick base-pairing interaction with tRNA 3'-CCA is a common feature of the majority of bacterial type A and type B RNase P enzymes. This interaction is important but not necessarily essential for cellular function.

**SUPPLEMENTARY DATA**

Supplementary Data is available at NAR Online.

**ACKNOWLEDGEMENTS**

We like to thank Ciarán Condon for providing \textit{B. subtilis} strain SSB320, B.M. Fredrik Pettersson and Leif A. Kirsebom for providing genomic DNA of \textit{S. aureus}, Michal Marszalkowski for preparation of recombinant \textit{B. subtilis} RNase P protein and Dagmar K. Willkom for critical reading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (HA 1672/7-4) and the Fonds der Chemischen Industrie Funding to pay the Open Access publication charge was provided by Deutsche Forschungsgemeinschaft (GK 1384).

**Conflict of interest statement:** None declared.

**REFERENCES**

1. Hartmann, E. and Hartmann, R.K. (2003) The enigma of ribonuclease P evolution. \textit{Trends Genet.}, 19, 561–569.
2. Evans, D., Marquez,S.M. and Pace,N.R. (2006) RNase P: interface of the RNA and protein components. \textit{Trends Biochem. Sci.}, 31, 333–341.
3. Guerrier-Takada,C., Gardiner,K., Marsh,T., Pace,N. and Altman,S. (1983) The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. \textit{Cell}, 35, 849–857.
4. Schell,P., Prumakool,P. and Roberts,J. (1974) Processing of \textit{E. coli} tRNA precursors. \textit{Brookhaven Symp. Biol.}, 26, 53–76.
5. Gößinger,M., Kretschmar-Kazemir, Far,R. and Hartmann,R.K. (2006) Analysis of RNase P protein (rnpA) expression in \textit{Bacillus subtilis} utilizing strains with suppressible \textit{rnpA} expression. \textit{J. Bacteriol.}, 188, 6816–6823.
6. Hall,T.A. and Brown,J.W. (2001) The ribonuclease P family. \textit{Methods Enzymol.}, 341, 56–77.
7. Tsai,H.Y., Masquida,B., Biswas,R., Westhof,E. and Gopalan,V. (2005) Molecular modeling of the three-dimensional structure of the bacterial RNase P holoenzyme. \textit{J. Mol. Biol.}, 325, 661–675.
8. Kirsebom,L.A. and Svärd,S.G. (1994) Base pairing between \textit{Escherichia coli} RNase P RNA and its substrate. \textit{EMBO J.}, 13, 4870–4876.
9. Hartl,W.D., Schlegl,J., Erdmann,V.A. and Hartmann,R.K. (1995) Kinetics and thermodynamics of the RNase P RNA cleavage reaction: analysis of tRNA 3'-end variants. \textit{J. Mol. Biol.}, 247, 161–172.
10. Oh,B.K., Frank,D.N. and Pace,N.R. (1998) Participation of the 3'-CCA of tRNA in the binding of catalytic Mg2+ ions by ribonuclease P. \textit{Biochemistry}, 37, 7277–7283.
11. Busch,S., Kirsebom,L.A., Notbohm,H. and Hartmann,R.K. (2000) Differential role of the intermolecular base-pairs G292-C(75) and G293-C(74) in the reaction catalyzed by \textit{Escherichia coli} RNase P RNA. \textit{J. Mol. Biol.}, 299, 941–951.
12. Svärd,S.G., Kagardt,U. and Kirsebom,L.A. (1996) Phylogenetic comparative mutational analysis of the base-pairing between RNase P RNA and its substrate. \textit{RNA}, 2, 463–472.
13. Brännvall,M., Pettersson,B.M.F. and Kirsebom,L.A. (2003) Importance of the +73/294 interaction in \textit{Escherichia coli} RNase P RNA substrate complexes for cleavage and metal ion coordination. \textit{J. Mol. Biol.}, 325, 679–709.
14. Wegscheid,B. and Hartmann,R.K. (2006) The precursor tRNA 3'-CCA interaction with \textit{Escherichia coli} RNase P RNA is essential for catalysis by RNase P \textit{in vivo}. \textit{RNA}, 12, 2135–2148.
15. Oh,B.K. and Pace,N.R. (1994) Interaction of the 3'end of tRNA with ribonuclease P RNA. \textit{Nucleic Acids Res.}, 22, 4087–4094.
16. LaGrandeur,T.E., Hüttelhofer,A., Noller,H.F. and Pace,N.R. (1994) Phylogenetic comparative chemical footprint analysis of the interaction between ribonuclease P RNA and tRNA. \textit{EMBO J.}, 13, 3945–3952.
17. Wegscheid,B., Condon,C. and Hartmann,R.K. (2006) Type A and B RNase P RNAs are interchangeable \textit{in vivo} despite substantial biophysical differences. \textit{EMBO Rep.}, 7, 411–417.
18. Kurz,J.C., Niranjankumar,S. and Fierke,C.A. (1998) Protein component of \textit{Bacillus subtilis} RNase P specifically enhances the affinity for precursor-tRNAs. \textit{Biochemistry}, 37, 2393–2400.
19. Buck,A.H., Dalby,A.B., Poole,A.W., Kazantzsev,A.V. and Pace,N.R. (2005) Protein activation of a ribozyme: the role of bacterial RNase P protein. \textit{EMBO J.}, 24, 3360–3368.
20. Pellegrini,O., Nezzar,J., Marchfelder,A., Putzer,H. and Condon,C. (2003) Endonucleolytic processing of CCA-less tRNA precursors by RNase Z in \textit{Bacillus subtilis}. \textit{EMBO J.}, 22, 4534–4543.
21. Dinos,G., Wilson,D.N., Teraoka,Y., Szafarski,W., Fucini,P., Kalpaxis,D. and Nierhaus,K.H. (2004) Dissecting the ribosomal inhibition mechanisms of edeine and pactamycin: the universally conserved residues G693 and C795 regulate P-site RNA binding. \textit{Mol. Cell}, 13, 113–124.
22. Kurz,J.C. and Fierke,C.A. (2002) The affinity of magnesium binding sites in the \textit{Bacillus subtilis} RNase P x pre-tRNA complex is enhanced by the protein subunit. \textit{Biochemistry}, 41, 9545–9558.
23. Alatossava,T., Jutte,H., Kuhn,A. and Kellenberger,E. (1985) Manipulation of intracellular magnesium content in polymyxin B nonapeptide-sensitized \textit{Escherichia coli} by ionophore A23187. \textit{J. Bacteriol.}, 162, 413–419.
24. Zarrinkar,P.P., Wang,J. and Williamson,J.R. (1996) Slow folding kinetics of RNase P RNA. \textit{RNA}, 2, 564–573.
25. Kim,K.S., Sim,S., Ko,J.H. and Lee,Y. (2005) Processing of M1 RNA at the 3' end protects its primary transcript from degradation. \textit{J. Biol. Chem.}, 280, 34667–34674.
26. Loria,A. and Pan,T. (2000) The 3' substrate determinants for the catalytic efficiency of the \textit{Bacillus subtilis} RNase P holoenzyme.
suggest autolytic processing of the RNase P RNA in vivo. *RNA, 6*, 1413–1422.

27. Lundberg, U. and Altman, S. (1995) Processing of the precursor to the catalytic RNA subunit of RNase P from *Escherichia coli*. *RNA, 1*, 327–334.

28. Heide, C., Feltens, R. and Hartmann, R.K. (2001) Purine N7 groups that are crucial to the interaction of *Escherichia coli* RNase P RNA with tRNA. *RNA, 7*, 958–968.

29. Kufel, J. and Kirsebom, L.A. (1998) The P15-loop of *Escherichia coli* RNase P RNA is an autonomous divalent metal ion binding domain. *RNA, 4*, 777–788.

30. Ciesiolka, J., Hardt, W.D., Schlegl, J., Erdmann, V.A. and Hartmann, R.K. (1994) Lead-ion induced cleavage of RNase P RNA. *Eur. J. Biochem.*, 219, 49–56.

31. Lindell, M., Brännvall, M., Wagner, E.G. and Kirsebom, L.A. (2005) Lead(II) cleavage analysis of RNase P RNA in vivo. *RNA, 11*, 1348–1354.

32. Zito, K., Hüttenhofer, A. and Pace, N.R. (1993) Lead-catalyzed cleavage of ribonuclease P RNA as a probe for integrity of tertiary structure. *Nucleic Acids Res.*, 21, 5916–5920.

33. Kazantsev, A.V., Krivenko, A.A., Harrington, D.J., Holbrook, S.R., Adams, P.D. and Pace, N.R. (2005) Crystal structure of a bacterial ribonuclease P RNA. *Proc. Natl. Acad. Sci. U.S.A.*, 102, 13392–13397.

34. Day-Storms, J.J., Niranjanakumari, S. and Fierke, C.A. (2004) Ionic interactions between PRNA and P protein in *Bacillus subtilis* RNase P characterized using a magnetocapture-based assay. *RNA, 10*, 1595–1608.