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The CCA anticodon specifies separate functions inside and outside translation in *Bacillus cereus*

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Abstract

Bacillus cereus 14579 encodes two tRNAs with the CCA anticodon, $tRNA^{Trp}$ and $tRNA^{Other}$. $tRNA^{Trp}$ was separately aminoacylated by two enzymes, TrpRS1 and TrpRS2, which share only 34% similarity and display different catalytic capacities and specificities. TrpRS1 was 18-fold more proficient at aminoacylating $tRNA^{Trp}$ with Trp, while TrpRS2 more efficiently utilizes the Trp analog 5-hydroxy Trp. $tRNA^{Other}$ was not aminoacylated by either TrpRS but instead by the combined activity of LysRS1 and LysRS2, which recognized sequence elements absent from $tRNA^{Trp}$. Polysomes were found to contain $tRNA^{Trp}$, consistent with its role in translation, but not $tRNA^{Other}$ suggesting a function outside protein synthesis. Regulation of the genes encoding TrpRS1 and TrpRS2 (*trpS1* and *trpS2*) is dependent on riboswitch-mediated recognition of the CCA anticodon, and the role of $tRNA^{Other}$ in this process was investigated. Deletion of $tRNA^{Other}$ led to up to a 50 fold drop in *trpS1* expression, which resulted in the loss of differential regulation of the *trpS1* and *trpS2* genes in stationary phase. These findings reveal that sequence-specific interactions with a tRNA anticodon can be confined to processes outside translation, suggesting a means by which such RNAs may evolve non-coding functions.

Keywords

Bacillus cereus; lysyl-tRNA synthetase; $tRNA^{Other}$; tryptophanyl-tRNA synthetase

Introduction

Accurate translation of mRNA is a central facet of gene expression. Proteins are made by pairing mRNA codons with aa-tRNA on the ribosome, resulting in the synthesis of a polypeptide whose sequence corresponds to that encoded in the respective mRNA. Aminoacylation of tRNAs is catalyzed by the aminoacyl-tRNA synthetases (aaRSs), which faithfully match cognate amino acid:tRNA pairs from among the vast number of non-cognate molecules in the cell.[1] After aa-tRNAs are synthesized, they are screened and delivered to the ribosome by elongation factor Tu (EF-Tu;[2]) where they are further checked[3] and then used for protein synthesis. Accurate recognition by aaRSs requires the identification of a unique set of elements, most commonly nucleotides or modified nucleotides, at particular positions in a given tRNA.[4] These identity elements are normally found in the acceptor and anticodon stems,

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the anticodon loop and the variable arm of the tRNA. Due to their size and complexity, different tRNAs provide sufficiently diverse identity elements to allow their specific selection by the corresponding aaRS. Amino acids are less easily distinguished and some aaRSs contain proofreading activities directed against non-cognate substrates.[5,6] Other strategies that allow discrimination against non-cognate amino acids include aaRS-derived trans-editing factors[7,8] and functional duplication of aaRSs.[1] Duplication of synthetase activities is widespread and occurs mostly within the same family, as for example in the acquisition of antibiotic resistant synthetase alleles by certain bacteria.[9] More rarely, non-orthologous duplication of synthetase activities is seen, as in the case of the two unrelated forms of lysyl-tRNA synthetase (LysRS1 and LysRS2). Analysis of the distribution of LysRSs shows LysRS2 encoded in all eukaryotes, most bacteria and some archaea, and LysRS1 in some bacteria, most archaea but not in eukaryotes.[10]

Although structurally unrelated, LysRS1 and LysRS2 recognize lysine and $tRNA^{Lys}$ in much the same way.[11–17] For example, the elements in $tRNA^{Lys}$ recognized by LysRS1 and LysRS2 are the same, namely the anticodon, acceptor stem and discriminator bases.[11,18–22] LysRS1 and LysRS2 are not usually found together, their co-existence being restricted to one archaeal group, the Methanosarcinaceae, and a few Bacilli.[10,23,24] *Methanosarcina barkeri* LysRS1 and LysRS2 can together aminoacylate the rare $tRNA^{Pyl}$ species in vitro, although whether this activity plays any role in vivo remains unclear.[25] In vivo $tRNA^{Pyl}$ aminoacylation by pyrrolysyl-tRNA synthetase is an essential step during methylamine methyltransferase synthesis,[26] and deletion of the gene encoding $tRNA^{Pyl}$ renders Methanosarcina unable to grow on methylamines.[27] The pathogen *Bacillus cereus* also encodes both forms of LysRS, but genome sequence analysis does not identify $tRNA^{Pyl}$ or any other components of the pyrrolysine insertion pathway.[28,29] Instead the two LysRSs in *B. cereus* 14579 act together, but not separately, to aminoacylate a previously uncharacterized species named $tRNA^{Other}$, but the function of this RNA and its possible participation in translation remained unclear.[23] We now show that $tRNA^{Other}$ is confined to regulatory processes outside translation, suggesting how some RNAs may have evolved non-coding functions.

Results

Sequence-specific recognition of $tRNA^{Other}$

The presence of numerous non-canonical Watson-Crick base pairs in the predicted secondary structure of $tRNA^{Other}$ suggested that editing and/or modification might be necessary for the folding and maturation of $tRNA^{Other}$ (Fig. 1). To investigate post-transcriptional editing of $tRNA^{Other}$ in *B. cereus*, RNA samples were amplified by RT-PCR, sub-cloned and sequenced. From 50 independent isolates, one contained a G16A replacement, while all others contained the genetically encoded sequence for $tRNA^{Other}$. The apparent absence of extensive editing, or modifications that could interfere with RT-PCR, and the very low aminoacylation activity of the in vitro transcribed tRNA,[23] suggest that mature $tRNA^{Other}$ may contain non-canonical base-pairs required for function.

In an effort to generate more stable $tRNA^{Other}$ variants to use in functional studies, mutants were constructed where non-canonical base pairs were replaced with Watson-Crick pairs. Aminoacylation by LysRS1:LysRS2 was then used as a screen for improved folding and stability. The importance of the three A-C base pairs at the ends of the acceptor stem, the D stem and the anticodon stem were investigated with A to G replacements to generate canonical Watson-Crick base pairs and evaluate the importance of A at these positions. C to U replacements were also generated, allowing the formation of Watson-Crick base pairs and evaluation of the effect of a C at each position. The role of the bulge created by the G2:A71 pair in the acceptor stem of $tRNA^{Other}$ was evaluated by replacing the G with U or the A with C, which allowed for canonical pairing at the second base pair of the acceptor stem. Since G:U

at the second base pair is a negative determinant for LysRS2 but a positive secondary identity element for LysRS1,[11] the G2:U71 replacement was also tested. Other mutants tested were G37A (A37 is a hyper-modified base in $tRNA^{Lys}$, t6A), C34U to allow comparison with the LysRS1:LysRS2 complex of *Methanosarcina barkeri*, and G16A to evaluate possible effects of editing of the D loop.

In vitro transcribed $tRNA^{Other}$ variants were charged with LysRS1 and LysRS2 separately and with both enzymes together. Aminoacylation levels were too low to allow quantification by conventional filter binding, consistent with the poor activity previously observed with in vitro transcribed substrates. We attempted to improve the poor activity displayed by the in vitro transcribed variants by overexpressing $tRNA^{Other}$ in *E. coli* and using unfractionated small RNAs from these cells as a source of substrate. This modified procedure allowed the detection and quantification of activity for the $tRNA^{Other}$ variants (Fig. 1). Analysis of the different positions in $tRNA^{Other}$ indicated that some are insensitive to change, such as A10:C24 in the D stem and A39 in the anticodon stem. Replacements at the A7:C66 base pair in the acceptor stem changed activity, with C66U reducing tRNA aminoacylation 70%. The only increase in activity resulted from the C32U replacement, which elevated charging by 50%, suggesting that a Watson-Crick base pair at the top of the anticodon loop might stabilize $tRNA^{Other}$. Most modifications to the bulge at the second base pair of the acceptor stem only caused a mild loss in activity, although A71U decreased the charging level by 40%. Mutations G37A and A16G resulted in 15–17% decreases in charging, while C34U reduced charging by 23%, indicating modest roles for these positions during aminoacylation. Overall, none of the mutants tested showed dramatic improvements in activity or expression compared to wild-type; this suggests that either $tRNA^{Other}$ is recognized by an aaRS other than LysRS1:LysRS2, or that it functions outside protein synthesis and may not require aminoacylation.

$tRNA^{Other}$ and $tRNA^{Trp}$ aminoacylation pathways are mutually exclusive

$tRNA^{Other}$, which contains a Trp anticodon (CCA), is not aminoacylated by TrpRS1.[23] Re-analysis of the genome sequence of *B. cereus* 14579 identified a second copy of TrpRS, named TrpRS2, which displays only 34% similarity with TrpRS1. TrpRS2 homologs have previously been shown to harbor a broad range of functions including interaction with the Trp modification enzyme NOS, difference in indolmycin sensitivity, and the ability to utilize 5-hydroxy-L-tryptophan (5OHW) as a substrate for aminoacylation.[30,31] *B. cereus* TrpRS2 aminoacylation activity was not inhibited by 5OHW at concentrations up to 1 mM, while TrpRS1 was inhibited at 500 μM and above (data not shown). The K_M for Trp for TrpRS1 and TrpRS2 was about the same while the k_{cat} for TrpRS1 was 18-fold higher than for TrpRS2 (Table 1). The difference in activity between the two TrpRSs, and the earlier finding that TrpRS1 cannot charge in vitro transcribed $tRNA^{Other}$ with Trp, led us to investigate whether either TrpRS could charge $tRNA^{Trp}$ and/or $tRNA^{Other}$ with Trp and/or 5OHW. Both TrpRSs are able to charge the cognate $tRNA^{Trp}$ with 5OHW, but neither could aminoacylate $tRNA^{Other}$ with either Trp or 5OHW (Fig. 2). These data indicate that the TrpRS aminoacylation machinery of *B. cereus* is unable to utilize $tRNA^{Other}$ despite the fact that this tRNA contains a Trp anticodon (CCA).

$tRNA^{Other}$ is not essential for protein synthesis in *B. cereus*

In vitro aminoacylation data indicates that $tRNA^{Other}$ does not function as an atypical Trp isoacceptor tRNA during protein synthesis. While earlier data showed $tRNA^{Other}$ could bind to heterologous EF-Tu under non-physiological conditions, this did not directly indicate a role in translation.[23] To investigate if $tRNA^{Other}$ is required for another, as yet unknown, role in protein synthesis, we constructed a new strain of *B. cereus* ($\Delta tRNA^{Other}$) in which the $tRNA^{Other}$ gene was replaced by two restriction sites, *Not* I and *Sac* II. Deletion of $tRNA^{Other}$ was not lethal to *B. cereus*, and viable strains were obtained. The growth rates of *B. cereus* $\Delta tRNA^{Other}$ and wild-type were virtually the same on rich and sporulation media,

while the mutant strain grew ~30% more slowly on minimal media (Table 2). To determine if aminoacylated $tRNA^{Other}$ is used in translation, *B. cereus* 14579 polysomes were isolated from exponential and stationary growth phases, and the presence of $tRNA^{Other}$ was determined by RT-PCR.[32,33] Positive controls included the presence of RT-PCR product of $tRNA^{Other}$ from free tRNA fractions and $tRNA^{Trp}$ from both free tRNA and polysome fractions. To control for reverse transcription of the unprocessed transcripts (pre-$tRNA^{Other}$ and pre-$tRNA^{Trp}$), primers complementary to 21 nucleotides upstream of the 5′ end of each tRNA gene were used along with the 3′primers to produce larger products. RT-PCR products for $tRNA^{Trp}$ and $tRNA^{Other}$ were initially found in all fractions, but only $tRNA^{Trp}$ was detected in polysomes (pre-$tRNA^{Trp}$ could not be detected under these conditions). RT-PCR product for $tRNA^{Other}$ did not exceed that of pre-$tRNA^{Other}$, confirming that $tRNA^{Other}$ is not present as a mature species in polysomes (Fig. 3). The absence of $tRNA^{Other}$ in polysomes implicates this RNA in functions outside translation.

$tRNA^{Other}$ and regulation of *trpS1* and *trpS2* gene expression

Many small RNAs function as regulators of transcription or translation in bacteria.[34] Among these is the T box transcription termination system, in which uncharged tRNAs interact directly with an mRNA leader region to stabilize an antiterminator, preventing the formation of a terminator, and allowing transcription to continue.[35,36] Direct interactions occur between the tRNA and mRNA leader at two main tRNA locations: the anticodon and the last four nucleotides including the discriminator base and CCA end. Genes commonly regulated by this mechanism are involved in amino acid metabolism, including those encoding many aminoacyl-tRNA synthetases.[37] Since $tRNA^{Other}$ has a tryptophan anticodon and *B. cereus* has two genomically encoded TrpRSs, we tested the involvement in vivo of $tRNA^{Other}$ in regulating the T boxes found in the leader regions of the corresponding *trpS* transcripts. Results from real-time RT-PCR revealed that transcript levels for both *trpS1* and *trpS2* were highest during exponential growth and reduced as cells entered stationary phase (Fig. 4). Comparing wild-type and *B. cereus* Δ$tRNA^{Other}$, *trpS2* transcript levels remained the same at all time points, whereas *trpS1* transcript levels diverge in stationary phase: *trpS1* transcript levels from wild-type cells are 10 to 100-fold higher than from *B. cereus* Δ$tRNA^{Other}$ cells (Fig. 4). This change in transcript levels provides a preliminary indication of a possible role for $tRNA^{Other}$ outside protein synthesis in T box regulation. The low activity of in vitro transcribed $tRNA^{Other}$ currently precludes further investigation of these findings in vitro.

Discussion

$tRNA^{Other}$ lacks canonical transfer RNA structure and functions

Poor aminoacylation activity and low expression levels prevented the determination of steady-state kinetic parameters for Lys-$tRNA^{Other}$ synthesis, and recognition was instead investigated by approximating the effects of mutations on total tRNA charging. The most significant changes were seen upon mutation of A7:C66 or G2:A71, suggesting that the $tRNA^{Other}$ acceptor-stem is recognized by the LysRS1:LysRS2 complex. This is consistent with the lack of cross-reaction with TrpRS, which specifically recognizes positions G73, G2:C71, G3:C70 and G4:C69 in Bacillus $tRNA^{Trp}$,[38] none of which are conserved in $tRNA^{Other}$. The other major identity element in $tRNA^{Trp}$ is the CCA anticodon,[39] which is conserved in $tRNA^{Other}$ but does not appear to be a major element for recognition by LysRS1:LysRS2, compatible with the lack of aminoacylation by TrpRS. While several elements do appear to modestly contribute to recognition by LysRS1:LysRS2, most of the unique non-canonical features of $tRNA^{Other}$ have little or no effect on charging levels. The relatively small number of recognition elements in $tRNA^{Other}$, and the retention of 30% or more aminoacylation activity by all mutants tested, is not typical for canonical tRNAs. This may be a result of recognition being focused on indirect

readout of the atypical structure of $tRNA^{Other}$ by LysRS1:LysRS2, rather than by direct binding to individual nucleotides as is the case for most aaRSs.

The observations that $tRNA^{Other}$ is orthogonal to Trp-tRNA synthesis and is predominantly made after exponential growth led us to initially speculate that it might substitute for $tRNA^{Trp}$ during stationary phase. Analysis of $tRNA^{Trp}$ charging levels indicated that it is over 80% aminoacylated throughout both the exponential and stationary phases of growth, excluding widespread substitution during translation of canonical Trp codons as a potential role for $tRNA^{Other}$ (ref. [23]; Ataide SF and Ibba M, unpublished results). Alternatively, $tRNA^{Other}$ could allow nonsense suppression of UGA stop codons in certain contexts, or might be aminoacylated with an as yet unknown non-canonical amino acid or aaRS as is the case for pyrrolysine. While both of these possibilities require that aminoacyl-$tRNA^{Other}$ functions in protein synthesis, polysome analysis provided no evidence of $tRNA^{Other}$ association with active ribosomes. Taken together, the atypical aminoacylation data and absence from polysomes suggest that $tRNA^{Other}$ may instead have a non-canonical function outside translation.

A role for $tRNA^{Other}$ as a trans-acting small RNA

Small non-coding RNAs (sRNAs) control gene expression at the level of both transcription and translation.[34] While most characterized sRNAs function by binding directly to complementary nucleic acid target sites, a few, such as tmRNA and some plant viral genomes, mimic the aminoacylation function of tRNA.[40,41] Both aminoacylated sRNAs and tRNAs have been shown to participate in numerous processes outside protein synthesis including transcription, protein degradation, and various macromolecule biosyntheses.[42,43] Despite these wide-ranging functions, nearly all aminoacylated sRNAs and tRNAs are also active in ribosomal translation of the genetic code. This includes the many tRNAs that extensively regulate transcription of aaRS and amino acid metabolism genes via the T-box mechanism in bacteria.[36] The only notable exception is the pseudo-$tRNA^{Gly}$ found in some Staphylococci species, which directly participates in cell wall biosynthesis but is excluded from translation.[44] Our data provide initial evidence for a comparable role for $tRNA^{Other}$ acting as an sRNA, as it does not participate in protein synthesis but does impact gene expression. The mechanism by which $tRNA^{Other}$ affects *trpS1* and *trpS2* expression in stationary phase is unclear; the presence of U73 in $tRNA^{Other}$, and the resulting mismatch with the corresponding U of the antiterminator,[45] may provide one key difference. $tRNA^{Other}$ might also impact the possible formation and regulation of TrpRS1/TrpRS2 heterologous dimers, which could in turn control non-canonical functions such as nitric oxide metabolism.[46] How this might be coordinated with canonical regulation by $tRNA^{Trp}$, and whether $tRNA^{Other}$ has more widespread effects beyond the *trpS* genes, now requires further investigation. The reduction in exponential growth rate on minimal medium when $tRNA^{Other}$ is deleted suggests that the observed effects on *trpS* may be part of a wider response rather than changes in T-box regulation alone. Regardless of the exact mode of action of $tRNA^{Other}$, our data show that its function is independent of the corresponding mRNA codon. This raises the possibility that other pseudo tRNA genes, the roles of which remain mostly unknown, may also encode sRNAs that exploit aspects of tRNA structure for functions outside translation.

Materials and Methods

RNA extraction and RT-PCR

B. cereus 14579 was grown to OD_{600} = 6.0, cells were then harvested and the pellet was suspended in 0.5 ml 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, mixed with 0.3 ml glass beads and snap frozen in an ethanol/dry ice bath. Phenol (0.5 ml, pH 6.6) was added to each tube and the cells were disrupted by vortexing (4 × 30 s pulses), centrifuged at 10,000 ×g for 5 min and

the aqueous layer collected. This extraction was repeated once with phenol and once with chloroform. The RNA was then ethanol precipitated, the pellet suspended in 50 μl water containing 10 u of DNase I (Invitrogen) and incubated at room temperature for 30 min. The reaction was stopped by the addition of 2.5 mM EDTA and heat inactivation of DNase I. The RNA was then phenol:chloroform extracted, ethanol precipitated, resuspended in 70 μl of water and quantitated spectrophometrically.

RT-PCR was performed with 15 μg of total RNA annealed with 1 μM oligonucleotide complementary to the 3′ end of $tRNA^{Other}$ and 1 mM dNTPs. The mixture was heated at 80° C for 2 min followed by incubation at 50°C for 15 min. To the mixture were added 10 mM DTT, first strand buffer, 200 u Superscript Reverse Transcriptase II (Invitrogen) and 20 u RNAsin (Promega), followed by incubation at 50°C for 35 min. PCR was performed upon addition of 3′-end oligonucleotide (1 μM), 5′ end oligonucleotide (2 μM), Pfu DNA polymerase buffer (20 mM Tris HCl, pH 8; 10 mM $(NH_4)_2SO_4$; 2 mM $MgSO_4$; 0.1% triton X100; 0.1 mg/ml BSA, 10 mM KCl), 0.3 mM dNTPs and 5 u Pfu DNA polymerase (Stratagene) to the RT mixture, followed by 25 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 1 min, and 1 cycle at 72°C for 10 min. The product of RT-PCR was resolved on a 2.5% agarose gel and visualized by staining with ethidium bromide.

In vitro and in vivo transcription of $tRNA^{Other}$ variants

$tRNA^{Other}$ mutants were cloned into pUC18 using *BamH*I and *Hind*III restriction sites and contained a *Bst*NI site for run-off in vitro transcription. The $tRNA^{Other}$ mutants were generated by the oligonucleotide annealing technique using specific pairs of oligonucleotides for each mutation inserted. For in vivo transcription of $tRNA^{Other}$ and mutants, the $tRNA^{Other}$ gene was cloned into vector pKK223-3 (*ptac* promoter)[47] into *Nde*I and *Hind*III sites after reconstruction with oligonucleotides pKK-bcother3 (5′TAT GGA TGG GGG TAT AAT TTA AGG GT3′) at the 5′end and pKK-bcother10 (5′AGC TTT TGA ATT TGG AGT GGA TGG C3′) at the 3′ end of the gene and their complementary primers. After transforming *E. coli* DH5α, plasmids were sequenced and PCR using the primers pKK-S (5′-TCT AGA CCG GCG TAG AGG ATC CGG GC-3′) and pKK-SA (5′-TCT AGA AAC GCA AAA AGG CCA TCC GTC A-3′) was used to amplify the $tRNA^{Other}$ sequence together with the promoter, terminator and tRNA processing sequence. The amplified product was digested with *Xba*I and cloned into the vector pSU2719.[48] The resulting plasmid pSU-bcotherWT was sequenced and used to transform DH5α cells for overexpression. The $tRNA^{Other}$ mutants were made by mutagenic PCR with the plasmid pSU-bcotherWT using pairs of complementary oligonucleotides with the desired changes. Plasmids were sequenced and used to transform DH5α cells for overexpression. The overexpression of $tRNA^{Other}$ and mutants was performed by growing transformed DH5α cells in LB supplemented with chloramphenicol (34 μg/ml) to a cell density of OD_{600} = 0.6 at 37° C. The expression of $tRNA^{Other}$ was then induced by IPTG at a final concentration of 1 mM for 16 h at 37°C. The cells were harvested by centrifugation and washed with phosphate-buffered saline; this and subsequent steps were performed at 4°C. Extraction of unfractionated tRNA was performed as described above.

Aminoacylation assays with LysRS

Aminoacylation assays were performed at 37°C in 100 mM HEPES (pH 7.2), 50 mM KCl, 20 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 30 μM [^{14}C]-Lys (312 mCi/mmol Amersham Biosciences UK Limited), 1 mg/ml of unfractionated RNA or 5 μM of in vitro transcribed $tRNA^{Other}$, and 1 μM LysRS1 and/or 50 nM LysRS2 prepared as described previously.[23] The reactions were first incubated for 5 min on ice with LysRS1 and/or LysRS2 without amino acid. All reactions were started by addition of the amino acid. Time points were taken for each reaction and spotted on a 3 MM filter paper pre-soaked with 5% TCA and 0.05% [^{12}C]-Lys, washed and counted. Unfractionated small RNAs containing in vivo transcribed $tRNA^{Other}$ mutants were

aminoacylated with Lys and separated on an acid urea gel for detection of aminoacylated $tRNA^{Other}$ and *E. coli* $tRNA^{Lys}$. As a control, unfractionated small RNAs from *B. cereus* 14579 and DH5α harboring empty PSU vector were charged with Lys and separated on the same gel used for detection of $tRNA^{Other}$ and $tRNA^{Lys}$. Cross hybridization with *E. coli* tRNA was quantified, and this value was subtracted from the uncharged species for each sample. The $tRNA^{Lys}$ probe was specific for the *E. coli* $tRNA^{Lys}$ and is unable to detect *B. cereus* $tRNA^{Lys}$.

Aminoacylation assays with TrpRS

TrpRS1 was purified as previously described.[23] Genomic DNA from *B. cereus* 14579 was used as template for amplification of the *trpS2* gene (encoding TrpRS2). Primers were designed to generate products flanked by *Nde*I and *EcoR*I sites for *trpS2*. PCR was carried out using Pfu DNA polymerase (Stratagene) and the product was cloned into TOPO-TA blunt end (Invitrogen). The gene was then sequenced with two times coverage. The *trpS2* gene was excised and inserted into the pTYB12 vector to allow production of a TrpRS2-intein fusion protein (IMPACT System, New England Biolabs). *B. cereus trpS2* cloned into the pTYB12 vector was overexpressed in *E. coli* BL21 cells. Transformants were grown at room temperature in LB supplemented with ampicillin (100 μg/ml) to cell density OD_{600} = 0.6. Expression of *trpS2* was induced by IPTG (1 mM) for 16 h at room temperature. Subsequent steps were performed at 4°C. Cells were harvested by centrifugation and washed in column buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 1 mM $MgCl_2$ and 10% glycerol). Cells were resuspended in column buffer supplemented with protease inhibitor cocktail (Hoffman-La Roche), passed through a french pressure cell, and then centrifuged at 20,000 ×g for 30 min. The resulting supernatant was loaded onto a chitin affinity column (New England Biolabs) according to the manufacturer's instructions. Protein was eluted from the chitin affinity column in a buffer of 50 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 150 mM NaCl, 10% glycerol and 20 mM 2-mercaptoethanol. The fractions containing TrpRS (judged to be >99% pure by Coomassie blue staining after SDS/PAGE) were pooled, concentrated by ultrafiltration using Amicon Ultra-15 (Millipore), dialyzed against storage buffer (50 mM Tris-HCl [pH 8.0], 1 mM $MgCl_2$, 150 mM NaCl, 10% glycerol and 10 mM 2-mercaptoethanol), and stored at −80° C.

Aminoacylation assays were performed at 37°C in 100 mM HEPES (pH 7.2), 25 mM KCl, 20 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 50 μM [5-^{3}H]-Trp (27 Ci/mol; Amersham Biosciences UK Limited), 10 μM of in vitro transcribed $tRNA^{Trp}$ or $tRNA^{Other}$, and 50 nM TrpRS. The reactions were first incubated for 5 min on ice with TrpRS without amino acid. All reactions were started by addition of the amino acid and aliquots spotted onto 3 MM filter disks presoaked in 5% TCA (w/v) containing 0.05% (w/v) [^{12}C]-Trp. Sample disks were washed three times in 5% TCA (w/v), dried and radioactivity counted. For Trp K_M determination, Trp was added at concentrations varying between 0.2 to 5 times K_M. Aliquots of 10 μl were taken every 15–60 s and spotted onto 3 MM filter disks presoaked in 5% TCA (w/v) containing 0.5% (w/v) [^{12}C]-Trp.

Acid urea gel electrophoresis of RNA and aminoacyl-tRNA

Unfractionated *B. cereus* small RNAs and $tRNA^{Other}$ transcript were aminoacylated with Lys (by *B. cereus* LysRS1, LysRS2, or both enzymes together) or with Trp (by TrpRS1 and TrpRS2 separately) as described above. The reaction was stopped with 1 volume of 0.3 M NaOAc (pH 4.5), 10 mM EDTA. After phenol (pH 4.3) and chloroform extraction followed by ethanol precipitation, the aminoacyl-tRNAs were dissolved in 2x loading buffer (7 M urea, 0.3 M NAOAc [pH 4.5], 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) and loaded on a 6.5% polyacrylamide gel (50 × 32 cm, 0.4 mm thick) containing 7 M urea, 0.1 M NaOAc (pH 5.0), and run at 4°C, 16 watts in 0.1 M NaOAc (pH 5.0) for 16 h. Detection of the RNAs

was performed by blotting and hybridization. The portion of the gel containing the tRNAs was electroblotted onto a Hybond N^+ membrane (Amersham Biosciences) using a Hoefer electroblot apparatus (Amersham Biosciences) at 20 V for 20 min and then at 40 V for 2 h with 10 mM Tris-OAc (pH 8.0), 5 mM sodium acetate, and 0.5 mM Na-EDTA as transfer buffer. The membranes were baked at 80°C for 2 h. tRNAs were detected by hybridization with 5′ [^{32}P]-labeled oligonucleotide probes. The probes were complementary to nucleotides 56–76 of $tRNA^{Other}$, 26–46 of $tRNA^{Lys}$ and 30–50 of $tRNA^{Trp}$.

Construction of *B. cereus* ΔtRNA^{Other}

To construct strain *B. cereus* Δ$tRNA^{Other}$, the first 600 nucleotides upstream of the 5′ end and downstream of the 3′ end of the $tRNA^{Other}$ gene were amplified from genomic DNA using Pfu DNA polymerase (Stratagene) to insert *Not*I and *Sac*II restriction sites in replacement of the $tRNA^{Other}$ sequence. Oligonucleotides bcΔ1 (5′GCT GCT AAG CTT GGT AGA AAT CAT CCA AAT AGT AAA A3′) and bcΔ2 (5′TAT AGC GGC CGC CGC GGT CGT GGT CGG AAT GAC AG3′) were used to amplify the 600 nt upstream of the 5′ end of $tRNA^{Other}$ and bcΔ3 (5′ACG ACC GCG GCG GCC GCT ATA CTT GTT CAT TAA AAG GTT TCA3′) and bcΔ4 (5′GCT GGA TCC GAT GTT TTA GTA GTA GAG AAT CAT TAA G3′) were used to amplify the 600 nt downstream of the 3′end of $tRNA^{Other}$. The two fragments were rejoined by overlapping PCR using bcΔ1 and bcΔ4 primers to insert *Hind*III and *BamH*I restriction sites. The assembled ~1,200 nt Δother was cloned into *Hind*III and *BamH*I sites of plasmid pMUTIN4,[49] resulting in pBCΔother. The plasmid was sequenced to confirm $tRNA^{Other}$ deletion and restriction sites prior to *B. cereus* transformation. After integration of pBCΔother into the *B. cereus* chromosome using a 600 nt flanking sequence around $tRNA^{Other}$, the new strain BCpBCΔother was confirmed by PCR, restriction digestion and Southern analysis. During the screen, media contained IPTG in order to maintain the transcription of downstream genes and avoid polar effects from the presence of the plasmid. Strains BCpBCΔother 2, 8 and 10, confirmed to be the correct construct, were subjected to excision of the plasmid by a single cross-over event.[50] In order to revert the insertion, five passages at 1/100 dilution in LB and growth until saturation in media without erythromycin were performed followed by serial dilution and plating. Cells were then replica-plated onto LB and LB supplemented with 20 μg/ml erythromycin and grown for 10 h. Colonies that were not present on LB supplemented with 20 μg/ml of erythromycin after 8 h were selected and screened. To confirm the excision of $tRNA^{Other}$, the strain was characterized by PCR, restriction digestion and Southern analysis of genomic DNA in comparison with wt strain. To further characterize the RNA content of the deletion strain (*B. cereus* Δ$tRNA^{Other}$), RT-PCR and northern blot analysis was performed in comparison with *B. cereus* wt. The RT-PCR also included *E. coli* as a negative control and in vitro transcribed $tRNA^{Other}$ as a positive control. The absence of a band corresponding to $tRNA^{Other}$ from the RT-PCR and in the northern blot confirmed the deletion from the chromosome.

Transformation of *B. cereus*

B. cereus electroporation with pBCΔother was performed using a variation of the protocol previously described in.[51] An overnight, saturated culture of *B. cereus* was diluted 1/100 in 600 ml of LB and grown at 30°C to OD_{600} = 0.3. Cells were harvested by centrifugation at 3,000 ×g at 4°C and washed with 72 ml ice cold EP buffer (0.1 mM K_2HPO_4/KH_2PO_4, 0.5 mM $MgCl_2$ and 260 mM sucrose). Cells were pelleted again and resuspended in 1.2 ml of ice cold EP and kept on ice for 30 min. Plasmid DNA (pBCΔother, 10 μg) was mixed with 100 μl *B. cereus* cells, suspended in EP, placed in a cuvette, and incubated on ice for 10 min. Cells were electroporated with a pulse of 1.5 kV, 335 Ω and 15 μF and incubated with 1.5 ml of LB for 5 h at 30°C. Recovered cells (500 μl) were spread on LB plates supplemented with 1 μg/ml of erythromycin, 25 μg/ml lincomycin and 1 mM IPTG, and plates were incubated at 30°C for 48 h. Colonies were transferred to LB plates supplemented with the same concentrations

of erythromycin, lincomycin and IPTG, grown at 30°C for 24 h and transferred to a new plate containing the same media. Plates were than replica-plated on LB containing 10 μg/ml of erythromycin, 25 μg/ml of lincomycin and 1 mM IPTG and grown at 30°C for 24 h. For the next round of replica-plating the concentration of erythromycin was 20 μg/ml and the final replica-plating was on LB supplemented with 20 μg/ml of erythromycin, 25 μg/ml of lincomycin and grown at 30°C for 24 h.

Polysome preparation and analysis

B. cereus strain ATCC 14579 was grown in 500 ml LB broth at 37°C to mid exponential or stationary phase. Chloramphenicol (5 μg/ml final concentration) was added to the cultures and incubated at 37°C for five minutes prior to sample collection. Polysomes were isolated as described,[32] except *B. cereus* cells were lysed by bead-beating with acid-washed glass beads in a multi-vortexer for 8 min at 4°C. RNA-containing sucrose gradient fractions were precipitated with 0.3 M sodium actetate, pH 6.5 and one volume isopropanol, centrifuged for 15 min at 14,000 rpm at 4°C, resuspended in 20 μl extraction buffer (0.3 M NaOAc, pH 6.5, 0.5% SDS, 5 mM EDTA), and pooled into free tRNA, 30S, 70S, and polysome fractions. Samples were treated with DNase I (Invitrogen) for 1 h at 25°C with RNase Inhibitor (Roche) added to each reaction, then phenol:chloroform extracted followed by ethanol precipitation.

Presence of $tRNA^{Other}$, $tRNA^{Trp}$, pre-$tRNA^{Other}$ and pre-$tRNA^{Trp}$ in polysomes was determined by RT-PCR of polysome fractions. All primers were purchased from Integrated DNA Technologies except pre-BCW5, purchased from Sigma-Aldrich. First strand cDNA was synthesized using SuperScript™ II reverse transcriptase (Invitrogen) and primer BCO3 (5′-TGG AGT GGA TGG CGG GAA TT-3′) for $tRNA^{Other}$ and pre-$tRNA^{Other}$ and primer BCW3 (5′-TGG CAG GGG CAG TAG G-3′) for $tRNA^{Trp}$ and pre-$tRNA^{Trp}$. Primer annealing was performed at 80°C for 3 min then 50°C for 15 min, and reverse transcription was performed at 50°C for 35 min to prevent tRNA secondary structure formation. PCR was performed in 10 μl reactions with 5 μl cDNA, 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 0.25 mM each of dATP, dCTP, dGTP and dTTP, 0.5 U Taq DNA polymerase (Invitrogen), 1 mM of the appropriate 3′ primer, and 1 mM of BCO5 (5′-GGG GGT ATA ATT TAA GGG TAA AAC-3′) for $tRNA^{Other}$, Pre-BCO5 (5′-GTC CTG TCA TTC CGA CCA CGA-3′) for pre-$tRNA^{Other}$, BCW5 (5′-AGG GGC ATA GTT TAA AGG TAG AAC-3′) for $tRNA^{Trp}$, and pre-BCW1 (CCC CCC TCC ACC ATT TAC AT) and 2 (GTC CTC TAG CCG GCA TCA TT) for pre-$tRNA^{Trp}$. PCR consisted of 95°C for 5 min, 30 repeats of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, then a final incubation of 72°C for 5 min.

Real time RT-PCR

B. cereus strain ATCC 14579 was grown in 500 ml LB broth at 37°C, and samples containing 1×10^8 cells (determined by absorbance readings at 600 nm) were removed at various time points. RNA was extracted using RNeasy Mini Kits (Qiagen) with DNase I treatment performed on the column. Following elution of RNA from the column, a second DNA digestion was performed with 2 μg RNA, DNase I (Invitrogen) and Alu I (Invitrogen) using Alu I buffer at 25°C for 30 min. DNA digestions were heated to 65°C for 10 min following addition of 1 μl 25 mM EDTA.

Transcript abundance of *trpS1* and *trpS2* was determined by real-time RT-PCR. All primers and probes were designed using GenScript Real-time PCR (TaqMan) Primer Design (https://www.genscript.com/ssl-bin/app/primer) and purchased from Integrated DNA Technologies. First strand cDNA was synthesized using SuperScript™ II reverse transcriptase (Invitrogen) and primer TrpRT1 (5′-TGA GTT AGC TGG ACC TCG TG-3′) for *trpS1* and primer TruntrpRT3 (5′-AAA CGT TAC CGC GAA ATC-3′) for *trpS2*. Primer annealing was performed at 80°C for 2 min then 50°C for 15 min, and reverse transcription was performed

at 42°C for 50 min. Real-time PCR was performed in an Opticon2 DNA Engine (BioRad) using TaqMan Universal PCR Master Mix (Applied Biosystems), 700 nM of primers TrpRT1 and TrpRT2 (5′-CCT CTC AAA TCC AAT CCG TT-3′) and 250 nM probe TrpRT-FAM (5′-6-FAM-TCT TCG CAA TAG CGC GTG CC-6-TAMARA-3′) for *trpS1*, 700 nM of primers TruntrpRT3 and TrunTrpRT4 (5′-TGA CAT AAC GCG AGC TCC TA-3′) and 150 mM TruntrpRT-FAM (5′-6-FAM-CAA TGC CTG AAA TTC GCA TTC CA-6-TAMARA-3′) for *trpS2*. Real-time PCR protocol was 95°C for 10 min and 40 repeats of 95°C for 15 sec, 57.2° C for 1 min, followed by plate reading.

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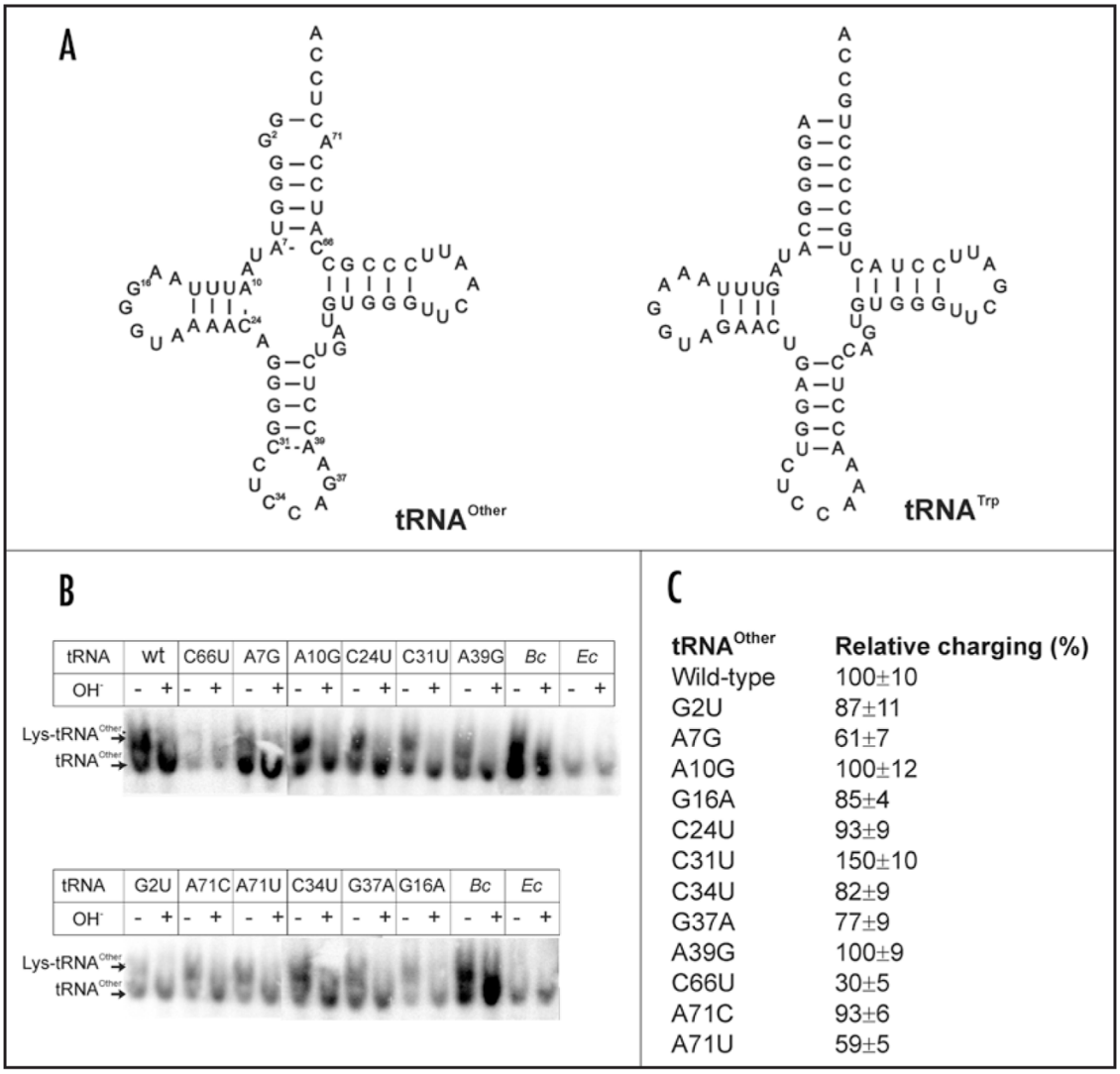

Figure 1.

Aminoacylation of tRNA^{Other}. (A) Predicted secondary structures of tRNA^{Other} and tRNA^{Trp}. (B) Aminoacylation of tRNA^{Other} variants by LysRS1:LysRS2. After aminoacylation of unfractionated RNA, half of the sample was deacylated (OH^-), and 25 μg of each fraction loaded. WT, wild-type; *BC*, unfractionated small RNAs from late stationary phase *B. cereus; EC*, unfractionated small RNA from *E. coli* DH5α cells containing empty PSU vector. (C) Percentage of charged tRNA compared to wild type, as an average of 3 independent experiments.

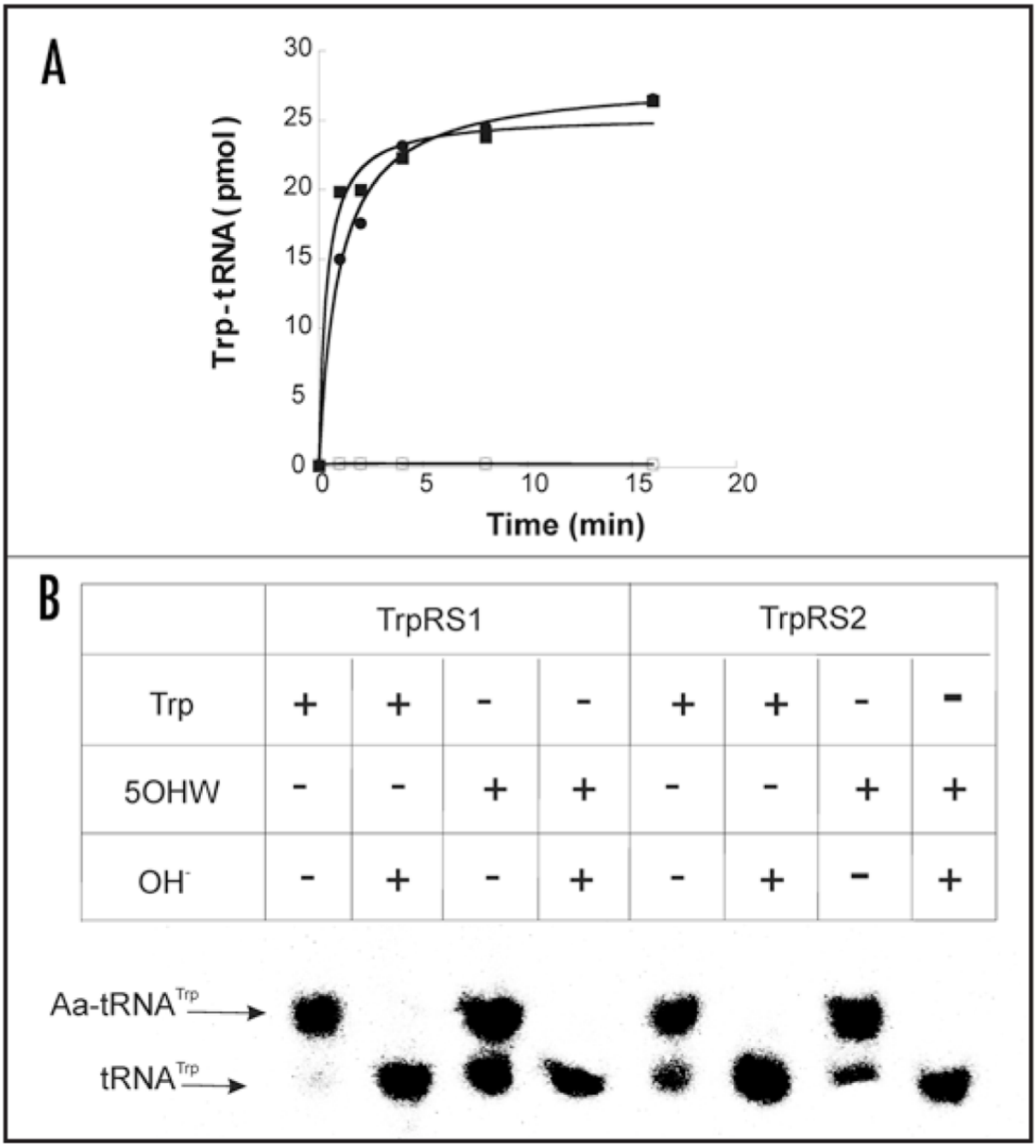

Figure 2.

Aminoacylation of $tRNA^{Trp}$ and $tRNA^{Other}$ by TrpRS1 and TrpRS2. (A) Aminoacylation of in vitro transcribed $tRNA^{Trp}$ by 50 nm TrpRS1 (■), 50 nM of TrpRS2 (●) and $tRNA^{Other}$ by 50 nm TrpRS1 (□), or 50 nM of TrpRS2 (○). The background level of aminoacylation determined in the absence of enzyme was ~0.2 pmol. (B) Analysis of $tRNA^{Trp}$ aminoacylation with 2 mM Trp or 2 mM 5OHW by 50 nM TrpRS1 or TrpRS2. OH^- −, no treatment after aminoacylation; OH^- +, deacylated. No aminoacylation of $tRNA^{Other}$ was observed under these conditions.

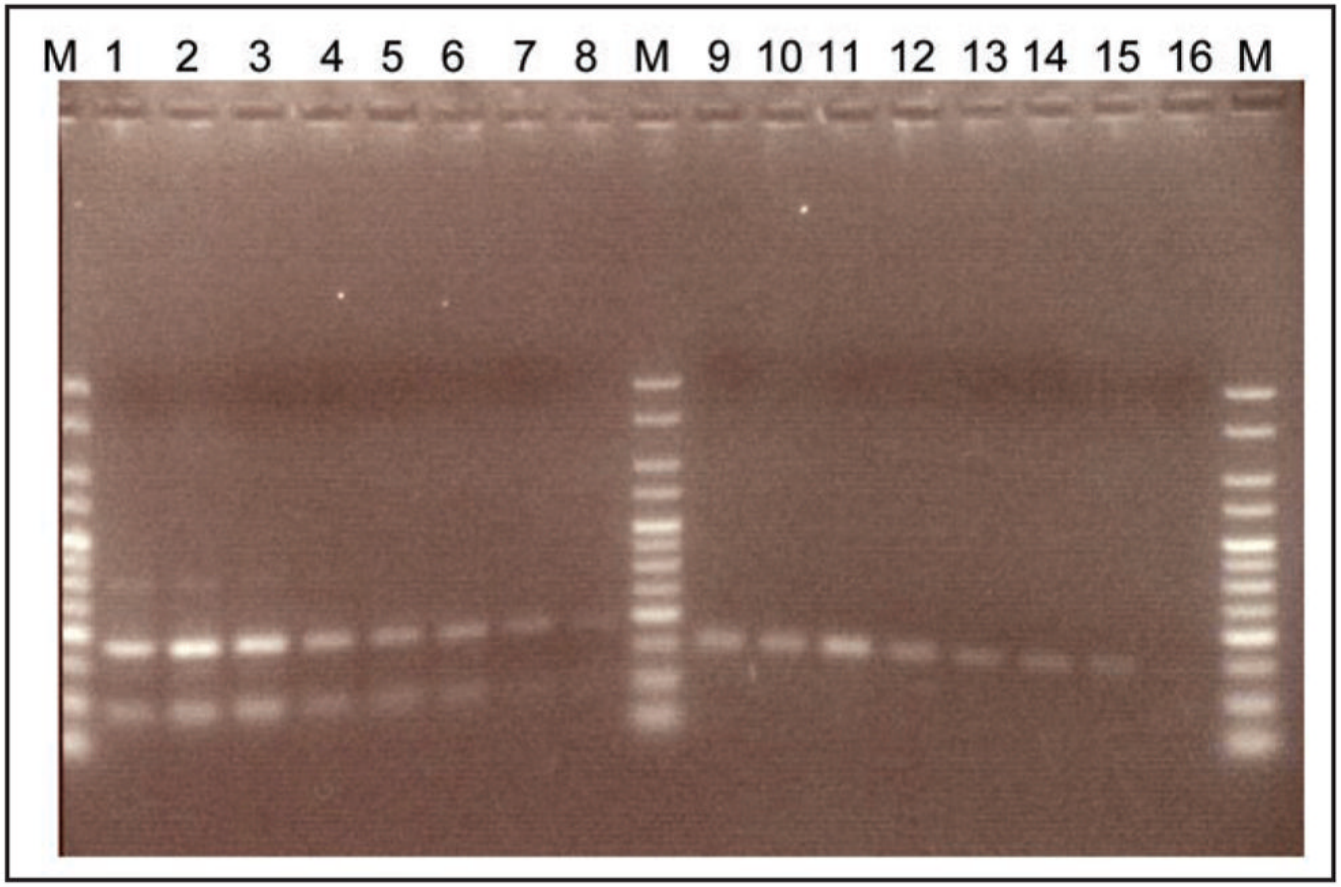

Figure 3.
tRNA^{Other} is not detected in *B. cereus* polysomes. RT-PCR of Pre-tRNA^{Other} and tRNA^{Other} from polysomes. Polysomes fractions were collected from cell lysate fractionated in 10–40% sucrose gradients. Lanes 1–8, 2-fold dilutions of Pre-tRNA^{Other} cDNA. Lanes 9–16, 2-fold dilutions of tRNA^{Other} cDNA. Expected product sizes (bp): tRNA^{Other}, 74; pre-tRNA^{Other}, 95. M, DNA ladder with following sizes (top to bottom): 500, 400, 300, 250, 200, 175, 150, 125, 100, 75, 50 and 25 bp.

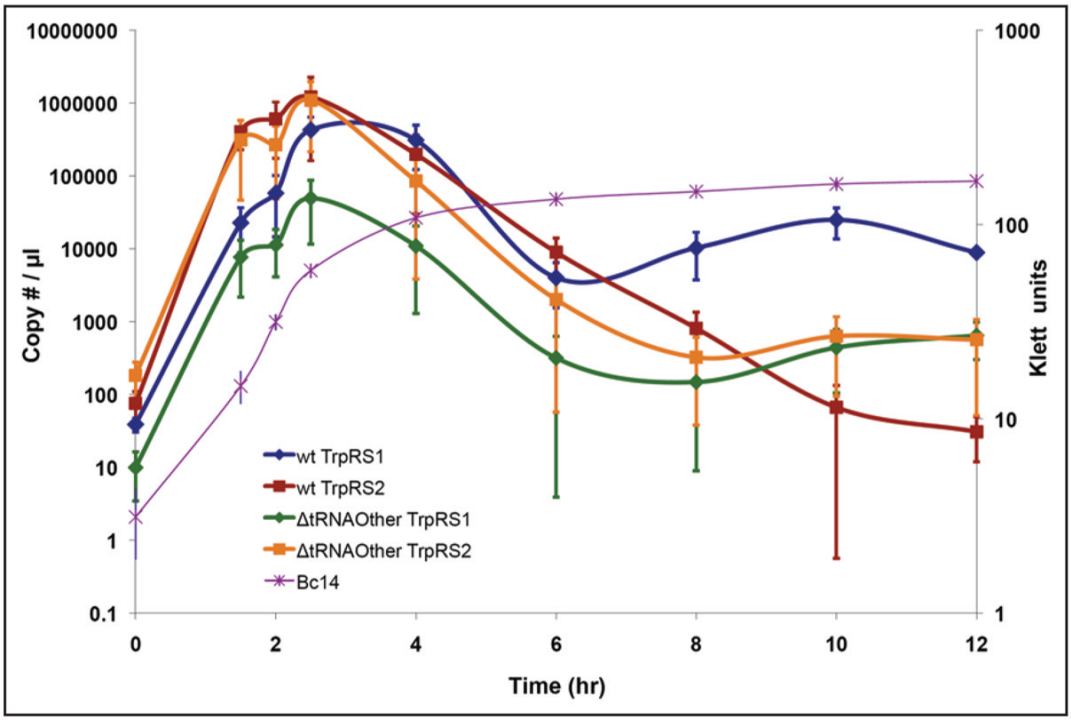

Figure 4.

Determination of *trpS1* and *trpS2* transcript levels. Real time RT-PCR was used to quantify the amounts of the *trpS1* and *trpS2* transcripts in *B. cereus* 14579 (wt) and *B. cereus* $\Delta tRNA^{Other}$ at various stages of growth. Cells were grown in LB broth at 37°C with agitation. Bc14, growth curve for wild-type *B. cereus* 14579.

Table 1

Aminoacylation kinetic parameters of *B. cereus* TrpRSs for Trp

aaRS	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} μM^{-1})
TrpRS1	4.2 ± 0.4	150 ± 2	37 ± 0.6
TrpRS2	7.6 ± 0.7	8 ± 0.05	1.09 ± 0.02

Table 2

Growth rate of *B. cereus* 14579 wild-type and ΔtRNA^{Other} strains in liquid culture

Growth medium	Doubling time (min)	
	***B. cereus* wild-type**	***B. cereus* ΔtRNA^{Other}**
Complete (LB)	33 ± 3	36 ± 2
Minimal	87 ± 4	114 ± 7
Sporulation	50 ± 4	54 ± 3