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Stationary-phase expression and aminoacylation of a transfer-RNA-like small RNA

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Genome-scale analyses have shown numerous functional duplications in the canonical translational machinery. One of the most striking examples is the occurrence of unrelated class I and class II lysyl-transfer RNA synthetases (LysRS), which together may aminoacylate non-canonical tRNAs. We show that, in *Bacillus cereus*, the two LysRSs together aminoacylate a small RNA of unknown function named tRNA^{Other}, and that the aminoacylated product stably binds translation elongation factor Tu. *In vitro* reconstitution of a defined lysylation system showed that Lys-tRNA^{Other} is synthesized in the presence of both LysRSs, but not by either alone. *In vivo* analyses showed that the class 2 LysRS was present both during and after exponential growth, whereas the class I enzyme and tRNA^{Other} were predominantly produced during the stationary phase. Aminoacylation of tRNA^{Other} was also found to be confined to the stationary phase, which suggests a role for this non-canonical tRNA in growth-phase-specific protein synthesis.

Keywords: aminoacyl-tRNA; *Bacillus cereus*; stationary phase; transfer RNA; translation

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INTRODUCTION

The fidelity of ribosomal protein synthesis depends on two key events: the matching of messenger RNA codons with the corresponding transfer RNA anticodons and the aminoacylation of these tRNAs with the correct amino acid. The aminoacylation of tRNAs with their cognate amino acids is catalysed by the aminoacyl-tRNA synthetase (aaRS) protein family, the accuracy of which is crucial in defining the genetic code (Ibba & Söll, 2004). In the aaRSs, there exist two structurally unrelated groups known as class I and class II (Eriani *et al*, 1990; Cusack *et al*, 1991; Ribas De Pouplana & Schimmel, 2001). Structural, functional and genomic analyses have shown that aaRSs of particular specificity are consistently found as members of one or other of these classes, regardless of their source organism. It was initially assumed that the essential function of aaRSs would lead to their conservation as a family with little evolutionary variation. Comparative genomics has instead shown widespread divergence in aminoacyl-tRNA synthesis (Woese *et al*, 2000). This includes the replacement of certain aaRSs by indirect pathways (Ibba *et al*, 2000), aaRS specificities for non-canonical amino acids such as pyrrolysine and phosphoserine (Blight *et al*, 2004; Sauerwald *et al*, 2005), highly diverged aaRS orthologues (Brown *et al*, 2003) and paralogues that may function outside protein synthesis (Sissler *et al*, 1999; Roy *et al*, 2003).

Two pathways synthesize lysyl-tRNA^{Lys}, each of which uses an unrelated lysyl-tRNA synthetase (LysRS). LysRS1 is a class I aaRS found in archaea and bacteria, whereas LysRS2 is a member of class II aaRS found mainly in bacteria and eukaryotes (Ambrogelly *et al*, 2002). LysRS1 and LysRS2 are not normally found together, and each form of the protein is resistant to inhibition by particular lysine analogues (Jester *et al*, 2003; Levengood *et al*, 2004). From the more than 250 publicly available genome sequences, the only instances in which both LysRS1 and LysRS2 are found together are the *Methanosarcineae* in the archaea and certain *Bacilli* among the bacteria. It was shown that *Methanosarcina barkeri* LysRS1 and LysRS2 together can aminoacylate the rare tRNA^{Pyl} species, although the role of this activity remains unclear (Polycarpo *et al*, 2003, 2004). In the pathogen *Bacillus cereus*, both LysRSs are also encoded; however, genome sequence analysis does not identify tRNA^{Pyl} or any other components of the pyrrolysine insertion pathway (Ivanova *et al*, 2003; Rasko *et al*, 2004). To understand the role of the two LysRSs in *B. cereus*, we investigated their RNA substrate specificities. This showed that they are able to act together, but not separately, to aminoacylate a previously uncharacterized species named tRNA^{Other}.

RESULTS AND DISCUSSION

Occurrence of LysRS1 and LysRS2 in *Bacilli*

We investigated the possibility that LysRS1 and LysRS2 might function together in the *B. cereus* strain 14579, which is the first

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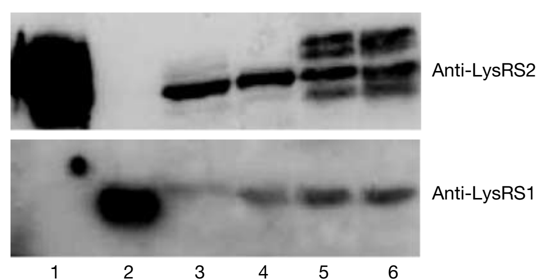

Fig 1 | Lysyl-transfer RNA synthetase 1 (LysRS1) and LysRS2 in *Bacillus cereus.* A 20 μg portion of total cell protein was separated by SDS–polyacrylamide gel electrophoresis, transferred to membranes and detected using either anti-LysRS1 or anti-LysRS2 polyclonal antibodies. The different growth stages are as follows: optical density $(OD)_{600} = 0.5$ (1 h, lane 3), $OD_{600} = 1.6$ (2 h, lane 4), $OD_{600} = 6.0$ (10 h, lane 5), $OD_{600} = 6.7$ (13 h, lane 6). A 0.1 μg portion each of LysRS1 (lane 2) and LysRS2 (lane 1) was loaded as a control.

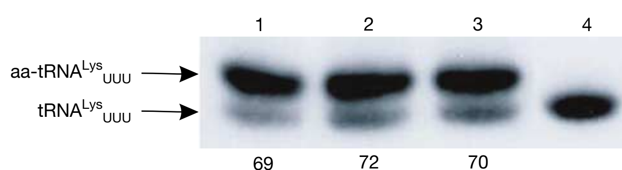

Fig 2 | Lysylation of tRNA^{Lys} *in vivo* in *Bacillus subtilis.* The level of charging is shown below each lane: *B. subtilis* 168 (lane 1), *B. subtilis* BCJ237.14 (lane 2), *B. subtilis* BCJ 239.31 (lane 3) and *B. subtilis* 168, OH^- treated (lane 4).

sequenced bacterial genome to encode LysRS1 and LysRS2. To detect whether LysRS1 and LysRS2 are present at the same time, we performed immunoblot analyses at different stages of growth (Fig 1). LysRS1 and LysRS2 were observed during and after exponential growth, but at different levels. LysRS2 predominated during exponential growth, but its level declined during the stationary phase. The appearance of further crossreacting species suggests that LysRS2 is modified in the stationary phase, although the nature of such modifications is unclear. LysRS1 has the reciprocal profile, with a low level during exponential growth, which increased substantially during the later stages of the growth cycle. The genomic contexts of the genes encoding LysRS1 (*lysK*) and LysRS2 (*lysS*) indicate differences in their regulation: *lysK* is preceded by a canonical T box with a lysine specifier codon (supplementary Fig X1 online), a form of regulation found in 14 of the 24 *Bacillus subtilis* aaRS genes. The *B. cereus lysS* gene has the same genomic context as *B. subtilis*, being the distal gene in the 9-cistron folate biosynthetic operon. In *B. subtilis*, expression of this operon is complex with several promoters, RNA processing and RNA stability contributing to the cellular level of LysRS2 (B.C.J. & K.M.D., unpublished results). These differences, both in regulation and production, led us to investigate in more detail the *in vivo* and *in vitro* activities of the two *B. cereus* LysRSs.

B. cereus LysRS1 and LysRS2 activities

To verify the activity of each LysRS *in vivo*, genomic replacements were constructed in the *B. subtilis* strain 168. Strain BCJ237.14 contains *lysK* from the *B. cereus* strain 14579 at the *rpsD* locus, expressed using the *rpsD* promoter with subsequent deletion of the endogenous *lysS*. In strain BCJ239.31, *lysS* from the *B. cereus* strain 10987 replaces the endogenous *lysS* gene and is therefore expressed normally. The endogenous *B. subtilis lysS* was subsequently deleted from both strains (supplementary Figs X2,X3 online). There is no significant difference between the doubling times of strains BCJ237.14, BCJ239.31 and strain 168 in cultures grown either in Luria broth (~26 min) or in minimal medium (~100 min). The level of tRNA^{Lys} charging was also similar, with 69% tRNA^{Lys} charged in the wild-type strain 168, whereas 72% and 70% of tRNA^{Lys} were charged in strains BCJ237.14 and BCJ239.31, respectively (Fig 2). To ascertain whether *B. cereus* LysRS1 and LysRS2 had canonical *S*-(2-aminoethyl)-L-cysteine (AEC) inhibition profiles, we performed disc assays (Fig 3). The *B. cereus* strain 14579 and the *B. subtilis* strains 168, BCJ2391.31 and BCJ270 (containing LysRS1 from *B. cereus* 14579 and the endogenous LysRS2) all showed similar and significant zones of inhibition. However, strain BCJ237.14, which has only LysRS1 from *B. cereus* 14579, has almost no zone of inhibition, a characteristic specific to class-I-type LysRSs (Jester *et al*, 2003; Levengood *et al*, 2004). These data show that both LysRS1 and LysRS2 from *B. cereus* are fully functional *in vivo*, and are able to act alone as canonical synthetases. To investigate possible differences in substrate binding and turnover between the two enzymes, LysRS1 and LysRS2 (*B. cereus* 14579; used in all further experiments) were produced heterologously and used to lysylate total small RNA extracted from late stationary-phase *B. cereus* cells. LysRS2 was considerably more active than LysRS1, as tenfold less enzyme was required to achieve full aminoacylation under the conditions used (Fig 4A). When both enzymes were used together, the plateau charging level was consistently higher than that for either LysRS alone, although in all cases, tRNA^{Lys} was being aminoacylated (Fig 4B). This suggested that RNA species, in addition to tRNA^{Lys}, may have been aminoacylated in the presence of both enzymes.

Identification of LysRS1:LysRS2 RNA substrates

To identify the RNAs aminoacylated by LysRS1:LysRS2, two separation procedures were used. After charging RNA from a total pool using the same conditions as described above (Fig 4A), aminoacylated species were purified by binding to immobilized *Thermus thermophilus* elongation factor Tu (Ribeiro *et al*, 1995). Aminoacylated species were eluted and fractionated by two-dimensional (2D) gel electrophoresis, and individual species were extracted and reverse transcribed to complementary DNA using the oligo-anchoring technique (Kapushoc *et al*, 2002). The cDNAs were sequenced, and the data used to search the *B. cereus* genome. This identified one species as tRNA^{Lys} and the other as the product of a gene of unknown function, previously annotated as tRNA^{Other}. Estimation of the abundance of each species from examination of the corresponding 2D gels (above) indicates that tRNA^{Lys} accounts for about 80% of the charged pool and tRNA^{Other} for about 20%. The secondary fold of tRNA^{Other}, which contains a tryptophan anticodon, is unusual and requires non-canonical base pairings to adopt a canonical fold, perhaps indicating the presence of nucleotide modifications in the native molecule (Fig 5). One other striking feature is the G2:A71 bulge, reminiscent of the G2:U71 wobble position previously implicated

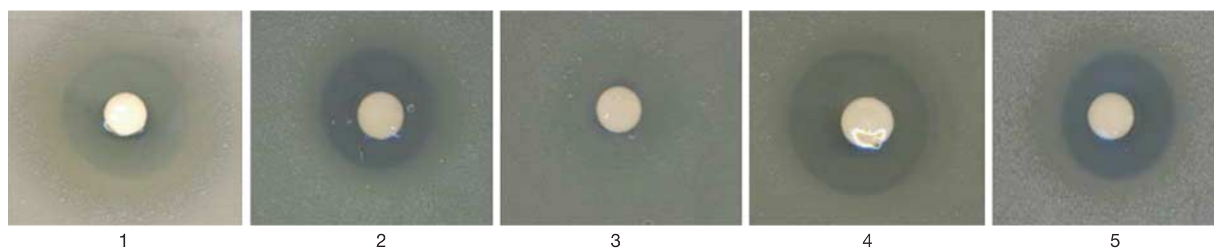

Fig 3 | Inhibition of growth by *S*-(2-aminoethyl)-L-cysteine. *Bacillus cereus* 14579 (lane 1), *Bacillus subtilis* 168 (lane 2), *B. subtilis* BCJ237.14 (lane 3), *B. subtilis* BCJ 239.31 (lane 4) and *B. subtilis* BCJ270 (lane 5).

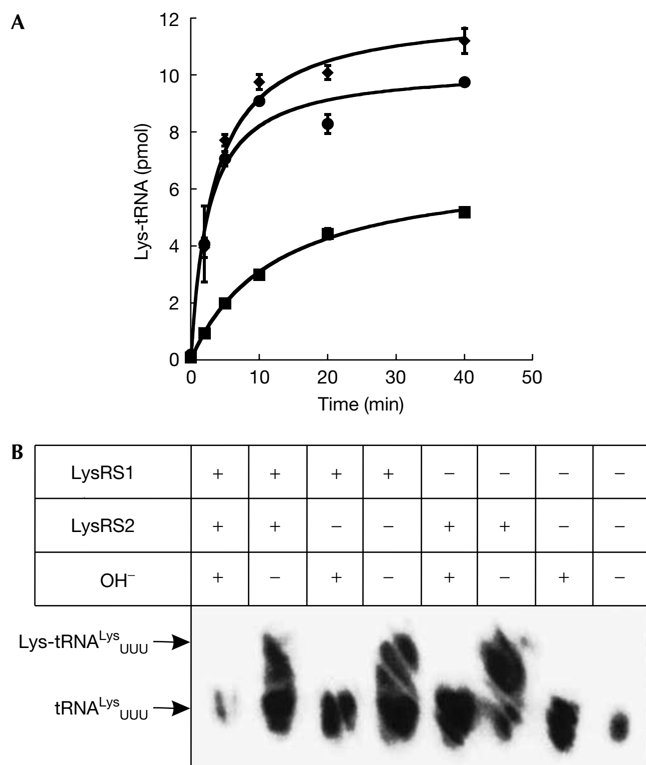

Fig 4 | Lysylation of total transfer RNA from *Bacillus cereus*. **(A)** Aminoacylation of total tRNA with [^{14}C]lysine with 1 μM lysyl-transfer RNA synthetase 1 (LysRS1; squares), 50 nM LysRS2 (circles) or both together (diamonds). Values shown are the means of three independent experiments, with error bars representing ±one standard deviation. **(B)** Analysis of tRNA$^{Lys}_{UUU}$ aminoacylation by hybridization against a tRNA^{Lys}-specific probe. $-$OH$^-$, no treatment after aminoacylation; $+$OH$^-$, deacylation with 100 mM Tris–OAc (pH 9) at 37 °C for 30 min. Slight reductions in tRNA abundance were routinely observed after deacylation because of the further sample processing involved, compared with untreated samples.

in tRNA^{Lys} substrate differentiation by LysRS1 and LysRS2 (Ibba *et al*, 1999). The aminoacylation of tRNA^{Other} was further investigated *in vivo* and *in vitro*.

Expression and aminoacylation of tRNA^{Other}

The presence of tRNA^{Other} during different phases of *B. cereus* growth was assessed by reverse transcription–PCR (RT–PCR), using RNA extracted at various times. tRNA^{Other} was first detected in the early stationary phase and continued to increase into the late stationary phase (Fig 6A). We then determined whether this correlated with the aminoacylation of tRNA^{Other} in the stationary phase. Acidic extraction and separation differentiates aminoacylated tRNAs from deacylated tRNAs, with the caveat that aminoacylation at the 3′ end cannot be assumed (see, for example, Salazar *et al*, 2004). $NaIO_4$ treatment and analysis of aminoacylated samples confirmed that aminoacylation was indeed occurring at the 3′ end in this case (data not shown). tRNA^{Other} was aminoacylated throughout the stationary phase, but was not produced or charged during the exponential phase (Fig 6), mirroring the change in the LysRS1:LysRS2 ratio from the exponential to the late stationary phase (Fig 1). Although the amino acid attached to tRNA^{Other} *in vivo* has yet to be determined, these data are consistent with aminoacylation with lysine by the concerted action of LysRS1 and LysRS2. To test this hypothesis, we reconstituted tRNA^{Other} aminoacylation *in vitro*.

LysRS1 and LysRS2 act together to charge tRNA^{Other}

In vitro-transcribed tRNA^{Other} was used for lysylation with combinations of LysRS1 and LysRS2, and aminoacylation was monitored by hybridization (Fig 7A). tRNA^{Other} was charged with lysine when both LysRSs were present, but not by either alone. To test further the specificity of this reaction, *in vitro*-transcribed tRNA^{Other} was also used as the RNA substrate in aminoacylation time-course assays with LysRS1, LysRS2 and both together (Fig 7B). *In vitro*-transcribed tRNA^{Other} was a relatively poor substrate (10% of the product could be aminoacylated), perhaps reflecting the need for nucleotide modifications to stabilize the unusual secondary structure of this non-canonical tRNA (Fig 5). Nevertheless, charging of tRNA^{Other} was clearly observed when LysRS1 and LysRS2 were present, but not with either LysRS alone, which supports the proposal that both enzymes act together. Although the complete lack of activity of *in vitro*-transcribed *B. cereus* tRNA^{Lys} prevents direct comparisons, estimation of the rates of tRNA^{Lys} charging by LysRS2 (Fig 4A) and tRNA^{Other} by LysRS1:LysRS2 (Fig 7B) suggests that the canonical tRNA^{Lys} is by far the more active of the two tRNAs. A similar pattern was reported for charging by seryl-tRNA synthetase of tRNA^{Sec}, which is about 100-fold less active than the canonical substrate tRNA^{Ser} (Baron & Böck, 1991). As tRNA^{Other} contains a Trp anticodon, we also attempted to charge *in vitro*-transcribed tRNA^{Other} with tryptophan using *B. cereus* tryptophanyl-tRNA synthetase (TrpRS). TrpRS was able to charge efficiently its cognate tRNA with tryptophan, but showed no such activity towards tRNA^{Other},

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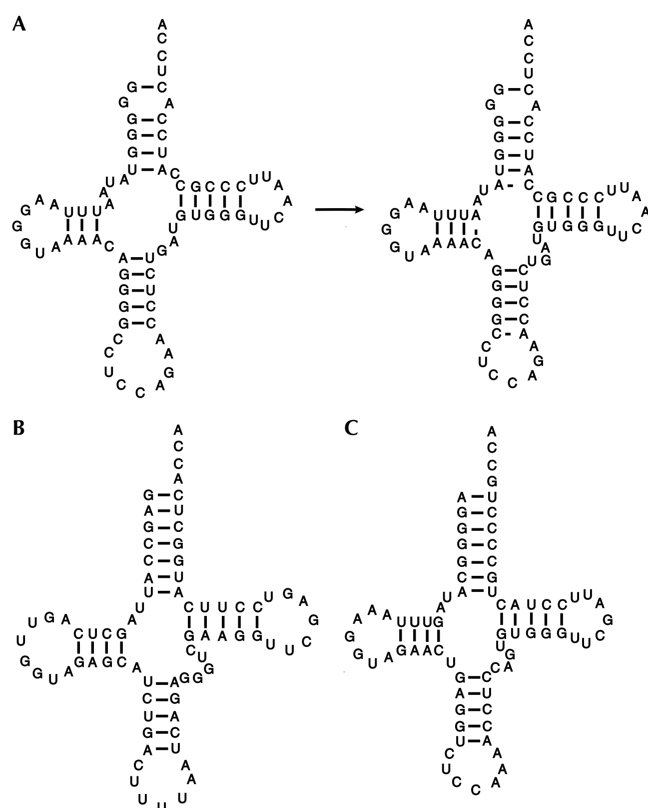

Fig 5 | Secondary structure of *Bacillus cereus* transfer RNAs. (**A**) The non-canonical structure of tRNA^{Other} as predicted by tRNAScan is shown on the left and an alternative canonical fold on the right. See text for details. Dashed lines indicate putative interactions that may depend on nucleotide modifications. (**B**) Predicted secondary structure of tRNA^{Lys}. (**C**) Predicted secondary structure of tRNA^{Trp}.

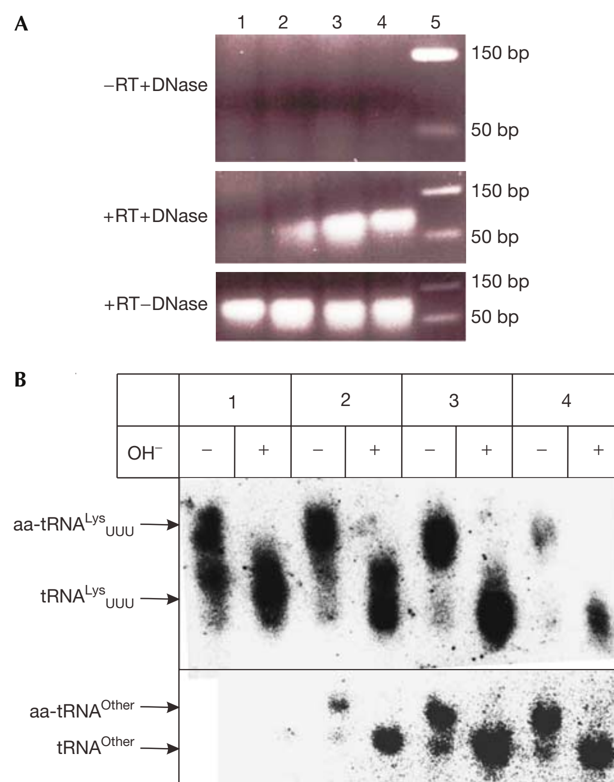

Fig 6 | Growth-phase-dependent expression and aminoacylation of tRNA^{Other}. RNA samples were extracted at optical density $(OD)_{600} = 0.5$ (1 h, lane 1), $OD_{600} = 1.6$ (2 h, lane 2), $OD_{600} = 6.0$ (10 h, lane 3) and $OD_{600} = 6.7$ (13 h, lane 4). (**A**) Reverse transcription–PCR using primers specific for tRNA^{Other} and total RNA extracts (lane 5, markers). Top, negative control; middle, amplication of tRNA^{Other}; bottom, amplification of tRNA^{Other} and the gene that encodes it. RT, reverse transcription; DNase, deoxyribonuclease. (**B**) Analysis of $tRNA^{Lys}_{UUU}$ and tRNA^{Other} aminoacylation by hybridization against specific probes. OH^- treatments are as in Fig 2.

confirming that tRNA^{Other} is aminoacylated specifically by LysRS1:LysRS2 (Fig 7C).

LysRS1 is essential in *B. cereus*

tRNA^{Other} was found to be synthesized and aminoacylated in the stationary phase but not in the exponential phase. A similar profile was observed for *bldA*, a rare codon-specifying tRNA^{Leu} in *Streptomyces coelicolor* that is expressed predominantly in the late stationary phase (Leskiw *et al*, 1993). *bldA* is required for the translation of rare leucine codons in several proteins that are involved in colonial differentiation, aerial mycelium formation and antibiotic production. tRNA^{Other} is encoded downstream of genes coding for a large peptide synthetase that is responsible for the production of a bacteriocin-like inhibitory substance (BLIS). BLIS is a broad-range antimicrobial produced in the late stationary phase (Risoen *et al*, 2004), providing a possible link to tRNA^{Other}, similar to that between *bldA* and antibiotic production. To investigate further this possible connection, we attempted to disrupt aminoacylation of tRNA^{Other} by deleting the LysRS1-encoding gene in *B. cereus*. The only transformants obtained were merodiploid, which suggests a requirement for LysRS1. Further analysis is necessary to reconcile this requirement with the observed patterns of LysRS1 and tRNA^{Other} production.

Possible roles for tRNA^{Other}

Many aaRSs have been found as duplicated orthologues in the same organism, a phenomenon associated with resistance to amino-acid analogues and responses to changes in cellular physiology (Brevet *et al*, 1995; Brown *et al*, 2003). In contrast, non-orthologous duplication of LysRS is still unique among aaRSs, and only a small minority of characterized organisms harbours both classes of the protein. In *B. cereus*, LysRS2 is a housekeeping enzyme, with LysRS1 and a non-canonical tRNA expressed only under certain conditions. In *M. barkeri*, a non-canonical tRNA is primarily aminoacylated with pyrrolysine by its own aaRS (pyrrolysyl-tRNA synthetase), and the role of the LysRS1:LysRS2 pathway may be to prevent ribosomal stalling when pyrrolysine is scarce. Charging of tRNA^{Other}, which contains a Trp anticodon, could fulfil a similar function by ensuring that Trp codons are still translated during the stationary phase when tryptophan may be in short supply. However, analysis of tRNA^{Trp} charging indicated that it is over 80% aminoacylated throughout the exponential and stationary phases (S.F.A. and M.I., unpublished results), excluding tryptophan limitation as a potential stimulus for tRNA^{Other}

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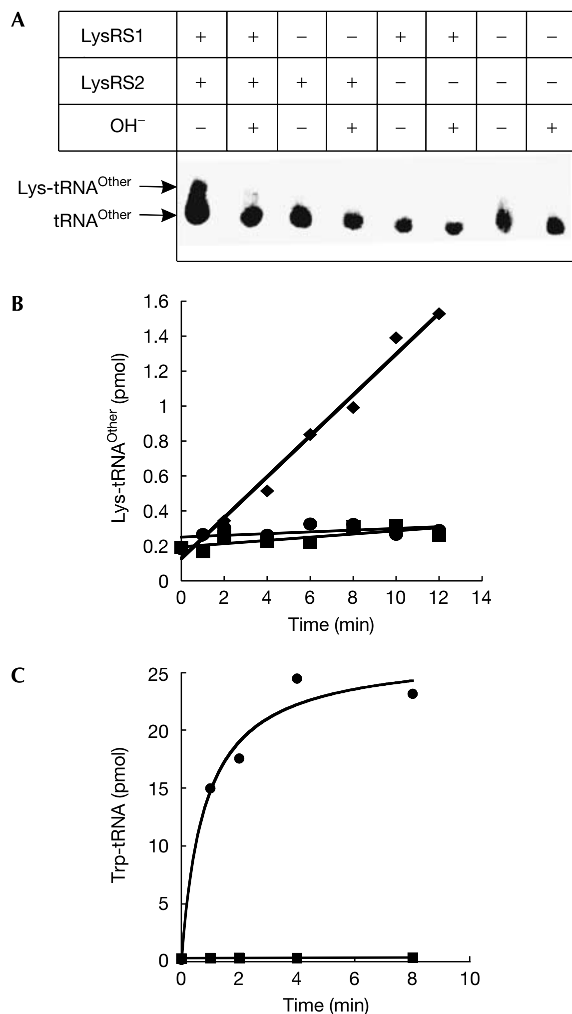

Fig 7 | Aminoacylation of tRNA^{Other} requires both lysyl-transfer RNA synthetase 1 (LysRS1) and LysRS2. **(A)** *In vitro*-transcribed tRNA^{Other} aminoacylation analyses by hybridization against a specific probe. OH$^-$ treatments are as in Fig 2. **(B)** Aminoacylation of *in vitro*-transcribed tRNA^{Other} with [^{14}C]lysine with 1 μM of LysRS1 (squares), 50 nM of LysRS2 (circles) or both together (diamonds). The background level of aminoacylation determined in the absence of enzyme was ~0.2 pmol. **(C)** Aminoacylation of *in vitro*-transcribed tRNA^{Trp} (circles) and tRNA^{Other} (squares) with [^{3}H]tryptophan and 50 nM of *Bacillus cereus* TrpRS.

aminoacylation. Conversely, LysRS1:LysRS2 may provide an alternative to charging tRNA^{Other} with an unknown non-canonical amino acid by another aaRS, as is the case for pyrrolysine. One candidate aaRS is the tryptophanyl-tRNA synthetase found in *B. cereus*, the equivalent of which from *Deinococcus radiodurans* can use 4-nitrotryptophan and 5-hydroxytryptophan (Buddha & Crane, 2005). Further investigation of these hypotheses now requires an understanding of whether aminoacyl-tRNA^{Other} can function in protein synthesis, as indirectly suggested by its ability to bind EF-Tu, and if it can, what codons it reads and what amino acids these ultimately specify.

METHODS

Preparation of bacterial strains, production and purification of enzymes and RNA, immunoblotting, RT–PCR, aminoacylation assays and gel electrophoresis of nucleic acids are described in the supplementary information online.

Isolation and characterization of chargeable species by EF-Tu affinity chromatography. Total tRNA (10 mg) from *B. cereus* was charged with 2 mM L-lysine in the presence of LysRS1 and LysRS2 for 20 min. Aminoacylated RNA species were purified using a *T. thermophilus* EF-Tu column, as described (Ribeiro *et al*, 1995). The eluate was deacylated and separated by 2D denaturing gel electrophoresis. Five bands were extracted and ethanol precipitated. RNA species (300 ng) were ligated with 40 pmol of oligo O1 (5′-AGGATCCTGCAGGCTCTTCC-3′, 5′ phosphorylated and 3′ blocked with dideoxy cytosine) using 20 U of T4 RNA ligase (New England BioLabs, Ipswich, MA, USA). Anchored tRNAs were reverse transcribed using oligo O1 (−; complementary to O1) and Superscript Reverse Transcriptase II (Invitrogen, Carlsbad, CA, USA), as described previously (Kapushoc *et al*, 2002). The product was digested with ribonuclease (RNase) H and RNase A (Invitrogen), phenol and chloroform extracted and ethanol precipitated. Single-stranded cDNA was ligated with 40 pmol of oligo O2 (5′-GTAAGCTTAATACGACTCACTATAG-3′, 5′ phosphorylated and 3′ blocked with dideoxy cytosine) using 20 U of T4 RNA ligase. After phenol and chloroform extraction, the DNA was ethanol precipitated and PCR was performed using oligos O1 (−) and O2 (−; complementary to O2). The PCR product was separated in a 2.5% agarose gel and fragments between 60 and 180 nucleotides were gel extracted, cloned into TOPO-TA blunt end (Invitrogen) and sequenced.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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