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Citation: Thiaville, Patrick C. et al. "Essentiality of Threonylcarbamoyladenine (T⁶A), a Universal tRNA Modification, in Bacteria: T⁶A Essentiality." *Molecular Microbiology* 98.6 (2015): 1199–1221.

As Published: <http://dx.doi.org/10.1111/mmi.13209>

Publisher: Wiley Blackwell

Persistent URL: <http://hdl.handle.net/1721.1/105541>

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

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Published in final edited form as:

Mol Microbiol. 2015 December ; 98(6): 1199–1221. doi:10.1111/mmi.13209.

Essentiality of threonylcarbamoyladenine (t⁶A), a universal tRNA modification, in bacteria

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Abstract

Threonylcarbamoyladenine (t⁶A) is a modified nucleoside universally conserved in tRNAs in all three kingdoms of life. The recently discovered genes for t⁶A synthesis, including *tsaC* and *tsaD*, are essential in model prokaryotes but not essential in yeast. These genes had been identified as antibacterial targets even before their functions were known. However, the molecular basis for this prokaryotic-specific essentiality has remained a mystery. Here, we show that t⁶A is a strong positive determinant for aminoacylation of tRNA by bacterial-type but not by eukaryotic-type isoleucyl-tRNA synthetases and might also be a determinant for the essential enzyme tRNA^{Ile}-lysidine synthetase. We confirm that t⁶A is essential in *Escherichia coli* and a survey of genome-wide essentiality studies shows that genes for t⁶A synthesis are essential in most prokaryotes. This essentiality phenotype is not universal in Bacteria as t⁶A is dispensable in *Deinococcus*

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radiodurans, *Thermus thermophilus*, *Synechocystis* PCC6803 and *Streptococcus mutans*. Proteomic analysis of t⁶A⁻ *D. radiodurans* strains revealed an induction of the proteotoxic stress response and identified genes whose translation is most affected by the absence of t⁶A in tRNAs. Thus, although t⁶A is universally conserved in tRNAs, its role in translation might vary greatly between organisms.

Keywords

tRNA; maturation; translation; modified nucleosides; t⁶A

Introduction

Complex tRNA modifications, often requiring multiple enzymes for their synthesis, are found in the anticodon loop at positions 34 or 37 of many tRNAs, and these modifications are critical for accurate decoding of mRNA codons (Agris *et al.*, 2007, El Yacoubi *et al.*, 2012). Threonylcarbamoyladenine (t⁶A₃₇) is one of the few universal complex modifications in the anticodon loop at position 37 (Jühling *et al.*, 2009). It is found in virtually all tRNAs decoding ANN codons, and its biosynthetic pathway was recently elucidated (Deutsch *et al.*, 2012), revealing major differences between the three domains of life (Thiaville *et al.*, 2014b).

Two core enzyme families are required for t⁶A synthesis in all domains of life (Figure 1) (El Yacoubi *et al.*, 2011 El Yacoubi *et al.*, 2009, Wan *et al.*, 2013, Thiaville *et al.*, 2014a). The first is TsaC (Tcs1 in Archaea and Eukarya) or its ortholog Sua5 [TsaC2 in Bacteria or Tcs2 in Archaea and Eukarya; see (Thiaville *et al.*, 2014b) for new gene nomenclature of t⁶A synthetic genes]; TsaC2 differs from TsaC by an additional C-terminal Rossman fold domain. The second is TsaD or its orthologs Kae1 (Tcs3), part of the KEOPS (Kinase Endopeptidase and Other Proteins of Small size) complex (renamed TCTC – threonylcarbamoyl transferase complex), or Qri7 (Tcs4) found in the mitochondria (Wan *et al.*, 2013, Thiaville *et al.*, 2014a). In addition, bacteria generally require TsaB and TsaE enzymes to synthesize t⁶A (Deutsch *et al.*, 2012, Lauhon, 2012), whereas archaea and eukaryotes require the other subunits of the KEOPS (TCTC) complex [Cgi121 (Tcs7), Pcc1 (Tcs6), Bud32 (Tcs5) and the yeast specific Gon7 (Tcs8) (Daugeron *et al.*, 2011, Perrochia *et al.*, 2013, Zhang *et al.*, 2015)].

Another major difference between kingdoms in t⁶A biosynthetic enzymes is the essentiality of the corresponding genes. None of the t⁶A synthesis genes are essential in *Saccharomyces cerevisiae*, although deletion of *SUA5* or *KAE1* leads to severe growth phenotypes (Na *et al.*, 1992, Kisseleva-Romanova *et al.*, 2006). t⁶A synthesis genes could also be deleted in *Schizosaccharomyces pombe* (Kim *et al.*, 2010, Spirek *et al.*, 2010), and T-DNA insertions have been isolated in most t⁶A synthesis genes in *Arabidopsis thaliana* (Alonso *et al.*, 2003) (Table 1), however, follow up studies are required to rigorously assess their dispensability. Although t⁶A synthesis genes are dispensable in eukaryotes, this is not the case in most prokaryotes. Indeed, all t⁶A synthesis genes are essential in the methanogenic archaeon *Methanococcus maripaludis* (Sarmiento *et al.*, 2013). In the halophilic archaeon *Haloflexax*

volcanii, only *pcc1* could be deleted, reducing the t⁶A content of tRNAs only slightly (Naor *et al.*, 2012) (Table 1). The four *tsaBCDE* genes are individually essential in *Escherichia coli* and many other gram-positive and gram-negative bacteria (Table 2). Because of their prokaryotic-specific essentiality, these genes had been identified as potential antibacterial targets even before their role in t⁶A synthesis was established, and inhibitors of TsaE were developed based on its ATP binding capabilities (Arigoni *et al.*, 1998, Freiberg *et al.*, 2001, Allali-Hassani *et al.*, 2004, Lerner *et al.*, 2007, Handford *et al.*, 2009). However, for t⁶A synthesis proteins to be considered viable targets for antibiotics, it is critical to understand their distribution profile as well as the reasons underlying their essentiality in bacteria.

In terms of phylogenetic distribution, 90% of the bacteria sequenced to date contain homologs of the four t⁶A synthesis proteins (Thiaville *et al.*, 2014b). A subset (2.2%) lack TsaB (Thiaville *et al.*, 2014b) and a few lack both TsaB and TsaE (Grosjean *et al.*, 2014). These latter organisms, which include pathogenic bacteria such as *Mycoplasma haemofelis* and *Mycoplasma suis* strain Illinois, would, therefore, not be sensitive to inhibitors targeting TsaB. Saturating mutagenesis or whole genome single gene deletion studies showed that t⁶A synthesis genes were essential in 11 out of 13 cases, with the possible exceptions being two reports on *Vibrio cholerae* (Table 2). Interestingly, the *V. cholerae* genomes contain both the *tsaC* and *sua5* (*tsaC2*) forms, and either gene alone is sufficient for growth (Chao *et al.*, 2013, Kamp *et al.*, 2013). Finally, t⁶A synthesis genes can be deleted in some bacteria, for example, *tsaD* and *tsaB* in *Deinococcus radiodurans* (Onodera *et al.*, 2013); *tsaD* in *Synechocystis* PCC 6803 (Zuther *et al.*, 1998); and, *tsaE* in *Streptococcus mutans* (Bitoun *et al.*, 2014). Some discrepancies were found in *Bacillus subtilis*, where all four t⁶A genes were found to be essential in a global transposon study (Table 2), but two of them, *ywIC* (*sua5* homolog) and *ydiB* (*tsaE* homolog) were found to be dispensable in follow-up targeted studies (Hunt *et al.*, 2006). Yet again, because these studies were performed before the t⁶A biosynthetic pathway had been fully elucidated, it is not known if t⁶A is actually absent in these mutants, or whether other routes for t⁶A synthesis exist in these specific organisms.

Comparison of the translation machineries in the different kingdoms could explain the essentiality of t⁶A synthesis enzymes in bacteria and archaea but not eukaryotes. t⁶A has been proposed to be a determinant for charging of tRNA^{Ile}_{GAU} by isoleucyl tRNA synthetase (IleRS) in *E. coli* (Nureki *et al.*, 1994, Giegé & Lapointe, 2009), while it is not a major determinant for the yeast IleRS, which requires Inosine 34 for the major tRNA^{Ile}_{AAU} and Ψ₃₄ (and perhaps Ψ₃₆) for the minor tRNA^{Ile}_{UAU} (even though t⁶A might contribute somewhat to the charging efficiency) (Senger *et al.*, 1997, Giegé & Lapointe, 2009). While a difference in the role of t⁶A as a determinant for the bacterial-type but not the eukaryotic-type IleRSs could explain its essentiality in Bacteria and not in yeast, it would not account for the essentiality of t⁶A in Archaea, which harbor eukaryotic-type IleRS enzymes (Ibba *et al.*, 1999).

Another potential basis for the prokaryotic specific essentiality of t⁶A is if it is a determinant for tRNA^{Ile} lysidine synthase (TilS) and agmatidine synthase (TiaS). In most prokaryotes, but not eukaryotes, the codon AUA is decoded by a tRNA^{Ile} bearing a CAU anticodon in which C is modified to lysidine (k²C) by TilS in Bacteria or to agmatidine (agm²C) by TiaS in Archaea (Soma *et al.*, 2003, Ikeuchi *et al.*, 2010, Mandal *et al.*, 2010). Since the

corresponding genes, *tisS* and *tiaS*, are essential (Soma *et al.*, 2003, Blaby *et al.*, 2010), the essentiality of t⁶A in prokaryotes could be due to an indirect role in k²C and agm²C synthesis. However, published biochemical studies seem to rule out such a role as unmodified transcripts of tRNA^{Ile}_{CAU} are efficiently recognized by the TisS and TiaS enzymes (Ikeuchi *et al.*, 2005, Ikeuchi *et al.*, 2010, Osawa *et al.*, 2011, Köhrer *et al.*, 2008). Finally, since a *tsaC* mutant can act as a suppressor of *prfA1* encoding a thermosensitive (ts) Release Factor 1 (RF1) in *E. coli* (Kaczanowska & Ryden-Aulin, 2004, Kaczanowska & Rydén-Aulin, 2005), and the absence of t⁶A in tRNAs is known to increase read-through of termination codons in yeast (Lin *et al.*, 2009), the essentiality of t⁶A in bacteria could also be due to an unforeseen interplay with translation termination (Dreyfus & Heurgue-Hamard, 2011).

In *E. coli*, it has been reported that the growth phenotype caused by the depletion of TsaB, TsaD or TsaE was partially suppressed by overexpression of *rstA*, part of a two-component-system response regulator (Handford *et al.*, 2009, Campbell *et al.*, 2007). This suggested that these genes belonged to a complex regulatory network (Msadek, 2009), but the *rstA* suppression phenotype has never been really explained. Overexpression of other genes such as *rho*, encoding a transcription terminator, or *dnaG*, encoding DNA primase, have also been reported to suppress the essentiality phenotype caused by depletion of the t⁶A synthesis enzymes (Bergmiller *et al.*, 2012, Hashimoto *et al.*, 2011, Hashimoto *et al.*, 2013), but it is unknown if t⁶A is absent from tRNA isolated from these suppressor strains. Lack of t⁶A-modified tRNA in these strains would compromise the development of antibacterials targeting the enzymes of t⁶A machinery, as resistant clones could be selected at a high frequency.

Finally, phenotypes deriving from the absence or reduction of t⁶A enzymes in prokaryotes are very diverse, and include defects in cell division or in stress responses, increased glycation of proteins, or cyanophycin accumulation (Handford *et al.*, 2009, Allali-Hassani *et al.*, 2004, Bitoun *et al.*, 2014, Zuther *et al.*, 1998, Oberto *et al.*, 2009, Katz *et al.*, 2010, Bergmiller *et al.*, 2011). However, the molecular basis for these pleiotropic phenotypes has not been established, and it is unknown whether these phenotypes are all due to translation defects caused by the absence of the modification, whether t⁶A has roles in the cell not linked to translation, or whether the genes for t⁶A biosynthesis have roles in the cell unrelated to t⁶A synthesis.

In view of these uncertainties and conflicting reports, and the importance of t⁶A enzymes in the bacteria analyzed to date, t⁶A levels were measured in different derivatives of *E. coli* reported to have circumvented the essentiality of t⁶A synthesis enzymes and in t⁶A gene deletion mutants of *Synechocystis*, *D. radiodurans* and *S. mutans*. This analysis revealed that t⁶A is strictly essential in *E. coli* but not in all bacteria. Through a combination of biochemical, genetic and proteomic experiments, we set out to understand these differences in t⁶A function along the bacterial phylogenetic tree.

Results

t⁶A is present in *E. coli* derivatives reported to have suppressed the essentiality of t⁶A genes

As discussed above, conditions in which *E. coli* survives in the absence of t⁶A synthesis enzymes have been reported. However, since most of these reports were published before the role of the *tsaBCDE* genes in t⁶A synthesis was established, we set out to measure t⁶A levels in these *E. coli* derivatives.

Deletion of the first four amino acids of TsaC was shown to suppress the growth defects of a *prfA1* strain at non-permissive temperature (*prfA1* encodes a thermosensitive RF1) (Kaczanowska & Ryden-Aulin, 2004). The two mutations $\Delta 1-4tsaC$ and *prfA1* could not be separated, and the presence of the $\Delta 1-4tsaC$ allele led to defects in ribosome maturation, hence the name *rimN* (Kaczanowska & Rydén-Aulin, 2005). In order to assess if this suppression was accompanied by a change in t⁶A levels, we compared t⁶A levels in the single mutant *prfA1* (US477), the double mutant $\Delta 1-4tsaC$ *prfA1* (MRA100), and the isogenic wild-type *E. coli* strain (US475). As shown in Figure 2, cells carrying the *prfA1* allele or both $\Delta 1-4tsaC$, *prfA1* alleles contained t⁶A in tRNAs. In fact, in two independent experiments it was found that both strains contained a higher level of t⁶A (~2-3 times more) than the parental strain. Eliminating the first four amino acids of TsaC clearly does not lower t⁶A levels in tRNAs and, therefore, suppression of the *prfA1* thermosensitive phenotype is not due to a global loss or reduction of t⁶A modification in tRNAs.

Overexpression of the response regulator *rstA* has been reported to partially suppress the essentiality phenotype of TsaB, TsaD, or TsaE depletion (Campbell *et al.*, 2007, Handford *et al.*, 2009). First, we sought to duplicate these results using the strains from the above-mentioned studies. In these strains, the chromosomal copies of the *tsaB*, *tsaD*, or *tsaE* genes, respectively, have been placed under the control of the arabinose-inducible P_{BAD} promoter and hence the cells do not grow in the absence of arabinose. Transforming these strains with *prstA* that expresses the *rstA* gene under the control of an IPTG inducible promoter allowed growth of the three strains on LB agar in the absence of the arabinose inducer (Figure 3A). This suppression was not observed with the parent control vector pKK223-3 (Campbell *et al.*, 2007). t⁶A levels were then analyzed from tRNAs of *tsaD* P_{BAD}:*tsaD* (*prstA*) (VDC9607) cells grown in absence of arabinose under conditions where the control *tsaD* P_{BAD}:*tsaD* (pKK223-3) (VDC9606) strain did not grow (data not shown). As shown in Figure 3B, tRNAs extracted from *tsaD* P_{BAD}:*tsaD* (*prstA*) grown in absence of arabinose contain t⁶A, even if levels under non-induced conditions are lower (33% less).

These results suggested that the known leakiness of the P_{BAD} promoter in the absence of the inducer arabinose (Campbell *et al.*, 2007) is sufficient to sustain cells with enough of the TsaD enzyme to assure t⁶A synthesis and growth. Therefore, a P_{TET} promoter, which is known to be more tightly controlled, was used for subsequent studies. *prstA* was used to transform a P_{TET}:*tsaD* strain where the native *tsaD* promoter was replaced by the anhydrotetracycline (aTc) inducible P_{TET} promoter (VDC5801) (El Yacoubi *et al.*, 2011). In this genetic set up, over-expression of *rstA* in P_{TET}:*tsaD* did not allow growth in the absence of the inducer (aTc) (Figure 4A). A similar result was observed in P_{TET}:*tsaC* strain

(VDC5684) where the *tsaC* gene encoding the first enzyme of the t⁶A pathway is under P_{TET} control (Gerdes *et al.*) (Figure 4B). The P_{BAD}:*tsaC* plasmid can complement growth of the P_{TET}:*tsaC* strain in the absence of any inducer [(as previously shown (Gerdes *et al.*)], confirming that low TsaC levels allow growth of *E. coli* (Figure 4B). Our results therefore suggest that high expression of *rstA* allows cells to grow with low levels of t⁶A (P_{BAD} backgrounds) but not with zero t⁶A (P_{TET} backgrounds).

In summary, our results suggest that to date the essentiality of t⁶A has never been suppressed in *E. coli* K12. These results, in combination with the observation that all four t⁶A synthesis genes are individually essential in *E. coli* K12 (Table 2), strongly support the essential nature of this universal modification in this model organism.

The essentiality of the t⁶A modification of tRNA in *E. coli* is not strain dependent

Since many of the suppression studies described above had been performed in different *E. coli* K12 strains, we therefore explored if the essentiality phenotype was dependent on the strain background. The P_{TET}:*tsaD* and P_{TET}:*tsaC* alleles from BW25113 were transferred by P1 transduction into *E. coli* B and *E. coli* C600 [VDC7061/62, VDC7065/66, VDC7069/70 and VDC7073/74, respectively]. The transduced alleles were checked by PCR (Figure S1), and tested for β-galactosidase activity (donors are LacZ⁻ and both recipients are LacZ⁺) and for growth in the absence of aTc. In the C600 background, P_{TET}:*tsaC* and P_{TET}:*tsaD* transductants required aTc for growth, like in the BW25113 donor derivatives (Table 3). However, the *E. coli* B transductants of both P_{TET}:*tsaC* and P_{TET}:*tsaD* grew in the absence of aTc (Table 3). To test if this growth was due to mutations rendering the P_{TET} promoter constitutive, these strains were used as donors for P1 transduction back into BW25113. Growth of four BW25113 P_{TET}:*tsaC* back transductants remained aTc independent (Figure S2), suggesting that mutations in the P_{TET} promoter made it constitutive, or that suppressor mutations that allow growth in the absence of t⁶A and are co-transduced with the P_{TET}:*tsaC* allele are present in the *E. coli* B P_{TET}:*tsaC* derivatives. Analysis of bulk tRNA extracted from *E. coli* B P_{TET}:*tsaC* (VDC7066) grown in LB without aTc inducer shows wild-type levels of t⁶A (Figure S1), suggesting mutations making P_{TET} constitutive had occurred. Whole genome sequencing of VDC7066, as detailed in the Supplemental Results Section, revealed that the *tetR* allele contains a T1173G change, creating a non-synonymous Q38P mutation. This mutation is located in the TetR DNA binding site (Ramos *et al.*, 2005) and most likely makes the expression of P_{TET} promoter constitutive. On the other hand, growth of four back-transductants of BW25113 P_{TET}:*tsaD* were dependent on aTc, unlike the *E. coli* B P_{TET}:*tsaD* donor (VDC7062) (Figure S2), hence the P_{TET} promoter was still regulated. This left three possible explanations: 1) *tsaD*, and therefore t⁶A in tRNA, is not essential in the *E. coli* B background; 2) a suppressor mutation that allows expression of the P_{TET}:*tsaD* allele in the absence of aTc is present in the *E. coli* B donor but did not transduce with the P_{TET}:*tsaD* allele; 3) a suppressor mutations makes *tsaD* dispensable for t⁶A synthesis. Here too, the presence of wild-type levels of t⁶A in bulk tRNA extracted from VDC7062 grown without inducer suggested the first explanation explanation is not correct (Figure S1). The whole genome sequence of VDC7062 was determined as reported in the Supplemental Results Section but this did not

provide any obvious explanation as to why VDC7062 does not require aTc for growth, so we could not discriminate between that last two hypotheses.

Our results show that t⁶A genes are essential in a least two, and maybe three different *E. coli* backgrounds, and show that the genetic set-ups that limit the expression of t⁶A synthesis genes are unstable under high selective pressure to express these essential genes.

t⁶A is a positive determinant for *E. coli* IleRS

The identity elements for the 20 aminoacylation systems of *E. coli* have been analyzed, and the isoleucine system was the only one proposed to be dependent on t⁶A for identity determination [for review see (Giegé *et al.*, 1998)]. The recent discovery of the t⁶A machinery and the ability to modify transcripts *in vitro* now allow addressing this experimentally.

To test whether t⁶A at position 37 is required for *E. coli* IleRS to charge the major *E. coli* isoleucine tRNA (tRNA^{Ile}_{GAU}), the corresponding T7 transcript was generated with and without t⁶A at position 37. As shown in Figure 5A, under the specific conditions used in here and at early time points, *in vitro* aminoacylation of the t⁶A-containing tRNA^{Ile}_{GAU} transcript using *E. coli* IleRS has a significantly faster aminoacylation rate (at least 25-fold higher) than that of unmodified tRNA^{Ile}_{GAU} transcript. Basically a similar result was obtained with bulk tRNA from *S. cerevisiae* wild-type and bulk tRNA from a yeast mutant specifically lacking t⁶A (Figure 5B), supporting the hypothesis that t⁶A at position 37 acts as a strong positive determinant for *E. coli* IleRS. In contrast, *S. cerevisiae* IleRS present in a total S100 extract aminoacylates bulk tRNA from yeast with similar rates irrespective of the t⁶A modification state and shows only a moderate preference for unmodified over modified *E. coli* tRNA^{Ile}_{GAU} transcript (Figure 5C-D).

t⁶A improves the efficiency of the *E. coli* TilS enzyme *in vitro*

In most bacteria, the anticodon of the minor isoleucine tRNA (tRNA^{Ile}_{CAU}) is k²CAU, where k²C stands for lysidine. tRNA^{Ile}_{CAU} is transcribed as a pre-tRNA with the anticodon CAU, which is subsequently modified by TilS to k²CAU (Muramatsu *et al.*, 1988b, Muramatsu *et al.*, 1988a). Lysidine at the wobble position is required for recognition of the tRNA by bacterial IleRS (instead of by MetRS) and for the faithful decoding of AUA codons. The C-terminal domain of TilS binds the anticodon of tRNA^{Ile}_{CAU}, and mutation of A37 to G37 was shown to reduce the activity of TilS (Ikeuchi *et al.*, 2005), suggesting that t⁶A might also be important for TilS activity.

A T7 transcript of *E. coli* tRNA^{Ile}_{CAU} was generated and modified *in vitro* to harbor t⁶A at position 37. As shown in Figure 5E, the t⁶A-containing transcript is a better substrate (approx. 13-fold) for *E. coli* TilS compared to the unmodified transcript. Thus, although the t⁶A-mediated enhancement of TilS activity is not as pronounced as that of IleRS activity described above, t⁶A could also be a positive determinant for *E. coli* TilS in the context of an otherwise unmodified *E. coli* tRNA^{Ile}_{CAU} transcript.

The t⁶A essentiality phenotype of *E. coli* cannot be suppressed

In a final attempt to dissect t⁶A essentiality genetically, a direct search for suppressors of t⁶A absence using the P_{TET}: *tsaC* strain (VDC5684) was performed. In this strain, *tsaC* is cotranscribed with the *aroE* gene (Serina *et al.*, 2004), so any mutation that makes the P_{TET} promoter constitutive would eliminate the auxotrophy for aromatic amino acids. Derivatives with suppressor mutations not affecting the promoter would remain auxotrophic for aromatic amino acids. In repeated attempts, we failed to isolate any auxotrophic suppressors; all the aTc independent mutants isolated grew in the absence of amino acids (data not shown).

The failure to identify true suppressors lacking t⁶A in tRNA in our laboratory and in others as discussed above, together with the requirement of t⁶A for both *E. coli* IleRS charging and TilS activity *in vitro* suggests that t⁶A may be required for at least two essential cellular processes in *E. coli*. To separate decoding of AUA by a lysidine-modified tRNA from charging by IleRS, we designed several genetic strategies to circumvent these two essentiality factors in order to attempt the generation of a viable t⁶A depleted strain. These strategies relied on: 1) the observation that eukaryotic-type IleRSs from yeast and other organisms are functional in *E. coli* (Racher *et al.*, 1991, Sassanfar *et al.*, 1996); 2) yeast IleRS (and possibly other bacterial IleRS of eukaryotic-type) do not require t⁶A as a determinant for efficient charging while *E. coli* IleRS does [(Giegé & Lapointe, 2009) and results above], and; 3) expressing *Mycoplasma mobile* tRNA^{Ile3}_{UAU}:IleRS pair under the P_{LAC} promoter (pNB26'2) (Taniguchi *et al.*, 2013, Bohlke & Budisa, 2014) can complement the *E. coli* *tilS* lethality phenotype and allows growth in the absence of k²C. Consistent with findings observed so far, repeated attempts to circumvent t⁶A essentiality in *E. coli* by co-expression of t⁶A independent IleRS and k²C independent tRNAs failed (Supplemental Results).

These negative results strongly support the possibility that the causes for t⁶A essentiality in *E. coli* are multiple and not yet fully understood and led us to explore other bacterial model organisms.

t⁶A is not essential in all bacteria

While, the results above confirmed that, at least for *E. coli*, t⁶A is strictly required for growth, derivatives harboring mutations in t⁶A genes have been reported for *D. radiodurans* R1, *Synechocystis* sp. PCC 6308 and *S. mutans* UA159 (Table 2). In order to test whether the deletion of t⁶A biosynthesis genes in these bacteria correlates with the loss of t⁶A in tRNAs, the levels of t⁶A were measured in *D. radiodurans* R1, *tsaD*(XYD), *tsaB*(XYZ) and *tsaD tsaB*(WDZ); *Synechocystis* sp. PCC 6803 and *tsaD*(gcp); as well as *S. mutans* UA159, *tsaE*(JB409) and *tsaE*(*ptsaE*)(JB409c) derivatives. In all cases, t⁶A was not detectable in tRNAs extracted from any of the mutant strains, while t⁶A was readily detected in tRNA extracted from the corresponding wild-type and complemented strains (Figure 6). Therefore, these bacteria are able to cope with the absence of t⁶A in tRNA (at least in laboratory conditions), while others, like *E. coli*, cannot (Table 2). We then set out to construct a *tsaC* null mutant in *Thermus thermophilus* HB8 under the assumption that, as this organism belongs to the Deinococcus-Thermus clade, it would be viable like *D.*

radiodurans. As predicted, the Δ *TtsaC* strain was viable and tRNA analysis showed that this mutant also lacked detectable levels of t⁶A (Figure 7).

t⁶A is not a determinant for IleRS and TilS in all Bacteria

It is possible to partially rationalize the dispensability of t⁶A in *D. radiodurans* and *T. thermophilus* as these bacteria carry a eukaryotic-like and not a bacterial-like IleRS (Woese *et al.*, 2000). However, both *Synechocystis* and *S. mutans* have bacterial-like IleRSs (Woese *et al.*, 2000). To test the possibility that in the course of deleting the t⁶A synthesis genes suppressor mutations alleviating the requirement of t⁶A for IleRS charging occurred in these latter organisms, the *S. mutans* *tsaE* mutant (SMU_409) and the parental *S. mutans* UA159 were sequenced. The parental strain contained 33 mutations when compared to the reference sequence (Dataset 1). These 33 variations were also present in *tsaE* (SMU_409).

Interestingly, one of these variations is a change of G to A at position 224 conferring a non-synonymous amino acid change of R75G in SMU_13 (TilS). This residue is not located in the tRNA binding region, hence further biochemical characterization studies are required to test if this mutation makes the *S. mutans* TilS t⁶A independent. In addition to these 33 variations, the *tsaE* (SMU_409) strain contained a mutation conferring a K520N change in the ATP-dependent nuclease Rex A (SMU_1499). Given that this mutation is not obviously linked to a change in determinants for the *S. mutans* IleRS, we concluded that t⁶A is not a positive determinant for IleRS in *S. mutans*, and is the most parsimonious explanation for the growth seen in *Synechocystis* *tsaD*.

The *D. radiodurans* TilS protein, like its relative from the *Deinococcus-Thermus* phylum clade, is fused to a cytidine deaminase domain of unknown function that could affect its recognition of target tRNA (Figure 8). To address the question of whether t⁶A is a positive determinant for the TilS enzyme in *D. radiodurans* and *T. thermophilus*, purified bulk tRNAs from the *D. radiodurans* R1 and *T. thermophilus* t⁶A⁻ mutants were analyzed, and k²C was present both in *D. radiodurans* and *T. thermophilus* t⁶A⁻ strains (Figure 9). LC-MS detection and subsequent MS/MS verification confirmed the presence of k²C at positions 34 in the anticodon loop of the minor tRNA^{Ile}_{CAU} in both wild-type and mutant *T. thermophilus* (Figure 9D-E). Thus, t⁶A is clearly not a determinant for TilS in these organisms.

Consequences of t⁶A deficiency on the *D. radiodurans* proteome

At the phenotypic level, the initial Onodera study had found that t⁶A deficient *D. radiodurans* strains were more sensitive to mitomycin C, which induces inter-strand DNA cross-links, but had not observed differences in growth rates between the t⁶A mutants and the parental strain (Onodera *et al.*, 2013). We reproduced this observation both in microtiter plates and in flasks, although the mutants consistently showed a longer lag time (data not shown). To gain further insight into the proteome changes in response to the absence of t⁶A, we performed a shotgun proteomics comparison of the *D. radiodurans* wild-type and *tsaD* strains at mid-log and late-log growth stages and sample were normalized for protein content. A total of 998 proteins were identified when merging the sixteen samples (4 conditions × 4 biological replicates) and quantified by spectral count. At mid-log growth, 53 proteins were significantly increased in the mutant compared to wild-type, while 68 were decreased (Table 4 and S4). At late-log growth, 103 proteins were significantly increased in

the mutant, while 66 were decreased (Table 5 and S5). The most reduced protein in the mutant at mid log is ribosomal protein L27p (DR_0085) (Table 4). The corresponding gene in *E. coli* is not essential, but mutants have greatly impaired growth rates, and are cold and heat sensitive (Wower *et al.*, 1998). Thus, the absence of any observed growth phenotype in *D. radiodurans* mutants is surprising, considering that the predicted fitness cost of a DR_0085 deletion is high (Table 4).

Using the MetaCyc Omics viewer (Caspi *et al.*, 2014), enrichments in the down-regulated genes for the acetyl-coA to butyrate fermentation pathway (P -value: 2×10^{-4}) and isoleucine degradation pathway (P -value: 8×10^{-4}) were observed in the late-log experiment. No pathway enrichment with P -values $< 10^{-3}$ were observed in the up-regulated genes using the MetaCyc Omics viewer. As stress is not well covered by the MetaCyc pathway enrichment tools, we performed a manual analysis that revealed that $>20\%$ (4/19 in the mid-log and 5/21 in the late-log sets) of the proteins whose expression levels increased over three-fold in the t^6A^- strains were related to protein homeostasis or other types of stress (Table 4 and 5). A total of 83 stress proteins have been catalogued in *D. radiodurans* R1 [Table 3 of (Makarova *et al.*, 2001)], and while this is certainly an underestimation, it still represents 2.5% (83 of 3261 CDS) of the *D. radiodurans* theoretical proteome. Hence, stress proteins are significantly enriched in the mutant (χ^2 , $P > 0.001$). These include chaperones [DR_0128 (GrpE) and DR_0606 (GroES)], the peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8) protein (DR_1063), and the cold-shock DEAD-box protein (DR_1624). Arginase (DR_0651) is also highly induced at both mid-log and late log-growth in the t^6A^- deficient strain. Increasing arginase lowers nitric oxide (NO) levels, and it has been shown that NO up-regulates transcription of *obgE*, a gene involved in bacterial growth proliferation and stress response in *D. radiodurans* (Patel *et al.*, 2009).

To assess if proteins whose levels are reduced in the t^6A^- strains are enriched in t^6A (ANN) codons, several types of analyses were performed as detailed in the supplemental results section. We investigated enrichment of t^6A dependent Ser and Arg codons, as well as abundance of and length of stretches of t^6A dependent codons in all *D. radiodurans* coding sequences (Tables S6-S9). In summary, there was a small general enrichment of t^6A dependent codon stretches in down-regulated proteins, but the significance of this observation is not clear. However, a few down-regulated proteins were clearly enriched in t^6A codons. For example ClpB (DR_1046) has a stretch of seven t^6A dependent codons (Table S8) and its expression was reduced nearly 4-fold in the t^6A^- mutant (Table 4).

Discussion

Maintaining t^6A in tRNA must confer a fitness advantage as its biosynthetic pathway is present in genomes of all self-replicating organisms sequenced to date (Thiaville *et al.*, 2014b). The consequences of t^6A deficiency, however, vary greatly from one kingdom to another and even from one clade to another. Additionally, t^6A synthesis genes could play additional roles in the cell unrelated to t^6A biosynthesis, making the study of the genes very difficult. Here, we focused on the essentiality phenotype of t^6A^- strains in bacteria and showed that while t^6A is essential in most bacteria this is not a universal feature of the kingdom. An obvious practical consequence of this observation is that antibacterial

compounds targeting this pathway may not be broad spectrum, although in most organisms of clinical interest t⁶A is essential.

Notably, we clearly demonstrate that t⁶A is a positive determinant of the *E. coli* IleRS for charging the major tRNA^{Ile}_{GAU}. Previous work from the Suzuki laboratory demonstrated that this modification, in combination with k²C, is a determinant of both *E. coli* and *M. mobile* IleRS for the charging of the minor tRNA^{Ile}_{CAU} (Taniguchi *et al.*, 2013). Because t⁶A is not required for charging by yeast IleRS, this may be the basis for the bacteria-specific essentiality of t⁶A. However, the presence of a eukaryotic type IleRS in some bacteria, such as members of the *Thermus-Deinococcus* clade, may circumvent the necessity of t⁶A. Additional mechanisms circumventing this requirement in bacterial-like IleRS could occur and further biochemical characterization of the IleRS/tRNA^{Ile} pairs of *S. mutans* and *Synechocystis* will be required to test whether t⁶A is also a determinant for IleRS in these organisms. Kinetic measurements showing that transcripts are very poor substrates for *Streptococcus pneumoniae* IleRS suggest that t⁶A may be a determinant for IleRS in *Streptococci* (Shepherd & Ibba, 2014).

We also show that *E. coli* tRNA^{Ile}_{CAU} requires t⁶A for efficient lysidinylation by *E. coli* TilS *in vitro*. Ikeuchi *et al.* compared the kinetics of lysidinylation of purified *E. coli* tRNA^{Ile}_{CAU} lacking lysidine but possessing all the other modifications (s⁴U₈, G_{m18}, D_{20a}, D_{20b}, t⁶A₃₇, ψ ₃₉, m⁷G₄₆, acp³U₄₇, T₅₄, and ψ ₅₅) to that of unmodified tRNA^{Ile}_{CAU} prepared by *in vitro* run-off transcription and concluded that none of the other modifications are strictly required for lysidine formation *in vitro* (Ikeuchi *et al.*, 2005). However, mutation of A37 to G37 has been shown to reduce the $k_{cat}/K_m \sim 15$ -fold (Ikeuchi *et al.*, 2005), suggesting that A37 may play an in lysidinylation of tRNA^{Ile}_{CAU}, and we show here a significant enhancement in the rate of lysidine formation in the presence of t⁶A. Taken together, these data clearly demonstrate that t⁶A enhances the efficiency of TilS.

At odds with the data from *E. coli* TilS is the observation that in species such as *Deinococcus*, k²C is present in t⁶A deficient mutants. Two types of TilS have been structurally characterized. The type I enzymes, exemplified by *E. coli* TilS, comprise an N-terminal domain (NTD) and two C-terminal domains (CTD1, CTD2), while the type II enzymes possess the NTD and only CTD1 (Ikeuchi *et al.*, 2005, Soma *et al.*, 2003). A study on the molecular basis of recognition and catalysis of types I and II TilS enzymes revealed variances in recognition elements owing to the difference in CTDs (Nakanishi *et al.*, 2005). In type I enzymes a large conformational change accompanies tRNA binding, a phenomenon that is not observed in type II enzymes. Type I TilS recognizes the base pairs C4-G69 and C5-G68 in the acceptor stem of tRNA^{Ile}_{CAU} via CTD2, which induces allosteric changes on the NTD for optimum positioning of C34 in the active site for lysidinylation (Ikeuchi *et al.*, 2005, Nakanishi *et al.*, 2005, Suzuki & Miyauchi, 2010). *D. radiodurans* TilS belongs to a subset of type I enzymes that harbor a putative cytidine deaminase encoded C-terminal to CTD2 (Figure 8). The structure for this TilS subtype is yet to be solved. A homology model of the *D. radiodurans* TilS was generated using *Geobacillus kaustophilus* TilS as the starting structure (PDB: 3A2K). The homology model of full length *D. radiodurans* TilS shows deviations in folding from *G. kaustophilus* TilS, notably in the NTD and CTD2, likely due to the presence of the extra domain. Interestingly, upon deletion of the putative cytidine

deaminase domain, the NTD and CTD1 domains adopt identical folding as *G. kaustophilus* TilS, while the CTD2 remains distorted. Thus, the mechanism of tRNA recognition by *D. radiodurans* TilS may differ from that of type I and II TilS due to the presence of an extra domain, which may account for t⁶A independence.

In addition to a role as determinants for IleRS and possibly TilS, t⁶A directly influences translational fidelity and could be especially important for the translation of specific, essential proteins. We have previously shown in yeast that more frame-shifting occurs in proteins that have stretches of t⁶A dependent codons (El Yacoubi *et al.*, 2011). Interestingly, analysis of the *D. radiodurans* genome revealed a low use of AUA codons (0.09% of all codons) as compared to *E. coli* (0.42%) and *H. volcanii* (0.22%). In fact, *D. radiodurans* rarely uses an A or U in the third position of any codon. As a consequence, there is only one putative essential gene (DR_2087, Table S9) in *D. radiodurans* that possesses two consecutive AUA codons. The gene encodes translation initiation factor IF3, which is reduced 1.8 fold in the t⁶A⁻ strain at mid-log (Table S4). However, the AUA codon usage for *Synechocystis* and *S. mutans* (0.44% and 0.78%, respectively) is similar to *E. coli*, and instances of sequential AUA codons are found in ~80 genes in both *Synechocystis* and *S. mutans* (Tables S13 and S14, respectively), similar to *E. coli* (79 genes with runs of AUA codons, Table S15), hence low usage of AUA codons cannot be the sole factor explaining the dispensability of t⁶A. Interestingly, while many of the genes with runs of AUA codons in *E. coli* and *S. mutans* are involved in essential processes, in *Synechocystis* the genes are mainly mobile elements or hypothetical genes (Tables S13-S15).

Recent systems level approaches integrating proteomics, codon usage, and modification profiling (Dedon & Begley, 2014, Gu *et al.*, 2014), mainly in eukaryotes, have shown that modifications can modulate the expression of specific genes or gene sets under stress. For example, exposure of yeast to the alkylating agent methylmethane sulfonate (MMS) causes increases in wobble mcm⁵U in tRNA^{Arg}_{UCU} that leads to enhanced translation of proteins from AGA-enriched genes (Begley *et al.*, 2007, Patil *et al.*, 2012a, Patil *et al.*, 2012b), while H₂O₂-induced stress increases the wobble m⁵C in tRNA^{Leu}_{CAA}, leading to increased translation of mRNAs for UUG-enriched genes (Chan *et al.*, 2012). Both the threonine degradation enzyme 2-amino-3-ketobutyrate coenzyme A ligase (EC 2.3.1.29) and the isoleucine degradation enzyme 3-isopropylmalate dehydratase large subunit (EC 4.2.1.33) are reduced 2.6 fold in the t⁶A⁻ strain and have stretches of over five t⁶A dependent ANN codons (Table S4 and S5). This could be a regulatory response if inhibiting isoleucine or threonine degradation (threonine is the isoleucine precursor) was a mechanism to compensate for poor charging of target tRNAs by IleRS when t⁶A is low. Further experiments are required to evaluate if the lower amounts of these catabolic enzymes are caused by poor translation of the ANN codons or by other regulatory mechanisms.

Our analysis of the *D. radiodurans* t⁶A⁻ proteome does suggest that the *in vivo* role of t⁶A in translation is going to be difficult to decipher because of the number of tRNAs involved (12 tRNAs are modified in *E. coli*) and the absence of a systematic understanding of the effects of t⁶A on tRNA levels, decoding efficiency and accuracy *in vivo*. More generally, the difficulty in elucidating the molecular basis for the essentiality or phenotypic consequences of t⁶A deficiency stems from the combination of direct and indirect effects that are difficult

to separate. An obvious indirect effect is the induction of heat-shock proteins that fits with the emerging realization that, in yeast, the absence of modifications in the anticodon loop results in mistakes in protein synthesis, leading to misfolding and activation of the proteotoxic response in yeast (Patil *et al.*, 2012a, Patil *et al.*, 2012b, Rezgui *et al.*, 2013, Nedialkova & Leidel, 2015). Our results suggest that this might also be the case for t⁶A, but further experimental work is required to assess if and how the absence of this modification leads to protein misfolding in *D. radiodurans* and in other organisms.

In conclusion, this work has laid the foundation for understanding the essentiality of t⁶A in Bacteria. We have clearly shown that there is not one unique cause for essentiality, and for some organisms such as *E. coli*, there may be multiple causes making antibacterial agents directed against t⁶A biosynthesis quite effective since target-based resistance mechanisms should not occur. In contrast, organisms such as *S. mutans* may not be sensitive to antibacterial agents targeting t⁶A biosynthesis, and the underlying mechanism that makes t⁶A dispensable in these organisms will require detailed biochemical studies.

Experimental Procedures

Bioinformatics

All genomes were downloaded from the PATRIC database (Wattam *et al.*, 2014). To examine the codon usage of whole genomes, a Java script located at http://www.bioinformatics.org/sms2/codon_usage.html platform (Stothard, 2000) was modified. This modified script takes a fasta file containing the CDS for a whole genome and returns, for each CDS, counts of each codon and frequency per 1000 codons. Tabulation of counts and figures were performed using Excel. Codon usage was also calculated using the Gene-Specific Codon Counting Database at <http://www.cs.albany.edu/~tumu/GSCC.html> (Tumu *et al.*, 2012). To find specific codons or runs of specific codons, an in-house Perl script based on a previously published C⁺ program was used (El Yacoubi *et al.*, 2009). This program takes a fasta file and returns two results. One result is the location of any codon requested in the submitted fasta file. The second output returns runs of codons for each CDS, including length of the codon run and the number of occurrences of each run. Pathway enrichments were generated using the Metacyc omics viewer using the default parameters (Caspi *et al.*, 2014). The *E. coli* essentiality data was derived from Ecogene (Zhou & Rudd, 2013) and the *D. radiodurans* fitness data was extracted from <http://cefg.uestc.edu.cn/ifim/index.php>. The Phyre platform was used to generate the homology model of the *D. radiodurans* TisS using standard parameters (Kelley & Sternberg, 2009).

Media and strains and genetic manipulations

All strains, plasmids and oligonucleotides used in this study are listed in Table S1, Table S2, and Table S3 respectively. Bacterial growth media were solidified with 15 g/l agar (BD Diagnostics Systems) for the preparation of plates. *E. coli* were routinely grown on LB medium (BD Diagnostics Systems) at 37 °C unless otherwise stated. Transformations were performed following standard procedures. IPTG (100 µM), Ampicillin (Amp, 100 µg/ml), Kanamycin (Km, 50 µg/ml), l-Arabinose (Ara, 0.02–0.2%), and Chloramphenicol (Cm, 35 µg/ml) were used when appropriate. M9 Minimal medium (Sambrook *et al.*, 1989), 0.1%

(w/v) glucose was used with 20 µg/ml phenylalanine, tyrosine and tryptophan added when needed. P1 transduction was performed following the classical methods (Miller, 1972). Transductants from VDC5801 and VDC5684 into *E. coli* B and C600 were checked by PCR for transduction of the P_{TET} allele into the recipient strains using primer pairs Ptet2771-fwd/ygjD661-rev and Ptet2771-fwd/yrdC573-rev respectively. *T. thermophilus* HB8 was grown at 70 °C in TR medium (0.4% (w/v) tryptone (Difco), 0.2% (w/v) yeast extract (Oriental Yeast, Tokyo), and 0.1% (w/v) NaCl (pH 7.5) (adjusted with NaOH)). To prepare plates, 1.5% (w/v) gelatin gum (Wako, Osaka, Japan), 1.5 mM CaCl₂, and 1.5 mM MgCl₂ were added to the TR medium. *D. radiodurans* was grown in TGY media (0.5% (w/v) tryptone, 0.1% (w/v) glucose, and 0.3% (w/v) yeast extract (all from Difco) at 30 °C. *S. mutans* was grown in BHI media (Difco) at 37 °C with 5% CO₂.

Sequencing

tRNA genes from *D. radiodurans* were amplified by PCR using oligos listed in Table S3. PCR products were Sanger-sequenced by the University of Florida Interdisciplinary Center for Biotechnology Research. Sequences were compared to wild-type sequences using Blast at NCBI.

Disruption of TtsaC

The *tsaC* null mutant of *Thermus thermophilus* HB8 (TtTsaC) was generated by substituting the target gene (TTHA0793, gi:55980762) with the thermostable kanamycin resistance gene (*HTK*) through homologous recombination as described previously (Hashimoto *et al.*, 2001). The plasmid for gene disruption (PD010793-01) was a derivative of the pGEM-T Easy vector (Promega, Madison, WI), constructed by inserting HTK flanked by 500-bp upstream and downstream sequences of the target gene. The transformation of *T. thermophilus* HB8 was performed by the following procedure (Hashimoto *et al.* 2001). An overnight culture was diluted 1:40 with TR medium and shaken at 70 °C for 2 h. This culture (0.4 ml) was mixed with 2 µg of the plasmid DNA, incubated at 70 °C for 2 h, and then spread on plates containing 500 µg/ml of kanamycin, which were incubated at 70 °C for 15 h. A colony was selected, cultured in TR medium, and spread on plates containing 500 µg/ml of kanamycin again, to remove the heteroplasmic recombinant cells completely, as *T. thermophilus* is a polyploid organism (Ohtani *et al.*, 2010). Gene disruption was confirmed by PCR amplification, using the isolated genomic DNA as the template (Figure 7).

Source of aminoacylation and modification enzymes used in this study

A total S100 aminoacyl-tRNA synthetase extract (free of tRNAs) from yeast provided by T. A. Weil (Klekamp & Weil, 1982) and purified His6-tagged *E. coli* IleRS and TlsS (Köhler *et al.*, 2008) were available as laboratory stocks. The TsaBCDE enzymes were purified as described previously (Deutsch *et al.*, 2012).

Construction of tRNA Templates for in vitro transcription

The templates for producing tRNA^{Ile} transcripts were produced via a Klenow extension reaction as described (Deutsch *et al.*, 2012) with the primers FtRNA^{Ile}(cat), RtRNA^{Ile}(cat), FtRNA^{Ile}(gat), and RtRNA^{Ile}(gat) listed in Table S3.

RNA Transcription and Purification

Transcription reactions to produce RNA were run for 4 hours at 37 °C in 80 mM HEPES (pH 7.5), 2.0 mM spermidine, 24 mM MgCl₂, 2.0 mM NTPs, 300 pmol template, and 2.5 µg of T7 polymerase. Prior to reactions the templates were heated to 95 °C for five minutes and allowed to cool slowly to room temperature over one hour. The RNA products generated in the transcription reactions were precipitated by the addition of 0.1 volume ammonium acetate (8.0 M), three volumes of 100% ethanol, and cooling at -80 °C for 30 minutes, then pelleted by centrifugation at 15,000 × g for 30 minutes at 4 °C. After removing the supernatants, the pellets were resuspended in 50 mM HEPES (pH 7.5) and 2.0 mM EDTA, the solutions mixed 1:1 with formamide, boiled for five minutes, and snap cooled on ice before being purified via urea-PAGE electrophoresis (10% acrylamide). The RNA was extracted from the gel by crush and soak, then precipitated as above, and suspended in 50 mM HEPES (pH 7.5), 2 mM EDTA.

In vitro RNA Modification with TsABCDE

Purified tRNA was converted to t⁶A-modified tRNA in reactions containing 5 µM TsABCDE, 5.0 mM threonine, 5.0 mM bicarbonate, 10 mM ATP, 100 mM Tris-HCl (pH 8.0), 300 mM KCl, 20 mM MgCl₂, 1.0 mM MnCl₂, 1.0 µM ZnCl₂, 50 µM tRNA, and 5mM DTT at 37°C for 20 minutes. To monitor the extent of modification, reactions were run in parallel containing 500,000 DPM [¹⁴C]-L-threonine diluted to 5.0 mM with unlabeled threonine. The reactions were then extracted with phenol/chloroform, and the tRNA precipitated and suspended as described above. tRNA from reactions containing [¹⁴C]-L-threonine were then analyzed using scintillation counting to quantify the extent of modification. Under these assay conditions the extent of modification plateaued after 20 minutes, measuring 60% for tRNA^{Ile}_{CAT} and 51% for tRNA^{Ile}_{GAT}.

In vitro aminoacylation of tRNA with isoleucine

0.5 – 1 A₂₆₀ (16-32 µM) of bulk tRNA or 0.01 – 0.025 A₂₆₀ (0.32-0.8 µM) of tRNA transcripts were aminoacylated *in vitro* in 50 µl-reactions with L-isoleucine using purified *E. coli* IleRS (0.5 µM) or total S100 extracts (5 µg) prepared from yeast. Reaction mixtures were as follows: (i) 50 mM HEPES pH 7.6, 10 mM MgCl₂, 5 mM ATP, 0.1 µg/µL BSA, and 5 µM L-[³H]-isoleucine (ARC; ~60 Ci/mmole) using *E. coli* IleRS; (ii) 50 mM HEPES pH 7.6, 50 mM KCl, 10 mM MgCl₂, 5 mM ATP, 5 mM DTT, 0.1 µg/µL BSA, and 5 µM L-[³H]-isoleucine (ARC; ~60 Ci/mmole) for use with yeast extracts. Prior to the addition of enzymes, tRNAs were pre-incubated at 65 °C for 3 minutes, transferred to 37 °C for 10 minutes and then left at room temperature for 5 minutes. Aminoacylation reactions were carried out at 37 °C for 60 min. At various time points, aliquots were removed and analyzed by precipitation with TCA followed by liquid scintillation counting of TCA-precipitable counts. TCA-precipitable counts were normalized per 1 A₂₆₀ of tRNA (1600 pmoles) for all experiments. Background (obtained from reactions run without tRNA) was subtracted from all values. All assays were carried out at least in triplicate.

In vitro modification of tRNA with lysidine

The *in vitro* modification of C34 to lysidine using purified *E. coli* Tils has been described (Köhler *et al.*, 2008). 0.01 – 0.025 A₂₆₀ (0.32-0.8 μM) of tRNA transcript was modified in a 50 μl-reaction containing 50 mM HEPES pH 7.6, 10 mM KCl, 15 mM MgCl₂, 10 mM ATP, 0.1 μg/μL BSA, 3.54 μM L-[³H]-lysine (Perkin Elmer; ~84.8 Ci/mmol) and 0.5 μM *E. coli* Tils. Inorganic pyrophosphatase (New England Biolabs) was added to the reaction at a concentration of 0.04 U/μl. The modification reactions were carried out at 37 °C for 60 minutes. The pre-incubation of tRNA and the analysis of incorporation of radiolabeled amino acid into tRNA were as described above. Assays were carried out in triplicate.

Preparation of bulk tRNA and detection of t⁶A

Bulk tRNA was extracted from *S. cerevisiae*, *E. coli* strains, *Synechocystis* PCC 6803, and *S. mutans* strains as previously described (El Yacoubi *et al.*, 2009). *D. radiodurans* was subcultured 1:100 from a starter culture into 1 L of TGY and grown at 30°C until the culture reached late-log at an OD₆₀₀ of 2.5. The cells were harvested by centrifugation at 8000 x g for 10 minutes at 4°C. The weight of the cell pellet was determined, and the cells were suspended in 95% ethanol at 0.1 volumes/g of dry weight to remove the S-layer. The cells were then harvested by centrifugation as before, and the pellets were suspended in 50 mM sodium acetate, pH 5.5 at 3 mL/g of cell weight. Zymolyase was added to a final concentration of 10 μg/mL and the samples were incubated at 37 °C for 30 minutes. Following the incubation, a volume of acidic-buffered phenol (pH 5.5), equal to the amount of 50 mM sodium acetate added previously, was added and the sample was incubated at 70 °C for 30 minutes. The sample was then cooled to room temperature prior to centrifugation at 4000 x g for 10 minutes at RT. The aqueous layer was transferred to a new tube, and an equal volume of 25:24:1 acidic-buffered phenol (pH 5.5):chloroform:isoamyl alcohol was added, mixed, and the sample was centrifuged again at 4000 x g for 10 minutes at RT. The aqueous phase was transferred to a new tube, and to it, an equal volume of chloroform was added, mixed, and the sample was centrifuged again. The aqueous phase was transferred, and sodium chloride was added to a final concentration of 1 M and 0.2 volumes of isopropanol were added. The sample was mixed well, incubated at -20 °C for 1 hour, and centrifuged at 8000 x g for 20 minutes at 4 °C. The supernatant was transferred to a new tube, and 0.6 volume of isopropanol was added to the supernatant, and the sample was incubated overnight at -20 °C. The next day, the samples were allowed to warm on ice for 10 minutes prior to centrifugation at 8000 x g for 20 minutes at 4 °C. The pellet was washed with 20 mL of 80% v/v ethanol, and centrifuged at 8000 x g, before drying the pellet in a CentriVap Concentrator (Labconco, Kansas City, MO) for 30 minutes at 40 °C, and the RNA was recovered by ethanol precipitation. The final pellets were suspended in 200 μL TE and stored at -20 °C. All tRNA extractions were performed in triplicate from independent cultures.

Nucleoside preparations were prepared by incubating 100 μg of linearized bulk tRNA with 10 units of Nuclease P1 (Sigma) in 10 mM ammonium acetate (pH 5.3) overnight at 37°C. The next day, 0.1 volume of 1 M ammonium bicarbonate (pH 7.0) was added to give a final concentration of 100 mM ammonium bicarbonate. 0.01 units of Phosphodiesterase I (Sigma) and 3 μL *E. coli* alkaline phosphatase (Sigma) were added, and the samples were incubated

for an additional 2 hours at 37 °C. The hydrolyzed nucleosides were further purified by filtering through a 5 kD MWCO filter (Millipore) (to remove enzymes), dried in a CentriVap Concentrator, and suspended in 20 µL of water prior to analysis by HPLC or LC-MS/MS. Nucleoside preparations were prepared as previously described using Nuclease P1 (Sigma) Phosphodiesterase I (Sigma), and *E. coli* alkaline phosphatase (Sigma) (El Yacoubi *et al.*, 2009).

HPLC and LC-MS/MS Analysis

t⁶A was detected by HPLC as described by (Pomerantz & McCloskey, 1990) using a Waters 1525 HPLC with Empower 2 software and detected with a Waters 2487 UV-vis spectrophotometer at 254 nm. Separation was performed on an Ace C-18 column heated to 30 °C, using 250 mM ammonium acetate (Buffer A) and 40% acetonitrile (Buffer B) run at 1 mL/min. 100 µg of nucleosides were injected and separated using a isocratic complex step gradient. Levels of t⁶A were measured by integrating the peak area from the extraction ion chromatograms. The ratios of Ψ-modified base/m₂²G were used to normalize for tRNA concentration across samples. Levels for mutant strains were expressed relative to wild-type levels. Results were confirmed by LC-MS/MS at the Donald Danforth Plant Science Center, St. Louis MO. The MS/MS fragmentation data, as well as a t⁶A standard provided by D. Davis (University of Utah) were also used to confirm the presence of t⁶A.

Detection of k²C

Prior to enzymatic digestion, the tRNA was denatured at 100 °C for 3 min then chilled in an ice water bath. To lower the pH, 1/10 volume of 0.1 M ammonium acetate (pH 5.3) was added. For each 0.5 absorbency unity (AU) of tRNA, 2 units Nuclease P1 (Sigma) was added and incubated at 45 °C for 2 h. The pH was readjusted by adding 1/10 volume of 1.0 M ammonium bicarbonate, then 0.002 units of snake venom phosphodiesterase was added and incubated at 37 °C for 2 h. Finally, 0.5 units of Antarctic phosphatase (New England Biolabs) was added and incubated at 37 °C for 1 h. The nucleoside digests were stored at -80 °C for further analysis.

The nucleoside digests were analyzed using a Hitachi D-7000 HPLC system with a LC-18-S (Supelco) 2.1 × 250 mm, 5 µm particle column with a flow rate of 300 µL/min, connected directly to the mass spectrometer. Mobile phases used were as follows: 5mM ammonium acetate, pH 5.3 and 40% acetonitrile in water. The column eluent was split immediately after the column, 1/3 to the electrospray ion source and 2/3 to the UV detector. The gradient used follows that previously described (Russell & Limbach, 2013).

Mass spectral data was acquired on a Waters G2-S ion mobility time-of-flight mass spectrometer operated in sensitivity mode using MS^E data collection. The ion source parameters included a capillary voltage of 2.5 kV, sampling cone voltage of 40 V offset by 80 V with a temperature of 200°C, desolvation gas at a rate of 500L/hour and a nebulization pressure of 6 Bar. The ion mobility parameters were set using helium gas with a start wave height at 10 V ending at 40 V at a wave velocity starting at 1000 m/s and ending at 500 m/s. To avoid nucleoside fragmentation prior to detection, the bias energy was adjusted to 45 eV at the trap and 3 eV at the ion transfer. Fragmentation was obtained by ramping the transfer

energy (post ion mobility and prior to TOF analysis) from 15 to 45 eV. Low and high energy MS and MS/MS spectra were verified and time-aligned in Drift Scope™ ver 4.2. Masses were adjusted using the Lock Spray™ collected once every 30 s for 500 msec with the calibrant Leucine Enkephalin. Data analysis was performed using Masslinx™ ver 4.1.

Oligonucleotide Analysis

Total tRNA for all strains was analyzed as previously described (Puri *et al.*, 2014). Total tRNA was incubated with 50 U of RNase T1 (Worthington Biochemical) per mg of tRNA in 20 mM ammonium acetate for 2 h at 37 °C. Digestion products were separated using an XBridge™ C18 column (100 mm × 2 mm; 1.7µm, 120Å) from Waters (Santa Clara, CA.) with a Hitachi La Chrome Ultra UPLC containing two L-2160U pumps, L-2455U diode array detector and an L-2300 column oven (35 °C) connected in-line with a Thermo Scientific (Waltham, MA) LTQ™ linear ion trap mass spectrometer. Before each run the column was equilibrated for 10 min at 95% Buffer A (200 mM hexafluoroisopropanol (HFIP), 8.6 mM triethylamine (TEA), pH 7) and 15% Buffer B (200 mM HFIP, 16.3 mM TEA: methanol, 50:50 v:v, pH 7). The gradient started at 10 %B and increased at 8 % min⁻¹ for 10 min. The mobile phase was then increased to 95% for 5 min before re-equilibrating prior to the next analysis. The mass spectrometer operating parameters included a capillary temperature of 275 °C, spray voltage of 4 kV, source current of 100 µA, and sheath, auxiliary and sweep gases set to 40, 10 and 10 arbitrary units, respectively.

Each RNase digestion reaction was analyzed four times using an MS scan range of 600-2000. Each instrumental segment consisted of a full scan, collected in negative polarity, followed by three product ion scans (scans 2-4). Product ion scans were obtained using data dependent collision-induced dissociation (CID) at a normalized collision energy of 35% with an activation time of 10 ms. In data dependent mode, scans 2-4 were triggered by the three most abundant ions from scan 1 and isolated by a mass width of 2 ($\pm 1 m/z$). Each ion selected for CID was analyzed for up to 10 scans before it was added to a dynamic exclusion list for 15 s (typical chromatographic fwhm) for both modes of data acquisition. Each RNase digestion reaction was also analyzed four times using an MS scan range of 800-1200. This smaller scan range was accompanied by a targeted MS/MS list intended to maximize the opportunity to obtain full sequence coverage. Wild type *T. thermophilus* tRNA^{Ile}_{CAU} was purified from total tRNA using a biotinylated deoxyribonucleotide probe complementary to the 3'-end of the tRNA and analyzed as described above. Examination of the tRNA^{Ile}_{CAU} anticodon modification profiles for the Sua6 knockout and *D. radiodurans* samples was performed using a targeted MS/MS approach (Wetzel & Limbach, 2013) with a scan range of 800-1200 on RNase T1 digested total tRNA.

Proteomics

Proteomic analysis of *D. radiodurans* R1 and mutant XYD (*tsaD*) was performed as described previously (Bouthier de la Tour *et al.*, 2013). For each strain, 4 biological replicates were grown at 30 °C shaking, starting from a 1:20 dilution from an overnight. Cultures (125 mL) were harvested at mid-log growth (~5h) and at late-log (~7h). Since the two strains have very different growth rates in flasks with heavy aeration, wild-type and mutant cells were harvested at identical stages of logarithmic growth. Cells were harvested

by centrifugation at room-temperature, all media removed, and then frozen in liquid nitrogen and finally stored at -80°C. The cell pellets were lysed and an equal amount of proteins was loaded onto a SDS-PAGE gel. After a short migration, the gel bands containing the whole soluble proteome were excised and digested with trypsin (Hartmann *et al.*, 2014). The resulting peptides were extracted, and each sample was analyzed individually by nanoLC-ESI MS/MS with an Ultimate3000 - LTQ Orbitrap XL system (Thermo). Relative quantitation based on spectral counting was then performed using the Tfold module of the PatternLab software with fold change and p value thresholds at 1.5 and 0.05 (Carvalho *et al.*, 2012). Comparisons between the two strains at mid- and late-log phases were performed separately. The list of proteins for the samples at mid-log phase, their spectral counts and PatternLab comparison is provided in Table S4 and Table S5, for the samples at mid-log and late-log phases, respectively. The list of MS/MS spectra assigned for the samples in both experiments are available upon request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by the National Institutes of Health (R01 GM70641 to V.d.C.L. and D. I-R., S10 RR027671 to P.A.L., and R01 GM17151 to U.L.R.) and National Science Foundation (CHE1212625 to P.A.L.). This work was also supported in part by grants from the X-ray Free Electron Laser Priority Strategy Program from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (to Y.B.). P.C.T. was funded in part by a Chateaubriand Fellowship from the French Embassy in the United States. We thank Patrick Reddy, Lana McMillan, Aimi Osaki, Akemi Shibuya, Erica Hartmann and Jean-Charles Gaillard for technical help. We are grateful to Eric Brown, Monika Ryden-Ulin, Takefumi Onodera, Tracy Palmer and Thomas Wen for sending us strains and plasmids and to Martin Hagemann for sending *Synechocystis* cell paste. We thank Thomas Begley for help with the Gene-Specific Codon Counting Database, and Henri Grosjean for constant discussion and inspiration.

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Abbreviations

t⁶A or t⁶A₃₇	threonylcarbamoyladenine
ASL	Anticodon Stem Loop
TCTC	ThreonylCarbamoyl Transferase Complex
IleRS	Isoleucyl tRNA Synthetase
TilS	tRNA ^{Ile} -lysine synthetase
k²C	lysidine
agm²C	agmatidine
aTc	anhydrotetracycline

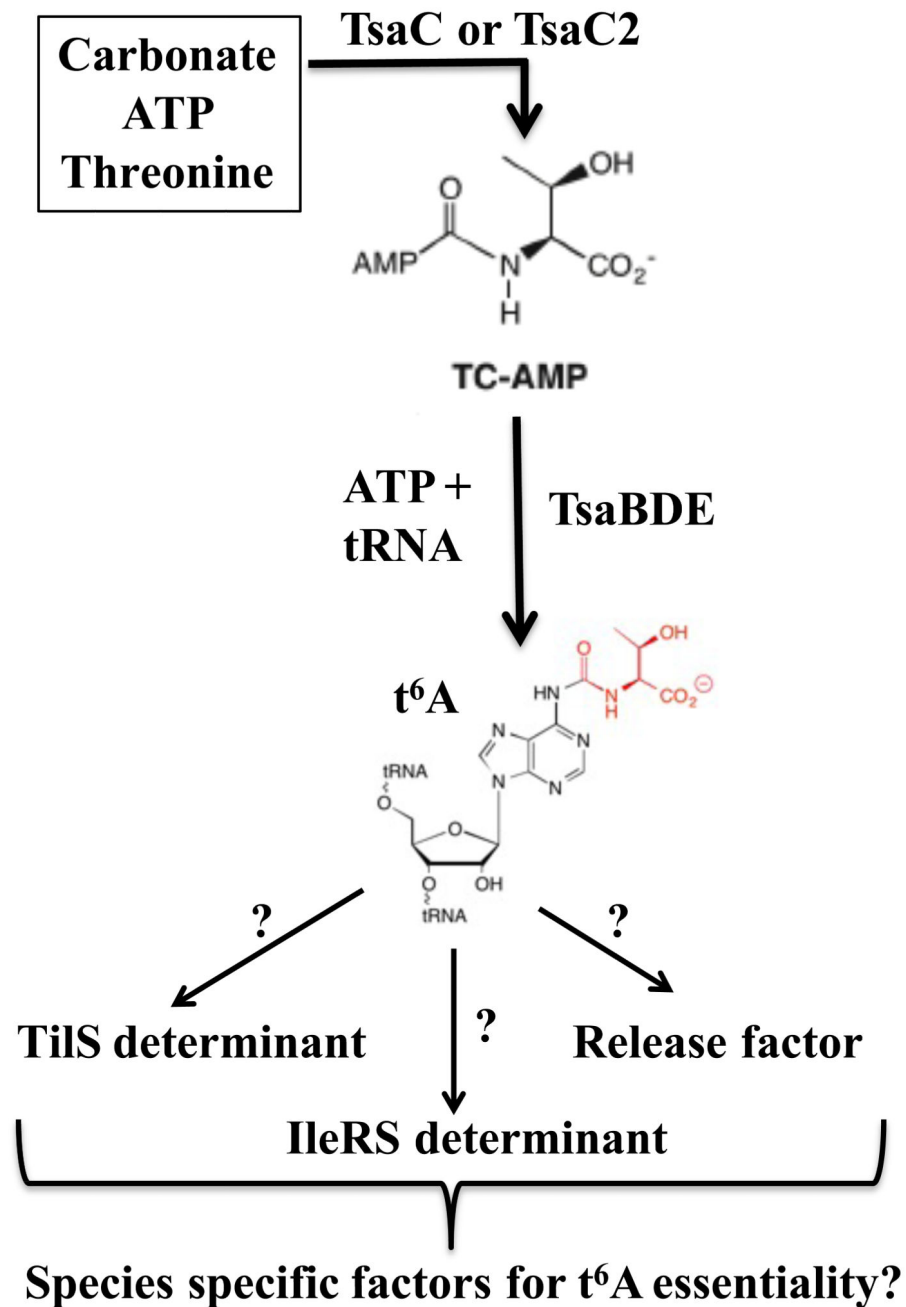
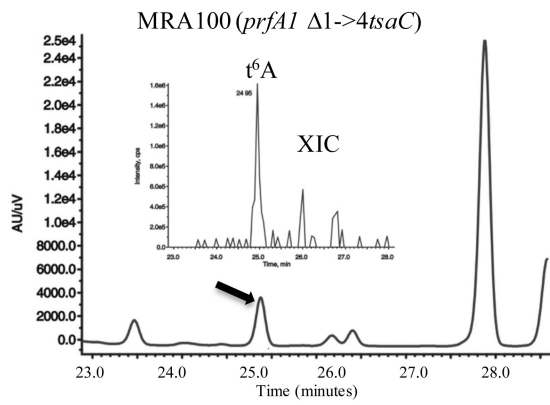


Figure 1. Pathway for biosynthesis of t⁶A₃₇ and its possible roles in tRNA function. TC-AMP, threonine-carbamoyl-AMP.



Strain	t ⁶ A ratio (Ψ/m_2^2G ratio)
US475 (Wild-type)	1
US477 (<i>prfA1</i>)	3.67
MRA100 (<i>prfA1</i> $\Delta 1 \rightarrow 4tsaC$)	2.81

Figure 2.

Evidence for presence of t⁶A in tRNAs isolated from *E. coli* carrying the *prfA1* allele (US477) or both *prfA1* 1->4*tsaC* alleles (MRA100) based on LC-MS/MS analysis. (left) Analysis of t⁶A levels in MRA100. Black arrow indicates t⁶A peak; inset shows extracted ion chromatogram (XIC) corresponding to the molecular ion for t⁶A. (right) Comparison of relative t⁶A levels in wild-type and mutant strains. The ratio of Ψ -modified base/ m_2^2G were used to normalize the level tRNAs between strains.

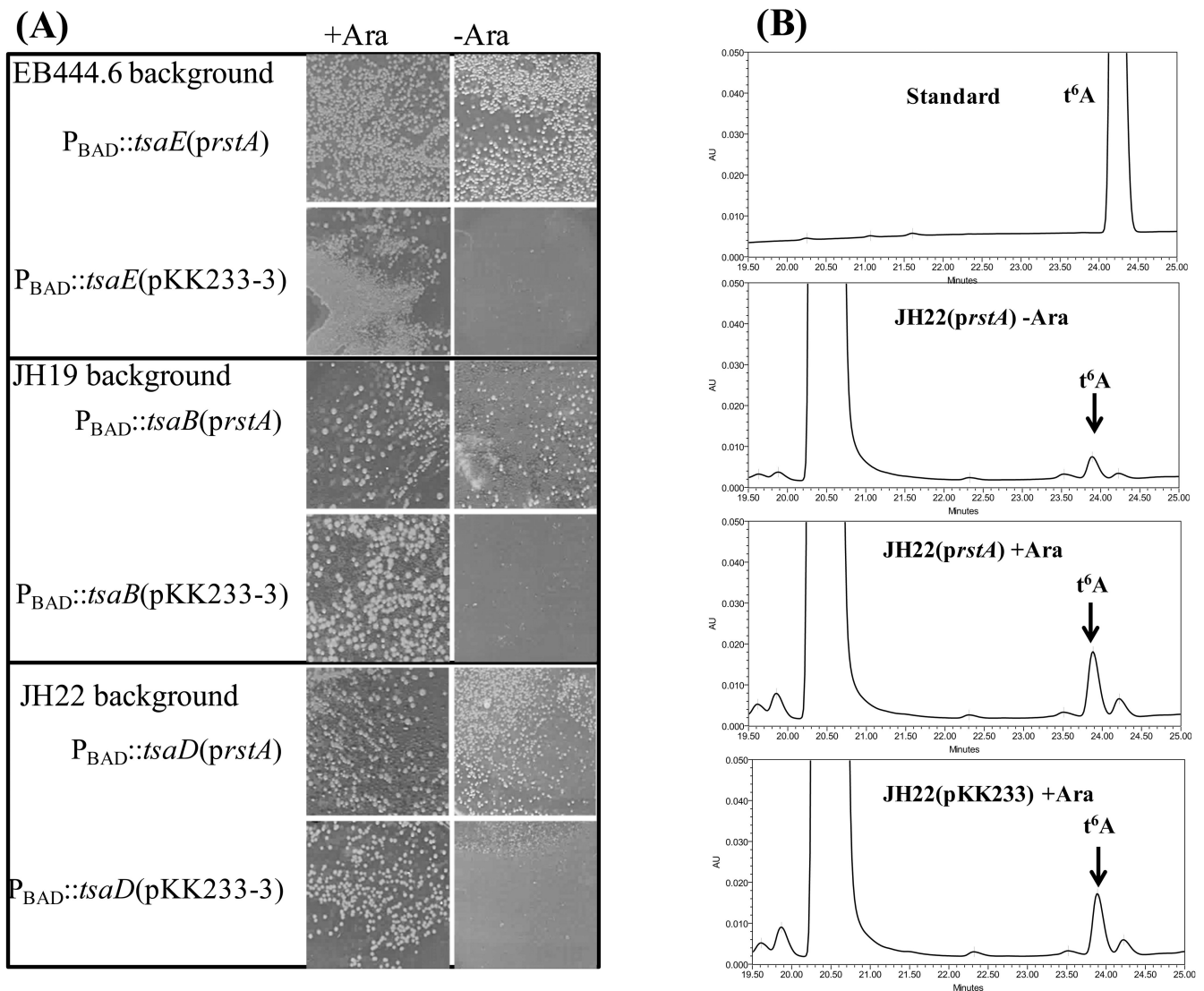


Figure 3.

Overexpression of *rstA* does not suppress the essentiality of t^6A in *E. coli*. (A) The chromosomal copies of *tsaE* (EB444.6), *tsaB* (JH19), or *tsaD* (JH22) have been placed under control of the arabinose-inducible P_{BAD} promoter. Each strain was transformed with an equal amount of plasmid *prstA* and plated with or without the inducer arabinose; pKK233-3, empty plasmid. (B) Analysis of t^6A content in tRNA isolated from various strain JH22 derivatives. Strains were grown static, overnight at 37 °C in LB-amp with 0.05% of the inducer arabinose. The following day, each culture was diluted 1:100 in LB-amp containing either 0.2% arabinose or 0.2% glucose. The cultures were grown at 37 °C with shaking for 4 hours, then the arabinose grown cultures were diluted 1:100 into LB-amp with 0.2% arabinose and the glucose-grown cultures were diluted into LB-amp with 0.2% glucose. The cultures were grown at 37 °C with shaking until the OD_{600nm} reached approximately 1.5. The bacteria were harvested by centrifugation and bulk tRNA was extracted and analyzed as described in Materials and Methods.

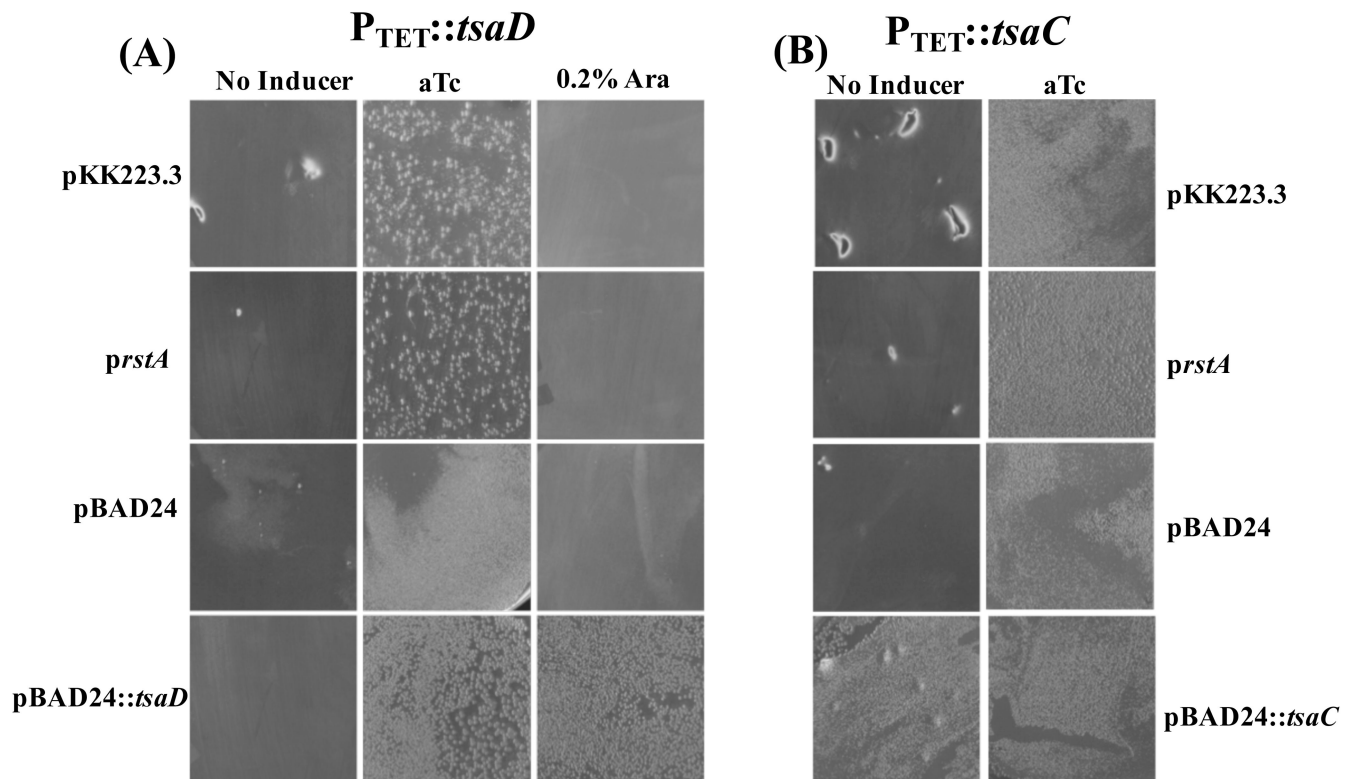
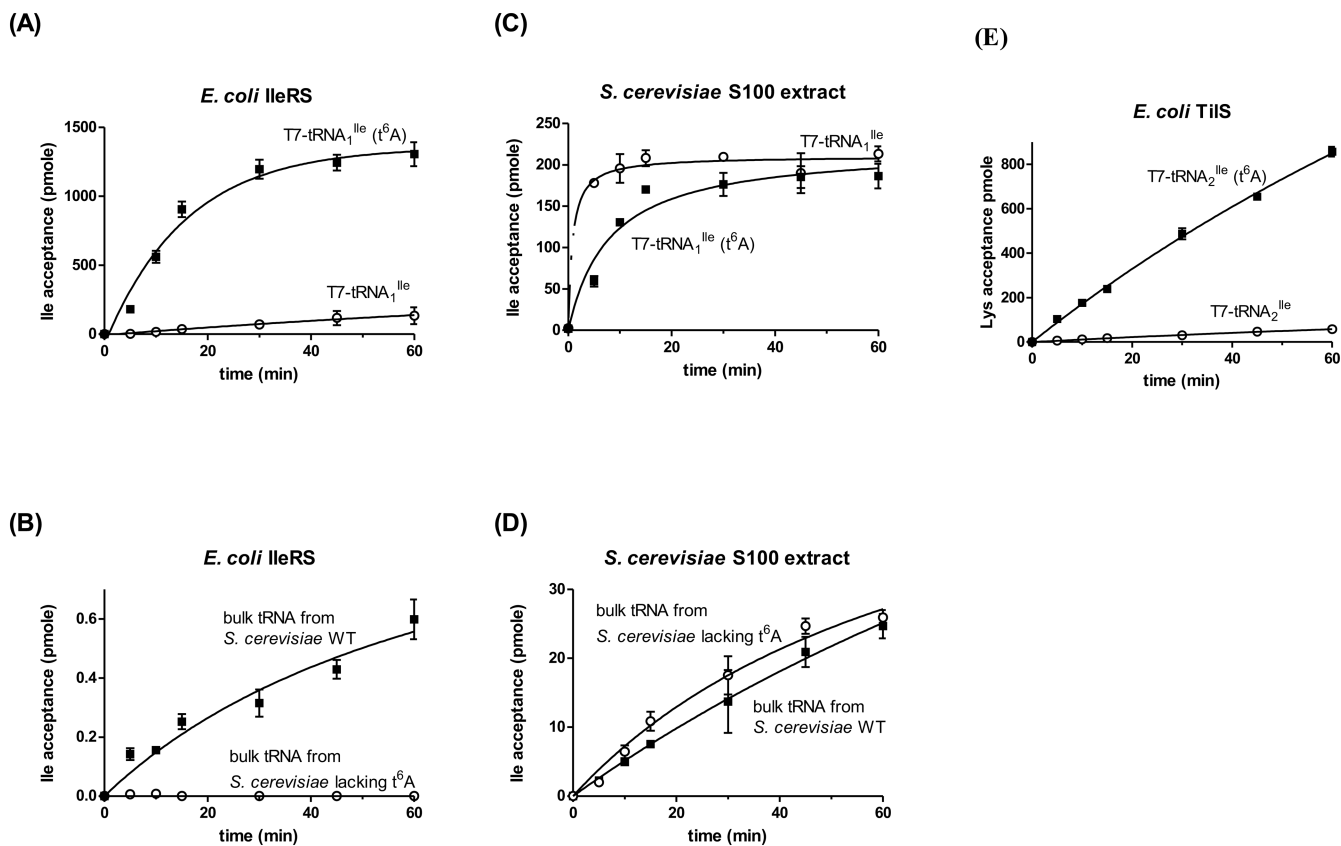


Figure 4.

Overexpression of *rstA* does not compensate for the absence of *tsuD* or *tsuC*. The chromosomal copies of *tsuD* ($P_{TET}::tsuD$) (A) or *tsuC* ($P_{TET}::tsuC$) (B) have been placed under control of the tightly regulated P_{TET} promoter. For complementation assays, cells were transformed with 100 ng of plasmid (*prstA*, pBAD24 or pBAD::*tsuD*) via electroporation, then recovered with 1 mL of LB and placed at 37°C for 1 hour with shaking. 200 μ L of cells were plated on LB-amp supplemented with or without aTc (50 ng/ml) or 0.02% arabinose.

**Figure 5.**

In vitro aminoacylation or lysidinylation of tRNA containing or lacking t⁶A. (A) Aminoacylation of a transcript of *E. coli* tRNA^{Ile}_{GAU} containing only t⁶A or no t⁶A using *E. coli* IleRS. (B) Aminoacylation of bulk tRNA from *S. cerevisiae* BY4741 (wild-type) or VDC9100 (*sua5*, t⁶A⁻) using *E. coli* IleRS. (C) Aminoacylation of a transcript of *E. coli* tRNA^{Ile}_{GAU} containing only t⁶A or no t⁶A using *S. cerevisiae* S100 extract. (D) Aminoacylation of bulk tRNA from *S. cerevisiae* BY4741 (wild-type) or VDC9100 (*sua5*, t⁶A⁻) using *S. cerevisiae* S100 extract. (E) Lysidinylation of a transcript of *E. coli* tRNA^{Lys}_{CAU} containing only t⁶A or no t⁶A using *E. coli* TilS. The relative rates of isoleucine or lysine acceptance in (A) and (E) were estimated by linear regression over the first 15 minutes of the reaction; 2.5 pmole/min for T7-tRNA^{Ile}_{GAU} and 62.0 pmole/min for T7-tRNA^{Ile}_{GAU} (t⁶A) using *E. coli* IleRS, 1.2 pmole/min for T7-tRNA^{Lys}_{CAU} and 15.8 pmole/min for T7-tRNA^{Lys}_{CAU} (t⁶A) using *E. coli* TilS, resulting in ~25-fold and ~13-fold higher rates for the respective modified transcript compared to the unmodified transcript. The actual fold increase of aminoacylation rates of modified versus unmodified transcript by *E. coli* IleRS is likely higher than 25-fold, since the enzyme concentration used was semi-saturating, as indicated by the time course shown in (A).

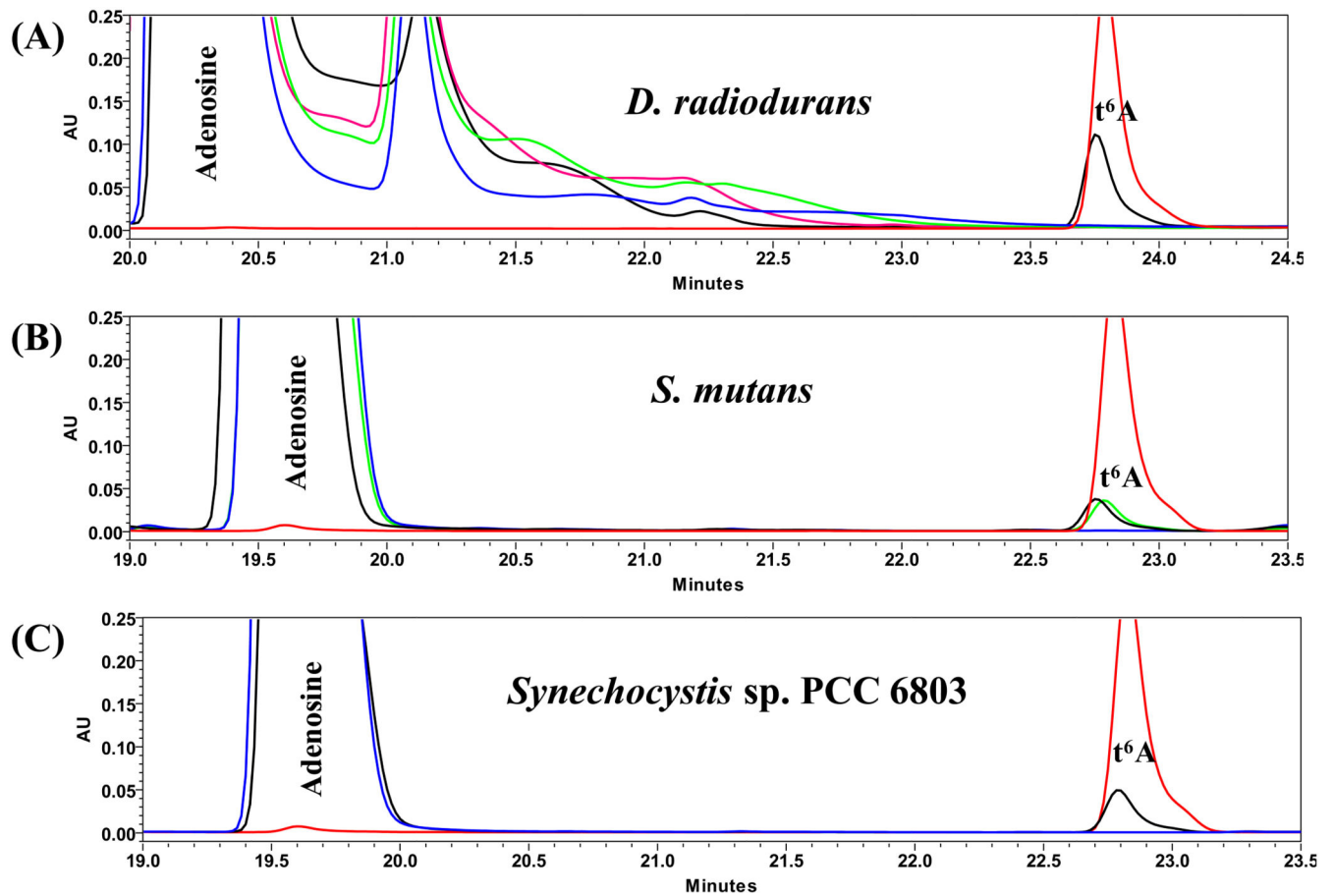


Figure 6.

HPLC analysis of nucleosides in digests of tRNAs extracted from wild-type and mutant strains of *D. radiodurans*, *S. mutans* and *Synechocystis* sp. PCC 6803. Red line in each panel indicates t⁶A synthetic standard. (A) *D. radiodurans* R1: wild-type (black line), *tsaD*, *tsaB* (WDZ) (blue line), *tsaD* (XYD) (green line), *tsaB* (XYZ) (pink line). (B) *S. mutans* UA159: wild-type (black line), *tsaE* (JB409) (blue line), *tsaE* (*ptsE*) (JB409c) (green line). (C) *Synechocystis* sp. PCC 6803: wild-type (black line) and *tsaD* (*gcp*) (blue line).

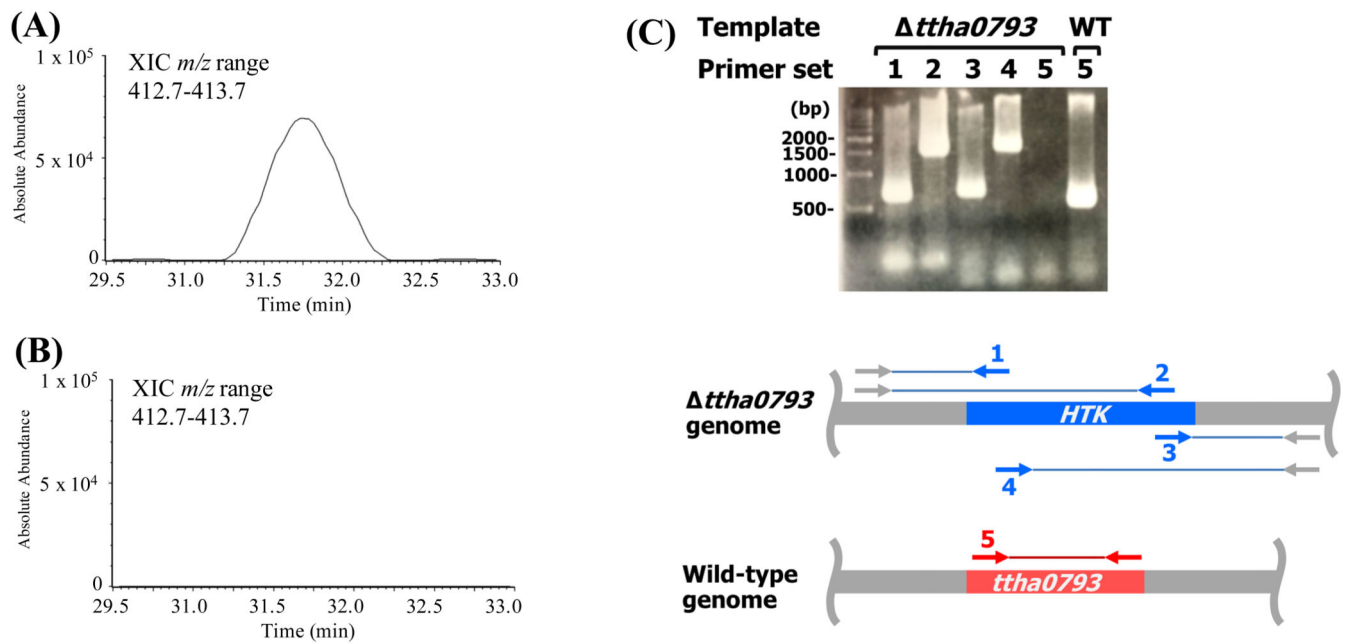


Figure 7. t^6A is absent in *T. thermophilus tsaC* cells. Representative LC-MS nucleoside data of t^6A obtained from total tRNA of *T. thermophilus* wild-type (A) and *tsaC* (*ttha0793*) mutant cells (B). Extracted ion chromatograms (XIC) for m/z range 412.7-413.7 corresponding to the molecular ion for t^6A . (C) PCR verification of the deletion of *tsaC* (*ttha0793*) in *T. thermophilus*. The various primer sets (1-5) used for analysis of the relevant region in the *ttha0793* and wild-type genome are indicated below. HTK, kanamycin nucleotidyltransferase.

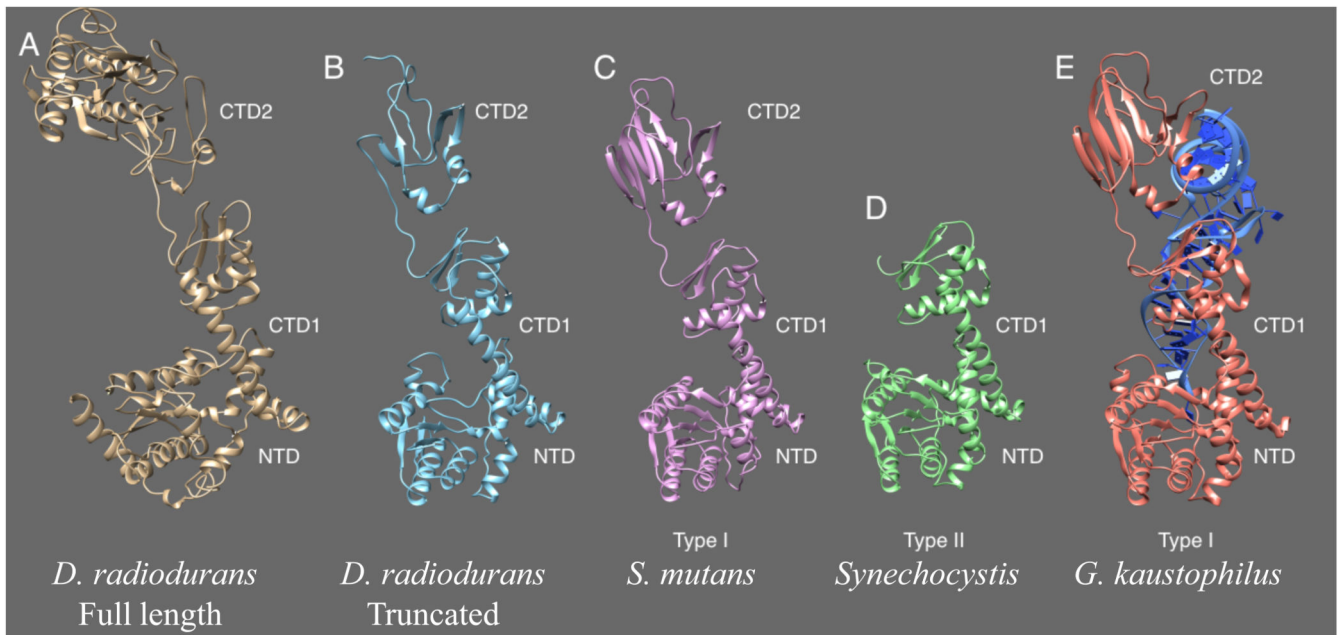
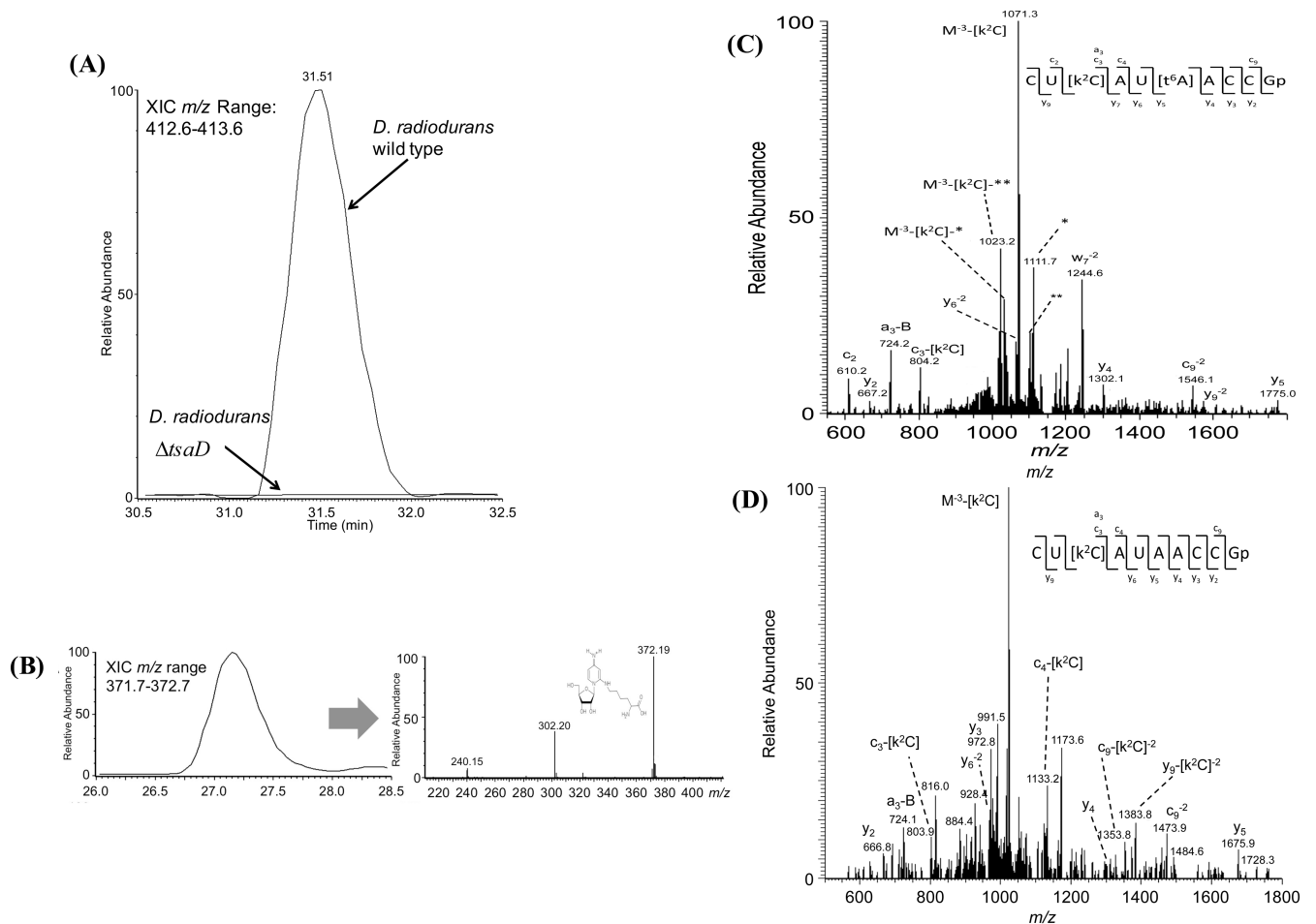


Figure 8.

Structural representation of TisS derived from various species. Phyre-generated homology models of TisS from (A) *D. radiodurans* (full-length, 600 aa), (B) *D. radiodurans* (truncated, 400 aa), (C) *S. mutans* and, and (D) *Synechocystis* sp. using (E) *G. kaustophilus* TisS (3A2K) in complex with tRNA (blue) as starting structure. Various domains are indicated as well as their respective TisS type. NTD, N-terminal domain; CTD 1 and 2, C-terminal domain 1 and 2.

**Figure 9.**

Representative LC-MS nucleoside analysis for N^6 -threonylcarbamoyladenine (t^6A) and lysidine (k^2C) isolated from total tRNA of *D. radiodurans* and *T. thermophilus*. (A) *D. radiodurans* wild-type and mutant (*tsaD*) samples. Extracted ion chromatograms (XIC) for m/z range 412.7-413.7 corresponding to the molecular ion for t^6A . (B) *D. radiodurans* *tsaD* (XYD) XIC for m/z range 371.7-373.7 corresponding to the molecular ion for k^2C [(M=H)⁺ = 372 Da]. Similar results were found in the *D. radiodurans* wild-type. (C) MS/MS spectrum obtained from RNase T1 digestion of tRNA from *T. thermophilus* wild-type and (D) *ttha0793* (*tsaC*) MS/MS verification of the presence or absence of k^2C and t^6A in tRNA^{Ile}_{CAU}.

Essentiality of t⁶A synthesis genes in eukaryotes and archaea

New nomenclatures for t⁶A biosynthetic genes are listed in parenthesis. All genes are essential except those highlighted in grey. Essentiality of gene names underlined has not been determined.

Table 1

Organism	TsaC (Tcs1)	Sua5 (Tcs2)	Kae1 (Tcs3)	Qri7 (Tcs4)	Bud32 (Tcs5)	Pcc1 (Tcs6)	Cgi121 (Tcs7)	Gon7 (Tcs8)	Type	Reference
<i>Haloferax volcanii</i> /DS2	HVO_0253		HVO_1895 ^I		HVO_1895 ^I	HVO_0652	HVO_0013		Single gene knockout	(Blaby <i>et al.</i> , 2010; Naor <i>et al.</i> , 2012)
<i>Methanococcus maripaludis</i>	MMP0186		MMP0415 [#]		MMP0415 [#]	MMP0246	MMP0967			(Sammiento <i>et al.</i>)
<i>Saccharomyces cerevisiae</i> /S228C		YGL169w	YKR038c	YDL104c	YCR262c	YKR095w-A	YML036w	YJL184w	Single gene knockout in haploid	(Winzeler, 1999; El Yacoubi <i>et al.</i> , 2009)
<i>Schizosaccharomyces pombe</i>		<u>SPCC895.03cc</u>	<u>SPBC16D10.03</u>	<u>SPCC1259.10</u>	<u>SPAP27G11.07c</u>	<u>SPAC4H3.13</u>	<u>SPCC24B10.12</u>	<u>SPAC6B12.18</u>	Single gene knockout	(Kim <i>et al.</i> , 2010; Spirek <i>et al.</i> , 2010)
<i>Arabidopsis thaliana</i>	<u>AT5G60590</u>		<u>AT4G22720</u>	<u>AT2G45270</u>	<u>AT5G26110</u>	<u>AT5G53045</u>	<u>AT4G34412</u>		tDNA mutagenesis	(Alonso <i>et al.</i> , 2003)

N.P. = gene not present in genome.

^I *H. volcanii* kae1 and bud32 occur as a gene fusion (HVO_1895)

Table 2

Essentiality phenotypes of *t*^oA synthesis genes in bacteria

All genes are essential except those highlighted in grey. Essentiality of gene names underlined has not been determined.

Organism	TsaC	Sua5 (TsaC2)	TsaB	TsaD	TsaE	Type	Reference
<i>E. coli</i> K12	b3282	b3064	b1807	b3064	b4168	Whole Genome, single gene knockout, non-polar	(Baba <i>et al.</i> , 2006, Kitagawa <i>et al.</i> , 2006)
<i>Vibrio cholerae</i> O1 El Tor E7946 and C6706	VC0054	VC1079	VC1989	VC0521	VC0343	Saturating transposon mutagenesis	(Chao <i>et al.</i> , 2013, Kamp <i>et al.</i> , 2013)
<i>Caulobacter crescentus</i> NA1000	CCNA_03501	CCNA_00057	CCNA_00069	CCNA_03648	CCNA_03648	Saturating transposon mutagenesis	(Christen <i>et al.</i> , 2011)
<i>Mycoplasma genitalium</i> G37	MG259*	MG046	MG208	MG046	N.P.	Saturating transposon mutagenesis	(Glass <i>et al.</i> , 2006)
<i>Mycoplasma pulmonis</i>	MYPUP_6130 [#]	MYPUP_1190	MYPUP_1180	MYPUP_1200	MYPUP_1200	Saturating transposon mutagenesis	(French <i>et al.</i> , 2008, Dybvig <i>et al.</i> , 2010)
<i>Bacillus subtilis</i> subsp. <i>subtilis</i> str. 168	BSU36950	BSU05920	BSU05920	BSU05940	BSU05910	Whole Genome, single gene knockout, non-polar	(Kobayashi <i>et al.</i> , 2003)
<i>Haemophilus influenzae</i> Rd	HI0656	HI0388	HI0388	HI0530/	HI0065	<i>mariner</i> -based minitransposon	(Akerley <i>et al.</i> , 2002)
<i>Acinetobacter baylyi</i> APD1	ACIAD0208	ACIAD1332 ²	ACIAD0677 ²	ACIAD1332 ²	ACIAD2376	Whole Genome, single gene knockout ³	(de Berardinis <i>et al.</i> , 2008)
<i>Salmonella</i> Typhii TY2	STY4395	STY3387	STY1950	STY3387	STY4714	Saturating transposon mutagenesis	(Langridge <i>et al.</i> , 2009)
<i>Francisella novicida</i> U112	FTN_0158	FTN_1565	FTN_1148	FTN_1565	FTN_0274	Saturating transposon mutagenesis	(Gallagher <i>et al.</i> , 2007)
<i>Pseudomonas aeruginosa</i> PA01	PA0022	PA0580	PA3685	PA0580	PA4948	Saturating transposon mutagenesis	(Jacobs <i>et al.</i> , 2003)
<i>Burkholderia thailandensis</i> E264	BTH_10669	BTH_110616	BTH_12001	BTH_110616	BTH_10723	Saturating transposon mutagenesis	(Gallagher <i>et al.</i> , 2013)
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> MW2	MW0860	MW1973	MW1975	MW1973	MW1976	Saturating transposon mutagenesis	(Chaudhuri <i>et al.</i> , 2009)
<i>Deinococcus radiodurans</i> R1	DR_1862	DR_0382	DR_0756	DR_0382	DR_2351	Single gene knockout	(Onodera <i>et al.</i> , 2013)
<i>Synechocystis</i> sp. PCC 6308	slr1866	slr0807	slr1063	slr0807	slr0257	Single gene knockout	(Zuther <i>et al.</i> , 1998)
<i>Streptococcus mutans</i> UA159	SMU.1083c	SMU.387	SMU.385	SMU.387	SMU.409	Single gene knockout	(Bitoun <i>et al.</i> , 2014)

N.P. = Gene not present in genome.

* *M. genitalium* MG259 is a TsaC/HemK fusion.

[#] *M. pulmonis* TsaC (MYPUP_6130) is essential, while HemK (MYPUP_1060) is not essential. (Dybvig *et al.*, 2010)

¹ Genomic map for TsaD (HI0530) mutation is not available and mutation is not confirmed.

² Mutations correspond with genomic duplication of the target gene.

³ Library was selected on minimal media.

Table 3
Growth phenotypes of strains carrying conditional *tsaC* and *tsaD* alleles

Strain	Lac	Growth -aTc	Growth +aTc	Kan
C600	+	+	+	S
<i>E. coli</i> B	+	+	+	S
VDC5684 BW25113 P _{TET} : <i>tsaC</i> : <i>aph</i>	-	+	+	R
VDC5801 BW25113 P _{TET} : <i>tsaD</i> : <i>aph</i>	-	-	+	R
VDC7073 C600 P _{TET} : <i>tsaC</i> : <i>aph</i>	+	-	+	R
VDC7069 C600 P _{TET} : <i>tsaD</i> : <i>aph</i>	+	-	+	R
VDC7065 <i>E. coli</i> B P _{TET} : <i>tsaC</i> : <i>aph</i>	+	+	+	R
VDC7061 <i>E. coli</i> B P _{TET} : <i>tsaD</i> : <i>aph</i>	+	+	+	R

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Table 4
Peptides significantly changed greater than 2-fold during mid-log growth phase

Full list of detected peptides can be found in supplementary Table S4. Stress proteins induced >3 fold are in red.

RefSeq Locus Tag	Functional annotation	Fold Change	pValue	Predicted Fitness
DR_0128	Heat shock protein GrpE	99.0	1.29E-04	0.233
DR_0907	Cold shock protein CspA	99.0	2.71E-03	0.043
DR_0989	cationic outer membrane protein OmpH, putative	99.0	1.24E-02	1
DR_0075	hypothetical protein	11.7	1.75E-03	0.922
DR_1705	hydrolase family protein	11.5	2.50E-03	0.775
DR_0630	Cell division protein FtsA	10.7	3.06E-04	0.182
DR_2394	N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	7.7	1.10E-03	1
DR_0651	Arginase (EC 3.5.3.1)	7.2	5.02E-05	1
DR_1447	hypothetical protein	6.3	1.00E-05	1
DR_0608	Histone acetyltransferase HPA2 and related acetyltransferases	5.0	1.64E-03	1
DR_A0047	Phosphomannomutase (EC 5.4.2.8) / Phosphoglucomutase (EC 5.4.2.2)	4.9	5.94E-03	1
DR_B0125	iron ABC transporter, periplasmic substrate-binding protein	4.3	3.49E-04	
DR_1335	Isoleucyl-tRNA synthetase (EC 6.1.1.5)	3.7	3.43E-03	1
DR_1063	Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8)	3.6	1.11E-02	0.775
DR_1199	ThiJ/PfpI family protein	3.5	1.08E-02	0.922
DR_0640	S-adenosylmethionine synthetase (EC 2.5.1.6)	3.4	8.29E-03	0.043
DR_A0184	Pyridoxal kinase (EC 2.7.1.35)	3.3	3.22E-03	1
DR_1915	hypothetical protein	3.1	1.67E-02	0.183
DR_2352	Porphobilinogen deaminase (EC 2.5.1.61)	3.1	2.78E-04	1
DR_0105	hypothetical protein	2.5	4.31E-03	1
DR_1422	hypothetical protein	2.5	9.43E-03	1
DR_A0018	5'-nucleotidase (EC 3.1.3.5)	2.5	5.20E-03	1
DR_0459	FIG00578356: hypothetical protein	2.3	1.01E-03	1
DR_1907	Predicted L-lactate dehydrogenase, Fe-S oxidoreductase subunit YkgE	2.3	7.41E-03	1
DR_B0014	Vitamin B12 ABC transporter, B12-binding component BtuF	2.2	1.25E-02	
DR_1277	ABC-type probable sulfate transporter, periplasmic binding protein	2.0	2.14E-03	0.043
DR_0006	Metal-dependent hydrolase (EC 3.-.-.-)	2.0	7.23E-03	1
DR_A0299	copper resistance protein, putative	2.0	5.32E-03	0.338
DR_1988	Phosphate starvation-inducible protein PhoH, predicted ATPase	-2.1	1.17E-02	1
DR_2346	2-amino-3-ketobutyrate coenzyme A ligase (EC 2.3.1.29)	-2.1	8.48E-03	1
DR_1027	amino acid ABC transporter, periplasmic amino acid-binding protein	-2.3	2.40E-05	1
DR_1055	Aspartyl-tRNA synthetase (EC 6.1.1.12) @ Aspartyl-tRNA(Asn) synthetase (EC 6.1.1.23)	-2.5	5.21E-03	0.705
DR_0921	Methionine gamma-lyase (EC 4.4.1.11)	-2.5	1.59E-02	0.043
DR_2123	Translation initiation factor 1	-2.5	8.73E-03	1
DR_1046	ClpB protein	-3.8	1.24E-02	1
DR_B0068	extracellular nuclease, putative	-21.5	5.24E-03	

RefSeq Locus Tag	Functional annotation	Fold Change	pValue	Predicted Fitness
DR_0085	LSU ribosomal protein L27p	-99.0	2.12E-04	0.135

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Table 5
Peptides significantly changed greater than 2-fold during late-log growth phase

Full list of detected peptides can be found in supplementary Table S5. Stress proteins induced > 3 fold are in red.

RefSeq Locus Tag	Functional annotation	Fold Change	pValue	Predicted Fitness
DR_0651	Arginase (EC 3.5.3.1)	99.0	2.79E-03	1
DR_0907	Cold shock protein CspA	99.0	6.68E-05	0.775
DR_2394	N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	43.5	1.21E-03	0.922
DR_1598	protease, putative	12.3	3.76E-05	0.848
DR_1370	FIG00579514: hypothetical protein	11.5	1.88E-03	1
DR_0009	Vancomycin B-type resistance protein VanW	10.0	5.56E-03	1
DR_0094	FIG00900003: hypothetical protein	9.3	6.67E-04	1
DR_1063	Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8)	8.8	1.49E-04	0.848
DR_1891	tetratricopeptide repeat family protein	7.3	1.93E-04	1
DR_1447	hypothetical protein	6.9	4.41E-05	1
DR_0612	Arginine utilization protein RocB	5.2	3.07E-04	1
DR_0606	Heat shock protein 60 family co-chaperone GroES	5.0	5.71E-04	0.182
DR_B0125	iron ABC transporter, periplasmic substrate-binding protein	4.7	6.12E-03	
DR_A0018	5'-nucleotidase (EC 3.1.3.5)	4.5	6.21E-04	1
DR_0121	FIG00578909: hypothetical protein	4.4	4.99E-03	1
DR_0640	S-adenosylmethionine synthetase (EC 2.5.1.6)	4.0	1.60E-03	0.043
DR_1624	Cold-shock DEAD-box protein A	3.9	2.53E-02	0.922
DR_1376	Hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8)	3.3	3.84E-03	0.706
DR_1335	Isoleucyl-tRNA synthetase (EC 6.1.1.5)	3.1	4.16E-03	0.134
DR_A0184	Pyridoxal kinase (EC 2.7.1.35)	3.0	3.86E-03	0.922
DR_0937	tetratricopeptide repeat family protein	3.0	3.29E-03	1
DR_0960	hypothetical protein	2.9	4.00E-03	1
DR_1915	hypothetical protein	2.9	1.36E-02	0.922
DR_A0333	FHA domain containing protein	2.8	3.59E-02	0.922
DR_B0014	Vitamin B12 ABC transporter, B12-binding component BtuF	2.8	7.90E-04	
DR_1379	Transcriptional regulator, TetR family	2.4	2.79E-03	1
DR_A0299	copper resistance protein, putative	2.3	8.07E-03	0.923
DR_1407	hypothetical protein	2.3	5.65E-03	1
DR_1465	FIG00577987: hypothetical protein	2.3	5.65E-03	1
DR_2278	amino acid ABC transporter, periplasmic amino acid-binding protein	2.2	1.81E-03	0.922
DR_1736	2',3'-cyclic-nucleotide 2'-phosphodiesterase (EC 3.1.4.16)	2.2	9.63E-04	0.922
DR_0686	Conserved repeat domain protein	2.1	8.48E-03	1
DR_1748	FIG00578260: hypothetical protein	2.1	5.80E-03	1
DR_1809	Glycine dehydrogenase [decarboxylating] (glycine cleavage system P protein) (EC 1.4.4.2)	2.1	2.79E-02	0.922
DR_0608	Histone acetyltransferase HPA2 and related acetyltransferases	2.0	4.27E-03	1
DR_0302	Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] (EC 2.6.1.16)	-2.0	1.59E-03	0.338

RefSeq Locus Tag	Functional annotation	Fold Change	pValue	Predicted Fitness
DR_0113	oxidoreductase, short-chain dehydrogenase/reductase family	-2.0	1.88E-04	0.922
DR_0456	MotA/TolQ/ExbB proton channel family protein	-2.0	6.70E-03	0.775
DR_2221	Tellurium resistance protein TerD	-2.0	1.63E-03	0.922
DR_A0005	Threonine dehydrogenase and related Zn-dependent dehydrogenases	-2.2	4.46E-02	0.922
DR_0859	Ribonuclease E inhibitor RraA	-2.2	4.40E-02	1
DR_A0237	Periplasmic aromatic aldehyde oxidoreductase, molybdenum binding subunit YagR @ 4-hydroxybenzoyl-CoA reductase, alpha subunit (EC 1.3.99.20)	-2.2	1.04E-03	0.923
DR_1132	Dihydroxy-acid dehydratase (EC 4.2.1.9)	-2.2	3.68E-03	0.922
DR_1544	Butyryl-CoA dehydrogenase (EC 1.3.99.2)	-2.2	8.73E-03	1
DR_0085	LSU ribosomal protein L27p	-2.3	3.70E-02	0.135
DR_2510	Enoyl-CoA hydratase (EC 4.2.1.17)	-2.5	1.00E-05	1
DR_1778	3-isopropylmalate dehydratase large subunit (EC 4.2.1.33)	-2.6	6.92E-05	0.847
DR_2346	2-amino-3-ketobutyrate coenzyme A ligase (EC 2.3.1.29)	-2.6	1.55E-03	0.775
DR_0362	D-alanine--D-alanine ligase A (EC 6.3.2.4)	-2.7	3.15E-02	0.232
DR_1074	(3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase (EC 4.2.1.-)	-2.7	4.27E-04	0.284
DR_0265	Predicted transcriptional regulator of N-Acetylglucosamine utilization, GntR family	-2.8	5.32E-03	1
DR_1082	Ribosomal subunit interface protein	-2.9	1.25E-04	0.922
DR_2033	Glutamine synthetase type III, GlnN (EC 6.3.1.2)	-3.3	1.24E-04	1
DR_1055	Aspartyl-tRNA synthetase (EC 6.1.1.12) @ Aspartyl-tRNA(Asn) synthetase (EC 6.1.1.23)	-3.4	1.21E-04	1
DR_1027	amino acid ABC transporter, periplasmic amino acid-binding protein	-3.7	1.61E-05	0.922
DR_1988	Phosphate starvation-inducible protein PhoH, predicted ATPase	-4.2	3.02E-03	0.922
DR_1072	Acetyl-CoA acetyltransferase (EC 2.3.1.9)	-5.0	1.07E-02	1
DR_1645	UDP-N-acetyl-D-mannosaminuronic acid transferase	-6.0	3.24E-03	0.847
DR_B0068	extracellular nuclease, putative	-9.3	4.56E-04	
DR_0459	FIG00578356: hypothetical protein	-99.0	1.00E-05	1