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Review

The t^{6}A modification acts as a positive determinant for the anticodon nuclease PrrC, and is distinctively nonessential in *Streptococcus mutans*

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Abstract

Endoribonuclease toxins (ribotoxins) are produced by bacteria and fungi to respond to stress, eliminate non-self competitor species, or interdict virus infection. PrrC is a bacterial ribotoxin that targets and cleaves $tRNA^{Lys}_{UUU}$ in the anticodon loop. *In vitro* studies suggested that the post-transcriptional modification threonylcarbamoyl adenosine (t^6A) is required for PrrC activity but this prediction had never been validated *in vivo*. Here, by using t^6A-deficient yeast derivatives, it is shown that t^6A is a positive determinant for PrrC proteins from various bacterial species. *Streptococcus mutans* is one of the few bacteria where the t^6A synthesis gene *tsaE* (*brpB*) is dispensable and its genome encodes a PrrC toxin. We had previously shown using an HPLC-based assay that the *S. mutans tsaE* mutant was devoid of t^6A. However, we describe here a novel and a more sensitive hybridization-based t^6A detection method (compared to HPLC) that showed t^6A was still present in the *S. mutans ΔtsaE,* albeit at greatly reduced levels (93% reduced compared to WT). Moreover, mutants in two other *S. mutans* t^6A synthesis genes (*tsaB* and *tsaC*) were shown to be totally devoid of the modification thus confirming its dispensability in this organism. Furthermore, analysis of t^6A modification ratios and of t^6A synthesis genes mRNAs levels in *S. mutans* suggest they may be regulated by growth phase.

Keywords

RNA maturation, translation, modified nucleosides, t^6A detection

Abbreviations

t^6A or t^6A_{37} threonylcarbamoyladenosine

ASL Anticodon Stem Loop

TCTC ThreonylCarbamoyl Transferase Complex

Introduction

tRNA molecules are heavily modified post-transcriptionally to ensure translational accuracy and cell survival[1]. tRNA Anticodon Stem Loops (ASLs) (Figure 1) make excellent targets for ribotoxins (or anticodon nucleases, ACNases) in toxin/antitoxin (TA) modules that can be utilized by organisms to eliminate competition or for suicidal functions under stress or phage attack[2,3]. Different ACNases have been characterized with very specific tRNA cleavage patterns. The *Escherichia coli* toxins Colicin E5 and Colicin D target tRNAs with a QUN anticodon (Q being the modified base Queuosine)[4] and $tRNA^{Arg}$ isoacceptors, respectively[5]. The *Mycobacterium tuberculosis* toxin VapC-mt4 targets $tRNA^{Ala}_{UGC}$, $tRNA^{Ser}_{GCU}$ and $tRNA^{Ser}_{GGA}$[6], while the enteric VapC proteins target $tRNA^{iniMet}_{CAU}$[7]. MazF-mt9, from the same organism, targets the ASL of $tRNA^{Lys}_{UUU}$[8]. Whereas none of these toxins require the presence of tRNA modifications for cleavage, there are other ACNases for which activity is dependent on such modifications. For example, the PaT toxin from *Pichia acacia* targets the methyl group of 5-methoxycarbonylmethyl-2-thiouridine ($mcm^5s^2U_{34}$) containing $tRNA^{Gln}_{UUG}$, as evidenced by the resistance of the $mcm^5s^2U_{34}$ deficient *Saccharomyces cerevisiae trm9*Δ strain[9]. Another example is the γ-toxin from *Kluyveromyces lactis,* which recognizes the wobble modification mcm^5U of $tRNA^{Glu}_{UUC}$[10].

One of the first identified ACNases was *E. coli* PrrC ($PrrC_{Ec}$)[11]. Infection of *E. coli* by bacteriophage T4 activates PrrC, which then cleaves the ASL of $tRNA^{Lys}_{UUU}$ to disrupt translation of late T4 proteins (primarily structural and assembly viral proteins)[12] and contain the infection in a suicidal gesture[2,13]. PrrC has been shown to cleave native $tRNA^{Lys}_{UUU}$ *in vitro* but not the corresponding unmodified transcript[14–16], leading to the hypothesis that a post-

transcriptional modification on $tRNA^{Lys}$ was a requirement for PrrC activity. Two complex post-transcriptional modifications are found in the ASL of $tRNA^{Lys}$ in both *E. coli* and *S. cerevisiae*[17]. First, the U at position 34 is modified to 5-methylaminomethyl-2-thiouridine (mnm^5s^2U) in *E. coli* and to 5-methoxycarbonylmethyluridine (mcm^5U) in *S. cerevisiae*. Second, the A at position 37 is modified to threonylcarbamoyl adenosine (t^6A) in both organisms (Figure 1). In some organisms, t^6A is further modified into complex derivatives such as cyclic-t^6A (ct^6A), N^6-methyl-t^6A (m^6t^6A)[6,7] and 2-methylthio-t^6A (ms^2t^6A)[18]. Unlike the γ-toxin from *K. lactis*, PrrC was still toxic when expressed in *S. cerevisiae elp3*Δ and *trm9*Δ derivatives (lacking mcm^5U_{34})[16]. This left the t^6A modification of $tRNA^{Lys}$ as a logical candidate for the missing positive determinant for cleavage by PrrC.

t^6A is one of the few universal modifications on the ASL found in most tRNAs that decode ANN codons[17]. The t^6A synthesis pathway has recently been elucidated in all domains of life (see[19] for review). In bacteria, the first enzyme L-threonylcarbamoyladenylate synthase (EC 2.7.7.87), which can be of type 1 (TsaC) or type 2 (TsaC2), produces L-threonylcarbamoyladenylate (TC-AMP). In yeast, the equivalent protein is encoded by the *TCS2* (*SUA5*) gene. The TC-AMP intermediate is then used by the bacterial tRNA adenosine (37) threonylcarbamoyltransferase complex, which is composed of the three subunits TsaD, TsaB and TsaE to transfer the threonylcarbamoyl moiety to the target adenosine-37 of the target tRNA. In the yeast cytoplasm, the transferase complex is composed of the KEOPS complex subunits (encoded by the *TCS3/KAE1, TCS5BUD32, TCS6/PCC1, TCS7CGI121, TCS8/GON7* genes) whereas TCS4 (QRI7) fulfills that role in yeast mitochondria. The *tsaBCDE* genes are generally essential in bacteria[20], and in *E. coli*, it was shown that t^6A is a strict determinant for the bacterial-type isoleucyl-tRNA synthetase (IleRS) and possibly for lysidine synthase (TilS), roles that easily explain the essentiality phenotype. However, the t^6A synthesis genes can be deleted in some bacteria, including *Streptococcus mutans*, an oral pathogen that thrives in multi-species biofilms on tooth surfaces[20]. In this organism, the *ΔtsaE (brpE/*SMU.409*)* strain is viable although growth rate was affected. The mutant is also compromised in biofilm formation, and is more sensitive than the wild-type to low pH and oxidative stress[21]. The *tsaE/brpE* gene is co-transcribed in *S. mutans* UA159 with *brpA*, which encodes a surface-associated protein with global effects on *S. mutans* biofilm formation and tolerance to antibiotic, acid, and oxidative stresses[22,23]. In a systematic study of a gene deletion library of *S. mutans*, *tsaC* (SMU.1083c) was found to be dispensable and its

deletion also led to poor acid survival and biofilm formation deficiencies[24]. Using an HPLC based assay, we previously showed that the *S. mutans ΔtsaE* strain lacked t^6A[20], but the t^6A levels in the *S. mutans tsaC* mutant have not been analyzed. More generally, the molecular rationale underlying the pH sensitivity and biofilm defects of t^6A deficient *S. mutans* strains is unknown.

Toxin/antitoxin modules such as MazE/F or RelB/E have been suggested to play a role in the survival of *S. mutans* in its harsh environment, possibly by triggering dormancy states[25,26]. A *prrC* homolog is also found in *S. mutans* (SMU.893/$PrrC_{Sm}$) just upstream of the *relBE* toxin/antitoxin genes[25]. The corresponding $PrrC_{Sm}$ protein is comparably active as a cytotoxin in yeast as its *E. coli* counterpart[27]. However, the role of PrrC in *S. mutans* physiology is not clear. The *prrC* gene is co-transcribed in an operon with the genes *hdsMSR* that encode the subunits of the type Ic DNA Restriction-Modification (R-M) complex prrI[28]. $PrrC_{Ec}$ is kept in an inactive state by binding to an EcoprrI/DNA complex, but the toxin can be activated by several mechanisms[28]. One such example is during infection by T4 phage, which results in the binding of *Eco*prrI by T4-encoded polypeptide STP, thus releasing active $PrrC_{Ec}$. Other stresses that affect the activity of type I DNA restriction endonucleases also appear to activate $PrrC_{Ec}$ and possibly disable protein synthesis[28]. Although, it is known that increased levels of dTTP combined with GTP hydrolysis activates the $PrrC_{Ec}$ ACNase, the triggers in other organisms such as *S. mutans* remain a mystery. Furthermore, it is unclear whether the PrrC toxin plays a role in dormancy or programed cell death (PCD), states known to be important for *S. mutans* survival[29].

In this work, we show that t^6A is a positive determinant for both the *E. coli* and *S. mutans* PrrC toxins. Using a newly developed and more sensitive than HPLC t^6A detection method (based on the previously described "positive hybridization in the absence of i^6A37" (*PHA6*) assay[30–32]), we demonstrate that t^6A is not absent in the *S. mutans ΔtsaE* strain as previously thought, but is present at greatly reduced levels compared to wild type. The *ΔtsaC* and *ΔtsaB mutants*, however, were also viable but appeared to completely lack this modification but are still viable. We also showed that in *S. mutans*, expression of both the *tsaBCE* genes and t^6A levels are both growth-phase dependent, being greatly reduced in stationary phase.

Results

The modification t^6A is a positive determinant for cleavage of tRNA by PrrC

If t^6A is a determinant for PrrC, then t^6A^- yeast strains should be resistant to the PrrC-induced growth inhibition previously observed in WT cells[16]. To test this hypothesis, a plasmid containing the *E. coli prrC* gene under the control of galactose inducible promoter (pYCPrrC$_{Ec}$)[16] was transformed into wild-type (WT) and t^6A deficient yeast strains. As shown Figure 2A, the BY4741 (pYCPrrC$_{Ec}$) cells did not grow in the presence of galactose whereas the t^6A deficient *tcs3*Δ (pYCPrrC$_{Ec}$) strain grew. The *tcs4*Δ strain (containing a deletion for the mitochondrial-specific t^6A synthesis gene but with a fully functional cytosolic t^6A biosynthesis machinery) was sensitive to the PrrC toxin similar to that observed in the WT strain. The strains carrying the control vector (pRS415) showed no difference in growth between induced and non-induced conditions.

A difficulty when working with most t^6A deficient yeast strains is their poor growth on minimal media supplemented with carbon sources other than glucose[33]. The *tcs8*Δ strain is an exception as it is devoid of t^6A but its growth on minimal media is robust enough for reproducible growth tests[33]. This strain was thus used to compare the toxicity of the PrrC$_{Ec}$ and *S. mutans* PrrC (PrrC$_{Sm}$) toxins in yeast and the role of t^6A in this toxicity. The empty vector pYCplac111 and a catalytically inactive variant of PrrC$_{Ec}$ bearing a Lys to Ala mutation at position 46 (PrrC$_{Ec}$ K46A)[16] were used as negative controls. As shown in Figure 2B, *tcs8Δ* is resistant to the expression of PrrC toxins from both *E. coli* and *S. mutans* while BY4741 is not. As expected, overexpression of any of the control plasmids did not affect growth of the BY4741or *tcs8Δ* strains. These results confirmed the prediction that t^6A is a positive determinant for the PrrC toxins from both *E. coli* and *S. mutans.*

Development of a sensitive hybridization based t^6A detection assay

To explore the role of t^6A in *S. mutans* physiology, a more sensitive detection assay was required. Inspired by the assay developed for the detection of i^6A by Northern blot (PHA6)[30], a Northern blot assay was developed for the detection of t^6A[34]. The premise of the assay is based on the carbamoylthreonyl group preventing hybridization of an ASL probe spanning position 37 (Figure 1). It is predicted that the extent of hybridization will increase as the levels of

t^6A modification decrease. A probe spanning the TΨC loop of the tRNA can be used as an internal control for tRNA quantification. For this assay, purified bulk tRNAs were spotted and cross-linked onto neutral nylon membranes and detected with biotinylated probes (Table S1) to allow detection with streptavidin-labeled Horseradish Peroxidase (HRP).

tRNA extracted from yeast BY4741, *tcs*2Δ, *tcs*4Δ, and *tcs*3Δ strains, were spotted onto nylon membranes in duplicate. One membrane was treated with a biotinylated probe annealing to the TΨC loop and the second membrane was treated with the ASL probe for $tRNA^{Ile}_{IAU}$. As shown in Figure 3A, only tRNAs from t^6A deficient strains *tcs2Δ* and *tcs3Δ* hybridized with the ASL probe, while no hybridization is observed with BY4741 and minimal hybridization with *tcs4Δ*. tRNAs from all strains hybridized with the TΨC probe showing relative quantities of tRNAs spotted on the membrane. These results are consistent with t^6A detection via HPLC[33]. The limit of detection of this method for yeast bulk tRNA was found to be 10 ng (Figure 3B).

This assay was then extended to tRNAs from the bacterial model *E. coli* and to different tRNA isoacceptors. t^6A is essential in *E. coli* making it impossible to isolate tRNAs from a t^6A deficient strain, therefore *in vitro* transcribed tRNA was used as negative control using probes specific for $tRNA^{Ile}_{GAU}$ and $tRNA^{Thr}_{GGU}$. As shown Figure 3C, both wild type and *in vitro* transcribed tRNAs anneal to the TΨC probe, while only the transcripts anneal to the ASL probe. Similar results were obtained with probes specific for with *E. coli* $tRNA^{Thr}_{GGU}$. This new assay was named Positive Hybridization in the Absence of t^6A, or PHAt6A Assay. This method allows one to detect t^6A in specific tRNAs with a sensitivity that now allows the exploration of the role of t^6A in bacterial physiology generally, and more specifically in *S. mutans*.

t^6A is not essential is *S. mutans* but the *tsaE* mutant does contain residual amounts of the modification

Previous analysis of bulk tRNA extracted from the *S. mutans ΔtsaE* strain using HLPC indicated that no t^6A was present in that background[20]. However, when using the PHAt6A method with probes specific for *S. mutans* $tRNA^{Ile}_{GAU}$, faint annealing was observed, which made us consider that t^6A may still be present (Figure 4A). To investigate this result further, each corresponding tRNA preparation was analyzed by mass spectrometry (MS), which demonstrated

that t^6A was present in the *ΔtsaE* strain but in lower amounts (~7% t^6A modified) than the wild-type (Figure 4B and Figure S1A). This result made us question our prior conclusions on the dispensability of t^6A in *S. mutans*[20]. We therefore obtained the *S. mutans* UA159 *ΔtsaC* and *ΔtsaB* strains (kind gift from Robert Quivey, University of Rochester)[24], and analyzed t^6A levels using PHAt6A with the probe targeting $tRNA^{Ile}_{GAU}$. As shown in Figure 4C, strong annealing of the ASL probe was observed in the tRNA extracted from the *ΔtsaC* and *ΔtsaB* strains but also from the *ΔtsaE* mutant. This ambiguity in the PHAt6A necessitated MS analysis (Figure 4B) of the tRNA samples. Indeed *ΔtsaC* and *ΔtsaB* are devoid of t^6A while *ΔtsaE* maintains trace amounts of t^6A modified tRNA. With only ~7% of the WT t^6A levels present in the *ΔtsaE* strain, we are at the limit of the detection of the PHAt6A modification and under the limit of regular HPLC[20]. Among the platforms for t^6A detection, only MS offers the sensitivity to detect such low levels of the modification.

We extended the method to other *S. mutans* tRNAs and found that $tRNA^{iniMet}_{CAU}$, a tRNA known to not be t^6A modified in bacteria[35], showed reduced annealing in the Δ*tsaE* strain compared to WT. This suggests *tsaE* might play a role in tRNA discrimination in the t^6A insertion machinery. It is possible that $tRNA^{iniMet}_{CAU}$ is targeted for t^6A modification by mistake when *tsaE* is absent. Further analysis is required to fully elucidate this observation.

Analysis of *tsaBCDE* expression levels and t^6A content in *S. mutans* in different growth conditions

At the time of the analysis (March 2017 release), 22 transcriptomics experiments for the *S. mutans* U159 strain had been integrated in the PATRIC[36] and 25 in the Microbesonline databases[37]. Using the transcriptomics heatmap analysis tools of pathway databases, we surveyed expression profiles of *prrC* (SMU.893) and of the four t^6A synthesis genes (*tsaC*/SMU.1083c; *tsaB*/SMU.385; *tsaD*/SMU.387; *tsaE*/SMU.409) and found a few conditions where these genes were differentially-expressed, such as biofilm vs. planktonic growth or when co-cultured with other oral bacteria (Table S2).

As mentioned above, the available transcriptomic data did suggest that the genes encoding for the t^6A synthesis proteins might be differentially expressed in biofilm vs planktonic growth. To explore this further, cells were grown in rich (BHI) media and in semi-defined media containing sucrose (which promotes biofilm growth)[38]. Cells were harvested from these two culture conditions at 4 different growth phases: early exponential (4 h growth), mid

exponential (6 h growth), late exponential (8 h growth), and stationary phase (12 h growth) (Figure S2). tRNA was extracted from cells harvested from each growth phase and growth condition, and analyzed via PHAt6A and by LC-MS/MS. The PHAt6A shows an increase in signal in the ASL from early exponential (4 h) to stationary phase (12 h) (Figure 5), suggesting that t^6A levels decrease in a growth-phase dependent manner. The samples were analyzed via MS on two separate occasions and both analysis showed a similar trend of decreasing t^6A levels along the growth curve for both BHI and biofilm media. However, there is no statistically significant difference in the degree of t^6A modification for each of these time points (Figure S3). This seeming disparity in results between the two methods arises from the scope of each analysis. MS quantifies t^6A modification from all tRNA isoacceptors where subtle variations in t^6A levels of a few tRNA species may be undetectable. In contrast, PHAt6A reports only on a specific tRNA species allowing for isoacceptor-specific monitoring of t^6A levels. Expression of *prrC* and of the t^6A biosynthesis genes were also measured by quantitative real-time PCR (qPCR) in the same set of biological samples. Expression of the t^6A synthesis genes correlated well with the PHAt6A results, whereby expression of most t^6A biosynthesis genes and of *prrC* decreased during stationary phase in both BHI and biofilm media (Figure 6A). The only exception to this pattern was in *tsaD* expression, which increased in stationary phase relative to exponential growth phase. The same pattern of growth phase dependent gene expression was observed in both BHI and biofilm media cultures (Figure 6A). Moreover, when directly comparing expression of each gene between growth conditions at each time point (Figure 6B), stationary phase *tsaC* expression was increased 2.5-fold in biofilm media relative to BHI.

Discussion

Recent studies in different model organisms have shown that levels of tRNA modification levels can be fine-tuned to specifically regulate the translation efficiency of specific genes[39,40]. To date, no such regulation has been observed with t^6A dependent codons, and it is not known whether t^6A levels are regulated in any model system. Indeed, the identity of the complete set of genes involved in t^6A synthesis were only discovered within the last five years[41]. In addition, the methods available for t^6A detection did not allow, until very recently, any physiological studies that could address potential regulation mechanisms. Advances in MS analytical methods, as well as the hybridization based

PHAt6A assay, described in this present paper, have now solved this issue. Using these tRNA modification profiling platforms, the ratios of specific modifications found in bulk tRNA extracted from different conditions can be captured. Recent studies have shown that levels of t^6A and ms^2t^6A vary accordingly to fluctuations in tRNA modification profiles in *Mycobacterium tuberculosis* under hypoxic conditions[40], and yeast under different stress conditions[43]. The MS platform is quantitative for the total amount of t^6A but is unable to discriminate between the different tRNAs. To identify the specific tRNA modifications in a sequence context, another MS-based platform called the RNA modification mapping approach can be used. This method involves base-specific RNase digestion to the oligonucleotide level. These RNase digestion products are separated and analyzed by LC-MS/MS from which the identity and site of modification can be determined[44]. Whereas MS-based platforms are accurate and robust, the cost of, and access to, such detection platforms is a major challenge. Herein lies the advantage of the PHAt6A assay, a cost-effective and universally accessible method for t^6A detection. This method allows detection of t^6A levels in specific tRNAs in very low sample quantities (only 10 ng of bulk tRNA are required) therefore facilitating analysis of samples from limited biological sources. Moreover, PHAt6A enables one to survey t^6A levels in many replicates over multiple growth conditions. In fact, this assay has already been used to monitor t^6A variations in *TCS3* mutants in *Drosophila melanogaster*[34]. With the recent discovery that mutations in the Human Tcs4 (Kae1) gene lead to severe disease[45], methods to easily detect t^6A levels in human cells could have diagnostic value.

The PHAt6A assay was used to analyze t^6A levels in different tRNAs extracted from *S. mutans* WT, *ΔtsaE*, *ΔtsaC* and *ΔtsaD* strains and showed that: 1) t^6A is dispensable in *S. mutans*; 2) *tsaC* and *tsaB* are strictly required for t^6A synthesis; 3) the absence of *tsaE* significantly reduces the amounts of t^6A, and TsaE is not strictly required for t^6A synthesis, instead possibly playing a role in specificity (targeting the correct tRNA) or in regulation of t^6A levels.

The first study on regulation of t^6A synthesis genes was recently published in the *Mycobacterium tuberculosis* model[46]. The *tsaD*, *tsaB* and *tsaE* genes are in the same operon with quite a complex regulation. Specific expression of *tsaD* was observed under a few conditions such as H_2O_2 exposure[46]. The *S. mutans tsaE* (*brpB*) gene is co-transcribed with the regulator gene *brpA*[22] (Figure S4), which plays a role in resistance to various antibiotic and environmental stressors[22,23]. The *S. mutans tsaB* and *tsaD* genes are in a predicted operon that encodes a MarR-type regulator

(SMU.384) of unknown function[37] (Figure S4). Results from available transcriptomic data (Table S2) and the qPCR results presented here (Figure 6 and Supplemental data 1), do suggest that the expression of *S. mutans* t^6A genes is growth-phase dependent, but further experiments will be required to fully understand the nature of this regulation.

The PrrC ACNases from *E. coli* or *S. mutans* were only toxic in yeast strains harboring t^6A. Combined with the prior results that showed that PrrC was toxic to strains missing the mcm^5U_{34} modification[47], it is clear t^6A is a positive determinant for PrrC. Attempts to purify recombinant WT PrrC for structure/function studies are confounded by the inhibition of protein synthesis elicited when PrrC begins to accumulate. The *tcs8Δ* yeast strain is resistant to PrrC but grows better than the other t^6A deficient yeast strains and could be used as a host for the large-scale expression of the toxin.

The presence of PrrC in *S. mutans*, an organism where t^6A is dispensable, raises questions and avenues for future work. Recent single cell analysis experiments have shown that subpopulations of *S. mutans* cells in biofilm have different fates: growth, dormancy or death depending on the expression of specific toxins[48]. Given that both *prrC* and *tsaE* expression are down-regulated in a *brpA* mutant (Table S2) and BrpA is thought to respond to cell envelope stress[22], could downregulation of t^6A levels protect a subpopulation from stress-induced PrrC? Further studies are required to explore the physiological role of PrrC in *S. mutans* and involvement of t^6A in mechanisms of toxin resistance.

Methods

Strains and Growth Conditions

A list of all organisms used in this study can be found in Table S3. Yeast strains were grown on YPD (DIFCO Laboratories) at 30°C. Synthetic minimal media, with or without agar, with or without dropout supplements (-uracil, -ura; -leucine, -leu; -histidine, -his) were purchased from Clontech (Palo Alto, CA) and prepared as recommended by the manufacturer. Glucose (Glu, 2% w/v), Glycerol (Gly, 4% w/v), 5-fluoro-orotic acid (5-FOA, 0.1% w/v) and G418 (300 μg/mL) were used when appropriate. Yeast transformations were carried out using frozen competent cells as

described[49] with plating onto the appropriate media. *E. coli* strains were grown in LB (1% tryptone w/v, 0.5% yeast extract w/v, and 1% salt w/v; 1.5% agar w/v was added for plates) at 37 °C, unless otherwise stated. When necessary, LB was supplemented with kanamycin (Kn, 50 μg/mL), ampicillin (Ap, 100 μg/mL), or chloramphenicol (Cm, 35 μg/mL). *S. mutans* was grown in Brain Heart Infusion media at 37°C in a CO_2 incubator. When necessary, 5 μg/μL of erythromycin, kanamycin (50 μg/mL), and spectinomycin was added. For phenotype screens, yeast cultures were grown in the media listed in the figure to saturation, washed, normalized to an OD_{600} of 1.0 and 5 μL of 1:10 serial dilutions were spotted on the listed media with the supplements listed in the figure and text. Galactose (2% w/v) and Raffinose (1% w/v) was added when needed.

Bioinformatics

Transcriptomics data was taken from the PATRIC database where as of March 2017, 22 experiments for *S. mutans* were available[36]. Microbesonline was also used as a resource for microarray data and operon prediction for *S. mutans*[37]. Resources at the National Center for Biotechnology Information (NCBI) and BLAST tools were used [50]. tRNA gene sequences were taken from the GtRNAdb: Genomic tRNA Database[51].

Extraction of bulk tRNAs and Preparation of *in vitro* transcribed tRNA

Bulk tRNA were prepared as previously described using acid buffered-phenol (phenol saturated with 50 mM sodium acetate, pH 5.8) and alcohol precipitation[52]. The template for producing E. coli $tRNA^{Ile}_{GAU}$ transcript was produced via a Klenow extension reaction[53] with the oligonucleotides 5'-AATTCCTGCAGTAATACGACTCACTATAAGGCTTGTAGCTCAGGT GGTTAGAGCGC-3' and 5'-TGGTAGGCCTGAGTGGACTTGAACCACCGACCTCACCCTT ATCAGGGGTGCGCTCTAAC-3'. The template for *E. coli* $tRNA^{Thr}_{GGU}$ utilized the plasmid pCDI147 which had been linearized by *Mva*I to allow for run-off transcription. pCDI147 was generated using two ligation events. First, the ligation of 6 oligonucleotides 5'-AGCTTTAATA CGACTCACTATAGGGGCTGATATGGCTCAG-3', 5'-TTGGTAGAGCGCACCCTTGGTAG GGGTGGGGTCCCCAGTTCGACTCTGGG-3', 5'-TATCAGCACCATATGCTAGTTATTGC TCAGG-3', 5'-GATCCCTGAGCAATAACTAGC-3', 5'-

ATATGGTGCTGATACCCAGAGTC GACTGGGGACCCCACCCCTACCAAGGG-3', and 5'-TGCCTCTACCAACTGAGCCATAT CAGCCCCTATAGTGAGTCGTATTAA-3'. This oligonucleotide was subsequently digested with *Bam*HI/*Hind*III before a second ligation into a similarly treated pUC18 plasmid to generate pCDI147. Transcription reactions were run for four hours at 37 °C in 80 mM HEPES (pH 7.4), 2.0 mM spermidine, 24 mM MgCl2, 2.0 mM ribonucleotide triphosphates (NTPs), 3 μM template, and 2.5 μg/mL of T7 polymerase. The RNA products generated in the transcription reactions were precipitated by the addition of 0.1 volume 8.0 M ammonium acetate, 3 volumes of 100% ethanol, and cooling at −80 °C for 30 minutes, then pelleted by centrifugation at 15,000 RCF for 30 minutes at 4 °C, and resuspended in 50 mM HEPES (pH 7.4), 2.0 mM EDTA. The solutions were mixed 1:1 with formamide, heated at 90 °C for 5 minutes, and snap cooled on ice before being purified via Urea-PAGE electrophoresis (10%). The RNA was extracted by cutting the excised band from the Urea-PAGE gel, slicing it into 1 cm cubes, followed by adding 10 mL HEPES (pH 7.4) 2 mM EDTA per 1 g of gel. This suspension was then placed at 4 °C with agitation overnight. The soluble portion of the suspension was then precipitated as previously described and resuspended in water. The tRNA solution was then frozen at -80 °C before lyophilization.

Positive Hybridization in the Absence of t6A Assay

Blotting. tRNAs were diluted to the appropriate concentration (3 μg – 1ng/μL) to which 3 volumes of denaturing solution (500 μL formamide, 162 μL 37% formaldehyde, 100 μL RNase-free 10X MOPS) was added. tRNAs were denatured at 85°C for 15 min and cooled to 4°C for 2 min and the final volume was adjusted to 30 μL with 10X SSC (1.5 M sodium chloride, 0.15 M sodium citrate). Biodyne® A membrane (Thermo Scientific) was rehydrated in 10X SSC for at least 10 min and placed in a dot blot vacuum manifold (Bio-Rad). Each of the wells were rinsed twice with 0.5 mL 10X SSC before applying the denatured tRNA samples. The wells were rinsed twice with 10X SSC before removing the membrane from the apparatus. The membrane was dried at in an 80˚C incubator for 30 seconds followed by RNA crosslinking at 120 mJ/cm^2 (optimal crosslink mode in Fisher Biotech UV Crosslinker FB-UV XL-1000).

Hybridization. The membrane was rehydrated in 10X SSC for 1 minute and pre-hybridized for 30--60 min at 42°C with pre-warmed Dig Easy Hyb (Roche). One μL of 100 μM biotinylated probes (Listed in Table S1) per 5 cm^2 membrane

was added to 80 µL DIG Easy Hyb and heat denatured at 95˚C for 10 minutes followed by cooling to 4°C for 2 min. The denatured probes were applied to the pre-hybridized membrane and incubated at 39°C for at least 16 hours with moderate rocking.

Washing. Hybridization solution was poured off and two types of washes was implemented: The low stringency wash consists of 3 washes with wash buffer (2x SSC/0.2% SDS) for 10 min while the high stringency wash adds 1 additional wash at room temp for 10 min and 1 final wash at 55°C for 15 min. For *S. cerevisiae* $tRNA^{Ile}$ GAU, membrane probed with TΨC was subjected to low stringency washing while the membrane probed with ASL required the high stringency wash to reduce background. For *E. coli* and *S. mutans* tRNAs, low stringency washing for both membranes were sufficient. Visualization was performed as described by the manufacturer of North2South Chemiluminescent Detection Kit (Thermo Scientific No. 17097)

Quantitative Real-time PCR (qPCR)

All *S. mutans* UA159 cultures were grown at 37 °C, 0 RPM in a 5% CO_2 incubator. For each experiment, *S. mutans* was freshly streaked from a 40% (vol vol^{-1}) glycerol stock (stored at -80ºC) onto Brain heart infusion (BHI) agar and grown for 48 h. A single colony was then inoculated into 40 ml BHI broth, and grown for 18 hours. For each growth experiment (n = 3), the *S. mutans* 18 hour culture was diluted to an optical density at 600 nm (OD_{600}) = 0.05, in a 0.4 media/flask volume ratio, and grown in BHI or Biofilm Media containing 11 mM glucose and 10 mM sucrose[38]. Culture samples were collected from each flask at 4, 6, and 12 hours growth (corresponding to early exponential, late exponential, and stationary phase), harvested by centrifugation, and cell pellets were stored at −80 °C in RNAlater (Thermo Fisher Scientific). RNA was subsequently isolated with the RNeasy Kit (Qiagen) and FASTPREP lysing matrix B tubes (MP Biomedical) using previously-described methods[54,55]. Each RNA sample was then subjected to a second DNAse treatment using the TURBO DNA-free™ Kit (Thermo Fisher Scientific) per the manufacturer's protocols. Lack of contaminating genomic DNA in each RNA sample was determined using PCR and *S. mutans gyrB* primers. RNA samples (0.750 µg) were subsequently converted to cDNA using the iScript Reverse Transcriptase kit (BioRad). Expression of genes of interest was measured in the cDNA from each sample by

quantitative real-time qPCR using iQ SYBR green supermix (BioRad) and the CFX Connect System (BioRad) following previously-published qRT-PCR protocols[56]. The Livak method ($2^{-\Delta\Delta Ct}$)[57] was used to calculate the relative fold change between the calibrator samples (indicated in each figure legend) and test samples. Primers specific to the housekeeping gene *gyrB* (gyrB-F/gyrB-R) were used as the reference gene (Table S4).

Mass Spectrometry

Total tRNA samples were digested with purified RNase T1 (50 U/µg tRNA) in a 220 mM ammonium acetate buffer for 2 h at 37°C. Samples were vacuum dried and resuspended with 10 µL mobile phase A for LC-MS/MS analysis. The RNase digestion products were separated on a Poroshell 120 EC-C18 column (1 × 50 mm and 2.7 µm pore size, column oven at 30 °C) using a Thermo Surveyor HPLC attached to a Thermo LTQ-XL (Thermo Scientific, Waltman, MA) linear ion trap mass spectrometer. Mobile phase A (MPA) consists of 8 mM TEA/200 mM HFIP, pH 7 and mobile phase B is 50% MPA and methanol with a flow rate set at 50 µL/min. The LC gradient initiated at 10%B then increased linearly to 60%B for 32 min, followed by 95%B for 5 min before a minimum 20 min re-equilibration period at 10%B. The source was set at the following conditions: capillary temperature was set at 275 °C, spray voltage of 4 kV and 35, 14 and 10 arbitrary flow units of sheath, auxiliary and sweep gas, respectively. The mass spectra were recorded in negative polarity. The entire run was divided into two segments, each with five scan events. The product ion's sequence information was obtained by collision induced dissociation (CID) in scan events 2--5. Data acquisition was through Thermo Xcalibur software.

Analysis of t^6A modifications in *S. mutans* grown in rich media and biofilm media

Bulk tRNA (1 µg) from each sample was hydrolyzed to ribonucleosides in a reaction containing Benzonase (0.375 U), calf intestine alkaline phosphatase (8.5 U), phosphodiesterase I (0.05 U), coformycin (3.5 µM; nucleobase deaminase inhibitor), deferoxamine (3 mM; antioxidant), butylated hydroxytoluene (0.3 mM; antioxidant) HEPES (500 mM, pH 8) and $MgCl_2$ (5 mM) in a final reaction volume of 50 µl. The reaction was allowed to proceed for 2 h at 37 °C and was stopped by removal of the enzymes by microfiltration with 10,000 Da spin filters. Following the addition of [$^{15}N_5$]-2'-deoxyadenosine as an internal standard for data normalization, ribonucleosides were resolved on a Synergy Fusion RP

HPLC column (2.5 µm particle size, 100 Å pore size, 100 mm length, 2 mm inner diameter; Phenomenex, Torrance, CA, USA) mounted on an Agilent 1290 series HPLC system equipped with a diode array detector (DAD). The ribonucleosides were eluted at a flow rate of 0.35 ml/min and a column temperature of 35 °C with a gradient consisting of 5 mM ammonium acetate (A) and acetonitrile (B) as follows: 0–1 min 100% A, 1–10 min 0–10% B, 10–24 min 10–40% B, 24–44 min 40–80% B, and 44–49 min 100% A to regenerate the column. The column, with its eluent directed through the DAD to record the 260 nM absorbance of canonical ribonucleosides, was coupled to an Agilent 6430 triple quadrupole mass spectrometer operated in positive ion mode with the following parameters: electro-spray ionization (ESI-MS), fragmentor voltage (average) 80 V, cell accelerator voltage 2 V, N_2-gas temperature 350 °C, N_2-gas flow 10 l/min, nebulizer 40 p.s.i., capillary 3500 V. Using dynamic multiple reaction monitoring (MRM), modified ribonucleosides were identified based on retention time (t^6A at 7.9–8.3 min) and mass transition (*m/z* 413→281 for loss of ribose from t^6A). The signal for t^6A was normalized by dividing by the peak area of the [$^{15}N_5$]-dA standard (inter-sample variation) and by the summed MRM peak areas of the canonical ribonucleosides (input RNA variation). The normalized peak areas of three biological replicates were then averaged.

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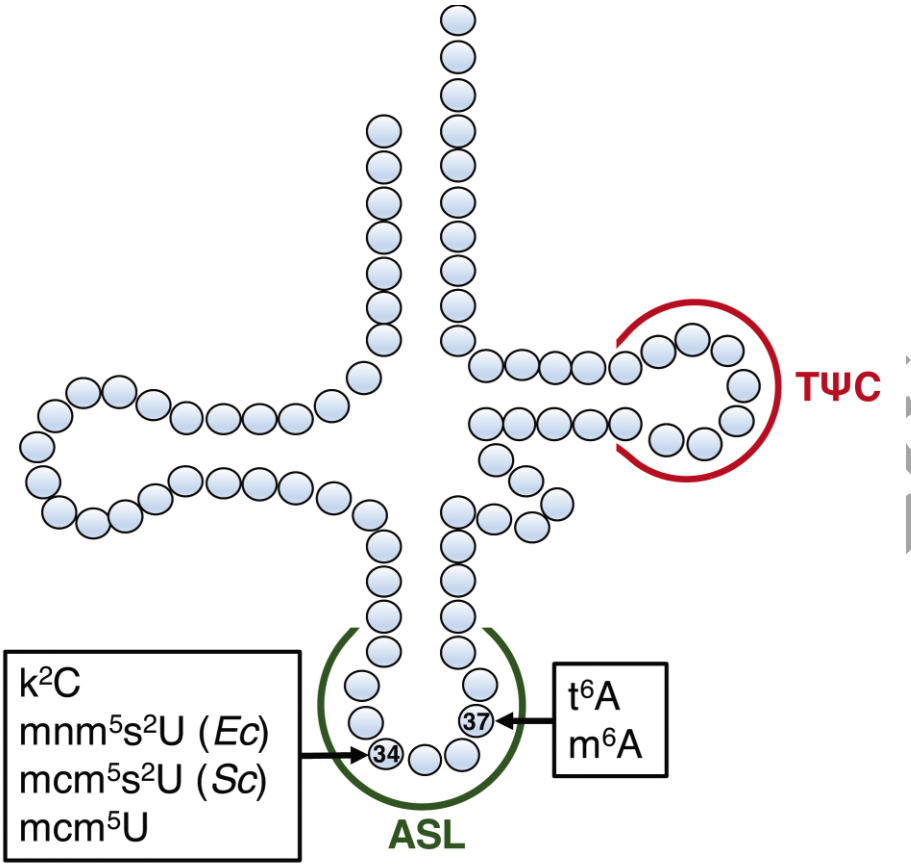

Figure 1. Mapping of nucleoside modifications and targets for oligo probes on tRNA. Modifications on positions 34 and 37 are listed in their respective boxes. Target for ASL probe is depicted in green and target for the TΨC probe is depicted in red.

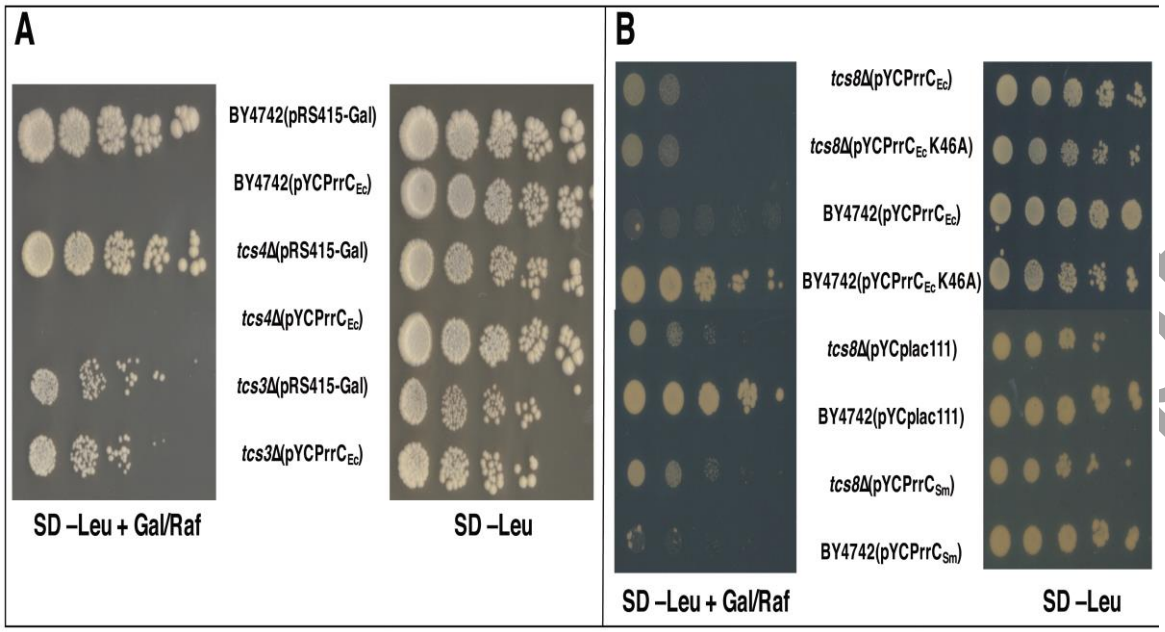

Figure 2. Resistance of t^6A deficient yeast strains to PrrC toxins. A. Cytosolic *tcs3Δ* is resistant to $PrrC_{Ec}$ while mitochondrial *tcs4Δ* is not. B. *tcs8Δ* is resistant to both $PrrC_{Ec}$ and $PrrC_{Sm}$, while the $PrrC_{Ec}$ K46A variant has no effect on growth. Cells were grown in synthetic minimal media containing agar and leucine dropout supplement (SD-Leu). Galactose (2% w/v) and raffinose (1% w/v) were added where necessary (SD-Leu Gal/Raf). Strains were incubated at 30°C for ~72 hours.

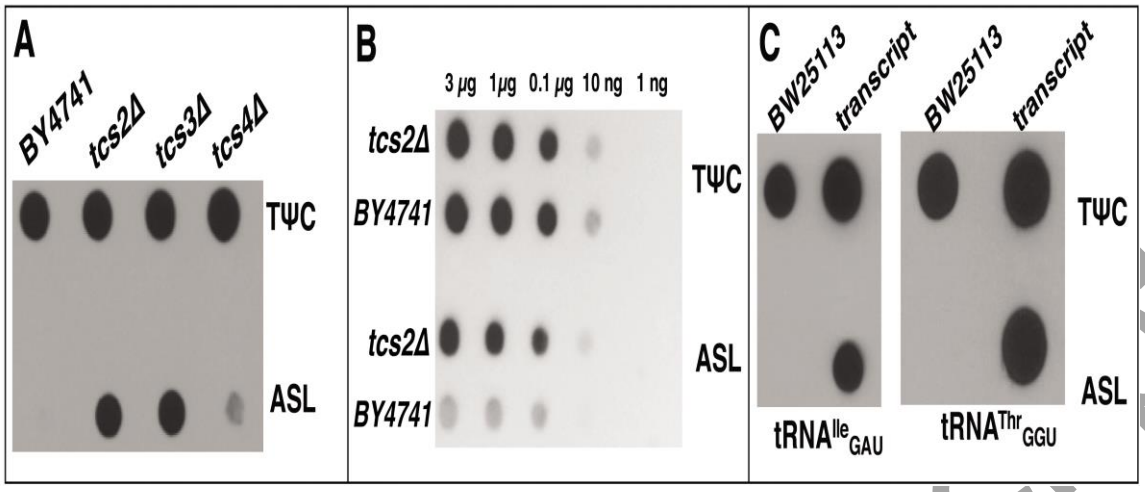

Figure 3. PHAt6A assay with yeast and *E. coli* bulk tRNAs. A. PHAt6A with tRNA isolated from wild type yeast and t^6A deficient strains. B. Sensitivity of the PHAt6A method using yeast tRNAs. C. PHAt6A with wild type *E. coli* and *in vitro* transcribed tRNAs.

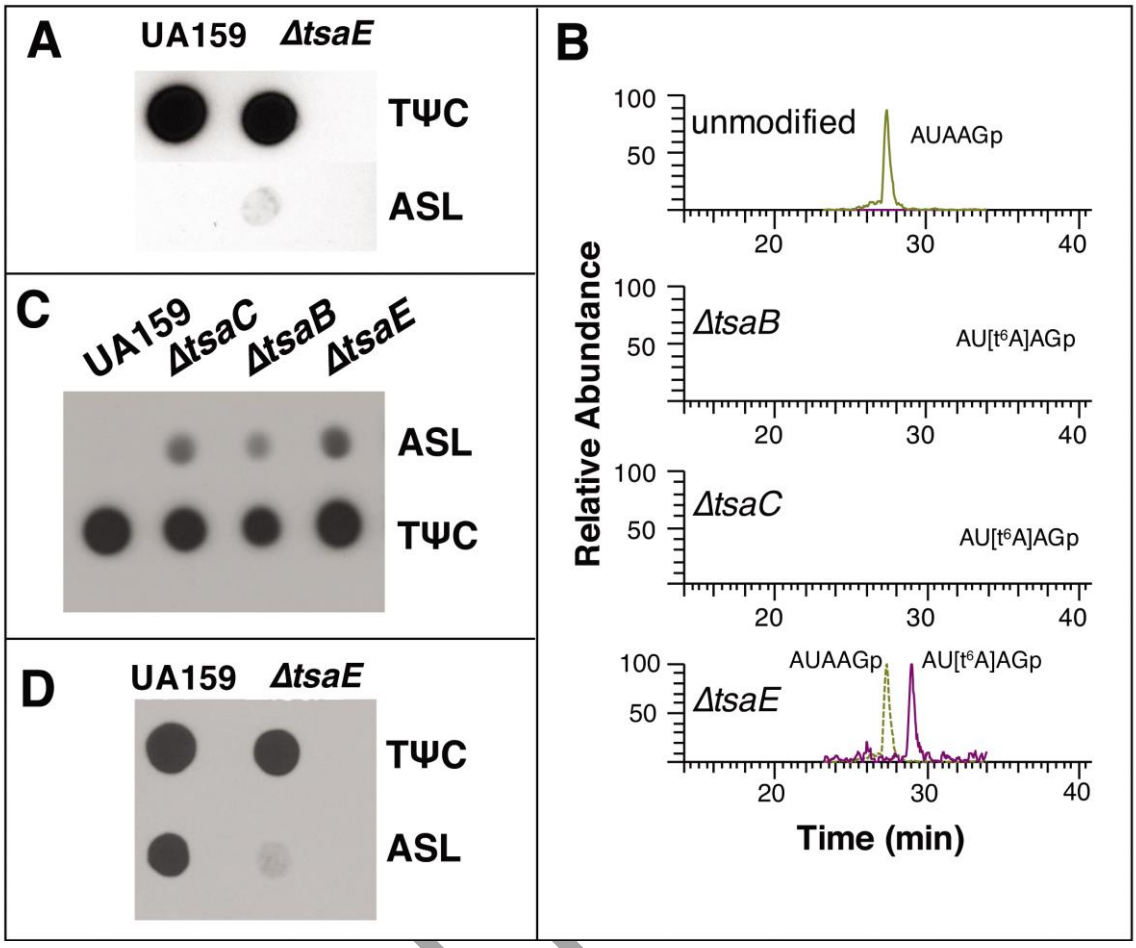

Figure 4. Detection of t^6A in *S. mutans* wild-type and mutant strains. A. PHAt6A with *S. mutans ΔtsaE*. B. Mass spec analysis of t^6A modification in $tRNA^{Ile}_{GAU}$ showing no t^6A in *ΔtsaC* and *ΔtsaB* but trace amounts are detected in *ΔtsaE.* C. PHAt6A with t^6A deficient strains *ΔtsaC, ΔtsaB, ΔtsaE* using oligonucleotides specific to $tRNA^{Ile}_{GAU}$. D. PHAt6A with wild type UA159 and *ΔtsaE* using probes for $tRNA^{iniMet}_{CAU}$.

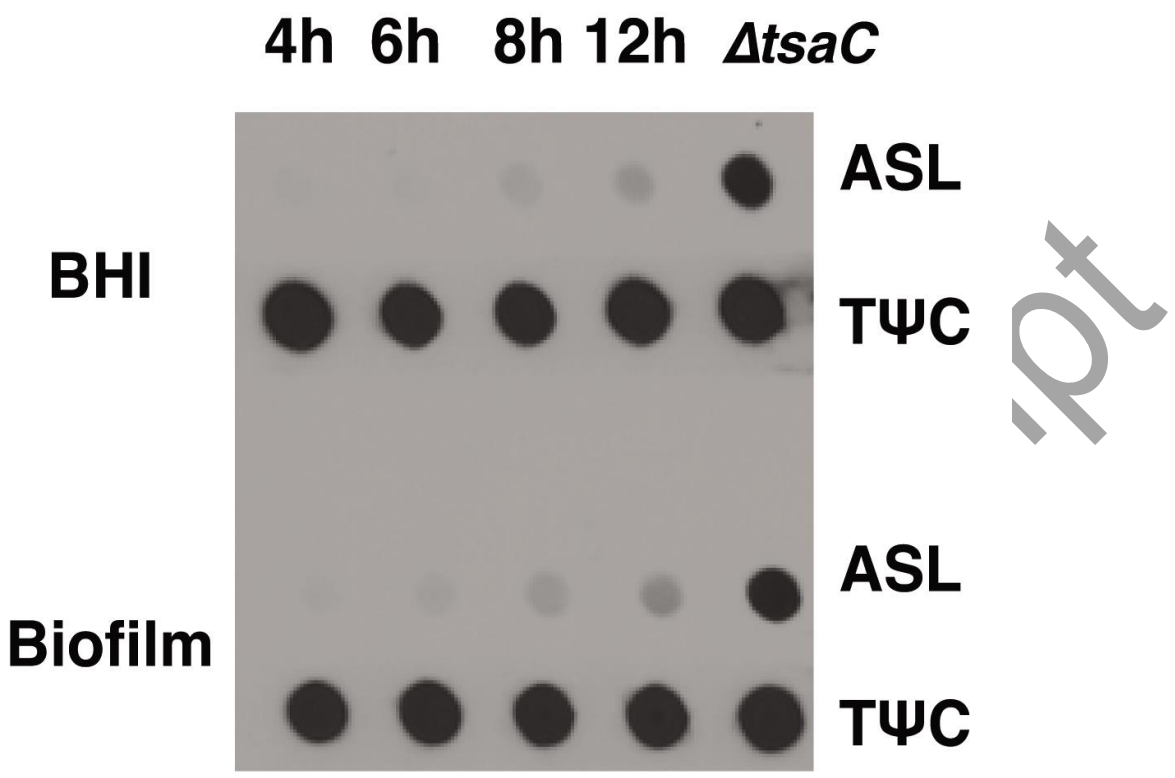

Figure 5. Detection of t^6A in *S. mutans* UA159 tRNA grown in Biofilm and Rich Media. PHAt6A of tRNA isolated from the following time points: 4h – Early-exponential, 6h – Mid-exponential, 8h – Late-exponential, and 12h – Stationary phase.

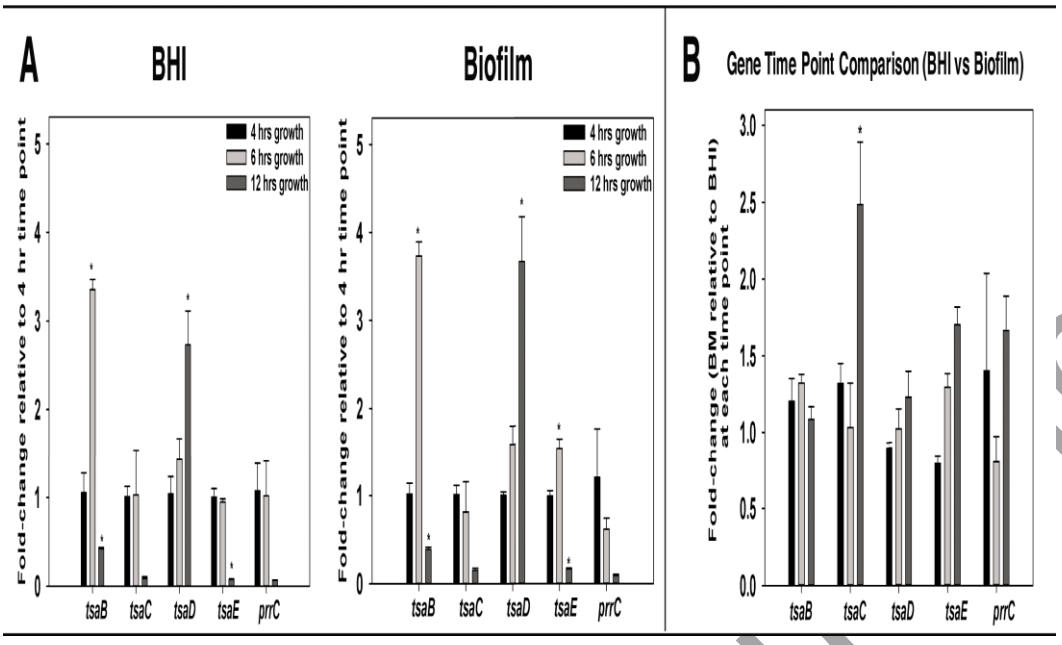

Figure 6. *S. mutans* UA159 gene expression profile for *ΔtsaC*, *ΔtsaB*, *ΔtsaD*, *ΔtsaE*, and *prrC* genes during growth in Biofilm and Rich media (BHI). A. Fold change difference of each of the genes with respect to 4h time point (calibrator). * indicates statistical significance ($P < 0.05$, Student-Newman-Keuls Test) relative to calibrator sample. B. Fold change difference in gene expression at each time point in Biofilm media with respect to Rich media (calibrator). * indicates statistical significance ($P < 0.05$, Two-tailed T-Test) relative to calibrator sample.