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RESEARCH ARTICLE

Analysis of tRNA^{Cys} processing under salt stress in *Bacillus subtilis* spore outgrowth using RNA sequencing data [version 1; peer review: 2 approved, 2 approved with reservations]

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Abstract

Background: In spore-forming bacteria, the molecular mechanisms of accumulation of transfer RNA (tRNA) during sporulation must be a priority as tRNAs play an essential role in protein synthesis during spore germination and outgrowth. However, tRNA processing has not been extensively studied in these conditions, and knowledge of these mechanisms is important to understand long-term stress survival.
Methods: To gain further insight into tRNA processing during spore germination and outgrowth, the expression of the single copy tRNA^{Cys} gene was analyzed in the presence and absence of 1.2 M NaCl in *Bacillus subtilis* using RNA-Seq data obtained from the Gene Expression Omnibus (GEO) database. The CLC Genomics work bench 12.0.2 (CLC Bio, Aarhus, Denmark, https://www.qiagenbioinformatics.com/) was used to analyze reads from the tRNA^{Cys} gene.
Results: The results show that spores store different populations of tRNA^{Cys}-related molecules. One such population, representing 60% of total tRNA^{Cys}, was composed of tRNA^{Cys} fragments. Half of these fragments (3′-tRF) possessed CC, CCA or incorrect additions at the 3′end. tRNA^{Cys} with correct CCA addition at the 3′end represented 23% of total tRNA^{Cys}, while with CC addition represented 9% of the total and with incorrect addition represented 7%. While an accumulation of tRNA^{Cys} precursors was induced by upregulation of the *rrnD* operon under the control of σ^{A}-dependent promoters under

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both conditions investigated, salt stress produced only a modest effect on tRNA^{Cys} expression and the accumulation of tRNA^{Cys} related species.
Conclusions: The results demonstrate that tRNA^{Cys} molecules resident in spores undergo dynamic processing to produce functional molecules that may play an essential role during protein synthesis.

Keywords
B. subtilis spore germination, outgrowth, salt stress, tRNACys processing, RNA-Seq data

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Introduction

When unfavorable environmental conditions affect cell growth, spore-forming bacteria activate and coordinate a temporal molecular program to assemble a dormant spore, which sequesters essential biomolecules for subsequent conversion back into growing cells. *Bacillus subtilis* sporulation has proved to be an extremely valuable model system for the study of the principal biomolecules that are stored and shielded during spore dormancy. Proteome analysis has revealed several categories of proteins that are stored in dormant *B. subtilis* spores, including proteins involved in ribosome biogenesis, carbon metabolism, RNA processing, and protein synthesis. These protein groups play important roles in spore germination and outgrowth (Swarge *et al.*, 2019). In addition, dormant spores store RNA, which suggests that these RNAs play specific roles during dormancy or germination and outgrowth or are used as ribonucleotide reservoirs for *de novo* RNA synthesis during spore germination. Some of the RNAs are transcripts involved in the spore formation process, such as those coding for membrane proteins, transporters, RNA processing enzymes, and proteins involved in protein synthesis, modification and degradation (Bassi *et al.*, 2016; Keijser *et al.*, 2007; Nagler *et al.*, 2016; Segev *et al.*, 2012). Among these RNA categories, tRNA has been detected in significant amounts in dormant spores, and represents an essential reservoir (Nagler *et al.*, 2016). One aspect of RNA metabolism that has not been well studied during spore germination and outgrowth is tRNA processing, and knowledge of those mechanisms will be essential to our understanding of long term stress survival by the spores and of the possible role of stored tRNAs in the generation of vegetative cells (Deutscher, 2006; Li & Deutscher, 1996; Li & Deutscher, 2002; Mohanty & Kushner, 2000; Ow & Kushner, 2002; Raynal *et al.*, 1998; Reuven *et al.*, 1997).

In *B. subtilis*, tRNA^{Cys} is encoded by a single copy gene and its study has significant relevance to aspects of processing and to the understanding of the global factors that affect cysteine metabolism. Thus, our previous studies have shown that tRNA^{Cys} processing during exponential growth in *B. subtilis* involves the ribonucleases RNase Z, PNPase, RNase R and the 3' end modifying enzyme, CCAase. The activities of these enzymes converge in ways that have not yet been completely elucidated to facilitate CCA addition and tRNA^{Cys} repair or degradation (Campos-Guillén *et al.*, 2010; Campos-Guillén *et al.*, 2019). To date, however, similar studies have not been performed on tRNA^{Cys} metabolism during spore germination and outgrowth in *B. subtilis*.

A dormant spore is highly organized within a dehydrated spore core, which is enveloped by a dense inner membrane, a germ cell wall, a cortex and a spore coat (Setlow, 2003). Each spore component is formed by molecules that play major roles in protecting the spore from a broad range of detrimental environmental conditions such as radiation, heat, desiccation and chemicals (Setlow, 2006). Under specific conditions, environmental nutrients are recognized by the germinant receptors (GRs) and promote biochemical changes such as the release of monovalent cations and Ca^{2+}-dipicolinate, and the cortex is hydrolyzed and rehydrated. These molecular changes initiate the germination process (Setlow, 2006). After this first step, a phase called outgrowth follows, in which metabolic activity and molecular organization ("ripening") convert the germinated spore into a growing cell. During the outgrowth phase, at least 30% of *B. subtilis* genes are activated (Horsburgh *et al.*, 2001; Keijser *et al.*, 2007).

One interesting aspect of spore germination is the effect of salt stress on the process and thus changes in the gene expression profile of outgrowing *B. subtilis* spores in the presence of high concentrations of NaCl have been analyzed by RNA sequencing (Nagler & Moeller, 2015; Nagler *et al.*, 2014; Nagler *et al.*, 2015; Nagler *et al.*, 2016). In the last study, the presence of tRNAs in high percentage was detected in dormant spores but the nature of the tRNA population was not analyzed in depth. Taking advantage of new methodologies in high-throughput RNA sequencing, the availability of datasets in the Gene Expression Omnibus (GEO) database and computational analysis, we have investigated the profile of tRNA^{Cys} in dormant and outgrowing *B. subtilis* spores in the presence of 1.2 M of NaCl. We were able to identify several categories of tRNA^{Cys} species in dormant spores and during the first minutes of their conversion into growing cells. This study provides valuable insights into tRNA^{Cys} processing during this state of transition.

Methods

All experimental conditions to obtain RNA from dormant and outgrowing *B. subtilis* spores in the presence and absence of NaCl were previously described (Nagler *et al.*, 2016). The raw RNA-Seq data indicated as SRR3488622 to SRR3488635 in Table 1, obtained from the Gene Expression Omnibus (GEO) database under the accession number GSE81238 were analyzed.

Table 1. Distribution of mapped reads for tRNA^{Cys} in dormant spore and outgrowth in presence and absence of 1.2 M NaCl.

GEO database	Experiment	Total reads	Mapped reads
SRR3488627	T0	7,395,066	243
SRR3488623	T0	9,593,083	349
SRR3488631	No NaCl T30	11,077,704	414
SRR3488628	No NaCl T30	8,808,097	566
SRR3488632	No NaCl T60	10,264,883	785
SRR3488629	No NaCl T60	10,487,594	823
SRR3488633	No NaCl T90	12,877,261	1,141
SRR3488630	No NaCl T90	8,069,190	683
SRR3488624	NaCl T30	10,215,022	462
SRR3488622	NaCl T30	10,294,457	882
SRR3488634	NaCl T60	9,182,674	760
SRR3488625	NaCl T60	9,468,524	530
SRR3488635	NaCl T90	12,497,964	813
SRR3488626	NaCl T90	9,377,023	712

GEO, Gene Expression Omnibus.

The RNA-seq raw data correspond to: T0 (dormant spore), outgrowth of spores during 30 (T30), 60 (T60) and 90 (T90) minutes after the initiation of germination in the presence and absence of 1.2 M NaCl. Each sample time was sequenced twice.

To avoid contributions to our results by tRNA isoacceptors whose genes do not encode CCA at the 3´end in *B. subtilis*, we examined products of the tRNA^{Cys} gene, which is present in single copy in the genome of *B. subtilis* and is located at the distal end of the *rrnD* operon. The gene does not encode the CCA at the 3´end, which must be added post-transcriptionally. The CLC Genomics work bench 12.0.2 (CLC Bio, Aarhus, Denmark) was used to analyze reads from the tRNA^{Cys} gene, and small RNA adapters and reads with ambiguous nucleotides were trimmed using default settings. Open source alternatives such as UniPro UGENE, UTAP or Galaxy could also have been used for this purpose. Trimmed reads were then were mapped to the *B. subtilis* 168 genome downloaded from the NCBI website (accession number NC_000964.3) with short read local alignment mapping using the default setting. We considered alignments with a length fraction of 0.8 and a similarity fraction of 0.8. Two mismatches and three insertions and deletions per read were allowed. The mapped reads results for all experiments ranged from 243 to 1,141.

Mapped reads were used in the sequence alignment program MUSCLE in MEGA X v10.1.7 with default settings (Kumar *et al.*, 2018) and high similarity aligned reads to the reference tRNA^{Cys} sequence were manually counted and categorized as the following subpopulations: I) precursors (containing genomically encoded nucleotides at the 5´or 3´ ends), II) tRNA immature (these species were 71 nucleotides in length and lacked only the CCA-end), III) mature tRNA with 3´-CCA, IV) immature tRNA with 3´-CC. We also observed short, tRNA^{Cys}-derived RNA fragments (tRFs) in this study. Reads representing such fragments were categorized as V) 3´-tRF fragments detected with CCA 3'ends, VI) 3´-tRF fragments detected with CC 3'ends, VII) fragments detected as 5´-tRF (71 bases − *n*, where *n* □ 0, from the 3' end) or internal fragments (between nucleotide positions 2 to 70), VIII) complete tRNA^{Cys} with incorrect 3´ tails and IX) 3´-tRF fragments with incorrect 3´ tails. The distribution analysis of tRNA^{Cys} subpopulations was done using the JMP 7 program (SAS Institute Inc., Cary, NC) with the frequency per group algorithm. Trend graphs were constructed in SigmaPlot software version 14.0 (Systat Software, San Jose, CA).

Results

In order to provide in-depth insight into tRNA^{Cys} processing in the dormant spore and during outgrowth in *B. subtilis*, RNA-Seq methodology and the GEO database (GSE81238) were utilized. The total number of reads for all experiments ranged from 7 to 12 million, but an interesting finding was that there is a distribution of reads mapped for tRNA^{Cys} in all experimental conditions tested (Table 1).

tRNA^{Cys} mapped reads from dormant spores

Reads that mapped to the region of the tRNA^{Cys} gene were used to distinguish between different stages of tRNA^{Cys} processing. To reduce contributions to the analysis of modification-induced polymerase fall-off during RNA-Seq methodology and due to the high percentage of short tRNA^{Cys}-derived RNA fragments (5´-tRFs or 3´-tRFs) that were observed in the analysis, for purposes of statistical analysis, all mapped reads were characterized.

The results show that the dormant spore stores a diverse population. An average of 296 mapped reads were analyzed in dormant spores (T0), and nine categories of tRNA^{Cys}-related species (see Methods) were characterized and are represented in Figure 1.

From these categories, we observed the following percentages of reads stored in dormant spores. A low percentage (1.01%) of reads represented tRNA with genomic encoded nucleotides at the 5´or 3´ ends, which were categorized as unprocessed transcripts (category I). The category with the lowest percentage of reads (0.34%) represented tRNAs with immature 3´ends (category II). The most abundant category of reads (22.85%) represented tRNAs with the correct CCA at the 3´ end (category III). An interesting finding was the relatively high percentage of mapped reads (9.47%) representing tRNAs with CC at the 3´end (category IV), which we suggest are immature tRNAs. We counted a high percentage of reads representing 3´-tRF fragments with correct CCA or CC 3´ends (23.19%) for categories V and VI. Due to the high number of mapped reads of fragments observed at time 0 (dormant spore) and compared with the lower number of mapped reads at the other times analyzed (see results below), we suggest that these reads are most likely fragments generated during spore formation. If these fragments are subsequently degraded, they might support *de novo* RNA synthesis during outgrowth. However, we cannot rule out the possibility that a percentage of them could be modification-induced polymerase fall-off byproducts resulting from the RNA-Seq methodology.

We observed a considerable percentage of reads (30.46%) of 5´-tRF or internal fragments, which are represented as category VII. The last categories observed, VIII and IX, were reads with incorrect post-transcriptionally added tails on complete tRNA^{Cys} or 3´-tRF fragments. The total percentage of these reads was 12.16% and the tails are shown in Figure 1 (table at right side). Both categories of molecules of complete tRNA^{Cys} or 3´-tRF fragments with CCA at the 3´end were observed to include species that carried the extra nucleotides C or CA. Some of them also had a second CCA terminus, while others possessed poly(A) tails.

Distribution of tRNA^{Cys} molecules during outgrowth

Our results show that the dormant spore stores mature tRNA^{Cys}, which could support the initial phases of translation, while tRNA^{Cys} processing progress during the outgrowth phase. On the other hand, fragments might be degraded so that the ribonucleotides obtained could be available for *de novo* RNA
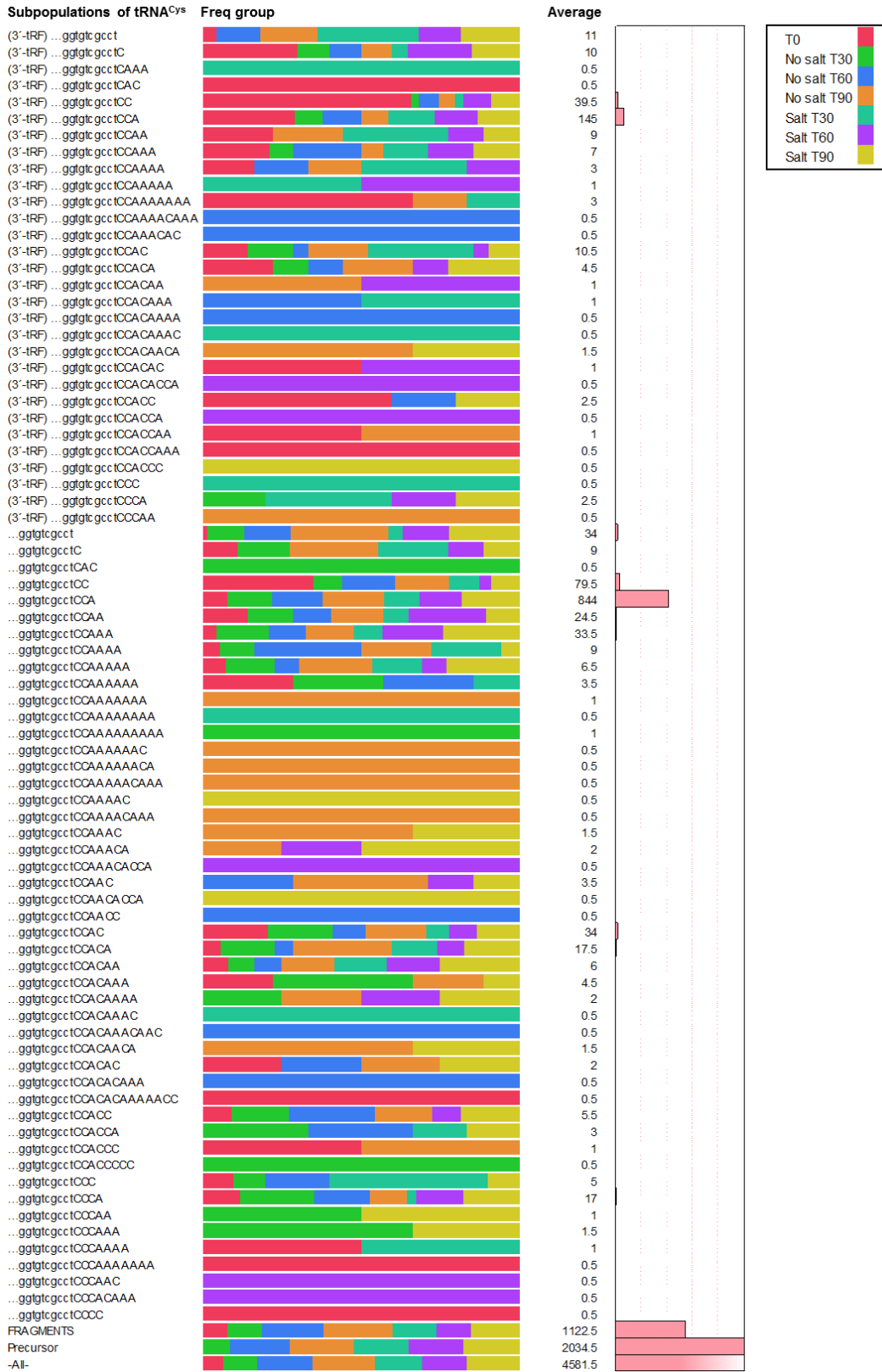
Figure 1. Overview of subpopulations of tRNA^Cys-related molecules in *B. subtilis* dormant spores. Reads categorized were: I) precursors, II) immature tRNA, III) mature tRNA with 3' CCA ends, IV) immature tRNA with 3' CC ends, V) 3'-tRF fragments detected with 3' CCA ends, VI) 3'-tRF fragments detected with 3' CC ends, VII) 5'-tRF or internal fragments, VIII) complete tRNA with incorrect 3´ tails added and IX) 3'-tRF fragments with incorrect 3´ tails added. The numbers after each category indicate the percentage of total tRNA^Cys represented by that species. The table at the right shows the tails associated with the various tRNA^Cys species in green.

synthesis during the outgrowth phase. Therefore, it is reasonable to suggest that during ripening phase, tRNA processing will progress and over time the population frequency of spore-stored tRNA^Cys molecules will change.

To understand the population dynamics of tRNA^Cys molecules during the outgrowth process, the mapped reads for tRNA^Cys at 30, 60 and 90 minutes after the initiation of germination in the absence and presence of 1.2 M of NaCl were analyzed. From a total of 9,163 mapped reads obtained (Table 1) during 90 minutes of outgrowth in both conditions, the distribution of each subpopulation of tRNA^Cys molecules was obtained, represented as the average at each sample time and visualized in Figure 2.

While some subpopulations of these molecules were observed at lower frequency and detected in at least one RNA sample and at one sampling time, other sub-populations were present at all analyzed time points. In agreement with previous reports, robust transcription was induced during outgrowth. Thus, the sub-population of precursors of tRNA^Cys are accumulated at all analyzed time points and represent a high frequency of tRNA molecules from the reads mapped with an average of 2034.5 mapped reads.

During ripening phase, it is reasonable to suppose that a high accumulation of the subpopulation of precursors during outgrowth will change the subpopulation of immature forms of tRNA^Cys (category II). However, the analysis of mapped reads shows a lower frequency, with an average of 34 mapped reads. These results may indicate that tRNA^Cys processing from precursors by endo or exo-ribonuclease activities progresses to some extent during the outgrowth phase to produce the immature forms of tRNA^Cys which are then available for CCA addition. In fact, the mature form of tRNA^Cys with CCA at its 3´end did increase in abundance during the outgrowth phase, with an average of 844 mapped reads, which means that CCA-adding activity is manifested during the ripening phase. Our results indicate that a significant fraction of the tRNA^Cys subpopulations with no CCA-end, or with C- or CC-ends are converted to mature tRNA^Cys during outgrowth. However, some immature species without complete CCA-ends were observed at all outgrowth time points.

An interesting subpopulation of tRNA^Cys was observed and represented by fragments or 3´-tRFs with different additions at the 3´end; together, these subpopulations represent an average of 1365.5 mapped reads. These results indicate that during ripening phase, degradation by RNases is an important

Figure 2. Distribution of subpopulations of tRNA^Cys molecules in dormant spores (T0) and during the outgrowth process 30, 60 and 90 minutes after the initiation of germination in the absence and presence of 1.2 M of NaCl. We show the last ten nucleotides of tRNA^Cys, with correct or incorrect additions at the 3´end. Fragments (total 5´-tRF and internal fragments), 3´tRF and precursors are indicated at the left side. The average of mapped reads for each subpopulation is indicated by a number and by the bar graph at the right side and its presence at each sampled time by a colored bar in the graph.

mechanism for obtaining ribonucleotides for RNA synthesis or that a mechanism of RNA quality control is operating in this phase. In this regard, an average of 194 mapped reads were observed for complete tRNA^{Cys} molecules and an average of 57 mapped reads for 3´-tRF fragments with incorrect additions at 3´end.

Distribution changes of tRNA^{Cys} molecules during outgrowth at high salinity

Effects on gene expression under conditions of high salinity have been reported for outgrowing spores (Nagler *et al.*, 2016). Therefore, to understand whether tRNA^{Cys} processing

and degradation might be affected by outgrowth in the presence of 1.2 M NaCl, mapped reads for each category mentioned above at the various sample times was contrasted with mapped reads from data obtained in the absence of NaCl. Our analysis results for mapped reads showed remarkably that precursors (category I) for tRNA^{Cys} strongly increased at 30 minutes after the initiation of germination in both conditions investigated compared with reads from dormant spores (T0), no statistical difference was observed in the presence of NaCl. At 60 and 90 minutes, accumulation of precursors remains relatively stable and both conditions show similar averages of reads mapped (Figure 3).
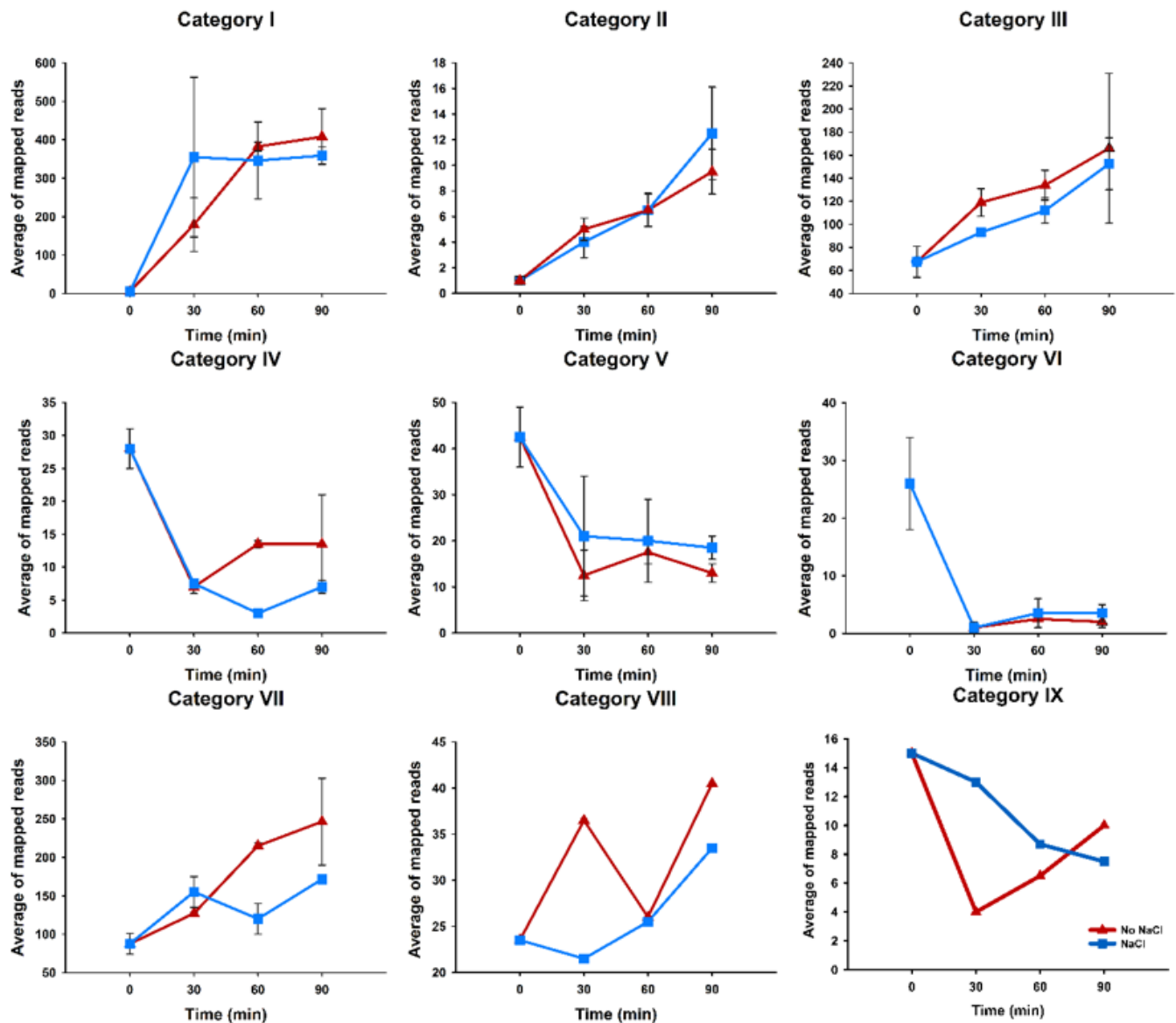
Figure 3. Overview of subpopulations of tRNA^{Cys} molecules in *B. subtilis* dormant spores and during the outgrowth phase in the presence and absence of 1.2 M NaCl. Average of reads mapped for each category were plotted. Error lines represent standard errors of means.

These results mean that during the ripening phase in outgrowing spores, tRNA[Cys] gene expression is upregulated. Given this observation, we investigated whether increased tRNA[Cys] transcription would differentially affect a particular subpopulation of tRNA[Cys] molecules related to the processing mechanisms. Beginning with category II, our results show that for the immature form of tRNA[Cys] there was an increase in abundance during outgrowth, but the frequency of reads mapped did not change significantly in the presence or absence of NaCl at any time point (Figure 3). The next step was to investigate whether the subpopulation of tRNA[Cys] with CCA at the 3´end (category III) would be affected during salt stress. No statistically significant differences in the abundance of category III species were observed in the presence or absence of NaCl at any time point (Figure 3).

An intriguing result was obtained in the analysis of immature tRNA with CC at the 3´end (category IV). While that subpopulation of tRNA[Cys] molecules decreased dramatically during the first 30 minutes after the initiation of germination in both conditions, in the presence of NaCl the abundance of this species continued to decrease, while in the absence of NaCl at 60 minutes this subpopulation of tRNA[Cys] molecules increased somewhat in abundance. These results suggest that CCA repair mechanisms may be operating in the absence of NaCl to produce mature tRNA[Cys] during this phase. If we consider the results for tRNA[Cys] stored in dormant spores at T0, categories III plus IV represent an average of 95.5±16 mapped reads, compared with an average of 93±1 and 119±12 mapped reads, respectively, for tRNA[Cys] with CCA in the presence and absence of NaCl at 30 minutes after the initiation of germination. These observations suggest that CCA-addition mechanisms are operative in the presence of NaCl, albeit less efficiently than in its absence. However, we cannot rule out the possibility that in the presence of NaCl RNase activity might be rerouted as a mechanism to obtain ribonucleotides to support transcription rather than CCA repair.

This last interpretation correlates with the results for the subpopulations of tRNA[Cys] 3´-tRF fragments with CCA or CC addition at the 3´end. The levels for both sub-populations (categories V and VI) decreased significantly, to near zero in the case of category VI, in both conditions during the outgrowth phase (Figure 3). Figure 3 shows further that category VII fragments increased in abundance during outgrowth in the presence and absence of NaCl, but to a lower level in the presence of NaCl than that observed in its absence.

The abundance of category VIII species increased at T30 then decreased somewhat thereafter in the absence of NaCl, while a continuous increase in this species was observed during outgrowth in the presence of NaCl. At T90, category VIII levels were essentially the same in the presence and absence of NaCl. Category IX levels decreased during outgrowth, initially more dramatically in the absence of NaCl than in its presence.

Discussion
In *B. subtilis,* tRNA genes are immersed in operons and tRNAs are therefore synthesized as precursors that undergo post-transcriptional modification to give functional molecules. These

modifications include 5´and 3´ processing by ribonucleases, addition of CCA at the 3´end of those tRNAs that do not encode this sequence and post-transcriptional editing of ribonucleosides.

The tRNA[Cys] gene is present in single copy in the genome of *B. subtilis* and is located at the distal end of the *rrnD* operon, which codes for three rRNA molecules; 16S, 23S and 5S rRNA, and a cluster of tRNA genes found downstream of the 5S rRNA gene (Vold, 1985; Wawrousek *et al.*, 1984). Regulation of expression of the *rrnD* operon is mediated by two cognate σ[A] consensus promoter sequences (P1 and P2) upstream of the 16S rRNA gene (Koga *et al.*, 2006). A third σ[A] consensus promotor is localized in the 23S-5S intergenic space, and is associated with mechanisms of the stringent response (Vold, 1985; Wawrousek *et al.*, 1984). A number of reports have revealed that the regulation of expression of P1 and P2 is maintained during spore formation, and that these promoters remain active even during the late stages of sporulation (de Hoon *et al.*, 2010; Koga *et al.*, 2006; Rosenberg *et al.*, 2012). Other reports observed slight differences in the expression of the P1 and P2 promoters under nutritional stress in vegetative cells (Natori *et al.*, 2009; Samarrai *et al.*, 2011).

Based on these reports, it is clear that tRNA[Cys] expression under the control of promoters P1 and P2 takes place during sporulation and that diverse sub-populations of tRNA[Cys] molecules might thus accumulate in dormant spores. To expand our knowledge of tRNA processing in dormant and germinating *B. subtilis* spores, RNA-Seq methodology was used to display the diversity of tRNA[Cys] species that were present in these conditions and in the spores germinated in the presence of high salt. Our results show for first time the diversity of the sub-populations of tRNA[Cys] stored in dormant spores in *B. subtilis* (Figure 1) and contribute to the knowledge of tRNA[Cys] processing during the dormancy and outgrowth phases.

Our results show a progressive generation and degradation of tRNA[Cys] fragments (Figure 3), which suggests that RNases are present and active at the various sampling times employed in these experiments. In fact, a recent report by Swarge *et al.* (2019) demonstrated via proteome analysis that several exo-ribonucleases are present in dormant spores, including PNPase, RNase R, RNase PH, YhaM, RNase Y and others. Transcripts for PNPase, YhaM and RNase Y were only observed at 15 minutes and for RNase R and RNAse PH at 30 minutes after the initiation of germination. Thus, these exo-ribonucleases remaining from sporulation are likely to play essential roles in the pathways of tRNA degradation and/or processing during the sporulation and ripening phases.

In the particular case of tRNA processing in *B. subtilis*, *in vitro* and *in vivo* studies of the endonucleolytic pathways of RNase P (Evans *et al.*, 2006; Kurz *et al.*, 1998) or RNase Z (Pellegrini *et al.*, 2003) and the exonucleolytic pathways of RNase PH, PNPase, RNase R or YhaM have been performed (Oussenko *et al.*, 2002; Oussenko *et al.*, 2005). From these studies it is clear that tRNA precursors with extensions at the 5´ end are eliminated by RNase P, while RNase Z processes the 3´end of almost all tRNAs lacking an encoded CCA (Pellegrini *et al.*, 2003) and the exonuclease RNase PH processes CCA-containing tRNA

precursors (Oussenko *et al.*, 2002; Oussenko *et al.*, 2005). The CCA motif at the 3´end is a key feature of all mature tRNAs and is essential for aminoacylation during protein synthesis (Hou, 2010; Wellner *et al.*, 2018). Our results show that an important subpopulation of mature tRNA with CCA at its 3' end is stored in the dormant spore and its levels increase during outgrowth. A delay in this increase was observed in the presence of NaCl, due perhaps to an effect of salt on the activities of enzymes related to tRNA^{Cys} processing. In fact, there are reports demonstrating the effects on CCA addition to tRNA^{Cys} of RNase Z, PNPase, RNase R and CCAase (Campos-Guillén *et al.*, 2010; Campos Guillén *et al.*, 2019). It remains to be seen how these enzymes are affected under salt stress conditions during spore outgrowth, but Nagler *et al.* (2016) reported the interesting observation that the *cca* gene was downregulated during 90 minutes of outgrowth in the presence of NaCl. A relevant feature is that the *cca* gene is immersed in the operon containing bacillithiol (a REDOX regulator and an α-anomeric glycoside of L-cysteinyl-D-glucosamine with L-malic acid) biosynthetic genes under σ^A-dependent promoter control, which suggests that *cca* expression and the integrity of the 3' terminus of tRNAs may be important during salt stress (Campos Guillen *et al.*, 2017; Gaballa *et al.*, 2010; Gaballa *et al.*, 2013).

According to previous reports, incorrect additions at the 3´end of tRNA^{Cys} were detected in *B. subtilis*, which suggests that alternative pathways of repair or degradation may exist in that organism (Campos-Guillén *et al.*, 2010; Campos Guillén *et al.*, 2019; Cruz Hernández *et al.,* 2013). Although a high percentage of mature tRNA with the CCA 3´end was observed in dormant spores, the species that would be available to support initial protein synthesis during outgrowth, it remains to be determined whether this stored tRNA^{Cys} is charged with cysteine. It should be noted in this regard that cysteinyl-tRNA synthetase was detected in dormant spores and its expression was also detected at 60 minutes after the initiation of germination (Nagler *et al.*, 2016; Swarge *et al.*, 2019).

Detrimental responses to salt during spore germination in *B. subtilis* have been analyzed previously (Nagler & Moeller, 2015;

Nagler *et al.*, 2014; Nagler *et al.*, 2015; Nagler *et al.*, 2016). From these studies, 402 genes related to the salt stress response were upregulated, some of them under σ^A-dependent promoter control (Nagler *et al.*, 2016). Therefore, the observed accumulation of tRNA^{Cys} precursors during the 90 minutes of outgrowth in our study suggests that upregulation of the *rrnD* operon, governed by σ^A-dependent promoters is important for macromolecular synthesis during outgrowth. An unanswered question is which of the three *rrnD* promoters is responsible for this upregulation.

Conclusions

The ability to quantitatively monitor tRNA abundance changes from dormant spore to germination and outgrowth will be critical to understanding these processes and the roles of transcription, post-transcriptional modification and translation in them. The results reported here using RNA-Seq and data analysis provide new insights into the dynamic changes in the subpopulations of RNA species related to tRNA^{Cys} molecules in dormant spores and during spore germination and outgrowth in the presence and absence of 1.2 M NaCl. An important unanswered question is what role these various subpopulations play in the metabolic economy of the spore and in the processes of germination and outgrowth.

Data availability

Source data

Raw RNA-Seq data on Gene Expression Omnibus, Accession number GSE81238: https://identifiers.org/geo:GSE81238

B. subtilis 168 genome from NCBI Reference Sequence, Accession number NC_000964.3: https://identifiers.org/refseq:NC_000964.3

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Version 1

Reviewer Report 10 May 2021

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✔ **José E. Barboza Corona** [iD]

Department of Food, Division of Life Sciences, Campus Irapuato-Salamanca, University of Guanajuato, Irapuato, Guanajuato, Mexico

This manuscript shows the different populations of tRNAcys-related molecules in dormant spores and during the first time of their conversion into growing cells in the presence or absence of saline stress. Different tRNA-cys species were categorized, and the percent of mature, immature or tRNA-cys fragments were determined.

It is common that in nature, dormant *Bacillus* spores germinate in the presence of nutrients. In this sense, it would be fascinating in future works to determine if similar behavior is found under the presence of saline stress and nutrients. In this regard, I would like to see in the introduction some words explaining the foundation to use saline stress in this work and not the presence of nutrients to pass from dormant to germinated spores. As the primary condition to germinate spores was the presence of NaCl, it would be essential to explain briefly how this experiment was done (beginning of the methods).

About the overview of the subpopulation of tRNA-cys in figure 3, I recommend using the annotation "No NaCl", "NaCl" showed in the category IX figure, in all figures, or well, show this annotation in the figure 3 legend. Alternatively, one of my primary concerns of this figure 3 is that standard deviations are considerable in more of the times, so it is difficult to consider that statistically significant differences are not found in categories II and III in the presence or absence of sodium chloride as you indicated in the manuscript. You need to clarify this point.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular studies with different Bacillus species.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 04 May 2021

https://doi.org/10.5256/f1000research.26240.r83556

?

Fabian M. Commichau 🆔
FG Synthetic Microbiology, Brandenburg University of Technology, Cottbus, Germany

In the present manuscript, Hernández and co-workers performed a bioinformatic analysis to uncover the modifications in the populations of $tRNA^{Cys}$-related molecules in the Gram-positive model bacterium *Bacillus subtilis*. The study is based on existing RNASeq data that have been generated with outgrowing spores in the absence and presence of sodium chloride.

The observation that the spores contain different $tRNA^{Cys}$ populations is very interesting. To the best of my knowledge, such an analysis has never been done before.

However, I have three critical points that I have to make:

(i) the whole analysis is based on only two data sets. It is therefore not possible to carry out a statistical evaluation. This should be corrected in the text and in the figures.

(ii) It is not entirely clear what functions the different tRNA populations could have in the cell. The authors could take up this point and speculate.

(iii) Is it known that the addition of NaCl affects the maturation/modification of tRNAs? If so, what could be the underlying molecular mechanism?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: I am not an expert in tRNA modification. My areas of expertise are bacterial physiology & genetics, gene regulation, second messengers.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 19 April 2021

https://doi.org/10.5256/f1000research.26240.r82204

Jungwook Kim 🆔
Department of Chemistry, Gwangju Institute of Science and Technology, Gwangju, South Korea

By analyzing RNA-Seq data, the authors demonstrated dynamic population changes of diverse tRNA species from dormant to outgrowth phases of *Bacilus subtilis*. In the dormant phase, precursor RNA (Category I) is present only at an insignificant level, however, it becomes the most dominant species during the outgrowth phase. Precursor RNA apparently undergoes efficient maturation steps, where a relatively low level of immature species (Category II) was detected at all time points of the ripening stage. Interestingly, 5'-tRFs (designated as Category VII) are the second most abundant species next to precursor RNA during the outgrowth phase, showing a gradual

increase in abundance over time. 3'-tRFs (Categories V, VI, VIII, and IX), on the other hand, appear to be formed at a much lower level in general compared to 5'-tRFs, and mature tRNA decrease sharply in the early stage of outgrowth. Authors proposed that these 3'-tRFs are intermediary species on the RNA degradation pathway during spore formation, products of which may serve as building blocks for de novo RNA synthesis in the outgrowth phase. The impact of a high concentration of salts (1.2M NaCl) on the distribution of tRNA subpopulation during the outgrowth phase was also investigated, but no significant effect was observed.

The RNA-Seq analysis on dynamics of tRNA metabolism offers interesting biological insights into the regulation of tRNA processing in dormant and outgrowth phases of *B. subtilis*. Being a non-expert on RNA-Seq, however, interpretation of some data was not clear to me, for example, whether tRNA species with a small number of reads (one or less) are biologically relevant or merely represent sequencing artifacts. If the former, have those species been detected previously in other cases? Secondly, despite Category VII being the second most abundant species in both spore and ripening stages, the discussion on the biological significance of this class in *B. subtilis* was largely lacking. In particular, the size distribution of 5'-tRFs is not clearly described compared to that of 3'-tRFs. A representative structure of Category VII displayed in Figure 1 implies that the cleavage occurred around the variable loop of mature tRNA^{Cys}. Interestingly, previously known 5'-tRFs in eukaryotes include species much shorter in length, which are typically generated from the endonuclease activities on D- or stem-loops. Lastly, can tRNA or its fragment with CC instead of CCA at the 3'-terminus (Category IV and VI, respectively) be considered as a degradation intermediate derived from mature tRNA (Category III) as opposed to an intermediate resulting from incomplete addition of CCA from immature tRNA (Category II) as authors claimed?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: tRNA modification

I confirm that I have read this submission and believe that I have an appropriate level of

expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 05 February 2021

?

Anne Moir
Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, UK

The authors present their *in silico* analysis of tRNA-Cys molecules in *B. subtilis* dormant spores, and during early outgrowth. The data analysed are those deposited by Nagler *et al.* (2016), in which they used RNA Seq to analyse gene expression during 90 min of outgrowth in an alanine glucose minimal medium. According to their published data, under these conditions the germinated spores do not yet show any increase in size, and this early stage of outgrowth has been referred to as "ripening". The authors here have examined all the data and have classified, for each relevant sequence read, the form of tRNA-Cys detected, from precursor through mature and degraded forms.

As the *in vivo* experiment was carried out in duplicate, with two samples at each time point, it is easy to overinterpret the absolute statistical significance of individual data points in Figure 3. I am not a statistics expert, but it seems that calculating a standard error of the mean of two values does highlight where the levels are particularly variable for an individual time point, but cannot be used in general to claim statistical significance. Such qualitative experiments are extremely useful nonetheless, as data from the succession of time points confirm the general trend.

The major conclusions are (i) a variety of forms of tRNA-Cys are present in dormant spores; the relative proportions are described (though to a spurious degree of accuracy, as a result of too many significant figures used in the calculated percentages), (ii) that during outgrowth, the total levels of this tRNA species increase approximately three fold, and the relative amounts of the various forms change - most notably, the precursor form increases dramatically, from almost negligible to approximately half of the total, and the mature CCA-tailed form increases approximately 2.5 fold in amount. There is a reduction in the level of several degradation products, except for that most commonly found (Class VII), the amount of which increases approximately three fold. There is no very strong evidence of differences between samples outgrowing in low and high salt in the graphs of Figure 3, although it may be the case for Category VII molecules.

This is the first detailed analysis of tRNA-Cys levels in spores, and the implications for its synthesis and processing are fully discussed.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Spore germination and outgrowth in Bacillus subtilis. I am not an expert in RNA Seq data or its analysis.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 17 Feb 2021

JUAN CAMPOS GUILLEN, UNIVERSIDAD AUTONOMA DE QUERETARO, Mexico

Thank you for your comments. We agree with you about the interpretation of statistical significance in figure 3. However, we decided to use standard errors of means for the next reasons:
1. When the mapped reads for all categories for tRNA^{Cys} were analyzed as percentage of total reads for each time, some data can easily be misunderstood. For example, for category III, from time 0 to time 30, a decrease percentage was observed when percentage of total was used. So that, the means for each category in each time was the better option for interpretation.

2. The raw RNA-Seq data were obtained from Nagler et al., 2016, so that only duplicated data were obtained in these experiments. We were curious to analyze other Gene Expression Omnibus (GEO) database for *Bacillus subtilis*, using different RNA protocol and sequencing methods, however mapped reads for tRNA^{cys} were not obtained. This mean that as the only one copy gene for tRNA^{Cys} in the genome, probably molar concentration could be less that other tRNAs isoacceptors with two or more copy genes. Additionally, RNA protocol and sequencing method could be affecting the total of mapped read, including in duplicated experiment.

3. In view of the above and for a comprehensive solution to this issue, we use standard

error as a mathematical tool used in statistics to measure variability. It is used to estimate the efficiency, accuracy, and consistency of a sample. In other words, it measures how precisely a sampling distribution represents a population. The standard error decrease as the sample size increases, in our case, two values were used, but standard error of means show the distribution of mapped read from these samples at each time for each experiment for a correct interpretation in the presence and absence of 1.2 M NaCl. From these results, no statistical difference was observed in the presence of NaCl.

Competing Interests: No competing interests were disclosed.

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