

Review

Critical Minireview: The Fate of tRNA^{Cys} during Oxidative Stress in *Bacillus subtilis*

Juan Campos Guillen ^{1,*}, George H. Jones ², Carlos Saldaña Gutierrez ³,
José Luis Hernández-Flores ⁴, Julio Alfonso Cruz Medina ¹, José Humberto Valenzuela Soto ⁵,
Sergio Pacheco Hernández ¹, Sergio Romero Gomez ¹ and Verónica Morales Tlalpan ⁶

¹ Facultad de Química, Universidad Autónoma de Querétaro, Cerro de las Campanas s/n, Querétaro Qro., 76010, Mexico; jacm022003@yahoo.com.mx (J.A.C.M.); serpacheco18@gmail.com (S.P.H.); ser69rom@gmail.com (S.R.G.)

² Department of Biology, Emory University, Atlanta, GA 30322, USA; ghjones@emory.edu

³ Facultad de Ciencias Naturales, Universidad Autónoma de Querétaro, Cerro de las Campanas s/n, Querétaro Qro., 76010, Mexico; doctorado.cs.uaq@gmail.com

⁴ Centro de Investigación y de Estudios Avanzados del IPN, P.O. Box 629, Irapuato 36500, GTO, México; jhernand@ira.cinvestav.mx

⁵ Centro de Investigación en Química Aplicada, Blvd. Enrique Reyna Hermsillo No.140, Saltillo 25294, Coahuila, México; humberto.valenzuela@ciqa.edu.mx

⁶ Facultad de Medicina, Universidad Autónoma de Querétaro, Cerro de las Campanas s/n, Querétaro Qro., 76010, Mexico; vmtlalpan@gmail.com

* Correspondence: juan.campos@uaq.mx; Tel.: +52-442-192-1200

Abstract: Oxidative stress occurs when cells are exposed to elevated levels of reactive oxygen species that could damage biological molecules. One bacterial response to oxidative stress involves disulfide bond formation either between protein thiols or between protein thiols and low-molecular-weight thiols. Bacillithiol was recently identified as a major low-molecular-weight thiol in *Bacillus subtilis* and related Firmicutes. Four genes (*bshA*, *bshB1*, *bshB2* and *bshC*) are involved in bacillithiol biosynthesis. The *bshA* and *bshB1* genes are part of a seven-gene operon (*ypjD*), which includes the essential gene *cca*, encoding CCA-tRNA nucleotidyltransferase. The inclusion of *cca* in the operon containing bacillithiol biosynthetic genes suggests that the integrity of the 3' terminus of tRNAs may also be important in oxidative stress. Addition of the 3' terminal CCA sequence by CCA-tRNA nucleotidyltransferase to give a mature tRNA and functional molecules ready for aminoacylation plays an essential role during translation and expression of the genetic code. Any defects in these processes, for example, the accumulation of shorter and defective tRNAs under oxidative stress, could exert a deleterious effect on cells. This review summarizes the physiological link between tRNA^{Cys} regulation and oxidative stress in *Bacillus*.

Keywords: bacillithiol; *Bacillus*; oxidative stress; tRNA

1. Introduction

Redox reactions are essential to the metabolic economy of living systems. However, when cells are exposed to elevated concentrations of reactive oxygen species numerous biochemical and physiological pathways may be affected, thus disrupting cellular homeostasis. For these reasons, organisms have developed strategies to limit the effects of oxidative stress on biological components. One such strategy involves protein protection through thiol regulation. Disulfide bonds are important in a number of metabolic pathways, one of which is the interaction between proteins and Low Molecular Weight (LMW) thiols during oxidative stress. Diverse prokaryotic and eukaryotic organisms have the ability to synthesize glutathione, bacillithiol or mycothiol as part of stress responses. These LMW thiols play an essential role in the maintenance of a reducing environment in the cytosol.

Another important cellular strategy involved in stress responses occurs at the level of tRNA metabolism, where tRNAs charged with their specific amino acids are essential for the biosynthesis of stress-related proteins. In addition, there is evidence to suggest that when cells do not have the capacity to effectively regulate the levels of reactive oxygen species, pathways are induced that lead to tRNA degradation as a mechanism for regulating the expression of particular genes. Although the role of tRNAs in cellular metabolism is extensive, in this minireview we will focus on the physiological link between bacillithiol biosynthesis and tRNA^{Cys} processing and regulation in *Bacillus subtilis*. Both molecules have molecular connections through the expression of genes from one operon (*ypjD*) which contains genes for both bacillithiol biosynthesis and tRNA maturation.

2. *Bacillus* under oxidative stress

Bacillus is a genus of Gram-positive bacteria which are members of the family Bacillaceae. These bacteria are endospore formers and are obligate aerobes or facultative anaerobes [1, 2]. Bacilli survive and disperse in the presence of other microorganisms in their natural environments. In the various environments in which they are found Bacilli are subject to multiple sources of toxic oxidizing agents that induce internal production of reactive oxygen species [3]. Those conditions promote decisions between survival and cell death when damage to biomolecules is severe.

Under stress conditions, initiation signals result in the activation of the master transcription regulator, sigma factor σ^B . Activated σ^B regulates the transcription of approximately 200 genes in response to multiple physical, chemical and other environmental stress stimuli [4]. *Bacillus* cells contain robust mechanisms to respond to and mitigate environmental stress. These responses include neutralization of stress stimuli by disulfide bonds, which play a major role in stabilizing protein structures or are part of their catalytic cycle. In addition, many proteins possess cysteine residues which function as redox switches, e.g. ribonucleotide reductase, RNase A, RNase T, methionine sulfoxide reductase, alkylhydroperoxide reductase, arsenate reductase, and the global repressor of the peroxide regulon PerR [5-8].

One important component of stress responses in *Bacillus* is the protection of exposed cysteine residues from fluctuations in the redox environment by bacillithiol (BSH), the α -anomeric glycoside of L-cysteinyl-D-glucosamine with L-malic acid. BSH is found in a wide range of bacteria including many firmicutes [9].

3. Bacillithiol biosynthesis and function

Bacillithiol biosynthesis (Figure 1) begins with the reaction of UDP-N-acetylglucosamine (UDP-GlcNAc) and L-malate to produce α -D-glucosaminyl L-malate (GlcNAc-Mal). This step is catalyzed by the enzyme BshA (N-acetyl- α -D-glucosaminyl L-malate synthase). The second step is deacetylation of GlcNAc-Mal to produce GlcN-Mal. This step is catalyzed by either of two redundant enzymes, BshB1 or BshB2 (N-acetyl- α -D-glucosaminyl L-malate deacetylase). The last step of BSH biosynthesis is achieved by coupling cysteine to GlcN-Mal, and is catalyzed by BshC (D-glucosaminyl L-malate cysteine ligase) [9, 10].

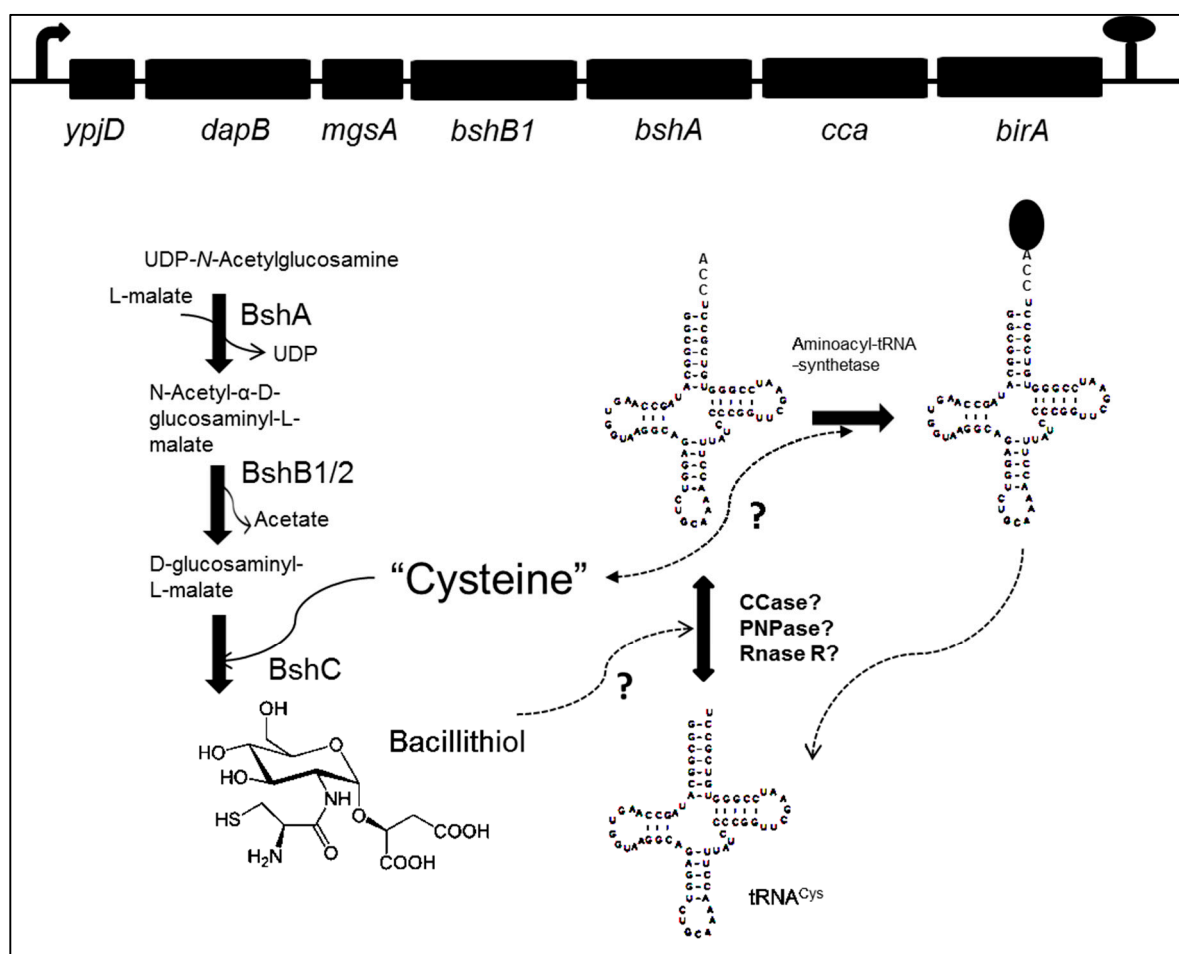


Figure 1. Possible relationship between tRNA^{Cys} and bacillithiol biosynthesis. Genes involved in bacillithiol biosynthesis are indicated in the operon (*bshB1* and *bshA*). The *cca* gene involved in tRNA maturation is indicated. A link between bacillithiol and tRNA^{Cys} maturation could have physiological relevance for *B. subtilis* under stress conditions. Thus the free cysteine pool would be necessary both for the synthesis of bacillithiol and for the aminoacylation of the single tRNA^{Cys} species in *B. subtilis*.

The genes *bshA* and *bshB1*, whose products are involved in first two steps in bacillithiol biosynthesis (Figure 1), are encoded in an operon [9, 10]. The operon begins with a putative pyrophosphohydrolase (*ypjD*), a dihydrodipicolinate reductase (*dapB*), and methylglyoxal synthase respectively (*mgsA*). These are followed by the *bshB1* and *bshA* genes. Downstream of *bshA* are two essential genes, *cca* and *birA*, encoding CCA-tRNA nucleotidyltransferase and biotin-protein ligase, respectively [11-13].

Once bacillithiol is synthesized during stress conditions, it is responsible for reducing intra- or intermolecular disulfide bonds in cytosolic proteins. It does this by attacking disulfide bonds and forming diverse BSH-disulfide products. This mechanism maintains a reducing cytoplasmic environment that protects exposed cysteine residues from oxidation and reduces accumulation of proteins with non-functional disulfide bonds [7].

Despite the established role of BSH in the maintenance of disulfide homeostasis, there is evidence to suggest that it is not essential for activating oxidative and disulfide stress responses for some stress-related genes in *Bacillus subtilis*. For instance, [14] used a 2D gel fluorescence-based thiol-modification assay to identify reversibly oxidized proteins during the induction of disulfide

stress in *B. subtilis* with diamide [diazenedicarboxylic acid bis(N,N-dimethylamide)], a specific oxidant for thiols. Their analysis demonstrated that protein redox status, including that of the Spx protein, a master regulator of disulfide (thioldepletion) stress [14], was unchanged in wild-type, *bshA*, and *bshB1bshB2* strains [6, 9]. Spx activity is controlled by a CXXC redox switch and, in its oxidized form, activates transcription of target promoters, including those of the essential thioredoxin/thioredoxin reductase system encoded by *trxA* and *trxB*, and promoters for the *ypjD* operon encoding genes for bacillithiol synthesis [10]. Possible candidate molecules for the maintenance of protein redox status in the experiments just described are free cysteine and perhaps tRNA^{Cys} (see below). With regard to a possible role for free cysteine, some redundancy in the function of LMW thiols in *B. subtilis* is expected [6]. In fact, both BSH and cysteine could regulate the activity of OhrR, a cysteine-based peroxide sensor [15].

Despite the apparent redundancy in disulfide stress protection mechanisms in *B. subtilis*, BSH null cells do show striking changes as compared with the wild type, e.g. reduced efficiency of sporulation, increased sensitivity to high concentrations of NaCl and low pH and dramatically increased sensitivity to the antibiotic fosfomycin [9]. The broad-spectrum activity of fosfomycin against bacteria is due to its function as a potent covalent inhibitor of MurA, a key enzyme involved in peptidoglycan biosynthesis [16]. One way to inactivate fosfomycin is through fosfomycin-resistance enzymes such as FosA, FosB and FosX. FosA is a glutathione transferase identified in various Gram-negative bacteria, which catalyzes the reaction between fosfomycin and glutathione (GSH), a low molecular weight thiol, to form an inactive GS–fosfomycin conjugate. FosB is a bacillithiol-S-transferase related to FosA identified in many low-G+C Gram-positive bacteria, which catalyzes formation of an inactive complex between BSH and the antibiotic [17, 18]. FosX is a metal-dependent hydrolase found in *Mesorhizobium loti* and *Listeria monocytogenes*, which catalyzes the hydrolysis of the antibiotic [16]. These activities are primarily responsible for conferring fosfomycin resistance on *Bacillus subtilis* and other Gram positive bacteria.

4. Role of CCA-tRNA nucleotidyltransferase

As is shown in Figure 1, *cca*, the gene encoding CCA, ATP(CTP): tRNA nucleotidyltransferase (CCAase), is a component of the operon that contains *bshA* and *bshB1*. The principal function of CCCase is the addition of CCA residues to the 3' end of tRNAs and tRNA-like transcripts, during maturation or recycling of these molecules. All mature tRNA molecules possess a functional CCA sequence at the 3' end, as this sequence is required for amino acid attachment [19, 20, 21].

The increased expression of genes involved in BSH biosynthesis (*bshB1* and *bshA*) under stress conditions in *Bacillus subtilis*, suggests the possibility that CCAase is also over-expressed in similar conditions. However, expression of *cca* and regulation of CCA addition at the 3' of tRNA during stress conditions has not received much attention, presumably because of the assumption that damaged tRNA molecules that might be produced under such conditions are degraded efficiently by RNases.

With regard to the latter possibility, there is evidence indicating that CCCase has the ability to distinguish between normal and damaged tRNAs, e.g. tRNAs with poly(A) tails or with CCACCA sequences, for efficient CCA addition at the 3' end. Such aberrant tRNAs are generally marked for degradation by RNases, as an important mechanism in successful quality control [22, 23]. Indeed, the cleavage of tRNAs by specific RNases in response to multiple physical, chemical and other environmental stresses has been detected in prokaryotes and eukaryotes and raises the possibility that this cleavage of tRNA, principally in the anticodon loop, could be a potentially important causative factor in the protection of cells from these stresses [24]. For instance, cleavage of tRNA^{Lys} in *Escherichia coli* by the PrrC nuclease, which is activated during Bacteriophage T4 infection, has been reported [25]. Similarly, cleavage of tRNAs by RNase T2 and members of the RNase A family have been detected under stress conditions in yeast and mammalian cells [26, 27]. tRNA cleavage during

amino acid starvation in various biological systems has also been demonstrated [24]. All these reports indicate that tRNA cleavage is a common phenomenon in biological systems; its significance, regulation and specific relationship to stress survival are unresolved questions.

5. A link between oxidative stress, bacillithiol and tRNA maturation?

The presence of *cca* in the *ypjD* operon, along with *bshA* and *bshB1* suggests the intriguing possibility that CCAase may be involved in the response of *B. subtilis* to oxidative stress. Evidence supporting this possibility was recently presented by Cruz Hernandez et al. [28]. In that study, *B. subtilis* was subjected to oxidative stress generated by exposure to mercury. The researchers then examined the integrity of six tRNAs, as judged by electrophoretic mobility on Northern blots. They found that mercury exposure had no effect on the mobility of five of the six tRNAs, those for alanine, tryptophan, valine, leucine and threonine. In contrast, Northern blots revealed a shorter species of tRNA^{Cys} that migrated more rapidly than the mature tRNA^{Cys}.

Unlike the situation in *Escherichia coli* and some other bacteria, the CCA end of tRNAs is not encoded in the genome of *B. subtilis* and must be added post-transcriptionally by CCAase. The smaller tRNA^{Cys} species observed in conditions of oxidative stress had an electrophoretic mobility identical to that previously reported for tRNA^{Cys} lacking the CCA end [29]. Thus, it seems likely that the smaller tRNA produced by oxidative stress induced by mercury exposure is CCA-less tRNA^{Cys}. After 4 hr of exposure to mercury the shorter tRNA^{Cys} species accounted for approximately 10% of total tRNA^{Cys} (mature tRNA^{Cys} plus the shorter species).

Cruz Hernandez et al. [28] also examined the effects of oxidative stress on the accumulation of the shorter tRNA^{Cys} in *B. subtilis* mutants lacking the ribonucleases polynucleotide phosphorylase (PNPase) and RNase R. They observed a significant increase in the relative amount of the shorter tRNA^{Cys} species in the *pnp* mutant and in the *pnp rnr* double mutant such that by 6 hr following exposure to mercury, the shorter tRNA^{Cys} species accounted for 70% of the total tRNA^{Cys} observed. The shorter tRNA^{Cys} species was not observed in the *rnr* single mutant, suggesting that PNPase may be involved in either the generation of mature tRNA^{Cys}, via its polymerization activity or in the degradation of the CCA-less tRNA^{Cys} via phosphorolysis.

Of additional interest was the observation that the relative amount of the shorter tRNA^{Cys} species decreased after 6 hr of oxidative stress. By 10 hr post exposure to mercury, the relative level of the shorter species had decreased to less than 1% of the total in the wild type strain and to ca. 10% of the total in the *pnp* mutant strain. The decrease in the relative amount of the putative CCA-less species with time of exposure to mercury can be rationalized via the operation of heavy metal detoxification mechanisms that are activated under conditions of heavy metal-induced oxidative stress. The failure to observe the shorter tRNA^{Cys} species in the *rnr* mutant remains unexplained.

How might CCAase activity be affected by oxidative stress? One possibility is suggested in Figure 1 whereby BSH inhibits the activity of CCAase, thereby decreasing the utilization of cysteine in protein synthesis, making it available for BSH synthesis and for the function of the free amino acid as an LMW thiol. Such a mechanism would compensate for any increase in the level of *cca* expression that might accompany increased expression of *bshA* and *B1* under stress conditions. Alternatively, BSH might stimulate the activity or the expression of nucleases such as PNPase and RNase R, leading to increased rates of tRNA^{Cys} degradation. If such mechanisms are operative in *B. subtilis*, there must be some feature of them that limits their effects to tRNA^{Cys}, at least among the tRNAs examined by Cruz Hernandez et al. [28]. One possible reason for this specificity might involve the requirement for cysteine in the formation of bacillithiol and the possible link between tRNA^{Cys} and cysteine levels (S-thiolation).

6. Concluding Remarks

Available evidence suggests the interesting possibility that there may be a biologically significant relationship between the maturation or decay of tRNA^{Cys} and the response of *B. subtilis* to oxidative stress. This evidence suggests the further possibility that tRNA^{Cys} may play a role in the organism's metabolic processes other than its role in translation. For example, tRNA^{Cys} might function as a store of cysteine for bacillithiol biosynthesis or might otherwise play a role in the protection of cysteine residues from oxidation in various stress-related proteins. These possibilities await experimental verification.

Acknowledgements: This work was partially financed by the Universidad Autónoma de Querétaro, México (project Fovin-UAQ, 2013, Clave 1299-Nue 3408 to J.C.G).

Author Contributions: All authors have contributed to the design, drafting and revision of the manuscript. Julio Alfonso Cruz Medina, José Humberto Valenzuela Soto, Sergio Pacheco Hernández and Sergio Romero Gomez were responsible for the figure design, edition and preparation. All authors approved the reviewed final version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Slepecky, R.A. and H.E. Hemphill, *The genus Bacillus—nonmedical*, in *The prokaryotes*. 2006, Springer. p. 530-562.
2. Mols, M. and T. Abee, *Primary and secondary oxidative stress in Bacillus*. *Environ Microbiol*, 2011. **13**(6): p. 1387-94.
3. Zuber, P., *Management of oxidative stress in Bacillus*. *Annu Rev Microbiol*, 2009. **63**: p. 575-97.
4. Reder, A., et al., *Contributions of Individual $\sigma(B)$ -Dependent General Stress Genes to Oxidative Stress Resistance of Bacillus subtilis*. *Journal of Bacteriology*, 2012. **194**(14): p. 3601-3610.
5. Herbig, A.F. and J.D. Helmann, *Roles of metal ions and hydrogen peroxide in modulating the interaction of the Bacillus subtilis PerR peroxide regulon repressor with operator DNA*. *Mol Microbiol*, 2001. **41**(4): p. 849-59.
6. Hochgrafe, F., et al., *Fluorescence thiol modification assay: oxidatively modified proteins in Bacillus subtilis*. *Mol Microbiol*, 2005. **58**(2): p. 409-25.
7. Leichert, L.I., C. Scharf, and M. Hecker, *Global characterization of disulfide stress in Bacillus subtilis*. *J Bacteriol*, 2003. **185**(6): p. 1967-75.
8. Narayan, M., et al., *Oxidative folding of proteins*. *Acc Chem Res*, 2000. **33**(11): p. 805-12.
9. Gaballa, A., et al., *Biosynthesis and functions of bacillithiol, a major low-molecular-weight thiol in Bacilli*. *Proc Natl Acad Sci U S A*, 2010. **107**(14): p. 6482-6.
10. Gaballa, A., et al., *Regulation of Bacillus subtilis bacillithiol biosynthesis operons by Spx*. *Microbiology*, 2013. **159**(Pt 10): p. 2025-35.
11. Bower, S., et al., *Cloning and characterization of the Bacillus subtilis birA gene encoding a repressor of the biotin operon*. *J Bacteriol*, 1995. **177**(9): p. 2572-5.
12. Kobayashi, K., et al., *Essential Bacillus subtilis genes*. *Proc Natl Acad Sci U S A*, 2003. **100**(8): p. 4678-83.
13. Raynal, L.C., H.M. Krisch, and A.J. Carpousis, *The Bacillus subtilis Nucleotidyltransferase Is a tRNA CCA-Adding Enzyme*. *Journal of Bacteriology*, 1998. **180**(23): p. 6276-6282.
14. Nakano, S., et al., *Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in Bacillus subtilis*. *Proceedings of the National Academy of Sciences*, 2003. **100**(23): p. 13603-13608.

15. Lee, J.W., S. Soonsanga, and J.D. Helmann, *A complex thiolate switch regulates the Bacillus subtilis organic peroxide sensor OhrR*. Proc Natl Acad Sci U S A, 2007. **104**(21): p. 8743-8.
16. Roberts, A.A., et al., *Mechanistic studies of FosB: a divalent-metal-dependent bacillithiol-S-transferase that mediates fosfomycin resistance in Staphylococcus aureus*. Biochem J, 2013. **451**(1): p. 69-79.
17. Cao, M., et al., *FosB, a cysteine-dependent fosfomycin resistance protein under the control of sigma(W), an extracytoplasmic-function sigma factor in Bacillus subtilis*. J Bacteriol, 2001. **183**(7): p. 2380-3.
18. Rigsby, R.E., et al., *Fosfomycin resistance proteins: a nexus of glutathione transferases and epoxide hydrolases in a metalloenzyme superfamily*. Methods Enzymol, 2005. **401**: p. 367-79.
19. Schürer, H., et al., *This Is the End: Processing, Editing and Repair at the tRNA 3-Terminus*, in *Biological Chemistry*. 2001. p. 1147.
20. Neuenfeldt, A., et al., *Evolution of tRNA nucleotidyltransferases: a small deletion generated CC-adding enzymes*. Proc Natl Acad Sci U S A, 2008. **105**(23): p. 7953-8.
21. Mörl, M. and A. Marchfelder, *The final cut: The importance of tRNA 3'-processing*. EMBO Reports, 2001. **2**(1): p. 17-20.
22. Li, Z., et al., *RNA quality control: degradation of defective transfer RNA*. EMBO J, 2002. **21**(5): p. 1132-8.
23. Wilusz, J.E., et al., *tRNAs marked with CCACCA are targeted for degradation*. Science, 2011. **334**(6057): p. 817-21.
24. Thompson, D.M. and R. Parker, *Stressing out over tRNA cleavage*. Cell, 2009. **138**(2): p. 215-9.
25. Levitz, R., et al., *The optional E. coli prr locus encodes a latent form of phage T4-induced anticodon nuclease*. EMBO J, 1990. **9**(5): p. 1383-9.
26. Fu, H., et al., *Stress induces tRNA cleavage by angiogenin in mammalian cells*. FEBS Lett, 2009. **583**(2): p. 437-42.
27. Thompson, D.M. and R. Parker, *The RNase Rny1p cleaves tRNAs and promotes cell death during oxidative stress in Saccharomyces cerevisiae*. J Cell Biol, 2009. **185**(1): p. 43-50.
28. Cruz Hernandez, A., et al., *Exposure of Bacillus subtilis to mercury induces accumulation of shorter tRNA Cys species*. Metallomics, 2013. **5**(4): p. 398-403.
29. Campos-Guillen, J., et al., *Characterization of tRNA(Cys) processing in a conditional Bacillus subtilis CCase mutant reveals the participation of RNase R in its quality control*. Microbiology, 2010. **156**(Pt 7): p. 2102-11.

