Elucidating the Function of a Pseudo-tRNA in *Bacillus cereus*

Dissertation

Presented in Partial Fulfillment of the Requirements for

The Degree Doctor of Philosophy in the Graduate School of

The Ohio State University

By

Theresa Elizabeth Rogers, M.S.

Graduate Program in Microbiology

The Ohio State University

2010

Dissertation Committee:

Michael Ibba, Advisor

Kurt Fredrick

Tina Henkin

Karin Musier-Forsyth
Copyright by

Theresa Elizabeth Rogers

2010
Abstract

Transfer RNAs (tRNAs) are primarily responsible for translation of the genetic code and function in numerous other processes such as viral replication, amino acid biosynthesis, and cell wall remodeling. tRNAs have also been shown to regulate gene expression by acting as sensors of the translation status of the cell, and it has been suggested that these highly stable molecules could provide a structural framework for small RNAs (sRNAs) that regulate other cellular processes. A pseudo-tRNA from *Bacillus cereus*, tRNA$\text{Other}^\text{Other}$, does not associate with polysomes, suggesting a role outside translation. Deletion of the corresponding gene leads to changes in cell wall morphology and significantly reduces resistance against a number of unrelated antibiotics including vancomycin, puromycin, novobiocin, and rifapentin. The deletion of the tRNA$\text{Other}^\text{Other}$ gene is also accompanied by significant changes in the expression of numerous genes involved in oxidative stress responses. Recent studies have shown that many bactericidal antibiotics act in part by inducing intracellular oxidative stress. Additional studies have shown that NO produced by bacterial nitric oxide synthase (NOS) can act as an antioxidant, thereby reducing oxidative stress. Consistent with a role in the oxidative stress response, deletion of tRNA$\text{Other}^\text{Other}$ significantly reduces nos transcript levels and intracellular NO production. Expression of tRNA$\text{Other}^\text{Other}$ changes in response to extracellular iron concentration, as predicted from the presence of three putative ferric uptake regulator (Fur) binding sites in the promoter region of the corresponding gene. Fur is a transcriptional regulator that functions to maintain intracellular iron homeostasis and is also involved in the oxidative stress response. Unbound intracellular
ferrous iron can generate hydroxyl radicals through the Fenton reaction, and therefore increase intracellular oxidative stress. Recent studies have demonstrated that many bactericidal antibiotics ultimately kill bacteria by inducing oxidative stress. Relieving intracellular oxidative stress increased resistance to vancomycin and puromycin, which was lost along with the deletion of the gene encoding tRNA\textsuperscript{Other}.

Several of the genes involved in the oxidative stress response contain significant sequence complementarities to tRNA\textsuperscript{Other}, suggesting a possible regulatory sRNA function. To explore the notion that tRNA\textsuperscript{Other} functions as a sRNA rather than a tRNA, the functional sequence and size of tRNA\textsuperscript{Other} was questioned. Mapping of tRNA\textsuperscript{Other} revealed a much larger RNA and possible variations due to transcription from different predicted promoters and potential processing sites. From these larger sequences, complementarity to additional mRNAs involved in the oxidative stress response was predicted. Taken together, these findings indicate that tRNA\textsuperscript{Other} is an iron-responsive sRNA that modulates antibiotic resistance by regulating the expression of multiple targets involved in oxidative stress response.
Acknowledgments

I wish to express my thanks to my advisor, Dr. Michael Ibba, for his guidance, patience, continual support, and endless encouragement throughout the years. It has been a pleasure to work with such a talented scientist and outstanding mentor.

I would like to thank the members of my committee, Dr. Kurt Fredrick, Dr. Tina Henkin, and Dr. Karin Musier-Forsyth for all of their helpful suggestions, clever insight, and time throughout my graduate career at The Ohio State University.

I wish to thank Dr. Mette Prætorius-Ibba for her patience, support, help, and encouragement when I began research in the field of microbiology. I thank Dr. Hervé Roy and Srujana Samhita Yadavalli for always being available and willing to answer or discuss my unending inquiries. I would like to thank Marla Gilreath for her help with optimizing the total RNA preps from B. cereus. I thank Kayla Humenanski for her work on B. cereus biofilms. I am grateful for all the help and support from other past and present members of the Ibba lab: Dr. Sandro Ataide, Dr. Rajat Banerjee, Dr. Tammy Bullwinkle, Dr. Jeffrey Levengood, Dr. Jiqiang Ling, Kiley Dare, Carolina Farah, Medha Raina, and Noah Reynolds.

I would also like to thank my family and friends for all their support and encouragement over the years.
Vita

June 2002 ......................................................... B.S. Biology, The Ohio State University

June 2002 to May 2003 ................................................................. Research Assistant,

Department of Microbiology, The Ohio State University

December 2005 ......................................................... M.S. Microbiology, University of Georgia

April 2006 to present ........................................ Graduate Research and Teaching Associate

Publications

1. Rogers, T.E., and Doran-Peterson, J.B. (2010) Analysis of cellulolytic and
hemicellulolytic enzyme activity within the Tipula abdominalis (Say) (Diptera;
Tipulidae) larval gut and characterization of Crocebacterium ilecola gen. nov., sp.


2. Banerjee, R., Chen, S., Dare, K., Gilreath, M., Praetorius-Ibba, M., Reina, M.,

functions inside and outside translation in Bacillus cereus. RNA Biology. 6:479-487.

xanthotipulae gen. nov., sp. nov., a novel member of the family Microbacteriaceae.

5. Ataide, S.F., Wilson, S.N., Dang, S., Rogers, T.E., Roy, B., Banerjee, R., Henkin,
targets translation. ACS Chemical Biology 2: 819-827.


Field of Study

Major Field: Microbiology
Table of Contents

Abstract ............................................................................................................................ ii
Acknowledgements ......................................................................................................... iv
Vita ..................................................................................................................................... v
List of Tables .................................................................................................................. xi
List of Figures .................................................................................................................. xii
List of Symbols and Abbreviations ................................................................................... xv

Chapter 1: The various functions of tRNAs .................................................................. 1
  1.1. Biosynthetic processes involving tRNAs ............................................................... 2
      1.1.1. Ribosomal protein synthesis ....................................................................... 2
      1.1.2. Amino acid biosynthesis ............................................................................. 3
      1.1.3. Tetrapyrrol synthesis .................................................................................... 6
      1.1.4. Antibiotic synthesis ...................................................................................... 6
      1.1.5. Peptidoglycan synthesis ............................................................................. 7
  1.2. Cell membrane modification .................................................................................. 8
  1.3. Protein degradation ............................................................................................... 10
  1.4. tRNA-like structures ........................................................................................... 11
  1.5. Regulation of gene expression .............................................................................. 13
      1.5.1. Regulation of gene expression by codon usage ......................................... 13
1.5.2. Regulation of gene expression by uncharged tRNAs .................. 14

1.6. Small RNA regulators in bacteria .................................................. 16

1.7. tRNA classification ...................................................................... 19

1.8. Purpose of study ........................................................................... 19

Chapter 2: Role of tRNA\textsuperscript{Other} in \textit{Bacillus cereus} ................................................. 21

2.1. Materials and methods ................................................................. 24

2.1.1. Bacterial strains and growth conditions .................................... 24

2.1.2. Preparation of polysome fractions ............................................. 25

2.1.3. RT-PCR of tRNA .................................................................... 26

2.1.4. Total RNA extraction and purification ...................................... 27

2.1.5. Transcriptional microarray ........................................................ 28

2.1.6. Quantitative RT-PCR of TrpRS1 and TrpRS2 transcripts .......... 30

2.1.7. Transmission electron microscopy ............................................ 34

2.2. Results ......................................................................................... 35

2.2.1. tRNA\textsuperscript{Other}-dependent phenotypic changes in \textit{B. cereus} .... 35

2.2.2. Role of tRNA\textsuperscript{Other} in ribosomal protein synthesis ........... 41

2.2.3. tRNA\textsuperscript{Other}–induced changes in transcriptional profiles .... 44

2.2.4. Prediction of mRNA targets for tRNA\textsuperscript{Other} ...................... 53

2.2.5. Role of tRNA\textsuperscript{Other} in cell wall morphology ................. 55

2.3. Discussion .................................................................................... 58

Chapter 3: Role of sRNA\textsuperscript{Other} in oxidative stress response .................. 63
3.1. Materials and Methods ........................................................................................................65
   3.1.1. Bacterial strains and growth conditions .................................................................65
   3.1.2. RNA extraction and purification ..............................................................................66
   3.1.3. RT-PCR ..................................................................................................................66
   3.1.4. Intracellular nitric oxide detection ............................................................................67
   3.1.5. Biofilm formation assays .........................................................................................67
3.2. Results ................................................................................................................................69
   3.2.1. sRNA$^{\text{Other}}$ expression is regulated by iron concentration .......................69
   3.2.2. Role of sRNA$^{\text{Other}}$ in oxidative stress response and antibiotic resistance .................................................................................................................................73
   3.2.3. Intracellular NO production in wt and B. cereus $\Delta$tRNA$^{\text{Other}}$ ............77
   3.2.4. Biofilm formation by wt and B. cereus $\Delta$tRNA$^{\text{Other}}$ .................................80
3.3. Discussion .........................................................................................................................82

Chapter 4: Mapping sRNA$^{\text{Other}}$ ..................................................................................87
4.1. Materials and Methods ......................................................................................................88
   4.1.1. Bacterial strains and growth conditions .................................................................88
   4.1.2. RNA extraction and purification ..............................................................................88
   4.1.3. 5’ RACE ................................................................................................................89
   4.1.4. 3’ RACE ...............................................................................................................92
4.2. Results ................................................................................................................................93
   4.2.1. 5’ end of sRNA$^{\text{Other}}$ ......................................................................................93
   4.2.2. 3’ end of sRNA$^{\text{Other}}$ ......................................................................................96
4.2.3. Prediction of mRNA targets for sRNA^{Other} ...........................................96

4.3. Discussion........................................................................................................103

Chapter 5: Conclusions..........................................................................................104

References..............................................................................................................110

Appendix A: Changes in transcript levels between wt and *B. cereus* ΔtRNA^{Other} under moderate and low iron conditions .........................................................128

Appendix B: Chromosomal disruption of *pbp1a* in *B. cereus*..............................130

Appendix C: Detection of sRNA^{Other} in free tRNA, 30S, 50S, 70S, and polysome fractions ........................................................................................................133
List of Tables

Table 2.1. Primers used for qRT-PCR.................................................................33

Table 2.2. Phenotypes of *B. cereus ΔtRNA<sub>Other</sub>* relative to wt determined by BIOLOG phenotypic array analysis .................................................................37

Table 2.3. Changes in transcript levels between wt and *B. cereus ΔtRNA<sub>Other</sub>* under moderate and low iron conditions .................................................................50

Table 2.4. Genes identified as potential mRNA targets of sRNA<sub>Other</sub> ..................54

Table 4.1. Primers used in initial 5’ mapping of sRNA<sub>Other</sub> ..................................91
List of Figures

Figure 1.1. The use of tRNAs in various cellular pathways ........................................... 2
Figure 1.2. The various biosynthetic pathways of Asn-tRNA$^{\text{Asn}}$ and Gln-tRNA$^{\text{Gln}}$ ....... 4
Figure 1.3. tRNA-dependent formation of LysPG .............................................................. 9
Figure 1.4. tmRNA rescues stalled ribosomes ................................................................. 12
Figure 1.5. Protein-binding RNA regulators ....................................................................... 17
Figure 1.6. True antisense and limited complementary base pairing sRNA ..................... 18

Figure 2.1. Proposed secondary structure of tRNA$^{\text{Other}}$ and comparison with tRNA$^{\text{Trp}}$ and tRNA$^{\text{Lys}}$ ........................................................................................................... 24
Figure 2.2. Preparation of ds-cDNA for transcriptional microarrays................................. 29
Figure 2.3. Standard curves for qRT-PCR of $trpS1$ and $trpS2$ ....................................... 31
Figure 2.4. Deletion of tRNA$^{\text{Other}}$ alters antibiotic resistance in wild-type (wt) and $B.\ cerus$ \Delta tRNA$^{\text{Other}}$ (Δ) ........................................................................................................... 38
Figure 2.5. tRNA$^{\text{Other}}$ is not detected in $B.\ cerus$ polysomes ......................................... 42
Figure 2.6. Transcript levels of $trpS1$ are derepressed during stationary phase in wt relative to $B.\ cerus$ \Delta tRNA$^{\text{Other}}$ ........................................................................................................... 47
Figure 2.7. Transcript profiles of wt and $B.\ cerus$ \Delta tRNA$^{\text{Other}}$ from cells collected during mid-exponential growth (A) and stationary phase (B) ......................... 48
Figure 2.8. Transcript profiles of mid-exponential vs. stationary phase growth of wt (A) and \textit{B. cereus ΔtRNA}^\text{Other} (B) and \textit{B. cereus ΔtRNA}^\text{Other} grown to mid-exponential phase with or without erythromycin (C)..........................49

Figure 2.9. Changes in gene expression due to the deletion of sRNA^\text{Other} ..................50

Figure 2.10. Protein sequence and structural alignments of \textit{B. cereus} TrpRS1 and TrpRS2........................................................................................................52

Figure 2.11. Transcript level profiles of \textit{nos} and \textit{trpS1} display a similar pattern over time in wt \textit{B. cereus}........................................................................................................53

Figure 2.12. Predicted interaction between sRNA^\text{Other} and its putative mRNA target, \textit{cymR}-homologue......................................................................................54

Figure 2.13. Lipid analysis of wt and \textit{B. cereus ΔtRNA}^\text{Other} ........................................55

Figure 2.14. Cell wall thickness of wt (A) and \textit{B. cereus ΔtRNA}^\text{Other} (B) ..............57

Figure 3.1. Antibiotic-induced stress leads to the production and accumulation of reactive oxygen species. .................................................................64

Figure 3.2. \textit{fur} transcript levels increase as extracellular iron concentration increases..70

Figure 3.3. sRNA^\text{Other} expression decreases as extracellular iron concentration increases.71

Figure 3.4. Deletion of sRNA^\text{Other} alters antibiotic resistance in wt and \textit{B. cereus ΔtRNA}^\text{Other} ........................................................................................................74

Figure 3.5. Endogenous nitric oxide production is higher in wt than \textit{B. cereus ΔtRNA}^\text{Other} during mid-exponential growth.................................................................78

Figure 3.6. Nitric oxide levels in wt and \textit{B. cereus ΔtRNA}^\text{Other} grown under various conditions.................................................................79

Figure 3.7. Biofilm formation in wt and \textit{B. cereus ΔtRNA}^\text{Other} ........................................81
Figure 3.8. Biofilm formation by wt and \textit{B. cereus} \textit{ΔtRNA}^{Other} grown under high and low iron conditions.................................................................82

Figure 3.9. Schematic of iron-induced regulation via the ferric uptake regulator, Fur....83

Figure 3.10. sRNA^{Other} as a small regulatory RNA with the predicted \textit{cymR} target......86

Figure 4.1. Mapping the 5’ end of sRNA^{Other}. .................................................................95

Figure 4.2. Mapping the 3’ end of sRNA^{Other}. .................................................................97

Figure 4.3. Predicted promoters and Fur binding sites in the upstream region of the gene encoding sRNA^{Other}, and predicted 5’- and 3’-ends of sRNA^{Other}. ...........98

Figure 4.4. Promoter predictions for \textit{σ}^A and \textit{σ}^B .................................................................99

Figure 4.5. Predicted interaction between sRNA^{Other} and two putative mRNA targets, \textit{nos} and \textit{sod}. .........................................................................................................................99

Figure 4.6. Predicted interaction between sRNA^{Other} and the \textit{cymR} mRNA targets......101

Figure 5.1. Schematic of sRNA^{Other} function in \textit{B. cereus}.........................................................109
List of Symbols and Abbreviations

°C  degrees Celsius (centigrade)
Å  angstrom (unit)
β  beta
δ  delta
σ  sigma factor
µ  micro
Δ  delta/deletion
2’  2’-hydroxyl of a nucleic acid
3’  3’-hydroxyl/end of a nucleic acid
5’  5’-phosphate/end of a nucleic acid
A  adenosine
A-site  aminoacyl-site of the ribosome
a.u.  arbitrary unit
aa  amino acid
aaPG  aminoacyl-phosphatidylglycerol
aaPGS  aminoacyl-phosphatidylglycerol synthase
aa-tRNA  aminoacyl-tRNA
aaRS  aminoacyl-tRNA synthetase
ADP  adenosine 5’-diphosphate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdT</td>
<td>tRNA-dependent amidotransferase</td>
</tr>
<tr>
<td>Ala</td>
<td>L-alanine</td>
</tr>
<tr>
<td>ALA</td>
<td>δ-aminolevulinic acid</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine 5’-monophosphate</td>
</tr>
<tr>
<td>Arg</td>
<td>L-arginine</td>
</tr>
<tr>
<td>Asn</td>
<td>L-asparagine</td>
</tr>
<tr>
<td>AsnRS</td>
<td>asparaginy-tRNA synthetase</td>
</tr>
<tr>
<td>Asp</td>
<td>L-aspartate</td>
</tr>
<tr>
<td>AspRS</td>
<td>aspartyl-tRNA synthetase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>B. anthracis</td>
<td><em>Bacillus anthracis</em></td>
</tr>
<tr>
<td>B. cereus</td>
<td><em>Bacillus cereus</em></td>
</tr>
<tr>
<td>B. subtilis</td>
<td><em>Bacillus subtilis</em></td>
</tr>
<tr>
<td>B. thuringiensis</td>
<td><em>Bacillus thuringiensis</em></td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>CAMP</td>
<td>cationic antimicrobial peptide</td>
</tr>
<tr>
<td>CDPS</td>
<td>cyclodipeptide synthase</td>
</tr>
<tr>
<td>Cys</td>
<td>L-cysteine</td>
</tr>
<tr>
<td>CysRS</td>
<td>cysteinyl-tRNA synthetase</td>
</tr>
<tr>
<td>DDT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DKP</td>
<td>diketopiperazine</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide 5’-triphosphate</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double-stranded RNA</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>E-site</td>
<td>exit-site of the ribosome</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EF-Tu</td>
<td>elongation factor Tu</td>
</tr>
<tr>
<td>fMet</td>
<td>formyl methionine</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>Ferric iron</td>
</tr>
<tr>
<td>Fe$^{3+}$</td>
<td>Ferrous iron</td>
</tr>
<tr>
<td>Fur</td>
<td>ferric uptake regulator</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>G</td>
<td>guanosine</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>$N$-acetylglucosamine</td>
</tr>
<tr>
<td>Gln</td>
<td>L-glutamine</td>
</tr>
<tr>
<td>GlnRS</td>
<td>glutaminyl-tRNA synthetase</td>
</tr>
<tr>
<td>Glu</td>
<td>L-glutamate</td>
</tr>
<tr>
<td>GluRS</td>
<td>glutamyl-tRNA synthetase</td>
</tr>
<tr>
<td>GluTR</td>
<td>glutamyl -tRNA reductatse</td>
</tr>
<tr>
<td>Gly</td>
<td>L-glycine</td>
</tr>
<tr>
<td>GSA</td>
<td>glutamate-semialdehyde</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5’ triphosphate</td>
</tr>
</tbody>
</table>
h  hour
H₂O  water
H₂O₂  hydrogen peroxide
His  L-histidine
Ile  L-isoleucine
LB  Luria-Bertani
Leu  L-leucine
L/F-transferase  leucyl/phenylalanyl-transferase
log  common logarithm
Lys  L-lysine
LysPG  lysyl-phosphatidylglycerol
LysPGS  lysyl-phosphatidylglycerol synthase
LysRS  lysyl-tRNA synthetase
lysRS  gene encoding LysRS
m  mili
M  molar
Met  L-methionine
min  minute
miRNA  mircoRNA
mRNA  messenger RNA
MurNAc  N-acetylmuramic acid
ND or nd  not determined
NO  nitric oxide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NRPS</td>
<td>non-ribosomal peptide synthase</td>
</tr>
<tr>
<td>p</td>
<td>pico</td>
</tr>
<tr>
<td>P-site</td>
<td>peptidyl-site of the ribosome</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PG</td>
<td>phosphatidylglycerol</td>
</tr>
<tr>
<td>Phe</td>
<td>L-phenylalanine</td>
</tr>
<tr>
<td>PLP</td>
<td>pyridoxal phosphate</td>
</tr>
<tr>
<td>PSTK</td>
<td>O-phosphoseryl-tRNA kinase</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative RT-PCR</td>
</tr>
<tr>
<td>R-transferase</td>
<td>arginyl-transferase</td>
</tr>
<tr>
<td>RACE</td>
<td>rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription-PCR</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>Sec</td>
<td>L-selenocysteine</td>
</tr>
<tr>
<td>SECIS</td>
<td>selenocysteine incorporation sequence</td>
</tr>
<tr>
<td>Sep</td>
<td>O-phosphoserine</td>
</tr>
<tr>
<td>SepCysS</td>
<td>Sep-tRNA:Cys-tRNA synthetase</td>
</tr>
<tr>
<td>SepRS</td>
<td>O-phosphoseryl-tRNA synthetase</td>
</tr>
<tr>
<td>SepSecS</td>
<td>Sep-tRNA:Sec-tRNA synthetase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Ser</td>
<td>L-serine</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>sRNA</td>
<td>small RNA (regulator)</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>tmRNA</td>
<td>transfer/messenger RNA</td>
</tr>
<tr>
<td>tRF</td>
<td>tRNA-derived fragment</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>Trp</td>
<td>L-tryptophan</td>
</tr>
<tr>
<td>TrpRS</td>
<td>tryptophanyl-tRNA synthetase</td>
</tr>
<tr>
<td><em>trpRS</em></td>
<td>gene encoding TrpRS</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>TyrRS</td>
<td>tyrosyl-tRNA synthetase</td>
</tr>
<tr>
<td>U</td>
<td>uracil</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>Val</td>
<td>L-valanline</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>Wt or wt</td>
<td>wild-type</td>
</tr>
</tbody>
</table>
Chapter 1

The various functions of tRNAs

Transfer RNAs (tRNAs) are well-known for their function in mRNA translation, yet recent studies have shown that canonical tRNAs can function in numerous processes outside translation (Fig. 1.1). For example, tRNAs can be used in the biosynthesis of amino acids, antibiotics, tetrapyrrols, and peptidoglycan of the bacterial cell wall. Aminoacyl-tRNA protein transferases use aminoacylated-tRNAs (aa-tRNAs) to tag the N-terminal end of a protein as a signal for proteolysis. tRNAs can also function in the regulation of gene expression in the stringent response and T box regulation. The range of possible functions for tRNAs has been further extended by the finding that they can serve as precursors for an abundant class of stable small RNAs, tRNA-derived RNA fragments (tRFs). In addition to canonical tRNAs, a number of non-standard variants have been identified that lack conserved structural features. While some of these non-canonical tRNA structures have canonical roles, for example certain mitochondrial forms that lack D- and T-arms (139), others do not function in translation and are classified as pseudo-tRNAs. While the function outside translation has been defined for some pseudo-tRNAs, most notably in peptidoglycan biosynthesis (48, 140), relatively little is known about the possible roles of these molecules in the cell. In this chapter, a review of tRNA functions within and outside translation is given.
Figure 1.1. The use of tRNAs in various cellular pathways. Examples in the green shaded box require the use of deacylated-tRNAs. Examples in the red shaded box require the use of misaminoacylated-tRNAs. Figure adapted from (11).

1.1. Biosynthetic processes involving tRNAs

1.1.1. mRNA translation

The transfer of genetic information encoded by DNA and expressed in the form of proteins is accomplished by transfer RNAs. To this end, tRNAs contain a complementary sequence to the genetic codon, the anticodon, and have an amino acid attached to their 3’-end through an aminoacyl-ester linkage (169). Central to the maintenance of the genetic code, the aminoacyl-tRNA synthetases (aaRS) catalyze the attachment of an amino acid to the 3’-CCA end of a tRNA, forming an aminoacyl-tRNA (aa-tRNA) (66, 167). There exists a separate aaRS for each translationally encoded
amino acid, and these enzymes must choose the correct amino acid from a pool of amino acids to pair with its canonical tRNAs chosen from a pool of tRNAs (66). The aminoacylation reaction occurs in a two-step process: (i) activation of the amino acid with ATP with subsequent release of pyrophosphate; and (ii) transfer of the amino acid to the 2’- or 3’-hydroxyl of the tRNA with release of AMP (66). The aa-tRNA is then bound by elongation factor Tu (EF-Tu), which delivers it to the aminoacyl-site (A-site) of the ribosome, where the aa-tRNA is subjected to a selection process, termed decoding, in which the anticodon is paired with the corresponding mRNA codon (52, 83, 105). When the cognate aa-tRNA is recognized in the 30S A-site of the ribosome, EF-Tu hydrolyzes GTP to release the aa-tRNA to be accommodated in the 50S A-site (52). The ribosome then catalyzes the transfer of the growing peptide on the peptidyl-tRNA (P-site tRNA) to the aa-tRNA in the A-site (104). Elongation factor G (EF-G) then catalyzes the translocation of P-site and A-site tRNAs to the adjacent sites, the exit- (E-) and P-sites, respectively (45). The ribosome is then prepared to accept another aa-tRNA in its A-site and continue elongation.

1.1.2. Amino acid biosynthesis

Although a separate aaRS exists for each of the 20 canonical genetically encoded amino acids, most bacteria and archaea can persist without asparaginyl-tRNA synthetase (AsnRS) and/or glutaminyl-tRNA synthetase (GlnRS) (64). For these organisms, non-discriminating aspartyl- and glutamyl-tRNA synthetases (AspRS and GluRS) aminoacylate tRNA^{Asn} with aspartate and tRNA^{Gln} with glutamate, respectively (Fig. 1.2) (135). Asp-tRNA^{Asn} and Glu-tRNA^{Gln} are then converted to Asn-tRNA^{Asn} and Gln-
tRNA$_{\text{Gln}}$ by the tRNA-dependent amidotransferases (AdT) (31, 32). AdT exists as the heterotrimeric enzyme GatCAB in bacteria and some archaea, and a homodimeric GatDE is present in some archaea (31, 150). The kinase subunit GatB/E activates the misacylated-tRNAs by phosphorylation and the glutaminase subunit GatA/D amidates Asp or Glu to Asn or Gln, respectively (150). The amidation step requires the transfer of ammonia from an amide donor through a 40 Å channel to the misacylated-tRNA (150). The misacylated-tRNAs avoid use in protein synthesis because they do not bind to EF-Tu as well as cognate aa-tRNAs, and also because substrate channeling can occur as in the formation of a transamidosome between AspRS, tRNA$_{\text{Asn}}$ and AdT (10, 116).

**Figure 1.2.** The various biosynthetic pathways of Asn-tRNA$_{\text{Asn}}$ and Gln-tRNA$_{\text{Gln}}$. To form the desired aa-tRNAs, direct pathways (top arrows) require only the aaRS and indirect pathways (bottom arrows) require an AdT and a non-discriminating aaRS, which can aminoacylate both tRNA$_{\text{Glu/Gln}}$ and tRNA$_{\text{Asp/Asn}}$. Figure from (66).
In some methanogenic archaea, cysteiny1-tRNA synthetase (CysRS) is absent and
cysteine biosynthesis also occurs in a tRNA-dependent manner (67, 129). O-
phosphoserine is transferred to tRNA\textsuperscript{Cys} by O-phosphoseryl-tRNA synthetase (SepRS) to
produce O-phosphoseryl-tRNA\textsuperscript{Cys} (Sep\textsuperscript{-}tRNA\textsuperscript{Cys}). Sep-tRNA\textsuperscript{Cys}-tRNA synthetase
(SepCysS) is dependent on the cofactor pyridoxal phosphate (PLP) for the conversion of
Sep\textsuperscript{-}tRNA\textsuperscript{Cys} to Cys-tRNA\textsuperscript{Cys} (129). Sep evades incorporation into proteins by the direct
shuttling of Sep\textsuperscript{-}tRNA\textsuperscript{Cys} from SepRS to SepCysS, which have been found to form a
binary complex (170).

Selenocysteine (Sec) is another amino acid that requires a tRNA-dependent
pathway for its synthesis (12). Although incorporation of selenocysteine into proteins via
the UGA codon has been described in the three domains of life, no aaRS specific to
selenocysteine has been found (12). In bacteria, selenocysteine biosynthesis begins with
the misaminoacylation of tRNA\textsuperscript{Sec} with serine by a seryl-tRNA synthetase (SerRS) to
form Ser\textsuperscript{-}tRNA\textsuperscript{Sec} (27, 38). Selenocysteine synthase (SelA) then uses the
selenophosphate to convert Ser\textsuperscript{-}tRNA\textsuperscript{Sec} to Sec\textsuperscript{-}tRNA\textsuperscript{Sec} (38). The selenocysteine
biosynthesis pathway in eukaryotes and archaea differ from the bacterial pathways in that
Ser\textsuperscript{-}tRNA\textsuperscript{Sec} is first converted to O-phosphoseryl-tRNA\textsuperscript{Sec} by O-phosphoseryl-tRNA
kinase (PSTK) (2, 24). PLP-dependent Sep\textsuperscript{-}tRNA\textsuperscript{Sec}-tRNA synthetase (SepSecS) then
converts O-phosphoseryl-tRNA\textsuperscript{Sec} to Sec\textsuperscript{-}tRNA\textsuperscript{Sec} using selenophosphate (2). Protein
synthesis using Sec\textsuperscript{-}tRNA\textsuperscript{Sec} via ribosomal translation at UGA codons requires the
downstream presence of both a selenocysteine incorporation sequence (SECIS) and the
translation factor SelB (40).
1.1.3. Tetrapyrrol synthesis

The syntheses of many tetrapyrroles, such as hemes and chlorophylls, require Glu-tRNA\textsuperscript{Glu} to begin synthesis of the universal tetrapyrrole biosynthetic precursor δ-aminolevulinic acid (ALA) (62, 63, 89, 131). Following formation of Glu-tRNA\textsuperscript{Glu} by glutamyl-tRNA synthetase (GluRS), the glutamate moiety is reduced by Glu-tRNA reductase (GluTR) to glutamate-semialdehyde (GSA) (63). GSA is subsequently transamidated by glutamate-semialdehyde aminomutase to ALA (63). The acidophilic bacterium *Acidithiobacillus ferrooxidans* has two tRNA\textsuperscript{Glu} isoacceptors and two GluRSs (GluRS1 and GluRS2) (89). GluRS1 aminoacylates tRNA\textsuperscript{Glu} and tRNA\textsuperscript{Gln} isoacceptors with an enlarged D-stem, while the non-discriminating GluRS2 favors the aminoacylation of a tRNA\textsuperscript{Gln} with a small (3 bp) D-stem. Studies suggest the two tRNA\textsuperscript{Glu} isoacceptors have separate functions in the biosynthesis of either protein or heme, and that GluRS1 expression is regulated by heme and Fe\textsuperscript{2+} concentrations (89).

1.1.4. Antibiotic synthesis

Many organisms have been found to use aa-tRNAs in the formation of antibiotics (46, 50, 156). For example, *Streptomyces viridifaciens* uses Ser-tRNA\textsuperscript{Ser} in one of the first steps to synthesize the antibiotic valanimycin (46). Interestingly, the seryl-tRNA synthetase (SerRS) encoded within the valanimycin gene cluster produces Ser-tRNA\textsuperscript{Ser} exclusively for valanimycin production (46).

A recently defined family of enzymes, the cyclodipeptide synthases (CDPS), utilizes aminoacyl-tRNAs to form peptide bonds in the production of cyclodipeptides (50). Cyclodipeptides belong to the diketopiperazine (DKP) family of secondary
metabolites, which have antibacterial, antiviral, antifungal, and immunosuppressive properties. Most known cyclodipeptides are synthesized by non-ribosomal peptide synthases (NRPSs) that activate free amino acids with ATP to form aminoacyl adenylates in preparation for peptide formation. The first enzyme described to produce cyclodipeptides with the use of aa-tRNAs, AlbC, catalyzes the formation of cyclo(L-Phe-L-Leu) in *Streptomyces noursei* (50). Many AlbC homologues have since been predicted via *in silico* analysis (50, 156). Recently, one of these homologues from *Mycobacterium tuberculosis*, Rv2275, was crystallized and found to have structural similarity to the catalytic domain of class Ic aaRSs, including tyrosyl- and tryprophanyl-tRNA synthetases (TyrRS and TrpRS, respectively) (156). Notably, CDPSs use only aa-tRNAs to form their product, whereas the aa-tRNA protein transferases transfer an amino acid from aa-tRNAs to a larger molecule (discussed in section 1.3) (50).

1.1.5. Peptidoglycan synthesis

Peptidoglycan, the major component of the bacterial cell wall, protects the cell against osmotic pressure of the cytoplasm and maintains cell shape (157). The basic peptidoglycan structure consists of alternating β-1,4-linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) forming a linear chain (47). Each chain is linked through the MurNAc units by peptide stems, which are cross-linked by aa-tRNA-dependent interpeptide bridges (22, 47). Modifications to the peptidoglycan structure by the tRNA-dependent addition of amino acids are often associated with an increase in antibiotic resistance (48, 60, 93). For example, peptidoglycan of *Staphylococcus aureus* is stabilized by pentaglycine bridges that require Gly-tRNA

Gly for
their formation (48), and in *Weissella viridescens*, peptidoglycan side chain formation is initiated by Ala-tRNA$^{\text{Ala}}$-dependent L-Ala incorporation (157). This process is mediated by the Fem factors (Factors Essential for Methicillin resistance, FemABX), a family of non-ribosomal peptidyl transferases (48, 60). An activated amino acid is transferred from an aa-tRNA to a hexapeptide lipid intermediate by FemX. FemA and FemB continue with the linkage of additional amino acids, and D,D-transpeptidases cross-link the interpeptide chains to ensure cell wall stability (48, 60).

1.2. **Cell membrane modification**

The aminoacyl-phosphatidylglycerol synthases (aaPGS) transfer amino acids from aa-tRNAs to the polar head of phosphatidylglycerol (PG), a component of the bacterial cell membrane (110, 126). First described in *Staphylococcus aureus*, the gene product of *mprF* (Multiple Peptide Resistance Factor) has a specificity for Lys-tRNA$^{\text{Lys}}$, forming lysyl-phosphatidylglycerol (LysPG), and is therefore known as lysyl-phosphatidylglycerol synthase (LysPGS) (126). Addition of the positively charged Lys to the negatively charged PG reduces the net negative charge of the membrane, which ultimately reduces the affinity of the membrane for cationic molecules. Many cationic antimicrobial molecules, such as cationic antimicrobial peptides (CAMPs), exploit the net negative charge of the bacterial cell membrane to target bacterial cells (122). Therefore, a reduced net negative charge on the cell membrane increases resistance to CAMPs. Other aa-tRNA specificities have been found for aaPGSs, including Arg-tRNA$^{\text{Arg}}$ and Ala-tRNA$^{\text{Ala}}$, forming ArgPG and AlaPG, respectively (124). The various membrane
modifications may contribute to altering more membrane-associated properties than just CAMP resistance, including acid resistance and membrane fluidity (122).

Figure 1.3. tRNA-dependent formation of LysPG. LysRS produces Lys-tRNA\textsuperscript{Lys}, which is utilized by the membrane-bound LysPGS to transfer Lys to PG, forming LysPG. Figure from (123).
1.3. Protein degradation

In addition to having functions in antibiotic resistance and the biosynthesis of antibiotics, aa-tRNAs are also involved in housekeeping functions such as promoting the degradation of certain proteins via the N-end rule (102). According to the N-end rule, which is conserved from bacteria through the so-called higher eukaryotes, the N-terminal residue of a protein is the main determinant for the half-life of a protein. According to this rule, certain amino acids are classified as stabilizing or destabilizing, and this classification differs between organisms. For example, the primary destabilizing amino acids in mammals are Arg, Lys, and His (type 1) or Phe, Leu, Trp, Ile, and Tyr (type 2) (155), whereas, in *E. coli*, the destabilizing residues are Phe, Tyr, Trp and Leu (148). In both eukaryotes and bacteria, the primary destabilizing amino acid residues can be added to the N-terminal end of a protein by the aa-transferases (102). In eukaryotes, an arginyl-transferase (R-transferase) recognizes the secondary destabilizing amino acid residues Asp and Glu, to which it transfers Arg from Arg-tRNA$^{\text{Arg}}$. Similarly, in bacteria, a leucyl/phenylalanyl-transferase (L/F-transferase) recognizes the secondary destabilizing amino acid residues Arg and Lys, to which it transfers a Leu or Phe residue from Leu-tRNA$^{\text{Leu}}$ or Phe-tRNA$^{\text{Phe}}$, respectively (102). In eukaryotes, the primary destabilizing residues are recognized by the protein N-recognin (E3) that associates with an ubiquitin ligase (E2) to ubiquitinate Lys residues (102). The ubiquitin tag is recognized by the 26S proteasome and subsequently degraded. In bacteria, the N-terminal primary destabilizing residues are recognized by ClpS, which delivers the tagged protein to the AAA+ ClpA•ClpP protease complex where the protein is degraded (102).
1.4. tRNA-like structures

The tRNA-like molecule, tmRNA, is also involved in protein degradation by tagging the C-terminus of a truncated polypeptide with a signal for degradation and plays a crucial role in the rescue of stalled ribosomes (Fig. 1.3.) (103). When encountering the 3’-end of a truncated mRNA, a ribosome will stall (75). For normal translation termination, the mRNA contains a stop codon that is recognized by a release factor, which catalyzes hydrolysis of the bond between the nascent polypeptide and P-site tRNA. The ribosome can then disassemble into its subunits and be reused in another round of translation (75, 103). An aminoacylated tmRNA rescues a stalled ribosome by acting as both a tRNA and an mRNA. The tmRNA is aminoacylated with alanine by alanyl-tRNA synthetase. The aminoacylated tmRNA then acts as a tRNA to add alanine to the C-terminal end of the truncated polypeptide. tmRNA then acts as an mRNA as the ribosome translates a portion of tmRNA to an 11 amino acid tail. The tagged polypeptide is marked for degradation, and the amino acids and ribosomal subunits are recycled (103).

tRNA-like molecules are often involved in viral genome replication. A tRNA-like structure is found on the 3’-end of many plant RNA viral genomes with various functions including increased infectivity, 3’-end maintenance, RNA encapsidation, and protein translation enhancement among others (35). Some of these tRNA-like structures have been shown to bind to EF-Tu, yet are not used in mRNA translation (58). As in the case of phage Qβ replicase, EF-Tu is used as a subunit of the replicase and interacts with the tRNA-like structure to initiate viral replication (58). Aminoacylation of these viral 3’-end tRNA-like structures is important for genome amplification of some viruses (149). The free –CCA\textsubscript{OH} 3’-end of the tRNA-like structures are important for both
aminoacylation and replication, and the maintenance of the \(-\text{CCA}_{\text{OH}}\) end by nucleotidyl-transferase is considered similar to telomerase activity (117).

**Figure 1.4. tmRNA rescues stalled ribosomes.** A ribosome will stall when it reaches the 3'-end of a truncated mRNA lacking a stop codon necessary for release factor binding. Ala-tmRNA can act as both a tRNA and an mRNA to rescue the stalled ribosome and concurrently tag the truncated polypeptide with a signal for degradation. tmRNA recognition of the truncated mRNA and stalled ribosome requires the additional factors SmpB and EF-Tu•GTP (not pictured). Figure modified from (103).
Retroviruses have been shown to use host tRNAs as primers for genome replication (76). The human immunodeficiency virus type 1 (HIV-1) uses one of the three human tRNA<sup>Lys</sup> isoacceptors (tRNA<sup>Lys,3</sup>) as a primer due to the perfect sequence complementarity between the 18 nt on the tRNA 3’-end and the primer binding site (159). tRNA<sup>Lys,3</sup> interaction with, and aminoacylation by, LysRS are both important for efficient packaging in HIV-1 (69). In addition, a recent study reveals for the first time that these viruses may have a tRNA-like structure directly attached to their genomes (Musier-Forsyth, unpublished).

### 1.5. Regulation of gene expression

#### 1.5.1. Regulation of gene expression by rare codon usage

The use of rare tRNAs can regulate expression of pathway-specific genes. For example, the expression of rare tRNAs is repressed by the protein Hha in *E. coli*, which is known to repress biofilm formation (44). The rare codons corresponding to these tRNA genes (AGG and AGA for arg<sub>U</sub>, ATA for ile<sub>X</sub> and ile<sub>Y</sub>, and CCC for pro<sub>L</sub>) are found in many genes involved in biofilm formation, including type I fimbriae regulators and structural gene clusters that promote surface adhesion (44). Biofilms are complex, three-dimensional communities of cells that are often important in pathogenesis (119). In *E. coli*, expression of *hha* is induced in mature biofilms, promoting cell dispersal from the biofilm by reducing surface adhesion (119). By regulating the expression of a few rare tRNA genes, *E. coli* is able to maintain a dynamic community to allow for biofilm formation and dispersion when appropriate under varying conditions.
Another example of gene expression regulation by rare tRNA expression is in the soil-dwelling bacterium *Streptomyces coelicolor*. Only a subset of genes in *S. coelicolor* A3 contain the rare leucine codon, UUA, which can be translated only by a rare tRNA encoded by the *bldA* gene (84, 87). Many of these genes are involved in *Streptomyces* morphological development and production of the antibiotics actinorhodin and undecylprodigiosin (87). Thus, regulation of *bldA* expression has downstream effects on cell morphology and antibiotic production.

### 1.5.2. Regulation of gene expression by uncharged tRNAs

Uncharged tRNAs function as sensors of amino acid concentration and as regulators of global gene expression in response to changes in amino acid concentration. In Gram-positive bacteria, this regulation often occurs via the T box transcription termination system in which an uncharged tRNA binds to the 5’-untranslated leader mRNA to induce formation of an antiterminator over the competing intrinsic transcriptional terminator structure (55). Formation of the antiterminator allows for continued transcription of the downstream genes, such as those encoding the aminoacyl-tRNA synthetases and proteins involved in amino acid biosynthesis and transport (55). In response to amino acid starvation in bacteria, such as *E. coli*, uncharged tRNAs stimulate the stringent response by inducing the production of the global transcriptional regulator ppGpp, which downregulates tRNA and rRNA concentration and increases expression of genes involved in amino acid biosynthesis (164). Similarly, in certain eukaryotic cells under amino acid starvation, uncharged tRNAs activate the protein Gcn2p, which reduces
overall protein translation by phosphorylating eIF2, which in turn increases amino acid production by activating the transcriptional regulator Gcn4p (168).

Uncharged tRNAs are often cleaved in response to amino acid starvation to rapidly reduce tRNA levels, thereby reducing protein synthesis (59, 71). However, tRNA cleavage is developmentally regulated, not induced by amino acid starvation, in the Gram-positive bacterium *Streptomyces coelicolor* (57). tRNA cleavage is also induced by oxidative stress in many eukaryotic cells, including mammals and plants (146). A recent study on *Giardia lamblia* found that aa-tRNAs are cleaved in response to stress and development into a dormant cyst (90). Unlike cleaved uncharged tRNAs, aa-tRNA cleavage products are retained at a constant level throughout the stress response, and therefore may have functions in addition to maintaining a low metabolic rate (90). It has been suggested that uncharged tRNAs and transfer RNA-derived fragments (tRFs) may act as regulators of gene expression, possibly as antisense RNAs (34, 91). In fact, a recent study in HeLa cells found that tRNA\textsuperscript{Gln} is cleaved by Dicer, an enzyme that cleaves double-stranded RNA (dsRNA) to produce short interfering RNAs (siRNAs) and microRNAs (miRNAs) (26). siRNAs and miRNAs modulate gene expression in eukaryotic cells by repressing translation and/or promoting mRNA decay (153). It has recently been proposed that tRNAs are the evolutionary ancestors of these regulatory RNAs (132). Further exploration is needed on the function of tRNAs and their cleavage products as small RNA regulators (sRNAs).
1.6. Small RNA regulators in bacteria

In bacteria, many small RNAs, which range in size from about 50 to 500 nt, have been found to possess regulatory roles in various cellular processes (161). Some of these sRNAs function as mimics of other nucleic acids to bind and regulate protein activity. For example, 6S RNA in *E. coli* binds and blocks activity of RNA polymerase with σ^{70} by mimicking an open promoter complex (Fig. 1.5) (160). CsrB and CsrC are sRNAs that sequester the protein CsrA that regulates carbon metabolism and motility by binding and altering expression from mRNAs (Fig. 1.5) (8). Many sRNAs bind directly to mRNAs through base pairing to modulate their expression (161). These sRNAs belong to two different groups: true antisense RNAs and sRNAs with limited complementarity to their mRNA targets (51). The true antisense RNAs are transcribed on the opposite strand of their target mRNAs. These *cis*-encoded sRNAs have extensive complementarity with which to bind their targets in order to repress mRNA translation or promote RNA degradation. This strategy is often used by plasmids, transposons, and bacteriophage to maintain a suitable copy number (18). In the cyanobacterium *Synechocystis*, *isiA* mRNA levels decrease in response to the expression of the *cis*-encoded sRNA IsrR (36). IsrR repression under iron-deficient conditions leads to an accumulation of *isiA* mRNAs. sRNAs that base pair with limited complementarity to their targets are *trans*-encoded, at a different location than their target mRNAs, and are often found to regulate mRNA translation and/or degradation of multiple targets (161). In general, these sRNAs require only small regions of base pairing (about 10-25 nt) with their targets, and this base pairing may include interruption of complementarity and non-Watson-Crick (G-U) base pairing (51, 161). This non-perfect base pairing often requires the RNA chaperone Hfq
to promote RNA-RNA interactions (19). Hfq has also been shown to bind tRNAs with comparable affinity to sRNAs and stimulate CCA-addition to the tRNA 3’-end, further supporting the notion that tRNAs may function as sRNAs (85, 130).

Figure 1.5. Protein-binding RNA regulators. CsrA and RNA polymerase with σ^{70} are sequestered by sRNAs CsrB and 6S RNA, respectively. The activity of these proteins is antagonized by these sRNA interactions. Figure modified from Waters and Storz, 2009 (161).
Figure 1.6. True antisense and limited complementarity base pairing sRNAs. A. Base pairing between true antisense (cis-encoded) sRNAs and their targets can result in no translation and subsequent mRNA degradation, mRNA cleavage, or transcription termination. B. Base pairing with limited complementarity between a sRNA and its target can result in an increase or decrease in translation or mRNA degradation. Black circles represent translation, and grey circles represent no translation. Figure modified from Waters and Storz, 2009 (161).
1.7. tRNA classification and pseudo-tRNAs

The function of a tRNA within translation can be predicted with software programs such as tRNAscan-SE, ARAGORN, and TFAM, among others (81, 94, 144). These programs base tRNA prediction on the anticodon sequence and predicted secondary structure, and TFAM uses additional identity elements to classify the tRNA. These bioinformatic approaches have led to the identification of large families of tRNAs in many organisms that exceed the expected needs for translation alone. Additionally, many of these tRNA genes lack canonical features of tRNAs, such as sequence and predicted secondary structure (94, 144). While some of these non-canonical tRNAs have canonical roles, for example certain mitochondrial forms lack D- and T- arms (139), others do not function in translation and are classified as pseudo-tRNAs (94, 144). These annotated pseudo-tRNAs may be relics of tRNAs that now maintain a different function, whether in biosynthesis of cell walls or antibiotics, regulation of gene expression, or genome replication.

1.8. Purpose of study

In this study, we aimed to determine the role of a predicted pseudo-tRNA in *B. cereus*, tRNA\textsuperscript{Other}. This tRNA-like RNA is aminoacylated *in vitro* by the concerted effort of unrelated class I and II lysyl-tRNA synthetases (LysRS), yet tRNA\textsuperscript{Other} has a CCA anticodon rather than the anticodons expected for tRNA\textsuperscript{Lys}, UUU or CUU. The CCA anticodon is complementary to the UGG codon for Trp in *B. cereus*. Deletion of the corresponding gene is not lethal when *B. cereus* is grown in rich media, but causes the cell to lose resistance to cationic antibiotics, ionophores, and detergents. In this study,
the function of tRNA$^\text{Other}$ within mRNA translation is questioned and various other functions of tRNAs and sRNAs are explored.
Chapter 2

Role of tRNA\textsuperscript{Other} in \textit{Bacillus cereus}

\textit{Bacillus cereus} is a Gram-type positive, facultative anaerobic, endospore-forming, rod-shaped bacterium found ubiquitously in soil (33, 68). As a common food pathogen, toxin-producing strains of \textit{B. cereus} are found in a vast array of foods, including rice, vegetables, and dairy products (33, 95). \textit{B. cereus}, along with \textit{Bacillus thuringiensis} (an insect pathogen) and \textit{Bacillus anthracis} (a mammalian pathogen), belongs to the \textit{cereus} group based on chromosomal phylogenetic similarity (68). The unique genes that separate these three group members into species are harbored mainly on their plasmids and include many genes involved in toxin production (68). The type strain \textit{B. cereus} ATCC 14579 is one of a handful of bacteria, including \textit{B. cereus} strains 10987, E33L, G9241, \textit{B. thuringiensis}, \textit{Photobacterium luminescens} subsp. \textit{laumondii} TTO1, \textit{Photobacterium asymbiotica}, and \textit{Symbiobacterium thermophilum}, that encode both class I and class II lysyl-tRNA synthetases (LysRS) within their genomes. The only other known occurrence of both LysRSs encoded within the same genome is in the genus \textit{Methanosarcina} of the Archaea (6).

As a general rule, a separate aminoacyl-tRNA synthetase (aaRS) exists for each of the 20 canonical amino acids (66), each belonging to one of two structurally distinct
groups: class I or class II (3, 66). Conserved structural features of the class I aaRS include a Rossmann fold in the active site and the His-Ile-Gly-His (HIGH) and Lys-Met-Ser-Lys-Ser (KMSKS) motifs, whereas class II aaRSs have a conserved active site consisting of an antiparallel β-sheet with motifs 1, 2, and 3 (66). Additionally, the two classes bind opposite sides of the tRNA acceptor stem: class I aaRSs bind the minor groove side while class II aaRSs bind the major groove side (66, 96). While most aaRSs belong to only one of these two classes, an exception is lysyl-tRNA synthetase, which can exist as a class I or class II aaRS (65, 66). Within the three domains of life, class I LysRSs are found mainly in the Archaea and some Bacteria, while class II LysRSs are found in all Eukarya and most Bacteria (70, 88).

The evolutionary retention of LysRS class I and II has been under investigation since the discovery of the class I LysRS encoded within the *Methanococcus maripaludis* genome (65), and the occasional presence of both classes of LysRS encoded within the same genome has been particularly intriguing (6, 113). The two enzymes have differential resistance to certain lysine analogues, which may aid in the survival of organisms with both classes of LysRS when exposed to these inhibitors (88). For example, the lysine analogue L-lysminimide inhibits class II but not class I LysRS, and L-γ-amino butyric acid inhibits class I but not class II LysRS (88). Additionally, docking models have demonstrated that the active sites of class I b and II b aaRSs, of which the LysRSs are members, can simultaneously bind the same tRNA acceptor stem without significant steric hindrance (120). A complex of the two LysRSs binding a single tRNA may provide a novel aminoacylation pathway, as had been suggested for lysylation of tRNA\(^{Pyl}\) in *Methanosarcina barkeri* (113). This novel aminoacylation pathway, requiring
the presence of both classes of LysRS to aminoacylate tRNA\textsuperscript{Pyl} with lysine, has been demonstrated \textit{in vitro} (113). However, lysylation of tRNA\textsuperscript{Pyl} by both class I and II LysRS \textit{in vivo} has yet to be determined, and pyrrolysyl-tRNA synthetase (PylS) has been shown to directly aminoacylate tRNA\textsuperscript{Pyl} with pyrrolysine (17, 112). Similarly, \textit{in vitro} studies with both classes of LysRS from \textit{B. cereus} ATCC 14579 have shown that class I and class II LysRS can act together, but not separately, to aminoacylate a tRNA-like small RNA, named tRNA\textsuperscript{Other} (Fig. 2.1) (6). tRNA\textsuperscript{Other} was originally identified as a substrate for both LysRSs by lysylation of a pool of \textit{B. cereus} total small RNAs. Although tRNA\textsuperscript{Other} has the anticodon CCA, which pairs with the UGG codon for tryptophan (Fig 2.1), neither of the two tryptophanyl-tRNA synthetases (TrpRSs) present in \textit{B. cereus} is able to aminoacylate tRNA\textsuperscript{Other} \textit{in vitro}. The presence of aminoacylated tRNA\textsuperscript{Other} in cell extracts was determined by acidic urea polyacrylamide gel electrophoresis followed by northern blot analysis (6). However, northern blot analyses were inconsistent between cell preps and probe design was problematic due to significant non-specific binding. \textit{In vitro} aminoacylation of tRNA\textsuperscript{Other} by the concerted effort of both class I and class II LysRS was extremely low in comparison to lysylation of tRNA\textsuperscript{Lys} by either LysRS alone, in that only 10\% of tRNA\textsuperscript{Other} \textit{in vitro} transcript was lysylated (6). These inconsistencies led us to question the role of tRNA\textsuperscript{Other} in ribosomal protein synthesis and to investigate possible functions outside translation. In this chapter, the role of tRNA\textsuperscript{Other} inside and outside of translation was explored.
2.1. Materials and methods

2.1.1. Bacterial strains, growth conditions, and BIOLOG analysis.

Wild-type (wt) *B. cereus* ATCC14597 and ΔtRNA<sub>Other</sub> were grown in Luria-Bertani (LB) broth with vigorous shaking at 37 °C. Growth was monitored by measuring absorbance at 600 nm (7). Overnight cultures were diluted 100-fold into 50 ml fresh, pre-warmed LB and incubated at 37 °C until they reached mid-exponential phase (OD<sub>600</sub> = 0.4 - 0.5). These cultures were subsequently diluted to an OD<sub>600</sub> of 0.1 to enrich for cells within mid-exponential phase prior to inoculation of larger (200 ml) batch cultures. When appropriate, vancomycin (2 µg/ml), rifampicin (1 µg/ml), novobiocin (0.4 µg/ml), nafcillin (2 µg/ml), 2,2-dipyridyl (DIP, 250 µM), and FeSO<sub>4</sub> (250 µM) were added to the culture media. The BIOLOG analysis was performed by comparing the changes in cell respiration of wt and *B. cereus* ΔtRNA<sub>Other</sub> in ~2,000 different culture conditions. Each condition was repeated twice and the BIOLOG analysis was performed.
by Biolog, Inc (Hayward, CA). To confirm certain differential phenotypes observed in the Biolog analyses, wild-type and ΔtRNA<sup>Other</sup> strains were grown in 200 ml batch cultures at 37 °C with shaking at 225 rpm.

### 2.1.2. Preparation of polysome fractions

The preparation of polysome fractions from *B. cereus* was adapted from Frederick, et al. 2000 and Algranati, et al. 1969 (1, 41). Briefly, cells were grown in LB broth at 37 °C to mid-exponential or stationary phase. Chloramphenicol (5 µg/ml final concentration) was added to the cultures, which were incubated at 37 °C for an additional five minutes. Cultures were then poured over loosely crushed ice and centrifuged at 10,000 x g for 5 min at 4 °C. Cells were washed in chilled lysis buffer (20 mM Tris-HCl (pH 7.5), 2 mM MgCl<sub>2</sub>, 10 mM NaCl), resuspended in 0.5 ml chilled lysis buffer per 100 ml original culture, added to microcentrifuge tubes containing 0.3 mg acid-washed glass beads, and stored at -80 °C until all samples were collected. 15 µl of sodium deoxycholate (10 %) and 1 µl RNase Inhibitor (Roche) were added to each sample after they were thawed on ice. Bead-beating was performed in a multi-vortexer for 8 min at 4 °C, cell lysate was centrifuged at 16,000 x g, 10 min at 4 °C, and the supernatant was transferred to a new tube. The supernatant (250-300 µl) was applied to the top of a 10-40% sucrose gradient (11 ml) containing 10 mM MgCl<sub>2</sub>, 20 mM Tris, pH 7.8, 100 mM NH<sub>4</sub>Cl<sub>2</sub>, and 2 mM DTT, and centrifuged 3 hr at 35,000 rpm in an SW-41 rotor (92,300 x g). Sucrose gradient fractions were collected in 500 µl aliquots using a syringe-pump system (ISCO Model 183) at 1.5 ml/min, and RNA was detected at 254 nm. RNA-containing fractions were precipitated with 0.3 M sodium acetate, pH 6.5 and one
volume isopropanol, centrifuged for 15 min at max speed at 4 °C, resuspended in 20 μl extraction buffer (0.3 M NaOAc, pH 6.5, 0.5 % SDS, 5 mM EDTA), and pooled into free tRNA, 30S, 70S, and polysome fractions. Samples were treated with DNase I (Invitrogen) for 1 h at 25 °C with RNase Inhibitor (Roche) added to each reaction, then phenol:chloroform extracted followed by ethanol precipitation.

2.1.3. RT-PCR of tRNA from polysome fractions

The presence of tRNA\textsuperscript{Other}, tRNA\textsuperscript{Trp}, pre-tRNA\textsuperscript{Other}, and pre-tRNA\textsuperscript{Trp} in polysome-containing fractions was determined by RT-PCR of extracted RNA. All primers were purchased from Integrated DNA Technologies except pre-BCW5, purchased from Sigma-Aldrich. First strand cDNA was synthesized using SuperScript\textsuperscript{TM} II reverse transcriptase (Invitrogen) and primer BCO3 (5’-TGGAGTGATGGCGGGAATT-3’) for tRNA\textsuperscript{Other} and pre-tRNA\textsuperscript{Other} and primer BCW3 (5’-TGGCAGGGGCAGTAGG-3’) for tRNA\textsuperscript{Trp} and pre-tRNA\textsuperscript{Trp}. Primer annealing was performed at 80 °C for 3 min then 50 °C for 15 min, and reverse transcription was performed at 50 °C for 35 min to prevent tRNA secondary structure formation. PCR was performed in 10 μl reactions with 5 μl cDNA, 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 5 mM MgCl\textsubscript{2}, 0.25 mM each of dATP, dCTP, dGTP, and dTTP, 0.5 U Taq DNA polymerase (Invitrogen), 1 mM of the appropriate 3’ primer, and 1 mM of BCO5 (5’-GGGGGTATAATTTAAGGGTAAAAC-3’) for tRNA\textsuperscript{Other}, Pre-BCO5 (5’-GTCCTGTCATTCCGACCACGA-3’) for pre-tRNA\textsuperscript{Other}, BCW5 (5’-AGGGGCATAGTTTAAAGGTAGAAC-3’) for tRNA\textsuperscript{Trp}, and pre-BCW5 (5’-CCCCCCTCCACCATTTCAT-3’) for pre-tRNA\textsuperscript{Trp}. 1 and (5’-
GTCCTCTAGCGGATCATATT-3’) for pre- tRNA^{Trp} 2. PCR was performed at 95 ºC for 5 min, 30 repeats of 95 ºC for 1 min, 55 ºC for 1 min, and 72 ºC for 1 min, then a final incubation of 72 ºC for 5 min.

### 2.1.4. Total RNA extraction and purification

For determining transcript abundance of *trpS1* and *trpS2*, *B. cereus* strain ATCC 14579 was grown in 500 ml LB broth at 37 ºC, and samples containing 1 x 10^8 cells (determined by absorbance readings at 600 nm) were removed at various time points. RNA was extracted using RNeasy Mini Kits (Qiagen) with DNase I treatment performed on the column. Following elution of RNA from the column, a second DNA digestion was performed with 2 µg RNA, DNase I (Invitrogen) and Alu I (Invitrogen) using Alu I buffer at 25 ºC for 30 min. DNA digestions were heated to 65 ºC for 10 min following addition of 1 µl 25 mM EDTA.

For transcriptional microarray analysis and subsequent confirmation of transcript levels via qRT-PCR, total RNA was stabilized with RNAprotect (QIAGEN) and isolated with an RNeasy mini kit (QIAGEN) according to the manufacturer’s instructions except as noted. Briefly, 5 ml of cells from the mid-exponential growth phase were pelleted at 5,000 x g, 4 ºC, resuspended in 1 ml RNAprotect, incubated at room temperature for 5 min, re-pelleted, and stored at -80 ºC. Cells were suspended in lysis buffer (30 mM Tris-HCl, pH 8.0, 1 mM EDTA, 15 mg/ml lysozyme) with 30 mg acid washed beads and vortexed in a multivortexer for 10 min at room temperature. Buffer RLT (700 µl) was added and cell suspension was vortexed for an additional 5 min before continuing with the manufacturer’s instructions. DNA digestion was performed with TURBO™ DNase
(Ambion) followed by a control PCR to check for residual contamination of the isolated RNA with DNA. RNA quality and concentration was determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc.) and visualized by formaldehyde-agarose gel electrophoresis (Fig. 2.2).

2.1.5. Transcriptional microarrays

Double-stranded cDNA (ds-cDNA) for transcriptional microarrays was prepared according to Roche NimbleGen, Inc. instructions using the SuperScript™ ds-cDNA synthesis kit (Invitrogen) (Fig. 2.2). For each transcriptional microarray, total RNA was isolated as described above and pooled from cultures grown in triplicate prior to ds-cDNA production. Transcriptional microarrays were performed once for wt and \( B.\ cereus \Delta tRNA^{Other} \) in stationary phase growth, once for mid-exponential phase \( B.\ cereus \Delta tRNA^{Other} \) grown in LB containing 100 uM erythromycin, and in duplicate for mid-exponential phase wt and \( B.\ cereus \Delta tRNA^{Other} \) (Fig. 2.2). Data analysis was performed using ArrayStar® v2.1 software.
Figure 2.2. Preparation of ds-cDNA for transcriptional microarrays. Purified RNA (A and C) and ds-cDNA (B and D) run on 2% agarose gels for the first (A and B) and second (C and D) set of transcriptional microarrays. For all gels, lanes 2 and 3 contain RNA or ds-cDNA for wt and ΔtRNA\textsuperscript{Other} strains, respectively. For gel B, lanes 4, 5, and 6 contain ds-cDNA from ΔtRNA\textsuperscript{Other} grown in LB + 10 µg/ml erythromycin, stationary phase wt cells, and stationary phase ΔtRNA\textsuperscript{Other} cells, respectively. DNA markers: Bioline EasyLadder I in lane 1 of A and B; Bioline EasyLadder II in lane 1 of C and D; Bioline HyperLadder™ V in lane 4 of A; and Fermentas GeneRuler™ 100 bp DNA Ladder in lane 4 of C and D.
2.1.6. Quantitative RT-PCR

Abundance of trpS1 and trpS2 mRNAs was determined by quantitative reverse transcription PCR (qRT-PCR) relative to a standard curve for each primer and probe set (Fig. 2.3). All primers and probes were designed using GenScript Real-time PCR (TaqMan) Primer Design (https://www.genscript.com/ssl-bin/app/primer) and purchased from Integrated DNA Technologies. First strand cDNA was synthesized using SuperScript™ II reverse transcriptase (Invitrogen) and primer TrpRT1 (5’-TGAGTTAGCTGGACCTCGTG-3’) for trpS1 and primer TruntrpRT3 (5’-AAACGTTACCGGAAATC-3’) for trpS2. Primer annealing was performed at 80 °C for 2 min, then at 50 °C for 15 min, and reverse transcription was performed at 42 °C for 50 min. Real-time PCR was performed in an Opticon2 DNA Engine (BioRad) using TaqMan Universal PCR Master Mix (Applied Biosystems), 700 nM of primers TrpRT1 and TrpRT2 (5’-CCTCTCAAATCCAATCCGTT-3’) and 250 nM probe TrpRT-FAM (5’-6-FAM-TCTTCGCAATAGCGCGTGCC-6-TAMARA-3’) for trpS1, 700 nM of primers TruntrpRT3 and TrunTrpRT4 (5’-TGACATAACGCGACTCCTA-3’) and 150 mM TruntrpRT-FAM (5’-6-FAM-CAATGCCTGAAATTCGCATTCCA-6-TAMARA-3’) for trpS2. The quantitative PCR protocol was 95 °C for 10 min and 40 repeats of 95 °C for 15 sec, 57.2 °C for 1 min, followed by a fluorescence reading of each reaction.
Figure 2.3. Standard curves for qRT-PCR of \textit{trpS1} and \textit{trpS2}. Standard curves were determined using 100-fold dilutions of genomic DNA for PCR with each primer set plus the TaqMan probe. Standard curves for \textit{trpS1} and \textit{trpS2} are presented in green and blue, respectively. Log-scale for the x-axis is in Log\textsubscript{10}.

To confirm differences in transcript levels between wt and \textit{B. cereus} \textit{ΔtRNA}\textsubscript{\textit{Other}} detected in the transcriptional microarrays, gene-specific primers were designed with GenScript Real-time PCR (TaqMan) Primer Design (https://www.genscript.com/ssl-bin/app/primer) to produce a single PCR product of 75 - 250 bp, based on amplification of wt \textit{B. cereus} genomic DNA (Table 2.1). Reverse transcription was performed using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions with gene-specific 3'-primers (1 µM) and total RNA (40 ng or 400 ng, see Table 2.1). Quantitative PCR was performed using iQ\textsuperscript{TM} SYBR\textsuperscript{®} Green Supermix with 1 µl cDNA and gene specific primers (300 nM). The Opticon2 DNA Engine (BioRad) was used with an initial 3 min incubation at 94 °C, 40 cycles of 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec followed by a fluorescence reading of each
reaction. Melting curves (50 – 95 °C) were performed for each PCR to confirm the presence of the correct amplicon and absence of primer dimers. For each reaction, a negative control was performed without reverse transcription. For each condition, qPCR was performed in triplicate for each total RNA preparation. RNA preparation and qRT-PCR were repeated three times and transcript level changes were averaged. Transcript level changes were determined by the Pfaffl method (Equation 1) (111). Similar to the ΔΔCt method, the change in the threshold crossing point (ΔCP or ΔCt) of the target cDNA is calculated between the control and sample conditions. Instead of assuming a PCR efficiency of 100 %, the Pfaffl method corrects for the actual PCR efficiency of specific primer sets (Equation 1). BC2458 and BC1232 (encoding thioesterase and anthranillate synthase, respectively) were chosen to serve as reference genes due to the lack of any transcript level change between wt and ΔtRNAOther grown with or without DIP, as shown in both the transcriptional microarray analysis and qRT-PCR. Reference gene transcript levels were quantified by comparing to data collected for genomic DNA standards for qRT-PCR.

\[
\text{ratio} = \frac{\left( E_{\text{target}} \right)^{\Delta CP_{\text{target}}(\text{control} - \text{sample})}}{\left( E_{\text{ref}} \right)^{\Delta CP_{\text{ref}}(\text{control} - \text{sample})}}
\]

**Equation 1. The Pfaffl method (111).** The Pfaffl method corrects for PCR efficiency \((E)\), whereas the ΔΔCt method assumes an efficiency of 2. \(E_{\text{target}}\), PCR efficiency of target cDNA; \(E_{\text{ref}}\), PCR efficiency of reference cDNA; \(\Delta CP_{\text{target}}(\text{control-sample})\), change in the threshold crossing point (also known as Ct) for the target cDNA between the control and experimental sample; \(\Delta CP_{\text{ref}}(\text{control-sample})\), change in the threshold crossing point (also known as Ct) for the reference cDNA between the control and experimental sample.
Table 2.1. Primers used for qRT-PCR*

<table>
<thead>
<tr>
<th>Set</th>
<th>Primer Name</th>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Primer Sequence</th>
<th>bp†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BC0787-FW</td>
<td>TGACTATTATCCAGCGCAAC</td>
<td>BC0787-RV</td>
<td>TAAGTACGGGAACCCACC</td>
<td>110</td>
</tr>
<tr>
<td>2</td>
<td>BC1368-FW</td>
<td>TTCTGATATCCAGCGCAAC</td>
<td>BC1368-RV</td>
<td>ACCTGGAAGAAACCGAC</td>
<td>101</td>
</tr>
<tr>
<td>3</td>
<td>BC5147-FW</td>
<td>TTCTGATATCCAGCGCAAC</td>
<td>BC5147-RV</td>
<td>ATGAGGTGCGGACTGTTG</td>
<td>94</td>
</tr>
<tr>
<td>4</td>
<td>BC5282-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC5282-RV</td>
<td>CATTTGCGGACTGTTG</td>
<td>117</td>
</tr>
<tr>
<td>5</td>
<td>BC2583-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC2583-RV</td>
<td>GGTGTGCGGACTGTTG</td>
<td>65</td>
</tr>
<tr>
<td>6</td>
<td>BC2773-FW</td>
<td>GCATTCTTCTTGGCAAGGCTA</td>
<td>BC2773-RV</td>
<td>TAAGTACGGGAACCCACC</td>
<td>161</td>
</tr>
<tr>
<td>7</td>
<td>BC4091-FW</td>
<td>GAATTAACCCAGCTTGGCAAGGCTA</td>
<td>BC4091-RV</td>
<td>GACCTTCTTGGCAAGGCTA</td>
<td>222</td>
</tr>
<tr>
<td>8</td>
<td>BC4194-FW</td>
<td>GGCGGACCTTGAGCTTTTTT</td>
<td>BC4194-RV</td>
<td>GACCTTCTTGGCAAGGCTA</td>
<td>163</td>
</tr>
<tr>
<td>9</td>
<td>BC1471-FW</td>
<td>CGTTATGGTTGGAAGTGAGT</td>
<td>BC1471-RV</td>
<td>TGCTACTGCTTATTCCCAT</td>
<td>240</td>
</tr>
<tr>
<td>10</td>
<td>BC2526-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC2526-RV</td>
<td>GAATTAACCCAGCTTGGCAAGGCTA</td>
<td>185</td>
</tr>
<tr>
<td>11</td>
<td>BC2632-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC2632-RV</td>
<td>GAATTAACCCAGCTTGGCAAGGCTA</td>
<td>185</td>
</tr>
<tr>
<td>12</td>
<td>BC3099-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC3099-RV</td>
<td>GAATTAACCCAGCTTGGCAAGGCTA</td>
<td>185</td>
</tr>
<tr>
<td>13</td>
<td>BC4007-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC4007-RV</td>
<td>GAATTAACCCAGCTTGGCAAGGCTA</td>
<td>185</td>
</tr>
<tr>
<td>14</td>
<td>BC5321-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC5321-RV</td>
<td>GAATTAACCCAGCTTGGCAAGGCTA</td>
<td>185</td>
</tr>
<tr>
<td>15</td>
<td>BC0458-FW</td>
<td>GACCTTCTTGGCAAGGCTA</td>
<td>BC0458-RV</td>
<td>GAATTAACCCAGCTTGGCAAGGCTA</td>
<td>185</td>
</tr>
<tr>
<td>16</td>
<td>BC1518-FW</td>
<td>GGCGGACCTTGAGCTTTTTT</td>
<td>BC1518-RV</td>
<td>GAATTAACCCAGCTTGGCAAGGCTA</td>
<td>185</td>
</tr>
<tr>
<td>17</td>
<td>BC1471-FW</td>
<td>CGTTATGGTTGGAAGTGAGT</td>
<td>BC1471-RV</td>
<td>TGCTACTGCTTATTCCCAT</td>
<td>240</td>
</tr>
<tr>
<td>18</td>
<td>BC0244-FW</td>
<td>TTCTGATATCCAGCGCAAC</td>
<td>BC0244-RV</td>
<td>TGCTACTGCTTATTCCCAT</td>
<td>240</td>
</tr>
<tr>
<td>19</td>
<td>BC4393-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC4393-RV</td>
<td>GAATTAACCCAGCTTGGCAAGGCTA</td>
<td>185</td>
</tr>
<tr>
<td>20</td>
<td>BC0244-FW</td>
<td>TTCTGATATCCAGCGCAAC</td>
<td>BC0244-RV</td>
<td>TGCTACTGCTTATTCCCAT</td>
<td>240</td>
</tr>
<tr>
<td>21</td>
<td>BC5444-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC5444-RV</td>
<td>GAATTAACCCAGCTTGGCAAGGCTA</td>
<td>185</td>
</tr>
<tr>
<td>22</td>
<td>BC2450-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC2450-RV</td>
<td>GAATTAACCCAGCTTGGCAAGGCTA</td>
<td>185</td>
</tr>
<tr>
<td>23</td>
<td>BC2458-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC2458-RV</td>
<td>GAATTAACCCAGCTTGGCAAGGCTA</td>
<td>185</td>
</tr>
<tr>
<td>24</td>
<td>BC1232-FW</td>
<td>GACCTTCTTGGCAAGGCTA</td>
<td>BC1232-RV</td>
<td>GAATTAACCCAGCTTGGCAAGGCTA</td>
<td>185</td>
</tr>
<tr>
<td>25</td>
<td>BC3718-FW</td>
<td>TATTATTGTGCGGCGCAATTGATA</td>
<td>BC3718-RV</td>
<td>TACGTACTGCTTATTCCCAT</td>
<td>240</td>
</tr>
<tr>
<td>26</td>
<td>BC4420-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC4420-RV</td>
<td>TGCTACTGCTTATTCCCAT</td>
<td>240</td>
</tr>
<tr>
<td>27</td>
<td>BC4651-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC4651-RV</td>
<td>TGCTACTGCTTATTCCCAT</td>
<td>240</td>
</tr>
<tr>
<td>28</td>
<td>BC4930-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC4930-RV</td>
<td>TGCTACTGCTTATTCCCAT</td>
<td>240</td>
</tr>
<tr>
<td>29</td>
<td>BC5293-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC5293-RV</td>
<td>TGCTACTGCTTATTCCCAT</td>
<td>240</td>
</tr>
<tr>
<td>30</td>
<td>BC1094-FW3</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC1094-RV</td>
<td>TGCTACTGCTTATTCCCAT</td>
<td>240</td>
</tr>
<tr>
<td>31</td>
<td>BC1550-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC1550-RV</td>
<td>TGCTACTGCTTATTCCCAT</td>
<td>240</td>
</tr>
<tr>
<td>32</td>
<td>BC2137-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC2137-RV</td>
<td>TGCTACTGCTTATTCCCAT</td>
<td>240</td>
</tr>
<tr>
<td>33</td>
<td>BC4336-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC4336-RV</td>
<td>TGCTACTGCTTATTCCCAT</td>
<td>240</td>
</tr>
<tr>
<td>34</td>
<td>BC5248-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC5248-RV</td>
<td>TGCTACTGCTTATTCCCAT</td>
<td>240</td>
</tr>
<tr>
<td>35</td>
<td>BC5350-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC5350-RV</td>
<td>TGCTACTGCTTATTCCCAT</td>
<td>240</td>
</tr>
<tr>
<td>36</td>
<td>BC5445-FW</td>
<td>TGCAGCGGAAGAAACCGAC</td>
<td>BC5445-RV</td>
<td>TGCTACTGCTTATTCCCAT</td>
<td>240</td>
</tr>
</tbody>
</table>

* Results for all primer sets are presented in Appendix A.
† Primer set amplicon size is in base pairs.
2.1.7. Transmission electron microscopy

Wild-type and *B. cereus* ΔtRNA<sup>other</sup> strains were grown as described above, except cells were grown to OD<sub>600</sub> = 0.3. Cells were then harvested and fixed overnight in 3 % glutaraldehyde, 0.1 M sucrose, and 0.1 M phosphate buffer, pH 7.4 at 4 °C. Cells were then washed 3x (0.1 M sucrose and 0.1 M phosphate buffer, pH 7.4) and postfixed in 1 % osmium tetroxide (Ted Pella, Inc., Redding, CA) for 1 hr at room temperature. Samples were *en bloc* stained in 1 % uranyl acetate and dehydrated in a graded series of ethanol and propylene oxide before embedding in Eponate 12 resin (Ted Pella, Inc., Redding, CA). Thin sections were cut on a Leica EM UC6 Ultramicrotome (Leica Microsystems, Exton PA), stained with lead citrate and observed on an FEI Tecnai G2 Spirit transmission electron microscope (FEI, Hillsboro, OR). Secondary fixation, staining and microscopy were performed at the Ohio State University Campus Microscopy and Imaging Facility. Cell wall thickness was measured using Adobe Photoshop CS3 Extended version 10.0.1. To obtain an average cell wall thickness for each cell, measurements were taken every 50 nm along the long sides of the rod-shaped cell at a perpendicular angle to its length. Cell poles and visible division sites were avoided in the measurements because cell wall thickness in these areas was variable relative to the long sides of the cell.
2.2. Results

2.2.1. tRNA\textsuperscript{Other}–dependent phenotypic changes in \textit{B. cereus}.

Previous comparisons of \textit{B. cereus} \textit{ΔtRNA}\textsuperscript{Other} with wild-type indicated that tRNA\textsuperscript{Other} is not an essential gene and has only a minor effect on growth rate under certain conditions (6). Deletion of tRNA\textsuperscript{Other} did not significantly affect sporulation, germination or the ability to produce a bacteriocin-like inhibitory substance (BLIS) characteristic of \textit{B. cereus} (6). To further investigate the role of tRNA\textsuperscript{Other}, a more extensive screening for changes in growth phenotypes (~1900 conditions) was carried out by performing phenotypic microarray analyses comparing the wild-type and \textit{ΔtRNA}\textsuperscript{Other} strains (Table 2.2). \textit{B. cereus} \textit{ΔtRNA}\textsuperscript{Other} acquired sensitivity to a number of compounds including numerous antibiotics, cationic detergents and positively charged ionophores. The increased resistance to several macrolide antibiotics (e.g., spiramycin, erythromycin, and oleandomycin) is an artifact of strain construction and was not further characterized. Decreased antibiotic resistance phenotypes resulting from the deletion of tRNA\textsuperscript{Other} were further characterized in batch cultures at various antibiotic concentrations.

For all antibiotic resistances further tested in batch culture, a moderate decrease in resistance was observed (Fig. 2.4). The largest decrease was observed for vancomycin resistance (Fig. 2.4 A). Both wt and \textit{B. cereus} \textit{ΔtRNA}\textsuperscript{Other} strains exhibited a decreased growth rate when grown in LB containing 2 \textmu g/ml vancomycin relative to both strains grown in LB lacking antibiotics. The decreased resistance to vancomycin is evident in the decreased growth rate relative to wt when exposed to the antibiotic. For growth in LB containing either 0.4 \textmu g/ml novobiocin or 2 \textmu g/ml nafcillin, delayed growth of about
4 to 5 hours is observed for both strains (Fig. 2.4 D and E). The growth rate for wt B. cereus increases prior to and at a higher rate than for the ΔtRNAOther strain. For confirmation of rifampicin resistance, rifapentin, another rifamycin derivative similar to rifampicin in RNA polymerase binding (4), was used for the batch cultures because rifapentin has a longer elimination half-life in animals, including humans, than rifampicin (14). For growth in LB containing 0.04 µg/ml rifapentin, growth is delayed about an hour longer for the ΔtRNAOther strain than for wt B. cereus (Fig. 2.4 C). Interestingly, once growth resumes, both strains have a similar growth rate. Relative growth of the two B. cereus strains in LB containing 15 µg/ml puromycin is quite different from growth in LB containing the other antibiotics tested in this study (Fig 2.4 B). From about 2 to 6 hours, B. cereus ΔtRNAOther maintains a similar growth rate as wt. The growth rate of wt increases after 6 hours, whereas B. cereus ΔtRNAOther maintains a similar growth rate throughout the 11 hour time course. Under the conditions described for all five antibiotics, both strains reach a similar cell density once in stationary phase after approximately eight to ten hours (Fig. 2.4 A). Under higher antibiotic concentrations (10 µg/ml vancomycin, 50 µg/ml puromycin, 0.25 µg/ml rifapentin, 40 µg/ml novobiocin, and 31 µg/ml nafcillin), B. cereus ΔtRNAOther was unable to grow after a 24 hr incubation at 37 °C, while wt maintained growth at lower rates than those presented in Figure 2.4 (data not shown).
**Table 2.2. Phenotypes of B. cereus ΔtRNA\(^{\text{Other}}\) relative to wt determined by BIOLOG phenotypic array analysis.**

<table>
<thead>
<tr>
<th>Resistances Lost</th>
<th>Mode of Action/Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzethonium Cl</td>
<td>Membrane, detergent, cationic</td>
</tr>
<tr>
<td>Chelerythrine</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>Dodecyltrimethyl NH4Br</td>
<td>Membrane, detergent, cationic</td>
</tr>
<tr>
<td>Domiphen bromide</td>
<td>Membrane, detergent, cationic, fungicide</td>
</tr>
<tr>
<td>Iodonitro Tetrazolium</td>
<td>Respiration</td>
</tr>
<tr>
<td>Lauryl sulfobetaine</td>
<td>Membrane, detergent, zwitterionic</td>
</tr>
<tr>
<td><strong>Nafcillin</strong></td>
<td><strong>Cell wall biosynthesis</strong></td>
</tr>
<tr>
<td>Niaproof</td>
<td>Membrane, detergent, anionic</td>
</tr>
<tr>
<td><strong>Novobiocin</strong></td>
<td><strong>DNA topoisomerase</strong></td>
</tr>
<tr>
<td>Pentachlorophenol</td>
<td>Respiration, ionophore, H(^+)</td>
</tr>
<tr>
<td><strong>Puromycin</strong></td>
<td><strong>Protein synthesis, 50S ribosomal subunit</strong></td>
</tr>
<tr>
<td><strong>Rifampicin</strong></td>
<td><strong>RNA polymerase</strong></td>
</tr>
<tr>
<td>Sanguinarine</td>
<td>ATPase, Na(^+)/K(^+) and Mg(^{2+})</td>
</tr>
<tr>
<td>Tetrazolium Violet</td>
<td>Respiration</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>Cell cycle modulation, DNA synthesis</td>
</tr>
<tr>
<td>Tylosin</td>
<td>Protein synthesis, 50S ribosomal subunit, macrolide</td>
</tr>
<tr>
<td><strong>Vancomycin</strong></td>
<td><strong>Cell wall biosynthesis</strong></td>
</tr>
</tbody>
</table>

* Only a subset of phenotype changes is shown. Data acquired by S.F. Ataide (5).

\(^{\text{a}}\)Bold lettering indicates antibiotics further investigated.
Figure 2.4. Deletion of tRNA<sub>Other</sub> alters antibiotic resistance in wt <i>B. cereus</i> and <i>B. cereus ΔtRNA<sub>Other</sub></i>. Wt (circles) and <i>B. cereus ΔtRNA<sub>Other</sub></i> (triangles) strains were grown in LB containing vancomycin (2 µg/ml) (A), puromycin (15 µg/ml) (B), rifapentin (0.04 µg/ml) (C), novobiocin (0.4 µg/ml) (D), nafcillin (2 µg/ml) (E). Wt cells were grown in LB (●) or LB + antibiotics (●) and ΔtRNA<sub>Other</sub> cells were grown in LB (▲) or LB + antibiotics (▲) at 37°C with shaking at 250 rpm. A and B are averages of three growth curves, and error bars represent standard deviations. C, D, and E are representative growth curves of at least three replicates.
Figure 2.4

A. Vancomycin

B. Puromycin

Continued
Figure 2.4 continued

C. Rifapentin

D. Novobiocin

E. Nafcillin
2.2.2. Role of tRNA\textsuperscript{Other} in mRNA translation

Early data demonstrated EF-Tu binding to tRNA\textsuperscript{Other}, but these data were performed under non-physiological conditions, and therefore a role for tRNA\textsuperscript{Other} in ribosomal protein synthesis was not confirmed (6). To investigate a possible role for tRNA\textsuperscript{Other} in translation, \textit{B. cereus} polysomes were isolated from exponential and stationary growth phases. RT-PCR was performed to detect the presence of tRNA\textsuperscript{Other}, tRNA\textsuperscript{Trp} as a positive control, and the unprocessed transcripts (pre-tRNA\textsuperscript{Other} and pre-tRNA\textsuperscript{Trp}), which served as negative controls. For the tRNA\textsuperscript{Trp} control reactions, an RT-PCR product for tRNA\textsuperscript{Trp} was always present in the polysome fractions, whereas a PCR product for pre-tRNA\textsuperscript{Trp} was present only when genomic DNA was used as the template. RT-PCR product for tRNA\textsuperscript{Other} was present in both the free tRNA and polysome fractions, yet serial dilutions of cDNA followed by PCR revealed that the concentration of tRNA\textsuperscript{Other} did not exceed that of pre-tRNA\textsuperscript{Other}, indicating that tRNA\textsuperscript{Other} is not present as a mature tRNA in polysomes (Fig. 2.5). The absence of tRNA\textsuperscript{Other} in polysomes suggests a function outside translation for this RNA.
Figure 2.5. tRNA\textsuperscript{Other} is not detected in \textit{B. cereus} polysomes. A. Cell lysate fractions separated on a 10-40% sucrose gradient to isolate the polysome fractions. B. Primers for RT-PCR were designed to amplify either tRNA\textsuperscript{Other} from its 5’- to 3’-ends or the unprocessed form, pre-tRNA\textsuperscript{Other}, using the same 3’-primer for cDNA synthesis and a second 5’-primer complementary to 20 nt upstream of tRNA\textsuperscript{Other}. C. RT-PCR of Pre-tRNA\textsuperscript{Other} and tRNA\textsuperscript{Other} from polysomes. Polysome fractions were collected from cell lysate fractionated in 10-40% sucrose gradients. Lanes 1 – 8, 2-fold dilutions of Pre-tRNA\textsuperscript{Other} cDNA. Lanes 10 – 17, 2-fold dilutions of tRNA\textsuperscript{Other} cDNA. M, marker: BioLine HyperLadder V with marker sizes (from top to bottom): 500, 400, 300, 250, 200, 175, 150, 125, 100, 75, 50, and 25 bp. Arrows indicate size of PCR products for pre-tRNA\textsuperscript{Other} and tRNA\textsuperscript{Other}. 

42
Figure 2.5

A

Free

30S

50S

70S

Polysomes

B

\[ \text{tRNA}^{\text{Other}} \]

\[ \text{pre-tRNA}^{\text{Other}} \]

C

\[ \text{pre-tRNA}^{\text{Other}} \]

\[ \text{tRNA}^{\text{Other}} \]
2.2.3. tRNA\textsuperscript{\textit{Other}}-induced transcriptional profile changes.

As mentioned in Chapter 1, tRNAs can function as regulators of gene expression through the T box transcription termination system. \textit{tRNA}\textsuperscript{\textit{Other}} has a Trp anticodon, and \textit{B. cereus} ATCC 14579 encodes two TrpRSs within its genome, each with a T box in the 5’ leader sequence of their respective mRNAs. A possible role for \textit{tRNA}\textsuperscript{\textit{Other}} in regulation of the \textit{trpS1} and \textit{trpS2} genes was investigated using qRT-PCR to observe differences in the transcription of these genes between wt and \textit{B. cereus \Delta}tRNA\textsuperscript{\textit{Other}} strains at various stages of growth in rich medium (Fig. 2.6). No significant changes were observed for \textit{trpS2} transcripts between the two strains during all stages of growth. However, \textit{trpS1} transcript levels for wt and \textit{B. cereus \Delta}tRNA\textsuperscript{\textit{Other}} strains were similar only during exponential growth and significantly diverged during stationary phase, resulting in a 50-fold increase in wt relative to \textit{B. cereus \Delta}tRNA\textsuperscript{\textit{Other}} (Fig. 2.6). Although these data are consistent with a role for \textit{tRNA}\textsuperscript{\textit{Other}} in T box transcription regulation, the discriminator base of \textit{tRNA}\textsuperscript{\textit{Other}} is not predicted to make contacts with the mRNA leader sequence that are strong enough to stabilize the antiterminator sequence, which allows transcription to continue. T box transcription termination is not the only process in which tRNAs have been shown to function as regulators of gene expression, therefore an exploration of broader regulatory functions was warranted.

The role of \textit{tRNA}\textsuperscript{\textit{Other}} in global regulation of gene expression was explored by comparing the transcriptomes of wt and \textit{B. cereus \Delta}tRNA\textsuperscript{\textit{Other}} strains by transcriptional microarray analysis. Although transcript level changes were expected to occur mainly during stationary phase, very few changes were observed during this stage of growth (Fig.
In contrast, many transcript level changes were observed during mid-exponential growth. Comparing the wild-type and ΔtRNA<sub>Other</sub> strains during exponential growth, significant transcript level changes occurred for 773 genes with a 2-fold change or higher, p-value ≤ 0.05 (see Appendix 1 for complete list). Of these, 53 genes had a 4-fold change or higher. The most notable change during mid-exponential growth, 10.8-fold lower in ΔtRNA<sub>Other</sub> compared to wt, is that of a transcript encoding a putative multimodular transpeptidase-transglycosylase, PBP 1a, which functions in peptidoglycan biosynthesis (49). The majority of the other significant transcript level changes ranged from 2- to 4-fold. Of these, a decrease in transcript levels occurred for a few genes encoding transcriptional regulators, including Fur, Spx, CymR, AraC, and DnrN, and many members of their regulons.
Figure 2.6. Transcript levels of *trpRS1* are derepressed during stationary phase in wt relative to *B. cereus* ΔtRNA*Other*. Transcript levels for *trpS1* and *trpS2* were determined by qRT-PCR for wt and *B. cereus* ΔtRNA*Other* strains. Growth was monitored using a klett meter. Error bars are standard deviations of qRT-PCR data.

Quantitative RT-PCR (qRT-PCR) was performed to confirm transcript level changes revealed in the transcriptional microarray analyses. An interesting pattern of transcript level changes was noted between the wt and ΔtRNA*Other* strains when grown in LB with or without the iron chelator DIP (Table 2.3). For cells grown in LB without DIP, transcript level changes for many genes were comparable between the qRT-PCR and microarray analyses. Surprisingly, the transcript level changes for three genes contradicted those determined by the transcriptional microarray analyses. These three genes, *spx*, *cymR*-homologue, and *fur*, encode transcriptional regulators involved in the oxidative stress response. While the transcriptional microarray results indicated a
decrease in transcript levels for these three genes in the tRNA\textsubscript{Other} deletion strain relative to wt, the levels determined by qRT-PCR from cells grown in LB without DIP were higher in \textit{B. cereus} ΔtRNA\textsubscript{Other} when compared to wt. However, when cells were grown in the presence of DIP, the transcript level changes for these genes were comparable to the changes observed in the transcriptional microarray analysis: transcript levels from all three genes were lower in ΔtRNA\textsubscript{Other} than in the wt strain (Table 2.3). The contradictory results suggest a difference in iron concentrations in LB medium batches used for the transcriptional microarray and qRT-PCR experiments. Under low iron conditions, \textit{spx}, cymR-homologue, and \textit{fur} transcript levels were significantly decreased in the ΔtRNA\textsubscript{Other} strain relative to wt, while an increase in these transcript levels was observed under higher iron conditions. These results indicate that the presence of tRNA\textsubscript{Other} is required for a significant reduction in the transcript levels of these genes when cells are grown under conditions with low iron concentration.

Some of the genes for which the qRT-PCR and microarray analyses were comparable are also involved in the oxidative stress response, such as those encoding superoxide dismutase [Fe/Mn] (SOD), nitric oxide synthase (NOS), and nitric oxide-dependent regulator (dnrN). \textit{sod} transcript levels were 2.0- to 3.7-fold lower in the ΔtRNA\textsubscript{Other} strain than the wt with or without DIP (Table 2.3 and Figure 2.9). Transcript levels for \textit{pbp1a} were comparable between microarray and qRT-PCR data for ΔtRNA\textsubscript{Other} relative to wt grown with or without DIP. A comparison of \textit{pbp1a} transcript levels between wt cells grown with or without DIP shows no significant change, indicating that the deletion of tRNA\textsubscript{Other} is the major contributing factor to the decrease in \textit{pbp1a} transcript levels (Table 2.3).
Figure 2.7. Transcript profiles of wt and B. cereus ΔtRNA<sub>Other</sub> from cells collected during mid-exponential growth (A) and stationary phase (B). Transcript level data for A are presented as the average of two microarrays. Each dot represents the transcript level for one gene. The three green lines, from top left to bottom right, indicate 2-fold higher, equal, and 2-fold lower transcript levels for ΔtRNA<sub>Other</sub> relative to wt B. cereus. White dots represent transcripts with a significant 2-fold change or greater.
Figure 2.8. Transcript profiles of mid-exponential vs. stationary phase growth of wt (A) and *B. cereus* ΔtRNA<sup>Other</sup> (B) and *B. cereus* ΔtRNA<sup>Other</sup> grown to mid-exponential phase with or without erythromycin (C). Transcript profiles were determined by transcriptional microarray (Roche Nimbelgen). Data presented are from one experiment. Each dot represents the transcript level for one gene. White dots represent transcripts with a 2-fold change or greater. The three green lines, from top left to bottom right, indicate 2-fold higher, equal, and 2-fold lower transcript levels for ΔtRNA<sup>Other</sup> relative to wt *B. cereus*. 
Table 2.3. Changes in transcript levels between wt and *B. cereus* ΔtRNA*Other* under moderate and low iron conditions.

<table>
<thead>
<tr>
<th>Gene #</th>
<th>Gene Product</th>
<th>Microarray</th>
<th>qRT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(Δ/wt)*</td>
<td>(Δ/wt)</td>
</tr>
<tr>
<td>BC1188</td>
<td>Spx</td>
<td>- 4.2</td>
<td>2.0 ± 1.0</td>
</tr>
<tr>
<td>BC2773</td>
<td>CymR-homologue</td>
<td>- 2.6</td>
<td>4.7 ± 2.5</td>
</tr>
<tr>
<td>BC4901</td>
<td>Fur</td>
<td>- 2.2</td>
<td>2.5 ± 1.1</td>
</tr>
<tr>
<td>BC2632</td>
<td>AraC</td>
<td>- 2.6</td>
<td>-2.2 ± 0.2</td>
</tr>
<tr>
<td>BC5445</td>
<td>SOD [Mn]</td>
<td>- 2.7</td>
<td>-3.7 ± 3.1</td>
</tr>
<tr>
<td>BC5444</td>
<td>NOS</td>
<td>- 1.6</td>
<td>-5.1 ± 2.2</td>
</tr>
<tr>
<td>BC2137</td>
<td>DnrN</td>
<td>- 3.4</td>
<td>-4.9 ± 1.7</td>
</tr>
<tr>
<td>BC1550</td>
<td>PBP 1a</td>
<td>- 11</td>
<td>-36 ± 18</td>
</tr>
<tr>
<td>BC2458</td>
<td>Thioesterase ‡</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>BC1232</td>
<td>Anthranilate synthase ‡</td>
<td>1.0</td>
<td>-1.1</td>
</tr>
</tbody>
</table>

*Δ, *B. cereus ΔtRNA*Other*; wt, wild-type *B. cereus* ATCC14579
†DIP, 2,2-dipyridyl, iron chelator
‡Negative control transcript levels were determined with a standard curve

Figure 2.9. Changes in gene expression due to the deletion of tRNA*Other*. Expression of genes in *B. cereus* ΔtRNA*Other* relative to wt grown in rich media (light green) and iron depleted media (dark green) was determined by qRT-PCR. Data presented in log2.
Like *B. cereus* ATCC 14579, *Deinococcus radiodurans* ATCC 13939 encodes two TrpRSs within its genome (20, 21). The two TrpRSs have about 40% and 29% identity to typical TrpRSs. *B. cereus* TrpRS1 and TrpRS2 share only 35% amino acid sequence identity with each other. Sequence alignments with *B. subtilis* 168 TrpRS and predicted structure alignment with the *Geobacillus stearothermophilus* TrpRS crystal structure (pdb1MAU) suggest that TrpRS2 is the more canonical *Bacillus* TrpRS than is TrpRS1 (Fig. 2.9 A and B). The observation that the *trpS1* transcript is maintained at a higher level than the *trpS2* transcript during stationary phase suggests an additional role for TrpRS1.

The unusual TrpRS (TrpRS II) from *D. radiodurans*, with only 29 % identity to typical TrpRSs, was found to interact with nitric oxide synthase (NOS), increasing NOS activity and affinity for its substrate L-arginine (21). Transcript levels of *B. cereus nos* were found to be about 5-fold higher in the wt relative to the ΔtRNA*"*strain by qRT-PCR when cells were grown in LB to mid-exponential phase. To explore a possible relationship between NOS and TrpRS1 in *B. cereus*, qRT-PCR was performed to determine nos transcript levels over time and compare these with *trpS1* transcript levels (Fig. 2.10). The transcript profile of *B. cereus nos* closely follows that of *trpS1* rather than *trpS2*, suggesting that NOS and TrpRS1 may be co-regulated. Changes in nitric oxide levels in the ΔtRNA*"*strain compared with wt are described in Chapter 3.
Figure 2.10. Protein sequence and structural alignments of \textit{B. cereus} TrpRS1 and TrpRS2. A. Multiple sequence alignment was performed by CLUSTAL W (1.81) (http://align.genome.jp/) with TrpRS from \textit{B. subtilis} 168 representing the typical \textit{Bacillus} TrpRS sequence. Alignment scores were 31 for \textit{B. subtilis} TrpRS and \textit{B. cereus} TrpRS1, 30 for \textit{B. cereus} TrpRS1 and \textit{B. cereus} TrpRS2, and 74 for \textit{B. subtilis} TrpRS and \textit{B. cereus} TrpRS2. Alignment is marked to indicate identical amino acids (*) and amino acids with high (:), and low (.) similarity. Predicted structures for \textit{B. cereus} TrpRS1 (B) and TrpRS2 (C) were performed using SWISS MODEL and the TrpRS crystal structure (pdb1MAU) from \textit{G. stearothermophilus}, which is binding with ATP and tryptophanamide in a pre-transition state conformation. TrpRS1 and TrpRS2 residues are orange and yellow, respectively. pdb1MAU is green (B) and blue (C), and the ligands are red.
Figure 2.11. Transcript level profiles of nos and trpS1 display a similar pattern over time in wt B. cereus. Transcript levels for trpS1 (blue), trpS2 (red), and nos (grey) were determined by qRT-PCR. Error bars indicate standard deviations of nos qRT-PCR data. Standard deviations for trpS1 and trpS2 are shown in Fig. 2.6.

2.2.4. Prediction of mRNA targets for tRNA$^{\text{Other}}$

To explore the possibility that tRNA$^{\text{Other}}$ acts as a small regulatory RNA, the TargetRNA and sRNATarget software programs were used to predict mRNA targets for the putative sRNA$^{\text{Other}}$ (23, 147). Twelve of the predicted targets also showed significant differences of 2-fold or greater from the transcriptional microarray data comparing ΔtRNA$^{\text{Other}}$ and wt B. cereus in exponential growth (Table 2.4 and Fig. 2.11). Of these targets, the cymR-homologue transcript level reduction of 2.6-fold in the ΔtRNA$^{\text{Other}}$ strain relative to wt was confirmed by qRT-PCR for cells growing exponentially in LB containing DIP (Table 2.3).
### Table 2.4. Genes identified as potential mRNA targets of sRNA\textsuperscript{Other}.

<table>
<thead>
<tr>
<th>Gene*</th>
<th>Function</th>
<th>Fold Change</th>
<th>p-value\textsuperscript{†}</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC0244</td>
<td>OppD, Oligopeptide transport ATP-binding protein</td>
<td>2.0 down</td>
<td>0.0015</td>
</tr>
<tr>
<td>BC0458</td>
<td>Alkaline phosphatase like protein</td>
<td>2.0 up</td>
<td>0.021</td>
</tr>
<tr>
<td>BC1094</td>
<td>CysA, Sulfate transport ATP-binding protein</td>
<td>2.7 up</td>
<td>0.026</td>
</tr>
<tr>
<td>BC1471</td>
<td>Spore maturation protein B</td>
<td>3.0 up</td>
<td>0.026</td>
</tr>
<tr>
<td>BC1528</td>
<td>Hypothetical membrane spanning protein</td>
<td>2.2 down</td>
<td>0.027</td>
</tr>
<tr>
<td>BC1888</td>
<td>Phage protein</td>
<td>3.3 up</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BC2632</td>
<td>Transcriptional regulator, AraC family</td>
<td>2.6 down</td>
<td>0.0045</td>
</tr>
<tr>
<td>BC2773</td>
<td>CymR-homologue, RRF2 family protein</td>
<td>2.6 down</td>
<td>0.023</td>
</tr>
<tr>
<td>BC3020</td>
<td>Hypothetical protein</td>
<td>2.7 down</td>
<td>0.0097</td>
</tr>
<tr>
<td>BC3260</td>
<td>Hypothetical protein</td>
<td>2.3 down</td>
<td>0.035</td>
</tr>
<tr>
<td>BC4194</td>
<td>Hypothetical protein (first orf in spolIIA operon)</td>
<td>2.1 up</td>
<td>0.014</td>
</tr>
<tr>
<td>BC5321</td>
<td>Hypothetical membrane spanning protein</td>
<td>3.7 up</td>
<td>0.026</td>
</tr>
</tbody>
</table>

* Listed genes have significant transcript level changes between wt and B. cereus ΔtRNA\textsuperscript{Other} based on data from the transcriptional microarrays of cells collected during exponential growth.

\textsuperscript{†} p-value is from T-test of microarray data.

---

### sRNA\textsuperscript{Other}

<table>
<thead>
<tr>
<th>9  AAUUAAGGUAAA–CAG 26</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
</tbody>
</table>

### cymR-homologue mRNA

| 20 UUAAUUUUAUUUGUC |

Alignment Score: -60, Pvalue: 0.007

---

**Figure 2.12. Predicted interaction between sRNA\textsuperscript{Other} and its putative mRNA target, cymR-homologue.** Target and RNA interaction predictions are from TargetRNA (147). Program parameters were focused around the stop codon of the cymR-homologue. Vertical lines (|) indicate a Watson-Crick base pair, and dots (:|) indicate a G-U wobble base pair. The sRNATarget program also predicted the cymR-homologue as a potential target with a score of 1, indicating that all 1000 classifiers were used for this prediction (23).
2.2.5. Role of tRNA\textsuperscript{\textit{Other}} in cell wall morphology

Lys-tRNA\textsuperscript{Lys}-dependent modification of membrane lipids confers resistance to a wide range of antibiotics and antimicrobial peptides (42, 163). Previous studies showed low levels of Lys-tRNA\textsuperscript{\textit{Other}} synthesis \textit{in vitro} (126), prompting the investigation of tRNA\textsuperscript{\textit{Other}} involvement in membrane lipid modification with lysine. Studies performed by H. Roy showed no difference in total lipid synthesis or the degree of modification in ΔtRNA\textsuperscript{\textit{Other}} relative to wt \textit{B. cereus}, indicating that tRNA\textsuperscript{\textit{Other}} does not modulate antibiotic resistance via tRNA-dependent membrane remodeling (Fig. 2.12) (125, 126).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{lipid_analysis.png}
\caption{Lipid analysis of wt and \textit{B. cereus} ΔtRNA\textsuperscript{\textit{Other}}. Black lines represent growth curves for wt (A) and \textit{B. cereus} ΔtRNA\textsuperscript{\textit{Other}} (B). Shaded areas represent percent lipid content. PE, phosphoethanolamine; CL, cardiolipin; PG, phosphatidylglycerol; Lys-PG, lysyl-phosphatidylglycerol. Lipid analysis performed by H. Roy.}
\end{figure}
The greatest transcript level change due to deletion of tRNA$^{\text{Other}}$ was a 10-fold reduction of the \textit{pbp1a} transcript, encoding a transglycosylase-transpeptidase involved in cell wall biosynthesis. Although deletion of \textit{pbp1a} is tolerated in both \textit{B. subtilis} and \textit{E. coli}, changes in cell morphology have been observed (49, 114). To investigate changes in the cell wall upon deletion of tRNA$^{\text{Other}}$, resulting morphological differences in \textit{B. cereus} were examined by transmission electron microscopy. The wt cell wall was found to be ~30% thicker than that of \textit{\Delta tRNA^{Other} B. cereus} (Fig. 2.13), consistent with reports from other systems linking increased cell wall thickness to improved antibiotic resistance (30, 114).
Figure 2.14. Cell wall thickness of wt (A) and *B. cereus ΔtRNA<sub>Other</sub> (B). TEM of wt (A) and *B. cereus ΔtRNA<sub>Other</sub> (B) cell wall. C. Average cell wall thickness, WT: 60 ± 5 nm, ΔtRNA<sub>Other</sub>: 45 ± 4 nm.
2.3. Discussion

Although the original discovery of tRNA$^{\text{Other}}$ was through a study aimed at finding new tRNA substrates for the LysRS class I and class II complex (6), the studies presented in this chapter reveal that tRNA$^{\text{Other}}$ does not function within ribosomal mRNA translation and instead may serve a role as a regulatory small RNA. As described in Chapter 1, tRNAs have many functions outside of translation. Also, it has been shown that tRNAs or tRNA-like molecules have evolved to function only outside translation, and therefore have abandoned the canonical role within ribosomal protein synthesis (35, 48, 127, 162).

The first indication that tRNA$^{\text{Other}}$ was not utilized by the ribosome for protein synthesis was the low level of aminoacylation by the LysRS class I and II complex and complete absence of aminoacylation by either of the two *B. cereus* TrpRSs. The lack of an RT-PCR signal in polysomes for tRNA$^{\text{Other}}$ different from that for pre-tRNA$^{\text{Other}}$ indicates that tRNA$^{\text{Other}}$ is not present in actively translating ribosomes. Pre-tRNA$^{\text{Trp}}$ was not detected in any of the polysome fractions, which is consistent with the immediate processing of tRNAs following transcription. In addition, pre- tRNA$^{\text{Other}}$ should only be detected in polysomes if associated with a translating mRNA. This suggests that tRNA$^{\text{Other}}$ may be cotranscribed with the upstream protein-coding gene or that the RT-PCR was sensitive enough to detect background levels of the unprocessed tRNA$^{\text{Other}}$. It is also possible that tRNA$^{\text{Other}}$ may associate with the ribosome in a manner different from a canonical tRNA.

The Trp anticodon of tRNA$^{\text{Other}}$, presence of T-box elements in the 5’ leader region of both *B. cereus trpS* mRNAs, and differential expression of *trpSl* during
stationary phase growth between wt and ΔtRNA\textsuperscript{Other} strains suggested a possible role for tRNA\textsuperscript{Other} in the T-box transcription termination system of \textit{trpS1}. However, binding of tRNA to the T-box element requires base pairing of the tRNA anticodon to the T-box specifier loop and the last four nucleotides on the acceptor stem, including the discriminator base and CCA 3’-end, to the T-box antiterminator (55). The discriminator base is different between tRNA\textsuperscript{Other} (U) and tRNA\textsuperscript{Trp} (G) (Fig. 2.1), and the T-box element for \textit{trpS1} mRNA contains a U. The discriminator base of tRNA\textsuperscript{Trp} can bind to the T-box by forming a G-U wobble base pair, thus stabilizing the antiterminator. Although stable U-U base pairing has been demonstrated (9, 86, 158), proper binding to stabilize the \textit{trpS1} antiterminator favors tRNA\textsuperscript{Trp} over tRNA\textsuperscript{Other} based on discriminator base identity. Therefore, tRNA\textsuperscript{Other} may be involved in the regulation of \textit{trpS1} expression via a different mechanism.

The \textit{B. cereus} protein coding transcriptome was analyzed by transcriptional microarrays to determine if tRNA\textsuperscript{Other} -dependent regulation of gene expression is specific for \textit{trpS1}, or if the presence of tRNA\textsuperscript{Other} also affects transcript profiles of other genes. Because \textit{trpS1} expression was differentially regulated between wt and \textit{B. cereus} ΔtRNA\textsuperscript{Other} during stationary phase, it was surprising that few transcript level changes were observed between the two strains during stationary phase relative to exponential growth by microarray analysis (Fig. 2.7). However, cells were collected during early stationary phase rather than later in stationary phase to obtain RNA of a high enough quantity and quality for the transcriptional microarray. On the other hand, high quality RNA could be obtained in the lower quantities necessary for qRT-PCR during late stationary phase. Analysis of qRT-PCR data indicates that transcript level changes for
*trpS1* in early stationary phase, the time period for which RNA was purified for transcriptional microarray analysis, are about 5-fold lower than one to two hours later in stationary phase (Fig. 2.6). It is also interesting that the transcript profiles between wt and *B. cereus ΔtRNA<sub>Other</sub>* differ greatly during exponential growth, yet realign as the cells enter stationary phase (Fig. 2.8). Comparing the amount of scatter observed in the transcript level plots between exponential and stationary phases for both wt and *B. cereus ΔtRNA<sub>Other</sub>* it is evident that there are fewer differences between these transcript profiles for *B. cereus ΔtRNA<sub>Other</sub>* than for wt (Fig. 2.8). Taken together, these data imply that the levels of many transcripts in exponentially growing *B. cereus ΔtRNA<sub>Other</sub>* are similar to the levels found in this strain during stationary phase (Fig. 2.7 and 2.8).

Significant changes in the level of transcripts encoding the transcriptional regulators Fur, Spx, and CymR and their regulons are apparent between exponentially growing wt and *B. cereus ΔtRNA<sub>Other</sub>*. Fur is the ferric uptake regulator involved in the regulation of iron homeostasis (107). Spx is a regulator of thiol homeostasis, which is activated by the oxidation of a disulfide bond (25). CymR is a member of the Spx regulon and regulates cysteine metabolism (39). The common thread between these three transcriptional regulators is that they are all involved in the oxidative stress response. The ratios of *fur, spx,* and *cymR*-homologue mRNAs between wt and *B. cereus ΔtRNA<sub>Other</sub>* changed from positive to negative when cells were grown under high or low iron conditions (Table 2.3). Therefore, the two strains may experience a different level of oxidative stress or regulation of the oxidative stress response may be altered by the deletion of tRNA<sub>Other</sub>.
Other proteins involved in the oxidative stress response that have significant changes in their transcript levels between wt and \textit{B. cereus} ΔtRNA\textsuperscript{Other} include SOD [Fe/Mn], NOS, and DnrN. SOD destroys highly reactive superoxides, converting them to the less reactive species H\textsubscript{2}O\textsubscript{2} (37, 53). Bacterial NOS produces intracellular nitric oxide in quantities that protect the cell from oxidative stress (53, 54). DnrN is an NO-responsive protein thought to protect the cell from NO-induced damage by Fe-S cluster repair and/or reduction of intracellular NO concentration (73, 138, 142). Removing free ferrous iron from the cytoplasm by repairing Fe-S clusters will reduce oxidative stress by preventing the Fenton reaction, in which Fe\textsuperscript{2+} reacts with H\textsubscript{2}O\textsubscript{2} to produce hydroxyl radicals (15, 16, 171).

Transcript levels for \textit{pbp1a} reduced ~35-fold in ΔtRNA\textsuperscript{Other} relative to wt \textit{B. cereus} grown to mid-exponential phase (Table 2.3). This was, by far, the largest transcript level change detected between wt and \textit{B. cereus} ΔtRNA\textsuperscript{Other}, with the majority of significant changes ranging from 2- to 5-fold. The reduced \textit{pbp1a} transcript levels are accompanied by a decrease in cell wall thickness in ΔtRNA\textsuperscript{Other} relative to wt \textit{B. cereus} (Fig. 2.14). An increased cell wall thickness has been correlated with an increase in resistance to certain antibiotics such as those targeting cell wall biosynthesis (30). An increased cell wall thickness may also increase resistance to antibiotics with a variety of targets by reducing the rate of transfer of the antibiotic across the cell wall. In addition, studies have demonstrated a correlation between a perturbed cell wall or membrane and an induction of stress responses, including the oxidative stress response (82, 137, 145, 151). Therefore, the reduced expression of \textit{pbp1a} may be a major contributing factor to the loss of antibiotic resistance in \textit{B. cereus} ΔtRNA\textsuperscript{Other} relative to wt.
Taken together, the data presented in this chapter suggest that tRNA\textsuperscript{Other} does not function within mRNA translation and instead may play a role in the regulation of peptidoglycan synthesis and the oxidative stress response. Therefore, we propose that tRNA\textsuperscript{Other} be reclassified as a sRNA and renamed sRNA\textsuperscript{Other}. The role of sRNA\textsuperscript{Other} in the response to oxidative stress is further examined in Chapter 3.
Chapter 3

Role of sRNA^Other in oxidative stress

Recent studies have shown that bactericidal antibiotics ultimately kill bacteria by inducing intracellular oxidative stress (Fig. 3.1) (78, 79). It has been proposed that antibiotic inhibition of processes such as DNA replication, transcription, and ribosomal protein synthesis disrupts the intracellular redox balance by altering the NAD^+/NADH ratio (77). As a normal byproduct of aerobic respiration, superoxide (O_2^-) is produced when oxygen accepts electrons from the electron transport chain (Fig. 3.1) (37). Superoxide dismutase (SOD) reduces O_2^- to hydrogen peroxide (H_2O_2), which in turn is reduced to H_2O by catalases or peroxidases. An increase in the NAD^+/NADH ratio during cellular respiration leads to an accumulation of endogenous O_2^- that can destabilize iron-sulfur clusters by oxidation, and subsequently increase the intracellular ferrous iron (Fe^{2+}) concentration. H_2O_2 can then react with Fe^{2+} through the Fenton reaction to produce hydroxyl radicals (OH'), which are highly destructive to DNA, proteins, and lipids (Fig. 3.1) (37). In the Fenton reaction, the redox active Fe^{2+} is oxidized to Fe^{3+}, which can be reduced back to Fe^{2+} by cellular reductants, such as cysteine, to be reused in OH' production (Fig. 3.1) (37). No known enzyme can eradicate OH', thus the cell avoids OH' production by a variety of means including repair of iron-
sulfur clusters, decreased cellular reductant production, reduced intracellular Fe$^{2+}$ concentration, and increased SOD and catalase or peroxidase production (15, 37, 98).

Figure 3.1. Antibiotic-induced stress leads to the production and accumulation of reactive oxygen species. Figure is from Dwyer, et al. 2009 (37).

To counteract oxidative stress, many bacteria produce endogenous nitric oxide (NO) (53, 54). NO is commonly thought to be an oxidant involved in bacterial killing, for example during the oxidative burst produced by mammalian immune cells (54, 115). NO forms nitrosyl complexes with free or bound redox-active iron, preventing the Fenton reaction, and oxidizes cellular reductants, such as cysteine, that continually supply ferrous iron (54). NO can also act as an antioxidant by preferentially reacting with
organic radicals such as lipid peroxyl and alkoxyl radicals, inhibiting lipid peroxidation (74). Several Gram-positive bacteria generate NO via a nitric oxide synthase (NOS) (54). Bacterial and eukaryal NOSs produce NO via the same overall reaction, in which L-arginine is oxidized to L-citrulline and NO (28, 29, 143). However, unlike the eukaryal NOS, which has a reductase domain, the bacterial NOS utilizes cellular reductants such as cysteine (28, 29). In addition to protecting bacterial cells from oxidative stress, NOS activity facilitates *Streptomyces turgidiscabies* infection in plants (72) and is required for *Bacillus anthracis* virulence (134). As described in Chapter 2, the deletion of sRNA$^{\text{Other}}$ in *B. cereus* reduces the expression of many genes involved in oxidative stress response, including *sod* and *nos*. In this chapter, the role of sRNA$^{\text{Other}}$ in the oxidative stress response is further explored.

3.1. Materials and Methods

3.1.1. Bacterial strains and growth conditions

*B. cereus* wt strain ATCC 14579 and ΔtRNA$^{\text{Other}}$ were grown overnight in 5 ml Luria-Bertani broth (LB) at 37 °C with shacking at 250 rpm. The overnight cultures were used to inoculate 25 ml of pre-warmed LB and incubated for 1.5 h at 37 °C. To enrich for cells in exponential growth, these cultures were used to inoculate another 25 ml of pre-warmed LB to an OD$_{600}$ of 0.1. The cultures were incubated for an additional 1.5 h at 37 °C and subsequently used to inoculate 100 ml LB plus appropriate antibiotics and/or DIP to an OD$_{600}$ of 0.05 or 0.1. When appropriate, vancomycin (2 µg/ml), DIP (250 µM, Sigma), and/or FeSO$_4$ (250 µM) were added to growth media.
3.1.2. RNA extraction and purification

Total RNA was stabilized with RNAprotect (QIAGEN) and isolated with RNaeasy mini kit (QIAGEN) according to the manufacturer’s instructions except as noted. Briefly, 5 ml of cells from mid-exponential growth phase were pelleted at 5,000 x g, 4 °C, resuspended in 1 ml RNAProtect (Invitrogen), incubated at room temperature for 5 min, re-pelleted, and stored at -80 °C. Cells were suspended in lysis buffer (30 mM Tris-Cl, pH 8.0, 1 mM EDTA, 15 mg/ml lysozyme) with 30 mg acid washed beads and vortexed in a multivortexer for 10 min at room temperature. Buffer RLT (700 µl) was added and the cell suspension was vortexed for an additional 5 min before continuing with the manufacturer’s instructions. DNA digestion was performed with TURBO™ DNase (Ambion), followed by a control PCR to check for residual DNA contamination. RNA quality and concentration was determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc.).

3.1.3. RT-PCR

Reverse transcription (RT) was performed with 400 ng of RNA with 1 pmol 3’ primer (see Table 2.1, for bcother and fur primers) and SuperScript II reverse transcriptase (Invitrogen) according to manufacturer’s instructions, except annealing and reverse transcription temperatures were altered for sRNAOther, 80 °C for 3 min then 50 °C for 15 min, and 50 °C for 35 min then 42 °C for 20 min, respectively. PCR was performed with 300 nM of each primer and Taq DNA polymerase (Invitrogen) for 35 cycles of 95 °C for 45 sec, 55 °C for 45 sec, and 72 °C for 45 sec.

3.1.4. Intracellular nitric oxide detection
At the appropriate growth phase (mid-exponential or stationary phase), wt and *B. cereus ΔtRNA<sub>Other</sub>* cells were pelleted at 4,000 x g for 7 min at 4 °C. Cells were washed three times in Hank’s Buffer (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 1.3 mM CaCl<sub>2</sub>, 1.0 mM MgSO<sub>4</sub>, 4.2 mM NaHCO<sub>3</sub>, 1 g/L glucose, without phenol). Cells were resuspended in Hank’s Buffer at room temperature to obtain a final volume of 1 ml and an OD<sub>600</sub> = 8.0. The NO probe 1,2-diaminoanthraquinone (DAA) was added to the resuspended cultures (50 µg/ml) and incubated in the dark at 37 °C with gentle agitation for 1 hr. Cells were washed three times in Hank’s Buffer at room temperature and resuspended each time with gentle pipetting. Cells were resuspended in 1 ml Hank’s Buffer and stored briefly at room temperature in the dark until visualized via fluorescence microscopy with a motorized, inverted epi-fluorescence microscope (Axio Observer D1, Zeiss) using a 300 W Xenon Arc bulb (Lambda DG-4, Sutter Instrument Company) (56). Total cells were viewed by phase-contrast and fluorescence was viewed using a rhodamine filter with a 580 nm cutoff (Chroma Technology Corporation). Average individual cell fluorescence and background subtraction was determined using Metamorph software (Molecular Devices). As a control for autofluorescence, cells were prepared as described, except without the addition of DAA.

### 3.1.5. Biofilm formation assays

Wt and *B. cereus ΔtRNA<sub>Other</sub>* were assayed for biofilm formation on glass slides at the liquid-air interface (165). Strains were grown to stationary phase (~18 h) in 5 ml of LB at 37 °C with shaking, then diluted to an OD<sub>600</sub> of 0.1 in fresh LB containing 500 µM DIP. Aliquots of 20 ml of the diluted culture were added to 50 ml conical vials
containing sterile glass microscope slides. The tubes were placed at a 45° angle and incubated at 30 °C and 37 °C for 24 and 48 hr. After the desired incubation period, the slides were rinsed with 5-7 ml LB, stained with safranin (2.5 %) for 15 min, and rinsed with distilled water. The extent of biofilm formation was examined by phase contrast microscopy.

To quantify biofilm formation, wt and B. cereus ΔtRNA<sub>other</sub> were prepared in the same manner as described above. Once the cultures had been diluted to an OD<sub>600</sub> of 0.1, aliquots of 200 µl were placed in each of the 350 µl wells of a 96-well microtiter plate. After incubation at 37 °C for 24 and 48 hr, wells were rinsed 5 times with phosphate buffered saline (PBS), stained with 250 µl crystal violet (1 %) for 15 min, then rinsed another 5 times with PBS. The crystal violet retained by the biofilm was dissolved with 250 µl of 95 % ethanol, and absorbance was measured at 590 nm. Three replicates were performed.
3.2. Results

3.2.1. sRNA\textsuperscript{Other} expression is regulated by iron concentration

Deletion of sRNA\textsuperscript{Other} led to widespread changes in the expression of genes from several major stress-response regulons. The largest of these groups is that of the ferric uptake regulator (Fur), which includes genes encoding iron transport and iron-sulfur cluster binding proteins. By utilizing DBTBS, a database of transcriptional regulation in \textit{Bacillus subtilis}, binding sites for the ferric uptake regulator (Fur) were predicted upstream of the gene encoding sRNA\textsuperscript{Other} and overlapping a putative sigma B (\(\sigma^B\)) promoter (136). Possible regulation of sRNA\textsuperscript{Other} by Fur was further implicated by comparing levels of sRNA\textsuperscript{Other} grown in excess or depleted iron. PCR of cDNA serial dilutions was performed, to determine relative concentrations of sRNA\textsuperscript{Other}. Although quantitative RT-PCR is usually the preferred method for determining RNA quantity, a low PCR efficiency is predicted for highly structured templates, as is predicted for sRNA\textsuperscript{Other}. As a control, \textit{fur} transcript level changes were also monitored under high and low iron concentrations. As expected, \textit{fur} transcript levels increased 2-fold after 30 min of growth in LB with high relative to low iron concentrations (Fig. 3.2). sRNA\textsuperscript{Other} levels decreased 2-fold after 30 min of growth in LB with high to low iron concentrations, and a 4-fold decrease was observed after 120 min (Fig. 3.3). The slow increase of sRNA\textsuperscript{Other} levels over 120 min is in contrast to the fast induction of the well-known \textit{E. coli} iron-regulated sRNA RyhB, which reaches a maximum concentration after about ten min (97).
Figure 3.2. *fur* transcript level increases as extracellular iron concentration increases. PCR of 2-fold dilutions of *fur* cDNA from cells grown for 20 and 30 min in LB with low or high iron concentrations. Low iron concentration was achieved by addition of the iron chelator DIP (250 µM). High iron concentration was achieved by the addition of FeSO₄ (250 µM). Green and red asterisks indicate presence and absence of a PCR signal, respectively. DNA marker is HyperLadder V (Bioline). Arrow indicates RT-PCR product for *fur* transcript at 222 bp.
Figure 3.3. sRNA<sub>Other</sub> expression decreases as extracellular iron concentration increases. PCR of 2-fold dilutions of sRNA<sub>Other</sub> cDNA from cells grown for 30, 90, 120, and 180 min in LB with low (lanes to the left of the DNA marker) or high (lanes to the right of the DNA marker) iron concentrations. Negative controls are shown in A, lane 12, and D, lane 14. Positive controls are shown in B, lane 19, and D, lanes 1 and 8. Low iron concentration was achieved by addition of the iron chelator DIP (250 µM) to LB, and FeSO₄ (250 µM) was added to LB for high iron concentration. Green and red asterisks indicate presence and absence of a PCR signal, respectively. DNA marker is HyperLadder V (Bioline). Arrows indicate RT-PCR product for sRNA<sub>Other</sub> at 74 bp.
Figure 3.3

A. 30 min
![Image of gel with lanes 1 to 12, labeled as Low Fe$^{2+}$ and High Fe$^{2+}$]

B. 90 min
![Image of gel with lanes 1 to 19]

C. 120 min
![Image of gel with lanes 1 to 13]

D. 180 min
![Image of gel with lanes 1 to 14]
3.2.2. **Role of sRNA\(^{\text{Other}}\) in oxidative stress response and antibiotic resistance**

The difference in transcript level changes for *spx*, *cymR*-homologue, and *fur* between wt and *B. cereus ΔtRNA\(^{\text{Other}}\)* cells grown in high or low iron (Chapter 2), and the recent studies linking induced oxidative stress to bactericidal antibiotic activity (77, 78) prompted a reinvestigation of antibiotic resistance changes in media containing the iron chelator DIP. DIP reduces free ferrous iron levels, thereby reducing cellular oxidative stress. The growth rate of *B. cereus ΔtRNA\(^{\text{Other}}\)* increased with the addition of DIP to LB with either vancomycin or puromycin (Fig. 3.4 A and B). This is consistent with oxidative stress involvement in vancomycin and puromycin bactericidal activity. However, addition of DIP did not significantly affect the growth rate of wt *B. cereus* when exposed to vancomycin. Also, DIP addition lowered the growth rate of wt *B. cereus* exposed to puromycin (Fig 3.4), suggesting that oxidative stress is not the only factor affecting cell growth upon exposure to these antibiotics.

Neither wt nor *B. cereus ΔtRNA\(^{\text{Other}}\)* resistance to rifapentin, novobiocin, and nafcillin increased with DIP addition (Fig. 3.4 C, D, and E). For rifapentin, the growth rate for wt was unaffected by DIP addition, while the *B. cereus ΔtRNA\(^{\text{Other}}\)* growth rate was reduced when iron concentration was low (Fig. 3.4 C). For novobiocin and nafcillin, growth rates were reduced for both wt and *B. cereus ΔtRNA\(^{\text{Other}}\)* when grown under low iron conditions (Fig. 3.4 D and E).
Figure 3.4. Deletion of sRNA\textsuperscript{Other} alters antibiotic resistance in wt \textit{B. cereus} and \textit{B. cereus} ΔtRNA\textsuperscript{Other}. Wt (circles) and \textit{B. cereus} ΔtRNA\textsuperscript{Other} (triangles) strains were grown in LB containing vancomycin (2 µg/ml) (A), puromycin (15 µg/ml) (B), rifapentin (0.04 µg/ml) (C), novobiocin (0.4 µg/ml) (D), nafcillin (2 µg/ml) (E). Wt cells were grown in LB + DIP (●), LB + antibiotics (●), LB + antibiotics + DIP (○), and ΔtRNA\textsuperscript{Other} cells were grown in LB + DIP (▲), LB + antibiotics (▲), LB + antibiotics + DIP (▲) at 37 °C with shaking at 250 rpm. A and B are averages of three growth curves, and error bars represent standard deviations. C, D, and E are representative growth curves of at least three replicates.
Figure 3.4

A  Vancomycin

B  Puromycin

Continued
Figure 3.4 continued

C  Rifapentin

D  Novobiocin

E  Nafcillin
3.2.3. Intracellular NO production in wt and \textit{B. cereus Δ}tRNA\textsubscript{Other}

To confirm that the decrease in \textit{nos} transcript levels between wt and \textit{B. cereus Δ}tRNA\textsubscript{Other} leads to a decrease in NO production, endogenous NO levels were monitored by the addition of the NO probe 1,2-diaminoanthraquinone (DAA). DAA reacts with NO to form a fluorescent insoluble compound that cannot escape an intact cell, allowing for measurement of NO levels within the cell. As predicted, NO levels are decreased in \textit{Δ}tRNA\textsubscript{Other} relative to wt \textit{B. cereus} (Fig. 3.5). Surprisingly, NO levels did not significantly change between cells grown in the presence or absence of vancomycin (Fig. 3.5). NO levels in both strains grown in LB + vancomycin + DIP were significantly reduced compared with cells grown in LB with or without vancomycin, yet NO levels were still higher than \textit{B. cereus} autofluorescence (Fig. 3.5 and 3.6). L-arginine was added to cultures to serve as a positive control for NO production and DAA transport into the cells, and to ensure that fluorescence was not reaching full saturation (Fig. 3.6). No significant difference in NO production was detected between wt and \textit{B. cereus Δ}tRNA\textsubscript{Other} grown under microaerobic conditions, in the presence of L-arginine, or in cells grown to stationary phase (Fig. 3.6). While wt cells grown to mid-exponential phase had diffuse fluorescence, stationary phase cells from both strains had a punctuate appearance (Fig. 3.5 and 3.6). Though not as evident, bright spots can also be seen in \textit{Δ}tRNA\textsubscript{Other} cells grown to mid-exponential phase (Fig. 3.5 and 3.6).
Figure 3.5. Endogenous nitric oxide production is higher in wt than B. cereus ΔtRNA<sub>Other</sub> during mid-exponential growth. Wt and B. cereus ΔtRNA<sub>Other</sub> were grown in LB (A and D), LB + vancomycin (B and E), and LB + vancomycin + DIP (C and F, respectively). G. Quantitative comparison of NO levels in wt (blue) and ΔtRNA<sub>Other</sub> (green) cells. Average fluorescence is presented in arbitrary units (a.u.).
Figure 3.6. Nitric oxide levels in wt and *B. cereus* ΔtRNA<sup>Other</sup> grown under various conditions. Wt (A-D) and *B. cereus* ΔtRNA<sup>Other</sup> (E-F) were grown in LB to mid-exponential phase (A, B, D-F, and H) or stationary phase (C and G). DAA (50 μM) was added to each cell preparation except A and E, which served as negative controls for autofluorescence. All cells were grown under aerobic conditions except B and F, for which cells were grown under microaerobic conditions. L-arginine was added to growth media for cells in D and H to serve as positive controls for NO production.
3.2.4. Biofilm formation by wt and \textit{B. cereus ΔtRNA}\textit{\textsuperscript{Other}}

For both wt and \textit{B. cereus ΔtRNA}\textit{\textsuperscript{Other}}, biofilm formation at the air-liquid interface was visible after 24 and 48 h of incubation at either 30 or 37 °C in LB with DIP addition, but not in LB alone. When grown at 30 °C, biofilms of both strains had a similar appearance at both 24 and 48 h (Fig. 3.7 A-D). Wt and \textit{B. cereus ΔtRNA}\textit{\textsuperscript{Other}} biofilms were strikingly different after 24 h at 37 °C (Fig. 3.7 E and F). Wt biofilms were sparse and less dense than biofilms of \textit{B. cereus ΔtRNA}\textit{\textsuperscript{Other}}. However, after 48 hours, the wt biofilms appeared to spread laterally along the air-liquid interface and in some areas had a high cell density (Fig. 3.7 G). \textit{B. cereus ΔtRNA}\textit{\textsuperscript{Other}} biofilms after 48 h appeared more dispersed, but lacked the cell density observed at 24 h (Fig. 3.7 H).

To quantify biofilm formation, cultures were grown in 96-well plates. Biofilm formation on the walls of the plastic 96-well plate was greatly reduced relative to that on glass slides, which was expected given that \textit{B. cereus} develops biofilms differently on various surfaces (165). However, it was still surprising to have a similar level of biofilm formation with or without addition of the iron chelator DIP (Fig. 3.8), given that no visible biofilm was detected on glass slides without DIP addition. Biofilm formation by \textit{B. cereus ΔtRNA}\textit{\textsuperscript{Other}} was significantly lower than wt when grown in either medium (Fig. 3.8).
Figure 3.7. Biofilm formation in wt and ΔtRNA<sup>Other</sup> B. cereus. Wt (A, C, E, and F) and B. cereus ΔtRNA<sup>Other</sup> (B, D, F, and G) biofilms were formed on glass slides after incubation at 30 °C (A-D) or 37 °C (E-H). Incubation times were for 24 h (A, B, E, and F) and 48 h (C, D, G, and H). Slides were washed to remove free cells and remaining cells were stained with safranin prior to viewing under phase contrast microscopy at 400x magnification. The two pictures per strain and condition are representative of the observed biofilm structures.
3.3. Discussion

The regulation of iron homeostasis is crucial to the prevention of intracellular oxidative stress (133). As the main transcriptional regulator of iron homeostasis in *Bacillus* spp., the ferric uptake regulator (Fur) represses transcription of genes encoding iron uptake proteins, siderophore biosynthesis proteins, and sRNAs when iron levels are high (Fig. 3.9) (107). In iron depleted conditions, Fur no longer represses the transcription of these genes, thus enabling their expression. Fur detects Fe$^{2+}$ concentrations by direct binding, which causes a conformational change that allows Fur to bind, and usually repress, specific DNA sequences. In recent years, several Fur-regulated sRNAs have been discovered in a variety of bacteria (43, 98-100, 106, 166). In general, these sRNAs are expressed during iron starvation and target the mRNAs of iron-
sulfur (Fe-S) cluster binding proteins (99). By promoting degradation of these mRNAs, Fe-S cluster binding proteins are not replenished as the old proteins are degraded during protein turnover, thus increasing the concentration of available intracellular iron (99).

Figure 3.9. Schematic of iron-induced regulation via Fur. In conditions of excess iron, Fur binds Fe$^{2+}$ and undergoes a conformational change allowing it to bind DNA. Fur-Fe$^{2+}$ represses expression of proteins involved in siderophore biosynthesis, iron uptake proteins, and sRNAs. When intracellular iron concentration is low, Fur no longer represses the expression of these genes. The Fur-regulated sRNAs repress the expression of Fe-S cluster binding proteins, and along with siderophores and iron uptake proteins, ultimately increase the intracellular iron concentration.

Not only does the deletion of sRNA$^{\text{Other}}$ affect the transcript profiles of many genes within the Fur regulon (as discussed in Chapter 2), but also sRNA$^{\text{Other}}$ itself is regulated by iron concentration, possibly by Fur. Although sRNA$^{\text{Other}}$ has almost no sequence homology to any of the other known Fur-regulated sRNAs, little to no sequence homology or similarity in RNA length exists between most of these sRNAs (99). The time to reach full expression is also highly variable, ranging from minutes to hours (97, 98, 100). While some of these sRNAs require Hfq to promote interaction with their target mRNA, others can form stable base pairs without any additional protein factor (99). These sRNAs have also been shown to function in systems in addition to iron
homeostasis. For example, the Fur-regulated RNAs were found to affect acid resistance in *Shigella flexerni* (106) and motility, chemotaxis, and biofilm formation in *Vibrio cholerae* (101). In this chapter, sRNA$^\text{Other}$ is shown to increase resistance to certain bactericidal antibiotics by altering the oxidative stress response in *B. cereus*. By reducing iron concentration in *B. cereus* growth medium by the addition of an iron chelator, the production of OH$^-$ via the Fenton reaction was prevented. The bactericidal activities of vancomycin and puromycin on *B. cereus ΔtRNA$^\text{Other}$* were reduced by relieving oxidative stress (Fig. 3.4), confirming a role for sRNA$^\text{Other}$ in reducing intracellular oxidative stress. However, the specific mechanism by which the presence of sRNA$^\text{Other}$ combats oxidative stress remains unknown.

As mentioned above, the production of endogenous NO via the bacterial NOS has been shown to reduce intracellular oxidative stress (53). As shown in Chapter 2, sRNA$^\text{Other}$ derepresses the expression of TrpRS1, the atypical enzyme of the two *B. cereus* TrpRSs. The atypical *D. radiodurans* TrpRS interacts with and enhances the activity of NOS to produce NO, which can act as an antioxidant to reduce intracellular oxidative stress. The expression pattern for the nos transcript is similar to that of trpS1, but not trpS2. In this chapter, it was shown that NO concentrations were significantly higher in wt relative to the *B. cereus ΔtRNA$^\text{Other}$* strain. NO levels did not significantly change between cells grown in the presence or absence of vancomycin, indicating that NO production is not significantly induced by vancomycin exposure and that the pre-existing endogenous NO concentration is the major factor in combating oxidative stress. This is consistent with studies demonstrating that *B. subtilis* cells are protected from oxidative stress following a brief exposure to exogenous NO (53).
Many Fur-regulated sRNAs function to reduce iron-rich protein concentration by targeting their mRNAs, but some have also been shown to have a function in acid resistance, chemotaxis and biofilm formation (99, 101). Recently, a study on clinical uropathogenic *E. coli* strains showed that biofilm formation and persistence is increased when exogenous iron levels are low and biofilms disperse upon iron addition (121). Although biofilm formation by wt and *B. cereus ΔtRNA*<sup>Other</sup> in plastic 96-well plates was not affected by lowering the iron concentration in the growth medium, formation of biofilms on glass slides after a 24 h incubation required DIP addition to LB. Under conditions that allowed biofilm formation, the deletion of sRNA<sup>Other</sup> resulted in a significant decrease in biofilm presence after 48 h at 37 °C (Fig. 3.7). Comparison between biofilm formation on glass slides verses the plastic walls of a 96-well plate showed that *B. cereus ΔtRNA*<sup>Other</sup> biofilms appear less dense after 48 h than after 24 h of incubation. Cellular detachment from a biofilm has been observed and can occur due to many factors including changes in nutrient availability (128). These data indicate that the deletion of sRNA<sup>Other</sup> leads to a loss in biofilm persistence rather than a change in initial biofilm formation.

One of the predicted mRNA targets for sRNA<sup>Other</sup> is the cymR-homologue transcript (refer to 2.2.3). As a regulator of cysteine metabolism, CymR represses the expression of genes involved in cysteine biosynthesis (39). If this target prediction is correct, sRNA<sup>Other</sup> would function as a regulatory sRNA to repress cymR-homologue expression under low iron conditions, thereby derepressing cysteine biosynthesis (Fig. 3.10). Under high iron conditions, sRNA<sup>Other</sup> expression would be repressed by Fur, thus derepressing cymR-homologue expression. The CymR-homologue would then repress
cysteine biosynthesis, decreasing intracellular cysteine, which acts as a cellular reductant to convert Fe$^{3+}$ to the redox active Fe$^{2+}$ (Fig. 3.10). Taken together, these data suggest that sRNA$^{\text{Other}}$ is an iron-regulated small regulatory RNA involved in the oxidative stress response and biofilm formation. In an attempt to more accurately predict mRNA targets of sRNA$^{\text{Other}}$, we mapped the mature and processed forms of sRNA$^{\text{Other}}$ (presented in Chapter 4).

**Figure 3.10.** sRNA$^{\text{Other}}$ as a small regulatory RNA with the predicted cymR target. sRNA$^{\text{Other}}$ is predicted to interact with the cymR-homologue transcript and repress its expression under iron-deplete conditions. Under iron-replete conditions, expression of sRNA$^{\text{Other}}$ is repressed allowing derepression of the cymR-homologue. CymR inhibits the production of proteins involved in cysteine biosynthesis. Through this system, when intracellular iron concentration is high, cysteine levels are low. This is beneficial to the cell given that cysteine acts as a cellular reductant converting Fe$^{3+}$ to the redox active form, Fe$^{2+}$. 

$\text{H}_2\text{O}_2 \rightarrow \text{OH}^- + \text{OH}^-$

$\text{Fe}^{2+} \rightarrow \text{Fe}^{3+}$

sRNA$^{\text{Other}}$  Cysteine$_{\text{Ox}}$  Cysteine$_{\text{Red}}$

$cymR$  Cysteine biosynthesis
Chapter 4

Mapping \textit{sRNA}^{\text{Other}}

To complement \textit{B. cereus} \(\Delta\textit{sRNA}^{\text{Other}}\), the proposed 74 bases of the mature tRNA form (71 genomics encoded nucleotides with CCA added to its 3’ end) had been successfully cloned into the expression vectors, pHCMC04, pHCMC05, and pDG148 (data not shown). However, \textit{sRNA}^{\text{Other}} was not detected via RT-PCR when induced expression was attempted. The next step was to attempt to clone \textit{sRNA}^{\text{Other}} with its natural promoter, the identity of which was unknown. Promoter predictions via the database of transcriptional regulation in \textit{B. subtilis} (http://dbtbs.hgc.jp/) included a \(\sigma^B\) promoter overlapped by the second of three putative Fur binding sites (136), which is consistent with the iron-regulation of \textit{sRNA}^{\text{Other}} described in Chapter 3. The currently known iron-regulated sRNAs range in size from less than 100 nt to over 2000 nt (99). Since \textit{sRNA}^{\text{Other}} is expected to function as a regulatory sRNA rather than a canonical tRNA, mapping of both 5’- and 3’-ends of the RNA was warranted to determine the functional size of the sRNA.
4.1. Materials and Methods

4.1.1. Bacterial strains and growth conditions

*B. cereus* wt strain ATCC 14579 and ΔtRNA<sup>Other</sup> were grown overnight in 5 ml Luria-Bertani broth (LB) at 37 °C and shaken at 250 rpm. The overnight cultures were used to inoculate 25 ml of pre-warmed LB and incubated for 1.5 hr at 37 °C. To enrich for cells in exponential growth, these cultures were used to inoculate another 25 ml of pre-warmed LB to an OD<sub>600</sub> of 0.1. The cultures were incubated for an additional 1.5 h at 37 °C and subsequently used to inoculate 100 ml LB to an OD<sub>600</sub> of 0.05 or 0.1. When appropriate, DIP (250 μM, Sigma) was added to growth media.

4.1.2. RNA extraction and purification

Total RNA was extracted and purified using the QIAGEN RNeasy Mini Kit with a modified protocol. Pelleted *B. cereus* cells (5 ml of culture at an OD<sub>600</sub> ~0.5) were resuspended by vortexing in 100 μl lysis buffer (30 mM Tris-Cl, pH 8, 1 mM EDTA, 15 mg/ml Lysozyme). Acid-washed beads (30 mg, 150-600 μm diameter) were then added and vortexed at maximum speed in a multi-vortexer at RT for 10 min. Buffer RLT + β-mercaptoethanol (700 μl) was added and cells were vigorously vortexed for an additional 5 min at maximum speed in a multi-vortexer. Lysed cells were centrifuged for 2 min at maximum speed and the clear cell lysate (~760 μl) was transferred to new tube. 100 % ethanol (590 μl) was added to the clear cell lysate, mixed by pipetting, transferred to an RNeasy Mini spin column, and centrifuged 15 s at ≥8,000 x g. The spin column was washed with Buffer RW1 (700 μl) (centrifuged 15 s at ≥8,000 x g), then placed in a new collection tube and washed twice with 500 μl buffer RPE (centrifuge 15 s at ≥8,000 x g).
After the column was dried by centrifugation in a new collection tube at maximum speed for one min, the RNA was eluted twice with 30 µl RNase-free H₂O and centrifugation for 1 min at ≥8,000 x g.

Residual DNA was removed by TURBO™ DNase (Ambion) (3 U TURBO™ DNase, 2 U/µl). After a 30 min incubation at 37 °C, an additional 3 U of TURBO™ DNase was added and incubated for another 30 min at 37 °C. TURBO™ DNase was inactivated and removed by two subsequent phenol (pH 4.5) and chloroform washes and ethanol precipitation. RNA was resuspended in 50 µl RNase-free H₂O and RNA concentration and quality were determined using an ND-1000 spectrophotometer (Nanodrop Technologies, Inc.).

4.1.3. 5’ RACE.

Preliminary mapping of the 5’ end of sRNA<sup>Other</sup> was performed by using various sets of primers for RT-PCR, each set containing the same 3’ primer and a variable 5’ primer (Table 4.1). RT-PCR was performed as described in section 2.1.3, except the 5’ primer varied in each reaction. Successful RT-PCR yielded products of up to 574 bp and prompted a further investigation into the size of sRNA<sup>Other</sup> (Fig. 4.1.A).

Mapping of the 5’ end of sRNA<sup>Other</sup> was performed using the 3’/5’ RACE kit (Rapid Amplification of cDNA Ends, Roche). cDNA was synthesized from total RNA (isolated from cells grown with or without DIP) with primer BCother3 (5’-TGGAGTGGATGGCGGGAATT-3’, 0.625 µM) and Transcriptor Reverse Transcriptase in reaction buffer containing dNTP mix (1 mM each), 50 mM Tris-HCl, 8 mM MgCl₂, 30 mM KCl, 5 mM DTT, pH 8.5, and incubated at 55 °C for 60 min. cDNA purification
was performed with the High Pure PCR Purification kit (Roche), using an altered protocol specific for first strand cDNA (fs-cDNA). Briefly, 100 µl binding buffer was added to the 20 µl fs-cDNA, mixed, added to the filter tube and centrifuged for 30 s at 6,000 x g. Following two washing steps with 500 then 200 µl wash buffer, the fs-cDNA was eluted from the filter with 50 µl elution buffer. A poly (A) tail was added to the 5’ end of the fs-cDNA using recombinant Terminal Transferase (3.2 U, Roche) in buffer containing 0.2 mM dATP, 10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl, pH 8.3, and incubated for 30 min at 37 °C. The first PCR was performed using the Expand High FidelityPLUS PCR System (Roche) with a nested 3’ primer, BCother3 nest1 (5’-GAATTGAACCCACATCAGAGG-3’, 0.25 µM), and Oligo dT-anchor primer (5’-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTTTV-3’, where V = A, C, or G, 0.25 µM). The first PCR protocol was as follows: 94 °C for 2 min; 10 rounds of 94 °C for 15 sec, 58 °C for 30 sec, and 72 °C, 40 sec; 25 rounds of increasing the extension time (72 °C) 20 sec after each cycle; and 72 °C for 7 min. A second PCR protocol was performed using 1 µl of products of the first PCR as the template. All conditions were the same as for the first PCR, except the annealing temperature was raised from 58 to 60 °C, and the primers used were the 3’ nested primer BCother3 nest2 (5’-GTTCTGGAGGCCCCTGTTTT-3’) and the 5’ PCR anchor primer (5’-GACCACGCGTATCGATGTCGAC-3’) provided by Roche. PCR products were visualized after running an aliquot from each reaction on a 2% agarose gel. 5’ RACE was repeated as described above using RNA purified from a second set of cultures, except the total RNA was treated with tobacco acid phosphatase (TAP; EPICENTRE Biotechnologies; 50 mM sodium acetate, pH 6.0, 1 mM EDTA, 0.1% β-mercaptoethanol,
and 0.01% Triton® X-100) to convert any 5'-triphasphate ends to 5'-monophosphate ends. TAP was removed by two subsequent phenol (pH 4.5) and chloroform extractions and an ethanol precipitation prior to cDNA synthesis.

Aliquots of both the first and second PCRs were cloned with the Zero Blunt TOPO-cloning vector (Invitrogen). In addition, DNA bands visible by agarose gel electrophoresis (Fig. 4.1) were purified using the QIAGEN Gel Purification kit and subsequently cloned with the Zero Blunt TOPO-cloning vector. Plasmids of successful clones were purified using a QIAGEN Mini-plasmid purification kit and digested with EcoRI (100 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, 0.025 % Triton X-100, pH 7.5; NEB) to determine insert size. Twenty-nine plasmids with various insert sizes were sequenced by the Plant-Microbe Genomics Facility (PMGF) at The Ohio State University.

<table>
<thead>
<tr>
<th>3' Primer:</th>
<th>Sequence</th>
<th>Amplicon Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCother3</td>
<td>5' TGGAGTGGATGGCGGAATT 3'</td>
<td></td>
</tr>
<tr>
<td><strong>5' Primers:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCother5</td>
<td>5' GGGGGTATAATTTAAGGGTAAAAAC 3'</td>
<td>74 bp</td>
</tr>
<tr>
<td>Bc14mRNAOther</td>
<td>5' GTCTCTGTCATTCCGACCACGA 3'</td>
<td>95 bp</td>
</tr>
<tr>
<td>Pre BCOther 2</td>
<td>5' ACTTGCTAGATTGTGGATCTGC 3’</td>
<td>125 bp</td>
</tr>
<tr>
<td>Pre BCOther 3</td>
<td>5’ GCATGGCAAAAAAGACAAATCGAGT 3’</td>
<td>198 bp</td>
</tr>
<tr>
<td>Pre BCOther 4</td>
<td>5’ GGTITGAAGTATACACTCTTTGTG 3’</td>
<td>229 bp</td>
</tr>
<tr>
<td>Pre BCOther 5</td>
<td>5’ CGATAACAAGTAAAAACGTGTCG 3’</td>
<td>398 bp</td>
</tr>
<tr>
<td>Pre BCOther 6</td>
<td>5’ CTACATATGATACGATAAATAAATGGG 3’</td>
<td>427 bp</td>
</tr>
<tr>
<td>Pre BCOther 7</td>
<td>5’ TACACAACCATGTGATATTGATTC 3’</td>
<td>483 bp</td>
</tr>
<tr>
<td>Pre BCOther 8</td>
<td>5’ GGTACTGCAATCAGAAGAATTAA 3’</td>
<td>574 bp</td>
</tr>
<tr>
<td>Pre BCOther 9</td>
<td>5’ GTAAGAGAAATTACATGAGAAG 3’</td>
<td>1004 bp</td>
</tr>
<tr>
<td>Pre BCOther 10</td>
<td>5’ CACGCTGTGGAAAATACCAAGAA 3’</td>
<td>1686 bp</td>
</tr>
<tr>
<td>Pre BCOther 11</td>
<td>5’ CTGATGATATTACTGAGGGAA 3’</td>
<td>1842 bp</td>
</tr>
<tr>
<td>Pre BCOther 12</td>
<td>5’ GATCACATGAGAAATAGTAGAC 3’</td>
<td>2111 bp</td>
</tr>
</tbody>
</table>

Table 4.1. Primers used in initial 5' mapping of sRNA<sup>Other</sup>.
4.1.4. 3’ RACE.

Mapping of the sRNA

3’ end was performed using the 3’/5’ RACE kit (Rapid

Amplification of cDNA Ends, Roche). Total RNA (isolated from cells grown with or

without DIP) was treated with E. coli poly (A) polymerase (NEB) to produce a poly (A)
tail at the 3’-ends (4 µg RNA, 50 mM Tris-HCl, 250 mM NaCl, 10 mM MgCl₂, 1 mM

ATP, pH 7.9, incubated for 10 min at 37 °C). Poly (A) polymerase was removed by two

subsequent phenol (pH 4.5) and chloroform extractions followed by an ethanol

precipitation. cDNA was synthesized from 3’-poly (A) tailed total RNA (2 µg) with

Oligo dT-anchor primer (5’-

GACCACGCGTATCGATGTCGAC

TTTTTTTTTTTTTTTTTV-3’, where V = A, C, or G,

0.625 µM) and Transcriptor Reverse Transcriptase in reaction buffer containing dNTP

mix (1 mM each), 50 mM Tris-HCl, 8 mM MgCl₂, 30 mM KCl, 5 mM DTT, pH 8.5, and

incubated at 55 °C for 60 min. The first PCR was performed using the Expand High

FidelityPLUS PCR System (Roche) with BCother5 nest1 primer (5’-

CCTCTGATGTGGGTTCAATTC

-3’, 0.25 µM) and PCR anchor primer (5’-

GACCACGCGTATCGATGTCGAC-3’, 0.25 µM). The first PCR protocol was as

follows: 94 °C for 2 min; 10 rounds of 94 °C for 15 sec, 60 °C for 30 sec, and 72 °C, 40

sec; 25 rounds of increasing the extension time (72 °C) 20 sec after each cycle; and 72 °C

for 7 min. Because only a light smear of DNA was visible by agarose gel electrophoresis

of the first PCR products, a second PCR protocol was performed using 1 µl of the first

PCR as the template. All conditions were the same as for the first PCR, except the

annealing temperature was raised from 60 to 65 °C, and the 5’ primer used was BCother5
nest3 (5’-CAATTCCGCCATCCACTTATACTTG-3’). 3’ RACE was repeated as described above using RNA extracted from a second set of cultures.

Aliquots of the second PCRs were cloned with the Zero Blunt TOPO-cloning vector (Invitrogen). Sections of the DNA smear visible by agarose gel electrophoresis (Fig. 4.2), were purified using the QIAGEN Gel Purification kit and subsequently cloned with the Zero Blunt TOPO-cloning vector. Sixty-six plasmids of successful clones were purified using a QIAGEN Mini-plasmid purification kit and digested with EcoRI (100 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl$_2$, 0.025 % Triton X-100, pH 7.5; NEB) to determine insert size. Forty-five plasmids with various insert sizes were sequenced by the Plant-Microbe Genomics Facility (PMGF) at The Ohio State University.

4.2. Results

4.2.1. 5’ end of sRNA$^\text{Other}$

An initial set of RT-PCR reactions used the same reverse primer and various forward primers (see Table 4.1) in an exploration of possible 5’ extensions for sRNA$^\text{Other}$. RT-PCR products were formed for many primer sets, with the largest PCR product consisting of 574 bp (Fig. 4.1.A.). No RT-PCR product was formed using primers BCOther3 with Pre BCOther 9 through BCOther 12, corresponding to genomic DNA PCR products of 1004 bp to 2111 bp. This suggested that the full, unprocessed mRNA form of sRNA$^\text{Other}$ was at least 574 bp and no more than 1003 bp longer on its 5’-end.

5’-rapid amplification of cDNA ends (5’-RACE) was performed to further investigate the full length sRNA$^\text{Other}$, and to detect possible processing sites. No products
were visible from the initial PCR other than primer dimers (at about 65 bp), suggesting a low abundance of sRNA<sup>Other</sup> in the total RNA fraction extracted from <i>B. cereus</i> (Fig. 4.1.B and C). After a second round of PCR with nested primers, bands were clearly visible at about 675, 490, and 105 bp. Interestingly, the ~675 and 490 bp bands are not detectable in the total RNA samples from cells grown in LB plus the iron chelator DIP.

Sequencing of TOPO-cloned 5’-RACE products confirmed the presence of the three main 5’-RACE products visualized by agarose gel electrophoresis (Fig. 4.3). The longest cDNA fragment, beginning at 565 or 566 nt upstream of the original sRNA<sup>Other</sup> 5’-end, corresponds to a site eight or nine nucleotides downstream of a predicted σ<sup>A</sup> promoter (Fig.s 4.3 and 4.4.A and D) (136). The exact transcriptional start site of this sequence cannot be determined by the 5’-RACE method used because the 5’-RACE product was produced using terminal transferase, which adds adenosines to the 3’-end cDNA (Fig. 4.4.D). Therefore, the transcriptional start site cannot be resolved between a sequence that ends at or just before a thymine. It is unusual for transcription to begin at a pyrimidine, yet the potential start sites are at either a thymine or cytosine. A second sequence begins 349 nt upstream of the original sRNA<sup>Other</sup> 5’-end and corresponds to a site 16 nt downstream of a predicted σ<sup>B</sup> promoter (Fig.s 4.3 and 4.4b-d) (136). A distance of 16 nt between the the -10 region of a σ<sup>B</sup> promoter sequence and a transcription start site is abnormal (154), and it is suspected that this is not the true start site (Fig. 4.4.D). A third 5’-RACE product appearing in many of the TOPO-cloned sequences was 17 nt shorter than the original sRNA<sup>Other</sup> size prediction (Fig. 4.3.A and B). No promoter sequence was identified upstream of this sequence, indicating that the 5’-end may have been formed by RNA processing.
Figure 4.1. Mapping the 5’ end of sRNA\textsuperscript{Other}. A. Initial mapping of the 5’ end of sRNA\textsuperscript{Other} by RT-PCR using various 5’ primers. Lanes 2-10: Amplicon sizes 74, 95, 125, 198, 229, 398, 427, 483, 574 bp. Lanes 1 and 11, DNA Marker. B. 5’ RACE of sRNA\textsuperscript{Other} from cells grown in LB (lanes 2 and 4) and LB + DIP (lanes 3 and 5). Lanes 2 and 3, first PCR. Lanes 4 and 5, second PCR. Lanes 1 and 6, DNA markers. C. 5’ RACE with TAP-treated RNA from cells grown in LB (lanes 1 and 4) and LB + DIP (lanes 2 and 3). Lanes 5 and 6, DNA markers. DNA bands marked with an arrow at about 65 bp were primer dimers.
4.2.2. 3’ end of sRNA\textsuperscript{Other}

3’-RACE products were not as distinct at those for 5’-RACE. A smear of DNA product could be seen after the first PCR, ranging from approximately 125 to 600 bp (Fig. 4.3.A and B). The second PCR, using nested primers, showed more defined DNA bands, yet still within a large range of sizes. The largest bands at approximately 1.75 and 3.0 kb were TOPO-cloned and sequenced to reveal 23S and 16S rRNA sequences rather than sequences specific to sRNA\textsuperscript{Other} (Fig. 4.3.B). Of the sequences that did correspond to the 3’-end of sRNA\textsuperscript{Other}, only a few had sequences with similar 3’-ends. The majority of these sequences end within a predicted intrinsic transcriptional terminator that ends at 523 nt downstream of the original sRNA\textsuperscript{Other} sequence (4.2). Other sequences ended approximately 340 and 210 nt downstream of the original sRNA\textsuperscript{Other} sequence, but are variable in their exact 3’-end site and do not have any predicted transcriptional terminators. These sequences are predicted to be the result of non-specific RNA degradation at the 3’-end.

4.2.3. Prediction of mRNA targets for sRNA\textsuperscript{Other}

Although results for sRNA\textsuperscript{Other} target predictions were presented in Chapter 2, the predictions were made with the original predicted size for sRNA\textsuperscript{Other}, which was only 74 nt. TargetRNA and sRNA\textsuperscript{Target} software programs were used to predict mRNA targets for the various forms of sRNA\textsuperscript{Other} predicted by the 5’- and 3’-RACE results (Fig. 4.3) (23, 147). The most consistent target predictions were made using the form of sRNA\textsuperscript{Other} with a 5’-end 17 nt shorter and a 3’-end 531 nt longer than the original 74 nt tRNA\textsuperscript{Other} sequence (Fig. 4.3). This sequence corresponds to the predicted 5’ RNA processing site
and the transcriptional terminator. In addition to retaining the target prediction of the cymR-homologue mRNA, both programs predicted nos and sod mRNAs as targets (Fig. 4.6). The predicted site of interaction between sRNA\textsuperscript{Other} and sod mRNA is focused around the ribosome binding site and translational start site, which is a common binding site for sRNAs to block ribosome recruitment (Fig. 4.5. C and D) (13).

Figure 4.2. Mapping the 3’ end of sRNA\textsuperscript{Other}. A. First 3’ RACE of sRNA\textsuperscript{Other} from cells grown in LB (lanes 1 and 5) and LB + DIP (lanes 2 and 6). Lanes 1 and 2, first PCR. Lanes 5 and 6, second PCR. Lanes 3 and 4, DNA markers. B. Second 3’ RACE of sRNA\textsuperscript{Other} from cells grown in LB (lanes 2 and 4) and LB + DIP (lanes 3 and 5). Lanes 2 and 3, first PCR. Lanes 4 and 5, second PCR. Lanes 1 and 6, DNA markers.
Figure 4.3. Predicted promoters and Fur binding sites in the upstream region of the gene encoding sRNA\textsuperscript{Other} (A), and predicted 5’- and 3’-ends of sRNA\textsuperscript{Other} (B). A. Promoter and Fur binding site predictions were performed using the DBTBS database (136). B. 5’- and 3’- were determined by 5’- and 3’-RACE, respectively. Circle with a vertical line at the bottom indicates an intrinsic terminator. Numbers indicate upstream (on left) and downstream (on right) relative to the originally predicted 71 nt of sRNA\textsuperscript{Other} i. 565 nt upstream; ii. 349 nt upstream; iii. 17 nt shorter than sRNA\textsuperscript{Other}; iv. 531 nt downstream; v. approximately 340 nt downstream; and vi. approximately 210 nt downstream.
A. $\sigma^A$ promoter consensus sequence (B. subtilis)

B. $\sigma^B$ promoter consensus sequence (B. subtilis)

C. $\sigma^B$ promoter consensus sequence (B. cereus)

D. Predicted promoter sequences for sRNA$^{other}$

$\sigma^A$:  
\[
\begin{array}{c}
\text{-35} \\
\text{-10} \\
\uparrow +1
\end{array}
\]

$\sigma^B$:  
\[
\begin{array}{c}
\text{-35} \\
\text{-10} \\
\uparrow +1
\end{array}
\]

Figure 4.4. Promoter predictions for $\sigma^A$ and $\sigma^B$. Weblogos A and B are from DBTBS (136), and weblogo C is from van Schaik, 2007 (154). Larger letters indicate higher occurrence in the consensus sequence. D. Predicted $\sigma^A$ and $\sigma^B$ promoters for sRNA$^{other}$. The -10 and -35 regions and +1 indicates the transcription start site, determined by 5’-RACE, are in bold lettering and underlined. The arrows pointing to the red, bold “A”s indicate the expected transcriptional start sites.
A. Alignment focus on stop codon of nos mRNA; Score based on P-value ≤0.05

sRNA\textsuperscript{Other} 241 AAAAAACUA----UAAAUUGUUUGUGUAGAUGUGUG 277
nos mRNA (reverse) 18 UUUUGGAUGGUAAUUUA--AUACUU--UCUUUAAC -20

Alignment Score: -76, P-value: 0.006

B. Alignment focus on stop codon of nos mRNA; Thermodynamic score

sRNA\textsuperscript{Other} 241 AAAAAAC--UAUA----UAAAUUGUUUGUGUAGAUGUGUGUGUAGUAAA 287
nos mRNA (reverse) 19 UUUUGGAUGGUAAUUUA--AUACUU--UCUUUAAC--CGAAGUUUU -29

Thermodynamic Alignment Score: -25

C. Alignment focus on start codon of sod mRNA; Score based on P-value ≤0.05

sRNA\textsuperscript{Other} 110 ACAUAAUUUAUUGAUUUUC--AGUUAAAGUG 140
sod mRNA (reverse) 4 UGUAUUGUAAUGGAGAAGAUAUUUUCGA -28

Alignment Score: -70, P-value: 0.015

D. Alignment focus on start codon of sod mRNA; Thermodynamic score

sRNA\textsuperscript{Other} 292 UGAGUUGAAAGUACCGACACUUUAAUUUUGUCUAAGAAGA 341
sod mRNA (reverse) 14 ACUUUACU--C-UUGU--UUG--AAU--GGAGGAAAGAUAUU--UUC -27

Thermodynamic Alignment Score: -28

Figure 4.5. Predicted interaction between sRNA\textsuperscript{Other} and two putative mRNA targets, nos and sod. Target and RNA interaction predictions are from TargetsRNA (147). Target prediction parameters for nos (A and B) and sod (C and D) included terminator removal, a hybridization seed of 8, G:U basepairs included, and alignment score determined with a P-value set to 0.05 (A and C) or thermodynamic energy (B and D). Vertical lines (|) indicate a Watson-Crick base pair, and dots (:)) indicate a G-U wobble base pair. The numbered nucleotide positions are relative to the start and stop codons for sod and nos mRNAs, respectively. The sRNATarget program also predicted sod mRNA as a potential target with a score of 0.896, indicating that 896 of 1000 classifiers were used for this prediction (23). The ribosome binding site and start codon are underlined in C and D (note that sod sequence is written 3’ to 5’).
Figure 4.6. Predicted interaction between sRNA\textsuperscript{Other} and the cymR mRNA targets. Target and RNA interaction predictions are from TargetRNA (147). Target prediction parameters for cymR included terminator removal, a hybridization seed of 8, and G:U basepairs included. Alignment was focused on the start codon (A), the stop codon (B), or the coding sequence (C and D). Alignment score was determined with a P-value set to 0.01 (C) or thermodynamic energy (A, B, and D). Vertical lines (|) indicate a Watson-Crick base pair, and dots (:) indicate a G-U wobble base pair. The numbered nucleotide positions are relative to the start and stop codons for sod and nos mRNAs, respectively. The ribosome binding site and start codon are underlined in A (note that cymR-homologue sequence is written 3’ to 5’).
Figure 4.6

A. Alignment focus on start codon; Thermodynamic score

| sRNA Other | 421 | AGAOUUUAAGUGAAACCUA--AUUGCUACACGACU--UUUGUA  | 460 |
| cymR mRNA | 9  | UUAAGGUAACGUUCGGGAAUAC--A---UCUGAAAGUACAU | -29 |

Thermodynamic Alignment Score: -29

B. Alignment focus on stop codon; Thermodynamic score

| sRNA Other | 473 | AAAGUGAAAA--UAGAGAAUAGAAUUCAUU | 499 |
| cymR mRNA | 14 | UUUUAAUUCGCUA--AUUUUAAGGUGA | -13 |

Thermodynamic Alignment Score: -21

C. Alignment focus on coding sequence; Score based on P-value ≤0.01

| sRNA Other | 105 | UAUCUACAAAUUU--AUUGUA--UUU--UCAGUAAUAAGAUAUUUGGCAAUUAACG | 158 |
| cymR mRNA | 315 | AGGAA--GUAAGUUAUGGUGCAUUAACGAAUCUUGUGAAGA--GUU----UGU-U | 265 |
| sRNA Other | 159 | UUAUGUUAACCGUUAAUACUAAAAGUUGA--UGCAAAAUAC-----AU--AAAUUAUUGGUA | 211 |
| cymR mRNA | 264 | ACUCAAAAA--AAGGAA--G--UUAAGCUGUAAUUAAAGAAGUAAAA--A | 215 |
| sRNA Other | 212 | AUUUUAAUUGUUUA | 225 |
| cymR mRNA | 214 | GGGGUAAGAAGCAGU | 201 |

Alignment Score: -89, P-value: 0.008

D. Alignment focus on coding sequence; Thermodynamic score

| sRNA Other | 100 | UGGGCUUUCAAUAAAUUAUGCAUUUAAGUUGAAGUUGCAUUAACUGAAGUACCAAAUAA--AGAC | 154 |
| cymR mRNA | 401 | AUUUUAAUUGGUG--A---AAAAGA--AAAAG--GUC--UGA-AUGCUAAAAACUAAA | 354 |
| sRNA Other | 155 | AGGGAU--GUUGAUGUAAGAUAUUUGCAUAAUUGGCAUAAUAAGUUGAAGU--AU | 212 |
| cymR mRNA | 353 | AGCAGUGAAGGAA--GUUAAA--GAAGAAGCGGAA--C--GAU---AGAAGAAGAUAU | 304 |
| sRNA Other | 213 | UA--UU-UC-------UGUAUAAAAUGCUAA--AACUAIUAAUUAUUGAWUUG | 264 |
| cymR mRNA | 303 | AUUGUGUAGUUGCAAAACAGAUA----CAUUGU--UGUAAGUGUUG----UUACUACAAAAUGG | 253 |
| sRNA Other | 265 | UUAUGGAGGUGGUUGAUAUAAAGUGUACAUUAAGGUGCAUACACUAAUUAAUUGCC | 323 |
| cymR mRNA | 252 | AGGUAAGCUAACAGUAGUAGUAAUGGUAAAAAGUUGAAGAAGGUGACACACUUAACUAAUAAC | 212 |
| sRNA Other | 324 | UUUUUG--GUGAAUGAAGAAGACACUAAUAGGCGUUAAACGUACCUUAAA | 382 |
| cymR mRNA | 211 | UUAAGAAGAUAUGUGCUDA---UGGGGAG---AA--GUGGG-----UC----U--AC---- | 173 |
| sRNA Other | 383 | AAAGAAUGGCGU--UCUUCUCCAACACAAUUGAGUACAUAAU--A---AGAAGAAGGCU | 437 |
| cymR mRNA | 172 | UAC--UUCAUAUGGACAGAAACGCA--GUUGAAACAUAAUACAAGGUCUGAAG---CA-A | 117 |
| sRNA Other | 438 | AAGUGUUAACA--------CACA--CU-------UGUAACAU---AAIUCACUA-A--GGGAAGA | 480 |
| cymR mRNA | 116 | CC--ACUUGUGUAACAGGACAGGAACUAC--GUGUGAAGAUGUG--AAUAAACACAC-- | 62 |
| sRNA Other | 481 | UAG--AGA--UAAAAGU--CAUACACAUUUAAAGACUAA--CAGCU | 520 |
| cymR mRNA | 61 | ACGGAACCGGCUUUUUUCGUAAACAUACUCUCUCUGAUAUUAAACACG | 15 |

Thermodynamic Alignment Score: -110
4.3. Discussion

The 5'-RACE products that are 565 and 349 nt longer at the 5' end of the original predicted size for sRNA\textsuperscript{Other} correspond to sites downstream of the predicted σ\textsuperscript{A} and σ\textsuperscript{B} promoters, respectively. This suggests that sRNA\textsuperscript{Other} expression is regulated by two different σ transcription factors. The Fur binding sites present on and around the σ\textsuperscript{B} promoter are commonly observed for Fur regulated genes (107), thus supporting regulation of sRNA\textsuperscript{Other} by Fur. The sequence indicating an RNA form that is 17 nt smaller than the original sRNA\textsuperscript{Other} suggests that this is a possible site of RNA processing. The predicted intrinsic transcriptional terminator at the 3'-end of the most consistent 3'-RACE product suggests that this is the true end of the sRNA\textsuperscript{Other} transcript. The other sites may be specific processing sites, but the randomness in these sequences suggests that they are processed by non-specific RNases. Interestingly, none of the sequenced 5'- and 3' RACE products indicated processing at the 5'- and 3'-ends of a typical tRNA. These data support the notion that sRNA\textsuperscript{Other} does not function as a tRNA.

To confirm the full size and any processed variants of sRNA\textsuperscript{Other}, Northern blot assays will be performed. Once the various forms of sRNA\textsuperscript{Other} are discerned, they can be used to complement \textit{B. cereus ΔtRNA\textsuperscript{Other}} and confirm sRNA\textsuperscript{Other} -specific phenotypes. From the 5'-RACE data presented in this chapter and data from Chapter 3 on iron regulation of sRNA\textsuperscript{Other}, it is anticipated that various forms of sRNA\textsuperscript{Other} are expressed under different growth conditions, specifically varying iron concentration. Collectively, these data support the function of sRNA\textsuperscript{Other} as a small regulatory RNA.
Chapter 5

Conclusions and Perspectives

5.1. Conclusions

Since their discovery over 50 years ago, tRNAs have been known to play an essential role in the translation of the genetic code. Recently, studies have shown that tRNAs have additional functions outside mRNA translation (11). These functions vary greatly from the biosynthesis of amino acids such as selenocysteine (12) to the regulation of global transcript profiles as in the stringent response (164). A new function for tRNAs as antisense small RNAs to regulate gene expression, whether as whole or cleaved tRNAs, has been predicted recently (34, 57, 80, 90, 91). One of the latest studies demonstrated that the tRNA-like RNA, tmRNA, can act as an antisense sRNA as well as function in the rescue of stalled ribosomes (92). In this study, Staphylococcus aureus tmRNA was found to have sequence complementarity and bind to the 5’-untranslated region of crtMN mRNA (92). It also has been suggested that sRNAs are derived from ancestral tRNAs (109). tRNA gene sequences are common recombination sites for virus DNA and often disrupt the tRNA sequence, forming a pseudo-tRNA gene (61, 118). These sequences may be eliminated through genome reduction. However, the pseudo-tRNA sequence instead may acquire a new function, such as a regulatory function, which allows the gene to persist within the genome. Pseudo-tRNA genes are found in virtually every sequenced
genome, yet very rarely are their gene products studied in order to identify their functions (80). The data presented within this document suggest that a predicted pseudo-tRNA from *B. cereus* has evolved a regulatory function acting as a small RNA with limited complementarity to its target(s), and is thus renamed sRNA$^{\text{Other}}$.

sRNA$^{\text{Other}}$ is one of many recently discovered iron-regulated small RNAs, which are usually found to be regulated by the transcriptional regulator, Fur. Under high levels of ferrous iron (Fe$^{2+}$), Fur binds excess iron and is activated for DNA binding. Fur usually acts as a repressor to reduce transcription of genes, such as sRNA$^{\text{Other}}$ described here. sRNA$^{\text{Other}}$ is expressed under low iron conditions and subsequently increases the mRNA levels of *pbp1a*, *trpRS1*, *nos*, *cymR*, *spx*, and *sod* by an undetermined mechanism. An increase in *pbp1a* expression is predicted to increase cell wall thickness, which can reduce susceptibility to certain antibiotics (Fig. 5.1). Deletion of *pbp1a* from the *B. cereus* genome is underway and comparative growth curves with wt *B. cereus* in the presence and absence of antibiotics will test this hypothesis.

The deletion of sRNA$^{\text{Other}}$ affects the oxidative stress response in *B. cereus*. There are many paths sRNA$^{\text{Other}}$ can take to alter the transcript profiles of genes involved in oxidative stress (Fig. 5.1). For example, the derepression of *trpS1* is expected to increase TrpRS1 levels. As with the unusual TrpRS from *D. radiodurans*, TrpRS1 may interact with and induce NO production by NOS. An increase in *nos* mRNA levels would be expected to increase NOS levels and subsequently increase production of NO. Transcripts of *nos* are predicted targets of sRNA$^{\text{Other}}$. sRNAs are most commonly known to reduce transcript levels by promoting RNA degradation or affect translation by blocking or revealing the ribosome binding site (141). However, sRNA regulators can
also stabilize mRNAs by decreasing accessibility of the mRNAs to RNases (141). In this manner, nos mRNA may be stabilized by sRNA<sup>Other</sup> binding.

Additionally, sod mRNA has been predicted as a target for sRNA<sup>Other</sup> regulatory RNA function by occluding the ribosome binding site. When ribosome binding is blocked, the mRNA is more susceptible to degradation by RNases, which will lead to a decrease in transcript level. This SOD is predicted to bind iron for its activity, and may be targeted by sRNA<sup>Other</sup> to reduce SOD production, thereby increasing intracellular iron levels. However, this prediction contrasts the qRT-PCR data, which indicates a reduction in sod transcript levels when sRNA<sup>Other</sup> is deleted from the <i>B. cereus</i> genome.

The <i>cymR</i>-homologue mRNA is predicted as a regulatory target for sRNA<sup>Other</sup>. Complementarity was predicted between sRNA<sup>Other</sup> and the start codon, stop codon, and coding sequence of the <i>cymR</i>-homologue mRNA using the target prediction programs TargetRNA and sRNATarget (Fig. 4.6) (23, 147). The complementarity around the start codon is predicted to block the ribosome binding site of the <i>cymR</i>-homologue mRNA (Fig. 4.6). This prediction is counterintuitive based on the reduction in <i>cymR</i>-homologue transcript levels in <i>B. cereus</i> ∆sRNA<sup>Other</sup> relative to wt. In contrast, extensive complementarity between the <i>cymR</i>-homologue mRNA and sRNA<sup>Other</sup> is predicted around the mRNA coding sequence. This RNA-RNA interaction may stabilize the RNA by blocking RNase attack and subsequent RNA degradation (141).

As is the case for NOS and SOD, CymR also is involved in reducing endogenous oxidative stress. CymR is a member of the Spx regulon, and both transcriptional regulators are involved in regulating gene expression in the response to oxidative stress. CymR regulates the biosynthesis of cysteine, which acts as a cellular reductant in <i>B.</i>
*cereus* to convert $\text{Fe}^{3+}$ to the redox active $\text{Fe}^{2+}$. The reduction of intracellular cysteine can lower the conversion of $\text{Fe}^{3+}$ to $\text{Fe}^{2+}$, which will prevent the production of hydroxyl radicals. This leads to a reduction in oxidative stress, which can increase resistance to certain bactericidal antibiotics, as was shown for vancomycin (Fig. 3.4). Ultimately, this study has shown that sRNA$^\text{Other}$ is an iron-regulated sRNA that regulates the expression of numerous genes responsible for reducing intracellular oxidative stress.

5.2. Future Perspectives

Several questions about the function of sRNA$^\text{Other}$ in *B. cereus* remain. Does sRNA$^\text{Other}$ function as a trans-encoded sRNA by limited base pair complementarity? If so, is Hfq required for its RNA-RNA interactions? Two Hfq-homologues are encoded within the *B. cereus* genome. If sRNA$^\text{Other}$ requires Hfq for its regulatory function, can either or both Hfq proteins serve this function? From the 5’ and 3’ RACE data, sRNA$^\text{Other}$ appears to be processed into various forms. Is sRNA$^\text{Other}$ only processed under certain conditions, such as low iron, and do all of the processed forms have a function?

To determine the size or sizes of sRNA$^\text{Other}$, northern blot analysis will be performed. This method will allow us to determine the relative quantities of each form of sRNA$^\text{Other}$ if more than one size of the sRNA is detected. Detection of various sized RNAs are expected because sRNA$^\text{Other}$ is predicted to be transcribed from two different promoters and may also be processed at its 5’-end. Iron-dependent expression and processing also can be monitored via northern blot analysis. The various forms of sRNA$^\text{Other}$ can then be tested for function. RNA-RNA interactions between sRNA$^\text{Other}$ and its targets can be determined by an electromobility shift assay (EMSA). To
circumvent the need for Hfq-RNA binding to promote RNA-RNA interactions, the RNAs can be heated and then allowed to anneal to each other as the temperature cools. By using these methods, the functional size(s) of sRNA\textsuperscript{Other} can be determined, and expression of the various forms of sRNA\textsuperscript{Other} can be compared between high and low iron conditions.

Analysis of the transcript sequence predicted to originate from the \(\sigma^A\) promoter 565 nt upstream of the predicted original tRNA\textsuperscript{Other} sequence revealed a putative ribosome binding site (GAGAAG) eight nucleotides upstream of an AUG start codon. The promoter is located within a gene sequence coding for a thioesterase (Fig. 4.3). Predicted translation from this start codon could produce a protein domain, an alpha/beta hydrolase fold (108). This domain contains a catalytic triad that is similar to a mirror-image of a serine protease catalytic triad (108). Translation from this transcript, would be consistent with the presence of the “unprocessed tRNA” detected in \(B.\ cerus\) polysome fractions. Transcription of from the \(\sigma^B\) promoter, which is 349 nt upstream of the predicted original tRNA\textsuperscript{Other} sequence and overlapped by a Fur binding site, and possible RNA processing would be consistent with expression of an iron-regulated small RNA.
Figure 5.1. Schematic of sRNA\textsuperscript{Other} function in \textit{B. cereus}. High levels of ferrous iron (Fe\textsuperscript{2+}) activate DNA binding of Fur, which repressed expression of sRNA\textsuperscript{Other}. When expressed under low iron conditions, sRNA\textsuperscript{Other} induces the mRNA levels of \textit{pbp1a}, \textit{trpRS1}, \textit{nos}, \textit{cymR}, \textit{spx}, and \textit{sod} by an undetermined mechanism. An increase in \textit{pbp1a} expression leads to an increase in cell wall thickness, which can reduce susceptibility to certain antibiotics. TrpRS1 may interact with and induce activity of NOS. Both NOS and SOD reduce endogenous oxidative stress. CymR and Spx are both involved in regulation of gene expression in the response to, and in order to combat, oxidative stress. A reduction in oxidative stress can increase resistance to certain bactericidal antibiotics. Arrows (\(\rightarrow\)) indicate induction and perpendicular lines (\(\perp\)) indicate repression.
References


5. Ataide, S. F. 2006. Rationale for the evolutionary retention of two unrelated lysyl-tRNA synthetases. Dissertation. The Ohio State University, Columbus, OH.


42. **Friedman, L., J. D. Alder, and J. A. Silverman.** 2006. Genetic changes that correlate with reduced susceptibility to daptomycin in *Staphylococcus aureus*. Antimicrob Agents Chemother **50**:2137-45.


Appendix A

Changes in transcript levels between wt and \textit{B. cereus ΔtRNA}\textsuperscript{Other} under moderate and low iron conditions.

<table>
<thead>
<tr>
<th>Gene #</th>
<th>Gene Product</th>
<th>Microarray (Δ/wt)*</th>
<th>qRT-PCR (Δ/wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC1188</td>
<td>Spx, transcriptional regulator</td>
<td>-4.2</td>
<td>2.0 ± 1.0</td>
</tr>
<tr>
<td>BC2773</td>
<td>CymR-homologue, RRF2 transcriptional regulator</td>
<td>-2.6</td>
<td>4.7 ± 2.5</td>
</tr>
<tr>
<td>BC4901</td>
<td>Fur, ferric uptake regulator</td>
<td>-2.2</td>
<td>2.5 ± 1.1</td>
</tr>
<tr>
<td>BC2632</td>
<td>AraC family transcriptional regulator</td>
<td>-2.6</td>
<td>-2.2 ± 0.2</td>
</tr>
<tr>
<td>BC5445</td>
<td>SOD [Mn], superoxide dismutase</td>
<td>-2.7</td>
<td>-3.7 ± 3.1</td>
</tr>
<tr>
<td>BC5444</td>
<td>NOS, nitric oxide synthase</td>
<td>-1.6</td>
<td>-5.1 ± 2.2</td>
</tr>
<tr>
<td>BC2137</td>
<td>DnrN, nitric oxide dependent regulator</td>
<td>-3.4</td>
<td>-4.9 ± 1.7</td>
</tr>
<tr>
<td>BC1550</td>
<td>PBP 1a, multimodular transpeptidase-transglycosylase</td>
<td>-11</td>
<td>-36 ± 18</td>
</tr>
<tr>
<td>BC2458</td>
<td>Thioesterase †</td>
<td>1.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>BC1232</td>
<td>Anthrinillate synthase †</td>
<td>1.0</td>
<td>-1.1 ± 0.5</td>
</tr>
<tr>
<td>BC0787</td>
<td>SpoVR, stage V sporulation protein R</td>
<td>6.1</td>
<td>2.7 ± 0.7</td>
</tr>
<tr>
<td>BC5147</td>
<td>SpoVAC, stage V sporulation protein AC</td>
<td>5.3</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>BC5282</td>
<td>SpoIID, stage III sporulation protein D</td>
<td>4.3</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>BC5283</td>
<td>SpoIIQ, stage II sporulation protein Q</td>
<td>6.4</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>BC4194</td>
<td>Hypothetical protein \textit{(spoIIIA operon)}</td>
<td>2.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>BC1471</td>
<td>Sporulation maturation protein</td>
<td>3.0</td>
<td>1.4 ± 0.9</td>
</tr>
<tr>
<td>BC3099</td>
<td>Sporulation Germination Protein</td>
<td>1.9</td>
<td>-1.6 ± 0.2</td>
</tr>
<tr>
<td>BC4007</td>
<td>Sporulation Kinase B</td>
<td>-1.9</td>
<td>-1.6 ± 0.7</td>
</tr>
<tr>
<td>BC4420</td>
<td>SafA, SpoVID-dependent spore coat assembly factor</td>
<td>6.0</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>BC4336</td>
<td>RNA polymerase \textit{σK} factor</td>
<td>2.3</td>
<td>-1.1 ± 0.1</td>
</tr>
<tr>
<td>BC0244</td>
<td>OppD, oligopeptide transport ATP-binding protein</td>
<td>-2.0</td>
<td>-3.1 ± 1.5</td>
</tr>
</tbody>
</table>

Continued
Appendix A. continued.

<table>
<thead>
<tr>
<th>Gene #</th>
<th>Gene Product</th>
<th>Microarray (Δ/wt)*</th>
<th>qRT-PCR (Δ/wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC5350</td>
<td>PlcR, transcriptional regulator</td>
<td>-2.0</td>
<td>-1.2 ± 0.03</td>
</tr>
<tr>
<td>BC2526</td>
<td>GntR family transcriptional regulator</td>
<td>-1.5</td>
<td>-1.7 ± 0.1</td>
</tr>
<tr>
<td>BC4930</td>
<td>DeoR family transcriptional regulator</td>
<td>4.5</td>
<td>1.3 ± 0.02</td>
</tr>
<tr>
<td>BC4393</td>
<td>RRF2 family transcriptional regulator</td>
<td>-1.3</td>
<td>-1.6 ± 0.03</td>
</tr>
<tr>
<td>BC1094</td>
<td>CysA, Sulfate transport ATP-binding protein</td>
<td>2.7</td>
<td>1.2 ± 0.8</td>
</tr>
<tr>
<td>BC1412</td>
<td>HisI, histidine metabolism</td>
<td>-1.8</td>
<td>-1.5 ± 0.3</td>
</tr>
<tr>
<td>BC5293</td>
<td>NADH dehydrogenase subunit L</td>
<td>4.1</td>
<td>1.5 ± 0.001</td>
</tr>
<tr>
<td>BC0458</td>
<td>Alkaline phosphatase-like protein</td>
<td>2.0</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>BC3718</td>
<td>PTS system, fructose-specific II ABC component</td>
<td>4.8</td>
<td>-9.8 ± 2.0</td>
</tr>
<tr>
<td>BC4651</td>
<td>Transporter, drug/metabolite exporter family</td>
<td>4.7</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>BC2450</td>
<td>Macrolide efflux protein</td>
<td>1.8</td>
<td>-1.6 ± 0.3</td>
</tr>
<tr>
<td>BC3020</td>
<td>Hypothetical protein</td>
<td>-2.7</td>
<td>-20 ± 14</td>
</tr>
<tr>
<td>BC5321</td>
<td>Hypothetical membrane protein</td>
<td>3.7</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>BC5248</td>
<td>Hypothetical protein</td>
<td>5.8</td>
<td>1.2 ± 0.3</td>
</tr>
</tbody>
</table>

*Δ, B. cereus ΔtRNA^Met*; wt, wild-type B. cereus ATCC14579

† Negative control transcript levels were determined with a standard curve.
Appendix B

Chromosomal disruption of *pbp1a* in *B. cereus* ATCC 14579

To disrupt the chromosomal *pbp1a* gene in *B. cereus* ATCC 14579, 312 nt corresponding to +81 nt to +392 nt within the *pbp1a* gene were amplified using Pfu Turbo® DNA polymerase (Stratagene) (0.025 u/µl Pfu, 20 mM Tris-HCl (pH 8.8), 2 mM MgSO₄, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton® X-100, 100 µg/ml BSA, 0.25 µM of each dNTP, and 0.25 µM of each primer). Primers were designed to incorporate NotI and BamHI restriction sites in FWintBC1550 (5’-GGAGCGGCGCGTGCTGGACGTAAGAAGAG-3’) and RVintBC1550 (5’-GCCAGGATCCAAGTCTGTCGTGCTGTC-3’), respectively. PCR products were purified by agarose gel electrophoresis followed by gel extraction (NucleoSpin® Extract II, Macherey-Nagel) and TOPO-cloning via the Zero Blunt® TOPO® PCR cloning kit. Resulting TOPO-clones and the plasmid pMUTIN4 (152) were digested with BamHI (0.3 u/µl BamHI, 50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 1 µg/ml BSA, pH 7.9; NEB) at 37 °C for 1 hr. NotI (0.3 u/µl) was then added to the reaction and incubated at 37 °C for an additional hour. Following agarose gel electrophoresis and extraction, the linearized pMUTIN4 and TOPO-cloned inserts were ligated by T4 DNA ligase (Invitrogen) to produce pM4-intBC1550 and subsequently used to transform XL1-Blue *E. coli*. To check for successful ligation and transformation
of pM4-intBC1550, PCR was performed using primers FWintBC1550 and RVintBC1550 with the conditions described above.

Wt *B. cereus* cells were transformed with 10 µg pM4-intBC1550 by electroporation as follows. To prepare for electroporation, a culture of wt *B. cereus* was grown in 5 ml LB, 37 °C, shaken at 250 rpm was used to inoculate 250 ml pre-warmed LB to an OD$_{600}$ of 0.05. Cells were incubated at 37 °C with shaking at 250 rpm until the culture reached an OD$_{600}$ of 0.2 – 0.4. The culture was cooled on ice for 5 – 10 min, and then cells were sedimented by centrifugation at 1,000 x g for 10 min at 4 °C. Cells were washed five times in 10 ml cold electroporation buffer (250 mM sucrose, 1 mM HEPES (pH 7.0), 1 mM MgCl$_2$, and 10 % glycerol) then resuspended to achieve an OD$_{600}$ of 30 – 60, which corresponds to a 150x concentration of the original cell suspension. pM4-intBC1550 (10 µg) was mixed with 100 µl cells and added to a 2 mm electroporation cuvette (Apollo). Electroporation was performed using the CelljecT Duo (Thermo Electron) with a pulse of 2.5 kV, 15 µF, and 355 Ω. Cells were immediately resuspended in 1.5 ml LB and incubated at 30 °C for 5 hours with gentle agitation. Cells were then sedimented by centrifugation at 1,000 x g for 10 min, resuspended in 100 – 200 µl LB, plated on LBA + 2 µg/ml erythromycin, and incubated at 30 °C until colonies appeared after 24 to 48 h. Colonies were streaked for isolation onto LBA + 4 µg/ml erythromycin.

Chromosomal integration of plasmid pM4-intBC1550 was confirmed by PCR and restriction digestion. Primers FW1550check (5’-GATGCTCAAGATTACGCCGAC-3’) and Mut-Rev (5’-CCACAGTAGTTCCACCACC-3’) were used in PCR to confirm integration at the 5’-end of *pbp1a*. Digestion of the 466 bp PCR products with HindIII (NEB) produced 119 and 347 bp bands. Primers RV1550check (5’-
GGTCATGCCTAAACCTTCAGC-3’) and Mut-For (5’-TCCTAACAGCACAAGAGC-3’) were used in PCR to confirm integration at the 3’-end of BC1550. The resulting 493 bp PCR product was digested with EcoRI to produce 150 and 343 bp bands.
Appendix C

Detection of sRNA<sub>Other</sub> in free tRNA, 30S, 50S, 70S, and polysome fractions