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N-TERMINAL PROCESSING OF RIBOSOMAL PROTEIN L27 IN STAPHYLOCOCCUS AUREUS

J. Harry Caufield

Virginia Commonwealth University

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

J. HARRY CAUFIELD
B.S., University of Delaware, 2008

Director: Dr. Gail Christie
Department of Microbiology and Immunology
Virginia Commonwealth University
Richmond, Virginia

Virginia Commonwealth University
Richmond, Virginia

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LIST OF ABBREVIATIONS

°C	Degrees Centigrade
Å	angstrom
aa	Amino acid
BSA	Bovine serum albumin
B. subtilis	*Bacillus subtilis*
Da	Dalton
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
D. radiodurans	*Deinococcus radiodurans*
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
g	gram
gDNA	Genomic DNA
gp	Gene product
IPTG	Isopropyl-B-D-Thiogalactopyranoside
Kb	Kilobase
L	liter
L27	Large ribosomal protein L27
LB	Lysogeny broth, also known as Luria-Bertani media
M	molar
ml	milliliter
mol	mole
mRNA	Messenger RNA
MRSA	Methicillin-resistant *Staphlyococcus aureus*
MW	Molecular weight
nm	nanometer
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PTC	Peptidyl transferase center
RBS	Ribosome binding site
RNA	Ribonucleic acid
rRNA	Ribosomal RNA

S. aureus	*Staphylococcus aureus*
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate- protein acrylamide gel electrophoresis
SOC	Super Optimal Broth with catabolite repression
TAE	Tris acetate EDTA
TE	Tris EDTA
tRNA	Transfer RNA
TSB	Tryptic soy broth
U	Unit of enzyme activity
wt/vol	Weight per volume

ABSTRACT

N-terminal processing of ribosomal protein L27 in *Staphylococcus aureus*

By J. Harry Caufield

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2012.

Principal Investigator: Dr. Gail Christie, Department of Microbiology and Immunology

The bacterial ribosome is essential to cell growth yet little is known about how its proteins attain their mature structures. Recent studies indicate that certain *Staphlyococcus aureus* bacteriophage protein sequences contain specific sites that may be cleaved by a non-bacteriophage enzyme (Poliakov et al. 2008). The phage cleavage site was found to bear sequence similarity to the N-terminus of *S. aureus* ribosomal protein L27. Previous studies in *E. coli* (Wower et al.1998; Maguire et al. 2005) found that L27 is situated adjacent to the ribosomal peptidyl transferase site, where it likely aids in new peptide formation. The predicted *S. aureus* L27 protein contains an additional N-terminal sequence not observed within the N-terminus of the otherwise similar *E. coli* L27; this sequence appears to be cleaved, indicating yet-unobserved ribosomal protein post-translational processing and use of host processes by phage. Phylogenetic analysis shows that L27 processing has the potential to be highly conserved. Further study of this phenomenon may aid antibiotic development.

CHAPTER 1

INTRODUCTION

Life depends upon ribosomes. All cells must scrupulously control translation of mRNA-encoded genes into useful proteins or risk wasting precious cellular resources on unnecessary or potentially toxic peptides. Living things at the periphery of our definition of life depend upon bacterial ribosomes as well. The bacteriophages, encoding no ribosomes of their own, must take advantage of their hosts' protein-producing machinery to manufacture new viral components. Such exploitation of host processes by bacteriophages can provide new insight into bacterial biology. This work describes one such example: a yet-unexplored cellular process involving post-translational processing of a ribosomal protein. It specifically focuses on processing of ribosomal protein L27 within *Staphlyococcus aureus* and shows the potential extent of this activity among numerous other bacterial species.

Staphlyococcus aureus is a Gram-positive coccus of the bacterial family Staphylococcaceae. This catalase-positive, facultative anaerobe requires a growth environment rich in amino acids and other complex nutrient resources. A commensal colonist of the human respiratory tract, mucous membranes and skin, it generally causes only limited irritation or infection. Ten distinct lineages compose the commensal *S. aureus* population (Lindsay, 2010), though acquisition of mobile genetic elements can cause any of these strains to become highly pathogenic. Pathogenic *S. aureus* strains are responsible for a variety of diseases: the toxins responsible for toxic shock syndrome, scalded skin syndrome, and necrotizing pneumonia are all

found in pathogenic *S. aureus.* Especially virulent strain of this species can also cause pervasive infections of the skin, deep tissues, and cardiac valves (Stefani and Goglio, 2010).

Infections by *S. aureus* strains resistant to the beta-lactam antibiotic methicillin (MRSA) have gained particular notoriety in recent years. MRSA infections have reached epidemic status and limit the number of effective antibiotics available. These antibiotic-resistant strains remain especially prevalent in clinical environments; MRSA remains a primary cause of hospital-associated infections, especially among the elderly (Klevens et al., 2007). Antibiotic resistance has led to use of alternative antibiotics such as the glycopeptide vancomycin. This provides only a temporary strategy for combating pathogenic *S. aureus,* as human cases of fully vancomycin-resistant *S. aureus* (VRSA) infection have been appearing for several years (Zhu et al., 2008).

Studies of *S. aureus* antibiotic resistance and pathogenicity show that mobile genetic elements are responsible for the transfer of many virulence factors of *S. aureus*. Horizontal transfer events, mediated by plasmids, transposons, temperate bacteriophages and other mechanisms, can lead to insertion of pathogenicity determinants. Resistance to methicillin is conferred by insertion of a 52 Kb DNA cassette known as Staphylococcus cassette chromosome *mec* (SCC*mec*) into the *S. aureus* chromosome (Katayama et al., 2000). Vancomycin resistance may be the result of horizontal transfer by a plasmid from *Enterococcus faecalis* (Zhu et al., 2008). *S. aureus* strains are also known to harbor a class of phage-related, superantigen-encoding pathogenicity islands (reviewed in Novick et al., 2010). These superantigen pathogenicity islands, or SaPIs, are phage-like genetic elements resident within some *S. aureus* strains that require infection by helper phages for replication and mobilization. SaPIs were originally discovered to carry the gene for toxic shock syndrome toxin-1 (TSST-1) (Lindsay et al.,

1998) and as such are highly relevant to *S. aureus* pathogenicity, but mobilize only in the presence of phage, as their genomes spread in phage-protein-composed particles.

Bacteriophages – or simply phages – are bacterial viruses, usually containing little more than their own genomes within a protein capsid. A variety of phage types and morphologies exist within a global population estimated at more than 10^{31} viral particles (Wommack and Colwell, 2000). These viruses exist freely in the environment and must inject their DNA into a host bacterium to replicate. Depending upon the phage in question and its local population, its life cycle may continue down one of two specific paths. A lytic pathway may be taken, in which the phage quickly subverts the host's protein production, shifting it to phage proteins. Some phages enter a lysogenic cycle instead, in which the phage genome integrates with the host chromosome and enters a lytic phase upon exposure to stimuli such as the host SOS response.

Most known bacteriophages are tailed bacteriophages of the order *Caudovirales.* These viruses all possess icosahedral heads known as capsids, as well as tails and connector proteins. They contain genomes of linear dsDNA. Individual tailed bacteriophage particles come together in a generally conserved process of macromolecular self-assembly, though the assembly process requires accessory proteins for stability during maturation. Once the host begins to produce phage proteins, structural proteins will begin to form the required phage particle structures as a function of their own conformation, requiring limited energy beyond that required for initial protein production. The immature capsid structure is known as a procapsid. Studies of two of the dsDNA phages of *E. coli*, P22 and T4, found that mature capsid assembly depended upon production of a precursor or 'scaffold' protein. Scaffold proteins were later found to be essential to the assembly of most other dsDNA phages and many other viruses (Dokland, 1999). Studies

of the phage P22 have shown that the scaffold protein is responsible for reducing the concentration of capsid protein required for phage particle assembly, as well as guiding formation of the icosahedral structure and recruiting other phage proteins to the maturing particle. In many phages these actions require capsid and/or scaffold proteins to be cleaved by a virally-encoded protease (Johnson 2010).

The study presented here emerged from observations of bacteriophage 80α, a helper phage responsible for transduction of one of the SaPI genetic elements mentioned above (Tallent et al., 2007). Studies of bacteriophage 80α of *S. aureus* showed that it assembled in much the same manner as other tailed bacteriophages, yet contained no virally-encoded protease for processing of its capsid and scaffold proteins. Furthermore, analysis of capsid (gp46) and scaffold (gp47) proteins showed they were cleaved when isolated from 80α procapsids in *S. aureus* but not when isolated from *E. coli* plasmid expression systems; see Figure 1 for protein size comparison by SDS-PAGE (Poliakov et al., 2008). Figure 2 compares the sequences of these proteins and indicates where the cleavage occurs. Scaffold protein is significantly smaller than capsid protein, but both proteins are N-terminally cleaved within a consensus sequence of NLQFFA. In their mature forms, both proteins begin with an alanine, indicating a site of cleavage immediately after a phenylalanine. Further work by P.K. Damle (unpublished) showed that plasmid-based expression of gp46 and gp47 alone in *S. aureus* also yielded cleaved proteins like those purified from 80α procapsids (Fig. 1b). These observations led to the conclusion that the protease involved in 80α capsid assembly must originate from the host. This protease presumably has a normal, non-bacteriophage-related function within *S. aureus*.

Figure 1. Comparison of bacteriophage 80α capsid and scaffold proteins by SDS-PAGE.
a) Bacteriophage 80α capsid protein (CP) and scaffold protein (SP) purified from bacteriophage 80α procapsids in *S. aureus* and the same proteins expressed from plasmid pPD2 in *E. coli.* Marker shown on left denotes protein size in KDa. Adapated from Poliakov et al., 2008.

b) Bacteriophage 80α capsid and scaffold proteins purified from overexpression in *E. coli* and *S. aureus.* Marker shown on right denotes protein size in KDa. P.K. Damle, unpub.

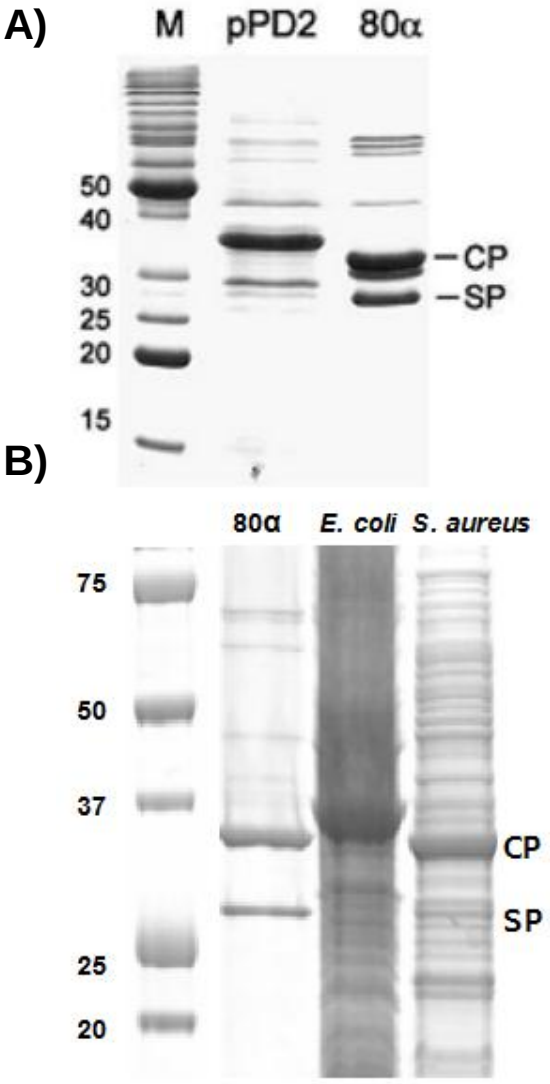

A)
M
pPD2
80α
50
40
30
25
20
15
CP
SP
B)
80α
E. coli
S. aureus
75
50
37
25
20
CP
SP

Figure 2. Protein sequence alignment of the N-terminal regions of bacteriophage 80α capsid protein (CP), bacteriophage 80α scaffold protein (SP), and *S. aureus* L27. All sequences from NCBI protein database – see Materials and Methods for accession numbers. Red line indicates predicted cleavage site. Amino acids on colored background indicate identity between at least one other sequence.


10
20
SP -MEENKLKFNLQFFADQSDDPDEPGGD- ...
CP MEQTQKLKLNLQHFASNNVKPQVFNPDN ...
L27 -----MLKLNLQFFASKKGVSSTKNGRD ...

Searching the *S. aureus* genome with the conserved sequence of the 80α capsid and scaffold protein cleavage site revealed one potential protease target. NCBI protein-protein BLAST was used to search the non-redundant protein sequence database for protein sequences in *S. aureus* similar to those of phage 80α capsid and scaffold proteins. The single confident result from each BLAST search was ribosomal protein L27. (Original search results courtesy of Terje Dokland lab, unpub.) This protein is the product of the gene *rpmA* and codes for a 94 amino acid protein of the 50S ribosomal subunit. The core L27 protein sequence shows very little similarity to that of the phage 80α capsid and scaffold proteins; no discernible alignment exists after the 30th amino acid of each sequence. The N-terminus of L27 as coded for by *rpmA* does appear to bear similarity to the conserved sequence seen in the phage proteins (see Figure 2). Most notably, this region does contain the conserved cleavage site seen in the phage proteins. If a host protease exists within *S. aureus* and cleaves phage 80α structural proteins, its usual target is likely to be this N-terminal region of the L27 protein. This activity would be a highly novel phenomenon as no examples of post-translational ribosomal protein processing are currently known.

The bacterial ribosome requires both RNA and a collection of proteins for its assembly and stability (reviewed in Shajani et al., 2010). The complete ribosome sediments as a 70S particle made up of a small 30S subunit and a large 50S subunit. The small subunit contains a 16S ribsosomal RNA molecule and 21 distinct proteins. The large subunit contains a 23S rRNA and a 5S rRNA as well as 33 proteins, one of which is L27. Early work by Traub and Nomura (1968) showed that *E. coli* ribosomal particles required no additional components for assembly: rRNA and ribosomal proteins were found to assemble into 30S particles *in vitro* without the

addition of any other cellular components. Furthermore, this work shows that the ribosomal proteins bind to 16S rRNA in a distinct order, with a set of protein-RNA interactions required for the stable binding of other ribosomal proteins. Peptidyl transferase activity depends upon sites within the rRNA.

Bacteria can evidently tolerate lack of some ribosomal proteins but not without impacts on growth. Treatment of ribosomes with proteinase K or SDS – presumably disrupting ribosomal proteins – has been found to severely limit but not abolish peptidyl transferase activity in *E. coli* (Noller et al., 1992). Individual knockouts of the 30S ribosomal protein genes *rpsF, rpsI, rpsM, rpsO, rpsQ,* and *rpsT* still remained viable, though the mutants were temperature sensitive (Bubunenko et al., 2007). As much of the work exploring ribosomal proteins concerns *E. coli* only, some of these results may not extend to ribosomes of other bacterial species.

Despite an ability to function without a full complement of ribosomal proteins, stable 70S ribosomes require these accessories to reach full efficiency. A substantial body of evidence suggests that ribsosomal protein L27 in particular aids both ribosome assembly and the peptidyl transferase reaction. The Keio collection of *E. coli* K-12 deletion mutants does not contain an *rpmA* mutant – the study behind this collection considered it essential for growth (Baba et al., 2006). Earlier work by Wower et al (1998), however, showed that *rpmA* deletions are not lethal in *E. coli.* Rather, lack of *rpmA* slows growth rates at 37ºC to nearly a fifth of that seen with the parent strain (*E. coli* LG90). These mutants did not grow at all at 25ºC or 43ºC. In addition to these growth defects, *E. coli rpmA* deletion strains formed only 40S precursors to the ribosomal 50S subunit. These malformed subunits lack the proteins L16, L20, and L21 as well, though they do still appear to form 70S particles *in vivo* (Wower et al., 1998). Notably, the L21 protein

is still produced when L27 is deleted (Wower et al., 1998) and though they appear to be located within the same operon, transcription of one of their respective genes does not appear to cause post-transcriptional regulation of the other (Nomura et al., 1984). In *Staphyloccus aureus*, *rpmA* was shown to be essential by transposon mutagenesis (Chaudhuri et al. 2009) and antisense RNA ablation of *rpmA* was found to be lethal (Ji et al., 2001).

Work by Lotti et al. (1987) used immuno-electron microscopy to find that L27 is located immediately adjacent to the peptidyl transferase center (PTC). Later, a crystal structure of the 50S ribosomal subunit from *D. radiodurans* (Harms et al., 2001) showed that L27 was one of the few proteins extending into the PTC. Its N-terminal region – though disordered in this model – was found to hold a position ideal for interaction with the P-site tRNA. Another structural study attempted to examine the L27 structure independent of ribosomal RNA. It described the free-protein form as a tetramer of four identical monomers (Wang et al., 2004). This work omitted 19 N-terminal amino acids from the modeled structure, as the region is very flexible and disordered in crystal structures. More recent structural examinations of the full 70S ribosome of *Thermus thermophilus* have confirmed the potential for interaction at the PTC (Selmer et al., 2006, also Trobro and Aqvist, 2008). The majority of the L27 structure appears to be conserved between *E. coli*, *D. radiodurans*, *T. thermophilus*, and likely most other members of Eubacteria, though it should be noted that the most similar ribosomal protein in Archaea by sequence, L21e (Fox, 2010), appears to have a different structure and a different set of interactions with its protein and RNA neighbors in the ribosome (Ban et al., 2000, also Harms et al., 2001).

Regulation of *rpmA* transcription may depend upon environmental factors. Studies of the *Bacillus subtilis* cold-shock response observed how some ribosomal protein genes, including

rpmA, saw doubled transcript levels after a temperature change from 37°C to 18°C. Two upstream genes occupying the same operon showed no change in levels of transcript under the same change in conditions. These results suggest that *rpmA* may be regulated by its own temperature-dependent promoter immediately upstream of the gene (Kaan et al., 2002). Work by Ohashi et al. (2003), also in *B. subtilis*, found that *rpmA* mutants had difficulty entering sporulation at 47°C. Ribosomal protein L27 may provide some bacterial species with an increased ability to handle temperature shifts.

A substantial body of work exists regarding N-terminal processing during the maturation of non-ribosomal proteins in bacteria. It is known that N-terminal methionines are frequently removed from proteins, for example. Statistical analyses of protein sequences by Flinta et al. (1986) found that N-terminal methionine removal is most likely to occur when the penultimate N-terminal residue is Ala, Gly, Pro, Ser, or Thr. Longer N-terminal peptides are also cleaved from peptides to direct their transport in the cell by the Sec pathway. These processing reactions generally occur while most of the new protein remains within the ribosome. Still further examples of post-translational structural modifications are seen in eukaryotes.

Wower et al. (1998) and Maguire et al. (2005) showed that L27 is an important precursor in ribosome assembly. If *S. aureus* L27 is sufficiently similar in sequence to *E. coli* L27 in all but the N-terminus, *E. coli* without L27 might be complemented by *S. aureus* L27 lacking the characteristic N-terminal sequence. This peptide sequence should be identical to the polypeptide existing in *S. aureus* after L27 is post-translationally processed. Evidence for L27 processing exists for *B. subtilis*, as Lauber et al. (2009) have shown that purified *B. subtilis* L27 is missing

the first 9 amino acids (MLRLDLQFF) coded for by the predicted gene sequence[1]. Lotti et al. (1987) also claimed to reconstitute 50S subunits from *E. coli* mutants lacking L27 by adding purified *Bacillus subtilis* L27. It must be assumed that the purified *B. subtilis* protein has already been subjected to the post-translational processing proposed here.

Processing of a ribosomal protein is a previously unrecognized phenomenon. While much is known about the structure of the ribosome and its constituent parts, the manner in which each element attains its final position remains unclear. The discovery of post-translational ribosomal protein processing reveals how the seemingly unrelated studies of bacteriophage capsid assembly and ribosomal proteins intersect at genetics; in both cases, sequences of mature proteins do not agree with their respective genes. In the context of the bacterial ribosome, however, protein processing suggests the possibility of unidentified regulatory mechanisms. This study was performed in order to investigate post-translational processing of *S. aureus* L27 and to determine how widely this phenomenon may be conserved among other bacterial species.

The work described here combined traditional microbiology methods with broad comparisons of genomic data from a variety of diverent bacterial species. Several *in vivo* methods were used to show how mature forms of L27 differ after expressing this protein within *E. coli* and *S. aureus*. (Maguire et al. (2001) performed a similar study in an attempt to replace *E. coli* L27 with that of thermophilic bacterium *Aquifex aeolicus.*) A potentially cleavage-deficient L27 mutant protein was expressed in *S. aureus* to explore the effects of restricted L27 processing. Lastly, bacterial species were compared on the basis of their respective 16S rRNA sequences and predicted L27 protein sequences.

[1]Lauber et al. suggested this discrepancy is due to errors annotating the reference sequence of the *B. subtilis* 168 genome.

STATEMENT OF OBJECTIVES

The Aims of this Study are:

1. Establish that *Staphylococcus aureus* ribosomal protein L27 is post-translationally processed by the removal of amino acids from its N-terminus.

2. Establish how the potential for N-terminal processing of L27 compares with phylogenetic distribution of bacterial species by 16S rRNA.

The overall objective of this study is to confirm the prediction that the mature form of ribosomal protein L27 differs from the gene product coded for by gene *rpmA* in *Staphylococcus aureus*.

CHAPTER 2

MATERIALS AND METHODS

Bacterial culture. Bacterial strains used in this study are described in Table 2. All *E. coli* strains were cultured in lysogeny broth (LB) as prepared using LB-Miller (Fisher Scientific, Fair Lawn, NJ) or were plated on LB agar consisting of LB-Miller broth and 1% (wt/vol) agar (both Fisher Scientific, Fair Lawn, NJ). All *E. coli* cultures were incubated at 37°C overnight unless otherwise noted and liquid cultures were grown on a platform shaker rotating at 200 RPM. Media was supplemented with ampicillin (100 µg/ml), kanamycin (60 µg/ml), or chloramphenicol (12.5 µg/ml) as appropriate.

S. aureus strains used in this study were cultured in tryptic soy broth (TSB) (Remel, Lenexa, KS) or on tryptic soy agar (TSA) (Becton, Dickinson, Sparks, MD) and incubated at 30°C overnight unless otherwise noted and liquid cultures were grown on a platform shaker rotating at 200 RPM. Media was supplemented with chloramphenicol (5 µg/ml) and tetracycline (2 µg/ml) as appropriate.

Genomic DNA extraction. Genomic DNA was isolated from *E. coli* strain DH5α by first growing 5 ml of culture in LB media overnight as described above. The culture was pelleted using an Eppendorf 5418 benchtop centrifuge at 14,000 rpm and removing the supernatant by micropipette. The cell pellet was resuspended in 500 µl of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). 50 µl of 10% (wt/vol) SDS and 25 µl of 20 mg/ml proteinase K were added to the resuspended pellet and mixed by inversion. The solution was incubated at 65°C for 30

minutes. The solution was then mixed by inversion with 500 µl of 1:1 phenol/chloroform and spun in the benchtop centrifuge for 10 minutes at 14,000 rpm. The resulting top aqueous phase was removed by micropipette and transferred to a fresh tube to which 500 µl of 1:1 phenol/chloroform was added and mixed by inversion. The solution was spun in the benchtop centrifuge again for 10 minutes at 14,000 rpm and the resulting top aqueous phase was extracted by micropipette. 50 µl of 3M sodium acetate (pH 5.2) was added to the solution and gently mixed, followed by 300 µl 100% isopropanol and mixed again, causing DNA precipitation. The DNA was spooled onto a micropipette tip and resuspended in 50 µl TE buffer and then incubated with 1 µl RNase A (10mg/ml) at 37°C for 15 minutes. Prepared gDNA was stored at 4°C until use.

DNA manipulations. DNA was purified from agarose gel using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) as per manufacturer's instructions. All restriction enzymes, T4 DNA Ligase, and enzyme buffers, including BSA, were purchased from New England Biolabs (Ipswitch, MA) and used as per manufacturer's instructions. PCR amplification products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) as per manufacturer's instructions. All plasmid minilysates were prepared with the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) according to manufacturer's instructions using overnight *E. coli* cultures grown in 5 ml of LB media with antibiotic as described above. Subsequent plasmid preparations for use in *S. aureus* transformations were prepared with the QIAfilter Plasmid Midiprep Kit (Qiagen, Valencia, CA) to ensure DNA concentrations above 1 µg/µl.

Agarose gels. Agarose gels were prepared by dissolving agarose (Bioline USA, Boston, MA) in 1X TAE buffer (American Bioanalytical, Natick, MA) to a concentration of 1% for all use except occasional comparisons of plasmid inserts, when 2% agarose gels were used. Ethidium bromide was added to gels at 0.1 µg/ml before polymerization. 5X loading dye was added to DNA prior to loading. Hyperladder DNA ladders I and IV (Bioline USA, Boston, MA) as well as Supercoiled DNA Ladder (New England Biolabs, Ipswitch, MA) were loaded and used as molecular markers to determine DNA size and concentration. Gels were run at 16.25 V/cm until the dye front had travelled approximately ¾ of the length of the gel, at which point DNA was visualized under UV light.

Plasmid screening. *E. coli* transformant candidate colonies were screened using a quick-check procedure (Akada, 1994). Colonies were selected from growth plates and grown in individual overnight cultures as described above. For each candidate, 100 µl of culture was added to 50 µl of phenol:chloroform (1:1) and 10 µl 0.1% bromophenol blue in microcentrifuge tubes. Each sample was mixed by vortexing for 10 seconds and spun in an Eppendorf 5418 benchtop centrifuge for 3 minutes at 13,000 rpm. Supernatant fractions were loaded in 10 µl volumes directly onto a 1% agarose in TAE gel alongside 2 µl of Supercoiled DNA Ladder (New England Biolabs, Ipswitch, MA) and empty vector. Transformant candidates were selected from those appearing larger than empty vector.

Polymerase Chain Reaction (PCR). PCR reactions were performed using a TGradient Thermoblock thermocycler (Whatman Biometra, Göttingen, Germany). Primers are shown in

Table 1 and were produced by Integrated DNA Technologies (Coralville, IA). Primer stocks were resuspended at first use in HPLC-grade water (JT Baker, Phillipsburg, NJ) to 100 µM. PCR amplifications were prepared as follows: 0.2 mM of each dNTP from a 10 mM dNTP mix (Invitrogen, Carlsbad, CA), 0.2 mM of each primer from 1:10 dilutions of 100 µM stocks in MQ water, 1X HF Phusion Buffer from a 5X stock and 1 to 2 U of Phusion DNA Polymerase (both from Finnzymes, Vantaa, Finland). Phusion polymerase required use of melting temps 3 degrees higher than those otherwise calculated for each primer. DNA template was added in concentrations of less than 50 ng/µl and the reaction volume was adjusted to 50 µl with MQ water.

The thermocycler program was optimized for each amplification. In general, the program was as follows: 98°C for 1 minute; 30 cycles of 96°C for 30 seconds, Tm(+3) for 30 seconds and 72°C for 80 seconds; 72°C for 5 minutes, and a holding temperature of 4°C.

Preparation of competent cells. *E. coli* used for cloning in this study were NEB 5-alpha chemically competent cells purchased from New England Biolabs (Ipswitch, MA) unless stated otherwise. Electrocompetent DH5α and IW312 cells were also prepared using the following procedure, adapted from the operating instructions for the Bio-Rad (Hercules, CA) Micropulser Electroporation Apparatus. Liquid overnight culture was prepared as described above in LB media. The following day, 24 ml of LB media without antibiotic was inoculated with 1 ml of overnight culture and placed at 37°C on a shaking platform at 200 rpm. The culture was allowed to grow to the middle of logarithmic-phase growth as determined by optical density; this corresponds to a Klett reading of 95 or an OD_{600} value of approximately 0.6. The culture was

transferred to ice and chilled for 20 minutes; all subsequent steps were kept cold as well. The culture was then transferred to conical centrifuge tubes and spun in an Eppendorf 5810R centrifuge at 4000 rpm for 5 minutes at 4°C. The resulting supernatant was discarded and the pellet was washed with a volume of cold, sterile 10% glycerol equivalent to the initial culture volume. This spin was repeated three times with subsequent glycerol wash volumes decreasing by half and the last centrifuge spin requiring only 5 minutes. After the last spin, supernatant was discarded, the pellet was resuspended in 0.5 to 1.0 ml cold 10% glycerol and separated into 50 µl aliquots. All freshly competent cells were stored at -80°C for no more than four months.

A similar procedure (adapated from that of McLaughlin and Ferretti, 1995) was used to prepare electrocompetent *S. aureus* cells, with initial resuspension of the pellet in cold sterile MQ water instead of cold 10% glycerol. The exact protocol was as follows: A bacterial culture was first grown overnight in TSB or BHI media. Media was inoculated with overnight culture as 50 µl culture in 500 ml of liquid media. The culture was grown at 32°C with gentle shaking to a point at which the optical density was determined by a reading on a Klett colorimeter to be approximately 95. At this point, the culture was chilled on ice for 15 minutes and pelleted in an Eppendorf 5810R centrifuge at 4000 rpm for 15 minutes at 4°C. The resulting supernatant was discarded, the pellet was resuspended in 25 ml of cold, sterile MQ water, and the spin was repeated. This wash step was performed twice. The wash step was then repeated twice with 30 ml and 15 ml of cold, sterile 10% glycerol. Following the final wash step, the supernatant was discarded and the cell pellet was resuspended in 500 µl of cold 10% glycerol and separated into 50 µl aliquots. Each aliquot was stored at -70°C.

Transformation. Chemically competent *E. coli* NEB 5-alpha (New England Biolabs, Ipswitch, MA) was transformed by first adding 1 µl of plasmid DNA or 2 µl of a 10 µl ligation reaction to a 50 µl aliquot of cells thawed on ice. Cells and DNA were incubated on ice for 30 minutes then placed in a 42°C water bath for 30 seconds. Heat-shocked cells were placed on ice for 5 minutes before addition of 750 µl SOC media and incubation at 37°C for 1 hour with shaking at 200 rpm. Cells were plated on solid selective media after the outgrowth incubation.

Electrocompetent *E. coli* and *S. aureus* were also transformed by electroporation. Competent *E. coli* were either prepared as described above or were TOP10 cells purchased from Invitrogen (Carlsbad, CA). Electrocompetent *S. aureus* SA178RI cells were prepared as described above. All cells were electroporated using a Bio-Rad (Hercules, CA) Micropulser Electroporation Apparatus and 2 µl of DNA for each 50 µl aliquot of cells thawed on ice. *E. coli* cells were electroporated in cold 0.1 cm electroporation cuvettes with the "Ec1" setting (for a time constant of about 5 msec). Electroporation was followed with addition of 750 µl SOC medium (prewarmed to 37°C) and incubation at 37°C for 1 hour with shaking at 200 rpm. Cells were plated on solid selective media after the outgrowth incubation. Electroporation of *S. aureus* was performed in the same manner but with cold 0.2 cm electroporation cuvettes and 750 µl TSB for the post-electroporation outgrowth medium

Complementation assays in Δ*rpmA E. coli.* Full-length and truncated forms of *Staphylococcus aureus rpmA* gene were tested for complementation in *E. coli* strain IW312, in which genomic *rpmA* had been replaced with the gene for kanamycin resistance (Wower *et al.* 1998). Complementation was assayed by the ability of cells to grow overnight at 37ºC or 42ºC on solid

media. The higher temperature was reported by Wower *et al.* (1998) to be non-permissive for growth in IW312 *E. coli* lacking functional L27 but permissive for growth in IW312 in which the deletion has been complemented, though uncomplemented IW312 also grows slowly at 37ºC.

Expression of proteins in *S. aureus.* Growth of bacterial strains used in this study were monitored by measurement of optical density on a Klett Model 900 colorimeter. All optical density readings at any one point in time were performed after calibrating the Klett reader against a blank containing media only.

Plasmid constructs based on the pG164 backbone (originally created by D'Elia et al., 2006) contain the phage T7 promoter and T7 terminator and as such require a host strain with the ability to produce T7 RNA polymerase. The strain used for this purpose was *S. aureus* SA178RI (D'Elia et al., 2006). Cultures of this strain were grown overnight in TSB media with chloramphenicol (5 µg/ml) and tetracycline (2 µg/ml) at 30ºC with shaking at 200 RPM. Overnight cultures were then diluted (1:100) in fresh TSB with the same antibiotic concentrations and grown to the middle of logarithmic-phase growth, as determined by a Klett reading of 95 or an OD_{600} value of approximately 0.6. At this point, 0.5 ml pre-induction samples were removed from each total sample volume and pelleted using an Eppendorf 5418 benchtop centrifuge at 14,000 rpm. The supernatant was removed from each sample and the remaining cell pellet was stored at 4ºC for no longer than 48 hrs. Protein overexpression was induced at this point through the addition of 1 mM IPTG. Post-induction samples were collected at 1, 2, and 3 hours after the addition of IPTG; each sample was collected in the same manner as the pre-induction sample. After collection of the final 0.5 ml sample the remaining sample

volume was pelleted in a Sorvall centrifuge with GSA rotor (10000 x g, 30 min, 4ºC) and stored at -20ºC.

Proteins were isolated and purified from collected cell pellets. Cells were lysed using an Emulsiflex C3 homogenizer. Each cell pellet was resuspended in 50 ml of lysis buffer containing a single Complete EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN) and passed through the homogenizer three times at a pressure of 30000 PSI. Cell lysates were clarified in a Sorvall centrifuge with a GSA rotor (20000 x g, 30 min, 4ºC) and separated into aliquots such that half of the volume was returned to 4ºC and the remaining volume was stored at -20ºC. All samples stored at 4ºC were purified within the following 48 hours.

Each protein sample was initially analyzed on Criterion XT pre-cast 12% bis-tris acrylamide gels on a Criterion electrophoresis apparatus filled with 1X XT MES running buffer (all from Bio-Rad, Hercules, CA) using a constant voltage of 120V for approximately 1 hour and 20 minutes. Further size comparisons were performed in the same way but with 16.5% tris-tricine acrylamide gels in tris-tricine-SDS buffer.

Phylogenetic comparison of protein sequences. The sequences of the predicted gene products of L27 genes from 37 bacterial species were compared to the arrangement of the 16S rRNA sequences. Gene sequences annotated as coding for L27 were located in the NCBI Genbank Gene database and verified for sequence similarity by nucleotide BLAST alignment. A nucleotide range of 500 bases upstream and 500 bases downstream of the predicted gene was extracted from the genome sequence. A 16S rRNA sequence each species examined was also

obtained from the Ribosome Database Project (RDP). Each L27 gene region was compared by nucleotide sequence alignment, adjusted by hand, and examined to verify the position of start codons. Individual DNA and protein sequences were examined using BioEdit software (Hall, 1999). Sequences of putative ORFs upstream of *rpmA* in each bacterial genome were compared with *Staphylococcus aureus ysxB*; sequences sharing more than 20% protein sequence identity with *S. aureus* predicted *ysxB* gene product were determined to have *ysxB* as well.

16S rRNA sequences were used to establish neighbor-joining phylogenic trees using MEGA version 5 (Tamura et al., 2011) with *Nanoarchaeum equitans* as a root. Each protein sequence was also aligned at the N-terminus. Finally, L27 sequences were clustered to establish whether differences at the N-terminus coordinated with the evolutionary patterns seen with 16S rRNA comparisons. Further phylogenetic arrangements were prepared using publicly available sequences aggregated from the Genbank set and sequences present in the PhAnToMe/PhageSEED database, an extension of the SEED database (Overbeek et al., 2005).

Table 1. Primers used in this study.

Primer	Sequence	Purpose	DNA Template
JHCECL27F2	5- CCA AGA ATT CAA GGA GAT ATA CAT ATG GCA CAT AAA AAG GCT GGC -3	Amplification of *E. coli rpmA*	*E. coli* DH5α
JHCECL27R2	5- GGC AAG CTT TTA TTC AGC TTC GAT GCT GAT AAA TTT ACG -3	Amplification of *E. coli rpmA*	*E. coli* DH5α
Ptac-F	5 - TAT AAT GTG TGG AAT TGT GAG CGG ATA ACA ATT -3	Sequencing and reamplification of pGZ119EH inserts	pGZ119EH constructs
RrnB-R	5 - GTC TTT CGA CTG AGC CTT TCG TTT TAT -3	Sequencing and reamplification of pGZ119EH inserts	pGZ119EH constructs
JHCL27F3	5- CCA AGA ATT CAA GGA GAT ATA CAT ATG TTA AAA TTA AAC TTA CAA TTC TTC GCA TC -3	Amplification of *Staph aureus rpmA*	*S. aureus* RN4220
JHCL27F3_A10	5- CCA AGA ATT CAA GGA GAT ATA CAT ATG GCA TCT AAA AAA GGG GTA AGT TCT AC -3	Amplification of *Staph aureus rpmA* with 9-codon truncation	*S. aureus* RN4220
JHCL27R3	5- GGC AAG CTT AGG TAG TTA TTC AGC TAC TGC ATA TAC AGA AAC T -3	Amplification of *Staph aureus rpmA*	*S. aureus* RN4220

Table 2. Sources of DNA and protein sequences specifically mentioned in this study.

Name	Source	Accession Number
ribosomal protein L27 [*Staphylococcus aureus* subsp. aureus JH1]	NCBI	ABR52581.1
ribosomal protein L27 [*Escherichia coli* str. K-12 substr. MG1655]	NCBI	AAC76217.1
ribosomal protein L27 [*Bacillus subtilis* subsp. subtilis str. 168]	NCBI	NP_390672.1
scaffold protein [*Staphylococcus* phage 80alpha]	NCBI	YP_001285360.1
major head protein [*Staphylococcus* phage 80alpha]	NCBI	YP_001285361.1
Staphylococcus phage 80alpha, complete genome	NCBI	NC_009526.1

Table 3. Bacterial strains and plasmids used in this study.

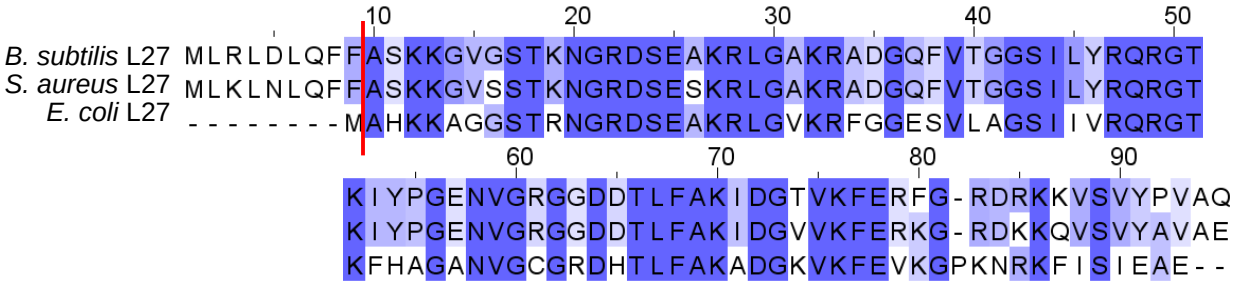
***S. aureus* Strain**	**Description**	**Source**
RN4220	Restriction deficient RN450 derivative	Novick et al. (1991).
SA178RI	Derivative of RN4220 carrying the T7 polymerase gene from λDE3 under control of the Pspac promoter	D'elia et al (2006).
***E.coli* Strain**	**Description**	**Source**
NEB 5-alpha	DH5α derivative cloning strain. *fhuA2* Δ(*argF-lacZ)U169 phoA glnV44* Φ*80*Δ (*lacZ*)M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs, Ipswitch, MA
BL21(DE3)	T7 Expression strain. *fhuA2 [lon] ompT gal (λ DE3) [dcm]* Δ*hsdS λ DE3 = λ sBamHIo* Δ*EcoRI-B int::(lacI::PlacUV5::T7 gene1) i21* Δ*nin5*	New England Biolabs, Ipswitch, MA
IW312	Δ*rpmA::kan*	Wower et al. (1998).
LG90	Wild-type parent strain of strain IW312. *F-,* Δ*lac pro XII*	Wower et al. (1998), originally in Guarente et al. (1980).
Plasmid	**Description**	**Source**
pGZ119EH	*E. coli* cloning and expression plasmid.	Lessl et al. (1992).
pPOT1AE	*E. coli* cloning and expression plasmid.	Tenson et al. (1997)
pL27	Plasmid pPOT1AE containing *E. coli rpmA*	Maguire et al (2005).
pG164	*E. coli* / *S. aureus* cloning and expression plasmid with T7 promoter	D'elia et al (2006).
pJHC23	Plasmid pGZ199EH containing *S. aureus rpmA*	This study
pJHC24	Plasmid pGZ199EH containing *S. aureus rpmA* truncated at 10th codon	This study
pJHC25	Plasmid pGZ199EH containing *E. coli rpmA*	This study
pJHC27	Plasmid pG164 containing *S. aureus rpmA*	This study
pJHC28	Plasmid pG164 containing *S. aureus rpmA* truncated at 10th codon	This study
pJHC29	Plasmid pG164 containing *E. coli rpmA*	This study
pJHC35M	Plasmid pG164 containing *S. aureus rpmA* coding for mutation in putative cleavage site (MLKLNLQFFASKK... → MLKLNLQAAASKK...)	This study (site-directed mutagenesis performed by Mutagenex, Hillsborough, NJ)

CHAPTER 3

Post-translational processing of ribosomal protein L27 in *Staphylococcus aureus* and implications for its expression in *Escherichia coli*

E. coli L27 shows strong sequence similarity with other L27 proteins in most bacterial species, yet a critical difference exists at the N-terminal region. Post-translational processing of *S. aureus* L27 at the N-terminus would leave mature proteins resembling those seen in *E. coli.* If *S. aureus* L27 is cleaved after the two phenylalanines in its predicted cleavage site, the mature protein should align at the N-terminus with the protein sequence of *E. coli* L27 following removal of its N-terminal methionine. Bacterial species with L27 sequences similar to *S. aureus* (that is, containing the "extended" N-terminus and the putative cleavage site) may also process this ribosomal protein. Comparisons of L27 from different species present the likelihood of this activity (Figure 3). The mature L27 protein sequence in nearly all bacterial species should then resemble that of *E. coli*, even in species coding for L27 with a longer N-terminus. The proximity of the L27 N-terminus to the P-site within the 50S ribosomal subunit suggests that failure to remove the additional amino acids from longer L27 proteins may impede peptide transfer. Maguire et al. (2005) have shown that extended modification at the *E. coli* L27 N-terminus leads to growth defects and charge differences, though tRNA retains its ability to bind at the P site.

Figure 3. Protein sequence alignment of *Bacillus subtilis* L27, *Staphylococcus aureus* L27, and *Escherichia coli* L27. All sequences from NCBI protein database – see Materials and Methods for accession numbers. Red line indicates predicted cleavage site. Amino acids on colored background indicate identity between at least one other sequence.


10
20
30
40
50
B. subtilis L27 MLRLDLQFFASKKGVGSTKNGRDSEAKRLGAKRADGQFVTGGSILYRQRGT
S. aureus L27 MLKLNLQFFASKKGVSSTKNGRDSESKRLGAKRADGQFVTGGSILYRQRGT
E. coli L27 --------MAHKKAGGSTRNGRDSEAKRLGVKRFGGESVLAGSIIVRQRGT
60
70
80
90
KIYPGENVGRGGDDTLFAKIDGTVKFERFG-RDRKKVSVYPVAQ
KIYPGENVGRGGDDTLFAKIDGVVKFERKG-RDKKQVSVYAVAE
KFHAGANVGCGRDHTLFAKADGKVKFEVKGPKNRKFISIEAE--

My initial attempts to show a requirement for post-translational processing of *S. aureus* ribosomal protein L27 relied upon complementation in an *E. coli* strain missing *rpmA*, the gene coding for L27. The hypothesis was as follows: if *S. aureus* L27 in its full-length form is expressed in the *E. coli* L27 deletion mutant (IW312), the defect will not be complemented, but expression of *S. aureus* L27 with a 10 amino acid truncation in the protein sequence will complement the deletion. This hypothesis was primarily based on sequence alignments, as the L27 protein sequences from *S. aureus* and *E. coli* were found to have more than 50% sequence identity when the predicted N-terminal extension is ignored. Strain IW312 is unable to grow at 42ºC, so growth at this temperature, or robust growth at 37ºC, was expected to signify complementation.

Subsequent experiments investigating L27 processing relied upon the effects of expression of mutant forms of the protein in *S. aureus*. Protein expression was checked by 1-dimensional gel electrophoresis of His-tagged proteins purified from *S. aureus* lysates. Plasmids were induced in *E. coli* with and without *rpmA* and in *S. aureus* with *rpmA* (deletion of the L27 gene is presumed to be lethal in *S. aureus*; a knockout of gene expression in *S. aureus* by antisense RNA proved lethal (Ji et al. 2001)).

Plasmid constructs were designed to express different forms of *S. aureus* and *E. coli rpmA* within *E.coli* (see Table 3 for the full list). Though a plasmid designed by Maguire et al. (2005) containing *E. coli rpmA* reportedly complements the *rpmA* deletion at 37ºC, attempts to construct vectors with the same pPOT1AE backbone were unsuccessful. The cloning vector pGZ119EH was used instead as the backbone for pJHC23, pJHC24, and pJHC25. It was chosen

primarily for its *tac* promoter, which was the same promoter used in plasmids constructed by Maguire et al. (2005) for their complementation assays in *rpmA* deletion strain IW312. The same work had found that high levels of overexpression of L27 appear to cause growth deficiencies, even in IW312. These vector inserts contained a specific ribosome binding site (AGGAAG) to more closely match expression conditions seen in wild-type *E. coli*. No further additions to each gene except for restriction sites were included unless otherwise specified. Plasmid pJHC23 contains *S. aureus rpmA* as amplified from *S. aureus* RN4220 genomic DNA (prepared by K. Lane). Plasmid pJHC24 is identical to pJHC23 with the exception of a gene truncation of 10 aa corresponding to the N-terminus of L27. The product of this gene as coded is expected to resemble *E. coli rpmA* at its amino terminus and was expected to complement the *rpmA* deletion in strain IW312. The N-terminus of this protein, by hypothesis, should also resemble that of the mature *S. aureus* L27 after N-terminal processing. Plasmid pJHC25 contains *E. coli rpmA* as amplified from strain NEB5-alpha (New England Biolabs, Ipswitch, MA). This plasmid was intended to be a positive control.

When expressed in *E. coli*, it was expected that the plasmid pJHC23 (coding for full-length *S. aureus* L27) would prove deleterious or lethal, as *E. coli* has not shown any ability to post-translationally process L27. The expressed proteins were expected be useless at best and incorporate into 50S ribosomal subunits at worst, hampering growth by interfering with peptidyl transferase activity. Conversely, pJHC24 (coding for 9-amino acid truncated *S. aureus* L27) and pJHC25 (coding for wild-type *E. coli* L27) were expected to complement the *rpmA* deletion in IW312 and allow growth at 42ºC.

Results of complementation assays were inconclusive. Complementation did occur with

plasmid pL27, coding for wild-type *E. coli rpmA*, at 37ºC as seen by Maguire et al. (2005) (see Figure 4). Growth at 42ºC under induction conditions showed only limited growth with this plasmid; Maguire et al. had not tested pL27 at this temperature but it was expected to complement the temperature sensitivity.

The empty pGZ119EH vector alone did not complement (see Figure 5). Transformation of IW312 *E. coli* with plasmids containing wild-type *E. coli rpmA* may complement the growth defect, as after an overnight incubation at 37ºC, pJHC25 transformants yielded larger colonies than the same transformants under induction conditions (Fig. 6). This could be the result of a leaky promoter enabling limited expression of wild-type *E. coli* L27. All colonies were approximately the same size (about 1 mm in diameter) after 36 hours at 37ºC. Re-streaking single colonies of IW312 transformants onto media containing 1 mM IPTG was expected to induce plasmids, hastening growth at 37ºC and permitting growth at 42ºC. Induction conditions do appear to enhance growth at 37ºC for truncated *S. aureus rpmA*-containing strains after 18 hours (Fig. 6). Under the same conditions, IW312 with full-length *S. aureus rpmA* yieded no colonies and wild-type *E. coli rpmA* constructs yielded colonies less than 1 mm in diameter. No transformant was found to grow at 42ºC under induction conditions, even after incubation times of 48 hours or more. Lower temperatures – between 30ºC and 32ºC – also prohibited growth of all transformants.

Plated colonies of the IW312 parent strain, LG90, were prepared as a control. LG90 grew to single colonies within 12 hours at both 37ºC or 42ºC with or without addition of 1 mM IPTG. LG90 transformed with pGZ119EH plasmids containing full-length *E. coli rpmA* or 9-codon truncated *S. aureus rpmA* showed no difference in growth from untransformed LG90.

Transforming this strain with a pGZ119EH plasmid encoding full-length *S. aureus rpmA* resulted in no growth in one out of every three transformation attempts. While this may be the result of leaky expression of a toxic peptide (or, in the case of unaffected growth, gene inactivation), the cloning strains of *E. coli* used to prepare plasmid lysates showed no impact on growth.

Cultures of *rpmA* deletion strain IW312 may be subject to compensatory mutations. The deletion of the L27 gene is highly deleterious yet nonlethal, so cultures of the strain subjected to further selection by antibiotics may drive generation of mutations in genes coding for other ribosome-associated proteins. These mutations may even arise during preparation of chemically competent cells of the deletion strain. This possibility also means that growth rates of complemented deletion strains may be difficult or impossible to accurately compare.

Technical limitations prevent further interpretations of the effects of mutant or non-native L27 expression in *E. coli*. It is clear that providing Δ*rpmA E. coli* with a copy of the wild-type gene on a plasmid can complement its growth deficiency at 37ºC yet growth at 42ºC still remains weaker than the parental strain. Repeating the assay with pGZ119EH plasmids encoding an identical wild-type gene did not appear to complement, though a *S. aureus rpmA* mutant similar in sequence to the *E. coli* gene did appear to restore growth at 37ºC. A plasmid encoding full-length *S. aureus rpmA* did not complement the defect but rather appeared toxic. Despite these results, it remains possible that these plasmids experienced mutations, creating pseudorevertants and presenting a false-positive complementation phenotype.

Figure 4. Complementation of *E. coli rpmA*-containing plasmids in *E. coli* Δ*rpmA* strain IW312. Both plates contain LB medium with ampicillin (100 µg/ml), kanamycin (60 µg/ml) and IPTG (1 mM). Left plate was incubated overnight at 42ºC; right plate was incubated overnight at 37ºC. Bottom of each plate shows highly limited growth of empty plasmid pPOT1AE. Top of left plate shows complementation but limited growth of one sample of IW312 with plasmid carrying *E. coli rpmA*. Top of right plate shows shows complementation of two samples of IW312 with plasmid carrying *E. coli rpmA*.

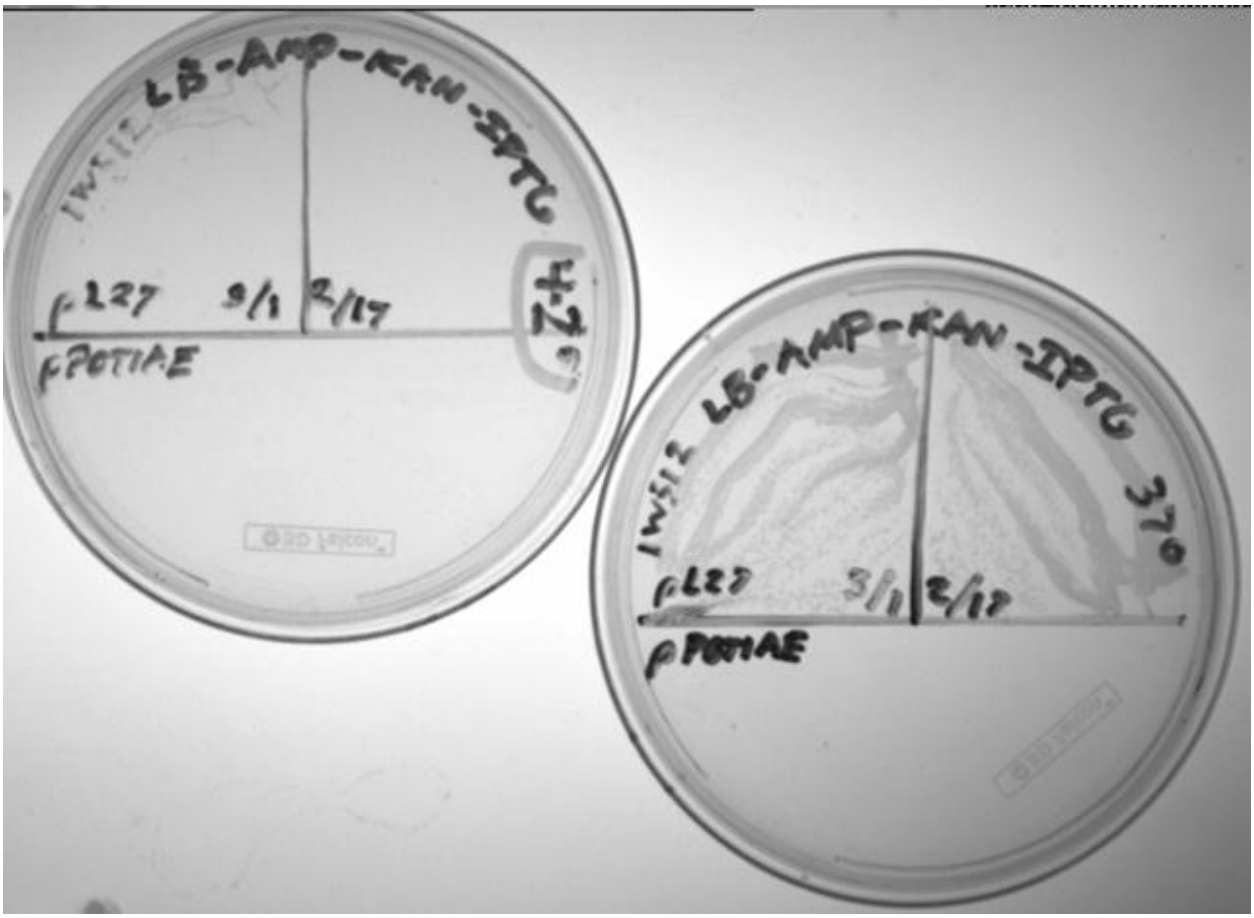

LB-AMP-KAN-IPTG
pL27
3/1
2/17
pPOTIAE
LB-AMP-KAN-IPTG 37°
pL27
3/1
2/17
pPOTIAE

Figure 5. Transformation of *E. coli* Δ*rpmA* strain IW312 with empty vector pGZ119EH. Plate contains LB medium with chloramphenicol (12.5 µg/ml) and was incubated at 37ºC overnight.

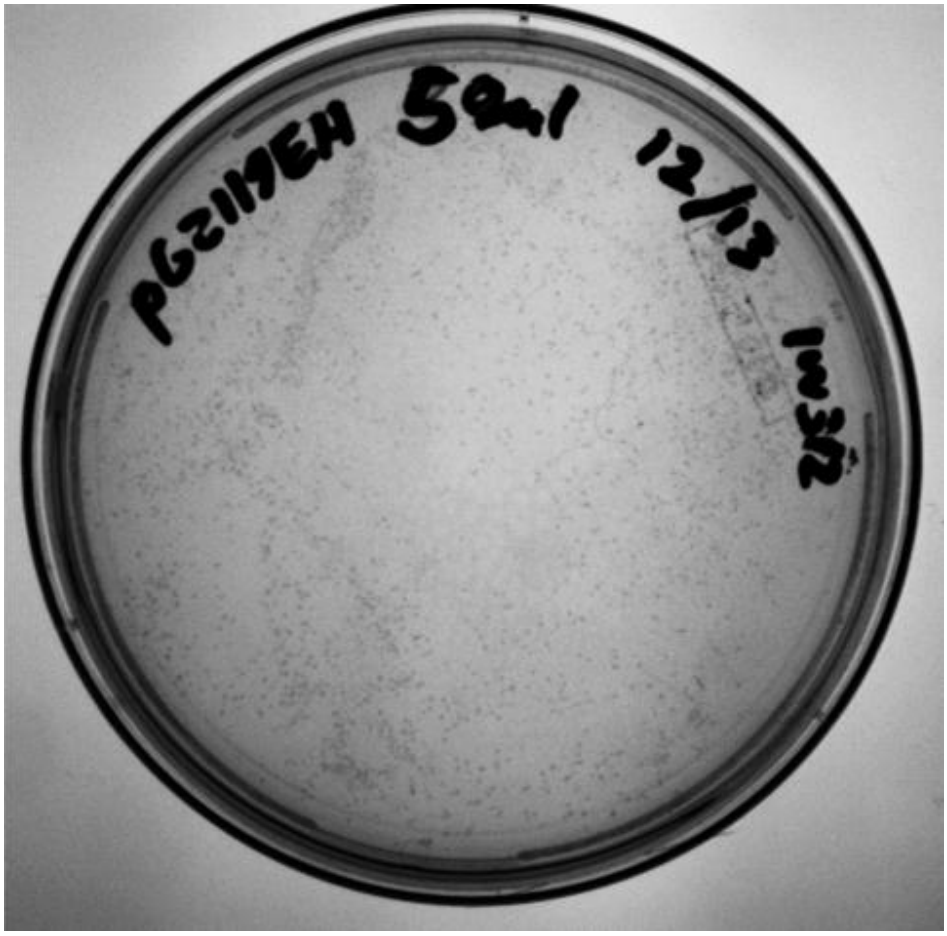
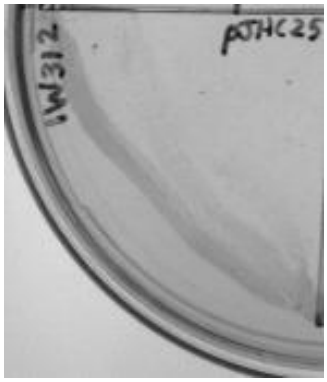
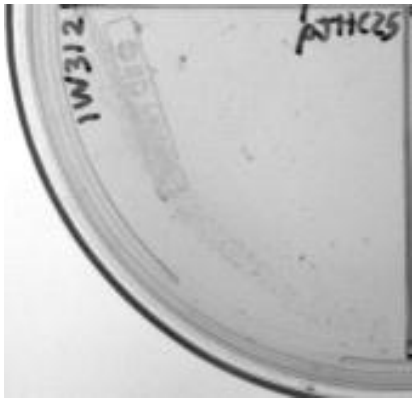

pGZ119EH 5ml 12/13 1ml 2ml

Figure 6. Complementation assays of pGZ119EH plasmids carrying *rpmA* in *E. coli* Δ*rpmA* strain IW312. Plates contain LB medium with chloramphenicol (12.5 µg/ml) and were incubated at 37ºC overnight.

	No IPTG	1 mM IPTG
Full-length *S. aureus rpmA*		
9-codon truncated *S. aureus rpmA*		
Full-length *E. coli rpmA*		

A second approach to the issue of L27 cleavage involved examination of proteins expressed in *S. aureus*. A set of plasmids was constructed using the pG164 backbone to express L27 mutants under control of the T7 promoter system. The pG164 plasmid also includes the ability to incorporate a C-terminal 6xHis tag for protein purification (this His tag is not codon-optimized for *S. aureus*, however, potentially reducing tagged protein concentrations). A means of purifying expressed protein is necessary to distinguish the plasmid-encoded versions of *rpmA* from the native one. Constructs pJHC27 (wild-type *S. aureus rpmA*), pJHC28 (9-codon truncated *S. aureus rpmA*), pJHC29 (wild-type *E. coli rpmA*), and pJHC35M (*S. aureus rpmA* with cleavage site mutation) were assembled using sequences from the plasmids detailed above (except for pJHC35M, which encodes *S. aureus* L27 with point mutations causing two phenylalanine codons to instead code for alanine; see Table 3).

The growth of cultures containing the L27 mutant-encoding plasmids was measured over time in order to monitor any negative impact expression of these proteins may have on viability. It was expected that expressing wild-type *S. aureus* L27 would not produce any deleterious effect. Overexpression of mutant forms of *S. aureus* L27 also appeared to produce no observable impact on growth. Plasmids were induced in *S. aureus* strain SA178RI with 1 mM IPTG for 3 hours, pelleted, and lysed by high-pressure homogenizer. The 6xHis-tagged proteins were then purified by Ni-NTA column and viewed on an acrylamide gel. See Fig. 7 for initial purification results.

Further examination of purified 6xHis-tagged proteins (Fig. 8) on a 16.5% tris-tricine acrylamide gel reveals that the peptide expressed from pJHC27 appears as two distinct bands, likely indicating that some of the overexpressed wild-type L27 was cleaved though a portion of uncleaved peptide remains. Only one band is visible in this range for overexpressed truncated

L27 from pJHC28, corresponding to the shorter polypeptide. Interestingly, only the smaller band is seen for the overexpressed L27 with the predicted cleavage site mutation, encoded by pJHC35M. This suggests that the dual phenylalanine to alanine mutation in the cleavage site does not prevent cleavage or is cleaved by a protease different from that cleaving wild-type L27. All fractions from this overexpression, including those shown in Fig. 6, also contain additional material at the <10 kDa range. These bands have been seen in multiple sets of lysates of pJHC35M overexpressions in SA187RI and have only been observed in those lysates. It is possible that this mutant is still cleaved but produces an excess of leftover peptides (the cleaved N-terminal sequence is about 1.1 KDa) not recognized by any downstream protein interactions.

Figure 7. PAGE of protein fractions from expression from plasmids pJHC27, pJHC28, and pJHC35M in *S. aureus* strain SA178RI. Gel is XT Criterion 12% bis-tris acrylamide (Bio-Rad). Marker is Bio-Rad Dual Color Precision Plus protein marker (Bio-Rad). Samples are marked as follows: FT are column flow-through, W is wash fraction, and E is elute fraction. Lower inset shows bands of purified protein. Overexpression products are denoted as follows: 27 is from full-length *S. aureus rpmA*, 28 is from 9-codon truncated *S. aureus rpmA*, and 35M is from *S. aureus rpmA* with cleavage site mutation.

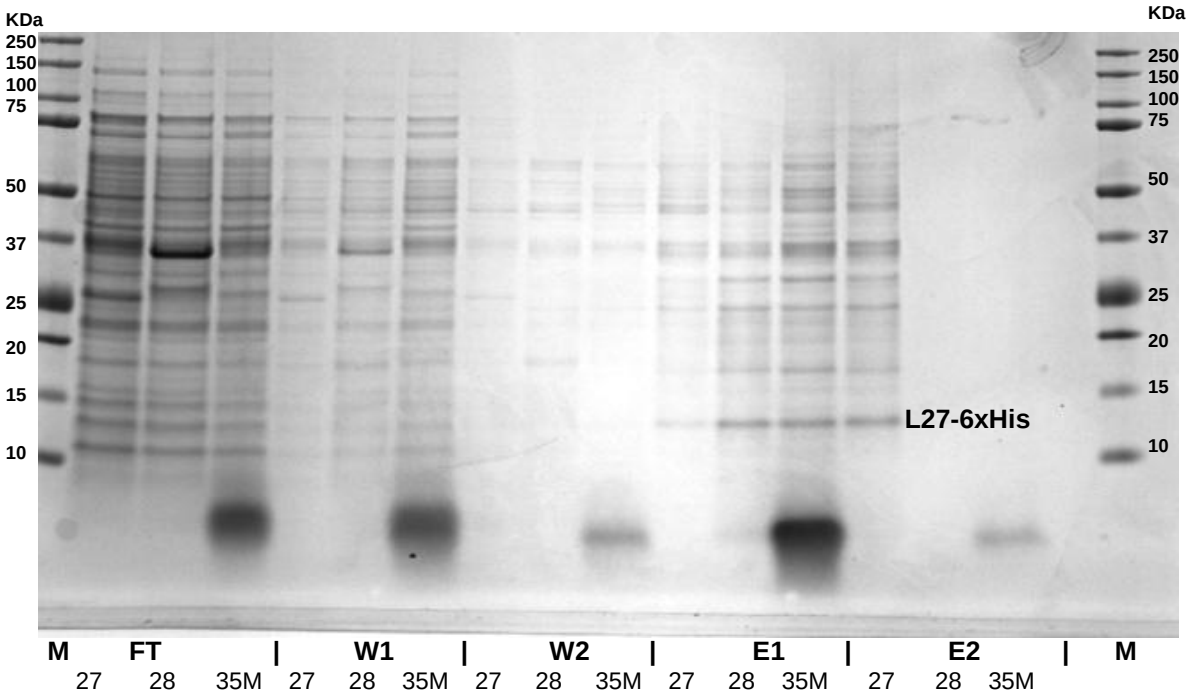
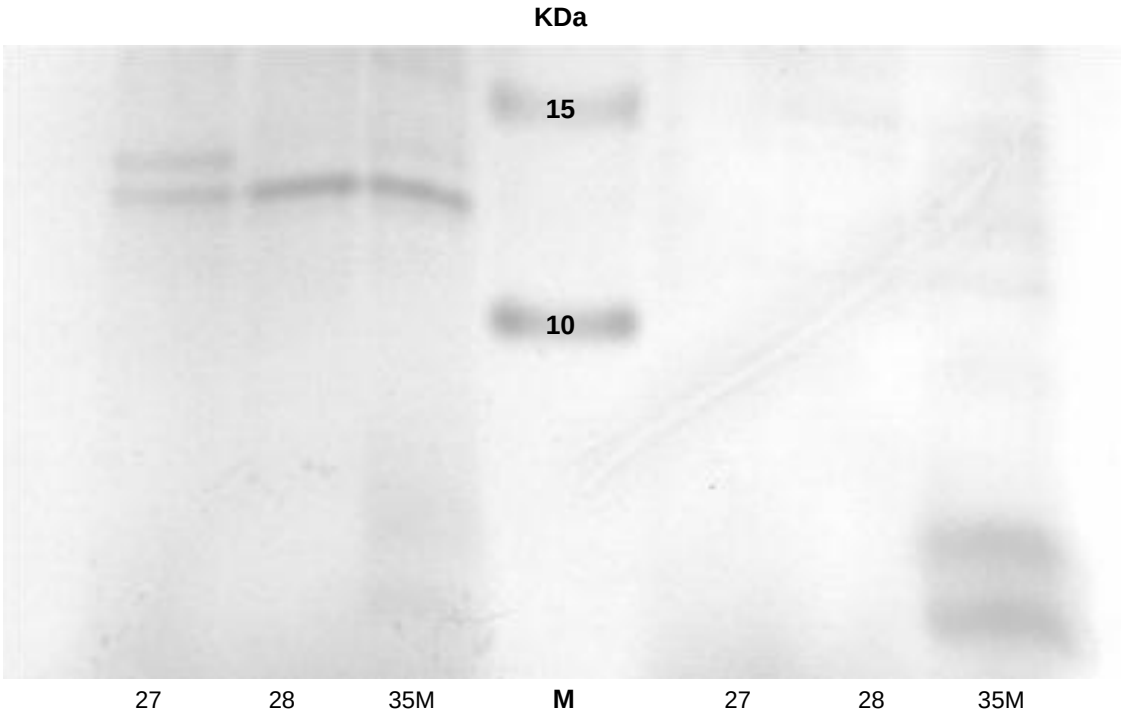

KDa
250
150
100
75
50
37
25
20
15
10
KDa
250
150
100
75
50
37
25
20
15
10
L27-6xHis
M FT | W1 | W2 | E1 | E2 | M
27 28 35M 27 28 35M 27 28 35M 27 28 35M 27 28 35M

Figure 8. High-resolution PAGE of protein fractions from expression from plasmids pJHC27, pJHC28, and pJHC35M in *S. aureus* strain SA178RI compared by peptide size. Gel is XT Criterion 16.5% tris-tricine acrylamide (Bio-Rad). Marker is Bio-Rad Dual Color Precision Plus protein marker (Bio-Rad). Overexpression products are denoted as follows: 27 is from full-length *S. aureus rpmA*, 28 is from 9-codon truncated *S. aureus rpmA*, and 35M is from *S. aureus rpmA* with cleavage site mutation.


KDa
15
10
27
28
35M
M
27
28
35M

CHAPTER 4

Extent of potential for L27 processing across divergent bacterial species

Differences between L27 protein sequences across bacterial species allow for grouping of species into two main groups: those coding for "long" L27 with an N-terminus resembling that coded for in *S. aureus*, or "short" L27 with an N-terminus resembling that coded for in *E. coli.* Species with similar 16S rRNA were found to be more likely to share a long or short L27 N-terminus (see Figure 9 for phylogenic tree), with a few notable exceptions. Interestingly, most long L27 species, like *S. aureus*, are Gram-positive. The Mycoplasma species were found to divert from this trend; the *M. mycoides* and *M. genitalium* genomes both appear to code for long L27, while *M. hyopneumoniae rpmA* appears to code for short L27. *M. hyopneumoniae* has been previously found to be similar to *M. suipneumoniae*, a species characteristically different from most other Mycoplasma known at the time (Goodwin et al., 1967) but a physical dissimilarity within the otherwise highly conserved ribosome structure suggests a need for further phylogenic clarification of the Mycoplasma species. This difference is especially interesting when viewed in context of *M. genitalium,* as the minimal genome of this species contains very few nonessential genes.

Bacterial species in which *rpmA* codes for a longer protein also carry the gene in a different genetic context from those coding for a shorter protein. This could simply be due to differences in entire ribosomal structures, but each context nonetheless appears to be conserved (see Figures 9 and 10). Genomes containing *rpmA* coding for L27 with a short or *E. coli*-like

L27 appear to carry a bicistronic operon containing the gene and *rplU*, coding for ribosomal protein L21. In *E. coli*, deletion of L27 leads to formation of ribosomal particles deficient in several other ribosomal proteins including L21 (Wower et al., 1998). Genomes containing *rpmA* coding for L27 with a longer N-terminus – with a few exceptions noted below – contain the gene as the last member of a set of three genes including not only *rplU* but also an ortholog of the *Bacillus subtilis* gene *ysxB* (see NCBI Gene ID 937961) (Fig. 10). The gene *ysxB* may have a ribosome-associated function as it is sandwiched by two ribosomal protein genes. It is highly conserved in sequence yet bears little similarity to any known protein or functional domain. It may have a function in the stringent response in *B. subtilis*, as *ysxB* transcription in this species is downregulated by the ribosome-bound (p)ppGpp synthetase RelA, a sensor of amino acid starvation (Eymann et al., 2002). It is possible that *rpmA*, *ysxB*, and *rplU* are transcribed together, though there may not be a direct transcriptional relationship between *rpmA* and *rplU* (Nomura et al., 1984).

Mycoplasma hyopneumoniae appears to be an outlier in the phylogeny, with an L27 N-terminus unlike the other *Mycoplasma* species examined. This was included as an interesting exception, as it may be genetically related to other *Mycoplasma* species in 16S rRNA sequence but has phenotypic differences distinguishing it from other mycoplasmas (Goodwin et al., 1967). Interestingly, work by Garcia-Vallvé et al. (2002) shows how *Arthrobacter* species may have acquired an L27 gene from a *Bacillus* species through horizontal gene transfer. Horizontal transfer may be responsible for acquisition of ribosomal proteins in other species as well.

It should be noted that the genomes of *Thermotogae* species *Thermotoga maratima* and *Thermosipho africanus* do appear to contain a *ysxB* gene between those coding for L27 and L21.

In both cases, this gene bears little sequence identity to that of *ysxB* in *S. aureus* or *B. subtilis* but does show some sequence similarity to *ysxB* in other members of *Firmicutes* such as *Eubacterium eligens*. Work by Nesbø et al. (2009) observed numerous instances of potential lateral genetic transfer between *Thermosipho africanus* and *Firmicutes*. This gene of unknown function may have originated with the *Firmicutes* and was later transferred laterally to a species of *Thermotogae.*

The bacterial species examined here demonstrate a clear dichotomy of L27 types. Most species within *Firmicutes* appear to code for L27 with an N-terminal extension while other bacterial species do not code for this extension. The sequence of this extension is highly conserved but is not identical in all species. Additionally, presence of the gene *ysxB* is almost exclusive to *Firmicutes* and may be related to presence of long L27, if only in proximity to the gene *rpmA.*

Figure 9. Phylogenic comparison of bacterial species by 16S rRNA gene, L27 protein N-terminus, and presence of *ysxB*. Strain names in blue contain *rpmA* coding for an L27 protein with no N-terminal extension. Strain names in red contain *rpmA* coding for an L27 protein with an N-terminal extension. The column '*ysxB*' indicates whether a given genome appears to contain a *ysxB* gene immediately adjacent to *rpmA*. 'L27 N-terminus' displays an alignment of the L27 protein from its N-terminal end to one amino acid after its predicted cleavage site. Tree was produced by neighbor-joining method with Jukes-Cantor distance model using MEGA 5 software package (Tamura et al., 2011). *Nanoarchaeum equitans Kin4-M* sequence is included as root.

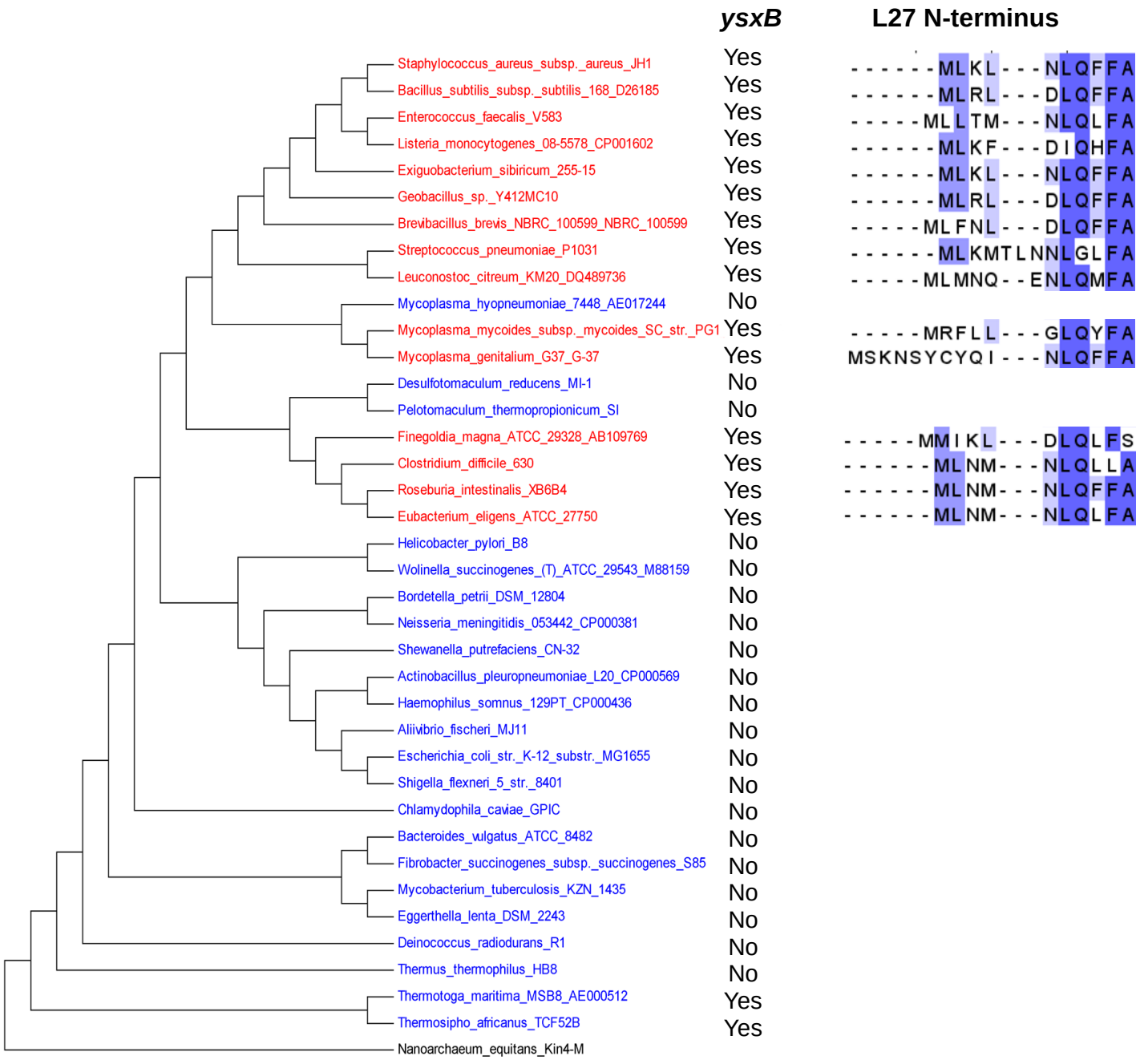
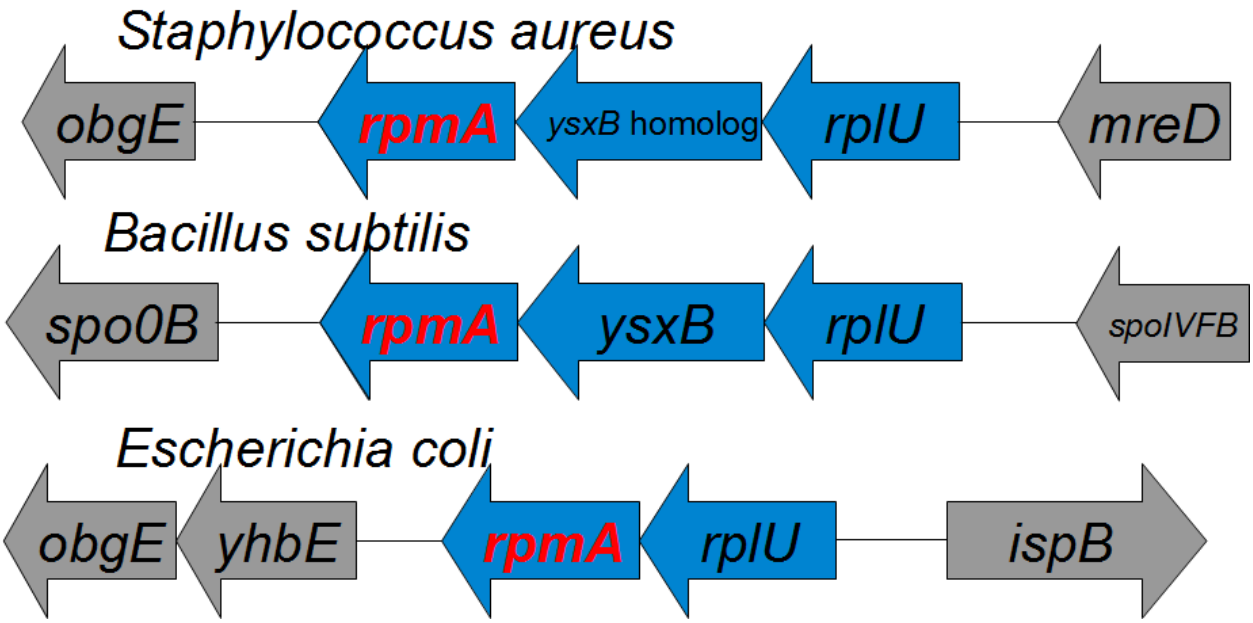

ysxB
L27 N-terminus
Staphylococcus_aureus_subsp._aureus_JH1
Bacillus_subtilis_subsp._subtilis_168_D26185
Enterococcus_faecalis_V583
Listeria_monocytogenes_08-5578_CP001602
Exiguobacterium_sibiricum_255-15
Geobacillus_sp._Y412MC10
Brevibacillus_brevis_NBRC_100599_NBRC_100599
Streptococcus_pneumoniae_P1031
Leuconostoc_citreum_KM20_DQ489736
Mycoplasma_hyopneumoniae_7448_AE017244
Mycoplasma_mycoides_subsp._mycoides_SC_str._PG1
Mycoplasma_genitalium_G37_G-37
Desulfotomaculum_reducens_MI-1
Pelotomaculum_thermopropionicum_SI
Finegoldia_magna_ATCC_29328_AB109769
Clostridium_difficile_630
Roseburia_intestinalis_XB6B4
Eubacterium_eligens_ATCC_27750
Helicobacter_pylori_B8
Wolinella_succinogenes_(T)_ATCC_29543_M88159
Bordetella_petrii_DSM_12804
Neisseria_meningitidis_053442_CP000381
Shewanella_putrefaciens_CN-32
Actinobacillus_pleuropneumoniae_L20_CP000569
Haemophilus_somnus_129PT_CP000436
Aliivibrio_fischeri_MJ11
Escherichia_coli_str._K-12_substr._MG1655
Shigella_flexneri_5_str._8401
Chlamydophila_caviae_GPIC
Bacteroides_vulgatus_ATCC_8482
Fibrobacter_succinogenes_subsp._succinogenes_S85
Mycobacterium_tuberculosis_KZN_1435
Eggerthella_lenta_DSM_2243
Deinococcus_radiodurans_R1
Thermus_thermophilus_HB8
Thermotoga_maritima_MSB8_AE000512
Thermosipho_africanus_TCF52B
Nanoarchaeum_equitans_Kin4-M
Yes
Yes
Yes
Yes
Yes
Yes
Yes
Yes
Yes
No
Yes
Yes
No
No
Yes
Yes
Yes
Yes
No
No
No
No
No
No
No
No
No
No
No
No
No
No
No
Yes
Yes
------MLKL---NLQFFA
------MLRL---DLQFFA
-----MLLTM---NLQLFA
------MLKF---DIQHFA
------MLKL---NLQFFA
------MLRL---DLQFFA
-----MLFNL---DLQFFA
------MLKMTLNNLGLFA
-----MLMNQ--ENLQMFA
-----MRFLL---GLQYFA
MSKNSYCYQI---NLQFFA
-----MMIKL---DLQLFS
------MLNM---NLQLLA
------MLNM---NLQFFA
------MLNM---NLQLFA

Figure 10. Genomic context of *rpmA* genes in three representative species. Genomes continue beyond these segments to the left and the right.


Staphylococcus aureus
obgE
rpmA
ysxB homolog
rplU
mreD
Bacillus subtilis
spo0B
rpmA
ysxB
rplU
spoIVFB
Escherichia coli
obgE
yhbE
rpmA
rplU
ispB

CHAPTER 5

DISCUSSION

It is clear that the ribosome serves a purpose critical to the life of every bacterial cell. This complex incorporates RNA and proteins in a flexible structure with numerous transitional states. It does appear that not every 70S ribosome is equivalent: while the division of ribosomal subtypes into the eukaryotic 80S ribosome and the prokaryotic 70S ribosome may provide a convenient classification method, 70S ribosomes may show important distinctions among divergent bacterial species. The genes coding for ribosomal RNAs are known to be more highly conserved in some sequence regions than others when compared across species. This work shows how a ribosomal protein may be processed in some, but not all, bacterial species.

The results of this study show that ribosomal protein L27 likely undergoes processing in *S. aureus*. The extent of this processing may vary by species. *E. coli* appears to code for a shorter L27 protein sufficiently similar to *S. aureus* L27 to allow an N-terminally truncated version of the *S. aureus* protein to be used in place of the native protein. Unmodified *S. aureus* L27 cannot be used in the same way, potentially due to poor fit into the ribosome at the peptidyl transferase center. It is clear that extensive further study will be necessary to properly explain how L27 reaches its mature position in ribosomes.

The ribosome structure is conserved throughout bacteria, so conservation of the structure of each ribosomal protein is expected. The comparisons in this study show how otherwise conserved proteins may participate in different interactions within different branches of a phylogenetic tree. *E. coli* appears to produce an L27 protein corresponding to the size of the

gene *rpmA*. *S. aureus* and *B. subtilis*, however, carry a longer gene but produce an L27 protein of the same size as that produced by *E. coli*. Bacterial species related to *S. aureus* and *B. subtilis* carry L27 genes coding for the same conserved N-terminal extension though the full length of the coded sequence would be unlikely to fit into a ribosomal structure (see Ban et al., 2000). An overall conclusion can be made: species more genetically similar to *S. aureus* code for L27 with an extended N-terminal domain not seen in species more genetically similar to *E. coli*. Most if not all of the extended L27 proteins likely undergo post-translational cleavage of their extensions, yielding proteins similar in sequence to that of *E. coli*. Extrapolating from observations of bacteriophage 80α, it also appears possible that phages of other hosts may require this post-translational modification during their own assembly stages.

Genomic context may be critical to understanding the relationships between L27 and other gene products. Upstream of *rpmA* is a very short IGR (intergenic region) and a hypothetical protein-coding gene. Further upstream is the gene for ribosomal protein L21, *rplU*. Further upstream from this point is an IGR of approximately 250 bp, followed by a non-ribosomal protein gene (rod-shape determining protein MreD). The hypothetical gene is conserved in *S. aureus* and has protein sequence similarity to putative ribosomal protein genes in a number of other organisms, including *Staphylococcus lugdunensis* and *Bacillus amylolique*, as determined by tBLASTx search. This gene is not present in *E. coli* or most other Gram negative organisms. Downstream of *rpmA* in *Staphylococcus aureus* is a roughly 400 bp IGR and a non-ribosomal protein coding gene (*obgE*, for GTPase ObgE). The genes *rplU* and *rpmA* may form an operon with the hypothetical protein-coding gene. If this gene is a protease and is essential to processing of the ribosomal protein, it would make sense for it to be transcribed along with the

ribosomal protein (and at the high rate of transcription expected for that a ribosomal protein gene). This gene is also highly conserved throughout species of *Firmicutes*.

Post-translational processing of a bacterial ribosomal protein would qualify as a novel observation not only for bacteriologists but for phage researchers. This particular instance of post-translational protein cleavage is likely that which enables cleavage of the capsid and scaffold proteins in bacteriophage 80α. While other bacteriophages are known to require protein cleavage during their assembly and maturation, this protein processing is generally thought to require a phage-encoded prohead protease. Phages such as 80α may have lost a prohead protease in exchange for a conserved ribosome-associated protease, enabling them to use smaller genomes.

The next step in this study is to determine which protease in *S. aureus* may act upon ribosomal proteins. Attempts to produce a distinct phenotype in complementation assays were unsuccessful, so other methods may be required to isolate this enzyme. *E. coli rpmA* deletion strains other than IW312, such as one used by Shoji et al. (2011), exist and have been complemented effectively. These strains may be useful for further complementation assays with *S. aureus* genes. Assaying for protein-protein interactions may prove fruitful. This could be done through a combination of bioinformatics and biomolecular methods: potential *S. aureus* protease genes could be identified and assayed for binding and/or cleavage of L27. Expressing these genes as part of a bacterial two-hybrid system along with *S. aureus* L27 – or even the bacteriophage 80α capsid and scaffold proteins – could reveal some of the non-transient interactions that may occur during N-terminal processing.

Any potential protease could currently be a protein of unknown function or functions

other than ribosome modification. Work by Cooper et al. (2009) found that deletion of the gene for GTPase YsxC in *S. aureus* is lethal. This enzyme was also found to interact with ribosomal proteins of the 50S subunit. Previous work with YsxC in *Bacillus subtilis* suggested that this protein binds to L27 (Wicker-Planquart et al. 2008) and that ribosomal precursors isolated from cells depleted of YsxC lacked L27 (Schaefer et al. 2006). Proteases modifying ribosomal proteins may not cleave those proteins exclusively – just as they may also cleave bacteriophage proteins.

Isolation of the protease in question and assaying its activity could conclusively prove its role in post-translational processing. Expression of the protease concurrent with *S. aureus* L27 in *E. coli* could provide one option, though the *E. coli* background may differ sufficiently from that of *S. aureus* to limit enzyme activity. Zymography may serve as a better alternative as it may permit a greater degree of control over digestion conditions.

Clarification of any mechanisms involving ribosomal protein L27 will reveal further details about how bacterial ribosomes assemble and how they perform their essential function in cells. L27 is involved in 50S ribosomal subunit assembly and the peptidyl transferase reaction (Wower et al., 1998), stabilization of other ribosomal proteins (Voorhees et al., 2009) as well as RNA processing and conformational changes within mature ribosomes (Wower et al., 2005). These findings are also relevant to bacteriophage research, as they demonstrate how phages may use conserved host processes during virion assembly.

The location of this protein in the ribosome and its role in stabilizing the peptidyl transferase center makes its post-translational processing a potential antibiotic target, especially if L27 processing is found to be essential for cell viability. Indeed, oxazolidinone antibiotics

such as linezolid appear to cross-link with L27, interfering with peptide assembly within the *S. aureus* ribosome (Colca et al., 2003). Linezolid may also interfere with tRNAs during peptide formation (Wilson et al., 2007). Linezolid has been shown to be effective against highly pathogenic strains of Gram-positive bacterial species (Perry and Jarvis, 2001). Studies using combinations of linezolid, vancomycin, and imipenem have also proven effective in a mouse model controlling *S. aureus* infections (Ribes et al., 2010).

Even the oxazolidinone compounds are not "silver bullet" antibiotics, as some clinical isolates of *S. aureus* have shown resistance to linezolid after just three weeks of treatment (Wilson et al., 2003). Understanding how to reliably control pathogenic *Staphylococcus aureus* infections remains a challenge, so L27 processing may serve as a novel additional target for potential antibiotic therapies. Life depends upon ribosomes, so we may need to depend upon the differences between ribosomal proteins to control *Staphylococcus* or other bacterial infections.

LITERATURE CITED

Akada, R. (1994). Quick-check method to test the size of *Escherichia coli* plasmids. *BioTechniques*, *17*(1), 58.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., et al. (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular Systems Biology*, *2*(1), 2006.0008.

Ban, N., Nissen, P., Hansen, J., Moore, P. B., & Steitz, T. A. (2000). The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science*, *289*(5481), 905-20.

Bubunenko, M., Baker, T., & Court, D. L. (2007). Essentiality of ribosomal and transcription antitermination proteins analyzed by systematic gene replacement in *Escherichia coli*. *Journal of Bacteriology*, *189*(7), 2844-53.

Chaudhuri, R. R., Allen, A. G., Owen, P. J., Shalom, G., Stone, K., Harrison, M., Burgis, T. A., et al. (2009). Comprehensive identification of essential Staphylococcus aureus genes using Transposon-Mediated Differential Hybridisation (TMDH). *BMC Genomics*, *10*(1), 291.

Christie, G. E., Anders, D. L., McAlister, V., Goodwin, T. S., Julien, B., & Calendar, R. (2003). Identification of Upstream Sequences Essential for Activation of a Bacteriophage P2 Late Promoter. *Journal of Bacteriology*, *185*(15), 4609-4614.

Colca, J. R., McDonald, W. G., Waldon, D. J., Thomasco, L. M., Gadwood, R. C., Lund, E. T., et al. (2003). Cross-linking in the living cell locates the site of action of oxazolidinone

antibiotics. *The Journal of Biological Chemistry*, *278*(24), 21972-9.

Cooper, E. L., García-Lara, J., & Foster, S. J. (2009). YsxC, an essential protein in Staphylococcus aureus crucial for ribosome assembly/stability. *BMC Microbiology*, *9*, 266.

Dokland, T. (1999). Scaffolding proteins and their role in viral assembly. *Cellular and Molecular Life Sciences*, *56*(7-8), 580-603.

D'Elia, M. A., Pereira, M. P., Chung, Y. S., Zhao, W., Chau, A., Kenney, T. J., et al. (2006). Lesions in teichoic acid biosynthesis in *Staphylococcus aureus* lead to a lethal gain of function in the otherwise dispensable pathway. *Journal of Bacteriology*, *188*(12), 4183-9.

Eymann, C., Homuth, G., Scharf, C., & Hecker, M. (2002). *Bacillus subtilis* functional genomics: global characterization of the stringent response by proteome and transcriptome analysis. *Journal of Bacteriology*, *184*(9), 2500-20.

Flinta, C., Persson, B., Jornvall, H., & Heijne, G. V. (1986). Sequence determinants of cytosolic N-terminal protein processing. *European Journal of Biochemistry*, *154*(1), 193-196.

Fox, G. E. (2010). Origin and Evolution of the Ribosome. *Cold Spring Harbor Perspectives in Biology*, *2*: a003483.

Garcia-Vallvé, S., Simó, F. X., Montero, M. A., Arola, L., & Romeu, A. (2002). Simultaneous horizontal gene transfer of a gene coding for ribosomal protein l27 and operational genes in *Arthrobacter* sp. *Journal of Molecular Evolution*, *55*(6), 632-7.

Glass, J. I., Assad-Garcia, N., Alperovich, N., Yooseph, S., Lewis, M. R., Maruf, M., Hutchison, C. A., et al. (2006). Essential genes of a minimal bacterium. *Proceedings of the National*

Academy of Sciences of the United States of America, *103*(2), 425-30.

Goodwin, R. F. W., Pomeroy, A. P., & Whittlestone, P. (1967). Characterization of *Mycoplasma suipneumoniae*: a mycoplasma causing enzootic pneumonia of pigs. *Journal of Hygiene*, *65*(01), 85-96.

Guarente, L. L., G; Roberts, TM; Ptashne, M. (1980). Improved methods for maximizing expression of a cloned gene: a bacterium that synthesizes rabbit β-globin. *Cell*, *20*(2), 543-553.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, *41*, 95–98.

Harms, J., Schluenzen, F., Zarivach, R., Bashan, A., Gat, S., Agmon, I., et al. (2001). High Resolution Structure of the Large Ribosomal Subunit from a Mesophilic Eubacterium. *Cell*, *107*(5), 679-688.

Ji, Y., Zhang, B., Van Horn, S. F., Warren, P., Woodnutt, G., Burnham, M. K., et al. (2001). Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA. *Science, 293*(5538), 2266-9.

Johnson, J. E. (2010). Virus particle maturation: insights into elegantly programmed nanomachines. *Current Opinion in Structural Biology*, *20*(2), 210-6.

Kaan, T., Homuth, G., Mader, U., Bandow, J., & Schweder, T. (2002). Genome-wide transcriptional profiling of the *Bacillus subtilis* cold-shock response. *Microbiology*, *148*(11), 3441-3455.

Katayama, Y., Ito, T., & Hiramatsu, K. (2000). A New Class of Genetic Element, *Staphylococcus* Cassette Chromosome mec, Encodes Methicillin Resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, *44*(6), 1549-1555.

Klevens, R. M., Morrison, M. A., Nadle, J., Petit, S., Gershman, K., Ray, S., et al. (2007). Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *Journal of the American Medical Association*, *298*(15), 1763-71.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, *23*(21), 2947-8.

Lauber, M. A., Running, W. E., & Reilly, J. P. (2009). *B. subtilis* ribosomal proteins: structural homology and post-translational modifications. *Journal of Proteome Research*, *8*(9), 4193-206.

Lessl, M., Balzer, D., Lurz, R., Waters, V. L., Guiney, D. G., & Lanka, E. (1992). Dissection of IncP conjugative plasmid transfer: definition of the transfer region Tra2 by mobilization of the Tra1 region in trans. *Journal of Bacteriology*, *174*(8), 2493-500.

Lindsay, J. A., Ruzin, A., Ross, H. F., Kurepina, N., & Novick, R P. (1998). The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*. *Molecular Microbiology*, *29*(2), 527-43.

Lotti, M., Stöffler-Meilicke, M., & Stöffler, G. (1987). Localization of ribosomal protein L27 at the peptidyl transferase centre of the 50 S subunit, as determined by immuno-electron microscopy. *Molecular & General Genetics*, *210*(3), 498-503.

Maguire, B. A., Manuilov, A. V., & Zimmermann, R A. (2001). Differential effects of replacing

Escherichia coli ribosomal protein L27 with its homologue from *Aquifex aeolicus*. *Journal of Bacteriology, 183*(22), 6565-72.

Maguire, Bruce A, Beniaminov, A. D., Ramu, H., Mankin, A. S., & Zimmermann, Robert A. (2005). A protein component at the heart of an RNA machine: the importance of protein l27 for the function of the bacterial ribosome. *Molecular Cell, 20*(3), 427-35.

Nesbø, C. L., Bapteste, E., Curtis, B., Dahle, H., Lopez, P., Macleod, D., et al. (2009). The Genome of *Thermosipho africanus* TCF52B: Lateral Genetic Connections to the *Firmicutes* and *Archaea*. *Journal of Bacteriology*, *191*(6), 1974–1978.

Noller, H. F., Hoffarth, V., & Zimniak, L. (1992). Unusual resistance of peptidyl transferase to protein extraction procedures. *Science, 256*(5062), 1416-9.

Nomura, M., Gourse, R., & Baughman, G. (1984). Regulation of the synthesis of ribosomes and ribosomal components. *Annual Review of Biochemistry, 53*, 75-117.

Novick, Richard P, Christie, G. E., & Penadés, J. R. (2010). The phage-related chromosomal islands of Gram-positive bacteria. *Nature Reviews. Microbiology*, *8*(8), 541-51.

Ohashi, Y., Inaoka, T., Kasai, K., Ito, Y., Okamoto, S., Satsu, H., et al. (2003). Expression Profiling of Translation-associated Genes in Sporulating *Bacillus subtilis* and Consequence of Sporulation by Gene Inactivation. *Bioscience, Biotechnology, and Biochemistry*, *67*(10), 2245-2253.

Overbeek, R., Begley, T., Butler, R. M., Choudhuri, J. V., Chuang, H.-Y., Cohoon, M., de Crécy-Lagard, V., et al. (2005). The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. *Nucleic Acids Research*, *33*(17), 5691-702.

Perry, C. M., & Jarvis, B. (2001). Linezolid: a review of its use in the management of serious gram-positive infections. *Drugs, 61*(4), 525-51.

Poliakov, A., Chang, J. R., Spilman, M. S., Damle, P. K., Christie, G. E., Mobley, J. A., et al. (2008). Capsid size determination by *Staphylococcus aureus* pathogenicity island SaPI1 involves specific incorporation of SaPI1 proteins into procapsids. *Journal of Molecular Biology, 380*(3), 465-75.

Ribes, S., Pachón-Ibáñez, M. E., Domínguez, M. A., Fernández, R., Tubau, F., Ariza, J., et al. (2010). In vitro and in vivo activities of linezolid alone and combined with vancomycin and imipenem against *Staphylococcus aureus* with reduced susceptibility to glycopeptides. *European Journal of Clinical Microbiology & Infectious Diseases.*

Schaefer, L., Uicker, W. C., Wicker-Planquart, C., Foucher, A.-E., Jault, J.-M., & Britton, R. A. (2006). Multiple GTPases participate in the assembly of the large ribosomal subunit in *Bacillus subtilis*. *Journal of Bacteriology, 188*(23), 8252-8.

Selmer, M., Dunham, C. M., Murphy, F. V., Weixlbaumer, A., Petry, S., Kelley, A. C., et al. (2006). Structure of the 70S ribosome complexed with mRNA and tRNA. *Science, 313*(5795), 1935-42.

Shajani, Z., Sykes, M. T., & Williamson, J. R. (2010). Assembly of Bacterial Ribosomes. *Annual Review of Biochemistry*.

Shoji, S., Dambacher, C. M., Shajani, Z., Williamson, J. R., & Schultz, P. G. (2011). Systematic Chromosomal Deletion of Bacterial Ribosomal Protein Genes. *Journal of Molecular Biology, 413*(4), 751‑761.

Stefani, S., & Goglio, A. (2010). Methicillin-resistant *Staphylococcus aureus*: related infections and antibiotic resistance. *International Journal of Infectious Diseases*, *14*, S19-S22.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*, *29*(5).

Tallent, S. M., Langston, T. B., Moran, R. G., & Christie, G. E. (2007). Transducing particles of *Staphylococcus aureus* pathogenicity island SaPI1 are comprised of helper phage-encoded proteins. *Journal of Bacteriology*, *189*(20), 7520-4.

Tenson, T., Xiong, L., Kloss, P., Mankin, A. S. (1997). Erythromycin resistance peptides selected from random peptide libraries. *Journal of Biological Chemistry*, *272*(28)**,** 17425-17430.

Traub, P., & Nomura, M. (1968). Structure and function of *E. coli* ribosomes. V. Reconstitution of functionally active 30S ribosomal particles from RNA and proteins. *Proceedings of the National Academy of Sciences of the United States of America*, *59*(3), 777-84.

Trobro, S., & Aqvist, J. (2008). Role of ribosomal protein L27 in peptidyl transfer. *Biochemistry*, *47*(17), 4898-906.

Voorhees, R. M., Weixlbaumer, A., Loakes, D., Kelley, A. C., & Ramakrishnan, V. (2009). Insights into substrate stabilization from snapshots of the peptidyl transferase center of the intact 70S ribosome. *Nature Structural & Molecular biology*, *16*(5), 528-33.

Wang, H., Takemoto, C. H., Murayama, K., Sakai, H., Tatsuguchi, A., Terada, T., et al. (2004). Crystal structure of ribosomal protein L27 from *Thermus thermophilus* HB8. *Protein Science*, *13*(10), 2806-10.

Wicker-Planquart, C., Foucher, A.-E., Louwagie, M., Britton, R. A., & Jault, J.-M. (2008). Interactions of an essential *Bacillus subtilis* GTPase, YsxC, with ribosomes. *Journal of Bacteriology*, *190*(2), 681-90.

Wilson, D. N., & Nierhaus, K. H. (2007). The oxazolidinone class of drugs find their orientation on the ribosome. *Molecular Cell*, *26*(4), 460-2.

Wilson, P., Andrews, J. A., Charlesworth, R., Walesby, R., Singer, M., Farrell, D. J., et al. (2003). Linezolid resistance in clinical isolates of *Staphylococcus aureus*. *The Journal of Antimicrobial Chemotherapy*, *51*(1), 186-8.

Wommack, K. E., & Colwell, R. R. (2000). Virioplankton: Viruses in Aquatic Ecosystems. *Microbiology and Molecular Biology Reviews*, *64*(1), 69-114.

Wower, I. K., Wower, J., & Zimmermann, R. A. (1998). Ribosomal protein L27 participates in both 50 S subunit assembly and the peptidyl transferase reaction. *The Journal of Biological Chemistry*, *273*(31), 19847-52. doi:10.1074/jbc.273.31.19847

Wower, I. K, Zwieb, C., & Wower, J. (2005). Transfer-messenger RNA unfolds as it transits the ribosome. *RNA, 11*(5), 668-73.

Zhu, W., Clark, N. C., McDougal, L. K., Hageman, J., McDonald, L. C., & Patel, J. B. (2008). Vancomycin-resistant *Staphylococcus aureus* isolates associated with Inc18-like *vanA* plasmids in Michigan. *Antimicrobial Agents and Chemotherapy*, *52*(2), 452-7.

VITA

John Harry Caufield was born on March 23, 1986 in Chester County, Pennsylvania. He graduated from Downingtown East High School in Lionville, Pennsylvania in 2004. He received his Bachelor of Science in Biological Sciences with a concentration in Biotechnology from the University of Delaware, Newark, Delaware in 2008.