Investigation of the cfr and rlmN genes in linezolid resistance

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DISSERTATION
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This dissertation is dedicated to my parents, Dr. Arthur and Jane LaMarre, and my fiancé Dr. Eugene Tarasov. Without the love, support, guidance and comfort they have shown me, I would not be the person I am today.
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JML
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LIST OF ABBREVIATIONS

[4Fe-4S] Four iron-Four sulfur cluster
ACME Arginine catabolic mobile element
AMV Avian Myeloblastosis Virus
ATP Adenosine triphosphate
BHI Brain heart infusion
bp Base pair
C2 Carbon 2
C8 Carbon 8
CA-MRSA Community acquired methicillin-resistant *Staphylococcus aureus*
cDNA Complementary deoxyribonucleic acid
dATP Deoxyadenosine triphosphate
dCTP Deoxycytidine triphosphate
ddGTP Dideoxyguanosine triphosphate
dGTP Deoxyguanosine triphosphate
DNA Deoxyribonucleic acid
dNTP Deoxynucleoside triphosphate
DTT Dithiothreitol
dTTP Deoxythymidine triphosphate
EDTA Ethylenediaminetetraacetic acid
FeCl₃ Iron (III) chloride
FPLC Fast protein liquid chromatography
HA-MRSA Hospital acquired methicillin-resistant *Staphylococcus aureus*
HCl Hydrogen chloride
HPLC High performance liquid chromatography
IPTG Isopropyl β-D-1-thiogalactopyranoside
K₂HPO₄ Dipotassium phosphate
kb Kilobase
KCl Potassium chloride
LB Luria-Bertani
LEADER Linezolid experience and accurate determination of resistance
MgCl₂ Magnesium chloride
MIC Minimum inhibitory concentration
MRSA Methicillin-resistant *Staphylococcus aureus*
Na₂S Sodium sulfide
NaCl Sodium chloride
NaOH Sodium hydroxide
NH₄Cl Ammonium chloride
Ni-NTA Nickel-nitriloacetic acid
NTP Nucleoside triphosphate
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<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PSM</td>
<td>Phenol soluble modulin</td>
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<td>PTC</td>
<td>Peptidyl transferase center</td>
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<td>PVL</td>
<td>Panton-Valentine leukocidin</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
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<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
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<td>SCC</td>
<td>Staphylococcal chromosomal cassette</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>SDT</td>
<td>Sodium dithionite</td>
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<td>spp</td>
<td>Species</td>
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<td>TCA</td>
<td>Trichloroacetic acid</td>
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<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
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<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
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<td>UV</td>
<td>Ultra violet</td>
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<td>VRE</td>
<td>Vancomycin-resistant enterococci</td>
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<tr>
<td>VRSA</td>
<td>Vancomycin-resistant <em>Staphylococcus aureus</em></td>
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SUMMARY

*Staphylococcus aureus* infections are a major concern in the healthcare field. Methicillin-resistant *Staphylococcus aureus* is of particular concern, as it is a common cause of nosocomial and community-acquired infections that can lead to infective endocarditis, pneumonia, bacteremia, sepsis and death. The development of new antibiotics has been slow over the past three decades, and the emergence of resistance mechanisms to the existing antimicrobials has highlighted the need for novel therapeutic molecules.

Linezolid is an oxazolidinone antibiotic that binds to the 23S ribosomal RNA (rRNA) of the ribosome. Since its release into the market in 2000, it has proven itself a very useful antibiotic due to its potency against Gram-positive pathogens, its ease of administration and its low incidence of side effects. Resistance to linezolid was assumed to be slow due to the synthetic nature of the drug and the novel core structure, to which bacteria had never been previously exposed. However, in 2005, a patient from Colombia harbored a linezolid resistant *S. aureus* isolate that carried the *cfr* gene. Cfr confers resistance to linezolid by attaching a methyl group at C8 of adenine 2503 (A2503) in the rRNA of the large ribosomal subunit. A2503 is located near the peptidyl transferase center and the polypeptide exit tunnel, which is the target of many clinically useful classes of antibiotics. Therefore, besides conferring linezolid resistance, Cfr renders cells resistant to a broad array of other antibiotics including phenicols, lincosamides, streptogramin A, pleuromutilins and some macrolides.

Interestingly, A2503 is the target of action of an endogenous methyltransferase encoded in the chromosomal gene *rlmN* (for ribosomal RNA large subunit methyltransferase gene N), which methylates A2503 at the C2 position. Cfr and RlmN are evolutionarily related enzymes that share 32% identity with one another, and are distinctive in that they are the only known
RNA methyltransferases of the Radical S-adenosylmethionine superfamily of enzymes, which utilizes a [4Fe-4S] cluster to produce radicals for carrying out various chemical reactions.

In order to comprehend the important aspects of the mechanisms of antibiotic resistance associated with modification of A2503 in 23S rRNA, we addressed the following topics: understanding the enzymatic properties of these proteins, the role of A2503 modification by the indigenous and acquired methyltransferases in antibiotic resistance, the genetic environment of the cfr gene and the fitness cost associated with its acquisition.

The rate of dissemination and the efficiency of maintenance of a resistance gene depend on the fitness cost associated with its acquisition. We investigated the fitness cost of cfr expression and found that acquisition of the cfr gene does not produce any appreciable reduction in the cell growth rate. Only in a cogrowth competition experiment was some loss of fitness observed. Cells expressing wild type and catalytically inactive Cfr had similar growth characteristics, indicating that the slight fitness cost associated with cfr acquisition stems from the expression of the Cfr polypeptide, rather than from the modification of the conserved rRNA residue. The generally low fitness cost of cfr acquisition offers a microbiological explanation for the apparent spread of the cfr gene among pathogens. In some clinical isolates, cfr is coexpressed with the erm gene, which encodes a methyltransferase targeting another 23S rRNA residue, A2058. We found that dimethylation of A2058 by Erm notably increases the fitness cost associated with the Cfr-mediated methylation of A2503.

Acquisition of the cfr gene is usually associated with a fairly low level of resistance to linezolid. One of the recent isolates of Staphylococcus epidermidis, designated 426-3147L, taken from a patient in the United States, stands out in this sense because it exhibited the unusually
SUMMARY (continued)

high linezolid MIC of >256 µg/ml. In order to understand the reason for such a high level of resistance, we examined the genetic environment of cfr in *S. epidermidis* 426-3147L and assessed other possible resistance mechanisms. We found that the cfr gene in 426-3147L is associated with a transposon and that transcription from the transposon promoter contributes to the high level of cfr expression. The transposon-cfr cassette is located on a multicopy plasmid, which increases the cfr gene dosage. In addition, we analyzed whether the 426-3147L-specific alterations in the upstream region of the cfr gene affect the proposed inducibility of cfr. However, the mutation in the putative regulatory region does not seem to elevate the expression level of Cfr in 426-3147L. The presence of mutations in rRNA and ribosomal protein genes which likely contribute to the resistance were also identified in this isolate. Thus, a combination of chromosomal mutations, the presence of the acquired cfr gene and the specific genetic environment of cfr account for the unusually high linezolid resistance of this *S. epidermidis* isolate.

The RlmN methyltransferase is responsible for the native modification of A2503 at C2. A recently described *rlmN* mutation in a clinical *S. aureus* isolate from a patient subjected to prolonged linezolid therapy decreases susceptibility to linezolid. Originally, this mutation was thought to increase the extent of A2503 modification. However, we show that the mutation in fact abolishes RlmN activity, resulting in a lack of A2503 modification. This is the first report of a link between RlmN activity and linezolid resistance in the clinical setting. Since many mutations could inactivate the *rlmN* gene, our findings unveil a potential mechanism for future linezolid resistance in clinical strains.

Both Cfr and RlmN belong to the special and poorly understood class of Radical SAM proteins.
enzymes. In collaboration with the Jomaa laboratory at Justus-Liebig-Universität, Giessen and the Galonić-Fujimori laboratory at the University of California San Francisco, we characterized the distinctive enzymatic activity of Cfr and RlmN, focusing on the rRNA substrate requirements and specificity. Having purified the active forms of both enzymes, we undertook a detailed analysis of the rRNA requirements to show that the enzymes can utilize protein-free 23S rRNA as a substrate, but not the fully assembled large ribosomal subunit, suggesting that the methylations take place during the early steps of ribosome assembly. The key recognition elements in the 23S rRNA are helices 90-92 and the adjacent single stranded RNA that encompasses A2503. Furthermore, by providing information on both the timing of methylation and its substrate requirements, our findings have important implications for the functional consequences of Cfr-mediated modification of rRNA in the acquisition of antibiotic resistance.
I. BACKGROUND AND SIGNIFICANCE

A. *Staphylococcus aureus Infections*

*Staphylococcus aureus* is a major pathogen, found in both humans and animals. It was discovered in the 1880s (Deurenberg and Stobberingh, 2008), and was associated with a mortality rate over 80% prior to the use of penicillin in the 1940s (Skinner, 1941). Although penicillin initially provided a useful cure, the 1950s saw outbreaks of penicillin-resistant *S. aureus* in both patients and healthcare workers of maternity and neonatal wards (Grundmann et al., 2006). The development of methicillin was supposed to assuage any concerns about the virulent *Staphylococcus*, but by 1961, the first methicillin-resistant *S. aureus* (MRSA) was documented in the healthcare setting (Jevons, 1961). In more recent years, MRSA has emerged as a serious threat worldwide. Klein et al. estimate that over 270,000 infections per year are MRSA-related in the United States, resulting in ~7,000 deaths annually (Klein et al., 2007). In the United States and Japan, 50% or more of the population are estimated to carry strains of MRSA, and 60% of hospital-associated *Staphylococcal* infections are due to methicillin-resistant strains (2004).

MRSA is responsible for a wide array of clinical problems, including skin and soft tissue infections, surgical site infections, endocarditis, bacteremia, sepsis and pneumonia and is the cause of the majority of nosocomial infections globally (Deurenberg and Stobberingh, 2008; Fowler et al., 2003; Grundmann et al., 2006; Hsu and Chu, 2004; Nubel et al., 2008; Yao et al., 2010). In addition to the hospital-acquired MRSA strains (HA-MRSA), community-acquired MRSA (CA-MRSA) is also a danger to the general population (Prevention, 1999; Udo et al., 1993). CA-MRSA tends to be clonally different from most of the HA-MRSA strains, and is often characterized as being more virulent, due to the expression of various virulence factors such as
Panton-Valentine leukocidin (PVL), the arginine catabolic mobile element (ACME), and phenol-soluble modulins (PSM), although much work is still necessary to understand the mode of pathogenesis (Chambers, 2001; Diep et al., 2008; Etienne, 2005; Löffler et al., 2010; Wang et al., 2007). CA-MRSA strains also differ from their HA counterparts in the Staphylococcal Chromosomal Cassette (SCC) type that they carry, which harbors the *mecA* gene, responsible for methicillin resistance. In general, HA-MRSA strains carry SCCmec type II or III and CA-MRSA most commonly carries SCCmec IV (Boyle-Vavra and Daum, 2007; Diep et al., 2008; Voyich et al., 2006). SCCmec IV tends to be smaller in size and lacks other drug resistance genes, whereas type II and III are large elements that increase the fitness cost of multiple drug resistances, perhaps indicating why HA strains are prevalent in healthcare settings where the use of antibiotics is widespread, but not as prevalent in community settings (Boyle-Vavra and Daum, 2007).

CA-MRSA is emerging as a particular concern because it tends to be more virulent than HA-MRSA, and it affects the general population with strong immune systems and no known risk factors. In particular, CA-MRSA has been documented to infect small children, athletes, military recruits and prison inmates, where it is easily transmissible through skin-to-skin contact, athletic injuries, whirlpools and the sharing of soap and towels (Baillargeon et al., 2004; Begier et al., 2004; Campbell et al., 2004; Kazakova et al., 2005; Lindemayer et al., 1998; Nguyen et al., 2005; Prevention, 1999; Zinderman et al., 2004).

Since MRSA strains often carry more than one resistance gene, or have cross-resistance to several types of antibiotics, physicians have oftentimes needed to use drugs of last resort, such as vancomycin. However, vancomycin resistance in *S. aureus* (VRSA) and enterococci (VRE) has raised concerns over the efficacy of the present antibiotics (Henwood et al., 2000;
Livermore, 2003; Reacher et al., 2000; Sahm et al., 1999). Therefore, it has become increasingly clear that there is an urgent need for other antimicrobial agents in the field.

B. The History of Linezolid

In April of 2000, Pharmacia marketed linezolid, the first antibacterial of the oxazolidinone family (Hutchinson, 2003). This was significant not only because linezolid was the first oxazolidinone to make it through clinical trials, but also because it was the first antimicrobial agent with a novel structure to be released on the market in almost thirty years (Hutchinson, 2003; Livermore, 2003). Oxazolidinones are small antimicrobials characterized by an aromatic ring attached to an oxazolidinone ring by the nitrogen atom; some derivatives also include a ring attached to the distal end of the aromatic ring, as well as modified side chains (Hutchinson, 2003) (Figure 1). Oxazolidinones were first discovered by scientists at E. I. Du Pont de Nemours & Company, and in the late 1980s Slee and coworkers characterized two compounds, DuP 721 and DuP 105, which were potent against Gram-positive organisms (Brickner et al., 2008; Slee et al., 1987).

Du Pont dropped their oxazolidinone project after issues with the toxicity of DuP 721 and DuP 105 arised. However oxazolidinone derivatives were simultaneously being studied by Upjohn Company (later Pharmacia and now Pfizer), who after designing several oxazolidinone compounds, advanced eperezolid and linezolid through development and Phase I clinical trials (Brickner et al., 2008; Hutchinson, 2003). Since linezolid had overall better pharmacokinetic properties and required less dosaging than eperezolid, linezolid was chosen to complete clinical trials (Brickner et al., 2008; Livermore, 2003). Linezolid includes three rings, with an acetamidomethyl tail attached to the oxazolidinone ring (Figure 1) (Wilson et al., 2008).
Figure 1. The structure of linezolid.
Linezolid (commercial name Zyvox™) is an attractive drug due to its efficacy and pharmacokinetic properties, including near 100% bioavailability in humans, easy formulation in intravenous form or oral form, and excellent tissue penetration (Brickner et al., 2008; Dryden, 2011; Rana et al., 2002). Linezolid has been shown to be active against skin and soft tissue infections and bone and central nervous system infections caused by Gram-positive organisms (Moylett et al., 2003; Rana et al., 2002). The toxicity profile of linezolid is also appealing, as the effects of treatment are relatively mild. The most common side effects are diarrhea and nausea, with reversible anemia and thrombocytopenia emerging after prolonged exposure (Brickner et al., 2008).

C. **The Mode of Action and Target of Linezolid**

Linezolid is an antibiotic that binds to the ribosome (Matassova et al., 1999; Wilson et al., 2008; Xiong et al., 2000). The ribosome is the macromolecular apparatus that is responsible for protein synthesis in the cell. In bacteria, it is a massive 2.5 million Dalton machine composed of two subunits, the 50S (large) subunit and the 30S (small) subunit. The 30S subunit, which is comprised of 16S ribosomal RNA (rRNA) and ~20 proteins, is responsible for decoding the messenger RNA (mRNA) and ensuring that the proper aminoacyl-tRNA is accommodated into the ribosome. Peptide bond formation then occurs in the peptidyltransferase center (PTC) of the 50S subunit, which is composed of 23S rRNA, 5S rRNA and ~30 proteins. Finally, the newly synthesized polypeptide departs the large subunit through a channel called the exit tunnel. Since the ribosome is a vital and ancient component of the cell, molecules that target functional centers such as the PTC or exit tunnel are among the most common antibiotics and are used as antibacterials.
While early reports on the binding of oxazolidinones were controversial, the binding and mechanism of action of these antibiotics have been more clearly detailed in recent years. Reports agree that oxazolidinones bind to the large subunit of the bacterial ribosome, although early studies by Matassova suggested that the compound family bound to both the 16S and 23S rRNAs (Matassova et al., 1999). Random mutational studies in the halophilic archaeon *Halobacterium halobium* demonstrated that linezolid resistance mutations, involving such nucleotides as C2499 and U2504 (*E. coli* numbering throughout), clustered in Domain V of 23S rRNA, close to the PTC (Kloss et al., 1999; Xiong et al., 2000). Subsequent studies with *Escherichia coli* by Xiong and colleagues showed that G2032A conferred linezolid resistance (Kloss et al., 1999; Xiong et al., 2000). Furthermore, crosslinking experiments verified that large ribosomal subunit protein L27, located near the PTC and 23S rRNA residues A2602, A2451 and U2506, is found in close proximity to the linezolid binding site. (Colca et al., 2003; Leach et al., 2007).

Confirmation of the antibiotic binding site and its mode of action came from the crystal structures of linezolid bound to the *Deinococcus radiodurans* and *Haloarcula marismortui* 50S subunits, diffracted to 3.3 Å and 2.7 Å, respectively (Ippolito et al., 2008; Wilson et al., 2008). The crystal structure of the *D. radiodurans* subunit demonstrates that linezolid is bound to the 23S rRNA such that the distal ring points toward the PTC and the oxazolidinone ring and the acetamidomethyl tail point down towards the nascent peptide exit tunnel, in close proximity to the nucleotide A2503 (Figure 2). Importantly, U2585, a normally flexible nucleotide, is seen stabilized by interactions coming from the distal ring. The drug overlaps with the aminoacyl group of the tRNA in the A-site. This overlap, along with the inhibition of U2585 to swing freely, seems to prevent proper A-site tRNA accommodation, thereby preventing translation (Wilson et al., 2008).
Figure 2. The binding of linezolid to the ribosome. Linezolid is shown in magenta, and U2585 is shown in blue. Several nucleotides involved in linezolid binding are shown in beige. The bottom panel depicts the same nucleotides in space-filling form. The coordinates are from the *D. radiodurans* structure with linezolid, which is deposited in the Protein Data Bank as 3DLL.
D. **Resistance to Linezolid**

Linezolid is a synthetic drug. It was thought, therefore, that since bacteria had no known natural resistance mechanisms against linezolid, development of resistance would be slow. For a number of years, indeed, the main mechanism of resistance was associated with spontaneous mutations in rRNA. Since linezolid binds to the ribosome, any target site mutations involving 23S rRNA need to be present in several of the multiple ribosomal DNA alleles in order to confer an appreciable level of resistance. However, prolonged treatment with linezolid can provide the selective conditions for dissemination of rRNA mutations to multiple alleles, resulting in clinically-significant resistance to the drug.

1. **Ribosomal ribonucleic acid mutations**

In the previous section, the mutations in *E. coli* and *H. halobium* selected in the laboratory were discussed. In laboratory experiments with enterococci, C2610, G2576, C2512, A2513 and G2505 mutations were discovered, as well as the G2447T mutation in *Mycobacterium smegmatis* (Prystowsky et al., 2001; Sander et al., 2002). Interestingly, in human cytoplasmic ribosomes, G2032 is replaced with C and C2499 is substituted with U, perhaps accounting for the selectivity of linezolid action (Leach et al., 2007; Wilson et al., 2008).

While the above ribosomal mutations identified by artificial selection of the resistant mutants were helpful in determining the linezolid binding region, they did not necessarily reflect what was occurring in clinical isolates. In 2001, Tsiodras et al. reported the first case of linezolid resistance in a clinical *S. aureus* strain. This strain, taken from an 85-year old patient being treated for MRSA-associated peritonitis, was found to carry a G2576T mutation in 5 ribosomal DNA alleles (Meka et al., 2004a; Tsiodras et al., 2001). The same authors later described clinical isolates of *S. aureus* with the T2500A mutation, also conferring linezolid resistance (Meka et al.,
2004b). The G2576T mutation is regarded as the most common mutation leading to a decrease in linezolid susceptibility in clinical strains of staphylococci and enterococci (Auckland et al., 2002; Farrell et al., 2009; Johnson et al., 2002; Jones et al., 2002; Marshall et al., 2002; Meka et al., 2004a). Later studies have shown that clinical strains of *S. aureus* and *Staphylococcus epidermidis* may contain G2447T, C2534T, G2215A and T2504A, either individually or combined with each other or other resistance mechanisms (Kosowska-Shick et al., 2010; Liakopoulos et al., 2009; Wong et al., 2010).

Since bacteria have multiple copies of *rrn* alleles, a mutation in only one copy may not have a large impact on resistance because only a fraction of the ribosomes will contain the mutation. In fact, it has been shown that resistance is directly correlated with a gene dosage effect. Marshall et al. have shown that the minimum inhibitory concentration (MIC) of linezolid increases in proportion to the number of *rrn* genes with the G2576T mutation in clinical strains of *Enterococcus faecium* (Marshall et al., 2002). Wild type cells (0 out of 6 mutated alleles) had an MIC of linezolid of 2 µg/mL, whereas strains with 3 out of 6 mutated alleles had an MIC of 32 µg/mL and the MIC of strains with 4-5 mutant alleles was 64 µg/mL (Marshall et al., 2002). Other studies confirm the gene dosage effect in *Enterococcus faecalis* and *S. aureus* as well (Livermore et al., 2007; Ruggero et al., 2003). The host recombinational machinery is likely responsible for the conversion of multiple alleles from wild type to mutant (Lobritz et al., 2003; Prammananan et al., 1999). Lobritz infers that the rate-limiting step of developing resistance is the occurrence of the mutation in the first 23S locus, but that subsequent recombinational events can be quicker, leading to a rapid rise in resistance levels (Lobritz et al., 2003). The G2576T mutation carries some fitness cost for the bacteria, most likely from adversely interfering with translation in some way (Meka et al., 2004a). In the absence of antibiotic pressure, a strain with
four out of five alleles mutated to G2576T reverted back to wild type in all but one allele (strains that are mutated in all alleles cannot revert back to wild type easily because of the lack of the wild type recombination template) (Pillai et al., 2002). Interestingly, the last mutant allele in the strain with 4 out of five alleles mutated to G2576T never reverted to wild type, indicating that this strain could easily have the G2576T mutation spread back into the other alleles, giving higher resistance, if under selective pressure to do so (Meka et al., 2004a). The above data suggest that while the G2576T mutation (or other rRNA mutations) may carry a fitness cost, a mutation in just one allele can rapidly spread to other alleles.

2. **Mutations in ribosomal proteins**

Aside from ribosomal rRNA mutations, there have been reports of mutations in ribosomal proteins L4 and L3 that may play a role in resistance to linezolid. Wolter et al. reported a mutation in L4 in clinical strains of *Streptococcus pneumoniae*, where double mutations Δ65TrpArg66 or Δ68LysGly69 were found (Wolter et al., 2005). These same mutations also confer resistance to macrolides and chloramphenicol, consistent with the fact that this region of L4 reaches up near the exit tunnel and PTC. When these mutations were engineered into a naïve strain, an increase in MIC was observed, documenting the causative relationship between the L4 mutations and linezolid resistance. It is unknown whether these mutations affect linezolid susceptibility in staphylococci. Interestingly, the engineered streptococcal strains grew slower than the mutant clinical strains, suggesting that in the clinical isolates, the deleterious effect of resistance is compensated by some secondary site mutations or additional mechanisms (Wolter et al., 2005).

Other ribosomal protein mutations in staphylococci have been described, but it is unclear if the individual mutations are responsible for linezolid resistance or if they are necessary to
offset the fitness cost of rRNA mutations that confer drug resistance. Therefore, direct mutagenesis studies are needed to confirm the role of these mutations in linezolid resistance. Nevertheless, since several clinical strains which show decreased susceptibility to linezolid carry mutations in L3 and L4, it is an important topic to address. In experiments selecting for oxazolidinone resistance, the novel mutation Gly155Arg in L3 was shown to occur in *S. aureus* (Locke et al., 2009b). This mutation also gives resistance to pleuromutilins, which also bind to the PTC, but it has not been found in clinical strains resistant to linezolid (Locke et al., 2009b). A deletion of Phe127-His146 in L3 found in a laboratory-selected oxazolidinone-resistant *S. aureus* strain is also suggested to affect binding of oxazolidinones by disturbing the tertiary structure of rRNA near the linezolid binding site (Locke et al., 2009b).

Ribosomal protein mutations in clinical strains have also been documented. In *S. aureus*, a ΔSer145 mutation was identified in L3, and an *S. epidermidis* strain carried an Ala157Arg mutation in L3 as well. It was hypothesized that this mutation may affect the positioning of nucleotides 2505 and 2506 in the linezolid binding site (Locke et al., 2009a). The deletion of Met169-Gly174 in L3 of linezolid-resistant clinical *S. aureus* strains may also force rearrangements near the PTC and disrupt linezolid binding (Locke et al., 2010). Other mutations in protein L3 were found in several clinical isolates of linezolid-resistant *S. epidermidis*. These included the mutations Lys68Asn, Leu101Val, His146Arg/Gln, Met156Thr, Phe147Ile, Val154Leu and the insertion 71GlyGlyArg72 (Kosowska-Shick et al., 2010; Wong et al., 2010). Interestingly, many of these mutations have been described in combination with each other or with other mutations in 23S rRNA, causing higher resistance to linezolid (Kosowska-Shick et al., 2010; Locke et al., 2009a; Locke et al., 2010; Wong et al., 2010). However, for most of the
ribosomal protein mutations, the causative relationships with resistance to linezolid have not
been affirmatively established.

3. Efflux

Efflux pumps are a common mechanism of antibiotic resistance for bacteria. The pumps force out antibacterials and prevent a buildup of antibiotic concentration inside the cell.

Linezolid, which can bind and inhibit E. coli ribosomes, is fairly ineffective for the treatment of Gram-negative infections (Livermore, 2003; Xiong et al., 2000). Multidrug efflux pumps are thought to play a role in the inherent resistance of Gram-negative bacteria to linezolid. For instance, the E. coli acrAB mutant, which is an efflux mutant, is much more sensitive to linezolid than wild type (Xiong et al., 2000). Inhibition of efflux pumps in E. coli by 1-(1-naphthylmethyl)-piperazine resulted in a 16-32 fold lower MIC of linezolid, suggesting that efflux pumps play a role in inhibiting cellular accumulation of the drug (Bohnert and Kern, 2005). This effect was also verified in other Gram-negative organisms including Enterobacter, Acinetobacter and Citrobacter spp (Schumacher et al., 2007).

Efflux pumps in Gram-positive organisms are also of importance for linezolid resistance. Recently, the lmrS gene from S. aureus was identified as a putative efflux pump of the major facilitator superfamily (Floyd et al., 2010). Cloning this gene into a hypersensitive E. coli strain increased the MICs of several antibiotics, including kanamycin, lincosamides and linezolid (Floyd et al., 2010). Homologs of lmrS were found in several bacteria, including Bacillus and Listeria spp., indicating that this efflux pump may play a role in linezolid resistance in other pathogens as well. Furthermore, it has been theorized that some unknown efflux mechanism played a role in the decreased amount of linezolid uptake in a passaged S. epidermidis strain.
selected for linezolid resistance (Sierra et al., 2009). These data indicate that efflux mechanisms in staphylococci may play a significant role in linezolid resistance.

E. **Cfr**

1. **The identification of the cfr gene and its role in drug resistance**

A new linezolid resistance mechanism that has been found in clinical strains in recent years is the presence of the *cfr* gene. Since its appearance in staphylococcal isolates from healthcare facilities, much has been done to attempt to characterize this gene, its mode of action and its spread among bacterial pathogens.

*Cfr* is an acquired gene first described in 2000, when it was found in a *Staphylococcus sciuri* strain isolated from the nasal swab of a calf suffering from a respiratory tract infection (Schwarz et al., 2000). This gene was initially shown to give resistance to chloramphenicol and florfenicol, and was thus named *cfr* for chloramphenicol florfenicol resistance. *Cfr* was of particular interest because it was the first gene described in Gram-positive bacteria that conferred resistance to the combination of florfenicol and chloramphenicol, indicating that it had a unique mechanism of action (Kehrenberg et al., 2005). In addition, footprinting studies showed that *cfr* action prevents the binding of another PTC-targeting antibiotic, clindamycin (Kehrenberg et al., 2005).

*Cfr* had no homology to any efflux pumps or drug-modification proteins, and the mechanism through which the enzyme conferred resistance was still unknown. Therefore, Kehrenberg and colleagues hypothesized that *cfr* acted by modifying the drug target site in the ribosome (Kehrenberg et al., 2005). A specific stop at A2503 in 23S rRNA observed by primer extension experiments and subsequent mass spectrometry, proved that *cfr* added an extra methyl group to this nucleotide, thereby affecting the binding of phenicols and lincosamides to the
ribosome (Kehrenberg et al., 2005). Later studies revealed that *cfr* not only conferred resistance to the three previously mentioned antibiotics (florfenicol, chloramphenicol and clindamycin), but also a wider range of ribosomal antibiotics, all of which have binding sites close to the PTC. In addition to phenicols and lincosamides, *cfr* gives resistance to oxazolidinones, pleuromutilins and streptogramin A antimicrobials, as well as 16-member ring macrolides (Long et al., 2006; Smith and Mankin, 2008).

Toh et al. proposed that Cfr targets C8 in A2503 (Toh et al., 2007). Subsequently, using a 5-tandem mass spectrometry analysis, Giessing et al. confirmed that A2503 was indeed specifically methylated on C8 of the adenine ring by Cfr (Figure 3) (Giessing et al., 2009). A methylation at this position would affect the binding of several classes of antibiotics by either perturbing their binding directly or by eliciting conformational changes in the binding pockets. A2503 is found at the junction of the PTC and peptide exit tunnel. Due to the functional importance of this site, several antibiotic classes bind to this area. It is not surprising, therefore, that the methylation conferred by Cfr gives resistance to so many classes of antibacterials. Due to its multi-drug resistant nature, the movements of *cfr* from staphylococcal animal isolates to the clinical setting, first reported in 2007, as well as its spread within healthcare facilities, have been topics of much discussion in the field (Toh et al., 2007).

2. **The genetic environment of *cfr* in bacterial isolates**

In the *S. sciuri* strain where *cfr* was first described, the gene was present on a 17.1 kb plasmid called pSCFS1, where it was preceded by two overlapping reading frames (ORF), *orf1* and *orf2*, and a putative promoter (Figure 4). *orf1* and *orf2* code for polypeptides of 59 and 44 amino acids long, respectively (Schwarz et al., 2000). It was hypothesized that these two ORFs may play a role in *cfr* expression or inducibility, although no studies have conclusively
Figure 3. Site of modification of adenine 2503 by Cfr. Cfr methylates A2503 at position C8; the methyl is shown in red.
Figure 4. A scheme of the cfr gene organization in pSCFS1. The cfr gene is preceded by two overlapping open reading frames, orf1 and orf2, which could be involved in regulation of cfr expression. The hypothetical cfr promoter is indicated by an arrow.
determined if the expression of \textit{cfr} is inducible. The location of the transcription start site has not been experimentally determined, but a putative computationally predicted promoter was proposed to be located 402 nucleotides upstream of \textit{cfr}.

Since the discovery of \textit{cfr} on pSCFS1, it has been identified in other staphylococcal isolates from animal origins, where resistance to florfenicol is of veterinary importance (Figure 5). Some isolates carried pSCFS3, a 35 kb plasmid which, in addition to \textit{cfr}, also contained the \textit{fexA} gene, a florfenicol-chloramphenicol efflux pump (Kehrenberg et al., 2007; Kehrenberg and Schwarz, 2006). The genetic environment of \textit{cfr} in pSCFS3 was similar to that found in pSCFS1, with some differences (Figure 5). Both plasmids contained the same putative promoter and the two overlapping reading frames, although the intergenic region between \textit{orf2} and \textit{cfr} in pSCFS3 was truncated by \textasciitilde35 bp compared to pSCFS1 (Kehrenberg and Schwarz, 2006). In addition, there was an Ala254Asp point mutation within the Cfr protein, although the Cfr enzyme encoded in the genes of both plasmids was active (Kehrenberg et al., 2005).

pSCFS1 was later identified in \textit{Staphylococcus simulans} strains, and pSCFS3 was also isolated from \textit{Staphylolococcus lentus} and \textit{S. aureus} strains, indicating that \textit{cfr} easily moved between staphylococcal species (Kehrenberg and Schwarz, 2006). Another survey of \textasciitilde25 other staphylococcal isolates uncovered a plasmid designated pSCFS6, which contained the \textit{cfr} and \textit{fexA} genes (Kehrenberg et al., 2007). While the sequence of \textit{cfr} in pSCFS6 matched to the one found in pSCFS1, this plasmid contained the IS21-558 insertion also found in pSCFS3, which may play a role in the mobility of \textit{cfr} (Kehrenberg et al., 2007).

Recently, the presence of \textit{cfr} has been detected in non-staphylococcal species, which either indicates the spread of this resistance mechanism or that the knowledge of \textit{cfr} importance prompted more stringent surveillance. In 2008, a surveillance study for the susceptibility of
Figure 5. The genetic environment of \textit{cfr} in various bacterial isolates from animal hosts. The names of the constructs are listed to the right of the schemes. The \textit{cfr} gene is often associated with other resistance genes including \textit{erm33}, \textit{spc}, \textit{lsbA} and \textit{fexA}. Mobile genetic elements such as IS sequences or \textit{tnp} genes of the transposase family are also found in close proximity to \textit{cfr}.
bacteria to phenicols in Chinese farm animals uncovered a novel *Bacillus* species (BS-01) that contained *cfr* on a plasmid (Dai et al., 2010). This was the first report of *cfr* in a non-staphylococcal strain. In this strain, *cfr* is located on the plasmid along with *ermB* (Figure 5). In 2010, another surveillance study described the isolation of yet another *cfr*-positive *Bacillus* strain (BS-02). Clonal typing suggested that the species were identical, but the plasmid in the BS-02 isolate differed slightly from the plasmid in BS-01 (Zhang et al., 2011). It included the IS256 element, which contains a transposase that most likely was responsible for the mobility of *cfr* (Figure 5) (Zhang et al., 2011).

A recent report by Wang and colleagues identified the presence of *cfr* in a *Proteus vulgaris* strain from a pig, which is the first description of *cfr* in a naturally-occurring Gram-negative organism (Wang et al., 2011). This strain, PV-01, has been shown to contain *cfr* inside the putative *fimD* gene on the chromosome (Figure 5). IS26 is thought to have assisted in integrating the *cfr* gene in the chromosome of this isolate. This observation is of concern because IS26 is present in several Gram-negative organisms, but also shares homology with IS431 in Gram-positive organisms, indicating that this element may be able to not only spread to other Gram-negative strains, but also reintegrate into Gram-positive bacteria (Wang et al., 2011).

The first report of *cfr* in clinical pathogens appeared in 2007, when the *cfr* gene was found to be present in a linezolid-resistant MRSA strain isolated in a hospital in Medellin, Colombia (Toh et al., 2007). There was indication that *cfr* in the CM05 isolate was somehow transferred from staphylococci in the veterinary setting, however the exact route of *cfr* acquisition by CM05 remains unknown. No other patients in the hospital carried CM05, and no one living at home with the patient carried the strain (Arias et al., 2008).
Subsequent studies of the genetic surroundings of \textit{cfr} in the CM05 strain revealed an environment that was previously undescribed for \textit{cfr} in other isolates (Figure 6). Southern blot analysis and full genome sequencing demonstrated that \textit{cfr} was present in the chromosome in one of the 23S rDNA alleles (\textit{rrn4}) (Locke et al., 2011; Toh et al., 2007). The putative \textit{cfr} promoter and part of \textit{orfl} had been disrupted by the insertion of \textit{ermB} from Tn917 (Figure 6) (Smith and Mankin, 2008; Toh et al., 2007). In this setting, \textit{cfr} was under the control of the \textit{ermB} promoter, where both genes (\textit{ermB} and \textit{cfr}) were constitutively expressed (Smith and Mankin, 2008). Since \textit{ermB} also renders cells resistant to several classes of antibiotics, the \textit{ermB-cfr} operon in CM05 (dubbed the \textit{mlr} operon for modification of large ribosomal subunit) renders cells resistant to all major classes of clinical antibiotics to target the large ribosomal subunit (Smith and Mankin, 2008; Toh et al., 2007).

These reports stress that \textit{cfr} is not only spreading among the bacterial population, but that the use of antibiotics in veterinary and agricultural practices can affect the persistence and spread of resistance mechanisms in the clinical setting. Since the description of the CM05 strain, \textit{cfr} has been identified in several other clinical isolates worldwide, highlighting the importance of monitoring this resistance mechanism in the clinical setting.

In 2004, the Linezolid Experience and Accurate Determination of Resistance (LEADER) program was launched as a means to monitor linezolid resistance in medical centers in the United States. In 2007, the LEADER program reported the first strain in the United States found to carry \textit{cfr} (Jones et al., 2002). This strain, designated 426-3147L, was a \textit{S. epidermidis} strain isolated from a 79 year-old female patient in Arizona. Interestingly, no linezolid therapy had been documented for this patient (Mendes et al., 2008). The LEADER data from the same year also detected a \textit{S. aureus} strain (004-373X) that was also \textit{cfr}–positive.
Figure 6. A comparison of the genetic environment of \textit{cfr} in the pSCFS1 plasmid and the chromosome of the \textit{S. aureus} CM05 strain. A. In the plasmid pSCFS1, \textit{cfr} is preceded by two overlapping open reading frames (\textit{orf1} and \textit{orf2}) and its promoter. B. In CM05, the insertion of the Tn917 transposon introduced the \textit{ermB} methyltransferase gene (together with its promoter and a regulatory leader ORF \textit{ermBL}) upstream of \textit{cfr}, thereby disrupting \textit{orf1} and placing \textit{cfr} under the control of the \textit{ermB} promoter, \textit{P}_{erm}. The transposon inverted repeats are indicated by triangles.
This strain was isolated from a 45 year-old female patient in Ohio who had previous exposure to linezolid (Jones et al., 2002; Mendes et al., 2008). Southern blot analysis suggested that \textit{cfr} was carried on plasmids in both of these strains (Mendes et al., 2008). The \textit{S. aureus} strain from Ohio had a downstream region similar to that found in pSCFS3, however the upstream region proved to be unique, as PCR with primers used in pSCFS3 failed (Mendes et al., 2008). The upstream region of 426-3147L also proved to be unique, but further analysis is necessary in both of these strains to determine the environment of \textit{cfr}.

LEADER program data from 2008 and 2009 showed very interesting results. In 2008, only one strain was found to carry \textit{cfr}, and this strain was found in the same healthcare facility in Arizona as the 426-3147L strain isolated in 2007; in addition, the strain was identical to the previous one, indicating that this \textit{cfr}-positive strain could persist in this facility. In 2009, four strains were found to contain \textit{cfr}. One \textit{S. aureus} strain was isolated in Kentucky from a 53 year-old male and one \textit{Staphylococcus capitis} strain was isolated in Michigan from a 74 year-old female; a \textit{S. aureus} strain with \textit{cfr} was isolated in the same facility in Ohio as the strain found in 2007. A \textit{S. epidermidis} strain containing \textit{cfr} was isolated again from the same facility in 2010, and more \textit{S. epidermidis} strains were recovered from the Arizona facility in 2008 and 2009, which appeared to be the same clone as the one described previously (Cercenado, 2010; Farrell et al., 2011). These data indicate that the dissemination of \textit{cfr} may not be quick in the United States, but that this mechanism can persistently reside in strains that have acquired it.

More \textit{cfr}-positive staphylococcal isolates were found in Maryland, Utah and Missouri from 2007-2010 (Cercenado, 2010). Interestingly, the \textit{cfr}-positive \textit{S. aureus} isolates had wild type 23S rRNA and ribosomal proteins L3 and L4; in contrast, the \textit{S. epidermidis} strains
contained a combination of mutations in 23S rRNA, L3 and L4 in addition to cfr (Cercenado, 2010). This often resulted in higher MICs of 128 µg/mL or more for S. epidermidis, compared to the 4-16 µg/mL MIC in the S. aureus strain. Up to now, a total of 3 cfr-carrying clinical strains were reported in the United States in 2007-2008, and 11 cfr-positive strains in 2009-2010.

3. **Cfr in clinics worldwide**

Aside from the cfr-containing strains described in the United States and the CM05 isolate from Colombia, several outbreaks of cfr-carrying pathogens have been reported worldwide, indicating the wide global distribution of cfr-positive clones.

In 2010, isolates were found in Italy, where nine S. epidermidis strains contained cfr in combination with *ermA* (macrolide, lincosamide and streptogramin B resistance) and *vga* (streptogramin A resistance) (Bongiorno et al., 2010). *Cfr* apparently was located on a 50 kb plasmid, but characterization of the environment of cfr has not been further elucidated. Another S. epidermidis isolate from Rome did not have vga, but did carry the *lsaB* gene responsible for lincosamide resistance (Mendes et al., 2010b). This isolate also had mutations in ribosomal protein L3 (Leu101Val, Phe147Leu, Ala157Arg) and in L4 (Asn158Ser) (Mendes et al., 2010b).

Three cfr-containing strains from Guadalajara, Mexico were described in 2010 (Mendes et al., 2010a). Two of these strains were S. epidermidis, and one was *Staphylococcus cohnii*. All of these isolates also contained mutations in L3, L4 or both. It is believed that cfr is present on plasmids in these isolates, with the two S. epidermidis plasmids identical to each other but different from the S. cohnii plasmid (Mendes et al., 2010a). Interestingly enough, only one of the patients had a documented history of linezolid therapy (Biedenbach et al., 2010). An intensive care unit in a hospital in Madrid, Spain has also documented an outbreak of cfr-positive isolates. Over the course of approximately three months, twelve patients in the intensive care unit and
three from other units in the same hospital had *cfr*-positive strains of *S. aureus* (Morales et al., 2010; Sanchez Garcia et al., 2010).

F. **The Mechanism of Action of Cfr and its Indigenous Evolutionary Relative RlmN**

The acquired antibiotic resistance gene *cfr* exhibits a significant degree of similarity (32% identity) to the indigenous bacterial gene *rlmN* (Figure 7) (Kaminska et al., 2010; Toh et al., 2008). RlmN is responsible for one of the native posttranscriptional modifications found in the RNA ribosome (Kowalak et al., 1995; Toh et al., 2008). These posttranscriptional modifications, primarily methylations and pseudouridylations of rRNA, are fairly conserved; however the exact function of most of these modifications remain elusive, and are thought to fine-tune the function of the ribosome (Chow et al., 2007; Toh and Mankin, 2008).

RlmN is a methyltransferase that appends a methyl group to C2 of A2503 (Figure 8) (Kowalak et al., 1995; Toh et al., 2008). While most methylations in rRNA occur at chemically-reactive nitrogen or oxygen centers, RlmN is unusual in that it targets a relatively unreactive carbon (Yan et al., 2010). The exact function of the RlmN-mediated methylation is unclear. A2503 is located at the junction of the PTC and the polypeptide exit tunnel, which is a critical region in the ribosome. The adenine in this position is highly conserved; this fact, along with the unique posttranscriptional modification, which is found in bacteria, uni-cellular eukaryotes and at least one archaeal species, underscores the functional role of A2503 in translation. However, the *rlmN* knock out strain of *E. coli* exhibits only a marginal growth defect, indicating that this modification is not essential to survival in laboratory conditions (Toh et al., 2008). It is unknown, however, if the essentiality of this enzyme and the methylation of A2503 is increased under different circumstances. Vázquez-Laslop and colleagues have shown that the identity of A2503 plays a crucial role in the communication between the nascent peptide in the exit tunnel.
Figure 7. The amino acid sequence alignment of Cfr and RlmN. The conserved cysteines of the CysXXXCysXXXCys Radical SAM motif are boxed in orange. The amino acids are color coordinated. Red: small, hydrophobic; Blue: acidic; Magenta: basic; Green: hydroxyl, sulfhydryl, amine or hydrogen. The alignment was generated using ClustalW.
and the PTC (Vazquez-Laslop et al., 2010). It is thought that A2503 is part of the system that conveys messages to the PTC during active translation of specific peptides such as ErmCL, ErmAL1 and SecM (Vazquez-Laslop et al., 2010). It is possible that the posttranscriptional methylation in this relay system might facilitate the positioning of A2503, thereby affecting its structural neighbors U2504 or A2062. The rearrangement of these nucleotides could be responsible for inducing an inactive conformation of the PTC during nascent peptide-dependent ribosomal stalling (Vazquez-Laslop et al., 2010). However, the complete understanding of the role of this modification is still unclear. Knowledge of the rRNA substrate of the RlmN methyltransferase is also lacking, although it could help identify the stage of ribosomal assembly on which RlmN works, possibly shedding light on the function of the methylation.

The acquired Cfr protein and the indigenous RlmN act on two different carbon centers of the same nucleotide, A2503 (Figure 8). They also are members of the same enzyme superfamily. A phylogenetic comparison of Cfr and RlmN with over 700 other closely related sequences has determined that RlmN is found in species from most bacterial taxons, whereas Cfr is represented in only a few taxons, namely green plants and alveolates in eukaryotes and chlamydiae and staphylococci in bacteria; the transfer of the Cfr/RlmN family into plants most likely occurred through the chloroplast endosymbiont, although the function of these proteins in eukaryotes is unknown (Kaminska et al., 2010). These data suggest that RlmN’s evolution has been shaped by vertical transfer and that it is most likely the ancestral form, whereas Cfr may have evolved in a horizontal manner following rlmA gene duplication (Kaminska et al., 2010). This also suggests that an enzyme conferring multi-drug resistance evolved from a chromosomal housekeeping gene simply by changing its specificity from a C2 carbon center to a C8 carbon center on the same nucleotide.
Figure 8. A2503 methylated by RlmN and Cfr. The top panel depicts the methyl group at C2 of A2503 (added by RlmN) in green and the methyl group at C8 (added by Cfr) in red. The bottom panel depicts linezolid (magenta) bound to the ribosome, with A2503 in orange. The methyl groups added by RlmN and Cfr are shown in green and red, respectively. U2585 (blue) is shown for orientation.
The specific signature CysXXXCysXXCys indicates that the two proteins belong to the Radical SAM (for S-adenosylmethionine) superfamily of enzymes, which is a diverse group of enzymes that carry out several different reactions in the cell. The Radical SAM superfamily of enzymes is estimated to have over 2,000 members in all domains of life (Duschene et al., 2009; Sofia et al., 2001). The common theme among all these enzymes is the reductive cleavage of SAM to a 5'-deoxyadenosyl radical. Central to the production of this radical is the use of a [4Fe-4S] cluster; the three-cysteine motif that binds it is found in all Radical SAM enzymes. The general reaction mechanism of Radical SAM enzymes involves the reduction of the [4Fe-4S] cluster, which cleaves SAM into methionine and a 5'-deoxyadenosyl radical. This radical is highly reactive and ends up abstracting a hydrogen atom from the substrate (e.g. a carbon center), which is then used to carry out the specific reaction characteristic for the particular enzyme (Berkovitch et al., 2004; Wang and Frey, 2007; Yan et al., 2010).

Cfr and RlmN use the mechanism to methylate the C8 or C2 of A2503, respectively (Giessing et al., 2009; Kowalak et al., 1995). Prior to our work (see Results below), nothing was known about the rRNA substrate requirement of these two enzymes. Yet knowledge of the substrate at which these enzymes act may have a critical impact on understanding the functional consequences of their actions. Some enzymes can modify fully assembled ribosomal subunits (Andersen and Douthwaite, 2006; Lesnyak et al., 2007). Others catalyze the rRNA modification only during ribosome assembly because the target site of their action becomes sequestered in the mature ribosome. The extent of rRNA modification, therefore, may be dramatically influenced by the mode of action of the enzyme and the rate of ribosome assembly. Modulating the degree of the rRNA modification could be used as an epigenetic mechanism of controlling gene
expression, and in the case of resistance rRNA methyltransferases such as Cfr or Erm, may significantly affect the level of antibiotic resistance.

In this work, we have studied the fitness cost of the expression of the *cfr* gene, alone and in combination with *ermB*, which has implications on the spread of *cfr* to other bacteria. We have further characterized a *cfr*-containing *S. epidermidis* clinical isolate. We have determined how a mutation in the indigenous *rlmN* gene may affect cell susceptibility to linezolid in the clinical setting. Finally, we have been the first to elucidate purification schemes and activity assays for RlmN and Cfr, and characterized the substrate requirements of these enzymes.
II. MATERIALS AND METHODS

A. Bacterial Strains and Primers

1. Bacterial strains

The bacterial strains of *E. coli*, *S. epidermidis* or *S. aureus* used for all experiments and procedures are listed in Table I.

2. Primers

The primers used in this study are listed in Table II. All of the primers are listed under the name as it appears in this text. The name of the primers as it appears in the laboratory database is included for the future use of the laboratory members. All DNA oligonucleotides were synthesized by Integrated DNA Technologies, Inc (Coralville, IA).

B. Plasmids

All plasmids either constructed in this study or used to construct the plasmids in this study are listed in Table III, and the constructed plasmids are shown in Figure 9.

1. Construction of plasmids for the expression of Cfr and RlmN

The *rlmN* gene was PCR-amplified from genomic DNA of the *E. coli* strain K12 using primers Primer 1 and Primer 2, which included the NdeI and XhoI restriction sites. The PCR product was purified, digested with NdeI and XhoI, and cloned into the corresponding sites of the vector pET-21a (Novagen, Madison, WI). The RlmN protein encoded in the resulting construct, pRlmN, is C-terminally His<sub>6</sub>-tagged.

The *cfr* gene was PCR-amplified from the plasmid pMS2 (Smith and Mankin, 2008), using Primer 3 and Primer 4 which carried the Bpu1102I and NdeI sites. The PCR product was initially cloned into the pGEM-T vector (Promega, Madison, WI). Once the *cfr*-positive clone was identified and the cloned gene sequenced, *cfr* was excised using
Table I. Bacterial strains used in this study

<table>
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<tr>
<th>Strain</th>
<th>Organism</th>
<th>Description</th>
<th>Source</th>
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<tr>
<td>BW25113</td>
<td><em>E. coli</em></td>
<td>Wild type K12</td>
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<tr>
<td>BL21De(3)</td>
<td><em>E. coli</em></td>
<td>Allows expression of proteins under the T7 promoter.</td>
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<td>XL10 Gold</td>
<td><em>E. coli</em></td>
<td>Allows for increased transformation efficiency.</td>
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<tr>
<td>RN4220</td>
<td><em>S. aureus</em></td>
<td>Derived from NCTC8325. Laboratory strain. Restriction deficient.</td>
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<tr>
<td>TOP10</td>
<td><em>E. coli</em></td>
<td>Medium-efficiency chemically competent cells.</td>
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<td>Keio xyla-</td>
<td><em>E. coli</em></td>
<td>JW3537 cells with an in-frame knockout of <em>xylA</em>. Kanamycin&lt;sup&gt;R&lt;/sup&gt;</td>
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<tr>
<td>Keio rlmN-</td>
<td><em>E. coli</em></td>
<td>JW2501 cells with an in-frame knockout of <em>rlmN</em>. Kanamycin&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>426-3147L</td>
<td><em>S. epidermidis</em></td>
<td>Clinical strain. Contains <em>cfr</em>.</td>
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<td>SAV1986</td>
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<td>Newman-derived strain with a transposon insertion in <em>xylA</em>. Erythromycin&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>SAV1218</td>
<td><em>S. aureus</em></td>
<td>Newman-derived strain with a transposon insertion in <em>rlmN</em>. Erythromycin&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>Newman</td>
<td><em>S. aureus</em></td>
<td>Clinical strain. ATCC 25904.</td>
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<td>JKD6210</td>
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<td>Clinical strain. Linezolid&lt;sup&gt;S&lt;/sup&gt;.</td>
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<td>JKD6300</td>
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<td>Clinical strain with a codon insertion in <em>rlmN</em>. Linezolid&lt;sup&gt;R&lt;/sup&gt;.</td>
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1. (Datsenko and Wanner, 2000)
2. Stratagene
3. Stratagene
4. (Giachino et al., 2001)
5. Invitrogen
6. (Baba et al., 2006)
7. (Baba et al., 2006)
8. (Mendes et al., 2008)
9. (Bae et al., 2004)
10. (Bae et al., 2004)
11. (Duthie and Lorenz, 1952)
12. (Gao et al., 2010)
13. (Gao et al., 2010)
Table II. DNA primers used in this study

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<td>Yfgb Forward</td>
<td>GGCCATATGTCTGAACAAATTAGTCAC</td>
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<td>Cfr Forward</td>
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<td>Primer 8</td>
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<td>Primer 9</td>
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<td>Primer 11</td>
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<td>Primer 12</td>
<td>Cfr_C119A</td>
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<td>Primer 19</td>
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<td>Primer 24</td>
<td>Hind Cfr Fwd</td>
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32
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<td>L2585</td>
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<td>Gent Tail Fwd</td>
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<td>Prom Cfr1</td>
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<td>Primer 45</td>
<td>Tn1 Out B</td>
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Table III. Plasmids used in this study

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<tr>
<th>Constructed Plasmids</th>
<th>Selection</th>
<th>Description</th>
<th>Figure</th>
<th>Source</th>
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<tr>
<td>pCfr</td>
<td>Ampicillin</td>
<td>Based on pET15b. <em>cfr</em> is cloned under the T7 promoter and is N-terminally His&lt;sub&gt;6&lt;/sub&gt;-tagged.</td>
<td>9</td>
<td>This study</td>
</tr>
<tr>
<td>pRlmN</td>
<td>Ampicillin</td>
<td>Based on pET21a. <em>rlmN</em> is cloned under the T7 promoter and is C-terminally His&lt;sub&gt;6&lt;/sub&gt;-tagged.</td>
<td>9</td>
<td>This study</td>
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<tr>
<td>pJL1</td>
<td>Ampicillin: <em>E. coli</em>; Chloramphenicol: <em>S. aureus</em></td>
<td>Based on pLI50. Contains <em>cfr</em>, <em>orf1</em>, <em>orf2</em>, the putative <em>cfr</em> promoter and 123 nt upstream of the promoter as seen in pSCFS1</td>
<td>9</td>
<td>This study</td>
</tr>
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<td>pJL1M</td>
<td>Ampicillin: <em>E. coli</em>; Chloramphenicol: <em>S. aureus</em></td>
<td>Identical to pJL1, except that codon 119 is mutated from TGT to GCT (Cys to Ala).</td>
<td>9</td>
<td>This study</td>
</tr>
<tr>
<td>pMS2M</td>
<td>Ampicillin: <em>E. coli</em>; Chloramphenicol: <em>S. aureus</em></td>
<td>Based on pMS2. Contains the <em>ermB</em> promoter, <em>ermBL</em>, <em>ermB</em> and <em>cfr</em> as it appears in pMS2, except that codon 119 of <em>cfr</em> is mutated from TGT to GCT (Cys to Ala).</td>
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<td>This study</td>
</tr>
<tr>
<td>pJL1-7L</td>
<td>Ampicillin: <em>E. coli</em>; Chloramphenicol: <em>S. aureus</em></td>
<td>Based on pLI50. Contains <em>cfr</em>, the <em>orf1</em> partial deletion, <em>orf2</em>, the putative <em>cfr</em> promoter and 17 nt upstream of the promoter as seen in isolate 426-3147L.</td>
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<td>pJL1B</td>
<td>Ampicillin: <em>E. coli</em>; Chloramphenicol: <em>S. aureus</em></td>
<td>Based on pLI50. Contains <em>cfr</em>, <em>orf1</em> (as seen in pSCFS1), <em>orf2</em>, the putative <em>cfr</em> promoter and 17 nt upstream of the promoter.</td>
<td>9</td>
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<th>Used Plasmids</th>
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<td>pET15b</td>
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<td>For expression and purification purposes</td>
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<td>pLI50</td>
<td>Ampicillin: <em>E. coli</em>; Chloramphenicol: <em>S. aureus</em></td>
<td>Shuttle plasmid for use in <em>E. coli</em> and <em>Staphylococcus aureus</em></td>
<td>9</td>
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<td>pMS2</td>
<td>Ampicillin: <em>E. coli</em>; Chloramphenicol: <em>S. aureus</em></td>
<td>Based on pLI50. Contains the <em>ermB</em> promoter, <em>ermBL</em>, <em>ermB</em> and <em>cfr</em> as it appears in isolate CM05</td>
<td>9</td>
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<tr>
<td>pSCFS1</td>
<td>Multiple</td>
<td>Isolated from <em>S. sciuri</em> in a calf. Contains <em>cfr</em> and multiple other resistance genes.</td>
<td>4, 6A</td>
<td>(Schwarz et al., 2000)</td>
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Figure 9. Constructed plasmids in this work. Upper panel: all plasmids constructed with the pLI50 plasmid as a backbone are shown. The location of the HindIII and XbaI restriction sites are depicted in blue. The mutated codon 119 of cfr is shown as a red band. Lower panel: the pRlmN and pCfr plasmids are shown. The backbone of pRlmN is pET21a, and the backbone of pCfr is pET15b. The NdeI and XhoI restriction sites are depicted in blue, and the His$_6$-tag is purple.
NdeI and Bpu1102I and cloned into the corresponding sites of the pET-15b vector (Novagen). A spontaneous mutation in the cfr stop codon that was incidentally generated during the cloning procedure was corrected by site-directed mutagenesis using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The Cfr protein expressed from the resulting construct, pCfr, is N-terminally His6-tagged.

2. **pJL1**

The pJL1 plasmid was constructed for the expression of the cfr gene in *S. aureus* cells. The nucleotide sequence of cfr with its putative promoter as is found in pSCFS1 (Schwarz et al., 2000; Schwarz et al., 2004) was synthesized by BioBasics, Inc. (Markham, Ontario, Canada), and cloned into the HindIII and XbaI sites of the pLI50 shuttle plasmid to produce the plasmid pJL1 (Lee et al., 1991; Schwarz et al., 2004; Schwarz et al., 2000).

3. **pJL1M**

To engineer the pJL1M plasmid, which carries a catalytically inactive version of Cfr, a mutation was introduced in the cfr gene of the pJL1 plasmid. Specifically, the wild type cfr codon 119 (TGT), encoding a catalytic cysteine, was mutated to GCT (alanine) by using a QuikChange XL site-directed mutagenesis kit and Primer 12 (Giessing et al., 2009).

4. **pMS2M**

In order to construct a plasmid that expressed wild type ErmB but mutant Cfr, the previously constructed plasmid pMS2, which contains the cfr and ermB genes in the genetic context present in the clinical CM05 strain, was used to produce the pMS2M plasmid by mutating the 119th cfr codon TGT to GCT as described above (Toh et al., 2007).

5. **pJL1-7L**
The cfr gene, including the 5'-upstream region until the insertion of IS256 found in S. epidermidis isolate 426-3147L, was cloned into the shuttle plasmid pJL1 (see above), creating plasmid pJL1-7L. This construct contains the deletion in orf1 as is found in isolate 426-3147L. The backbone for this construct was prepared by digesting 0.5 µg of pJL1 with 25 units each of HindIII and SphI (Fermentas, Glen Burnie, MD) in the presence of 1x Tango buffer (50 µL total volume) for 3 h at 37 °C. The mixture was heat inactivated at 80 °C for 20 min. Two units of shrimp alkaline phosphatase (Fermentas) were added for 1 h at 37 °C, and the enzyme was heat inactivated at 65 °C for 15 min.

The insert containing cfr and its regulatory region was amplified by PCR. In a total volume of 120 µL, 700 ng of total DNA from isolate 426-3147L was mixed with 240 pmol each of Primer 24 and Primer 25, Accuprime 1x Buffer II and 3 U of HiFi Taq polymerase (Invitrogen, Carlsbad, CA). The amplification reaction consisted of a denaturation step at 94 °C for 120 sec, 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 68 °C for 80 sec. A final extension step was carried out at 68 °C for 60 sec. The resulting PCR products were cleaned using the QIAquick PCR purification kit (Qiagen, Germantown, MD). The cleaned PCR products were digested in a total volume of 50 µL by digesting 0.5 µg of each PCR product with 25 units each of HindIII and SphI in the presence of 1x Tango buffer overnight at 37 °C. The enzymes were heat inactivated at 80 °C for 20 min.

To ligate the vector backbone to the PCR inserts, a 1:5 molar ratio of vector: insert was incubated overnight at 16 °C in the presence of 5 U of T4 DNA ligase (Fermentas) in a total volume of 20 µL. Two µL of the ligation product were transformed into E. coli XL10 Gold cells (Stratagene) and selected on Luria-Bertani (LB) agar (BD Diagnostics, Sparks, MD) plates containing 100 µg/mL ampicillin. After characterization, the plasmid was transformed into S.
**aureus** (see below). The transformants were selected using brain heart infusion (BHI) agar (BD Diagnostics) plates containing 30 µg/mL chloramphenicol.

6. **pJL1B**

The plasmid pJL1B, to be used as a control with plasmid pJL1-7L, was constructed from the pJL1 plasmid. pJL1B differs from pJL1 in that it contains the 5'-upstream region and *cfr* gene found in pSCFS1, but only up until the same point as pJL1-7L. To construct pJL1B, 0.5 µg of pJL1 was digested with 25 units each of HindIII and SphI in the presence of 1x Tango buffer (50 µL total volume) for 3 h at 37 °C. The mixture was heat inactivated at 80 °C for 20 min. Two units of shrimp alkaline phosphatase were added for 1 h at 37 °C, and the enzyme was heat inactivated at 65 °C for 15 min.

The insert containing the putative promoter, the entirety of *orf1*, *orf2* and *cfr* was amplified by PCR. In a total volume of 120 µL, 500 ng of pJL1 was mixed with 240 pmol each of primers Primer 24 and Primer 25, Accuprime 1 x Buffer II and 3 U of HiFi Taq polymerase. The amplification cycle consisted of a denaturation step at 94 °C for 120 sec, 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 68 °C for 80 sec. A final extension step was carried out at 68 °C for 60 sec. The resulting PCR products were cleaned using the QIAquick PCR purification kit. In a total volume of 50 µL, 0.5 µg of each PCR product was digested with 25 units each of HindIII and SphI in the presence of 1 x Tango buffer overnight at 37 °C. Ten U of DpnI (Stratagene) were added to the sample for one additional hour. The enzymes were heat inactivated at 80 °C for 20 min.

To ligate the plasmid backbone to the PCR inserts, a 1:5 molar ratio of vector: insert was incubated overnight at 16 °C in the presence of 5 U of T4 DNA ligase in a total volume of 20 µL. Two µL of the ligation product were transformed into *E. coli* XL10 Gold cells and selected on
LB agar supplemented with 100 µg/mL ampicillin. After characterization, the plasmid was transformed into *S. aureus* cells. Transformants were selected using BHI agar supplemented with 30 µg/mL chloramphenicol.

C. **Transformation of *Staphylococcus aureus* RN4220**

1. **Preparation of electrocompetent *Staphylococcus aureus* cells**

   *S. aureus* RN4220 cells were made competent by the following procedure. An overnight culture of cells was grown in 10 mL of tryptic soy broth (TSB) medium (BD Diagnostics) at 37 °C with constant shaking at 250 rpm. The culture was then diluted 1:100 in 1 L of fresh TSB broth, and shaken at 37 °C until the cells reached OD$_{600}$ = 0.5. The cells were harvested by centrifugation at 4,000 x g at 4 °C for 15 min. The pellets were washed with 200 mL of chilled wash solution (filter-sterilized 0.5 M sucrose) and repelleted as above. The cells were washed three more times under the same conditions with 30 mL of the cold wash solution. Finally, the cells were resuspended in 10 mL of chilled wash solution, aliquoted into 50 µL batches, flash-frozen and stored at -80 °C until further use.

2. **Electroporation procedure**

   Fifty µL of the electrocompetent cells were thawed on ice. Meanwhile, 0.2-1 µg of plasmid DNA in 5 µL was gently pipetted onto a MF-Membrane 0.025 µm filter (Millipore, Billerica, MA) that was floating in a Petri dish filled with molecular biology grade water. The DNA was dialyzed for 5 min, and then was added to the thawed cells. The mixture was transferred to a chilled 0.1 cm electroporation cuvette (Bio-Rad, Hercules, CA), and was electroporated at 2.5 kV, 100 Ω, and 25 µF (BTX Harvard Apparatus, Holliston, MA). Immediately following electroporation, 750 µL of TSB broth were added to the cells, and the suspension was transferred to 15 mL round bottom tubes (BD Biosciences, Durham, NC) and
incubated at 37 °C for 1 h with shaking. One hundred µL of the cells were plated on BHI agar supplemented with the proper antibiotics and incubated overnight at 37 °C.

The procedure for transforming p7LC into RN4220 was based on the protocol by Lofblom et al., with minor modifications (Lofblom et al., 2007). One µg of plasmid DNA was dialyzed on the Millipore filters as stated above, and then mixed with 50 µL of thawed electrocompetent RN4220 cells. The mixture was incubated for 10 min on ice before being transferred to a 0.1 cm electroporation cuvette and electroporated as already described. One mL of B2 medium (10 g casein hydrolysate, 25 g yeast extract, 5 g glucose, 25 g NaCl, and 1 g K₂HPO₄ per liter, pH 7.5) was added to the porated cells, and they were incubated at 37 °C for 2 h with shaking. The cells were plated on BHI agar supplemented with 10 µg/mL florfenicol. The plates were incubated at 37 °C for 30 h.

D. Microbiological Testing

Minimal inhibitory concentration (MIC) testing was carried out using the CLSI-approved broth microdilution protocol (Clinical, 2006). Overnight cultures of the cells, grown in BHI medium, were diluted 1:100 in fresh media. The cells were grown to an OD₆₀₀ = 0.05 at 37 °C while shaking, and then diluted to an OD₆₀₀ = 0.004. Meanwhile, 50 µL of BHI medium were added to every well of a 96-well plate. Fifty µL of various antibiotics at 4 x the maximum concentration tested were added to the first well. Fifty µL were taken out of the first well and added to the second well. This serial-dilution process was repeated for all the wells, except for the last well of the row, which was a no-antibiotic control. Fifty µL of cells at OD₆₀₀ = 0.004 were added to each well, and the plate was covered and placed inside a container with damp paper towels. The plate was incubated at 37 °C overnight.

E. Isolation of Genomic DNA, Plasmid DNA and Cellular RNA
1. **Isolation of genomic DNA from staphylococci**

Isolation of total genomic DNA from staphylococci was performed using the Master Pure Gram Positive DNA Purification Kit (Epicentre Biotechnologies, Madison, WI), with minor modifications. Five mL of an overnight culture was centrifuged at 1,600 x g for 5 min, and the supernatant was discarded. One hundred fifty µl TE buffer (supplied in the kit) were added and the cells were resuspended by pipetting. The solution was transferred to a clean microcentrifuge tube and 1 µl of Ready-Lyse Lysozyme and 15 µl of 2 mg/mL lysostaphin (Sigma Aldrich, St. Louis, MO) were added to each resuspended pellet. The solution was incubated at 37 °C for 1.5 to 2 h. One µl of Proteinase K was diluted into 150 µl of Gram Positive Lysis solution, and 150 µl of the Proteinase K/ Gram Positive Lysis mixture was added to the sample. The mixture was transferred to a tube containing ~100 µL of <106 µm beads (Sigma Aldrich) and processed in a bead-beater for 5 min (Scientific Industries, Bohemia, NY). The tubes were spun for 1 min at a top speed in a desktop centrifuge, and the resulting supernatant was transferred to a clean microcentrifuge tube. The solution was then incubated at 70 °C for 15 min, with mixing occurring every 5 min. The samples were then placed on ice for 5 min, followed by the addition of 175 µl of MPC Protein Precipitation Reagent and vigorous vortexing for 10 sec. The tubes were spun at 4 °C for 10 min at top speed in a desktop centrifuge to pellet the debris. The supernatant was transferred to a clean tube and the pellet was discarded. One µl of RNase A was added to each sample and mixed thoroughly. The solution was then incubated at 37 °C for 30 min. Five hundred µL of isopropanol were added to the recovered supernatant and the solution was mixed gently by inverting the tube several times. The tube was spun at 4 °C for 10 min at top speed to pellet the DNA. The isopropanol was removed, and 200 µL of 70% ethanol were added to the
pellet to rinse the DNA. The 70% ethanol was carefully removed, and the pellet was air dried for 15 min. The isolated DNA was resuspended in 100 µL of water.

2. **Isolation of plasmid DNA from staphylococci (minipreparation)**

Isolation of plasmids from *Staphylococcus* spp. was based on the procedure described in the Wizard plasmid kit, which was used with some modifications (Promega). Five mL of overnight cultures were pelleted and resuspended in 190 µL of cell resuspension solution. Five µL of 2 mg/mL lysostaphin and 5 µL of 2 mg/mL lysozyme (Sigma Aldrich) were added, and the solution was incubated at 37°C for 1 h. Two hundred fifty µL of the cell lysis solution were added, and the remaining steps of the procedure were followed as stated in the kit manual.

3. **Isolation of plasmid DNA from staphylococci (midipreparation)**

Total plasmid was isolated using the PureYield Plasmid Midiprep System (Promega), with minor modifications. Briefly, 50 mL of cells were pelleted and resuspended in 2.88 mL Resuspension Buffer (supplied in the kit) supplemented with 120 µg lysostaphin and 120 µg lysozyme. The mixture was incubated at 37 °C for 1 h, and the remaining steps were followed as instructed by the kit manual.

4. **Isolation of cellular RNA from staphylococci**

Total RNA was isolated from staphylococci cells using the RNeasy Mini Kit (Qiagen) with some modifications. Briefly, overnight cultures of cells grown in BHI medium supplemented with 30 µg/mL chloramphenicol were diluted 1:100 in fresh media and grown to an OD$_{600} = 0.5$. Five mL of cells were pelleted, washed with 500 µl water and resuspended in 200 µl of lysis buffer (Tris-HCl, pH 7.5, 30 mM MgCl$_2$, 30 mM NH$_4$Cl) containing 0.5 mg/mL of lysostaphin. Lysis was carried out for 30 min at 37 °C. Three hundred fifty µl of buffer RLT
from the RNeasy Mini Kit were then added, and the remaining steps of RNA isolation were followed as instructed by the kit manual.

5. **Isolation of plasmid DNA from *Escherichia coli***

Five mL of culture were grown at 37 °C overnight with constant shaking. Plasmid DNA was isolated using the High Pure Plasmid kit (Roche Applied Science, Branford, CT).

F. **Primer Extension Analysis**

1. **General primer extension procedure**

The general primer extension analysis procedure described by Merryman et al. was used for rRNA analysis with specific modifications outlined in the sections below (Merryman et al., 1999). The primers were labeled with \([\gamma^{32}P]\) ATP in 10 µl of 1 x PNK buffer A (Fermentas) containing 25 µCi of 6,000 Ci/mmol \([\gamma^{32}P]\) ATP, various pmol of primer and 5 U T4 Polynucleotide kinase (Fermentas). The reaction was incubated at 37 °C for 30 min, followed by a heat-inactivating step (90 °C for 2 min).

One µL of 4.5 x hybridization buffer (225 mM K-Hepes, pH 7.0, 450 mM KCl), 1 µL of the labeled primer and 1.5 µg in 2.5 µL of the total isolated RNA template were combined. The mixture was incubated for 1 min at 90 °C and cooled down to ca. 45 °C over the course of 30 min in order to anneal the primer to the RNA.

Meanwhile, 0.65 µL of 10 x extension buffer (1.3 M Tris-HCl, pH 8.5, 100 mM MgCl₂, 100 mM DTT), 1.5 µL of the dNTP mixture (the composition of the mixture was adjusted for individual experiments, see below), 0.75 µL water, and 0.1 µL AMV reverse transcriptase (Seikagaku America, Falmouth, MA) were thoroughly mixed and 3 µL were added to the annealing mixture. The samples were incubated at 40 °C for 30 min to allow cDNA synthesis by reverse transcriptase. One hundred twenty µL precipitation buffer (84 mM sodium acetate, 0.8
mM EDTA, 70% ethanol) were added, and the solution was incubated in a mixture of dry ice and ethanol for 10 min to precipitate the DNA. The DNA pellet was collected by centrifuging the tubes for 30 min at 4 °C at 14,000 rpm. The supernatant was removed, the pellet was rinsed with 70% ethanol and dried in the SpeedVac for 3 min until all traces of the ethanol were removed. The pellet was then resuspended in 3 µL of formamide dye (80% formamide, 10 mM EDTA, 1 mg/mL xylene cyanol, 1 mg/mL bromophenol blue), heated at 90 °C for 1 min, and loaded onto a 40 cm long, 0.4 mm thick 6% polyacrylamide sequencing gel. The gel was run at 40 W for approximately 120 min and then transferred to chromatographic paper (Thermo Fisher Scientific, Waltham, MA). After drying, the gel was exposed overnight to a phosphorimager screen. Quantification of primer extension bands was carried out at various exposure times so that the bands were not overly exposed.

2. **The extent of A2503 modification in staphylococci**

For the analysis of modification of A2503 in 23S rRNA, 3 pmol of [5'-32P]-labeled DNA Primer 26 was annealed to 1.5 µg of *S. aureus* total RNA. The primer was extended with AMV reverse transcriptase in the presence of a dNTP mixture composed of 1 mM concentrations of dGTP, dATP, and dCTP and 0.25 mM dTTP.

3. **Determination of the transcription start site**

Primer extension was carried out as stated above, with minor differences. Twenty µg of total RNA prepared from cells carrying different plasmid constructs were annealed with 0.75 pmol of [5'-32P]-labeled DNA Primer 27. The primer was extended with AMV Reverse Transcriptase in the presence of 1 mM each dGTP, dATP, dCTP and dTTP. One µL of 10 N NaOH was then added to each reaction and incubated for 15 min at 37 °C. The solution was
subsequently neutralized with HCl. The cDNA products were resolved in a denaturing 6% polyacrylamide sequencing gel as stated above.

G. **Growth Rate Determination**

RN4220 cells transformed with the pLI50-derived plasmids were grown overnight at 37 °C in BHI medium supplemented with 10 µg/mL chloramphenicol. Cultures were diluted to an OD$_{600}$ of 0.05 in 10 mL of fresh medium and grown in 250-mL flasks with shaking (230 rpm) at 37 °C. The optical densities of the cultures were measured every 30 min. The doubling times and standard deviations were determined from two independent experiments.

H. **Competition Growth**

1. **Competition assays for the determination of cfr fitness cost**

Cultures of cells transformed with different plasmids were grown overnight in BHI medium supplemented with 10 µg/mL chloramphenicol. The cultures were diluted to an OD$_{600}$ of 0.05 in fresh BHI/chloramphenicol medium and shaken at 37 °C until the OD$_{600}$ of the culture reached 0.5. Portions (5 mL) of each culture (OD$_{600}$ of 2.5) in the strain pairs (pJL1/pLI50, pJL1/pJL1M, or pMS2/pMS2M) were mixed and grown with shaking at 37 °C for 18 h. Cells were then diluted 1,000-fold into fresh BHI/chloramphenicol medium and grown again for 24 h to stationary phase. Three passages were done in total, corresponding to ~40 cell generations. At each passage, the ratio of cells was determined by plating dilutions on BHI-agar plates containing 10 µg/mL chloramphenicol, with or without 10 µg/mL florfenicol. Colonies were counted after 18 h growth at 37 °C. The fitness cost per generation was calculated as previously described (Lenski, 1991; Sander et al., 2002).

2. **Growth competition of RlmN$^+$ and RlmN$^-$ strains**
S. aureus strains SAV1218 (where rlmN has been replaced with erm), SAV1986 (xylA has been replaced with erm), Newman (wild type), JKD6210 (wild type clinical), and JKD6300 (where rlmN contained the insertion of Gln at position 357) were grown in BHI medium overnight at 37 °C with shaking (during this stage, the SAV1218 and SAV1986 cultures were supplemented with 10 µg/mL erythromycin) (Bae et al., 2004; Gao et al., 2010). The overnight cultures were diluted to an OD$_{600}$ = 0.05 in BHI medium supplemented with 0.5 µg/mL linezolid, grown to an OD$_{600}$ = 0.5, and the following pairs were mixed in equal amounts at an OD$_{600}$ = 0.05: SAV1218/SAV1986, Newman/SAV1218, and JKD6210/JKD6300. Two mL of the mixes were pelleted and frozen at -80 °C for further use as the beginning time point. The mixed cultures were grown overnight at 37 °C with constant shaking; in the morning, they were diluted 1:2,000 in fresh BHI-0.5 µg/mL linezolid, and 2 mL of the culture were pelleted and saved in the -80 °C freezer. This cycle was repeated for 2 more days, collecting cells at each cycle. All of the saved cell pellets were thawed, washed with water, repelleted, and resuspended with 200 µl of lysis buffer (Tris-HCl, pH 7.5, 30 mM MgCl$_2$, 30 mM NH$_4$Cl) containing 0.5 mg/mL of lysostaphin. Lysis was carried out for 30 min at 37 °C. Three hundred fifty µl of buffer RLT from the RNeasy Mini Kit were then added, and the remaining steps of RNA isolation were followed as instructed by the kit manual. The RNA was then used in a primer extension analysis to quantify the ratio of RlmN$^+$:RlmN$^-$ cells at each time point as described below.

The primer extension analysis used to this end is similar to the primer extension procedure described previously, except that the nucleotide mix contains a dideoxy-NTP. Specifically, Primer 28 was labeled with [$\gamma$-$^{32}$P] ATP in 10 µl of 1 x PNK buffer A containing 25 µCi of 6,000 Ci/mmol [$\gamma$-$^{32}$P] ATP, 6 pmol of primer and 5 U T4 Polynucleotide kinase. The
solution was incubated at 37 °C for 30 min, followed by a heat-inactivation step at 90 °C for 2 min.

One µL of 4.5 x hybridization buffer (225 mM K-Hepes, pH 7.0, 450 mM KCl), 1 µL (1.4 pmol) of the labeled primer and 2.5 µL (0.5 µg) of total RNA were combined. The mixture was heated at 90 °C for 1 min and cooled down to ca. 45 °C over the course of 30 min in order to anneal the primer to the RNA.

Meanwhile, 0.65 µL of 10 x extension buffer (1.3 M Tris-HCl, pH 8.5, 100 mM MgCl₂, 100 mM DTT), 1.5 µL of the dNTP chase mixture (1 mM dATP, 1 mM dCTP, 1 µM dTTP, and 0.2 mM ddGTP), 0.75 µL water, and 0.1 µL AMV reverse transcriptase were mixed and 3 µL was added to the annealing mixture. The sample was incubated at 40 °C for 30 min. One hundred twenty µL precipitation buffer (84 mM sodium acetate, 0.8 mM EDTA, 70% ethanol) were added, and the solution was incubated in a mixture of dry ice and ethanol for 10 min to precipitate the DNA. The sample was centrifuged for 30 min at 4 °C at 14,000 rpm to pellet the DNA. The supernatant was removed, and the pellet was rinsed with 70% ethanol and dried in the SpeedVac for 3 min, until all traces of the ethanol were removed. The pellet was then resuspended in 3 µL of formamide dye, heated at 90 °C for 1 min, and loaded onto a 0.4 mm thick, 20 cm long 12% polyacrylamide sequencing gel. The gel was run at 20 W for approximately 60 min, then transferred to a sheet of X-ray film, wrapped with plastic wrap and exposed to a phosphorimager screen for 40 min.

I. Southern Blot Analysis

One µg of total DNA or plasmid isolated from S. epidermidis 426-3147L was digested in a total volume of 20 µl with 20 U of HindIII for 3 h. The Southern blot was carried out as described (Toh et al., 2007). Briefly, the digest products were electrophoresed at 4 °C on a 14 cm
x 11 cm 0.9% agarose gel, run between 100-140 V. The excess gel was cut away, and holes were punched with a pipette tip to designate the marker bands. The gel was soaked in 0.25 M HCl for 10 min with gentle shaking to reduce the size of the large DNA fragments. The gel was rinsed briefly with water and soaked in Alkaline Transfer Buffer (0.4 N NaOH, 1 M NaCl) for 15 min while shaking. The buffer was replaced with fresh Alkaline Transfer Buffer and agitated again for 20 min. Meanwhile, a strip of 3MM paper (Whatman, Piscataway, NJ) was cut to the width of the gel and prewet first with water and then 10x SSC (0.3 M sodium citrate, 3 M NaCl, pH 7.2). The gel was placed face down on the moist blot paper with the paper ends soaking in the chamber with transfer buffer. A Hybond-N+ filter membrane (Amersham, Arlington Heights, IL) was cut to the size of the gel, prewetted in water, then soaked in 10x SSC and placed carefully on top of the gel. Another piece of 3MM paper was moistened in 10x SSC and placed on top. A 6-in stack of paper towels were cut to the size of the gel and placed on top of the 3MM paper. A 400 g weight was placed carefully over the towels. Transfer occurred overnight. The paper towels were carefully peeled off and the assembly was disassembled. Holes corresponding to the marker were punched with a needle in the membrane. The membrane was carefully peeled from the gel and soaked in 6x SSC buffer for 5 min to remove extra pieces of agarose. The damp membrane was placed on plastic wrap, DNA-side down, and UV irradiated to fix the DNA for 5 min on a transilluminator (Spectroline, Westbury, NY) equipped with a 254 nm filter. The membrane was then placed at 37 °C for several hours to dry. The dry membrane was placed in a roller bottle and 5 mL of prewarmed prehybridization buffer (6x SSC, 5x Denhardt’s reagent (Sigma Aldrich), 0.5% SDS, 100 µg/mL herring sperm DNA (Invitrogen)) was added. The bottle was incubated on a roller in a hybridization oven at 65 °C for 30 min. Labeled probes specific to cfr and the chromosomal control, se0011 (see below), were denatured at 90 °C for 30 sec and added to the
hybridization tubes. Hybridization was continued at 65 °C for 3 h. The membrane was then washed in a tray for 5 min with 2x SSC, 0.5% SDS at room temperature with gentle rocking. The buffer was replenished and the membrane was washed again for 15 min at room temperature. The membrane was then washed with 0.1x SSC, 0.1% SDS for 30 min at 65 °C, followed by a brief wash at room temperature with 0.1x SSC. The membrane was covered in plastic wrap and exposed overnight to a phosphorimager screen.

Hybridization probe synthesis and labeling was carried out as follows. In a 50 µl reaction, 50 ng of total DNA from the S. epidermidis isolate 426-3147L was used as a template in the PCR reaction with 1 pmol each of primers specific for cfr or for the chromosomal gene se0011 (Primers 29-30 and Primers 31-32 respectively). The PCR product was cleaned with the SV Wizard Kit (Promega). One hundred ng of the cleaned PCR products were then labeled by using the HexaLabel DNA Labeling kit (Fermentas), following the protocol included in the manual. The labeled probes were finally cleaned by passing them through a G50 Illustra Minispin Column (GE Healthcare Life Sciences, Piscataway, NJ).

J. Curing of p7LC from Staphylococcus epidermidis 426-31467L

A culture of 426-3147L cells was grown overnight in BHI broth with no antibiotics. This culture was streaked onto BHI agar plates and incubated at 42 °C for 48 h. The culture was restreaked onto fresh BHI agar and incubated for 24 h at 42 °C. The culture was once again restreaked onto a fresh BHI plate and maintained at 42 °C for 48 h. Colonies from this plate were replica plated on pairs of BHI agar plates, with or without 20 µg/mL florfenicol plates.

K. Analytical Polymerase Chain Reactions

1. Multiplex Polymerase Chain Reaction
Multiplex PCR was carried out on 426-3147L cells or 426-3147L cells cured of the p7LC plasmid. Briefly, a dab of cells from a plated colony was dipped in 10 µL of water, and the sample was boiled for 10 min. The boiled cells were spun down on a benchtop centrifuge at maximum speed for 1 min, and 1 µL of the resulting supernatant was used as a template in the PCR reaction. Two primers (20 pmol each) specific for \textit{cfr} (Primer 39 and Primer 40) and 2 primers specific for ribosomal protein L3 (Primer 37 and Primer 38) were used in a 20 µL reaction with Fermentas 1x buffer, 0.2 mM dNTPs, 1.5 mM MgCl$_2$, and 0.5 U Taq DNA polymerase. The PCR cycle consisted of a denaturing step of 2 min at 94 °C; 30 cycles of 94 °C for 30 sec, 53 °C for 30 sec and 72 °C for 45 sec; a final extension at 72 °C for 60 sec. In control reactions that contained only one primer pair, 40 pmol of each primer was used.

2. **Detection of the presence of \textit{cfr} in staphylococcal isolates**

PCR for the presence of \textit{cfr} in \textit{S. epidermidis}, \textit{S. aureus}, or \textit{S. sciuri} was carried out on whole cells. Briefly, cells from a colony were incubated for 10 min in 10 µl of water at 100 °C. After 1 min centrifugation, 1 µl of the resulting supernatant was used as a template in the PCR reaction. PCR for the presence of \textit{cfr} in the plasmid fractions of \textit{S. epidermidis} 426-3147L or \textit{S. aureus} RN4220 transformed with p7LC was carried out on isolated plasmid DNA. In both cases, the PCR reaction was carried out with two \textit{cfr}-specific primers (Primer 39 and Primer 40). The PCR conditions were: 120 sec at 94 °C; 30 cycles of 94 °C for 30 sec, 45 °C for 30 sec and 72 °C for 45 sec; a final extension at 72 °C for 60 sec was included.

3. **For sequencing the upstream region of \textit{cfr} and the Tn4001 region**

The \textit{cfr}- and Tn4001-containing region of \textit{S. epidermidis} 426-3147L was sequenced using several primers either in a PCR reaction (under conditions stated above, except with
primer-specific annealing temperatures and amplification-specific extension times) or by primer walking. The primers used included Primers 33 and 34 and Primers 41-45.

L. Analysis of cfr Inducibility

Cells from glycerol stocks were streaked on BHI agar plates containing 40 µg/mL spectinomycin (for S. sciuri/pSCFS1) or 30 µg/mL chloramphenicol (for RN4220/pJL1B and RN4220/pJL1-7L) and were incubated at 37 °C overnight. The following day, the cells were re-streaked on BHI agar with the respective antibiotic supplemented with 0.5 µg/mL florfenicol or BHI agar with the respective antibiotic alone and incubated overnight at 37 °C. Resultant colonies were then grown in BHI broth under the antibiotic conditions listed above. RNA was extracted from these cultures, and primer extension was carried out as previously described to analyze the extent of cfr-mediated modification of A2503.

M. Expression, Purification and Reconstitution of Cfr and RlmN

A 5 mL culture of E. coli BL21 (DE3)/pRlmN or pCfr was grown overnight at 37 °C in LB medium containing ampicillin (100 µg/mL) and was used to inoculate 2 L of the same medium. When OD_{600} reached 0.4-0.6, the incubation temperature was lowered to 18 °C and isopropyl-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.2 mM to induce gene expression. FeCl₃ was added to a final concentration of 0.2 mM to provide a sufficient amount of iron for iron-sulfur cluster assembly. After incubation for an additional 24 h at 18 °C, cells were harvested by centrifugation (5,000 × g, 10 min) at 4 °C and stored at -80 °C. The typical yield was 5 g of wet cells per liter of culture. Subsequent purification was carried out at 4 °C, and all buffers were degassed and saturated with nitrogen before use in an anaerobic glovebox.
Thawed cell pellets (~10 g) were resuspended in 50 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 300 mM NaCl) and subjected to 10 × 30 sec ultrasonic bursts (50% cycle, power level 5), with a 1 min cooling interval between each blast. Cell debris was removed by centrifugation (30,000 × g, 30 min), and the supernatant was mixed by slow agitation with 10 mL of Ni-NTA resin (Qiagen) for 1 h at 4°C. The slurry was poured into a column and subsequently washed with lysis buffer supplemented with 20 mM and then 40 mM imidazole (50 mL each). The brownish protein was eluted with elution buffer (50 mM Tris-HCl, 10% glycerol, 300 mM NaCl, 250 mM imidazole, pH 8.0).

The brownish fractions that contained the desired proteins were separately pooled (~20 mL). The [4Fe-4S] cluster was further reconstituted by treating the fractions with 350 µM DTT, 140 µM cysteine, and 150 µM Na₂S. While the mixture was gently stirred, FeCl₃ was slowly added to a final concentration of 450 µM. The stirring was continued for another 2 h at room temperature, and the proteins were subsequently concentrated in an Amicon concentrator with YM-10 membrane (Millipore) to less than 1 mL prior to loading on a 30 mL P6 desalting column (Bio-Rad). The reconstituted proteins were eluted from the column using 50 mM Tris-HCl buffer (pH 8.0) containing 50 mM NaCl and 10% glycerol. The collected proteins were further purified by FPLC on a 5 mL Hi-Trap Q HP column (GE Healthcare Life Sciences) using buffer A (50 mM Tris-HCl, pH 8.0, 50 mM NaCl) and buffer B (50 mM Tris-HCl, pH 8.0, 1 M NaCl). The FPLC buffers for Cfr purification also contained 10% glycerol. The column was run with 100% buffer A for 5 mL, followed by a linear gradient of 0 to 40% B in 20 mL. The flow rate was 1.5 mL/min, and the detector was set to 280 and 410 nm. The eluted proteins were diluted with FPLC buffer A and concentrated in an Amicon concentrator prior to storage at -80 °C. The concentrations of the purified proteins were determined by the Bradford method using bovine
serum albumin as the standard (Bradford, 1976). The relative molecular weight and purity of enzyme samples were determined using SDS-PAGE (Laemmli, 1970). The average protein yield was ~1.2 mg/L for RlmN and ~0.3 mg/L for Cfr.

N. **Generation of Cfr and RlmN Mutants**

Mutagenesis of conserved cysteine residues in the CysXXXCysXXCys motif of RlmN and Cfr to alanines in pRlmN and pCfr was carried out using the QuikChange Site-Directed Mutagenesis Kit. The oligonucleotides (Primers 7-12, Table II) used for mutagenesis were purchased from Bioneer Inc (Alameda, CA). The constructed mutant plasmids were transformed into *E. coli* TOP10 cells, purified with the Qiaprep Spin Miniprep Kit (Qiagen) and subsequently verified by DNA sequencing. The mutant plasmids were then used to transform *E. coli* BL21(DE3) cells (Novagen) which were used for protein expression. The RlmN and Cfr mutants were expressed, purified, and subjected to iron-sulfur cluster reconstitution in the same manner as that for the wild type enzymes, except that the final FPLC purification step was omitted due to the small quantities of proteins obtained.

O. **Isolation of Ribosomes from *Escherichia coli***

1. **Isolation of 70S ribosomes**

70S ribosomes from the xylA- and rlmN- strains from the *E. coli* Keio collection (Baba et al., 2006) were isolated as previously described (Baba et al., 2006; Moazed and Noller, 1989), with only minor differences. Twenty mL of an overnight culture of the cells, grown in LB broth and supplemented with 30 µg/mL kanamycin, were diluted into 2 L of fresh media. The culture was grown with vigorous shaking at 37 °C until the cells reached an OD<sub>600</sub> = 0.6. The cells were harvested at 4 °C, 4,600 x g for 10 min. The pellets were washed with 20 mL cold buffer A (20 mM Tris-HCl, pH7.5, 100 mM NH<sub>4</sub>Cl, 10 mM MgCl<sub>2</sub>, 0.5 mM EDTA and 6 mM β-
mercaptoethanol). The cell pellet was resuspended in 20 mL buffer A and lysed using a French Press set to 16,000 psi. The lysed cells were centrifuged at 31,000 x g at 4 °C for 15 min. Ten U RQI DNase (Promega) were added to the supernatant, and the mixture was spun for 10 min at 70,000 x g at 4 °C. Ten mL of cushion buffer (20 mM Tris-HCl, pH 7.5, 500 mM NH₄Cl, 10 mM MgCl₂, 0.5 mM EDTA, 1.1 M sucrose) was placed in a 32 mL QuickSeal polyallomeric centrifuge tube (Beckman, Fullerton, CA). The cell supernatant was carefully layered over the cushion with a syringe. The tube was filled to the top with buffer B (20 mM Tris-HCl, pH 7.5, 500 mM NH₄Cl, 10 mM MgCl₂, 0.5 mM EDTA) and capped off with a rubber stopper. The tubes were ultracentrifuged in a Ti-70 rotor at 33,000 rpm for 18-22 h at 4 °C. After centrifugation, the supernatant was discarded and the pellet and sides of the tube were gently rinsed with 1 mL resuspension buffer (50 mM Tris-HCl, pH 7.5, 150 mM NH₄Cl, 5 mM MgCl₂, and 6 mM β-mercaptoethanol). The rinse was repeated 3 times. Three hundred µL of resuspension buffer and a small magnet were added to the pellet, and the pellet was kept at 4 °C with gentle stirring over a magnetic plate for 2 h, or until the pellet was dissolved.

2. **Isolation of 50S and 30S subunits**

For isolation of the 50S and 30S subunits, 70S ribosomes isolated from above were dialyzed against the buffer 50 mM Tris-HCl, pH 7.6, 100 mM NH₄Cl, 1 mM MgCl₂, 6 mM β-mercaptoethanol for 6 h in a dialysis bag with a 3500 Da cut off. Meanwhile, a 10-40% sucrose gradient was created by the Gradient Master 107 ip (Biocomp, Fredericton, NB) using 10% (w/v) sucrose and 40% (w/v) sucrose in the above buffer. One hundred OD₂₆₀ were layered onto the sucrose gradient, and centrifuged in a swinging bucket rotor (Sorvall AH-629) for 16 h, 25,000 rpm, 4 °C. Gradients were fractionated using a syringe pump through a UV monitor. The fractions corresponding to the 30S and 50S subunits were pooled, the concentration of MgCl₂
was increased to 10 mM, and TNM buffer (50 mM Tris-HCl, pH 7.6, 100 mM NH₄Cl, 10 mM MgCl₂, 6 mM β-mercaptoethanol) was added to 40 mL. Solutions were placed in the tubes of the Beckman Ti-70 rotor and ultracentrifuged at 40,000 rpm, 16 h, 4 °C. Pellets were rinsed with TNM buffer and resuspended in 0.5 mL of the same buffer.

3. **Isolation of 23S and 16S rRNA**

Six to ten OD₂₆₀ of 50S or 30S subunits were diluted into 100 µl of TNM buffer. Samples were then phenol extracted using an equal volume of phenol (pH 6.7), shaken for at least 5 min and spun in a centrifuge for 10 min. The aqueous phase was recovered, and an equal volume of phenol/chloroform was added. Samples were again shaken for 5 min, centrifuged for 10 min and the aqueous phase was extracted with an equal volume of chloroform. The rRNA was precipitated with 3 volumes of ethanol overnight at -20 °C, pelleted, washed with 70% ethanol, and resuspended in water.

To obtain total rRNA, 70S ribosomes were isolated as described above and diluted to 30 OD₂₆₀ U/mL in the extraction buffer: 0.3 M sodium acetate, pH 5.5, 5 mM EDTA, 0.5% SDS. Samples were extracted with phenol, phenol/chloroform, and chloroform as stated above. The samples were precipitated with 3 volumes of ethanol overnight at -20 °C. RNA pellets were resuspended in 350 µL 0.3 M sodium acetate and ethanol precipitated again at -80 °C for 10 min. Pellets were washed with 70% ethanol and resuspended in water.

P. **Preparation of Truncated Ribosomal RNA Substrates for Cfr and RlmN Activity Assays**

23S rRNA fragments that encompassed A2503 were generated by *in vitro* transcription using PCR products as templates. All forward PCR primers included the T7 RNA polymerase promoter sequence TAATACGACTCACTATAGG, followed by several nucleotides.
corresponding to specific segments of 23S rRNA. The primers used for PCR amplifications are listed in Table II under the names Primers 13-23. Segments of the 23S rRNA gene were amplified using the plasmid pKK3535, carrying the E. coli rrnB operon as a template (Brosius et al., 1981). PCR products were cleaned using the SV Wizard Kit and subsequently used for in vitro transcription. For the shortest truncation, nucleotide positions 2496-2507, the template for in vitro transcription was prepared by annealing 400 pmol each of Primer 17 and Primer 19 in 40 µL of water. The mixture of the two primers was incubated at 100 °C for 1 min and then allowed to cool down to room temperature over 20 min.

For the in vitro transcription, 10 µg of DNA template were placed in 100 µL of transcription buffer (40 mM Tris-HCl, pH 8.0, 22 mM MgCl₂, 5 mM DTT, 1 mM spermidine) supplemented with 2.5 mM each rNTPs, 20 mM DTT, 0.2 U/µL RiboLock RNase inhibitor, and in-house prepared T7 RNA polymerase. Reactions were incubated at 37 °C for 3 h, when they had turned cloudy. Twenty U of RQ1 DNase were added, and incubation at 37 °C continued for 30 min. Then, 102 µL of water were added to each reaction, followed by 22.6 µL of Stop Solution (5 M ammonium acetate, 100 mM EDTA). Once the solutions turned clear, 1 volume of isopropanol (for transcripts longer than 86 nucleotides) or 2.5 volumes of ethanol (for transcripts shorter than 86 nucleotides) were added, and the reactions were incubated at -20 °C for 20 min or at -80 °C overnight. Samples were spun at 4 °C for 20 min at 21,000 × g. Pellets were resuspended in 200 µL of 0.3 M sodium acetate, pH 5.5 and extracted with phenol, phenol/chloroform, and chloroform. RNA was precipitated with 3 volumes of ethanol overnight at -80 °C. The samples were pelleted, washed with 70% ethanol, resuspended in water, and stored at -80 °C.

Q. **Activity Assays for Cfr and RlmN**
A typical activity assay reaction for RlmN/Cfr contained 10 mM MgCl₂, 2 mM sodium dithionite, 1.5 µCi S-adenosyl-L-[³H-methyl]methionine ([³H-methyl]-SAM, 10.0 Ci/mmol), 1 µM RlmN (or 2 µM Cfr), and 0.2 µM (10 pmol) of purified rRNA in 50 µL of 100 mM Tris-HCl buffer, pH 8.0. All the reaction components were made anaerobic by bubbling or purging with argon prior to mixing in an MBraun glovebox (Stratham, NH). The reaction was initiated by addition of [³H-methyl]-SAM, and after 30 min of incubation at 37 °C, the reaction mixture was transferred onto a 23 mm Whatman DE81 filter paper disk (Whatman). The paper discs were thoroughly washed with a 5% trichloroacetic acid (TCA) solution by gentle swirling (3 × 5 min), air-dried, and placed in scintillation vials. Five mL of scintillation fluid were added, and the amount of retained radioactivity was determined in a LS6500 scintillation counter (Beckman).
III. RESULTS AND DISCUSSION

A. **Low Fitness Cost of the Multidrug Resistance Gene cfr**

1. **Introduction**

One of the most important mechanisms of resistance to ribosomal antibiotics operates through target site modification. In principle, this can be achieved by rRNA mutations, but the redundancy of rRNA genes makes it difficult to reach sufficient levels of resistance by a mutation in a single *rrn* allele (Lobritz et al., 2003). Furthermore, rRNA mutations often negatively affect ribosome functions and are rapidly reversed in the absence of selection (Meka et al., 2004a; Wolter et al., 2006). Therefore, a more common resistance mechanism is based on the chemical modification of rRNA. Cfr-mediated methylation of the C8 position of the adenine base renders bacteria resistant to a broad array of protein synthesis inhibitors, including oxazolidinones such as linezolid (Giessing et al., 2009; Kehrenberg et al., 2005; Long et al., 2006; Schwarz et al., 2000; Smith and Mankin, 2008). As discussed previously, the *cfr* gene was originally found in staphylococcal strains isolated from pigs and cattle (Kehrenberg and Schwarz, 2006; Schwarz et al., 2000). In the first *S. aureus* clinical strain CM05, *cfr* is present on the chromosome on a transposable genetic element and is preceded by the *ermB* gene. The *ermB* gene encodes another rRNA methyltransferase that targets A2058 in 23S rRNA (Figure 10). The *ermB* and *cfr* genes are coexpressed under the P_{erm} promoter in the *mlr* operon. Expression of *mlr* results in modification of A2058 and A2503 in 23S rRNA and renders cells resistant to all clinically relevant antibiotics that target the large ribosomal subunit (Smith and Mankin, 2008; Toh et al., 2007).
Figure 10. The simultaneous methylations of A2503 and A2058 by Cfr and ErmB, respectively, may affect ribosomal function. Left panel: Locations of A2503 (orange) and A2058 (cyan) in the large ribosomal subunit. Peptidyl-tRNA (pale green) with the nascent peptide (bright green) and aminoacyl-tRNA (blue) are shown. Right panel: Close-up view of the close contacts of A2058 and A2503 with the nascent peptide. The methyl groups added to C8 and C2 of A2503 by Cfr and RlmN, respectively, and to the exocyclic amine of 2058 by ErmB are highlighted in red.
A number of other reports of new cfr-positive clinical isolates have appeared (Bonilla et al., 2010; Cercenado, 2010; Farrell et al., 2009; Locke et al., 2010; Mendes et al., 2010a; Mendes et al., 2008; Mendes et al., 2010b; Morales et al., 2010; Sanchez Garcia et al., 2010; Shore et al., 2010). Although it is difficult to conclude whether this trend indicates the recent spread of the cfr gene or simply the fact that its presence has been overlooked previously, the ongoing rapid dissemination of cfr among pathogenic strains remains a real possibility.

One important question that remains unclear about Cfr-based resistance is the fitness cost associated with the acquisition of the cfr gene. The target of Cfr action, A2503, is located in a functionally critical region of the ribosome. rRNA residues located in this region participate in the catalysis of peptide bond formation, as well as in interactions with the nascent peptide (Polacek and Mankin, 2005; Vazquez-Laslop et al., 2010). A2503 is naturally methylated at C2 in the ribosomes of many bacteria by the indigenous enzyme RlmN (Kowalak et al., 1995; Toh et al., 2008). The natural posttranscriptional modification of A2503 underscores its possible functional importance in protein synthesis (Vazquez-Laslop et al., 2010). It would not be surprising if the Cfr-mediated addition of an extra methyl group at the C8 position of A2503 negatively affects translation, and thus, cell fitness. The presence of yet another modification at a neighboring rRNA residue (A2058) conferred by the Erm methyltransferase, as in the mlr operon, may potentially further reduce the fitness of the resistant strains. The spread and maintenance of a resistance gene are directly linked to the fitness cost associated with the gene expression. Genes whose presence significantly reduces cell fitness are rapidly lost in the absence of selection, whereas those that come at a low cost can stably persist in the cells, even when pathogens are not exposed to antibiotics (Andersson and Hughes, 2010; Bjorkholm et al., 2001; Foucault et al., 2010; Lee and Edlin, 1985; Marciano et al., 2007; Marcusson et al., 2009;
Rodriguez-Rojas et al., 2010; Sundqvist et al., 2010). Therefore, knowing the fitness cost of a resistance mechanism is important for predicting the efficiency of its maintenance and the rate of expansion. With this goal in mind, we assessed the fitness cost associated with the expression in S. aureus cells of the cfr gene alone or in combination with ermB.

2. **Experimental results**

a. **Expression of Cfr has little effect on growth rate**

In order to determine whether the expression of the Cfr protein significantly affects cell fitness, we constructed the pJL1 plasmid in which the cfr gene is expressed under the control of its native promoter present in the originally described cfr-carrying plasmid, pSCFS1 (Figure 11) (Schwarz et al., 2000). Expression of the active cfr gene in S. aureus strain RN4220 transformed with pJL1 was verified by MIC testing and analysis of the modification status of A2503 in 23S rRNA (Table IV, Figure 12). While the florfenicol MIC of cells transformed with the empty vector was 4 µg/mL, cells carrying pJL1 exhibited a much higher MIC (128 µg/mL), suggesting the Cfr protein is expressed and modifies 23S rRNA. In agreement with this conclusion, primer extension analysis showed the appearance of a strong reverse transcriptase stop at A2503 in rRNA isolated from pJL1-transformed cells, indicating hypermethylation of this nucleotide (Figure 12). As an initial assessment of the fitness cost associated with Cfr expression, we compared the growth rates of cells transformed with either pJL1 or the empty vector. The doubling times of cells transformed with any of the two plasmids were rather similar (30 ± 0.4 min for pLI50 and 36 ± 0.4 min for pJL1), indicating that the impact of expression of Cfr on cell fitness was fairly low. We followed this up with a more sensitive assay in which cells carrying pJL1 (cfr-positive) were cocultured with cells transformed with the empty pLI50 control vector. An equal number of pJL1- and pLI50-transformed cells were grown together in
Figure 11. The maps of the plasmids used in fitness cost cogrowth experiments. The shuttle plasmid pLI50 was used as the backbone for both pJL1 and pMS2. pJL1 includes the $cfr$ promoter, $P_{cfr}$, and the $cfr$ gene. pMS2 includes the $mlr$ operon from the CM05 strain of $S. aureus$ in which the $cfr$ and $ermB$ genes are coexpressed from the $P_{erm}$ promoter.
Table IV. Florfenicol minimal inhibitory concentrations for *S. aureus* RN4220 cells transformed with different constructs.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>MIC (ug/ml)</th>
</tr>
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<tbody>
<tr>
<td>pLI50</td>
<td>4</td>
</tr>
<tr>
<td>pJL1</td>
<td>128</td>
</tr>
<tr>
<td>pJL1M</td>
<td>4</td>
</tr>
<tr>
<td>pMS2</td>
<td>128</td>
</tr>
<tr>
<td>pMS2M</td>
<td>4</td>
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</tbody>
</table>
Figure 12. The extent of A2503 modification in strains carrying various cfr-containing constructs. Primer extension analysis reveals the presence of an extra modification at A2503. The strong band at A2503 in 23S rRNA isolated from cells transformed with the pJL1 or pMS2 plasmids shows a high degree of hypermodification of A2503 by Cfr (arrow). No difference in the intensity of the band compared to the untransformed RN4220 control is seen in the cells transformed with pJL1M or pMS2 that carry the catalytically inactive mutant of Cfr.
chloramphenicol-containing BHI medium and passaged four times, allowing the cultures to reach saturation at each cycle (altogether, the cells were cogrown for ca. 40 generations). At each cycle, the ratio of cells transformed with pLI50 or pJL1 was assessed by plating aliquots of exponential cells onto BHI-agar plates containing either no selective antibiotic (total number of colony forming units) or supplemented with 10 µg/mL of florfenicol (the number of colony forming units of pJL1-transformed cells). The data showed that in the competition growth setting, cells expressing Cfr are outcompeted by the control cells, albeit at a slow rate. The estimated fitness cost of Cfr expression is only 4.6% per generation (Figure 13 A).

The observed minor decrease in cell fitness could result from expression of a foreign protein in the cell or, alternatively, could be caused by the introduction of an additional methyl group to a functionally important nucleotide located in a critical region of the ribosome. To distinguish between these possibilities, we engineered a catalytically inactive mutant of Cfr and examined the growth competition between *S. aureus* cells expressing active and inactive Cfr protein. In the mutant (pJL1M), the catalytically important cysteine 119 of Cfr was replaced with alanine (Kaminska et al., 2010; Yan et al., 2010). The florfenicol MIC of cells transformed with pJL1M was indistinguishable from that of cells carrying the empty pLI50 vector and primer extension did not show any difference compared to the control, thereby confirming previous biochemical data that the Cys119Ala mutant of Cfr is inactive (Figure 12 and Table IV) (Kaminska et al., 2010; Yan et al., 2010). When cells expressing active Cfr and its inactive mutant were cogrown in culture for ca. 40 generations, their ratio remained essentially unchanged (Figure 13 B). This result suggests that under our experimental conditions, the introduction of an additional methyl group at C8 of A2503 in 23S rRNA has little impact, if any, upon cell growth rate. Thus, the fitness cost associated with expression of active Cfr likely
Figure 13. Growth competition of strains transformed with cfr-containing constructs. pLI50 and pJL1 (A), pJL1 and pJL1M (B), and pMS2 and pMS2M (C). The fraction of cells expressing active Cfr was estimated by spreading cells on plates with or without florfenicol over a period of ca. 40 generations.
comes from the mere expression of the Cfr protein, rather than from the resulting rRNA modification.

b. Dimethylation of A2058 by the Erm methyltransferase increases the fitness cost associated with Cfr-mediated modification of A2503

In some clinical isolates, the cfr gene is coexpressed with the ermB gene which encodes the Erm methyltransferase targeting A2058 in 23S rRNA (Bongiorno et al., 2010; Smith and Mankin, 2008; Toh et al., 2007). Although our results showed that C8 methylation of A2503 has only a slight effect on cell fitness, and thus, on translation, it was possible that altering the structure of a neighboring nucleotide would alter the cost of A2503 modification. In order to test this hypothesis, we used the previously constructed plasmid, pMS2, which contains the mlr operon cloned into the pLI50 backbone (Toh et al., 2007). In this construct, ermB and cfr are coexpressed from the ermB promoter. Similar to pJL1M, the cfr gene in pMS2 was mutated by introducing the Cys119Ala mutation. We verified that the mutant plasmid (pMS2M) does not provide resistance to florfenicol, nor do pMS2M-transformed cells show any detectable Cfr-mediated modification of A2503 (Figure 12 and Table IV). Interestingly, in the cogrowth experiment, cells transformed with pMS2M, and thus expressing only active ErmB, rapidly outcompeted cells with the wild type pMS2, which expressed a combination of ErmB and Cfr (Figure 13 C). The fitness cost associated with cfr expression in the presence of active ermB was 10.4% per generation. This result shows that when A2058 is dimethylated by Erm, the addition of an extra methyl group to C8 of A2503 becomes sufficiently deleterious to notably reduce the ability of cells to compete with their peers lacking active Cfr protein.

3. Discussion
Since A2503 in 23S rRNA is functionally important, we initially expected that the expression of the multidrug resistance Cfr methyltransferase that alters the chemical structure of this nucleotide would come at a significant cost for the cell. However, our experimental data show that the acquisition of the cfr gene has in fact only a small effect upon the growth rate of S. aureus cells, indicating that the fitness of the cfr-positive cells is affected only slightly. In agreement with this result, in a cogrowth setting, cells transformed with the plasmid that carries the active cfr gene were slowly outcompeted by the control cells carrying an empty vector. The estimated fitness cost of cfr presence (4.6% per generation) is relatively low compared to some other resistance mechanisms whose fitness costs can be in the 15 to 35% range (Sander et al., 2002; Schrag et al., 1997).

Comparison of the strain possessing active Cfr with the strain expressing the catalytically inactive mutant showed that the fitness cost is approximately the same for Cfr and its mutant, arguing that C8 methylation of A2503 per se does not cause growth rate reduction. The slight fitness loss observed in our experiments apparently stems from a property of the Cfr protein unrelated to the catalysis of rRNA methylation. Our other studies have shown that Cfr acts during ribosomal assembly (see section below). Interaction of either active Cfr or its catalytically inactive mutant with the ribosomal precursor may compete with the binding of indigenous modifying enzymes (e.g., RlmN), ribosomal proteins, assembly chaperones, etc., possibly interfering with the normal process of ribosomal assembly. Slowing the ribosome assembly rate by even a small margin could reduce cell fitness to the extent that they start to lose in growth competition with cells lacking Cfr.

Although the C8 methylation of the single residue A2503 does not seem to interfere with translation, the situation dramatically changes when the Erm methyltransferase modifies a
neighboring nucleotide, A2058. *S. aureus* cells carrying the *ermB* gene and expressing active Cfr more readily lose in competition to the *ermB*-positive cells expressing the catalytically inactive Cfr mutant. Thus, the presence of two extra methyl groups at the exocyclic amine of A2058 notably increases the fitness cost of the additional methyl group at C8 of A2503. Both of the adenines, A2058 and A2503, are located in a close proximity to each other (at a distance of 6.6 Å) in the ribosomal nascent peptide exit tunnel (see Figure 10), and both are involved in functional interactions with the regulatory nascent peptides (Cruz-Vera et al., 2005; Nakatogawa and Ito, 2002; Vazquez-Laslop et al., 2010). Experimental evidence indicates that monitoring the nascent peptide structure can involve several redundant sensors whose integrated signals trigger a functional ribosomal response to regulatory nascent peptides (Seidelt et al., 2009; Vazquez-Laslop et al., 2010). Interfering with the operation of one sensor (A2503) by Cfr-mediated methylation may have little effect on the functional ribosomal response. However, if sensing of the nascent peptide in the tunnel is already crippled by Erm-mediated modification of A2058, then C8 methylation of A2503 may become more deleterious. In agreement with this hypothesis, in the CM05 methicillin-resistant *S. aureus* isolate where *cfr* is coexpressed with *ermB*, the loss of *cfr* appears to provide notable growth advantage in the absence of antibiotic selection because *cfr*-negative clones appear with a high frequency upon plating (Locke et al., 2011).

Since our conclusions about the fitness cost associated with Cfr expression have been drawn from a laboratory model system based on an artificially-generated *S. aureus* strain, we want to add a cautionary note. Recent genome sequencing of the laboratory *S. aureus* strain RN4220 used in our study showed that it carries several mutations that, on their own, might negatively affect its fitness (Nair et al., 2011). Because the initially less-fit cells are expected to be hyper-sensitive to a potential burden associated with *cfr* expression, the low fitness cost
determined in our experiments could be potentially overestimated. Obviously, these considerations do not compromise, but rather reinforce our conclusion about the low fitness cost of \textit{cfr}. Nevertheless, one needs to keep in mind that the response of clinical strains could be somewhat different. It should also be noted that in our experiments, the low fitness cost of \textit{cfr} was manifested in optimized laboratory conditions in a nutrient-rich medium. We cannot exclude the general possibility that the fitness cost could be higher in natural environments, or that the fitness loss associated with \textit{cfr} acquisition could be more pronounced in organisms other than \textit{S. aureus}. These possibilities could be addressed in future studies. From the epidemiological standpoint, the low cost of \textit{cfr} in staphylococci, especially in the absence of an associated \textit{erm} gene, is troubling. The \textit{cfr} gene is often linked with mobile genetic elements (plasmids or transposons) and thus is prone to mobilization and horizontal gene transfer (Bongiorno et al., 2010; Kehrenberg et al., 2007; Schwarz et al., 2000; Toh et al., 2007). Furthermore, since Cfr confers resistance to a very broad array of ribosomal antibiotics, including phenicols, lincosamides, oxazolidinones, pleuromutilins, streptogramin A, and even some macrolides, the use of any of these drugs can select for cells that have acquired \textit{cfr} and promote maintenance of this gene in the population. The negligible loss of fitness associated with the presence of \textit{cfr} in the genomes of pathogens suggests that cells can easily maintain the gene even in the absence of selection. This could greatly increase the probability and the rate of the spread of \textit{cfr}-based resistance. Indeed, after the first description of the \textit{cfr} gene in the CM05 strain in 2007, a growing number of reports indicate the presence of this gene among clinical strains of different species and in various locations worldwide (Bongiorno et al., 2010; Bonilla et al., 2010; Cercenado, 2010; Mendes et al., 2010a; Mendes et al., 2008; Morales et al., 2010). These
observations may reflect the ongoing spread of the cfr gene. Our study offers a possible microbiological explanation for this alarming trend.

B. **Unusually High Linezolid Resistance in the Clinical *Staphylococcus epidermidis* Strain 426-3147L: Characterization of the Transcription and Translation Signals Controlling cfr Expression**

1. **Introduction**

The gene encoding Cfr was originally reported to be present on the 17 kb plasmid pSCFS1 from a *Staphylococcus sciuri* strain isolated from a calf (Schwarz et al., 2000). In this strain, cfr is preceded by two overlapping open reading frames (ORFs), which code for peptides of 59 and 44 amino acids, respectively (see Figure 4) (Schwarz et al., 2000). While the role of these ORFs has not been elucidated, it has been suggested that cfr may be inducible, and these ORFs may have a function in the regulation of cfr expression. Since the first description of pSCFS1 in 2000, several other staphylococcal isolates from animals have been reported which harbor cfr, where it is often associated with mobile genetic elements such as transposons and insertion elements. In 2007, the first clinical isolate of *S. aureus* containing cfr was described (Toh et al., 2007). Later on, a number of other cfr-positive clinical and veterinary strains of *S. aureus*, *S. epidermidis*, *P. vulgaris* and *Bacillus* spp. have been reported (Dai et al., 2010; Mendes et al., 2008; Mendes et al., 2010b; Wang et al., 2011).

In contrast to the majority of cfr-containing clinical staphylococcal isolates which exhibit linezolid MICs in the range of 8-32 µg/ml, a recent clinical isolate of *S. epidermidis* (426-3147L), has a linezolid MIC that exceeded 256 µg/ml (Mendes et al., 2008). Although the strain was positive for cfr, the reason for such an exceedingly high linezolid MIC was unknown. We examined the genetic environment of cfr in *S. epidermidis* 426-3147L, its expression and the
presence of other genetic modifications that can contribute to linezolid resistance. We found that a combination of several chromosomal mutations and specifics of \( cfr \) expression contribute to an unusually high resistance of the strain to linezolid.

2. **Experimental results**

   a. **Increased extent of A2503 modification in the \( S. \) epidermidis isolate 426-3147L**

   Initial PCR analysis showed the presence of the \( cfr \) gene in the genome of the isolate (Figure 14 A). This prompted us to investigate whether the possible overexpression of the \( cfr \) gene contributes to the unusually high level of linezolid resistance of 426-3147L. In our first approach, we evaluated the extent of Cfr-mediated modification of A2503 in 23S rRNA of this isolate. Cfr methylates C8 of the A2503 base in 23S rRNA (Giessing et al., 2009). This modification interferes with the progression of reverse transcriptase, especially at a reduced concentration of dTTP (Kehrenberg et al., 2005). Therefore, the extent of A2503 modification can be semi-quantitatively assessed by primer extension (Kehrenberg et al., 2005; Toh et al., 2007). Primer extension with a 23S rRNA-specific primer was carried out on total RNA isolated from the \( S. \) epidermidis 426-3147L strain, the \( S. \) sciuri strain carrying \( cfr \) on the plasmid pSCFS1, the \( cfr \)-negative \( S. \) epidermidis ATCC 12228 control strain and the \( cfr \)-negative \( S. \) aureus strain RN4220 (Figure 14 B) (Schwarz et al., 2000). The appearance of a strong reverse transcriptase stop at A2503 in the \( cfr \)-positive strains confirmed the modification of this nucleotide by the Cfr methyltransferase. The arrest of reverse transcriptase progression at A2503 in the 426-3147L 23S rRNA was ca. 3-fold more efficient than in the rRNA of \( cfr \)-negative strains. Furthermore, the band reflecting the reverse transcriptase stop at A2503 in 426-3147L rRNA was 1.5% stronger than the corresponding band in the rRNA sample extracted from the \( S. \)
Figure 14. The presence of the *cfr* gene in *S. epidermidis* 426-3147L leads to rRNA modification. A. PCR analysis that reveals the presence of *cfr* (arrow) in 426-3147L. pSCFS1 was used as a positive control; *S. aureus* RN4220 and *S. epidermidis* ATCC 12228 were used as negative controls. B. Primer extension analysis of the extent of A2503 methylation. Isolate 426-3147L exhibits a notably higher extent of A2503 modification than the negative controls or the *S. sciuri* strain with pSCFS1. Quantification of the A2503 band from the gel in panel B is included in the right panel. In both panels, SA: *S. aureus*; SE: *S. epidermidis*; SS: *S. sciuri*. 
scuiri strain carrying *cfr* on the pSCFS1 plasmid. This result suggests that an extensive Cfr-mediated modification of A2503 contributes to linezolid resistance in the *S. epidermidis* isolate 426-3147L and that either the genetic organization or expression pattern of the *cfr* gene may account for the elevated level of Cfr activity in this strain.

b. **In the *S. epidermidis* 426-3147L strain, the *cfr* gene is located on a plasmid**

In the first *cfr*-positive clinical isolate and in a subsequently isolated *cfr*-containing strain, the *cfr* gene resides in the chromosome (Locke et al., 2011; Toh et al., 2007; Wang et al., 2011). However, it can also be found on plasmids (Dai et al., 2010; Kehrenberg et al., 2004; Kehrenberg and Schwarz, 2006; Mendes et al., 2008). Our preliminary analysis showed that the *S. epidermidis* isolate 426-3147L carries one or several plasmids that could potentially host the *cfr* gene. A standard plasmid preparation protocol produced a fraction significantly enriched in the plasmid material, although we were unable to effectively eliminate substantial contamination with chromosomal DNA. In order to understand whether *cfr* was located on a plasmid or the chromosome, total DNA or plasmid-enriched DNA were digested with the HindIII restriction enzyme and, after Southern blotting, hybridized with a *cfr*-specific probe (Figure 15 A and 15 B). A probe specific for the chromosomal gene *se0011*, encoding homoserine-o-acetyltransferase, was included as a hybridization control. If *cfr* was located on the chromosome in this strain, the relative intensities of both probes would remain the same in the total DNA sample and the plasmid-enriched sample; if *cfr* was located on a plasmid, the intensity of one of the bands would increase in the plasmid-enriched DNA sample relative to the other band. Analysis of the Southern blot showed that while the total DNA and the plasmid-enriched fraction contained DNA bands that hybridized with both probes, the relative intensity of the *cfr*-specific
Figure 15. The cfr gene is located on a plasmid in the *S. epidermidis* isolate 426-3147L. Southern blot analysis of the HindIII digest on total DNA (lane 1) or total plasmid DNA (lane 2) from 426-3147L. Probes specific for cfr or a genomic gene (*se0011*) were used. A. Ethidium bromide staining; B. Autoradiography.
band was significantly stronger in the plasmid-enriched fraction, suggesting that the cfr gene most likely resides on the plasmid.

In order to verify the presence of cfr on a plasmid, the plasmid-enriched DNA fraction from S. epidermidis 426-3147L was used to transform the S. aureus strain RN4220. Transformants were selected on plates containing 10 µg/ml florfenicol, and a number of florfenicol-resistant colonies were recovered. Analysis of the plasmid prepared from the transformed RN4220 cells showed a restriction pattern that partially matched that observed for the original total plasmid prepared from S. epidermidis 426-3147L (Figure 16 A). The size of the plasmid transferred to the RN4220 cells, designated p7LC, was estimated from the sum of the lengths of the restriction fragments to be approximately 30,000 bp. The presence of additional bands in the plasmid preparation from 426-3147L that were missing in the p7LC preparation from the transformed S. aureus RN4220 cells suggests that S. epidermidis 426-3147L has several plasmids, only one of which (p7LC) likely carries the cfr gene. The high florfenicol and linezolid MICs of the resultant S. aureus transformants (Table V) and PCR analysis (Figure 16 B) confirmed that the cfr gene was successfully transferred with the plasmid. This result clearly established that in the linezolid-hyperresistant S. epidermidis isolate 426-3147L, the cfr gene resides on the p7LC plasmid.

Although we were unable to accurately determine the copy number of the cfr-bearing plasmid p7LC in S. epidermidis 426-3147L, the relative intensity of the plasmid-specific bands in the HindIII digest of the total DNA preparation (Figure 15 A) argue that the plasmid copy number can be in the range of 5-7 copies per chromosome, accounting for a high dosage of the cfr gene in the cells, thus likely contributing to the elevated linezolid MIC in this strain.
Table V. Minimal inhibitory concentrations of different staphylococcal cells.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain</th>
<th>Linezolid µg/ml</th>
<th>Florfenicol µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. epidermidis</td>
<td>ATCC 12228</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>426-3147L</td>
<td>&gt;256</td>
<td>1024</td>
</tr>
<tr>
<td>S. aureus</td>
<td>RN4220</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>S. aureus</td>
<td>RN4220/p7LC</td>
<td>8</td>
<td>512</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>426-3147L/cured of p7LC</td>
<td>64</td>
<td>16</td>
</tr>
</tbody>
</table>
Figure 16. Restriction digest of plasmid isolated from RN4220 transformed with p7LC. A. Plasmid isolated from RN4220 transformants exhibits the HindIII digest pattern that partially matches that of the total plasmid from *S. epidermidis* 426-3147L. The green stars indicate the bands associated with p7LC in both samples. B. PCR reveals the presence of *cfr* in both strains, confirming the presence of *cfr* on p7LC. The empty pLI50 plasmid is used as a negative control.
c. **Unusual transcriptional and genetic organization of the cfr gene in the p7LC plasmid**

In the originally described cfr-bearing plasmid pSCFS1, expression of the cfr gene was proposed to be driven by a predicted promoter located 402 bp upstream of the gene start codon (Schwarz et al., 2000). Since transcription from this promoter has not been experimentally verified, we wanted to test whether it indeed drives cfr expression and whether additional transcriptional signals could contribute to the high level of cfr expression in p7LC. We first verified that the predicted promoter directs the transcription of cfr in the pSCFS1 plasmid. Primer extension carried out on total RNA prepared from *S. scuiri* cells carrying the pSCFS1 plasmid showed an indicative band placing transcription start 32 nt upstream of the start of orf1 in pSCFS1, which corresponds to position 5,924 of the reported sequence (Figure 17) (Kehrenberg et al., 2004; Schwarz et al., 2000). This observation confirmed the transcriptional activity of the predicted cfr promoter and showed that this promoter accounts for cfr expression in the *S. scuiri/pSCFS1* strain. We then proceeded with analyzing the transcription pattern in the *S. epidermidis* strain. While the band corresponding to transcripts initiated at the cfr promoter were clearly detected, several longer primer extension products were observed (marked by asterisks in Figure 17), suggesting that some of the transcripts which encompass the cfr gene in the *S. epidermidis* 426-3147L strain originated upstream of the cfr promoter. The presence of these transcripts clearly indicated that additional promoters, located farther upstream from the conventional cfr promoter, contribute to the overall expression of cfr in p7LC. This observation prompted us to further explore the structure of the upstream region of cfr.

Preliminary sequencing efforts showed that ca. 300 bp of the DNA segment in the p7LC plasmid upstream of the cfr gene closely matched the sequence of the 5'-transcribed region of cfr.
Figure 17. Mapping of the transcription start site in cfr-containing strains. Primer extension analysis shows that cfr transcription in all three cfr-positive strains S. sciuri/pSCFS1, S. lentus/pSCFS3 and S. epidermidis 426-3147L/p7LC starts from the same promoter Pcfr. The difference in the position of the transcription start band is due to the presence of the 51 bp in the 5’-untranslated region in pSCFS1 and pSCFS3 that are missing in p7LC. Additional transcripts originating at a promoter located upstream from Pcfr contributes to the cfr expression in S. epidermidis 426-3147L (indicated by the asterisks). RN4220 served as the negative control. SS: S. sciuri; SL: Staphylococcus lentus; SA: S. aureus; SE: S. epidermidis.
in pSCFS1, including the cfr promoter (although a 51 bp deletion and one nucleotide mutation were found within one of the upstream ORFs) (Figure 18 B). The initial attempt to amplify a more extended upstream region of cfr using the primer pSCFS1R located at the end of the cfr gene and the direct primer pSCFS1F, corresponding to the sequence found in pSCFS1 654 bp upstream from cfr gave no PCR product. Subsequent primer walking experiments using Primers 33, 34 and 41-45 showed that this region contains a complete copy of the Tn4001 transposon, including two inversely oriented transposase genes flanking the aminoglycoside resistance gene aacA-aphD (Figure 18 A). The 26 bp inverted repeats that form the transposase recognition sites and are characteristic to the transposon ends are present in isolate 426-3147L, demonstrating the intact nature of Tn4001 in this isolate (Byrne et al., 1989). Furthermore, the presence of an outward-facing promoter originating in the IS256 gene may contribute to the longer cfr-containing transcripts seen from the primer extension experiment (Byrne et al., 1989).

While in the canonical Tn4001 sequence the two inversely oriented transposase genes are identical to each other, in 426-3147L, the transposase gene closest to cfr carries a short region (660 nt) which is substantially different from the canonical IS256 transposase gene found on the other end of the transposon. A similar structure of the Tn4001-type region, complete with one wild type IS256 gene, aacA-aphD, a IS256-like gene and cfr, has recently been described on a plasmid whose sequence was deposited on October 6, 2011 to the NCBI database. This plasmid, pSS-01, identified in a Staphylococcus cohnii strain from a healthy pig, also contains the fexA gene (see Figure 5). FexA confers resistance to phenicols, and has been associated with cfr in other plasmids. However, PCR reactions specific for fexA yielded no product when total 426-3147L DNA was used as a template (data not shown), suggesting that fexA is not present in the p7LC plasmid. Furthermore, a predicted HindIII restriction digest of pSS-01 does not match the
Figure 18. The map of the DNA region adjacent to the \textit{cfr} gene in the p7LC plasmid. A. The Tn4001-like element is found upstream of \textit{cfr}. The \textit{IS256-1} and \textit{IS256-2} transposase genes flank the aminoglycoside resistance gene \textit{aacA-aphD}. \textit{Cfr} contains the promoter \textit{Pcfr}, the truncated \textit{orf1} and the full \textit{orf2}. B. The amino acid alignment of ORF1 in \textit{S. epidermidis} 426-3147L and pSCFS1. A point mutation is shown in red, and the start codon of ORF 2 is shown in orange.
experimental digest pattern of p7LC, indicating that the Tn4001-cfr cassette has inserted into different plasmid backbones to produce pSS-01 and p7LC. Further characterization of the p7LC sequence is needed to accurately compare it with pSS-01. Nevertheless, the finding that the same arrangement of cfr with other resistance genes is found in veterinary and clinical isolates suggest that selective pressures caused by veterinary antibiotics may play an important role in drug resistance mechanisms found in the clinics.

d. **Alterations in the 5'-transcribed region of the cfr gene may negatively affect its inducibility**

The originally described cfr-positive isolate of *S. scuiri* with the cfr gene on the plasmid pSCFS1, showed signs of inducible expression of the cfr gene. Pre-incubation of the cells with subinhibitory concentrations of florfenicol was reported to increase the florfenicol MIC from 64 µg/ml for non-induced cells to 512 µg/ml for the induced cells (Schwarz et al., 2000). The possibly inducible nature of the cfr gene was tentatively associated with the presence of the two overlapping short ORFs in the 5'-transcribed region, because the regulation of several antibiotic resistance genes is known to be controlled by upstream leader ORFs (Ramu et al., 2011). The upstream region of the cfr gene in the *S. epidermidis* isolate carries a 51 bp deletion that alters orf1 (Figure 18 B). In order to understand whether this alteration in the putative regulatory region affects cfr expression, we compared the expression of the cfr gene preceded by the 5'-transcribed spacer of pSCFS1 to that present in the *S. epidermidis* isolate. The cfr gene and the upstream region, including the promoter as they appear in isolate *S. epidermidis* 426-3147L and *S. sciuiri/pSCFS1*, were cloned into a shuttle plasmid pJL1 to produce plasmids pJL1-7L and pJL1B (Figure 19A) (LaMarre et al., 2011). pJL1B and pJL1-7L were transformed into *S. aureus* RN4220 cells and the extent of Cfr-mediated modification of A2503 in 23S rRNA under non-
Figure 19. Inducibility of $cfr$ in different strains. A. A map of $cfr$ and its upstream region in the plasmids used in induction experiments. Both constructs contain the promoter $P_{cfr}$, $orf2$ and $cfr$, but they differ in the sequence of $orf1$. $orf1$ in pJL1B is the same as in pSCFS1, and the $orf1$ in pJL1-7L is the same as in $S. \text{epidermidis}$ 426-3147L, including a 51 bp deletion. B. Primer extension reveals that the extent of A2503 modification increases in pSCFS1 and pJL1B-transformed cells, but remains unchanged in p7LC-transformed cells. A bar graph below the gel represents the intensity of the A2503-specific band; the graph to the right indicates the level of induction by florfenicol. SS: $S. \text{sciuri}$; SA: $S. \text{aureus}$.
inducing conditions and after the pre-incubation of cells in the presence of 0.5 µg/ml florfenicol was analyzed by primer extension (Figure 19 B). In addition, *S. sciuri* cells containing pSCFS1 were analyzed in similar experiments (Figure 19 B). A2503 was modified substantially more extensively in the cells transformed with the pJL constructs compared to pSCFS1 transformants, possibly due to a higher copy number of the pLI50-derived pJL plasmids. Pre-incubation of cells with a subinhibitory concentration of florfenicol results in a 2.8-fold increase in A2503 modification in cells transformed with pSCFS1, thus confirming the previous conclusion about the inducible nature of *cfr* in pSCFS1 derived from microbiological testing. However, we failed to observe any significant induction in the cells transformed with the pJL constructs, irrespective of whether the *cfr* upstream region corresponded to that in pSCFS1 (as in the pJL1B transformants) or carried the 51 bp deletion present in *S. epidermidis* 426-3147L (as in the pJL1-7L transformants). A possible explanation is that overexpression of *cfr* in the pJL transformants may mask its inducibility due to a high level of A2503 modification even in the absence of induction. Yet the cells carrying pJL1B showed a slight induction (ca 1.4-fold) of A2503 modification, whereas the cells transformed with pJL1-7L did not show such a trend. Thus, the deletion of 51 bp in the 5'-transcribed region of the *cfr* gene observed in the *S. epidermidis* 426-3147L isolate might negatively affect *cfr* inducibility. Nevertheless, in order to strengthen this conclusion, the experiments will need to be repeated in a low copy number plasmid, or the corresponding 51 bp deletion in *orfI* needs to be engineered directly in the pSCFS1 plasmid.

e. **Determinants other than *cfr* contribute to the high level of linezolid resistance in *S. epidermidis* 426-3147L.**

Our analysis of the *cfr* genetic organization in the *S. epidermidis* strain revealed several factors that could affect its expression, including its presence on a multicopy plasmid and the
contribution of an upstream transposon element to cfr transcription. However, none of these findings appear to be sufficient to explain an exceptionally high level of linezolid resistance observed in the 426-3147L strain. While S. epidermidis 426-3147L showed linezolid MIC of >256 µg/ml, S. aureus cells transformed with the cfr-containing plasmid isolated from S. epidermidis 426-3147L showed a linezolid MIC of only 8 µg/ml. Although this difference could be attributed to the host genetic background, it was likely that other resistance determinants were operating in the S. epidermidis isolate. To verify this possibility, 426-3147L was cured of the p7LC plasmid by incubating cells at 42 °C. The loss of p7LC was confirmed by multiplex PCR, which showed the lack of the cfr gene in the cured strain, but the presence of the control band corresponding to the rplC gene (Figure 20). The cured strain exhibited a linezolid MIC of 64 µg/ml which was reduced compared to the original, cfr-positive cells, but was substantially higher than the MIC of the linezolid-susceptible control, S. epidermidis strain ATCC 12228 (Table V). This result shows that additional resistance mechanisms likely contribute to linezolid resistance of the S. epidermidis 426-3147L isolate.

Besides the presence of the cfr gene, linezolid resistance can be conferred by mutations in 23S rRNA as well as in ribosomal proteins L3 and L4 (Locke et al., 2009a, b; Wolter et al., 2005). To test for the presence of other resistance mechanisms, our collaborators at the Jones Microbiology Institute sequenced Domain V of six individual alleles of 23S rRNA gene as well as the genes rplC and rplD encoding ribosomal proteins L3 and L4.

The C2534T mutation (E. coli numbering) was identified in two of the six ribosomal rrn alleles. This mutation was previously associated with linezolid resistance in S. epidermidis isolates (Liakopoulos et al., 2010; Wong et al., 2010). Three missense mutations, altering amino acid residues His146Gln, Val154Leu and Ala157Arg, were identified in the rplC gene, and the
Figure 20. PCR analysis revealing the successful loss of p7LC from the 426-3147L strain. PCR reactions were run with primer pairs specific for either cfr or ribosomal protein L3, or both pairs together. Template DNA was prepared from the original 426-3147L strain or the strain cured from p7LC (cured).
gene *rplD* had an insertion of a glycine codon between amino acid residues 71 and 72. We could conclude that the ribosomal mutations, in combination with the presence of the *cfr* gene, account for the unusually high level of linezolid resistance in the *S. epidermidis* 426-3147L isolate.

3. **Discussion**

We have described a combination of resistance mechanisms that account for the high resistance of the clinical *S. epidermidis* strain 426-3147L to linezolid. The finding of identical isolates in the same healthcare facility in subsequent years stresses the ability of this strain to persist in a clinical setting, which highlights the concern of understanding linezolid resistance in hospitals (Farrell et al., 2009; Farrell et al., 2011; Mendes et al., 2008; Mendes et al., 2010b).

One of the key resistance factors in the strain is the presence of the *cfr* gene that encodes the methyltransferase which targets rRNA residue A2503 located in the linezolid binding site. Although several *cfr*-positive clinical isolates have been reported since the first description of the *cfr* gene in a hospital MRSA strain in 2007, the genetic organization of *cfr* in the *S. epidermidis* strain 426-3147L is unusual.

Primer extension analysis revealed a higher degree of A2503 modification in *S. epidermidis* 426-3147L compared to *S. sciuri* carrying another *cfr*-containing plasmid pSCFS1. We think that elevated activity of *cfr* in 426-3147L contributes to the high resistance to linezolid. Several factors may account for the high level of *cfr* expression in the 426-3147L strain. Firstly, the presence of the *cfr* gene on a multicopy plasmid increases the gene dosage. Although it was impossible to accurately determine the plasmid copy number, our estimates suggest that there are at least 5-7 plasmid copies per chromosome in *S. epidermidis* 426-3147L. Secondly, the *cfr* gene is located directly downstream of a complete copy of the Tn4001 transposon. A promoter found within the transposase gene likely contributes to the transcription of *cfr*. In addition, the
alteration in the structure of the \textit{cfr} upstream region may render the putatively-inducible \textit{cfr} gene constitutive.

In the course of analyzing \textit{cfr} expression in \textit{S. epidermidis} 426-3147L, we obtained two important pieces of information pertaining to the general mechanism of its expression. We were able to experimentally verify that the putative \textit{cfr} promoter, previously predicted only computationally on the basis of analysis of the nucleotide sequence of the pSCFS1 plasmid, indeed directs \textit{cfr} transcription (Kehrenberg et al., 2004; Schwarz et al., 2000). Secondly, we obtained biochemical evidence that preincubation of the staphylococcal strain carrying the pSCFS1 plasmid with florfenicol results in a higher degree of A2503 modification. This observation is compatible with the proposed inducible nature of \textit{cfr}. However, it should be noted that the observed increase in rRNA modification in response to florfenicol treatment was only modest, especially in comparison with such well-characterized inducible resistance genes as the \textit{erm} family, which encode methyltransferases targeting A2058 in 23S rRNA (Vester et al., 1998; Villsen et al., 1999). Either the \textit{cfr} regulatory region in pSCFS1 is already altered compared to the original native \textit{cfr} version that possibly exists in one of the antibiotic producers, or florfenicol is not a potent inducer of \textit{cfr}. Our attempts to identify a more potent \textit{cfr} inducer using such compounds as chloramphenicol, tiamulin and pristinamycin failed to pinpoint an antibiotic with a more potent inducing activity (unpublished). It is also possible that \textit{cfr} expression has been evolutionarily optimized to be induced only moderately. Although the induction of \textit{cfr} in the cells carrying the pSCFS1 plasmid was easily detectable, cells transformed with the multicopy pLI50-based plasmid that carries \textit{cfr} under the control of the same regulatory region showed only minimal induction of \textit{cfr}, possibly due to high level of \textit{cfr} activity in the uninduced state. Yet the deletion in the upstream region found in the 426-3147L isolate seems to eliminate even this
slight induction, therefore arguing that the deletion of 51 bp from the upstream ORF1 could render the gene constitutive in 426-3147L.

In the first cfr-positive MRSA isolate from a human, cfr was genetically and transcriptionally linked to the ermB gene. Co-expression of the Cfr and Erm methyltransferases rendered cells resistant to all the clinically-relevant antibiotics targeting the large ribosomal subunit, including drugs belonging to the macrolide, lincosamide, pleuromutilin, phenicol, oxazolidinone and streptogramin families of compounds. The presence of the transposon Tn4001 that carries the aacA-aphD gene (which renders cells resistant to multiple aminoglycosides) upstream of cfr in 426-3147L links together two multidrug resistance genes that protect cells from antibiotics acting upon both ribosomal subunits. Given that Tn4001 is known to be a highly mobile element that possesses random insertion specificity, and that the cfr-Tn4001 cassette is present on a plasmid, it is reasonable to expect dissemination of this cassette to other hosts and genetic locales, as is evident by its presence in plasmid pSS-01 from S. cohnii.

The cfr gene is not the sole determinant of linezolid resistance in S. epidermidis 426-3147L. Curing the strain from the cfr-containing plasmid reduced resistance from >256 µg/ml to 64 µg/ml, indicating that other mechanisms contribute to the extremely high linezolid MIC of the original isolate. Sequencing of the rDNA and ribosomal protein genes rplC and rplID showed the presence of the C2534T mutation in two alleles of rDNA in the 426-3147L strain as well as several mutations in proteins L3 and L4. The C2534T mutation has been previously found in S. epidermidis which, when present in two alleles like S. epidermidis 426-3147L, was reported to give a linezolid MIC of 8 µg/ml (Liakopoulos et al., 2010). The mutations found in S. epidermidis 426-3147L genes encoding proteins L3 and L4 also have been previously noted in clinical strains of staphylococci (Kosowska-Shick et al., 2010; Locke et al., 2009a). The frequent
occurrence of L3 and L4 mutations in resistant clinical strains argues that alterations in these proteins either provide a certain level of linezolid resistance as such or that they decrease any fitness cost associated with rRNA mutations or cfr-mediated rRNA modification. Locke and colleagues have obtained *S. aureus* mutants under oxazolidinone selection with either the Gly155Arg or ΔPhe127-His146 mutations (Locke et al., 2009b). These mutations are close in proximity to the mutations found in *S. epidermidis* 426-3147L, further indicating that they might play a role in linezolid resistance. In addition, deletion mutations of amino acids 65-66 or 68-69 in protein L4 in *Streptococcus pneumoniae* resulted in a decrease of linezolid susceptibility, which was verified by engineering naïve bacteria with these changes.

In conclusion, the extremely high level of linezolid resistance of the *S. epidermidis* 426-3147L isolate is accounted for by a combination of resistance mechanisms, at least some of which (such as the cfr-mediated rRNA modification, the rRNA and ribosomal protein mutations) operate through alteration of the drug target. The combined action of these mechanisms can be sufficient to provide the level of resistance observed in the isolate. Nevertheless, we cannot rule out the involvement of other resistance mechanisms, such as drug efflux of drug modification. Altogether, our findings reinforce a growing understanding that in spite of the synthetic nature of linezolid, the pathogens targeted by this antibiotic have sufficient genetic flexibility to acquire very significant levels of resistance by combining the effects of several different resistance mechanisms.

C. **Inactivation of the Indigenous Methyltransferase RlmN in *Staphylococcus aureus***

1. **Introduction**
During the early years of linezolid’s use, clinically relevant resistance occurred only rarely and was mostly limited to target site mutations in rRNA and ribosomal proteins (Locke et al., 2009a; Meka et al., 2004b; Prystowsky et al., 2001; Wolter et al., 2005). The first clinical linezolid-resistant MRSA isolate with an acquired linezolid resistance gene was CM05, which carries the cfr gene (Toh et al., 2007). Besides being the target for the Cfr resistance enzyme, A2503 is also naturally methylated at C2 by the action of an indigenous methyltransferase, RlmN, which is highly homologous to Cfr and utilizes the same Radical SAM mechanism (Toh et al., 2008; Yan et al., 2010). The rlmN gene is widespread and is found in the genomes of most bacterial pathogens. Although rlmN is thought to play a role in the interaction of the ribosome with the nascent peptide, its inactivation in E. coli or S. aureus has little effect upon cell growth (Vazquez-Laslop et al., 2010). S. aureus (but not E. coli) cells lacking the rlmN gene showed slight (1 dilution or less) elevation in susceptibility to linezolid (Toh and Mankin, 2008; Toh et al., 2008). A new mechanism of decreased susceptibility to linezolid was described in a recent study (Gao et al., 2010). Upon prolonged treatment of a MRSA-infected patient with linezolid, a mutation (an insertion of an additional Gln codon after codon 353) arose in the rlmN gene. This mutation increased the linezolid MIC from 0.75 µg/mL observed in the original MRSA strain (JKD6210) to 2 µg/mL in the mutant (JKD6229) when a high-level inoculum (2 McFarland units) was used in the Etest. Engineered into the original S. aureus strain JKD6210, this mutation also increased the linezolid MIC to 2 µg/mL in the resulting strain (JKD6300), revealing the causative relationship of the reduced susceptibility with the genetic change in rlmN. In view of the previous report that rlmN inactivation causes a slight increase in linezolid susceptibility, it was proposed that the resistance mutation in rlmN increases the degree of A2503 methylation (Gao et al., 2010). For example, the mutation could change the enzyme specificity to allow
RlmN to methylate not only C2 but also C8 of A2503, as Cfr does. To test this hypothesis and to provide a molecular explanation for the rlmN-associated resistance mechanism, we investigated the status of A2503 modification in the mutant strains.

2. **Experimental results**

   a. **The rlmN mutation in JKD6229 inactivates enzyme activity**

   Primer extension analysis can distinguish between C2-monomethylated adenine, C2- and C8-dimethylated adenine, or the completely nonmethylated nucleotide. Methylation at C2 results in a moderate reverse transcriptase stop which generates an extra band of a moderate intensity on a sequencing gel, C2- and C8-dimethylated A2503 produces a strong stop, and the complete lack of nucleotide modification allows for unimpeded progression of reverse transcriptase (Kehrenberg et al., 2005; Toh et al., 2007; Toh et al., 2008). To our surprise, the rlmN mutation found in the selected (JKD6229) or engineered (JKD6300) S. aureus strain which decreased susceptibility to linezolid did not cause an increase in the methylation at 2503, as originally suspected, but rather the complete abatement of modification (Figure 21). Thus, the insertion of an additional Gln codon at position 353 in the rlmN gene results in an inactive form of the RlmN enzyme. This result seemingly contradicted the previous report where an rlmN knockout led to a slight decrease in linezolid resistance (Toh et al., 2008). Therefore, we revisited those results.

   b. **Cogrowth experiments with RlmN+ and RlmN- strains**

   Despite multiple attempts, direct MIC testing in which we compared the linezolid susceptibility of the rlmN knockout mutant of the S. aureus Newman strain (SAV1218) with that of the strain with a knockout of the neutral gene xylA (SAV1986) did not provide a definitive answer whether the lack of rlmN causes resistance or hypersusceptibility to linezolid. The MIC difference was so small (one 2-fold dilution or less) that it varied between experiments, making it
Figure 21. Mutation in the *rlmN* gene found in the clinical MRSA strains JKD6229 prevents A2503 modification. Primer extension analysis of 23S rRNA isolated from the control laboratory *S. aureus* strain RN4220, the original MRSA strain JKD6210, and its selected (JKD6229) or engineered (JKD6300) derivative illustrates that strains containing the codon insertion in RlmN abolishes A2503 methylation.
difficult to obtain conclusive results. Therefore, we decided to mimic the clinical “natural selection” setting where the spontaneous mutant needs to compete with the wild type under antibiotic pressure. A mixture of the \textit{rlmN} knockout (SAV1218) cells and neutral knockout control (SAV1986) cells was cocultured in BHI medium in the presence of 0.5 µg/mL linezolid, and the ratio of the cells was monitored over ca. 30 cell generations. In the control cells, A2503 is fully C2 modified, and primer extension results in a pronounced reverse transcriptase stop at this position, whereas in the mutant that lacks modification, no stop is observed. Therefore, primer extension on 23S rRNA isolated from the mixed population can be used as an efficient way to monitor the ratio of RlmN\(^+\) and RlmN\(^-\) cells. As Figure 2 indicates, the strain lacking A2503 methylation was more prevalent by the end of the experiment, indicating that under selective pressure, cells lacking functional RlmN do indeed outcompete those with active RlmN. We observed the same results when growth of the original MRSA cells (JKD6210) and of the engineered codon-insertion mutant (JKD6300) was monitored in the same experimental setting. These data are in agreement with the results of the clinical study where cells with the mutation that inactivated the RlmN methyltransferase took over the population upon prolonged linezolid therapy and were associated with linezolid treatment failure (Gao et al., 2010).

3. **Discussion**

The possible clinical impact of subtle changes in linezolid susceptibility in other pathogens due to \textit{rlmN} inactivation has not been defined but, by analogy with other
Figure 22. The *S. aureus* strain lacking functional RlmN wins in growth competition with wild type cells in the presence of linezolid. Mixed cultures of RlmN-positive (SAV1986) and RlmN-negative (SAV1218) Newman strains were grown in the presence of 0.5 µg/ml linezolid, and the ratio of RlmN+/RlmN- cells was quantified every 10 generations by primer extension.
antibiotics, might significantly affect treatment outcomes (Lodise et al., 2008). In the originally described clinical strain JKD6229, the elevated resistance to linezolid was associated with a codon insertion in the \textit{rlmN} gene which, as our findings demonstrate, inactivates the encoded enzyme. However, as our studies of the \textit{rlmN} knockout show, the same effect could be expected from any mutation that prevents expression of functional RlmN. Any nonsense mutation in the gene, missense mutation at functionally critical positions of the protein, or promoter mutation is expected to decrease susceptibility to linezolid. Given that the lack of the RlmN-mediated modification has little effect on cell growth in the absence of antibiotic, such resistance mechanisms can be easily maintained even after discontinuation of antibiotic therapy (Toh et al., 2008). Our findings expand the list of resistance mechanisms based on the lack of natural rRNA modification and call for monitoring the sequence of the \textit{rlmN} gene and its regulatory regions in clinical isolates subjected to linezolid exposure (Helser et al., 1971; Johansen et al., 2006; Lazaro et al., 1996; Okamoto et al., 2007).

D. \textbf{Functional Properties of RlmN and Cfr, Radical S-adenosylmethionine Enzymes Involved in Methylation of Ribosomal RNA}

1. \textbf{Introduction}

Evidence obtained in \textit{in vivo} studies indicates that Cfr methylates the C8 atom of A2503 of 23S rRNA (\textit{E. coli} numbering), located in the peptidyl transferase center of the bacterial large ribosomal subunit (Giessing et al., 2009). Such modification renders cells resistant to several important classes of ribosomal antibiotics that act upon the peptidyl transferase center, including phenicols, pleuromutilins, streptogramins A, lincosamides, and the recently developed oxazolidinones (Kehrenberg et al., 2005; Long et al., 2006; Schwarz et al., 2000; Smith and Mankin, 2008). The spread of \textit{cfr}-based resistance may compromise several key antibiotics,
including the oxazolidinone linezolid, which is often used as the last line of defense against multidrug-resistant bacterial infections (Hutchinson, 2003; Livermore, 2003). The drug resistant methyltransferase Cfr is a close homolog of a methyltransferase RlmN (Figure 7) (Toh et al., 2008). RlmN is an endogenous cellular enzyme which modifies the same nucleotide (A2503), but at the C2 atom of the adenine base. Such native posttranscriptional modifications of rRNA residues are presumed to play an important role in modulating and optimizing ribosomal function, although the precise roles of most modifications, including the RlmN-mediated C2 methylation of A2503, remain largely obscure (Chow et al., 2007; Toh et al., 2008).

RlmN and Cfr represent an intriguing pair of evolutionarily related rRNA modifying enzymes with completely different functions: one (RlmN) is an endogenous enzyme used by the cell to refine functions of the ribosome in protein synthesis, and the other (Cfr) is an acquired methyltransferase that protects cells from the action of antibiotics. Besides the important biological functions of the two enzymes, the enzymatic mechanisms which are thought to be used by these methyltransferases place RlmN and Cfr among the most interesting types of RNA-modifying enzymes. Most of the enzymes which methylate cellular RNA targets use SAM as a donor of electrophilic methyl groups to methylate nucleophilic oxygen atoms within the ribose moieties or nitrogen atoms within RNA nucleotide bases. RlmN and Cfr are unique as they perform chemically-difficult methyl transfer to sp^2^-hybridized carbon atoms of an adenosine nucleotide. RlmN and Cfr belong to the Radical SAM superfamily, which is characterized by the presence of the cysteine-rich CysXXXCysXXCys motif (Booker, 2009; Frey et al., 2008; Frey and Magnusson, 2003; Sofia et al., 2001). Enzymes belonging to the Radical SAM superfamily use this motif to bind a unique four iron-four sulfur cluster, which, upon reduction to the +1 oxidation state, donates one electron to the bound SAM, leading to the formation of a 5′-
deoxyadenosyl radical and methionine. The resulting 5′-deoxyadenosyl radical is a potent oxidant which then initiates a radical transformation by performing hydrogen atom abstraction from its substrate. Subsequent catalytic steps, specific to the given Radical SAM enzyme, result in a variety of outcomes such as amine shifts (lysine aminomutases), nucleotide reduction (ribonucleoside triphosphate reductase III), sulfur insertion (biotin synthase, lipoyl synthase), oxidative decarboxylation (coproporphyrinogen oxidase), methylthiolation, and complex rearrangements, among others (Anton et al., 2008; Ballinger et al., 1992a; Ballinger et al., 1992b; Baraniak et al., 1989; Chang et al., 1996; Chatterjee et al., 2008; Cicchillo et al., 2004a; Cicchillo et al., 2004b; Eliasson et al., 1990; Eliasson et al., 1992; Hernandez et al., 2007; King and Reichard, 1995; Layer et al., 2005; Layer et al., 2003; Layer et al., 2006; Lee et al., 2009; Moss and Frey, 1987; Pierrel et al., 2004; Tse Sum Bui et al., 2003; Tse Sum Bui et al., 2004; Ugulava et al., 2001a; Ugulava et al., 2001b). Overall, members of this superfamily participate in more than 40 distinct biochemical transformations, and the full catalog of functions carried out by these enzymes is yet to be determined (Frey and Magnusson, 2003).

Despite the importance of RlmN in modulating protein synthesis and Cfr in the generation of multiantibiotic-resistant phenotypes, no biochemical studies on the in vitro activity of Radical SAM methyltransferases had been reported. Purification of these enzymes in their active forms and elucidation of their catalytic mechanism would provide critical insight into the functional principles of Radical SAM methyltransferases and may pave the way for combating Cfr-based antibiotic resistance. Understanding the substrate requirements of RlmN and Cfr could further lead to detailed structural analyses of methyltransferase-RNA recognition features. These considerations prompted us to initiate an effort in cloning and purifying Cfr and RlmN and in collaboration with the laboratories of Hassan Jomaa (Justus-Liebig-Universität, Giessen,
Germany) and Danica Galonic-Fujimori (University of California San Francisco) to investigate their enzymatic activity.

2. **Experimental results**

a. **Expression, purification, and reconstitution of RlmN and Cfr**

The *rlmN* gene was amplified from *E. coli* genomic DNA and cloned into a pET-21a expression vector, and the resultant recombinant plasmid (pRlmN) was introduced into the *E. coli* BL21(DE3) strain, allowing for the production of C-terminally His₆-tagged RlmN. To prepare N-terminally His₆-tagged Cfr protein (attempts to clone *cfr* into a C-terminally His₆-tagged construct failed), the *cfr* gene was amplified from the plasmid pMS2, cloned into a pET-15b expression vector, and the resulting pCfr plasmid was used to transform *E. coli* BL21(DE3) cells (Smith and Mankin, 2008). Protein expression from both constructs was induced as described in Materials and Methods, and the efficient expression of both proteins was verified by gel electrophoresis analysis. After that, both proteins were purified under anaerobic conditions by nickel affinity chromatography, resulting in the isolation of brownish proteins. For each of the proteins, the isolated protein fractions were treated with DTT, cysteine, Na₂S and FeCl₃ to allow for reconstitution of the iron-sulfur cluster. The reconstituted proteins were further purified by ion-exchange chromatography under anaerobic conditions, resulting in homogeneous dark brown proteins. The purified and reconstituted proteins migrated at approximately 43 kDa (RlmN) and 37 kDa (Cfr) on an SDS-PAGE gel, in good agreement with the calculated molecular weight of the polypeptides (43.1 and 39.9 kDa, respectively) (Figure 23).

b. **In vitro activity and rRNA substrates of RlmN and Cfr**

To determine whether RlmN and Cfr display methyltransferase activity in vitro, a series of rRNAs was incubated with each of the methyltransferases. In a typical assay, the enzyme was
Figure 23. Analytical gel of the purified RlmN and Cfr proteins. SDS-PAGE analysis shows the purified RlmN and Cfr proteins migrate approximately at their predicted molecular weights of 41 and 39 kDa, respectively.
incubated under anaerobic conditions with purified rRNA, ribosomal subunits or intact ribosomes in the presence of sodium dithionite an electron source for the reduction of the [4Fe-4S] cluster to the +1 oxidation state and S-adenosyl-L-[methyl-\(^3\)H] methionine ([\(^3\)H-methyl]-SAM) as a methyl donor. The intact 70S *E. coli* ribosome, the large (50S) and small (30S) ribosomal subunits, and the rRNA components of both of these subunits, 23S rRNA (with 5S rRNA) and 16S rRNA, were tested as candidate substrates for RlmN and Cfr.

These candidate substrates were prepared either from the wild type *E.coli* or from the *rlmN* knockout strain. In the wild type 23S rRNA, A2503 is supposed to be C2 methylated by the action of endogenous RlmN, whereas A2503 remains unmodified in 23S rRNA from the *rlmN* knockout strain. Following the incubation with the methyltransferase and \([\(^3\)H-methyl]-SAM\), rRNA was recovered from the reaction and analyzed for the presence of tritium-derived radioactivity by filter binding and scintillation counting. 23S rRNA from the *rlmN* knockout strain was the only substrate methylated by RlmN (Figure 24 1A), while both the wild type and *rlmN* knockout 23S rRNA were substrates for Cfr (Figure 24, 1B). Only protein-free rRNA, but not large ribosomal subunits or 70S ribosomes, could serve as RlmN or Cfr substrates. The inability of Cfr or RlmN to modify these latter substrates is likely a consequence of steric hindrance precluding the access of the enzymes to A2503 in the context of the mature 50S subunit. To better establish the specificity of both enzymes, we prepared rRNA with an A2503 to G mutation. Neither Cfr nor RlmN could catalyze transfer of the label from \([\(^3\)H-methyl]-SAM\) to the mutant 23S rRNA, indicating that the activity of both methyltransferases is targeted toward A2503 (Figure 24 1A and 1B insets).

The CysXXXCysXXCys motif, found in both of the methyltransferases, is a characteristic feature of Radical SAM enzymes where it ligates an iron-sulfur cluster. To
Figure 24. RNA substrate requirements of RlmN and Cfr. Panel 1 shows the activity of RlmN (A) and Cfr (B) toward ribosomes, their subunits and various rRNA species. Candidate substrates isolated from the rlmN knockout strain of E. coli are prefaced with KO, while those isolated from the wild type strain are labeled as WT. SDT, sodium dithionite. Inserts show that 23S rRNA with the A2503G mutation cannot serve as RlmN or Cfr substrates. Panel 2 illustrates that mutations of conserved cysteines in the characteristic CysXXXCysXXCys Radical SAM motif inactivate both Cfr and RlmN.
investigate the importance of this motif in RlmN and Cfr catalysis, we prepared a series of mutants in which cysteines in the CysXXXCysXXCys motifs were individually replaced with alanines. The Cys125Ala, Cys129Ala, and Cys132Ala mutants of RlmN as well as the Cys112Ala, Cys116Ala, and Cys119Ala mutants of Cfr were overproduced, purified, and reconstituted in the same manner as that for the wild type enzymes. All mutants provided soluble proteins, although a substantial decrease in stability as compared to the wild type enzymes was observed during their purification. None of the mutants were active toward their respective 23S rRNA substrates (Figure 24.2), strongly implicating a crucial role for the cysteine-rich motif and the associated iron-sulfur cluster in the methyl transfer reactions.

In 23S rRNA, A2503 is located in a single-stranded region flanked by helices 89 and 90-92 (Figure 25 A-C). To further define rRNA substrate requirements for the RlmN- and Cfr-catalyzed methyl transfer, several fragments of 23S rRNA were prepared by in vitro transcription and tested in methylation assays with purified enzymes. 23S rRNA fragments encompassing parts of Domain V (pos. 2018-2788, 2018-2625, and 2447-2625) were readily used by RlmN and Cfr as substrates for methylation (Figure 25 D-F). Removal of a larger part of Domain V did not prevent RlmN or Cfr from modifying their RNA target as long as the helix structure H90-H92, which includes the functionally important A-loop, was present in the substrate construct (Figure 25, G-H). The activity of both enzymes was somewhat attenuated with shorter constructs, suggesting that some of the deleted rRNA regions may stabilize the conformation of the RNA substrate recognized by the methyltransferases. The construct that contained helix 89 but lacked helices 90-92 was inactive, underscoring the importance of the H90-H92 segment as the RlmN and Cfr recognition element (Figure 25 I).
Figure 25. Analysis of the rRNA substrate of RlmN and Cfr. (A) Schematic representation of 23S rRNA secondary structure. The position of A2503 is shown as an orange dot, and the region of 23S rRNA used in the truncations below is boxed in red. (B) Close-up of the 23S rRNA secondary structure encompassing A2503. Helix 89 is pale green, helices 90-92 are yellow, and helix 93 is violet. (C) Folding of the rRNA segment shown in B in the context of the large ribosomal subunit. Coloring of the helices is the same as in B. A2503 is shown in orange, with the methyl groups of C2 and C8 colored as dark purple and magenta, respectively. (D-K) Activities of Cfr (dark gray) and RlmN (light gray) for the specific truncations. The enzyme activities are graphed relative to the activity for full 23S rRNA as the substrate, with 100% delineated with a dashed line.
3. **Discussion**

Having cloned and isolated functionally-active RlmN and Cfr, we have determined that neither of these methyltransferases can act on the assembled 50S subunit or the 70S ribosome. In contrast, protein-free 23S rRNA proved to be a good substrate. This observation is fully compatible with the location of A2503 deep in the peptidyltransferase cavity of the mature large ribosomal subunit, where it is poorly accessible to the modification enzymes.

Thus, RlmN and Cfr-catalyzed methylation of A2503 most likely takes place during ribosome assembly (Kaczanowska and Ryden-Aulin, 2007). Many intermediate assembly steps separate naked 23S rRNA and the mature 50S subunit in the assembly pathway, thus limiting the knowledge of the precise ribosomal assembly step at which the two enzymes may act. However, the observation that the enzymes are not functional with the mature 50S subunit substrate indicates that there is only a narrow time frame during the course of the subunit assembly when A2503 can be modified. This conclusion has important ramifications for the Cfr-mediated mechanism of antibiotic resistance because the extent of modification (and thus, the extent of resistance) may critically depend on the rate of ribosomal assembly. Other factors, such as pretreatment of cells with antibiotics that slow ribosome biogenesis or the activity of other modification enzymes that utilize adjacent segments of rRNA in the heavily modified peptidyl transferase center, may influence the window of opportunity for the Cfr and RlmN enzymes to act. The environmental conditions and growth phase of the cell can also have effects on the extent of A2503 modification, since ribosomes need to be actively assembling in order for Cfr and RlmN to methylate the rRNA. Following this logic, it is also possible that organisms with different rates of ribosomal biogenesis may then be modified at A2503 to different extents. Investigation of the species-specific variation of Cfr-mediated A2503 modification and its correlation with pretreatment with other antibiotics may pave the way for better antibiotic
regimens and new approaches for combating antibiotic resistance.

Studies of the RNA substrate of RlmN and Cfr suggest that the 23S rRNA segment required for moderate methylation of A2503 is limited to the helix system H90-H92 and the adjacent single-stranded stretch of RNA that includes A2503. Removal of helices 90-92 from the RNA substrate precluded both RlmN and Cfr from modifying A2503, indicating that this helical structure is the key recognition element for both enzymes. At the late steps of ribosomal assembly, proper folding of this structure may be assisted by an RNA helicase DbpA, which directly interacts with helix 92 (Brunelle et al., 2006; Nicol and Fuller-Pace, 1995). Therefore, depending on the sequence of events, DbpA could also influence the extent of A2503 modification by RlmN and/or Cfr. Nevertheless, we did not observe any difference in resistance to florfenicol when Cfr was expressed in wild type or in dbpA- cells (unpublished) and thus concluded that either Cfr (and probably RlmN) acts upon RNA prior to DbpA action or the 90-92 helical element of 23S rRNA can be recognized by the methyltransferases irrespective of its DbpA-mediated transformation. Knowledge of the minimal RNA substrate of the RlmN and Cfr Radical SAM methyltransferases should facilitate subsequent structural and kinetic studies of both enzymes. The role of the RlmN-catalyzed C2 methylation of A2503 in the process of translation is unknown. Previous work has demonstrated that while growth rates of wild type and ΔrlmN cells are comparable, cells lacking the RlmN methyltransferase slowly lose in growth competition with wild type cells (Toh et al., 2008). The rlmN gene is present in many bacterial species, and its homologs are found in some single-celled eukaryotes and archaea, indicating that the A2503 modification may assist ribosome functions in all three major evolutionary domains. A2503 is located at the junction between the peptidyl transferase center and the nascent peptide exit tunnel and is apparently critical for the ability of the ribosome to sense and respond to
specific nascent peptide sequences (Vazquez-Laslop et al., 2010). Posttranscriptional modification of A2503 may be required to optimize this function. In addition, helix 92, which is located close to A2503 and is a part of an rRNA element recognized by RlmN, contains the highly conserved A-loop, which makes critical contacts with the A-site tRNA (Brunelle et al., 2006). It is therefore possible that the A2503 C2 methylation serves as one of the check points of proper ribosome assembly and gives a stamp of approval for the subsequent assembly steps (possibly involving DbpA-mediated refolding of helix 92). The role of posttranscriptional modifications as indicators of correct assembly has been proposed in the case of some modifying enzymes such as KsgA and RluD, highlighting the possibility that the endogenous C2 methylation might play a similar function (Gutgsell et al., 2001; Xu et al., 2008).

Within the scope of these studies, our collaborators (Yan and Galonic-Fujimori, University of California San Francisco) determined the nature of the modifications of Cfr and RlmN. Interestingly, when the in vitro methylated 23S rRNA was analyzed by HPLC, 23S rRNA isolated from the rlmN knockout strain incubated with RlmN resulted in the formation of a radioactive product that coeluted with 2-methyladenosine, and no modification of the wild type 23S rRNA with this enzyme was observed (Yan et al., 2010). In contrast, when Cfr-treated rRNA from the rlmN knockout strain was isolated and analyzed in the same manner, the appearance of two new products was noted: 8-methyladenosine and 2,8-dimethyladenosine, with the physiologically relevant C8 as the preferred substrate. Incubation of the same methyltransferase with the wild type 23S rRNA, already modified at the C2 position by the endogenous RlmN, provided 2,8-dimethyladenosine as the sole radiolabeled product (Yan et al., 2010). This observation also indicates that RlmN action is not a prerequisite for Cfr activity and that the two enzymes may even compete for the unmethylated rRNA substrate during ribosomal
assembly. It also indicates that Cfr has retained some of its putative ancestral ability to methylate C2, albeit less avidly than RlmN (Yan et al., 2010).

The work of our collaborators, as well as other groups, has sought to elucidate the mechanism of Cfr and RlmN in depth. These studies, even those carried out in collaboration with us, were not included in this thesis. In the course of the investigation of the Cfr and RlmN enzymatic mechanisms, it became clear that the methyl group from SAM is not directly added to A2503 as previously thought (Grove et al., 2011; Yan and Fujimori, 2011). While there is some debate as to the fate of the methyl molecule, one group hypothesizes that the methyl group is transferred from SAM to a cysteine in the enzyme itself before being added to the adenine target (Grove et al., 2011). The recent crystal structure of RlmN in complex with SAM appears to contain this methylated cysteine, adding support to this hypothesis (Boal et al., 2011). Isolation of functionally active Cfr and RlmN opened an opportunity to study both the intriguing enzymology of Radical SAM methylation, as well as to answer key questions about the RNA substrate requirement for these enzymes. Our findings, along with results obtained in other labs, pave the way for a better understanding of how these enzymes, both the indigenous and the acquired, work in the cell. The understanding of substrate specificity provided important information for future directions in investigating how these enzymes affect cell physiology and resistance to antibiotics.
IV. CONCLUSIONS

In this work, we have established several important aspects of the genetics and biochemistry of the Cfr and RlmN enzymes, both of which can mediate linezolid resistance. First, our study established that, under laboratory conditions, methylation of A2503 by Cfr incurs little cost to the cell. In other words, the addition of a single methyl group to C8 of A2503 does not significantly affect cell growth. It appears that the ribosome has enough innate flexibility in this area to avoid translation problems when an additional methyl group is added to one of the functionally-important nucleotide residues. The fitness cost of cfr is not as drastic as the cost of other resistance mechanisms. This finding has important implications for the spread of cfr in the clinic, as well as the maintenance of this gene in the absence of selective pressure. Our data may give plausible explanations why cfr-positive strains can be isolated from patients with no known linezolid therapy and why cfr-positive strains have been found to persist in healthcare facilities for years.

When the expression of Cfr is combined with a ribosomal dimethyltransferase, Erm, the fitness cost of A2503 methylation increases. This implies that three methyl groups in this region of the ribosome are unfavorable to the process of translation. From the animal and clinical cfr-containing strains studied so far, cfr has been shown to associate with several types of erm genes, indicating that these isolates may act under selective pressure differently than strains containing only cfr.

We have characterized a highly linezolid-resistant S. epidermidis clinical isolate and showed that active cfr expression is one of the key contributions to the unusually high drug resistance. In this strain, cfr is on the multicopy plasmid p7LC, where it is positioned next to the transposon Tn4001. Both the high gene copy number and active transcription, enhanced by the
presence of a transposon promoter, are factors that stimulate active expression of $cfr$ in *S. epidermidis* 426-3147L. The region of p7LC containing the Tn4001-$cfr$ cassette matches a segment of a different $cfr$-carrying plasmid isolated from swine. This implies that antibiotic use in the veterinary setting may influence the spread and maintenance of resistance genes in the clinical setting. Other factors, namely ribosomal RNA and protein mutations may also add to the linezolid resistance of the *S. epidermidis* 426-3147L strain. Our work has experimentally established the promoter that controls $cfr$ transcription ($P_{cfr}$) and has provided experimental evidence for $cfr$ inducibility in some genetic settings. Overall, the data obtained about the genetic environment of $cfr$ in 426-3147L demonstrates $cfr$’s ability to travel among various pathogenic strains and associate with factors that can increase its expression.

In addition to studies of Cfr-based antibiotic resistance, we demonstrated that alterations in the indigenous Cfr-homolog, RlmN, may also alter cell susceptibility to linezolid. We showed that in contrast to the original proposal, a mutation in the *rlmN* gene found in a clinical isolate with reduced susceptibility to linezolid did not activate, but rather abolished RlmN function. This study, therefore, was the first to link the lack of indigenous C2 methylation at A2503 to an increase in linezolid resistance. Our findings have important implications for the surveillance of linezolid resistance and the role of indigenous rRNA modifications in the action of clinically-relevant antibiotics.

We were the first to clone, overexpress and purify functionally-active Cfr and RlmN. The activity assay that we developed allowed detailed studies of the enzymology of these rRNA methyltransferases. We demonstrated that inactivation of the key CysXXXCysXXCys motif in Cfr or RlmN abolished activity of these methyltransferases, thereby justifying their classification as Radical SAM enzymes. Analysis of Cfr and RlmN expanded the repertoire of the known
activities of the Radical SAM superfamily. In our work, we determined the substrate requirements for Cfr and RlmN, demonstrating that these enzymes act during ribosomal assembly. This finding has important implications for understanding how other factors, such as cell growth conditions or treatment with other antibiotics, could affect the extent of rRNA modification by either of these enzymes, and thus sheds light both on the mechanism of linezolid resistance conferred by Cfr and the possible functional importance of RlmN.

Several other studies, such as the investigation of the details of Cfr- or RlmN-mediated methyltransfer catalysis, mapping the location of the cfr gene in the chromosome of the first cfr-positive clinical isolate CM05, and the structure of the plasmid from this isolate, which were carried out in collaboration with other groups, were not included in this thesis.


Enterococcus faecalis and Enterococcus faecium isolated from two Austrian patients in the same intensive care unit. Eur J Clin Microbiol Infect Dis 21, 751-754.


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