Low Fitness Cost of the Multidrug Resistance Gene cfr\textsuperscript{\textdagger}

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The recently described rRNA methyltransferase Cfr that methylates the conserved 23S rRNA residue A2503, located in a functionally critical region of the ribosome, confers resistance to an array of ribosomal antibiotics, including linezolid. A number of reports of linezolid-resistant cfr-positive clinical strains indicate the possible rapid spread of this resistance mechanism. Since 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 in a laboratory Staphylococcus aureus strain. We 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 because Cfr-expressing cells slowly lose to the cfr-negative control strain. Interestingly, cells expressing wild-type and catalytically inactive Cfr had very similar growth characteristics, indicating that the slight fitness cost associated with cfr acquisition stems from expression of the Cfr polypeptide rather than from the modification of the conserved rRNA residue. In some clinical isolates, cfr is coexpressed with the erm gene, which encodes a methyltransferase targeting another 23S rRNA residue, A2058. Dimethylation of A2058 by Erm notably increases the fitness cost associated with the Cfr-mediated methylation of A2503. The generally low fitness cost of cfr acquisition observed in our experiments with the laboratory S. aureus strain offers a microbiological explanation for the apparent spread of the cfr gene among pathogens.

A number of clinically useful antibiotics target the large ribosomal subunit (33). One of the most important mechanisms of resistance to these compounds 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 (20). Furthermore, rRNA mutations often negatively affect ribosome functions and are rapidly reversed in the absence of selection (25, 48). Therefore, a more common resistance mechanism is based on the chemical modification of rRNA. One of the most recently discovered multidrug resistance genes, cfr, encodes an RNA methyltransferase that modifies a conserved adenine at position 2503 of the 23S rRNA (15, 45) (Fig. 1). Cfr-mediated methylation of the C8 position of the adenine base renders bacteria resistant to a broad array of protein synthesis inhibitors, including penicilins, lincosamides, pleuromutilins, streptogramins A, 16-member ring macrolides, and oxazolidinones (11, 15, 22, 40, 43).

The cfr gene was originally found in staphylococcal strains isolated from pigs and cattle (14, 40). In 2007, the first cfr-positive clinical strain of methicillin-resistant Staphylococcus aureus was described previously (2, 45). In this strain, designated 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 (Fig. 1). The ermB and cfr genes are coexpressed under the P\textsubscript{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 (43, 45). Since 2007, a number of reports of new cfr-positive clinical isolates have appeared (5, 6, 9, 21, 26–29, 36, 42). 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 (34, 47). A2503 is naturally methylated at C2 in the ribosomes of many bacteria by the indigenous enzyme RlmN (Fig. 1) (16, 46). The natural posttranscriptional modification of A2503 underscores its possible functional importance in protein synthesis (47). 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.

Spread and maintenance of a resistance gene is 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 (1, 3, 10, 18, 23, 24, 35, 44). 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.

MATERIALS AND METHODS

Strains and plasmids. The laboratory strain S. aureus RN4220 was used in all experiments (32). The nucleotide sequence of cfr with its putative promoter as is found in pSCSFI (39, 40) was synthesized by BioBasics, Inc. (Markham, Ontario, Canada), and cloned into the HindIII and XbaI sites of the pLI50 shuttle plasmid (17) to produce the plasmid pJL1. To engineer the pJL1M plasmid, which carries a catalytically inactive version of Cfr, the wild-type cfr codon 119 (TGT), encoding a catalytic cysteine, was mutated to GCT (alanine) by using a QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Plasmid pMS2, which contains the cfr and ermB genes in the context of the clinical CM05 strain, was constructed previously (45). pMS2M was constructed by mutating the 119th cfr codon TGT to GCT as described above.

Growth rate determination. RN4220 cells transformed with the pLI50-derived plasmids were grown overnight at 37°C in brain heart infusion (BHI) media (BD Diagnostics, Sparks, MD) supplemented with 10 μg of chloramphenicol (CHL) ml. Cultures were diluted to an optical density at 600 nm of 0.05 in fresh BHI/CHL medium and grown again for 24 h to stationary phase. Three passages were done in total, corresponding to ~40 cell generations. The ratio of cells was determined by plating dilutions on BHI-agar plates containing 10 μg of CHL/ml, with or without 10 μg of florfenicol (FLR)/ml. Colonies were counted after 18 h growth at 37°C. The doubling times and standard deviations were determined from two independent experiments.

Competition growth. Cultures of cells transformed with different plasmids were grown overnight in BHI supplemented with 10 μg of CHL/ml. The cultures were diluted to an optical density at 600 nm of 0.05 in fresh BHI/CHL medium and shaken at 37°C until the optical density of the culture reached 0.5. Portions (5 ml) of each culture were diluted 1,000-fold into fresh BHI/CHL medium and grown again for 24 h to stationary phase. Three passages were done in total, corresponding to ~40 cell generations. The ratio of cells was determined by plating dilutions on BHI-agar plates containing 10 μg of CHL/ml, with or without 10 μg of florfenicol (FLR)/ml. Colonies were counted after 18 h growth at 37°C. The doubling times and standard deviations were determined from two independent experiments.

Microbiological testing. MICs were determined by the broth microdilution method as established by the Clinical and Laboratory Standards Institute (7).

RNA isolation and assessment of A2503 modification. Total RNA was isolated from S. aureus cells grown again for 24 h to stationary phase. Total RNA was isolated from S. aureus cells using a Qiagen RNeasy minikit (Valencia, CA) with some modifications. Briefly, overnight cultures of cells grown in BHI/CHL medium were diluted 1:100 in fresh medium and grown to an A600 of 0.5. Then, 5-ml portions of cells were pelleted, washed with 500 μl of H2O, and resuspended in 200 μl of lysis buffer (Tris-HCl [pH 7.5], 30 mM MgCl2, 30 mM NH4Cl) containing 0.5 mg of lysostaphin (Sigma, St. Louis, MO)/ml. Lysis was carried out for 30 min at 37°C. Portions (350 μl) of buffer RLT from the RNeasy minikit were then added, and the remaining steps of RNA isolation were followed as instructed by the kit manual.

The analysis of modification of A2503 in 23S rRNA was carried out by primer extension as described previously (15, 45) with minor modifications. Specifically, 3 pmol of [5’-32P]-labeled DNA primer L2585 (ACTGTCTCAC GACGTTCT) was annealed to 1.5 μg of S. aureus total RNA. The primer was extended with AMV reverse transcriptase (Seikagaku America, Falmouth, MA) in the presence of 1 mM concentrations of dGTP, dATP, and dCTP and 0.25 mM dTTP. The cDNA products were resolved in a denaturing 6% polyacrylamide sequencing gel.

RESULTS

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, pSCSFI (40). 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. While the FLR 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 (Table 1). 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 (see Fig. 3).

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
TABLE 1. FLR MICs for S. aureus RN4220 cells transformed with different constructs

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>MIC (µg/ml)</th>
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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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pJL1), indicating that the impact of expression of the Cfr protein 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 CHL-containing BHI medium and passaged four times, allowing the cultures to reach saturation at each cycle (altogether, the cells were co-grown 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 CFU) or supplemented with 10 µg of FLR/ml (CFU of pJL1-transformed cells). The data (see Fig. 4A) showed that in the competition growth setting, cells expressing Cfr are outcompeted by the control cells, albeit only at a slow rate. The estimated fitness cost of Cfr expression is only 4.6% per generation.

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 (12, 49). The FLR MIC of cells transformed with pJL1M was indistinguishable from that of cells carrying the empty pLI50 vector (Table 1) and primer extension did not show any difference compared to the control (Fig. 3), thereby confirming previous biochemical data that the C119A mutant of Cfr is inactive (12, 49).

When cells expressing active Cfr and its inactive mutant were co-grown in culture for ca. 40 generations, their ratio remained unchanged (Fig. 4B). 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. Thus, the slight fitness cost associated with expression of active Cfr likely comes from the mere expression of the Cfr protein, rather than from the resulting rRNA modification.

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 that encodes the Erm methyltransferase targeting A2058 in 23S rRNA (4, 43, 45). 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 (Fig. 2) (45). 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 C119A mutation. We verified that the mutant plasmid (pMS2M) does not provide resistance to FLR (Table 1), nor do pMS2M-transformed cells show any detectable Cfr-mediated modification of A2503 (Fig. 3).

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 (Fig. 4C). 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, 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.

![FIG. 2. Plasmids used in the present study. 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.](image-url)
Because 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 \textit{cfr} gene has in fact only a small effect upon the growth rate of \textit{S. aureus} cells, indicating that the fitness of the \textit{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 \textit{cfr} gene were slowly outcompeted by the control cells carrying an empty vector. The estimated fitness cost of \textit{cfr} presence was 4.6\% per generation, which is relatively low compared to some other resistance mechanisms whose fitness costs can be in the 15 to 35\% range (37, 38).

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 \textit{per se} does not cause growth rate reduction. The slight fitness loss observed in our experiments stems from a property of the Cfr protein unrelated to the catalysis of rRNA methylation. Our previous studies have shown that Cfr acts during ribosomal assembly (49). 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 ribosome 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 a single A2503 residue does not seem to interfere with translation, the situation dramatically changes when the Erm methyltransferase modifies a neighboring nucleotide, A2058. \textit{S. aureus} cells carrying the \textit{ermB} gene and expressing active Cfr readily lose in competition to the \textit{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 (6.6 Å) in the ribosomal nascent peptide exit tunnel (Fig. 1), and both are involved in functional interactions with the regulatory nascent peptides (8, 31, 47). 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 (41, 47). Interfering with the operation of only 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 \textit{S. aureus} isolate where \textit{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 (J. B. Locke et al., unpublished data).

Our conclusions about the fitness cost associated with Cfr expression have been drawn from a laboratory model system and thus need to be treated with certain caution. 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 (30). Because the initially less-fit cells are expected to be even more sensitive to a potential burden associated with *cfr* expression, the presence of these mutations should not compromise our conclusion to a potential burden associated with the initially less-fit cells are expected to be even more sensitive.

Moreover, the low cost of *cfr* acquisition could be more pronounced in organisms other than *S. aureus*. These possibilities could be addressed in future studies.

From the epidemiological standpoint, the low cost of *cfr* in staphylococci, especially in the absence of an associated *erm* gene, is troubling. The *cfr* gene is often associated with mobile genetic elements (plasmids or transposons) (4, 13, 40, 45) and gene mobility in microorganisms is a concern in the spread of antibiotic resistance among clinical strains of different species and in various locations worldwide (4–6, 26, 27, 29). These observations may reflect the ongoing spread of the *cfr* gene. Our study offers a possible microbiological explanation for this alarming trend.

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