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Combined Effect of the Cfr Methyltransferase and Ribosomal Protein L3 Mutations on Resistance to Ribosome-Targeting Antibiotics

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ABSTRACT Several groups of antibiotics inhibit bacterial growth by binding to bacterial ribosomes. Mutations in ribosomal protein L3 have been associated with resistance to linezolid and tiamulin, which both bind at the peptidyl transferase center in the ribosome. Resistance to these and other antibiotics also occurs through methylation of 23S rRNA at position A2503 by the methyltransferase Cfr. The mutations in L3 and the *cfr* gene have been found together in clinical isolates, raising the question of whether they have a combined effect on antibiotic resistance or growth. We transformed a plasmid-borne *cfr* gene into a uL3-depleted *Escherichia coli* strain containing either wild-type L3 or L3 with one of seven mutations, G147R, Q148F, N149S, N149D, N149R, Q150L, or T151P, expressed from plasmid-carried *rplC* genes. The L3 mutations are well tolerated, with small to moderate growth rate decreases. The presence of Cfr has a very minor influence on the growth rate. The resistance of the transformants to linezolid, tiamulin, florfenicol, and Synercid (a combination of quinupristin and dalfopristin [Q-D]) was measured by MIC assays. The resistance from Cfr was, in all cases, stronger than the effects of the L3 mutations, but various effects were obtained with the combinations of Cfr and L3 mutations ranging from a synergistic to an antagonistic effect. Linezolid and tiamulin susceptibility varied greatly among the L3 mutations, while no significant effects on florfenicol and Q-D susceptibility were seen. This study underscores the complex interplay between various resistance mechanisms and cross-resistance, even from antibiotics with overlapping binding sites.

KEYWORDS Cfr, L3 mutations, antibiotic resistance, linezolid resistance, tiamulin resistance

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Over time, more and more mutations in bacterial ribosomal protein L3 (renamed uL3 in accordance with the new universal naming of ribosomal proteins [1]) have been associated with resistance to the antibiotics linezolid (LZD, an oxazolidinone) and tiamulin (TIA, a pleuromutilin). These drugs have overlapping binding sites at the peptidyl transferase center (PTC) in the ribosome (Fig. 1). Specific mutations of 23S rRNA and methylation at position 2503 also cause resistance to these antibiotics, as reviewed in references 2–4. The main part of L3 is positioned on the surface of the 50S ribosomal subunit, but a branched loop extends close to the PTC (Fig. 1), the binding site for various ribosomal antibiotics. The first L3 resistance mutation reported in bacteria was from *Escherichia coli* selected with TIA, and its role in resistance was verified by genetic evidence (5). Since then, L3 mutations have been associated with resistance to LZD, TIA/valnemulin, and anisomycin, as reviewed in references 2 and 6. Relationships between L3 mutations and antibiotic resistance have been reported in *Brachyspira*, *Staphylococcus*, *E. coli*, and *Mycobacterium tuberculosis*, but many of the findings lack genetic verification. Our recent study (6) demonstrated a clear antibiotic

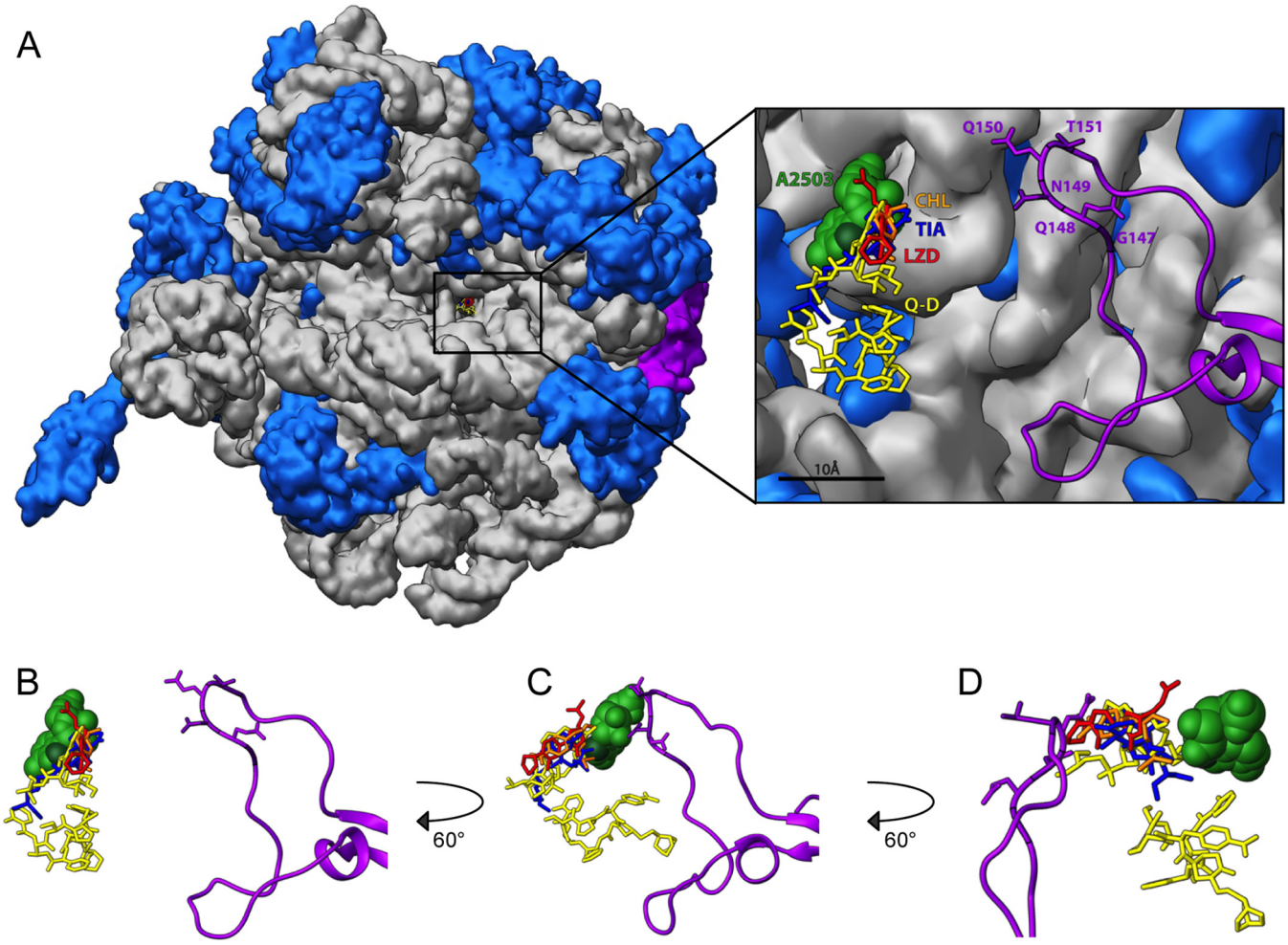

FIG 1 Relative positions of L3 mutations, methylation of the 23S rRNA at position A2503, and antibiotics. (A) The *E. coli* 50S ribosomal subunit (PDB code 4TP9) showing the rRNA in gray, ribosomal proteins in blue, and the surface portion of L3 in purple. The subunit is viewed directly into the tunnel for the nascent peptide. The clipped zoom to the right shows a cutaway representation of the PTC with the antibiotics LZD (red), TIA (blue), CHL (orange), and Q-D (yellow) superimposed on top of each other (PDB codes 1XBP, 4V7T, 4TP9, and 4WFA). CHL represents phenicols, as no structural model of FF binding is available. 23S RNA A2503 is shown as green spheres with C-8 (the site of Cfr methylation) in darker green. The central part of L3 is shown as a purple ribbon with side chains specified for mutated positions. (B to D) Views of A2503, the antibiotics, and L3 in the same orientation as in panel A (B), vertically rotated 60° relative to the view in panel B (C), and vertically rotated 120° relative to the view in panel B (D).

resistance effect of 6 of 10 mutations in the central loop of L3 in *E. coli*, one to LZD and five to TIA. The mutations correspond to mutations observed in pathogenic bacteria, and that study thus supports, in general, the presumed or proven effects from other studies. For example, an LZD resistance effect from L3 N149R in *E. coli* (6) correlates well with a resistance effect of the L3 C154R substitution in *M. tuberculosis* at the corresponding position (7–9).

Also, Cfr is an important resistance factor for antibiotics binding at the PTC. The *cfr* gene encodes an rRNA methyltransferase (10) that adds a methyl group at the C-8 position of 23S rRNA nucleotide A2503 at the PTC (11) (Fig. 1). This m^8A2503 modification confers resistance to more than six classes of antibiotics that bind at overlapping nonidentical sites at the PTC (12–14). The *cfr* gene was originally discovered on multiresistance plasmids isolated during surveillance studies of florfenicol (FF) resistance in *Staphylococcus* spp. of animal origin (15). Since then, a large number of clinical and veterinary staphylococcal isolates containing *cfr* in different genetic contexts have been found around the world (2, 16–18, and references therein). In some cases, a connection between the resistant isolates and prior LZD treatment has been documented. In addition, some of the *cfr*-carrying isolates have also been shown

to harbor mutations in L3, and therefore it has been suggested that L3 mutations and *cfr* have a coordinated effect on antibiotic resistance (19). The *cfr* gene has, in addition to staphylococcal isolates, been found in other pathogenic bacteria, both Gram positive and Gram negative (18, 20, and references therein). The presence of *cfr* on mobile genetic elements such as plasmids and transposons at different geographic locations strongly suggests that it can be disseminated within the microbial community and spread among bacteria, thus conferring resistance without prior exposure to a drug.

In this study, we investigated seven L3 mutations in *E. coli* and their effect on antibiotic resistance when combined with Cfr-mediated 23S A2503 methylation. For this, we constructed a *cfr*-encoding plasmid termed pKPCfr that is compatible with the plasmids encoding the mutated L3 genes constructed by Klitgaard et al. (6). We measured the doubling times of the L3 mutant strains with and without Cfr to assay the effect of the L3 mutations and Cfr on growth rates. To analyze antibiotic resistance, the MICs of LZD, TIA, FF, and Synercid (a combination of quinupristin and dalfopristin [Q-D]) were determined for each strain with and without Cfr. These results were then compared to those for the L3 wild-type (WT) strains to establish the combined effect of L3 mutations and the methylation of 23S rRNA A2503 on antibiotic susceptibility. We show that L3 mutations can affect the degrees of TIA and LZD resistance achieved with the A2503 methylation, either in a synergistic or in an antagonistic way, depending on the actual mutation. The effects of the L3 mutations vary greatly between the positions and the nature of the amino acids changes.

RESULTS

Various clinical data point to the coexistence of L3 mutations and either 23S RNA mutations or the presence of the *cfr* gene in strains resistant to PTC antibiotics (6, 19). This raises the question of a synergistic effect of L3 mutations and Cfr on antibiotic resistance. Having a collection of relevant L3 mutations in *E. coli* and the *cfr* gene, we are able to explore this hypothesis. Nucleotide A2503 of the 23S rRNA, the target site of Cfr, is located at the PTC, in close proximity to the binding sites of the antibiotics investigated in this study, as illustrated in Fig. 1. Furthermore, the branched loop of L3 also extends close to this site, although the crystal structure of the *E. coli* 50S subunit shows that a part of the 23S subunit obstructs direct interaction between L3 and the PTC (Fig. 1).

Primer extension to confirm 23S A2503 methylation by Cfr. To ensure that the effect of adding the *cfr* gene is actually from m^8A2503 methylation by Cfr, we examined A2503 of the 23S rRNA by primer extension (see the supplemental material), as done in previous studies of Cfr (10, 11). The primer extension assay cannot be used for precise quantification of the extent of m^8A modification because part of the stop is originating from an m^2A methylation caused by an intrinsic enzyme and, in general, this assay is better suited for qualification rather than quantification. The gel data clearly show that Cfr modifies A2503 of the 23S rRNA of all of the mutants tested.

Antibiotic susceptibilities of the L3 mutant strains with and without Cfr expression. The effects of the L3 mutations and Cfr on antibiotic susceptibility were investigated by MIC assays. We measured the MICs of LZD (an oxazolidinone), TIA (a pleuromutilin), FF (a phenicol), and Q-D (a streptogramin mixture) for WT L3 and the seven L3 mutant strains with and without Cfr. For LZD, only the N149R and Q150L mutations demonstrated a significant (>2-fold) MIC increase and decrease, respectively, in the absence of Cfr relative to the WT (Table 1). With TIA, all of the mutations except Q148F and N149R yielded significant MIC increases without Cfr. FF and Q-D bind in the ribosomes at sites overlapping the binding sites of TIA and LZD (Fig. 1). Determinants of resistance to such overlapping antibiotics might give cross-resistance (4, 12, 21), and therefore, we included these antibiotics in this study. For the minimal distances between the mutated amino acids and the antibiotics and A2503, see the supplemental material. As shown in Table 1, none of the L3 mutations investigated had a significant effect on sensitivity to FF or Q-D.

TABLE 1 Growth rates and antibiotic susceptibilities of *E. coli* AS19ΔL3 strains with plasmids encoding L3

L3 amino acid mutation located on a pBR322L3 plasmid	Doubling time (min) with/without Cfr	MIC (μg/ml) with/without Cfr			
		TIA	LZD	FF	Q-D
None (WT)	25/26	0.5/16	8/128	1/16	4/32
G147R	41/44	2/48	4/32	1/16–32	4/32
Q148F	28/29	1/24	8/64	1/16	8/32
N149S	27/27	8/64	8/64	1/16	4/32
N149D	28/29	4/96	4/16	1/16	4–8/32
N149R	27/28	1/24	32/>128	2/32	8/32
Q150L	31/31	4/96	2/16	1/16	8/32
T151P	32/33	2/48	4/32	2/16–32	8/32

As expected, the MICs for most strains changed dramatically upon the addition of a plasmid with the *cfr* gene expressing Cfr (Table 1). For FF and Q-D MICs, there seem to be no synergistic or antagonistic effect of the L3 mutations plus Cfr. The TIA and LZD data are more interesting and are plotted as columns with and without Cfr in Fig. 2 to more easily examine the effects. It is clear that the effects of Cfr are much more prominent than the effects of the L3 mutations. Surprisingly, the effects of Cfr vary considerable among the mutations and show very different patterns for the two antibiotics. The data for TIA show that all mutations plus Cfr increased the MICs by more than the sum of the effects of the individual L3 mutation and Cfr. For LZD, only the N149R mutation produced higher values than the WT, while all of the other L3 mutations decreased the effect of Cfr on antibiotic susceptibility.

The five L3 mutations, G147R, N149S, N149D, Q150L, and T151P, all caused a reduced susceptibility to TIA, resulting in a higher MIC than that of the WT (Table 1). These data are in accordance with our previous study (6). The effect is retained in the presence of Cfr. The combined effect of N149D and Q150L is a 6-fold MIC increase, from 16 to 96 μg/ml (Fig. 2). An opposite effect was seen with LZD, where three mutations (G147R, N149D, and Q150L) enhanced susceptibility to LZD relative to the WT, resulting in a lower MIC than that of the WT (Table 1 and Fig. 2). Adding Cfr reduced susceptibility to LZD, as expected, but to various degrees. For six of the seven L3 mutations, the combined effect with Cfr is thus lower than the effect of Cfr alone. N149R produced reduced susceptibility to LZD (MIC of 32 μg/ml compared to 8 μg/ml for the WT), and this effect was also seen in the presence of Cfr, although the MIC reached the upper limit of the assay (>128 μg/ml).

Growth rates of the L3 mutant strains with and without Cfr expression. Whether or not a mutation appears in a natural setting is, of course, related to its fitness cost, competitors, and benefits such as antibiotic resistance. The doubling times of the L3

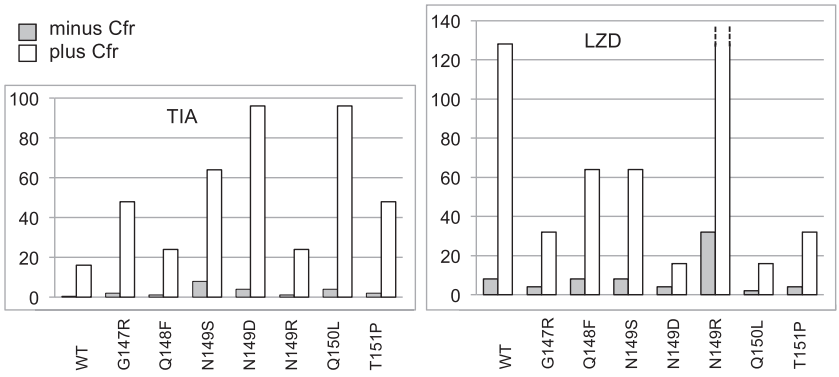

FIG 2 Graphic illustration of the effects of L3 mutations alone and combined with Cfr methylation on TIA and LZD susceptibility. The values on the vertical axes are the MICs from Table 1 in micrograms per milliliter.

mutant strains were determined with and without Cfr to assess the effect of combining L3 mutations and 23S rRNA methylation by Cfr on growth. In accordance with the previous results (6), an increase in the doubling times of all seven mutant L3 strains was observed. Most of the doubling time increases were moderate (2 to 6 min), but the G147R mutation resulted in a more severe 25- to 41-min doubling time increase (Table 1). Addition of the Cfr-expressing plasmid to the strains produced only a tiny doubling time increase (1 to 3 min). This is as expected from a study of *Staphylococcus aureus* (22) and our unpublished results obtained with *E. coli*, showing that Cfr expression does not significantly affect the bacterial growth rate.

DISCUSSION

The finding of L3 mutations together with the *cfr* gene carried on plasmids in clinical strains showing antibiotic resistance and the suggestion of a synergistic effect of this combination prompted us to investigate this hypothesis. Resistance to LZD is of special interest, as it is a highly effective drug used as an alternative for the treatment of difficult infections. TIA resistance is also of interest because it binds at the same site in the ribosomes, it is used in the veterinary field and can thus be selective for antibiotic resistance determinants that can spread to clinical strains. Also, there is an interest in developing pleuromutilin derivatives as clinical drugs, and one is already in use.

As reviewed in reference 6, a number of L3 mutations are detected especially in *Staphylococcus* species, but only some of them have been proven to be related antibiotic resistance. In all likelihood, not all of the L3 mutations observed are relevant to antibiotic resistance, so the role of these mutations needs to be examined. The role of L3 mutations in *M. tuberculosis* resistance to LZD treatment has recently been verified (7–9). In this context, it should be emphasized that both the L3 sequence and the molecular interactions of L3 in the ribosome vary from one bacterium to another, so findings from one bacteria are not proof of the effect in others but do suggest the possibility of an effect and can support a suggested relationship. This is also emphasized in the study of the binding of LZD and other PTC antibiotics to the *S. aureus* ribosome (23), showing deviations to ribosomes from other bacteria.

Of the mutated L3 amino acid residues investigated in this study, G147 is located farthest from Cfr-methylated 23S rRNA A2503 (Fig. 1). The G147R mutation replaces the small glycine residue with a larger, positively charged arginine, which might possibly interact with the 23S rRNA, indirectly affecting the antibiotic binding sites at the PTC. It is equivalent to L3 G155R in *S. aureus*, which has been related to resistance to both LZD and TIA (21, 24, 25) and was shown to be a single determinant that can confer cross-resistance to these antibiotics. In our experiments, the *E. coli* G147R mutation, on its own, did not significantly affect LZD susceptibility but caused some resistance to TIA; however, the G147R mutation heavily impaired the resistance provided by Cfr. The effect of G147R plus Cfr on TIA relative to the WT with Cfr is an increase in the MIC, while the effect with LZD is the opposite; the G147R mutation decreases the effect of Cfr by decreasing the MIC 4-fold (Table 1 and Fig. 2). The G147R mutation also yielded the largest doubling time increase, from 25 to 41 min (Table 1). Thus, for *E. coli*, this mutation seems expensive (in doubling time), but with TIA, the mutation will increase the effect of Cfr while with LZD it will decrease the effect of Cfr.

In general, the Q148F, Q150L, and T151P mutations have effects on the MICs similar to that of G147R, although there are some variations. The biggest difference from the effect of G147R is that they are well tolerated, with only small doubling time increases. The *E. coli* Q150 L3 position is equivalent to S158 in L3 of *Staphylococcus cohnii*, where a change from an S158Y mutation to an S158F mutation in strains with *cfr*-mediated LZD resistance produces a MIC change from 16 to 64 μg/ml (19). The same strains showed LZD MICs of 4 and 2 μg/ml, respectively, after clearance of the *cfr* plasmid (19). The LZD MIC of WT S158 L3 in *S. cohnii* is not reported, but the plus/minus effect of Cfr is comparable to the 8-fold plus/minus effect of Cfr (2 to 16 μg/ml) that we observed for Q150L L3 in *E. coli*.

N149 is the L3 amino acid located closest to the PTC with its side chain pointing

toward it (Fig. 1). Thus, mutations at N149 would likely affect antibiotic susceptibility and mutations here have been found and related to antibiotic resistance in *E. coli*, *Brachyspira hyodysenteriae*, *Staphylococcus epidermidis*, and *M. tuberculosis* (reviewed in references 6 and 8). The three N149 mutations investigated are well tolerated but have different antibiotic resistance and Cfr effects, underlining the influence of specific amino acids. N149S replaces one uncharged side chain with another, N149D inserts a negatively charged side chain, and N149R insert a positive, longer side chain. Mutation N149D affects TIA sensitivity only moderately (MIC increase from 0.5 to 4 μg/ml), but when combined with *cfr* expression, the MIC increases to 96 μg/ml. It also provides no resistance to LZD alone, but in combination with Cfr, it lowers the effect of Cfr from an MIC of 128 μg/ml to 16 μg/ml. This L3 mutation thus lowers the effect of Cfr on LZD but increases its effect on TIA. The almost opposite is caused by N149R, which does not affect the TIA MIC or the effect of Cfr on the TIA MIC but provides resistance to LZD alone and in combination with Cfr. The third N149 mutation, N149S, provides TIA but not LZD resistance and it enhances the effect of Cfr on TIA but reduces its effect on LZD.

Conclusion. Our previous results demonstrated that mutations in ribosomal protein L3 can have vastly different effects on the response to antibiotics, even though the mutated residues investigated are located close to each other and the antibiotics affected have overlapping binding sites. When these mutations are combined with m^{8}A2503 methylation, the effects are variable and show that L3 mutations can affect the degree of resistance achieved from m^{8}A2503 methylation by Cfr. It is a bit surprising that some L3 mutations can decrease the effect of the m^{8}A2503 methylation and lower the MIC, thus making the bacteria more susceptible to an antibiotic. The combined effect of L3 mutations and A2503 methylation is different from various positions of the mutations and from changes to various amino acids. It is thus also to be expected that the effects of L3 mutations vary between bacterial strains.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* TOP10 strain (Invitrogen, Carlsbad, CA, USA) was used for transformation of ligated plasmids. Derivatives of hyperpermeable *E. coli* AS19 (26) were used for MIC analysis because of its higher antibiotic sensitivity than other *E. coli* strains. *E. coli* AS19ΔL3 (6) with plasmids encoding L3 mutations and tetracycline (TET) resistance was grown in LB medium with 5 μg/ml kanamycin (KAN), 5 μg/ml TET, and 0.2% glucose. The *E. coli* AS19ΔL3 strains with plasmid-encoded mutated L3 and pKPCfr were grown in LB medium with 4 μg/ml chloramphenicol (CHL).

E. coli AS19ΔL3 containing plasmid-encoded WT or mutant L3 (as L3 is an essential ribosomal protein) was constructed in a previous study (6). The seven mutant L3-encoding plasmids used were pBR322L3G147R, pBR322L3Q148F, pBR322L3N149S, pBR322L3N149D, pBR322L3N149R, pBR322L3Q150L, and pBR322L3T151P, where the last numbers and letters refer to the L3 mutations.

Construction of pKPCfr, a pACYC184 derivative with the *cfr* gene. For construction of plasmid pKPCfr, the *cfr* gene was amplified from a pBRCfr vector (a pBR322 derivative containing *cfr* with codons optimized for *E. coli* [27]) with primers 5′-CTGTACCTCAGGATAGGGGTTCCGCGCACATT-3′ and 5′-TCATG CCTGAGGGGAGTGGTGAATCCGTTAGC-3′ containing Eco81I restriction sites. The PCR product was further expanded by six nucleotides at either end by using primers 5′-ATGCGTCTGTACCTCAGGATAGGG-3′ and 5′-TACATGTCATGCCTGAGGGGAGTG-3′ to ensure that enough nucleotides were available for the restriction endonuclease to bind. The pACYC184 vector, containing a single Eco81I restriction site, and the PCR product were then digested with Eco81I, and the two fragments were ligated together with T4 DNA ligase (Roche). pACYC184 contains a *cat* gene providing CHL resistance but not FF resistance. The ligated plasmid was transformed into *E. coli* TOP10, and colonies containing the plasmid were selected with 5 μg/ml TET. Finally, the plasmid was extracted and the sequence was verified by DNA sequencing with primers 5′-TGGGAGGCAGAATAAATGA-3′ and 5′-GCTTATGTCTATTGCTGGTT-3′.

Transformation of pKPCfr into the L3 mutant strains. The *E. coli* AS19ΔL3/plasmid L3 mutant strains were grown overnight in 5 ml of LB containing 5 μg/ml KAN and 5 μg/ml TET and made competent by a standard method with $MgCl_2$ and $CaCl_2$. The competent cells were transformed with pKPCfr, and colonies carrying the plasmid were selected with 4 μg/ml CHL.

Antibiotic susceptibility testing. The combined effect of the L3 mutations and Cfr was investigated by MIC assays. L3 mutant strains with and without Cfr-expressing plasmids were grown overnight in LB medium at 37°C and diluted, and growth was restarted. At an optical density at 450 nm (OD_{450}) of approximately 0.01, a 100-μl bacterial suspension was mixed with 100 μl of antibiotic solution in a series with 2-fold dilution steps. The antibiotics tested were LZD (1 to 128 μg/ml), TIA (0.125 to 64 μg/ml), FF (0.5 to 32 μg/ml), and Q-D (1 to 64 μg/ml). MICs were determined by OD_{450} measurement with a Victor 3 plate reader (Perkin-Elmer). For some strains, the TIA MICs varied between experiments and therefore we added intermediate concentrations ranging from 6 to 96 μg/ml to obtain stable readings. The MIC was defined as the lowest antibiotic concentration at which growth was absent following 24 h of

incubation at 37°C. The results are presented in Table 1 as representative values of at least two independent experiments.

Determination of strain growth rates. The impact of the L3 mutations on the growth rate of the strains with and without Cfr was assayed by measuring OD_{450} for growth in LB medium at 37°C with shaking. Doubling times were determined by performing regression analysis of the exponential phase, and the results are presented in Table 1.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00862-17.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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