

## Influence of Recombination on Development of Mutational Resistance to Linezolid in *Enterococcus faecalis* JH2-2<sup>∇</sup>

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**We compared the propensities of *Enterococcus faecalis* JH2-2 and of the recombination-deficient JH2-2 *recA* strain to develop mutational resistance to linezolid. In both organisms, a mutation in a single *rrl* copy conferred resistance to linezolid. Delay in acquisition of the mutation by other *rrl* copies in JH2-2 *recA* showed that gene conversion contributed to the acquisition of resistance.**

The oxazolidinone antimicrobial agent linezolid interferes with protein synthesis and is active against multidrug-resistant gram-positive organisms (11).

Linezolid resistance in *Enterococcus faecalis* has been associated with a variety of mutations in domain V of the 23S rRNA genes, in particular G2447T, T2500A, G2505A, G2512U, G2513U, G2576U, and C2610G (4, 5, 7, 10) (*Escherichia coli* numbering). Reports of in vitro investigations and clinical studies assigned a major role to the G2576U mutation in linezolid resistance in *E. faecalis*, *Enterococcus faecium*, and *Staphylococcus aureus* (4, 6, 8, 10). Further, studies showed a direct correlation between the level of resistance and the proportion of mutated *rrl* (23S rRNA) genes (4, 6). Mutation in one, several, or all of the *rrl* gene copies may be associated with gradually increasing levels of resistance. The gene copy number ranges from four in *Streptococcus pneumoniae* and *E. faecalis* to six in *E. faecium* and five or six in *S. aureus*. However, the frequency with which clinical isolates having the mutation in multiple gene copies are found suggests that homologous recombination between mutated and wild-type 23S rRNA genes may promote the development of high-level resistance under antimicrobial selective pressure.

Gene conversion has already been implicated in increasing the levels of resistance of *Mycobacterium smegmatis* to aminoglycosides (9) and of *S. pneumoniae* to evernimicin (1). To test the role of homologous recombination in the acquisition of resistance, we compared the abilities of *E. faecalis* JH2-2 and its recombination-deficient relative *E. faecalis* JH2-2 *recA* to acquire in vitro stepwise resistance to linezolid.

To inactivate the *recA* gene in *E. faecalis* JH2-2, we used the thermosensitive shuttle vector pMAD, which confers resistance to erythromycin (3). We constructed a derivative of the pMAD vector carrying a 404-bp DNA fragment from the 5' end of the *recA* gene, an antibiotic (spectinomycin) resistance cassette, and a 398-bp DNA fragment from the 3' end of the gene. The recombinant pMAD/*recA* vector was extracted from

*E. coli*, and a two-step procedure was then used for allele replacement in *E. faecalis*. Briefly, the double-recombination event was obtained by subjecting the strain to several subcultures at 44.5°C under the selective pressure of spectinomycin, as described previously (3). The recombinant *E. faecalis recA* strain was finally retrieved from among spectinomycin-resistant, erythromycin-susceptible colonies (recognized by counterselection), indicating successful replacement recombination. Efficient inactivation was verified by PCR.

The strain JH2-2 *recA* was tested for the ability to repair DNA damaged by UV-induced degradation. For the UV sensitivity assay, serial dilutions of *E. faecalis* JH2-2 and JH2-2 *recA* were spotted onto brain heart infusion (BHI) plates and exposed to UV as described previously (12). After 10 s of exposure, survivor counts for *E. faecalis* JH2-2 *recA* were 200-fold lower than those for the parental strain. This result confirmed the deficiency in recombinational repair related to RecA protein inactivation. No evidence for the impairment of the biological fitness was found, since no significant difference between the growth curves for *E. faecalis* JH2-2 and *E. faecalis* JH2-2 *recA* in BHI medium was observed.

Linezolid-resistant mutants of *E. faecalis* JH2-2 and JH2-2 *recA* were obtained by serial passages in BHI broth (Becton-Dickinson, Le-Pont-de-Claix, France) containing increasing concentrations (0.25 to 64 µg/ml) of linezolid. A minimum of 11 passages was performed.

At each selection step, 50 µl of bacterial culture growing at the highest antibiotic concentration was streaked onto antibiotic-free BHI agar. Five to 10 colonies were then suspended in saline for PCR and sequencing and for the determination of MICs of linezolid by the agar dilution method (<http://www.sfm.asso.fr>).

The initial linezolid MIC for both JH2-2 and JH2-2 *recA* was 2 µg/ml. Serial passages on doubling concentrations of linezolid resulted in progressive MIC increases for both strains (Table 1). A first level of resistance, with the MIC equal to 8 µg/ml, was reached after 4 days for JH2-2 and 5 days for JH2-2 *recA*. After this point, the MIC for JH2-2 increased daily to reach 32 µg/ml, remained stable for 3 days, and then increased to 64 µg/ml (Fig. 1). For JH2-2 *recA*, the MIC of linezolid also increased in a stepwise pattern but only to a maximum of

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TABLE 1. Correlation of the presence of point mutation G2576T in the 23S rRNA genes with the level of linezolid resistance in *E. faecalis*

| <i>E. faecalis</i> strain | No. of passages <sup>a</sup> | Linezolid MIC ( $\mu\text{g/ml}$ ) | Nucleotide at position 2576 <sup>b</sup> in: |             |             |             |
|---------------------------|------------------------------|------------------------------------|--|-------------|-------------|-------------|
|                           |                              |                                    | <i>rrlA</i>                                  | <i>rrlB</i> | <i>rrlC</i> | <i>rrlD</i> |
| JH2-2                     | 0                            | 2                                  | G  | G           | G           | G           |
|                           | 4                            | 8                                  | G  | G           | G           | T           |
|                           | 5                            | 16                                 | T  | G           | G           | T           |
|                           | 6, 8                         | 32                                 | T  | G           | G/T         | T           |
|                           | 9, 10, 11                    | 64                                 | T  | G           | T           | T           |
| JH2-2 <i>recA</i>         | 0                            | 2                                  | G  | G           | G           | G           |
|                           | 5                            | 8                                  | G  | G           | G           | T           |
|                           | 6                            | 8                                  | G  | G           | G/T         | T           |
|                           | 7, 8, 9                      | 16                                 | G  | G           | T           | T           |
|                           | 10, 11                       | 32                                 | T  | G           | T           | T           |

<sup>a</sup> Number of passages under linezolid selective pressure.

<sup>b</sup> *E. coli* numbering. Mutations are in boldface.

32  $\mu\text{g/ml}$  and it remained stable after 17 passages (data not shown). In addition, for each increase in the MIC for JH2-2 *recA*, a minimum of 2 days was required.

To determine the number of *rrl* copies that carried mutations, a DNA fragment encompassing the region associated with linezolid resistance (nucleotides 2254 to 2683; *E. coli* numbering) in each *rrl* gene copy from the wild type and mutants was amplified. One common primer and one operon-specific primer for every copy were designed on the basis of the published *E. faecalis* V583 genome (GenBank accession number AE016830). The primers used are those described previously (4), except for the specific primer for *rrlD*, which was changed to 5'-CCTTTTAAACTTAGTCAATC-3'. Amplified DNA fragments were then sequenced in both directions.

DNA sequences of *rrl* copies showed that JH2-2 and JH2-2 *recA* mutants contained the major G2576T mutation (Table 1). No other mutation in positions critical for linezolid resistance (2505, 2512, 2513, and 2610) could be detected, in contrast to the data in previous reports (4, 5, 7, 10). The first G2576T mutation occurred on the *rrlD* copy in both JH2-2 and JH2-2 *recA* and was sufficient for the acquisition of linezolid resis-

tance (MIC = 8  $\mu\text{g/ml}$ ). This result confirms previous data obtained from an animal model of enterococcal infection by Bourgeois-Nicolaos et al. (4). In contrast, Lobritz et al. (5) were unable to identify a resistant JH2-2 mutant selected in vitro with the G2576T mutation at only one locus. In our study, additional mutations occurred in *rrlA* and *rrlC* copies. The relationship between the number of mutated copies and the expression of resistance was unclear, since the level of linezolid resistance did not strictly correlate with the number of 23S rRNA gene copies carrying the nucleotide exchange.

At certain intermediate mutation steps, the selected bacterial population contained mixtures of wild-type and mutated *rrlC* copies (Table 1): the plating of culture samples and the sequencing of *rrlC* copies in 10 individual colonies showed that only a small proportion (2 of 10) of the colonies were mutated. This result may reflect partial replacement of the parental population by secondary-step mutants under linezolid selective pressure.

The delays in the selection of the secondary mutations in the recombination-deficient strain were only one passage for the acquisition of the first and second mutations but six passages for the acquisition of the mutation on the third copy. This finding suggested a role for gene conversion in the acquisition of resistance to linezolid. However, the observed delays were relatively minor, suggesting that recombination systems other than *recA* were active in this strain and could compensate partially for *recA* inactivation.

A previous study by Lobritz et al. (5) was designed to explore the role of recombination in linezolid resistance in *E. faecalis* and led to similar conclusions. However, that study was carried out with the *E. faecalis* UV202 strain, which is a recombination-deficient mutant of JH2-2 (12). The UV202 mutant was obtained from the JH2-2 strain by chemical mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine on the basis of sensitivity to UV irradiation. Although the derivative was found to contain a single amino acid mutation in the RecA protein (2), the presence of other mutations, including some in genes other than *recA* involved in recombinational repair, could not be ruled out. In addition, revertants are easily obtained at a frequency of  $10^{-8}$  (12). Therefore, the use of a strain in which the *recA* gene has been specifically and definitely inactivated seemed preferable. Moreover, Lobritz et al. (5) selected a G2576U mutation in JH2-2, as in our study, but only a G2505A mutation in *E. faecalis* UV202, which renders comparisons between the two strains difficult.

The G2576T mutations were still present in an *E. faecalis* JH2-2 mutant and a JH2-2 *recA* mutant after 15 serial passages in antibiotic-free medium.

In conclusion, the data presented here confirm that the successive accumulation of a single point mutation, G2576T, in the *rrl* gene copies of *E. faecalis* is associated with a stepwise increase in the level of linezolid resistance and show that this accumulation is favored by homologous recombination.

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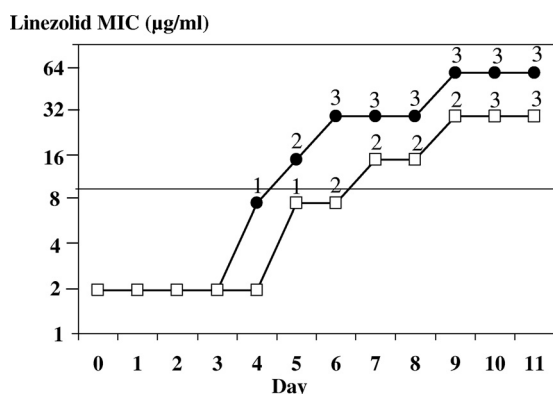


FIG. 1. Selection of mutants of *E. faecalis* JH2-2 (closed circles) and *E. faecalis* JH2-2 *recA* (open squares) resistant to linezolid by serial passages in BHI broth containing linezolid. The numbers above the symbols indicate the numbers of mutated *rrl* gene copies.

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