Dose Dependence of Emergence of Resistance to Linezolid in Enterococcus faecalis In Vivo

Nadège Bourgeois-Nicolaos,1,2,3 Laurent Massias,4 Brigitte Couson,5 Marie-José Butel,2 Antoine Andremont,1,3 and Florence Doucet-Populaire2,5

1Laboratoire de Bactériologie, Hôpital Bichat Claude Bernard (Assistance Publique des Hôpitaux de Paris), 2Équipe d’Accueil 4065, Faculté des Sciences Pharmaceutiques et Biologiques, Université René Descartes–Paris 5, 3Équipe d’Accueil 3964, Université Diderot–Paris 7, 4Laboratoire de Toxicologie Hôpital Bichat Claude Bernard, Paris, and 5Microbiologie Centre Hospitalier Versailles, Le Chesnay, France

Background. The emergence of resistance to antibiotics in vivo, particularly in commensal, potentially pathogenic bacteria, is a factor that is key to the future of antibiotics. To better document the circumstances favoring the emergence of resistance to linezolid (the first of a new class of antibiotics, the oxazolidinones), we modeled the effect of different regimens of linezolid on Enterococcus faecalis in gnotobiotic mice.

Methods. We studied the rate of emergence of linezolid-resistant E. faecalis mutants in the digestive tract of gnotobiotic mice monoassociated with linezolid-susceptible E. faecalis and fed with water containing linezolid (0.5, 0.05, or 0.005 g/L). 23S Ribosomal RNA (rRNA) mutations were characterized by sequencing each of the 4 copies of the rRNA genes individually.

Results. Mutants were readily obtained in vivo, but the frequencies, persistence, and type of mutants were all dependent on the linezolid regimen. Mutations conferring resistance, either the G2505A or G2576U mutation, were present in domain V of the 23S rRNA gene of all resistant isolates. Levels of resistance increased with the number of mutated copies of the 23S rRNA gene and with duration of exposure.

Conclusion. The antibiotic dose appears to be critical in the dynamics and molecular basis of resistance.

The rise in the prevalence of antibiotic-resistant bacteria is a public health problem. The impact of antibiotics on the commensal flora during treatment is a major driving force in the emergence of resistance [1, 2]. In the intestinal flora, resistant bacteria selected during treatment, such as enterobacteria or enterococci, are potentially pathogenic [3–5]. The dynamics of the emergence of resistance in vivo, which are not well known, are an issue for new antibiotics, because such emergence could jeopardize the usefulness and life span of antibiotics.

Linezolid, active against gram-positive bacteria, is such a new antibiotic that has been recently approved for clinical use [6]. The first of a new class of antibiotics, the oxazolidinones, linezolid can be used against multiple-drug–resistant gram-positive cocci, including methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococci (VRE) [7]. It inhibits bacterial protein synthesis by binding specifically to domain V of the 23S rRNA of the 50S ribosomal subunit [8] and is not affected by the resistance mechanisms that affect other antibiotics [9].

Mutants resistant to linezolid are not selected easily in vitro [10], and emergence of resistance, although rare [11], has been observed among clinical isolates of Staphylococcus aureus and enterococci [12–16]. Although resistance has been observed in enterococci without prior exposure to linezolid [17, 18], it is usually associated with prior and prolonged exposure [19]. The emergence of resistance in vitro is due to the presence of various mutations in the central loop of the domain V region of the ribosome [10], but it results, in vivo, from a single type of mutation (the G2576U mutation [Escherichia coli numbering]) [13–16, 20]. E. faecalis contains 4 copies of rRNA operons (rrn) on its chromosome [21]. The level of resistance correlates with
the number of the 4 copies of the 23S rRNA gene that are mutated [22–24].

Here, we report on the circumstances that favor the emergence of resistance to linezolid in vivo. However, because of the difficulties associated with human studies, we used gnotobiotic mice, a model previously used to study in vivo the impact of antibiotics on commensal bacteria [25, 26] and resistance [27–31]. E. faecalis was chosen as the target both because it is more prone to generate linezolid-resistant mutants than is E. faecium [10] and because it is the most abundant enterococcal species in the human gut [32], where mutants are most likely to emerge, despite the fact that only 0.2% of the administered dose of linezolid is excreted in the feces [33]. Mean (±SD) fecal concentrations of linezolid in humans were 7.1 ± 2.6 mg/kg after 4 days of linezolid treatment and 3.0 ± 2.0 mg/kg after 8 days of treatment [34]. We modeled the effect of various linezolid regimens and found that the dynamics of emergence and the types of mutants selected were dependent on both the dose and length of treatment.

METHODS

Bacterial Strains
Linezolid-susceptible E. faecalis JH2-2 [35] was used as the target strain. Aliquots of the bacteria were stored in brain-heart infusion (BHI) broth (bioMérieux) with glycerol at −80°C. For each study, fresh isolates were grown on BHI agar plates (bioMérieux) at 37°C overnight.

Drug
Linezolid (Pfizer) solutions of 2 mg/mL were aliquoted and stored at −20°C. For each study, a sample was thawed and diluted to the desired concentration with 0.9% NaCl. The MIC and mutant prevention concentration (MPC) of linezolid were determined as described elsewhere [36]. The MIC and MPC of linezolid for wild-type E. faecalis JH2-2 were both 2 mg/L. Susceptibility to linezolid (=4 mg/L) was defined according to Clinical Laboratory Standards Institute breakpoints [37].

Selection of Linezolid-Resistant E. faecalis in Gnotobiotic Mice
At the time of arrival, adult, female, germ-free, consanguineous C3H mice (mean weight, 25 g) (Charles River Laboratories) were randomly assigned to groups of 6 mice, with each group housed in separate sterile isolators (JCE Biotechnology) with ad libitum sterile food (Dietex France) and water. The germ-free status of the mice was ensured before the beginning of the experiment by testing fecal samples for aerobic and anaerobic growth of bacteria and yeast. The local ad hoc committee approved the protocol for in vivo experiments. After ~1 week of acclimatization, each mouse was inoculated intragastrically with 300 µL of a broth culture containing 10⁴ cfu of E. faecalis JH2-2. One week later, after the target strain had become established at high density in the intestine, oral treatment was initiated by adding 0, 0.005, 0.05, or 0.5 g of linezolid per liter of drinking water and was continued for 21 days. Water bottles were changed every 72 h. Antibiotic activity in the bottles remained stable between the changes (data not shown).

Counts of Bacteria
Mouse pellets were collected individually from each mouse after spontaneous emission in sterile tubes, 5 days a week. The fresh pellets were weighed, and 10-fold dilutions were made in re-generated peptone broth. Linezolid-resistant and total E. faecalis were enumerated using the automatic spiral system (AES Laboratoires) on Mueller-Hinton agar containing or not containing linezolid (4 mg/L) and expressed as log10 per gram of pellet. The limit of detection was 2 log10 cfu/g of pellet.

Molecular Characterization of Mutants
Identification of possible mutation in domain V of the 23S rRNA gene. The domain V region (bp 2254–2683) (E. coli numbering) associated with in vitro linezolid resistance was amplified for 5, 10, and 5 linezolid-resistant E. faecalis isolates recovered from mice receiving linezolid at 0.5, 0.05, and 0.005 g/L, respectively, by use of primers 23S-V/F (5'-AGTTTGACT-GGGCGGTC-3') and 23S-V/R (5'-CCGGCTCCTCTCGTA-3'). The 100-µL polymerase chain reaction (PCR) mixture contained 10 µL of 10× standard buffer without MgCl2 (Invitrogen), 0.25 mmol/L MgCl2, 500 µmol/L dNTP, 2.5 U Taq DNA polymerase (Invitrogen), 5 µL each primer (10 pmol/µL), 250 ng of DNA, and sterile distilled water up to 100 µL. Cycling parameters were 94°C for 5 min; 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min for 25 cycles; and 72°C for 10 min. The 429-bp PCR products were sequenced using Big Dye PCR (Perkin-Elmer/Cetus) and were analyzed on an ABI Prism 310 DNA Genetic Analyzer (Perkin-Elmer Applied Biosystems).

Determination of copy numbers of the 23S rRNA gene carrying a mutation. The 4 different copies of the 23S rRNA gene were amplified separately (figure 1). For each copy, one common primer and one operon-specific primer were designed on the basis of the published E. faecalis genome VS83 (GenBank accession number AE016830) (see table 1). The 100-µL PCR mixture contained 10 µL of 10× standard buffer without MgCl2 (Invitrogen), 0.25 mmol/L MgCl2, 500 µmol/L dNTP, 2.5 U Taq DNA polymerase (Invitrogen), 5 µL each primer (10 pmol/µL), 250 ng of DNA, and sterile distilled water up to 100 µL. Cycling parameters were 94°C for 5 min; 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min for 25 cycles; and 72°C for 10 min. The domain V region (bp 2254–2683) of each 23S rRNA gene was amplified and sequenced as described above.
Figure 1. Schematic diagram of the determination of the no. of copies of the 23S rRNA gene (rrn) that carried mutations. The 4 different copies were amplified separately. For each copy, 1 common primer (23S-F) and 1 operon-specific primer (23S-1R, 23S-2R, 23S-3R, or 23S-4R) were designed from the published Enterococcus faecalis genome V583 (GenBank accession number AE016830). The domain V region (bp 2254–2683) of each copy was amplified using primers 23S-V/F and 23S-V/R and was sequenced.

### Physiological Characteristics of Linezolid-Resistant E. faecalis

#### Stability of resistance

A single colony of linezolid-resistant E. faecalis was serially subcultured 22 times (over the course of 1 month) on antibiotic-free Columbia cysteine agar (bio-Mérieux). The MICs of linezolid for the original linezolid-resistant E. faecalis and for subcultured strains were determined.

#### Growth curves

Single colonies of linezolid-susceptible E. faecalis JH2-2 and linezolid-resistant E. faecalis mutants were used to inoculate 10 mL of BHI broth and were incubated at 37°C overnight. Growth studies were performed in triplicate by inoculating BHI broth (1:100 vol/vol), and the optical density at 600 nm was measured every hour for 8 h. Growth rates (expressed as μ) were calculated by multiplying the slope of the regression equation of the linear portion of the curves.
pretreatment count (with water containing 0.5, 0.05, or 0.005 g/L of linezolid. The
in an initial experiment, 3 groups of 6 mice were provided
Resistant
effects of linezolid regimen on the selection of linezolid-
genome copy
Sequence (5’–3’)
Temperature, °C
Size, bp

Table 1. Primers pairs used for amplification of the 4 copies of the 23S rRNA gene

<table>
<thead>
<tr>
<th>23S rRNA gene copy no. primer</th>
<th>Sequence (5’–3’)</th>
<th>Temperature, °C</th>
<th>Size, bp</th>
</tr>
</thead>
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<tr>
<td>1 23S-F</td>
<td>GGGCTGTTGGGATACTA</td>
<td>58</td>
<td>1898</td>
</tr>
<tr>
<td>1 23S-1R</td>
<td>GGACGTATAGGCCTGC</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>2 23S-F</td>
<td>GGGCTGTTGGGATACTA</td>
<td>58</td>
<td>1415</td>
</tr>
<tr>
<td>2 23S-2R</td>
<td>GCGATCTCTGCCTGAC</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>3 23S-F</td>
<td>GGGCTGTTGGGATACTA</td>
<td>58</td>
<td>1111</td>
</tr>
<tr>
<td>3 23S-3R</td>
<td>CCCCCTTCAAGCTTAC</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>4 23S-F</td>
<td>GGGCTGTTGGGATACTA</td>
<td>58</td>
<td>1952</td>
</tr>
<tr>
<td>4 23S-4R</td>
<td>CCACAGTGAATTTGCCCA</td>
<td>54</td>
<td></td>
</tr>
</tbody>
</table>

relating log optical density to time (in hours) by 2.303 [38].
Generation time (expressed as ß) was calculated according to the
formula ß = ln2/μ. Student’s t test was used for comparisons.

Linezolid High-Performance Liquid Chromatography (HPLC)
Assay of Pellets
Linezolid was extracted from pellets (20 mg) with the use of
1 mL of acetonitrile. After mixing, the sample was centrifuged
at 2000g for 10 min. The supernatant was evaporated under
nitrogen, and the residue was reconstituted with 100
µL mobile phase (15% acetonitrile and 85% water [vol/vol]) and then was
transferred in injection vials. A Novapack C8 3.9-mm × 150-
mm column (Waters) was used for HPLC, with a flow of 0.8
mL/min. Linezolid was detected at 251 nm, and an injection
volume of 40 µL was used. Chromatograms were analyzed using
Millennium 32 software (Waters). The following linezolid
quantities were used for calibration: 0.005, 0.01, 0.05, 0.1, 0.5,
and 1 µg in 50 µL of deionized water added to ~20 mg of mice
pellets. The lower limit of detection of HPLC was 0.05 µg of
linezolid/g of pellet. Samples in which linezolid was undetect-
able were scored as 0.05 µg/g of pellet, for calculation of mean
values.

RESULTS
Effects of Linezolid Regimen on the Selection of Linezolid-
Resistant \textit{E. faecalis} in the Digestive Tract of Gnotobiotic Mice
In an initial experiment, 3 groups of 6 mice were provided
with water containing 0.5, 0.05, or 0.005 g/L of linezolid. The
pretreatment count (± SD) of wild-type \textit{E. faecalis} JH2-2 was
9.94 ± 0.26 log10 cfu/g of pellets for all 3 groups (figure 2), and
it remained stable in all nontreated control mice. No resistant
mutant was isolated at any time from any nontreated mouse.

Median (± SD) fecal concentrations of linezolid were 11.04 ± 11.65, 1.07 ± 1.05, and 0.19 ± 0.22 µg/g, respectively (figure
2, left). Effects on counts of total and linezolid-resistant fecal
\textit{E. faecalis} and on the susceptibility of the isolates to linezolid
are shown in figure 2 (middle and right panels), respectively.

Twenty-five linezolid-resistant mutants were isolated from
the group receiving treatment with 0.5 g/L. One mutant (2.5
log10 cfu/g of pellet) with an MIC of linezolid of 8 mg/L was
isolated from 1 mouse on day 2 of treatment, and 24 mutants
(mean [± SD], 3.6 ± 0.5 log10 cfu/g of pellet), all with an MIC
of linezolid of 16 mg/L, were isolated from 2 mice (including
the one mouse colonized on day 2) after 21 days.

In the group receiving treatment with 0.05 g/L, linezolid-
resistant \textit{E. faecalis} counts were high in all mice after 5 days of
treatment and throughout the experiment (mean [± SD], 9.5
± 0.33 log10 cfu/g of pellet). The MICs of linezolid, studied in
36 mutants, increased with length of treatment, to 16 mg/L
between day 5 and day 14, to 32 mg/L between day 14 and
day 17, and to 64 mg/L after day 18.

In the group receiving treatment with 0.005 g/L, a mutant
(MIC of linezolid, 8 mg/L) appeared after day 4 in one mouse,
close to the limit of detection. The mutant was isolated at various
times thereafter (mean concentration [± SD], 2.4 ±
0.25 log10 cfu/g of pellet).

In the second experiment (figure 3), mice were initially
treated with 0.005 mg/L for 21 days and then with 0.05 mg/L.
Mutants were found in mean concentrations (± SD) of 2.4
± 0.25 log10 cfu/g during the first phase of treatment and in
concentrations of 9.2 ± 0.3 log10 cfu/g during the second phase
of treatment. For 36 resistant isolates studied, the MICs of
linezolid increased to 16 mg/L on day 2 of treatment, to 32
mg/L between day 8 and day 15, and to 64 mg/L by day 16.

Molecular Characteristics of Linezolid-Resistant Mutants
The linezolid-susceptible \textit{E. faecalis} (JH2-2) isolate had the
same domain V region sequence as did \textit{E. faecalis} V583 (Gen-
Bank accession number AJ295306). All linezolid-resistant \textit{E. fae-
calis} isolates had mutations in this region that differed according
to the concentrations of linezolid administered. In the group
receiving treatment with 0.5 g/L, linezolid-resistant mutants (5
of 5 isolates tested) had a G2505A mutation. In the group
receiving treatment with 0.05 g/L, 0 of 10 mutants tested had that
mutation, but all had the G2576U mutation. In the group
receiving treatment with 0.005 g/L, 4 of 5 mutants tested (MIC, 8
mg/L) had the G2576U mutation, and 1 mutant also had mu-
tation G2505A. Each isolate carried only 1 type of mutation in
the 4 copies of the 23S rRNA gene. MICs of linezolid increased
with the increased numbers of mutated copies (table 2).
**Figure 2.** Effect of 3 different linezolid regimens (0.5, 0.05, and 0.005 g/L) on bacterial survival and mutant enrichment in the digestive tracts of gnotobiotic mice: the group receiving 0.5 g/L (A), the group receiving 0.05 g/L (B), and the group receiving 0.005 g/L (C). The left column of panels shows the linezolid concentration in fecal samples obtained from the 3 groups. Circles denote the linezolid concentrations determined by high-performance liquid chromatography (HPLC) assay. The lower horizontal line denotes the limit of detection of the HPLC method (0.05 µg of linezolid/g of pellet). The upper horizontal line denotes the MIC and mutant prevention concentration (MPC) of linezolid to linezolid-susceptible *Enterococcus faecalis* (JH2-2). The center column of panels shows the effect of the linezolid treatments on total *E. faecalis* (H18567) and linezolid-resistant *E. faecalis* (H17005) populations over 21 days. Data are the mean of the log10 cfu/g of feces for total *E. faecalis* and for linezolid-resistant *E. faecalis*, for the 6 mice. The error bars denote the SD. The horizontal line denotes the limit of detection (2 log10 cfu/g of pellet). The right column of panels shows the effect of each linezolid regimen on the susceptibility of *E. faecalis* isolates.

**Physiological Characteristics of Linezolid-Resistant Mutants**

Twenty mutants obtained in vitro were tested. Only one (a G2505A mutation in one copy of the 23S rRNA gene) reverted to susceptibility after 22 passages (over 30 days) on antibiotic-free medium. The MIC reverted from 8 to 2 mg/L, which is the MIC of the parental strain. However, the revertant retained the G2505A mutation in one copy of the 23S RNA gene, and there was no mutation noted elsewhere in the domain V region (bp 2254–2683). The growth characteristics of the mutants were compared with those of the wild-type parent (JH2-2) (table 3). The presence of one mutated copy of the 23S rRNA gene (either the G2505A or G2576U mutant) was associated with a significantly longer generation time; conversely, the presence of 3 or 4 copies was associated with a significantly shorter generation time (table 3). This could only be tested for G2576U mutants, because no mutants with >2 G2505A-mutated copies were isolated.

**DISCUSSION**

The present study was performed in an artificial intestinal ecosystem in monoassociated gnotobiotic mice. This model is different from the normal flora of humans, because it lacks anaerobes. However, it appears to be relevant to the study of the emergence of mutants, because the total number of target bacteria is similar to that present in a healthy human colon. The emergence, concentrations, and persistence of linezolid-resistant *E. faecalis* mutants were highly regimen dependent. Mutants emerged as early as 2, 5, and 4 days after treatment with linezolid in the groups given 0.5, 0.05, and 0.005 g/L, respectively. No mutants were isolated at any time in the absence of linezolid, which is consistent with the following findings: (1) de novo resistance to linezolid is uncommon in enterococci, (2) the frequency of spontaneous mutation toward linezolid resistance is low in vitro [39], and (3) mutants are rarely isolated from patients without previous exposure to linezolid [17, 40];
in such instances, cross-contamination may explain colonization [17, 18].

That mutants readily emerged under selective pressure in the present study was consistent with what has been previously reported in patients [19], although, in those patients, most resistant enterococci isolates reported were *E. faecium*. This finding reflects the fact that linezolid is mostly used to treat VRE-infected patients, in whom *E. faecium* are more prevalent than *E. faecalis* [41]. The time elapsed before colonization has been reported to be 16 and 48 days after initiation of linezolid treatment in 2 patients colonized with VRE [42], which is later than what we observed in mice; however, the patients were sampled only once, and the sensitivity of detection is unknown.

In mice exposed to the highest concentrations of linezolid (0.5 g/L), the intestinal concentrations were the most similar to those noted in humans receiving 1200 mg/day [34]; they were greater than the MIC for the susceptible target strain JH2-2, which explains why it was almost fully eliminated. Mutants had difficulties in emerging because first-step mutants—those with only one copy of the 23S RNA gene mutated—are still susceptible to concentrations of linezolid present in the intestinal lumen of these mice. This may explain why colonization by resistant enterococci is rarely observed in patients. The sharp decrease in counts of enterococci in this group of mice is consistent with observations in linezolid-treated volunteers [34], further suggesting the validity of the model.

By contrast, the fecal concentrations in mice treated with 10 times less linezolid were just below the MIC for the target strain, which explains why the JH2-2 counts decreased only slightly and why first-step mutants could emerge and cohabitate simultaneously at high density with the parental strain. Interestingly, mutants with 1 mutated copy appeared over time, and the MICs increased accordingly.

Table 2. Phenotypic and genotypic characteristics of linezolid-resistant *Enterococcus faecalis* obtained from 3 groups of mice treated with linezolid at 0.5, 0.05, or 0.005 g/L.

<table>
<thead>
<tr>
<th>Linezolid dose (g/L), treatment day</th>
<th>MIC of linezolid, mg/L</th>
<th>Linezolid-resistant <em>E. faecalis</em> isolates, no.</th>
<th>Mutation in domain V of the 23S rRNA gene</th>
<th>23S rRNA gene copy no(s).</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
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<tr>
<td>2</td>
<td>8</td>
<td>1</td>
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<td>M</td>
</tr>
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</tr>
<tr>
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<td>G2576U</td>
<td>M</td>
</tr>
<tr>
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</tr>
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</tr>
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</tr>
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<tr>
<td>21</td>
<td>8</td>
<td>2</td>
<td>G2576U</td>
<td>W</td>
</tr>
</tbody>
</table>

NOTE. M, mutated; W, wild type.

* No. analyzed.

* Escherichia coli numbering.
Table 3. Generation times of linezolid-susceptible Enterococcus faecalis (JH2-2) in brain-heart infusion (BHI) broth and various genotypes of linezolid-resistant E. faecalis.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Copies of the 23S rRNA gene with a mutation, no.</th>
<th>Generation time, mean ± SD, min</th>
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<tr>
<td>Wild type</td>
<td>0</td>
<td>31.7 ± 0.3</td>
</tr>
<tr>
<td>G2505A</td>
<td>1</td>
<td>37.3 ± 0.4*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>40.1 ± 1.1*</td>
</tr>
<tr>
<td>G2576U</td>
<td>1</td>
<td>38.9 ± 0.5*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>32.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>29.6 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>29.2 ± 0.3*</td>
</tr>
</tbody>
</table>

* P < 0.05 (Student’s t-test) for the comparison of the generation time of linezolid-resistant E. faecalis with that of linezolid-susceptible E. faecalis (JH2-2).

Last, when the selective pressure was low (as in the group receiving treatment with 0.005 g/L), the concentrations of linezolid were, at all times, below the MIC for the target strain, and the counts were not significantly affected. Mutants with relatively low MICs emerged, but they remained at low concentrations, close to the limit of detection (probably because of the barrier effect exerted by the susceptible enterococci present in high counts). The effect of such intraspecies barriers has been described in the past in E. coli [43], although its mechanism is not well explained. This inference was confirmed in the experiment in which mice were initially treated with 0.005 g/L and then with 0.05 g/L, where levels of colonization by the experiment in which mice were initially treated with 0.005 g/L, the group with 0.05 g/L, and in one isolate from the group receiving treatment with 0.005 g/L, demonstrating that the G2505A mutation could be selected in vivo as well.

For all previously described linezolid-resistant E. faecalis strains, at least 2 of 4 copies of the 23S rRNA genes mutated, suggesting that mutation of only one copy did not confer a significant level of resistance [48]. In the present study, however, mutants with a mutation in only one copy of the gene could be selected in vivo by 2 of 3 regimens tested. Such single-copy mutants were much easier to select than were mutants with 2 mutated copies, suggesting that selection of single-copy mutants in commensal E. faecalis could be an event which is thus far undetected in patients but which could be a concern when the drug becomes more widely used. We confirmed the association of the number of mutated copies with the MIC for G2576U mutants [22, 48] and extended it for G2505A mutants. Most likely, mutations spread from one 23S rRNA gene to others by homologous recombination [48]. That no mutant with 3 or 4 copies with the G2505A mutation was isolated suggests that such mutants might not be viable. In isolates from the group receiving treatment with 0.05 g/L, the group with the highest number of mutants, resistance increased with the number of mutated copies of the 23S rRNA gene and, also, with the duration of exposure to linezolid. To our knowledge, this is the first clear experimental evidence that prolonged treatment favors the emergence of resistance in the intestinal flora. Our findings thus strongly support the current recommendations for reducing the length of antibiotic treatments as much as possible.

For G2576U mutants, the number of copies of 23S rRNA genes mutated was inversely proportional to the generation time, except that single-copy mutants grew slower than the parental strain. That a single G2576U mutation confers a selective disadvantage explains not only the low level of intestinal colonization achieved by such strains in our model but, also, why this type of mutant has not been reported in clinical isolates. Overall, this suggests that mutation of a single 23S rRNA gene is the critical and, maybe, the rate-limiting step in the emergence of linezolid resistance. Primary colonization with
such single-mutation G2576U mutants, as was observed as early as 5 days after treatment initiation in mice receiving treatment with 0.05 g/L, was probably what facilitated further emergence of linezolid-resistant *E. faecalis* with 2, 3, and 4 mutated copies and, therefore, increased levels of resistance. In patients, enterococcal infections mostly originate from the intestinal tract of colonized patients [49], and it is thus not surprising that mutants with at least 2 mutated copies of the 23S rRNA gene have been frequently isolated [19].

In conclusion, our in vivo experiments involving mice help explain the pattern of emergence of resistance to linezolid observed in clinical isolates. We showed that dose is critical to the dynamics of resistance and that the molecular basis of resistance, the number of mutated copies of the target gene, confers specific traits to this set of events. Our findings should help define the best therapeutic strategies to minimize the emergence of resistance in the clinical setting, and similar studies would be beneficial for any new antibiotic appearing on the market.

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**References**

31. Perrin-Guyomard A, Poul JM, Corpet DE, Sanders P, Fernandez AH, Bartholomew M. Impact of residual and therapeutic doses of cipro-