MAJOR ARTICLE

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

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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

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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 ($\pm$SD) fecal concentrations of linezolid in humans are 7.1 $\pm$ 2.6 mg/kg after 4 days of linezolid treatment and 3.0 $\pm$ 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 ($\leq$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 10^8 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 regenerated 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 $\log_{10}$ per gram of pellet. The limit of detection was 2 $\log_{10}$ 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′-AGTTTGACTGGGGCGGTC-3′) and 23S-V/R (5′-CCGGTCCTCTCGTACTA-3′). The 100-μL polymerase chain reaction (PCR) mixture contained 10 μL of 10× standard buffer without $MgCl_2$ (Invitrogen), 0.25 mmol/L $MgCl_2$, 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 V583 (GenBank accession number AE016830) (see table 1). The 100-μL PCR mixture contained 10 μL of 10× standard buffer without $MgCl_2$ (Invitrogen), 0.25 mmol/L $MgCl_2$, 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 35 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.

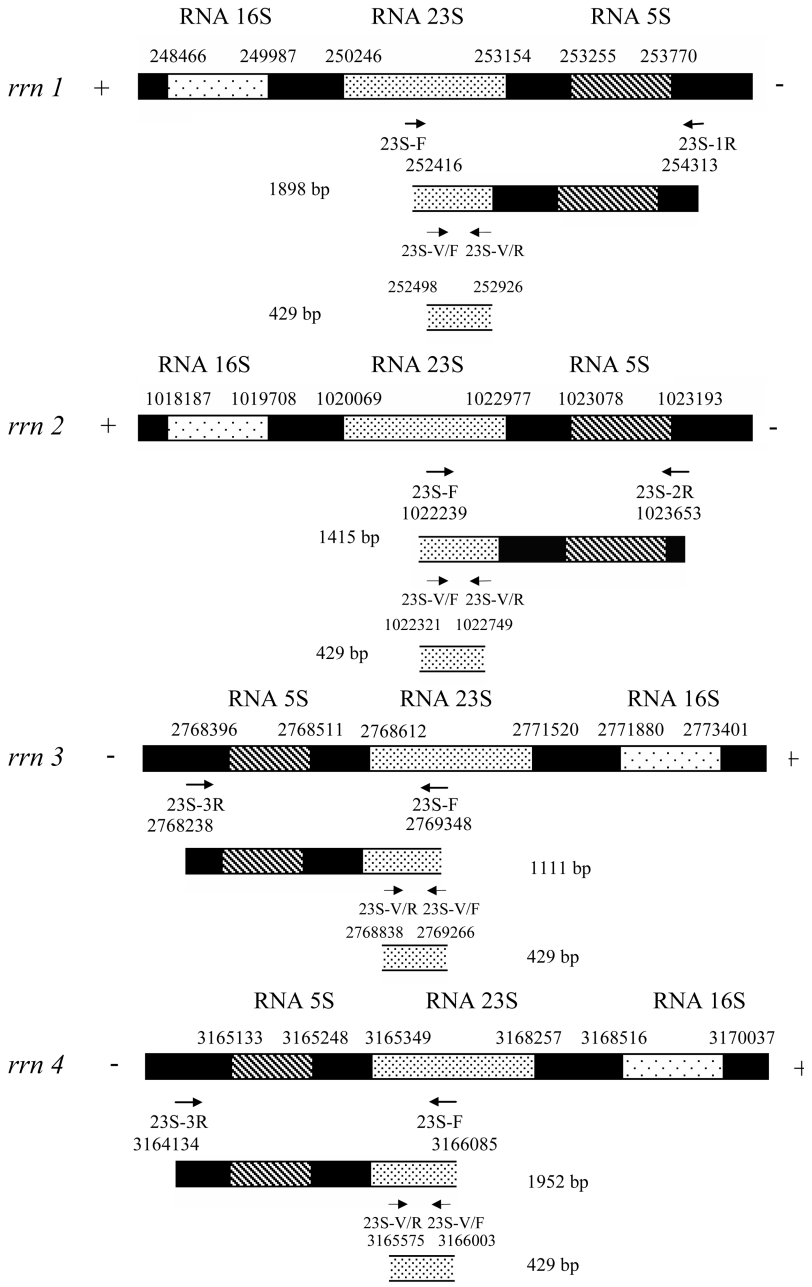

Figure 1. Schematic diagram of the determination of the no. of copies of the 23S rRNA gene *(rrn)* that carried mutations. The 4 different copies were amplified separately. For each copy, 1 common primer (23S-F) and 1 operon-specific primer (23S-1R, 23S-2R, 23S-3R, or 23S-4R) were designed from the published *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 (bioMérieux). 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

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

23S rRNA gene copy no., primer	Sequence (5′→3′)	Temperature, °C	Size, bp
1			
23S-F	GGCGCTGGTGGGATACTA	58	1898
23S-1R	GGACGGTTATGAGCCGTC	58	
2			
23S-F	GGCGCTGGTGGGATACTA	58	1415
23S-2R	GCGATCTCCTGCGTGAC	56	
3			
23S-F	GGCGCTGGTGGGATACTA	58	1111
23S-3R	CCCTTCTTCAAGCTTATC	52	
4			
23S-F	GGCGCTGGTGGGATACTA	58	1952
23S-4R	CCACAGTGATTTTGCCCA	54	

relating log optical density to time (in hours) by 2.303 [38]. Generation time (expressed as θ) was calculated according to the formula $\theta = \ln 2/\mu$. 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 2000*g* 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 undetectable 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 *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 *E. faecalis* JH2-2 was 9.94 ± 0.26 $\log_{10}$ 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 *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 $\log_{10}$ 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 $\log_{10}$ 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 *E. faecalis* counts were high in all mice after 5 days of treatment and throughout the experiment (mean [±SD], 9.5 ± 0.33 $\log_{10}$ 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 $\log_{10}$ 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 $\log_{10}$ cfu/g during the first phase of treatment and in concentrations of 9.2 ± 0.3 $\log_{10}$ 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 *E. faecalis* (JH2-2) isolate had the same domain V region sequence as did *E. faecalis* V583 (GenBank accession number AJ295306). All linezolid-resistant *E. faecalis* 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 mutation 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).

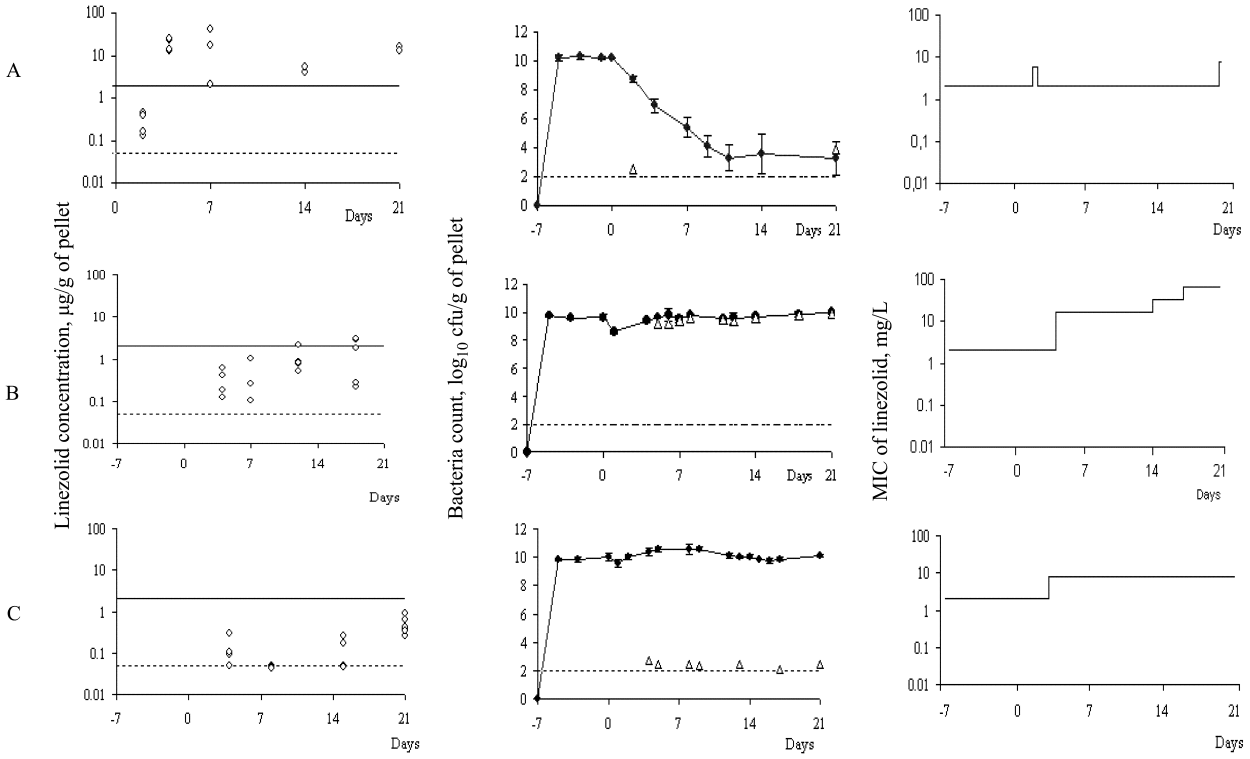

Figure 2. Effect of 3 different linezolid regimens (0.5, 0.05, and 0.005 g/L) on bacterial survival and mutant enrichment in the digestive tracts of gnotobiotic mice: the group receiving 0.5 g/L *(A),* the group receiving 0.05 g/L *(B),* and the group receiving 0.005 g/L *(C).* The left column of panels 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* (●) and linezolid-resistant *E. faecalis* (△) populations over 21 days. Data are the mean of the $\log_{10}$ 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 $\log_{10}$ 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];

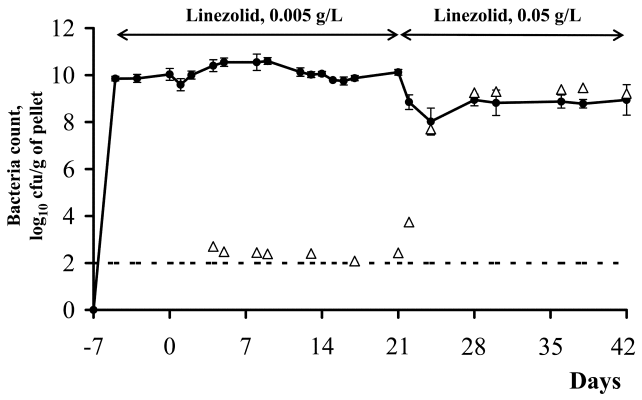

Figure 3. Effect of linezolid, 0.05 g/L, in drinking water on total *Enterococcus faecalis* (●) and linezolid-resistant *E. faecalis* (△) populations in the digestive tracts of gnotobiotic mice initially treated with linezolid, 0.005 mg/L, for 21 days. Data are the mean of the $\log_{10}$ cfu/g of pellet for total *E. faecalis* and linezolid-resistant *E. faecalis,* for the 6 mice. The error bars denote the SD. The horizontal line denotes the detection limit (2 $\log_{10}$ cfu/g of pellet).

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.

Linezolid dose (g/L), treatment day	MIC of linezolid, mg/L	Linezolid-resistant *E. faecalis* isolates, no.[a]	Mutation in domain V of the 23S rRNA gene[b]	23S rRNA gene copy no(s).			
				1	2	3	4
0.5							
2	8	1	G2505A	M	W	W	W
21	16	4	G2505A	M	W	W	M
0.05							
5	16	2	G2576U	M	W	W	M
7	16	1	G2576U	M	W	W	M
8	16	1	G2576U	W	M	W	M
11	16	1	G2576U	W	W	M	M
15	32	2	G2576U	M	W	M	M
21	64	3	G2576U	M	M	M	M
0.005							
4	8	1	G2576U	W	W	M	W
8	8	1	G2576U	W	W	W	M
15	8	1	G2505A	W	W	M	W
21	8	2	G2576U	W	W	W	M

NOTE. M, mutated; W, wild type.

[a] No. analyzed.

[b] *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*.

Genotype	Copies of the 23S rRNA gene with a mutation, no.	Generation time, mean ± SD, min
Wild type	0	31.7 ± 0.3
G2505A	1	37.3 ± 0.4[a]
	2	40.1 ± 1.1[a]
G2576U	1	38.9 ± 0.5[a]
	2	32.4 ± 0.7
	3	29.6 ± 0.2[a]
	4	29.2 ± 0.3[a]

[a] $P < .05$ (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 linezolid-resistant *E. faecalis* were initially close to the detection limit and were at a level close to that of the susceptible strain, when the antibiotic pressure increased, and remained so thereafter (figure 3).

The MIC and MPC of linezolid were equal for the susceptible target strain (JH2-2), which is different from what has been reported with mycobacteria [44], a very different genus phylogenetically. So far, to our knowledge, the molecular basis for this still remains unexplored at this time. Our findings do not support the opinion that the so-called "in vitro–determined selection window," actually the MIC-MPC range, observed with fluoroquinolones [45, 46] is also observed with linezolid. On the other hand, the MPC determined in vitro is usually based on constant concentrations over a long time, in contrast to the in vivo situation. Therefore, it is very difficult to predict concentrations at the site of mutation, and these in vivo results may not be contradictory to in vitro findings. This may be because the selection window of linezolid with regard to our target strain was nonexistent. Nevertheless, mutants were easily selected in vivo, when the antibiotic concentrations were close to the MIC and the MPC, as in mice from the group treated with 0.05 g/L. Our data also support the notion that resistant mutants are selectively enriched when antimicrobial concentrations are just below their MIC [47].

All the mutants with a MIC >4 mg/L that we isolated carried either a G2505A or G2576U substitution in the domain V region of the 23S rRNA gene. No other mutations were observed within the region. The G2576U mutation was observed in all mutants from the group treated with 0.05 g/L and in 4 of 5 mutants from the group treated with 0.005 g/L. All reported clinical isolates of linezolid-resistant enterococci harbor mutation G2576U. By contrast, the G2505A mutation has previously been described only after in vitro selection [10, 48]. Here, it was found in all resistant isolates from the group receiving treatment with 5 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 treatments favor 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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