

Acquired Gentamicin Resistance by Permeability Impairment in *Enterococcus faecalis*

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Received 30 March 2006/Returned for modification 10 May 2006/Accepted 31 July 2006

Enterococci are intrinsically resistant to low levels of aminoglycosides. We previously selected in vitro and in vivo *Enterococcus faecalis* with intermediate-level resistance to gentamicin that did not abolish synergism with a cell-wall-active agent (E. Aslangul et al., *Antimicrob. Agents Chemother.* 49:4144–4148, 2005). The aim of this study was to investigate the mechanism of resistance to gentamicin in the 1688-G3 third-step mutant (MIC, 512 µg/ml) of *E. faecalis* JH2-2. No mutations were found in the genes for L6 ribosomal protein and the four copies of 16S rRNA. Production of a known aminoglycoside-modifying enzyme was unlikely due to the distinct resistance phenotype and absence of the corresponding genes. Efflux was also unlikely since ethidium bromide MICs were similar for JH2-2 and 1688-G3 and since the pump inhibitors reserpine and verapamil had no effect on gentamicin resistance in both strains. To study gentamicin accumulation, we developed a nonisotopic method based on a fluorescent polarization immunoassay. Impaired gentamicin accumulation was observed in 1688-G3 compared to JH2-2 and was only partially reversible by the *N,N'*-dicyclohexylcarbodiimide (DCCD) uncoupler agent. The lower sensitivity of 1688-G3 to DCCD suggested alteration of the F_oF_1 -ATPase. However, no mutations were detected in the structural genes (*atp*) for the F_o channel and no difference in transcript levels of *atpB* and *atpE* was found between 1688-G3 and JH2-2. Our data are compatible with acquisition of intermediate-level gentamicin resistance by uptake impairment in *E. faecalis*.

Enterococci are intrinsically resistant to low levels of aminoglycosides. Gentamicin acts synergistically with cell-wall-active antibiotics such as beta-lactams and glycopeptides (39). However, synergism is abolished when enterococci have acquired high-level resistance to gentamicin (MIC, >512 µg/ml) (6).

We previously isolated mutants with intermediate-level gentamicin resistance (64 µg/ml < MIC ≤ 512 µg/ml) in the rabbit endocarditis model when animals were infected with *Enterococcus faecalis* strain BM4275 or BM4281, either of which displays VanB-type glycopeptide resistance, and treated with a combination of vancomycin and gentamicin (28). Gentamicin-resistant mutants of glycopeptide-susceptible *E. faecalis* JH2-2 were also isolated during gentamicin monotherapy in the same animal model (28). Against JH2-2 mutants selected in vitro with intermediate-level gentamicin resistance, gentamicin remained synergistic with amoxicillin in the rabbit endocarditis model (1, 27).

The main acquired aminoglycoside resistance mechanism is enzymatic inactivation of the drugs. Other mechanisms include target modification by mutations in ribosomal proteins or 16S rRNA, methylation of 16S rRNA, efflux, and diminished uptake impairment (4, 19, 34, 41, 44, 45). In *E. faecalis*, only four aminoglycoside-modifying enzymes confer resistance to gentamicin (7, 12, 24, 48). Except for streptomycin, no target modifications are known to affect aminoglycoside susceptibility in

E. faecalis. Efflux pumps have been described in enterococci, but aminoglycosides are not substrates (22, 29). However, 34 genes in the *E. faecalis* genome encode putative efflux pumps. Aminoglycoside uptake, which relies on the proton motive force through the F_oF_1 -ATPase, has been studied in streptococci but not in *Enterococcus* spp. (16). This enzyme couples the electrochemical proton gradient to ATP synthesis from ADP or from oxidative phosphorylation. The resulting proton potential (proton motive force) is used to transport a variety of cations, either in the same direction as the protons (symport) or in the opposite direction (antiport). Aminoglycosides are cationic molecules that are transported in symport mode by the F_oF_1 -ATPase. In bacteria with a deficient respiratory chain, such as enterococci, the proton potential is generated solely following ATP hydrolysis by F_oF_1 -ATPase. The *E. faecalis* enzyme is composed of the F_o membrane proton channel and of the F_1 cytoplasmic catalytic site (23). The subunit distribution in each portion of the ATPase follows a known stoichiometric ratio in F_1 (a3, b3, g1, d1, e1) while it is less clear for F_o (a1, b2, c10 + 1). Since the *N,N'*-dicyclohexylcarbodiimide (DCCD) receptor is located on the c subunit, treatment of strains with this uncoupler agent was used to reveal gentamicin uptake differences between JH2-2 and 1688-G3 (25). Mutations in the F_oF_1 -ATPase gene (*atp*) could potentially be responsible for gentamicin resistance in *E. faecalis* or a difference in their transcription level.

The aim of this study was to determine the mechanism of gentamicin resistance in *E. faecalis* JH2-2 mutants selected in vitro. Since the mutants with intermediate- and high-level gentamicin resistance were obtained in a stepwise manner, ac-

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TABLE 1. Organisms and susceptibilities

<i>E. faecalis</i> strain	Gentamicin MIC ($\mu\text{g/ml}$)		Value for mutant of JH2-2	
	Geometric	Arithmetic	Gentamicin concn for selection ^a ($\mu\text{g/ml}$)	Frequency of mutation
JH2-2	64	40		
1477-G1	128	80	80	1.7×10^{-5} – 2.6×10^{-6}
1573-G2	256	200	160	2×10^{-8} – 7×10^{-8}
1688-G3	512	400	400	3.3×10^{-8} – 1×10^{-9}

^a Selection on BHI agar.

quired resistance was likely to be due to mutations in chromosomal genes. Mutational resistance to gentamicin has not been studied in enterococci. We therefore screened for mechanisms described in other bacterial species and for mutations in known gentamicin targets, namely, ribosomal protein L6 and 16S rRNA. We sought for the presence of active gentamicin efflux and gentamicin-modifying enzymes. We also screened for the presence of mutations in the F_0F_1 -ATPase gene and their transcript level. Along with these studies, we developed a nonisotopic intracellular gentamicin assay to evaluate gentamicin uptake.

MATERIALS AND METHODS

Bacterial strains. *E. faecalis* JH2-2 is susceptible to amoxicillin and exhibits low-level resistance to gentamicin (MIC, 32 to 64 $\mu\text{g/ml}$) (20). Three successive step mutants resistant to gentamicin were selected in vitro on brain heart infusion (BHI) agar (Difco) containing a gentamicin concentration corresponding to twice the MIC of the parental strains, as described previously (1). The frequency of mutant selection was approximately 10^{-5} , 10^{-8} , and 10^{-9} during the first, second, and third steps, respectively. At each step, one colony was randomly selected (1477-G1, 1573-G2, and 1688-G3, respectively) (Table 1). The stability

of the mutants was tested by three successive passages on gentamicin-free BHI agar (1).

Escherichia coli strains DH5 α (pAM6306) (7), NC95 (48), and KHE5-2 (24) and *Enterococcus faecium* JH7 (9) were used as positive controls for amplification of genes encoding aminoglycoside-modifying enzymes (see below). BHI broth and agar were used in all experiments. All cultures were incubated at 37°C.

MIC determination. MICs were determined by dilution in BHI agar (40, 46). *E. faecalis* strains were grown overnight in BHI broth at 37°C. Plates were inoculated with a Steers replicator delivering approximately 10^4 to 10^5 CFU per spot (10 μl) and incubated at 37°C for 24 h. All assays were performed at least in duplicate.

For determination of gentamicin MICs, in addition to the geometric distribution of concentrations tested from 4 to 2,048 $\mu\text{g/ml}$, an arithmetic distribution was used by adding two intermediate concentrations between every usual value. The final concentrations were as follows: 16, 20, 24, 32, 40, 50, 64, 80, 100, 128, 160, 200, 256, 320, 400, 512, 700, 850, and 1,024 $\mu\text{g/ml}$. These dilutions were more sensitive in detecting moderately increased MICs.

To detect a putative active efflux, gentamicin MICs were also determined in the presence of the efflux pump inhibitors reserpine (Sigma, Issy les Moulineaux, France) and verapamil (Laboratoires Knoll, Levallois Perret, France) at final concentrations of 100 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$, respectively. The MICs of ethidium bromide (EtBr), known to be a substrate for efflux pumps, were also determined (22, 36). After dilution in sterile water, EtBr was used at final concentrations of 1 to 10 $\mu\text{g/ml}$.

Susceptibility to amoxicillin, erythromycin, tetracycline, ciprofloxacin, gentamicin, tobramycin, amikacin, kanamycin, streptomycin, spectinomycin, and paromomycin (Sigma) was also determined by agar dilution.

DNA cloning. Whole-cell DNA from two first-generation mutants and two second-generation mutants of strain JH2-2 digested by HindIII, BamHI, EcoRI, Sau3A, and PstI was cloned into pUC18 digested similarly. The ligation products were transformed into *E. coli* JM83 with selection on Trypticase soy agar containing either 100 $\mu\text{g/ml}$ of ampicillin or 4 $\mu\text{g/ml}$ or 8 $\mu\text{g/ml}$ of gentamicin plus ampicillin at 100 $\mu\text{g/ml}$. The plates were incubated for 72 h at 37°C. All experiments have been made at least in duplicate.

DNA amplification and sequencing. DNA was extracted using Pure Magna LC (Roche, Mannheim, Germany). Genes for aminoglycoside-modifying enzymes, ribosomal protein L6 (*rplF* gene), and F_0F_1 -ATPase (*atp*) and the four 16S rRNA genes (Efl16SA, Efl16SB, Efl16SC, and Efl16SD) were amplified in an I-Cycler (Bio-Rad, Marnes-la-Coquette, France) using the specific primers listed in Table 2 and those already described (7, 12, 24, 48). The PCR products were

TABLE 2. Nucleotide sequences of primers used for amplification and probes used for real-time PCR

Target or probe	Sequence	Position ^a	Direction ^b
Target			
<i>rplF</i>	5'-TCTAAACCAGGTTTACG-3'	205402	F
	5'-ACTCAGCAGTACCAGAG-3'	206354	R
Efl16SA	5'-AAGTTCTTGACATTCGAACGA-3'	248256	F
	5'-GGTGTCTCGGTTTGTGTTG-3'	250129	R
Efl16SB	5'-AGACGACAACGACCGAGCGG-3'	1017931	F
	5'-AAGGCCCCGAATTTATTTGA-3'	1019808	R
Efl16SC	5'-AAGGCCCCGAATTTATTTGA-3'	2771761	F
	5'-CAGACAACAACAACCAAGCAA-3'	2773637	R
Efl16SD	5'-GGTGTCTCGGTTTGTGTTG-3'	3168355	F
	5'-CAGACAACAACAACCAAGCAA-3'	3170274	R
Efl16S ^c	5'-ACGATCCGAAAACCTTCTTCAC-3'	2772962	R
	5'-TCGGCAATGGACGAAAAGTC-3'	2773023	F
<i>atp</i>	5'-CTGCCAACTCAAACAAGCCT-3'	2528854	F
	5'-GGTACGATAGCTTTGCCAGGG-3'	2530619	R
<i>atpE</i> ^c	5'-GACACCTAAAATTGGCACAGCTT-3'	2529599	R
	5'-TGTCTGGTCAATTAAGAACAACAATG-3'	2529669	F
<i>atpB</i> ^c	5'-TCGGCGGTTGGACTTTTC-3'	2530221	R
	5'-CTTGGGTCTCGTTACAAAATCG-3'	2530286	F
Probe ^c			
<i>atpE</i>	5'-ATTAAAGCGACCCCGATAA-3'	2529624	F
<i>atpB</i>	5'-AAACGCTGAGATCGTCGCCACA-3'	2530240	F
Efl16S	5'-CACGCGGCGTTGCTCGGTC-3'	2772995	F

^a Corresponding *E. faecalis* V583 position.

^b F, forward; R, reverse.

^c Used for real-time PCR.

electrophoresed in agarose gels (2%, wt/vol) containing EtBr (0.5 µg/ml), visualized under UV illumination, purified with the QIAquick kit (QIAGEN), quantified by agarose gel electrophoresis with known mass markers (Low DNA Mass Ladder; Boehringer GmbH, Germany), and sequenced with the primers described in Table 2 and an ABI Prism sequencing kit (Applied) according to the manufacturer's recommendations. The sequences were aligned using the BioEdit biological sequence editor 5.0.6 and compared with JH2-2 sequences.

RNA extraction and purification. RNA was extracted in triplicate from the strains 1688 and JH2-2. One colony of each strain was inoculated into 5 ml of Luria-Bertani broth and grown overnight at 37°C with shaking. Five hundred microliters of this overnight culture was inoculated into 200 ml of fresh broth and incubated under the same growth conditions. After 180 min of growth, a volume of culture corresponding to 2×10^8 bacteria was centrifuged for 5 min at $6,000 \times g$ at 4°C. The bacterial pellet was immediately suspended in 100 µl of TE lysis buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) containing 400 µg/ml lysozyme (Sigma-Aldrich, Saint Quentin Fallavier, France) and incubated for 3 min at room temperature. RNA was then extracted using the RNeasy minikit (QIAGEN, Courtaboeuf, France) plus a treatment with DNase following the manufacturer's recommendations. RNA was quantified at an optical density of 260 nm with a Gene Quant II spectrophotometer (Amersham-Pharmacia Biotech, Orsay, France).

Reverse transcription. Immediately after extraction, reverse transcription was performed on 500 ng RNA using the Taqman reverse transcription reagent kit (Applied) according to the manufacturer's recommendations.

Real time-PCR. The amounts of cDNA obtained by reverse transcription were quantified with the real-time fluorogenic 5' nuclease assay using an ABI Prism 7000 sequence detector (Applied). We determined the level of transcripts of the *atpB* and *atpE* genes and of the small-subunit rRNA gene. The probes and primers (Table 2) used to quantify their expression were designed using the Primer Express ABI Prism program (Applied). The probes were obtained from Applied and labeled 5' with the 6-carboxyfluorescein fluorescent dye as reporter and 3' with 6-carboxytetramethylrhodamine as quencher. Amplification was performed in a final volume of 20 µl in MicroAmp optical plates (Applied) as described previously (5). The amount of DNA in the sample was calculated by comparing it with the values obtained with standards comprising 10-fold dilutions of 1688 and JH2-2 DNA ranging from 50 ng to 0.00005 ng per ml. Negative controls, consisting of distilled water or total RNA, were included in each test to detect DNA contamination. Data were analyzed with Sequence Detector version 16 application software (Applied) on a personal computer linked directly to the ABI Prism 7000 Sequence Detection system, as recommended by the manufacturer. The mRNAs of *atpB* and *atpE* genes extracted from each strain were normalized on the basis of the small-subunit rRNA levels, which were determined in each of the real-time PCR experiments. The PCRs were repeated three times for each strain.

Preparation of ¹²⁵I-gentamicin. Gentamicin was iodinated by the chloramine T method, as described previously for gentamicin radioimmunoassays (35). Briefly, gentamicin sulfate (Sigma, Bonneuil sur Marne, France) at a concentration of 20 µg/100 µl in distilled water was added to 1 mCi of iodine-125 (Cisra). Then, chloramine T (500 µg in 50 µl of distilled water) was added and mixed for 2 minutes. The reaction was stopped by adding sodium pyrosulfite (Aldrich) at a concentration of 1 µg in 100 µl of distilled water. To remove excess radioactive iodine, the solution was filtered through a silver cation-exchange resin (On Guard II Ag IH; 2-2-ml cartridge; Dionex). Four hundred microliters (2×10^6 dpm) of ¹²⁵I-gentamicin was added to 40 µl of nonisotopic gentamicin at concentrations ranging from 32 to 128 µg/ml. The entire process was monitored by gamma counting (Cobra II, AutoGamma; Packard), and the specific activity of the iodinated gentamicin was 18 mCi/mg.

Gentamicin dosage with the FPIA method. The gentamicin concentration was measured by automated fluorescence immunopolarization (FPIA; AxSYM; Abbott, Rungis, France). The FPIA technique combines competitive antibody binding with fluorescence polarization (21, 42). The competing antigens are gentamicin contained in the test sample and fluorescein-labeled gentamicin provided with the assay kit, and the target is sheep antigentamicin antiserum. The assay technique, validated by the manufacturer for human plasma and serum, was found to offer similar performance when tested with bacterial lysates (see below). The detection limit was 0.3 mg/liter, and the coefficients of variation were below 7% at the different concentrations tested.

Gentamicin uptake studies with ¹²⁵I-gentamicin. One CFU of each *E. faecalis* strain was grown overnight in 10 ml of BHI broth to a density of approximately 10^9 CFU/ml. Aliquots of 500 µl of the overnight culture were transferred in five tubes containing 4.5 ml of BHI broth at 37°C to allow for exponential growth. The mixture of ¹²⁵I-gentamicin and nonisotopic gentamicin was then added at fixed concentrations ranging from 32 to 128 µg/ml. Gentamicin addition defined

the start of the kinetic study (T_0). Every 15 min for 1 hour, one aliquot was centrifuged for 10 min at 3,500 rpm at 10°C. The supernatant was discarded, and radioactivity in the pellet was counted.

Gentamicin uptake studies with the FPIA method. Large sample volumes were necessary to detect gentamicin with the nonisotopic method. One CFU of each strain was cultured overnight in 50 ml of BHI broth, and 5 ml was added to 45 ml of BHI broth to obtain at least 10^{10} to 10^{11} bacteria per tube. Gentamicin was then added at fixed concentrations ranging from 32 to 1,024 µg/ml (T_0 of the kinetic study). One tube was then collected every 15 min for 1 h and centrifuged at 3,500 rpm for 10 min. The pellet was washed with 5 ml of sterile water, centrifuged, resuspended in 200 µl of sterile water, and incubated at 95°C for 10 min to explode bacteria by heat shock. The concentration of intracellular gentamicin in the solution obtained was determined by the FPIA method.

At each time point of the kinetic studies, the bacteria were enumerated by being plated on BHI agar. The results were expressed as the amount of gentamicin per CFU (dpm/CFU or µg/CFU).

Kinetic studies with DCCD. DCCD (Sigma, Issy les Moulineaux, France) was used to increase the electrical potential of bacteria during kinetic studies (31). DCCD was dissolved in dichloromethane (Carlo Erba, La Chaussée en Vexin, France) in glass tubes and then diluted in sterile water at final concentrations ranging from 10 to 100 µg/ml. Kinetic studies were performed by the FPIA method.

RESULTS

Characteristics of in vitro mutants with acquired gentamicin resistance. *E. faecalis* JH2-2 yielded mutants with low (<128 µg/ml), intermediate (≤ 128 µg/ml to ≤ 500 µg/ml), and high (MIC, >500 µg/ml) gentamicin resistance, according to CLSI criteria. The gentamicin MICs for strains JH2-2, 1477-G1, 1573-G2, and 1688-G3 were 64, 128, 256, and 512 µg/ml in the geometric assay and 40, 80, 200, and 400 µg/ml in the arithmetic assay, respectively (Table 1). MICs of amikacin, kanamycin, tobramycin, streptomycin, netilmicin, paromomycin, and neomycin were increased for each generation of mutants, with MICs of >500 µg/ml for 1688-G3. All mutants remained susceptible to spectinomycin. When the MIC ratios between the mutant and JH2-2 were considered, the most affected aminoglycosides at each selection step were gentamicin and kanamycin. The MICs of amoxicillin, vancomycin, erythromycin, tetracycline, and ciprofloxacin remained unchanged. The growth rate of 1477-G1, 1573-G2, and 1688-G3 was not altered by acquired gentamicin resistance, compared with JH2-2.

Cloning of digested DNA. Gentamicin resistance from the four mutants could not be cloned into *E. coli* despite the use of several restriction enzymes in three independent experiments.

Sequence of the *rplF* gene. The *rplF* gene for ribosomal protein L6 was amplified, and sequence analysis of the PCR product did not show any mutation relative to JH2-2 in first- and third-generation mutants. No mutations in ribosomal proteins conferring gentamicin resistance have been reported to date in *E. faecalis*.

Search for genes encoding aminoglycoside-modifying enzymes. DNA corresponding to the *aph(2'')Ib*, *aph(2'')Ic*, *aph(2'')Id*, and *aac(6')-aph(2'')* genes for aminoglycoside-modifying enzymes known to confer gentamicin resistance in enterococci could not be amplified from total DNA of JH2-2, 1477-G1, 1573-G2, and 1688-G3.

Sequencing of the 16S rRNA genes. The four 16S rRNA genes (Ef16SA, Ef16SB, Ef16SC, and Ef16SD) of *E. faecalis* 1688-G3 and JH2-2 were amplified separately and sequenced. No mutations were found in the third-generation mutant.

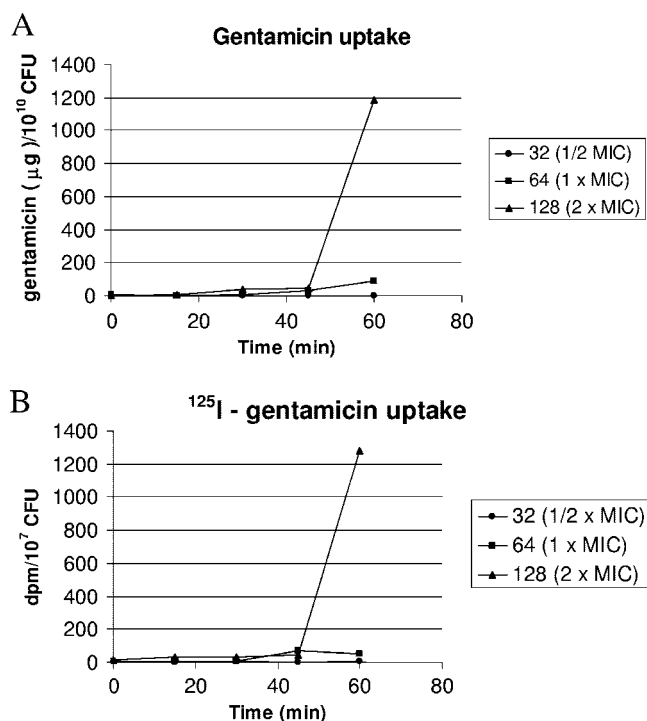


FIG. 1. Gentamicin uptake by *E. faecalis* JH2-2 at a concentration of 32 (circles), 64 (squares), and 128 (triangles) $\mu\text{g}/\text{ml}$ of gentamicin (A) and ^{125}I -gentamicin (B). The number of viable bacteria ranged from 7.5×10^9 to 1.65×10^{10} for gentamicin at 32 $\mu\text{g}/\text{ml}$, from 2×10^8 to 3.2×10^9 for gentamicin at 64 $\mu\text{g}/\text{ml}$, and from 9.5×10^9 to 1.55×10^7 for gentamicin at 128 $\mu\text{g}/\text{ml}$.

atp mutation and transcription. No mutations were retrieved in *atpB* and *atpE*, encoding the a and c F_0 subunits, respectively. Using real-time PCR, the transcript levels of *atpB* and *atpE* genes were not different between 1688 and JH2-2.

Efflux of gentamicin. To detect a putative active efflux in the mutants, the gentamicin MICs for strains JH2-2, 1477-G1, 1573-G2, and 1688-G3 were determined in the presence and in the absence of two efflux pump inhibitors (reserpine at 20 $\mu\text{g}/\text{ml}$ and verapamil at 100 $\mu\text{g}/\text{ml}$). No differences were observed with the two inhibitors.

Ethidium bromide is a substrate for efflux pumps in gram-negative bacteria and in *E. faecalis* (22, 30, 36). It was found to be toxic for strain JH2-2 at concentrations above 20 $\mu\text{g}/\text{ml}$. Plates containing EtBr concentration gradients from 0 to 10 $\mu\text{g}/\text{ml}$ in BHI agar were streaked with strains JH2-2, 1477-G1, 1573-G2, and 1688-G3 (30). No differences in growth of the strains could be noted after 24 or 48 h at 37°C.

The EtBr MICs for *E. faecalis* JH2-2 and its three derivatives were determined with and without verapamil and reserpine. The MICs ranged between 1.25 and 2.5 $\mu\text{g}/\text{ml}$ and were not affected by either inhibitor.

Gentamicin uptake by JH2-2. Gentamicin uptake was first measured by the isotopic method (Fig. 1). Accumulation curves with gentamicin concentrations of 32, 64, and 128 $\mu\text{g}/\text{ml}$ (0.5, 1, and 2 MICs, respectively) suggested an external gentamicin concentration-dependent uptake. When the external gentamicin concentration was at least equal to the MIC, rapid accumulation started. At the MIC and above, gentamicin ac-

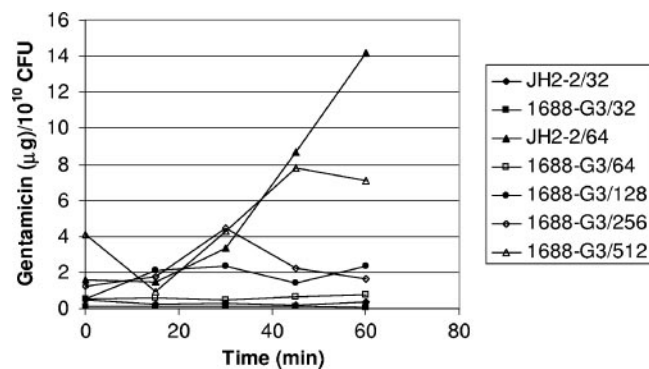


FIG. 2. Gentamicin uptake by *E. faecalis* JH2-2 and derivative *E. faecalis* 1688-G3 at various gentamicin concentrations (micrograms per milliliter) below the MIC of each strain.

cumulation in strain JH2-2 was slow during the first 30 min and then accelerated until the end of the first hour, resulting in a biphasic uptake (Fig. 1B). Intracellular gentamicin concentrations were 100-fold greater at twice the MIC than at the MIC. The detection limit of the method is the passive adsorption of ^{125}I on bacteria, up to 70% of total radioactivity detected. The detection limit was 50 dpm.

Gentamicin uptake was also studied with an adapted FPIA method (Fig. 1A). The features of gentamicin transport observed with the FPIA method were the same as those with the isotopic method, (i) the lag in accumulation and (ii) intracellular gentamicin concentrations. The threshold for gentamicin detection was 0.3 $\mu\text{g}/\text{ml}$, and the best correlation was obtained for gentamicin concentrations ranging between 0.3 and 10 $\mu\text{g}/\text{ml}$ by FPIA. All experiments allowed us to detect gentamicin concentrations in this range. However, the isotopic method was more sensitive and required an inoculum of only 10^7 CFU compared to at least 10^{10} CFU for the nonisotopic one.

Gentamicin uptake by *E. faecalis* JH2-2 and mutant 1688-G3. Gentamicin accumulation kinetics were characterized as dose-response curves in strain JH2-2 (MIC, 32 to 64 $\mu\text{g}/\text{ml}$) and its mutant 1688 (MIC, 512 $\mu\text{g}/\text{ml}$) (Fig. 2). Strain 1688-G3 thus appeared to be more impermeable to gentamicin than JH2-2. The concentration cutoff determining gentamicin entry in bacteria was the MIC of each strain. However, gentamicin accumulation in 1688-G3 was lower than that in JH2-2 at the respective MICs for the strains (Fig. 2). No killing of either strain occurred during the first hour when the gentamicin concentration was lower than or equal to the MIC. In contrast, when the external gentamicin concentration was above the MIC, JH2-2 numbers fell by 2 \log_{10} CFU/ml after the first hour.

Effect of DCCD on gentamicin uptake and on killing. The DCCD uncoupler was tested against strains JH2-2 and 1688-G3 (Fig. 3). At 80 μM , no effect on gentamicin uptake was found for either strain (data not shown). In contrast, 100 μM DCCD led to gentamicin accumulation in JH2-2 (2,500 μg per 10^{10} CFU compared to only 14 μg per 10^{10} CFU without DCCD). Addition of 100 μM DCCD caused resistant strain 1688-G3 to take up gentamicin at a rate comparable to that of susceptible JH2-2 in the absence of DCCD. Nevertheless, the intensity of the DCCD effect on gentamicin uptake differed

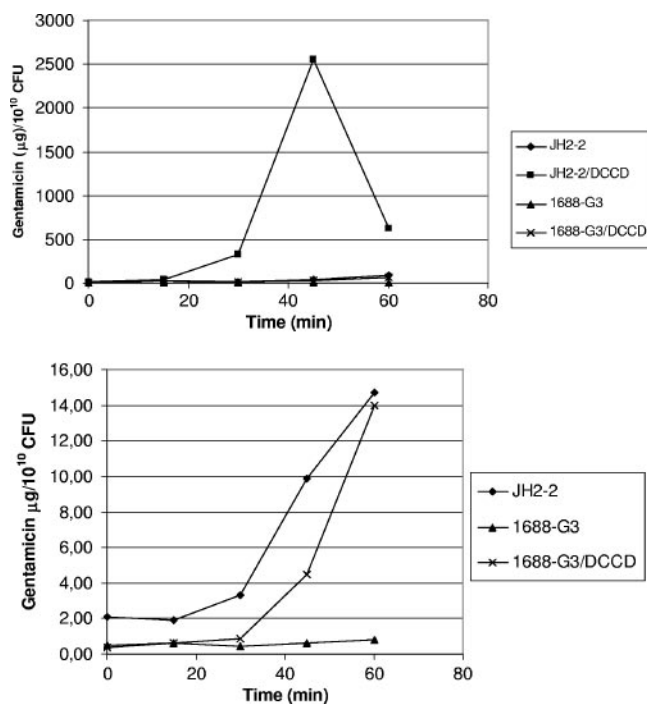


FIG. 3. Effect of 100 μM of DCCD on gentamicin uptake by *E. faecalis* JH2-2 (top) and mutant *E. faecalis* 1688-G3 (bottom) at a fixed gentamicin concentration of 64 $\mu\text{g/ml}$.

between the two strains. Gentamicin uptake increased 18-fold in the resistant mutant (from 0.8 to 14 $\mu\text{g}/10^{10}$ CFU in 1688-G3) while it increased 178-fold in JH2-2 (from 14 to 2,500 $\mu\text{g}/10^{10}$ CFU) when the strains were cultured at the same gentamicin concentration. Moreover, DCCD diminished the lag time of gentamicin penetration into JH2-2 by 15 min while it had no effect on 1688-G3. Killing was also enhanced by 100 μM DCCD: the CFU count fell from 1×10^9 CFU/ml to 3×10^7 CFU/ml for JH2-2 and from 2.5×10^9 to 1.65×10^8 CFU/ml for 1688-G3.

F₀F₁-ATPase. The portion of the *atp* operon that encodes F₀ subgroups a, b, and c was amplified. Sequence comparison of the genes in strains JH2-2 and 1688-G3 did not reveal any mutation.

DISCUSSION

Three generations of *E. faecalis* JH2-2 mutants (1477-G1, 1573-G2, and 1688-G3) with increased gentamicin resistance were selected in vitro. The mutants were resistant to all deoxystreptamines and to streptomycin. Since stepwise selection of the derivatives suggests that resistance was secondary to mutational events, we screened for mutations in the known gentamicin targets.

Low-level gentamicin resistance in *E. coli* can be due to mutations in ribosomal protein L6 (4, 26). The resistance phenotype of mutant 1477-G1 was similar to that of the one-step *E. coli* mutants obtained in vitro as it included tobramycin, kanamycin, streptomycin, neomycin, and kasugamycin. However, the search for a mutation in protein L6 of the third-step mutant 1688-G3 was unsuccessful. Mutations in 16S rRNA

confer resistance to streptomycin and deoxystreptamines, including gentamicin, in mycobacteria (34, 43). The 4,6-deoxystreptamine aminoglycosides bind to the A site of 16S rRNA (49), guanosine 1405, guanosine 1491, and cytosine 1496 of 16S rRNA being part of the gentamicin binding site (49). *E. faecalis* possesses four copies of the 16S rRNA gene. The third-generation mutant was obtained in three consecutive steps, and it is thus conceivable that the increasing level of resistance to gentamicin could be linked to accumulation of mutations in the rRNA portion of the target of the antibiotic. However, no mutations were found in the four copies of 16S rRNA of the mutant.

Cloning can be useful to identify aminoglycoside resistance due to drug or ribosomal modification, since there is no barrier to heterospecific expression of the corresponding genes (47). Enzymatic modification of aminoglycoside molecules is the most common resistance mechanism in enterococci, but multiple cloning attempts failed at detecting a resistance gene, arguing against this type of resistance. The cloning approach can also identify aminoglycoside resistance due to posttranscriptional modification of rRNA (13, 17, 18) and to efflux (3, 8). Methylation of G1405 of 16S rRNA is the means of protection used by bacteria that produce aminoglycosides (2, 18). However, the *E. faecalis* genome does not contain candidate genes for this function.

The first efflux pump was identified in *Enterococcus hirae*, and the substrate was EtBr (36). Efflux of chloramphenicol and norfloxacin was reported in *E. faecalis* and *E. faecium*, but the pumps were not identified (29). The first efflux pump identified in *E. faecalis* was EmeA, which shared homology with NorA of *Staphylococcus aureus* (22). In enterococci, 34 genes potentially code for multidrug resistance pumps (10). Efflux seems unlikely in our mutants because this mechanism involves resistance to multiple antibiotics and because experiments with EtBr and efflux inhibitors yielded negative results.

Gentamicin resistance in *E. faecalis* 1688-G3 appears to be due to defective antibiotic uptake since intrabacterial concentrations are lower in 1688-G3 than in JH2-2 when cultured in the same conditions. We designed a nonisotopic method to study gentamicin uptake. Intrabacterial gentamicin dosage with FPIA was compared with a ¹²⁵I isotopic method since ³H- and ¹⁴C-radiolabeled gentamicin are not available. ¹²⁵I is incorporated in gentamicin molecules at a 1/1 ratio, and incorporation does not modify the electrical load but increases molecular weight by one-third. The main limitation with ¹²⁵I-gentamicin is up to 70% passive adsorption on bacteria. The FPIA-derived method allowed detection of gentamicin in bacterial lysates, and the curves obtained were comparable to those obtained with ¹²⁵I-gentamicin. It is a simple and reproducible method allowing dosage of all aminoglycosides. The major limitation is the large size of the inoculum needed to detect gentamicin concentrations above 0.3 $\mu\text{g/ml}$.

The gentamicin accumulation kinetic is biphasic in *E. faecalis* as in *S. aureus*, whereas three phases have been described for streptomycin accumulation in *E. coli* (11, 38). To the best of our knowledge, gentamicin uptake kinetics in enterococci has not been described.

The DCCD uncoupler agent increases the magnitude of the $\Delta\Psi$ electrical potential in a concentration-dependent manner (11). In *S. aureus*, gentamicin uptake is enhanced by DCCD

(11, 14, 15, 31, 32), and this effect has not been described in other bacterial species. Miller et al. (37) described gentamicin uptake in several *S. aureus* mutants. DCCD had no effect on gentamicin uptake in *S. aureus* menadione auxotroph mutants or mutants defective in the respiratory chain (37). In contrast, DCCD allows enhancement of gentamicin accumulation in an *S. aureus* mutant obtained in the endocarditis model and the final intrabacterial concentration was similar to that obtained in the wild-type strain without DCCD (37). Unfortunately, the mechanism of resistance of the mutant was not studied. The similar effect of DCCD on gentamicin uptake in *S. aureus* nonrespiratory mutants and in *E. faecalis* 1688-G3 (Fig. 3) suggests that gentamicin permeability impairment is probably not restricted to the *Enterococcus* genus and that this mechanism of resistance can be easily selected in vivo.

The DCCD receptor is located on the c subunit of the F_0F_1 -ATPase (25). The genes for the eight subgroups of the F_0F_1 -ATPase are part of the *atp* operon and are cotranscribed (23). Regulation of expression of *atp* is not fully understood, and subunit combination is posttranscriptionally regulated. Poor affinity of DCCD for its target may explain its lower effect on gentamicin uptake by *E. faecalis* 1688-G3. We found no mutations in the genes encoding the F_0 portion and no difference in the level of transcription of the F_0 subunits, including the DCCD c receptor subunit. However, the impermeability of mutant 1688-G3 might be due to an inappropriate stoichiometric ratio of the F_0 subunits secondary to dysregulation of posttranscriptional combination of subunits (33, 45).

In conclusion, we have selected mutants expressing intermediate gentamicin resistance due primarily to uptake impairment, which, to the best of our knowledge, has not been described in enterococci. In addition, our study allowed development of a nonisotopic method for measuring intracellular gentamicin concentrations. The assay is simple and reproducible and can be extended to all antibiotics that are detected by the FPIA method (amikacin and tobramycin) and to other bacterial genera.

ACKNOWLEDGMENTS

We thank M. Arthur who contributed to initiating this study and T. Lambert and F. Depardieu for technical help.

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