ABSTRACT  Bacteria can survive antibiotic treatment both by acquiring antibiotic resistance genes and through mechanisms of tolerance that are based on phenotypic changes and the formation of metabolically inactive cells. Here, we report an Enterococcus faecalis strain (E. faecalis UM001B) that was isolated from a cystic fibrosis patient and had no increase in resistance but extremely high-level tolerance to ampicillin, vancomycin, and tetracycline. Specifically, the percentages of cells that survived 3.5-h antibiotic treatment (at 100 μg · ml⁻¹) were 25.4% for ampicillin and 4.3% and 51.9% ± 4.0% for vancomycin and tetracycline, respectively; vancomycin did not exhibit any significant killing. Consistent with the changes in antibiotic susceptibility, UM001B was found to have reduced penetration of ampicillin and vancomycin and accumulation of tetracycline compared to the reference strain ATCC 29212. Based on whole-genome sequencing, four amino acid substitutions were identified in one of the tetracycline efflux pump repressors (TetRs), compared to ATCC 29212. Results of molecular simulations and experimental assays revealed that these mutations could lead to higher levels of tetracycline efflux activity. Consistently, replicating these mutations in Escherichia coli MG1655 increased its tolerance to tetracycline. Overall, these findings provide new insights into the development of multidrug tolerance in E. faecalis, which can facilitate future studies to better control enterococcal infections.

IMPORTANCE Enterococcus faecalis represents a major group of pathogens causing nosocomial infections that are resistant to multiple classes of antibiotics. An important challenge associated with E. faecalis infection is the emergence of multidrug-tolerant strains, which have normal MICs but do not respond to antibiotic treatment. Here, we report a strain of E. faecalis that was isolated from a cystic fibrosis patient and demonstrated high-level tolerance to ampicillin, vancomycin, and tetracycline. Whole-genome sequencing revealed critical substitutions in one of the tetracycline efflux pump repressors that are consistent with the increased tolerance of E. faecalis UM001B to tetracycline. These findings provide new information about bacterial antibiotic tolerance and may help develop more effective therapeutics.

KEYWORDS Enterococcus faecalis, multidrug tolerance, antibiotic penetration, biofilm formation, whole-genome sequencing, tetR

Enterococcus faecalis is a Gram-positive bacterium that commonly inhabits the gastrointestinal tract of mammals (1, 2). When they enter a wound, bloodstream, or urinary tract, however, E. faecalis cells can cause serious infections (1, 3). According to
the National Healthcare Safety Network, *E. faecalis* is one of the top five most frequently isolated pathogens in adults with health care-associated infections (4). Recently, *E. faecalis* cells have also been identified in the lungs of patients with respiratory diseases, complicating the outcomes of antibiotic therapies (5–7). The threat of *E. faecalis* to human health is further emphasized by its intrinsic low-level susceptibility to most of the available antibiotics, making treatment challenging (1).

Bacteria can survive antibiotic treatment through both resistance and tolerance (8). Resistance is defined as the inherited ability of microorganisms to grow in the presence of an antibiotic even at high concentrations, as indicated by high MIC values (9). Enterococci could develop resistance to β-lactams through the production of low-affinity penicillin-binding proteins (PBPs) (10, 11), to lincosamides and trimethoprim-sulfamethoxazole due to efflux pumps encoded by the *lsa* genes (10–12), and to aminoglycosides as the result of reduced membrane permeability (10, 11, 13). Besides these intrinsic mechanisms, enterococcal strains are highly capable of horizontal gene transfer to acquire drug resistance genes (14, 15).

Unlike resistance, antibiotic tolerance does not change the MIC (9). Clinically, however, tolerance results in poor responses to antibiotic treatment, as resistance does (16, 17). Failing to clear these infections also provides an environment for the development of antibiotic resistance over time (8).

In addition to the mechanisms at the cellular level, bacteria can survive antibiotic attack by forming biofilms, which are slimy structures with cells embedded in an extracellular matrix (18, 19). Due to multiple mechanisms such as the protection by extracellular polymeric substrates and the slow growth of biofilm cells, mature biofilms are up to 1,000 times more tolerant to antimicrobial agents than are their planktonic counterparts (18–20). Clinically, *E. faecalis* is a prominent bacterium found in biofilms on infected vascular ports, urinary stones, and orthopedic devices and is also associated with bloodstream infections and enterococcal endodontic infections (21, 22).

Recently, we isolated an *E. faecalis* strain (*E. faecalis* UM001B, henceforth UM001B) from a 35-year-old male cystic fibrosis patient with a 6-year history of *Pseudomonas aeruginosa* infection. By the time of isolation, the patient had received 1 year of intensive antibiotic treatment that included tobramycin (Tob) and ciprofloxacin (Cip), at the State University of New York (SUNY) Upstate University Hospital, to control *P. aeruginosa*-mediated infections. The identification of an *E. faecalis* strain in addition to *P. aeruginosa* motivated us to study the antibiotic susceptibility of UM001B. UM001B demonstrated a record high-level tolerance to ampicillin (Amp), tetracycline (Tet), and vancomycin (Van). When challenged with these three antibiotics at extremely high concentrations, 17.9% ± 2.3% and 42.4% ± 12.9% of UM001B cells survived 3.5-h treatment with Amp (500 μg · ml⁻¹) and Tet (250 μg · ml⁻¹), respectively; Van did not show significant killing after 3.5-h treatment when the concentration was as high as 500 μg · ml⁻¹. This high-level multidrug tolerance further motivated us to investigate the underlying mechanisms. Our data indicate that UM001B has reduced penetration of Amp and Van and less accumulation of Tet in the cytoplasm, compared to the reference strain ATCC 29212. These changes could help explain the high-level multidrug tolerance of UM001B.

**RESULTS**

**UM001B demonstrates high-level multidrug tolerance.** We first investigated the susceptibility of UM001B to representative antibiotics, including Amp (β-lactam), Van, ofloxacin (Ofx) (fluoroquinolone), Tet, and Cip (fluoroquinolone) (Table 1). Among these agents, Amp, Ofx, Cip, and Tet are broad-spectrum antibiotics and Van is a potent antibiotic against Gram-positive bacteria specifically. Amp and Van are clinically used to treat *E. faecalis* infections (23, 24). Although Tet is no longer used to treat enterococcal infections, it is still used against some other microbial infections, such as upper respiratory tract infections caused by *Streptococcus pyogenes* (25). A wild-type (WT) *E. faecalis* strain (*E. faecalis* ATCC 29212, henceforth ATCC 29212) and a laboratory strain (*Bacillus subtilis* 168) were used as controls. Strain ATCC 29212 was chosen because the
Clinical and Laboratory Standards Institute recommended it for quality control and reference due to its close relevance to UM001B, based on the 16S rRNA gene sequence (see Fig. S1 in the supplemental material). Strain B. subtilis 168 was included as a common Gram-positive laboratory strain. The Cip MIC for UM001B (90 μg · ml⁻¹) is higher than those for B. subtilis 168 (0.05 μg · ml⁻¹) and ATCC 29212 (0.5 μg · ml⁻¹).

According to the threshold of resistance (8 μg · ml⁻¹) defined by Cotter and Adley (26), UM001B is resistant to Cip. Unlike that for Cip, the Amp, Tet, Van, and Ofx MICs for UM001B are all lower than or comparable to those of B. subtilis 168 and ATCC 29212 (Table 1), suggesting that UM001B is not resistant to these antibiotics.

Although UM001B is not resistant to Amp, Tet, Van, and Ofx, it is highly tolerant to these antibiotics, as indicated by high minimum bactericidal concentration (MBC)/MIC ratios (>250, >32, >250, and >133, respectively) (Table 1), which have been used to define tolerance (27). MBC is defined here as the lowest concentration that can achieve >99.9% (3-log₁₀ unit) reduction in CFU per milliliter, as described previously (27). Among the antibiotics tested, the highest tolerance was observed for Van (Fig. 1A); e.g., no significant killing of UM001B was observed even at a high concentration of 500 μg · ml⁻¹. In comparison, this condition (500 μg · ml⁻¹ Van) killed ATCC 29212 and B. subtilis 168 by 93.3% ± 2.2% and 98.0% ± 0.5%, respectively. The tolerance of UM001B to Amp and Tet is also much higher than that of B. subtilis 168 and ATCC 29212. The percentages of the clinical isolate (UM001B) that survived Amp (500 μg · ml⁻¹) and Tet (250 μg · ml⁻¹) treatment were 27.1 and 14,262.5 times higher than those of B. subtilis 168 and 48.1 and 8.2 times higher than those of ATCC 29212, respectively (P < 0.005, two-way analysis of variance [ANOVA] adjusted by Tukey’s test [n ≥ 3]) (Fig. 1). Such high-level multidrug tolerance is remarkable and deserves further study.

**UM001B has lower membrane permeability to antibiotics than does ATCC 29212.** Amp and Van kill bacteria by inhibiting cell wall synthesis (28, 29), while Tet stops protein synthesis (30). Although these three antibiotics have different mechanisms of action, their targets (enzyme transpeptidase for Amp, d-alanyl-d-alanine terminus of pentapeptide precursors for Van, and 30S ribosomal subunit for Tet) are synthesized intracellularly in Gram-positive bacteria (28–30), where internalized antibiotics can target. Therefore, to understand why UM001B has high-level multidrug tolerance, we compared the penetration of these three antibiotics into the cytoplasm of UM001B and ATCC 29212. The concentration of an antibiotic in the cytoplasm of treated cells was quantified by lysing the cells with chloroform and testing the killing effects of released antibiotics using appropriate reporter strains (Escherichia coli MG1655 for Amp and Tet and B. subtilis 168 for Van). A standard curve was established for each antibiotic by testing the reporter strain with a cell lysate of the untreated E. faecalis strain spiked with known amounts of antibiotics (Fig. S2 and S3). By fitting the killing results with the standard curve, the amount of antibiotic in the cytoplasm of E. faecalis was quantified. We developed this method to quantify antibiotic penetration instead of using the traditional analytical method, liquid chromatography with tandem mass spectroscopy (LC-MS), due to the limitations of LC-MS in detecting antibiotics such as Amp observed in this study (data not shown) and previously reported by Richter et al. (31). We validated this new method using antibiotics that can be detected by LC-MS, e.g., the penetration of minocycline (an antibiotic that belongs to the Tet

**TABLE 1** MICs and MBCs for E. faecalis UM001B, E. faecalis ATCC 29212, and B. subtilis 168

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC or MBC (μg · ml⁻¹) for:</th>
<th>Van</th>
<th>Amp</th>
<th>Tet</th>
<th>Ofx</th>
<th>Cip</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
<td>MBC</td>
</tr>
<tr>
<td>B. subtilis 168</td>
<td>2</td>
<td>10</td>
<td>50</td>
<td>100</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>E. faecalis ATCC 29212</td>
<td>1</td>
<td>10</td>
<td>4</td>
<td>10</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td>E. faecalis UM001B</td>
<td>2</td>
<td>&gt;500</td>
<td>2</td>
<td>&gt;500</td>
<td>8</td>
<td>&gt;250</td>
</tr>
</tbody>
</table>

aResistant because MICs higher than 8 μg · ml⁻¹ are defined as clinically resistant for Cip (26).

E. faecalis
family) into *E. coli* (data not shown). In this study, to evaluate antibiotic penetration, we incubated *E. faecalis* cells with an antibiotic for 15 min and then quantified the cytoplasmic antibiotic concentration. To evaluate antibiotic accumulation, similar experiments were conducted with 1-h incubation. These two time points (15 min and 1 h) were chosen because it is documented that antibiotic penetration takes around 15 min to reach equilibrium across bacterial membranes, after which bacterial activities such as efflux pumps start to affect antibiotic concentration in the cytoplasm and the final amount of accumulation (31). While the concentrations of Amp and Van were found to be 76.5 ± 9.6 and 43.5 ± 6.3 μg·mL⁻¹, respectively, in ATCC 29212 cells after a 15-min incubation with the antibiotic (100 μg·mL⁻¹), no detectable level of either antibiotic was found in UM001B cells after the same treatment time (Fig. 2A and B). This indicates that these two antibiotics were unable to penetrate, which is consistent with the high-level tolerance of UM001B to these two antibiotics (Fig. 1A and C). The penetration of Tet did not differ between ATCC 29212 and UM001B after 15 min of incubation (P = 0.8459, one-way ANOVA adjusted by Tukey’s test for the rest of the statistical analyses in this study unless indicated otherwise [n = 3]) (Fig. 2C); however, more (2.9 times) Tet accumulated in ATCC 29212 cells than in UM001B cells after 1-h incubation (20.8 ± 4.8 versus 5.9 ± 3.9 μg·mL⁻¹; P = 0.0259 [n ≥ 3]) (Fig. 2C). This result indicates that other mechanisms, such as efflux, are involved in the tolerance of UM001B to Tet (Fig. 1B).

**Mutations in a tetR gene increase Tet tolerance.** To reveal the mechanisms behind the high-level multidrug tolerance of UM001B, we conducted whole-genome sequencing (WGS) of UM001B and compared the results with those for the reference strain ATCC 29212. The results revealed one gap (confirmed using PCR with the primers listed in Table 2) and
3,044 variations leading to changes in amino acids, including single-nucleotide polymorphisms (SNPs), insertion-deletions (indels), multiple-nucleotide polymorphisms (MNPs), and microindels (Fig. S4). Most of the changes were associated with unidentified/hypothetical proteins (646 variations). Among annotated variations, we identified 18 variations in 10 genes related to antibiotic tolerance, 252 variations in 55 genes related to cell wall/membrane synthesis, 74 variations in 45 genes related to membrane permeability, and 44 variations in 5 genes related to biofilm formation (Fig. 3). Some of these variations may contribute to the differences in antibiotic susceptibility between UM001B and ATCC 29212 observed in this study.

![FIG 2](image-url) Penetration and accumulation of Amp (A), Van (B), and Tet (C) into exponential-phase planktonic UM001B or ATCC 29212 cells grown in LB medium. Antibiotics in the cytoplasm of E. faecalis cells were harvested after lysis of the cells with chloroform. Antibiotic concentrations after 15 min or 1 h of incubation were determined using a reporter strain, by fitting the killing results to the corresponding standard curve (Fig. S2 and S3). Each condition was tested with at least three biological replicates ($n \geq 3$).

### TABLE 2

<table>
<thead>
<tr>
<th>Molecule</th>
<th>DNA sequence (5' to 3')</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer for 16S rRNA gene amplification</td>
<td>ATTCTAGAGTTTGATCATGGCTCA</td>
<td>71</td>
</tr>
<tr>
<td>Reverse primer for 16S rRNA gene amplification</td>
<td>ATGGTACCGTGACGGGCGGTGTGA</td>
<td>71</td>
</tr>
<tr>
<td>Forward primer for gene encoding LPxTG cell wall anchor domain protein</td>
<td>AGACAGCAAAGGAAGGACGG</td>
<td>This study</td>
</tr>
<tr>
<td>Reverse primer for gene encoding LPxTG cell wall anchor domain protein</td>
<td>AATCGTTGTAGCACCACAGGG</td>
<td>This study</td>
</tr>
<tr>
<td>Paired-end sequencing adaptor 1</td>
<td>AGATCGGAAGAGCGTGTTGCTGAGGGAAGAGGAGTGTGA</td>
<td>Omega Bioservices</td>
</tr>
<tr>
<td>Paired-end sequencing adaptor 2</td>
<td>AGATCGGAAGAGCGTGTTGCTGAGGGAAGAGGAGTGTGA</td>
<td>Omega Bioservices</td>
</tr>
<tr>
<td>Forward primer for tetR amplification</td>
<td>TTTTATGGAAGATGTTGTGA</td>
<td>This study</td>
</tr>
<tr>
<td>Reverse primer for tetR amplification</td>
<td>ATGGAACCAAAATTATCAAA</td>
<td>This study</td>
</tr>
</tbody>
</table>
Among the variations, 12 MNPs in one of the nine genes encoding Tet repressor (TetR) family proteins caught our attention since UM001B has high-level Tet tolerance. We were particularly interested in the tetR gene because it is the only gene that harbors more than 10 variations, leading to changes in four amino acids (Ser156Leu, Glu180Gly, His192Arg, and Asp214Tyr) (Fig. S5). TetR family proteins regulate the expression of efflux pumps, such as TetA (32, 33) and EfrCD (34). The TetR homodimer blocks the promoter of efflux pump genes by binding to the repetitive palindromic sequences in the upstream intergenic region using helixes $\alpha 9$ and $\alpha 10$ (32, 33). In the presence of Tet, the TetR homodimer interacts with Tet along with magnesium to form a protein-ligand complex (Fig. 4A). This interaction leads to a conformational change in the Tet-ligand complex and thus the release of TetR homodimer from the promoter of efflux pump genes. This detachment activates expression of the Tet efflux pump, extruding Tet from bacterial cells. The four changed amino acids are located in the $\alpha 9$ and $\alpha 10$ helixes of the C-terminal domain of TetR in UM001B (Fig. S5). Interestingly, these four substitutions are unique in UM001B, since they are not found in any of the other 14 E. faecalis isolates listed in the National Center for Biotechnology Information (NCBI) database (Fig. S5).

To understand the role of these mutations in the Tet tolerance of UM001B, we calculated the root mean square deviation (RMSD) between Tet and the UM001B TetR homodimer and compared it with that of ATCC 29212 using the GROMACS molecular dynamics (MD) package (version 2016.4) (Fig. 4B) (35). The RMSD reveals the conformational changes due to the binding between Tet and the TetR homodimer (36). A larger RMSD value means that the ligand-protein binding can cause larger fluctuations upon binding and is more prone to dissociation. The RMSD for Tet and the UM001B TetR homodimer is significantly larger than those for the controls, and the four amino acid substitutions in UM001B TetR were found to cause the TetR-ligand (Tet) interaction to be more prone to conformational changes, compared to that in the reference strain ATCC 29212 (Fig. 4B and Table 3). This conformational change could result in greater
expression of the Tet efflux pumps, which helps explain the decrease in Tet accumulation and the high-level tolerance of UM001B (Fig. 1B).

To further verify whether these mutations increased Tet tolerance, we introduced the tetR gene of UM001B into *E. coli* MG1655 using the plasmid pGEM-T (Fig. 4C). We chose *E. coli* MG1655 because it has a TetR-TetA regulatory system homologous to that in UM001B (according to the SWISS-MODEL analysis) (37). The *E. coli* MG1655 strain with the plasmid pGEM-T carrying tetR from UM001B showed 22.3 and 13.3 times higher Tet tolerance, compared to the empty vector control and the strain with the tetR gene from

**TABLE 3** System details for CAGE simulations

<table>
<thead>
<tr>
<th>System</th>
<th>No. in simulation of:</th>
<th>Tet</th>
<th>Water</th>
<th>Counter ions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT with Tet</td>
<td>1</td>
<td>2</td>
<td>14,500</td>
<td>17</td>
</tr>
<tr>
<td>WT without Tet</td>
<td>1</td>
<td>0</td>
<td>14,500</td>
<td>17</td>
</tr>
<tr>
<td>CI with Tet</td>
<td>1</td>
<td>2</td>
<td>14,500</td>
<td>11</td>
</tr>
<tr>
<td>CI without Tet</td>
<td>1</td>
<td>0</td>
<td>14,500</td>
<td>11</td>
</tr>
</tbody>
</table>

CI, clinical isolate UM001B.
ATCC 29212, respectively. This verifies that the variations in the tetR gene do contribute to Tet tolerance.

**UM001B biofilms are less susceptible to Van treatment, compared to ATCC 29212.** Since bacterial biofilms play an important role in chronic infections and antibiotic tolerance (18–22), we compared the biofilm formation of UM001B with that of ATCC 29212. To better mimic the host environment, we coated the substrate glass surfaces with mucin and cultured the biofilms in brain heart infusion (BHI) medium (Fig. 5). UM001B and ATCC 29212 did not exhibit significant differences in biofilm formation at 24 h after inoculation ($P = 0.8$ for biomass [n = 3]) (Fig. 5). However, UM001B biofilms are more tolerant to Van treatment ($200 \mu g \cdot m l^{-1}$) than are those of ATCC 29212. Specifically, 3.5-h treatment with Van did not show significant effects on 24-h biofilms of UM001B ($P = 0.56$ for biomass [n = 3]) (Fig. 5). In comparison, the same treatment reduced the biomass of ATCC 29212 biofilms by 76% ($1.4 \pm 0.3$ versus $0.3 \pm 0.05 \mu m^3$, before versus after treatment; $P = 0.000004$ [n = 3]) (Fig. 5).

**DISCUSSION**

The emergence of multidrug-resistant enterococci has been on the rise globally (38, 39). Such infections are difficult to treat and are associated with high morbidity and mortality rates, putting a heavy burden on the health care system (10, 39). *E. faecalis* is the primary cause of enterococcal infections and is among the most commonly occurring antimicrobial-resistant nosocomial pathogens (39).

UM001B is highly tolerant, but not resistant, to Amp, Van, and Tet. According to nucleotide Basic Local Alignment Search Tool (BLASTn) analysis, currently reported Amp and Van resistance mechanisms such as PBPs, $\beta$-lactamase, and Van resistance clusters on mobile plasmids (10, 17, 40) do not exist in UM001B (see Table S1 in the supplemental material). Although SNPs were identified in other *pbp*-associated genes encoding the PBP transpeptidase domain (A349S) and PBP1A family (T412A), compared to those in ATCC 29212, they showed no impact on protein structure, protein pocket structure, and binding affinity for Amp or Van, based on molecular docking (Table S2). Besides the known resistance mechanisms, a broad search using BLASTn with the database of resistance determinants using ResFinder version 3.2 also did not identify any mutations or newly acquired resistance determinants in UM001B. Together, these results support our finding that UM001B has high-level tolerance but not resistance to these antibiotics.

Unlike ATCC 29212, Amp and Van barely reached the cytoplasm of UM001B cells.
after 15 min of antibiotic treatment. Although the targets of both Amp and Van (transpeptidase and d-alanyl-d-alanine terminus, respectively) are in the peptidoglycan of *E. faecalis*, the synthesis of targets takes place in the cytoplasm, and the reduced penetration of Amp and Van could have contributed to the high-level tolerance of UM001B to these two antibiotics, compared to ATCC 29212. Using WGS, a large number of variations between the genomes of UM001B and ATCC 29212 were identified, many of which were in the groups of transporter proteins, including ABC transporters, multidrug transporters, and membrane proteins involved in the transportation of metabolites such as nutrients and ions. The former ones may be associated with antibiotic tolerance, while the latter ones may cause differences in growth, as observed in this study. The growth of UM001B is slower than that of ATCC 29212 in both lysogeny broth (LB) and BHI medium (data not shown).

Regarding the Tet tolerance of UM001B, currently reported Tet resistance mechanisms such as the ribosome protection protein TetM (41) and efflux pump protein TetL (42) do not exist in UM001B (Table S1). This is consistent with the Tet tolerance, instead of resistance, of UM001B. Variations in membrane proteins did not affect drug penetration (Fig. 2C and 3). Instead, 12 variations in one of the genes encoding TetR family proteins may be contributors. TetR is well documented to affect Tet tolerance by controlling the expression of the Tet efflux pump genes, such as *tetA* (32, 33) and *efrCD* (34). In the absence of Tet, TetRs form dimers and inhibit the transcription of efflux pump genes by blocking the shared promoter with its N-terminal domains. During Tet treatment, Tet directly interacts with the C-terminal domains, triggers a conformational change in the α1 to α3 helices involved in binding to the target promoter region, and frees the promoter for transcription of efflux pump genes, leading to the activation of efflux pumps that extrude Tet from the bacterial cytoplasm (33, 43). The increase in binding of Tet to TetR homodimers in UM001B at the α9 position due to the variations may promote the breakage of the TetR-promoter complex and the activation of Tet efflux pumps, leading to reduced accumulation of Tet in UM001B cells, compared to that in ATCC 29212 cells. Since the target of Tet, the 30S ribosome, is in the bacterial cytoplasm, this helps explain the increase in Tet tolerance observed in UM001B (Fig. 1B).

The growth of UM001B is slower than that of ATCC 29212 in both LB and BHI media, possibly due to the substitutions in membrane proteins for the transport of metabolites (Fig. 3). However, UM001B and ATCC 29212 formed similar biofilms on mucin-coated glass substrates after 24 h of biofilm growth in BHI medium, a nutrient-rich medium with host factors such as gelatin and hemoglobin (44). Compared to the genome of ATCC 29212, a major deletion (amino acids 1 to 388) was identified in a LysM domain that is conserved in major autolysins (Table S3). Autolysin is essential for programmed cell death and subsequent extracellular DNA release in the biofilm matrix, contributing to biofilm structural stability (45). This could have contributed to the different patterns of dead cells and the three-dimensional (3D) structure of UM001B colonies, compared to ATCC 29212 colonies, on LB agar plates (Fig. S6). LB agar plates were used here to mimic the Young’s modulus of lung tissues (~10 versus 1 to 5 kPa) (46, 47).

In conclusion, we isolated an *E. faecalis* strain, UM001B, from a cystic fibrosis patient that demonstrated resistance to Cip. For Amp, Ofx, Van, and Tet, UM001B has normal MICs but remarkably high-level tolerance. This clinical isolate demonstrated a capability for biofilm formation on mucin-coated surfaces and high-level tolerance to Van similar to those of the reference strain ATCC 29212. The tolerance of UM001B to Amp and Van was attributed to the decrease in antibiotic penetration, which is consistent with the variations in genes encoding membrane proteins and transporters. The changes in one of the TetR proteins and their roles in Tet tolerance were corroborated by GROMACS simulation and biological assays (by cloning the gene tetR into an *E. coli* strain). Further studies on the gene expression in UM001B using methods such as whole-transcriptome analysis with total RNA sequencing and quantitative PCR will help better understand tolerance/resistance to the antibiotics described in this paper and develop more effective controls.
MATERIALS AND METHODS

Bacterial strains and media. E. faecalis UM001B was isolated at SUNY Upstate University Hospital from a sputum sample from a cystic fibrosis patient (SUNY Upstate University Hospital Institutional Review Board protocol number 405389). Briefly, E. faecalis UM001B was isolated from a sputum sample from the patient following a standard isolation protocol developed by the Microbiology Laboratories at SUNY Upstate University Hospital with guidance from previously published protocols (48–50). During the isolation, blood agar (5% sheep blood), manniitol salt agar, chocolate agar, MacConkey agar, and Burkholderia cepacia selective agar plates (Remel Inc., San Diego, CA, USA) were used to culture microorganisms.

E. faecalis UM001B, E. faecalis ATCC 29212, E. coli MG1655, and B. subtilis 168 were routinely grown in LB medium containing 10 g · liter⁻¹ tryptone, 10 g · liter⁻¹ sodium chloride, and 5 g · liter⁻¹ yeast extract (S1). To study the biofilms in the presence of host factors, ATCC 29212 and UM001B were also grown in BHI medium (37 g · liter⁻¹ BHI). E. coli MG1655 and B. subtilis 168 were used as reporter strains to quantify intracellular antibiotic concentrations in this study.

Antibiotic treatment. UM001B, ATCC 29212, and B. subtilis 168 were tested for their Cip, Amp, Ofx, Van, and Tet MICs. Briefly, an overnight culture of each strain was used to inoculate LB medium with added antibiotic at different concentrations to an optical density at 600 nm (OD₆₀₀) of 0.01 in a 96-well plate. The OD₆₀₀ of the bacterial suspension was measured using a microplate reader (Epoch 2; BioTek, Winooski, VT, USA) after overnight incubation at 37°C, with shaking at 200 rpm. The MIC was defined as the concentration that yielded >90% inhibition of the OD₆₀₀ after overnight growth, compared to the untreated control.

To evaluate antibiotic tolerance, UM001B, ATCC 29212, and the reporter strains (E. coli MG1655 for Amp and Tet and B. subtilis 168 for Van) were treated with corresponding antibiotics, and the numbers of CFU were determined before and after treatment. For Cip and Ofx, stationary-phase UM001B, ATCC 29212, and P. aeruginosa PA01 cells were washed three times with phosphate-buffered saline (PBS) and resuspended in PBS to an OD₆₀₀ of 0.5. Bacterial cells were then incubated with various concentrations of each antibiotic (0, 0.01, 0.05, 0.1, 0.5, 1, 10, 50, 100, 200, 500, or 800 μg · ml⁻¹) for 3.5 h (Cip) or 8 h (Ofx) at 37°C, with shaking at 200 rpm. For Amp, Van, or Tet, exponential-phase cells (OD₆₀₀ of 0.2 to 0.4) of ATCC 29212, UM001B, B. subtilis 168 (for Van), or E. coli MG1655 (for Amp or Tet) were washed three times with PBS and resuspended in LB medium to an OD₆₀₀ of 0.5. The cells were then treated with various concentrations of Amp (0, 10, 100, or 500 μg · ml⁻¹), Van (0, 1, 5, 10, 50, 100, 200, or 500 μg · ml⁻¹), or Tet (0, 0.5, 1, 5, 15, 20, 25, 50, 100, 150, or 250 μg · ml⁻¹) for 3.5 h in LB medium at 37°C, with shaking at 200 rpm. After antibiotic treatment, cells were washed three times with PBS and plated to count CFU using the drop plate method (S2). Tet, Amp, and Van were tested in LB medium rather than PBS because these antibiotics are not effective against nongrowing cells (S3).

Antibiotic penetration and accumulation. To evaluate antibiotic penetration and accumulation, stationary- or exponential-phase cells of E. faecalis strains (5 ml of cultures with an OD₆₀₀ of 0.5) were washed three times with PBS and then treated with different antibiotics (100 μg · ml⁻¹ Amp or Van or 150 μg · ml⁻¹ Tet) for 15 min or 1 h. Cells were then washed once with PBS and resuspended in 50 μl PBS. We washed cells only once for penetration and accumulation, instead of three times as described above for antibiotic tolerance testing, to prevent the antibiotics in E. faecalis cells from diffusing out. E. faecalis cells that went through the same process but were treated without antibiotics were used as controls. Chloroform (500 μl) was used to lyse the cells and to release intracellular antibiotics. After centrifugation at 2,000 × g for 4.5 min, cell debris was removed, and the lysates were evaporated in a vacuum desiccator overnight. After evaporation, 50 μl PBS was added to dissolve E. faecalis lysates and antibiotics by vortex-mixing for 5 min. A corresponding reporter strain (B. subtilis 168 for Van and E. coli MG1655 for Amp and Tet) was used to quantify the concentration of antibiotic in each sample. Specifically, reporter strains were cultured to early exponential phase with an OD₆₀₀ of 0.2 to 0.5. The cell density was adjusted to an OD₆₀₀ of 0.5 to establish a standard curve for each antibiotic and to quantify the concentration in unknown samples. To establish the standard curve, the reporter strain was treated with the lysates of E. faecalis cells spiked with a known amount of antibiotic for 1 h. Using the standard curves (see Fig. S2 and S3 in the supplemental material), the amount of antibiotic extracted from the cytoplasm of E. faecalis cells can be calculated with equation 1,

\[
p = a \cdot e^{b \cdot c}
\]

in which p is the percentage of survival, a is the slope of the killing curve, b is the killing constant, and c is the concentration of antibiotic extracted from the cytoplasm of 5 ml E. faecalis cells (OD₆₀₀ of 0.5).

The intracellular antibiotic concentration was further calculated by normalizing c with the number and average volume of E. faecalis cells. The average volume of the E. faecalis cells used in this study was found to be 0.13 μm³, based on microscopic images.

Biofilm formation and Van treatment. To test biofilm formation on surfaces with a Young’s modulus similar to that of lung tissues, we grew UM001B and ATCC 29212 biofilms on LB agar plates. The LB agar plates were prepared by adding 15 g · liter⁻¹ agar to the LB medium prepared as described above. Bacterial suspensions (100 μl each, with an OD₆₀₀ of 0.01) were evenly plated onto agar plates and incubated for 6 h. Then, individual colonies were labeled with the Live/Dead BacLight bacterial viability kit (Life Technologies Inc., Carlsbad, CA, USA), and 3D images were taken using an Axio Imager M1 fluorescence microscope (Carl Zeiss Inc., Berlin, Germany), with 1-μm intervals in the z direction. To investigate biofilm formation on mucin (a host factor)-coated surfaces, we prepared mucin-coated glass surfaces by following the protocol described previously by Landry et al. (S4), with slight modifications. Briefly, glass slides (7.0 mm in width, 10.0 mm in length, and 1 mm in thickness) were first
Antibiotic Tolerance of Enterococcus faecalis

cleaned by soaking in piranha solution (a mixture of 3% 

and H2O2 at 7:3 [vol/vol]) at 80°C for 10 min. These 

slides were then further cleaned at room temperature in 2 M HCl solution for 5 min, followed by 

washing with Millipore water and drying with nitrogen. Once the glass slides were dried, their surfaces 

were primed with a 4% [vol/vol] solution of (3-aminopropyl)trimethoxysilane (APMTS) (Sigma-Aldrich, St. 

Louis, MO, USA) in dry acetone. This process was conducted at 50°C for 12 h. After sequential washes with 

acetone and Millipore water, glass slides were soaked in a 1% [vol/vol] solution of electron microscopy-

grade glutaraldehyde for 1 h. Then, the surfaces were coated with mucin by soaking in 100 µg · ml⁻¹ 

mucin from porcine stomach (Sigma-Aldrich) for at least 3 h. Glass slides were washed three times with 

PBS solution before biofilm inoculation in BHI medium. The biofilms were cultured in 20 ml BHI medium 

for 24 h without shaking, and inoculated with a starting OD600 of 0.05 using an overnight culture. 

For biofilms formed on mucin-coated surfaces, we also compared their susceptibility to Van (200 µg · 

ml⁻¹) in PBS. After growth, slides with biofilms were gently washed three times with PBS. For control, 

biofilms were incubated in PBS without Van for 3.5 h at 37°C without shaking. For Van treatment, biofilms 

on glass slides were incubated in PBS with Van (200 µg · ml⁻¹) for 3.5 h at 37°C. After gentle washing, 

biofilms (both controls and treated) were stained using the Live/Dead BacLight bacterial viability kit (Life 

Technologies Inc.) in 3 ml PBS solution supplemented with 4.5 µl of SYTO 9 and propidium iodide. 

Fluorescence images of biofilms (3D) were taken using an Axio Imager M1 fluorescence microscope (Carl 

Zeiss Inc.) with 1-µm intervals in the z direction and analyzed using COMSTAT (55).

WGS and data analysis. The identity of UM001B was determined by sequencing its 16S rRNA using 

the primers listed in Table 2 and the cloning vector pGEM-T vector system (Promega, Madison, WI). The 

cloned vector was sequenced at the Institute of Biotechnology, Cornell University (Ithaca, NY). The 16S 

rRNA gene sequence of this isolate showed 100% query alignment and 99.7% identity with that of E. 

faecalis, based on BLASTn analysis using the NCBI database.

To sequence the whole genome of UM001B, chromosomal DNA from two biological replicates was 

isolated by following the protocol described previously (56). DNA was dried in a vacuum desiccator and 

analyzed with next-generation sequencing (Omega Bioservices, Norcross, GA). CLC Genomics Workbench 

(Qiagen Bioinformatics, Hilden, German) was used to analyze the sequence by following the workflow 

described in Fig. S7, with 200× coverage. First, paired-end raw data were trimmed to remove the 

adaptors before assembly (Fig. 57A). After quality control, the genome of UM001B was assembled using 

both reference-guided de novo assembly and assembly-guided assembly with the genome of ATCC 

29212 as the reference (57–59). The whole-genome sequence was first run against the public database 

using PubMed to confirm that the multilocus sequence typing (MLST) results for UM001B indicated E. 

faecalis. The relationship between the genomes of UM001B and ATCC 29212 was corroborated using a 

phylogenetic tree (16S rRNA gene sequence-based comparison) (Fig. S1) and BRIG (whole-genome 

sequence-based comparison) (Fig. S4) (60). The gaps and mutations that existed in both reference-

guided de novo and reference-guided-only assemblies were considered for further analysis.

To identify the potential variations for further analysis, we only selected the variations that had a 

variation frequency of 100% and average quality higher than 30% to ensure that sequences were of high 

quality. We then applied a filter to select SNPs, MNPd, indels, and microinDels that cause protein 

sequence changes. In this study, we focused only on the variations in genes that are involved in cell 

wall/membrane synthesis, membrane transportation, biofilm formation, and antibiotic tolerance/resis-

tance. The effects of variations on amino acid sequences were identified using the SWISS-MODEL server 

(37). We also checked whether UM001B harbors the reported mechanisms of resistance against Amp, 

Van, and Tet. By aligning both the DNA and amino acid sequences against the corresponding sequences 

of ATCC 29212 and UM001B, we checked for the presence of genes that are known to lead to antibiotic 

resistance. In this process, we defined the presence of the corresponding genes when the alignment 
of either the DNA or amino acid sequence reached a percentage of identity of >60% while the percentage 
of coverage was >90%. The results are summarized in Table S1 in the supplemental material.

MD methods. All four MD simulations were performed using the GROMACS MD package (version 

2016.4) (35) and the Gromos54a7 force field (61). The topology files of Tet were generated using an 

Automated Topology Builder (ATB) force field and repository (62). The PDB files for the TetR protein 

downloaded from the RCSB databank (PDB accession number 120X), and the topology files were 

obtained using GROMACS utilities. The mutated TetR protein was created with YASARA (63). Four 

protein-ligand water systems (Table 3) were generated using in-built GROMACS utilities in a cubic 

simulation box with 8-nm box dimensions.

The MD simulations were performed using the GROMACS simulation package. The energy minimi-

zation of each system was performed using the steepest descent algorithm (63) until the force on each 

atom was below the tolerance parameter of 100 kJ · mol⁻¹ · nm⁻¹. Three-dimensional periodic boundary 

conditions were applied to all systems. Isothermal-isochoric and isothermal-isobaric equilibration runs 

were performed for 0.1 ns and 0.05 ns, respectively. The particle mesh Ewald method was used to 

compute long-range electrostatic interactions (64). The neighbor list was updated every 5 steps using 

1.0 nm for short-range van der Waals and electrostatic cutoff values. The systems were maintained at 
a pressure of 1.01 × 10⁶ Pa using an isotropic Parrinello-Rahman barostat (65) with a coupling constant 

τp of 2.0 ps and a compressibility factor of 4.5 Pa⁻¹. The temperature was maintained at 310 K by 
independently coupling the water and protein-ligand to an external velocity rescaling thermostat (66) 

with a τv of 0.1 ps. The LINCS algorithm (67) was used to constrain the bonds with H atoms. The 

production runs were performed in triplicate, each for 500 ns. The simulation analyses were performed 

using GROMACS utilities. Molecular visualization and graphics were generated using visual PyMOL 
(PyMOL Molecular Graphics System, version 2.2; Schrödinger, LLC).
Prediction of binding affinity. Molecular Docking is a computational technique that is commonly used to predict noncovalent binding affinity of a small molecule and a macromolecule (68). AutoDock Vina, which was developed to perform molecular docking and virtual screening more efficiently than its older version, was used to determine the different binding modes for Van, Amp (ligand), and PBP (receptor). AutoDock Vina used pdbqt files for both ligand and receptor generated by scripts from the AutoDock Tools package. After preparing the input files, AutoDock Vina uses rectangular boxes to define the binding sites of interest in the receptor. The centers of the boxes were defined using the x, y, and z coordinates. The 3D dimensions of each box were based on the size of the binding sites of interest (69). Once the docking sites were determined, docking runs were performed. By default, nine binding modes were generated, and corresponding binding affinity and RMSD of those modes, compared with the first binding mode, were provided. Binding modes that shared similar RMSDs were considered one mode.

Cloning. The pGEM-T vector system (Promega Corp.) was used to clone the tetR gene from UM001B and ATCC 29212 into E. coli MG1655, the tetR sequences were amplified using colony PCR with the primers listed in Table 2. The cloned vectors were transformed into E. coli DH5α using the heat shock method (70). Correct colonies were selected using blue-white screening with LB/isopropyl β-D-thiogalactopyranoside (IPTG)/5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)/Amp agar plates (100 μg · ml⁻¹ Amp, 0.5 mM IPTG, and 80 μg · ml⁻¹ X-Gal). Plasmids from white colonies were isolated for confirmation by single- and double-restriction analyses using SpHl and NotI and then transformed into E. coli MG1655. The susceptibility to Tetr of different cells (E. coli MG1655/pGEM-T [control plasmid], E. coli MG1655/pGEM-tetRWT, and E. coli MG1655 tetRCl) was tested as described above. Amp was added to the growth medium at 100 μg · ml⁻¹ to maintain the plasmids.

Statistics. SAS, Windows version 9.1.3 (SAS, Cary, NC, USA), was used for all statistical analyses. One-way ANOVA adjusted by Tukey’s test was used to analyze the raw data, unless stated otherwise. Results with P values of < 0.05 were considered statistically significant.

Data availability. The genome sequence of UM001B was deposited at NCBI with accession number CP053026.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only. SUPPLEMENTAL FILE 1, PDF file, 2.2 MB.

ACKNOWLEDGMENTS

This work was supported by the Hill Collaboration program. We are grateful to Run Anbar, Mary Forell, Donna Lindner, Carol Matkoski, and Scott Riddell at SUNY Upstate University Hospital for helping collect the clinical isolate used in this study. We also thank Roy Welch at Syracuse University for providing access to CLC Genomics Workbench and Arne Heydorn at the Technical University of Denmark for providing the COMSTAT software. H.G. and D.R. conceived the study and designed the experiments. C.F. and Z.S. isolated the strain. H.G., S.R., X.Z., and T.G. carried out the experiments. H.M. and S.N. conducted the simulations. H.G., H.M., C.F., Z.S., S.N., and D.R. cowrote the paper. All authors discussed the results and comments on the manuscript.

We declare no competing financial interests.

REFERENCES

5. Bonten MJ, van Tiel FH, van der Geest S, Stobberingh EE, Gaillard CA. 1993. Enterococcus faecalis pneumonia complicating topical antimicrobial ATCC 29212 into E. coli MG1655. The tetR sequences were amplified using colony PCR with the primers listed in Table 2. The cloned vectors were transformed into E. coli DH5α using the heat shock method (70). Correct colonies were selected using blue-white screening with LB/isopropyl β-D-thiogalactopyranoside (IPTG)/5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)/Amp agar plates (100 μg · ml⁻¹ Amp, 0.5 mM IPTG, and 80 μg · ml⁻¹ X-Gal). Plasmids from white colonies were isolated for confirmation by single- and double-restriction analyses using SpHl and NotI and then transformed into E. coli MG1655. The susceptibility to Tet of different cells (E. coli MG1655/pGEM-T [control plasmid], E. coli MG1655/pGEM-tetRWT, and E. coli MG1655 tetRCl) was tested as described above. Amp was added to the growth medium at 100 μg · ml⁻¹ to maintain the plasmids.


