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Supplementary Discussion

Sequencing of the strain and identifying the biosynthetic cluster. The estimated genome size of *E. terrae* is 6.6 Mb. We performed the assembly using the in house pipeline of TUCF Genomics. The assembled draft genome was then screened for sequences related to NRPS-coding genes, *i.e.* adenylation domains. Contigs putatively coding for the teixobactin biosynthetic pathway were ordered and manual editing was performed, which enabled the combination of some contigs which were assembled separately. Closure of the remaining gaps was performed by designing bridging fragments by PCR and Sanger sequencing using the same primers as for the amplification. This enabled gap closure between the contigs.

Predicted DNA-DNA hybridization values between *E. terrae* and closest relatives with published genomes in RAST. The predicted DNA-DNA hybridization values between *E. terrae* and the other strains were as follows: *A. denitrificans* (21.50% ± 2.34), *L. cholodnii* (23.00% ± 2.37), *M. petroleiphilum* (23.80% ± 2.38), and *R. gelatinosus* (22.90% ± 2.37). These values are much less than the threshold of 70%, the widely accepted cutoff for relatedness between different species.

Toxicity studies. hERG inhibition testing as performed with quinidine as a positive control. The IC50 of quinidine was 1.44 µg/mL, consistent with published data, and IC50 for teixobactin was >100 µg/mL, showing a lack of activity against hERG.

In testing for activity against cytochromes P450 (CYP), teixobactin (12.5 µg/mL) showed minimal inhibition against all five CYPs; 16% inhibition of five of the six CYP isozymes (1A2, 2C9, 2C19, and 3A4/5-testosterone, 3A4/5-midazolam) and 32.9% inhibition against CYP2D6.

Teixobactin did not exhibit genotoxicity when present at 125 µg/mL in an *in vitro* micronucleus test that employs fluorescent cell imaging to assess cytotoxicity and quantify micronuclei.

In vitro plasma half life. In order to determine whether the half life of teixobactin *in vivo* was compatible with a therapeutic application, teixobactin and control compounds (propantheline, enalapril , and warfarin) at 5 µM were incubated in two biological replicates with plasma from different species (human, rat, dog, and mouse) at 37 ºC. At different time points (0, 20, 40, 80, and 120 minutes) an aliquot was removed and mixed with stop solution (methanol containing propranolol, diclofenac, or other internal MS standard). The samples were centrifuged to remove precipitated protein, and the supernatants analyzed by LC/MS/MS to quantitate the remaining compound. Data were fit to a first-order decay model to determine half-life. The half-life of teixobactin was 190 min in human plasma, 211 min in rat plasma, 187 min in dog plasma, and 151 min in mouse plasma. These are reasonable residence times for an antimicrobial compound.

Structure determination. The 2D structure of teixobactin was determined using data from multiple NMR experiments (COSY, TOCSY, HMBC, HSQC, NOESY) – see Extended Data Figure 4 for the spectra. The ^{13}C-NMR data and assignments are shown in Extended Data Figure 3 and 4. Additionally, a quasi-molecular ion peak of *m/z* 1242.7253 for $C_{58}H_{96}N_{15}O_{15}$ $[M+H]^+$ (calculated 1242.7205 for $C_{58}H_{96}N_{15}O_{15}$) was determined by high resolution electrospray ionization mass spectroscopy, revealing $C_{58}H_{95}N_{15}O_{15}$ as the molecular formula.

In order to determine the 3D structure of teixobactin, the compound was subjected to an advanced Marfey's analysis and authentic samples of all four diastereomers of enduracididine were synthesized since they were not commercially available for comparison. In all but one case, unambiguous data was generated to determine the configuration of the amino acids. The stereochemistry of the four isoleucines was determined to be non-identical by Marfey's analysis in the ratio 3:1. Furthermore, the allo- and non-allo- forms were not chromatographically resolved under reverse phase conditions. Teixobactin was fully hydrolysed and the L-FDLA derivatives

were assessed. A normal-phase gradient LC-MS technique was developed, using a Phenomenex Lux-Cellulose column, and eluting the compounds with a gradient system of hexane, and isopropanol with 0.2% formic acid. All four isoleucine stereoisomers were resolved using this method. Using this technique as well as spiking in isoleucine standards (L-isoleucine standard was purchased from TCI UK Ltd., and the allo-L, allo-D- and D- isoleucine standards were purchased from BACHEM), it was determined that teixobactin contained 3 x L-isoleucines and 1 x allo-D-isoleucine.

Resistance studies. In order to determine a suitable concentration of teixobactin for single-step selection for resistance, we first determined a minimal concentration for which no resistant mutants are observed, usually referred to as MPC.

The MPC for *S. aureus* ATCC 29213 was determined by plating 10^9 cells per plate onto 10 MHA plates containing 1xMIC, 1.25xMIC, 1.5xMIC, 1.75xMIC, 2xMIC, 2.25xMIC, and 2.5xMIC teixobactin. After 48 hours of incubation, the plates were examined for colonies. There were no colonies on plates with ≥2xMIC teixobactin. Note that the intrinsic variability in determining MIC is also 2 fold, this means that even at a very low concentration of the compound, there was no resistance development.

Next, we performed sequential culturing of *S. aureus* in the presence of subinhibitory levels of teixobactin in order to increase the probability of obtaining resistant mutants. *S. aureus* ATCC 29213 cells were grown in 1 mL of media (MHB with 0.002% polysorbate 80) containing teixobactin at different concentrations. Ofloxacin was included as a control.

Cells were added to teixobactin present at 0.25xMIC, 0.5xMIC, 1xMIC, 2xMIC and 4xMIC. At 24 hour intervals, the cultures were checked for growth. Cultures from the second highest concentrations that allowed growth (OD_{600} ≥2) were diluted 1:100 into fresh media containing 0.25xMIC, 0.5xMIC, 1xMIC, 2xMIC and 4xMIC of teixobactin. This serial passaging was repeated daily for 30 days. Any cultures that grew at higher than the MIC levels were passaged on drug free MHA plates and the MIC was then determined by broth microdilution. No resistant mutants were obtained. This experiment was repeated, and produced the same negative result.

In order to maximize the chance of obtaining a resistant mutant, we performed an additional experiment with very small incremental increases in the drug concentration. Cells were added to a series of tubes with small differences in the concentration of teixobactin (0.06xMIC, 0.25xMIC, 0.5xMIC, 0.75xMIC, 1xMIC, 1.25xMIC, 1.5xMIC, and 2xMIC). At 24 hour intervals, cultures from the highest concentration that allowed growth to a minimum OD_{600} of 0.2 were diluted 1:100 into fresh medium containing 0.06xMIC, 0.25xMIC, 0.5xMIC, 0.75xMIC, 1xMIC, 1.25xMIC, 1.5xMIC, and 2xMIC. This passaging was repeated for 27 days. Cultures that grew at levels higher than the MIC were passaged on drug free MHA plates, and the MIC was determined. For teixobactin, there were no mutants with an MIC greater than the parent *S. aureus* ATCC 29213.