In vivo Imaging and Tracking of VRE-microbiota Interactions via Anaerobic Fluorescent Reporters in Extremely Drug-resistant Bacteria

Benjamin R. Treat[1], Joseph Hobeika[1], Marygrace Duggar[1,3], Trinity Fields[1], Cliff Guy[2], Mollie Black[3,4], Christina M. Kohler[1], and Elisa B. Margolis[1,4]

[1] Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee
[2] Department of Immunology, St. Jude Children's Research Hospital, Memphis, Tennessee
[3] St. Jude Graduate School of Biomedical Sciences, St. Jude Children's Research Hospital, Memphis, Tennessee
[4] Department of Host Microbe Interactions, St. Jude Children's Research Hospital, Memphis, Tennessee

Keywords: Vancomycin Resistant Enterococcus faecium (VREfm), Multidrug-resistant organisms (MDROs), Microbiome, Microbiology Tools, Anaerobic Fluorescent Reporters

Abstract

Vancomycin-resistant *Enterococcus faecium* (VREfm) has emerged as a major nosocomial pathogen, but studying its host and microbiome interactions remains challenging due to limited genetic tools. Current obstacles include extensive intrinsic and acquired resistance that precludes use of conventional selection markers, poor plasmid maintenance, and oxygen-dependent reporters unsuitable for gut environments. Here we present an integrated toolkit that enables robust genetic manipulation and *in vivo* tracking of VREfm. We developed a universal puromycin selection system effective across diverse clinical isolates and an enhanced *pheS*** counterselection marker for stable genomic integration. We identified two neutral genomic loci that support reporter genes insertion without fitness cost. Using this system, we demonstrate long-term tracking of VREfm colonization, strain competition and host-microbiome interactions in mouse models via chromosomally-integrated anaerobic fluorescent proteins (eUnaG2 and smURFP). This toolkit advances the study of VREfm pathogenesis by enabling stable genetic manipulation of even the most resistant clinical isolates and direct visualization of host-microbe interactions in ecologically and immunologically relevant *in vivo* environments.

Introduction

Vancomycin-resistant *Enterococcus faecium* (VREfm) has emerged as a significant nosocomial pathogen, responsible for an increasing number of hospital-acquired infections globally. Infections caused by *VREfm* are particularly concerning in immunocompromised populations, including critically ill patients in intensive care units, where this opportunist can contribute to high morbidity and mortality[1–3]. The rising prevalence of antibiotic resistance in *E. faecium*, particularly to vancomycin—a last-line therapy for Gram-positive infections—poses a substantial challenge to public health due to limited therapeutic options and the pathogen's ability to acquire resistance to other classes of antibiotics, such as aminoglycosides and linezolid[4–7].

Understanding VREfm colonization, persistence, and transmission is critical to curbing its spread. VREfm is known to colonize the human gastrointestinal tract, from where it can disseminate to other body sites and serve as a reservoir for nosocomial transmission, often via the hands of healthcare workers or contaminated surfaces[4,8,9]. However, current methods for studying *VREfm*–microbiota interactions, including the mechanisms of colonization and persistence in the gut, remain inadequate. There remains a need for robust models to investigate these dynamics and guide better strategies for preventing the spread of *VREfm* in hospital settings.

Three major technical barriers limit VRE research. First, the microbe's extensive inherent resistance profile renders conventional selection markers ineffective[10], while rapid acquisition of new resistance determinants complicates strain-specific tracking[11]. Second, clinical VREfm strains rarely maintain plasmids stably, necessitating genomic integration, which in turn is hindered by lack of known insertion sites and reliable counterselection strategies for this purpose. Third, distinguishing specific VREfm strains within complex microbial communities is challenging, as both endogenous related enterococci and bacteria sharing similar antibiotic resistance profiles coexist in these environments[11]. Additionally, traditional fluorescent and bioluminescent tracking methods require oxygen, and do not work in the anaerobic gut environment[12–14].

To overcome these limitations, we developed an integrated genetic system combining novel selection markers, counterselection and anaerobic fluorescent reporters. Our

approach leverages VREfm universal susceptibility to puromycin and an improved counterselection system to enable genomic insertion of bright anaerobic fluorescent reporters. This strategy enables real-time visualization of extensively-drug resistant (XDR) *VREfm* colonization, monitoring of niche competition with commensals, and long-term tracking of clinical isolate strains in experimental animal models.

Results

Development of Puromycin Selection System for Extensively-Resistant Clinical VRE Strains

Genetic manipulation of extensively drug-resistant VREfm requires reliable positive selection markers to identify successful transformants. To develop selection that works with a myriad of clinical strains, we evaluated a diverse panel of VRE isolates (CDC AR Isolate Bank). Common selection markers for gram positives, including aminoglycoside modifying enzymes, 23S ribosomal RNA methylases, tetracycline resistance proteins, and chloramphenicol acetyltransferases are frequently present in clinical VREfm isolates (**Figure 1**). This widespread resistance necessitates the use of multiple selection strategies for genetically modifying *VREfm*. In cases of XDR clinical isolates, where selection options are either limited or absent, sequential genetic modifications become particularly challenging.

To address this limitation, we tested VREfm susceptibility to several broad-spectrum antibiotics that are not suitable for clinical use (Zeocin™, hygromycin B, and puromycin)[15–17] (**Supplemental Figure 1A**). Among these, we discovered that XDR isolates of VREfm were susceptible to puromycin (**Table 1**), which led us to develop a universal selection system utilizing a puromycin acetyltransferase (*pac*) gene. Puromycin, an aminonucleoside antibiotic that terminates protein synthesis through tRNA mimicry, represents an ideal selection marker for VREfm due to its clinical disuse (tRNA conserved across prokaryotes and eukaryotes) and target conservation (important for XDR organisms)[17–19]. We evaluated puromycin susceptibility across a panel of drug resistant VRE isolates (including other *Enterococci* such as *E. faecalis*

and *E. casseliflavus*) from the CDC (Antimicrobial Resistance Isolate Bank) and found all to be susceptible with MICs <18 ug/mL.

To validate the puromycin selection system, we tested transformation across 13 VRE strains (from CDC AR Isolate Bank), including 9 VREfm strains with distinct resistance profiles, plus *E. faecalis, E. casseliflavus*, and *E. lactis* strains. All strains successfully transformed and exhibited elevated puromycin resistance, with MICS increasing from 8.5-16 ug/mL in wild-type strains to 43-98 ug/ml in transformants (**Table 1, Supplemental Figure 1C**). This universal puromycin selection system enables consistent transformation across diverse XDR clinical isolates. The system's reliability and broad applicability make it particularly valuable for studying MDROs where conventional selection options are limited.

Developing Anaerobic Reporters for use in VREfm

Traditional fluorescent and bioluminescent reporters require oxygen for chromophore formation or light production, limiting their utility in studying VREfm under physiologically relevant anaerobic conditions[12–14]. We evaluated several oxygen-independent reporters including bile pigment binding proteins (eUnaG[20,21], eUnaG2[22], smURFP[23]) and self-labeling protein tags (HaloTag9[24]) for use in VREfm (**Figure 2A**).

Initial screening in *E. faecalis* strain OG1/CK111 revealed superior performance of bile-pigment binding proteins (**Figure 2B**). The eUnaG2[22] and smURFP reporters demonstrated comparable performance when test in VREfm clinical isolates (**Figure 2C-D**). These reporters offer key advantages for *in vivo* applications- their cofactors are naturally present as heme breakdown products in the intestinal tract, enabling fluorescence without exogenous supplementation and their spectral separation permits dual-color labeling for strain differentiation studies.

In a mouse model of VRE colonization, we detected smURFP fluorescence by flow cytometry of stool and by confocal microscopy (**Figure 3**). eUnaG2 plasmid reporters similarly worked in mice (data not shown). This suggests that biliverdin and bilirubin-binding proteins have sufficient access to unmodified biliverdin and bilirubin in the large intestine, and are well-suited to be robust anaerobic fluorescent reporters *in vivo*.

Genomic Integration and Counterselection

These anaerobic reporter plasmids, while very bright, are unstable over time in VREfm isolates without maintaining selection (**Supplemental Figure 2**). Therefore, we next sought to generate long-term stable VREfm reporters, which requires robust counterselection. While initially testing *pheS**-based counterselection using the established *E. faecalis pheS** A312G mutation[25], we observed poor counterselection efficiency in several VREfm strains in rich media (due to competition between amino acids in the media and the counterselection compound p-chloro-phenylalanine (PCPA)). To overcome this limitation, we engineered a second mutation in *pheS*, yielding *pheS*** (T258A/A312G), based on recent improvements to the *E. coli pheS*-based counterselection system[26]. Using *pheS* derived from *E. faecalis* (80.9% sequence homology to *E. faecium*) minimizes off-target recombination in VREfm strains. The enhanced *pheS*** counterselection gene showed significantly improved counterselection efficiency compared to single mutants when tested in both *E. faecalis* and VREfm (**Supplemental Figure 3**).

To generate stable genomic insertions, we identified optimal integration sites using four key criteria: location between conserved ORFs, proximity to C-terminal regions and terminators, adjacency to active transcription, and tolerance of natural transposon insertions. These criteria were necessary due to the genetic diversity and plasticity of VREfm strains. From 15 candidate sites, we validated two high-priority loci (657/680 and 12650/12655) that support stable integration without impacting bacterial fitness (**Figure 4**).

In Vivo Tracking Applications

Taken together, these tools enable long-term tracking of any clinical VRE isolates. Using a dual eUnaG2/smURFP reporter integrated into the 675/680 locus of clinical VREfm strain VRECG19, we demonstrated stable tracking for 21 days in mice. VREfm colonization remained detectable throughout the experiment, with fluorescent colonies maintaining reporter expression (**Figure 5A-B**). Flow cytometric analysis of filtered stool enabled direct detection of this strain up to 21 days after inoculation (**Figure 5C**) in these mice and for more than one year in a long-term *in vivo* colonization experiment (data not shown).

Discussion

Development of genetic tools for studying extensively drug-resistant organisms presents unique challenges. Our puromycin selection system addresses a fundamental barrier in VREfm research- the lack of reliable selection markers for clinical isolates. By leveraging universal puromycin susceptibility and a codon-optimized *pac* gene, we enable genetic manipulation of previously intractable strains.

The enhanced *pheS*** counterselection system significantly improves genomic integration efficiency compared to existing methods. In the future, this will reap additional benefits as it is not dependent on minimal media, allowing its application to manipulation of metabolic pathways and building high-depth transposon libraries. Our identification of two neutral insertion sites provides stable chromosomal integration without fitness costs. This advance enables long-term strain tracking without maintaining selection pressure, critical for studying colonization, pathogenesis and host interactions.

Anaerobic fluorescent reporters address the oxygen-dependence limitation of traditional tools. The eUnaG2 and smURFP reporters function robustly in the gut environment without exogenous supplementation, as their cofactors derive from natural heme breakdown. Their spectral separation would allow for simultaneous tracking of multiple strains, essential for studying strain replacement and microbiome interactions.

This toolkit enabled direct investigation of VREfm population dynamics and microbial interactions *in vivo*. The dual-reporter system allows simultaneous tracking of different strains to study competition/succession. The ability to distinguish and track VREfm along with members of the microbiome opens new avenues for understanding the establishment and maintenance of gut colonization in the context of diverse microbiomes. This system could be particularly valuable for evaluating therapeutic approaches that aim for gut decolonization or manipulate the microbiota to prevent VREfm.

Successful application of these tools requires a few considerations of several technical limitations. Diet autofluorescence can interfere with *in vivo* fluorescence detection,

necessitating careful sample processing and controls. Among clinical isolates genomic insertion efficiencies vary widely between strains (generally 10-30%). While puromycin selection is broadly effective, optimal concentrations require narrow strain-specific MIC determination. Finally, reporter brightness depends on local biliverdin/bilirubin breakdown product availability, which may differ in different infection models.

These tools enable new research directions in VREfm biology. Stable strain labeling permits direct visualization of colonization sites, investigation of niche competition with commensals, and tracking of transmission dynamics. The ability to manipulate clinical isolates allows genetic studies in relevant strain backgrounds. This resource advances our capability to understand VREfm pathogenesis and interactions.

Materials and Methods

Bacterial strains

Bacterial strains used in this study include a panel of VRE isolates provided by the CDC Antimicrobial Resistance (AR) Isolate Bank, which include a diverse set of highly antibiotic resistant *Enterococcus* isolates(https://wwwn.cdc.gov/arisolatebank/). Additional VREfm isolates were obtained from BEI (EnGen0312, ERV102, HF50104, TX0082), or previously described isolates from St. Jude Children's Research Hospital (VRECG13-10-S, VRECG19-10-S)[27]. *E. faecalis* conjugal donor CK111 was developed by Kristich, et. al.[25], and was a gift from Danielle Garsin. All strains were grown and maintained in Brain Heart Infusion media (Difco BHI, BD Biosciences). For selection in Enterococci, the following selection conditions were maintained – chloramphenicol 30ug/mL, spectinomycin 300-500ug/mL, puromycin 20-30ug/mL, and gentamycin 300ug/mL. *E. coli* Stellar cells (Takara Bio USA) were used to maintain plasmids, under selection at the following concentrations– chloramphenicol 50ug/mL, spectinomycin 100ug/mL, puromycin 100ug/mL, and gentamycin 25ug/mL

Plasmid Construction

All plasmids were constructed using Infusion cloning (Takara Bio USA, Inc.) and maintained in Stellar competent *E. coli* (Takara Bio USA). Reporter plasmids were

based on the gram positive reporter plasmid pBSU101-EGFP[28], with addition of the chloramphenicol resistance gene catA9 and the pCF10 origin for increased compatibility with difficult VREfm strains. Anaerobic reporter genes were obtained from Addgene(https://www.addgene.org/), including UnaG (Addgene#163125), eUnaG2(Addgene #162457), smURFP(Addgene #80341), and HaloTag9(Addgene #169324)—and replaced EGFP to be under control of the *S. agalactiae* cfb promoter. For UnaG, a point mutation in cloning primer mutated the second amino acid (V2L) to yield eUnaG[20]. For smURFP/eUnaG2 dual reporters, an additional ribosome-binding site was placed after smURFP stop codon, and before eUnaG2, to allow for dual expression from the *cfb* promoter.

For puromycin selection validation, we constructed pBRT38, an *Enterococcus-E. coli* shuttle vector expressing codon-optimized pac under control of the catA9 promoter (**Supplemental Figure 1B**). The puromycin resistance *pac* gene was codon optimized for *E. faecium* and obtained from GenScript Biotech.

Genomic insertion plasmids were based on the pGPA1 temperature-sensitive transposon delivery plasmid[29] (Addgene#115476), with transposase and transposable elements replaced with VREfm homologous regions amplified from VREfm TX0082, along with dual smURFP/eUnaG2 under control of the *cfb* promoter. Additionally, the temperature sensitive origin of this plasmid was fixed by removing a premature stop codon in pWV01^{ts} origin copG, and *pheS*** was added for effective counterselection on BHI supplemented with 5-10mM PCPA.

Plasmids generated for this work are listed in **Table 2** and will be made available on Addgene prior to full publication of research.

Plasmid Transformation

Electrocompetent cells were prepared as follows: Bacterial cultures were inoculated from single colonies and grown overnight in BHI at 37°C with shaking before 1:1000 dilution into 25mL BHI supplemented with 200mM sucrose and glycine (5% w/v for *E. faecalis*, 1% w/v for *E. faecium*), and grown overnight. The next morning, cultures were centrifuged 4000g to pellet, resuspended in 25mL fresh BHI supplemented with 200mM sucrose and glycine, and incubated an additional hour at 37°C. Cells were next

harvested by centrifugation, washed three times with ice-cold wash buffer (500mM sucrose, 10% glycerol), and resuspended ~500uL of wash buffer. For electroporation, 50 µL of competent cells were mixed with plasmid DNA (500 ng–2 µg), incubated on ice for 30 min, and pulsed at 1.8 kV (1-mm cuvette). Post-electroporation, cells were resuspended in recovery medium (BHI with 500mM sucrose) and incubated at 37°C for 2h (or at 28°C for 6-8 h for temperature-sensitive plasmids). Transformants were selected on BHI agar with appropriate antibiotics and incubated overnight, with chloramphenicol or puromycin selection plates requiring up to 48 h.

The *E. faecalis* conjugal-delivery strain OG1 derivative, CK111, was employed to transform difficult strains via conjugation[25]. CK111 was first transformed via electroporation as above with plasmid DNA that contains the pCF10 conjugal origin. CK111 containing plasmid was grown overnight under selection, washed 2x in fresh media, then CK111 donor was mixed at a ratio of 10:1 with fresh cultures of VREfm recipient. This mixture was incubated overnight on filter paper on BHI agar without selection. Filter paper matings were then removed and resuspend in BHI prior to plating on agar containing 20ug/mL vancomycin and appropriate antibiotics.

Genomic Integration

To generate stable genomic insertions, dual smURFP/eUnaG2 reporter plasmids containing homology to the 675/680 chromosomal region (pBRT48, pBRT49) or the 12650/12655 chromosomal region (pBRT62) were transformed into the VREfm strain as described above, and single colonies were picked and maintained at 28°C under dual selection (gentamycin/chloramphenicol for pBRT48 and pBRT62) for one passage. Next cultures were subcultured twice at 1:1000 in BHI containing only 300ug/mL gentamycin at 37°C to encourage recombination and plasmid loss. Finally, cultures were serially diluted and plated onto BHI supplemented with 300ug/mL gentamycin and 5-10mM PCPA. Successful integration was confirmed by screening single colonies for genomic insertion by PCR. (675-detect-F, 5'-catgacaagtaaggacagctatatcgtccc ; 680-detect-R, 5'-cgtggcaaacgagtcactagcaag ; 12650-detect-F, 5'-cgtttatagattggctgtggctttg ; 12655-detect-R, 5'-ctcactgaaagaacaactcgcatc). For plasmid pBRT49, 20ug/mL puromycin was maintained throughout to maintain the insertion element.

Mouse colonization model

6-8 week old female C57BL/6NTac (Taconic Biosciences) mice were given oral vancomycin hydrochloride (0.5mg/mL) in drinking water for 2 days prior to inoculation with VREfm. Mice were inoculated with $1x10^8$ CFU of VREfm AR0803 containing pBRT31 (smURFP expressing reporter plasmid) or VRECG19 675-DUAL reporter by oral gavage and monitored for colonization by plating fresh stool. Briefly, stool was resuspended 10% w/v in sterile 1xPBS, serially diluted, and plated onto BHI supplemented with 30ug/mL vancomycin and 20ug/mL meropenem to select for VREfm growth. Colonies were enumerated and the number of colonies per 5mg stool calculated. Further confirmation of fluorescence included stool plated onto VRE-selective medium supplemented with 5nM biliverdin and 10% (w/v) stool filtered through 40 uM filters prior to flow cytometry.

Minimum inhibitory concentration (MIC) assay

The MIC of antimicrobial agents was determined using the broth microdilution method. Briefly, bacterial strains were grown overnight in BHI at 37°C with shaking and adjusted to a standardized inoculum of ~5×10^5 CFU/mL in fresh BHI. Bacteria were added to two-fold serial dilutions of antibiotic-containing media in flat-bottom 96-well microtiter plates, with final concentrations of 0.8ug/mL to 400 µg/mL. Negative controls contained BHI only, while positive growth controls included bacteria without antibiotics. Plates were incubated at 37°C for 18–24 h under aerobic conditions. MIC values were determined as the lowest concentration of the antimicrobial agent that completely inhibited visible bacterial growth, calculated by nonlinear fit of OD_{600} measurements, and visually confirmed. All assays were performed in biological triplicates.

Anaerobic Reporter Fluorescence

Vancomycin-resistant *Enterococcus faecium* (VREfm) transformants using reporters expressed from the *S. agalactiae cfb* promoter expressing HaloTag9, eUnaG, eUnaG2, or smURFP were cultured in BHI medium at 37°C with shaking. For HaloTag labeling, after overnight cultures were grown, 1mL of culture was pelleted and resuspended in 1XPBS supplemented with 1 µM HaloTag® TMR Ligand for 1 hour at 25°C, followed by washing to remove unbound ligand. Briefly, cells were pelleted at 2,000 × g for 5 min,

resuspended in 1mL of 1X PBS. Cells were then pelleted and resuspended in 1mL fresh 1XPBS. This wash was repeated 2–3 times to remove any unbound HaloTag reagent, before fluorescence measurement. For eUnaG and eUnaG2 fluorescence, overnight cultures were supplemented with 10–25 nM bilirubin. Similarly, for smURFP fluorescence, overnight cultures were supplemented with 2.5–5 nM biliverdin. As BHI media has some background fluorescence, cells pelleted and resuspended in 1X PBS before fluorescence measurements. Advantages of eUnaG2[22] and smURFP reporters are they require supplementation (10-25nM bilirubin for eUnaG, 2.5-5nM biliverdin for smURFP) only *in vitro* and eliminate washing steps needed for self-labeling tags such as HaloTag9

Flow cytometry

Fresh stool was collected from mice 1-21 days post colonization with VREfm strains, and resuspended in 1xPBS at 10%w/v. Resuspended stool was filtered through 40uM cell strainers. Samples were then diluted 10x in 1XPBS and 50uL run on and ApogeeFlow MicroPLUS. Small Angle Light Scatter(SALS) and Large Angle Light Scatter(LALS) [equivalent to forward scatter and side scatter] were detected using a 405nm laser. For eUnaG2, a 488nm excitation laser with a 530/40 bandpass filter was used for detection. For smURFP, a 638nm laser with 676/36 bandpass filter was used for detection. Analysis and gating were performed using FlowJo™ v10.10 Software (BD Life Sciences).

Fluorescent Imaging

Tissues were collected, embedded in tissue freezing medium and immediately frozen in liquid-nitrogen cooled isopentane. 10µm thick cryosections were mounted on charged slides and dried for 1 hr at RT prior to rehydration in PBS, followed by blocking in PBS containing 1% (wt/vol) BSA and 0.05% Tween-20. Sections were subsequently incubated with 1ug/mL wheat germ agglutinin conjugated with alexa fluor 555 (ThermoFisher; cat W32464) for 1hr at RT. Sections were washed in PBS and were subsequently mounted with Vectashield mounting media with DAPI (Vector labs; cat H-1800). Images were acquired using an inverted Ti2 microscope (Nikon Instruments) equipped with a 60X plan Apo oil objective, Spectra/AuraII illumination source

(Lumencor) with 395/555/640 excitation lines and emission filters as appropriate, and Orca Fusion digital CMOS camera (Hamamatsu). 3D images were acquired with a 0.2μM step size, followed by deconvolution and analysis using NISElements software (ver 5.42.04).

Acknowledgements

We would like to thank Daria van Tyne (University of Pittsburgh) and Jason Rosch (St. Jude Children's Research Hospital) for protocols, strains[27], and helpful advice in working with difficult VREfm. CK111, the conjugal donor *E. faecalis* strain[25], as well as several plasmids were a kind gift of Danielle Garsin. Plasmid pBSU101-EGFP, which was the basis for anaerobic reporter plasmid constructs, was a kind gift from Kevin Wood[28,30]. Plasmid pMAL-c5x_UnaG was a gift from Julie Biteen (Addgene#163125). Plasmid pJYDN3p was a gift from Gideon Schreiber (Addgene#162457). Plasmid pBAD-smURFP-RBS-HO-1 was a gift from Erik Rodriguez and Roger Tsien (Addgene# 80341). Plasmid pET51b(+)HaloTag9 was a gift from Kai Johnsson (Addgene#169324). Plasmid pGPA1, which was the basis of the temperature-sensitive genomic insertion plasmids, was a gift from Willem van Schaik (Addgene#115476). This work was supported by The American Lebanese Syrian Associated Charities (ALSAC) and by the National Institute of Health (NIH) grant R21AI180489-01.

1. Xie, O. *et al.* Epidemiology, treatment and outcomes of bloodstream infection due to vancomycin-resistant enterococci in cancer patients in a vanB endemic setting. *BMC Infect. Dis.* **20**, 228 (2020).

2. Taur, Y. *et al.* Intestinal Domination and the Risk of Bacteremia in Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation. *Clin. Infect. Dis.* **55**, 905–914 (2012).

3. Zirakzadeh, A. *et al.* Vancomycin-resistant enterococcal colonization appears associated with increased mortality among allogeneic hematopoietic stem cell transplant recipients. *Bone Marrow Transplant.* **41**, 385–392 (2008).

4. Faron, M. L., Ledeboer, N. A. & Buchan, B. W. Resistance Mechanisms, Epidemiology, and Approaches to Screening for Vancomycin-Resistant Enterococcus in the Health Care Setting. *J. Clin. Microbiol.* **54**, 2436–2447 (2016).

5. Olearo, F. *et al.* Emergence of linezolid-resistance in vancomycin-resistant *Enterococcus faecium* ST117 associated with increased linezolid-consumption. *Int. J. Med. Microbiol.* **311**, 151477 (2021).

6. Hourigan, D., Stefanovic, E., Hill, C. & Ross, R. P. Promiscuous, persistent and problematic: insights into current enterococcal genomics to guide therapeutic strategy. *BMC Microbiol.* **24**, 103 (2024).

7. Chow, J. W. Aminoglycoside Resistance in Enterococci. *Clin. Infect. Dis.* **31**, 586–589 (2000).

8. De Angelis, G. *et al.* Infection control and prevention measures to reduce the spread of vancomycin-resistant enterococci in hospitalized patients: a systematic review and meta-analysis. *J. Antimicrob. Chemother.* **69**, 1185–1192 (2014).

9. Eichel, V. M. *et al.* Epidemiology and outcomes of vancomycin-resistant enterococcus infections: a systematic review and meta-analysis. *J. Hosp. Infect.* **141**, 119–128 (2023).

10. Shepard, B. D. & Gilmore, M. S. Antibiotic-resistant enterococci: the mechanisms and dynamics of drug introduction and resistance. *Microbes Infect.* **4**, 215–224 (2002).

11. García-Solache, M. & Rice, L. B. The Enterococcus: a Model of Adaptability to Its Environment. *Clin. Microbiol. Rev.* **32**, e00058-18 (2019).

12. Tsien, R. Y. The green fluorescent protein. *Annu. Rev. Biochem.* **67**, 509–544 (1998).

13. Gould, S. J. & Subramani, S. Firefly luciferase as a tool in molecular and cell biology. *Anal. Biochem.* **175**, 5–13 (1988).

14. Ozbakir, H. F., Anderson, N. T., Fan, K.-C. & Mukherjee, A. Beyond the Green Fluorescent Protein: Biomolecular Reporters for Anaerobic and Deep-Tissue Imaging. *Bioconjug. Chem.* **31**, 293–302 (2020).

15. Gritz, L. & Davies, J. Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. *Gene* **25**, 179–188 (1983).

16. Baron, M., Reynes, J.-P., Stassi, D. & Tiraby, G. A selectable bifunctional β-galactosidase::phleomycin-resistance fusion protein as a potential marker for eukaryotic cells. *Gene* **114**, 239–243 (1992).

17. Vara, J. A., Portela, A., Ortín, J. & Jiménez, A. Expression in mammalian cells of a gene from Streptomyces alboniger conferring puromycin resistance. *Nucleic Acids Res.* **14**, 4617–4624 (1986).

18. Vara, J., Perez-Gonzalez, J. A. & Jimenez, A. Biosynthesis of puromycin by Streptomyces alboniger: Characterization of puromycin N-acetyltransferase. *Biochemistry* **24**, 8074–8081 (1985).

19. Aviner, R. The science of puromycin: From studies of ribosome function to applications in biotechnology. *Comput. Struct. Biotechnol. J.* **18**, 1074–1083 (2020).

20. Ko, S., Kwon, J. & Shim, S.-H. Enhanced UnaG With Minimal Labeling Artifact for Single-Molecule Localization Microscopy. *Front. Mol. Biosci.* **8**, (2021).

21. Kumagai, A. *et al.* A Bilirubin-Inducible Fluorescent Protein from Eel Muscle. *Cell* **153**, 1602–1611 (2013).

22. Zahradník, J. *et al.* A Protein-Engineered, Enhanced Yeast Display Platform for Rapid Evolution of Challenging Targets. *ACS Synth. Biol.* **10**, 3445–3460 (2021).

23. Rodriguez, E. A. *et al.* A far-red fluorescent protein evolved from a cyanobacterial phycobiliprotein. *Nat. Methods* **13**, 763–769 (2016).

24. Frei, M. S. *et al.* Engineered HaloTag variants for fluorescence lifetime multiplexing. *Nat. Methods* **19**, 65–70 (2022).

25. Kristich, C. J., Chandler, J. R. & Dunny, G. M. Development of a host-genotype-independent counterselectable marker and a high-frequency conjugative delivery system and their use in genetic analysis of Enterococcus faecalis. *Plasmid* **57**, 131–144 (2007).

26. Miyazaki, K. Molecular engineering of a PheS counterselection marker for improved operating efficiency in Escherichia coli. *BioTechniques* **58**, 86–88 (2015).

27. Chilambi, G. S. *et al.* Evolution of vancomycin-resistant Enterococcus faecium during colonization and infection in immunocompromised pediatric patients. *Proc. Natl. Acad. Sci.* **117**, 11703–11714 (2020).

28. Aymanns, S., Mauerer, S., Zandbergen, G. van, Wolz, C. & Spellerberg, B. High-Level Fluorescence Labeling of Gram-Positive Pathogens. *PLOS ONE* **6**, e19822 (2011).

29. Zhang, X. *et al.* RNA-seq and Tn-seq reveal fitness determinants of vancomycin-resistant Enterococcus faecium during growth in human serum. *BMC Genomics* **18**, 893 (2017).

30. Hallinen, K. M., Guardiola-Flores, K. A. & Wood, K. B. Fluorescent reporter plasmids for single-cell and bulk-level composition assays in E. faecalis. *PLoS ONE* **15**, e0232539 (2020).

31. Michaux, C. *et al.* Single-Nucleotide RNA Maps for the Two Major Nosocomial Pathogens Enterococcus faecalis and Enterococcus faecium. *Front. Cell. Infect. Microbiol.* **10**, 600325 (2020).

Isolate	Species	Puromycin MIC (ug/mL) wt (pac)	+pBRT38
AR0783	*E. faecium*	11.5	46.9
AR0787	*E. faecium*	11.5	96.2
AR0789	*E. faecium*	12.4	43
AR0790	*E. faecium*	12	47.3
AR0792	*E. faecium*	16	77.7
AR0794	*E. faecium*	14.2	47.2
AR0802	*E. faecium*	14.1	45.1
AR0803	*E. faecium*	12	48.3
AR0807	*E. faecium*	16	45.8
AR0781	*E. faecalis*	13	98.4
AR0782	*E. faecalis*	12.2	45.3
AR0801	*E. casseliflavus*	11.3	45.2
AR0806	*E. lactis*	8.5	47.1

Table 1. VRE AR Isolates are Universally Compatible with Puromycin Selection

Plasmid	Purpose	Selection
pBRT25-eUnaG	eUnaG expression	Spectinomycin, Chloramphenicol
pBRT26-Halo-eUnaG2	HaloTag9-eUnaG2 expression	Spectinomycin, Chloramphenicol
pBRT27-smURFP/eUnaG2	smURFP/eUnaG2 expression	Spectinomycin, Chloramphenicol
pBRT31-smURFP	smURFP expression	Spectinomycin, Chloramphenicol
pBRT32-eUnaG2	eUnaG2 expression	Spectinomycin, Chloramphenicol
pBRT33-Tn-*pheS**	*pheS** counterselection test	Gentamycin, Chloramphenicol
pBRT34-Tn-*pheS***	*pheS*** counterselection test	Gentamycin, Chloramphenicol
pBRT38-pac-eUnaG2	*pac* gene; eUnaG2 expression	Puromycin, Chloramphenicol
pBRT48-675-DUAL-gent	675/680 DUAL smURFP/eUnaG2 genomic insertion	Gentamycin, Chloramphenicol
pBRT49-675-DUAL-pac	675/680 DUAL smURFP/eUnaG2 genomic insertion	Puromycin, Chloramphenicol
pBRT63-12650-DUAL-gent	12650/12655 DUAL smURFP/eUnaG2 genomic insertion	Gentamycin, Chloramphenicol

Table 2. Plasmids used in this publication

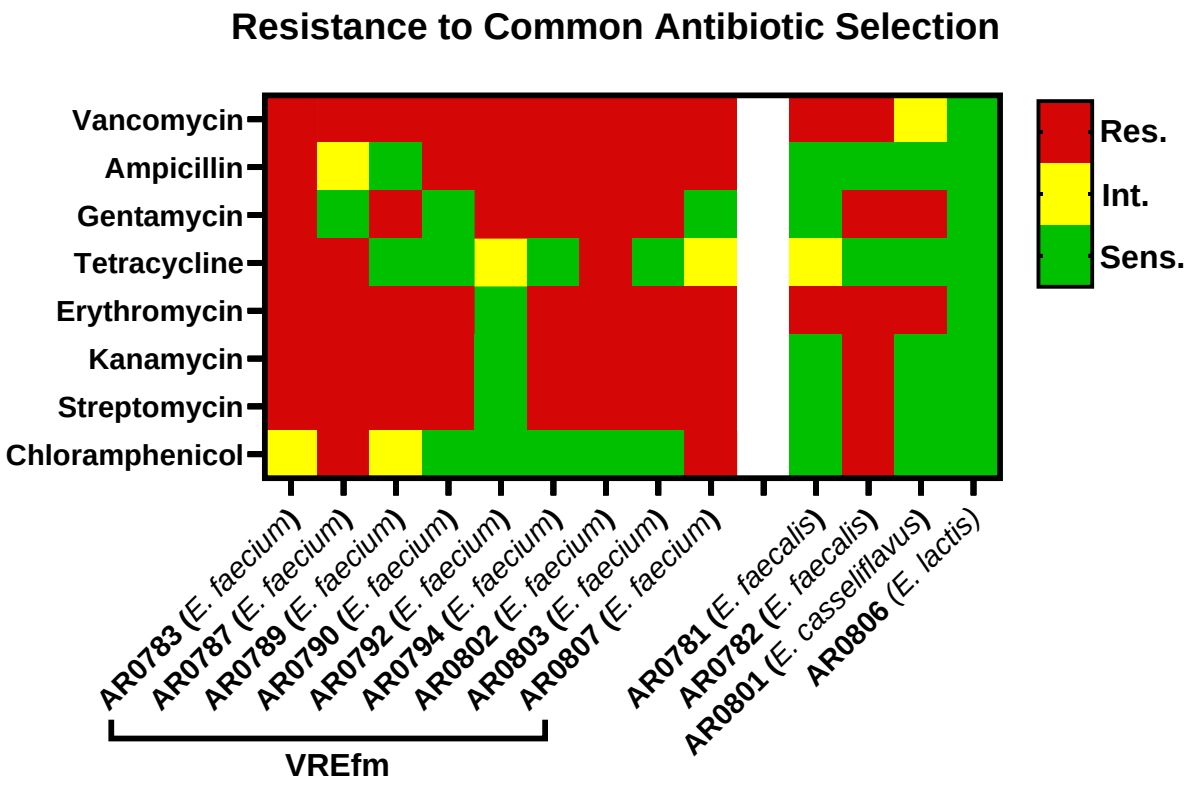


Figure 1. VREfm Isolates Are Resistant to Many Common Selection Agents. Heatmap of resistances to many common antibiotics for selection in Enterococci. All Isolates obtained from the CDC VRE AR Isolate Bank. Red indicates high-level resistance, yellow intermediate resistance, and green indicates sensitivity

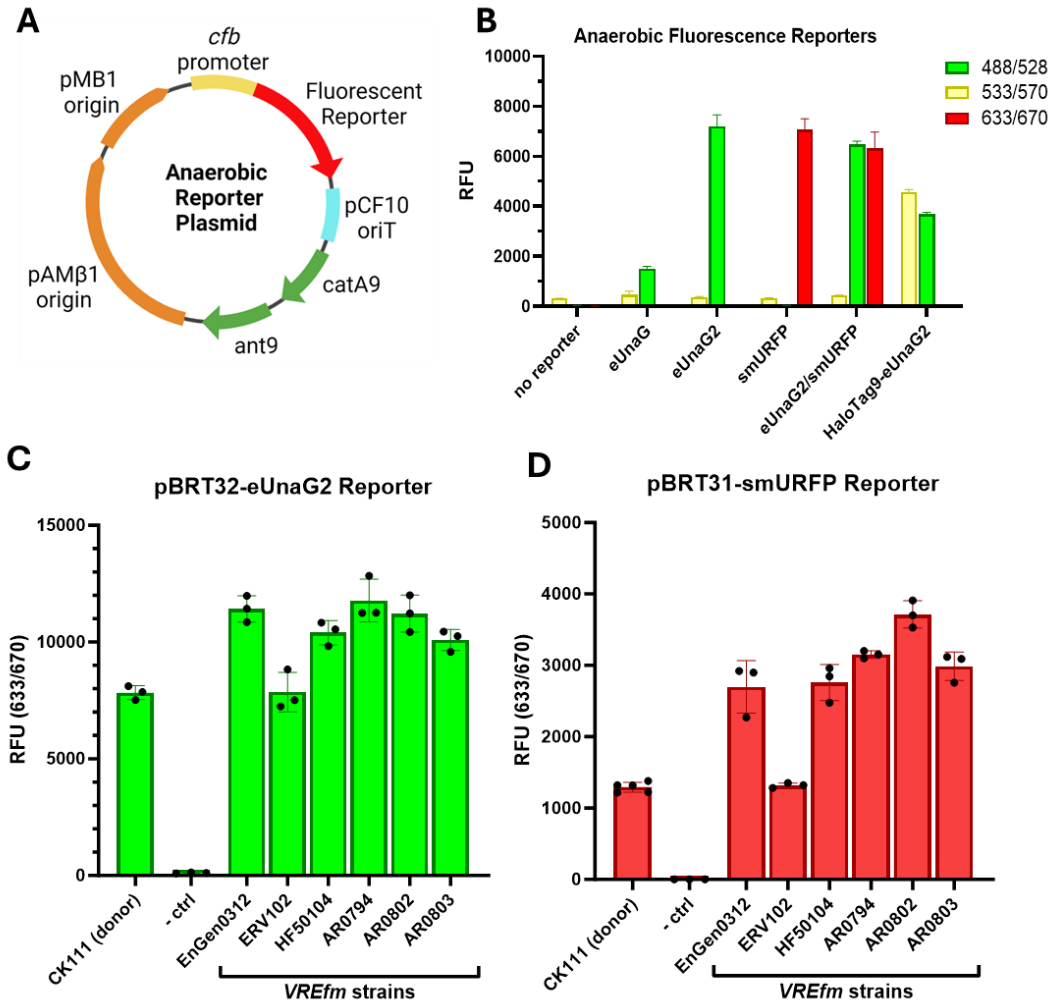


Figure 2. Anaerobic Fluorescent Reporters designed for VRE *E. faecium.* (**A**) Anaerobic Reporter Plasmid includes an E. coli origin (pMB1), an Enterococcus origin (pAMβ1), the S. *agalactiae cfb* promoter, anaerobic fluorescent reporter genes (eUnaG, eUnaG2, smURFP, eUnaG2/smURFP dual expression, or a HaloTag9-eUnaG2 fusion gene), the pCF10 origin of transfer (oriT), and antibiotic resistance markers (catA9/chloramphenicol resistance, ant9/spectinomycin resistance). (**B**) Relative fluorescence of reporter plasmids in conjugal donor strain *E. faecalis* CK111. Two independent transformants are represented for each group. (**C**) and (**D**) Relative fluorescence of VREfm strains transformed with eUnaG2 or smURFP reporter plasmids. Fluorescence measures performed after overnight growth in BHI supplemented with 5nM biliverdin and 25nM bilirubin. Cultures were centrifuged at 6,000g and pellets resuspended in BHI prior to fluorescence readings on a BioTek Synergy H1 microplate reader. For fluorescence detection, 2-4 independent transformants were analyzed. Data plotted as mean with SD in Graphpad Prism 10.4. Plasmid schematic created with

BioRender.com.

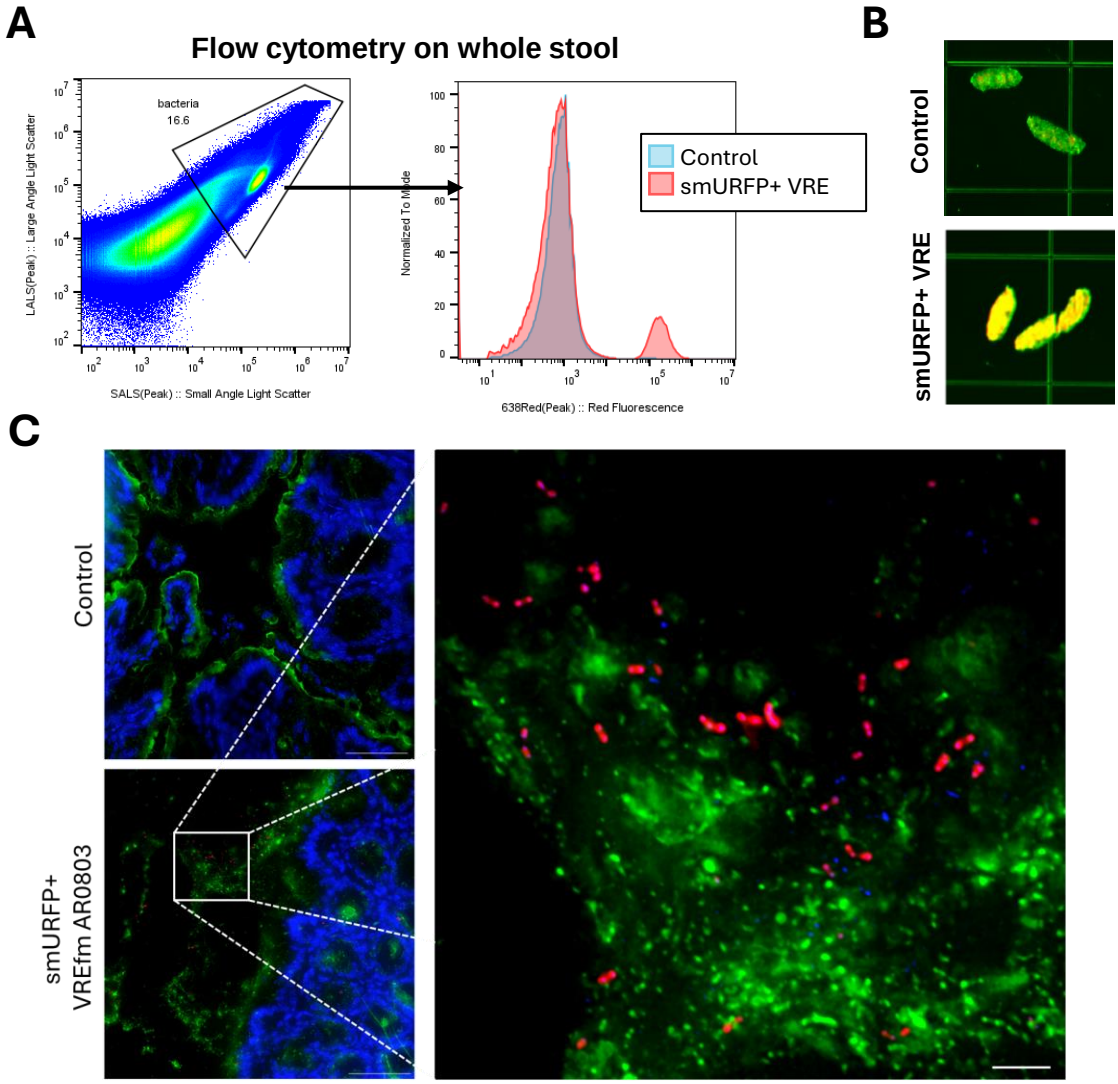
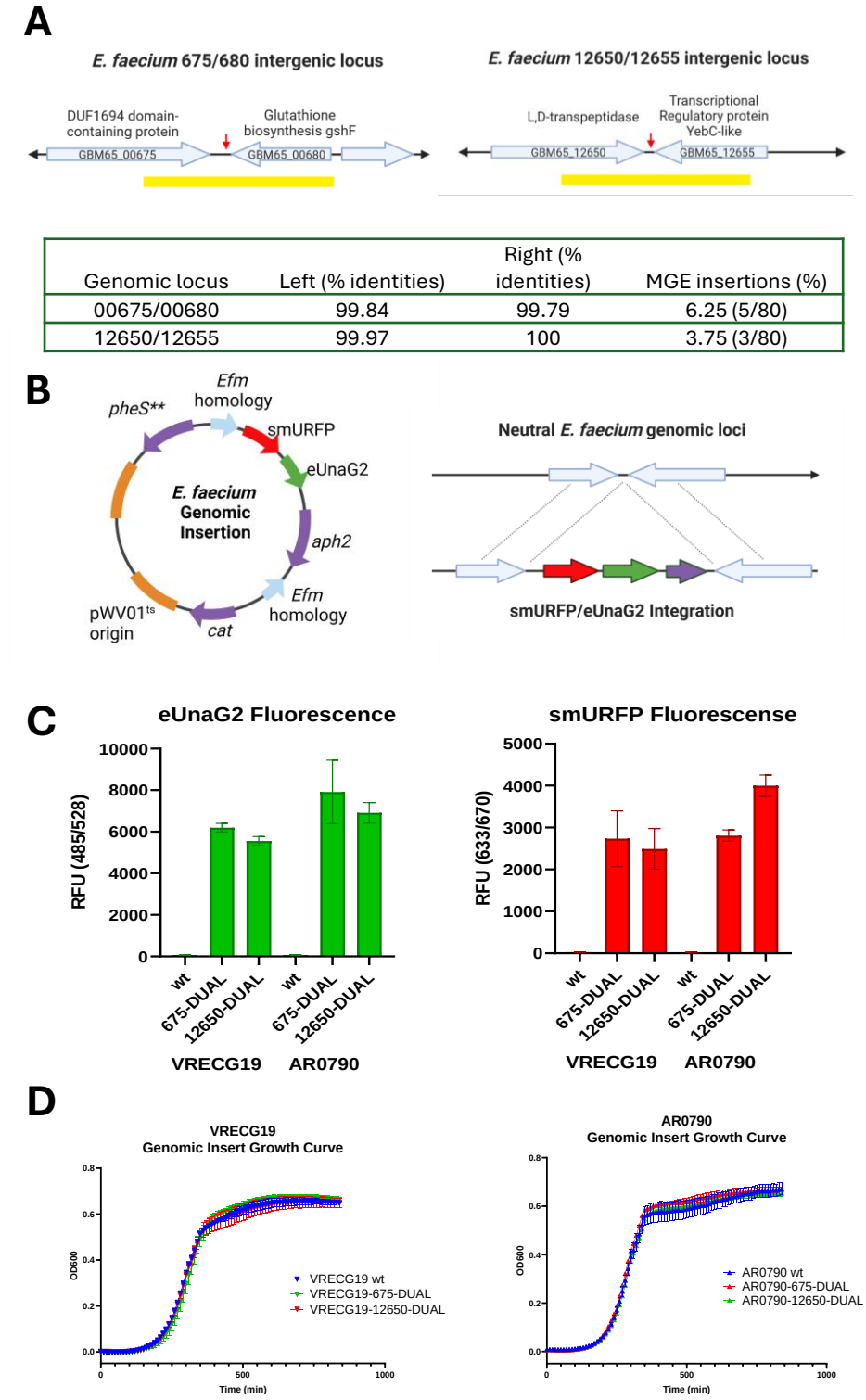


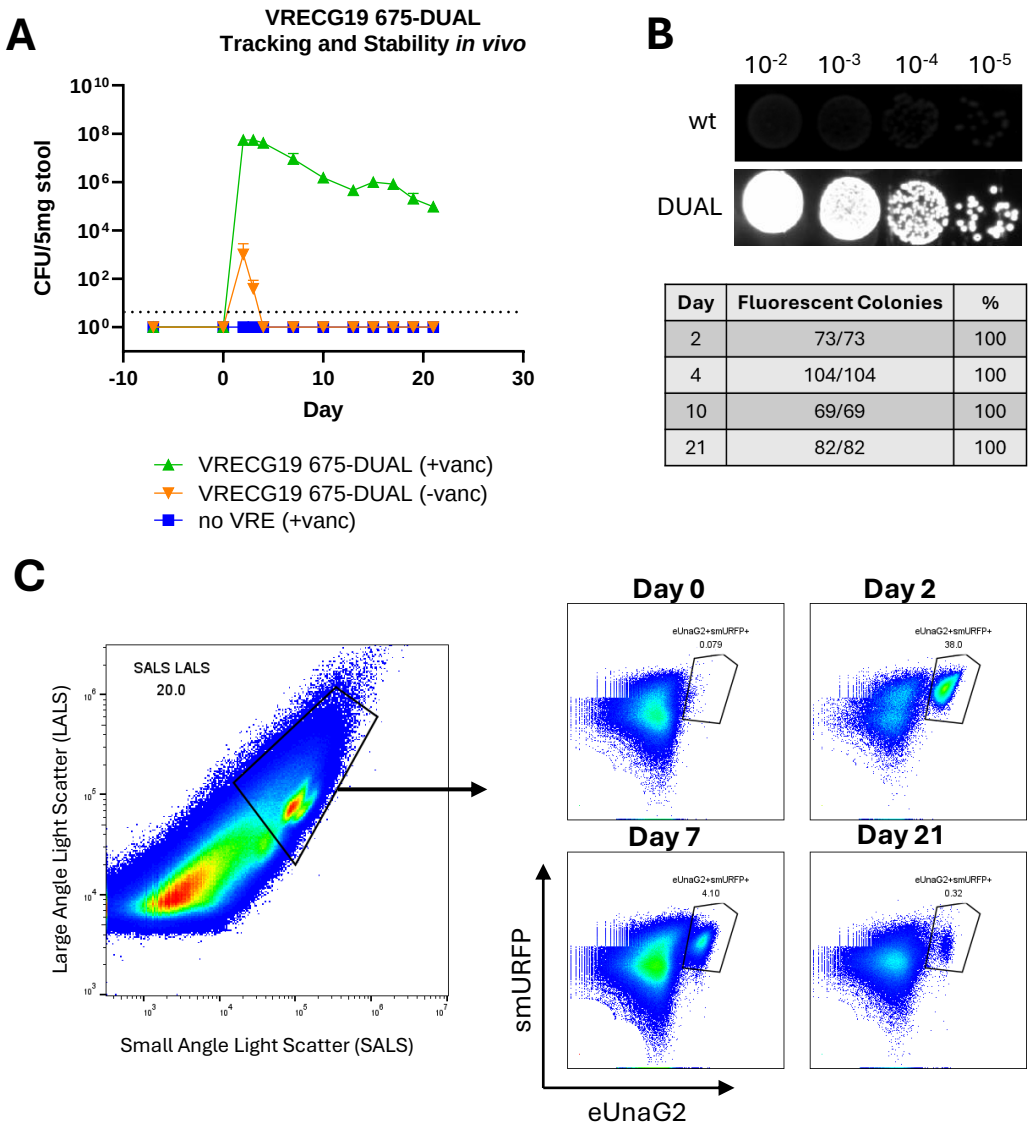
Figure 3. Tracking Anaerobic VREfm Fluorescence in Mice. 6-8 week old female C57Bl/6 mice (Taconic Biosciences) were placed on Vancomycin (0.5mg/mL in water) for 48 hours prior to inoculation with $1x10^8$ CFU *E. faecium* AR0803 containing pBRT31 (smURFP reporter). (**A**) Fresh stool from mice was collected 24 hours post inoculation (hpi), resuspended in PBS at 10% w/v, 40uM filtered, and run on a flow cytometer (ApogeeFlow MicroPLUS). (**B**) Whole stool fluorescence at 24 hpi (BioRad ChemiDoc MP, green indicates 488nm with 532/28 filter, red indicates 647nm with 700/50 filter) (**C**) Confocal microscopy of 10µM unfixed intestinal sections stained with wheat germ agglutinin (WGA) AF555 and DAPI.



Genomic locus	Left (% identities)	Right (% identities)	MGE insertions (%)
00675/00680	99.84	99.79	6.25 (5/80)
12650/12655	99.97	100	3.75 (3/80)

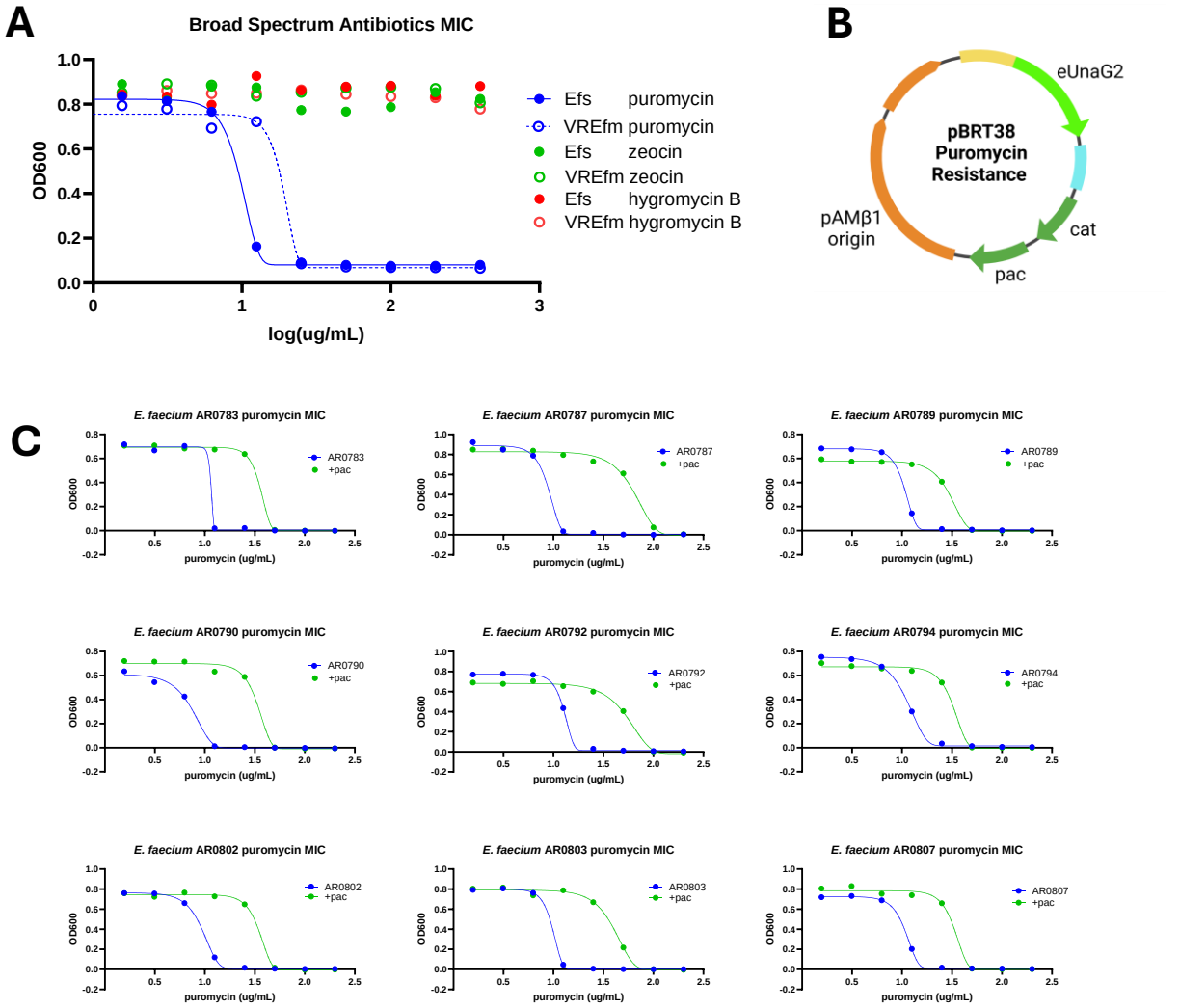
Figure 4. Neutral Genomic Integration Sites in *E. faecium.* (**A**) Schematic of 675/680 and 12650/12655 insertion loci (*E. faecium* VRECG19-10-S reference sequence; Genbank: WEFX01000001.1). The 675/680 intergenic locus, situated between a

glutathione biosynthesis bifunctional protein (gshF) and an uncharacterized conserved protein (YueI, DUF2278 family), showed high transcriptional activity[31] and strong conservation across VREfm strains. Transposon insertions in 5 (6.25%) of 80 sequenced VREfm isolates suggested minimal fitness impact. The 12650/12655 intergenic locus, between an L,D-transpeptidase and a probable transcriptional regulatory protein (YebC), demonstrated moderate transcriptional activity and 3 (3.75%) natural transposon insertion frequency across 80 isolates. Table indicates sequence conservation on the left and right homology arms and intergenic mobile genetic element (MGE) insertions detected in panel of 80 clinical *E. faecium* isolates. Red arrows indicate location of MGE insertions detected in panel of 80 isolates. Yellow bars indicate homology region used in genomic insertion plasmids. (**B**) Plasmid map and schematic of recombination for pBRT48 and pBRT62 genomic insertion plasmids. Plasmids contain 1) homology arms for the 675/680 locus (pBRT48) or the 12650/12655 locus (pBRT62), 2) anaerobic fluorescent reporter genes to be inserted, 3) gentamicin resistance gene, 4) pWV01 temperature sensitive origin, 5) chloramphenicol resistance gene (cat), and 6) *pheS*** counterselection to ensure plasmid loss. (**C**) eUnaG2 and smURFP fluorescence reporter activity of two VREfm strains (VRECG19 and AR0790) with genomic insertions in the two insertion loci. Overnight growth in BHI supplemented with 5nM biliverdin and 25nM bilirubin. Cultures were centrifuged at 6,000g and pellets resuspended in BHI prior to fluorescence readings on a BioTek Synergy H1 microplate reader. (**D**) Overnight growth curves of wild-type, 675-DUAL, or 12650-DUAL genomic insertions of two VREfm strains (VRECG19 and AR0790). Growth curves were performed in a 96-well plate in triplicate on a BioTek Synergy H1 microplate reader overnight at 37C in an anaerobic chamber (Coy Laboratory Products). Data plotted as mean with SD in Graphpad Prism 10.4.

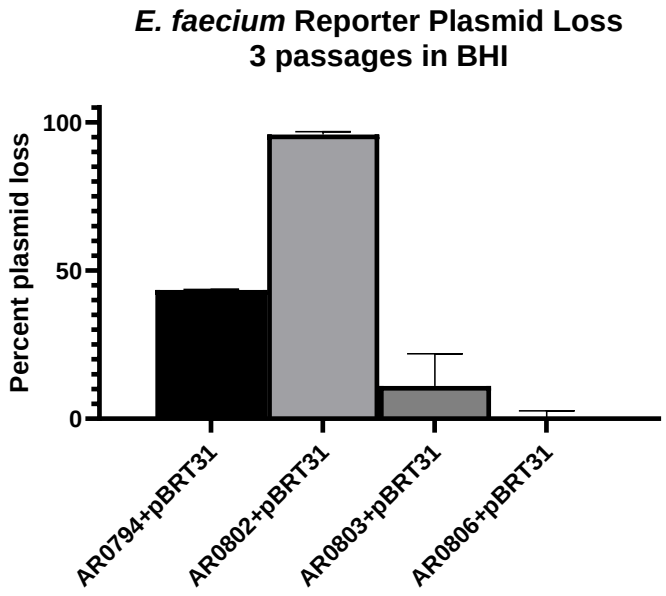


Day	Fluorescent Colonies	%
2	73/73	100
4	104/104	100
10	69/69	100
21	82/82	100

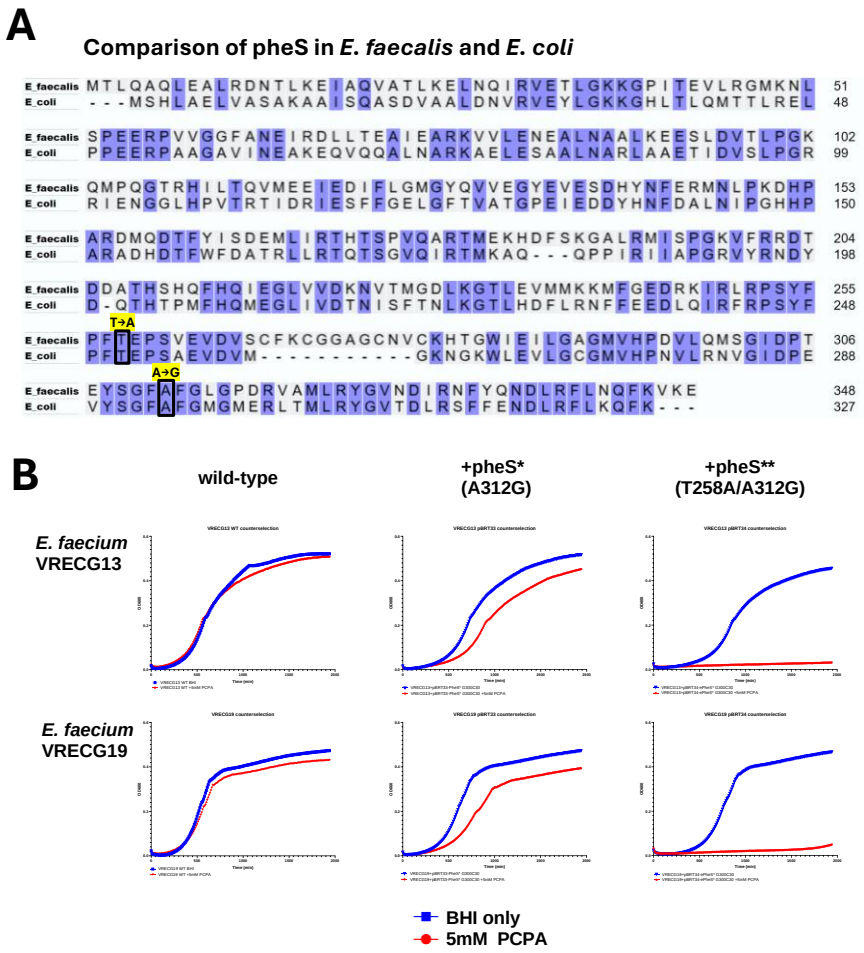
Figure 5. Long-term Tracking of Dual-Fluorescent VREfm in Mice. 6-8 week old female C57Bl/6 mice (5 per group, Taconic Biosciences) were placed on Vancomycin (0.5mg/mL in water) for 7 days prior to inoculation with $1x10^8$ CFU *E. faecium* VRECG19-675-DUAL. (**A**) Fresh stool from mice was collected at indicated days post inoculation (dpi), resuspended in PBS at 10% w/v, serially diluted, and plated onto selective agar (BHI supplemented with 30ug/mL Vancomycin and 20ug/mL Meropenem) containing biliverdin (5nM). (**B**) Colonies were confirmed to be VRECG19-675-DUAL strain by fluorescent imaging of whole agar plates (BioRad ChemiDoc MP, 647nm with 700/50 filter), with numbers of fluorescent colonies enumerated at days 2, 4, 10, and 21) (**C**) Representative data of resuspended stool from 0, 2, 7, and 21 dpi, 40uM filtered, and run on a flow cytometer (ApogeeFlow Micro-PLUS)



Supplemental Figure 1. Puromycin as Universal Selection in VREfm. (**A**) Minimum Inhibitory Concentration (MIC) assays of puromycin, zeocin, and hygromycin B were performed on *E. faecalis* (Efs) OG1Rf, and VREfm strain TX0082. (**B**) Plasmid schematic of pBRT38 plasmid containing eUnaG2 reporter, a chloramphenicol resistance gene, a codon-optimized *pac* gene under control of a duplicate chloramphenicol promoter. (**C**) MIC assays for puromycin of 9 VREfm strains from CDC AR Isolate Bank, wild-type are in blue, and transformants expressing *pac* from pBRT38 are in green.



Supplemental Figure 2. Plasmid Instability in VREfm. Four *E. faecium* strains from the CDC AR Isolate Bank were transformed with plasmid pBRT31 (smURFP expressing), maintained under strict selection (500ug/mL spectinomycin, 30ug/mL chloramphenicol) prior to passaging (1:1000) single colony isolates 3 times in BHI without selection. Cultures were then serially diluted and plated on BHI agar alone or BHI agar supplemented with 500ug/mL spectinomycin and 30ug/mL chloramphenicol. Colonies were enumerated and the percent plasmid loss calculated. Assays were performed in triplicate, and the percentage of colonies that maintained resistance plotted as a mean with S.D.



Supplemental Figure 3. Improved *PheS* Counterselection in *E. faecium*** (**A**) Partial amino acid sequence comparison of *E. coli* and *E. faecalis pheS*, highlighting mutations to generate *pheS**(A312G) and *pheS***(T258A/A312G) counterselection genes. (**B**) Growth curves of three E. faecium strains of wild-type and transformants containing plasmid pBRT33(*pheS**) or pBRT34 (*pheS***). Transformants were grown in media containing BHI only or 5mM p-chlorophenylalanine (PCPA). Sequence alignment generated using Uniprot Align tool (https://www.uniprot.org/align).