File S1. Expanded Materials and Methods.

Bhardwaj, et al. Chlorhexidine induces VanA-type vancomycin resistance genes in enterococci

Electroporation of *E. faecium*. A modification of a previously published protocol (1) was used. After overnight growth, *E. faecium* cultures were diluted 10-fold into 50 mL of pre-warmed BHI broth. The cultures were grown at 37°C without shaking until the OD_{600nm} reached 0.6-0.7. The cells were collected by centrifugation at 10,000 rpm at 4°C for 10 mins. The pellet was resuspended in 10 mL of filter-sterilized lysozyme buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 50 mM NaCl) supplemented with 25 µg/mL lysozyme and 83 µL of a 2.5 KU/mL mutanolysin stock. The cells were incubated at 37°C for 30 mins, then pelleted as above and washed 3X with ice-cold filter-sterilized electroporation buffer (0.5 M sucrose and 10% glycerol). The cells were resuspended in a final volume of 500 µL ice-cold electroporation buffer, aliquoted, and stored at -80°C. For transformation, 100-150 µL cells were transformed by electroporation at 2.5 kV with a minimum of 1 µg of plasmid DNA. Immediately after electroporation, 1 mL ice-cold BHI broth was added, and the cells were incubated at 30°C for 2.5 hours. The cells were then plated on BHI plates supplemented with appropriate antibiotics to select for transformants.

RNA isolation for RNA sequencing. *E. faecium* 1,231,410 cultures treated for 15 mins with 0X (control) or 1X MIC H-CHG were harvested and mixed with two volumes of RNAProtect Bacteria reagent (Qiagen) according to the manufacturer's recommendation. The pellet was resuspended in 500 µL IHB-1 solution (50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8.0) supplemented with 125 µL of a 50 mg/mL lysozyme stock and 25 µL of a 2.5 KU/mL mutanolysin stock and incubated at 37°C for 20 mins. RNA was isolated using RNA Bee reagent (Tel-Test) and treated with DNase I (Roche) for 2 hours at 37°C. The RNA samples

were again purified using RNA Bee. RNA integrity was verified by agarose gel electrophoresis. Absence of DNA contamination was confirmed by PCR using 100 ng of RNA and primers for amplification of a 16S ribosomal RNA (rRNA) gene (Table S1).

Generation of pPB101. A 946 bp amplicon containing the chloramphenicol (*cat*) resistance cassette from pLT06 (2) was cloned into the pTCV-*lac* plasmid at the PstI site, resulting in the plasmid pPB101.

Generation of pHA101. For generation of pHA101, the origin of transfer (*oriT*) from plasmid pHOU2 (3) was amplified (Table S1). The 292 bp product and pLT06 (2) were digested with BamHI and PstI, ligated, and propagated in *E. coli* EC1000 (4).

***E. faecium* 1,231,410 β-galactosidase assays.** *E. faecium* PB103 and PB104 were grown overnight in BHI broth with chloramphenicol and diluted next day to an OD_{600nm} of 0.05 in fresh BHI broth with chloramphenicol. Cultures were incubated with shaking at 100 rpm. At an OD_{600nm} of 0.4-0.5, 25 mL of the culture was added to 25 mL of BHI-chloramphenicol containing either vancomycin (2 µg/mL final concentration; positive control) or different concentrations of H-CHG, such that final concentrations of 0X, 1/4X, 1/2X or 1X MIC H-CHG were attained. 100 µL of each culture was sampled after 0, 30, 60, 90 and 120 mins of incubation. The cells were permeabilized using 0.1% SDS and chloroform, and β-galactosidase activity was measured utilizing *ortho*-nitrophenyl-β-galactoside (ONPG) as previously described (5).

Generation of a VanX-hexahistidine fusion. A hexahistidine tag was added in-frame to the C-terminal end of VanX (EFTG_02040) by knock-in of DNA sequence into the *E. faecium* 1,231,410 genome. For this, 999 bases upstream and downstream of the *vanX* stop codon were amplified (Table S1). The two products were reamplified by overlap extension PCR, resulting in

a 2 kb product. The Arm1_rev and Arm2_for primers used for the reaction contained 21 complementary base pairs, such that an internal hexahistidine coding sequence was generated by overlap extension PCR. The product was digested with EcoRI, ligated with EcoRI-digested pHA101, and transformed into *E. coli* EC1000. The construct, pPB201, was sequence verified and electroporated into *E. faecium* 1,231,410. Temperature shifting at the non-permissible temperature of 42°C and counter-selection with *p*-chlorophenylalanine was followed according to a previously published protocol (2). The *E. faecium* 1,231,410 strain with a hexahistidine coding sequence inserted in-frame upstream of the *vanX* stop codon, referred to as strain PB221, was confirmed by PCR and DNA sequencing of the region of interest.

Protein isolation for assessment of VanX levels in *E. faecium* 1,231,410 cultures. For protein isolation, 50 mL culture was pelleted by centrifugation (8000 x *g* for 10 mins at 4°C). The pellet was washed with 1 mL of ice-cold wash buffer (0.1 M phosphate buffer, pH 7.0 and 1 M NaCl) and re-centrifuged. The pellet was again resuspended in 1 mL wash buffer supplemented with 50 µL of a 50 mg/mL lysozyme stock and 125 µL of a 2.5 KU/mL mutanolysin stock and incubated at 37°C for 2 hours. Roche anti-protease solution per the manufacturer's instructions and 45 mM imidazole were added. Next, the cells were lysed by bead-beating (eight times for 45 sec each), and the resulting lysate was centrifuged (13,300 x *g* for 25 mins at 4°C) to remove insoluble material. The protein concentration of the supernatant was measured using the Biorad protein reagent according to the manufacturer's instructions.

Generation of *vanR* and *vanRS* deletions in *E. faecium* 1,231,410. The *vanR* (EFTG_02044) and *vanRS* genes (EFTG_02043-44) were deleted in-frame utilizing plasmid pHA101**.** For this, 999 bp of flanking sequence upstream and downstream of the regions of interest were amplified and digested with BamHI, ligated with BamHI-digested pHA101, and propagated in *E. coli* EC1000. The deletion constructs pPB202 (Δ*vanR*) and pPB203 (Δ*vanRS*) were confirmed by

DNA sequencing and electroporated into *E. faecium* 1,231,410. The *E. faecium* 1,231,410 Δ*vanR* (PB222) and Δ*vanRS* (PB223) deletion mutants were obtained by temperature shift and counterselection, as described above. The deletion mutants were confirmed by DNA sequencing of the region of interest. PB222 retains 3 amino acids from the N-terminus and 8 amino acids from the C-terminus of VanR, with ~95% of the *vanR* gene deleted. PB223 encodes 3 amino acids from the N-terminus of VanR fused with 3 amino acids from the C-terminus of VanS, with ~99% of *vanR* and *vanS* genes deleted.

1. **Bae T, Kozlowicz B, Dunny GM.** 2002. Two targets in pCF10 DNA for PrgX binding: their role in production of Qa and *prgX* mRNA and in regulation of pheromone-inducible conjugation. *Journal of Molecular Biology* **315:**995-1007.
2. **Thurlow LR, Thomas VC, Hancock LE.** 2009. Capsular polysaccharide production in *Enterococcus faecalis* and contribution of CpsF to capsule serospecificity. *Journal of Bacteriology* **191:**6203-6210.
3. **Panesso D, Montealegre MC, Rincon S, Mojica MF, Rice LB, Singh KV, Murray BE, Arias CA.** 2011. The *hylEfm* gene in pHylEfm of *Enterococcus faecium* is not required in pathogenesis of murine peritonitis. *BMC Microbiology* **11:**20.
4. **Leenhouts K, Buist G, Bolhuis A, ten Berge A, Kiel J, Mierau I, Dabrowska M, Venema G, Kok J.** 1996. A general system for generating unlabelled gene replacements in bacterial chromosomes. *Molecular & General Genetics* **253:**217-224.
5. **Miller J.** 1972. p. p. 352–355, Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Table S1: Primers used in this study.

Primer name	Sequence
1.Real time primers	
410 *clpX* for	GCAAAACGCTCGTTAGCTGT
410 *clpX* rev	CCAGACCCTGTAGGTCCGAT
410 *vanA* for	CCCAGCATTTTTCGCAACGA
410 *vanA* rev	TGCATGGCAAGTCAGGTGAA
502 *clpX* for	CCCTGTAGGTCCGATCAAGC
502 *clpX* rev	AAACGCTCGTTAGCTGTTGC
502 *vanA* for	AGTCAATAGCGCGGACGAAT
502 *vanA* rev	GCGGCACTGTTTCCCAATAC
933 *clpX* for	GTACGGCTGTAAGCCCCAAT
933 *clpX* rev	AGACGGTGCTTTGCTCTTCA
933 *vanA* for	GGCTCATCCTTCGGTGTGAA
933 *vanA* rev	GCACTGTTTCCCAATACCGC
TUH4-64 *clpX* for	GCTCGTTAGCTGTTGCCGTA
TUH4-64 *clpX* rev	CCAGACCCTGTAGGTCCGA
TUH4-64 *vanB* for	TGCATGGACAAATCACTGGC
TUH4-64 *vanB* rev	CAGGGTAGGTAAGCGTCCTC
HIP11704 *clpX* for	TTTGCAATCGCTGATGCGAC
HIP11704 *clpX* rev	GATAATGCCCTTTTCCGCACG
HIP11704 *vanA* for	AAGTCAATAGCGCGGACGAA
HIP11704 *vanA* rev	GCGGCACTGTTTCCCAATAC
V583 *clpX* for	TTTCAACATCTTCCCCTACATAACC
V583 *clpX* rev	AGGTAAAACTTTCTTGGCTCAAACG
V583 *vanB* for	CCCCGGATAGGAAAACGCAT
V583 *vanB* rev	CTGTATCGCACCATCCTCCC
2. Construction of *vanH* pTCV-*lac-cat*	
410vanH for_EcoRI	ATGCGAATTCATAATTTTTTAGGAAAATCTCAA
410*vanH* rev_BamHI	ATGCGGATCCACAGTAATGCCGATGTTATTCATAA
pTCV MCS for	GTTGAATAACACTTATTCCTATC
pTCV MCS rev	CTTCCACAGTAGTTCACCACC

3. Construction of *vanX* Histag

vanX flanking arm1 for_EcoRI	ATGCATGAATTCCGGTTGTGCGGTATTGGGAAA
vanX flanking arm1 rev_Histag	TCAGTGGTGGTGGTGGTGGTGTTTAACGGGGAAATCAAA
vanX flanking arm2 for_Histag	CACCACCACCACCACCACTGAACTTTTAACCGTTGCACG
vanX flanking arm2 rev_EcoRI	ATGCATGAATTCCGCATTTTCTTCTATCCAC
vanX seq_for	ACACTTTCTTGGCGAACAGGA
vanX seq_rev	TTCCCGAACTGATTGACCGC

4. Construction of *vanRS* Knockout

vanRS flanking arm1 for_BamHI	ATGCATGGATCCATAAAAGCCTGTCTCTGTTCC
vanRS flanking arm1rev_PstI	ATGCATCTGCAGATCGCTCATAGTTATCACCCT
vanRS flanking arm2 for_PstI	ATGCATCTGCAGAGGTCCTAAGAGATGTATATA
vanRS flanking arm2 rev_BamHI	ATGCATGGATCCTGGTAATCTTGTCAAATCTTT
vanRS seq_for	ATAAAAGCCTGTCTCTGTTCC
vanRS seq_rev	TGGTAATCTTGTCAAATCTTT

5. Construction of *vanR* Knockout

vanR flanking arm1 for_BamHI	ATGCATGGATCCATAAAAGCCTGTCTCTGTTCC
vanR flanking arm1 rev_SphI	ATGCATGCATGCATCGCTCATAGTTATCACCCT
vanR flanking arm2 for_SphI	ATGCATGCATGCGTTGGTTATAAAATTGAAAA
vanR flanking arm2 rev_BamHI	ATGCATGGATCCGAACGAGCATTGTCCAGCCTA
vanR seq_for	ATAAAAGCCTGTCTCTGTTCC
vanR seq_rev	GAACGAGCATTGTCCAGCCTA

6. DNA contamination check primers

16S universal check_for	AGAGTTTGATCCTGGCTCAG
16S universal check_rev	GGTTACCTTGTTACGACTT

7. Amplification of chloramphenicol (*cat*) gene and *oriT*

cat for_PstI	ATGCTCTGCAGAAGCGAACGAAAAACAATTGC
cat rev_PstI	ATGCTCTGCAGAAAATGTGGTTGTTATACGTTC
*oriT*_for BamHI	TACGAAGGATCCCGCCAACGAATCGCCAAC
*oriT*_rev PstI	CACTAGCTGCAGCGTTTCTTTGAATAGGACGTT

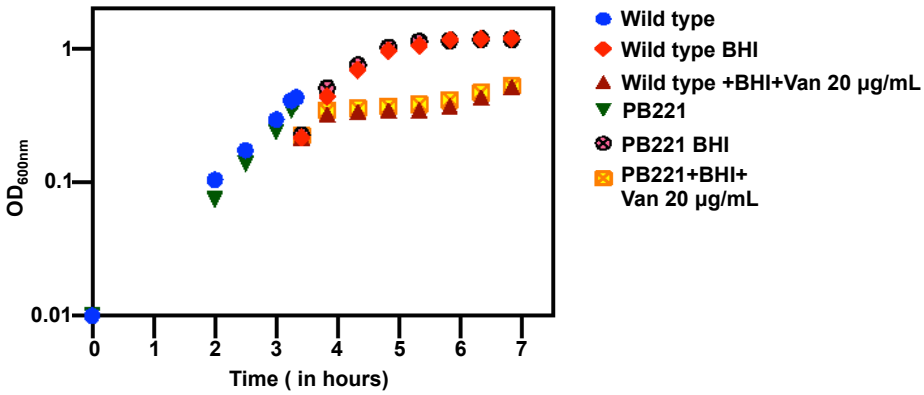

Figure S1. Representative growth curve of wild-type *E. faecium* 1,231,410 and PB221. Overnight cultures of each strain were diluted in BHI and grown at 37°C in BHI with shaking at 100 rpm until the OD_{600nm} reached 0.3-0.4. 25 mL culture from each strain was added to an equal volume of pre-warmed BHI with or without vancomycin supplementation at 20 µg/mL final concentration. The cultures were incubated at 37°C with shaking, and the OD_{600nm} was monitored for 3 hours.

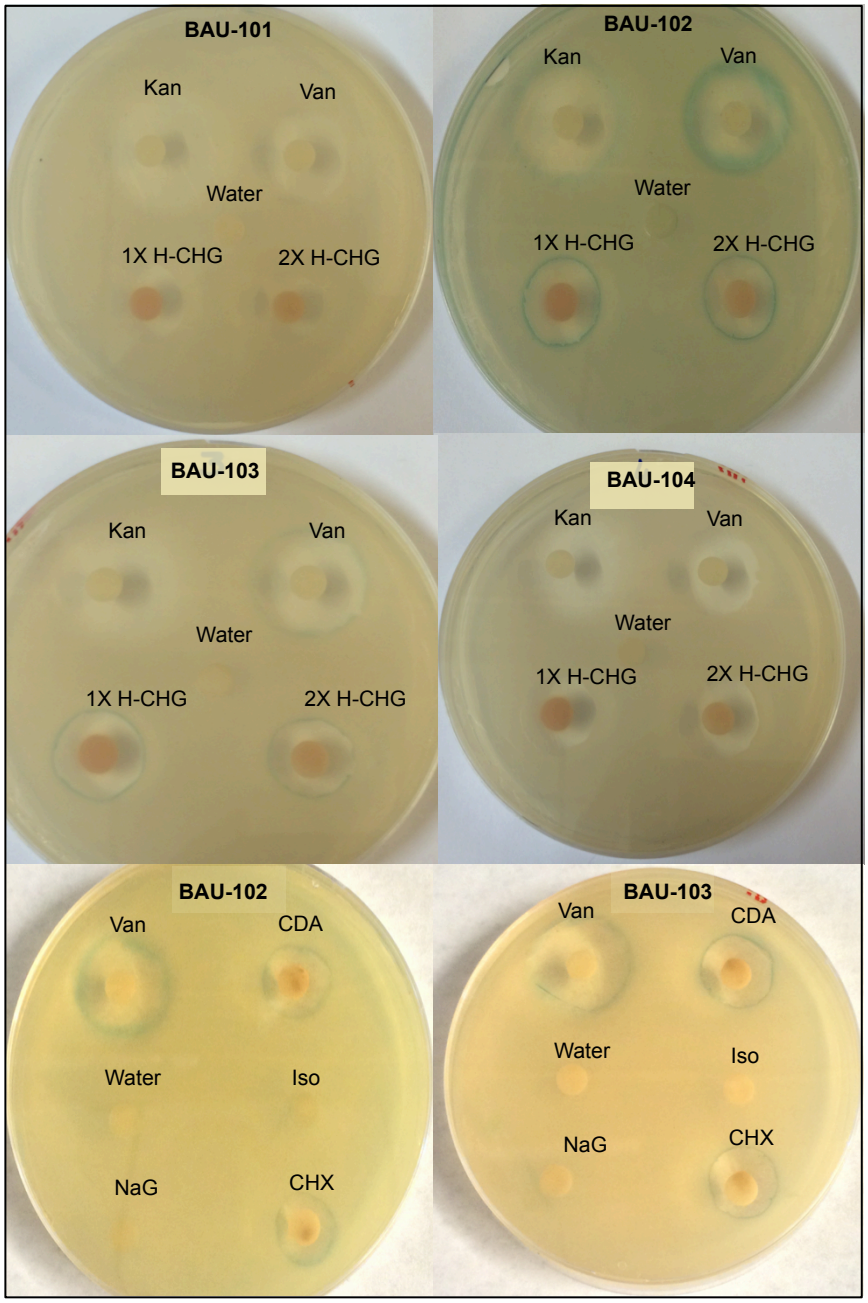

Figure S2. *Bacillus subtilis* reporter strain results. For the qualitative LacZ assays, 300 µL of overnight culture of the reporter strain was spread on LB+X-gal (indicator dye; 40 µg/mL). Discs containing test compounds were placed on plates. The plates were incubated overnight at 37°C and transferred to 4°C next day for additional color development. Positive induction of *vanH* promoter would be indicated by the formation of blue halo around the zone of inhibition. Compounds tested, Kan: Kanamycin (50 mg/mL) 5 µL; Van: Vancomycin (50 mg/mL) 5 µL; water 5 µL; 1X MIC H-CHG 6.10 µL; 2X MIC H-CHG 12.20 µL. To find out which component of H-CHG induced the *vanH* promoter, different component of H-CHG were tested on reporter strains BAU-102 and BAU-103. 5 µL of each of the following compounds were tested. Van: vancomycin (positive control); CDA: chlorhexidine diacetate salt; water; Iso: isopropanol; NaG: sodium gluconate; CHX: chlorhexidine.