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Chlorhexidine Induces VanA-Type Vancomycin Resistance Genes in Enterococci

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Chlorhexidine is a bisbiguanide antiseptic used for infection control. Vancomycin-resistant *E. faecium* (VREfm) is among the leading causes of hospital-acquired infections. VREfm may be exposed to chlorhexidine at supra- and subinhibitory concentrations as a result of chlorhexidine bathing and chlorhexidine-impregnated central venous catheter use. We used RNA sequencing to investigate how VREfm responds to chlorhexidine gluconate exposure. Among the 35 genes upregulated ≥10-fold after 15 min of exposure to the MIC of chlorhexidine gluconate were those encoding VanA-type vancomycin resistance (*vanHAX*) and those associated with reduced daptomycin susceptibility (*liaXYZ*). We confirmed that *vanA* upregulation was not strain or species specific by querying other VanA-type VRE. VanB-type genes were not induced. The *vanH* promoter was found to be responsive to subinhibitory chlorhexidine gluconate in VREfm, as was production of the VanX protein. Using *vanH* reporter experiments with *Bacillus subtilis* and deletion analysis in VREfm, we found that this phenomenon is VanR dependent. Deletion of *vanR* did not result in increased chlorhexidine susceptibility, demonstrating that *vanHAX* induction is not protective against chlorhexidine. As expected, VanA-type VRE is more susceptible to ceftriaxone in the presence of sub-MIC chlorhexidine. Unexpectedly, VREfm is also more susceptible to vancomycin in the presence of subinhibitory chlorhexidine, suggesting that chlorhexidine-induced gene expression changes lead to additional alterations in cell wall synthesis. We conclude that chlorhexidine induces expression of VanA-type vancomycin resistance genes and genes associated with daptomycin nonsusceptibility. Overall, our results indicate that the impacts of subinhibitory chlorhexidine exposure on hospital-associated pathogens should be further investigated in laboratory studies.

Enterococcus faecium and *Enterococcus faecalis* are Gram-positive bacteria and gastrointestinal tract colonizers that opportunistically colonize wounds and the bloodstream, causing life-threatening infections, including bacteremia and endocarditis (1, 2). They are particularly associated with central-line associated bloodstream infection (CLABSI), a type of hospital-acquired infection (HAI) that arises from central venous catheter use. Enterococci are associated with 18% of CLABSIs in the United States (3).

Of particular concern for CLABSI treatment are vancomycin-resistant enterococci (VRE), which are resistant to the glycopeptide antibiotic vancomycin. Vancomycin forms complexes with the terminal D-alanyl–D-alanine (D-Ala–D-Ala) residues of peptidoglycan precursors, thereby halting peptidoglycan synthesis (4, 5). VanA- and VanB-type VRE have an alternate pathway of cell wall synthesis due to their acquisition of transposons containing vancomycin resistance genes. The genes enable enterococci to form modified peptidoglycan precursors that terminate in D-alanyl–D-lactate (D-Ala–D-Lac) instead of D-Ala–D-Ala (6–8). Vancomycin has a lower affinity for D-Ala–D-Lac termini (9), and cross-links in the cell wall can be formed using these precursors. By this mechanism, the enterococcal cell wall becomes highly resistant to the action of vancomycin.

To attempt to reduce the number of hospital-acquired infections, including those caused by VRE, infection control practices are implemented by health care facilities. Chlorhexidine is a bisbiguanide antiseptic (10) that is incorporated into a number of infection control products, including chlorhexidine- and silver-impregnated central venous catheters (11, 12). The practice of chlorhexidine bathing is recommended for all acute-care hospitals to reduce CLABSI occurrence (13). For chlorhexidine bathing, patients are bathed daily with a no-rinse chlorhexidine preparation or chlorhexidine-impregnated washcloths (14). The chlorhexidine remains on the skin, providing an antimicrobial coating that is replenished with each bathing. Chlorhexidine is amphipathic, and it likely interacts with both phospholipids and proteins on the bacterial cell surface (15, 16). Its interaction with the membrane is reported to be similar to that of antimicrobial peptides (15). These interactions disrupt membrane integrity and potential, leading to leakage of cytoplasmic constituents; at high chlorhexidine concentrations, cytoplasm congealing and complete breakdown of the cell membrane occur, conferring a bactericidal effect (17–19). For *Bacillus subtilis*, a rod-shaped Gram-positive bacterium, chlorhexidine at the MIC induces the formation of dented spots on the cell surface near the cell poles, leading to the hypothesis that chlorhexidine preferentially interacts with anionic lipids located at the *B. subtilis* cell poles (20).

A recent clinical trial that reported no impact of chlorhexidine bathing on hospital-acquired infection occurrence (21) raised concerns about the effects of chlorhexidine bathing on hospital-associated pathogens, including selection for reduced chlorhexidine susceptibility and for cross-resistance to antibiotics in clinical use (22, 23). A recent study semiquantitatively evaluated chlo-

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
Bacterial strains		
E. faecium 1,231,410	Skin and soft tissue infection isolate; VanA-type VRE	35
E. faecium 1,231,502	Bloodstream isolate; VanA-type VRE	35
E. faecium 1,230,933	Wound isolate; VanA-type VRE	35
E. faecium TUH4-64	Human clinical isolate; VanB-type VRE	77
E. faecalis HIP11704	VanA-type VRE; coisolated with vancomycin-resistant *Staphylococcus aureus*	78
E. faecalis V583	Bloodstream isolate; VanB-type VRE	79
PB103	*E. faecium* 1,231,410 transformed with pPB101	This study
PB104	*E. faecium* 1,231,410 transformed with pPB102	This study
PB221	*E. faecium* 1,231,410 with hexahistidine coding sequence integrated upstream of *vanX* (EFTG_02040) stop codon	This study
PB222	*E. faecium* 1,231,410 Δ*vanR* (EFTG_02044)	This study
PB223	*E. faecium* 1,231,410 Δ*vanRS* (EFTG_02043-44)	This study
B. subtilis BAU-101	*B. subtilis* harboring *vanH*::*lacZ* cassette integrated into the *amyE* locus of chromosome	34
B. subtilis BAU-102	BAU-101 harboring *vanRS* cassette inserted 81 bp downstream of the *cat-86* promoter on plasmid pHB201	34
B. subtilis BAU-103	BAU-101 harboring *vanR* cassette inserted 81 bp downstream of the *cat-86* promoter on plasmid pHB201	34
B. subtilis BAU-104	BAU-101 harboring *vanS* cassette inserted 81 bp downstream of the *cat-86* promoter on plasmid pHB201	34
E. coli EC1000	*E. coli* cloning host; provides *repA* in *trans*; F^- *araD139* (*ara ABC-leu*)*7679 galU galK lacX74 rspL thi*; *repA* of pWV01 in *glgB*; Km	80
E. coli DH5α	*E. coli* cloning host; F^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *hsdR17*($r_K^-m_K^+$) λ^-	
E. coli BW23474	Cloning host for pTCV-*lac* and pPB101; Δ*lac-169 robA1 cre C510 hsdR514 endA recA1* Δ*uidA*::*pir-116*	81
Plasmids		
pLT06	Markerless, counterselectable exchange plasmid; confers chloramphenicol resistance	82
pHOU2	Derivative of pCJK47 in which the *erm*(C) gene was replaced by *aph-2′-ID* and *cat* was incorporated in the cloning site for allelic replacements; confers gentamicin resistance	83
pHA101	pLT06 plasmid with *oriT* from pHOU2 inserted at PstI; confers chloramphenicol resistance	This study
pTCV-*lac*	Expression vector for Gram-positive bacteria; confers kanamycin and erythromycin resistance	33
pPB101	pTCV-*lac-cat*; expression vector for Gram-positive bacteria; confers kanamycin, erythromycin, and chloramphenicol resistance	This study
pPB102	pPB101 containing 248-bp EcoRI/BamHI-digested *vanH* (EFTG_02042) promoter region	This study
pPB201	pHA101 containing a 2.043-kb EcoRI/EcoRI-digested fragment flanking upstream and downstream of *E. faecium* 410 *vanX* gene EFTG_02040	This study
pPB202	pHA101 containing a 2.028-kb BamHI/BamHI-digested fragment flanking upstream and downstream of *E. faecium* 410 *vanR* EFTG_02044	This study
pPB203	pHA101 containing a 2.028-kb BamHI/BamHI-digested fragment flanking upstream and downstream of *vanRS* EFTG_02043-44	This study

rhexidine levels on the skin of 20 patients pre- and post-chlorhexidine bathing, finding that the levels varied depending on body site and time postbath (24). Levels within the reported range of chlorhexidine MIC for enterococci (25–29) were detected on patient skin (24). In another study, chlorhexidine susceptibilities were monitored for CLABSI enterococcal isolates obtained from hospital wards using chlorhexidine bathing (30). It was observed that the chlorhexidine MIC increased significantly in those isolates compared to CLABSI isolates from nonbathing wards (30). The results of both studies indicate that enterococci are exposed to subinhibitory chlorhexidine concentrations in clinical settings as a result of chlorhexidine bathing.

Motivated by studies indicating that VRE are exposed to subinhibitory levels of chlorhexidine, in this study, we used RNA sequencing to assess the global transcriptional responses of *E. faecium* 1,231,410, a VanA-type vancomycin-resistant *E. faecium* (VREfm) strain, to exposure to a chlorhexidine gluconate (CHG)-containing consumer product. To our knowledge, this is the first study to evaluate *E. faecium* global transcriptional response to an antiseptic. We observed a potent induction of VanA-type vancomycin resistance genes and genes associated with daptomycin resistance upon exposure to the MIC of CHG. Induction of vancomycin resistance genes by CHG was found to be dependent upon VanR, and resulted in increased susceptibility to ceftriaxone in the presence of subinhibitory CHG. Our results suggest that the long-term impact of chlorhexidine bathing on HAI pathogens such as VRE should be further investigated in laboratory studies.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are shown in Table 1. *E. faecium* and *E. faecalis* were cultured at 37°C on brain heart infusion (BHI) agar or in BHI broth without agitation unless otherwise stated. *Bacillus subtilis* was cultured at 37°C on lysogeny broth (LB) with agar or in LB broth with shaking at 225 rpm unless otherwise stated. *Escherichia coli* strains were cultured on LB. Antibiotics were used at the following concentrations: chloramphenicol, 15 μg/ml for *E. coli* and *E. faecium* and 34 μg/ml for *B. subtilis*, and erythromycin, 10 μg/ml for *B. subtilis*. Vancomycin concentrations used are stated for specific experiments below.

Routine molecular biology techniques. *E. faecium* genomic DNA (gDNA) was isolated using a previously published protocol (31). Electroporation of *E. faecium* is described in the supplemental material. Plasmids were purified using the Qiagen Miniprep kit. DNA fragments were puri-

fied using the Qiagen QIAquick PCR purification kit. *Taq* polymerase (New England BioLabs [NEB]) was used for routine PCRs. Phusion polymerase (Fisher) was used for cloning applications. Restriction endonuclease and T4 DNA ligase reactions were performed per the manufacturer's instructions (NEB). Routine DNA sequencing was performed by the Massachusetts General Hospital DNA core facility (Boston, MA). Primers used in this study are shown in Table S1 in the supplemental material.

MIC determinations. MICs were determined by broth microdilution. Twofold serial dilutions of drug were made with BHI broth in a 96-well microtiter plate. An overnight culture of bacteria was diluted to an optical density at 600 nm (OD_{600}) of 0.01, and 5 μl of the diluted culture was used to inoculate wells of the plate. The OD_{600} of the cultures was monitored every 30 min for 24 h using a microtiter plate reader (Synergy MX; Biotek). The MIC was defined as the lowest drug concentration at which the OD_{600} of the well matched the OD_{600} of the negative-control well (uninoculated BHI medium).

Growth kinetic assays. An over-the-counter chlorhexidine gluconate (CHG) product, Hibiclens (4% [wt/vol] CHG with 4% isopropyl alcohol solution, referred to as H-CHG here), was used for growth kinetic assays and RNA sequencing experiments. Overnight cultures of *E. faecium* 1,231,410 were diluted to an OD_{600} of 0.01 in BHI broth and incubated at 37°C with agitation at 100 rpm until the OD_{600} reached 0.4 to 0.5. Twenty-five milliliters of the culture was added to equal volumes of prewarmed BHI broth containing different concentrations of H-CHG such that final concentrations of 0×, 0.5×, 1× or 2× MIC of H-CHG were attained. OD_{600} values were monitored for 24 h. For viability counts, 100 μl of culture obtained at each time point was serially diluted in 1× phosphate-buffered saline, and appropriate dilutions were spread on BHI agar plates.

RNA sequencing. RNA was harvested from *E. faecium* 1,231,410 cultures, then treated with DNase, and verified for integrity (see the supplemental material). RNA samples were submitted for RNA sequencing to the Tufts University Core Facility (Boston, MA). Library preparation for Illumina HiSeq 2000 sequencing was performed using the Illumina TruSeq Stranded RNA Sample Preparation kit with RiboZero treatment for rRNA removal. The kit allowed for strand-specific transcript detection. Fifty base reads were obtained from single-end sequencing. The RNA sequencing experiment was independently performed twice.

RNA sequencing results were analyzed using the CLC Genomics Workbench version 7.5. Raw sequencing reads were mapped to *E. faecium* DO (GenBank accession number NC_017960) rRNA and tRNA genes using default parameters. Next, the remaining unassembled reads were mapped to the *E. faecium* 1,231,410 draft reference genome (whole-genome sequencing [WGS]; GenBank accession number NZ_ACBA00000000.1) using default parameters. Read mappings for control and test cultures were compared using the RNA-Seq analysis algorithm using default parameters. The gene expression values were quantified on the basis of RPKM (reads per kilobase of transcript per millions of reads mapped), and these values were compared between control and H-CHG treatment conditions to calculate fold change. Kal's Z-test was used to calculate *P* value. Genes upregulated in H-CHG-treated cultures with a fold change of ≥10 and *P* value of <0.05 in each of the two trials were considered for further analysis in this study.

RT-qPCR. One hundred nanograms of RNA was used to synthesize cDNA with Superscript II (Life Technologies) and random hexamers according to the manufacturer's instructions. RNase H (NEB) was added to remove RNA, and cDNA was purified using the QIAquick PCR purification kit (Qiagen). Five nanograms of cDNA was used as the template in quantitative reverse transcription-PCR (RT-qPCR) with primers to amplify internal regions of *vanA*, *vanB*, or *clpX* (see Table S1 in the supplemental material). Primers for RT-qPCR were designed using NCBI Primer-BLAST (32). RT-qPCR was performed with a Cepheid Smart Cycler and SYBR green I (Sigma-Aldrich). *vanA* and *-B* gene expression was internally normalized to *clpX*. Threshold cycle (C_T) values were used to calculate the fold change of *vanA* and *-B* gene expression between H-CHG-treated cultures and control cultures according to the formula FC = $2^{-(\Delta\Delta CT)}$, where $\Delta\Delta C_T$ = (C_T of *vanA* or *-B* in H-CHG-treated cultures − C_T of *clpX* in H-CHG-treated cultures) − (C_T of *vanA* or *-B* in control cultures − C_T of *clpX* in control cultures). The expression of *vanA* and *-B* in the control culture was set to 1. The relative fold changes in *vanA* and *-B* expression from two independent experiments (trials 1 and 2) were quantified.

Assessment of $vanH_A$ promoter activity in *E. faecium* 1,231,410. *E. faecium* 1,231,410 *vanH* promoter activity was evaluated using the expression plasmid pTCV-*lac* (33) modified to express chloramphenicol resistance (pPB101) (see the supplemental material). A 248-bp region containing the *vanH* promoter region was amplified from *E. faecium* 1,231,410 gDNA, digested, and ligated with EcoRI- and BamHI-digested pPB101, resulting in plasmid pPB102. pPB101 and pPB102 were introduced into *E. faecium* 1,231,410 via electroporation, resulting in strains PB103 and PB104, respectively. β-Galactosidase assays were performed to assess *vanH* promoter activity upon exposure to vancomycin and different concentrations of H-CHG (see the supplemental material). The activity was measured in duplicate for each time point, and the experiment was performed independently four times.

Assessment of VanX levels in *E. faecium* 1,231,410 cultures. 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, generating strain *E. faecium* PB221 (see the supplemental material). An overnight culture of *E. faecium* PB221 was diluted into BHI broth and incubated with shaking at 100 rpm until an OD_{600} of 0.4 to 0.5 was reached. The culture was split into BHI broth with different concentrations of H-CHG or vancomycin such that final concentrations of 0×, 1/4×, 1/2×, or 1× MIC of H-CHG or 20 μg/ml of vancomycin were attained. Cultures were sampled for analysis after 1.5 and 2 h of incubation. Total soluble protein was isolated from each sample as described in the supplemental material. Equal amounts (250 μg) of total soluble protein from each culture sample were loaded onto 100 μl of washed nickel-nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen). The beads were washed twice with 1 ml of wash buffer supplemented with 45 mM imidazole. The proteins and beads were incubated together for 2 h at 4°C. After incubation, the beads were centrifuged (13,300 × *g* for 2 min at room temperature) and washed twice with 1 ml of wash buffer supplemented with 75 mM imidazole to remove nonspecific proteins. Next, 6× SDS loading dye was added directly to the beads and boiled for 10 min vigorously. The samples were analyzed by 12% SDS-PAGE, and VanX protein levels were evaluated by Western blotting on a polyvinylidene difluoride (PVDF) membrane with an alkaline phosphatase-conjugated monoclonal anti-polyhistidine clone His-1 antibody (Sigma-Aldrich) and Western Blue stabilized substrate for alkaline phosphatase (Promega) per the manufacturer's instructions to confirm the presence of His-tagged VanX proteins. VanX protein levels were quantified by calculating the integrated density value (IDV) of the protein bands using the Alphaimager spot density tool.

***B. subtilis* reporter assays.** A previously developed *vanH* promoter reporter system in the heterologous host *Bacillus subtilis* 168 (34) was used to test *vanH* promoter responsiveness to specific components of H-CHG as well as the roles of *vanR* and *vanS* in induction. For qualitative β-galactosidase assays, 0.3 ml of an overnight culture of each reporter strain was spread on LB agar containing 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal; 40 μg/ml) with chloramphenicol for BAU-101, chloramphenicol and erythromycin for BAU-102, and erythromycin for BAU-103 and BAU-104. Paper discs containing different amounts of H-CHG (1× or 2× MIC) or 5 μl of a 40-mg/ml vancomycin stock (positive control) or water or a 40-mg/ml kanamycin stock (negative controls) were placed on the plates. The plates were incubated overnight at 37°C and on the next day were transferred to 4°C for the complete development of blue color around the discs. Chlorhexidine diacetate solution (Sigma-Aldrich), chlorhexidine powder (Sigma-Aldrich), sodium D-gluconate salt (Sigma-Aldrich), and isopropyl alcohol were also assessed for their abilities to induce the *vanH* promoter.

Assessment of *vanA* expression in *E. faecium* 1,231,410 Δ*vanR* and Δ*vanRS*. The *vanR* (EFTG_02044) and *vanRS* genes (EFTG_02043-44) were deleted in frame utilizing plasmid pHA101 (see the supplemental material). Broth microdilution in BHI broth was used to determine the vancomycin and H-CHG MICs for the *E. faecium* 1,231,410 Δ*vanR* and Δ*vanRS* strains. RNA was isolated from cultures treated with 0× and 1× MIC of H-CHG for 15 min as described above. RNA was also isolated from cultures treated with 50 μg/ml of vancomycin for 2 h. RT-qPCR was performed as described above to assess *vanA* and *clpX* expression.

Synergy assays. Broth microdilution was utilized to test for synergism between CHG and ceftriaxone or vancomycin. For synergy tests with ceftriaxone, 2-fold serial dilutions of a fresh 1-mg/ml stock of ceftriaxone disodium salt in water (TCI) for *E. faecalis* and 50 mg/ml for *E. faecium* were made in BHI broth (control), BHI broth supplemented with 2, 5, or 20 μg/ml of vancomycin (positive control), and BHI broth supplemented with different concentrations of H-CHG or chlorhexidine digluconate solution (Sigma-Aldrich; diluted to 5% prior to use) in 96-well microtiter plates. For synergy tests with vancomycin, 2-fold dilutions of a fresh 40-mg/ml vancomycin stock were made in BHI broth or BHI broth supplemented with different concentrations of H-CHG. Overnight cultures of *E. faecalis* and *E. faecium* were diluted to an OD_{600} of 0.01, and 5 μl of the diluted culture was used to inoculate wells of the plate. The OD_{600} of the cultures was measured after 24 h of incubation at 37°C.

Sequence accession number. Raw Illumina RNA sequencing data generated in this study are available in the Sequence Read Archive under accession number SRP065084.

RESULTS

Chlorhexidine MICs for enterococci used in this study. *E. faecium* 1,231,410 was isolated in 2005 and is a clade A1 skin and soft tissue infection isolate harboring VanA-type vancomycin resistance genes (35, 36). *E. faecium* 1,231,410 was the model strain used for our chlorhexidine experiments.

Previous studies have reported that *E. faecium* and *E. faecalis* chlorhexidine MICs range from 0.5 to 16 μg/ml and that chlorhexidine MICs for VRE and vancomycin-sensitive enterococci are similar (25–29). The over-the-counter chlorhexidine gluconate product Hibiclens (referred to as H-CHG here) was selected for our studies because we sought to evaluate a widely available chlorhexidine-containing consumer product. The H-CHG MIC for *E. faecium* 1,231,410 was determined to be a 1/8,192 dilution of H-CHG, corresponding to 4.9 μg/ml of chlorhexidine. H-CHG MICs for all enterococci queried (Table 1) ranged from 2.5 to 9.8 μg/ml. These values are in the range expected based on previous literature (25–29). For further confirmation, the MIC of a chlorhexidine digluconate solution from Sigma-Aldrich was determined for *E. faecium* 1,231,410 and found to be identical to the H-CHG MIC.

Growth kinetics of *E. faecium* 1,231,410 with H-CHG. Next, the growth kinetics of *E. faecium* 1,231,410 cultures exposed to different concentrations of H-CHG were studied. For these experiments, an exponentially growing culture of *E. faecium* 1,231,410 in BHI broth was split into flasks containing BHI with H-CHG such that final concentrations of 1/2× MIC, 1× MIC, and 2× MIC of H-CHG were attained. The OD_{600} of cultures exposed to 1× MIC and 2× MIC decreased compared to that of cultures not exposed to H-CHG (Fig. 1A). The OD_{600} of the cultures growing in 1/2× MIC decreased for 30 min after H-CHG exposure but began to increase afterwards. After 24 h of incubation, the OD_{600} of *E. faecium* 1,231,410 cultures exposed to 1× MIC of H-CHG were equivalent to those of control cultures; *E. faecium* 1,231,410 cultures exposed to 2× MIC did not recover (data not shown).

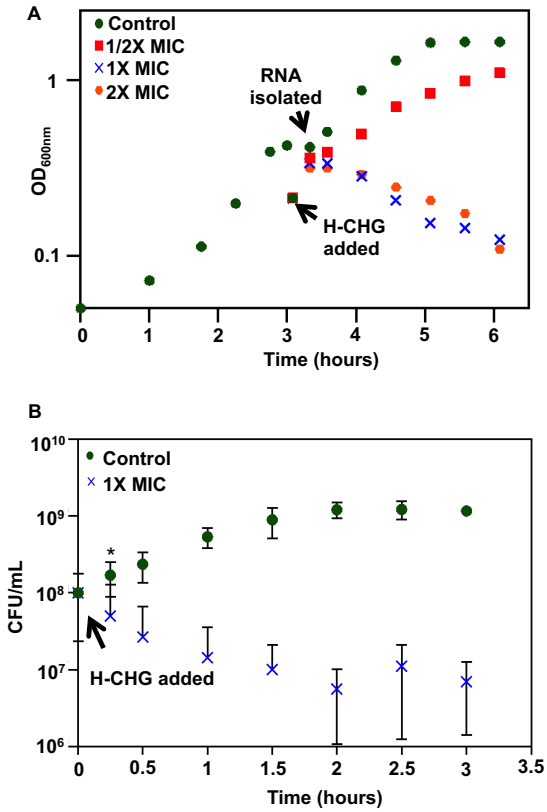

FIG 1 Growth kinetics of *E. faecium* 1,231,410 after H-CHG treatment. (A) Representative optical density curve. *E. faecium* was grown at 37°C in BHI with shaking at 100 rpm until the optical density at 600 nm (OD_{600}) reached 0.3 to 0.4. Twenty-five milliliters of culture was added to an equal volume of prewarmed BHI containing different concentrations of H-CHG such that final concentrations of 0× (control; green circles), 1/2× (red squares), 1× (blue crosses), or 2× MIC (orange hexagons) were attained. The cultures were incubated at 37°C with shaking, and OD_{600} was monitored. (B) Viability curve. Viable cell count (CFU per milliliter) for 1× MIC-treated cultures (blue crosses) and control cultures (green circles) was assessed. Error bars indicate standard deviations from 3 independent experiments. *, $P < 0.05$ by the Student one-tailed *t* test; significance was assessed only for the 15-min time point. For transcriptomic analyses, RNA was harvested from cultures exposed to 0× (control) and 1× MIC of H-CHG for 15 min.

The viability of cultures treated with 1× MIC of H-CHG and control cultures was assessed, and a significant reduction in viable cells was observed after 15 min of exposure to H-CHG (Fig. 1B). The average CFU per milliliter for control cultures at this time point was 1.7×10^8, while for H-CHG-treated cultures it was 5.0×10^7.

RNA sequencing analysis of *E. faecium* 1,231,410 response to H-CHG. Illumina RNA sequencing was performed for cultures exposed to 0× (control) and 1× (test) MIC of H-CHG for 15 min, indicated by an arrow in Fig. 1A. Genes differentially expressed shortly after H-CHG exposure may be representative of the *E. faecium* response to contact with patient skin contaminated with subbactericidal CHG, which is a point of interest for our research.

The RNA sequencing experiment was performed twice independently. We identified 35 genes upregulated ≥10-fold in H-CHG-exposed cells in both of the two RNA sequencing trials (Table 2). We focused on highly upregulated genes in this study because we reasoned that these genes could be protective for H-

TABLE 2 Differentially upregulated genes in cultures treated with 1× MIC of H-CHG versus control cultures identified by two independent RNA sequencing trials

E. faecium ORF	V583 ortholog[a]	Trial 1 FC[b]	Trial 2 FC[b]	Description	Overlap[c]
Predicted transport systems					
EFTG_01192	EF1057	11.4	14.6	*mntH2*; natural resistance-associated macrophage protein	□α
EFTG_01315		13.2	26.7	Zinc/iron permease	
EFTG_02616	EF1262	27.5	43.3	Conserved hypothetical protein	ϕα
EFTG_02617		45.5	29.7	Conserved hypothetical protein	
EFTG_02287	EF2226	22.0	21.8	ABC transporter	□
EFTG_02288	EF2227	16.0	18.6	ABC transporter	□
EFTG_02514	EF0575	20.5	63.2	Cationic ABC transporter	α
EFTG_02515		25.6	48.0	Transposase	
EFTG_02516	EF0576	10.4	39.3	Cation ABC transporter	α
EFTG_02517	EF0577	14.7	61.2	Adhesion lipoprotein	α
EFTG_02518	EF0578	13.5	55.9	Iron-dependent repressor	α
EFTG_02519	EF0579	11.7	17.8	Conserved hypothetical protein	α
EFTG_02682		43.7	28.2	Extracellular solute-binding protein family 1	
EFTG_02683		29.7	29.1	Conserved hypothetical protein	
EFTG_02684		26.7	11.3	ABC transporter permease	
EFTG_02685		26.7	21.6	Predicted protein	
EFTG_02686		25.1	17.9	ABC transporter system ATP-binding protein	
Vancomycin resistance genes					
EFTG_02038		19.0	8.4[d]	*vanZ*; VanZ protein	
EFTG_02039		15.4	8.4[d]	*vanY*; D-alanyl–D-alanine carboxypeptidase	
EFTG_02040	EF2293	104.3	61.1	*vanX*; D-Ala–D-Ala dipeptidase	
EFTG_02041	EF2294	78.7	37.1	*vanA*; D-Ala–D-lactate ligase	
EFTG_02042	EF2295	82.6	58.9	*vanH*; D-lactate dehydrogenase	
EFTG_02043		6.4[d]	7.6[d]	*vanS*; sensor histidine kinase	
EFTG_02044		7.1[d]	9.8[d]	*vanR*; vancomycin response regulator	
Extracytoplasmic stress-associated genes					
EFTG_00421	EF1533	10.5	19.3	Conserved hypothetical protein	□ϕ
EFTG_00736	EF2698	9.8[d]	28.0	*telA*; toxic anion resistance protein	△ϕ
EFTG_00737	EF2697	35.8	55.9	*xpaC*; conserved hypothetical protein	△ϕ
EFTG_00904	EF2477	11.9	10.5	Conserved hypothetical protein	ϕ
EFTG_00974	EF1006	15.9	15.2	Conserved hypothetical protein	ϕ
EFTG_01178	EF1753	69.0	117.9	*liaX*; conserved hypothetical protein	△ϕ
EFTG_01179	EF1752	32.0	28.5	*liaY*; conserved hypothetical protein	△□ϕ
EFTG_01180	EF1751	31.1	33.1	*liaZ*; integral membrane protein	ϕ
EFTG_01316	EF0026	52.6	34.6	Predicted protein	ϕ
EFTG_01545	EF3027	19.9	15.2	*htrA*; serine protease	ϕ
EFTG_01950	EF0932	23.1	10.7	Conserved hypothetical protein	□ϕ
Miscellaneous genes					
EFTG_00189		11.0	30.8	*spx*; arsenate reductase	
EFTG_01407		58.2	45.1	Conserved hypothetical protein	
EFTG_01778	EF0466	14.7	15.1	*nagB*; glucosamine-6-phosphate isomerase	
EFTG_01890		10.6	13.4	Predicted protein	
EFTG_02731		18.9	12.5	Predicted protein	

[a] Identified by previous comparative genome analysis (35).
[b] Fold change (FC) in gene expression in H-CHG cells relative to control cells as assessed by RNA sequencing.
[c] See Data Set S1 in the supplemental material and the text for references. Triangles indicate that the gene is associated with daptomycin nonsusceptibility. Squares indicate that the gene is associated with stress response to overexpression of the Fst toxin. Alpha symbols indicate that the gene is associated with metal stress response. Phi symbols indicate that the gene is upregulated in response to cell wall-active antibiotics.
[d] Fold changes of <10.

CHG exposure. We segregated the 35 genes into 4 groups: vancomycin resistance genes, extracytoplasmic stress-associated genes, predicted transport systems, and miscellaneous genes. Data Set S1 in the supplemental material is an expanded version of Table 2 showing read mapping data, cotranscription predictions, and conserved domain analysis of protein sequences.

Among the most highly upregulated genes in H-CHG-treated cultures were the VanA-type vancomycin resistance gene cluster (Table 2; see also Data Set S1). *vanHAX*, whose expression is regulated by the two-component system VanRS (37), includes the structural genes required for vancomycin resistance (reviewed in reference 5). The VanH dehydrogenase converts pyruvate into

D-lactate, which is utilized by the VanA ligase to form D-Ala–D-Lac cell wall precursors. D-Ala–D-Ala generated by the chromosomally encoded D-Ala–D-Ala ligase, Ddl, is hydrolyzed by the D-Ala–D-Ala dipeptidase VanX. The accessory genes *vanY* and *vanZ* encode a carboxypeptidase that cleaves D-Ala from late cell wall precursors terminating in D-Ala–D-Ala and a protein of unknown function, respectively (5). The induction of these genes in the presence of H-CHG suggests that chlorhexidine and/or chlorhexidine-induced stress induces VanA-type vancomycin resistance gene expression in *E. faecium*.

Other highly upregulated genes have previously been implicated in the enterococcal extracytoplasmic stress response (Table 2; see also Data Set S1). We identified overlap between the H-CHG transcriptomic response in *E. faecium* 1,231,410 with the transcriptomic response of *E. faecalis* OG1RF to the cell wall-active antibiotics ampicillin, bacitracin, cephalothin, and vancomycin (38) and with the *E. faecalis* OG1X response to the plasmid-encoded Fst toxin, which likely interacts with the OG1X cell membrane, causing stress (39, 40). Further, mutations in four genes in our data set are associated with daptomycin nonsusceptibility in *E. faecalis* and *E. faecium* (41–44). Of specific interest, the *liaXYZ* genes, which are directly regulated by the cell envelope stress regulator LiaR in *E. faecalis* (45), are highly upregulated in H-CHG-treated *E. faecium* cells. Finally, *htrA*, encoding a predicted membrane-anchored cell surface serine protease, was also upregulated. HtrA family proteins are important for the perception and turnover of misfolded and mislocalized proteins in phylogenetically diverse organisms (46). The *E. coli* HtrA family protein DegS participates in the activation of RpoE, an extracytoplasmic function sigma factor, in response to mislocalized proteins (46). Upregulation of *htrA* suggests that the perception and/or turnover of misfolded or mislocalized proteins on the cell surface is important for the H-CHG stress response. Overall, these results indicate that within 15 min of H-CHG exposure, *E. faecium* 1,231,410 mounts a transcriptional response to extracytoplasmic stress. This is consistent with chlorhexidine causing cell wall and/or cell membrane damage in *E. faecium* 1,231,410.

Several predicted membrane transport systems were highly upregulated in response to H-CHG exposure (Table 2; see also Data Set S1 in the supplemental material). Seven of these genes have previously been implicated in metal stress response in *E. faecalis*; specifically, they are upregulated in response to zinc (47, 48). In addition, the ortholog of EFTG_01192 in *E. faecalis* V583, referred to as EF1057, is downregulated in response to iron chloride excess (49). Orthologs of another predicted transport system, encoded by EFTG_02287-02288 in *E. faecium* 1,231,410 and EF2226-EF2227 in *E. faecalis* V583, were upregulated in Fst toxin-treated *E. faecalis* OG1X (39), indicating overlap with the extracytoplasmic stress response.

A putative transport system encoded by a predicted 5-gene operon (EFTG_02682-EFTG_02686) was highly upregulated in *E. faecium* 1,231,410 exposed to H-CHG (Table 2; see also Data Set S1). Interestingly, this operon is not present in 18 *E. faecalis* or 3 clade B (commensal clade) *E. faecium* genomes previously compared by whole-genome analysis (35), nor is it present in *E. faecium* DO, a common reference strain for *E. faecium* studies. However, the operon is present on the 131-kbp p1 plasmid present in the VanB-type VRE *E. faecium* bloodstream isolate Aus0085 (open reading frames [ORFs] EFAU085_p1045 to EFAU085_p1041) (50) and is present in 8 clade A1 and 6 clade A2 *E. faecium* isolates from a recent comparative genome study (36). This result is significant because it demonstrates that additional genes in the auxiliary (i.e., noncore) *E. faecium* genome, other than the VanA-type vancomycin resistance genes, are responsive to H-CHG exposure. As for function, conserved domain analysis indicates that the operon codes for an ATP-binding cassette (ABC) transport system, potentially transporting polyamines or iron (see Data Set S1).

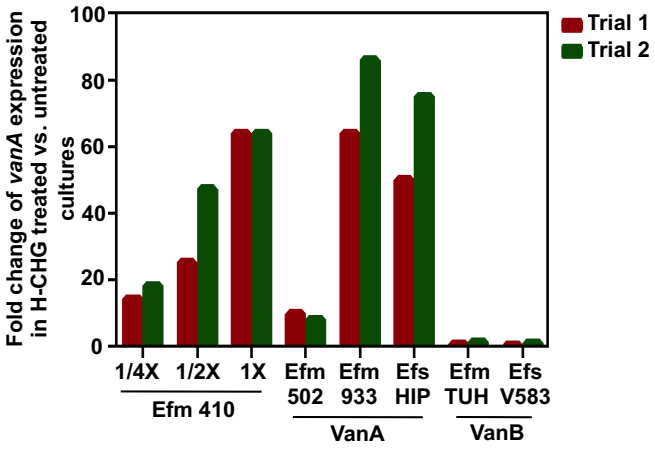

FIG 2 H-CHG induces VanA-type vancomycin resistance genes in *E. faecium* and *E. faecalis*. RT-qPCR was used to quantify the expression of *vanA* or *vanB* upon exposure to H-CHG for 15 min compared to control conditions. Expression of *vanA* and -*B* was internally normalized to *clpX* as described in the text. Expression of *vanA* or -*B* in control cultures was set to 1 (not shown). The fold change in *vanA* or -*B* expression in cultures treated with 1× MIC of H-CHG relative to the control was quantified for two independent experiments for all strains queried. *E. faecium* 1,231,410 *vanA* expression upon exposure to 1/4× or 1/2× MIC of H-CHG for 15 min was additionally queried. Efm, *E. faecium*; Efs, *E. faecalis*; HIP, HIP11704; TUH, TUH4-64.

Other upregulated genes include EFTG_00189, encoding a putative redox-responsive transcriptional regulator, and EFTG_01890, encoding a protein with a rhodanese-like domain that may also be involved in redox stress response. EFTG_01778, encoding a putative glucosamine-6-phosphate deaminase, is also upregulated. This gene is likely involved in *N*-acetylglucosamine metabolism in *E. faecium* (51).

***vanA* upregulation in response to H-CHG occurs in other VanA-type VRE and is not strain or species specific.** By RNA sequencing, we observed up to 104-fold upregulation of vancomycin resistance genes (*vanHAX*) in *E. faecium* 1,231,410 exposed to 1× MIC of H-CHG for 15 min. Because of the clinical significance of vancomycin resistance in enterococci, we further studied *vanHAX* induction by H-CHG. RT-qPCR for *vanA* expression was performed to confirm the RNA sequencing results (Fig. 2). *vanA* expression was internally normalized to the housekeeping gene *clpX*, which encodes the ATPase subunit of the housekeeping ClpXP protease (52), and was not found to be differentially regulated in RNA sequencing trials (data not shown). Using RT-qPCR, *E. faecium* 1,231,410 *vanA* was 64-fold upregulated in cultures exposed to 1× MIC of H-CHG for 15 min, compared to unexposed cultures (Fig. 2), confirming the RNA sequencing results. *vanA* was up to 47-fold and 18-fold upregulated in cultures exposed to 1/2× MIC and 1/4× MIC of H-CHG, respectively, for 15 min (Fig. 2).

We next evaluated whether H-CHG induction of *vanA* was

strain or species specific by RT-qPCR analysis of VanA-type VRE *E. faecium* 1,231,502, the VanA-type VRE *E. faecium* 1,230,933, and the VanA-type VRE *E. faecalis* HIP11704. Induction of *vanA* in response to H-CHG was observed for all three strains, although the fold upregulation of *vanA* in *E. faecium* 1,231,502 was modest (at least 8.6-fold) compared to that in the other strains (at least 50-fold) (Fig. 2). From these results, we conclude that *vanA* induction by H-CHG is not strain or species specific.

Interestingly, no upregulation of *vanB* was observed after exposure to 1× MIC of H-CHG for the VanB-type VRE isolate *E. faecium* TUH4-64 or in the VanB-type VRE isolate *E. faecalis* V583 (Fig. 2). The maximum fold change in *vanB* expression observed was 1.3 for one of the TUH4-64 trials. A key difference between VanA- and VanB-type systems is specificity of induction. VanB-type systems are induced only by vancomycin, while VanA-type systems are induced by vancomycin, teicoplanin, and other compounds (discussed further below). This difference in specificity is linked to their respective VanRS regulatory two-component systems, which share little amino acid sequence identity (53). The induction of *vanA* but not *vanB* by H-CHG suggests that the VanA-type VanRS system is responsive to chlorhexidine and/or chlorhexidine-induced stress.

The *E. faecium* 1,231,410 *vanH* promoter is responsive to H-CHG. The *vanHAX* genes have a common promoter upstream of *vanH* (37, 54). We sought to determine whether increased *vanH* promoter activity contributed to *vanHAX* induction in response to H-CHG exposure. The previously identified *vanH* transcription start site, predicted sigma factor binding sites, and inverted repeat sequences and predicted VanR binding sites upstream of the *vanH* coding region (37, 54) are conserved in *E. faecium* 1,231,410 (data not shown). However, a partial IS*1251* sequence is inserted at position −102 relative to the *vanH* transcription start site. This insertion disrupts the 5′ 15 bp of the ~80-bp phosphorylated VanR DNA footprint previously identified by Holman et al. (54), although sigma factor and predicted regulator binding sites are intact. The sequence occurring between the IS*1251* insertion and *vanH* was amplified and used to generate a reporter construct. Plasmid pPB102 contains a transcriptional fusion of the *vanH* promoter to the *lacZ* gene in pPB101 (pTCV-*lac-cat*).

β-Galactosidase activities of *E. faecium* 1,231,410 strains harboring pPB101 or pPB102 were assessed in the presence of vancomycin and in the presence of different concentrations of H-CHG at 0, 30, 60, 90, and 120 min postexposure (Fig. 3). For *E. faecium* 1,231,410 harboring pPB102, *vanH* promoter activity increased over the growth curve under control conditions, as expected based on previous studies of *vanH* promoter activity (55). Addition of vancomycin stimulated *vanH* promoter activity, as expected. Addition of 1/4× MIC of H-CHG to the cultures resulted in a significant increase in *vanH* promoter activity with time compared to that of the control (Fig. 3). β-Galactosidase activity was equivalent to that of the control for cultures treated with 1/2× MIC of H-CHG and was negligible for cultures treated with 1× MIC of H-CHG (data not shown). These results are likely due to the inhibitory action of H-CHG on growth of the reporter strain at 1/2× and 1× MIC (Fig. 3A). No β-galactosidase activity was detected for *E. faecium* 1,231,410 transformed with pPB101 (data not shown). We conclude from these results that *vanH* promoter activity is directly impacted by the addition of H-CHG, leading to increased *vanHAX* transcription. These results indicate that *vanHAX* induction by H-CHG is dependent on the VanRS two-component system.

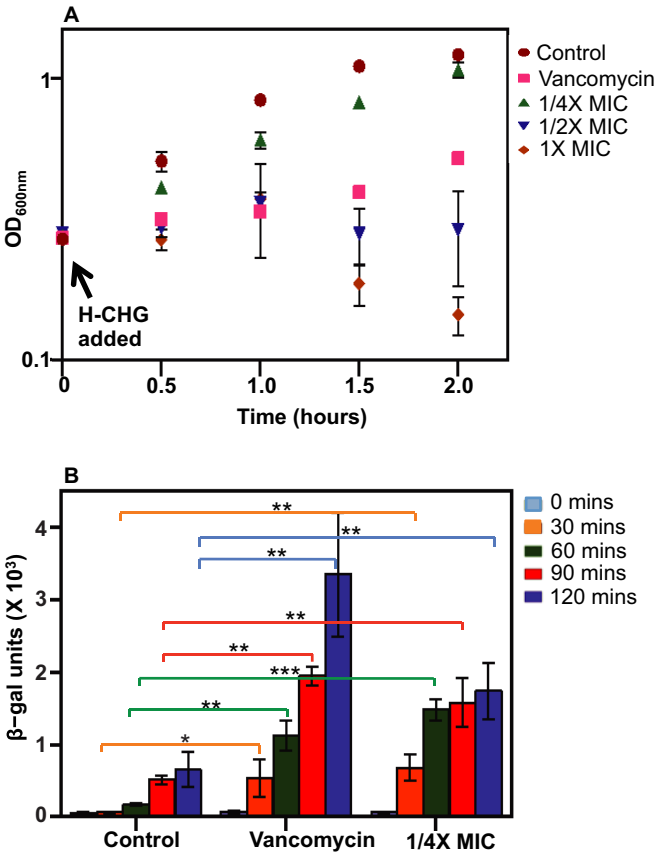

FIG 3 *vanH* promoter activity in *E. faecium* 1,231,410. (A) Average growth curve of *E. faecium* PB104. Aliquots of an exponentially growing *E. faecium* PB104 culture at an OD_{600} of 0.4 to 0.5 were added to 25 ml of BHI-chloramphenicol (control; red circles) or BHI-chloramphenicol containing H-CHG or vancomycin such that 1/4× MIC of H-CHG (green triangles), 1/2× MIC of H-CHG (blue triangles), 1× MIC of H-CHG (orange diamonds), or 2 μg/ml of vancomycin (pink squares) were attained. Cultures were incubated with shaking at 100 rpm at 37°C, and the OD_{600} was monitored. Error bars indicate standard deviations from 4 independent experiments. (B) β-Galactosidase activity assays. Enzyme activity assays were performed as described in the text. Samples were harvested from *E. faecium* PB104 cultures at multiple time points after exposure to H-CHG, vancomycin, or control conditions. Error bars indicate standard deviations from 4 independent experiments. The one-tailed Student *t* test was used to assess significance. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

VanX protein levels are elevated after H-CHG exposure. We sought to determine whether increased transcription of *vanHAX* resulted in increased levels of *van*-encoded proteins. Previous studies indicated that increased *vanHAX* promoter activity did not necessarily result in vancomycin resistance, since high-level expression of the genes is required (56, 57). Since vancomycin is also an inducer of *vanHAX*, it was not possible to use vancomycin resistance as a phenotypic output. To assess translation resulting from increased *vanHAX* transcription with H-CHG, an 18-bp hexahistidine coding sequence was knocked into the *E. faecium* 1,231,410 genome, upstream of the *vanX* stop codon. Previous studies have reported using a VanX dipeptidase enzyme assay (56–58) or a VanX-specific antibody (59) for detecting increased VanX activity or protein levels in cultures treated with vancomycin or

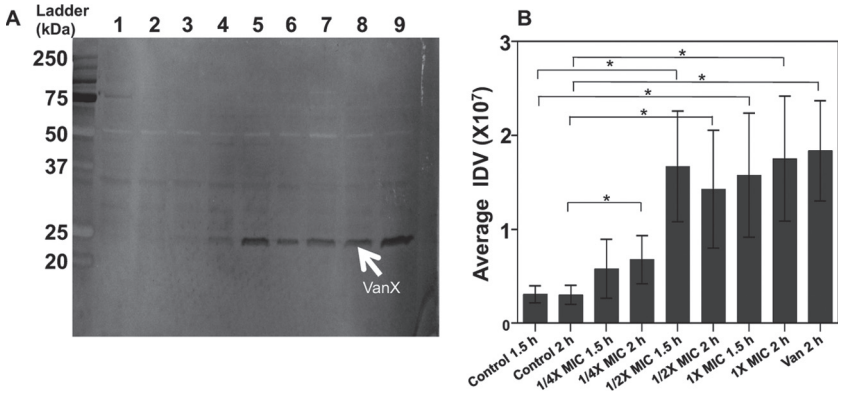

FIG 4 Detection and quantification of VanX protein levels in *E. faecium* cultures. (A) Representative Western blot. A total of 250 μg of protein extracted from *E. faecium* PB221 cultures was analyzed by Western blotting with anti-polyhistidine antibody as described in the text. A 23-kDa protein (indicated by arrow) was detected in cultures treated with H-CHG and vancomycin. Lanes: 1, control cells after 1.5 h of incubation; 2, control cells after 2 h of incubation; 3, cells after 1.5 h of incubation with 1/4× MIC of H-CHG; 4, cells after 2 h of incubation with 1/4× MIC of H-CHG; 5, cells after 1.5 h of incubation with 1/2× MIC of H-CHG; 6, cells after 2 h of incubation with 1/2× MIC of H-CHG; 7, cells after 1.5 h of incubation with 1× MIC of H-CHG; 8, cells after 2 h of incubation with 1× MIC of H-CHG; 9, cells after 2 h of incubation with 20 μg/ml of vancomycin. (B) Quantification of VanX protein levels. The amount of VanX protein was quantified by calculating the IDV (integrated density value) of each of the protein band by using Alphaimager spot density tool. Average values are shown. Error bar represent the standard deviations from 3 experiments. The one-tailed Student *t* test was used to assess significance. *, $P < 0.05$.

other test compounds. Therefore, there is a precedent for VanX detection as a proxy for vancomycin resistance.

We first compared the growth kinetics and vancomycin MIC of the hexahistidine knock-in strain, *E. faecium* PB221, compared to the wild-type strain. These assays were performed to verify that the sequence knock-in did not affect the vancomycin resistance of the strain or confer a growth defect. No difference in growth rate or yield was observed during growth in BHI media and BHI media supplemented with vancomycin (see Fig. S1 in the supplemental material), and the vancomycin MICs for the two strains were the same (312.5 μg/ml). The H-CHG MIC was also unaffected. Next, Western blotting was used to detect VanX levels in *E. faecium* PB221 cultures exposed to vancomycin, H-CHG, or water for 1.5 or 2 h (Fig. 4). These time points were chosen to allow for translation of the Van proteins after exposure to a promoter inducer. A representative Western blot is shown in Fig. 4A; the 23-kDa VanX-His_6 protein is indicated by an arrow. The average VanX band intensity values (integrated density values) for three independent experiments are shown in Fig. 4B. The results demonstrate that significantly more VanX protein was produced in vancomycin- and H-CHG-treated cells than in control cells.

***vanHAX* promoter induction by H-CHG requires VanR in a *B. subtilis* heterologous expression system.** To further extend the study of the induction of vancomycin resistance genes by H-CHG, we used a previously developed *vanH* promoter reporter system in *B. subtilis* 168 (34). In strain BAU-101, the *vanH* promoter from the VanA-type VRE *E. faecium* A624 is fused to *lacZ* and chromosomally integrated. Derivatives of this strain express plasmid-borne *vanR* and/or *vanS* genes (Table 1). If a compound is an inducer of the *vanH* promoter, in the presence of a chromogenic indicator for β-galactosidase activity, a blue halo will be observed around the zone of inhibition.

We used the *B. subtilis* BAU reporter strain series to assess *vanH* promoter activity in response to vancomycin (positive control), kanamycin or water (negative controls), and H-CHG (Table 3; see also Fig. S2 in the supplemental material). For the reporter strains BAU-102 and BAU-103, which express *vanRS* or *vanR*, respectively, blue haloes were observed around H-CHG and vancomycin zones of inhibition. The faint blue haloes observed around H-CHG and vancomycin zones of inhibition for strain BAU-103 could result from cross talk between VanR and heterologous two-component membrane sensors and/or gene dosage effects of *vanR* expression from a multicopy plasmid.

The *B. subtilis* BAU reporter strain series was also used to determine the specific component of H-CHG responsible for *vanH* promoter induction (Table 3; see also Fig. S2). A chlorhexidine-containing solution (chlorhexidine diacetate) and individual components of the H-CHG antiseptic (powdered chlorhexidine, isopropyl alcohol, and sodium D-gluconate salt) were tested for the ability to induce blue halo formation in reporter strains BAU-102 and BAU-103. H-CHG, powdered chlorhexidine, and chlorhexidine diacetate were the only substances tested that induced the *vanH* promoter.

Induction of *vanA* by H-CHG is VanR dependent. To confirm that induction of vancomycin resistance genes by H-CHG is dependent upon VanR, we constructed *E. faecium* 1,231,410 *vanR* and *vanRS* deletion mutants. Because deletion of *vanS* alone leads to constitutive expression of vancomycin resistance (60), the contribution of VanR in a VanS deletion mutant was not assessed. The vancomycin MICs for the Δ*vanR* and Δ*vanRS* mutants were re-

TABLE 3 *Bacillus subtilis* reporter strain results

B. subtilis strain	Promoter activity in response to[a,b]:							
	Van	Kan	Water	H-CHG	CDA	CHX	NaG	Isopropanol
BAU-101	−	−	−	−	ND	ND	ND	ND
BAU-102	+	−	−	+	+	+	−	−
BAU-103	+	−	−	+	+	+	−	−
BAU-104	−	−	−	−	ND	ND	ND	ND

[a] Abbreviations: Van, vancomycin (positive control); Kan, kanamycin (negative control); H-CHG, Hibiclens; CDA, chlorhexidine diacetate salt; NaG, sodium gluconate; CHX, chlorhexidine powder.
[b] +, blue halo observed around the compound; −, no blue halo observed; ND, not determined.

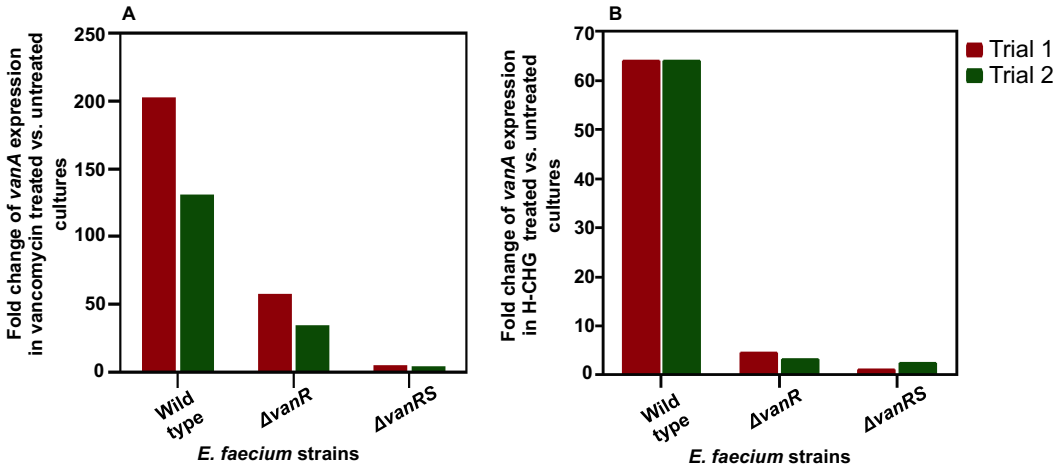

FIG 5 RT-qPCR analysis of *vanA* gene expression in response to vancomycin and H-CHG. RT-qPCR was used to quantify the expression of *vanA* ligase upon exposure to vancomycin (50 μg/ml) for 2 h (A) and 1× MIC H-CHG for 15 min (B) versus unexposed cultures for the *E. faecium* 410 wild type and Δ*vanR* and Δ*vanRS* deletion mutants. The expression of the *vanA* gene was internally normalized to *clpX*. The expression of *vanA* in control cultures was set to 1 (not shown), and relative fold change expression in vancomycin and H-CHG treated cultures from two independent experiments was quantified (trial 1, red bars; trial 2, green bars).

duced compared to that for the wild-type *E. faecium* 1,231,410 (2.4 μg/ml versus 312.5 μg/ml), but the H-CHG MIC was unaltered.

RT-qPCR was used to assess *vanA* expression in cultures treated with vancomycin for 2 h (Fig. 5A) or 1× MIC of H-CHG for 15 min (Fig. 5B). As expected, *vanA* expression was reduced in the Δ*vanR* and Δ*vanRS* mutants relative to the wild-type strain for both vancomycin- and H-CHG-treated cultures. For Fig. 5A, the difference in *vanA* expression observed between the Δ*vanR* and Δ*vanRS* strains suggests that VanS plays some role in activation of *vanA* expression in the absence of VanR. However, this gene expression pattern does not result in vancomycin resistance, since, as noted above, the Δ*vanR* and Δ*vanRS* mutants have identical vancomycin MICs. From results shown in Fig. 5B, we conclude that H-CHG induction of *vanA* is VanR dependent. Additionally, since the H-CHG MIC is unaffected by *vanR* or *vanRS* deletion, induction of VanA-type vancomycin resistance genes is not protective against H-CHG.

***E. faecalis* HIP11704 is resensitized to ceftriaxone in the presence of sub-MIC chlorhexidine.** Enterococci are intrinsically resistant to most cephalosporins, which are β-lactam antibiotics. β-Lactam antibiotics inhibit cell wall biosynthesis by binding to penicillin-binding proteins (PBPs), which cross-link peptidoglycan precursors (61). The mechanism for cephalosporin resistance in enterococci is multifactorial and incompletely understood, but production of PBP5, which has a low affinity for β-lactams, is involved (61). Previous studies on vancomycin-resistant enterococci have reported synergism between β-lactams and vancomycin (62–65). Induction of the vancomycin resistance genes, resulting in carboxypeptidase expression and hydrolysis of D-Ala–D-Ala termini from peptidoglycan precursors, is required for this synergy (64). In this study, we have shown that H-CHG induces vancomycin resistance gene expression in VanA-type *E. faecium* and *E. faecalis* (Fig. 2). Therefore, an expected phenotype of H-CHG-treated VRE is increased cephalosporin susceptibility.

We tested VanA-type *E. faecium* 1,231,410 and *E. faecalis* HIP11704 for synergism with ceftriaxone, a broad-spectrum cephalosporin, using a broth microdilution assay (Table 4). Interestingly, a reduction in ceftriaxone MIC (from 500 μg/ml to 2 μg/ml) was observed for *E. faecalis* HIP11704 in the presence of subinhibitory H-CHG or CHG, demonstrating synergism between chlorhexidine and ceftriaxone. As expected (64), vancomycin also induced ceftriaxone susceptibility in *E. faecalis* HIP11704.

The ceftriaxone MIC was significantly higher for *E. faecium* 1,231,410 than for *E. faecalis* HIP11704. As noted previously (66), *E. faecium* 1,231,410 has an elevated ampicillin MIC (>4 μg/ml), which may be due to sequence variations in *pbp5* and other, as-yet-unidentified loci. While the ceftriaxone MIC did decrease for *E. faecium* 1,231,410 in the presence of either vancomycin or H-CHG (Table 4), the extent of the reduction is likely of little clinical significance.

***E. faecium* 1,231,410 is more susceptible to vancomycin in the presence of sub-MIC chlorhexidine.** We performed a second set of synergy assays to assess whether vancomycin and H-CHG could act additively to increase the vancomycin resistance of *E. faecium* 1,231,410. Unexpectedly, the strain became more sensitive to vancomycin in the presence of sub-MIC H-CHG (Table 5). A drop in vancomycin MIC of similar magnitude was not observed for the *E. faecium* 1,231,410 Δ*vanR* mutant. This result indicates that upregulation of the vancomycin resistance genes is required for vancomycin sensitization in the presence of chlorhexidine.

TABLE 4 Ceftriaxone MIC for VanA-type VRE

Strain	Ceftriaxone MIC (mg/ml)[a]					
	No Van	+Van, 2 μg/ml	+Van, 20 μg/ml	+1/4× MIC	+1/2× MIC	+0.75× MIC
E. faecium 410	>50	ND	12.5	25	6.25	No growth
E. faecalis HIP11704	0.50	0.002	ND	0.25	0.002	0.002

[a] Average MICs were determined in BHI media after 24 h of incubation from 3 independent biological replicates. ND, not determined.

TABLE 5 Vancomycin MIC for *E. faecium* 1,231,410

Strain	Vancomycin MIC (μg/ml)[a]		
	No H-CHG	+1/2× MIC H-CHG	+1× MIC H-CHG
E. faecium 1,231,410	312.5	0.6–4.8	No growth
Δ*vanR* strain	1.2–2.4	0.3–0.6	No growth

[a] MICs were determined in BHI media after 24 h of incubation from 3 (wild-type strain) and 4 (Δ*vanR* strain) independent biological replicates. The range of MICs observed is shown where applicable.

DISCUSSION

The goal of this study was to investigate the transcriptional responses of *E. faecium* 1,231,410, a vancomycin-resistant clinical isolate, to MIC levels of a CHG-containing consumer product (H-CHG). Among the highly upregulated genes after 15 min of exposure to the product was the VanA-type vancomycin resistance gene cluster. Because of the clinical relevance of vancomycin resistance, the rest of the current study focused on this aspect of the *E. faecium* 1,231,410 transcriptional response. However, other genes of note were induced by H-CHG exposure. These include *liaXYZ*, which are associated with daptomycin nonsusceptibility in enterococci (41–44), as well as other genes associated with extracytoplasmic stress. Presumably, these genes are upregulated in response to chlorhexidine-induced cell surface stress. Other highly upregulated genes include predicted metal transport systems and a predicted ABC transport system of unknown function that appears to be encoded by a mobile genetic element. The specific roles of these transport systems in the H-CHG stress response remain to be determined; possibilities include chlorhexidine efflux (note that a new family of chlorhexidine efflux proteins was recently identified for Gram-negative bacteria [67, 68]), transport of metals to maintain redox balance in the cell, or transport of cell wall-related metabolites. Future studies will compare the transcriptomic response of *E. faecium* to H-CHG to that for CHG and sodium gluconate, which will help to determine which specific components of H-CHG are responsible for the transcriptional changes observed.

We observed that MIC and sub-MIC levels of H-CHG induced expression of the VanA-type vancomycin resistance gene cluster in *E. faecium* 1,231,410. Induction of *vanA* by H-CHG occurred in VanA-type *E. faecalis* and *E. faecium*, but *vanB* was not induced by H-CHG in VanB-type *E. faecalis* and *E. faecium*. Using a *vanH* promoter reporter, we determined that exposure to H-CHG resulted in increased *vanH* promoter activity. An *E. faecium* 1,231,410 derivative expressing a hexahistidine-tagged VanX protein was used in Western blotting experiments to show that increased *vanH* promoter activity with H-CHG resulted in significantly increased VanX protein levels. Using a combination of approaches in *E. faecium* 1,231,410 and the heterologous host *B. subtilis*, we determined that VanR is required for induction of *vanHAX* by H-CHG. Experiments with *B. subtilis* demonstrated that chlorhexidine is the specific component of H-CHG responsible for *vanH* promoter induction. Importantly, expression of the vancomycin resistance genes is not protective against chlorhexidine, since the H-CHG MIC is unaffected by *vanR* or *vanRS* deletion.

Collectively, our results indicate that the VanRS two-component system senses either chlorhexidine or chlorhexidine-induced cell surface stress and activates *vanHAX* expression. Induction of VanA-type vancomycin resistance genes by compounds other than vancomycin has been well studied, although with conflicting results (34, 55, 56, 69, 70). These conflicting results could be attributed to the different methods used to assess induction (multicopy expression plasmids, expression of *van* genes outside or inside their native context, resistance phenotype, and VanX enzymatic activity). For this reason, we endeavored to use multiple approaches to demonstrate that chlorhexidine is an inducer of VanA-type vancomycin resistance. Despite the conflicts noted above, there is consensus that vancomycin, teicoplanin, and moenomycin are inducers of VanA-type vancomycin resistance, while only vancomycin is an inducer of VanB-type resistance. For VanB-type vancomycin resistance (responsive only to vancomycin), direct binding of vancomycin to the VanS sensor has been experimentally demonstrated for a *Streptomyces* host (71). For VanA-type vancomycin resistance, an alternative model has been proposed to explain its relaxed specificity of induction relative to the VanB-type resistance. This model posits that the VanA-type VanS protein is responsive to cell surface destabilization and, specifically, inhibition of transglycosylation (56, 69). This model is supported by the observation that structurally unrelated drug classes activate VanA-type resistance expression, including glycopeptides, moenomycin, and now chlorhexidine. Further study is required to elucidate the specific aspect of chlorhexidine or chlorhexidine-induced stress that leads to induction of VanA-type resistance.

As expected for VRE actively expressing vancomycin resistance (64), VanA-type VRE were more sensitive to ceftriaxone in the presence of subinhibitory CHG. The magnitude of this effect was small for *E. faecium* 1,231,410, which has elevated ceftriaxone (Table 4) and ampicillin (66) MICs. Unexpectedly, sub-MIC H-CHG increased *E. faecium* 1,231,410 susceptibility to vancomycin, but a similar increase in vancomycin susceptibility was not observed for a Δ*vanR* derivative (Table 5). This suggests that this effect is dependent upon expression of the vancomycin resistance genes. We identified two explanations for vancomycin sensitivity of *E. faecium* 1,231,410 in the presence of both vancomycin and chlorhexidine. The first explanation is that in the presence of both vancomycin and chlorhexidine, *E. faecium* 1,231,410 synthesizes peptidoglycan precursors that terminate in neither D-Ala–D-Ala (synthesized by Ddl) nor D-Ala–D-Lac (synthesized by VanA). Instead, precursors that terminate in a structure that is sensitive to vancomycin binding are synthesized by VanA. The mechanism underlying this would be the relaxed substrate specificity of the VanA ligase (72, 73) combined with chlorhexidine-dependent gene expression changes that alter substrate pools and lead to the incorporation of amino or short acids that weaken the cell wall, as has been previously reported (74). Based on an analysis of predicted amino acid racemases and D-isomer-specific dehydrogenases (see Data Set S2 in the supplemental material), this hypothesis is not supported by our RNA sequencing data. An alternative explanation is that in the presence of both vancomycin and chlorhexidine, *E. faecium* 1,231,410 synthesizes peptidoglycan precursors that terminate in D-Ala–D-Lac, as expected. However, these termini fail to be cross-linked due to chlorhexidine-induced changes in transpeptidase expression. Although not achieving our ≥10-fold change threshold used in this study, several transpeptidases (encoded by *pbpF*, *ddcP*, and *pbpA*) are upregulated after 15 min of exposure to chlorhexidine (see Data Set S2). These data

suggest that transpeptidase ratios are altered in the presence of chlorhexidine, which supports the second hypothesis. Analyses of *E. faecium* 1,231,410 peptidoglycan structure are of interest for future work, as are deletion of *pbpF*, *ddcP*, and *pbpA* to assess their roles in the vancomycin sensitization we observe for *E. faecium* 1,231,410 in the presence of chlorhexidine. Understanding this mechanism could be informative for novel strategies to treat VREfm infections.

What are possible clinical impacts of VRE exposures to sub-MIC chlorhexidine? Based on our results, *E. faecium* and *E. faecalis* isolates harboring VanA-type resistance genes will synthesize modified cell walls in response to subbactericidal levels of chlorhexidine. Glycopeptide-dependent VRE have been isolated from patients undergoing vancomycin therapy (5). These isolates have mutations in *ddl* and depend upon the exogenous presence of vancomycin to induce *vanA* or *vanB* such that a cell wall can be formed (75). It would be of interest to investigate whether sub-MIC chlorhexidine exposure can rescue VanA-type glycopeptide-dependent VRE. It is also significant that genes protective against daptomycin (*liaXYZ*) are highly upregulated in response to chlorhexidine. It was recently demonstrated that deletion of *liaR*, encoding an activator of *liaXYZ* expression (45), restores daptomycin susceptibility to daptomycin-nonsusceptible enterococci (76). Further studies will be required to determine whether gene expression "priming" by chlorhexidine impacts treatment outcomes with daptomycin, or if frequent exposure to subinhibitory chlorhexidine is selective for strains that constitutively activate *liaXYZ*.

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P.B. and K.L.P. designed experiments, P.B. and E.Z. performed experiments, P.B. and K.L.P. analyzed data, and P.B. and K.L.P. wrote the manuscript.

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