School of Natural Sciences and Mathematics

Chlorhexidine Induces VanA-Type Vancomycin Resistance Genes in Enterococci

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Citation:
Chlorhexidine Induces VanA-Type Vancomycin Resistance Genes in Enterococci

POOJA BHARDWAJ, ELIZABETH ZIEGLER, KELLI L. PALMER
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Chlorhexidine is a bisbiguanide antiseptic used for infection control. Vancomycin-resistant Enterococcus 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 ceftiraxone 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 CLABSIIs 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 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.

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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 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 agitation (225 rpm) unless otherwise stated. *Escherichia coli* strains were cultured on LB. Antibiotics were used at the following concentrations: chloramphenicol, 15 μg/ml; kanamycin, 15 μg/ml; ampicillin, 100 μg/ml; erythromycin, 5 μg/ml; gentamicin, 10 μg/ml.

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-

### TABLE 1 Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. faecium</em> 1,231,410</td>
<td>Skin and soft tissue infection isolate; VanA-type VRE</td>
<td>35</td>
</tr>
<tr>
<td><em>E. faecium</em> 1,231,502</td>
<td>Bloodstream isolate; VanA-type VRE</td>
<td>35</td>
</tr>
<tr>
<td><em>E. faecium</em> 1,230,923</td>
<td>Wound isolate; VanA-type VRE</td>
<td>35</td>
</tr>
<tr>
<td><em>E. faecium</em> TUH4-64</td>
<td>Human clinical isolate; VanB-type VRE</td>
<td>77</td>
</tr>
<tr>
<td><em>E. faecalis</em> HIP1704</td>
<td>VanA-type VRE; coisolated with vancomycin-resistant <em>Staphylococcus aureus</em></td>
<td>78</td>
</tr>
<tr>
<td><em>E. faecalis</em> V583</td>
<td>Bloodstream isolate; VanB-type VRE</td>
<td>79</td>
</tr>
<tr>
<td>PB103</td>
<td><em>E. faecium</em> 1,231,410 transformed with pPB101</td>
<td>This study</td>
</tr>
<tr>
<td>PB104</td>
<td><em>E. faecium</em> 1,231,410 transformed with pPB102</td>
<td>This study</td>
</tr>
<tr>
<td>PB221</td>
<td><em>E. faecium</em> 1,231,410 with hexahistidine coding sequence integrated upstream of vanX (EFTG_02040)</td>
<td>This study</td>
</tr>
<tr>
<td>PB222</td>
<td><em>E. faecium</em> 1,231,410 ΔvanR (EFTG_02044)</td>
<td>This study</td>
</tr>
<tr>
<td>PB223</td>
<td><em>E. faecium</em> 1,231,410 ΔvanRS (EFTG_02043-44)</td>
<td>This study</td>
</tr>
<tr>
<td><em>B. subtilis</em> BAU-101</td>
<td><em>B. subtilis</em> harboring vanH: lacZ cassette integrated into the amyE locus of chromosome</td>
<td>34</td>
</tr>
<tr>
<td><em>B. subtilis</em> BAU-102</td>
<td>BAU-101 harboring vanRS cassette inserted 81 bp downstream of the cat-86 promoter on plasmid pH2</td>
<td>34</td>
</tr>
<tr>
<td><em>B. subtilis</em> BAU-103</td>
<td>BAU-101 harboring vanR cassette inserted 81 bp downstream of the cat-86 promoter on plasmid pH2</td>
<td>34</td>
</tr>
<tr>
<td><em>B. subtilis</em> BAU-104</td>
<td>BAU-101 harboring vanS cassette inserted 81 bp downstream of the cat-86 promoter on plasmid pH2</td>
<td>34</td>
</tr>
<tr>
<td><em>E. coli</em> EC100</td>
<td><em>E. coli</em> cloning host; provides repA in trans; F− araD139 (ara ABC-leu)/7679 gaiU galK lacX74 rpsL thi-1 repA of pWV01 in ggg8; Km</td>
<td>80</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td><em>E. coli</em> cloning host; F− endA1 glnV44 thi-1 recA1 gyrA96 deoR nupG/Φ80d lacZΔM15 (lacZYA-argF)U169 hsdR7(rK mK) λ</td>
<td>81</td>
</tr>
<tr>
<td><em>E. coli</em> BW23474</td>
<td>Cloning host for pTCV-lac and pH2</td>
<td>81</td>
</tr>
</tbody>
</table>

**Plasmids**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLT06</td>
<td>Markerless, counterselectable exchange plasmid; confers chloramphenicol resistance</td>
<td>82</td>
</tr>
<tr>
<td>pHOU2</td>
<td>Derivative of pJC17 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</td>
<td>83</td>
</tr>
<tr>
<td>pH101</td>
<td>pLT06 plasmid with oriT from pHOU2 inserted at PstI; confers chloramphenicol resistance</td>
<td>This study</td>
</tr>
<tr>
<td>pTCV-lac</td>
<td>Expression vector for Gram-positive bacteria; confers kanamycin and erythromycin resistance</td>
<td>33</td>
</tr>
<tr>
<td>pPB101</td>
<td>pTCV-lac-cat; expression vector for Gram-positive bacteria; confers kanamycin, erythromycin, and chloramphenicol resistance</td>
<td>This study</td>
</tr>
<tr>
<td>pPB102</td>
<td>pPB101 containing 248-bp EcoRI/BamHI-digested vanH (EFTG_02042) promoter region</td>
<td>This study</td>
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<tr>
<td>pPB201</td>
<td>pHA101 containing a 2.043-kb EcoRI/EcoRI-digested fragment flanking upstream and downstream of <em>E. faecium</em> vanX gene (EFTG_02040)</td>
<td>This study</td>
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<tr>
<td>pPB202</td>
<td>pHA101 containing a 2.028-kb BamHI/BamHI-digested fragment flanking upstream and downstream of <em>E. faecium</em> vanX (EFTG_02044)</td>
<td>This study</td>
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<tr>
<td>pPB203</td>
<td>pHA101 containing a 2.028-kb BamHI/BamHI-digested fragment flanking upstream and downstream of vanRS (EFTG_02043-44)</td>
<td>This study</td>
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</table>
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, 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. One hundred nanograms of RNA was used to synthesize cDNA, 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.

**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 = $2^{-\Delta \Delta C_T}$, where $\Delta C_T = (C_T$ 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 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 EcoR1- 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 Alphalager 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 vanK 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-indolyl-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 n-glucurate 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 ΔvanR and ΔvanRS. The vanR (EFTG_02044) and vanA 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 ΔvanR and ΔvanRS 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 30 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* 1,231,400 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).

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-
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

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."
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 daptoycin 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 turn-over 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_p1043 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).

Other upregulated genes include EFTG_00189, encoding a putative redox-responsive transcriptional regulator, and EFTG_01880, encoding a protein with a rhodanese-like domain that may also be involved in redox stress response. EFTG_01778, encoding a putative glucoseamine-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 cpxA, 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 Enterococcus 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 1X 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 IS1251 insertion is positioned at position 102 relative to the vanH transcription start site. This insertion disrupts the 5’ 15 bp of the 80-bp phosphorlylated 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 IS1251 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 (pTCE-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 1/4X 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 1/2X MIC of H-CHG and was negligible for cultures treated with 1X 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 1/2X and 1X 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.

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 hexabistidine 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...
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* isopropyl alcohol, and sodium D-gluconate salt) were tested for the ability to induce blue halo formation in reporter strains BAU-101 through 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 ΔvanR and ΔvanRS mutants were re-

**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-polylhistidine 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.

**TABLE 3** *Bacillus subtilis* reporter strain results

<table>
<thead>
<tr>
<th>B. subtilis strain</th>
<th>Promoter activity in response to a,b</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAU-101</td>
<td>Van</td>
</tr>
<tr>
<td>BAU-102</td>
<td>+</td>
</tr>
<tr>
<td>BAU-103</td>
<td>+</td>
</tr>
<tr>
<td>BAU-104</td>
<td>−</td>
</tr>
</tbody>
</table>

**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.
Enterococcus faecium Response to 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 is multifactorial and incompletely understood, but the presence of sub-MIC chlorhexidine.

### TABLE 4 Ceftriaxone MIC for VanA-type VRE

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ceftriaxone MIC (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Van, 2 µg/ml</td>
</tr>
<tr>
<td>E. faecium 410</td>
<td>&gt;50</td>
</tr>
<tr>
<td>E. faecalis HIP11704</td>
<td>0.50</td>
</tr>
</tbody>
</table>

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

FIG 5 RT-qPCR analysis of vanA 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 ΔvanR and ΔvanRS 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).
H-CHG in VanB-type E. faecalis flux (note that a new family of chlorhexidine efflux proteins was remain to be determined; possibilities include chlorhexidine ef-
trasmers and a predicted ABC transport system of unknown function in response to chlorhexidine-induced cell surface stress. Other the es observed where applicable.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Vancomycin MIC (µg/ml)*</th>
<th>+1/2× MIC</th>
<th>+1× MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. faecium 1,231,410</td>
<td>312.5</td>
<td>0.6–4.8</td>
<td>No growth</td>
</tr>
<tr>
<td>ΔvanR strain</td>
<td>1.2–2.4</td>
<td>0.3–0.6</td>
<td>No growth</td>
</tr>
</tbody>
</table>

*MICs were determined in BHI media after 24 h of incubation from 3 (wild-type strain) and 4 (ΔvanR 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 excracytoplasmic 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 (mucopy 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 ∆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 on 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 precursors 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 pbfP, ddxP, and pbP) 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 dlt 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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