EfrEF and the transcription regulator ChlR are required for chlorhexidine stress response in Enterococcus faecalis V583

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Abstract

*Enterococcus faecalis* is an opportunistic pathogen and leading cause of healthcare-associated infections. Daily chlorhexidine gluconate (CHG) bathing of patients is generally regarded as an effective strategy to reduce the occurrence of healthcare-associated infections. It is likely that *E. faecalis* are frequently exposed to inhibitory and sub-inhibitory CHG in clinical settings. The goal of this study was to investigate how the vancomycin-resistant strain *E. faecalis* V583 transcriptionally responds to and tolerates stress from CHG. We used transcriptome (microarray) analysis to identify genes up-regulated by *E. faecalis* V583 in response to CHG. The genes *efrE* (EF2226) and *efrF* (EF2227), encoding a heterodimeric ABC transport system, were the most highly up-regulated genes. *efrEF* expression was induced by CHG at concentrations several two-fold dilutions below the MIC. Deletion of *efrEF* increased *E. faecalis* V583 susceptibility to CHG. We found that ChlR, a MerR-like regulator encoded upstream of *efrEF*, mediated the CHG-dependent up-regulation of *efrEF*, and deletion of *chlR* also increased chlorhexidine susceptibility. Overall, our study gives insight into *E. faecalis* stress responses to a commonly used antiseptic.
Enterococci are Gram-positive bacteria and colonizers of the gastrointestinal tracts of humans and animals. *Enterococcus faecalis*, an opportunistic pathogen, is one of the leading causes of healthcare-associated infections, including bloodstream infections, surgical wound infections, and urinary tract infections (1). The intrinsic antibiotic resistance of *E. faecalis* combined with horizontal acquisition of antibiotic resistance genes often complicates treatment of these infections (2). Vancomycin-resistant enterococci (VRE) are particularly of concern.

Chlorhexidine is a bisbiguanide disinfectant and antiseptic with broad-spectrum antimicrobial efficiency against bacteria. Chlorhexidine gluconate (CHG), a chlorhexidine salt solution, is used for infection control, including whole-body rinsing of patients in intensive care units (ICUs), oral cleansing, and surgical hand washes. The mechanism of action and efficacy of chlorhexidine against bacteria has been studied for decades (3). It is generally postulated that the antimicrobial activity of chlorhexidine stems from its cationic nature. Chlorhexidine attaches to the negatively charged cell envelope, resulting in breakage of the outer leaflet. High concentrations of chlorhexidine severely compromise the cytoplasmic membrane, leading to cell lysis. At lower concentrations, near the MIC, chlorhexidine distorts the cell walls of Gram-positive and Gram-negative bacteria, leading to morphological changes in the cell surface (4). Uptake of chlorhexidine into the cytoplasm causes precipitation of the cytoplasmic components and inhibits ATPase activity (5, 6).

Daily CHG bathing of ICU patients is used to control VRE and other nosocomial infections (7-9). CHG is typically detectable on patients’ skin for 24 hours post-bath (10). However, VRE recover to pre-bath density on patients’ bodies in less than 24 hours (10). This finding indicates that VRE are frequently exposed to inhibitory and sub-inhibitory CHG as a result of CHG bathing. Recent *E. faecalis* isolates from an ICU demonstrated a high prevalence...
of reduced chlorhexidine susceptibility (11). It is conceivable that extensive use of CHG bathing could select for strains with reduced chlorhexidine susceptibilities.

It is currently not well-understood how VRE respond to and tolerate stress from sub-inhibitory CHG. By using microarray analysis, we find that gene expression in *E. faecalis* V583 is altered after CHG exposure. Of particular interest is that EF2226 and EF2227, which encode the heterodimeric ATP-binding cassette (ABC) transporter EfrEF (12), are the most up-regulated genes in *E. faecalis* V583 upon exposure to CHG. By deletion analysis, we show that *efrEF* expression confers protection from CHG. Further, EF2225 (referred to as chlR here), a putative MerR family transcription regulator encoded upstream of *efrEF*, mediates the up-regulation of *efrEF* in response to CHG exposure.

Materials and Methods

**Bacterial strains and routine molecular biology procedures.** Bacterial strains and plasmids used in this study are listed in Table 1. *E. faecalis* was routinely cultured in Brain Heart Infusion (BHI) medium with or without agar at 37°C unless otherwise noted. *Escherichia coli* was routinely cultured in lysogeny broth or agar at 37°C unless otherwise noted. Chloramphenicol was used at 15 μg/mL. PCR was performed with Taq polymerase (New England Biolabs) or Phusion (Fisher Scientific). Plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen). Inserts in engineered plasmids were sequenced (Massachusetts General Hospital DNA Core) to ensure that no mutations occurred during cloning. Primer sequences used in this study are in Table S1.

**Susceptibility testing.** Unless otherwise noted, the CHG used in this study was commercially available Hibiclens® (referred to H-CHG hereafter). The H-CHG MIC was determined in BHI broth using broth microdilution. The MIC was defined as the lowest concentration of H-CHG that inhibited visible cell growth. MIC values were independently confirmed using chlorhexidine.
digluconate (Sigma). For all experiments in this study, 1X MIC refers to the *E. faecalis* V583 H-CHG MIC as determined by broth microdilution.

**Growth kinetic assays with H-CHG.** *E. faecalis* V583 growth was monitored by recording optical density of cultures at 600 nm (OD<sub>600</sub>) using a spectrophotometer. Overnight culture was diluted to an OD<sub>600</sub> of 0.01 in BHI broth and incubated at 37°C with agitation at 100 rpm. At mid-log phase (OD<sub>600</sub> between 0.4 and 0.5), 25 mL of culture was split into flasks with pre-warmed medium with or without H-CHG such that concentrations of 1X MIC, 1/2X MIC, and no H-CHG (control) were attained. Growth was then monitored at 15-min intervals for the first half hour and 30-min intervals for the subsequent 3 hours.

**Transcriptomic analysis.** Total RNA was extracted from *E. faecalis* V583 cultures after 15 minutes exposure to 1X MIC H-CHG or no H-CHG. Briefly, 10 mL culture were transferred to 20 mL RNA Protect Bacteria Reagent (Qiagen) and incubated at room temperature for 10 mins. Cells were then pelleted by centrifugation at 11,000 x g, resuspended in IHB-1 buffer (50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8.0) supplemented with 125 µL of a 50 mg/mL lysozyme stock and 25 µL of a 2.5 kU/mL mutanolysin stock, and incubated at 37°C for 20 mins. Total RNA was isolated by RNA Bee (Tel-Test) extraction following the manufacturer’s protocol. RNA was dissolved in 50 µL RNase free water (Ambion). RNA samples were treated with RNase-free DNase I (Roche) to remove contaminating DNA and purified using the Qiagen RNeasy kit. DNA contamination was monitored by PCR with primers targeting a 16S rRNA gene (Table S1). RNA integrity was confirmed by visualization of intact 23S and 16S rRNA bands on a 1% agarose gel. RNA was quantified with Nanodrop. cDNA was synthesized using Supercript II (Invitrogen) and random hexamers (Qiagen). 3 µg cDNA were fragmented with Roche DNase I and 3’ end-labeled using the Bioarray Terminal Labeling Kit (Enzo). Labeled, fragmented cDNA was hybridized to custom Affymetrix GeneChips probing *E. faecalis* V583 gene sequences.
(Gilmorea520187F; (13)). Processing of Affymetrix GeneChips was performed at the University of Iowa DNA facility. Two independent transcriptome experiments were performed.

**Microarray data analysis.** Microarray data was processed by the bioconductor package based on the R statistical programming environment. The .CEL files were pre-processed by the RMA algorithm, and the processed data were subjected to gene expression analysis by the linear models for microarray data (Limma) package (14). All codes utilized for gene expression analysis followed the Limma user’s guide. Statistical analysis was performed utilizing two independent microarray data sets. Genes with fold change ≥4 and FDR-adjusted p-value <0.05 were considered further. The microarray data have been deposited in the EMBL-EBI databank (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5181.

**Semi-quantitative and quantitative reverse transcriptase PCR (RT-PCR).** RT-PCR was performed to confirm select microarray results and to determine whether efrE and efrF are co-transcribed. For semi-quantitative RT-PCR, RNA (100 ng) was used as template for cDNA synthesis with 250 ng random hexamers and Superscript II (Invitrogen). Five ng of the resulting purified cDNA was used as template for PCR. The housekeeping gene clpX was used as a control. Quantitative RT-PCR (RT-qPCR) was performed with AzuraQuart™ green fast qPCR mix per the manufacturer’s recommendations. RT-qPCR experiments were performed independently three times. The gyrB housekeeping gene was used as control, and expression was normalized to this gene. Statistical significance was assessed by the Student's t test.

**Construction of deletion mutants.** Vector pHA101 (15), a derivative of pLT06 (16), was used to create deletion mutants of *E. faecalis* V583. Mutants were generated by markerless in-frame deletion as previously described (15). Briefly, ~1.0 kb regions upstream and downstream of the gene(s) targeted for deletion were amplified by PCR. Products were digested by restriction enzymes as described in Table 1 and ligated with pHA101. Plasmid constructs were propagated
in *E. coli* EC1000 with chloramphenicol selection. Plasmids were transformed into *E. faecalis* V583 cells by electroporation (17). Deletions were generated using temperature shift and p-chlorophenylalanine counterselection as previously described (15, 16).

**Complementation of deletion mutants.** Deletion mutant strains were complemented *in trans* using the shuttle vector pCAT28, a derivative of pAT28 (18) that confers chloramphenicol resistance. For complementation, the *chlR* or *efrEF* complete ORFs with predicted promoter regions were amplified by PCR, treated with restriction enzymes as indicated in Table 1 and Table S1, and ligated into pCAT28. Plasmids were propagated in *E. coli* DH5α and electroporated into *E. faecalis*.

**Primer extension.** Total RNA was obtained as described above. Primer extension was performed using 6-carboxyfluorescein-labeled primers as previously described (19). DNA fragment analysis was processed at the University of Oklahoma Health Sciences Center Laboratory for Genomics and Bioinformatics. Data were analyzed by Peak Scanner™ software version 1.0 (Thermo Fisher). The size of the most abundant cDNA product was used to determine the transcription start site.

**Viability assay.** Broth cultures were adjusted to an OD$_{600}$ of 0.3 and serially diluted in PBS. 10 μl of each dilution were spotted on agar plates containing different concentrations of H-CHG. Colonies were counted after overnight incubation in 37°C. Counts of colonies between 20 and 200 were taken into consideration and normalized.

**β-galactosidase assay.** The putative promoter regions of the *efrEF* operon and the *chlR* gene were amplified using PCR. Products were digested by EcoRI and BamHI, and ligated into pPB101 (15). pPB101 and derivatives were propagated in *E. coli* BW23474 and then transformed into *E. faecalis* strains by electroporation. For the β-galactosidase assay, a qualitative assay was used. Stationary phase cultures were adjusted to an OD$_{600}$ of 0.3 and
diluted in PBS buffer. 10 μl of each dilution were spotted on BHI agar plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and different concentrations of H-CHG.

Results

**E. faecalis V583 growth kinetics after H-CHG exposure.** E. faecalis V583 is a VanB-type vancomycin-resistant bloodstream infection isolate and model strain for E. faecalis studies (20, 21). The broth microdilution H-CHG MIC for E. faecalis V583 is 9.8 μg/ml, which is within the lowest range of CHG residual concentrations detected on patients' bodies (0-18.75 μg/ml) (10).

We assessed the growth of E. faecalis V583 in response to different concentrations of H-CHG by spiking H-CHG into cultures in exponential phase (Figure 1). We used the H-CHG MIC obtained by broth microdilution as a reference for the amount of H-CHG spiked into cultures. V583 stops growing after exposure to 1X MIC H-CHG, but cells remain viable (Figure 1A and 1B). V583 is initially growth-inhibited but recovers to normal growth after exposure to 1/2X MIC H-CHG (Figure 1A). After 20 h incubation, the optical densities of cultures exposed to 1X MIC H-CHG are identical (OD$_{600}$ ~1.8) to that of untreated control cultures.

**efrEF** are highly up-regulated in response to H-CHG. We used custom Affymetrix GeneChips with probes targeting E. faecalis V583 open reading frames (ORFs) (13) to assess the transcriptional response of V583 to H-CHG exposure. Across two independent experimental trials, exposure to 1X MIC H-CHG for 15 mins resulted in the ≥4-fold up-regulation of 75 genes, compared to their expression levels in untreated cells (Dataset S1). Of the 75 genes, 39 (52%) are predicted by PSORTb version 3.0 (22) to encode membrane proteins (Dataset S1). In comparison, only 28.4% of the V583 proteome (884 of 3112 proteins) are predicted to be membrane proteins (23). This is a significant enrichment for membrane proteins in the H-CHG stress response ($\chi^2$(df = 1, N = 3187) = 18.685, p < 0.0001).
Of genes up-regulated in response to H-CHG, efrE and efrF stood out with 286- and 326-fold up-regulation, respectively. Semi-quantitative RT-PCR (Figure S1) analyses confirmed the microarray results for these two genes, and quantitative RT-PCR (Figure 2) confirmed the up-regulation of efrF in the presence of H-CHG. We also assessed efrEF expression in the vancomycin-susceptible E. faecalis strain, OG1RF, which we determined has the same broth microdilution H-CHG MIC (9.8 μg/ml) as E. faecalis V583. In the presence of H-CHG, the efrEF orthologs OG1RF_11766 and OG1RF_11767 are up-regulated (Figure 2 and Figure S2).

ABC transporters typically consist of two subunits which function as homo- or heterodimers. A previous study purified EfrE and EfrF, and determined that the proteins formed a heterodimer (12). It is therefore likely that efrE and efrF are co-transcribed and co-regulated. To test this, RT-PCR was performed using RNA isolated from E. faecalis V583 grown in the presence of H-CHG. Primers were designed to amplify the 52 bp intergenic region between efrE and efrF, along with parts of the efrE and efrF coding regions. The results demonstrate that a transcript containing efrE and efrF is present in E. faecalis V583 (Figure S2).

**Deletion of efrEF increases H-CHG susceptibility.** To investigate how efrE and efrF impact H-CHG susceptibility in E. faecalis, we constructed an efrEF deletion mutant. Growth on BHI was not affected when efrEF were deleted (Figure 3A). However, the deletion mutant is more susceptible to H-CHG than the wild-type strain in broth microdilution assays (MIC of 2.4 μg/mL H-CHG) and in agar plate assays (Figure 3A). We complemented the ΔefrEF mutant by expressing efrEF from its native promoter on a multicopy plasmid. Complementation restored H-CHG susceptibility to wild-type levels (Figure 3A).

**ChiR, a MerR family regulator, mediates efrEF up-regulation in response to H-CHG.** We were interested in how efrEF are regulated. NCBI Conserved Domains analysis assigns EF2225
to the MerR-like family of transcription regulators. The EF2225 locus is encoded upstream of efrEF and is divergently transcribed. The layout of EF2225 and efrEF is representative of a MerR regulon (24, 25). We refer to EF2225 as chlR hereafter.

To investigate if ChlR regulates efrEF expression, a ΔchlR strain was constructed. The ΔchlR strain was complemented in trans by cloning the complete chlR gene and putative promoter into a multi-copy vector. The ΔchlR mutant strain shows increased susceptibility to H-CHG (Figure 3B), and its broth microdilution H-CHG MIC (4.9 μg/mL) is half that of the wild-type strain. H-CHG susceptibility is restored to the wild-type level in the complemented strain (Figure 3B).

To further substantiate the relationship between ChlR and efrEF, efrEF expression was assessed in the ΔchlR and complemented strains by RT-qPCR. Upon 15 min exposure to 1/2X MIC H-CHG, the transcription levels of efrE and efrF in the ΔchlR mutant remain the same regardless of the presence of H-CHG, whereas H-CHG induces efrE and efrF expression in the chlR complemented strain (Figure 4). This result demonstrates that ChlR is required for the up-regulation of efrEF in response to H-CHG.

To investigate the efrEF promoter, primer extension was used to find the transcription start site (TSS) of efrE. Primer extension identified two transcription start sites. Under standard culture conditions in BHI broth, we identified a TSS at -78 bp upstream of the efrE ORF. When cells were exposed to H-CHG, an additional TSS was detected at -33 bp upstream of the efrE ORF; its corresponding promoter is designated as P_EF hereafter. On the basis of this evidence, we propose that the efrEF operon has two promoters. One promoter, corresponding to the -78 bp TSS, is constitutive and is responsible for basal transcription of efrEF. P_EF is a ChlR-dependent promoter (Figure 5).
Based on existing knowledge of MerR family regulators (24), the chlR promoter is predicted to be oriented opposite to $P_{EF}$. We obtained inconsistent results across multiple primer extension trials for the chlR TSS. The presumptive chlR promoter is designated $P_R$ hereafter.

**H-CHG treatment induces ChlR to activate the efrEF promoter.** In our microarray trials, the expression of chlR was not affected by H-CHG (fold change = 0.3; $p$-value = 0.57). This evidence indicates that H-CHG may directly or indirectly trigger the activation of ChlR.

We performed β-galactosidase assays to assess responses of the efrEF promoter $P_{EF}$ to H-CHG. Promoter reporter strains were spotted on agar plates supplemented with X-Gal and different concentrations of H-CHG (Figure 6). As expected, the control strain lacking a promoter for lacZ, *E. faecalis* V583 pPB101 (strain FL101), displayed no detectable β-galactosidase activity in the presence or absence of H-CHG (Figure 6A). For cultures without H-CHG, $P_{EF}$ promoter activity was not detected. Sub-inhibitory concentrations of H-CHG elicited increases in $P_{EF}$ promoter activity (Figure 6A). These results demonstrate that H-CHG is required to stimulate efrEF promoter activity, and that H-CHG concentrations several two-fold dilutions below the MIC still elicit this response. Conversely, $P_{EF}$ induction by H-CHG was absent in V583 $ΔchlR$ (Figure 6A). We conclude that ChlR is required for activation of the efrEF promoter upon H-CHG exposure.

We constructed a chlR promoter reporter, pFL202 (Figure 4B), to assess responses of the $P_R$ promoter to H-CHG. Unlike $P_{EF}$, $P_R$ was active regardless of the presence or absence of H-CHG. Moreover, $P_R$ was active in V583 $ΔchlR$ irrespective of H-CHG (Figure 6B).

The $P_{EF}$ promoter includes a consensus -35 sequence (TTGACA) and a near-consensus -10 region (TACAAT) for binding by a housekeeping sigma factor. The -10 and -35 sequences are separated by 19 bp. This 19 bp spacing is typical for MerR family promoters; MerR regulators recruit RNA polymerase holoenzyme to non-optimal promoters (24, 25). Unusually,
however, the TSS of $P_{EF}$ occurs 13 bp downstream from the 3’ end of the -10 region. Non-standard TSS spacing was also observed for MerR regulation of $merA$ in the archaeon *Sulfolobus solfataricus* (26), but to our knowledge has not been observed for other MerR regulators in bacteria.

Typical MerR-regulated promoters harbor a palindromic MerR binding motif between the -35 and -10 promoter regions (24). Within the $P_{EF}$ promoter region, we identified a palindromic motif, TTCAAGTTACTTGAA (Figure 5), which does not occur elsewhere on the V583 chromosome. Because the 5’ half of the motif lies directly adjacent to the predicted -35 region (Figure 5), alteration of that sequence may prevent RNA polymerase binding. We modified the 3’ motif from ACTTGAA to CAGCTAC to determine if this motif affects $efrEF$ promoter inducibility. H-CHG induction was abolished for the mutant $P_{EF}$ promoter construct (Figure 6C).

**Discussion**

In this study, we performed transcriptomic analysis to identify genes that are differentially regulated when *E. faecalis* V583 is exposed to H-CHG. The genes $efrEF$ were the most highly up-regulated. We found that $efrEF$ and the transcription regulator ChlR are required for H-CHG stress response in *E. faecalis* V583. ChlR activates $efrEF$ expression in response to H-CHG. These results are consistent with and identify new features of the chlorhexidine stress response in enterococci. The $efrEF$ orthologs in a VanA-type VRE strain, *E. faecium* 1,231,410, were also up-regulated in the presence of H-CHG (15). Moreover, sequential sub-inhibitory H-CHG exposure selected for *E. faecium* 1,231,410 $efrE$ mutants that conferred reduced H-CHG susceptibility (27). Finally, deletion of $efrE$ in *E. faecalis* OG1RF conferred decreased susceptibility to chlorhexidine and pentamidine (28, 29). Our results deepen our understanding of $efrEF$ by identifying a transcriptional regulator that is required for the induction of $efrEF$ expression in response to H-CHG stress.
Gaps in knowledge remain about the enterococcal response to chlorhexidine stress. Specifically, what does EfrEF transport, and what ligand activates ChlR? These processes are significant because they reduce enterococcal susceptibility to chlorhexidine. Hassan et al. discovered chlorhexidine efflux proteins in Gram-negative bacteria (30, 31), but EfrEF does not belong to this protein family. Overexpression of efrEF in *Lactococcus lactis* conferred enhanced efflux of fluorescent dyes and decreased susceptibility to multiple antibiotics (12). Monitoring C14-chlorhexidine transport in wild-type and ΔefrEF *E. faecalis* would be required to determine whether chlorhexidine is a substrate for EfrEF. Alternatively, EfrEF may transport a metabolite that is required for the cell to survive stress imposed by H-CHG and other antimicrobials.

ChlR belongs to the MerR regulator family. MerR was first identified as a transcriptional activator of the mercury resistance (*mer*) operon in Gram-negative bacteria. An activated MerR dimer bound at a dyad symmetrical motif sequence in the *mer* promoter region drives a conformational change in DNA that results in induction of *mer* operon expression (24, 25). Generally, MerR family proteins possess two domains: a highly conserved N-terminal DNA binding region and a poorly conserved C-terminal ligand binding region (24, 25). The functionality of the N-terminal region depends on ligand binding by the C terminus. The variable C-terminal sequences of the MerR protein family recognize different ligands including metals and dyes, and therefore lack amino acid sequence conservation (24, 25). The C-terminal region of ChlR possesses no predicted conserved domains. As shown by our microarray and β-galactosidase reporter analyses, *chlR* expression is not induced by H-CHG. It appears that ChlR requires H-CHG or metabolites associated with H-CHG stress to induce *efrEF* expression. Our study did not determine whether chlorhexidine directly interacts with ChlR. The observation that *efrEF* is up-regulated in response to the plasmid post-segregational killing toxin Fst (32) confirms that *efrEF* up-regulation is not specific to H-CHG stress. It is unknown whether ChlR
mediates the Fst-dependent up-regulation of efrEF. Identifying the specific ligand of ChlR will be a topic of future studies.

In conclusion, our study provides novel insights into the transcriptomic response of vancomycin-resistant *E. faecalis* to chlorhexidine. The *chlR* and *efrEF* genes play key roles in *E. faecalis* survival of H-CHG exposure at concentrations near the MIC. Notably, 1/8X MIC H-CHG activated ChlR-dependent *efrEF* expression; induction at lower concentrations may occur but we did not test this. In a hospital environment, *E. faecalis* is likely exposed to sub-inhibitory concentrations of H-CHG which are not lethal but are sufficient to elicit a transcriptional response. It remains to be determined whether this transcriptional response impacts susceptibility to other clinically relevant antimicrobials.

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Figure legends.

Figure 1. Growth curves. a) Optical density at 600 nm (OD₆₀₀) is shown on the y-axis. Mid-exponential phase *E. faecalis* V583 cultures (OD₆₀₀ of 0.4-0.5) were split into fresh, pre-warmed media to achieve different H-CHG concentrations (indicated with arrow). For all experiments, 1X MIC is the broth microdilution MIC of *E. faecalis* V583. The time point at which RNA was harvested is also indicated. b) Viable cell counts (CFU/mL) for 1X MIC-treated cultures and control cultures are shown. For this curve, the time at which cultures were split is set to 0 hr. For both panels, error bars indicate standard deviations from 3 independent experiments.

Figure 2. Quantitative RT-PCR confirms H-CHG-dependent up-regulation of *efrF*. Primers were designed to amplify ~600 bp internal regions of *efrF*. RNA was harvested from *E. faecalis* V583 after 15 mins exposure to no CHG, 1/2X MIC H-CHG or 1X MIC H-CHG. The gyr"b (gyrB) gene was amplified as a control gene. *, p < 0.05; **, p < 0.005; ***, p < 0.0005.

Figure 3. *efrEF* and *chlR* deletion mutants are more susceptible to H-CHG. Overnight cultures were adjusted to an OD₆₀₀ of ~0.3 and serially diluted in 10-fold dilutions. 10 μl of each dilution (from 10⁻¹ to 10⁻⁶; left to right in each image) were spotted on BHI agar supplemented with different concentrations of H-CHG. Figures are representative of three independent trials. Experiments assessing the contributions of *efrEF* (panel A) and *chlR* (panel B) to chlorhexidine susceptibility are shown.

Figure 4. *chlR* is required for H-CHG-dependent up-regulation of *efrF*. qRT-PCR was used to investigate *efrF* expression in wild-type V583, Δ*chlR*, Δ*chlR* with the empty complementation
vector pCAT28, and complemented ΔchlR (ΔchlR pFL102) cultures after 15 mins exposure to 1/2X MIC H-CHG or no H-CHG. The gyrase B (gyrB) gene was amplified as a control gene. *, p < 0.05.

Figure 5. Organization of chlR-efrE intergenic region. The organization of the chlR and efrEF genes is shown (not drawn to scale). Transcription start sites (TSS) were detected 33 and 78 bp upstream of the efrE ORF. Consensus -35 and near-consensus -10 housekeeping sigma factor promoter sequences upstream of the H-CHG-responsive efrE promoter (PEF) are shown in bold. Putative ChlR binding motifs are underlined. The predicted promoter for the -78 bp TSS is not shown for clarity.

Figure 6. β-galactosidase assays. Cultures were spotted on BHI agar plates supplemented with X-Gal and different concentrations of H-CHG. Wild-type V583 with pPB101 (promoterless lacZ) was used as the negative control for all panels. a) efrE promoter activity (pFL201) in wild-type V583 and the ΔchlR strain. b) chlR promoter activity (pFL202) in wild-type V583 and the ΔchlR strain. c) efrE promoter activity in wild-type V583 with (pFL204) or without (pFL203) mutation of the 3’ region of the putative ChlR binding motif. Figures shown are representative of three independent trials.
Table 1. Bacterial strains and plasmids used in this study.

| Strain or plasmid | Description | Reference |
|-------------------|-------------|-----------|
| **Bacterial strains** | | |
| *E. faecalis* strains | | |
| V583 | Bloodstream isolate; VanB-type VRE | (20) |
| OG1RF | Human oral isolate | (33) |
| ΔchlR | *E. faecalis* V583 ΔchlR | This study |
| ΔefrEF | *E. faecalis* V583 ΔefrEF | This study |
| ΔefrEF pFL103 | ΔefrEF transformed with pFL103 | This study |
| FL101 | *E. faecalis* V583 transformed with pPB101 | This study |
| FL201 | *E. faecalis* V583 transformed with pFL201 | This study |
| FL202 | *E. faecalis* V583 transformed with pFL202 | This study |
| FL203 | *E. faecalis* V583 transformed with pFL203 | This study |
| FL204 | *E. faecalis* V583 transformed with pFL204 | This study |
| ΔchlR pFL102 | ΔchlR transformed with pFL102 | This study |
| ΔchlR pFL201 | ΔchlR transformed with pFL201 | This study |
| ΔchlR pFL202 | ΔchlR transformed with pFL202 | This study |
| **E. coli** strains | | |
| EC1000 | Cloning host; provides repA in trans; F- araD139 (ara ABC-leu)7679 galU galK lacX74 rpsL thi; repA of pWV01 in glgB; Km | (34) |
| DH5α | Cloning host; F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR supE44 thi-1 endA1 relA1 gyrA96 thsB16 (lacZYA-argF)U169 hsdR17 (rK- mK+) λ- | (35) |
| BW23474 | Cloning host for pPB101 and derivatives; Δlac-169 robA1 cre C510 hsdR514 endA recA1 ΔuidA::pir-116 | (36) |
| **Plasmids** | | |
| pHA101 | pLT06 plasmid with oriT from pHO2 inserted at PstI | (15) |
| pCAT28 | Shuttle vector; pUC and pAMβ1 origins; confers chloramphenicol resistance | M. Rodrigues and K. Palmer |
| Construct   | Description                                                                 | Reference |
|-------------|------------------------------------------------------------------------------|-----------|
| pFL102      | pCAT28 containing 882-bp EcoRI/BamHI-digested chlR ORF and promoter region  | This study|
| pFL103      | pCAT28 containing 3673-bp EcoRI/BamHI-digested efrEF ORF and promoter region | This study|
| pPB101      | pTCV-lac-cat; expression vector for Gram-positive bacteria; confers kanamycin, erythromycin, and chloramphenicol resistance | (15)      |
| pFL201      | pPB101 with 114-bp EcoRI/BamHI-digested efrEF promoter region               | This study|
| pFL202      | pPB101 with 114-bp EcoRI/BamHI-digested chlR promoter region                | This study|
| pFL203      | pPB101 with 98-bp EcoRI/BamHI-digested efrEF promoter region               | This study|
| pFL204      | pPB101 with 98-bp EcoRI/BamHI-digested efrEF promoter region with modified motif CAGCTAC | This study|
Table 2. Broth microdilution H-CHG MICs

| Strain                  | H-CHG MIC (μg/mL) |
|-------------------------|-------------------|
| *E. faecalis* V583      | 9.8               |
| *E. faecalis* V583 ΔefrEF | 2.4               |
| *E. faecalis* V583 ΔchlR | 4.9               |
| *E. faecalis* OG1RF     | 9.8               |
Fold change relative to gyrB

V583
OG1RF
Control
½X MIC
1X MIC

* p < 0.05
** p < 0.005
*** p < 0.0005
Fold change relative to gyrB

|        | V583 | ΔchlR | ΔchlR + pCAT28 | ΔchlR + pFL102 |
|--------|------|-------|---------------|----------------|
| 2^10   |      |       |               |                |
| 2^5    |      |       |               |                |
| 2^0    |      |       |               |                |
| 2^-5   |      |       |               |                |
| 2^-10  |      |       |               |                |

* p < 0.05
a) V583 pPB101
b) V583 ΔchlR pFL201
c) V583 pPB101

No CHG

1/2X MIC

1/4X MIC

1/8X MIC

V583 pFL201
V583 pFL203
V583 V583 pFL204