



# The Two-Component System ChtRS Contributes to Chlorhexidine Tolerance in *Enterococcus faecium*

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**ABSTRACT** *Enterococcus faecium* is one of the primary causes of nosocomial infections. Disinfectants are commonly used to prevent infections with multidrug-resistant *E. faecium* in hospitals. Worryingly, *E. faecium* strains that exhibit tolerance to disinfectants have already been described. We aimed to identify and characterize *E. faecium* genes that contribute to tolerance to the disinfectant chlorhexidine (CHX). We used a transposon mutant library, constructed in a multidrug-resistant *E. faecium* bloodstream isolate, to perform a genome-wide screen to identify genetic determinants involved in tolerance to CHX. We identified a putative two-component system (2CS), composed of a putative sensor histidine kinase (ChtS) and a cognate DNA-binding response regulator (ChtR), which contributed to CHX tolerance in *E. faecium*. Targeted *chtR* and *chtS* deletion mutants exhibited compromised growth in the presence of CHX. Growth of the *chtR* and *chtS* mutants was also affected in the presence of the antibiotic bacitracin. The CHX- and bacitracin-tolerant phenotype of *E. faecium* E1162 was linked to a unique, nonsynonymous single nucleotide polymorphism in *chtR*. Transmission electron microscopy showed that upon challenge with CHX, the  $\Delta$ *chtR* and  $\Delta$ *chtS* mutants failed to divide properly and formed long chains. Normal growth and cell morphology were restored when the mutations were complemented in *trans*. Morphological abnormalities were also observed upon exposure of the  $\Delta$ *chtR* and  $\Delta$ *chtS* mutants to bacitracin. The tolerance to both chlorhexidine and bacitracin provided by ChtRS in *E. faecium* highlights the overlap between responses to disinfectants and antibiotics and the potential for the development of cross-tolerance for these classes of antimicrobials.

**KEYWORDS** *Enterococcus*, bacitracin, biocides, chlorhexidine, disinfectants, tolerance

*Enterococcus faecium* is a facultative anaerobic Gram-positive bacterium that naturally colonizes the gastrointestinal tract of humans and animals. Since the 1990s, *E. faecium* has also emerged as one of the leading causes of nosocomial infections (1, 2). The population of *E. faecium* is currently divided into clade A-1, containing most clinical isolates, clade A-2, with most animal-derived strains, and clade B, in which most isolates of healthy humans are clustered (3). Whether clade A-1 and clade A-2 are monophyletic and can be reliably distinguished from each other has recently been questioned (4). Nosocomial *E. faecium* strains are frequently resistant to glycopeptides and  $\beta$ -lactam antibiotics (5, 6), complicating the treatment of clinical infections. Since the late 1990s, and despite the worldwide spread of vancomycin-resistant enterococci (VRE), only two antibiotics (daptomycin and linezolid) have been approved by the FDA for use against VRE. Other antibiotics (quinupristin-dalfopristin, tigecycline, oritavancin, tedizolid, telavancin, and dalbavancin) have been suggested as alternatives for treatment of infections caused by VRE in clinical practice. They have, however, not been approved by the FDA for the treatment of VRE infections (7–9). The use of the polypeptide antibiotic

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bacitracin, in combination with other antibiotics, in the treatment of VRE infections has also been proposed (10).

While antibiotics are gradually losing their effectiveness against *E. faecium*, antiseptics and disinfectants are becoming increasingly important to prevent the spread of multidrug-resistant *E. faecium* in health care settings (11). Chlorhexidine (CHX) is a bisbiguanide agent and has diverse applications as a disinfectant for surfaces and as an antiseptic for topical applications (12). The mode of action of CHX is poorly understood. CHX, which is positively charged at neutral pH, is thought to be attracted to the bacterial cell surface, where it may electrostatically interact with negatively charged phospholipids. Depending on the concentration of CHX, it can reduce bacterial membrane fluidity or disrupt the structural integrity of the membrane, causing increased permeability and leakage of cell contents and, ultimately, cell death (13–15). In health care, CHX is often used in surgical scrubs for preoperative skin preparation, impregnated wash cloths for postoperative wound care, daily patient bathing, and oral care of intubated patients (16, 17). Regular bathing of patients with CHX significantly reduces the colonization by VRE and other multiresistant organisms in intensive care units and general medicine wards (18–23). Recently, increased tolerance to these compounds has been reported for Gram-positive cocci, and this could contribute to future co- or cross-selection for antibiotic resistance (24–30). In addition, subinhibitory concentrations of CHX induce the expression of genes involved in vancomycin and daptomycin resistance in enterococci (31).

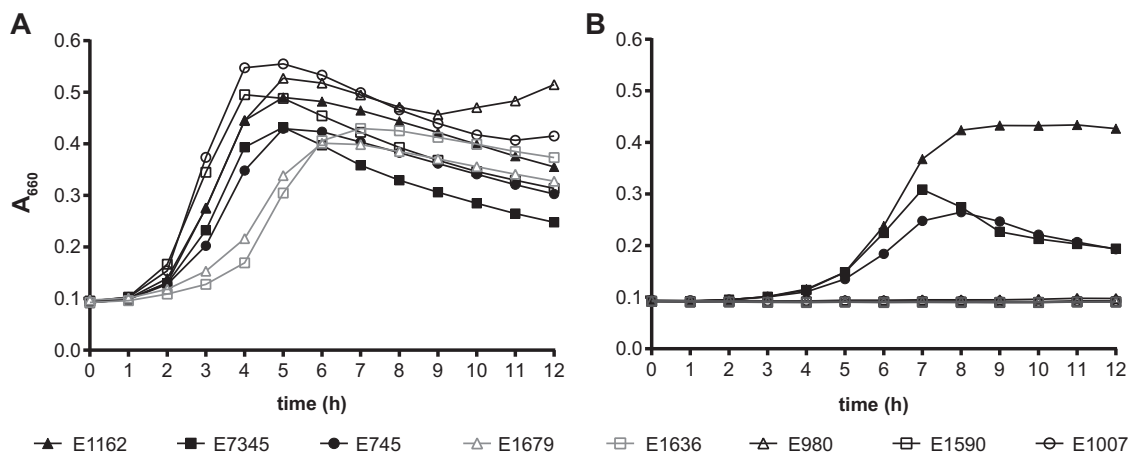
In this study, we used microarray-based transposon mapping (M-TraM [32]) to perform a genome-wide screening of a transposon mutant library to identify genes involved in the tolerance to CHX in *E. faecium*. Two genes that were identified in the M-TraM screening were predicted to encode a two-component regulatory system (2CS). 2CSs are signal transduction systems in bacteria consisting of a sensor histidine kinase and its response regulator. They play important roles in the adaptation of bacteria to changes in the environment and have been implicated in orchestrating cellular responses that lead to increase of tolerance to antimicrobials in different Gram-positive bacteria, including enterococci (33–35). The two genes encoding the 2CS were further characterized to define their role in tolerance to CHX.

## RESULTS

***E. faecium* strains from different phylogenetic backgrounds differ in their tolerance to CHX.** First, we assayed the tolerance to CHX of the *E. faecium* strain E1162, a multidrug-resistant bloodstream isolate, previously assigned to clade A-1 (3, 32), by measuring growth in Mueller-Hinton broth (MHB) supplemented with different concentrations of CHX (see Fig. S1 in the supplemental material). We found that at CHX concentrations of 1.7  $\mu\text{g ml}^{-1}$  or higher, growth was essentially inhibited completely. In follow-up experiments, CHX was used at 1.2  $\mu\text{g ml}^{-1}$ , as this concentration led to an extended lag phase and lower growth rate of *E. faecium* E1162.

Next, we compared the abilities of seven other *E. faecium* strains (two strains from clade A-1, two strains from clade A-2, and three strains from clade B [3]) to grow in the presence of CHX (Fig. 1). We found that the strains from clade A-1, all of which were isolated from bloodstream infections in hospitalized patients, were able to grow in medium containing 1.2  $\mu\text{g ml}^{-1}$  CHX, while the strains from clade A-2 or clade B could not (Fig. 1). E1162 had the highest growth rate in the presence of the disinfectant, and therefore it was chosen for the follow-up experiments into the mechanism of CHX tolerance in *E. faecium*.

**Identification of a CHX tolerance locus in *E. faecium* E1162 by M-TraM.** We identified conditionally essential genes in the *E. faecium* E1162 transposon mutant library, during growth in the presence of 1.2  $\mu\text{g ml}^{-1}$  CHX, through microarray-based transposon mapping (M-TraM) (32). While the M-TraM analysis hinted at a functional contribution of several genes in CHX tolerance (Table 1), we focused on the gene with locus tag EfmE1162\_2203, of which the transposon mutant was significantly affected (8.7-fold lower abundance in the CHX-exposed library compared to the untreated



**FIG 1** Growth of *E. faecium* strains challenged with CHX. Shown are the growth curves for different *E. faecium* strains in MHB alone (A) and MHB in the presence of 1.2 µg ml<sup>-1</sup> CHX (B). Clade A-1 strains are indicated by solid black symbols, and clade A-2 and clade B are indicated by open gray and open black symbols, respectively. The growth curves represent the averages from three replicates.

library) during growth in the presence of the disinfectant. EfmE1162\_2203 is annotated as encoding a DNA-binding response regulator. EfmE1162\_2203 is located adjacent to a gene (Efm E1162\_2202) encoding a histidine kinase of which the transposon mutant abundance was moderately (3.0-fold) reduced upon exposure to CHX. The proteins encoded by EfmE1162\_2202 and EfmE1162\_2203 likely form a 2CS in *E. faecium* E1162. We have renamed EfmE1162\_2203 and EfmE1162\_2202 *chtR* and *chtS*, for chlorhexidine tolerance response regulator and chlorhexidine tolerance sensor histidine kinase, respectively.

To determine the distribution of *chtR* and *chtS* among *E. faecium* strains, we assessed the presence of these two genes in all of the strains tested for CHX tolerance and in 85 previously published genome sequences, which were previously assigned to clade A-1 (*n* = 41), clade A-2 (*n* = 31), and clade B (*n* = 13) (3, 36). The analysis showed that the ChtRS 2CS is conserved in all analyzed *E. faecium* strains. By analyzing the nucleotide sequences of the *chtR* and *chtS* genes of the eight *E. faecium* strains tested for their tolerance to CHX (Fig. 1), a single nonsynonymous nucleotide change, leading to an amino acid substitution (P102H), was found in the ChtR protein of all CHX-tolerant clade A-1 strains, compared to the non-CHX-tolerant clade A-1 and B strains. This amino acid is located in a predicted dimerization interface located in the signal receiver domain of ChtR.

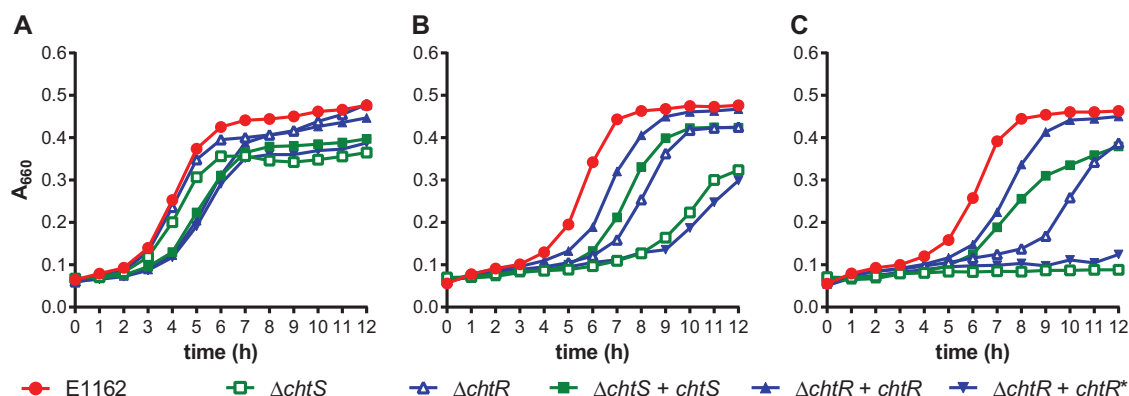
**TABLE 1** *E. faecium* genes implicated in tolerance to chlorhexidine by M-TraM analysis<sup>a</sup>

Locus tag <sup>b</sup>	Gene name	Annotation	Avg fold change <sup>c</sup>
<b>EfmE1162_2203</b>	<b><i>chrR</i></b>	<b>DNA-binding response regulator</b>	<b>8.7</b>
EfmE1162_0264		Permease protein, putative	4.6
EfmE1162_0996		Hypothetical protein	4.2
EfmE1162_2026		Lactose phosphotransferase system repressor	3.9
EfmE1162_0997		Conserved hypothetical protein	3.9
EfmE1162_2510		Holliday junction DNA helicase RuvA	3.8
EfmE1162_0300		PrgW	3.6
EfmE1162_2635		Esterase	3.6
EfmE1162_0431		Conserved hypothetical protein	3.2
EfmE1162_0394		PrgO	3.2
<b>EfmE1162_2202</b>	<b><i>chrS</i></b>	<b>Sensor histidine kinase</b>	<b>3.0</b>

<sup>a</sup>Boldface type indicates the *chtRS* system targeted for further analysis.

<sup>b</sup>The locus tag represents the gene containing the transposon insertion.

<sup>c</sup>Fold change in expression of the gene as determined by a ratio of the library grown under the control condition to that under the CHX-challenged condition. Results were averaged from four replicates.

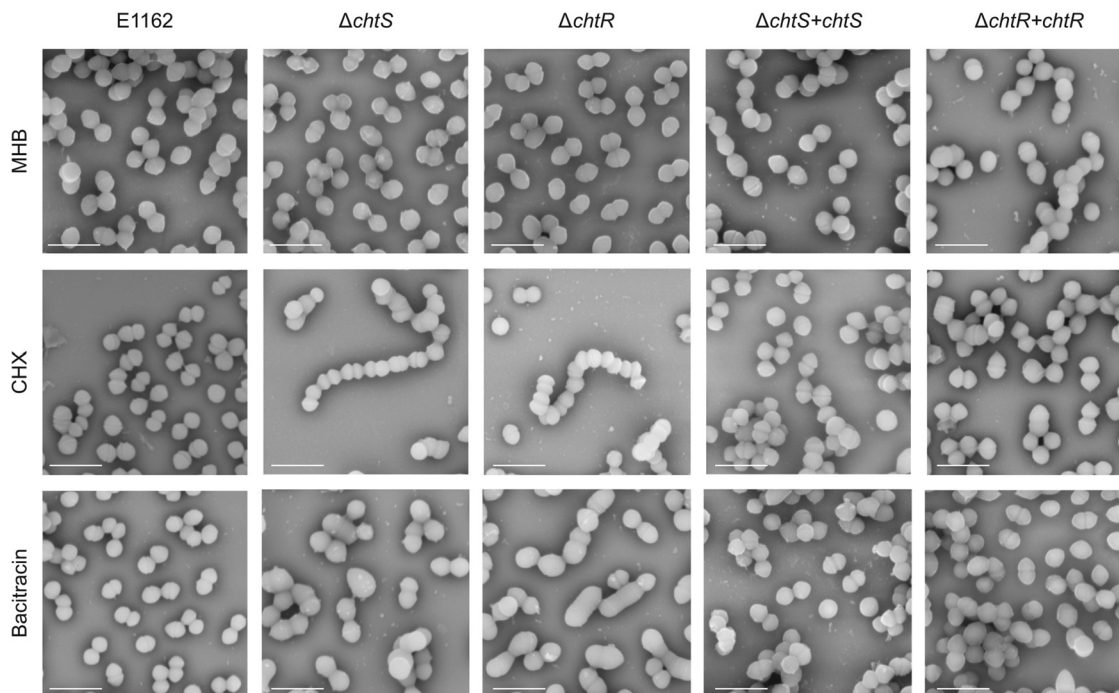


**FIG 2** Effect of targeted mutations in the  $\Delta chtR$  and  $\Delta chtS$  mutants upon challenge with CHX and bacitracin. The growth curves shown in panel A correspond to strains growing in MH broth. Panels B and C correspond to the same strains growing in MH broth supplemented with  $1.2 \mu\text{g ml}^{-1}$  CHX and  $4 \mu\text{g ml}^{-1}$  bacitracin, respectively. Wild-type strain E1162 is shown in red, while the  $chtS$  and  $chtR$  targeted deletion mutants are shown in green and blue open symbols, respectively. Solid green and blue symbols represent the growth curves of the *in trans* complemented strains (the  $\Delta chtS + chtS$ ,  $\Delta chtR + chtR$ , and  $\Delta chtR + chtR^*$  strains). The growth curves represent the averages from three experiments.

**The ChtRS 2CS contributes to CHX and bacitracin tolerance.** To validate the M-TraM results, we constructed  $chtR$  and  $chtS$  markerless deletion ( $\Delta chtR$  and  $\Delta chtS$ ) mutants. We also constructed two strains in which these mutations were complemented *in trans*, which were named the  $\Delta chtR + chtR$  and  $\Delta chtS + chtS$  strains. No differences in growth were observed between E1162 and the two targeted mutants or the complemented strains when grown in MHB (Fig. 2A). In the presence of  $1.2 \mu\text{g ml}^{-1}$  CHX, E1162 had a lag phase of approximately 4 h. Under the same conditions, the  $\Delta chtR$  and  $\Delta chtS$  mutants had a lag phase of almost 8 h and exhibited slower exponential growth than E1162 (Fig. 2B). The  $\Delta chtR + chtR$  and  $\Delta chtS + chtS$  complemented strains exhibited wild-type levels of growth in the presence of CHX. These results confirm that both  $chtR$  and  $chtS$  are involved in CHX tolerance of *E. faecium* E1162. When the *in trans* copy of  $chtR$  was mutated (resulting in  $chtR^*$ ) to engineer a proline at position 102 of ChtR, as is characteristic for ChtR in CHX-susceptible strains, it could no longer complement the growth defect caused by the  $chtR$  deletion.

In addition, we decided to test the effect of the deletions in  $chrR$  and  $chrS$  on the tolerance of *E. faecium* E1162 to the antibiotic bacitracin, as a homologous 2CS (EF0926-EF0927) has previously been described to have a minor role in bacitracin tolerance in *Enterococcus faecalis* V583 (37). Upon exposure of the  $\Delta chtR$  and  $\Delta chtS$  mutants to  $4 \mu\text{g ml}^{-1}$  bacitracin, the growth of both mutants was completely inhibited, while in the *in trans* complemented strains, growth was restored to near wild-type levels (Fig. 2C). The  $chtR$  deletion mutant complemented with the  $chtR^*$  allele remained inhibited in its growth in the presence of bacitracin.

**CHX and bacitracin challenge affects cellular morphology in the  $\Delta chtR$  and  $\Delta chtS$  mutants but not in *E. faecium* E1162.** In order to further characterize the effects of CHX and bacitracin on *E. faecium* E1162 and its  $chtR$  and  $chtS$  mutants, cells were analyzed by scanning electron microscopy (SEM) (Fig. 3). In these experiments, bacitracin was added to the medium at  $1 \mu\text{g ml}^{-1}$ , as this concentration is permissive for growth of the  $chtR$  and the  $chtS$  deletion mutants. No apparent changes in cellular morphology were found when E1162 was challenged with CHX or bacitracin, compared to growth in MHB. However, when the  $\Delta chtS$  and  $\Delta chtR$  mutants were challenged with CHX, the cells failed to divide properly and formed chains. Exposure of the  $\Delta chtS$  and  $\Delta chtR$  mutants to bacitracin resulted in swollen cells with various cellular abnormalities. The chaining phenotype and the cellular abnormalities found in CHX and bacitracin-challenged  $\Delta chtS$  and  $\Delta chtR$  mutants, respectively, were not observed in the  $\Delta chtR + chtR$  and  $\Delta chtS + chtS$  *in trans* complemented strains.



**FIG 3** Cell morphology of *E. faecium* E1162, the  $\Delta$ *chtS* and  $\Delta$ *chtR* mutants, and the  $\Delta$ *chtS* + *chtS* and  $\Delta$ *chtR* + *chtR* complemented strains upon exposure to CHX and bacitracin. E1162, the  $\Delta$ *chtS* and  $\Delta$ *chtR* mutants, and the  $\Delta$ *chtS* + *chtS* and  $\Delta$ *chtR* + *chtR* complemented strains were grown to an OD<sub>660</sub> of 0.2. The cells were grown in Mueller-Hinton broth (MHB) alone, in MHB with 1.2  $\mu$ g ml<sup>-1</sup> chlorhexidine (CHX), or in MHB with 1  $\mu$ g ml<sup>-1</sup> bacitracin. Specimens were coated with 1-nm-diameter gold particles. Images were taken at a magnification of 35,000 $\times$ . The scale bars correspond to 2  $\mu$ m.

## DISCUSSION

Enterococci have recently become important nosocomial pathogens (4). The ability of *E. faecium* to rapidly acquire drug resistance determinants threatens the treatment of infections caused by this organism. Antiseptics and disinfectants, including CHX, have been used for decades to prevent the spread of multidrug-resistant pathogens, including enterococci, in health care settings (19). In the present study, we found that clinical multidrug-resistant *E. faecium* isolates belonging to a distinct subpopulation of hospital-associated strains that are contained in clade A-1 were able to tolerate CHX, while *E. faecium* strains belonging to the other clades (A-2 and B) were more susceptible to CHX. The increased tolerance to CHX in clade A-1 strains, compared to strains from other *E. faecium* clades, may have been selected by the exposure to antiseptics and disinfectants, which are commonly used in health care settings. As clade A-1 strains appear to have specifically evolved to thrive in hospitalized patients, the increased tolerance to disinfectants may form an additional adaptation to this specific niche and could contribute to the success of these isolates as hospital-acquired opportunistic pathogens. Using M-TraM, we identified the 2CS ChtRS as being essential for CHX tolerance in the drug-resistant clinical isolate *E. faecium* E1162.

The *chtS* and *chtR* genes putatively encode a histidine kinase and a response regulator, together forming a 2CS. 2CSs regulate the expression of genes as a response to environmental cues (38–40). The signal is received at the extracellular sensor domain, and its transduction occurs via ATP-dependent phosphorylation. The phosphoryl group is transferred from the histidine phosphotransfer domain to the conserved signal receiver domain of the response regulator (41). The single amino acid substitution (P102H) that distinguishes ChtR in CHX-tolerant clade A-1 strains from ChtR in CHX-susceptible clade A-2 and clade B strains is located in the predicted dimerization interface of the REC domain in ChtR. The activation and regulation of response regulators by dimerization through receiver domains have previously been studied in other bacteria (42–44), including Gram-positive organisms (45), and changes in the

dimerization interface of the signal receiver domain of ChtR could affect the function of the response regulator, thereby altering the control of gene expression upon CHX exposure by ChtRS. The inability of the construct with the mutated allele of *chtR* (encoding the ChtR protein with a proline residue at position 102) to complement a *chtR* deletion suggests a crucial role for this SNP in the CHX-tolerant phenotype of clade A-1 strains. Mechanistic studies of the proteins encoded by the different *chtR* alleles, including their dimerization and phosphotransfer characteristics, may be the topic of future studies.

In the present study, E1162 mutants deficient in *chtRS* exhibited decreased tolerance to CHX and, in addition, were more susceptible to bacitracin. In other Gram-positive bacteria, including *E. faecalis*, 2CSs also contribute to the protective response against low concentrations of bacitracin (46–50). Bacitracin is an antibiotic that targets peptidoglycan biosynthesis by binding to undecaprenol pyrophosphate (UPP), blocking its recycling during peptidoglycan synthesis. This, in turn, interferes with the transport of new peptidoglycan building blocks, leading to disruption of cell wall synthesis (51). Since 2CSs exert their effect through the regulation of expression of effector genes, the observed loss of CHX and bacitracin tolerance in the *chtR* and *chtS* deletion mutants is most likely due to altered expression of genes regulated by ChtRS. The effector genes regulated by ChtRS remain to be elucidated.

Bacitracin can decrease colonization by vancomycin-resistant *E. faecium* in the gastrointestinal tract (52, 53) and may be used orally to control the dissemination of vancomycin-resistant enterococci (10). However, therapeutic failure of bacitracin to treat VRE colonization has also been reported (54, 55) and may be caused by intrinsic bacitracin resistance in enterococci, to which ChtRS contributes in *E. faecium*. *E. faecium* has a multitude of intrinsic and acquired resistance mechanisms that allow it to survive the selective pressures imposed by the nosocomial environment, including antibiotic therapy and the use of disinfectants. Further studies should be performed to characterize the mechanisms by which different antiseptics and disinfectants may cross-select or coselect for clinically relevant antibiotics. Information resulting from this line of inquiry may be used to develop efficient disinfection protocols, while minimizing the risk of further resistance development in *E. faecium*.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The strains and plasmids used in this study are listed in Table S1 in the supplemental material. *Escherichia coli* was grown in lysogeny broth (LB), and *E. faecium* strains were grown in Muller-Hinton broth (MHB) at 37°C with shaking at 200 rpm, unless mentioned otherwise. When appropriate, antibiotics were added at the following concentrations: spectinomycin at 300  $\mu\text{g ml}^{-1}$  for *E. faecium* and 100  $\mu\text{g ml}^{-1}$  for *E. coli* and gentamicin at 300  $\mu\text{g ml}^{-1}$  for *E. faecium* and 25  $\mu\text{g ml}^{-1}$  for *E. coli*. CHX was used at 1.2  $\mu\text{g ml}^{-1}$ , unless mentioned otherwise. Media were obtained from Oxoid (Basingstoke, United Kingdom). Antibiotics and disinfectants were obtained from Sigma-Aldrich (Saint Louis, MO).

**M-TraM to identify genes involved in CHX tolerance.** The *mariner* transposon mutant library of *E. faecium* E1162 and the M-TraM method have been described previously (32). In brief, four overnight cultures of the E1162 mutant library were cultured at 37°C in MHB, diluted to an optical density at 660 nm ( $\text{OD}_{660}$ ) of 0.025 in 20 ml of prewarmed MHB supplemented with CHX, and then grown at 37°C until the mid-exponential phase ( $\text{OD}_{660}$  of 0.3). M-TraM was performed with four biological replicates following previously described procedures (32). Statistical analysis of hybridization signals between the conditions was performed using Cyber-T (56). Genes were considered differentially expressed when all four probes showed a Bayesian *P* value of  $<0.001$  and the abundance of a gene was  $\leq 0.2$  or  $\geq 5.0$  compared to the untreated control (32).

**Construction of markerless deletion mutant in *chtS* and *chtR* and in *trans* complementation.** *chtS* and *chtR* markerless deletion mutants were generated by previously described methods (32). Brain heart infusion broth or agar was used as the growth medium for *E. faecium* during all genetic manipulations. In brief, the 5' and 3' flanking regions (approximately 500 bp) of each gene were PCR amplified with two sets of primers as follows: Up-*chtS*\_2202-F-XhoI and Up-*chtS*\_2202-R-EcoRI for the upstream fragment of *chtS* and Down-*chtS*\_2202-F-EcoRI and Down-*chtS*\_2202-R-XmaI for the downstream fragment of *chtS* and Up-*chtR*\_2203-F-XhoI and Up-*chtR*\_2203-R-EcoRI for the upstream fragment of *chtR* and Down-*chtR*\_2203-F-EcoRI and Down-*chtR*\_2203-R-XmaI for the downstream fragment of *chtR*. The primer sequences are listed in Table S2 in the supplemental material. The two flanking regions of each gene were then fused together by PCR and cloned into pWS3 (57). A gentamicin resistance cassette flanked by *lox66* and *lox71* sites was amplified by PCR using the primers pAT392\_EcoRI\_lox66\_genta\_F and pAT392\_EcoRI\_lox71\_genta\_R and cloned into the EcoRI site that

was generated between the 5' and 3' flanking regions of each gene in the pWS3 construct, as described previously (32). Finally, the two constructs, named pWJ1 and pWJ2, were electrotransformed individually into *E. faecium* E1162, and the *chtS* ( $\Delta$ *chtS*) and *chtR* ( $\Delta$ *chtR*) markerless deletion mutants were generated as described before (32).

For in *trans* complementation of the  $\Delta$ *chtS* and  $\Delta$ *chtR* mutants, the full-length genes and upstream regions of 356 and 390 nucleotides, respectively, were amplified by PCR using Accuprime high-fidelity *Taq* polymerase (Life Technologies, Bleiswijk, The Netherlands). The primers for these PCRs were named Comp2202\_Fw\_SacI and Comp2202\_Rv\_SmaI for the amplification of *chtS* and Comp2203\_Fw\_SacI and Comp2203\_Rv\_SmaI for the amplification of *chtR*. These primers introduce SacI and SmaI restriction sites, and after digestion with these enzymes, the resulting products were cloned into pEF25. The constructs were sequenced to confirm the absence of mutations and electrotransformed into the  $\Delta$ *chtS* and  $\Delta$ *chtR* mutants as described previously (32), generating the  $\Delta$ *chtS* + *chtS* and  $\Delta$ *chtR* + *chtR* complemented strains.

To determine the role of a nonsynonymous SNP in *chtR*, leading to a P102H amino acid substitution in ChtR in clade A-1 strains, we ordered the *chtR* gene of E1162 and its promoter as a genomic block (gBlock [Integrated DNA Technologies, Leuven, Belgium]) but made a specific base change leading to a proline residue at position 102 in the translated protein. The construct was otherwise identical (confirmed by sequencing) to the PCR product used to complement the  $\Delta$ *chtR* mutant in E1162. SacI and SmaI sites at the end of the gBlock were used to clone the fragment into pEF25. The construct was then electrotransformed into the  $\Delta$ *chtR* mutant, resulting in the  $\Delta$ *chtR* + *chtR*\* strain.

**Growth inhibition assays.** The MIC of CHX was determined by broth microdilution in MHB, according to standard methodologies (58). MICs of CHX were not more than one dilution step different from each other for all strains in this study (data not shown). For this reason, we focused on kinetic growth assays, which provide more quantitative information than the endpoint measurements used in MIC determinations. Growth curves were determined using a BioScreen C instrument (Oy Growth Curves AB, Helsinki, Finland). Cultures of *E. faecium* E1162, the  $\Delta$ *chtR* and  $\Delta$ *chtS* mutants, and the in *trans* complemented strains were inoculated in MHB, with appropriate antibiotics, and incubated overnight at 37°C. Overnight cultures were then diluted to an OD<sub>660</sub> of 0.0025 in 300  $\mu$ l MHB and challenged with CHX or bacitracin. The cultures were incubated in the BioScreen C system at 37°C with continuous shaking. The absorbance at 660 nm ( $A_{660}$ ) was recorded every 15 min for 12 h. Each experiment was performed in triplicate.

**SEM.** E1162 and the  $\Delta$ *chtS* and  $\Delta$ *chtR* mutants were grown overnight in MHB. Subsequently, they were diluted to an OD<sub>660</sub> of 0.0025 in MHB and MHB supplemented with 1.2  $\mu$ g ml<sup>-1</sup> CHX or 1  $\mu$ g ml<sup>-1</sup> bacitracin and further grown until the OD<sub>660</sub> reached 0.2. Bacteria were immediately fixed with 1% glutaraldehyde (Sigma) onto poly-L-lysine-coated glass slides and prepared for SEM, as previously described (59). In brief, the cells were serially dehydrated by consecutive incubations of 5 min in 25% ethanol in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub> [pH 7.4]) and 50% ethanol in PBS, 75% and 90% ethanol, and twice in 100% ethanol, followed by 15- to 20-min incubations in 50% ethanol-hexamethyldisilazane (HMDS) and 100% HMDS. After overnight evaporation of HMDS at room temperature, samples were mounted onto specimen mounts and coated with 1-nm gold particles, using a Quorum Q150R sputter coater at 20 mA. Microscopy was performed using the Phenom PRO tabletop scanning electron microscope (PhenomWorld, Eindhoven, The Netherlands).

**Accession number(s).** The microarray data generated in the M-TrAM experiment have been deposited in the ArrayExpress database (<http://www.ebi.ac.uk/arrayexpress>) under accession no. E-MTAB-4173.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.02122-16>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.1 MB.

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A.M.G.P., R.J.L.W. and W.v.S. designed the study. A.M.G.P., J.W., J.C.B., M.R., E.C.B., E.M., and X.Z. performed experiments. J.R.B. contributed bioinformatic analyses. All authors contributed to data interpretation. The manuscript was written by A.M.G.P., M.J.M.B., R.J.L.W., and W.v.S.

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