Factors mediating synergism between vancomycin and chlorhexidine in VanA-type vancomycin-resistant *Enterococcus faecium*

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Running title: Vancomycin and chlorhexidine synergism in VRE

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Abstract

Enterococci are Gram-positive gastrointestinal tract colonizers of humans and animals. Vancomycin-resistant enterococci (VRE) are important nosocomial pathogens and can cause life-threatening infections. To control hospital-associated infections, skin antisepsis and bathing utilizing chlorhexidine is recommended for VRE patients in acute care hospitals. Previously, we reported that exposure to inhibitory chlorhexidine levels induced the expression of vancomycin resistance genes in VanA-type *Enterococcus faecium*. However, vancomycin susceptibility actually increased for VanA-type *E. faecium* in the presence of chlorhexidine. Hence, a synergistic effect of the two antimicrobials was observed. In this study, we tested various models to elucidate the mechanism(s) of synergism between chlorhexidine and vancomycin. We deleted each of the *pbp* genes from a model VanA-type VRE *E. faecium* strain. We found that deletion of *ddcP*, a membrane-bound carboxypeptidase, resulted in partial loss of synergism. Interestingly, addition of excess D-lactate, but not D-alanine, enhanced synergism. Furthermore, we isolated a synergy escaper mutant in *E. faecium* and utilized whole genome sequencing to determine that a mutation in a gene encoding an ATPase of phosphate-specific transporters (*pstB*) also resulted in loss of synergism. Our study is significant because understanding the mechanisms for chlorhexidine-induced vancomycin resensitization in VRE could lead to new combinatorial therapeutics to treat VRE infections.

Introduction

Enterococcus faecium and *E. faecalis* are Gram-positive commensal bacteria inhabiting the gastrointestinal tracts of humans and animals (1). A recently published evolutionary history of the enterococci elucidated how these bacteria became the leading causes of hospital-associated infections (2). The ability to survive in harsh environmental conditions including starvation and desiccation facilitated the emergence of hospital-adapted strains which are resistant to the action of antibiotics and disinfectants. Hospital-adapted enterococcal strains have limited treatment options and are typically characterized by high-level resistance to vancomycin, a glycopeptide antibiotic which inhibits the process of peptidoglycan synthesis (3, 4). Vancomycin-resistant enterococci (VRE) synthesize peptidoglycan precursors for which vancomycin has low affinity (5-8). Vancomycin resistance in hospital-adapted enterococcal isolates occurs through the horizontal acquisition of resistance genes (9, 10). For VanA-type VRE, vancomycin resistance is conferred and controlled by the activities encoded by the *vanRS, vanHAX*, and *vanYZ* genes.

Patients in critical care units are frequently bathed or cleansed with chlorhexidine, a cationic cell membrane-targeting antimicrobial, to reduce the occurrence of hospital-associated infections (11-13). Chlorhexidine interacts with the negatively charged phospholipids and proteins on the cell membrane after primary adsorption by the cell (14, 15). Low chlorhexidine levels disrupt the membrane potential and integrity whereas high chlorhexidine levels can cause a complete precipitation of the cytoplasm (16-18). We performed transcriptomic analysis of a VanA-type vancomycin-resistant *E. faecium* exposed to inhibitory levels of chlorhexidine and found that chlorhexidine stress induced the expression of genes associated with vancomycin and daptomycin resistance (19, 20). However, vancomycin MIC decreased when chlorhexidine was present in broth microdilution assays (19). In this study, we identified molecular factors that alter

this phenotype, with the ultimate aim to exploit synergism between vancomycin and chlorhexidine.

Here, we investigated three models we previously proposed (19) to explain chlorhexidine-induced vancomycin sensitization despite transcriptional activation of VanA-type vancomycin resistance genes by chlorhexidine. Vancomycin resistance genes code for the synthesis of alternative peptidoglycan precursors that terminate in D-alanine-D-lactate (D-Ala-D-Lac), for which vancomycin has lower affinity compared to the normal D-alanine-D-alanine (D-Ala-D-Ala). Model 1 is that altered penicillin-binding protein *(pbp)* expression in the presence of chlorhexidine prevents D-Ala-D-Lac precursors from being cross-linked. Model 2 proposes that chlorhexidine alters substrate pools for peptidoglycan synthesis, resulting in vancomycin-sensitive termini that are neither D-Ala-D-Ala nor D-Ala-D-Lac. Model 3 is that post-translational regulation of VanX and/or VanY prevents depletion of D-Ala-D-Ala termini from peptidoglycan precursors in the presence of chlorhexidine. Overall, elucidating mechanisms for chlorhexidine-induced vancomycin susceptibility in VRE could lead to new therapeutics for the treatment of VRE infections.

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 broth without agitation unless otherwise stated. *Escherichia coli* was cultured at 37°C in lysogeny broth (LB) broth with shaking at 225 rpm or on LB with 1.5% agar unless otherwise stated. The chlorhexidine product used for all experiments was Hibiclens (4% wt/vol chlorhexidine gluconate with 4% isopropyl alcohol). We refer to Hibiclens as H-CHG in this study. Antibiotics were added at the following concentrations: vancomycin, 50 µg/ml for *E.*

105 *faecium*, and chloramphenicol, 15 µg/ml for *E. coli* and *E. faecium*.

106

107 **Routine molecular biology techniques**. *E. faecium* genomic DNA (gDNA) was isolated using
108 a previously published protocol (21). Electroporation of *E. faecium* was performed as described
109 previously (19). Plasmids were purified using the GeneJET Miniprep kit (Thermo Scientific).
110 DNA fragments were purified using the Purelink PCR purification kit (Invitrogen). *Taq*
111 polymerase (New England Biolabs; NEB) was used for routine PCR reactions. Phusion
112 polymerase (Fisher) was used for cloning applications. Restriction endonucleases (NEB) and T4
113 DNA ligase (NEB) reactions were performed per the manufacturer's instructions. Routine DNA
114 sequencing was performed by the Massachusetts General Hospital DNA core facility (Boston,
115 MA). All genetic constructs were validated by DNA sequencing. Primers used in this study are
116 shown in Table S1.

117

118 **MIC determinations**. The minimum inhibitory concentration (MIC) was defined as the lowest
119 drug concentration at which the OD_{600nm} of the bacterial culture matched the OD_{600nm} of the
120 negative control (uninoculated BHI broth). For this study, we refer to synergy MIC as the
121 vancomycin MIC of enterococci in BHI supplemented with H-CHG. The synergy MIC was
122 determined by slightly modifying our previously published protocol (19). 5 µl of vancomycin
123 stock solution (40 mg/ml in water) was added to 195 µl of BHI supplemented with H-CHG in the
124 first well of a 96-well microtiter plate. Next, 100 µl was transferred to the next well containing
125 100 µl of BHI supplemented with H-CHG to make two-fold serial dilutions of vancomycin drug.
126 Overnight cultures of *E. faecium* or *E. faecalis* were diluted to OD_{600} of 0.01 in fresh BHI, and 5
127 µl of the diluted culture was used to inoculate the wells of the plate. The OD_{600} of the cultures
128 was measured after 24 h incubation at 37°C. For determining synergy MIC in the presence of D-
129 lactate or D-alanine, D-lactate or D-alanine were solubilized to a final concentration of 0.2 M in
130 BHI and the solutions were filter sterilized. Two-fold serial dilutions of vancomycin were made in

BHI supplemented with D-lactate/D-alanine and H-CHG as described above. Fold decrease was calculated by dividing the vancomycin MIC in the absence of H-CHG by the vancomycin MIC in the presence of the highest H-CHG concentration at which visible growth was observed. The experiment was performed at least three times.

Deletion of genes in *E. faecium*. Genes were deleted in-frame utilizing plasmid pHA101 as described previously (19). Briefly, ~1 kb regions upstream and downstream of the target gene were amplified and ligated to pHA101. The sequence of the deletion construct plasmid was verified by Sanger sequencing and introduced into *E. faecium* by electroporation. Temperature shifting at the non-permissible temperature of 42°C and counter-selection with *p*-chlorophenylalanine was followed according to a previously published protocol (22). Deletion mutants were confirmed by DNA sequencing of the region of interest.

After several unsuccessful attempts to generate unmarked deletions of *pbpA* and *pbp5* genes in *E. faecium* 1,231,410 (*E. faecium* 410), we introduced a tetracycline resistance marker (*tetL*) between the flanking upstream and downstream arms of the deletion constructs to select for deletion mutants. Briefly, *tetL* was amplified from pLT06-*tet* using primers *tetL* For and Rev. The deletion constructs were linearized via PCR using Phusion DNA polymerase (Fisher) and primers *pbpA/5*-linear For and Rev (Table S1). The linearized PCR products were dephosphorylated using Shrimp Alkaline phosphatase (New England Biolabs) per the manufacturer's instructions and then ligated with *tetL* to generate the deletion constructs PB408 and PB409. The deletion construct was propagated in EC1000 and sequenced using Sanger sequencing prior to transformation into *E. faecium* 410.

Complementation of *ddcP* deletion. The *ddcP* gene was restored to the chromosome of the *E. faecium* 410 *ddcP* deletion mutant. The *ddcP* gene and ~1 kb regions up- and downstream

were amplified from *E. faecium* 410 wild-type gDNA, and the amplicon was digested and inserted into pHA101. The knock-in plasmid construct (pMI101) was transformed into the *ddcP* deletion mutant by electroporation. The temperature shifting and counter-selection protocol was followed as described previously (22). The chromosomal integration of the gene was confirmed by Sanger sequencing.

Growth kinetics of *E. faecium* in the presence of H-CHG and vancomycin. Overnight cultures of *E. faecium* were diluted to an OD_{600} of 0.01 in fresh, pre-warmed BHI and incubated at 37°C with shaking at 100 rpm. The cultures were grown until OD_{600} reached 0.5 to 0.6. Twenty-five milliliters of the culture were added to equal amounts of pre-warmed BHI containing vancomycin (50 µg/ml) and/or H-CHG (4.9 µg/ml), or only BHI (control). OD_{600} values were monitored for 6 h, and an OD_{600} reading was taken at the 24 h time point. The growth curves were repeated independently three times. For assessing synergy between vancomycin and glycine, the same experimental design was used, except that H-CHG was replaced with 0.2 M glycine.

Isolation of *E. faecium* 1,231,410 synergy escaper mutant. An *E. faecium* 410 wild-type culture treated with vancomycin and H-CHG was incubated for 24 h, when turbidity was observed. The recovered culture was used as an inoculum for a second growth curve experiment with vancomycin and H-CHG. OD_{600} values were monitored for 6 h, and at the end of 6 h, the cultures were cryopreserved at -80°C. The stocked populations were struck on BHI agar, and the synergy MIC was determined for well-isolated colonies using the broth microdilution assay described above. Colonies with elevated synergy MIC as compared to the parental *E. faecium* 410 strain were passaged three times in BHI broth and the synergy MICs determined again. A strain with a stably elevated synergy MIC was isolated; this strain is referred to as SE101.

Genome sequencing and analysis. SE101 gDNA was isolated according to a previously published protocol (21) and sequenced with Illumina technology at Molecular Research LP (Shallowater, Texas). Paired end, 2x150 reads were obtained. For the analyses, sequence reads were assembled locally to the *E. faecium* 410 draft reference genome (GenBank accession number NZ_ACBA00000000.1) using default parameters in CLC Genomics Workbench (Qiagen). Polymorphisms in the resequencing assemblies were detected using basic variant mapping using default settings with a minimum variant frequency of 50%. To detect transposon/IS element hopping, the assembly parameters were changed to global instead of local alignment, and regions with sequential polymorphisms were manually analyzed for potential transposon/IS element hopping. Sanger sequencing was utilized to confirm polymorphisms.

Phosphate levels measurement. A commercially available kit (Sigma MAK030) and previously published protocol (20, 23) was utilized to measure intracellular inorganic phosphate (P_i) levels at five time points (OD_{600} from 0.4-0.5, 0.6-0.7, 0.7-0.8, 0.8-0.9, 1.0-1.5) from *E. faecium* 410 and SE101 cultures. The phosphate levels were normalized by CFU and five independent trials were performed.

Accession number. Raw Illumina sequencing reads generated in this study are available in the Sequence Read Archive under the accession number SRP113791.

Results and Discussion

Effect of sub-inhibitory H-CHG on vancomycin MIC in enterococci with different vancomycin resistance phenotypes. In enterococci, vancomycin resistance can be of

different types depending on the ligase gene (4, 24-26); however, all types rely on modification of the terminal peptidoglycan precursor to confer resistance. The VanA and VanB phenotypes are the most prevalent in VRE clinical isolates in the U.S. and confer a D-Ala-D-Lac substitution, whereas VanC-type enterococci less frequently cause clinical infections and have a D-Ala-D-Serine substitution (27). We previously reported induction of vancomycin resistance genes upon exposure to H-CHG in VanA-type enterococci (19). Utilizing broth microdilution assays, we sought to determine the effect of varying levels of H-CHG on vancomycin MIC in enterococci with different vancomycin resistance phenotypes (Table 1). As shown in Table 2, H-CHG increased the susceptibility of the VanA-type *E. faecium* strains 1,231,410 (*E. faecium* 410) and 1,231,502 and the *E. faecalis* HIP11704 to vancomycin, with 1024-, 16- and 42-fold decrease in vancomycin MIC observed, respectively. The results for *E. faecium* 410 are consistent with our previously reported results (19). Vancomycin MIC decreased 85-fold for the VanB-type *E. faecium* strain TUH4-64 and only 2-fold for the VanB-type *E. faecalis* V583 (Table 2). Only a 2-4 fold change in vancomycin MIC was observed for VRE with the VanC phenotype and for vancomycin-susceptible enterococci. Furthermore, as we previously reported (19), in the absence of *vanR* response regulator, vancomycin MIC decreased by only 4-fold for a Δ*vanR* derivative of the VanA-type *E. faecium* strain 410 (Table 2). Overall, we conclude that the effects of H-CHG/vancomycin co-treatment are strain-variable for reasons that are as yet unclear.

Addition of D-lactate enhances chlorhexidine-induced vancomycin sensitization. It was previously observed that culture supplementation with 0.2 M of glycine or select D-amino acids (including D-methionine, D-serine, D-alanine, or D-phenylalanine) increased VRE susceptibility to vancomycin (28). Consistent with this, Aart et al recently reported that excess D-Ala substrate competes with D-Lac, thereby increasing the ratio of cell wall termini ending at D-Ala and the efficacy of vancomycin against *Streptomyces* and *E. faecium* (29). We reasoned that if H-CHG

stress resulted in an alteration of substrate pools and therefore vancomycin-sensitive termini that are neither D-Ala-D-Ala nor D-Ala-D-Lac (Model 2), an excess of D-lactate could compete with this alternative pathway, thereby increasing the number of D-Ala-D-Lac termini and resulting in loss of synergism between vancomycin and H-CHG.

To test this, synergy assays were performed with the VanA-type VRE strain *E. faecium* 410 in the presence of 0.2 M D-lactate or D-alanine (Table 3). *E. faecium* 410 was selected because we observed the strongest synergism phenotype with this strain (Table 2). Addition of D-lactate to BHI broth lacking H-CHG resulted in a 4-fold increase in vancomycin MIC (Table 3), demonstrating that excess D-lactate does in fact result in reduced vancomycin susceptibility. However, and counter to our expectations, in the presence of both D-lactate and H-CHG, the H-CHG-induced vancomycin resensitization phenotype was enhanced (Table 3). This result indicates that the synergism phenotype is dependent upon D-Ala-D-Lac termini and is enhanced by increased abundance of D-Ala-D-Lac termini. As expected based on the results of Aart et al (29), vancomycin MIC decreased in the presence of D-alanine, and we observed only a 2-fold additional MIC decrease in the presence of both D-alanine and H-CHG (Table 3).

Deletion of *ddcP* results in partial loss of synergism against *E. faecium* 1,231,410. The enterococcal cell wall is a multi-layered network and is characterized by the presence of peptidoglycan, teichoic acid, and polysaccharides (30, 31). The main component of the cell wall is peptidoglycan, which is a mesh-like structure (30) and consists of parallel glycan chains cross-linked by amino acids. The glycan chains consist of two alternating amino sugars, N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), connected by β-1,4 linkages (32-34). In *E. faecium*, each MurNAc sugar is linked to short stem pentapeptides (L-alanine^{1}-D-isoglutamic acid^{2}-L-lysine^{3}-D-alanine^{4}-D-alanine^{5}), which alternate between L- and D-amino acids (33, 35). The MurNAc and GlcNAc glycan sugars are synthesized as a UDP (Uridine

diphosphate) derivative in a step-wise fashion in the cytosol (36). Next, MurNAc sugars containing short peptides are transferred to a lipid carrier Lipid I (C55-undecaprenol) (37, 38) and added to UDP-derivative GlcNAc to build a disaccharide, GlcNAc-MurNAc-pentapeptide-C55 pyrophosphate, also known as Lipid II (36, 37). Lipid II units are translocated from the cytosol to the outer side of the cell membrane (39) and polymerized through an ordered rate of two processes, transglycosylation (condensation of linear glycan chains) and transpeptidation (cross-linking between carboxyl group of one pentapeptide and amino acid of an adjacent pentapeptide). The disaccharide units are integrated into the growing peptidoglycan layers to form the cell wall (40, 41) and the lipid carrier is recycled back into the cytosol.

Two classes of penicillin-binding proteins (Pbps) mediate the transpeptidation process (42-44). Class A Pbps (encoded by *ponA*, *pbpF,* and *pbpZ*) are bifunctional, multimodular, high-molecular mass proteins, and catalyze both transpeptidation and transglycosylation reactions. Class B Pbps (encoded by *pbpB*, *pbpA,* and *pbp5*) are monofunctional, low-molecular mass proteins, and catalyze only transpeptidation reactions. Class A and B Pbps mediate 4,3 cross-links (D,D-transpeptidation) between cell wall precursors and these cross-links constitute the majority of the mature cell wall (41). However, 3,3 cross-links are also present in the enterococcal cell wall. The combined activities of DdcP or DdcY (D,D-carboxypeptidase) and the L,D transpeptidase Ldt_{fm} can bypass conventional D,D-transpeptidation and mediate 3,3 cross-linking (45-47). DdcP and/or DdcY generates tetrapeptides and reduces the availability of pentapeptide precursors by trimming the terminal D-Ala. Next, Ldt_{fm} mediates cross-links between these cell wall termini.

To determine whether Pbps contribute to vancomycin-chlorhexidine synergism against VRE (Model 1), we deleted each of these genes in *E. faecium* 410 (Table 1). We utilized growth curves to assess phenotypes of the deletion mutants in the presence and absence of

vancomycin and H-CHG. For these experiments, vancomycin (50 µg/ml) and H-CHG (4.9 µg/ml) were added to exponentially growing *E. faecium* cultures; a no-drug control was also performed. As shown in Fig. 1A, the OD_{600} of *E. faecium* 410 wild-type cultures decreased after addition of vancomycin and H-CHG, consistent with cell lysis. After 24 h, the cultures treated with vancomycin and H-CHG were visibly turbid, indicating that *E. faecium* can recover from the effects of the antimicrobials in this experimental condition.

A Δ*ddcP* mutant had a different phenotype from the wild-type strain in this assay (Fig. 1B). After treatment with vancomycin and H-CHG, the OD_{600} values for the *ddcP* deletion mutant did not decrease; rather, a bacteriostatic effect was observed. We conclude that deletion of *ddcP* results in a partial loss of synergism between vancomycin and H-CHG. The difference in the OD_{600} values was statistically significant between the wild-type and the Δ*ddcP* mutant for all time points post-H-CHG and vancomycin addition (*P*-value < 0.05, one-tailed Student's *t* test). The Δ*ddcP* mutant was complemented by restoration of the *ddcP* gene in *cis*. The growth phenotype of the complemented strain in the presence of vancomycin and H-CHG was similar to the wild-type (Fig. 1C). No statistically significant differences in growth were observed between the wild-type and the *ddcP* complemented strain in the presence of vancomycin and H-CHG.

The growth phenotypes of the Δ*pbpF*, Δ*ponA*, Δ*pbpZ*, Δ*pbpA,* Δ*pbp5,* Δ*ddcY,* Δldt_{fm}, and Δ*vanY* deletion mutants were comparable to the parental strain (Fig. S1A-L) with one exception. In the absence of the *pbpB* transpeptidase (Fig. S1H), the cultures did not recover from the effect of vancomycin and H-CHG after up to 48 h of incubation.

A Δ*ddcP* mutant is resistant to vancomycin-glycine synergy. Synergism between glycine and vancomycin was previously reported for VRE (28). To determine if the *E. faecium* Δ*ddcP*

313 mutant is resistant to the synergistic combination of glycine and vancomycin, we carried out
314 growth curves in the presence of vancomycin (50 µg/ml) and 0.2 M glycine. A lytic effect was
315 observed for the wild-type strain cultured with vancomycin and glycine, with the most
316 pronounced effect observed at the 24 h time point (Fig. 2A). However, this lytic effect was not
317 observed for the Δ*ddcP* mutant (*P*-value < 0.05, assessed by one-tailed Student's *t*-test) (Fig.
318 2B).
319
320 **VanY$_A$ does not contribute to the synergy phenotype.** Previously, Kristich et al investigated
321 the genetic basis of synergism between vancomycin and cephalosporins (a class of β-lactam
322 antibiotics) in the VanB-type VRE strain *E. faecalis* V583 (48). The synergism was mediated by
323 VanY$_B$, a carboxypeptidase that reduces the availability of precursors ending at D-Ala-D-Ala by
324 trimming the terminal D-Ala, thereby eliminating the target of vancomycin. In the absence of
325 *vanY$_B$*, cross-linking of cell wall precursors was mediated by low-affinity Pbps and synergism
326 between vancomycin and cephalosporins was lost (48). To determine whether *vanY$_A$*
327 contributed to vancomycin-chlorhexidine synergism against VRE, which is a component of our
328 Model 3, we deleted *vanY$_A$* in *E. faecium* 410. We observed no effect on the synergy phenotype
329 (Fig. S1C). Moreover, deletion of *ddcP* in a Δ*vanY* background did not further enhance the
330 phenotype of a Δ*ddcP* mutant (Fig. S2E).
331
332 ***E. faecium* 1,231,410 can escape from vancomycin-chlorhexidine synergy.** We observed
333 the growth kinetics of *E. faecium* 410 cultures exposed to no, either, or both 50 µg/ml
334 vancomycin and 4.9 µg/ml H-CHG over a two-day growth curve experiment. As shown in Fig.
335 3A, cultures exposed to vancomycin were growth-inhibited for the first 2.5 h after exposure, and
336 after 2.5 h, OD_{600} began to increase, consistent with the induction of vancomycin resistance
337 genes and synthesis of modified cell walls, as previously observed (49, 50). The cultures
338 exposed to H-CHG were also temporarily growth-inhibited. Consistent with the experiments

339 shown in Fig. 1A, the OD_{600} of cultures exposed to both vancomycin and H-CHG declined,
340 consistent with cell lysis, and after 24 h, the cultures recovered.
341
342 The next day, the recovered culture (from the vancomycin + H-CHG growth curve) was used as
343 an inoculum to repeat the growth curve experiment (Fig. 3B). Interestingly, the growth inhibition
344 phenotypes observed for the first growth curve experiment were not observed in this second
345 passage. Most strikingly, cell lysis was no longer observed for the vancomycin- and
346 chlorhexidine-treated culture. This is an important observation since it indicates that synergy
347 mutant(s) that do not lyse in the presence of vancomycin and H-CHG can readily emerge.
348
349 **Synergy escape mutants have a mutation in *pstB*.** We colony-purified a synergy escaper
350 mutant (SE101) from the second growth curve cycle, as described in the materials and
351 methods. The growth kinetics of SE101 in the presence of vancomycin and H-CHG confirmed
352 that the synergism phenotype is altered in this strain (Fig. 4A and B). SE101 was initially
353 growth-inhibited in the presence of vancomycin and H-CHG, but after 3 h, OD_{600} values began
354 to increase, unlike what is observed for the wild-type. Significant differences in OD_{600} values
355 were observed for SE101 compared to the wild-type for time points 3 h after addition of
356 vancomycin and H-CHG (P-value < 0.05 using one-tailed Student's t test).
357
358 Utilizing whole genome sequencing, we identified a mutation conferring a S199L substitution in
359 PstB (EFTG_01173) in SE101. As a result of this substitution, the protein is predicted to fold into
360 a beta-strand instead of a coil (51). The *pst* (phosphate-specific transport) operon has been well
361 characterized in *E. coli* and consists of *pstSCAB* and *phoU* (a regulator). In phosphate-
362 starvation conditions, inorganic phosphate (Pi) binds PstS and is released into the cytoplasm by
363 the inner membrane channel formed by PstA-PstC. PstB energizes this channel by hydrolyzing
364 ATP (52). We quantified the levels of intracellular inorganic phosphate (Pi) in the wild-type strain

and in SE101. However, the levels were not significantly different for any time point assayed (Fig. S2).

To determine if the *pstB* mutation resulted in a gain or loss of function, we deleted the complete *pst* transport system (EFTG_01170-74) in SE101 and in the *E. faecium* 410 wild-type. The growth observed in the presence of vancomycin and H-CHG for the SE101Δ*pst* deletion mutant was significantly different as compared to SE101 (Fig. 4B and D*; P*-value < 0.05 using one-tailed Student's *t* test). Specifically, unlike SE101, the OD_{600} values did not increase for the SE101Δ*pst* deletion mutant after 3 h in the presence of vancomycin and H-CHG. We conclude that the *pstB* mutation in SE101 resulted in a gain of function; however, the exact mechanism(s) for this is unclear.

Conclusions and perspective. Combinatorial therapeutics that exploit specific vulnerabilities of VRE are possible novel therapies against VRE. We previously reported that VRE exhibit increased susceptibility to vancomycin in the presence of chlorhexidine (19). The goal of the current study was to identify molecular contributors to this phenotype. The long-term goal is to use this information to identify less toxic compounds that could be compounded with vancomycin to exploit this vulnerability. That said, products incorporating chlorhexidine with antibiotics have been previously reported. Synergism between vancomycin and chlorhexidine was previously reported in methicillin-resistant *Staphylococcus aureus*, where chitosan-based sponges were utilized for localized delivery of these two synergistic compounds that inhibited *S. aureus* growth for 21 days (53). Another study exploited synergism between chlorhexidine and β-lactam antibiotics and synthesized hybrid organic salts (GUMBOS), which were effective against clinical isolates of Gram-positive and Gram-negative bacteria (54).

In our previous report, we proposed three models that are not mutually exclusive that could

391 explain this phenotype. The models are reiterated here. Model 1 is that altered Pbp levels in the
392 presence of chlorhexidine prevent D-Ala-D-Lac precursors from being cross-linked. Model 2
393 proposes that the chlorhexidine stress response alters substrate pools for peptidoglycan
394 synthesis, resulting in vancomycin-sensitive termini that are neither D-Ala-D-Ala nor D-Ala-D-
395 Lac. Finally, model 3 is that post-translational regulation of VanX and/or VanY prevents
396 depletion of D-Ala-D-Ala termini from peptidoglycan precursors in the presence of chlorhexidine,
397 thereby causing cells to be sensitive to vancomycin. In terms of Model 2, the results of the D-
398 lactate amendment study (Table 3) indicate that D-Ala-D-Lac termini (and therefore, induction of
399 the vancomycin resistance genes) are required for the synergy phenotype. Model 3 is not
400 supported by our observation that *vanY*$_A$ deletion has no impact on the synergy phenotype (Fig.
401 S1). Note that *vanX*$_A$ was not investigated in this study.
402
403 Growth analyses of the *E. faecium pbp* deletion mutants provide support for Model 1. To our
404 knowledge, this is the first study to report successful deletion of all *pbp* genes in *E. faecium*.
405 Upon *ddcP* deletion, which is predicted to result in increased availability of pentapeptide
406 precursors for cross-linking, *E. faecium* 410 was less susceptible to the synergistic action of
407 vancomycin and chlorhexidine. Our previously published transcriptomic study identified up to 5-
408 fold induction of *ddcP* in *E. faecium* 410 cultures treated with the MIC of H-CHG for 15 minutes,
409 as compared to untreated cultures (19). Overall, our data suggest that the combined activities of
410 DdcP and the vancomycin resistance proteins are incompatible and result in cell lysis,
411 presumably by autolysin activity, when both H-CHG and vancomycin are present. Interestingly,
412 the *ddcP* deletion mutant displays similar growth kinetics in presence of vancomycin plus H-
413 CHG (Figure 1B) and vancomycin plus 0.2 M D-glycine (Figure 2B). This suggests that
414 substrate availability may also play some role in the synergy phenotype observed, but this is
415 speculative. It is possible that in the presence of H-CHG, DdcP actively trims the terminal D-Ala
416 from peptidoglycan precursors and generates tetrapeptides. At the same time, in the presence

417 of vancomycin, the combined activities of the vancomycin resistance genes result in
418 pentapeptides terminating in D-Lac. The relative availability of penta- and tetrapeptides with
419 chemically different termini likely impacts the overall efficiency of cross-linking and the strength
420 of the cell wall. Since all Pbps do not have the same affinity for tetra- versus pentapeptides,
421 unacceptable precursors for transpeptidation are synthesized in the presence of both
422 vancomycin and chlorhexidine, and the cells lyse. However, complicating this explanation, we
423 did not observe any difference in growth phenotype between the L,D transpeptidase (*ldt*$_{fm}$)
424 deletion mutant and the wild-type in the presence of vancomycin and H-CHG (Fig. S1F and G).
425 As reported previously, activity of Ldt$_{fm}$ is dependent on availability of tetrapeptides (45, 46). If
426 (and how) Ldt$_{fm}$ activity changes in the presence of vancomycin and H-CHG remains to be
427 elucidated. A critical set of experiments will be to analyze peptidoglycan precursor pools and
428 mature peptidoglycan structures in *E. faecium* cultures exposed to vancomycin and
429 chlorhexidine. This analysis would allow us to analyze the relative balance of tetra- versus
430 pentapeptide termini, as well as their chemical compositions.
431
432 We also found that synergy escaper mutants (i.e., cells that failed to lyse) arose after 24 h of
433 exposure to both vancomycin and chlorhexidine. A non-synonymous mutation in *pstB* conferred
434 a survival advantage in the presence of the two antimicrobials. Deletion of the *pst* system and
435 antimicrobial susceptibility has been previously linked. An *E. coli pst* deletion mutant has
436 increased sensitivity to vancomycin and cationic antimicrobial peptides due to modification of
437 lipid A (55). A non-synonymous mutation in *phoU,* a negative regulator of the *pst* operon, was
438 present in a chlorhexidine-adapted *E. faecium* 410 derivative with reduced chlorhexidine and
439 daptomycin susceptibilities and decreased intracellular Pi levels (20). However, the exact
440 mechanism(s) of how the Pst system impacts antimicrobial susceptibility is unknown.
441
442 Our results indicate that the mechanism of synergy between vancomycin and chlorhexidine is

multi-factorial. Interestingly, *in vitro*, *E. faecium* rapidly adapted and loss of synergism arose after just 24 h of exposure to vancomycin and chlorhexidine. Overall, our study highlights the complexity of the enterococcal cell wall stress response in response to combination antimicrobial therapy and identifies a novel contributor (*pstB*) to this response.

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458 **Table 1. Bacterial strains and plasmids used in the study.**

Strain or plasmid	Description	Reference
Bacterial strains		
E. faecium 1,231,410	Clade A clinical isolate from skin and soft tissue infection; VanA-type VRE	(56)
E. faecium 1,141,733	Clade B clinical isolate from blood; Van-susceptible	(56)
E. faecium TUH4-64	Human clinical isolate; VanB-type VRE	(57)
E. faecium 1,231,502	Clade A clinical isolate from blood; VanA-type VRE	(56)
E. faecalis HIP11704	VanA-type clinical isolate from wound	(58)
E. faecalis V583	Clinical isolate from blood; VanB-type VRE	(59)
E. gallinarum EG2	VanC-type clinical isolate from blood	(56)
E. casseliflavus EC10	VanC-type clinical isolate from blood	(56)
PBPs deletions/insertions mutants		
PB411	*E. faecium* 1,231,410 Δ*pbpF* (EFTG_02258)	This study
PB412	*E. faecium* 1,231,410 Δ*ddcP* (EFTG_01253)	This study
PB413	*E. faecium* 1,231,410 Δ*vanY* (EFTG_02039)	This study
PB414	*E. faecium* 1,231,410 Δ*ldtfm* (EFTG_02461)	This study
PB415	*E. faecium* 1,231,410 Δ*pbpB* (EFTG_00959)	This study
PB416	*E. faecium* 1,231,410 Δ*ponA* (EFTG_00370)	This study
PB417	*E. faecium* 1,231,410 Δ*pbpZ* (EFTG_01189)	This study
PB418	*E. faecium* 1,231,410 Δ*ddcP* Δ*vanY*	This study
PB419	*E. faecium* 1,231,410 Δ*pbpA* (EFTG_02132); marked deletion with *tetL*	This study
PB420	*E. faecium* 1,231,410 Δ*pbp5* (EFTG_00244); marked deletion with *tetL*	This study
MI111	*E. faecium* PB412 with reconstituted *ddcP*	This study
MI112	*E. faecium* 1,231,410 Δ*ddcP* Δ*ldtfm*	This study
MI113	*E. faecium* 1,231,410 Δ*ddcY* (EFTG_00498)	This study
Synergy escaper mutants		
SE101	Synergy escaper mutant; has Ser199Leu substitution in EFTG_01173	This study
PB430	*E. faecium* 410 Δ*pst* transporter (EFTG_01170-74)	This study
PB431	SE101 Δ*pst* transporter (EFTG_01170-74)	This study
Plasmids		
pHA101	Markerless, counterselectable exchange plasmid; confers chloramphenicol resistance (Cam^R)	(19)
pPB401	pHA101 containing a 2.028-kb EcoRI/EcoRI-digested fragment flanking upstream and downstream of *E. faecium* 410 *pbpF* (EFTG_02258), Cam^R	This study
pPB402	pHA101 containing a 2.019-kb EcoRI/EcoRI-digested fragment flanking upstream and downstream of	This study

	fragment flanking upstream and downstream of *E. faecium* 410 *ddcP* (EFTG_01253), Cam^R	
pPB403	pHA101 containing a 2.010-kb EcoRI/EcoRI-digested fragment flanking upstream and downstream of *E. faecium* 410 *vanY* (EFTG_02039), Cam^R	This study
pPB404	pHA101 containing a 2.010-kb EcoRI/EcoRI-digested fragment flanking upstream and downstream of *E. faecium* 410 *ldtfm (*EFTG_02461), Cam^R	This study
pPB405	pHA101 containing a 2.028-kb EcoRI/EcoRI-digested fragment flanking upstream and downstream of *E. faecium* 410 *pbpB (*EFTG_00959), Cam^R	This study
pPB406	pHA101 containing a 2.028-kb EcoRI/EcoRI-digested fragment flanking upstream and downstream of *E. faecium* 410 *ponA (*EFTG_00370), Cam^R	This study
pPB407	pHA101 containing a 2.028-kb EcoRI/EcoRI-digested fragment flanking upstream and downstream of *E. faecium* 410 *pbpZ (*EFTG_01189), Cam^R	This study
pPB408	pHA101 containing a 4.317-kb fragment containing flanking upstream, tetracycline resistance gene (*tetL*), downstream arms of *E. faecium* 410 *pbpA* EFTG_02132, confers tetracycline antibiotic resistance	This study
pPB409	pHA101 containing a 3.555-kb fragment containing flanking upstream, tetracycline resistance gene (*tetL*), downstream arms of *E. faecium* 410 *pbp5* EFTG_00244, confers tetracycline antibiotic resistance	This study
pPB410	pHA101 containing a 2.028-kb BamH1/BamHI-digested fragment flanking upstream and downstream of *E. faecium* 410 *ddcY (*EFTG_00498), Cam^R	This study
pMI101	pHA101 containing a 3.303-kb EcoRI/EcoRI-digested fragment flanking upstream and downstream arms with *ddcP* gene (EFTG_01253), Cam^R	This study
pPB411	pHA101 containing a 2.028-kb EcoRI/EcoRI-digested fragment flanking upstream and downstream of *E. faecium* 410 *pst* transporters *(*EFTG_01170-74), Cam^R	This study

Table 2. Median vancomycin MIC in the presence of H-CHG.

	(Vancomycin MIC µg/ml)[a]					
Strain	**BHI**	**BHI + 1.2 µg/ml H-CHG**	**BHI + 2.4 µg/ml H-CHG**	**BHI + 4.9 µg/ml H-CHG**	**Van-type**	**(Fold decrease)[b]**
E. faecium 1,231,410	250	62.5	0.2	No growth	VanA	1024
E. faecium 1,231,502	625	39.1	No growth	No growth	VanA	16
E. faecium TUH4-64	78.2	24.4	0.9	No growth	VanB	85
E. faecalis HIP11704	625	312.5	156.5	14.67	VanA	42
E. faecalis V583	39.1	39.1	39.1	19.56	VanB	2
E. gallinarum EG2	9.8	4.9	No growth	No growth	VanC	2
E. casseliflavus EC10	2.5	2.4	0.6	No growth	VanC	4
E. faecium 1,141,733	1.2	0.6	No growth	No growth	Van^S	2
E. faecium 1,231,410 Δ*vanR*	1.2	0.9	0.3	No growth	Van^S	4

[a]Vancomycin MICs (µg/ml) at 24 h post-inoculation from at least three independent experiments.

[b]Fold decrease was calculated by dividing the vancomycin MIC in the absence of H-CHG by the vancomycin MIC in the presence of highest concentration of H-CHG where growth was observed. Van^S, Vancomycin susceptible.

Table 3. Median vancomycin MICs in *E. faecium* 1,231,410.

	Vancomycin MIC (µg/ml)[a]		
H-CHG (µg/ml)[b]	**BHI**	**BHI + 0.2M D-Lactate**	**BHI + 0.2M D-Alanine**
0	250	1000	7.8
1.2	62.5	1.0	3.9
2.4	0.2	No growth	No growth
4.9	No growth	No growth	No growth

[a]Vancomycin MICs (µg/ml) at 24 h post-inoculation from at least three independent experiments.
[b]4.9 µg/ml H-CHG is the MIC for *E. faecium* 1,231,410; 1.2 µg/ml H-CHG and 2.4 µg/ml H-CHG are 1/4X MIC and 1/2X MIC, respectively.

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Figure legends

Figure 1. A Δ*ddcP* mutant has reduced susceptibility to vancomycin/H-CHG synergy. Optical density (OD_{600nm}) of (A) *E. faecium* 1,231,410 wild-type (*E. faecium* 410), (B) the *ddcP* deletion mutant, and (C) the *ddcP* complemented strain with and without vancomycin and H-CHG treatment. *E. faecium* was cultured in BHI broth until the OD_{600} reached 0.6, as described in materials and methods. Equal volumes of cultures were split into BHI (control; red circles) or BHI containing vancomycin (50 µg/ml) and H-CHG (4.9 µg/ml) (blue squares). OD_{600} values were monitored for 6 h. Error bars indicate standard deviations from n=3 independent experiments. Significance was assessed using the one-tailed Student's *t*-test. * denotes *P*-value < 0.05. Stars indicate significant differences between vancomycin- and H-CHG-treated cultures in panel B versus A, and in panel C versus B. Note that growth curve of *E. faecium* 410 wild-type in the presence of vancomycin and chlorhexidine from Fig. 1A has been shown again in Fig. S1A for comparison with the *pbp* deletion mutants.

Figure 2. A Δ*ddcP* mutant has reduced susceptibility to vancomycin/glycine synergy. (A) *E. faecium* 410 wild-type and (B) *ddcP* deletion mutant cultures were grown at 37°C in BHI until OD_{600} reached 0.6 as described in materials and methods. Equal volumes of cultures were split into BHI (control; red circles) or BHI containing vancomycin (50 µg/ml) and glycine (0.2 M) (green squares). OD_{600} values were monitored for 6 h and a reading at 24 h was recorded. Error bars indicate standard deviations from n=3 independent experiments. Significance was assessed using the one-tailed Student's *t*-test. * denotes *P*-value < 0.05. Stars indicate significant differences between vancomycin- and glycine-treated cultures in panel B versus A.

Figure 3. *E. faecium* 410 can adapt to vancomycin/H-CHG synergy. The growth kinetics of *E. faecium* 410 in the presence of vancomycin and H-CHG were observed over a two-day (40 h)

759 growth curve. Panel (A) Representative OD_{600} of *E. faecium* 410 after treatment with 0X
760 (control; red circles), vancomycin (orange squares), H-CHG (green triangles) or vancomycin
761 and H-CHG (inverted blue triangles). *E. faecium* culture was grown at 37°C in BHI until OD_{600}
762 reached 0.6 and equal volumes of cultures were split into BHI with different antimicrobials
763 (shown by arrow) as described in materials and methods. OD_{600} values were monitored for 6 h
764 and after 24 h, the vancomycin and H-CHG-treated recovered culture (circled and indicated with
765 dashed arrow) was used as an inoculum to repeat the growth curve (shown in panel B).
766
767 **Figure 4. Mutations in the phosphate-specific transport (*pst*) operon result in escape**
768 **from vancomycin-H-CHG synergy.** Growth of (A) *E. faecium* 410 wild-type, (B) SE101, (C) *E.*
769 *faecium* 410Δ*pst*, and (D) SE101Δ*pst*. *E. faecium* was cultured in BHI until the OD_{600} reached
770 0.6. Equal volumes of cultures were split into BHI (control; red circles) or BHI containing
771 vancomycin (50 µg/ml) and H-CHG (4.9 µg/ml) (blue squares). OD_{600} values were monitored for
772 6 h and the 24 h time point was recorded. Error bars indicate standard deviations from n=3
773 independent experiments. Significance was assessed using the one-tailed Student's *t*-test. *
774 denotes *P*-value < 0.05. Stars indicate significant differences between vancomycin- and H-
775 CHG-treated cultures in panel B versus A, in panel C versus A, and in panel D versus B.
776
777 **Supplemental Figures and Tables**
778
779 **Figure S1. Representative optical density (OD_{600}) of (A) *E. faecium* 410 wild-type, (B)**
780 **Δ*pbpZ*, (C) Δ*vanY*, (D) Δ*ponA,* (E) Δ*ddcP* Δ*vanY*, (F) Δldt_{fm}, (G) Δ*ddcP* Δldt_{fm}, (H) Δ*pbpB*,**
781 **(I) Δ*pbpF*, (J) Δ*pbp5*, (K) Δ*ddcY,* and (L) Δ*pbpA* after vancomycin and chlorhexidine**
782 **treatment.** *E. faecium* was cultured at 37°C in BHI broth until the OD_{600} reached 0.6 as
783 described in the materials and methods. Equal volumes of culture were split into BHI containing
784 0X (control; red circles) or vancomycin and chlorhexidine (Van and H-CHG; blue squares).

785 OD_{600} values were monitored for 6 h. Error bars indicate standard deviations from three
786 independent experiments.

787

788 **Figure S2. Quantification of intracellular organic phosphate (Pi) levels in *E. faecium* 410**
789 **wild-type and SE101 synergy escaper mutant**. Intracellular Pi levels were measured for both
790 strains at different growth time points (OD_{600} 0.4-1.0) as described in materials and methods.
791 The levels (pmoles) were normalized using CFU count. Standard deviation was calculated from
792 n=5 independent experiments and significance value was calculated using one-tailed Student's *t*
793 test. Time points: 1, OD_{600} 0.4-0.5; 2, OD_{600} 0.6-0.7; 3, OD_{600} 0.7-0.8; 4, OD_{600} 0.8-0.9; OD_{600}
794 1.0-1.5.

795

796 **Table S1. List of primers used in the study.**

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