

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

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27 **Abstract**

28

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

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53 Introduction

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

68

69 Patients in critical care units are frequently bathed or cleansed with chlorhexidine, a cationic cell
70 membrane-targeting antimicrobial, to reduce the occurrence of hospital-associated infections
71 (11-13). Chlorhexidine interacts with the negatively charged phospholipids and proteins on the
72 cell membrane after primary adsorption by the cell (14, 15). Low chlorhexidine levels disrupt the
73 membrane potential and integrity whereas high chlorhexidine levels can cause a complete
74 precipitation of the cytoplasm (16-18). We performed transcriptomic analysis of a VanA-type
75 vancomycin-resistant *E. faecium* exposed to inhibitory levels of chlorhexidine and found that
76 chlorhexidine stress induced the expression of genes associated with vancomycin and
77 daptomycin resistance (19, 20). However, vancomycin MIC decreased when chlorhexidine was
78 present in broth microdilution assays (19). In this study, we identified molecular factors that alter

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

81

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

95

96 **Materials and Methods**

97

98 **Bacterial strains and growth conditions.** Bacterial strains used in this study are shown in
99 Table 1. *E. faecium* and *E. faecalis* were cultured at 37°C on brain heart infusion (BHI) agar or
100 in broth without agitation unless otherwise stated. *Escherichia coli* was cultured at 37°C in
101 lysogeny broth (LB) broth with shaking at 225 rpm or on LB with 1.5% agar unless otherwise
102 stated. The chlorhexidine product used for all experiments was Hibiclens (4% wt/vol
103 chlorhexidine gluconate with 4% isopropyl alcohol). We refer to Hibiclens as H-CHG in this
104 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 OD₆₀₀ of 0.01 in fresh BHI, and 5
127 µl of the diluted culture was used to inoculate the wells of the plate. The OD₆₀₀ 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

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

135

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

143

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

154

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

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

162

163 **Growth kinetics of *E. faecium* in the presence of H-CHG and vancomycin.** Overnight
164 cultures of *E. faecium* were diluted to an OD₆₀₀ of 0.01 in fresh, pre-warmed BHI and incubated
165 at 37°C with shaking at 100 rpm. The cultures were grown until OD₆₀₀ reached 0.5 to 0.6.
166 Twenty-five milliliters of the culture were added to equal amounts of pre-warmed BHI containing
167 vancomycin (50 µg/ml) and/or H-CHG (4.9 µg/ml), or only BHI (control). OD₆₀₀ values were
168 monitored for 6 h, and an OD₆₀₀ reading was taken at the 24 h time point. The growth curves
169 were repeated independently three times. For assessing synergy between vancomycin and
170 glycine, the same experimental design was used, except that H-CHG was replaced with 0.2 M
171 glycine.

172

173 **Isolation of *E. faecium* 1,231,410 synergy escaper mutant.** An *E. faecium* 410 wild-type
174 culture treated with vancomycin and H-CHG was incubated for 24 h, when turbidity was
175 observed. The recovered culture was used as an inoculum for a second growth curve
176 experiment with vancomycin and H-CHG. OD₆₀₀ values were monitored for 6 h, and at the end
177 of 6 h, the cultures were cryopreserved at -80°C. The stocked populations were struck on BHI
178 agar, and the synergy MIC was determined for well-isolated colonies using the broth
179 microdilution assay described above. Colonies with elevated synergy MIC as compared to the
180 parental *E. faecium* 410 strain were passaged three times in BHI broth and the synergy MICs
181 determined again. A strain with a stably elevated synergy MIC was isolated; this strain is
182 referred to as SE101.

183

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

195

196 **Phosphate levels measurement.** A commercially available kit (Sigma MAK030) and previously
197 published protocol (20, 23) was utilized to measure intracellular inorganic phosphate (P_i) levels
198 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
199 and SE101 cultures. The phosphate levels were normalized by CFU and five independent trials
200 were performed.

201

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

204

205 **Results and Discussion**

206

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

209 different types depending on the ligase gene (4, 24-26); however, all types rely on modification
210 of the terminal peptidoglycan precursor to confer resistance. The VanA and VanB phenotypes
211 are the most prevalent in VRE clinical isolates in the U.S. and confer a D-Ala-D-Lac substitution,
212 whereas VanC-type enterococci less frequently cause clinical infections and have a D-Ala-D-
213 Serine substitution (27). We previously reported induction of vancomycin resistance genes upon
214 exposure to H-CHG in VanA-type enterococci (19). Utilizing broth microdilution assays, we
215 sought to determine the effect of varying levels of H-CHG on vancomycin MIC in enterococci
216 with different vancomycin resistance phenotypes (Table 1). As shown in Table 2, H-CHG
217 increased the susceptibility of the VanA-type *E. faecium* strains 1,231,410 (*E. faecium* 410) and
218 1,231,502 and the *E. faecalis* HIP11704 to vancomycin, with 1024-, 16- and 42-fold decrease in
219 vancomycin MIC observed, respectively. The results for *E. faecium* 410 are consistent with our
220 previously reported results (19). Vancomycin MIC decreased 85-fold for the VanB-type *E.*
221 *faecium* strain TUH4-64 and only 2-fold for the VanB-type *E. faecalis* V583 (Table 2). Only a 2-4
222 fold change in vancomycin MIC was observed for VRE with the VanC phenotype and for
223 vancomycin-susceptible enterococci. Furthermore, as we previously reported (19), in the
224 absence of *vanR* response regulator, vancomycin MIC decreased by only 4-fold for a Δ *vanR*
225 derivative of the VanA-type *E. faecium* strain 410 (Table 2). Overall, we conclude that the
226 effects of H-CHG/vancomycin co-treatment are strain-variable for reasons that are as yet
227 unclear.

228
229 **Addition of D-lactate enhances chlorhexidine-induced vancomycin sensitization.** It was
230 previously observed that culture supplementation with 0.2 M of glycine or select D-amino acids
231 (including D-methionine, D-serine, D-alanine, or D-phenylalanine) increased VRE susceptibility
232 to vancomycin (28). Consistent with this, Aart et al recently reported that excess D-Ala substrate
233 competes with D-Lac, thereby increasing the ratio of cell wall termini ending at D-Ala and the
234 efficacy of vancomycin against *Streptomyces* and *E. faecium* (29). We reasoned that if H-CHG

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

239
240 To test this, synergy assays were performed with the VanA-type VRE strain *E. faecium* 410 in
241 the presence of 0.2 M D-lactate or D-alanine (Table 3). *E. faecium* 410 was selected because
242 we observed the strongest synergism phenotype with this strain (Table 2). Addition of D-lactate
243 to BHI broth lacking H-CHG resulted in a 4-fold increase in vancomycin MIC (Table 3),
244 demonstrating that excess D-lactate does in fact result in reduced vancomycin susceptibility.
245 However, and counter to our expectations, in the presence of both D-lactate and H-CHG, the H-
246 CHG-induced vancomycin resensitization phenotype was enhanced (Table 3). This result
247 indicates that the synergism phenotype is dependent upon D-Ala-D-Lac termini and is enhanced
248 by increased abundance of D-Ala-D-Lac termini. As expected based on the results of Aart et al
249 (29), vancomycin MIC decreased in the presence of D-alanine, and we observed only a 2-fold
250 additional MIC decrease in the presence of both D-alanine and H-CHG (Table 3).

251
252 **Deletion of *ddcP* results in partial loss of synergism against *E. faecium* 1,231,410.** The
253 enterococcal cell wall is a multi-layered network and is characterized by the presence of
254 peptidoglycan, teichoic acid, and polysaccharides (30, 31). The main component of the cell wall
255 is peptidoglycan, which is a mesh-like structure (30) and consists of parallel glycan chains
256 cross-linked by amino acids. The glycan chains consist of two alternating amino sugars, N-
257 acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), connected by β -1,4 linkages
258 (32-34). In *E. faecium*, each MurNAc sugar is linked to short stem pentapeptides (L-alanine¹-D-
259 isoglutamic acid²-L-lysine³-D-alanine⁴-D-alanine⁵), which alternate between L- and D-amino
260 acids (33, 35). The MurNAc and GlcNAc glycan sugars are synthesized as a UDP (Uridine

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

270

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

283

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

287 vancomycin and H-CHG. For these experiments, vancomycin (50 µg/ml) and H-CHG (4.9 µg/ml)
288 were added to exponentially growing *E. faecium* cultures; a no-drug control was also performed.
289 As shown in Fig. 1A, the OD₆₀₀ of *E. faecium* 410 wild-type cultures decreased after addition of
290 vancomycin and H-CHG, consistent with cell lysis. After 24 h, the cultures treated with
291 vancomycin and H-CHG were visibly turbid, indicating that *E. faecium* can recover from the
292 effects of the antimicrobials in this experimental condition.

293
294 A $\Delta ddcP$ mutant had a different phenotype from the wild-type strain in this assay (Fig. 1B). After
295 treatment with vancomycin and H-CHG, the OD₆₀₀ values for the *ddcP* deletion mutant did not
296 decrease; rather, a bacteriostatic effect was observed. We conclude that deletion of *ddcP*
297 results in a partial loss of synergism between vancomycin and H-CHG. The difference in the
298 OD₆₀₀ values was statistically significant between the wild-type and the $\Delta ddcP$ mutant for all
299 time points post-H-CHG and vancomycin addition (P -value < 0.05, one-tailed Student's t test).
300 The $\Delta ddcP$ mutant was complemented by restoration of the *ddcP* gene in *cis*. The growth
301 phenotype of the complemented strain in the presence of vancomycin and H-CHG was similar
302 to the wild-type (Fig. 1C). No statistically significant differences in growth were observed
303 between the wild-type and the *ddcP* complemented strain in the presence of vancomycin and H-
304 CHG.

305
306 The growth phenotypes of the $\Delta pbpF$, $\Delta ponA$, $\Delta pbpZ$, $\Delta pbpA$, $\Delta pbp5$, $\Delta ddcY$, ΔIdt_{im} , and $\Delta vanY$
307 deletion mutants were comparable to the parental strain (Fig. S1A-L) with one exception. In the
308 absence of the *pbpB* transpeptidase (Fig. S1H), the cultures did not recover from the effect of
309 vancomycin and H-CHG after up to 48 h of incubation.

310
311 **A $\Delta ddcP$ mutant is resistant to vancomycin-glycine synergy.** Synergism between glycine
312 and vancomycin was previously reported for VRE (28). To determine if the *E. faecium* $\Delta ddcP$

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 $\Delta ddcP$ 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 $\Delta vanY$ background did not further enhance the
330 phenotype of a $\Delta ddcP$ 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, OD₆₀₀ 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 OD₆₀₀ 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, OD₆₀₀ values began
354 to increase, unlike what is observed for the wild-type. Significant differences in OD₆₀₀ 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

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

367
368 To determine if the *pstB* mutation resulted in a gain or loss of function, we deleted the complete
369 *pst* transport system (EFTG_01170-74) in SE101 and in the *E. faecium* 410 wild-type. The
370 growth observed in the presence of vancomycin and H-CHG for the SE101 Δ *pst* deletion mutant
371 was significantly different as compared to SE101 (Fig. 4B and D; *P*-value < 0.05 using one-
372 tailed Student's *t* test). Specifically, unlike SE101, the OD₆₀₀ values did not increase for the
373 SE101 Δ *pst* deletion mutant after 3 h in the presence of vancomycin and H-CHG. We conclude
374 that the *pstB* mutation in SE101 resulted in a gain of function; however, the exact mechanism(s)
375 for this is unclear.

376
377 **Conclusions and perspective.** Combinatorial therapeutics that exploit specific vulnerabilities of
378 VRE are possible novel therapies against VRE. We previously reported that VRE exhibit
379 increased susceptibility to vancomycin in the presence of chlorhexidine (19). The goal of the
380 current study was to identify molecular contributors to this phenotype. The long-term goal is to
381 use this information to identify less toxic compounds that could be compounded with
382 vancomycin to exploit this vulnerability. That said, products incorporating chlorhexidine with
383 antibiotics have been previously reported. Synergism between vancomycin and chlorhexidine
384 was previously reported in methicillin-resistant *Staphylococcus aureus*, where chitosan-based
385 sponges were utilized for localized delivery of these two synergistic compounds that inhibited *S.*
386 *aureus* growth for 21 days (53). Another study exploited synergism between chlorhexidine and
387 β -lactam antibiotics and synthesized hybrid organic salts (GUMBOS), which were effective
388 against clinical isolates of Gram-positive and Gram-negative bacteria (54).

389
390 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

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

447

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

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

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

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

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

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463 ^aVancomycin MICs ($\mu\text{g/ml}$) at 24 h post-inoculation from at least three independent
464 experiments.

465 ^bFold decrease was calculated by dividing the vancomycin MIC in the absence of H-CHG by the
466 vancomycin MIC in the presence of highest concentration of H-CHG where growth was
467 observed. Van^S, Vancomycin susceptible.

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

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H-CHG ($\mu\text{g/ml}$) ^b	Vancomycin MIC ($\mu\text{g/ml}$) ^a		
	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

516

517 ^aVancomycin MICs ($\mu\text{g/ml}$) at 24 h post-inoculation from at least three independent
518 experiments.

519 ^b4.9 $\mu\text{g/ml}$ H-CHG is the MIC for *E. faecium* 1,231,410; 1.2 $\mu\text{g/ml}$ H-CHG and 2.4 $\mu\text{g/ml}$ H-CHG
520 are 1/4X MIC and 1/2X MIC, respectively.

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733 **Figure legends**

734

735 **Figure 1. A $\Delta ddcP$ mutant has reduced susceptibility to vancomycin/H-CHG synergy.**

736 Optical density (OD_{600nm}) of (A) *E. faecium* 1,231,410 wild-type (*E. faecium* 410), (B) the *ddcP*
737 deletion mutant, and (C) the *ddcP* complemented strain with and without vancomycin and H-
738 CHG treatment. *E. faecium* was cultured in BHI broth until the OD_{600} reached 0.6, as described
739 in materials and methods. Equal volumes of cultures were split into BHI (control; red circles) or
740 BHI containing vancomycin (50 μ g/ml) and H-CHG (4.9 μ g/ml) (blue squares). OD_{600} values
741 were monitored for 6 h. Error bars indicate standard deviations from n=3 independent
742 experiments. Significance was assessed using the one-tailed Student's *t*-test. * denotes *P*-value
743 < 0.05. Stars indicate significant differences between vancomycin- and H-CHG-treated cultures
744 in panel B versus A, and in panel C versus B. Note that growth curve of *E. faecium* 410 wild-
745 type in the presence of vancomycin and chlorhexidine from Fig. 1A has been shown again in
746 Fig. S1A for comparison with the *pbp* deletion mutants.

747

748 **Figure 2. A $\Delta ddcP$ mutant has reduced susceptibility to vancomycin/glycine synergy. (A)**

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

756

757 **Figure 3. *E. faecium* 410 can adapt to vancomycin/H-CHG synergy.** The growth kinetics of

758 *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 OD₆₀₀ 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 OD₆₀₀
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. OD₆₀₀ 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 OD₆₀₀ 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). OD₆₀₀ 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 (OD₆₀₀) of (A) *E. faecium* 410 wild-type, (B)**
780 **Δ*pbpZ*, (C) Δ*vanY*, (D) Δ*ponA*, (E) Δ*ddcP* Δ*vanY*, (F) Δ*ldt_{fm}*, (G) Δ*ddcP* Δ*ldt_{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 OD₆₀₀ 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 OD₆₀₀ 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 (OD₆₀₀ 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, OD₆₀₀ 0.4-0.5; 2, OD₆₀₀ 0.6-0.7; 3, OD₆₀₀ 0.7-0.8; 4, OD₆₀₀ 0.8-0.9; OD₆₀₀
794 1.0-1.5.

795

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

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