

1 **Reduced chlorhexidine and daptomycin susceptibility arises in vancomycin-resistant**

2 ***Enterococcus faecium* after serial chlorhexidine exposure**

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14 Running title: Chlorhexidine adaptation in *E. faecium*

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25 **Abstract**

26

27 Vancomycin-resistant *Enterococcus faecium* (VREfm) are critical public health concerns  
28 because they are among the leading causes of hospital-acquired bloodstream infections.  
29 Chlorhexidine (CHX) is a bisbiguanide cationic antiseptic that is routinely used for patient  
30 bathing and other infection control practices. VREfm are likely frequently exposed to CHX;  
31 however, the long-term effects of CHX exposure have not been studied in enterococci. In this  
32 study, we serially exposed VREfm to increasing concentrations of CHX for a period of 21 days  
33 in two independent experimental evolution trials. Reduced CHX susceptibility emerged (4-fold  
34 shift in CHX MIC). Sub-populations with reduced daptomycin (DAP) susceptibility were  
35 detected, which were further analyzed by genome sequencing and lipidomic analysis. Across  
36 the trials, we identified adaptive changes in genes with predicted or experimentally confirmed  
37 roles in chlorhexidine susceptibility (*efrE*), global nutritional stress response (*relA*), nucleotide  
38 metabolism (*cmk*), phosphate acquisition (*phoU*), and glycolipid biosynthesis (*bgsB*), among  
39 others. Moreover, significant alterations in membrane phospholipids were identified. Our results  
40 are clinically significant because they identify a link between serial sub-inhibitory CHX exposure  
41 and reduced DAP susceptibility. In addition, the CHX-induced genetic and lipidomic changes  
42 described in this study offer new insights into the mechanisms underlying the emergence of  
43 antibiotic resistance in VREfm.

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47 **Introduction**

48

49 *Enterococcus faecium* is a Gram-positive bacterium that naturally colonizes the human  
50 gastrointestinal tract and is an opportunistic pathogen associated with bacteremia, urinary tract  
51 infections, endocarditis, and wound infections (1-3). Vancomycin-resistant *E. faecium* (VREfm)  
52 are of particular concern for infection treatment. VREfm are among the primary etiological  
53 agents of central line-associated bloodstream infections (CLABSIs), a type of healthcare-  
54 associated infection (HAI) that arises from central venous catheter use and is associated with  
55 high mortality in the United States (4, 5). *E. faecium* contamination on indwelling venous  
56 catheters, surgical instruments, and hospital surfaces is challenging to eradicate (2, 6-8). In  
57 hospital and clinical settings, improper infection control practices, contaminated surfaces, and  
58 indiscriminate use of antibiotics contribute to persistence of *E. faecium* (6, 9).

59

60 Chlorhexidine (CHX) is a cationic antiseptic and membrane-active antimicrobial (10-12). The  
61 primary mechanism of action of CHX is to disrupt the bacterial cell membrane and cause  
62 leakage of cytoplasmic contents and precipitation of cytoplasm (13-15). CHX is recommended  
63 by the Society for Healthcare Epidemiology of America to reduce CLABSI occurrence in acute  
64 care hospitals (16). Specifically, CHX bathing and CHX-impregnated cardiovascular catheters  
65 are used for CLABSI control (16-18). Clinical reports have raised concerns about the long-term  
66 effects of CHX bathing on hospital-associated pathogens (19-22). The CHX concentrations on  
67 patient skin can fall below the MIC for VREfm between bathings (22). Frequent exposure to sub-  
68 inhibitory CHX could select for VREfm mutants with reduced susceptibility to CHX and other  
69 antimicrobials that also interact with the bacterial cell surface. It was recently reported that  
70 colistin resistance emerged in the Gram-negative pathogen *Klebsiella pneumoniae* after  
71 exposure to CHX (23).

72

73 In a previous study, we used RNA sequencing to study the global transcriptomic responses of a  
74 VanA-type VREfm strain to CHX (24). We found that CHX exposure elicited expression of  
75 genes associated with antibiotic resistance and extracytoplasmic stress, including genes  
76 associated with vancomycin resistance (*vanHAX*) and reduced daptomycin (DAP) susceptibility  
77 (*liaXYZ*) (24). In the current study, we test the hypothesis that serial exposure to sub-MIC CHX  
78 selects for VREfm mutants with reduced susceptibilities to CHX and other membrane and cell  
79 wall-targeting antimicrobials, with particular focus on DAP.

80

## 81 **Materials and Methods**

82

83 **Bacterial strains and growth conditions.** Bacterial strains used in this study are shown in  
84 Table 1. *E. faecium* was cultured at 37°C on brain heart infusion (BHI) agar or in BHI broth  
85 without agitation unless otherwise stated. Unless otherwise stated, the CHX product used for  
86 experiments was Hibiclens (4% wt/vol chlorhexidine gluconate with 4% isopropyl alcohol).  
87 Chloramphenicol was used at 15 µg/ml for *E. coli* and *E. faecium*.

88

89 **Routine molecular biology techniques.** *E. faecium* genomic DNA (gDNA) was isolated using  
90 a previously published protocol (25). DNA fragments were purified using the Purelink PCR  
91 purification kit (Thermo Fisher). *Taq* polymerase (New England Biolabs; NEB) was used for  
92 routine PCR. Routine DNA sequencing was performed by the Massachusetts General Hospital  
93 DNA core facility (Boston, MA). Primers used are shown in Table S1.

94

95 **MIC determinations.** Vancomycin, ampicillin, and CHX MICs were determined by broth  
96 microdilution in BHI as described previously (24). CHX MICs were recorded at 48 hr post-  
97 inoculation. DAP MICs were measured using Etest (BioMérieux, Inc.) strips on Mueller-Hinton  
98 agar (MHA) plates per the manufacturer's instructions. MICs were independently assessed at

99 least three times.

100

101 **Quantitative RT-PCR (RT-qPCR).** *E. faecium* 1,231,410 (*E. faecium* 410) and 1,141,733  
102 cultures treated for 15 min with 0X (control) and 1X MIC CHX were harvested and mixed with  
103 two volumes of RNAProtect Bacteria reagent (Qiagen) according to the manufacturer's  
104 recommendation and a previously published protocol (24). 100 ng RNA was used to synthesize  
105 cDNA with Superscript II (Life Technologies) and 5 ng cDNA was used as template in RT-qPCR  
106 with primers to amplify internal regions of *liaX* or *clpX*. Threshold cycle ( $C_T$ ) values were used to  
107 calculate the fold change of *liaX* gene expression between 1X MIC CHX-treated cultures and  
108 control cultures (n=2 independent trials).

109

110 To assess *liaX* expression in the DAP mutants and the *E. faecium* 410 wild-type, overnight  
111 cultures inoculated from glycerol stocks were diluted into fresh pre-warmed BHI. The cultures  
112 were incubated at 37°C until an optical density at 600 nm ( $OD_{600}$ ) of ~ 0.6 was reached. 15 ml of  
113 the cultures were harvested as described above. The experiment was performed independently  
114 three times and one-tailed Student's *t* test was used to assess significance.

115

116 **Serial passage experiments.** *E. faecium* 410 wild-type was used for *in vitro* serial passage  
117 according to a previously published protocol (26). The CHX 1X MIC value for *E. faecium* 410  
118 was 4.9 µg/ml on day 1. For serial passage, overnight culture was adjusted to  $OD_{600}$  0.1 and  
119 exposed to CHX below, at, and above the MIC in BHI broth. The cultures were incubated at  
120 37°C, and after 24 hr, the cultures with visible growth in the highest drug concentration were  
121 used as inoculum for the next round of passaging on day 2. Again, the inoculum  $OD_{600}$  was  
122 adjusted to 0.1, and cultures were exposed to CHX as described above. Passages were  
123 performed for 21 days. The serial passage experiment was performed independently twice,  
124 referred to as Experiments A and B. Cultures from the 22<sup>nd</sup> day of experiment A (referred to as

125 Population A) and experiment B (referred to as Population B) were analyzed in this study. *E.*  
126 *faecium* 410 cultures were also passaged in BHI without CHX for 21 days in two independent  
127 trials. Populations from the 21<sup>st</sup> day of these trials are referred to as 410-P1 and P2 in this  
128 study.

129

130 **Agar daptomycin susceptibility assay.** Cultures of Population A and Population B were  
131 inoculated directly from glycerol stocks into BHI broth and incubated overnight. Cultures were  
132 serially diluted in 1X phosphate buffered saline (PBS) and spotted on MHA plates supplemented  
133 with calcium (50 µg/ml) with or without 10 µg/ml DAP. Plates were incubated for 24-36 hr at  
134 37°C prior to colony counting. Three (for Population A) or four (for Population B) independent  
135 trials were performed. One-tailed Student's *t* test was used to assess significance. *E. faecium*  
136 410 unpassaged (7 trials) and no-drug passaged populations (3 trials each) were assayed as  
137 controls. Colonies arising on DAP plates from two independent trials each for Populations A and  
138 B were pooled, inoculated in BHI broth, and cryopreserved.

139

140 **Genome sequencing and analysis.** Genomic DNA was isolated from overnight broth cultures.  
141 DAP MIC was confirmed for these cultures by Etest assay. Library preparation and 2x150  
142 paired end Illumina sequencing was performed by Molecular Research LP (Shallowater, Texas).  
143 The *E. faecium* 410 wild-type strain was also sequenced as a control. Sequence reads were  
144 first assembled to the previously published *E. faecium* 410 draft genome sequence  
145 (NZ\_ACBA00000000.1) using default parameters for local alignment in CLC Genomics  
146 Workbench (Qiagen). Polymorphisms in the read assemblies were detected using the basic  
147 variant mapping tool for sites with ≥10-fold coverage. Variations occurring with ≥50% frequency  
148 were compared with the *E. faecium* 410 wild-type read assembly to find mutant-specific SNPs.  
149 To detect putative transposon hops, the read mapping parameters were changed to global  
150 instead of local alignment, and regions of interest were manually analyzed. ≥98.8% of

151 nucleotide positions in the *E. faecium* 410 reference were covered  $\geq 10$ -fold and included in  
152 variation analyses. Variants unique to the DAP-resistant mutants were confirmed by Sanger  
153 sequencing.

154

155 **Phosphate assay.** A commercially available kit (Sigma MAK030) and a previously published  
156 protocol (27) were utilized to measure intracellular inorganic phosphate ( $P_i$ ) levels. Overnight  
157 cultures were diluted to an  $OD_{600}$  0.01 in 50 ml pre-warmed BHI and incubated at 37°C with  
158 shaking at 100 rpm. The phosphate levels were measured at four time points, time point 1:  
159  $OD_{600}$  0.4 to 0.5; 2,  $OD_{600}$  0.5 to 0.6; 3,  $OD_{600}$  0.6 to 0.7; 4,  $OD_{600}$  0.7 to 0.8. For each timepoint,  
160 100  $\mu$ l culture was serially diluted in 0.9% sterile NaCl and spotted on BHI plates for CFU  
161 determination. Also, 1 ml of the culture was incubated on ice for 5 min and then pelleted at  
162 13,300 X g for 2 min at 4°C. The pellet was washed twice in 1 ml double-distilled water,  
163 resuspended in 0.5 ml double-distilled water, and then disrupted thrice (Fast-Prep-24; MPBio) at  
164 6.5 m/s for 30 s. The homogenized samples were centrifuged at 13,300 X g for 15 min at 4°C.  
165 Twenty-five  $\mu$ l of the supernatant was diluted with 25  $\mu$ l double distilled water, and the  $P_i$  levels  
166 were measured per the manufacturer's instructions. The phosphate levels were normalized by  
167 CFU. One-tailed Student's *t* test was used to assess significance from three independent trials.

168

169 **Bacterial growth curves.** Overnight cultures from glycerol stocks were diluted to an  $OD_{600}$  0.01  
170 in 50 ml pre-warmed BHI and incubated at 37°C and shaking at 100 rpm. The  $OD_{600}$  values  
171 were measured for 6 hr. The experiment was performed independently three times and one-  
172 tailed Student's *t* test was used to assess significance.

173

174 **Rifampin resistance frequency.** Overnight cultures of *E. faecium* 410 wild-type and DAP-  
175 resistant mutants were serially diluted in 1X PBS and spot-plated on BHI agar to obtain total  
176 CFU counts. Three ml of the cultures were pelleted and spread on BHI agar plates

177 supplemented with 50 µg/ml rifampin to obtain rifampin-resistant CFU counts. Plates were  
178 incubated for 24-48 hr. Four independent trials were performed and the significance value was  
179 calculated using one-tailed Student's *t* test.

180

181 **Gene deletion and complementation.** Loci (EFTG\_02287-02288) encoding the predicted ABC  
182 transport system EfrEF were deleted in-frame utilizing plasmid pHA101. Briefly, 999 bp flanking  
183 upstream and downstream regions of the genes of interest were amplified using primers in  
184 Table S1 and ligated with the plasmid pHA101, and propagated in *E. coli* EC1000. The  
185 construct, pPB301, was sequence-verified and electroporated into *E. faecium* 410 using a  
186 previously published protocol (24). Temperature shift at non-permissible temperature of 42°C  
187 and counter-selection with p-chlorophenylalanine was followed according to a previously  
188 published protocol (28). Deletion of EFTG\_02287-02288 in the mutant strain (*E. faecium*  
189 PB301) was confirmed by Sanger sequencing. For complementing this deletion *in trans*,  
190 EFTG\_02287-02288 with their putative native promoter were amplified and ligated with pLZ12  
191 (29). The complementation construct (pAH201) was sequence-verified and electroporated into  
192 the deletion mutant to generate the strain AH102. The empty pLZ12 plasmid was also  
193 transformed into the deletion mutant as a control (strain AH101).

194

195 **Complementation assay for CHX susceptibility.** Overnight cultures of AH101 and AH102  
196 were serially diluted and spot plated on BHI-chloramphenicol (15 µg/ml) plates supplemented  
197 with or without 1/8X MIC CHX. The plates were incubated at 37°C for 24-36 hr. The CFU/ml of  
198 three independent trials was quantified and one-tailed Student's *t* test was used to assess  
199 significance.

200

201 **Lipidomic analysis.** Overnight cultures from the glycerol stock were inoculated into 10 ml BHI  
202 and incubated at 37°C. The 10 ml cultures were added to pre-warmed 250 ml BHI and



203 incubated until an OD<sub>600</sub> of ~ 0.6 was obtained. 100 µl was removed for DAP Etest testing on  
204 MHA plates. Also, 15 ml of the cultures was added to 2 volumes of RNAProtect Bacteria  
205 reagent (Qiagen) according to manufacturer's recommendation and a previously published  
206 protocol (24), and samples were used to assess *cls*, *cmk*, *clpX* or *liaX* expression by RT-qPCR  
207 as described above. The remaining culture was pelleted at 10,000 rpm at 4°C. Cell pellets were  
208 stored at -80°C prior to lipid extraction by the Bligh and Dyer method (30). Lipid analysis by  
209 normal phase LC-electrospray ionization (ESI) MS was performed using an Agilent 1200  
210 Quaternary LC system coupled to a high resolution TripleTOF5600 mass spectrometer (Sciex,  
211 Framingham, MA), as previously described (31, 32). Normal phase LC was performed on an  
212 Agilent 1200 Quaternary LC system using an Ascentis Silica HPLC column, (5 µm; 25 cm x 2.1  
213 mm; Sigma-Aldrich). Mobile phase A and B solvents, flow conditions, and instrumental settings  
214 for ESI/MS and MS/MS are as previously published (33). Data analysis was performed using  
215 Analyst TF1.5 software (Sciex, Framingham, MA).

216

217 **Accession number.** Raw Illumina sequencing reads generated in this study is available in the  
218 SRA under the accession number SRP108331.

219

## 220 **Results**

221

222 ***In vitro* evolution of reduced CHX susceptibility in VREfm.** Previous RNA sequencing  
223 analysis identified up to 118-fold up-regulation of *liaXYZ* in CHX-treated *E. faecium* 1,231,410  
224 (*E. faecium* 410) (24), a VanA-type vancomycin- and ampicillin-resistant blood isolate and  
225 member of the hospital-adapted Clade A1 (34, 35). This was of interest because *liaXYZ* is  
226 protective against DAP (26, 36-40). We confirmed the CHX-stimulated up-regulation of *liaX* in *E.*  
227 *faecium* 410 and a commensal Clade B strain, *E. faecium* 1,141,733 (35) using RT-qPCR (Fig.  
228 S1).

229

230 We hypothesized that repeat exposure to sub-inhibitory CHX could select for mutants with  
231 reduced susceptibility to CHX and concomitant reduced susceptibility to other antimicrobials. To  
232 test this, we performed *in vitro* serial passaging of *E. faecium* 410 with CHX for a period of 21  
233 days, starting with a sub-MIC concentration of 2.9 µg/ml (Fig. 1). Similar patterns of MIC shifts  
234 were observed for two independent trials over the course of 21 days. The CHX MICs of the  
235 evolved populations (referred to as populations A and B) recovered after one drug-free passage  
236 was confirmed to be increased (19.6 µg/ml) as compared to the parental strain (Table 2). The  
237 CHX MIC was not altered in *E. faecium* 410 passaged for 21 days in medium without CHX. We  
238 conclude that reduced CHX susceptibility emerges in VREfm after serial *in vitro* CHX exposure.

239

240 The populations A and B had variable but significantly higher DAP MICs relative to the wild-type  
241 strain (Table 2), with the DAP MIC in some experimental trials meeting the  $\geq 4$  µg/ml breakpoint  
242 for DAP resistance (41). Vancomycin MIC was 2-fold higher for both populations relative to wild-  
243 type, and ampicillin MIC was 2-fold higher for population A.

244

245 **Reduced DAP susceptibility is present in the CHX-passaged populations.** We sought to  
246 further quantify and investigate the basis for elevated DAP MIC in the CHX-passaged  
247 populations. The CHX-passaged populations (A and B) were cultured on agar with and without  
248 10 µg/ml DAP to quantify CFU (Fig. 2). *E. faecium* 410 wild-type stock cultures (410-1 and -2)  
249 and *E. faecium* 410 passaged for 21 days in the absence of CHX (410-P1 and -P2) were used  
250 as controls. Sub-populations with reduced DAP susceptibility were detected in the CHX-  
251 passaged populations (Fig. 2).

252

253 Next, isolated colonies arising on DAP plates from each of two independent DAP plating trials  
254 were pooled and stocked for further analysis. These mutants will be referred to as DAP-A1 and

255 DAP-A2 (from population A; 14 and 11 colonies were pooled, respectively) and DAP-B1 and  
256 DAP-B2 (from population B; 18 and 5 colonies were pooled, respectively) hereafter. The DAP  
257 MICs of these mutants after two DAP-free passages were found to be variable but significantly  
258 higher than the *E. faecium* 410 wild-type (Table 2). The DAP mutants, with the exception of  
259 DAP-B2, have significantly longer generation times than *E. faecium* 410 wild-type in BHI broth  
260 (Table S2). No significant differences in spontaneous rifampin resistance frequencies were  
261 observed between the DAP mutants and *E. faecium* 410 wild-type (Table S2), indicating that  
262 these strains are not hyper-mutators. RT-qPCR analysis identified a small but statistically  
263 significant increase in *liaX* expression in the DAP-A1 and A2 mutants relative to wild-type. The  
264 increase was insignificant for DAP-B1 and B2 (Fig. S2).

265

266 **DAP strains have mutations in genes previously associated with reduced antimicrobial**  
267 **susceptibilities.** Genome sequencing identified mutations occurring in the DAP strains relative  
268 to *E. faecium* 410 wild-type (Table 3). All four share a common mutation in *efrE* (EFTG\_02287),  
269 which encodes one subunit of the heterodimeric ABC transporter EfrEF (42). *efrE* is up-  
270 regulated 22-fold by *E. faecium* 410 in response to CHX (24), and deletion of *efrE* from *E.*  
271 *faecalis* OG1RF confers increased susceptibility to CHX (43, 44). No mutations other than in  
272 *efrE* were common to all strains.

273

274 DAP-A1 and DAP-A2 share IS1251 insertions in the promoter region of *cmk*, which encodes  
275 cytidylate kinase (Table 3). RT-qPCR confirmed the down-regulation of *cmk* in DAP-A2 in the  
276 two trials but not in DAP-A1 (Fig. S3). Population heterogeneity in regards to IS1251 insertion at  
277 the *cmk* promoter was observed in DAP-A1 by gel electrophoresis analysis of *cmk* promoter  
278 amplicons (data not shown).

279

280 DAP-A1 and DAP-A2 each have different mutations in *relA* and in a gene encoding a predicted

281 alpha/beta hydrolase (Table 3). Production of (p)ppGpp, a bacterial alarmone, is controlled by  
282 RelA. Changes in ppGpp concentration modulate the stringent stress response and impact  
283 antibiotic tolerance and virulence in *Enterococcus* (45, 46). Mutations in *relA* have previously  
284 been associated with *in vitro* (47) and *in vivo* (48) emergence of DAP resistance in *Bacillus*  
285 *subtilis* and *E. faecium*, respectively. In DAP-A1, deletion of 11 bases (CTAGGATTTAC) from  
286 *relA* results in synthesis of a truncated, 505 amino acid protein that lacks the C-terminal ACT  
287 regulatory domain. The function of ACT domain as suggested is that it regulates the catalytic  
288 activities of the amino terminal domain to ensure that synthesis and degradation of ppGpp are  
289 not co-stimulated (49, 50). In DAP-A2, the A58E substitution is predicted by EMBOSS (51) to  
290 convert a beta-strand fold into an alpha helix in the HD4 metal-dependent phosphohydrolase  
291 domain of RelA. DAP-A1 and DAP-A2 also possess nonsense and frameshift mutations,  
292 respectively, in a gene encoding a predicted alpha/beta hydrolase. To our knowledge, this gene  
293 has not previously been linked with antimicrobial susceptibility.

294  
295 Different mutations were identified in DAP-B1 and DAP-B2. In DAP-B1, a E61K substitution  
296 occurs in PhoU. PhoU regulates phosphate intake by high-affinity inorganic phosphate-specific  
297 transporters (*pst* transporters encoded by EFTG\_01170-74 in *E. faecium*) in *E. coli* (52-56).  
298 The *pst* transport system is up-regulated in *Streptococcus pneumoniae* in response to penicillin  
299 (57), and mutations in the *pst* system led to decreased accumulation of reactive oxygen species  
300 after exposure to penicillin (58). Moreover, an adaptive point mutation in a putative phosphate  
301 transporter gene (*pitA*) in *S. aureus* conferred growth phase-dependent tolerance to DAP (27).  
302 However, the exact molecular mechanism(s) of how inorganic phosphate levels alter  
303 susceptibility to these antimicrobials is unclear. We confirmed that inorganic phosphate  
304 concentrations were significantly lower in the DAP-B1 mutant relative to the wild-type for two of  
305 four time-points assayed (Fig. S4).

306

307 In DAP-B2, a V57M substitution occurs in BgsB (59). BgsB, a glycosyltransferase (GT),  
308 catalyzes the transfer of glucose from UDP-glucose to 1,2-diacylglycerol (DAG) forming 3-D-  
309 glucosyl-1,2-diacylglycerol (MGlcDAG). Further, MGlcDAG is converted to diglucosyl DAG  
310 (DGlcDAG) by BgsA (60). MGlcDAG and DGlcDAG are important glycolipids for cell membrane  
311 fluidity and lipoteichoic acid (LTA) synthesis. Deletion of *bgsB* in *E. faecalis* 12030 led to a  
312 complete loss of cell membrane glycolipids and affected the chain length of glycerol phosphate  
313 polymer of LTA. It also led to 2-fold increased sensitivity to antimicrobial peptides (colistin and  
314 polymixin B), and reduced virulence in a rat model of endocarditis (59, 61). Deletion of other cell  
315 wall glycosyltransferases (*epaOX* and *epaI*) in *E. faecalis* enhances susceptibility to DAP (62)  
316 through perturbations in the cell envelope. A role for GTs in the production of immature  
317 polysaccharides, which prevented DAP binding, was suggested.

318

319 ***efrE* impacts CHX susceptibility.** Because *efrE* mutations were shared by all DAP strains, we  
320 deleted the EfrEF transport system from *E. faecium* 410 to assess its role in CHX and DAP  
321 susceptibility. Deletion of *efrEF* resulted in a 4-fold decrease in CHX MIC as compared to *E.*  
322 *faecium* 410 (n=3 independent trials). The median DAP MIC of the  $\Delta$ *efrEF* mutant was  
323 determined to be 2  $\mu$ g/ml (range, 2-3  $\mu$ g/ml; n=5 independent trials; *p*-value = 0.09 compared to  
324 *E. faecium* 410 wild-type using one-tailed Student's *t* test). Complementation *in trans* with *efrEF*  
325 on a multi-copy vector (AH102) resulted in a 2- to 4-fold increase in CHX MIC as compared to  
326 an empty vector control strain (AH101) using broth microdilution assays (n=2 independent  
327 trials). However, we observed a growth inhibition effect when chloramphenicol was added to the  
328 broth microdilution assay for vector selection. Hence, we utilized a spot-plating assay to quantify  
329 CFU differences between strains AH101 and AH102 in the presence of CHX and  
330 chloramphenicol (Fig. S5). Complementation of ABC transporter genes *in trans* resulted in  
331 significant increase in CFU count in the presence of CHX compared to control cultures.

332

333 To identify a putative function for EfrEF, we performed comparative lipidomic analysis of *E.*  
334 *faecium* 410 and the *efrEF* deletion mutant. The most striking differences observed were for two  
335 species whose positive ion  $[M+H]^+$  signals are detected at  $m/z$  288 and  $m/z$  316 by ESI/MS.  
336 These two species are present in *E. faecium* 410 wild-type, but absent in the *efrEF* deletion  
337 mutant (Fig. S6). High resolution mass measurement and tandem MS analysis identified these  
338 species as ethoxylated fatty amines previously identified as components of the anti-static  
339 additive Atmer-163 (63) commonly used in consumer products such as plastics. The  $m/z$  288  
340 and 316 ions correspond to the  $[M+H]^+$  ions of Atmer-163 containing  $C_{13}$  and  $C_{15}$  fatty alkyl  
341 chains, respectively. The absence of Atmer-163 ( $C_{13}$ ) and Atmer-16 ( $C_{15}$ ) in the *efrEF* mutant  
342 suggests that this ABC transporter is involved in transporting the Atmer-163 species, with lipid-  
343 like structures, from media into the cells.

344

345 **Distinct alterations of membrane phospholipid compositions in DAP mutants.** We next  
346 compared lipid profiles between the DAP mutants and *E. faecium* 410 wild-type. The lipid  
347 extracts were subjected to normal phase LC-ESI/MS using a silica column for lipid separation.  
348 As shown by the total negative ion chromatogram data in Fig. 3 and Table S3, cardiolipin  
349 (bisphosphatidylglycerol; CL), an anionic phospholipid, was dramatically reduced in DAP-A  
350 mutants (10-fold in DAP-A1 and 12-fold in DAP-A2) as compared to *E. faecium* 410. As  
351 expected, an increased accumulation of the precursor phosphatidic acid (PA) (4-fold in DAP-A1  
352 and 5-fold in DAP-A2) was also detected. The expression levels of two predicted cardiolipin  
353 synthase (*cls*) genes (EFTG\_00614 and EFTG\_01168), which mediate reversible  
354 transphosphatidylation of PG molecules to synthesize cardiolipin, were not significantly altered  
355 compared to *E. faecium* 410 (data not shown). In contrast to DAP-A mutants, CL and PA  
356 contents were not significantly altered in DAP-B mutants (Table S3), suggesting that DAP-A and  
357 DAP-B mutants do not share identical molecular mechanisms for reduced DAP susceptibility  
358 despite their similar DAP phenotypes.

359

360 Significant changes were observed in several other lipid species in DAP mutants. As shown in  
361 Table S3, in DAP-B1, levels of GPDD (glycerolphosphate DGlcDAG), a LTA precursor, were  
362 about 4-fold and lysylphosphatidylglycerol (LPG) were 4-fold higher than *E. faecium* 410 wild-  
363 type. In DAP-B2, amounts of monoglucosyl DAG (MGlcDAG or MHDAG) were 2-fold higher,  
364 potentially as a result of the *bgsB* mutation. The levels of GPDD were at least 2-fold higher for  
365 all DAP-A and -B strains.

366

## 367 **Discussion**

368

369 The goal of this study was to test the hypothesis that serial exposure to sub-MIC CHX selects  
370 for VREfm mutants with reduced susceptibilities to CHX, with concomitant effects on  
371 susceptibility to other membrane and cell wall-targeting antimicrobials. Our serial passage  
372 experiments demonstrate that reduced CHX susceptibility can emerge in VREfm after repeat  
373 sub-inhibitory exposure. Moreover, our DAP plating experiments demonstrate that reduced DAP  
374 susceptibility concomitantly emerges in a sub-population of CHX-passaged cells. Using  
375 genomics and lipidomics, we identified genetic and physiological changes occurring in these  
376 sub-populations with reduced DAP susceptibility.

377

378 CHX, a cationic antiseptic, interacts with the bacterial cell membrane (12). Various mechanisms  
379 of reduced CHX susceptibility in Gram-negative and -positive bacteria have been reported. The  
380 two main mechanisms include CHX efflux (23, 42-44, 64-67) and changes in outer membrane  
381 content (68, 69). The recently identified two-component system ChtRS contributes to CHX  
382 tolerance in *E. faecium*, presumably via regulation of expression of genes in its regulon, which is  
383 currently undefined (70). In this study, we have confirmed a role for the heterodimeric ABC  
384 transporter in CHX susceptibility in VREfm. Deletion of *efrEF* increases susceptibility of *E.*

385 *faecium* 410 to CHX, and an amino acid substitution in EfrE is associated with decreased  
386 susceptibility to CHX. The substrate of EfrEF was not assessed in prior studies. By lipidomic  
387 analysis, we found that presence of two ethoxylated fatty amine fatty alkyl diethanolamine  
388 compounds was abolished in the *efrEF* deletion mutant relative to wild-type. It remains to be  
389 determined if these compounds are protective against CHX. Nonetheless, their lipid-like  
390 structures may provide useful clues in identifying the molecular species that EfrEF transports, or  
391 in the functional studies of EfrEF.

392

393 Daptomycin (DAP) is a cyclic lipopeptide antibiotic used to treat infections caused by multidrug-  
394 resistant Gram-positive pathogens including VREfm (71-73). DAP resistance arises by mutation,  
395 leading to treatment failure (74). DAP is a negatively charged molecule that requires calcium  
396 ions for activity. Interaction of the cationic DAP-calcium complex with the membrane induces  
397 daptomycin oligomerization, membrane phospholipid remodeling, and other physiological  
398 alterations, ultimately leading to cell death (47, 75-79). Broadly speaking, alterations in cell  
399 surface composition and in cellular stress responses are associated with reduced DAP  
400 susceptibility in Gram-positive bacteria (80, 81).

401

402 In this study, we identified adaptive changes in genes with predicted or experimentally  
403 confirmed roles in chlorhexidine susceptibility (*efrE*), global nutritional stress response (*relA*),  
404 nucleotide metabolism (*cmk*), phosphate acquisition (*phoU*), and glycolipid biosynthesis (*bgsB*)  
405 occurring in the CHX-passaged mutants with reduced DAP susceptibilities. That the strains  
406 arising on DAP plates are not hyper-mutators indicates that these mutations arose as a result of  
407 CHX selection. It remains to be determined at what point in the CHX serial passage  
408 experiments reduced DAP susceptibility emerged, as only the beginning and end-points of the  
409 evolution experiments were assessed in this study. Deep sequencing of populations at  
410 beginning, mid- and end-points of the CHX passage experiments could be used in future studies



411 to further examine the diversity and frequency of genetic variations arising as a result of serial  
412 sub-inhibitory CHX exposure. Moreover, it remains to be determined whether susceptibility to  
413 host-associated cationic antimicrobial peptides is also altered as a result of serial CHX  
414 exposure.

415

416 Lipidomic analysis of the DAP mutants identified major alterations in membrane lipid  
417 compositions, underscoring a link between membrane lipid compositions and DAP  
418 susceptibility. CL, an anionic phospholipid, is present at the septal and polar regions in the  
419 bacterial cell membrane and is important for the regulation of processes like cell division,  
420 membrane transport, and localization of proteins to specific sites (82, 83). Since DAP  
421 preferentially binds to charged regions of the membrane, we hypothesize that reduced amounts  
422 of CL in DAP mutants from Population A can affect the binding of DAP antibiotic to the  
423 membrane regions. Tran et al showed that remodeling of CL can divert DAP away from division  
424 septum in *E. faecalis* (84). In Population B, we detected elevated levels of MHDAG and LTA  
425 precursor, implying that LTA synthesis is impaired in these DAP strains. Zorko et al showed  
426 direct binding and inhibition of LTA synthesis by CHX using fluorescence displacement and  
427 isothermal titration (85). Another study showed inactivation of LTA by CHX in *E. faecalis* (86).  
428 DAP mechanism of action by inhibition of LTA synthesis has also been suggested (87, 88) but  
429 recent reports have not supported this mechanism (89, 90). In future studies, we will assess the  
430 specific contributions of individual mutations identified in the DAP mutants to the lipid content  
431 alterations observed.

432

433 Our work has clinical implications. If sub-inhibitory CHX exposure selects for VREfm mutants  
434 with enhanced abilities to tolerate or resist DAP, these mutants could contribute to treatment  
435 failures with DAP. Frequent improper use of CHX (i.e., presence of sub-inhibitory concentrations  
436 on patient skin) may favor the emergence and persistence of these VREfm mutants in

437 healthcare settings. Surveillance of VREfm from hospital wards utilizing CHX bathing would be  
438 useful to monitor the long-term impact of CHX bathing on these organisms. Routine sub-  
439 inhibitory CHX exposure may be a contributing factor to the clinical emergence of DAP  
440 resistance in VREfm.

441

## 442 **Acknowledgements**

443

444 We would like to thank Dr. Ronda Akins for providing daptomycin. This work was supported by  
445 start up funds from the University of Texas at Dallas to K.L.P. The MS facility in the Department  
446 of Biochemistry at Duke University Medical Center and Z.G. were partially supported by grants  
447 GM-069338 and EY023666 from the National Institutes of Health.

448

449 **Table 1. Bacterial strains and plasmids used in the study.**  
450

<b>Strain or plasmid</b>	<b>Description</b>	<b>Reference</b>
<b><u>Bacterial strains</u></b>		
<i>E. faecium</i> 1,231,410	Clade A skin and soft tissue infection isolate; VanA-type VRE, DAP-susceptible	(35)
<i>E. faecium</i> 1,141,733	Clade B clinical isolate, Van-susceptible	(35)
Population A	<i>In vitro</i> evolved population with reduced CHX susceptibility from Experiment A, stocked from 22 <sup>nd</sup> Day	This study
Population B	<i>In vitro</i> evolved population with reduced CHX susceptibility from Experiment B, stocked from 22 <sup>nd</sup> Day	This study
410-P1	<i>E. faecium</i> 410 <i>in vitro</i> evolved population in BHI from Day 21 <sup>st</sup> , Trial 1	This study
410-P2	<i>E. faecium</i> 410 <i>in vitro</i> evolved population in BHI from Day 21 <sup>st</sup> , Trial 2	This study
DAP-A1	Mutants with reduced DAP susceptibility from Population A; Trial 2	This study
DAP-A2	Mutants with reduced DAP susceptibility, from Population A; Trial 3	This study
DAP-B1	Mutants with reduced DAP susceptibility, from Population B; Trial 2	This study
DAP-B2	Mutants with reduced DAP susceptibility, from Population B; Trial 3	This study
PB301	<i>E. faecium</i> 410 ABC transporter (Eftg_02287-88) deletion mutant	This study
AH101	<i>E. faecium</i> 410 ABC transporter (Eftg_02287-88) deletion mutant transformed with empty pLZ12 plasmid	This study
AH102	<i>E. faecium</i> 410 ABC transporter (Eftg_02287-88) deletion transformed with pAH201 complementation plasmid	This study
<i>E. coli</i> EC1000	<i>E. coli</i> cloning host; provides <i>repA</i> in <i>trans</i> ; <i>F</i> <sup>-</sup> <i>araD139</i> ( <i>ara ABC-leu</i> )7679 <i>galU galK lacX74 rspL thi</i> ; <i>repA</i> of pWV01 in <i>glgB</i> ; Km	(91)
<b><u>Plasmids</u></b>		
pLZ12	<i>E. coli</i> and <i>Streptococcus</i> shuttle vector, confers chloramphenicol resistance (Cam <sup>R</sup> )	(29)
pHA101	Markerless counter selectable exchange plasmid for <i>Enterococcus</i> , confers Cam <sup>R</sup>	(24)
pAH201	pLZ12 plasmid containing a 4.186-kb BamHI/BamHI-fragment with ABC transporter genes (Eftg_02287-88) under its native promoter	This study
pPB301	pHA101 containing a 1.998-kb EcoRI/BamHI-digested fragment with flanking upstream and downstream of ABC transporter genes (Eftg_02287-88)	This study

451

452 **Table 2. MIC values for CHX and cell wall-targeting antibiotics.**

<b>Strain or Population</b>	<b>CHX MIC<sup>a</sup></b>	<b>DAP MIC range, median (<i>p</i>-value)<sup>b</sup></b>	<b>AMP MIC</b>	<b>VAN MIC</b>
<i>E. faecium</i> 410	4.9	2, 2 (N/A)	195	250
Population A	19.6	2-6, 3 (0.0085)	390	500
Population B	19.6	3-4, 3.5 (0.0006)	195	500
DAP-A1	19.6	3-6, 4 (0.0043)	195	500
DAP-A2	19.6	3-6, 4 (0.0015)	390	1000
DAP-B1	19.6	3-4, 3.5 (0.0006)	195	1000
DAP-B2	19.6	3-4, 3.5 (0.0006)	195	1000

453

454 <sup>a</sup>All MICs in table expressed in µg/ml.

455 <sup>b</sup>DAP MICs were performed independently either 7 (for *E. faecium* 1,231,410 and Population A) or  
456 6 times. The one-tailed unpaired Student's *t* test was used to assess significance of the difference  
457 in MIC as compared to *E. faecium* 1,231,410. *E. faecium* 410, *E. faecium* 1,231,410; AMP,  
458 ampicillin; Van, vancomycin.

**Table 3. List of mutations identified by whole-genome sequencing in the DAP-resistant mutants.**

Mutant	Description of gene	Nucleotide variation (frequency) <sup>a</sup>	Amino acid change	<i>E. faecalis</i> V583 ortholog
<b>DAP-A1</b>	Eftg_02287 ABC transporter	C67008T (98.47%)	Ala290Val	EF2226
	Eftg_01724 RelA/SpoT ( <i>relA</i> )	CTAGGATTTAC30479Del (98.19%)	Leu505fs	EF1974
	Eftg_01135 Alpha/beta hydrolase	G137748A (55.64%)	Trp150*	EF1505
	Eftg_00577 Cytidylate kinase ( <i>cmk</i> ) promoter	A618699IS 1251 <sup>b</sup>	IS 1251 insertion	EF1547
<b>DAP-A2</b>	Eftg_02287 ABC transporter	C67008T (99.77%)	Ala290Val	EF2226
	Eftg_01724 RelA/SpoT ( <i>relA</i> )	CA29139AG (95.74%)	Ala58Glu	EF1974
	Eftg_01135 Alpha/beta hydrolase	137847G insertion (88.17%)	Ile184fs	EF1505
	Eftg_00577 Cytidylate kinase ( <i>cmk</i> ) promoter	A618699IS 1251 <sup>b</sup>	IS 1251 insertion	EF1547
<b>DAP-B1</b>	Eftg_02287 ABC transporter	C67008T (98.962%)	Ala290Val	EF2226
	Eftg_01175 PhoU regulator	G173845A (99.61%)	Glu61Lys	EF1754
	Eftg_02534 Transposase	C71844A (99.47%)	Lys206Asn	
<b>DAP-B2</b>	Eftg_02287 ABC transporter	C67008T (99.36%)	Ala290Val	EF2226
	Eftg_02162 Glycosyltransferase ( <i>bgsB</i> )	C50223T (67.09%)	Val57Met	EF2890

<sup>a</sup>Frequency of mutation in the read assembly as determined by variant detection in CLC Genomics Workbench.

<sup>b</sup>Precise location of insertion was mapped by Sanger sequencing.

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734 **Figure legends**

735

736 **Figure 1. *E. faecium* can adapt to CHX.** *In vitro* evolution of reduced CHX susceptibility in *E.*  
737 *faecium* 1,231,410 (*E. faecium* 410) by serial passaging in increasing concentrations of CHX for  
738 a period of 21 days. CHX passage MIC (y-axis) for each day of passage (x-axis) is shown for  
739 two independent experiments (A and B).

740

741 **Figure 2. Reduced DAP susceptibility in CHX-passaged *E. faecium* populations A and B.**  
742 The geometric mean and geometric standard deviation of CFU/ml count for n=3 or 4  
743 independent trials is shown. Strains and populations are described in the text. The dashed line  
744 represents limit of detection ( $10^3$  CFU/ml). The boxed populations were sent for whole genome  
745 sequencing. \*,  $P < 0.05$ ; one-tailed Student's *t* test.

746

747 **Figure 3. Cardiolipin levels are decreased in DAP-A1 and DAP-A2 mutants.** Lipidomic  
748 analysis of *E. faecium* 410 and DAP strains DAP-A1 and -A2 was performed by normal-phase  
749 LC-ESI/MS in the negative ion mode. The major lipids detected are phosphatidylglycerol (PG),  
750 cardiolipin (CL), diacylglycerol (DAG), monohexosyldiacylglycerol (MHDAG),  
751 dihexosyldiacylglycerol (DHDAG), phosphatidic acid (PA), and lysylphosphatidylglycerol (LPG).  
752 While the PG levels are similar in all three strains, the CL levels are significantly decreased and  
753 PA are increased in DAP-A1 and -A2 mutants compared to *E. faecium* 410. Shown are the total  
754 ion chromatograms (TIC) and the selected mass spectra of  $[M-H]^-$  ion species of PG and CL.

755

756 **Supplemental Figures and Tables**

757

758 **Figure S1. CHX induces *liaX* gene expression in *E. faecium*.** RT-qPCR was used to quantify

759 the expression of *liaX* upon exposure to CHX for 15 min as compared to control untreated  
760 condition. Expression of *liaX* was internally normalized to *clpX* and expression in control  
761 cultures was set to 1 (not shown). The fold change in *liaX* expression in cultures treated with 1X  
762 MIC CHX relative to the control was quantified for two independent (Trial 1 and 2) experiments.  
763 Efm 410, *E. faecium* 1,231,410; Efm 733, *E. faecium* 1,141,733.

764

765 **Figure S2. *liaX* gene expression in DAP strains vs. parental strain.** RT-qPCR was used to  
766 quantify the expression of *liaX* in the DAP strains at exponential phase ( $OD_{600} \sim 0.6$ ) vs. *E.*  
767 *faecium* 410 wild-type in three independent trials. Expression of *liaX* was internally normalized  
768 to *clpX* and *liaX* expression in control cultures was set to 1. The standard deviation was  
769 calculated from  $n=3$  independent experiments and one-tailed Student's *t*-test was used to  
770 calculate significance value. \*,  $P < 0.05$ . 410 wild-type, *E. faecium* 1,231,410.

771

772 **Figure S3. RT-qPCR for quantifying the expression levels of cytidylate kinase (*cmk*).** RT-  
773 qPCR was used to quantify the expression of *cmk* in DAP mutant DAP-A1 and DAP-A2 vs. *E.*  
774 *faecium* 410 during exponential growth ( $OD_{600} \sim 0.6$ ). Expression of *cmk* was internally  
775 normalized to *clpX*. Expression of *E. faecium* 410 *cmk* was set to 1 (not shown). The fold  
776 change in *cmk* expression was quantified for two independent experiments.

777

778 **Figure S4. Quantification of intracellular organic phosphate (Pi) levels in *E. faecium* 410**  
779 **wild-type and DAP-B1 mutant.** Intracellular Pi levels were measured for wild-type and DAP  
780 strain DAP-B1 at different growth time points ( $OD_{600}$  0.4-0.8) as described in materials and  
781 methods. The levels (pmoles) were normalized using CFU count. Standard deviation was  
782 calculated from  $n=3$  independent experiments and significance value was calculated using one-  
783 tailed Student's *t* test. Time points: 1,  $OD_{600}$  0.4-0.5; 2,  $OD_{600}$  0.5-0.6; 3,  $OD_{600}$  0.6-0.7 and 4,  
784  $OD_{600}$  0.7-0.8. \*,  $P < 0.05$ . 410 wt, *E. faecium* 1,231,410.

785

786 **Figure S5. Complementation of  $\Delta$ frEF deletion mutant.** The average CFU count of the  
787  $\Delta$ frEF deletion mutant transformed with empty pLZ12 vector and complementation vector  
788 pAH201 on BHI and chloramphenicol plate supplemented with or without 1/8X MIC levels of  
789 CHX from n=3 independent biological trials is shown. The CFU count was comparable for two  
790 strains on BHI plate supplemented with chloramphenicol. A significantly higher CFU count of  
791  $\Delta$ frEF deletion mutant transformed with complementation vector was observed. \*,  $P < 0.05$   
792 value was calculated using one-tailed Student's  $t$  test. Cam, chloramphenicol.

793

794 **Figure S6. Lipid-like compounds (Atmer-163) are detected in *E. faecium* 410 but not in**  
795  **$\Delta$ frEF mutant. A)** Positive ion ESI mass spectra showing the detection of Atmer-163 ( $C_{13}$ )  
796 ( $[M+H]^+$  at  $m/z$  288) and Atmer-163 ( $C_{15}$ ) ( $[M+H]^+$  ion at  $m/z$  316) in *E. faecium* 410, and their  
797 absence in the  $\Delta$ frEF mutant. **B)** Chemical structures and molecular formulae of Atmer-163  
798 ( $C_{13}$ ) and Atmer-163 ( $C_{15}$ ). **C)** MS/MS spectrum of Atmer-163 ( $C_{15}$ )  $[M+H]^+$  ion at  $m/z$  316. The  
799 fragment ion structures are depicted.

800

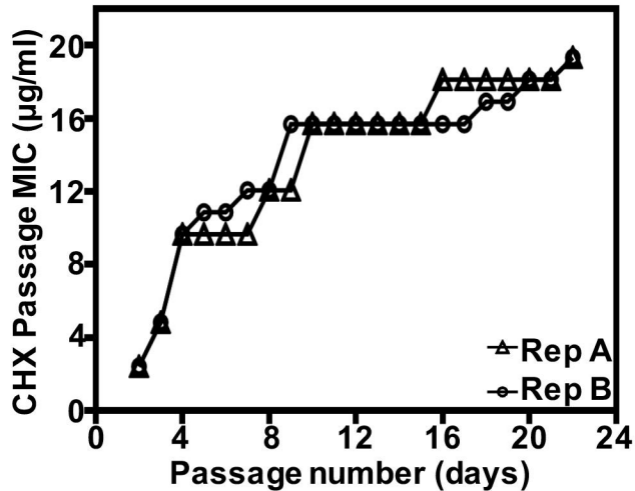
801 **Table S1.** List of primers used in the study.

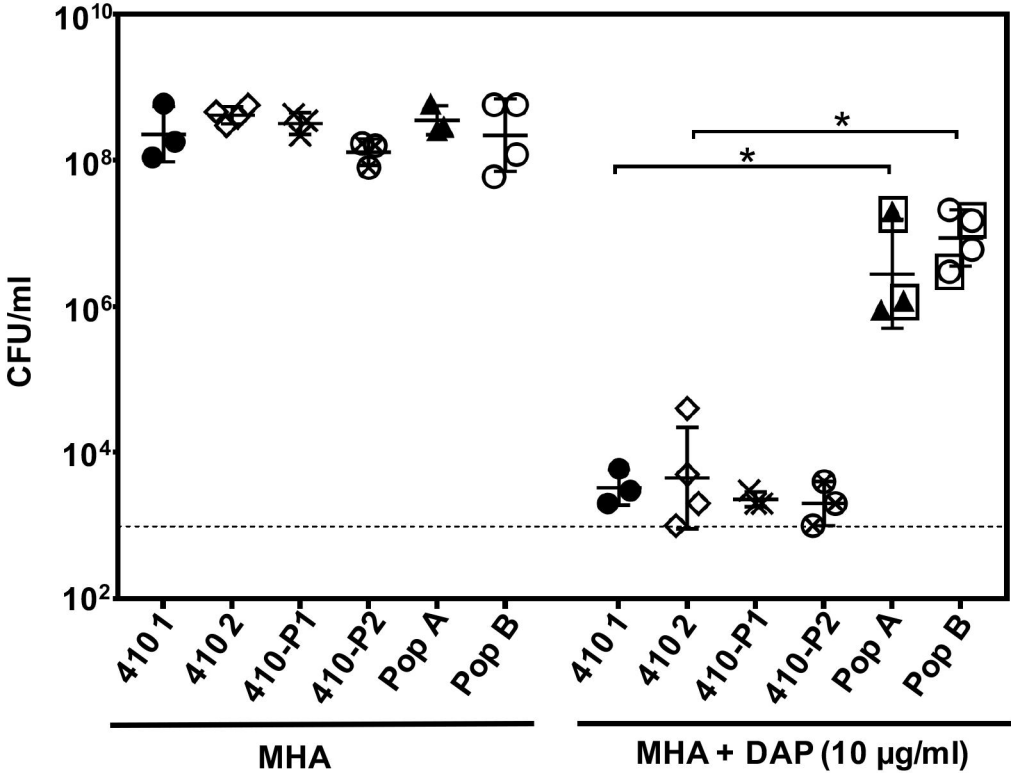
802

803 **Table S2.** Doubling time and Rifampin mutation frequency for the DAP strains vs. *E. faecium*  
804 410 wild-type.

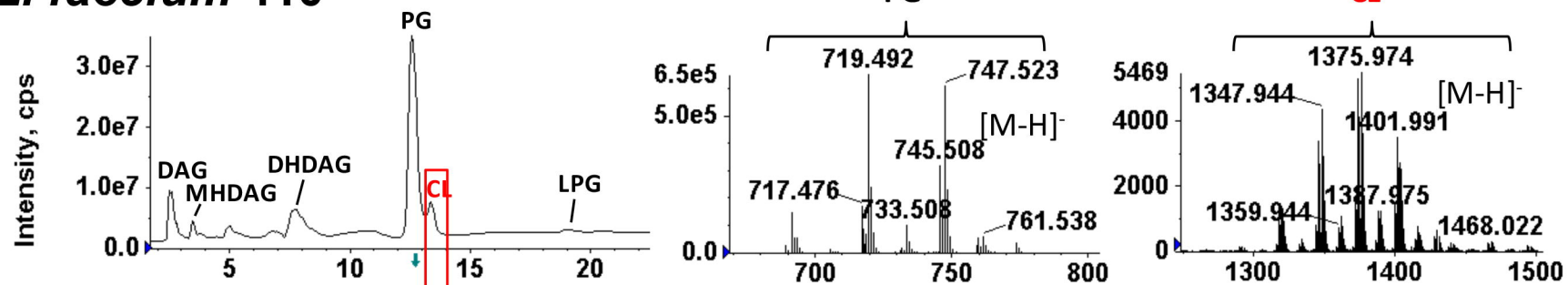
805

806 **Table S3.** The relative levels of major lipids identified in *E. faecium* 410 wild-type and DAP  
807 strains.

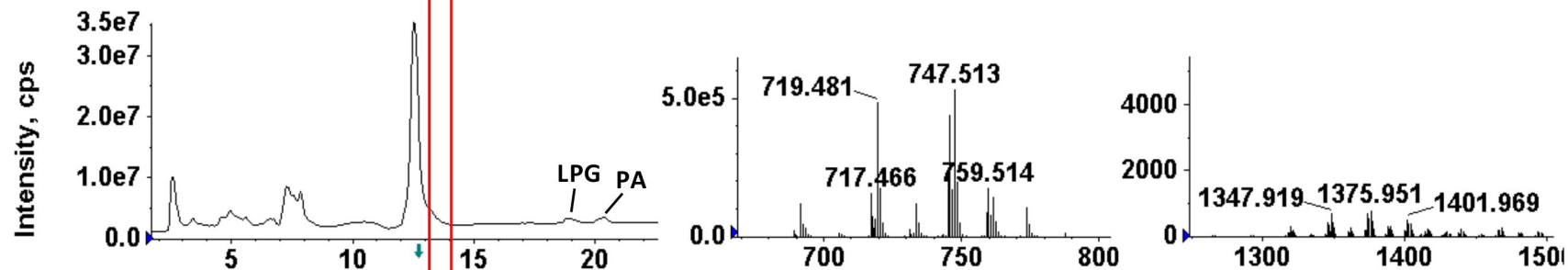




# A) *E. faecium* 410



# B) DAP-A1



# C) DAP-A2

