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**Unexpected cell wall alteration-mediated bactericidal activity of the
antifungal caspofungin against vancomycin-resistant *Enterococcus faecium***

Running title: In vitro activity of caspofungin against *E. faecium*

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27 **ABSTRACT**

28 *Enterococcus faecium* has become a major opportunistic pathogen with the emergence of
29 vancomycin-resistant enterococci (VRE). As part of the gut microbiota, they have to cope
30 with numerous stresses including effects of antibiotics and other xenobiotics, especially in
31 patients hospitalized in intensive care units (ICUs) who receive many medications. The aim of
32 this study was to investigate the impact of the most prescribed xenobiotics for ICU patients
33 on fitness, pathogenicity and antimicrobial resistance of the *vanB*-positive *E. faecium*
34 Aus0004 reference strain. Several phenotypic analyses were carried out and we observed
35 that caspofungin, an antifungal agent belonging to the echinocandins family, had an
36 important effect on *E. faecium* growth in vitro. We confirmed this effect by electron
37 microscopy and peptidoglycan analysis and showed that, even at a subinhibitory
38 concentration ($\frac{1}{4} \times \text{MIC}$, 8 mg/L), caspofungin had an impact on cell wall organization
39 especially in abundance of some muropeptide precursors. By RNA-seq, it was also shown
40 that around 20% of the transcriptome were altered in the presence of caspofungin with 321
41 and 259 significantly upregulated and downregulated genes, respectively. Since the fungal
42 target of caspofungin (i.e., beta-(1,3)-glucan synthase) was absent in bacteria, the
43 mechanistic pathway of caspofungin activity was investigated. The repression of genes
44 involved in the pyruvate metabolism seemed to have a drastic impact on bacterial cell
45 viability while decrease of glycerol metabolism could explain the conformational
46 modifications of peptidoglycan. This is the first report of caspofungin antibacterial activity
47 against *E. faecium*, highlighting the potential impact of non-antibiotic xenobiotics against
48 bacterial pathogens.

49 **INTRODUCTION**

50 Infections are one of the major threats for inpatients, especially for patients hospitalized in
51 intensive care units (ICUs). They are responsible for an important increase of morbidity and
52 mortality rates as well as a burst of medical costs since approximatively 50% of ICU patients
53 acquire an infection during their hospitalization with 60% of attributable deaths (1). While
54 approximatively 70% of non-cardiac ICU patients receive antibiotics for preventive or
55 curative indications during their stay, non-antibiotic molecules are also extensively used for
56 patient care, such as inotropic drugs, opioid and non-opioid analgesics, anxiolytics,
57 anticoagulants, antacids, curares or antifungal agents (2, 3). It is now well accepted that
58 bacteria must cope to survive numerous environmental stresses in their host leading to
59 significant physiological effects (4). Whereas numerous reports have been published in the
60 literature concerning the impact of antibiotics (especially at subinhibitory concentrations) on
61 bacterial cell physiology and pathogenicity (5), little is known about the direct effect of non-
62 antibiotic molecules on bacterial pathogens. Nonetheless, it has been demonstrated that
63 therapy with non-antibiotics may have significant effects upon bacterial cells physiology and
64 virulence (6). For instance, catecholamine administration raised the growth, the biofilm
65 formation and enhance survival traits in case of antibiotic therapy in *Pseudomonas*
66 *aeruginosa* in-vivo and ex-vivo models (7). Rise of virulence traits in the presence of
67 catecholamines has been described in other bacterial pathogens such as *Escherichia coli*,
68 *Salmonella* Typhimurium, *Staphylococcus epidermidis* and *Campylobacter jejuni* (8–15).
69 Recently, it has also been described that virulence of *P. aeruginosa* could be enhanced in the
70 presence of morphine, a major analgesic molecule largely prescribed in ICUs, using a murine
71 intestinal colonization model (16). In ICUs, patients are commonly colonized or infected by a
72 small contingent of multidrug-resistant (MDR) isolates gathered under the acronym “ESKAPE

73 bugs” for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*,
74 *Acinetobacter baumannii*, *P. aeruginosa* and *Enterobacter* spp. (1, 17).

75 Initially considered as part of the commensal gastrointestinal tract microbiota, *E. faecium*
76 has become an important issue in the therapeutic field since MDR isolates have spread in
77 hospital settings. Over the last decades, vancomycin-resistant enterococci (VRE) isolates
78 have emerged within the *E. faecium* species, particularly with the worldwide spread of
79 hospital-adapted isolates belonging to the clonal complex (CC) 17 (18–20). Actually, these
80 CC17 strains are part of a particular epidemic lineage of hospital-adapted strains described
81 as clade A1. This particular clade A1 is genetically distant from the community-associated
82 human lineage designated as clade B (20). Since most reports concerning xenobiotics
83 influence have been described in Gram-negative bacteria, the impact of non-antibiotic
84 xenobiotics used in ICU patients on *E. faecium* isolates remained undocumented.

85 The aim of this study was then to investigate the influence of highly-prescribed non-
86 antibiotic molecules in noncardiac ICUs, against a well characterized clinical, CC17 hospital-
87 adapted isolate of *E. faecium* (*E. faecium* Aus0004) (21). Since caspofungin (an antifungal
88 agent considered as a non-antibiotic molecule) was shown to have a significant impact on *E.*
89 *faecium*, different approaches (in vitro tests, electron microscopy, peptidoglycan analysis,
90 global transcriptomic analysis) were performed in order to decipher this unexpected effect.

RESULTS AND DISCUSSION

Impact of non-antibiotic molecules on *E. faecium* Aus0004 growth kinetics. The effect of eight different non-antibiotic molecules (norepinephrine, morphine, acetaminophen, midazolam, unfractionated heparin, pantoprazole, atracurium, and caspofungin) extensively used in ICU patients was first phenotypically evaluated. The growth kinetics of *E. faecium* Aus0004 was monitored in Tryptic-Soy broth (TSB) supplemented or not with xenobiotics at therapeutic free plasma concentrations (Figure 1A). Out of the eight molecules tested, only caspofungin had an important inhibitory effect on *E. faecium* Aus0004 since no growth was observed at the therapeutic concentration (data not shown). This conducted us to determine the MIC of caspofungin for Aus0004, which was at 32 mg/L (Table 1). Using a subinhibitory concentration ($\frac{1}{4} \times \text{MIC}$, 8 mg/L) of caspofungin, we showed a significant impact on bacterial growth with a lag time extension of about 1 hour (Figure 1A). Note that there was no difference on exponential growth rates between absence and presence of caspofungin ($1.487 \pm 0.034 \text{ h}^{-1}$ vs. $1.444 \pm 0.041 \text{ h}^{-1}$, respectively; $P = 0.2299$, unpaired t test). In the light of these unexpected results, we decided to confirm if this effect was specific to caspofungin or observed with other molecules belonging to the echinocandin family (e.g., micafungin). The growth kinetics of *E. faecium* Aus0004 was then evaluated in TSB with or without micafungin at 16 mg/L (corresponding to $\frac{1}{4} \times \text{MIC}$) (Table 1). A similar effect on bacterial growth (lag time extension) was observed with this second compound (Figure 1B). These preliminary results demonstrated that echinocandins had a significant impact on *E. faecium* Aus0004 fitness in vitro.

As opposed to what it has been described in *E. coli*, *S. Typhimurium* and *S. epidermidis* (12, 22–24), we did not observe any effect of catecholamines on *E. faecium* growth kinetics. We hypothesized here, the essential and critical impact of serum presence in the growth media,

115 since, as previously described in *Listeria monocytogenes*, overgrowth observed with
116 catecholamine was formerly linked with iron uptake, promoted by an increasing ferric
117 reductase activity (25). Another important non-antibiotic molecule largely prescribed in ICU
118 patients is morphine. In our study, no impact was observed on growth kinetics of *E. faecium*
119 Aus0004 with this opioid analgesic, whereas it has been described that chronic exposition to
120 morphine significantly increased pro-inflammatory interleukins serum and cecal levels that
121 enhanced biofilm formation and adhesion in a *P. aeruginosa* murine infection model (16).
122 Interestingly, the impact of caspofungin on bacteria has only been evaluated in one study on
123 *S. aureus*, where no impact was found on bacterial growth in vitro (26) whereas an
124 important impact on biofilm formation kinetics was observed both in vitro and in vivo, when
125 caspofungin was associated with moxifloxacin, a fluoroquinolone family antibiotic known to
126 be effective against *S. aureus*. Note that in this study, caspofungin was used at a higher
127 concentration (40 mg/L) as compared to the concentration used in our study (8 mg/L). As
128 described in fungi, we can hypothesize that caspofungin, when used at high-level
129 concentrations, exhibits a paradoxical effect that is characterized by the resumption of
130 growth of otherwise susceptible strains of *S. aureus* (27–29).

131

132 **In vitro activity of caspofungin on Gram-positive and -negative bacterial pathogens.** The
133 activity of caspofungin (at 8 mg/L) was evaluated in vitro on representative Gram-positive
134 and -negative bacterial species. The bacterial growth of Gram-positive bacteria (*S. aureus*
135 and *Enterococcus faecalis*) was impacted (Figure 1C) whereas that of Gram-negatives (*P.*
136 *aeruginosa*, *E. coli* and *Enterobacter cloacae*) was not (Figure 1D). Then, it appears that
137 caspofungin only has a significant impact on in-vitro bacterial growth of Gram-positive
138 bacteria. This is likely due to the difference of bacterial cell wall composition between Gram-

139 positive and -negative bacteria. Indeed, it is well known that Gram-negative bacteria have a
140 thin peptidoglycan layer surrounded by an outer membrane enriched with
141 lipopolysaccharide whereas Gram-positive bacteria lack the outer membrane and are
142 surrounded by murein layers many times thicker and negatively charged. We could
143 hypothesize here that the outer membrane of Gram-negative bacteria would be non-
144 permeable (30) to caspofungin, a high molecular weight negatively-charged molecule.

145

146 **Bactericidal activity of caspofungin against *E. faecium* Aus0004.** In order to compare their
147 antibacterial activities, MICs of different antibacterial and antifungal agents against *E.*
148 *faecium* Aus0004 were determined using the broth microdilution (BMD) reference method.
149 As expected, caspofungin remained less active than antibiotics tested (i.e., vancomycin,
150 teicoplanin, linezolid, daptomycin, and tigecycline) (Table 1). Interestingly, caspofungin was
151 ≥ 32 -fold-more active against *E. faecium* Aus0004 than other antifungals tested (i.e.,
152 amphotericin B, voriconazole, and 5-fluoro-cytosine) (Table 1).

153 The next step was to determine if caspofungin had a bacteriostatic or bactericidal activity
154 against *E. faecium* Aus0004 by time-kill curves analysis (at $8 \times \text{MIC}$) using anti-Gram-positive
155 antibiotics as comparators. Interestingly, we observed a rapid bactericidal effect ($> -3 \text{ Log}_{10}$
156 reduction) in the presence of caspofungin only after 3 hours of incubation (Figure 2). This
157 effect was sustained during the 24-hour period of the experiment without any regrowth
158 (Figure 2). Note that the bactericidal activity of caspofungin was even more rapid and
159 pronounced than that of daptomycin, known as a model of rapid bactericidal antibiotic (31,
160 32). Both vancomycin and linezolid did not exhibit a bactericidal activity (Figure 2). These
161 data are surprising since caspofungin and other beta-1,3-glucan synthase inhibitors are
162 known to be fungistatic molecules (33).

163

164 **Alterations of *E. faecium* Aus0004 cell wall under caspofungin exposure.** The impact of
165 caspofungin (at 8 mg/L) on cell wall components was first visualized using an ultrastructural
166 morphology analysis by scanning and transmission electron microscopy (SEM and TEM,
167 respectively). SEM experiments revealed that caspofungin was responsible for serious
168 morphological abnormalities with roughened surface and extrusions all around the cell
169 surface, suggesting a strong effect on bacterial envelope (Figure 3A). TEM experiments did
170 not reveal any change of the cell wall thickness in the presence of caspofungin (Figure 3B).
171 Analysis of the chemical composition of the peptidoglycan of *E. faecium* Aus0004 cultures
172 growing in the presence of subinhibitory concentration of caspofungin (8 mg/L) at different
173 growth points (mid-exponential, late-exponential, and early-stationary phases) by ultra-
174 performance liquid chromatography (UPLC) and MS/MS analysis showed a significant
175 decrease of four muropeptides (Figure 4). Interestingly, the relative abundance of those
176 muropeptides decreased between 20% and 60% depending on the type of muropeptides
177 and the incubation time of the cells (Figure 4). This dramatic drop is consistent with an
178 important impact of caspofungin on the peptidoglycan composition of *E. faecium*. Since
179 peptidoglycan biosynthesis is essential during the cell growth (34), the defect on bacterial
180 growth (Figure 1) and the bactericidal effect (Figure 2) observed in the presence of
181 caspofungin could be related with peptidoglycan modifications induced by the antifungal
182 drug.

183

184 **Whole transcriptome analysis of the response of *E. faecium* Aus0004 to caspofungin.** We
185 used a global transcriptomic approach by RNA-seq to decipher the impact of caspofungin at
186 subinhibitory concentration ($1/4 \times \text{MIC}$, 8 mg/L) on different metabolic pathways in *E.*

187 *faecium* Aus0004. Total RNAs were isolated in biological duplicates from *E. faecium* Aus0004
188 grown to the late-exponential phase in both cases (same optical densities), following rRNA
189 depletion. Between 7 and 16 million reads were obtained for each cDNA library, of which
190 more than 97% mapped to the genome of *E. faecium* Aus0004 (Table S1). Less than 2% of
191 reads mapped to sequences of the three plasmids while less than 0.6% of reads
192 corresponded to rRNA genes, confirming the high efficacy of rRNA depletion (Table S1). The
193 reproducibility of the duplicate RNA-seq experiments was satisfactory ($r^2 > 0.97$) under both
194 conditions (Figure S1).

195 Differential gene expression (DGE) analysis was performed only for chromosomal genes
196 (except for rRNA and tRNA genes) and not for plasmidic genes because of the low number of
197 reads and the absence of significant changes in gene expression (data not shown). The fold-
198 change (FC) of expression of each annotated gene in the chromosome of *E. faecium* Aus0004
199 between cells grown in the presence (+Cas) or absence (-Cas) of caspofungin (8 mg/L) is
200 presented as a MA-plot representation (Figure 5A) (see also Tables S2 in the supplemental
201 material). To assess the reliability of RNA-seq in determining DGEs, we determined by RT-
202 qPCR mRNA levels of three upregulated genes (*hupA*, *sodA*, EFAU004_02731) and five
203 downregulated genes (*dexB*, *glpK*, *pdhD*, *pdhB*, EFAU004_02122) (Table 2). Those genes
204 were chosen according to their levels of expression FCs and their putative functions. The
205 ratios of the transcripts from -Cas and +Cas samples determined by RNA-seq and compared
206 to those obtained by RT-qPCR resulted in an excellent concordance, with a Pearson
207 correlation value of 0.994 (Figure 5B). Therefore, this confirmed that RNA-seq was a reliable
208 method for global transcriptomic analysis in *E. faecium* under the conditions tested in this
209 study.

210 The analysis of transcriptomic data obtained by RNA-seq showed that 580 genes (20.3% of
211 the chromosomal genes) had statistically-significant alterations of their expression levels (FC
212 >2 or <-2, adjusted *P*-value <0.1), with 321 upregulated genes and 259 downregulated genes
213 (Tables S2B and S2C). All the genes presenting modified amounts of mRNA between the two
214 conditions were classified into functional categories using the COG and KEGG classifications
215 (35) (Figures S2 and S3). Among these 580 genes, more than 30% coded for proteins of
216 unknown function or not found in other species avoiding gene ontology research. In the
217 presence of caspofungin, the expression of genes encoding for proteins involved in
218 carbohydrate transport or metabolism and energy production or conversion was significantly
219 repressed whereas expression of genes coding for proteins involved in transcription,
220 replication, recombination and repair, and inorganic ion transport or metabolism was
221 significantly upregulated (Figures S2 and S3; Tables S2B and S2C).

222

223 **Impact of caspofungin on metabolism of *E. faecium* Aus0004.** Out of the 20 most repressed
224 genes by caspofungin, 13 were involved in carbohydrate transport or metabolism, especially
225 genes coding for phosphotransferase systems (PTS), systems that mediate sugar uptake in
226 bacteria and utilization as energy source (Table S2B). Since it has been demonstrated that
227 some PTS genes act as regulatory factors promoting adaption to stressful metabolic
228 conditions (36) and potentially enhanced the possibility of bacterial survival, we could
229 hypothesized that the severe alteration of PTS transcript levels might play a role in the
230 apparent lethality of caspofungin on *E. faecium* Aus0004. Interestingly, several genes
231 involved in glycerol metabolism in *E. faecium* showed a decrease of their transcript levels.
232 We observed a downregulation of the expression of genes composing the *glpKOF* operon
233 (EFAU004_00377-00379) (fold changes, - 6.0, - 5.8 and -4.6, respectively) as well as genes

234 composing the *dhaKLM* operon (EFAU004_00392-00394) (fold changes, -4.9, -4.2 and -5.1,
235 respectively) (Table S2C). It is well assumed now that glycerol is an essential precursor for
236 the synthesis of lipids and in many Gram-positive bacteria, including enterococci, for the
237 biosynthesis of lipoteichoic acids (37). Moreover, it has also been described that glycerol
238 metabolism pathways are under regulation of PTS system which transcripts levels are largely
239 impacted here (36). There also a strong repression of genes involved in the pyruvate
240 metabolism since the so-called *pdhABCD* operon (EFAU004_01091-01094) seemed to be
241 impact with an important downregulation notify in our transcriptomic analysis (fold changes,
242 -4.1, -8.6, -6.9, and -6.4, respectively). These enzymes allow the pyruvate transformation
243 into acetyl-CoA then proceed by a two-step reduction to generate ATP, or directly enters
244 into fatty acid biosynthesis (38). Since pyruvate dehydrogenation seemed to be impacted,
245 we could hypothesize that in the presence of caspofungin, a lack of ATP is generated. This
246 decrease of ATP formation may explain changes in bacterial fitness in the presence of
247 caspofungin. All these data pointed out that caspofungin presence induced an important
248 stress that modifies the carbohydrate metabolism and the cross-connected metabolic
249 pathways essential for *E. faecium* Aus0004 growth. Moreover, it was evidenced that
250 caspofungin likely induced an oxidative stress, since the *sodA* gene (EFAU004_01348) coding
251 for the manganese-dependent superoxide dismutase, a well-known protein involved in
252 oxidative stress regulation in *Enterococcaceae* (39), was significantly upregulated (fold
253 change, +4.1). Interestingly, the role of *sodA* was previously described as an important
254 pathway in tolerance of enterococci and *S. aureus* to cell wall active antibiotics (40).

255

256 **Impact of caspofungin on antimicrobial resistance of *E. faecium* Aus0004.** Regarding
257 findings on alterations of *E. faecium* Aus0004 cell wall and metabolism in the presence of

258 echinocandins, we addressed the role of caspofungin on antimicrobial resistance like
259 previously described for subinhibitory concentrations of antibiotics (5). Then, we determined
260 MICs of vancomycin, teicoplanin, daptomycin and ciprofloxacin against *E. faecium* Aus0004
261 by Etest strip method using Mueller-Hinton (MH) plates supplemented or not with 8 mg/L
262 caspofungin. Interestingly, we observed a four-fold increase in MICs of vancomycin in the
263 presence of caspofungin whereas no change was found for daptomycin, teicoplanin and
264 ciprofloxacin (Table 2). Note that, this impact was not observed with other antifungal agents
265 and seemed to be specific to echinocandins (data not shown). Since *E. faecium* Aus0004 is a
266 *vanB*-positive strain, we assumed that this increase of vancomycin MIC (and not that of
267 teicoplanin) was due to an upregulation of the *vanB* operon. However, RNA-seq data and RT-
268 qPCR (FC of *vanB* gene, -1.8 with an adjusted *P*-value >0.1) did not confirm this hypothesis
269 (Table S2A). Also, we observed a 4-to-8-fold increase in MICs of vancomycin among *vanA*-
270 and *vanB*-positive isolates (except for one strain) (Table 3). These findings are consistent
271 with the impact of echinocandins on cell wall components of *E. faecium*.

272

273 **Impact of caspofungin on biofilm formation.** This is well known that bacterial biofilms are a
274 significant medical challenge because they are difficult to treat using standard therapeutic
275 approaches, given that they are a major barrier to antibiotic effectiveness, especially in MDR
276 *E. faecium* isolates (41). In order to characterize the effect of caspofungin on biofilm
277 production, we evaluated levels of static biofilm formation of *E. faecium* Aus0004 (used as
278 biofilm non-producer) and *E. faecium* HM1070 Δ *asrR* (used as biofilm producer) (42) in the
279 presence of caspofungin subinhibitory concentration (8 mg/L). As previously described in
280 fungal models (43), caspofungin significantly reduced the ability of biofilm formation of
281 HM1070 Δ *asrR* whereas no difference was observed for *E. faecium* Aus0004 (Figure 6). These

282 data substantiate previous findings concerning caspofungin impact against bacterial biofilm
283 found in *S. aureus* (26). Interestingly, the authors of the latter study explained that this
284 biofilm formation shutdown in *S. aureus* through inhibition of *ica* operon by caspofungin and
285 in particular, IcaA, protein that shares an homology with the β -1-3-glucan synthase, the
286 caspofungin fungal target. Herein, the protein that shares the most homology with IcaA (i.e.,
287 EFAU004_00389) seemed to be not statistically impacted by the presence of caspofungin as
288 retrieved in our transcriptomic analysis (Table S2A).

289

290 **Mechanism of action of caspofungin against *E. faecium*.** Since the fungal target of
291 caspofungin in fungi is the β -1-3 glucan synthase that does not exist in prokaryotes, we
292 attempted to identify the bacterial target. To do this, we tried to select in vitro spontaneous
293 caspofungin-resistant mutants by serial passages in agar containing a caspofungin
294 concentration gradient. Unfortunately, after several sequential growth assays (i.e., 45 days
295 of subcultures), we did not obtain any *E. faecium* colony harboring an increase in
296 caspofungin MIC.

297

298

299 **CONCLUSION**

300 We reported herein that caspofungin seemed to have a strong bactericidal effect against *E.*
301 *faecium* notwithstanding the lack of similar protein to its fungal target, the β -1-3 glucan
302 synthase. Interestingly, we showed that bacterial growth in the presence of subinhibitory
303 concentration of caspofungin altered the transcripts level of approximatively 20% of the *E.*
304 *faecium* genome. Even though unlikely to be clinically relevant, the observed antagonism
305 with vancomycin would be helpful to understand the mechanism of action of caspofungin in
306 *E. faecium*. This present study is the first report of caspofungin antibacterial activity against
307 Gram-positive bacteria as *E. faecium*, and further investigations on effects of non-antibiotic
308 xenobiotics against VRE should be conducted in the future.

309

310 MATERIALS AND METHODS

311 **Bacterial isolates and growth conditions.** Bacterial strains used in this study are listed in
312 Table S3. The main strain used was the *vanB*-positive reference strain *E. faecium* Aus0004 of
313 which the complete genome sequence is available (GenBank accession number CP003351.1)
314 (21). UCN strains were obtained from the French Reference Centre for Enterococci for which
315 detection for *vanA/B* genes was performed as previously described (44). For biofilm
316 formation experiments, *E. faecium* HM1070 Δ *asrR* was used as positive control as previously
317 described (42).

318 For growth experiments, *E. faecium*, *S. aureus* and *E. faecalis* were cultured without shaking
319 at 35°C in TSB whereas *E. coli*, *E. cloacae* and *P. aeruginosa* strains were cultured with
320 shaking (200 rpm) at 35°C in Luria-Bertani (LB) broth. Bacteria were cultured in TSB with
321 adjunction of non-antibiotic molecules mostly prescribed in ICUs such as morphine (major
322 analgesic), norepinephrine (vasoactive amine), pantoprazole (proton pump inhibitor),
323 atracurium (neuromuscular blocking agent), paracetamol (minor analgesic), diazepam
324 (benzodiazepine), unfractionated heparin (anticoagulant agent) and caspofungin (antifungal
325 agent) at a concentration corresponding to their awaiting circulating blood level.

326 For phenotypic tests, a subinhibitory concentration of caspofungin corresponding to $1/4 \times$
327 MIC (8 mg/L) was used for *E. faecium* Aus0004. Growth rates during the exponential phase
328 were calculated for each condition (with or without caspofungin 8 mg/L) and expressed as
329 numbers of generations per hour (h^{-1}).

330 In vitro mutants with decreased caspofungin susceptibility have been tried to be obtained
331 from *E. faecium* Aus0004 after serial passages on MH agar supplemented with increasing
332 concentrations of caspofungin and by the gradient method on agar medium as previously
333 described (45).

334 **MIC determination.** MICs of five different antifungal agents (i.e., micafungin, 5-
335 fluorocytosine, voriconazole, amphotericin B, and caspofungin) against *E. faecium* Aus0004
336 were determined by the broth microdilution method (BMD) in Mueller-Hinton (MH) broth .
337 MICs of eight different antibiotic agents (i.e., ampicillin, erythromycin, vancomycin,
338 teicoplanin, daptomycin, tigecycline, ciprofloxacin and linezolid) against *E. faecium* Aus0004
339 were also determined by the BMD according to CA-SFM/EUCAST recommendations
340 (www.sfm-microbiologie.org). Note that for daptomycin MIC determination, calcium
341 chloride (50 mg/L) was added into MH broth. All determinations of MIC values were
342 performed in three independent experiments.

343 MICs of four antibiotics (vancomycin, teicoplanin, daptomycin, and ciprofloxacin) were
344 determined for *E. faecium* Aus0004 in the presence of 8 mg/L of caspofungin in the MH
345 medium using Etest strips following the manufacturer's instructions (bioMérieux, Marcy-
346 l'Etoile, France).

347 **Time-kill curves experiments.** Time-kill curves were determined to appreciate the
348 antibacterial activity (using an antibiotic concentration equal to 8 × the MIC) on *E. faecium*
349 Aus0004, as previously described (46). Briefly, 16-hour overnight cultures were inoculated
350 1:20 in 10 ml of fresh MH broth containing anti-Gram-positive antibiotics (vancomycin,
351 linezolid, daptomycin) or caspofungin and incubated at 35°C for 24 hours. Bacterial survival
352 was checked by CFU counts after 0, 3, 6, 9, and 24 h of incubation in three independent
353 experiments by plating the cultures on BHI agar plates. For daptomycin assay, 50 mg/L of
354 calcium chloride were added to the MH broth.

355 **Biofilm production assay.** The capacity of *E. faecium* Aus0004 and *E. faecium* HM1070Δ*asrR*
356 (a biofilm-positive strain) (42) to form biofilm in the presence of subinhibitory concentration
357 of caspofungin (8 mg/L) was evaluated at 24 h. Briefly, bacteria that had been grown

358 overnight were inoculated 1:100 in 10 ml of TSB with 0.25% glucose and shared into 96-
359 microwells polystyrene plates (NUNC, Denmark). After 24 h of static incubation at 35°C, the
360 plates were washed three times with PBS and stained with 1% crystal violet for 30 min. The
361 wells were rinsed with distilled water and ethanol-acetone (80:20, vol/vol). After drying,
362 optical density at 580 nm (OD₅₈₀) was determined using a microplate reader (Multiskan
363 Ascent, Thermo Electron Corporation). Biofilms were formed under static conditions and
364 each assay was performed in at least three independent experiments. Normalized biofilms
365 were calculated by dividing the total biofilm value (OD₅₈₀) by the bacterial growth for each
366 strain (expressed in Log₁₀ values of CFU counts).

367 **Cell-wall analysis by electron microscopy.** For SEM experiments, *E. faecium* Aus0004 cells
368 were cultured up to the late-exponential phase in TSB supplemented or not with a
369 concentration of 8 mg/L (1/4 × MIC) of caspofungin and then pelleted by centrifugation,
370 rinsed in PBS and fixed with 2.5% glutaraldehyde in cacodylate buffer 0.1M [pH 7.0] at 4°C
371 during 15h. The cells were rinsed in cacodylate buffer and then sedimented during 15 days
372 on Thermanox coverslip (Thermo Fischer Scientific, Villebon-sur-Yvette, France) coated with
373 poly-L-lysine, then dehydrated in progressive baths of ethanol (70-100%). Bacterial cells
374 were sputtered with platinum and observed with scanning electron microscope JEOL 6400F
375 (Jeol, Tokyo, Japan). For TEM experiments, the bacterial strain was cultured in the same
376 conditions than SEM experiments, but after PBS rinse, the cells were fixed with 2.5%
377 glutaraldehyde in cacodylate buffer 0.1M [pH 7.0] containing ruthenium red (0.4 mg/l) for
378 15h at 4°C. The cells were then rinsed and post-fixed 1 hour with 1% osmium tetroxyde in
379 cacodylate buffer 0.1M [pH 7.0] in presence of ruthenium red (0.4 mg/l) at 4°C protected
380 from light. The cells were rinsed, pelleted in 1.5% agar with a low melting point (40°C) and
381 then dehydrated in progressive ethanol baths (70-100%), embedded in resin Embed 812

(Electron Microscopy Sciences, Hatfield, PA, USA) and polymerised 24h at 60°C. Ultrathin sections were done and contrasted with uranyl acetate and lead citrate. The cells were observed with transmission electron microscope JEOL 1011 (Jeol, Tokyo, Japan) and images were taken with an ORIUS 200 CCD camera (Gatan France, Evry, France). Cell-wall analysis by electron microscopy was proceeded in three independent experiments.

Determination of peptidoglycan composition and muropeptides analysis. For the peptidoglycan isolation, *E. faecium* Aus0004 cells were cultured in TSB with and without caspofungin (8 mg/L) until mid-exponential phase, late exponential phase and early stationary phase), and then pelleted, resuspended in PBS and boiled while stirring in 10% SDS for 1 hour. Peptidoglycan isolation and digestion with muramidase was performed as previously described (47). Solubilized muropeptides were reduced by adding 0.5 M sodium borate pH 9.5 and sodium borohydride to a final concentration of 10 g/L. Finally, samples were adjusted to pH 3.5 with phosphoric acid. UPLC analyses were performed on a Waters UPLC system equipped with an ACQUITY UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm×150 mm (Water, USA) and detected at Abs. 204 nm. Muropeptides were separated mainly using a linear gradient from buffer A (phosphate buffer 50 mM pH 4.35) to buffer B (phosphate buffer 50 mM pH 4.95 methanol 15% (v/v)) in a 40 min run. Identity of the muropeptides was assigned by MS/MS spectrometry and for quantification, the area of each peak in the chromatogram was considered. Peptidoglycan analysis was performed using three independent cultures for each strain.

RNA isolation and transcriptomic analysis. *E. faecium* Aus0004 was cultured at 35°C until the late-exponential growth phase (to the same optical densities) in TSB (-Cas media) or in TSB supplemented with subinhibitory concentration of caspofungin (8 mg/L) (+Cas media) corresponding to an incubation of 6h30 and 7h30, respectively. Total RNA was extracted using the

406 ZR Fungal/Bacterial RNA mini-prep kit (Zymo Research, Irvine, CA, USA) in biological
407 duplicate. Residual chromosomal DNA was removed by treating samples with the TURBO
408 DNA-free kit (Life Technologies, Saint-Aubin, France). DNA-free RNA samples were quantified
409 using the NanoDrop One spectrophotometer (Thermo Scientific, Villebon-sur-Yvette, France)
410 and were then depleted from ribosomal RNAs (e.g. 23S, 16S, and 5S rRNAs) using the Ribo-
411 Zero rRNA Removal Kit (Gram-Positive Bacteria) (Illumina-Epicentre, Madison, WI, USA)
412 according to the manufacturer's instructions. Finally, the samples were washed using the
413 RNA Clean and Concentrator - 5 kit (Zymo Research, Irvine, CA, USA). The rRNA depletion
414 efficiency was evaluated by analyzing the samples using the Agilent 2100 bioanalyzer
415 (Agilent Technologies, Les Ulis, France). cDNA libraries were prepared with the strand-
416 specific NEXTflex Rapid Directional RNA-Seq Kit (dUTP-based) v2, and sequencing was
417 performed using an Illumina HiSeq 2500 instrument (Illumina, San Diego, CA, USA) with Run
418 Rapid Single Read of 50 bp multiplexing protocol (ProfileXpert-LCMT, Lyon, France).

419 For bioinformatic analysis, reads were mapped against the genome sequence of *E. faecium*
420 AUS0004 (GenBank accession numbers CP003351.1, CP003352.1, CP003353.1, and
421 CP003354.1) using the CLC Genomics Workbench software v10.0.1 (CLCbio, Qiagen, San
422 Diego, CA, USA). FC values, reads per kilobase per million mapped (RPKM) determination,
423 and statistical analysis were performed using the CLC Genomics Workbench and DESeq2 R
424 package (48). Gene expressions were identified using a \log_2 absolute fold change and values
425 greater or lower than 2 were considered as induced or repressed, respectively, and
426 statistical significance was accepted in case of an P value <0.1 . Mean expression and \log_2 FC
427 values for each gene were plotted and visualized as a MA plot figure.

428 Validation of RNA-seq FC was done by RT-qPCR with specific primers for eight differentially-
429 expressed genes (Table S4). Total RNAs were extracted as described above and residual DNA

430 was removed with the TURBO DNA-free kit. cDNAs were synthesized from total RNA
431 (approximately 1 µg) using the QuantiFast reverse transcription kit (Qiagen, San Diego, CA,
432 USA) according to the manufacturer's instructions, and transcript levels were determined by
433 the DeltaDelta Ct method using the *atpA* gene as a housekeeping control gene. Each
434 experiment was performed in triplicate including RNA-seq biological duplicates.

435 **Accession number.** Raw and processed data generated in this study have been submitted to
436 the Gene Expression Omnibus (GEO) repository at the National Center for Biotechnology
437 Information (NCBI) and are available under accession no. GSE100091.

438

439

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- 575

576 **LEGENDS OF THE FIGURES**

577 **Figure 1. (A)** *Enterococcus faecium* Aus0004 growth during 24 hours in Tryptic-Soy broth
578 (TSB) supplemented or not with eight non-antibiotic molecules commonly used in ICU
579 patients at their awaiting clinical concentrations (except for caspofungin used at 8 mg/L,
580 equivalent at $\frac{1}{4}$ x MIC). **(B)** *E. faecium* Aus0004 growth in TSB supplemented with
581 caspofungin (8 mg/L) and another echinocandin, micafungin (at a concentration of $\frac{1}{4}$ x MIC,
582 16 mg/L). **(C)** Growth curves of *E. faecium* Aus0004, *Enterococcus faecalis* ATCC 29212 and
583 *Staphylococcus aureus* ATCC 29213 in the presence or absence of caspofungin (8 mg/L). **(D)**
584 Growth in Luria-Bertani broth with or without adjunction of caspofungin (8 mg/L) of
585 *Escherichia coli* ATCC 25922, *Enterobacter cloacae* ATCC 13047 and *Pseudomonas*
586 *aeruginosa* ATCC 27853.

587

588 **Figure 2.** Time-kill curves of different anti-Gram-positive antibiotics and caspofungin against
589 *E. faecium* Aus0004 (8 x MIC) in Mueller-Hinton broth (MHB).

590

591 **Figure 3.** Cell wall analysis of *E. faecium* Aus0004 in the presence of caspofungin (8 mg/L) by
592 electron microscopy. **(A)** Scanning electron microscopy (SEM) images of *E. faecium* Aus0004
593 cells growth in Tryptic-Soy broth (TSB) (top) or TSB + caspofungin (bottom). Magnification x
594 20,000. Morphological abnormalities on cell surface (roughened surface, extrusions) are easily
595 visible. **(B)** Transmission electron microscopy (TEM) images of *E. faecium* Aus0004 cells
596 growth in TSB (top) or TSB + caspofungin (bottom).

597

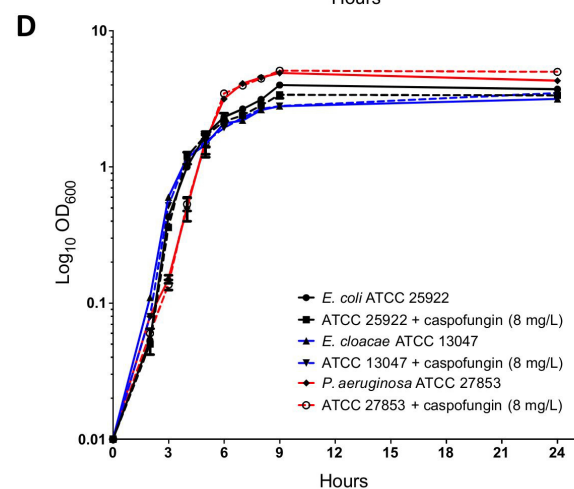
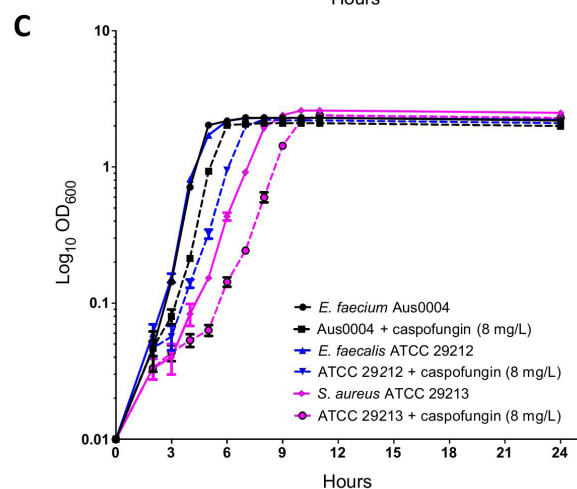
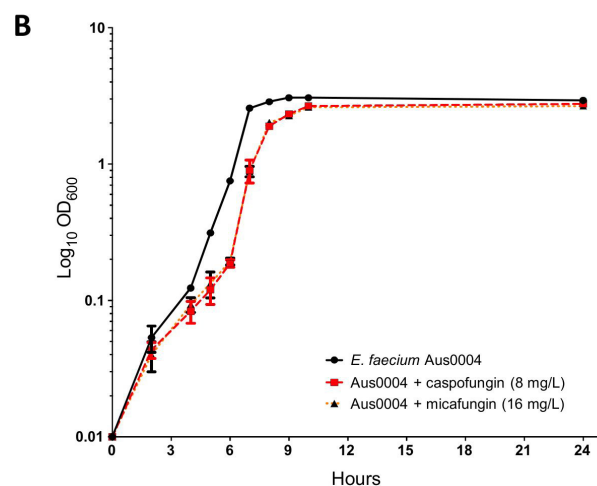
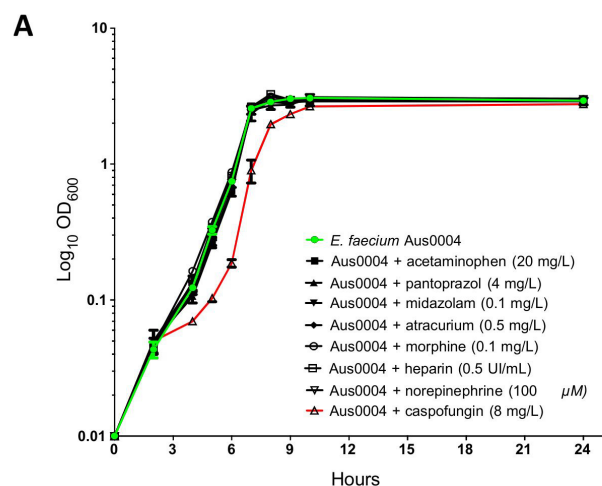
598 **Figure 4.** Peptidoglycan modifications produced by caspofungin exposure. **(A-B)**
599 Representative UPLC chromatogram of *E. faecium* Aus0004 peptidoglycan of a non-treated

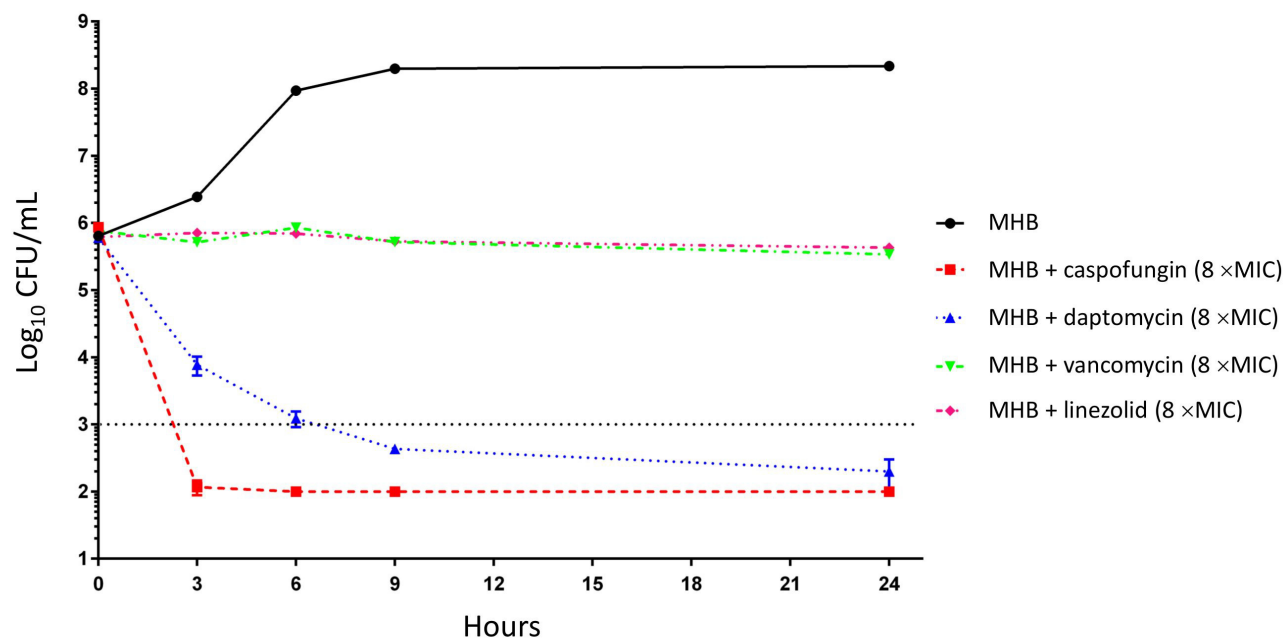
sample **(A)** and structure of the mucopeptides determined for each indicated peak that was confirmed by MS analysis **(B)** (M stands for monomer, D for dimer and the numbers refer to the length of the stem peptide). Peaks presenting significant changes after caspofungin treatment are labeled in red. **(C)** Quantification of the abundance of the mucopeptides determined under two growth conditions: Tryptic-Soy broth (TSB) (grey bars, untreated samples) and TSB + caspofungin 8 mg/L (black bars, treated samples). The experiment was realized at 3 different times of growth (mid-exponential phase, late exponential phase and early stationary phase). Statistical analysis was performed using the student t test. Asterisks represent significant p values from comparisons of treated with untreated samples: * <0.05 , ** <0.005 . Each experiment was performed in triplicate.

Figure 5. Transcriptional response of *E. faecium* Aus0004 growth with caspofungin. **(A)** Global analysis of transcript levels in *E. faecium* Aus0004 by RNA-seq represented by an MA-plot (caspofungin 8 mg/L vs. control) generated by the DESeq2 R package. Log₂ fold changes in expression of each chromosomal gene are shown on the y-axis versus the mean of normalized counts is shown on the x-axis (log₁₀ scale). Points with adjusted p-value less than 0.1 are indicated in red. **(B)** Validation of RNA-seq results by qRT-PCR of 10 genes. Mean log₂ ratios of values determined in the qRT-PCR experiments are plotted against the mean log₂ ratios of the RNA-seq experiments.

Figure 6. Normalized biofilm formation in the presence of caspofungin (8 mg/L) in *E. faecium* Aus0004 strain (a biofilm non-producer) and *E. faecium* HM1070Δ*asrR* (a biofilm producer). Normalized biofilms were calculated by dividing the total biofilm value (OD₅₈₀) by the

623 bacterial growth for each strain (expressed in Log_{10} values of CFU counts). Statistical
624 comparison was performed using the unpaired t test.

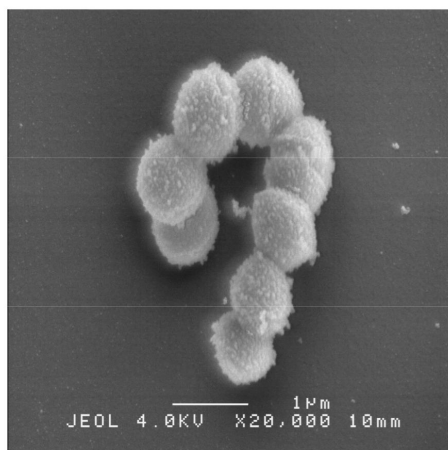




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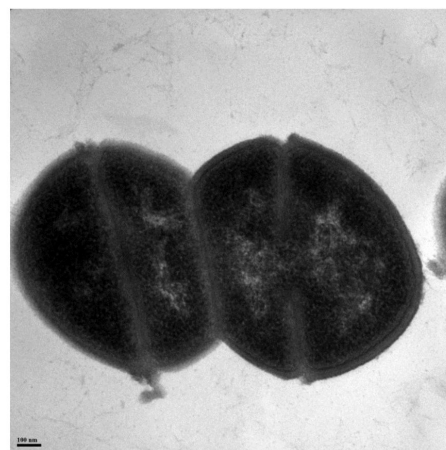


TSB

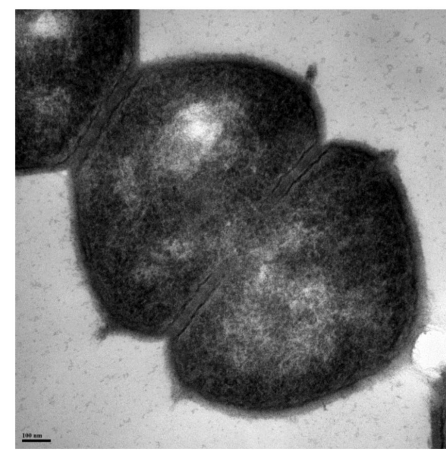


TSB + caspofungin (8 mg/L)

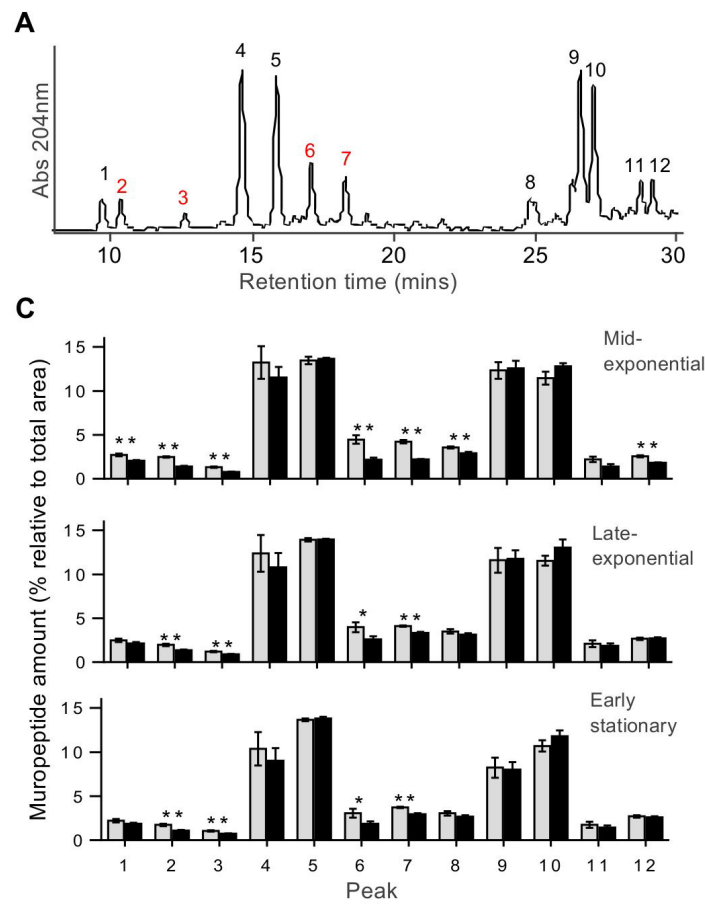
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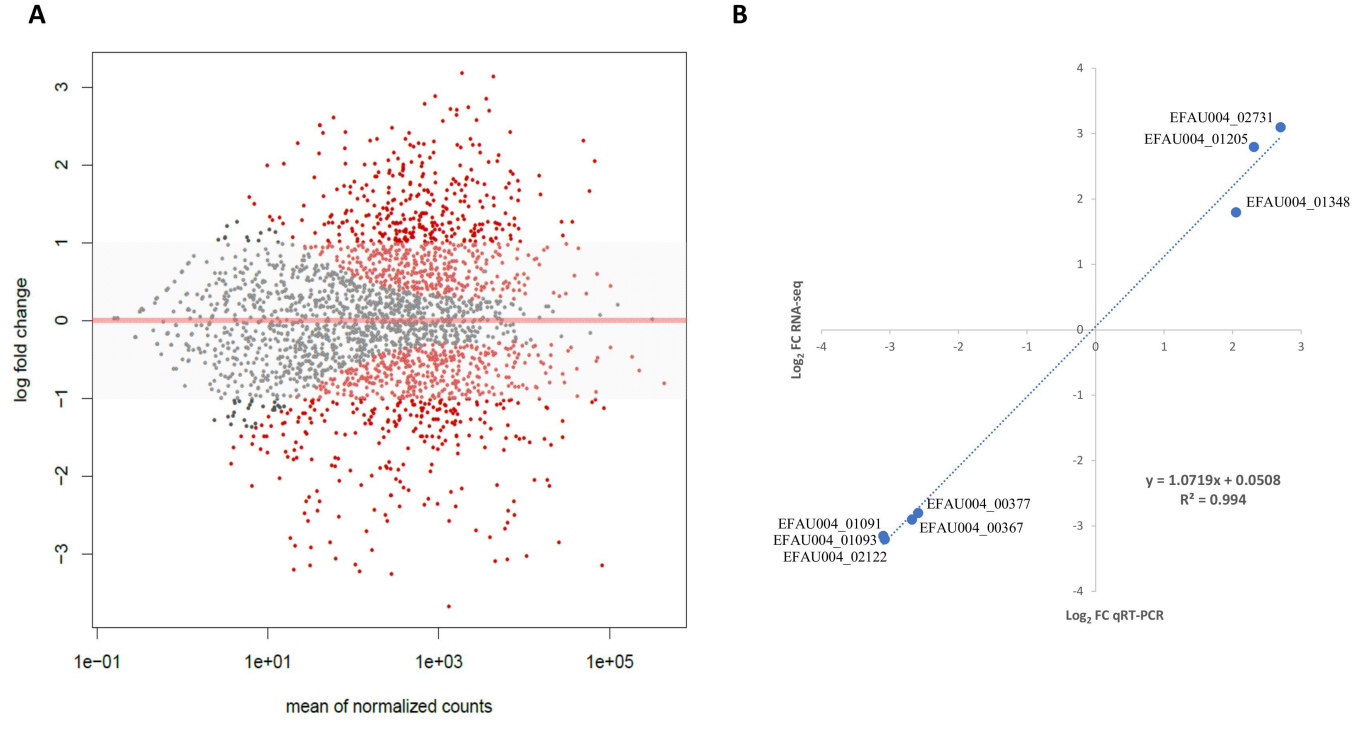


TSB



TSB + caspofungin (8 mg/L)





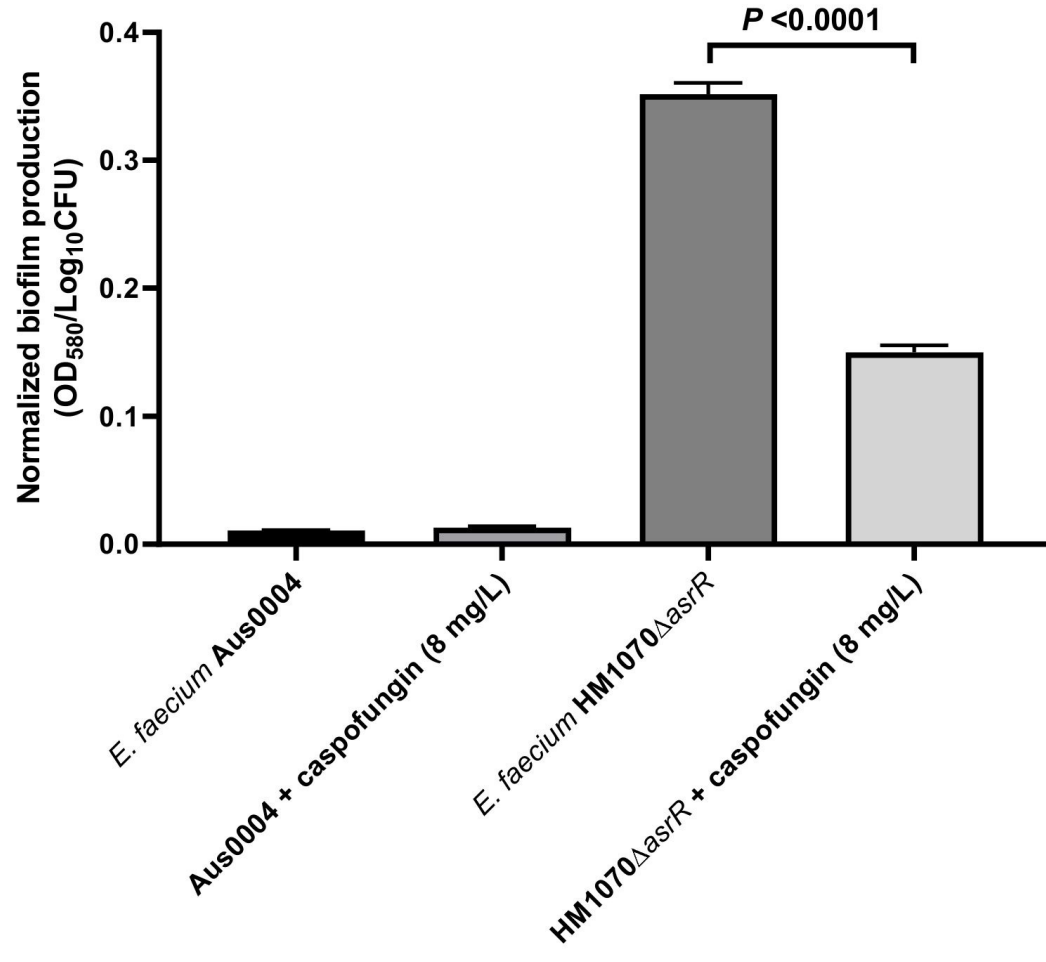


Table 1. MICs of several antibiotic and antifungal molecules for *E. faecium* Aus0004

| Antimicrobial | MIC (mg/L) |
|----------------------|------------|
| <i>Antibacterial</i> | |
| Ampicillin | >256 |
| Erythromycin | >256 |
| Vancomycin | 8 |
| Teicoplanin | 1 |
| Daptomycin | 2 |
| Ciprofloxacin | 2 |
| Linezolid | 1 |
| Tigecycline | 0.06 |
| <i>Antifungal</i> | |
| Caspofungin | 32 |
| Micafungin | 64 |
| Voriconazole | >1,024 |
| Amphotericin B | >1,024 |
| Flucytosine | >1,024 |

Table 2. Selected genes used for RNA-seq validation by qRT-PCR experiments.

| Gene no. | Gene name | Product name | Gene start | Gene end | RNA-seq fold change | Adjusted <i>P</i> -value |
|---------------|-------------|--|------------|----------|---------------------|--------------------------|
| EFAU004_00367 | <i>dexB</i> | Glucan 1,6- α -glucosidase | 364710 | 366333 | -9.6 | 1.20E-35 |
| EFAU004_00377 | <i>glpK</i> | Glycerol kinase | 379384 | 380881 | -6.0 | 5.60E-31 |
| EFAU004_01091 | <i>pdhD</i> | Dihydrolipoyl dehydrogenase | 1113699 | 1115106 | -6.4 | 4.70E-63 |
| EFAU004_01093 | <i>pdhB</i> | Transketolase | 1116781 | 1117759 | -8.6 | 4.32E-16 |
| EFAU004_01205 | <i>hupA</i> | DNA-binding protein HU | 1233614 | 1233890 | 4.9 | 3.76E-37 |
| EFAU004_01348 | <i>sodA</i> | Superoxide dismutase, Mn ²⁺ | 1395234 | 1395843 | 4,1 | 2,33E-42 |
| EFAU004_02122 | - | L-lactate oxidase | 2151833 | 2152937 | -8.4 | 8.70E-12 |
| EFAU004_02731 | - | Zeta toxin | 2792658 | 2793333 | 6,5 | 1,75E-53 |

Table 3. Antimicrobial susceptibility testing of *E. faecium* strains with different phenotypes of susceptibility or resistance to glycopeptides.

| <i>E. faecium</i> strains | <i>van</i> operon | MIC (mg/L) | | | | | | | |
|------------------------------|-------------------|------------|------|-------------|------|------------|------|---------------|------|
| | | Vancomycin | | Teicoplanin | | Daptomycin | | Ciprofloxacin | |
| | | MH | CAS8 | MH | CAS8 | MH | CAS8 | MH | CAS8 |
| UCN103 | <i>vanA</i> | 32 | 256 | 4 | 4 | 4 | 8 | 8 | 8 |
| UCN104 | <i>vanA</i> | 32 | 256 | 2 | 2 | 2 | 4 | >32 | >32 |
| UCN105 | <i>vanA</i> | 4 | 32 | 2 | 2 | 4 | 8 | >32 | >32 |
| AUS0004 | <i>vanB</i> | 8 | 32 | 1 | 1 | 2 | 8 | 2 | 2 |
| UCN 106 | <i>vanB</i> | 16 | 64 | 1 | 1 | 4 | 8 | >32 | >32 |
| UCN 107 | <i>vanB</i> | 16 | 64 | 0.5 | 0.5 | 4 | 8 | >32 | >32 |
| UCN 108 | <i>vanB</i> | 32 | 64 | 0.5 | 0.5 | 4 | 4 | >32 | >32 |
| UCN 109 | <i>vanB</i> | 64 | 256 | 0.25 | 0.5 | 2 | 4 | >32 | >32 |
| UCN 110 | - | 0.5 | 1 | 0.25 | 1 | 2 | 4 | 2 | 2 |
| UCN 111 | - | 0.5 | 1 | 0.12 | 0.25 | 2 | 4 | 4 | 4 |
| UCN 112 | - | 1 | 1 | 0.12 | 0.25 | 2 | 4 | 2 | 4 |
| UCN 113 | - | 0.5 | 1 | 0.25 | 1 | 2 | 4 | 8 | 8 |
| UCN 114 | - | 0.5 | 1 | 0.25 | 0.5 | 1 | 4 | >32 | >32 |

^a MH : Mueller-Hinton ; ^b CAS8 : MH + caspofungin (8 mg/L)