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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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ABSTRACT

Enterococcus faecium has become a major opportunistic pathogen with the emergence of vancomycin-resistant enterococci (VRE). As part of the gut microbiota, they have to cope with numerous stresses including effects of antibiotics and other xenobiotics, especially in patients hospitalized in intensive care units (ICUs) who receive many medications. The aim of this study was to investigate the impact of the most prescribed xenobiotics for ICU patients on fitness, pathogenicity and antimicrobial resistance of the *vanB*-positive *E. faecium* Aus0004 reference strain. Several phenotypic analyses were carried out and we observed that caspofungin, an antifungal agent belonging to the echinocandins family, had an important effect on *E. faecium* growth in vitro. We confirmed this effect by electron microscopy and peptidoglycan analysis and showed that, even at a subinhibitory concentration (¼ × MIC, 8 mg/L), caspofungin had an impact on cell wall organization especially in abundance of some muropeptide precursors. By RNA-seq, it was also shown that around 20% of the transcriptome were altered in the presence of caspofungin with 321 and 259 significantly upregulated and downregulated genes, respectively. Since the fungal target of caspofungin (i.e., beta-(1,3)-glucan synthase) was absent in bacteria, the mechanistic pathway of caspofungin activity was investigated. The repression of genes involved in the pyruvate metabolism seemed to have a drastic impact on bacterial cell viability while decrease of glycerol metabolism could explain the conformational modifications of peptidoglycan. This is the first report of caspofungin antibacterial activity against *E. faecium*, highlighting the potential impact of non-antibiotic xenobiotics against bacterial pathogens.

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INTRODUCTION

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

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

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

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

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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 (¼ × 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 ($1.487 \pm 0.034\ h^{-1}$ vs. $1.444 \pm 0.041\ h^{-1}$, 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 ¼ × 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,

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Impact of caspofungin on biofilm formation. This is well known that bacterial biofilms are a significant medical challenge because they are difficult to treat using standard therapeutic approaches, given that they are a major barrier to antibiotic effectiveness, especially in MDR *E. faecium* isolates (41). In order to characterize the effect of caspofungin on biofilm production, we evaluated levels of static biofilm formation of *E. faecium* Aus0004 (used as biofilm non-producer) and *E. faecium* HM1070Δ*asrR* (used as biofilm producer) (42) in the presence of caspofungin subinhibitory concentration (8 mg/L). As previously described in fungal models (43), caspofungin significantly reduced the ability of biofilm formation of HM1070Δ*asrR* whereas no difference was observed for *E. faecium* Aus0004 (Figure 6). These

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

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

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CONCLUSION

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

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MATERIALS AND METHODS

Bacterial isolates and growth conditions. Bacterial strains used in this study are listed in Table S3. The main strain used was the *vanB*-positive reference strain *E. faecium* Aus0004 of which the complete genome sequence is available (GenBank accession number CP003351.1) (21). UCN strains were obtained from the French Reference Centre for Enterococci for which detection for *vanA/B* genes was performed as previously described (44). For biofilm formation experiments, *E. faecium* HM1070Δ*asrR* was used as positive control as previously described (42).

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

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

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

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

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

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

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

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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 (OD_{580}) 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 (OD_{580}) by the bacterial growth for each
366 strain (expressed in Log_{10} 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

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(Electron Microscopy Sciences, Hatfield, PA, USA) and polymerised 24h at 60°C. Ultrathin sections were done and contrasted with uranyle 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

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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

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

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

ACKNOWLEDGEMENTS

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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 ¼ x MIC). **(B)** *E. faecium* Aus0004 growth in TSB supplemented with
581 caspofungin (8 mg/L) and another echinocandin, micafungin (at a concentration of ¼ 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

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sample **(A)** and structure of the muropeptides 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 muropeptides 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. Log_2 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 (log_{10} 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 log_2 ratios of values determined in the qRT-PCR experiments are plotted against the mean log_2 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 (OD_{580}) by the

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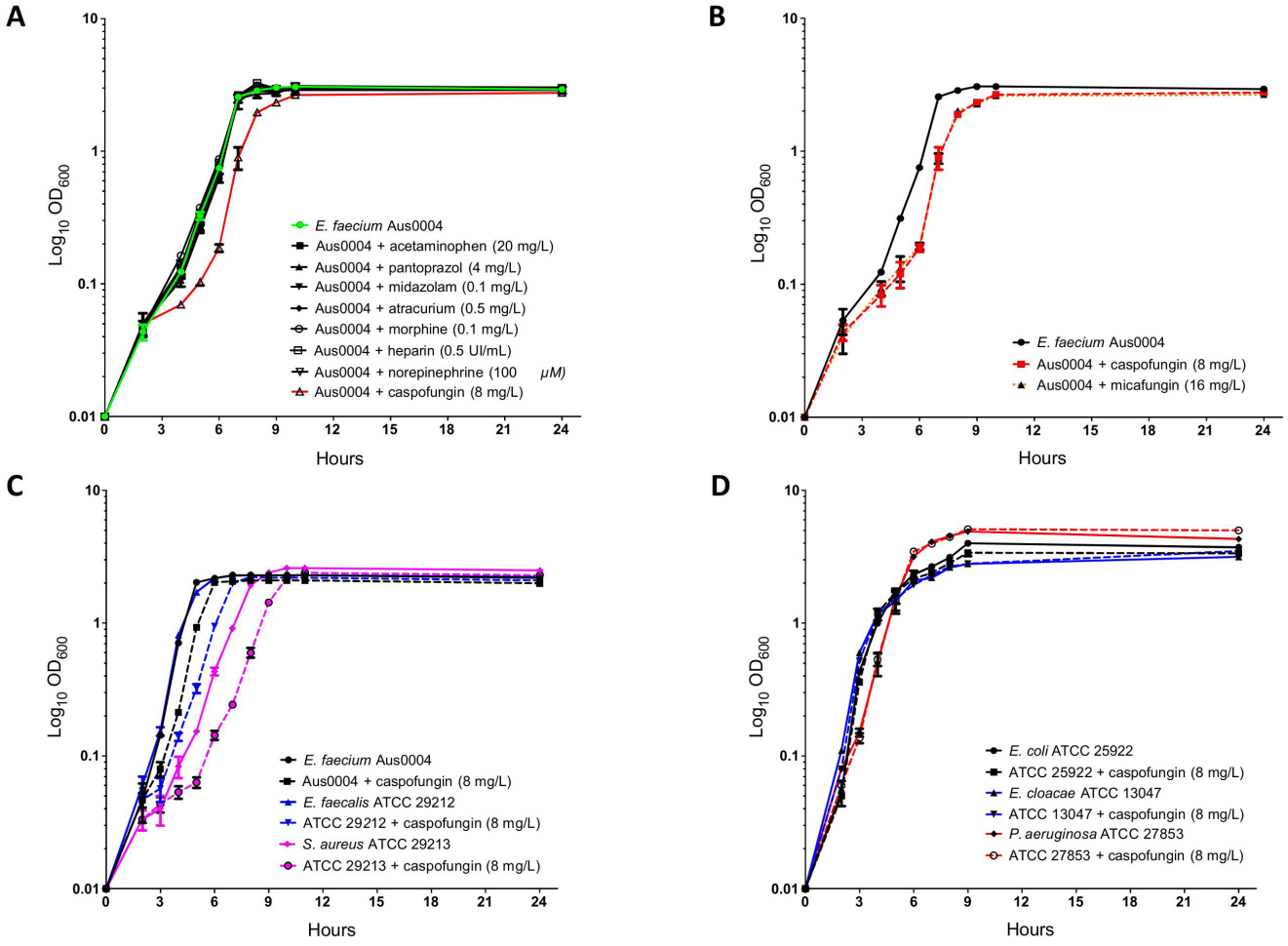
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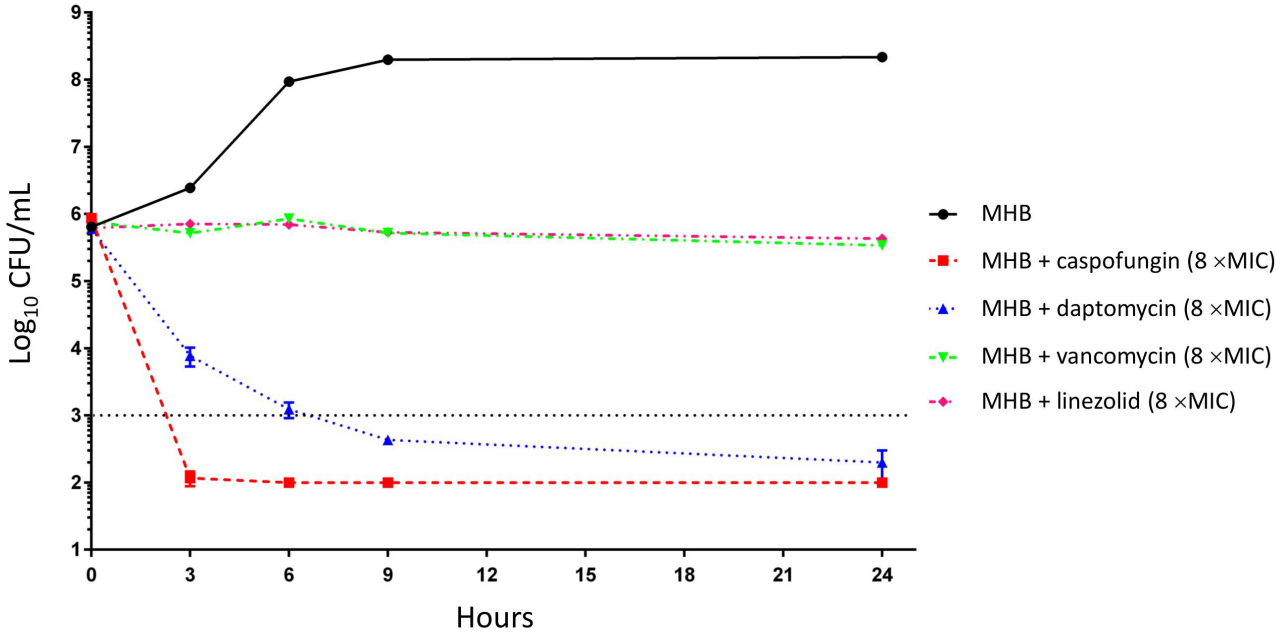
bacterial growth for each strain (expressed in Log_{10} values of CFU counts). Statistical comparison was performed using the unpaired *t* test.

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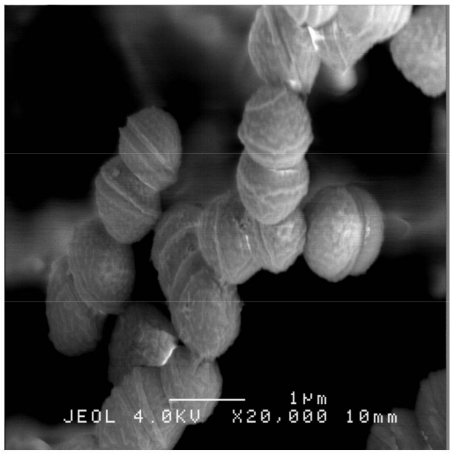


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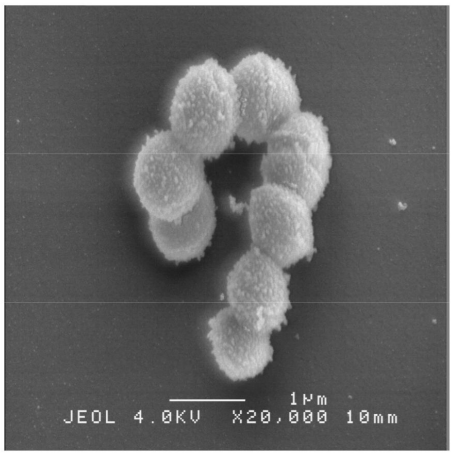


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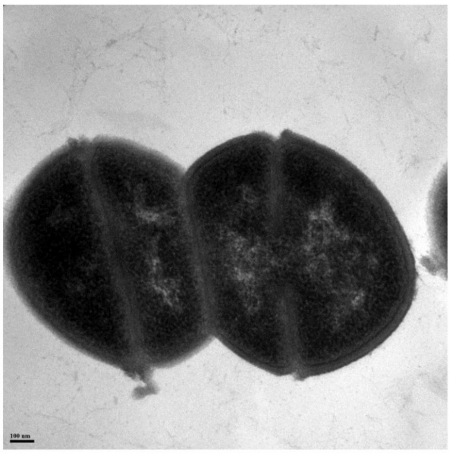


TSB

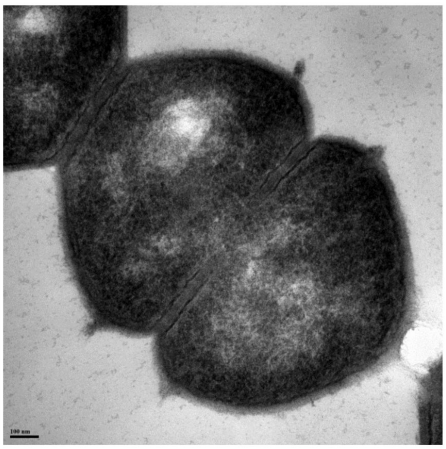


TSB + caspofungin (8 mg/L)

B

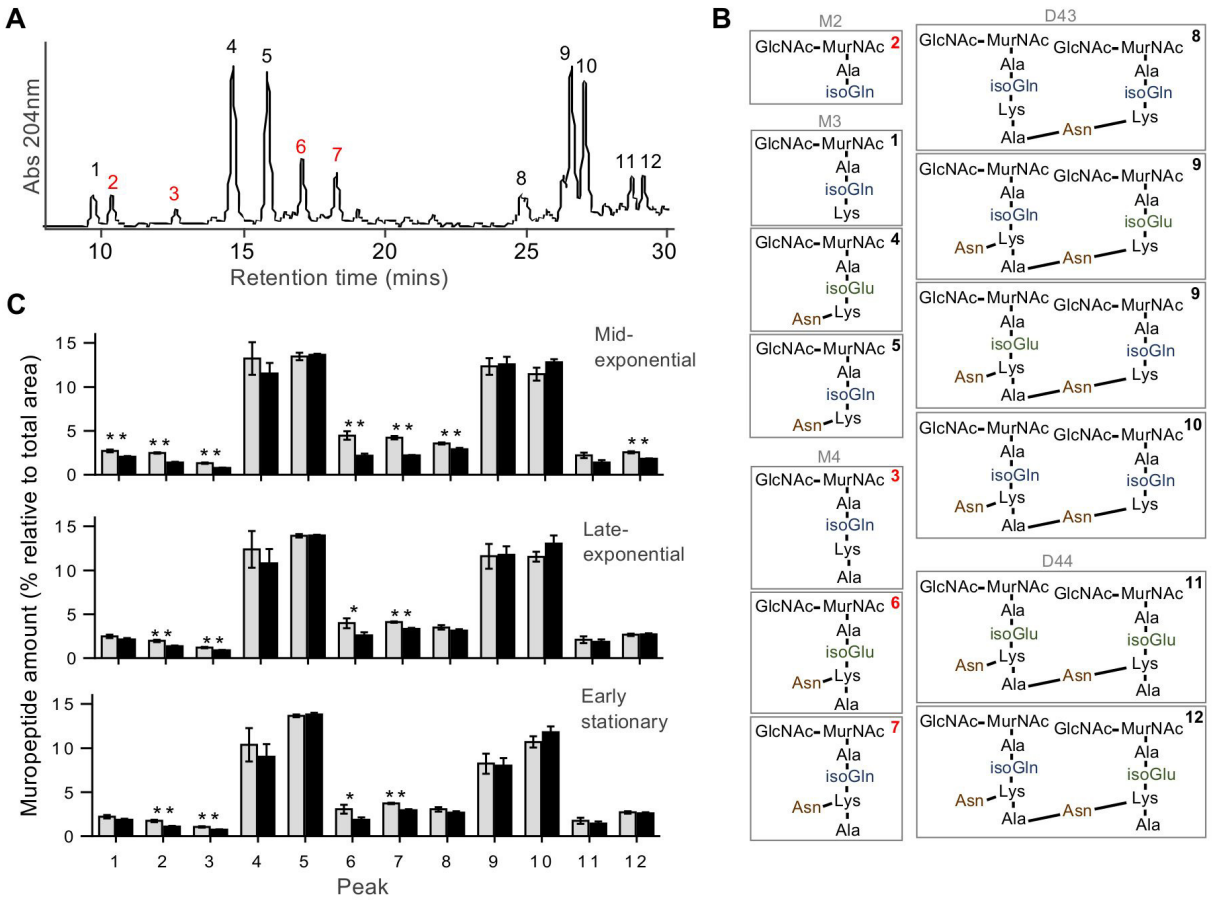


TSB



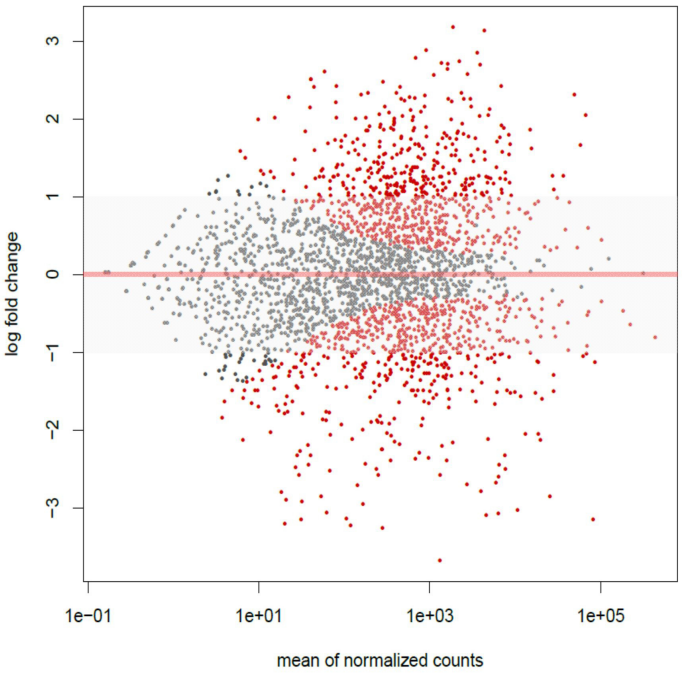
TSB + caspofungin (8 mg/L)

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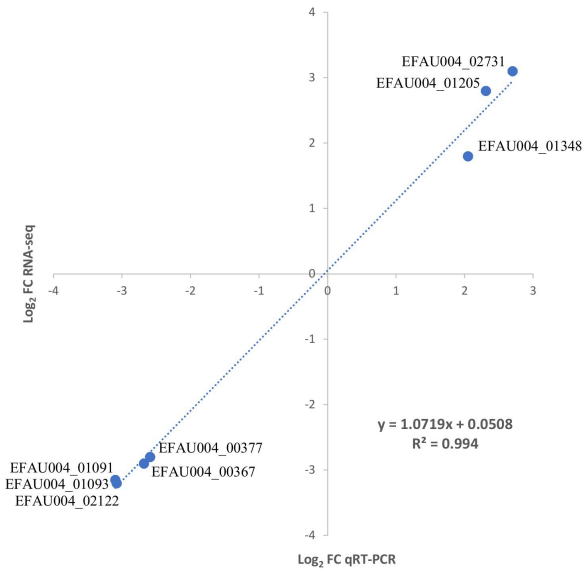


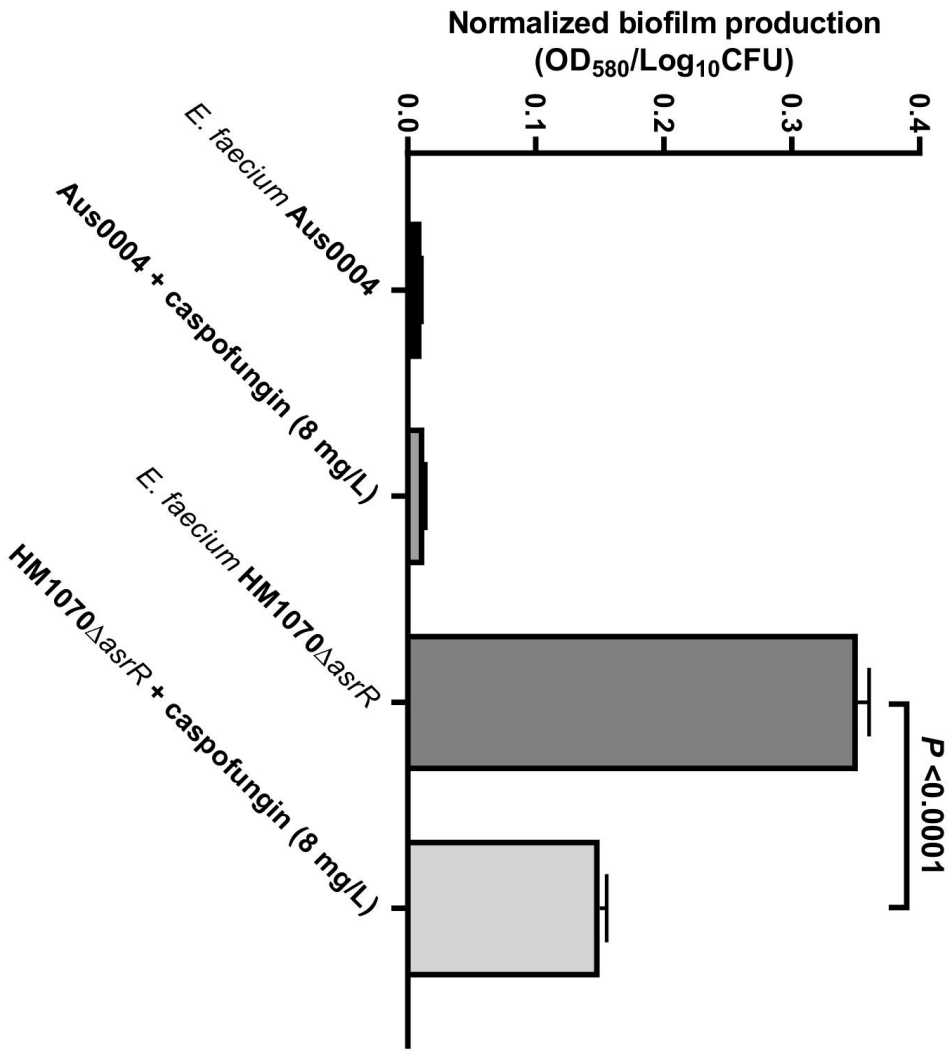
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A



B




Normalized biofilm production
(OD580/Log10CFU)
0.0
0.1
0.2
0.3
0.4
E. faecium Aus0004
Aus0004 + caspofungin (8 mg/L)
E. faecium HM1070ΔasrR
HM1070ΔasrR + caspofungin (8 mg/L)
P <0.0001

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

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

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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 *P*-value
EFAU004_00367	*dexB*	Glucan 1,6-alpha-glucosidase	364710	366333	-9.6	1.20E-35
EFAU004_00377	*glpK*	Glycerol kinase	379384	380881	-6.0	5.60E-31
EFAU004_01091	*pdhD*	Dihydrolipoyl dehydrogenase	1113699	1115106	-6.4	4.70E-63
EFAU004_01093	*pdhB*	Transketolase	1116781	1117759	-8.6	4.32E-16
EFAU004_01205	*hupA*	DNA-binding protein HU	1233614	1233890	4.9	3.76E-37
EFAU004_01348	*sodA*	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

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

***E. faecium* strains**	***van* operon**	**MIC (mg/L)**							
		Vancomycin		Teicoplanin		Daptomycin		Ciprofloxacin	
		MH	CAS8	MH	CAS8	MH	CAS8	MH	CAS8
UCN103	*vanA*	32	256	4	4	4	8	8	8
UCN104	*vanA*	32	256	2	2	2	4	>32	>32
UCN105	*vanA*	4	32	2	2	4	8	>32	>32
AUS0004	*vanB*	8	32	1	1	2	8	2	2
UCN 106	*vanB*	16	64	1	1	4	8	>32	>32
UCN 107	*vanB*	16	64	0.5	0.5	4	8	>32	>32
UCN 108	*vanB*	32	64	0.5	0.5	4	4	>32	>32
UCN 109	*vanB*	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)

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