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MECHANISMS OF ACTION: PHYSIOLOGICAL EFFECTS

Unexpected Cell Wall Alteration-Mediated Bactericidal Activity of the Antifungal Caspofungin against Vancomycin-Resistant *Enterococcus 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 frequently 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 family of echinocandins, 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 (1/4× MIC, 8 mg/liter), caspofungin had an impact on cell wall organization, especially with respect to the abundance of some muropeptide precursors. By transcriptome sequencing (RNA-seq), it was also shown that around 20% of the transcriptome was altered in the presence of caspofungin, with 321 and 259 significantly upregulated and downregulated genes, respectively. Since the fungal target of caspofungin (i.e., β-1,3-glucan synthase) was absent in bacteria, the mechanistic pathway of caspofungin activity was investigated. The repression of genes involved in the metabolism of pyruvate seemed to have a drastic impact on bacterial cell viability, while a 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 nonantibiotic xenobiotics against bacterial pathogens.

KEYWORDS *E. faecium*, VRE, antibacterial, caspofungin, echinocandins

Infections are among 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 in medical costs since approximately

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50% of ICU patients acquire an infection during their hospitalization, with 60% of deaths attributable to infections (1). While approximately 70% of noncardiac ICU patients receive antibiotics for preventive or curative indications during their stay, nonantibiotic molecules are also extensively used for patient care, including inotropic drugs, opioid and nonopioid analgesics, anxiolytics, anticoagulants, antacids, curares, or antifungal agents (2, 3). It is now well accepted that bacteria must cope with numerous environmental stresses to survive 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 nonantibiotic molecules on bacterial pathogens. Nonetheless, it has been demonstrated that therapy with nonantibiotics may have significant effects upon the physiology and virulence of bacterial cells (6). For instance, catecholamine administration raised the growth rate and affected biofilm formation and enhanced survival traits in cases of antibiotic therapy in *Pseudomonas aeruginosa in vivo* and *ex vivo* models (7). Enhancement of virulence traits in the presence of catecholamines has been described in other bacterial pathogens such as *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Staphylococcus epidermidis*, and *Campylobacter jejuni* (8–15). Recently, it was also reported that virulence of *P. aeruginosa* was 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 bugs" (for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* spp.) (1, 17).

Initially considered to be 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, strains of vancomycin-resistant enterococci (VRE) have emerged within the *E. faecium* species, particularly with the worldwide spread of hospital-adapted isolates belonging to the clonal complex 17 (CC17) (18–20). Actually, these CC17 strains are part of a particular epidemic lineage of hospital-adapted strains described as clade A1. This particular A1 clade is genetically distant from the community-associated human lineage designated clade B (20). Since most reports concerning the influence of xenobiotics have described Gram-negative bacteria, the impact of nonantibiotic xenobiotics used in ICU patients on *E. faecium* isolates remained undocumented.

The aim of this study was to investigate the influence of highly prescribed nonantibiotic 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 to be a nonantibiotic molecule) was shown to have a significant impact on *E. faecium*, different approaches (*in vitro* tests, electron microscopy [EM], peptidoglycan analysis, and global transcriptomic analysis) were taken in order to decipher this unexpected effect.

RESULTS AND DISCUSSION

Impact of nonantibiotic molecules on *E. faecium* Aus0004 growth kinetics. The effect of eight different nonantibiotic 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 (Fig. 1A). 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 induced us to determine the MIC of caspofungin for Aus0004, which was at 32 mg/liter (Table 1). Using a subinhibitory concentration (1/4× MIC, 8 mg/liter) of caspofungin, we found a significant impact on bacterial growth with a lag time extension of about 1 h (Fig. 1A). Note that there was no difference in exponential-

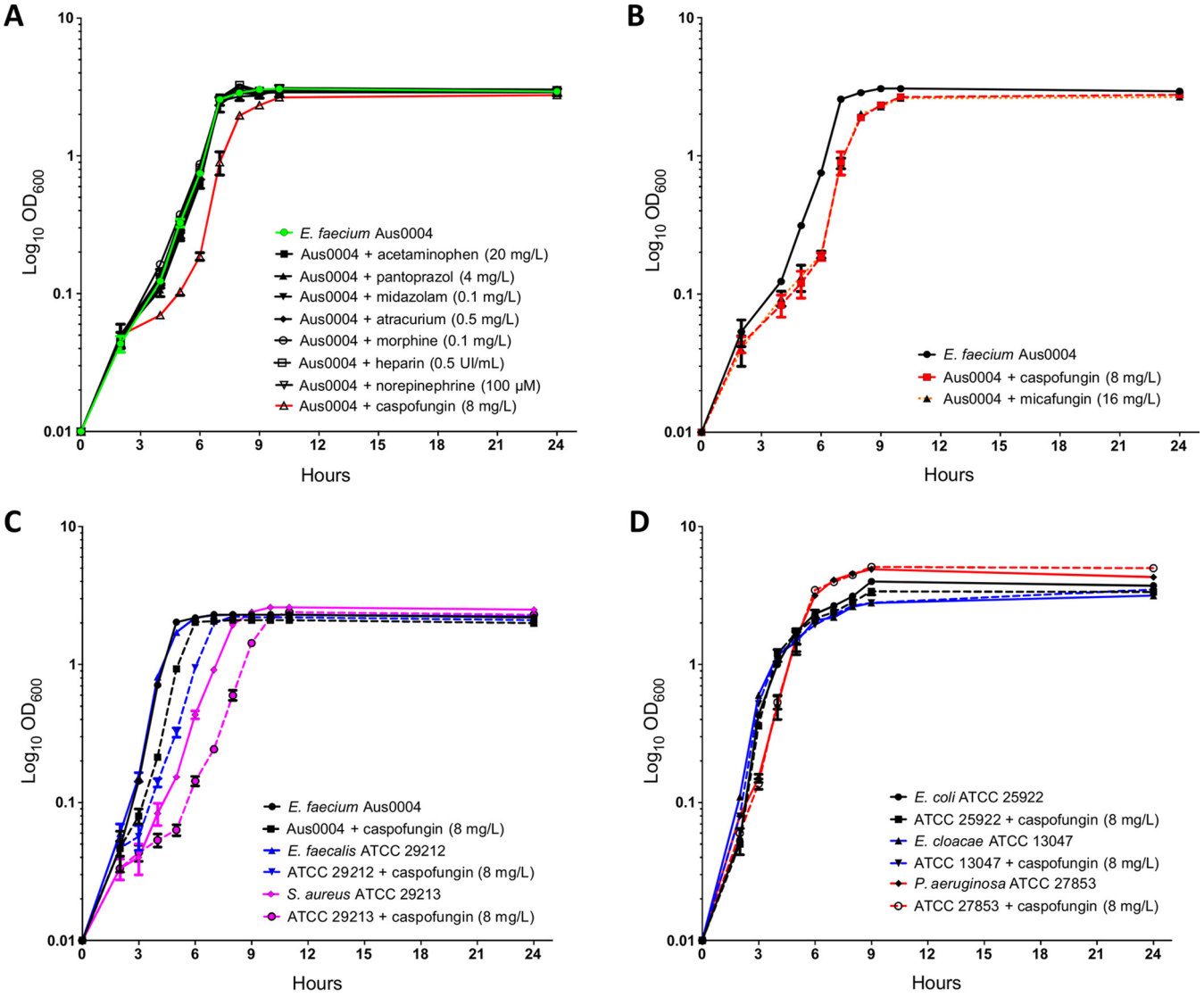

FIG 1 (A) *Enterococcus faecium* Aus0004 growth during 24 h in tryptic soy broth (TSB) supplemented or not with eight nonantibiotic molecules commonly used in ICU patients at the standard clinical concentrations (except for caspofungin used at 8 mg/liter, equivalent to 1/4× MIC). (B) *E. faecium* Aus0004 growth in TSB supplemented with caspofungin (8 mg/liter) and another echinocandin, micafungin (at a concentration of 1/4× MIC, 16 mg/liter). (C) Growth curves of *E. faecium* Aus0004, *Enterococcus faecalis* ATCC 29212, and *Staphylococcus aureus* ATCC 29213 in the presence or absence of caspofungin (8 mg/liter). (D) Growth in Luria-Bertani broth with or without addition of caspofungin (8 mg/liter) of *Escherichia coli* ATCC 25922, *Enterobacter cloacae* ATCC 13047, and *Pseudomonas aeruginosa* ATCC 27853.

growth rates under conditions that included the absence of caspofungin (−Cas) and presence of caspofungin (+Cas) (1.487 ± 0.034 h^{-1} and 1.444 ± 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/liter (corresponding to 1/4× MIC) (Table 1). A similar effect on bacterial growth (lag time extension) was observed with this second compound (Fig. 1B). These preliminary results demonstrated that echinocandins had a significant impact on *E. faecium* Aus0004 fitness *in vitro*.

In contrast to what it has previously 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, since, as previously described in *Listeria mono-*

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

Antimicrobial	MIC (mg/liter)
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

cytogenes, overgrowth observed with catecholamine was formerly linked with iron uptake, promoted by increased 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 proinflammatory 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 been evaluated in only 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 that study, caspofungin was used at a higher concentration (40 mg/liter) than that used in our study (8 mg/liter). As described in fungi, we can hypothesize that caspofungin, 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/liter) was evaluated *in vitro* with representative Gram-positive and -negative bacterial species. The bacterial growth of Gram-positive bacteria (*S. aureus* and *Enterococcus faecalis*) was impacted (Fig. 1C), whereas that of Gram-negatives (*P. aeruginosa*, *E. coli* and *Enterobacter cloacae*) was not (Fig. 1D). Then, it appears that caspofungin has a significant impact only on *in vitro* bacterial growth of Gram-positive bacteria. This is likely due to the differences of bacterial cell wall composition between Gram-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 nonpermeable (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 the 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-fluorocytosine) (Table 1).

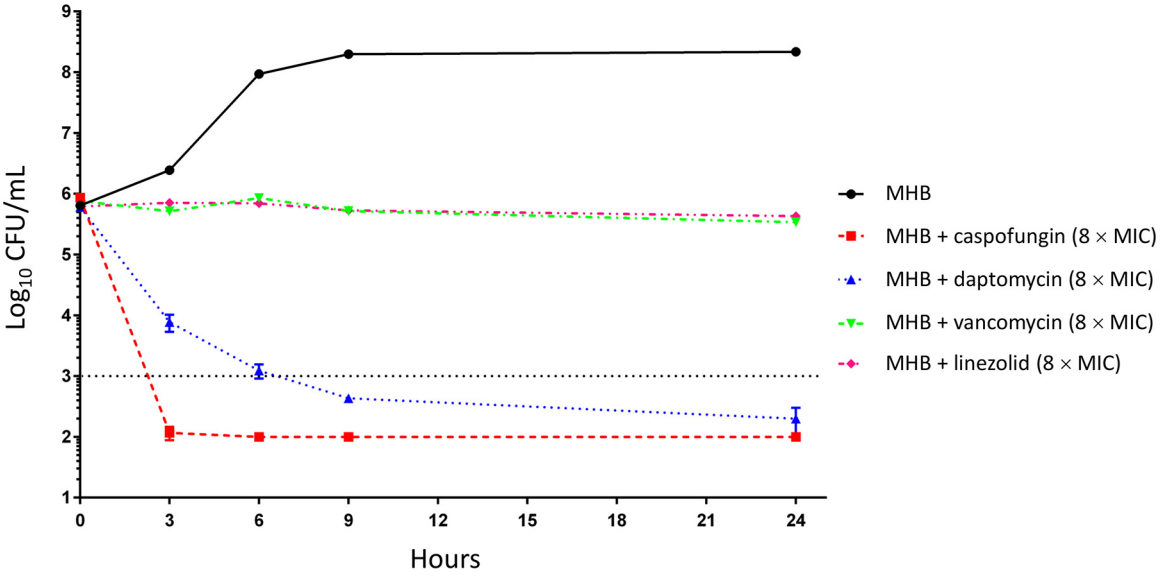

FIG 2 Time-kill curves of different anti-Gram-positive antibiotics and caspofungin against *E. faecium* Aus0004 (8× MIC) in Mueller-Hinton broth (MHB).

The next step was to determine if caspofungin had bacteriostatic or bactericidal activity against *E. faecium* Aus0004 by time-kill curve analysis (at 8× the MIC) using anti-Gram-positive antibiotics as comparators. Interestingly, we observed a rapid bactericidal effect (greater than -3 Log_{10} reduction) in the presence of caspofungin after only 3 h of incubation (Fig. 2). This effect was sustained during the 24-h period of the experiment without any regrowth (Fig. 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 bactericidal activity (Fig. 2). These data are surprising since caspofungin and other β-1,3-glucan synthase inhibitors are known to be fungistatic molecules (33).

Alterations of *E. faecium* Aus0004 cell wall under caspofungin exposure. The impact of caspofungin (at 8 mg/liter) on cell wall components was first visualized using an ultrastructural morphology analysis by scanning electron microscopy 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 (Fig. 3A). TEM experiments did not reveal any change of the cell wall thickness in the presence of caspofungin (Fig. 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/liter) at different growth time points (mid-exponential, late-exponential, and early stationary phases) by ultraperformance liquid chromatography (UPLC) and tandem mass spectrometry (MS/MS) analysis showed a significant decrease of four muropeptides (Fig. 4). Interestingly, relative abundances of those muropeptides decreased by between 20% and 60% depending on the type of muropeptides and the incubation time of the cells (Fig. 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 growth of the cell (34), the defects in bacterial growth (Fig. 1) and the bactericidal effect (Fig. 2) observed in the presence of caspofungin might be related to 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 that incorporated transcriptome sequencing (RNA-seq) to decipher the impact of caspofungin at subinhibitory concentration (1/4× MIC, 8 mg/liter) on different metabolic pathways in *E. faecium* Aus0004.

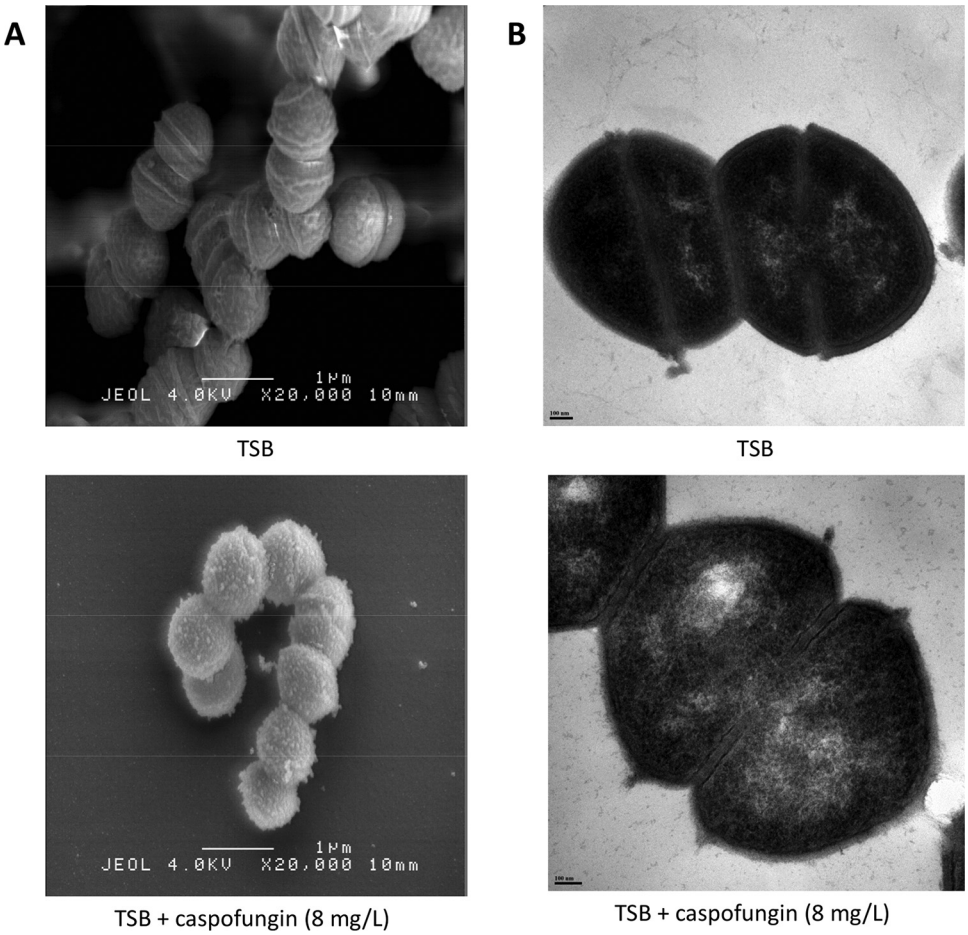

FIG 3 Cell wall analysis of *E. faecium* Aus0004 in the presence of caspofungin (8 mg/liter) by electron microscopy. (A) Scanning electron microscopy (SEM) images of *E. faecium* Aus0004 cells grown in tryptic soy broth (TSB) (top) or TSB plus caspofungin (bottom). Magnification, ×20,000. Morphological abnormalities on cell surface (roughened surface, extrusions) are easily visible. (B) Transmission electron microscopy (TEM) images of *E. faecium* Aus0004 cells grown in TSB (top) or TSB plus caspofungin (bottom).

Total RNAs were isolated in biological duplicate from *E. faecium* Aus0004 grown to the late-exponential phase in both cases (same optical densities [OD]), 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 (see Table S1 in the supplemental material). Less than 2% of reads mapped to sequences of the three plasmids whereas 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 sets of conditions (see Fig. S1 in the supplemental material).

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/liter) is presented as a MA plot representation (Fig. 5A) (see also Table S2 in the supplemental material). To assess the reliability of RNA-seq in determining DGEs, we determined by reverse transcription-quantitative PCR (RT-qPCR) mRNA levels of three upregulated genes (*hupA*, *sodA*, and EFAU004_02731) and five downregulated genes (*dexB*, *glpK*, *pdhD*, *pdhB*, and EFAU004_02122) (Table 2). Those genes were chosen according to their levels of expression FCs and their putative

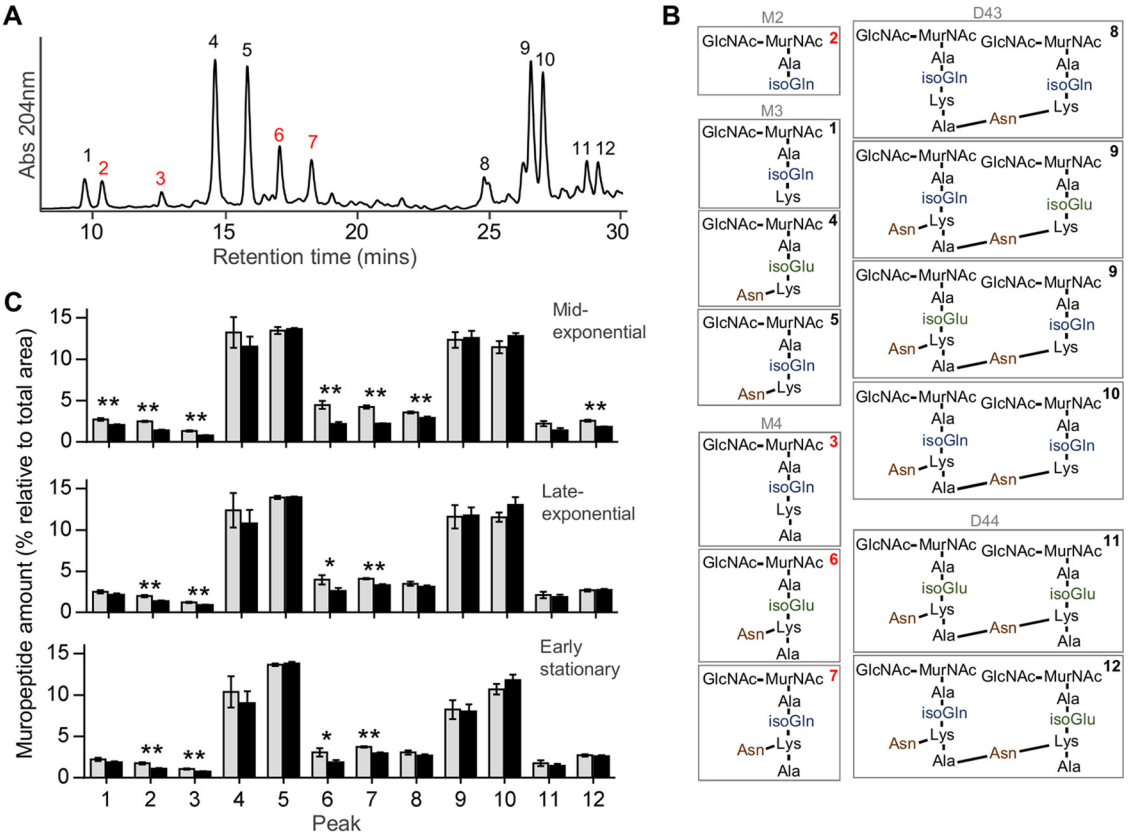

FIG 4 Peptidoglycan modifications produced by caspofungin exposure. (A and B) Representative UPLC chromatogram of *E. faecium* Aus0004 peptidoglycan of a nontreated sample (A) and structure of the muropeptides determined for each indicated peak that was confirmed by MS analysis (B) (M, monomer; D, dimer; the numbers refer to the length of the stem peptide). Peaks representing significant changes after caspofungin treatment are labeled in red. (C) Quantification of the abundance of the muropeptides determined under two sets of growth conditions: tryptic soy broth (TSB) (gray bars, untreated samples) and TSB plus caspofungin 8 mg/liter (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 Student's *t* test. Asterisks represent significant *P* values from comparisons of treated samples to untreated samples (*, $P < 0.05$; **, $P < 0.005$). Each experiment was performed in triplicate.

functions. The ratios of the transcripts from −Cas and +Cas samples determined by RNA-seq and compared to those obtained by RT-qPCR showed excellent concordance, with a Pearson correlation value of 0.994 (Fig. 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.

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 greater than 2 or less than −2, adjusted *P* value < 0.1), with 321 upregulated genes and 259 downregulated genes (Table S2B and C). All the genes presenting modified amounts of mRNA between the two conditions were classified into functional categories using the COG and KEGG classifications (35) (Fig. S2 and S3). Among these 580 genes, more than 30% coded for proteins of unknown function or not found in other species, not allowing gene ontology analysis. In the presence of caspofungin, expression of genes coding 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 (Fig. S2 and S3; see also Table S2B and C).

Impact of caspofungin on metabolism of *E. faecium* Aus0004. Of the 20 genes whose expression was most highly repressed by caspofungin, 13 were found to be

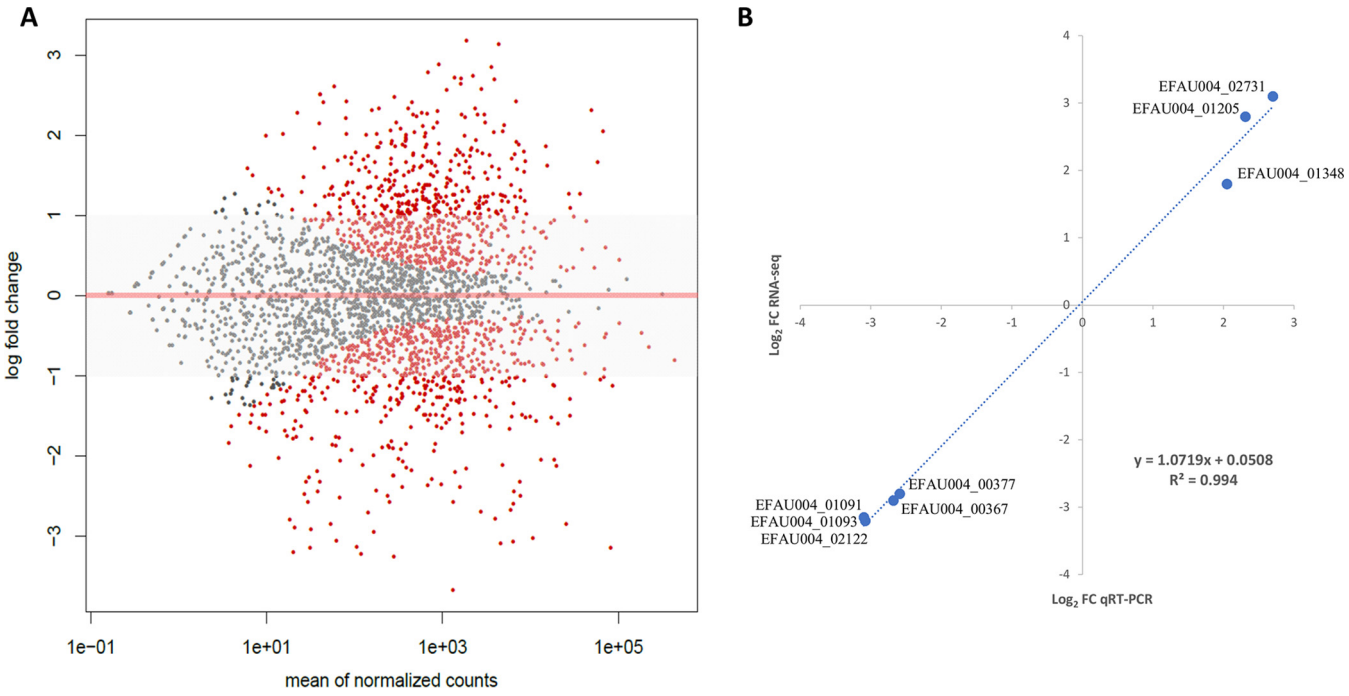

FIG 5 Transcriptional response of *E. faecium* Aus0004 growth with caspofungin. (A) Global analysis of transcript levels in *E. faecium* Aus0004 by RNA-seq represented by an MA plot (caspofungin 8 mg/liter versus control) generated by the DESeq2 R package. Log_2 fold change values representing expression of each chromosomal gene are shown on the *y* axis versus the mean of normalized counts that is shown on the *x* axis (Log_{10} scale). Points corresponding to an adjusted *P* value of less than 0.1 are indicated in red. (B) Validation of RNA-seq results by quantitative reverse transcription-PCR (qRT-PCR) analysis 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.

involved in carbohydrate transport or metabolism, in particular, genes coding for phosphotransferase systems (PTS), which are systems that mediate uptake and utilization of sugar as an energy source in bacteria (Table S2C). Since it has been demonstrated that some PTS genes act as regulatory factors promoting adaption to stressful metabolic conditions (36) and potentially enhance the possibility of bacterial survival, we hypothesized that the severe alteration of PTS transcript levels might play a role in the apparent lethality of caspofungin for *E. faecium* Aus0004. Interestingly, several genes involved in glycerol metabolism in *E. faecium* showed decreases in transcript levels. We observed downregulation of expression of genes composing the *glpKOF* operon (EFAU004_00377, EFAU004_00378, and EFAU004_00379) (fold changes, −6.0, −5.8, and −4.6, respectively) as well as genes composing the *dhaKLM* operon (EFAU004_00392, EFAU004_00393, and EFAU004_00394) (fold changes, −4.9, −4.2, and −5.1, respectively) (Table S2C). It is now widely assumed 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 reported that glycerol metabolism pathways are under the regulation of PTS

TABLE 2 Selected genes used for RNA-seq validation by qRT-PCR experiments[a]

Gene no.	Gene name	Product name	Gene start position	Gene end position	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

[a]qRT-PCR, quantitative reverse transcription-PCR.

and that the transcript levels are highly impacted (36). There was also a strong repression of genes involved in pyruvate metabolism since the so-called *pdhABCD* operon (EFAU004_01091, EFAU004_01092, EFAU004_01093, and EFAU004_01094) seemed to be impacted, with important downregulation results seen in our transcriptomic analysis (fold changes, −4.1, −8.6, −6.9, and −6.4, respectively) (Table S2C). These enzymes allow pyruvate transformation into acetyl coenzyme A (acetyl-CoA), which then proceeds by a two-step reduction process to generate ATP or directly enter into fatty acid biosynthesis (38). Since pyruvate dehydrogenation seemed to be impacted, we hypothesized that the presence of caspofungin results in a lack of ATP. This decrease of ATP formation may explain the changes in bacterial fitness that occurred in the presence of caspofungin. All these data pointed out that the presence of caspofungin induced an important form of stress that modified 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) (Table S2B). Interestingly, *sodA* was previously described as representing an important pathway with a role in tolerance of cell wall-active antibiotics in enterococci and *S. aureus* (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 echinocandins, we addressed the role of caspofungin in antimicrobial resistance as previously described (5) for subinhibitory concentrations of antibiotics. Then, we determined MICs of vancomycin, teicoplanin, daptomycin, and ciprofloxacin against *E. faecium* Aus0004 by the Etest strip method using Mueller-Hinton (MH) plates supplemented or not with 8 mg/liter caspofungin. Interestingly, we observed a 4-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 in the vancomycin MIC (and not in that of teicoplanin) was due to an upregulation of the *vanB* operon. However, RNA-seq data and RT-qPCR (FC in expression of *vanB* gene, −1.8, with an adjusted *P* value of >0.1) did not confirm this hypothesis (Table S2A). Also, we observed a 4-to-8-fold increase in the 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 a biofilm nonproducer) and *E. faecium* HM1070 Δ*asrR* (used as a biofilm producer) (42) in the presence of a subinhibitory caspofungin concentration (8 mg/liter). As previously described in fungal models (43), caspofungin significantly reduced the ability of HM1070 Δ*asrR* to form biofilms whereas no difference was observed for *E. faecium* Aus0004 (Fig. 6). These data substantiate previous findings concerning the impact of caspofungin against *S. aureus* bacterial biofilms (26). Interestingly, the authors of the latter study explained that this biofilm formation shutdown in *S. aureus* was mediated through inhibition of an *ica* operon by caspofungin and in particular, IcaA, a protein that shares homology with the β-1,3-glucan synthase, the caspofungin fungal target. Here, the protein that shares the most homology with IcaA (i.e., EFAU004_00389) seemed to be not statistically significantly impacted by the presence of caspofungin as retrieved in our transcriptomic analysis (Table S2A).

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

		MIC (mg/liter)[a]							
		Vancomycin		Teicoplanin		Daptomycin		Ciprofloxacin	
E. faecium strain	*van* operon	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; CAS8, MH plus caspofungin (8 mg/liter).

Mechanism of action of caspofungin against *E. faecium*. Since the fungal target of caspofungin in fungi is the β-1,3-glucan synthase, which 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.

Conclusion. We reported here that caspofungin seemed to have a strong bactericidal effect against *E. faecium* notwithstanding the lack of a protein similar to its fungal target, the β-1,3-glucan synthase. Interestingly, we showed that bacterial growth in the presence of a subinhibitory concentration of caspofungin altered the levels of tran-

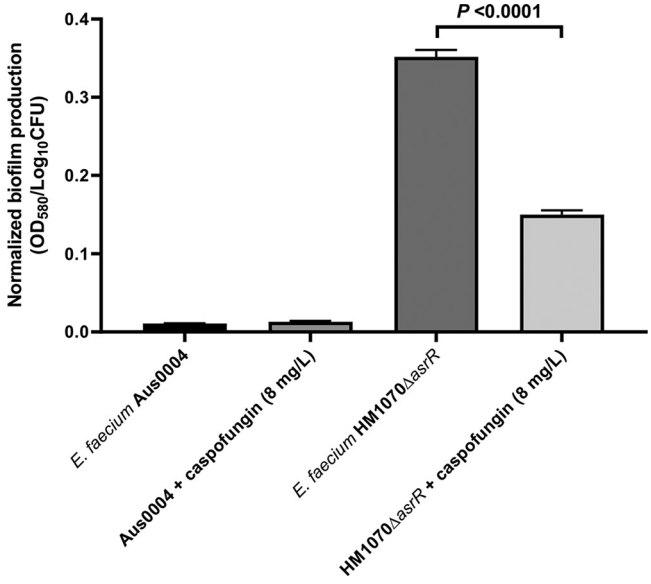

FIG 6 Normalized biofilm formation in the presence of caspofungin (8 mg/liter) in *E. faecium* Aus0004 strain (a biofilm nonproducer) and *E. faecium* HM1070 Δ*asrR* (a biofilm producer). Normalized biofilms were calculated by dividing the total biofilm value (OD_{580}) by the bacterial growth value for each strain (expressed in Log_{10} values of CFU counts). Statistical comparison was performed using the unpaired *t* test.

scripts to approximately 20% of the level seen with the *E. faecium* genome. Even though it is unlikely to be clinically relevant, greater knowledge of the observed antagonism with vancomycin would be helpful to understand the mechanism of action of caspofungin in *E. faecium*. The present article is the first report of caspofungin antibacterial activity against Gram-positive bacteria such as *E. faecium*, and further investigations of the effects of nonantibiotic xenobiotics against VRE should be conducted in the future.

MATERIALS AND METHODS

Bacterial isolates and growth conditions. The bacterial strains used in this study are listed in Table S3 in the supplemental material. The main strain used was the *vanB*-positive reference strain *E. faecium* Aus0004, the complete genome sequence of which is available (GenBank accession number CP003351.1) (21). UCN strains were obtained from the French Reference Centre for Enterococci and used for detection of *vanA/B* genes as previously described (44). For biofilm formation experiments, *E. faecium* HM1070 Δ*asrR* was used as a 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 the addition of nonantibiotic 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 the standard circulating blood level.

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

We attempted to obtain mutants with decreased caspofungin susceptibility from *E. faecium* Aus0004 *in vitro* after serial passages on MH agar supplemented with increasing concentrations of caspofungin and by the gradient method on agar medium as previously described (45).

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 (BMD) reference method 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 BMD according to Comité de l'antibiogramme de la Société Française de Microbiologie (CA-SFM)/EUCAST recommendations (www.sfm-microbiologie.org). Note that for daptomycin MIC determinations, calcium chloride (50 mg/liter) 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/liter of caspofungin in the MH medium using Etest strips following the manufacturer's instructions (bioMérieux, Marcy-l'Etoile, France).

Time-kill curve experiments. Time-kill curves were determined to determine antibacterial activity (using an antibiotic concentration equal to 8× the MIC) against *E. faecium* Aus0004, as previously described (46). Briefly, 16-h overnight cultures were inoculated 1:20 in 10 ml of fresh MH broth containing anti-Gram-positive antibiotics (vancomycin, linezolid, and daptomycin) or caspofungin and incubated at 35°C for 24 h. 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/liter of calcium chloride was added to the MH broth.

Biofilm production assay. The capacity of *E. faecium* Aus0004 and *E. faecium* HM1070 Δ*asrR* (a biofilm-positive strain) (42) to form biofilm in the presence of a subinhibitory concentration of caspofungin (8 mg/liter) was evaluated at 24 h. Briefly, bacteria that had been grown overnight were inoculated 1:100 in 10 ml of TSB with 0.25% glucose and apportioned into 96-microwell polystyrene plates (Nunc, Denmark). After 24 h of static incubation at 35°C, the plates were washed three times with phosphate-buffered saline (PBS) and stained with 1% crystal violet for 30 min. The wells were rinsed with distilled water and ethanol-acetone (80:20 [vol/vol]). After the wells had dried, optical density at 580 nm (OD_{580}) was determined using a microplate reader (Multiskan Ascent; Thermo Electron Corporation). Biofilms were formed under static conditions, and each assay was performed in at least three independent experiments. Normalized biofilm values were calculated by dividing the total biofilm value (OD_{580}) by the bacterial growth for each strain (expressed in Log_{10} values of CFU counts).

Cell wall analysis by electron microscopy. For SEM experiments, *E. faecium* Aus0004 cells were cultured up to the late-exponential phase in TSB supplemented or not with a concentration of 8 mg/liter (1/4× MIC) of caspofungin and then pelleted by centrifugation, rinsed in PBS, and fixed with 2.5% glutaraldehyde–cacodylate buffer 0.1 M (pH 7.0) at 4°C for 15 h. The cells were rinsed in cacodylate buffer and then sedimented for 15 days on Thermanox coverslips (Thermo Fisher Scientific, Villebon-sur-Yvette, France) coated with poly-L-lysine and dehydrated in progressive baths of ethanol (70% to 100%). Bacterial cells were subjected to sputter coating with platinum and observed with a scanning electron microscope (Jeol 6400F; Jeol, Tokyo, Japan). For TEM experiments, the bacterial strain was cultured under the same conditions as were used for the SEM experiments, but after the PBS rinse, the cells were fixed

with 2.5% glutaraldehyde–cacodylate buffer 0.1 M (pH 7.0) containing ruthenium red (0.4 mg/liter) for 15 h at 4°C. The cells were then rinsed and postfixed 1 h with 1% osmium tetroxide–cacodylate buffer 0.1 M (pH 7.0) in the presence of ruthenium red (0.4 mg/liter) at 4°C while protected from light. The cells were rinsed, pelleted in 1.5% agar with a low melting point (40°C), and then dehydrated in progressive ethanol baths (70% to 100%), embedded in resin (Embed 812; Electron Microscopy Sciences, Hatfield, PA, USA) and polymerized for 24 h at 60°C. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. The cells were observed with a transmission electron microscope (Jeol 1011; Jeol, Tokyo, Japan), and images were taken with an Orius 200 charge-coupled-device (CCD) camera (Gatan France, Evry, France). Cell wall analysis by electron microscopy was performed in three independent experiments.

Determination of peptidoglycan composition and muropeptide analysis. For peptidoglycan isolation, *E. faecium* Aus0004 cells were cultured in TSB with and without caspofungin (8 mg/liter) until mid-exponential phase, late-exponential phase, and early stationary phase and then pelleted, resuspended in PBS, and boiled while being stirred in 10% SDS for 1 h. Peptidoglycan isolation and digestion with muramidase were performed as previously described (47). Solubilized muropeptides were reduced by addition of 0.5 M sodium borate (pH 9.5) and sodium borohydride to reach a final concentration of 10 g/liter. 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 C_{18} column (Water, USA) (130 Å, 1.7-μm pore size, 2.1 mm by 150 mm) and detected at A_{204}. 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% [vol/vol]) in a 40-min run. The identity of the muropeptides was assigned by MS/MS, and for quantification, the area of each peak in the chromatogram was analyzed. 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 alone (−Cas media) or in TSB supplemented with a subinhibitory concentration of caspofungin (8 mg/liter) (+Cas media), corresponding to an incubation of 6 h 30 min or 7 h 30 min, respectively. Total RNA was extracted using a ZR fungal/bacterial RNA miniprep kit (Zymo Research, Irvine, CA, USA) in biological duplicate. Residual chromosomal DNA was removed by treating samples with the Turbo DNA-free kit (Life Technologies, Saint-Aubin, France). DNA-free RNA samples were quantified using a NanoDrop One spectrophotometer (Thermo Scientific, Villebon-sur-Yvette, France) and were then depleted of ribosomal RNAs (e.g., 23S, 16S, and 5S rRNAs) using a Ribo-Zero rRNA removal kit (Gram-positive bacteria) (Illumina-Epicentre, Madison, WI, USA) according to the manufacturer's instructions. Finally, the samples were washed using an RNA Clean & Concentrator-5 kit (Zymo Research, Irvine, CA, USA). The rRNA depletion efficiency was evaluated by analyzing the samples using an Agilent 2100 bioanalyzer (Agilent Technologies, Les Ulis, France). cDNA libraries were prepared with a strand-specific NEXTflex rapid directional (dUTP-based) RNA-Seq kit (v2), and sequencing was performed using an Illumina HiSeq 2500 instrument (Illumina, San Diego, CA, USA) with the Run Rapid Single Read of 50 bp multiplexing protocol (ProfileXpert-LCMT, Lyon, France).

For bioinformatic analysis, reads were mapped against the genome sequence of *E. faecium* AUS0004 (GenBank accession numbers CP003351.1, CP003352.1, CP003353.1, and CP003354.1) using CLC Genomics Workbench software v10.0.1 (CLCbio; Qiagen, San Diego, CA, USA). Determinations of FC values and numbers of mapped reads per kilobase per million (RPKM) and statistical analyses were performed using the CLC Genomics Workbench and DESeq2 R package (48). Gene expression levels were identified using the $\log_2$ absolute fold change method (values higher than or lower than 2 were considered to represent induction or repression, respectively), and statistical significance was accepted in the cases in which the *P* value was <0.1. Mean expression and $\log_2$ FC values for each gene were plotted and visualized as an MA plot figure using the DESeq R package (48).

Validation of RNA-seq FC was done by RT-qPCR with specific primers for eight differentially expressed genes (Table S4). Total RNAs were extracted as described above, and residual DNA was removed by the use of a Turbo DNA-free kit. cDNAs were synthesized from total RNA (approximately 1 μg) using a QuantiFast reverse transcription kit (Qiagen, San Diego, CA, USA) according to the manufacturer's instructions, and transcript levels were determined by the DeltaDelta threshold cycle ($\Delta\Delta C_T$) method using the *atpA* gene as a housekeeping control gene. Each experiment was performed in triplicate, including RNA-seq biological duplicate experiments.

Accession number(s). 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.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.7 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 5, XLSX file, 0.01 MB.

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