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Analysis of the tolerance of pathogenic enterococci and *Staphylococcus aureus* to cell wall active antibiotics

Rabia Ladjouzi[1,2], Alain Bizzini[1], François Lebreton[3]†, Nicolas Sauvageot[1], Alain Rincé[1], Abdellah Benachour[1] and Axel Hartke[1]*

[1]*Université de Caen Basse-Normandie, EA4655 U2RM-Stress and Virulence, F-14032 Caen, France;* [2]*Université de Béjaia, FSNV, Laboratoire de Biotechnologie Végétale et Ethnobotanique, 06000 Bejaia, Algérie;* [3]*Université de Caen Basse-Normandie, Faculté de Médecine, EA4655 U2RM-Antibioresistance, F-14032 Caen, France*

*Corresponding author. Tel: +33-2-31-56-54-04; Fax: +33-2-31-56-53-11; E-mail: axel.hartke@unicaen.fr
†Present address: Massachusetts Eye and Ear Infirmary, Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts, USA.

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Objectives: Tolerance refers to the phenomenon that bacteria do not significantly die when exposed to bactericidal antibiotics. Enterococci are known for their high tolerance to these drugs, but the molecular reasons why they resist killing are not understood. In a previous study we showed that the superoxide dismutase (SOD) is implicated in this tolerance. This conclusion was based on the results obtained with one particular strain of *Enterococcus faecalis* and therefore the objective of the present communication was to analyse whether dependence of tolerance on active SOD is a general phenomenon for enterococci and another Gram-positive pathogen, *Staphylococcus aureus*.

Methods: Mutants deficient in SOD activity were constructed in pathogenic enterococci. The wild-type *sodA* gene was cloned into an expression vector and transformed into SOD-deficient strains for complementation with varying levels of SOD activity. Previously constructed SOD-deficient strains of *S. aureus* were also included in this study. Tolerance to vancomycin and penicillin was then tested.

Results: We demonstrated that the dependence on SOD of tolerance to vancomycin and penicillin is a common trait of antibiotic-susceptible pathogenic enterococci. By varying the levels of expression we could also show that tolerance to vancomycin is directly correlated to SOD activity. Interestingly, deletion of the *sodA* gene in a non-tolerant *Enterococcus faecium* strain did not further sensitize the mutant to bactericidal antibiotics. Finally, we showed that the SOD enzymes of *S. aureus* are also implicated in tolerance to vancomycin.

Conclusion: High tolerance of enterococci to cell wall active antibiotics can be reversed by SOD deficiency.

Keywords: bactericidal antibiotics, oxidative stress, superoxide dismutases

Introduction

Resistance to antibiotics is continuously increasing. Therefore there is a constant demand for new compounds or innovative treatment strategies. However, the approval of new antibiotic compounds has decreased considerably during recent decades.[1] As a consequence, treatment strategies that try to increase the action of existing antimicrobials would be of great benefit. Of special interest in this respect are bactericidal drugs that efficiently kill pathogens. These drugs are expected to cure disease more rapidly and restrict the emergence of resistance better than bacteriostatic antibiotics. Despite the importance of bactericidal antibiotics for successful treatment, surprisingly little is known about the mechanisms of how these drugs actually kill bacteria. It is generally believed that killing is due to the primary action of antibiotics, i.e. through the disruption of cell wall synthesis by cell wall active compounds like penicillins or glycopeptides, or through the inhibition of DNA replication and induction of DNA double-strand breaks by gyrase inhibitors such as fluoroquinolones. In the case of cell wall active compounds it has been shown that peptidoglycan hydrolases contribute to killing in a cell density-dependent manner.[2,3] Recently another mechanism for antibiotic-induced cell death has been proposed.[4,5] Based on system-level analysis mainly conducted with *Escherichia coli*, this cell death pathway model proposes that bactericidal antibiotics regardless of their primary targets also induce cell death by generating reactive oxygen species (ROS).[5,6] ROS can block growth by inactivating key metabolic enzymes, but the actual lethal effects are thought to be mainly due to ROS-induced DNA damage.[4,6,7]

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Enterococci are widespread in nature and are found as intestinal commensals of humans and many other animals. Two species in particular, *Enterococcus faecalis* and *Enterococcus faecium*, have emerged over the past few decades as the second or third most common cause of nosocomial infections worldwide.[8] These organisms are causing increasing concerns in public health since they can exhibit high levels of resistance to common anti-Gram-positive antimicrobials.[8] In addition, *E. faecalis* exhibits tolerance to the bactericidal effects of cell wall active agents as well as gyrase inhibitors.[9–11] By definition, antibiotics are considered as bactericidal if viable counts are reduced by at least 99.9% after 24 h of exposure.[12] We showed recently that 80%, 7% and 7% of *E. faecalis* strain JH2-2 survived treatment for 24 h with 20-fold the MIC of vancomycin, penicillin and norfloxacin, respectively.[9] By testing single or multiple deficient mutants affected in oxidative stress defence activities or DNA repair, we demonstrated that the only mutant significantly killed by bactericidal but not bacteriostatic antibiotics was the one with an inactivated *sodA* gene.[9] Of note, *E. faecalis* harbours only one gene encoding a manganese-cofactored superoxide dismutase (SOD).[13] The most spectacular decrease in antibiotic tolerance was observed for treatments with vancomycin and penicillin with sensitization factors of the Δ*sodA* mutant of nearly 10^4-fold compared with the wild-type.[9] These results demonstrated that the hitherto unexplained tolerance of *E. faecalis* to bactericidal antibiotics seems to be linked to SOD activity and hence bactericidal antibiotics kill this species by inducing a superoxide burst. However, in contrast to our data, two studies reported that *E. coli* SodA/SodB-deficient mutants were significantly more resistant to killing by ampicillin, norfloxacin or gentamicin.[14,15] Another study reported that a single Δ*sodA* mutant of *E. coli* had no discernible phenotype when exposed to norfloxacin, but under these conditions the Δ*sodB* single mutant exhibited a decreased survival at longer treatment times compared with the wild-type.[6] Furthermore, survival of a Δ*sodA* mutant of *Listeria monocytogenes* was comparable to that of the wild-type when exposed to several bactericidal antibiotics.[14] So we could not exclude that the observed effect of SOD deficiency on tolerance was strain dependent and specific to the *E. faecalis* JH2-2 strain. We therefore decided to analyse the relationship between SOD deficiency and sensitivity to bactericidal cell wall active drugs in more detail.

In this communication we show that: (i) loss of tolerance to vancomycin and penicillin due to SOD deficiency is a general trait of susceptible strains of *E. faecalis* and *E. faecium*; (ii) this phenomenon is not observed in a non-tolerant *E. faecium* strain; (iii) tolerance is directly correlated with *sodA* expression level; and (iv) the Mn-SOD enzymes SodA and SodM of the major human pathogen *Staphylococcus aureus* also protect the cells against killing by vancomycin, albeit to a lesser extent than enterococci. Finally, since loss of tolerance has been shown hitherto only in laboratory media, we tested if killing of SOD-deficient strains also takes place under infection-mimicking conditions.

Materials and methods

Bacterial strains, plasmids, and growth conditions

Strains and plasmids used in this study are described in Table S1 (available as Supplementary data at *JAC* Online) and the oligonucleotides in Table S2 (available as Supplementary data at *JAC* Online). *E. faecalis* and *E. faecium* were grown in M17 medium[16] supplemented with 0.5% (w/v) glucose (GM17) and *S. aureus* strains were grown in tryptic soy broth (TSB) at 37°C under aerobic conditions (100 mL Erlenmeyer flasks filled with 10 mL of growth medium, stirred at 60 or 120 rpm). When necessary, the media were supplemented with 150 mg/L erythromycin or 15 mg/L chloramphenicol for *E. faecalis* and *E. faecium* strains. For *S. aureus* cultures, we used the following antibiotic concentrations when needed: 5 mg/L of erythromycin, 3 mg/L of tetracycline and 100 mg/L of kanamycin. The growth kinetics of the cultures were followed both by measurement of optical density at 600 nm (OD_{600}) with a biophotometer (Eppendorf, Hamburg, Germany) and by viable cfu counts on agar plates. *E. faecium* strains used for conjugation with *E. faecalis* were obtained from the national enterococcal strain collection of the University Hospital in Caen and are clinical isolates with natural conjugative plasmids harbouring the *vanB* operon. These strains are not listed in Table S1 (available as Supplementary data at *JAC* Online) because little information is available for them. The reference numbers of these strains are 07/005, 08/048 and 08/140. *E. coli* strains were cultivated at 37°C in Luria–Bertani (LB)[17] broth with vigorous shaking (160 rpm). When appropriate, 100 mg/L ampicillin, 150 mg/L erythromycin or 10 mg/L chloramphenicol was added to the medium. Bacterial stocks were stored at −80°C in GM17, TSB or LB broth supplemented with glycerol 15% (v/v).

Susceptibility testing and time–kill curves

The MICs were determined by the Etest® method. Time–kill curves were determined with exponentially growing cultures by adding vancomycin or penicillin to a final concentration of 20 mg/L for *E. faecalis and E. faecium* strains and 5 mg/L for *S. aureus* strains at an OD_{600} of 0.5 (corresponding to $\sim 4\times10^8$ cfu/mL). Growth was followed by measuring the OD_{600} at regular intervals and cfu counts were determined after 24 h of incubation by plating 10-fold serial dilutions of the cultures on GM17 agar plates. The number of survivors giving rise to colonies was determined after 48 h of incubation of the plates at 37°C. Antibiotic killing was assessed as the percentage of surviving colonies relative to the viable count at the time of drug addition. The sensitization factor is defined as the ratio of survival of a parent strain to that of a mutant strain.

General molecular methods

Molecular cloning and other standard techniques were performed as described.[17] Antibiotics, chemicals and enzymes were reagent-grade, commercially available products. *E. coli*, *E. faecalis* and *E. faecium* were transformed by electroporation[18] using the Gene Pulser apparatus (Bio-Rad Laboratories, Marnes la Coquette, France). Plasmids and PCR products were purified using Qiagen kits (Qiagen, Courtaboeuf, France).

Construction of the E. faecalis OG1RFΔsodA mutant

The *E. faecalis OG1RFΔsodA* mutant was constructed by allelic exchange using a method based on the conditional replication of the pLT06 vector.[19] For this purpose we used a previous *E. faecalis* JH2-2 Δ*sodA* mutant.[13] The JH2-2 Δ*sodA* mutant was used to prepare the DNA template for PCR amplification using Triple Master Polymerase Mix (Eppendorf, Hamburg, Germany) of a 2.7 kb DNA fragment harbouring a *sodA* deletion using the primers sodmadecoI and sodmad2 (Table S2, available as Supplementary data at *JAC* Online) containing *EcoRI* and *NcoI* restriction sites, respectively. The *sodA* mutation consists of the introduction of two stop codons separated by a *BamHI* site and a central deletion of 8 bp in the coding sequence of the *sodA* gene leading to a truncated protein of 112 amino acids.[13] The 2.7 kb PCR product was digested by *EcoRI* and *NcoI* and cloned into pLT06 vector already equally treated with the same two enzymes and finally transformed into electrocompetent cells of *E. coli* XL-1 blue. The resulting recombinant plasmid containing the mutated

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sodA gene was used to transform *E. faecalis OG1RF*. After electroporation the plasmid was maintained at the permissive temperature of 30°C by plating cells on GM17 agar medium containing chloramphenicol and X-Gal. The transformed colonies were verified by PCR for the presence of recombinant plasmid and then used to trigger the first crossing-over by applying the non-replicative condition of the vector (44°C/GM17 chloramphenicol). Single-crossover transformants (chloramphenicol-resistant) were used for temperature shifts (30°C/GM17 without antibiotic) in order to release the plasmid. Following this step, chloramphenicol-susceptible clones were screened for the presence of the mutated *sodA* allele by restriction analysis (*BamHI* digestion) of the PCR product, DNA sequencing and a zymogram for the absence of SOD activity.

Construction of ΔsodA mutants of E. faecium Com12 and E. faecium 1,141,733

Δ*sodA* mutants of *E. faecium* Com12 and *E. faecium* 1,141,733 were constructed by double crossing-over mutagenesis using the pLT06 vector as described below for the *E. faecalis OG1RFΔsodA* mutant. From the genomic DNA of *E. faecium* Com12 we amplified by PCR with Com1NCO1/StopRev primer pair (Table S2, available as Supplementary data at *JAC* Online) a 986 bp DNA fragment including the 228 bp of the 5′ part of the *sodA* gene and its upstream region and a second DNA fragment of 1341 bp using the Stopfor/Com4Rev primer pair (Table S2, available as Supplementary data at *JAC* Online) corresponding to the remaining 405 bp of the *sodA* gene and its downstream region (Figure S1, available as Supplementary data at *JAC* Online). The Stopfor and StopRev primers share a complemented sequence of 24 bp, where four stop codons were inserted (Figure S1, available as Supplementary data at *JAC* Online). To generate the entire DNA fragment harbouring the mutated *sodA* gene, a second PCR was performed using the primer pair Com1NCO1/Com4Rev and the mix of the two previous amplified fragments as template DNA. The resulting DNA fragment of 2.3 kb was excised from the agarose gel electrophoresis and purified. After digestion with appropriate restriction enzymes, this fragment was cloned into pLT06 plasmid to generate pLT06Δ*sodACom12*. This recombinant plasmid was used to transform both *E. faecium* Com12 and *E. faecium* 1,141,733. Comscreenfor primer (Table S2, available as Supplementary data at *JAC* Online) was designed on the basis of the modified sequence of the *sodA* gene (Figure S1, available as Supplementary data at *JAC* Online) to allow the screening by PCR of the chloramphenicol-susceptible clones obtained after the second crossing-over event. When Comscreenfor was used in combination with the Com1coRev primer (located outside of the construct; Figure S1, available as Supplementary data at *JAC* Online), only the Δ*sodA* mutant clones gave rise to a 1.34 kb DNA product. The absence of SOD enzymatic activity was also verified by zymogram.

Construction of the E. faecium HM1070ΔsodA mutant

The HM1070Δ*sodA* mutant was derived from *E. faecium* HM1070 by allelic exchange with a truncated copy of *sodA* and using conditional replication of the pG(+)Host9 vector as described.[20] Fragments upstream (680 bp) and downstream (686 bp) of *sodA* were amplified by PCR using HM1070 chromosome as template and primer pairs HMsod1NcoI/HMsod2BamH1 and HMsod3BamHI/HMsod4SalI (Table S2, available as Supplementary data at *JAC* Online). The forward primer binding to the 3′-end of *sodA* (HMsod3BamHI) and the reverse primer to the 5′-end (HMsod2BamH1) were modified to carry the same restriction site (Table S2, available as Supplementary data at *JAC* Online). Following restriction, ligation and reamplification using HMsod1NcoI/HMsod4SalI, the resulting fragment carrying the truncated *sodA* copy was cloned in the temperature-sensitive shuttle vector pG(+)Host9 to create the plasmid pG(+)host9Δ*sodA*HM1070 (Table S1, available as Supplementary data at *JAC* Online). The hybrid plasmid was introduced into HM1070 by electrotransformation and homologous recombination followed by excision of the wild-type copy, as described previously.[20] Deletion of 63% of the *sodA* gene was confirmed by PCR and DNA sequencing using the HMsodcont primer (Table S2, available as Supplementary data at *JAC* Online).

Complementation of the E. faecalis JH2-2 and OG1RF ΔsodA mutants

For the complementation assays, a DNA fragment containing the entire *sodA* gene of *E. faecalis* JH2-2 was obtained by PCR using primers SODCOMPBAM and SODCOMPPST (Table S2, available as Supplementary data at *JAC* Online) harbouring *BamHI* and *PstI* sites, respectively. The PCR product was cloned into the pMSP3535 plasmid[21] downstream of the nisin-inducible promoter (P*nis*). Recombinant plasmid was first transformed in *E. coli* Top10 cells, amplified and finally used to transform competent cells of the *E. faecalis ΔsodAJH2-2* and *ΔsodAOG1RF* mutants. After electroporation, 300 μL of cell suspension was plated onto GM17 agar containing erythromycin. The plates were incubated for 48 h at 37°C. The colonies obtained were analysed for the presence of the plasmid containing an intact *sodA* gene by DNA sequencing of the PCR product using primers p3535For1 and p3535Rev2 (Table S2, available as Supplementary data at *JAC* Online) and SOD activity was detected by zymogram.

Preparation of cell extracts, PAGE and SOD activity detection

Cultures of *E. faecalis*, *E. faecium*, *S. aureus* and their derivative *sod* mutants were harvested by centrifugation, the pellets were washed once in 50 mM Tris, 7 mM DTT, 1.06 mM EDTA (pH 7.0), and finally resuspended in 50mM Tris (*tris* hydroxymethyl aminomethane), 2 mM EDTA, 100 mM NaCl and 5 mM DTT (pH 7.0). Glass beads (0.1–0.25 mm diameter) were added and the cells were disrupted by vortexing for 5 min. The samples were chilled on ice, and the extraction procedure was repeated. After centrifugation for 10 min at 6000 **g**, cell debris and unbroken cells were removed. The supernatants were transferred to new tubes. The protein concentration was determined by the method of Bradford.[22] Non-denaturing PAGE was carried out according to the protocol of Laemmli,[23] except that SDS and mercaptoethanol were omitted. Equal amounts of proteins (30 μg) were loaded in each lane, and the gel was used to detect SOD activity according to the protocol of Beauchamp and Fridovich.[24]

Statistics

Differences among strains in killing by bactericidal antibiotics were calculated using the Student's *t*-test. *P* values <0.05 were considered to be significant.

Results

Antibiotic tolerance disappears in ΔsodA mutants of susceptible but not resistant E. faecalis strains

The exact molecular mechanisms explaining why *E. faecalis* is more tolerant to bactericidal antibiotics than other pathogenic cocci are not yet understood. In a previous study we showed that inactivation of the *sodA* gene encoding SOD led to the collapse of tolerance to the cell wall active antibiotics vancomycin, penicillin and, to a lesser extent, to the gyrase inhibitor norfloxacin in *E. faecalis* strain JH2-2.[9] Due to the well-known enzymatic activity of SOD, we proposed that bactericidal drugs induce a superoxide burst in *E. faecalis* and that superoxide anion accumulates to toxic levels in the Δ*sodA* mutant, explaining why survival dropped by several orders of magnitude in the SOD-deficient compared

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with the parental strain. The model has been experimentally supported since a catalase-peroxidase mutant accumulated H_2O_2 when treated with a bactericidal but not with a bacteriostatic antibiotic.[9]

E. faecalis is characterized by great genetic and phenotypic diversity.[25–27] Therefore we wondered if the loss of tolerance of a Δ*sodA* mutant is a strain-independent phenomenon and decided to construct Δ*sodA* deletion mutants in other *E. faecalis* strains. First we tried to delete the *sodA* gene in the vancomycin-resistant clinical isolate V583, which carries a chromosomal *vanB* operon.[28] The idea was to analyse if induction of the oxidative burst we postulated from our previous results is a consequence of disruption of cell wall synthesis provoked by vancomycin or if it is independent of its primary action. In *E. coli*, oxidative stress has been shown to be secondary to the primary modes of action of bactericidal antibiotics since ROS could be detected upon treatment of susceptible but not resistant bacteria.[6,29] However, all attempts to construct a Δ*sodA* mutant in strain V583 were unsuccessful. We concluded that for unknown reasons inactivation of the *sodA* gene in this vancomycin-resistant clinical isolate does not seem possible.

To answer the question whether primary action of the bactericidal antibiotics is a prerequisite for induction of the oxidative burst or if it is independent of it, we then transferred by conjugation VanB resistance into the Δ*sodA* mutant of *E. faecalis* strain JH2-2 using *E. faecium* strains as donors harbouring the resistance operons on plasmids. The transconjugants obtained not only survived exposure to 20 mg/L (corresponding to 20-fold MIC of susceptible strains) of vancomycin, but were able to grow in the presence of this concentration of the antibiotic (data not shown). This demonstrates that, like in *E. coli*, the oxidative stress is triggered by the primary mode of action of vancomycin.

Using the same tools as above for strain V583, we then tried to construct a Δ*sodA* mutant in another vancomycin- and penicillin-sensitive *E. faecalis* strain. We used the laboratory strain OG1RF and obtained the mutant without any difficulty. Of note, the MIC values of the mutant were unchanged in comparison to the parent strain. The sensitivity of the mutant to exposure to 20 mg/L of penicillin and vancomycin was then tested. Against both antibiotics the Δ*sodA* mutant was significantly more susceptible than the parent strain, with sensitization factors of $>10^3$ and $>10^4$, respectively (Figure 1), confirming the result obtained with strain JH2-2. Furthermore, complementation *in trans* restored tolerance to vancomycin and penicillin (Figure 1). These data indicate that efficient killing of SOD-deficient mutants by cell wall active bactericidal antibiotics is a strain-independent phenomenon in *E. faecalis*.

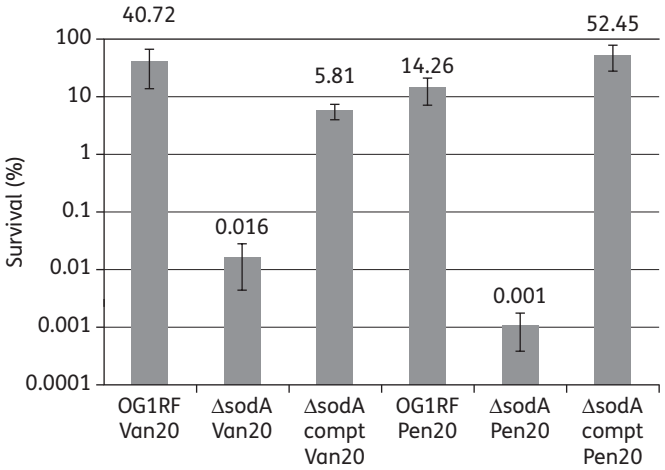

Figure 1. Relative survival of *E. faecalis* strain OG1RF, its isogenic Δ*sodA* mutant, and the complemented strain (Δ*sodA* compt) after 24 h of exposure to 20 mg/L of vancomycin (Van20) or penicillin (Pen20) in GM17 medium. The complemented strain harbours plasmid pMSP3535::*sodA* in which the wild-type *sodA* gene of strain *E. faecalis* JH2-2 is under control of the nisin-inducible promoter P*nis*. Expression of the *sodA* gene was induced by 0.5 mg/L of nisin. The mean values of at least three different experiments are represented, with error bars indicating standard deviations.

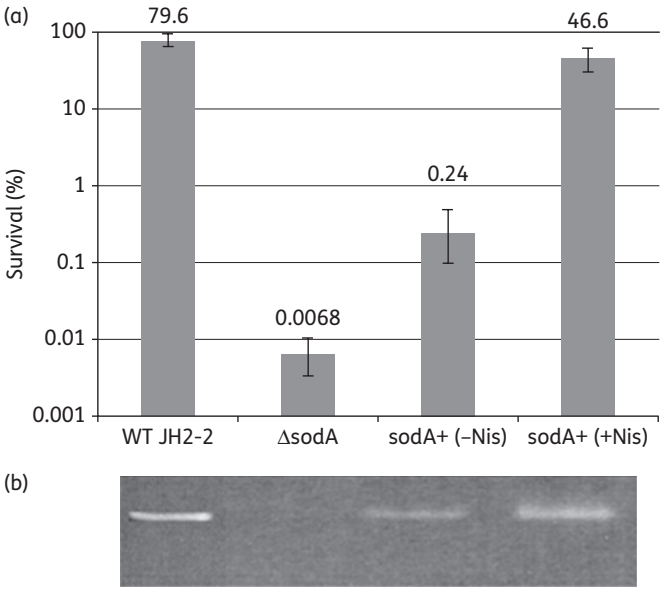

Figure 2. Correlation of SOD activity with antimicrobial tolerance. (a) Relative survival of *E. faecalis* strain JH2-2 wild-type (WT JH2-2), the JH2-2Δ*sodA*-deficient strain (Δ*sodA*), and the complemented JH2-2Δ*sodA* strain (*sodA*+) harbouring the plasmid pMSP3535::*sodA* after 24 h exposure to 20 mg/L vancomycin in GM17 medium in the presence or absence of 0.5 mg/L of nisin (+Nis or −Nis). No difference in survival was seen in the presence or absence of 0.5 mg/L of nisin for the wild-type and the Δ*sodA* strain harbouring the empty pMSP3535 vector. The mean values of at least three different experiments are represented, with error bars indicating standard deviations. (b) Zymogram reporting SOD activity for the different strains mentioned above. Lanes correspond to the strains indicated on the abscissa in (a).

Degree of tolerance is correlated with SOD activity in E. faecalis

The above-mentioned *in trans* complementation has been achieved by cloning the wild-type *sodA* allele of *E. faecalis* strain JH2-2 into plasmid pMSP3535[21] under the control of the nisin-inducible promoter of this vector. This construction opened the possibility of varying the level of expression of Mn-SOD by using different nisin concentrations for induction. As shown in Figure 2, SOD activity could be detected even without nisin induction, demonstrating that the P*nisA* promoter on this vector is somewhat leaky. However, the addition of nisin to the culture induced *sodA* expression since a stronger activity band was present on the zymogram under these conditions (Figure 2). We then tested

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survival against 20 mg/L of vancomycin of the Δ*sodA* mutant of strain JH2-2 harbouring either the empty pMSP3535 vector or the recombinant plasmid pMSP3535::*sodA* in the presence or absence of nisin. The Δ*sodA* mutant with the empty vector was highly susceptible to killing by vancomycin independent of the presence or absence of nisin (Figure 2). The SOD-deficient strain harbouring the recombinant plasmid in the absence of nisin significantly restored tolerance to the glycopeptide. However, tolerance of this strain was virtually fully restored to the wild-type level by inducing *sodA* expression by the addition of nisin. This demonstrates that the degree of tolerance to vancomycin is directly correlated with SOD activity.

SodA deficiency sensitizes vancomycin-tolerant but not non-tolerant E. faecium strains

E. faecium is the second enterococcal species of clinical importance.[8] Currently ∼40% of enterococcal nosocomial infections in Europe and US hospitals are caused by this bacterium.[30,31] So we wondered whether deletion of the *sodA* gene would have an effect on tolerance to bactericidal antibiotics in this species as well.

Before starting the construction of Δ*sodA* mutants, we tested the tolerance of different *E. faecium* strains to vancomycin (Figure 3). We chose five *E. faecium* strains from the Broad Institute and strain HM1070.[32] Of note, all these strains have MICs of ∼1 mg/L as determined by Etest. With the exception of the last strain, all isolates were highly tolerant against killing by 20 mg/L of vancomycin with survival rates of about 50% after 24 h of exposure (Figure 3). In contrast, >99.9% of cells of *E. faecium* strain HM1070 were killed by this treatment. Based on these results, we decided to construct Δ*sodA*-deficient mutants in two vancomycin-tolerant *E. faecium* strains (strains Com12 and 1,141,733) and in the non-tolerant strain HM1070. As for *E. faecalis*, the vancomycin MIC values of the mutant strains were unchanged compared with the respective wild-type strains. The Δ*sodA* mutants of both tolerant strains were significantly killed by exposure to 20 mg/L of vancomycin (Figure 4), although SodA deficiency seems to have a slightly greater sensitization effect in strain Com12 than in strain 1,141,733. The former strain was also tested for survival against 20 mg/L of penicillin. The wild-type strain proved tolerant to this treatment, with a survival rate of 42% of the population after 24 h of incubation (Figure 4). In contrast, the isogenic SOD-deficient strain lost its tolerance to this β-lactam antibiotic since >99.99% of the population was killed by this treatment.

The results of the *E. faecium* strain HM1070 and its isogenic Δ*sodA* mutant are different from those of the two other strains. As mentioned above, the HM1070 wild-type strain is, in contrast to the other *E. faecium* isolates tested, not tolerant to treatment with 20 mg/L of vancomycin. Interestingly, its survival rate is comparable to that of the Δ*sodA* mutant of strain 1,141,733 (Figure 4). Introduction of a mutation in the *sodA* gene in strain HM1070 showed no additional effect on survival against 10 mg/L of vancomycin in comparison to the control strain (Figure 4). A similar result has been obtained using 5 mg/L or 20 mg/L of this glycopeptide (data not shown). Furthermore, both strains were also exposed to 20 mg/L of penicillin, corresponding to 20-fold the MIC of this strain. Survival was extremely low under these conditions, but no difference between the mutant and the wild-type strain was observed (data not shown). From these results we speculated that strain HM1070 might have a natural deficiency of SOD activity.

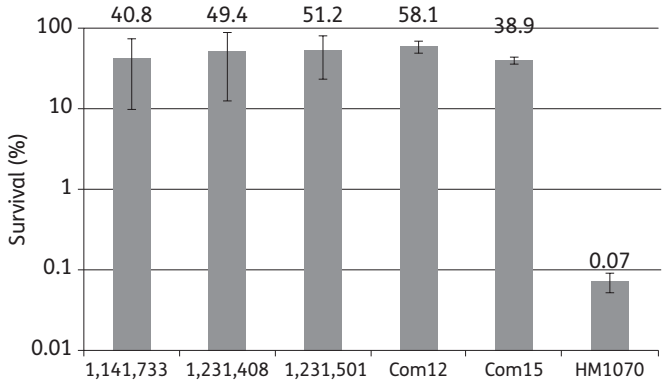

Figure 3. Relative survival of different *E. faecium* strains after 24 h treatment with 20 mg/L of vancomycin in GM17 medium. The mean values of at least three different experiments are represented with error bars indicating standard deviations.

However, this was not the case, since the SOD activity was revealed in this strain but not in the Δ*sodA* mutant by zymogram assay (data not shown). Furthermore, an increase of SOD activity by introduction of plasmid pMSP3535::*sodA* into the HM1070 strain did not significantly increase tolerance, demonstrating that the SodA level is also not the limiting factor (data not shown). However, we noticed an important decrease of the OD_{600} from 0.5 to 0.03 for the cultures of strain HM1070 after 24 h of treatment, whereas a decrease in OD_{600} is not observed for the tolerant *E. faecium* strains under these conditions. This decrease in OD_{600} for strain HM1070 was not observed in cultures incubated for 24 h without vancomycin. This suggests that in the case of the non-tolerant *E. faecium* strain HM1070, cell death is due to cell lysis triggered by vancomycin.

Tolerance to vancomycin is correlated with SOD activity in S. aureus

In contrast to *E. faecalis*, which has only one *sodA* gene, *S. aureus* harbours two *sod* genes, both of them encoding manganese-type SOD enzymes (SodA and SodM).[33] Therefore this was an interesting context to test whether one or both Mn-SODs are also implicated in tolerance to bactericidal antibiotics in this important human pathogen. We used vancomycin as a model for these experiments and tested three *S. aureus* strains: the wild-type strain SH1000 and isogenic Δ*sarA* and Δ*sarA*/Δ*sodA*/Δ*sodM* mutants.

SarA has been shown to be a pleiotropic transcriptional regulator of virulence factors[34] and, most important for the context of the present study, also regulates expression of *sodA* and *sodM* genes.[35] This last study showed that both genes, but in particular *sodM*, are overexpressed in a *sarA* mutant, leading to an increase in SOD activity in this strain. So we were interested to test if this is correlated with an increase in tolerance to vancomycin and if inactivation of the two *sod* genes also affects tolerance in *S. aureus*.

We first tested resistance of strain SH1000 and isogenic mutants to vancomycin by Etest. This showed that the MICs of all strains were ∼2 mg/L. Based on these results, we tested tolerance by exposing the wild-type strain for 24 h to 20-fold its MIC for vancomycin. However, survival rates were extremely low under these conditions, indicating that *S. aureus* seems to be less tolerant

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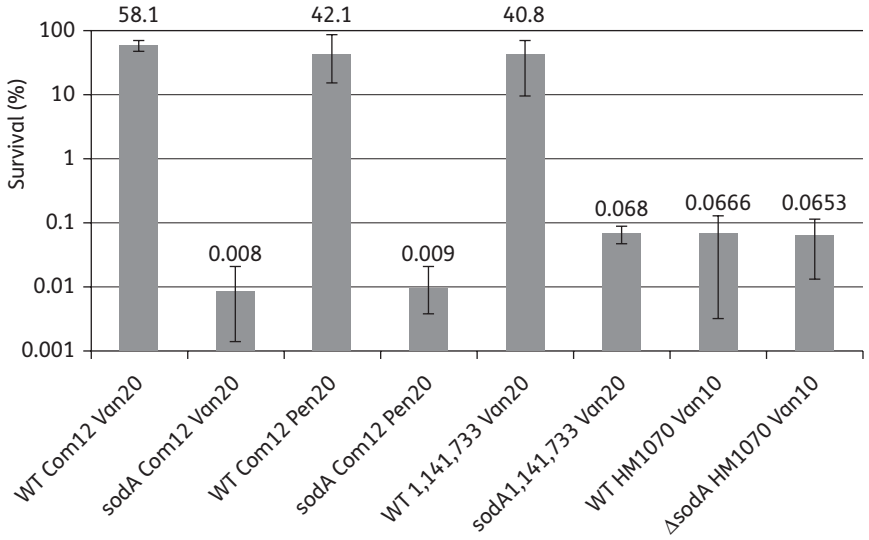

Figure 4. Relative survival of *E. faecium* wild-type strains and corresponding isogenic Δ*sodA* mutants after 24 h exposure to 20 mg/L (Van20) or 10 mg/L (Van10) of vancomycin in GM17 medium. The mean values of at least three different experiments are represented, with error bars indicating standard deviations.

than enterococci towards this glycopeptide. By testing different concentrations and exposure times, we finally conducted the following experiments with 5 mg/L vancomycin, which is only two to three times higher than the MIC of the different strains. Using these conditions, survival of the wild-type strain was ∼30% after 6 h of treatment (Figure 5). The survival of mutants affected in either one of the two *sod* genes was comparable to that of the parent strain (data not shown), whereas the *sarA* single mutant was more tolerant than the wild-type, which may be explained by the higher SOD activity in this mutant. However, a *P* value of 0.17 indicates that the difference between the two strains seems not statistically significant (Figure 5). Interestingly, the Δ*sarA*/Δ*sodA*/Δ*sodM* mutant was significantly more susceptible in comparison to the wild-type ($P<0.001$) and the *sarA* mutant strain ($P<0.05$) (Figure 5), with sensitization factors of 20-fold and nearly 10^2-fold, respectively. We concluded that SOD activity contributes to protect cells against killing by the bactericidal antibiotic of this pathogen as well, although to a much lesser extent than enterococci.

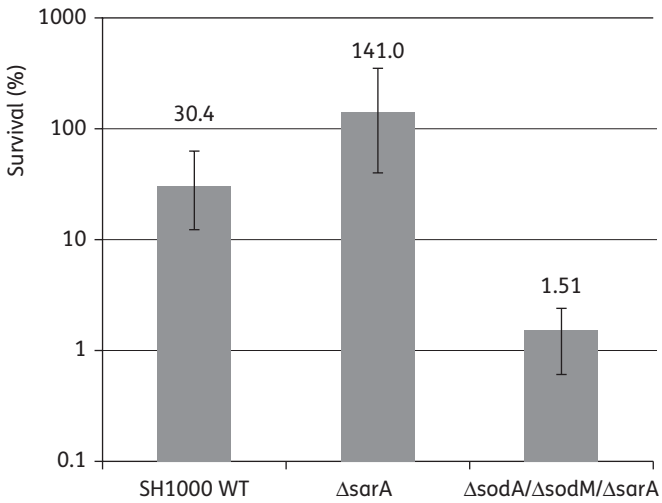

Figure 5. Relative survival of *S. aureus* strain SH1000 wild-type (WT), Δ*sarA* mutant and the triple mutant Δ*sodA*/Δ*sodM*/Δ*sarA* after 6 h exposure to 5 mg/L of vancomycin in TSB medium. The mean values of at least three different experiments are represented, with error bars indicating standard deviations.

Loss of tolerance is also observed under infection-mimicking conditions in enterococci

The experiments presented so far were all conducted in the laboratory medium GM17. Of note, in Brain Heart Infusion medium the Δ*sodA* mutant of strain JH2-2 was also more susceptible to vancomycin than the wild-type (data not shown). We concluded that loss of tolerance in SOD-deficient mutants is independent of the growth medium. Finally, we examined whether loss of tolerance of SOD-deficient enterococci could also be observed under infection-mimicking conditions. We used horse serum for these experiments and exposed wild-type bacteria and isogenic Δ*sodA* mutants to 20 mg/L of vancomycin. The Δ*sodA* mutants of *E. faecalis* (strains JH2-2 and OG1RF) and the tolerant *E. faecium* strains were significantly more susceptible to vancomycin than their isogenic wild-type control (Figure 6).

Discussion

The combined results of this study corroborate that tolerance to cell wall bactericidal antibiotics is intimately linked to SOD activity in enterococci and to a lesser extent in *S. aureus*. However, enterococci seem to be more suitable than *S. aureus* for tolerance studies since these bacteria are more tolerant than *S. aureus* to cell wall active drugs. It seems that the more a bacterium is tolerant, the greater is the negative effect of SOD deficiency on survival. This hypothesis would also provide an explanation for the absence of an effect of SOD deficiency on survival with norfloxacin, gentamicin

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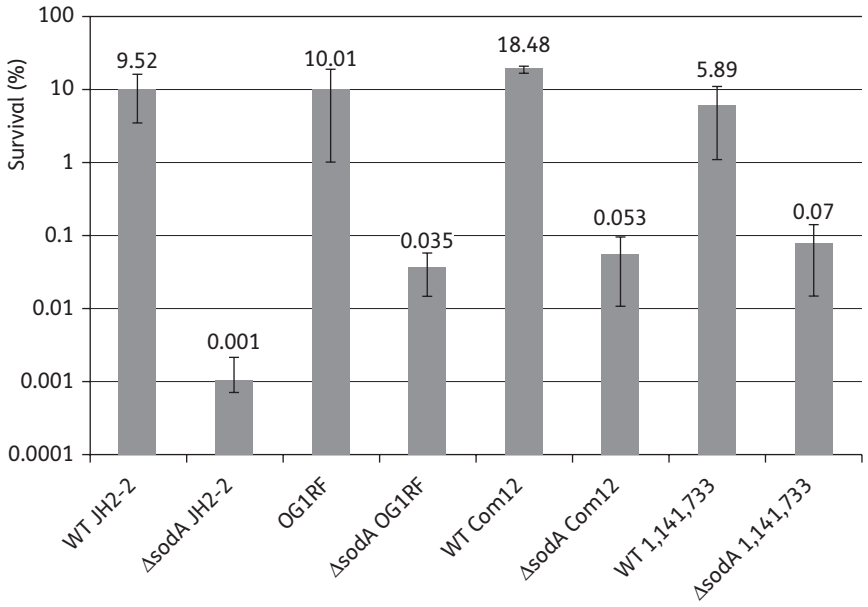

Figure 6. Relative survival of *E. faecalis* wild-type strains JH2-2 and OG1RF and wild-type *E. faecium* strains Com12 and 1,141,733 as well as their corresponding Δ*sodA* mutants after 24 h exposure to 20 mg/L of vancomycin in horse serum. The mean values of at least three different experiments are represented, with error bars indicating standard deviations.

or kanamycin in *L. monocytogenes*.[14] This pathogen is not tolerant to these antibiotics since it is rapidly killed by these drugs.[14] Thus, according to the hypothesis above, SOD activity should not have an additional role in survival against these agents.

Very contrasting results have been published for *E. coli*. One study found a slightly higher sensitivity to norfloxacin of a Δ*sodB* mutant in comparison to the wild-type strain,[6] but two other studies reported that individual SOD-deficient strains demonstrated no difference, whereas the corresponding double Δ*sodA*/Δ*sodB* mutant was significantly (10- to 100-fold) more tolerant to norfloxacin, ampicillin and kanamycin than the parental strain.[14,15] The Gram-negative enterobacteria are very different from enterococci. *E. coli* is not tolerant to bactericidal antibiotics and has a functional tricarboxylic acid (TCA) cycle and a respiration chain. TCA and the respiration chain are key components for the cell death pathway model that proposes a cascade of radical reactions triggered by the stimulation of central metabolism and respiration, leading to the killing of cells by bactericidal antibiotics.[5,36] However, the *E. coli* cell death pathway model, which has been called into question in two very recent publications,[37,38] cannot be extrapolated to enterococci since key elements like the TCA cycle and, in the case of *E. faecium*, the respiration chain are absent.[39] Overall, this excludes that perturbation of the respiratory chain is responsible for antibiotic-mediated generation of $O_2^{\cdot-}$ in enterococci. Because the mechanisms of induction of the superoxide burst are different in *E. coli* and enterococci, we suspect that these fundamental differences may account for the different behaviours of SOD-deficient mutants when exposed to bactericidal antibiotics in these bacteria. It would now be interesting to elucidate how these drugs actually provoke the generation of superoxide in enterococci and to define the cascade of events leading finally to cell death in SOD-deficient mutants.

An interesting result of this study was the identification of an *E. faecium* strain that was not tolerant to vancomycin. Loss of tolerance of the HM1070 isolate was independent of SOD activity since an isogenic Δ*sodA* mutant was not more susceptible than the wild-type and overexpression of SodA in the wild-type strain did not significantly increase tolerance. Recently it has been proposed that killing by the β-lactam antibiotic amoxicillin is due to two mechanisms in *E. faecalis* strain JH2-2. One seems to be based on the induction of an internal oxidative stress and the other is due to the action of autolysins.[2] We noticed for the non-tolerant *E. faecium* strain an important decrease in the OD_{600} after 24 h of treatment with vancomycin. Such a phenomenon was not observed with the tolerant *E. faecium* as well as for the *E. faecalis* strains used in this study. This suggests that strain HM1070, which seems to be atypical among enterococci, is predominantly killed by vancomycin via a lysis-dependent but SOD-independent cell death mechanism. However, cell lysis is not observed in cultures grown for 24 h in the absence of antibiotic, suggesting that lysis is due to the combined action of vancomycin and autolysins. A similar result has been described for the lysis of *Streptococcus pneumoniae* when exposed to penicillin.[3] It will now be important to deduce the underlying molecular mechanisms for the absence of lysis of tolerant *E. faecium* strains as well as the increased lysis of non-tolerant *E. faecium* strains in more detail. One hypothesis could be a differential expression of peptidoglycan hydrolases, as proposed for pneumococci,[3] in tolerant and non-tolerant enterococci. Another possibility is induction of a prophage by the antibiotic treatment. Such a possibility cannot be excluded at the moment, although our preliminary results do not support this concept.

In conclusion, the results presented in this study indicate that it is possible to abolish the tolerance of enterococci and *S. aureus* by deficiency in SOD activity. Therefore a drug targeting the Mn-SODs of these organisms should improve the chemotherapeutic efficacy of treatment of enterococcal infections with cell wall active antibiotics.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 and Tables S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Bumann D. Pathogen proteomes during infection: a basis for infection research and novel control strategies. *J Proteomics* 2010; **73**: 2267–76.

2 Dubée V, Chau F, Arthur M *et al*. The in vitro contribution of autolysins to bacterial killing elicited by amoxicillin increases with inoculum size in *Enterococcus faecalis*. *Antimicrob Agents Chemother* 2011; **55**: 910–2.

3 Tomasz A, Waks S. Mechanism of action of penicillin: triggering of the pneumococcal autolytic enzyme by inhibitors of cell wall synthesis. *Proc Natl Acad Sci USA* 1975; **72**: 4162–6.

4 Dwyer DJ, Kohanski MA, Collins JJ. Role of reactive oxygen species in antibiotic action and resistance. *Curr Opin Microbiol* 2009; **12**: 482–9.

5 Kohanski MA, Dwyer DJ, Hayete B *et al*. A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 2007; **130**: 797–810.

6 Dwyer DJ, Kohanski MA, Hayete B *et al*. Gyrase inhibitors induce an oxidative damage cellular death pathway in *Escherichia coli*. *Mol Syst Biol* 2007; **3**: 91.

7 Foti JJ, Devadoss B, Winkler JA *et al*. Oxidation of the guanine nucleotide pool underlies cell death by bactericidal antibiotics. *Science* 2012; **336**: 315–9.

8 Simonsen GS, Småbrekke L, Monnet DL *et al*. Prevalence of resistance to ampicillin, gentamicin and vancomycin in *Enterococcus faecalis* and *Enterococcus faecium* isolates from clinical specimens and use of antimicrobials in five Nordic hospitals. *J Antimicrob Chemother* 2003; **51**: 323–31.

9 Bizzini A, Zhao C, Auffray Y *et al*. The *Enterococcus faecalis* superoxide dismutase is essential for its tolerance to vancomycin and penicillin. *J Antimicrob Chemother* 2009; **64**: 1196–202.

10 Krogstad DJ, Pargwette AR. Defective killing of enterococci: a common property of antimicrobial agents acting on the cell wall. *Antimicrob Agents Chemother* 1980; **17**: 965–8.

11 Moellering RC Jr, Korzeniowski OM, Sande MA *et al*. Species-specific resistance to antimicrobial synergism in *Streptococcus faecium* and *Streptococcus faecalis*. *J Infect Dis* 1979; **140**: 203–8.

12 Finberg RW, Moellering RC, Tally FP *et al*. The importance of bactericidal drugs: future directions in infectious disease. *Clin Infect Dis* 2004; **39**: 1314–20.

13 Verneuil N, Mazé A, Sanguinetti M *et al*. Implication of (Mn)superoxide dismutase of *Enterococcus faecalis* in oxidative stress responses and survival inside macrophages. *Microbiology* 2006; **152**: 2579–89.

14 Feld L, Knudsen GM, Gram L. Bactericidal antibiotics do not appear to cause oxidative stress in *Listeria monocytogenes*. *Appl Environ Microbiol* 2012; **78**: 4353–7.

15 Wang X, Zhao X. Contribution of oxidative damage to antimicrobial lethality. *Antimicrob Agents Chemother* 2009; **53**: 1395–402.

16 Terzaghi BE, Sandine WE. Improved medium for lactic streptococci and their bacteriophages. *Appl Microbiol* 1975; **29**: 807–13.

17 Sambrook J, Russell DW. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 2001.

18 Cruz-Rodz AL, Gilmore MS. High efficiency introduction of plasmid DNA into glycine treated *Enterococcus faecalis* by electroporation. *Mol Gen Genet* 1990; **224**: 152–4.

19 Thurlow LR, Thomas VC, Hancock LE. Capsular polysaccharide production in *Enterococcus faecalis* and contribution of CpsF to capsule serospecificity. *J Bacteriol* 2009; **191**: 6203–10.

20 Maguin E, Duwat P, Hege T *et al*. New thermosensitive plasmid for gram-positive bacteria. *J Bacteriol* 1992; **174**: 5633–8.

21 Bryan EM, Bae T, Kleerebezem M *et al*. Improved vectors for nisin-controlled expression in gram-positive bacteria. *Plasmid* 2000; **44**: 183–90.

22 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248–54.

23 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–5.

24 Beauchamp C, Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* 1971; **44**: 276–87.

25 Bizzini A, Zhao C, Budin-Verneuil A *et al*. Glycerol is metabolized in a complex and strain-dependent manner in *Enterococcus faecalis*. *J Bacteriol* 2010; **192**: 779–85.

26 Bourgogne A, Garsin DA, Qin X *et al*. Large scale variation in *Enterococcus faecalis* illustrated by the genome analysis of strain OG1RF. *Genome Biol* 2008; **9**: R110.

27 McBride SM, Fischetti VA, Leblanc DJ *et al*. Genetic diversity among *Enterococcus faecalis*. *PLoS One* 2007; **2**: e582.

28 Sahm DF, Kissinger J, Gilmore MS *et al*. In vitro susceptibility studies of vancomycin-resistant *Enterococcus faecalis*. *Antimicrob Agents Chemother* 1989; **33**: 1588–91.

29 Albesa I, Becerra MC, Battán PC *et al*. Oxidative stress involved in the antibacterial action of different antibiotics. *Biochem Biophys Res Commun* 2004; **317**: 605–9.

30 Iwen PC, Kelly DM, Linder J *et al*. Change in prevalence and antibiotic resistance of *Enterococcus* species isolated from blood cultures over an 8-year period. *Antimicrob Agents Chemother* 1997; **41**: 494–5.

31 Treitman AN, Yarnold PR, Warren J *et al*. Emerging incidence of *Enterococcus faecium* among hospital isolates (1993 to 2002). *J Clin Microbiol* 2005; **43**: 462–3.

32 Bozdogan B, Leclercq R. Effects of genes encoding resistance to streptogramins A and B on the activity of quinupristin-dalfopristin

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against *Enterococcus faecium*. *Antimicrob Agents Chemother* 1999; **43**: 2720–5.

33 Valderas MW, Hart ME. Identification and characterization of a second superoxide dismutase gene (*sodM*) from *Staphylococcus aureus*. *J Bacteriol* 2001; **183**: 3399–407.

34 Chien Y, Manna AC, Projan SJ *et al*. SarA, a global regulator of virulence determinants in *Staphylococcus aureus*, binds to a conserved motif essential for *sar*-dependent gene regulation. *J Biol Chem* 1999; **274**: 37169–76.

35 Ballal A, Manna AC. Regulation of superoxide dismutase (*sod*) genes by SarA in *Staphylococcus aureus*. *J Bacteriol* 2009; **191**: 3301–10.

36 Kohanski MA, Dwyer DJ, Wierzbowski J *et al*. Mistranslation of membrane proteins and two-component system activation trigger antibiotic-mediated cell death. *Cell* 2008; **135**: 679–90.

37 Liu Y, Imlay JA. Cell death from antibiotics without the involvement of reactive oxygen species. *Science* 2013; **339**: 1210–3.

38 Keren I, Wu Y, Inocencio J *et al*. Killing by bactericidal antibiotics does not depend on reactive oxygen species. *Science* 2013; **339**: 1213–6.

39 Huycke MM. Physiology of enterococci. In: Gilmore MS, ed. *The Enterococci: Pathogenesis, Molecular Biology, Antibiotic Resistance and Infection Control*. Washington, DC: ASM Press, 2002: 301–54.

40 Yagi Y, Clewell DB. Recombination-deficient mutant of *Streptococcus faecalis*. *J Bacteriol* 1980; **143**: 966–70.

41 Murray BE, Singh KV, Ross RP *et al*. Generation of restriction map of *Enterococcus faecalis* OG1 and investigation of growth requirements and regions encoding biosynthetic function. *J Bacteriol* 1993; **175**: 5216–23.

42 Palmer KL, Carniol K, Manson JM *et al*. High-quality draft genome sequences of 28 *Enterococcus sp*. isolates. *J Bacteriol* 2010; **192**: 2469–70.

43 Horsburgh MJ, Aish JL, White IJ *et al*. sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325-4. *J Bacteriol* 2002; **184**: 5457–67.

44 Arnaud M, Chastanet A, Débarbouillé M. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. *Appl Environ Microbiol* 2004; **70**: 6887–91.

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