Development of in Vitro Resistance to Daptomycin in Enterococcus Faecium and Estimation of Its Fitness Cost

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Research

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Abstract

Background

Daptomycin has broad-spectrum antibacterial activity against Gram-positive pathogens, but recent studies have revealed cases where daptomycin has failed to treat multidrug-resistant bacteria, such as vancomycin-resistant *Enterococcus faecium*. However, the resistance evolution of *E. faecium* to daptomycin *in vitro* and fitness cost remain unclear. In this study, we sought to analyze the resistance development and mechanism of *E. faecium* to daptomycin, and further to investigate the relationship between daptomycin resistance and fitness cost.

Methods

To investigate the development of daptomycin resistance in *E. faecium*, 6 daptomycin-susceptible (DAP-S) clinical isolates, including 3 vancomycin-resistant *E. faecium* (VRE) and 3 vancomycin-susceptible *E. faecium* (VSE), were exposed to daptomycin *in vitro* by serial passage experiment. Then the different resistance mechanisms of daptomycin-resistant (DAP-R) mutants were analyzed by polymerase chain reaction (PCR), cytochrome C binding assay and transmission electron microscopy. Furthermore, we also estimated the changes of fitness cost among each highly DAP-R mutants by bacterial growth curve measurement, *in vitro* competition experiments, infection model of *Galleria mellonella* larvae and biofilm formation assays.

Results

In vitro, a total of 21 DAP-R mutants with minimal inhibitory concentration (MIC) of 4 to 512 µg/mL were obtained, and these mutants carried more than one mutation of LiaFSR and YycFG system encoding genes. More positive charges were detected among highly DAP-R mutants than parent isolates, and the cell walls of SC1174-D and SC1762-D mutants were remarkably thicker than those of the parent isolates. In comparison with parent isolates, besides, the growth, competition ability and virulence were significantly reduced, while the biofilm formation capacity was markedly elevated among each highly DAP-R mutants.

Conclusions

Our findings suggest that *E. faecium* isolates are able to rapidly acquire DAP resistance *in vitro* through different dynamic resistance mechanisms, which often accompany by significant fitness cost. Intriguingly, DAP and glycopeptide antibiotics may present collateral-sensitivity during *E. faecium* acquired DAP resistance *in vitro*.

Background

Enterococcus faecium is an ubiquitous bacteria in nature. It is also an opportunistic pathogen that can cause severe hospital infections, like bacteremia, as well as less severe infections, like urinary tract infections, in the hospital and community settings [1]. It is reported that infections caused by *E. faecium*

is ~20% and is increasing gradually [2]. Moreover, *E. faecium*, as a member of the 'ESKAPE' pathogens which have an ability to escape the biocidal action of antimicrobial agents, can develop antibiotic resistance and cause deadly clinical outbreaks, making significant challenges to the health care system [3]. In the past, vancomycin and teicoplanin were considered to be the most effective glycopeptides for the treatment of enterococcal infections. However, widespread use and unreasonable use of antibiotics have increased the emergence and prevalence of vancomycin-resistant *E. faecium* (VRE), which poses a great threat to human public health because of high mortality rate [3, 4].

Daptomycin (DAP) is a cyclic lipopeptide which targets bacterial cell membranes [5]. In 2003, the US Food and Drug Administration approved its use for treatment of complex skin and soft tissue infections, infective endocarditis and osteomyelitis [6], owing to its wide range of antibacterial activities to most Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *enterococci* [7]. Hence, DAP has been recognized as one of the last line antibiotics against multidrug-resistant Gram-positive pathogens, like VRE [8]. In recent years, an increase number of treatment failure cases with DAP has been reported abroad [9, 10].

To date, most studies in the field of DAP resistance focused on *Staphylococcus aureus* and *Enterococcus faecalis* [11, 12], only a few focus on *E. faecium*. Several resistance mechanisms to DAP in Gram-positive pathogens, like *Staphylococcus aureus* and *Enterococcus faecalis*, have been identified, including the alteration of the cell surface charge and cell membrane components [5, 13]. Mutations in encoding genes of three-component regulatory system LiaFSR, two-component regulatory system YycFG, cardiolipin synthase (CIs) and glycerophosphoryl diester phosphodiesterase (GdpD) have been shown responsible for the DAP resistance [14]. Additionally, increased thickness of the cell-wall and abnormal septations also contribute to DAP resistance in Gram-positive pathogens [15].

At present, it is not clear whether *E. faecium* can acquire DAP resistance *in vitro*, if so, by what pathways or mechanism. And what changes in biological characteristics of *E. faecium* will occur after obtaining daptomycin resistance. In this study, hencein, we investigated the development of DAP resistance by serial passage experiment *in vitro*, and further analyzed the resistance mechanism and fitness cost. We hope to deepen the understanding of the process and pathways of resistance to DAP in *E. faecium*, and to provide a new insight to mitigate or prevent DAP-R *E. faecium* that may appear in China in the future.

Methods

Bacterial strains and growth conditions

We collected and identified 3 VRE strains (SC1174, SC1379, SC1762) and 3 VSE strains (SC1543, SC1706, SC1726), which were daptomycin-susceptible (DAP-S) (DAP MIC=2.0 µg/mL) strains, isolated between 2017 and 2018 from the First Affiliated Hospital of Wenzhou Medical University, a comprehensive teaching hospital in China. These isolates were used as parent strains to screen DAP-R mutants *in vitro*. Isolates were stored at -80 °C, and incubated on the blood agar plates at 37°C for 18-24 h before using.

Serial passage experiment selection of DAP-R *E. faecium*

To investigate the development of daptomycin resistance in *E. faecium*, DAP-S strains, including 3 VRE isolates and 3 VSE isolates, were used to select DAP-R variants by serial passage experiments *in vitro*, according to prior published methods with some modifications [16]. Specifically, single pure colony was randomly selected, and then inoculated in 3 mL fresh Luria Broth (LB) with 50 µg/mL calcium (The LB used in this study contains 50 µg/mL calcium, unless otherwise specified) and allowed to grow to logarithmic period, 30 µL overnight cultures were transferred to 2.97 mL fresh LB with graded concentrations of DAP: 1/2×MIC, 1×MIC, 2×MIC and 4×MIC. And then the cultures were incubated at 37°C overnight without shaking. Strains were cultured at each concentration for 3 passages before their exposure to the next concentration. This process lasted 36 days.

Genetic stability testing and determination DAP susceptibility

Colonies from the highly DAP-R mutants of each parent isolates were picked, inoculated without DAP for 10 passages, and then MIC value against DAP was determined again to check the stability of the phenotype using broth microdilution method. The result was interpreted according to the latest guideline of Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute (CLSI, 2020) (susceptible, ≤ 4 µg/mL; resistant, ≥ 8 µg/mL). *E. faecium* ATCC 29212 was used as a control strain.

Homologous analysis

In this experiment, clone correlation analysis was also performed by multilocus sequence typing (MLST) for parent strains and all DAP-R mutants. The sequences of seven housekeeping genes (*gdh*, *purK*, *pstS*, *atpA*, *adk*, *ddl* and *gyd*) were amplified and sequence types (ST) were assigned based on the MLST databases (<https://pubmlst.org/efaecium/>). Primers were listed in Table S1 (see Additional file 1).

Antimicrobial susceptibility testing

Agar dilution method was carried out to measure the MIC of commonly used antibiotics among parent isolates and highly DAP-R mutants, including ampicillin, ciprofloxacin, levofloxacin, nitrofurantoin, penicillin, erythromycin, linezolid, tetracycline, vancomycin and teicoplanin. The results were interpreted according to the criteria established by CLSI. *E. faecium* ATCC 29212 was used as a control strain. The experiment was repeated in triplicates.

PCR amplification and DNA sequencing

DNA was extracted from parent strains, all mutants and ATCC 29212 strain using Biospin bacterial genome DNA extraction kit (shanghai boyun biotech co.,ltd), and used as the template in polymerase chain reactions (PCR). Genes were amplified with specific primers [17, 18]: (i) LiaFSR three-component regulatory system encoding gene *liaF*, *liaS* and *liaR*; (ii) YycFG two-component regulatory system encoding gene *yycF* and *yycG*. Primers were listed in Table S1 (see Additional file 1). The positive PCR products were sequenced by Shanghai MajorbioBio-Pharm Technology Co. (Shanghai, China). The

sequencing results were compared with parent strains and the standard strain *Enterococcus faecium* DO (Accession number: CP003583) using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>).

Cytochrome C binding assay

The relative positive surface charges of *E. faecium* strains were determined by previously published methods [12]. Briefly, bacterial suspensions of the parent strains and the highly DAP-R induced strains were allowed to regrow to logarithmic phase after overnight cultures were diluted 1:100 in 20 mL fresh LB. Then cells were washed twice with MOPS (morpholinepropanesulfonic acid) buffer (20 mM, pH=7.0) after centrifugation, and the bacterial suspension was adjusted to an OD₆₀₀ of 1.0. Cytochrome C, a positively charged molecule, was prepared in MOPS (5 mg/mL), and then 100 µL was added to 900 µL of bacterial suspension. After 30 min at room temperature, samples were centrifuged at 12,000 g for 5 min. Supernatant free cytochrome C were recovered and the OD₅₃₀ was measured spectrophotometrically. Less free cytochrome C that was detected in the supernatant means more net positively charge on the bacterial surface. The experiment was repeated at least three times.

Transmission electron microscopy

Two parent strains (SC1174 and SC1762) and their last generation of induced strains (SC1174-D and SC1762-D) were randomly selected to measure the cell wall thickness using TEM as described previously with some modifications [19]. After overnight culture in LB, 1 mL cultures were pelleted and washed 3 times using 0.1 M Millonig's phosphate buffer. The pellet was then resuspended in 1 mL glutaraldehyde in Millonig's phosphate buffer, and further processed in the Electron Microscope Room of Wenzhou medical University. For each strain, Cell wall thickness of at least 25 cells was measured at ×40,000 and 60,000 magnification, respectively. *P* values of <0.05 indicated statistical significance.

Growth kinetics measurement

Growth kinetics was assessed for all ancestral and the the highly DAP-R mutants. After overnight growth in LB at 37°C for 24 h, cultures were diluted into 20 mL LB with a final bacterial suspension concentration of 0.5 McFarland standard, and then incubated at 37°C for 24 h with agitation at 200 rpm. Growth curves were generated by plotting OD₆₀₀ over time. The experiment was repeated in triplicates, and the averages were used for estimating growth parameters.

***In vitro* competition experiments**

In vitro competition experiments were performed in triplicates to measure fitness cost between parent strains and last generation of DAP induced strains [20]. Exponentially growing cells of the DAP-S parent strains and DAP-R mutants were adjusted to 1×10^3 colony-forming units (CFU)/mL, and then equal volumes were combined, thus the initial ratio of the strainpairs was infinitely close to 1:1. Then 1 mL of the mixture was added to 19 mL LB broth and cultured at 37°C with agitation at 200 rpm for 24 h, which corresponds to approximately 20 cell generations. Serial 10-fold dilutions were plated in duplicate on

DAP-free LB agar and LB agar containing 4 µg/mL DAP (This concentration inhibited the growth of all parent strains). After overnight incubation at 37°C for 24 h, the CFU of the DAP-R colonies and parent strains were counted, respectively. The competition index (CI) means adaptive difference, which was defined as the ratio between the CFU of the DAP-R strains and the DAP-S strains. The CI values were calculated for each independent competition assay, and the median values were calculated too.

Crystal violet biofilm assays

Biofilm-bound crystal violet was quantified for parent strains and the last generation of DAP-R mutants. For the biofilm formation assays, isolates were incubated at 37°C with shaking at 200 rpm overnight, and culture suspensions were diluted 1:100 into fresh LB, then inoculated (200 µL per well) into 96-well polystyrene microtiter plate for 48 h at 37°C. After incubation, planktonic cells were removed, and 200 µL 0.1% crystal violet was added to each well. The plate was incubated for 20 min at 37°C before it was washed twice with sterile water. Finally, 95% ethanol was used to dissolve the stained biofilm and the absorbance at OD₆₀₀ was measured for quantification. Additionally, untreated well supplemented with sterile LB was served as control. All experiments were conducted in triplicates.

***Galleria mellonella* killing assays**

Infection model of *Galleria mellonella* larvae was used to compare the virulence differences between parent strain and the last generation of DAP-R mutants as described previously with slight modifications [21]. Twelve caterpillars weighing between 200 and 250 mg were randomly selected for each isolate. Bacterial suspension or normal saline (NS) were injected into the last left proleg by 25 µL Hamilton precision syringe. Larvae were injected with 10 µL of bacterial suspension containing 10⁸ CFU/mL as an experimental group, and injected with nothing or 10 µL NS as a control group. Worms were incubated at 37°C in the dark, and their survival or mortality rates were observed and recorded every 24 h over a period of 6 days to plot killing curve. Those worms that did not move when touched and their body colors were dark brown were considered dead. The result of infection model was rapidity and extent of mortality of *Galleria mellonella*, as assessed by Kaplan-Meier analysis and Logrank test. All experiments were performed in 3 independent replicates.

Statistical analysis

Statistical analysis of growth rate was performed with the GraphPad Prism 8.0 software using one-way analysis of variance (ANOVA). The independent Student's t-test (two-tailed) was performed using SPSS software version 26.0 for statistical analysis, *P* values of <0.05 were considered as significance.

Results

DAP-R *E. faecium* mutants

To identify the development and pathways of DAP resistance among *E. faecium* strains, 3 VRE isolates and 3 VSE isolates were exposed to graded levels of the DAP, with a starting concentration of 1 µg/mL. As shown in Table 1, a total of 21 mutants from the 6 original strains were obtained. The resistance of mutants to DAP was not changed after passaging without DAP for 10 days in LB, and the MIC of these strains against DAP enhanced by 16-256 folds (Table 1). Besides, mutants with low level of daptomycin resistance were isolated for the first time on the fourth day of daptomycin induction, and we also obviously observed that the resistance of mutants to daptomycin increased gradually with the prolongation of DAP exposure day (Figure 1), demonstrating that resistant mutants can be rapidly selected by serial passaging across sub-lethal gradient.

Moreover, the results of homologous analysis showed that the ST types of SC1174, SC1379, SC1762, SC1543, SC1706 and SC1726 were ST761, ST78, ST976, ST32, ST1025 and ST32, respectively (Table 1). The ST type of the parent strains was consistent with that of the respective induced strains, which illustrated the parent strains and their induced strains had high homology.

Antimicrobial susceptibility testing profiles

The agar dilution method was used to determine the MIC of both parent strains and derived strains, according to the latest guideline of CLSI. As shown in Table 2, after acquired daptomycin resistance, the resistance of DAP-R mutants to antibiotics did not change except SC1762-D mutant. In contrast to the parent strain SC1762, SC1762-D mutant was highly sensitive to vancomycin and teicoplanin, which presented collateral-sensitivity that bacteria acquiring resistance to one antibiotic can give rise to sensitivity to other drugs during adaptive evolution [22]. The MIC of vancomycin dropped from 512 to 2 µg/mL, and teicoplanin dropped from 128 to below 2 µg/mL, respectively.

LiaFSR and YycFG systems encoding genes mutations were related to DAP resistance

To investigate various DAP resistance evolutionary routes, we analyzed the gene mutations of *liaFSR* and *yycFG* by PCR and sequencing. Different gene mutations were observed in different mutants, compared with parent strains and standard strain *Enterococcus faecium* DO (Figure 2). Specifically, mutations of *liaS*, *liaR* and *yycF* genes were observed among SC1174 induced strains, it should be noted that mutations in *liaS* and *yycF* genes only occurred in the last generation induced strain SC1174-D with DAP MIC of 64 µg/mL. Similarly, the accumulation of *liaS* gene mutations was also found in the last generation induced strain SC1379-D. For SC1762 induced strains, mutations were detected in all genes except *liaR*, and accumulation of genes mutations was detected in *liaF*, *yycF* and *yycG*. For SC1543 induced strains, *liaF*, *liaS* and *yycG* genes mutations were noticed. SC1706 induced strains mainly carried *liaR* and *yycG* genes mutations. And the mutations of *liaS* and *yycF* genes were also found in SC1726 induced strains.

Increased cell surface charge and cell-wall thickness mediated DAP resistance

DAP resistance mechanisms were also analyzed by cytochrome C binding assay and thickness of the cell-wall assays. cytochrome C binding assays revealed that DAP-R mutants had significantly increased positive charge on surface relative to parent strains except SC1379-D ($P < 0.05$) (Figure 3). Furthermore, we found that SC1174-D mutant strain (55.09 ± 6.55 nm) exhibited thicker cell-wall than its parent strain SC1174 (19.97 ± 5.26 versus, $P < 0.0001$) (Figure 4A and Figure 4C). Similarly, SC1762-D mutant strain (46.456 ± 9.08 nm) presented significantly thicker cell-wall than its parent strain SC1762 (18.21 ± 3.36 nm, $P < 0.0001$) (Figure 4B and Figure 4C). Noticeably, the septum of cell-wall was obviously thickened both in SC1174-D and SC1762-D mutant strains compared to their corresponding parent strains.

***E. faecium* showed fitness costs after acquiring DAP resistance**

To assess whether the evolution of DAP resistance strains was accompanied by associated fitness costs, we analyzed bacterial growth curves, biofilm formation ability and competition experiments for the last generation mutants. Growth rate of DAP mutants slowed down and the bacterial density was lower than their original strains (All $P < 0.001$) (Figure 5). The results of competition experiments showed that DAP mutants had a marked decrease in fitness. The greatest decrease was observed for the SC1726 strain, with a median CI of 0.021, followed by the SC1379, SC1762, SC1706, SC1174 and SC1543 isolates, with median CI of 0.077, 0.096, 0.098, 0.111, 0.141 and respectively (Figure 6A). Moreover, the biofilm forming ability of tested DAP-induced strains was significantly higher than corresponding parent strain, especially SC1706-D ($P < 0.05$) (Figure 6B).

Infection model of *Galleria mellonella* larvae

To evaluate the virulence changes of induced strains, an infection model of *Galleria mellonella* larvae was constructed. Compared with the corresponding DAP-S strains, at 6 days post-infection, the mortality of larvae was lower for DAP-R mutants ($P < 0.001$), and no mortality was observed in the control injected with NS (Figure 7).

Discussion

Application of antibiotics has played a crucial role in controlling and prevention of bacterial infections in humans, but with a wide range of continuous and inappropriate use of antibiotics, bacterial resistance to antimicrobial agents has become increasingly common, which poses a serious threat to global public health [23]. Nevertheless, under the pressure of antimicrobial selectivity, the evolution of bacterial resistance to antibiotics largely depends on the changes of genomics background and adaptability. Thus, better understanding of the development and evolutionary trajectories of DAP resistance may potentially inform effective therapeutic strategies in the future.

Experimental evolution, like serial passage experiment *in vitro*, is an efficient tool to investigate the effects of environment and inherent genetic factors on specific phenotype changes during evolutionary adaptation, and to provide exact insights into the course of adaptation to antimicrobials, the frequency of

mutations, fitness costs associated with resistance, and the predictability of evolutionary trajectories and adaptive pathways [24].

In our study, 3 VRE and 3 VSE isolates were selected as parent isolates to evolve resistance to DAP *in vitro*. We found the DAP MIC of parent isolates was significantly and rapidly enhanced, and DAP-R strains were first detected only 4 days after exposure to daptomycin, indicating that *E. faecium* was easy to develop DAP acquired resistance *in vitro*. Therefore, it is necessary to take preventive and control measures to deal with DAP-R *E. faecium* that may appear in China in the future.

Previous study has been reported that laboratory evolution of bacteria to different antimicrobials can decrease or enhance the sensitivity to multiple other antimicrobials agents, called cross-resistance and collateral sensitivity, respectively [25]. Cross-resistance was not detected between DAP and other antimicrobials agents. But, intriguingly, it was surprising that DAP evolved resistance exhibited collateral-sensitivity to vancomycin and teicoplanin in SC1762-D. These data remind us that once DAP is invalid after a period of treatment in clinic, the sensitivity of glycopeptides should be paid close attention to, or the combination of DAP and glycopeptides may be an effective measure to deal with VRE strains.

Previous studies have shown that mutations in LiaFSR, a three-component regulatory system controlling cell-envelope stress response, and in YycFG, a two-component regulatory system regulating cell envelope homeostasis, were directly linked with the emergence of daptomycin (DAP) resistance in enterococci [14, 26]. Here, mutations in LiaFSR and YycFG systems encoding genes were found in DAP-induced strains, which were consistent with Munita JM et al. previous work which showed that mutations in these systems could be a pivotal initial event in the development of DAP resistance. [18]. But unfortunately, the same gene mutation sites were not observed in all the induced strains, which may explain the diversity and dynamics of daptomycin resistance evolution pathway in *E. faecium*.

Intriguingly, the occurrence time of genes mutations was not consistent in different strains, indicating that gene mutations were related to daptomycin exposure time. For example, *liaS* gene mutations were firstly detected in the first generation of SC1762 and SC1726 induced strains on the fourth day of daptomycin induction, while it was not detected until the last generation of SC1174 and SC1379 induced strains after the 36 days of daptomycin induction. Moreover, it was obvious that the frequency of genes mutations was different among LiaFSR and YycFG systems, the mutation frequency of *liaS* gene was the highest, followed by *yycG* and *liaF* genes, and finally *yycF* and *liaR* genes, which suggested that different genes played different roles in the evolution of daptomycin resistance. Contrary to earlier report, *liaS* and *yycG* genes were likely to play leading roles in mediating DAP resistance in these strains, which may be due to different pathways of daptomycin resistance in different strains [27]. In fact, our findings were not entirely consistent with previous study. There was no evidence showing a rule between gene mutations and increased the MIC of DAP among *E. faecium*, but accumulation of *liaS*, *liaF* and *yycF* genes mutations may be positively correlated with the MIC of DAP, which demonstrated evolved DAP resistance is dynamic [18].

Other mechanisms should be taken into consideration, such as charge rejection mechanism and cell membrane stress adaptation [11, 19]. It was described that DAP resistance is associated with distinct alterations of cell membrane phospholipid content in *enterococci* [28]. Indeed, we also found cell surface net positive charge and cell-wall thickness were dramatically increased in these mutants compared to parent strains, and the spacing of the cell-wall was markedly thickened.

Notably, laboratory evolution often lead to adapted strains having different physiological characteristics compared with their parent strains, which are usually related to a fitness cost that is typically observed as a reduced bacterial growth rate [29, 30]. Our results showed a statistically significant decreased growth rate and enhanced biofilm formation for DAP-R mutants, which were similar with previous findings that the ability of biofilm formation was associated with increased resistance [31]. And the lethality and pathogenicity in *Galleria mellonella* larvae were also weakened for mutants. Besides, the fitness cost is most commonly measured by pair-wise competition experiments [32]. Our data suggested that the median CI values for adaptive mutants were associated with a statistically markedly decreased fitness *in vitro*. In summary, we demonstrate that bacteria acquired daptomycin resistance through different evolutionary pathways *in vitro*, which would appear with varying degrees of fitness costs.

Conclusions

E. faecium tended to develop DAP resistance *in vitro*, which was likely to be related to the following three ways: (i) gene mutations in LiaFSR and YycFG systems; (ii) “repulsion” from the cell surface; (iii) thickened cell wall. Besides, this is the first study to report that DAP resistance of *E. faecium* conferred predictable sensitivities to glycopeptides, providing a new way to optimize treatments for multidrug-resistant *E. faecium* infection. It was also worth noting that with the increase of DAP resistance, the ability of resistant bacteria to propagate was weakened, suggesting that evolving DAP resistance was associated with fitness costs.

Abbreviations

ATCC: American Type Cultures Collection; ANOVA: One-way analysis of variance; CLs: Cardiolipin synthase; CLSI: Clinical and Laboratory Standards Institute; CFU: Colony-forming units; CI: Competition index; DAP: Daptomycin; DAP-R: Daptomycin-resistant; *E. faecium*: *Enterococcus faecium*; DAP-S: Daptomycin-susceptible (DAP-S); GdpD: Glycerophosphoryl diester phosphodiesterase; LB: Luria broth; MIC: Minimal inhibitory concentration; MLST: Multilocus sequence typing; MOPS: Morpholinepropanesulfonic acid; NS: Normal saline; PCR: Polymerase chain reaction; ST: Sequence types; TEM: Transmission electron microscopy; VRE: Vancomycin-resistant *E. faecium*; VSE: Vancomycin-susceptible *E. faecium*.

Declarations

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The need for ethics approval and consent is deemed unnecessary in this research according to the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Contributions

WLZ, TC, and QW carried out experiments. WLZ and YX analyzed the data. WLZ wrote the manuscript. SXL and KHY performed the results analysis and YZ directed the drawing. JMC and TLZ designed the study and revised the manuscript. All authors reviewed and approved the final version of the manuscript.

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References

1. Contreras GA, Munita JM, Arias CA. Novel Strategies for the Management of Vancomycin-Resistant Enterococcal Infections. *Curr Infect Dis Rep*. 2019;21(7):22; doi: 10.1007/s11908-019-0680-y.
2. Lebreton F, Manson AL, Saavedra JT, Straub TJ, Earl AM, Gilmore MS. Tracing the *Enterococci* from Paleozoic Origins to the Hospital. *Cell*. 2017;169(5); doi: 10.1016/j.cell.2017.04.027.
3. Mulani MS, Kamble EE, Kumkar SN, Tawre MS, Pardesi KR. Emerging Strategies to Combat ESKAPE Pathogens in the Era of Antimicrobial Resistance: A Review. *Frontiers in microbiology*. 2019;10:539; doi: 10.3389/fmicb.2019.00539.
4. Arias CA, Murray BE. The rise of the Enterococcus: beyond vancomycin resistance. *Nature reviews Microbiology*. 2012;10(4):266-78; doi: 10.1038/nrmicro2761.
5. Heidary M, Khosravi AD, Khoshnood S, Nasiri MJ, Soleimani S, Goudarzi M. Daptomycin. *The Journal of antimicrobial chemotherapy*. 2018;73(1); doi: 10.1093/jac/dkx349.
6. Cassini A, Högberg LD, Plachouras D, Quattrocchi A, Hoxha A, Simonsen GS, et al. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. *The Lancet Infectious diseases*. 2019;19(1):56-66; doi: 10.1016/S1473-3099(18)30605-4.
7. Rand KH, Houck HJ, Silverman JA. Daptomycin-reversible rifampicin resistance in vancomycin-resistant *Enterococcus faecium*. *J Antimicrob Chemother*. 2007;59(5):1017-20; doi: 10.1093/jac/dkm045.
8. Humphries RM, Pollett S, Sakoulas G. A current perspective on daptomycin for the clinical microbiologist. *Clin Microbiol Rev*. 2013;26(4):759-80; doi: 10.1128/CMR.00030-13.
9. Arnaiz de Las Revillas F, Fernandez-Sampedro M, Arnaiz-García AM, Gutierrez-Cuadra M, Armiñanzas C, Pulitani I, et al. Daptomycin treatment in Gram-positive vascular graft infections. *Int J Infect Dis*. 2018;68:69-73; doi: 10.1016/j.ijid.2018.01.009.
10. Revest M, Jacqueline C, Boudjemaa R, Caillon J, Le Mabecque V, Breteche A, et al. New in vitro and in vivo models to evaluate antibiotic efficacy in *Staphylococcus aureus* prosthetic vascular graft infection. *The Journal of antimicrobial chemotherapy*. 2016;71(5):1291-9; doi: 10.1093/jac/dkv496.
11. Berti AD, Baines SL, Howden BP, Sakoulas G, Nizet V, Proctor RA, et al. Heterogeneity of genetic pathways toward daptomycin nonsusceptibility in *Staphylococcus aureus* determined by adjunctive antibiotics. *Antimicrob Agents Chemother*. 2015;59(5):2799-806; doi: 10.1128/AAC.04990-14.
12. Yin Y, Chen H, Li S, Gao H, Sun S, Li H, et al. Daptomycin resistance in methicillin-resistant *Staphylococcus aureus* is conferred by IS256 insertion in the promoter of *mprF* along with mutations in *mprF* and *walK*. *Int J Antimicrob Agents*. 2019;54(6):673-80; doi: 10.1016/j.ijantimicag.2019.08.021.

13. Miller WR, Bayer AS, Arias CA. Mechanism of Action and Resistance to Daptomycin in *Staphylococcus aureus* and *Enterococci*. Cold Spring Harb Perspect Med. 2016;6(11); doi: 10.1101/cshperspect.a026997.
14. Tran TT, Panesso D, Gao H, Roh JH, Munita JM, Reyes J, et al. Whole-genome analysis of a daptomycin-susceptible *enterococcus faecium* strain and its daptomycin-resistant variant arising during therapy. Antimicrob Agents Chemother. 2013;57(1):261-8; doi: 10.1128/AAC.01454-12.
15. Bayer AS, Schneider T, Sahl H-G. Mechanisms of daptomycin resistance in *Staphylococcus aureus*: role of the cell membrane and cell wall. Ann N Y Acad Sci. 2013;1277:139-58; doi: 10.1111/j.1749-6632.2012.06819.x.
16. Bhardwaj P, Hans A, Ruikar K, Guan Z, Palmer KL. Reduced Chlorhexidine and Daptomycin Susceptibility in Vancomycin-Resistant *Enterococcus faecium* after Serial Chlorhexidine Exposure. Antimicrob Agents Chemother. 2018;62(1); doi: 10.1128/AAC.01235-17.
17. Leliek H, Franke GC, Ruckert C, Wolters M, Wolschke C, Christner M, et al. Emergence of daptomycin non-susceptibility in colonizing vancomycin-resistant *Enterococcus faecium* isolates during daptomycin therapy. Int J Med Microbiol. 2015;305(8):902-9; doi: 10.1016/j.ijmm.2015.09.005.
18. Munita JM, Panesso D, Diaz L, Tran TT, Reyes J, Wanger A, et al. Correlation between mutations in *liaFSR* of *Enterococcus faecium* and MIC of daptomycin: revisiting daptomycin breakpoints. Antimicrob Agents Chemother. 2012;56(8):4354-9; doi: 10.1128/AAC.00509-12.
19. Prater AG, Mehta HH, Kosgei AJ, Miller WR, Tran TT, Arias CA, et al. Environment Shapes the Accessible Daptomycin Resistance Mechanisms in *Enterococcus faecium*. Antimicrob Agents Chemother. 2019;63(10); doi: 10.1128/AAC.00790-19.
20. Li S, Yin Y, Chen H, Wang Q, Wang X, Wang H. Fitness Cost of Daptomycin-Resistant *Staphylococcus aureus* Obtained from in Vitro Daptomycin Selection Pressure. Front Microbiol. 2017;8:2199; doi: 10.3389/fmicb.2017.02199.
21. Taglialegna A, Varela MC, Rosato RR, Rosato AE. *VraSR* and Virulence Trait Modulation during Daptomycin Resistance in Methicillin-Resistant *Staphylococcus aureus* Infection. mSphere. 2019;4(1); doi: 10.1128/mSphere.00557-18.
22. Horinouchi T, Suzuki S, Kotani H, Tanabe K, Sakata N, Shimizu H, et al. Prediction of Cross-resistance and Collateral Sensitivity by Gene Expression profiles and Genomic Mutations. Sci Rep. 2017;7(1):14009; doi: 10.1038/s41598-017-14335-7.
23. Watkins RR, Bonomo RA. Overview: Global and Local Impact of Antibiotic Resistance. Infect Dis Clin North Am. 2016;30(2):313-22; doi: 10.1016/j.idc.2016.02.001.
24. Sandberg TE, Salazar MJ, Weng LL, Palsson BO, Feist AM. The emergence of adaptive laboratory evolution as an efficient tool for biological discovery and industrial biotechnology. Metab Eng. 2019;56; doi: 10.1016/j.ymben.2019.08.004.
25. Jahn LJ, Munck C, Ellabaan MMH, Sommer MOA. Adaptive Laboratory Evolution of Antibiotic Resistance Using Different Selection Regimes Lead to Similar Phenotypes and Genotypes. Frontiers in microbiology. 2017;8:816; doi: 10.3389/fmicb.2017.00816.

26. Wang G, Yu F, Lin H, Murugesan K, Huang W, Hoss AG, et al. Evolution and mutations predisposing to daptomycin resistance in vancomycin-resistant *Enterococcus faecium* ST736 strains. PLoS One. 2018;13(12):e0209785; doi: 10.1371/journal.pone.0209785.
27. Arias CA, Panesso D, McGrath DM, Qin X, Mojica MF, Miller C, et al. Genetic basis for in vivo daptomycin resistance in enterococci. N Engl J Med. 2011;365(10):892-900; doi: 10.1056/NEJMoa1011138.
28. Tran TT, Panesso D, Mishra NN, Mileykovskaya E, Guan Z, Munita JM, et al. Daptomycin-resistant *Enterococcus faecalis* diverts the antibiotic molecule from the division septum and remodels cell membrane phospholipids. mBio. 2013;4(4); doi: 10.1128/mBio.00281-13.
29. Andersson DI, Hughes D. Antibiotic resistance and its cost: is it possible to reverse resistance? Nature reviews Microbiology. 2010;8(4):260-71; doi: 10.1038/nrmicro2319.
30. Suzuki S, Horinouchi T, Furusawa C. Phenotypic changes associated with the fitness cost in antibiotic resistant *Escherichia coli* strains. Mol Biosyst. 2016;12(2):414-20; doi: 10.1039/c5mb00590f.
31. Saffari M, Karami S, Firoozeh F, Sehat M. Evaluation of biofilm-specific antimicrobial resistance genes in *Pseudomonas aeruginosa* isolates in Farabi Hospital. Journal of medical microbiology. 2017;66(7):905-9; doi: 10.1099/jmm.0.000521.
32. Guo B, Abdelraouf K, Ledesma KR, Nikolaou M, Tam VH. Predicting bacterial fitness cost associated with drug resistance. The Journal of antimicrobial chemotherapy. 2012;67(4):928-32; doi: 10.1093/jac/dkr560.

Tables

Table 1 Daptomycin MIC and ST type of parent strains and each generation daptomycin-induced mutants

Isolates		DAP MIC ^a (µg/mL)		ST ^b type	
Parent isolates	DAP-induced isolates	Parent isolates	DAP-induced isolates	Parent isolates	DAP-induced isolates
SC1174	SC1174-D1 ^c	2	8^d	761	761
	SC1174-D2		32		761
	SC1174-D		64		761
SC1379	SC1379-D1	2	16	78	78
	SC1379-D2		32		78
	SC1379-D		64		78
SC1762	SC1762-D1	2	4	976	976
	SC1762-D2		16		976
	SC1762-D3		32		976
	SC1762-D		64		976
SC1543	SC1543-D1	2	16	32	32
	SC1543-D2		32		32
SC1706	SC1706-D1	2	4	1025	1025
	SC1706-D2		8		1025
	SC1706-D3		32		1025
	SC1706-D		128		1025
SC1726	SC1726-D1	2	8	32	32
	SC1726-D2		32		32
	SC1726-D3		64		32
	SC1726-D4		128		32
	SC1726-D		512		32

^a DAP, Daptomycin; MIC, Minimum inhibitory concentration. ^b ST, Sequence types. ^c -D1~-D4, The first to the fourth generation of DAP-induced isolates; -D, The last generation of DAP-induced mutants or highly DAP-induced mutants; MIC, Minimal inhibitory concentration. ^d The values in bold font indicate resistance.

Table 2 Activity of daptomycin and other antibiotics against *E. faecium* parent strains and daptomycin-induced mutants

Isolates	Antibiotic MIC ^a (µg/mL)										
	DAP	AMP	CIP	LVX	NIT	PEN	ERY	LNZ	TCY	VAN	TEC
SC1174	2	512 ^b	512	128	128	512	0.25	2	0.5	512	128
SC1174-D ^c	64	512	512	128	128	512	0.25	2	0.5	512	128
SC1379	2	512	512	128	128	512	512	1	>16	512	256
SC1379-D	64	512	512	128	128	512	512	1	>16	512	256
SC1762	2	512	256	128	128	512	512	1	0.25	512	128
SC1762-D	64	512	256	128	128	512	512	1	0.25	2	≤2
SC1543	2	8	1	2	128	16	512	1	0.5	1	≤2
SC1543-D	32	8	1	2	128	16	512	1	0.5	1	≤2
SC1706	2	2	4	8	128	4	8	2	0.25	1	≤2
SC1706-D	128	2	8	8	128	4	8	2	0.25	1	≤2
SC1726	2	2	8	8	256	8	8	2	≤1	1	≤2
SC1726-D	512	2	8	8	256	8	8	2	≤1	1	≤2

^a MIC, Minimal inhibitory concentration; DAP, Daptomycin; AMP, Ampicillin; CIP, Ciprofloxacin; LVX, Levofloxacin; NIT, Nitrofurantoin; PEN, Penicillin; ERY, Erythromycin; LNZ, Linezolid; TCY, Tetracycline; VAN, Vancomycin; TEC, Teicoplanin. ^b The values in bold font indicate resistance. ^c -D, The last generation of DAP-induced mutants or highly DAP-induced mutants.

Figures

Figure 1

E. faecium adapts to daptomycin. In vitro evolution of reduced DAP susceptibility in *E. faecium* by serial passaging in increasing concentrations of DAP for a period of 36 days.

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Figure 2

Genes mutations in daptomycin-resistant mutants. (A) LiaFSR genes mutations in all DAP-R mutants. Different strains have different genes mutations distribution, and accumulation mutations of *liaF* and *liaS* were detected in SC1762-D2 ~ SC1762-D, and in SC1543-D, SC1726-D4 ~ SC1726-D, respectively. No cumulative mutations were observed in *liaR* gene. (B) *YycFG* genes mutations in all DAP-R mutants. Accumulation mutations of *yycG* was showed in SC1174-D and SC1762-D2 ~ SC1762-D, respectively. -

D1~-D4, the first to the fourth generation of DAP-induced isolates; -D, the last generation of DAP-induced mutants or highly DAP-induced mutants.

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Genes mutations in daptomycin-resistant mutants. (A) LiaFSR genes mutations in all DAP-R mutants. Different strains have different genes mutations distribution, and accumulation mutations of *liaF* and *liaS* were detected in SC1762-D2 ~ SC1762-D, and in SC1543-D, SC1726-D4 ~ SC1726-D, respectively. No

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Figure 3

Relative positive surface charge by cytochrome C binding. All DAP-R isolates had significantly increased positive surface relative to their parent strains except SC1379-D. *P <0.05, ***P <0.001, NS means no statistical difference. -D, the last generation of DAP-induced mutants or highly DAP-induced mutants.

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Figure 4

Cell-wall thickness by transmission electron microscopy. For each strain, cell wall thickness measurements were taken from a minimum of 25 cells at $\times 40,000$ and $\times 60,000$ magnification, respectively.

Compared to respective parent strains, induced strains SC1174-D (A) and SC1762-D (B) showed increased cell wall thickness and abnormal septation. (C) 25 cells were analyzed for each strain. Data were presented as mean and standard deviation. **** $P < 0.0001$. -D, the last generation of DAP-induced mutants or highly DAP-induced mutants.

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Cell-wall thickness by transmission electron microscopy. For each strain, cell wall thickness measurements were taken from a minimum of 25 cells at $\times 40,000$ and $\times 60,000$ magnification, respectively. Compared to respective parent strains, induced strains SC1174-D (A) and SC1762-D (B) showed increased cell wall thickness and abnormal septation. (C) 25 cells were analyzed for each strain. Data were presented as mean and standard deviation. **** $P < 0.0001$. -D, the last generation of DAP-induced mutants or highly DAP-induced mutants.

Figure 5

Bacterial growth curves for all strains in this study. A-F represent growth curves for SC1174 and SC1174-D, SC1379 and SC1379-D, SC1762 and SC1762-D, SC1543 and SC1543-D, SC1706 and SC1706-D, SC1726 and SC1726-D, respectively. -D, the last generation of DAP-induced mutants or highly DAP-induced mutants.

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Figure 6

The biofilm formation ability and the result of in vitro competition index. A. The result of in vitro competition index between parent and evolved isolates. B. Levels of biofilm formation between parent and evolved isolates. *P < 0.05, ***P < 0.001. -D, the last generation of DAP-induced mutants or highly DAP-induced mutants.

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The biofilm formation ability and the result of in vitro competition index. A. The result of in vitro competition index between parent and evolved isolates. B. Levels of biofilm formation between parent and evolved isolates. *P < 0.05, ***P < 0.001. -D, the last generation of DAP-induced mutants or highly DAP-induced mutants.

Figure 7

Infection model of *Galleria mellonella* larvae. A-F represents the survival curves of *Galleria mellonella* larvae infected with SC1174 and SC1174-D, SC1379 and SC1379-D, SC1762 and SC1762-D, SC1543 and SC1543-D, SC1706 and SC1706-D, SC1726 and SC1726-D, respectively. -D, the last generation of DAP-induced mutants or highly DAP-induced mutants.

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