Dawan et al. Annals of Microbiology (2020) 70:1
https://doi.org/10.1186/s13213-020-01552-1

Annals of Microbiology

UNIVERSITÀ DEGLI STUDI DI MILANO

ORIGINAL ARTICLE Open Access

Role of antibiotic stress in phenotypic switching to persister cells of antibiotic-resistant *Staphylococcus aureus*

Jirapat Dawan[1], Shuai Wei[1,2] and Juhee Ahn[1*]

Abstract

Purpose: This study was designed to evaluate phenotypic and genotypic properties of persister cells formed by *Staphylococcus aureus* ATCC 15564 (SA^{WT}), oxacillin-induced *S. aureus* (SA^{OXA}), ciprofloxacin-induced *S. aureus* (SA^{CIP}), and clinically isolated multidrug-resistant *S. aureus* CCARM 3080 (SA^{MDR}).

Methods: The dose-dependent biphasic killing patterns were observed for SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} in response to twofold minimum inhibitory concentrate (MIC) of ciprofloxacin. The surviving cells of SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} after twofold MIC of ciprofloxacin treatment were analyzed using a metabolic-based assay to estimate the fractions of persister cells.

Results: The least persister formation was induced in SA^{CIP} after twofold MIC of ciprofloxacin treatment, showing 58% of persistence. The lowest fitness cost of resistance was observed for the recovered persister cells of SA^{CIP} (relative fitness = 0.95), followed by SA^{MDR} (relative fitness = 0.70), while the highest fitness cost was observed for SA^{WT} (relative fitness = 0.26). The mRNA transcripts were analyzed by RT-PCR assay in recovered persister cells pre-incubated with ciprofloxacin. The highest expression levels of stress-related genes (*dnaK* and *groEL*) and efflux pump-related genes (*mepR*, *norA*, and *norB*) were observed in the recovered persister cells of SA^{OXA} and SA^{MDR}.

Conclusion: This study provides valuable information for understanding crosstalk between antibiotic resistance, tolerance, and persistence in different antibiotic-resistant *S. aureus* strains.

Keywords: *Staphylococcus*, Persistence, Resistance, Tolerance, Ciprofloxacin

Introduction

Over the past few decades, the overuse and misuse of antibiotics have accelerated the emergence and spread of antibiotic-resistant *Staphylococcus aureus*, specifically methicillin-resistant *S. aureus* (MRSA) (Deguchi et al. 2018). The frequent use of antibiotics potentially exerts selection pressure on *S. aureus*, resulting in an increased resistance to multiple antibiotics (Sakoulas et al. 2006). MRSA is the leading cause of hospital- and community-acquired infections, including sepsis, bacteremia, osteomyelitis, and endocarditis (Lowy 1998; Conlon 2014). The antibiotic selection pressure can provide a survival strategy for MRSA conferring fitness benefits and eventually lead to treatment failure (Sahukhal et al. 2017). The bacterial response to antibiotic stress causes phenotypic switching from normal to persister, tolerant, and resistant cells (Balaban et al. 2004; Lewis 2007; Kester and Fortune 2014). Persistence, tolerance, and resistance are mainly characterized by bacterial growth rates in the presence of antibiotic stress. Persistence is a metabolically inactive state that bacterial sub-population enters a dormant state under stressful conditions. Tolerance is a state that slow-growing bacteria dominate the population, while resistance is defined as the ability of bacteria to grow at the same rate in the presence of antibiotic stress (Fernández-García et al. 2018). Among them, however, the persistence has been relatively overlooked in the evolution of antibiotic resistance in bacteria.

* Correspondence: juheeahn@kangwon.ac.kr
[1]Department of Medical Biomaterials Engineering and Institute of Bioscience and Biotechnology, Kangwon National University, Chuncheon, Gangwon 24341, Republic of Korea
Full list of author information is available at the end of the article

Recently, the persistence has received growing attention as a new approach for controlling antibiotic-resistant bacteria (Fernández-García et al. 2018). The persistence is reversible phenotypic switching associated with heterogeneous bacterial populations (Balaban et al. 2004; Patra and Klumpp 2013). Persister cells also play an important role in the emergence of antibiotic resistance, resulting in antibiotic treatment failure (Dörr et al. 2010). Factors affecting the persister cell formation include efflux pump systems, SOS response, energy production, signal transduction, stringent response, protein degradation, and toxin-antitoxin (TA) systems (Lewis 2010; Fasani and Savageau 2013; Harms et al. 2016; Sahukhal et al. 2017). Understanding the characteristics of persistence is essential to optimize antibiotic chemotherapy (Girgis et al. 2012). However, the precise mechanism of persistence is not clearly understood. Therefore, the objectives of this study were to evaluate the phenotypic switching to persister cells of antibiotic-resistant *S. aureus* when exposed to a high level of ciprofloxacin.

Materials and methods

Bacterial strains and culture conditions

Strains of *Staphylococcus aureus* ATCC 15564 (SA^{WT}) and clinically isolated multidrug-resistant *S. aureus* CCARM 3080 (SA^{MDR}) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and the Culture Collection of Antibiotic Resistant Microbes (CCARM, Seoul, Korea), respectively. The strains were cultured in tryptic soy broth (TSB; Difco, Becton, Dickinson and Co., Sparks, MD, USA) at 37 °C for 20 h. The cultured cells were harvested by centrifugation at 3000×*g* for 20 min at 4 °C, washed twice with phosphate-buffered saline (PBS; pH 7.2), and then diluted with TSB prior to use.

Stepwise selection assay

SA^{WT} cells were exposed to oxacillin and ciprofloxacin to obtain experimentally induced antibiotic-resistant *S. aureus*. The minimum inhibitory concentrate (MIC) values of oxacillin and ciprofloxacin are 0.25 and 0.5 μg/ml, respectively. According to the serial passage method (Birošová and Mikulášová 2009), the concentrations of oxacillin and ciprofloxacin were increased from 0.125 (a half the MIC) to 8 μg/ml and from 0.25 (a half the MIC) to 32 μg/ml, respectively. The oxacillin- and ciprofloxacin-induced isogenic SA^{WT} strains were assigned to oxacillin-induced *S. aureus* (SA^{OXA}) and ciprofloxacin-induced *S. aureus* (SA^{CIP}), respectively. The antibiotic-induced resistance in SA^{OXA} and SA^{CIP} were extensively cultured for 10 passages in antibiotic-free TSB to confirm the stability of antibiotic resistance.

Antibiotic susceptibility assay

The antibiotic susceptibilities of SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} to the selected antibiotics were determined according to the Clinical Laboratory Standards Institute (CLSI) broth microdilution assay (CLSI 2019). Antibiotic stock solutions were prepared by dissolving in distilled water (ampicillin, cephalothin, gentamicin, oxacillin, and vancomycin) and glacial acetic acid (ciprofloxacin) to obtain a final concentration of 10.24 mg/ml. Each antibiotic stock solution was serially (1:2) diluted in 96-well microtiter plates and then inoculated with SA^{WT}, SA^{OXA}, SA^{CIP}, or SA^{MDR} (10^5 CFU/ml each). The inoculated plates were incubated at 37 °C for 18 h. The lowest antibiotic concentrations with no visible growth were defined as minimum inhibitory concentration (MIC).

Induction of persister cells

To induce persister cells, SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} cells were exposed to different concentrations (1/2 to twofold MIC) of ciprofloxacin. After 24 h of incubation at 37 °C, the cells were collected by centrifugation at 3000×*g* for 20 min at 4 °C, serially (1:10) diluted, and plated on TSA using an Autoplate Spiral Plating System (Spiral Biotech Inc., Norwood, MA, USA). The plates were incubated at 37 °C for 24–48 h to enumerate viable cells. In addition, the harvested cells were further analyzed to investigate phenotypic and genotypic properties of persister cells.

Measurement of persister cells

The metabolic activities of SA^{WT}, SA^{OXA}, SA^{CIP}, or SA^{MDR} cells exposed to twofold MIC of ciprofloxacin at 37 °C for 24 h were measured using a WST kit (iNtRON Biotechnology, MA, USA). In brief, the harvested cells (100 μl each) were placed in 96-well plates and mixed with 10 μl of the pre-warmed mixture of electro-connecting solution (10 μl) and WST-1 reagent (100 μl). The plates were incubated for 5 h. The absorbance was measured at 440 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) with a reference wavelength of 650 nm. A standard curve was prepared at serially diluted controls from 10^8 to 10^2 CFU/ml and measured as described above.

Estimation of fitness cost of resistance

The relative fitness was estimated to evaluate the pleiotropic cost of resistance of SA^{WT}, SA^{OXA}, SA^{CIP}, or SA^{MDR} cells exposed to twofold MIC of ciprofloxacin at 37 °C for 24 h. The persister cells were cultured at 37 °C for 24 h in antibiotic-free TSB. The relative fitness was expressed as the ratio of the growth (OD_{600}) of persister cells to that of control.

Disk diffusion assay

The ciprofloxacin-exposed SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} cells were regrown in fresh TSB at 37 °C for 20 h, which were used to evaluate the antibiotic susceptibilities by the disk diffusion test. The cultured cells were spread on TSA plates and the plates dried for 5 min. Antibiotic disks, including cefotaxime (CEF; 30 μg), cephalothin (CEP; 30 μg), chloramphenicol (CHL; 30 μg), ciprofloxacin (CIP; 5 μg), erythromycin (ERY; 15 μg), levofloxacin (LEV; 5 μg), and meropenem (MER; 10 μg), were placed onto TSA. After incubation at 37 °C for 24 h, the diameters of clear zone were measured using a digital vernier caliper (The L.S. Starrett Co., Athol, MA, USA). The changes in resistance were expressed as the equation: resistance (%) = [(inhibition zone of control – inhibition zone of persistence)/inhibition zone of control] × 100.

Quantitative RT-PCR assay

An RNeasy Protect Bacteria Mini kit (Qiagen, Hilden, Germany) was used to extract. Briefly, the re-growing cells of SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} treated with twofold MIC of ciprofloxacin were mixed with RNAprotect Bacteria Reagent (1 ml). After centrifugation at 3500×*g* for 20 min, the collected cells were lysed with lysozyme-containing buffer and then RNAs were extracted through an RNeasy mini column. The extracted RNAs washed with a Wipe buffer to remove the genomic DNA were mixed with RT buffer, RT primer mix, and a master mixture of reverse transcriptase. After incubation at 42 °C for 15 min, the master mixture was incubated at 42 °C for 15 min and consecutively 95 °C for 2 min and mixed with each primer (2 μl; Table 1), 2× QuantiTect SYBR Green PCR Master (10 μl), and cDNA (2 μl), and RNase-free water (4 μl). Each PCR mixture (20 μl) was run at 95 °C (30 s), 45 cycles of 95 °C (5 s), 55 °C (20 s), and then 72 °C (15 s) using an QuantStudio™ Real-Time PCR System (Applied Biosystems™, USA). The comparative method was used to estimate the relative expression levels of genes (Livak and Schmittgen 2001). The C_T values of target genes in SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} cells were compared to the C_T values obtained from the untreated respective control cells, which was normalized by the reference gene (16S rRNA).

Table 1 Primer sequences used in qPCR analysis for *Staphylococcus aureus*

Gene	Molecular function	Primer name and sequence*
16S rRNA	Reference gene	F: CATGCTGATCTACGATTACT R: CCATAAAGTTGTTCTCAGTT
dnaK	Chaperone proteins	F: ACTTCGTCCGGGTTTACTCC R: ACAATGGAACCTACACGCCA
groEL	Chaperone proteins	F: CAGTACCACCACCTGCAACA R: TGCAGCAAGTGAAACAGAGC
mepR	Efflux pump regulator	F: TCGATGCACAAGATACGAGA R: GCGATACGAGTGTTTGTTCC
norA	Multidrug efflux pump	F: TCGTCTTAGCGTTCGGTTTA R: TCCAGTAACCATCGGCAATA
norB	Multidrug efflux pump	F: AGCGCGTTGTCTATCTTTCC R: GCAGGTGGTCTTGCTGATAA

*F, forward; R, reverse

Statistical analysis

All experiments were carried out in three biological replicates. The obtained data were analyzed by the Statistical Analysis System (SAS) software. The general linear model (GLM) and least significant difference (LSD) procedures were applied to compare among SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} persister cells at $p < 0.05$.

Results and discussion

In this study, ciprofloxacin, the gyrase inhibitor, was used to induce persister cells. This study demonstrates the effect of ciprofloxacin treatment on the characteristic features of recovered persister cells formed by various antibiotic-resistant *S. aureus* strains (SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR}). Not only antibiotic-resistant bacteria but also antibiotic-tolerant persisters can cause serious health problems, resulting in antibiotic treatment failure (Dörr et al. 2010; Giraud et al. 2017). However, the formation of persister cells and their characteristics still remain unclear. Therefore, in this study, the phenotypic and transcriptomic properties were analyzed to characterize the persistence of various antibiotic-resistant *S. aureus* strains in response to ciprofloxacin.

Induction of persister cells

The inactivation characteristics of SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} were examined as a function of ciprofloxacin concentration as shown in Fig. 1. The dose-dependent biphasic killing patterns were observed for SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} treated with different ciprofloxacin concentrations ranging from zero- to twofold MIC, which were characterized by rapid initial inactivation followed by distinct tailing. The non-growing subpopulation in SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} remained persistent under ciprofloxacin treatment, showing 4–5 log CFU/ml after twofold MIC of ciprofloxacin treatment (Fig. 1). This suggests that the heterogeneous bacterial populations exposed to bactericidal antibiotics elicit dose-dependent biphasic killing patterns, which are characteristic features of persister cells (Keren et al. 2004; Dörr et al. 2010; Knudsen et al. 2013). To ensure the phenotypic switching to persister cells of SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR}, the ratios of surviving persister cells after treatment with twofold MIC of ciprofloxacin were measured using a metabolic-

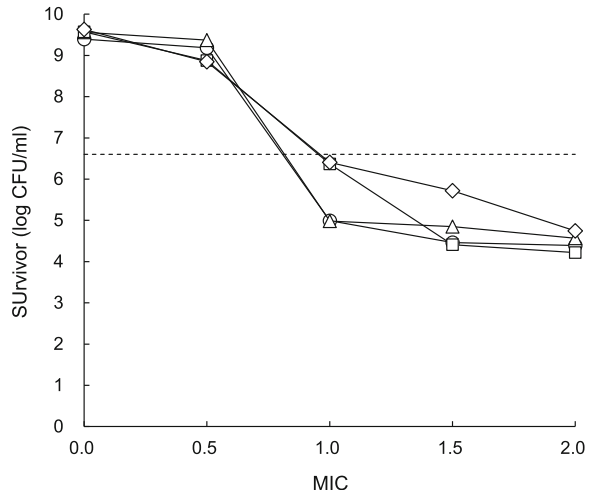

Fig. 1 Survivors of *Staphylococcus aureus* ATCC 15564 (SA^{WT}; circle), oxacillin-induced *S. aureus* (SA^{OXA}; triangle), ciprofloxacin-induced *S. aureus* (SA^{CIP}; square), and clinically isolated multidrug-resistant *S. aureus* CCARM 3080 (SA^{MDR}; diamond) treated with increasing minimum inhibitory concentrations (MICs) of ciprofloxacin at 37 °C. The MICs of ciprofloxacin against SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} were 2, 1, 32, and 32 μg/ml, respectively. The dotted line indicates inoculum levels

based assay for eliminating persister cells (Fig. 2). The highest levels of persistence frequency were SA^{MDR} (91%) and SA^{OXA} (86%) after twofold MIC of ciprofloxacin treatment, followed by SA^{WT} (78%). The least persister cells were induced in SA^{CIP} exposed to twofold MIC of ciprofloxacin, suggesting that SA^{CIP} was adapted to ciprofloxacin during the pre-exposure to ciprofloxacin. The persistence frequency varied with the classes of antibiotics and the degrees of antibiotic resistance in bacteria (Johnson and Levin 2013). The phenotypic variation is a bacterial survival strategy under unfavorable conditions, leading to the acquired resistance to stresses (Rahman et al. 2017).

Phenotypic resistance to antibiotics

The relative fitness was estimated by comparing the growth of persister cells to the growth of untreated control cells (Fig. 3). The lowest fitness cost was associated with the development of multidrug resistance that provides survival benefit during the antibiotic treatment (Patra and Klumpp 2013). The highest relative fitness of resistance was observed at SA^{CIP} persister cells (0.95), followed by SA^{MDR} (0.70) and SA^{OXA} (0.49). The least relative fitness of resistance was 0.26 for SA^{WT}, indicating the highest fitness cost. The antibiotic resistance of the re-growing persister cells of SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} treated with twofold MIC of ciprofloxacin was compared to the untreated control cells (Fig. 4). The recovered persister cells of SA^{WT} had high fitness cost (Fig. 3), resulting in the reduced frequency of resistance in the presence of antibiotics (Fig. 4) (Andersson and Hughes 2011). The various antibiotic resistances were observed for the cells recovered from the persisters of SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} exposed to twofold MIC of ciprofloxacin (Fig. 4), indicating the inherent resistance to antibiotics can affect the phenotypic properties of persister cells (Johnson and Levin 2013). The development of cross-resistance was noticeable in the recovered persister cells of SA^{OXA}, SA^{CIP}, and SA^{MDR}. The recovered persister cells of SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} showed highly enhanced resistance to erythromycin. No changes in the resistance to cephalothin,

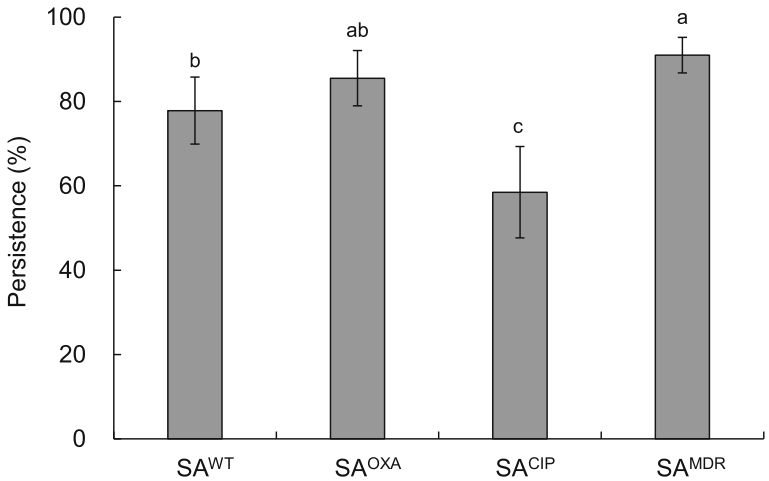

Fig. 2 Persistence rates of *Staphylococcus aureus* ATCC 15564 (SA^{WT}), oxacillin-induced *S. aureus* (SA^{OXA}), ciprofloxacin-induced *S. aureus* (SA^{CIP}), and clinically isolated multidrug-resistant *S. aureus* CCARM 3080 (SA^{MDR}) after 24-h exposure to twofold MIC of ciprofloxacin at 37 °C. The MICs of ciprofloxacin against SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} were 2, 1, 32, and 32 μg/ml, respectively. Different letters (a–c) on the bars indicate significant difference among strains at $p < 0.05$

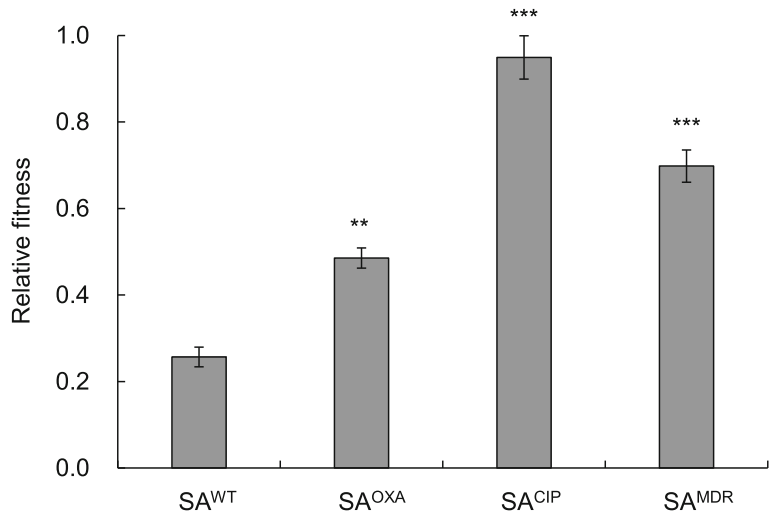

Fig. 3 Relative fitness of *Staphylococcus aureus* ATCC 15564 (SA^{WT}), oxacillin-induced *S. aureus* (SA^{OXA}), ciprofloxacin-induced *S. aureus* (SA^{CIP}), and clinically isolated multidrug-resistant *S. aureus* CCARM 3080 (SA^{MDR}) after 24-h exposure to twofold MIC of ciprofloxacin at 37 °C compared to the respective untreated control. Different letters (a–d) on the bars indicate significant difference among strains at $p < 0.05$

ciprofloxacin, and erythromycin were observed for recovered persister cells of SA^{MDR} because the untreated control cells previously acquired high-level resistance to those antibiotics. The phenotypic switching can be occurred by non-stress-mediated stochastic and stress-mediated responsive mechanisms (Kussell and Leibler 2005). The bacterial survival strategy in the presence of antibiotics is responsible for the increased mutation rates, leading to the development of antibiotic resistance (Li et al. 2017).

The antibiotic-resistant bacteria encoding pre-resistome genes can response to lethal stresses as survival mechanisms, inducing different bacterial phenotypes such as resistance, tolerance, and persistence (Morosini and Cantón 2011; Galán et al. 2013; Brauner et al. 2016). In general, the persister cells formed by environmental stresses are

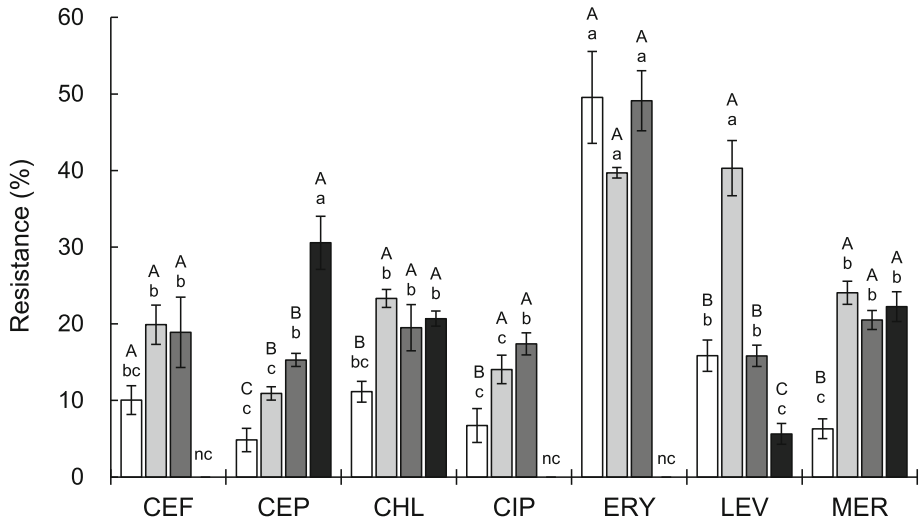

Fig. 4 Percentage resistance of the re-growing cells of *Staphylococcus aureus* ATCC 15564 (SA^{WT}), oxacillin-induced *S. aureus* (SA^{OXA}), ciprofloxacin-induced *S. aureus* (SA^{CIP}), and clinically isolated multidrug-resistant *S. aureus* CCARM 3080 (SA^{MDR}) exposed to twofold MIC of ciprofloxacin at 37 °C for 24 h compared to the respective untreated control. The MICs of ciprofloxacin against SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} were 2, 1, 32, and 32 μg/ml, respectively. Different letters (A–C) on the bars within strains are significantly different at $p < 0.05$ and different letters (a–c) on the bars within antibiotic are significantly different at $p < 0.05$. nc denotes no change in antibiotic resistance. CEF cefotaxime, CEP cephalothin, CHL chloramphenicol, CIP ciprofloxacin, ERY erythromycin, LEV levofloxacin, MER meropenem

reversible switching to parental state (Fisher et al. 2017; Nicol et al. 2019). The phenotypic changes to persister cells may play an important role in the antibiotic resistance (Rahman et al. 2017). The SOS-induced persister cells of SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} might be formed upon exposure to ciprofloxacin (Marques 2015). The phenotypic and genotypic variations of persister cells regrown in favorable condition are not distinguishable from those of antibiotic-sensitive parent cells (Fisher et al. 2017; Wu et al. 2017; Nicol et al. 2019). Persister cells have been known to be quiescent (Keren et al. 2004; Patra and Klumpp 2013; Wu et al. 2017). In contrast to previous studies, the results obtained from this study point out the variable persistence frequency, relative fitness, and resistance, depending on the nature of antibiotic resistance in *S. aureus* (SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR}).

Differential gene expression

The relative expression of stress-related genes (*dnaK* and *groEL*) and efflux pump-related genes (*mepR*, *norA*, and *norB*) was observed in the recovered persister cells of SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} treated with twofold MIC of ciprofloxacin compared to respective untreated controls (Fig. 5). The highest expression levels of *dnaK* and *groEL* genes were observed in SA^{OXA} and SA^{MDR}, showing more than fourfold increase, whereas the relative expression level of *danK* in SA^{CIP} was decreased by more than twofold (Fig. 5). The DnaK system (*dnaK*, *dnaJ*, and *grpE*) and the GroE system (*groES* and *groEL*) prevent stress-induced protein misfolding and aggregation, known as molecular chaperones (Cardoso et al. 2010). The transcriptional levels of *dnaK* and *groEL* mRNAs are increased in the presence of antibiotics, resulting in antibiotic resistance (Cardoso et al. 2010). The overexpression of *dnaK* gene in *S. aureus* can cause increases in the resistance to β-lactam antibiotics (Singh et al. 2007). The relative expression levels of efflux pump-related genes (*mepR*, *norA*, and *norB*) followed the similar pattern as those of stress-related genes (*dnaK* and *groEL*) in SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} with the exception of *norA* gene in SA^{WT}. The *norA* gene was suppressed by more than fivefold in SA^{WT}. The highest expression levels of *dnaK*, *groEL*, *mepR*, *norA*, and *norB* were observed in SA^{OXA} and SA^{MDR}, whereas those stress- and efflux pump-related genes were the least stable in SA^{CIP}. The overexpression of *mepR*, *norA*, and *norB* in SA^{OXA} and SA^{MDR} (Fig. 4) corresponds to the antibiotic resistance to different classes of antibiotics. This observation indicates a positive relationship between efflux pump activity and persistence (Rahman et al. 2017). The efflux pump systems are still active in persister cells (Pu et al. 2016; Rahman et al. 2017). The *norA* and *norB* genes encoding multidrug efflux pump belong to the major facilitator superfamily (MFS), which are mainly involved in transport of antibiotics such as norfloxacin and ciprofloxacin using the proton motive force (Costa et al. 2013). NorB plays an important role in the fitness of *Staphylococcus aureus*, leading to pathogenesis (Ding et al. 2008). The MepR, a regulatory protein, represses

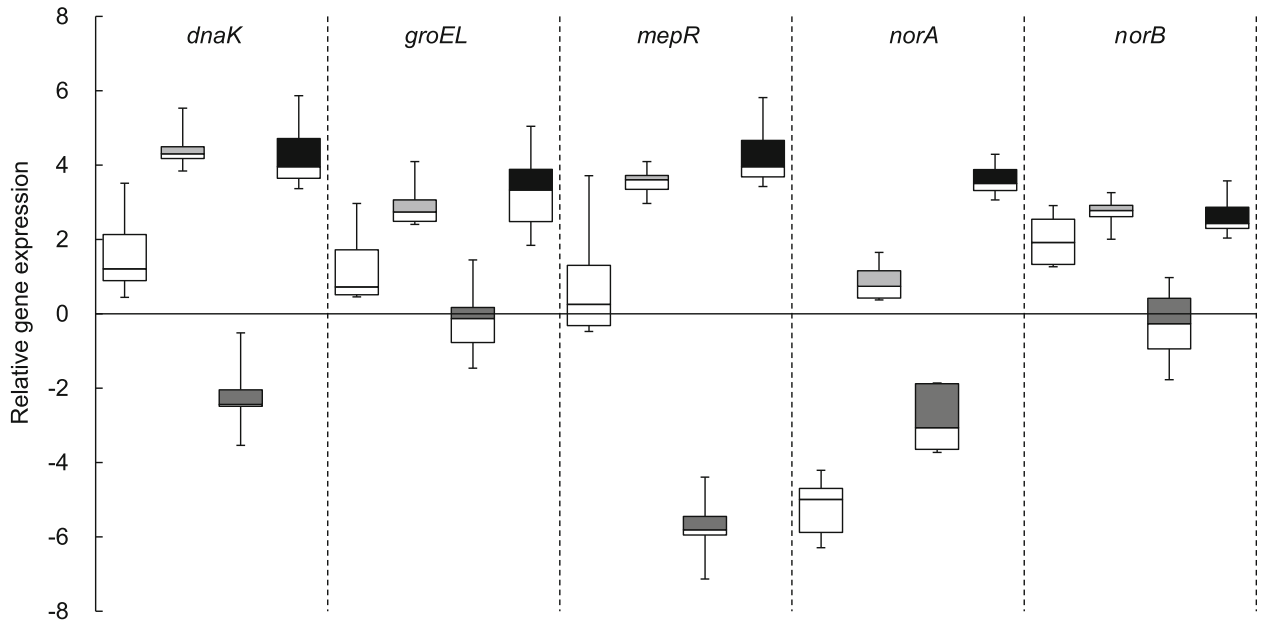

Fig. 5 Relative gene expression in the re-growing cells of *Staphylococcus aureus* ATCC 15564 (SA^{WT}; white square), oxacillin-induced *S. aureus* (SA^{OXA}; light gray square), ciprofloxacin-induced *S. aureus* (SA^{CIP}; dark gray square), and clinically isolated multidrug-resistant *S. aureus* CCARM 3080 (SA^{MDR}; black square) exposed to twofold MIC of ciprofloxacin at 37 °C for 24 h compared to the respective untreated control

the multidrug transporter (MepA), belonging to the multidrug and toxic compound extrusion (MATE) family (Kaatz et al. 2006; Costa et al. 2013).

In conclusion, this study highlights the dynamics of persister formation in different antibiotic-resistant SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR}, connecting between antibiotic resistance, tolerance, and persistence. The persister cells formed by different antibiotic-resistant SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR} exhibited the variable patterns of phenotypic and genotypic variations, showing the highest persistence frequencies in SA^{OXA} and SA^{MDR}, the lowest fitness cost in SA^{CIP}, and the development of multiple antibiotic resistance in SA^{OXA} and SA^{CIP}. The pre-existing antibiotic resistance can affect the phenotypic switching to persister cells of SA^{WT}, SA^{OXA}, SA^{CIP}, and SA^{MDR}. The persister cells formed by multidrug-resistant bacteria can easily acquire resistance to additional antibiotic treatment. The level of persistence is associated with the class and dose of antibiotics. The responses of different antibiotic-resistant bacteria to antibiotics can be useful information for designing effective antibiotic treatment regimens. Not only antibiotic-resistant bacteria but also persister cells can cause chemotherapeutic failure. Therefore, this provides valuable insight into the phenotypic and genotypic properties of persister cells.

Publisher's note

Acknowledgements

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Ethics approval

This article does not contain any studies involving human participants performed by any of the authors.

Consent to participate

Not applicable.

Authors' contributions

JD carried out all experiments and also contributed to the writing the manuscript. SW contributed to the experimental design and statistical analysis. JA conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

Funding

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2016R1D1A3B01008304).

Author details

[1]Department of Medical Biomaterials Engineering and Institute of Bioscience and Biotechnology, Kangwon National University, Chuncheon, Gangwon 24341, Republic of Korea. [2]Guangdong Provincial Key Laboratory of Aquatic Product Processing and Safety, College of Food Science and Technology, Guangdong Ocean University, Zhanjiang 524088, China.

Received: 12 September 2019 Accepted: 29 January 2020
Published online: 21 February 2020

References

Andersson DI, Hughes D (2011) Persistence of antibiotic resistance in bacterial populations. FEMS Microbiol Rev 35:901–911
Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S (2004) Bacterial persistence as a phenotypic switch. Science 305:1622–1625
Birošová L, Mikulášová M (2009) Development of triclosan and antibiotic resistance in *Salmonella enterica* serovar Typhimurium. J Med Microbiol 58: 436–441
Brauner A, Fridman O, Gefen O, Balaban NQ (2016) Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nat Rev Micro 14:320–330
Cardoso K, Gandra RF, Wisniewski ES, Osaku CA, Kadowaki MK, Felipach-Neto V, Haus LFA-Á, Simão RCG (2010) DnaK and GroEL are induced in response to antibiotic and heat shock in *Acinetobacter baumannii*. J Med Microbiol 59: 1061–1068
CLSI (ed) (2019) Performance standards for antimicrobial susceptibility testing. 29th ed. CLSI supplement M100, Wayne
Conlon BP (2014) *Staphylococcus aureus* chronic and relapsing infections: evidence of a role for persister cells. BioEssay 36:991–996
Costa SS, Viveiros M, Amaral L, Couto I (2013) Multidrug efflux pumps in *Staphylococcus aureus*: an update. Open Microbiol J 7:59–71
Deguchi H, Kitazawa K, Kayukawa K, Kondoh E, Fukumoto A, Yamasaki T, Kinoshita S, Sotozono C (2018) The trend of resistance to antibiotics for ocular infection of *Staphylococcus aureus*, coagulase-negative staphylococci, and *Corynebacterium* compared with 10-years previous: a retrospective observational study. Plos One 13:e0203705
Ding Y, Onodera Y, Lee JC, Hooper DC (2008) NorB, an efflux pump in *Staphylococcus aureus* strain MW2, contributes to bacterial fitness in abscesses. J Bacteriol 190:7123–7129
Dörr T, Vulić M, Lewis K (2010) Ciprofloxacin causes persister formation by inducing the TisB toxin in *Escherichia coli*. PLoS Biology 8:e1000317
Fasani RA, Savageau MA (2013) Molecular mechanisms of multiple toxin–antitoxin systems are coordinated to govern the persister phenotype. Proc Nat Acad Sci 110:E2528–E2537
Fernández-García L, Fernandez-Cuenca F, Blasco L, López-Rojas R, Ambroa A, Lopez M, Pascual Á, Bou G, Tomás M (2018) Relationship between tolerance and persistence mechanisms in *Acinetobacter baumannii* strains with AbkAB toxin-antitoxin system. Antimicrob Agent Chemother 62
Fisher RA, Gollan B, Helaine S (2017) Persistent bacterial infections and persister cells. Nat Rev Microbiol 15:453
Galán J-C, González-Candelas F, Rolain J-M, Cantón R (2013) Antibiotics as selectors and accelerators of diversity in the mechanisms of resistance: from the resistome to genetic plasticity in the β-lactamases world. Front Microbiol 4:9–9
Giraud E, Rychlik I, Cloeckaert A (2017) Antimicrobial resistance and virulence common mechanisms. Front Microbiol 8
Girgis HS, Harris K, Tavazoie S (2012) Large mutational target size for rapid emergence of bacterial persistence. Proc Nat Acad Sci 109:12740–12745
Harms A, Maisonneuve E, Gerdes K (2016) Mechanisms of bacterial persistence during stress and antibiotic exposure. Science 354:aaf4268
Johnson PJT, Levin BR (2013) Pharmacodynamics, population dynamics, and the evolution of persistence in *Staphylococcus aureus*. PLoS Genet 9:e1003123
Kaatz GW, DeMarco CE, Seo SM (2006) MepR, a repressor of the *Staphylococcus aureus* MATE family multidrug efflux pump MepA, is a substrate-responsive regulatory protein. Antimicrob Agent Chemother 50:1276–1281
Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K (2004) Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett 230:13–18
Kester JC, Fortune SM (2014) Persisters and beyond: mechanisms of phenotypic drug resistance and drug tolerance in bacteria. Crit Rev Biochem Mol Biol 49: 91–101
Knudsen GM, Ng Y, Gram L (2013) Survival of bactericidal antibiotic treatment by a persister subpopulation of *Listeria monocytogenes*. Appl Environ Microbiol 79:7390–7397
Kussell E, Leibler S (2005) Phenotypic diversity, population growth, and information in fluctuating environments. Science 309:2075–2078
Lewis K (2007) Persister cells, dormancy and infectious disease. Nat Rev Micro 5: 48–56
Lewis K (2010) Persister cells. Ann Rev Microbiol 64:357–372

Li J, Xie S, Ahmed S, Wang F, Gu Y, Zhang C, Chai X, Wu Y, Cai J, Cheng G (2017) Antimicrobial activity and resistance: influencing factors. Front Pharmacol 8
Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Method 25:402–408
Lowy FD (1998) *Staphylococcus aureus* infections. N Eng J Med 339:520–532
Marques CNH (2015) Isolation of persister cells from biofilm and planktonic populations of *Pseudomonas aeruginosa*. Bio-protocol 5:e1590
Morosini M-I, Cantón R (2011) Emergence and spread of antibiotic resistance following exposure to antibiotics. FEMS Microbiol Rev 35:977–991
Nicol M, Mlouka MAB, Berthe T, Di Martino P, Jouenne T, Brunel J-M, Dé E (2019) Anti-persister activity of squalamine against *Acinetobacter baumannii*. Int J Antimicrob Agent 53:337–342
Patra P, Klumpp S (2013) Population dynamics of bacterial persistence. PLoS One 8:e62814
Pu Y, Zhao Z, Li Y, Zou J, Ma Q, Zhao Y, Ke Y, Zhu Y, Chen H, Baker MAB, Ge H, Sun Y, Xie XS, Bai F (2016) Enhanced efflux activity facilitates drug tolerance in dormant bacterial cells. Mol Cell 62:284–294
Rahman T, Yarnall B, Doyle DA (2017) Efflux drug transporters at the forefront of antimicrobial resistance. Eur Biophys J 46:647–653
Sahukhal GS, Pandey S, Elasri MO (2017) msaABCR operon is involved in persister cell formation in Staphylococcus aureus. BMC Microbiol 17:218
Sakoulas G, Eliopoulos GM, Moellering RC Jr (2006) Adaptation of methicillin-resistant *Staphylococcus aureus* in the face of vancomycin therapy. Clin Infect Dis 42:S40–S50
Singh VK, Utaida S, Jackson LS, Jayaswal RK, Wilkinson BJ, Chamberlain NR (2007) Role for *dnaK* locus in tolerance of multiple stresses in *Staphylococcus aureus*. Microbiol 153:3162–3173
Wu S, Yu P-L, Flint S (2017) Persister cell formation of *Listeria monocytogenes* in response to natural antimicrobial agent nisin. Food Cont 77:243–250