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Review

Virulence alterations in staphylococcus aureus upon treatment with the sub-inhibitory concentrations of antibiotics

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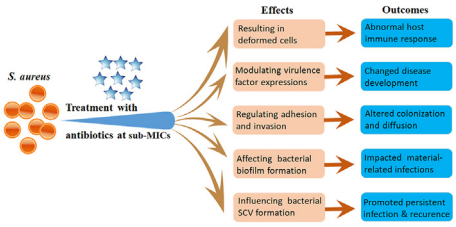
HIGHLIGHTS

- Sub-MICs of antibiotics cause deformed *S. aureus* cells.
- Antibiotics at sub-MICs affect the expression of *S. aureus* virulence factors.
- Sub-MICs of antibiotics impact on the adhesion and invasion of *S. aureus*.
- Sub-MICs of antibiotics moderate *S. aureus* biofilm formation.
- Antibiotics at sub-MIC levels affect SCV production of *S. aureus*.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: The treatment of patients with *Staphylococcus aureus* infections mainly relies on antistaphylococcal regimens that are established with effective antibiotics. In antibiotic therapy or while living in nature, pathogens often face the sub-inhibitory concentrations (sub-MICs) of antibiotics due to drug pharmacokinetics, diffusion barriers, waste emission, resistant organism formation, and farming application. Different categories of antibiotics at sub-MICs have diverse effects on the physiological and chemical properties of microorganisms. These effects can result in virulence alterations. However, the mechanisms underlying the actions of antibiotics at sub-MICs on *S. aureus* virulence are obscure.

Aim of review: In this review, we focus on the effects of sub-MICs of antibiotics on *S. aureus* virulence from the aspects of cell morphological change, virulence factor expression, bacterial adherence and invasion, staphylococcal biofilm formation, and small-colony variant (SCV) production. The possible mechanisms of antibiotic-induced *S. aureus* virulence alterations are also addressed.

Key scientific concepts of review: Five main aspects of bacterial virulence can be changed in *S. aureus* exposure to the sub-MIC levels of antibiotics, resulting in deformed bacterial cells to stimulate abnormal host immune responses, abnormally expressed virulence factors to alter disease development, changed bacterial adhesion and invasion abilities to affect colonization and diffusion, altered biofilm formation to potentate material-related infections, and increased SCV formation to achieve persistent infection and recurrence. These advanced findings expand our knowledge to rethink the molecular signaling roles of antibiotics beyond their actions as antimicrobial agents.

Introduction

The escalating crisis of antimicrobial resistance due to the extensive and indiscriminate use of antibiotics and their leakage

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into the environment has garnered appeal. The antibiotic resistance of a certain bacterium is phenotypically determined by minimum inhibitory concentrations (MICs), which are defined as the lowest antibiotic concentrations that can inhibit the growth of pathogens in a medium after 18–24 h of culture *in vitro.* During antibiotic therapy or while living in nature, however, pathogens may encounter the sub-inhibitory concentrations (sub-MICs) of antibiotics due to several situations [1,2]. First, antibiotic concentrations decrease to sub-MICs over time after administration as a result of the pharmacokinetics of antibiotics [3]. Second, the occurrence of antibiotic sub-MICs in tissues or the internal milieu of bacterial biofilms is attributed to physical and chemical barriers to antibiotic penetration, as well as to drug–drug interactions in sites other than in the blood where pathogens are in direct contact with antibiotics [4-8]. Third, antibiotic sub-MICs may be generated after waste emissions from hospitals and other treatment facilities or pharmaceutical manufacturers. Fourth, antibiotic sub-MICs can occur owing to bacterial evolution and drug-resistant organism formation [1]. Lastly, in stock farming, antimicrobial drugs used at sub-MICs are still allowed in some countries to promote animal production or prevent bacterial infections [9]. The important effects of sub-MICs of antibiotics on the morphology, biofilm formation, and virulence expression of Gram-positive and Gram-negative bacteria are of interest and have been intensively investigated in the last decade [10].

Staphylococcus aureus is a representative of Gram-positive bacteria and the culprit pathogen of human beings; this bacterium causes a variety of infections ranging from local suppurative infections to fetal pneumonia, pseudomembranous enteritis, pyelonephritis, pericarditis, sepsis, and brain abscess [11]. The co-infections of *S. aureus* with other prevalent pathogens, such as *Pseudomonas aeruginosa*, human immunodeficiency virus, and SARS-CoV-2, have been reported [12-14]. The pathogenicity of *S. aureus* mainly depends on the expression of multiple virulence factors, such as hemolytic toxins, including α-, β-, γ-, and δ-toxins, that can damage platelets, destroy lysosomes, and cause local ischemia and necrosis; Panton-Valentine leucocidin (PVL), a pore-forming toxin that destroys white blood cells and macrophages; eight serotypes of enterotoxins, namely, A, B, C_1, C_2, C_3, D, E, and F; toxic shock staphylococcal toxin 1 (TSST-1), coagulase, and staphylococcal protein A (SpA) [11]. In addition, the factors responsible for bacterial adherence, motility, and biofilm formation are associated with the virulence of *S. aureus*. Accumulated studies have demonstrated that *S. aureus* can develop genetic and phenotypic variations and alter its virulence expression to adapt to stress environments with the sub-MICs of antibiotics [1,2].

The effect of antibiotics at sub-MIC concentrations on *S. aureus* was first investigated in 1940 by Gardner, who observed the morphological changes of *S. aureus* upon exposure to 1/4 MIC of penicillin *in vitro* [15]. Different categories of antibiotics may have diverse effects on bacterial morphology and virulence production. However, some research results are contradictory, and the mechanisms underlying antibiotic actions at sub-MICs remain obscure. The sub-MICs of β-lactams have been shown to enhance the expression of *S. aureus* exotoxins and adhesion factors positively [16,17]. By contrast, the production of *S. aureus* toxins is significantly suppressed under exposure to the sub-MICs of antimicrobial agents, such as lincosamides and oxazolidinones, which target microbial ribosomes [18]. The most studies concerning the effects of antibiotics at sub-MIC concentrations on the virulence of *S. aureus* have been conducted *in vitro*, and the *in vivo* researches are rare and encouraging [1,4,5,18]. In this review, we summarized the *in vitro* effects of the sub-MICs of antibiotics on the virulence of *S. aureus* from the aspects of morphology, virulence expression, bacterial adherence and invasion, biofilm formation, and small-colony variant (SCV) production. The *in vivo* effects of antibiotics at sub-MICs on the virulence of *S. aureus* were also addressed in a separate section.

Effects of the sub-MICs of antibiotics on the morphology of *S. aureus*

In general, intact bacteria are rod-shaped (bacilli), spherical (cocci), and spiral-shaped (spirilla). Bacilli are most common, followed by cocci, and spirilla are relatively rare. *S. aureus* is spherical. When cultured in agar plates, *S. aureus* colonies present round and swollen shapes, smooth surfaces, and neat edges with or without golden yellow pigments. Treatment with antibiotics at sub-MICs can cause three main types of morphological changes in *S. aureus*, specifically, cell morphology deformation, cell wall component changes, and cell wall breakdown [19-21]. For example, enlarged or damaged methicillin-resistant *S. aureus* (MRSA) subsp. aureus USA300_FPR3757 and methicillin-susceptible *S. aureus* (MSSA) subsp. aureus Rosenbach American Type Culture Collection (ATCC®) 25923™ cells with reduced adhesiveness, double cells attached to each other, or *S. aureus* cells with emerging holes are observed upon exposure to the 1/2 to 1/8 MIC of dicloxacillin, cefodizime, cefotaxime, or ceftriaxone [19,20]. A 1/2 MIC concentration of cefodizime presents the greatest effect on the damage of *S. aureus* ATCC® 25923™, and the morphological changes are also observed at 1/4 and 1/8 MIC concentration of cefodizime but to a lesser extent [20]. The ciprofloxacin (CFX) at 1/2 MIC (64 μg/mL) and its membrane-targeting-modified derivatives CFX-ester-PPh3 (1/2 MIC = 5.56 μg/mL) and CFX-amide-PPh3 (1/2 MIC = 1.39 μg/mL) can induce dramatically morphological changes, such as thinned and irregular deformation, membrane disruption, loss of cell contents in the MRSA strain 5016 [22]. The cell membrane permeability increases in a dose-dependent manner in MRSA252 treated with berberine at sub-MICs (1/8 to 1/2 MIC), as a result, the doughnut shaped cells appear under the transmission electron microscope observation [23]. Juma *et al.* reported that a combination of 20% MIC of sophorolipid biosurfactant (0.4%, v/v) and 80% MIC of tetracycline (0.4 μg/mL) can result in significantly morphological changes in MRSA ATCC® 43300™ with larger cell diameters (from 758 ± 75 to 1276 ± 220 nm, $P < 0.01$) and increased bacterial core stiffness (from 205 ± 46 to 396 ± 66 mN/m, $P < 0.01$) compared with those in bacteria treated with tetracycline alone [24]. When exposure to 1/4 MIC of thioridazine (4 μg/mL), MRSA USA300_FPR3757 cells present thickened and irregular cell walls with a shortage of intracellular amino acids [19]. The 1/2 MIC of ceftaroline (0.25 μg/mL) can cause cell wall breakdown and cell-shape deformation in MRSA strains 06/1483 and 05/3291 [25]. Treatment with 1.5 μM of the membrane-active lipopeptide compound $C_{10}OOC_{12}O$, MRSA USA300 cells exhibit sustained mild membrane damages, and Hershkovits *et al.* speculated that such damaged bacterial membrane may not yet damaged enough at sub-MIC concentration of lipopeptide to the leakage of larger chemical molecules, such as ATP, which indeed happens at the MIC concentration of $C_{10}OOC_{12}O$ (12.5 μM in Luria Bertani medium) [26].

Different antibiotics at sub-MIC concentrations may have different effects on the morphology of *S. aureus* cells. The 1/2 MIC of desleucyl-oritavancin (10 μg/mL) can cause cell wall disorders with reduced cell wall cross-linking efficiencies and increased *N*-deacetylated muropeptides in *S. aureus* ATCC® 6538P™ [27], whereas the sub-MICs (1/2 to 1/32 MIC) of ciprofloxacin and amikacin have no effects on the morphology of a clinical *S. aureus* isolate [28]. In principle, the sub-MICs of certain antibiotics can weaken *S. aureus* cell walls through binding to penicillin-binding proteins (PBPs), such as methicillin and cefoxitin [29]. Moreover, some antimicrobial agents, such as vancomycin, may change peptidoglycan cross-linking via binding to

the D-alanyl-D-alanine moieties of lipid II, a precursor of cell wall biosynthesis [27,30]. Others, such as tunicamycin at sub-MICs of 2.5 and 5 μg/mL, can promote cell deformation by targeting and altering the cell wall teichoic acid in *S. aureus* strains ATCC® 29213™, ATCC® 43300™, ATCC® 25923™, and PRI 4656 [31]. Still others, like oritavancin, may cause cell wall disorders by binding to cross-linked peptidoglycans in cell walls to interfere with cell wall maturation [27]. All these possible mechanisms are summarized and presented in Fig. 1.

Although the effects of the sub-MICs of antibiotics on *S. aureus* morphology may exhibit strain-to-strain differences, three main effects are exerted after the formation of deformed *S. aureus* upon treatment with the sub-MICs of antibiotics. First, in combination with antibiotics, *S. aureus* cell deformations may hinder antibiotic resistance or facilitate death, thus resulting in sub-MIC concentration of drug-induced antibiotic sensitivity. Thorsing *et al.* reported that 1/4 MIC of thioridazine can be able to sensitize MRSA USA300 to the antimicrobial effect of dicloxaillin by reduction of the cell viability by 3 log10 CFU/mL compared with that under dicloxaillin-treatment alone [19]. The combination of sub-MIC concentrations of sodium new houttuyfonate and berberine chloride offers a synergistic action against MRSA strains ATCC® 43300™ and ATCC® 33591™, as well as a vancomycin-intermediate *S. aureus* strain Mu50 [32]. Second, *S. aureus* with poorly cross-linked cell walls after treatment with antibiotics at sub-MICs may release a large number of toxins and other pathogenic factors to aggravate the inflammatory response in the host [33]. Lastly, changes in *S. aureus* cell wall components after sub-MICs of antibiotic treatment may affect the adhesion and aggregation capabilities of staphylococcal cells, resulting in decreased colonization [19,21]. However, whether and how antimicrobial susceptibility, host immune response, and bacterial pathogenicity in deformed *S. aureus* bacteria are changed upon exposure to sub-MICs of antibiotics remain largely unknown, and further investigations are suggested to illustrate these points.

Effects of sub-MICs of antibiotics on the expression profiles of *S. aureus* virulence factors

Bacterial virulence refers to the degree of the pathogenicity of a certain pathogen to its host. Increasing data have shown that the sub-MICs of antimicrobial agents can alter the expression levels of bacterial toxins and those of factors responsible for colonization and invasion. Recently, Hodille *et al.* reviewed the effects of the diverse categories of antibiotics on *S. aureus* toxin production and host immune response [10]. They concluded that treatment with the sub-MICs of ribosome-targeting antibiotics and cell wall-active agents result in opposing manifestations of *S. aureus* virulence factor expression, with the former resulting in decreased production and the latter increasing expression. Such information is important for the establishment of valuable therapeutic regimens to improve patient outcomes in case of *S. aureus* infections. Table 1 lists the effects of the sub-MICs of different antibiotics on the expression of virulence factors in *S. aureus*.

The effects of antimicrobial agents on *S. aureus* virulence factor expression are commonly investigated by reporter fusion assays for specific gene promoters of interest, the mRNA quantitation of virulence factors, transcriptomic profiling, and the measurement of certain virulence proteins and proteomic profiles [10]. The effect of antibiotics on the production of α-toxin, a major virulence factor that contributes to staphylococcal disease, was first explored by Kernodle *et al.* [44]. They found that nafcillin-nonsusceptible and -susceptible *S. aureus* strains display increased α-toxin expression when treated with 0.006 to 0.1 μg/mL of nafcillin compared with the untreated bacteria. Further studies confirmed that α-toxin expression increases upon β-lactam exposure, and such β-lactams seemingly induce increased α-toxin production in MRSA by up to 30-fold relative to that in MSSA strains [45]. Other investigations have shown that α-toxin expression reduces after treatment with the sub-MICs of fusidic acid, erythromycin, or aminoglycosides (Table 1).

PVL is another *S. aureus*-produced pore-forming toxin that contributes to bacterial pathogenesis. The sub-MICs (ranging from 1/8 to 1/2 MIC) of β-lactams, including oxacillin and nafcillin, can induce an increase in the production of PVL against diverse *S. aureus* backgrounds (ST1, ST8, ST80, and ST59) [16,17,39], whereas other antibiotics, such as tedizolid and linezolid at 1/16 MIC, result in decreased PVL production in MRSA 1560, and the decreased mRNA levels of PVL are also observed in MRSA USA300 after treatment with sub-MICs (1/8 to 1/2 MIC) of clindamycin, linezolid, and tigecycline [2,39]. Studies have demonstrated that the antibiotics active in protein synthesis inhibition exert varied effects on TSST-1 production with the most remarkable inhibitory effects (Table 1). Most recently, our group revealed that only the sub-MICs of β-lactam antibiotics, such as oxacillin, methicillin, cefox-

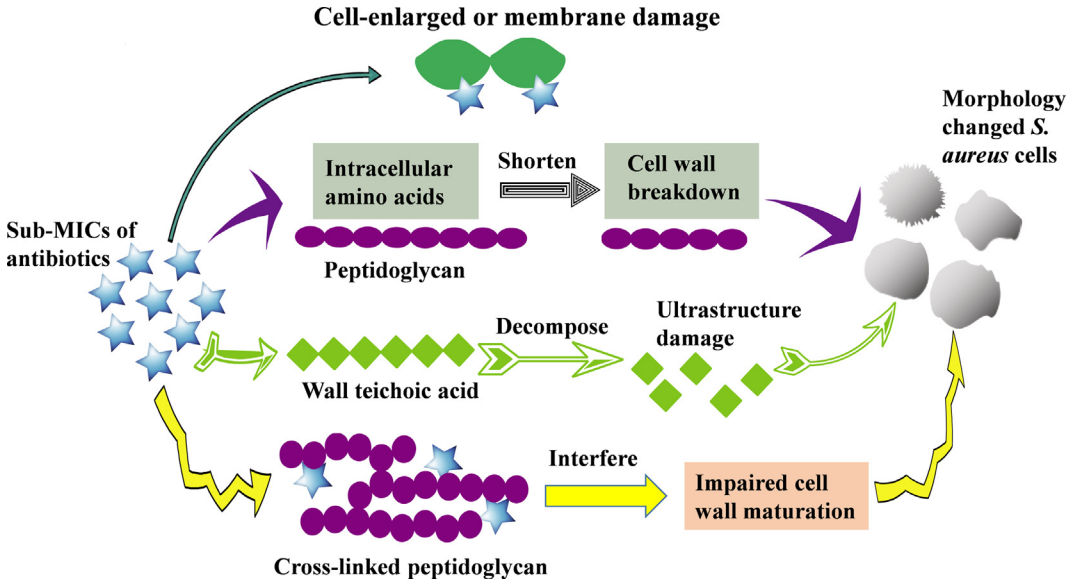

Fig. 1. The potential mechanisms underlying the formation of *S. aureus* deformation cells upon treatment with antibiotics at sub-MICs.

Table 1
The expression levels of virulence factors in *S. aureus* cultured in the presence of sub-MICs of antibiotics compared with those in *S. aureus* without antibiotic-treatment.

Antibiotics	Hla	PVL	TSST-1	PSM	SPA	Coagulase	Enterotoxins A and B	References
Penicillin	N	N	N	N	↑	N	N	[10]
Methicillin	↑	N	N	N	–	–	N	[34,35]
Oxacillin	N	↑	N	↓	↑	N	N	[10]
Nafcillin	↑	↑	N	N	N	N	N	[2,10]
Imipenem	N	↑	N	N	N	N	N	[10]
Gentamicin	N	N	↓	N	N	N	N	[10]
Erythromycin	↓	N	N	↑	N	N	N	[10]
Flucloxacillin	N	N	↓	N	N	N	N	[10]
Roxithromycin	N	N	N	N	N	↓	N	[35]
Enoxacin	↓	N	N	N	–	–	N	[34,35]
Ciprofloxacin	↓	N	N	N	–	–	N	[35]
Azithromycin	↓	N	N	N	N	N	N	[36]
Lincomycin	N	N	N	N	N	↓	N	[34]
Clindamycin	↓	↓	↓	↓	↓	↓	N	[10,34,36,37]
Fosfomycin (FOM)	↓	N	N	N	N	N	N	[38]
Tigecycline	↓	↓	↓	N	↓	N	N	[10,39]
Vancomycin	N	–	–	N	–	N	N	[39]
Daptomycin	N	–	N	N	–	N	N	[39]
Linezolid	↓	↓	↓	↑	↓	N	↓	[10,39]
Tedizolid	↓	↓	↓	↓	N	N	N	[2]
Mupirocin	↓	N	N	N	N	N	N	[37]
Costus oil	↓	N	↓	N	N	N	N	[21]
Fusidic acid	↓	N	N	N	N	N	N	[40]
C10OOc12O	↓	N	N	N	N	N	N	[26]
LP5	↓	N	N	N	↑	N	↓	[41]
Thymol	↓	N	N	N	N	N	↓	[21]
Chlorogenic Acid	↓	N	N	N	N	↓	N	[21]
Sclareol	↓	N	N	N	N	N	N	[42]
L-NPDNJ	↓	N	N	N	N	N	N	[43]

"↑", the expression level increased; "↓", the expression level decreased; "-", comparable in the expression level; N, not determined.

itin, imipenem, and meropenem, but not vancomycin, chloramphenicol, erythromycin, and kanamycin, can promote the expression of a cluster of lipoprotein-like genes (*sa2275-sa2273*) encoding important virulence factors that contribute to MRSA pathogenicity [29]. A natural terpenoid (+)- nootkatone at a sub-MIC of 50 μg/mL suppresses the expression levels of *sarA*, *agrA*, *RNAIII*, and *spa*, which regulate the expression of toxins, in both MRSA strain SJTUF 20758 and MSSA strain ATCC® 25923™ [46].

The mechanisms underlying the actions of antibiotics at sub-MICs on *S. aureus* virulence expression are multifaceted. Antibiotics at sub-MICs can not only regulate the expression of toxins (e.g., α-toxin, PVL, SpA, PSM, and enterotoxins) through quorum sensing systems; two-component systems; or other regulators, such as SaeRS, SarA, Rot, and CcpA, but also directly bind to some amino acids of toxin proteins to affect their functions. For example, fosfomycin inhibits the activity of *S. aureus* α-toxin by binding to its Lys154 and Asp108 [38]. Furthermore, antibiotics targeting the 50S ribosome of bacteria can impair bacterial transcription and result in the accumulation of mRNA and transcription complex intermediates, thereby resulting in the accumulation of toxin mRNA [47].

Virulence factors implement their pathogenic roles either by host cell destruction or cell regulation pathway utilization. For example, *S. aureus* α-toxin may promote host inflammatory necrosis through the MAPK-associated signaling pathway or inflammasome-mediated inflammation [38]. An *et al.* reported that fosfomycin at a sub-MIC concentration (4 μg/mL) reduces the expression of α-toxin in *S. aureus* 8325–4, and the phosphorylation levels of p38, ERK, and JNK are remarkably decreased in THP-1 cells infected by fosfomycin-treated *S. aureus* relative to the untreated strain [38]. They also revealed that fosfomycin treatment can downregulate inflammasome-NLRP3-mediated inflammation in macrophages infected by α-toxin-positive *S. aureus*. These data are highly consistent with those of Morikawa *et al.*, who observed that the sub-MICs of fosfomycin can greatly diminish IL-1β levels in LPS-treated human monocytes [48].

Effects of sub-MICs of antibiotics on the adhesion and invasion of *S. aureus*

The pathogenesis of *S. aureus* is closely associated with its capacity to adhere directly to host cells or to the extracellular matrix (ECM) [49]. Bacterial adhesion is the first step in the invasion of host cells and the formation of biofilms. A repertoire of adhesive molecules, including staphylococcal Fn-binding proteins A and B, clumping factors A and B, serine aspartate repeat-containing protein D, serine-rich adhesin for platelets, and staphylococcal autolysin, are responsible for *S. aureus* adhesion [50]. The effects of the sub-MIC levels of antibiotics on *S. aureus* adhesion and invasion may vary depending on the bacterial strain and host cell model used.

The overall roles of certain antibiotics at the sub-MICs in *S. aureus* adhesion and invasion are summarized in Table 2. Maioli *et al.* reported that the role of ceftibuten, a third-generation cephalosporin, at sub-MICs (1/4 MIC to 1/2 MIC) in the clinical treatment of bacterial infection is mainly attributed to its antiadherence characteristics [72]. Sasso *et al.* demonstrated that the fluoroquinolone agent gemifloxacin at the 1/32 MIC concentration significantly reduces the adhesiveness of *S. aureus* ATCC® 25923™ to primary human mucosal epithelial cells [3]. The strain-dependent modulatory effects of antibiotics at sub-MICs on the adhesion capabilities of *S. aureus* have been observed. Lázaro-Díez *et al.* revealed that the 1/2 to 1/4 MIC levels of ceftaroline can enhance bacterial adhesion of MRSA clinical isolates 06/1483 and 05/3291, whereas MRSA strain 05/2369 presents a reduced adhesive capability at the sub-MICs of ceftaroline tested [25]. The expression levels of adhesion-associated genes (*fnbA*, *fnbB*, *clfA*, *clfB*, and *icaD*) increase by more than 5-fold in *S. aureus* strain CCARM 3080 exposed to a half MIC of levofloxacin [51]. The relative expression levels of *clfB* and *fnbB* increase in *S. aureus* strain KACC 10778 treated with oxacillin (>3-fold), whereas those of *icaA* and *icaD* genes are downregulated by more than 5-fold under 1/2 MIC of levofloxacin or oxacillin treatment. When *S. aureus* ATCC® 15564™ is used, how-

Table 2
The biofilm formation, bacterial adherence, invasion, and SCV production of *S. aureus* cultured in the presence of sub-MICs of antibiotics compared with those in *S. aureus* without antibiotic-treatment.

Antibiotics	Adhesion	Invasion	Biofilm formation	SCVs	Reference
Oxacillin	↑	N	↑	N	[51-53]
Methicillin	N	N	N	–	[54]
Ampicillin	–	↑	N	N	[55]
Amoxicillin	N	N	N	–	[54]
Ceftaroline	↑	N	↑	N	[25]
Cefalexin	N	N	↑	N	[35]
Cefodizime	↓	N	N	N	[20]
Ceftriaxone	↓	N	N	N	[20]
Gentamicin	N	N	N	↑	[54]
Kanamycin	N	N	↓	N	[56]
Amikacin	–	↑	N	N	[55]
Gemifloxacin	↓	N	N	N	[3]
Telithromycin	N	N	↓	N	[50]
Azithromycin	N	N	↓	N	[50]
Erythromycin	N	N	N	–	[54]
Ciprofloxacin	N	N	↓	–	[22,54]
Enrofloxacin	N	N	↓	N	[56]
Moxifloxacin	↑	N	N	N	[52]
Gatifloxacin	↓	N	N	N	[57]
Rufloxacin	↓	N	N	N	[58]
Tetracyclin,	N	N	N	–	[54]
Clarithromycin	N	N	↓	–	[54,56]
Clindamycin	↑	N	↓	↑	[36,50,54]
Minocycline	N	N	–	N	[59]
Vancomycin	–	N	↓	–	[52-54]
Daptomycin	N	N	↓	N	[50]
Linezolid	–	N	N	N	[52]
Mupirocin	N	N	↑	–	[54,60]
Lincomycin	N	N	↓	N	[56]
Fusidic acid	↓	N	↓	N	[40]
Rifampicin	–	–	↑	↑	[52,54,59]
Tunicamycin	↓	↓	↓	N	[31]
Colistin sulfate	N	N	↓	N	[56]
Berberine	N	N	↓	N	[56]
RP557	N	N	↓	N	[61]
Nisin	↓	N	↓	N	[62]
Glyceryl trinitrate	N	N	↓	N	[63]
Alpiniapurpuratalectin	N	N	↓	N	[64]
Carboxymethyl chitosan	N	N	↓	N	[65]
Eugenol	N	N	↓	N	[66]
Chlorogenic acid	↓	N	N	N	[21]
Acetylisovaleryltylosin tartrate	N	N	↑	N	[56]
Plantaricin GZ1-27	N	N	↓	N	[67]
IMD0354	N	N	↓	N	[68]
L-NPDNJ	N	N	↓	N	[43]
(+)-Nootkatone	N	N	↓	N	[47]
ZnO-Ag NPs	N	N	↓	N	[69]
Chitosan(CS)	N	N	↓	N	[70]
Syph-1	N	N	↓	N	[71]

"↑", increased; "↓", decreased; "-", comparable (no effects); N, not determined.

ever, most adhesion-related genes are slightly upregulated in *S. aureus* cultured with levofloxacin or oxacillin at 1/2 MIC [51]. Sasso *et al*. showed that 1/32 and 1/64 MIC of gatifloxacin remarkably inhibits the adhesiveness of *S. aureus* ATCC® 25923™ to human epithelial cells [57]. Moreover, *S. aureus* may display an antibiotic-specific adhesion reaction under treatment with the sub-MICs of antimicrobial agents. Using fibronectin-coated microplates and human osteoblasts, Rasigade *et al*. observed that treatment with 1/2 MIC of oxacillin (0.125 μg/mL), linezolid (0.5 μg/mL), or moxifloxacin (0.06 μg/mL) results in a hyperadhesive phenotype of *S. aureus* reference strain 8325–4 with an increase in *fnbA*/*B* transcription. As a result, oxacillin-treated *S. aureus* cells present greatly increased adhesion capability (682% ± 374%) compared with the untreated strain (256% ± 128%) in the infection of osteoblastic MG-63 cells [52]. By contrast, exposure to 1/2 MIC of rifampin (0.003 μg/mL) decreases fibronectin adhesion in *S. aureus* 8325–4 and five clinical *S. aureus* isolates DU5883, SST2008 1028, ST2008 0563, HT2000 0594, and HT2001 0390. Gentamicin and vancomycin treatment exerts no effect on fibronectin binding or *fnbA*/*B* expression levels in *S. aureus* [52]. Braga *et al*. demonstrated that 1/2 MIC concentration of cefodizime or cefotaxime exhibits the greatest inhibitory effect on the adhesion of *S. aureus* ATCC® 25923™, with a gradual reduction from 1/2 to 1/16 MIC [20].

S. aureus can invade and propagate in mammalian cells [36]. After adhesion, subsequent invasion may occur during the infection of suitable host cells by *S. aureus*. However, few studies have been performed to investigate the effects of the sub-MICs of antibiotics on *S. aureus* invasion partially because bacterial invasion is a dynamic process and difficult to observe. Hu *et al*. found that 1/2 MIC of clindamycin (0.1 μg/mL) but not azithromycin (1 μg/mL) results in the loss of the invasive capacity of *S. aureus* ATCC® 51650™ to enter primary human nasal epithelial cells; the infection rate of antibiotic-treated bacteria is 6.3% ± 0.76%, whereas that of bacteria without antibiotic treatment is 49% ± 9.8% [36]. In some cases, the sub-MICs of antibiotics that promote *S. aureus* adhesion capacity do not correlate well with bacterial invasion capability.

Rasigade *et al*. observed that 1/2 MIC (0.125 μg/mL) of oxacillin-treated *S. aureus* strain 8325–4 presents increased bacterial adhesion to cultured osteoblasts, whereas oxacillin-treated bacteria exhibits comparable bacterial invasion capacity for osteoblast cells (9.2% ± 4.1%) relative to untreated *S. aureus* (6.0% ± 5.1%) [52]. These results imply that the relevance of *S. aureus* adhesion and invasion regulation during infections with or without antibiotic treatment remains unclear and requires further investigation.

Effects of sub-MICs of antibiotics on *S. aureus* biofilm formation

The biofilm is a microbial community that forms as microorganisms adhere to each other and grow on biotic or abiotic surfaces, such as artificial heart valves and catheters [73]. The developmental cycle of a biofilm usually includes four steps: (i) the attachment of microbial cells to a surface; (ii) the assembly of the attached cells into a microcolony; (iii) the growth of microbial cells into a mature biofilm; and (iv) the detachment of the fully developed biofilm [74,75]. In addition to microbial cells, a typical biofilm contains microorganism-secreted adhesive ECM, which constitutes 90% of the biofilm volume [76]. The constituent of the biofilm ECM may vary depending on microbial species. The ECM of *S. aureus* comprises polysaccharides, extracellular DNA, and proteins and can protect pathogens from inactivation by antimicrobial agents, including antibiotics and chemical disinfectants [77,78]. Killing pathogenic cells in biofilms with appropriate antibiotics is 1000-fold more difficult than killing planktonic cells [79]. Thus, pathogen-formed, biofilm-associated infections are commonly persistent and become a tremendous health burden in our world [80-82]. At least 65% of all chronic bacterial infections are related to biofilms, which can cause a wide variety of disease manifestations, including endocarditis, urinary tract infections, and catheter infections [83-86]. The sub-MICs of antibiotics may affect biofilm formation by interfering with certain developmental steps, and many exploratory findings on the effects of sub-MICs of antibiotics on biofilm formation are crucial for setting up new options to treat biofilm-related *S. aureus* infections [87].

The effects of antibiotics at sub-MIC concentrations on *S. aureus* biofilm formation can vary depending on the tested strain, associated biofilm stage, and antibiotic used. The sub-MICs of most antibiotics inhibit the development of *S. aureus* biofilm, whereas those of several antibiotics, such as oxacillin, ceftaroline, mupirocin, and rifampicin (Table 2), can promote staphylococcal biofilm formation [25,59,60]. Sritharadol *et al*. compared the effects of sub-MICs of mupirocin with those of cefazolin, levofloxacin, gentamicin, and erythromycin on the biofilm formation of MRSA and MSSA [60]. Confocal laser microscopy revealed that the biofilm structure, cell viability, and cell density of the mupirocin-treated groups increase compared with those of untreated MRSA strains USA300 and ATCC® 43300™, and MSSA strain ATCC® 29213™. The expression level of the *RNAIII* gene in biofilms of MRSA USA300 displays the highest increase upon initial exposure (3 h) to mupirocin at 1/6 MIC level (0.17 mg/mL), and is then downregulated (6 h), whereas the expression levels of the other two biofilm formation-related regulatory genes, *agrA* and *sarA*, in strain USA300 shows no obvious difference in the presence or absence of mupirocin [60]. These findings indicate that sub-MICs of mupirocin can promote the strain-dependent biofilm formation of *S. aureus* and that the *RNAIII* gene may play important roles in the early attachment stage of staphylococcal biofilm formation. However, the exact function of *RNAIII* factor and the mechanism underlying *S. aureus* biofilm promotion remains unknown and is of great interest for later investigations.

The sub-MIC levels of the macrocyclic antibiotic rifampicin greatly promotes biofilm formation in seven out of 10 *S. aureus* isolates that are nonbiofilm producers in common tryptic soy broth (TSB) [59]. Multiplex PCR detection revealed that *icaA* and *icaB* genes are present in all 10 investigated *S. aureus* strains. The role of the *ica* operon, which encodes enzymes in rifampicin-induced *S. aureus* biofilm production, has yet to be elucidated given that biofilm formation by *S. aureus* can be mediated by either *icaADBC*-independent or -dependent pathways [88]. Haddadin *et al*. showed that a wide range of the sub-MICs (2.81%–45%) of cefalexin has great potential to induce the formation of *S. aureus* National Collection of Type Cultures (NCTC) 11962 biofilm [35]. The authors proposed that cephalexin-induced *S. aureus* biofilm formation is associated with bacterial hydrophobicity. Owing to its inhibitory role in cell wall synthesis, cefalexin at 1/16 MIC can increase the hydrophobicity of the cell surface of *S. aureus* strain NCTC 11962; this effect may lead to increased cell adhesion during biofilm formation [35].

Except for a few antibiotics with biofilm-promoting potential, most antistaphylococcal agents show inhibitory effects on *S. aureus* biofilm formation. Jo and Ahn evaluated the biofilm-forming capacity of *S. aureus* strains KACC 10778, ATCC® 15564™, and CCARM 3080 grown in the presence of oxacillin and levofloxacin at sub-MICs and found that the number of the biofilm-forming *S. aureus* strain KACC 10778 cells decrease by approximately 2 log10 CFU/mL under oxacillin treatment, whereas those of strains ATCC® 15564™ and CCARM 3080 decrease by 0.5–1 log10 CFU/mL under levofloxacin and oxacillin treatment [51]. The biofilm of MRSA strain ATCC® 43300™ treated with plantaricin GZ1-27 at 1/2 and 1/4 MIC decreases by 55.3% and 40.2%, respectively, accompanying with reduction of cell to cell connections [67]. Kang *et al*. showed that 1/2 MIC of CFX or CFX-PPh3 inhibits the biofilm formation of MSSA strain ATCC® 29213™ and MRSA strains 5016, 5013, and 3416 by 43.9% to 89.5% relative to the untreated strains [22]. Escober *et al*. demonstrated that the nuclear factor-kappa B inhibitor N-[3,5-Bis(trifluoromethyl) phenyl]-5-chloro-2-hydroxy benzamide at sub-MIC of 0.0313 μg/mL can inhibit the initial cell attachment and biofilm formation of vancomycin-resistant *S. aureus* strain VRS1 in a dose-dependent manner [68]. The sub-MICs of other antimicrobial compounds, such as N-nonyloxypentyl-L-DNJ, N4-benzyl-N-2-phenylquinazoline-2,4-diamine, terpenoid (+)-nootkatone, chitosan, marine steroid siphonocholin, zinc oxide, and silver nanoparticles, are found to be able to inhibit the biofilm formation of diverse *S. aureus* strains [43,46,69-71,89].

S. aureus polysaccharide intercellular adhesin, the major component of biofilm ECM, is synthesized by *ica* operon enzymes, and their related regulators can be affected by certain antibiotics at sub-MICs. For example, Zheng *et al*. revealed that the 1/4 MIC of telithromycin is greatly superior to that of azithromycin and clindamycin, as well as that of vancomycin or daptomycin, in the inhibition of *S. aureus* biofilm formation; this antibiotic acts by downregulating the RNA expression levels of *sigB*, *agrA*, *clfA*, and *icaA* in MSSA and MRSA isolates [50]. The eDNA also plays important roles in the adhesion and maturation stages of biofilm-forming *S. aureus*. Andre *et al*. revealed that sub-MIC bacteriocin nisin effectively inhibits *S. aureus* biofilm by reducing eDNA amounts and polysaccharide compositions without significantly changing protein contents [62]. Exposure to the sub-MICs of tunicamycin (0.25–0.5 μg/mL) greatly reduces the biofilm-forming capability of *S. aureus* by decreasing eDNA levels [31].

In addition to polysaccharides and eDNA, many exoproteins secreted by *S. aureus* contribute to biofilm formation; these exoproteins include accumulation-related, biofilm-associated, fibronectin-binding, extracellular-adhesive, and ECM proteins; iron-regulated surface determinant protein C; and clumping factors [50]. Antibiotics at sub-MICs may influence the formation of *S. aureus* biofilms through direct or indirect moderation of exoprotein expression. Schilcher *et al*. showed that clindamycin at the

sub-MIC levels can trigger a transcriptional stress response in tested *S. aureus* strains via SigB and increases the expression levels of biofilm-associated genes, including *fnbA*, *fnbB*, *atlA*, *irgA*, *psm*, and *agrA* [90]. The effects of the sub-MIC levels of seven antimicrobial agents (kanamycin, enrofloxacin, lincomycin, clarithromycin, acetylisovaleryltylosin tartrate, colistin sulfate, and berberine) on biofilm formation by *S. aureus* were investigated in a recent study, and the results showed that all antibiotics tested, except for acetylisovaleryltylosin tartrate, can inhibit the biofilm formation of *S. aureus* clinical strain Hb0206 effectively [56]. RT-qPCR determination revealed that all antibiotics remarkably reduce the expression levels of six *S. aureus* biofilm-associated genes (*fnbA*, *rbf*, *eno*, *lrgA*, *cidA*, and *sarA*). However, acetylisovaleryltylosin tartrate promotes these genes. Taken together, antibiotics at the sub-MICs can affect *S. aureus* biofilm formation through directly regulating biofilm-associated exoprotein genes and global modulatory genes, such as *sigB*, *sarA*, and *agrA*, which then participate in biofilm formation regulation (Fig. 2). For example, SarA can induce the formation of *S. aureus* USA300 biofilms through promoting the synthesis of polymeric N-acetyl-glucosamine (PNAG), the major polysaccharide intercellular adhesions of staphylococcal ECM [60].

Although strain-to-strain differences can exist upon exposure to antibiotics at sub-MICs, several antibiotics do not exert effects on *S. aureus* biofilm formation. Examples of this phenomenon include the sub-MICs of minocycline on five clinical and five nonclinical *S. aureus* isolates [59], sub-MICs of rifampicin on nonclinical *S. aureus* H01 [59], and sub-MICs of ceftaroline on *S. aureus* clinical strain 05/3291 [25]. Providing a full explanation for the variable responses of biofilm formation by *S. aureus* strains to antibiotics at sub-MICs is difficult. *S. aureus* strains, including patient-derived clinical isolates and healthy volunteer-isolated strains, maintain heterogeneous biofilm producer phenotypes. Despite the existence of the *ica* operon, some *S. aureus* strains, such as clinical isolate C39, do not change their weak-biofilm producer phenotypes when exposed to sub-MICs of rifampicin but instead display increased biofilm production in medium supplemented with 1.0% glucose [59]. A clear understanding of the multiple biofilm production responses of *S. aureus* strains in the presence of antibiotics at sub-MIC levels may be established through the accumulation of additional exploratory data by using different strains, various antibiotics, and unified culture conditions.

Effects of sub-MICs of antibiotics on *S. aureus* SCV formation

Bacterial SCVs are characterized as subpopulations of bacteria that display slow growth rates and atypical colonies (nearly 1/10 of the wild-type strain) and possess unusual metabolic pathways (decreased oxidative phosphorylation, deficient electron transport, or insufficient thymidine biosynthesis) [91]. *S. aureus* SCVs were first investigated in 1951 by Dr. Hale, who described a mutant of *S. aureus* that requires carbon dioxide for normal growth [92]. Thereafter, *S. aureus* SCVs with deficiency of menaquinone-, heme-, thiamine-, and thymidine-biosynthetic pathways were identified, and such SCVs formed mainly due to the antibiotic-induced or spontaneous mutation of certain metabolic genes, including *har*, *hemB*, *ctaA*, and *thyA* [91,93-95]. Currently, increasing evidence has demonstrated that *S. aureus* SCVs are of medical importance for facilitating clinically persistent and recurrent infections [54]. Approximately 70% of patients who underwent long-term antibiotic treatment exhibit *S. aureus* SCV infection [94].

Studies have shown that *S. aureus* SCVs can be induced from their parental strains by exposure to various antibiotics *in vitro* [54,96]. Zhang *et al.* conducted an induction experiment wherein the capability of 11 antibiotics to stimulate SCVs in 66 clinical *S. aureus* isolates was investigated. They found that 26 out of 66 (33.3%) clinical strains grow small colonies (≤1 mm) after 24 h

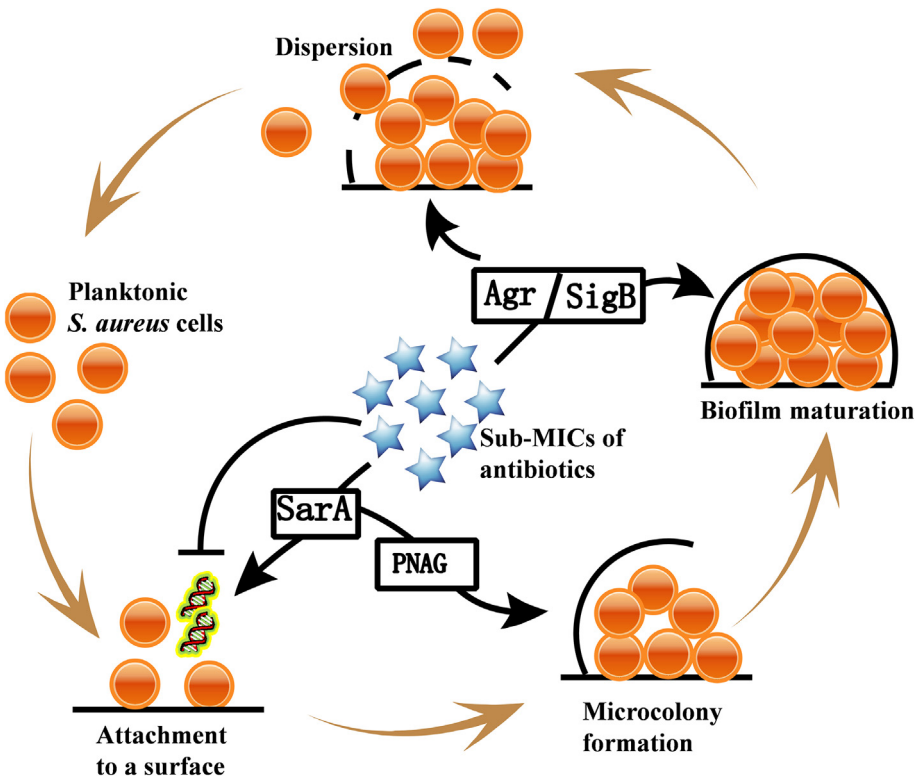

Fig. 2. The mechanisms of the effects of the sub-MICs of antibiotics on the biofilm formation of *S. aureus*. Antibiotics at sub-MIC levels affect the formation of *S. aureus* biofilms through directly modulating biofilm-associated genes or global regulatory genes, including *sigB*, *sarA*, and *agrA*, which then take part in the biofilm formation regulation. PNAG, polymeric N-acetyl-glucosamine.

of culture in TSB agar plates with the sub-MICs of gentamicin and that seven out of 66 (10.6%) strains display mixed populations containing large and small colonies [54]. Zhang *et al.* revealed that the reference *S. aureus* strain ATCC® 51650™ can switch to the SCV phenotype after 1 h of culture under treatment with 1/2 MIC of gentamicin, whereas other antibiotics can not induce *S. aureus* SCVs at short incubation times [54]. Moreover, high clindamycin and rifampicin concentrations induce *S. aureus* SCVs after 24 h of culture, whereas clarithromycin, ciprofloxacim, amoxicillin, methicillin, tetracyclin, erythromycin, vancomycin, and mupirocin may not participate in *S. aureus* SCV induction. Further studies showed that aminoglycoside gentamicin-induced *S. aureus* SCVs exhibit hemin-auxotrophic phenotypes [94]. Weaver *et al.* showed that *S. aureus* can rapidly increase resistance to rishexylaminomelamine–trisphenylguanide via the selection of a menaquinone-auxotroph SCV, which became an exclusively SCV phenotype after continuous cell passage in media with increasing drug concentrations [97]. By using skin fibroblast and lung epithelial cell infection models, Häffner *et al.* demonstrated that *S. aureus agr* mutants present a higher percentage of SCV formation in comparison with their wild-type strain 6850 [98].

The *in vivo* effects of sub-MICs of antibiotics on *S. aureus* virulence

In contrast to *in vitro* studies, the *in vivo* investigations of roles of antibiotics at sub-MICs in the virulence alterations of *S. aureus* are rare [38,61,68]. The reasons may be ascribed to the limitation of detection methods for *S. aureus in vivo*, the dynamic variation of sub-MICs of antibiotics administered, and the complicated internal physiological and chemical environments. Using an otitis media-rat model, Song *et al.* showed the effect of a sub-MIC concentration of the phyto-compound eugenol on *S. aureus* colonization *in vivo* [66]. The authors found that 1/2 MIC of eugenol is able to decrease 88% of *S. aureus* ATCC® 29213™ colonization in the middle ear of rat. Scanning electron microscopy observed that the whole middle ears of the only *S. aureus* treated rats are coated with bacterial biofilms, which are not visible in the middle ears of rats treated with *S. aureus* and the eugenol at a half MIC, however, the alteration of virulence factor expression in *S. aureus* of the infected rat model was not determined [66].

In patients under long-term antibiotic treatment, *S. aureus* SCVs are commonly isolated [99,100]. Loss *et al.* illustrated the evolution of a SCV from its wild-type strain in a patient fitted with a prosthetic joint and experiencing *S. aureus* infection relapse and rifampicin treatment [101]. Lånnergard *et al.* isolated menadione-auxotrophic *S. aureus* SCVs and their isogenic wild-type strains from three patients suffering from chronic osteomyelitis and receiving long-term antibiotic therapy [102]. Other studies reported thymidine-auxotrophic *S. aureus* SCVs from patients with cystic fibrosis and receiving trimethoprim-sulfamethoxazole therapy [103,104]. Kussmann *et al.* demonstrated the emergence of dalbavancin- nonsusceptible and teicoplanin-resistant *S. aureus* SCVs in a patient with cardiac device-related endocarditis undergoing long-term dalbavancin treatment [105]. Their findings implied the existence of a new lipoglycopeptide resistance mechanism because the *in vivo* induced *S. aureus* SCVs are formed in the absence of lipopeptide- or glycopeptide-antibiotic treatment. Such a mechanism requires further investigation.

Concluding remarks

In addition to their antimicrobial functions, the signal induction roles of antibiotics at sub-MICs have gained increasing interest in recent years. The major *in vitro* effects of the sub-MIC levels of antibiotics on the virulence of the important pathogen *S. aureus* include five aspects: (i) inducing bacterial cell deformation to stimulate abnormal host immune responses; (ii) modulating the expression levels of *S. aureus* virulence factors to alter disease development; (iii) regulating strain-specified adhesion and invasion capabilities to affect bacterial colonization and diffusion; (iv) altering *S. aureus* biofilm formation to potentate implantation material-related infections; and (v) influencing bacterial SCV formation to achieve persistent infection and recurrence. However, whether these *in vitro* effects of antibiotic sub-MICs on *S. aureus* virulence can be expected to present *in vivo* is unclear, and would be a fruitful research area for additional investigation.

The manifestations of the overall effects of antibiotics at sub-MIC concentrations on *S. aureus* virulence are dependent on strains and vary by antibiotic types, and inconsistent effects have been observed when a certain antimicrobial agent is used against different *S. aureus* strains or the same strain is treated with various types of antibiotics. The antibiotic susceptibility, virulence profile, growth stage, and culture condition of *S. aureus* and the time of antibiotic application may work independently or synergistically to contribute to divergent results. Mechanically, antibiotics at sub-MICs affect *S. aureus* virulence through direct binding to certain molecules, such as cell wall synthetic factors PBP1 and PBP2 and virulence factor PVL, and/or via modulating global regulators, including *sigB*, *sarA*, and *agrA*, which form a regulatory network to control *S. aureus* virulence. Therefore, modern omics technologies, such as proteomics, transcriptomics, metabonomics, and interactomics, should be applied to explore the complex effects of sub-MICs of antibiotics on *S. aureus* virulence. Hopeful findings should help construct novel therapeutic strategies that facilitate the treatment of infections caused by *S. aureus* in the future.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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Author contributions

Xiancai Rao and Rong Zhang proposed the idea for the article. Juan Chen and Huyue Zhou did the literature search and data analysis. Juan Chen drafted the work. Xiancai Rao, Jingbin Huang and Rong Zhang critically revised the manuscript.

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