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Sub-MIC streptomycin and tetracycline enhanced Staphylococcus aureus biofilm formation, an in-depth study mimicking food processing under antibiotics residue

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Sub-MIC streptomycin and tetracycline enhanced *Staphylococcus aureus* Guangzhou-SAU749 biofilm formation, an in-depth study on transcriptomics

Abstract:

Staphylococcus aureus is a major human pathogen, a potential "Super-bug" and a typical biofilm forming bacteria. With usage of large amount of antibiotics, the residual antibiotics in clinical settings further complicate the colonization, pathogenesis and resistance of *S. aureus*. This study aimed at investigating the phenotypical and global gene expression changes on biofilm formation of a clinical *S. aureus* isolate treated under different types of antibiotics. Firstly, an isolate Guangzhou-SAU749 was selected from a large sale of previously identified *S. aureus* isolates, which exhibited weak biofilm formation in terms of biomass and viability. Secondly, 9 commonly prescribed antibiotics for *S. aureus* infections treatment, together with 10 concentrations ranging from 1/128 to 4 minimum inhibitory concentration (MIC) with 2-fold serial dilution, were used as different antibiotic stress conditions. Then, biofilm formation of *S. aureus* Guangzhou-SAU749 at different stages including 8 h, 16 h, 24 h, and 48 h, was tested by crystal violet and MTS assays. Thirdly, the whole genome of *S. aureus* Guangzhou-SAU749 was investigated by genome sequencing on PacBio platform. Fourthly, since enhancement of biofilm formation occurred when treated with 1/2 MIC tetracycline (TCY) and 1/4 MIC streptomycin (STR) since 5 h, the relevant biofilm samples were selected and subjected to RNA-seq and bioinformatics analysis. Last, expression of two component system (TCS) and biofilm associated genes in 4 h, 8 h, 16 h, 24 h, and 48 h sub-MIC TCY and STR treated biofilm samples were performed by reverse

transcription-quantitative polymerase chain reaction (RT-qPCR). Although most antibiotics lowered the biomass and cell viability of Guangzhou-SAU749 biofilm at concentrations higher than MIC, certain antibiotics including TCY and STR promoted biofilm formation at sub-MICs. Additionally, upon genome sequencing, RNA-seq and RT-qPCR on biofilm samples treated with sub-MIC of TCY and STR at key time points, genes *lytR*, *arlR*, *hssR*, *tagA*, *clfB*, *atlA* and *cidA* related to TCS and biofilm formation were identified to contribute to the enhanced biofilm formation, providing a theoretical basis for further controlling on *S. aureus* biofilm formation.

Keywords: *Staphylococcus aureus*; biofilm; sub-minimum inhibitory concentration, tetracycline, streptomycin; two component system

1. Introduction

As one of the leading human pathogens, *Staphylococcus aureus* is responsible for a large variety of infections and diseases, such as skin and tissue infections, bacteremia, endocarditis, and food poisoning [1, 2]. In various acute and chronic infections, *S. aureus* causes higher medical care cost than other species [3]. As a microbial community, biofilm is a common form of microbes gathering on the surface of clinical environment and allows cells to persist in different environmental pressures (such as osmotic pressure, antibiotics and disinfectants) [4-6]. *S. aureus* is a typical biofilm former, and its biofilm can colonize different *in vivo* surfaces including a variety of host tissue, from endocardium and gastrointestinal tract, as well as *in vitro* surfaces of

medical devices and clinical environments [7-11]. Importantly, *S. aureus* biofilm mediated infections are difficult to therapeutically manage as cells in biofilm state exhibit strong resistance to antibiotics, which shows 1,000 times higher minimum inhibitory concentration (MIC) than that of planktonic state [12].

Antimicrobial resistance of clinical pathogens caused by universal antibiotics usage is a one of the major global problems [13-16], as antibiotics are commonly used in microbial infections. Along with antibiotics treatment, sub-therapeutic level of antibiotics exists in the systemic circulation of patients due to the reduced antibiotics bioavailability caused by drug-drug or host-drug interaction [17, 18]. In addition, higher dose of antibiotics application in livestock results in the accumulation of antibiotic residues in meats which are subsequently intaken by human beings [19]. Thus, the role of antibiotics at sub-MIC in affecting microorganisms has been considered to be a major concern [20-23]. Here, MIC refers to the antibiotic concentration which is the lowest concentration but sufficient for complete inhibition of bacterial growth, and sub-MIC refers to the concentration lower than MIC [67]. In general, antibiotics even at sub-MIC would lower the growth and reproduction of bacteria. Joanna et al. had found that aminoglycosides at the dose of sub-MIC could inhibit *Proteus mirabilis* biofilm formation with the absence of ascorbic acid [24]. It had also been observed that sub-MIC of clindamycin and azithromycin limited the production of exoprotein in *S. aureus*, resulting in reduced toxicity induced by exoprotein and additionally modulated cascade in inflammation [25]. However, antibiotics at sub-MIC had been shown to enhance microbial population diversity, biofilm formation, and expression of toxins and

virulence determinants. Rachid et al. found that sublethal concentration of tetracycline promoted biofilm formation of *Staphylococcus epidermidis* due to the promotion of *ica* operon expression [20]. Li et al. had found that sub-MIC of norfloxacin had varied influence on *Streptococcus suis* including increased biofilm formation in which higher viable cell number was induced by 1/4 minimum inhibitory concentration (MIC), and lowered growth rate induced by 1/2 MIC [26]. Nevertheless, the enhancement in biofilm formation caused by sub-MIC of some antibiotics has yet to be fully analyzed along with the underlying molecular mechanism.

It is noteworthy that, a significant discrepancy had been found in respect to the biofilm formation of *S. aureus* under sub-MIC of antibiotics [27-31]. A list of studies demonstrating that sub-MIC antibiotics induce bacterial biofilm formation had been reviewed [29]. Sub-MIC of oxacillin [32], cefalexin [33], cefazolin [34], vancomycin [32], and linezolid [35] had been identified to induce *S. aureus* biofilm formation. In contrary, azithromycin at sub-MIC (1/16 MIC, 1/8 MIC) reduced the biofilm formation of *S. aureus* [36]. A number of factors have not been taken into consideration in previous studies, as below. Firstly, *S. aureus* isolates are considerably diverse, with different phenotypic and genotypic characteristics. For phenotypes, antimicrobial susceptibility highly differs among *S. aureus* isolates, leading to different “sub-lethal” concentrations. Also, *S. aureus* isolates shows large diversity in biofilm forming capability, and mostly strains with strong biofilm formation were used in previous studies. Collectively, antimicrobial mechanisms on *S. aureus* significantly vary, and influence of different antibiotic types on biofilm formation of *S. aureus* largely remains

unclear.

Consequently, to address the concerns as above, this study firstly selected a clinical strain from a large scale of *S. aureus* isolates with phenotypic and genotypic characterization previously tested [37]. Secondly, change in biofilm formation was further determined under 10 different concentrations of 9 antibiotics which are commonly used to treat *S. aureus* infections. Thirdly, the whole genome of the strain was sequenced and analysed with focus on biofilm associated genes and virulence factors. Eventually, biofilm samples with biomass and cell viability significantly increased by sub-MIC of antibiotics were further subjected to transcriptomic analysis by RNA-seq and reverse transcript-quantitative polymerase chain reaction (RT-qPCR).

2. Material and methods

2.1 Bacterial strain

In 2011, *S. aureus* strain Guangzhou-SAU749 was isolated from the sputum of a patient suffering from respiratory disease in the First Affiliated Hospital of Guangzhou Medical University in Guangzhou, China. It was one of 12 multidrug resistance *S. aureus* strains selected from 524 clinical isolates. Bacterial identification was performed using a VITEK 2 Automated System (BioMérieux, Durham, NC). Maintained as glycerol stocks, *S. aureus* strain was transferred onto Trypticase soy agar (TSA) (Huankai, China) and adapted to incubation at 37°C for up to 24 h. To acquire stationary phase culture, single colony was sub-cultured in Trypticase soy broth (TSB) (Huankai, China) for approximately 16 h at 37°C. Stationing phase culture was sub-cultured (1:100) again in

TSB for approximately 2.5 h at 37°C to acquire logarithmic phase culture prior to subsequent experiments.

2.2 Antibiotic susceptibility investigation

Antimicrobial susceptibility testing of *S. aureus* strains were performed using a VITEK 2 Automated Susceptibility System (bioMérieux) and Etext strips (AB bioMérieux). MICs of 9 antibiotics (streptomycin (STR), kanamycin (KAN), gentamycin (GEN), tetracycline (TCY), oxytetracycline (OXY), ciprofloxacin (CIP), erythromycin (ERY), trimethoprim (TMP), vancomycin (VAN)) on *S. aureus* strain Guangzhou-SAU749 were further tested by broth dilution method [38-40]. Briefly, antibiotic stocking solution (at a concentration higher than the standard MIC value for *S. aureus*) was 1:10 serial diluted at a final volume of 100 μL with Müller-Hinton (MH) medium (Huankai, China) in 96 well plate with 100 μL bacterial cultures (approximately 1.5×10^6 CFU/mL) added subsequently. Fresh MH medium was added as negative control. The 96 well plate was incubated at 37°C with shaking for approximately 16 h and subjected to Optical density (OD) at 600 nm (OD_{600nm}) measuring by microplate reader. Each experiment was performed in triplicate. The MICs were interpreted based on Clinical and Laboratory Standards Institution (CLSI) guidelines 2016 and 2018 for *S. aureus* [39, 40].

2.3 Biomass and cell viability determination of biofilm

Biofilm formation ability of the *S. aureus* strains was determined via biomass and

cell viability examination. Biomass was assessed by crystal violet (CV) assay. In brief, after incubation, the attached biofilms in each well were washed by 200 μL of phosphate buffered saline (PBS) for at least 3 times after removing the supernatant. Subsequently, the biofilms were treated with 200 μL of 0.01% (w/v) CV (Kemiou, China) for 10 min. Again, the biofilms were washed by 200 μL of PBS for 5 times after removing the CV. Upon drying in room temperature for 15 min, the biofilms were treated with 200 μL of 95% ethanol to fully resolubilization for another 15 min. From each well, 125 μL liquid was transferred to a new plate for measurement of OD_{540nm}. Cell viability was tested via the MTS assay. Similarly, the attached biofilms were washed by 200 μL of PBS for at least 3 times after removing the supernatant. Subsequently, washed biofilms were treated with 200 μL of MTS reagent (Promega, China) in dark at 37°C for 2.5 h. Following incubation, 125 μL of the treated liquid was transferred to a new plate for measurement of OD_{490nm}. Each experiment was performed in triplicate.

2.4 Biofilm formation with different concentrations of antibiotics

Biofilm growth with antibiotics treatment was performed in a 96 well plate with the addition of 10 serial concentrations (1/128, 1/64, 1/32, 1/16, 1/8, 1/4, 1/2, 1, 2 and 4 MIC) of antibiotics. The antibiotic working solutions were at a concentration of 16 MIC and 1:2 serial diluted at a final volume of 100 μL with TSB medium in 96 well plate. 100 μL bacterial cultures were added subsequently. Fresh TSB medium was added instead of antibiotics as negative control. The biofilms were cultured at 37°C without shaking for 0 h, 8 h, 16 h, 24 h, and 48 h, respectively. Biomass and cell viability

of biofilms were assessed by CV and MTS assays, respectively. Each experiment was performed in triplicate.

2.5 Genome sequencing

A bacterial gDNA isolation kit designed for bacteria (Sigma-Aldrich, USA) was used in this study to prepare DNA samples of *S. aureus* strain Guangzhou-SAU749. Upon purification and quality assessment, the DNA samples were applied on 2nd generation sequencer Illumina HiSeq 2500 and 3rd generation sequencer PacBio RS II by GeneDenove Bio company (Guangzhou, China). A hierarchical genome assembly process (HGAP) was used for sequence assembly [41]. Gene prediction and amnotation were performed with softwares GeneMarkS (gene prediction), Basic Local Alignment Search Tool (BLAST) (from National Center for Biotechnology Information (NCBI), annotation), *tRNA*scan-SE (v.1.21, tRNA prediction), and RNAmmer (v.1.2, rRNA prediction) and databases UniProt and Nucleotide collection (Nr) [42, 43]. In addition, transposon and genome island were predicted by softwares TransposonPSI (version: 20100822) and IslandViewer4 (http://www.pathogenomics.sfu.ca/islandviewer/), respectively. The genes and proteins were further clustered according to their Gene Ontology (GO) terms, Clusters of Orthologous Genes [9] categories, and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways, based on corresponding databases [44-46]. Concerning the focus of this study was antibiotic and biofilm formation, antimicrobial resistance genes and virulence factors were predicted using Antibiotic Resistance Genes Database (ARDB), Comprehensive Antibiotic Resistance

177 Database (CARD) and Virulence Factors Database (VFDB).

178

179 **2.6 Transcriptomics analysis**

180 For antibiotics type and concentration selection, TCY at 1/2 MIC representing
181 antibiotics showing enhanced cell viability and STR at 1/4 MIC representing those
182 showing increased biomass were used. Since time point selection plays an essential role
183 in transcriptomic analysis, we performed an in-depth experiment to determine the time
184 point for transcriptomic analysis. Firstly, based on the first time point exhibiting
185 enhanced biofilm formation, biofilm formation was further examined with 1 h intervals
186 until significant increase in biomass or cell viability were obtained (between 0 and 8 h
187 in this study), followed by further examination with 0.5 h intervals (between 4 and 7 h
188 in this study). Biofilm samples of Guangzhou-SAU749 induced by TCY at 1/2 MIC
189 (TCY stressed sample), and STR at 1/4 MIC (STR stressed sample) were collected at 5
190 h. Wildtype biofilm samples (WT sample) of Guangzhou-SAU749 without antibiotic
191 treatment was used as control. Three biological replicates of biofilm samples were
192 included. Total RNAs of *S. aureus* biofilm samples were prepared by the application of
193 TRizol reagent (Sigma-Aldrich, USA) according to manufacturer's instruction. The
194 RNA samples were sent to a local sequencing company Gendenovo Bio Company
195 (Guangzhou, China) for further purification and quality assessment. A 2nd generation
196 sequencer Illumina Hiseq 2500 was applied for RNA-seq.

197 The clean reads yield from the sequencing platform was aligned to the genome
198 sequence of *S. aureus* strain Guangzhou-SAU749 using TopHat [47]. The 3 biofilm

199 samples were classified into 2 comparative groups (TCY stressed group and STR
200 stressed group) with TCY and STR samples compared to WT sample, respectively).
201 Differentially expressed genes (DEGs) in each group were determined by the
202 application of DEGseq software [48, 49] and based on expression level evaluated by
203 RPKM [50, 51]. Genes matching the standard of $|\log_2(\text{fold change})| > 1$ in
204 combination with P value < 0.05 in this study were designated as significant DEGs.
205 Enrichment analysis on DEGs with GO term and KEGG pathway annotation was
206 performed to identify enriched GO terms and KEGG pathways matching the standard
207 of P value < 0.05 (Fisher Exact Test) or < 0.01 (Hypergeometric Distribution) [52, 53].
208

209 **2.7 RT-qPCR**

210 RT-qPCR was performed to monitor the expression levels of selected genes during
211 the whole process of biofilm formation (4 h, 8 h, 16 h, 24 h, and 48 h) using a standard
212 equipment Light Cylcler 480 (Roche, Switzerland). Each reaction was conducted in a
213 25 μL system following the standard procedure suggested by manufacturer. Blank
214 control was induced in each plate with double distilled H_2O replacing template cDNA.
215 *16S rRNA* gene was applied for each reaction as a reference to calculate relative gene
216 expression level by $2^{-\Delta\Delta CT}$ method. Each reaction was run in triplicate.
217

218 **2.8 Statistical analysis**

219 The data were illustrated as the mean ± standard deviation from triplicate
220 experiments. Statistical analysis of experimental groups compared with control was

examined by one-way analysis of variance [54] followed by Tukey multiple intergroup comparison unless specially indicated. A P value < 0.05 was considered to be statistically significant unless specially indicated.

2.9 Nucleotide sequence accession number

The genome sequences of *S. aureus* strain Guangzhou-SAU749 were deposited in the NCBI GenBank database under the accession numbers CP053185-CP053186.

3. Results

3.1 Background information of *S. aureus* Guangzhou-SAU749

The *S. aureus* strain Guangzhou-SAU749 was selected from a large scale of *S. aureus* strains with phenotypic and genotypic characterization previously tested [37]. Firstly, 12 multidrug resistance *S. aureus* strains covering strong, medium and weak biofilm formation ability as well as major multilocus sequencing types (MLST) and SCC*mec* types were selected from 524 clinical *S. aureus* isolates (Table 1). Secondly, the biofilm formation ability (cell viability and biomass) of the 12 *S. aureus* strains with the treatment of sub-MIC of antibiotics were examined. Divergent changes were found with the treatment of different types and concentrations of antibiotic. The biofilm biomass of 6 strains was enhanced by sub-MIC of STR. The cell viability of biofilms of 2 strains was enhanced by sub-MIC of TCY. Amongst, Guangzhou-SAU749 was in both groups. Thus, Guangzhou-SAU749 was used in this study. In addition, Guangzhou-SAU749 belonged to the most common MLST type (ST239, exhibited

243 weak biofilm formation ability, and was resistant to all antibiotics tested in this study
244 except for VAN (Table 1).
245
246 **3.2 Biofilm biomass change with antibiotic treatment**
247 A total of 9 antibiotics from 6 subtypes were included in this study to examine the
248 biomass change of *S. aureus* Guangzhou-SAU749 during biofilm formation within 48
249 h (Fig. 2). The antibiotics were classified into 3 groups including aminoglycosides
250 (STR, KAN, GEN), tetracyclines (TCY and OXY), and others (CIP ERY TMP and
251 VAN).
252 Concerning aminoglycosides group (STR, KAN, and GEN) (Fig. 2A-2C), lower
253 biofilm biomass of Guangzhou-SAU749 was obtained within 48 h at high
254 concentrations including MIC, 2 MIC and 4 MIC. Remarkably, increase in biofilm
255 biomass of Guangzhou-SAU749 was obtained when treated with STR (at large
256 proportion of time points, especially at 1/4 MIC) (Fig. 2A) and GEN (at early time
257 points, especially at 1/8 MIC) (Fig. 2B). For KAN, increase in biofilm biomass of
258 Guangzhou-SAU749 was observed in 1/32 and 1/16 MIC at 8 h and in 1/2 MIC at 24 h
259 (Fig. 2C).
260 Concerning tetracyclines group (TCY and OXY) (Fig. 2D-2E), when *S. aureus*
261 Guangzhou-SAU749 cells were treated at high concentrations including MIC, 2 MIC
262 and 4 MIC, decrease in biomass was identified within 48 h. However, when *S. aureus*
263 Guangzhou-SAU749 treated with TCY at concentrations lower than MIC, no
264 significant decrease was found (Fig. 2D). Slightly increase in biomass was observed

265 under OXY treatment at concentrations ranging from 1/128 MIC to 1/4 MIC at 24 h
266 and in 1/64 MIC at 48 h (Fig. 2E).

267 A few of other antibiotics from quinolone, macrolide, sulfonamide, and
268 glycopeptide groups were also tested, including CIP, ERY, TMP, and VAN (Fig. 2F-2I).
269 According to the results, for CIP treatment, decrease in biomass was largely found only
270 when CIP concentrations were higher than 1/16 MIC (Fig. 2F). For ERY treatment,
271 decrease in biomass was obtained at high concentrations including MIC, 2 MIC and 4
272 MIC, and insignificant changes at concentrations ranging from 1/64 MIC to 1/8 MIC
273 (Fig. 2G). For TMP and VAN treatments, lower and insignificant change in biomass
274 were observed for all concentrations, respectively (Fig. 2I).

275 Overall, aminoglycosides and tetracyclines groups showed promotion on *S. aureus*
276 Guangzhou-SAU749 biofilm biomass to different levels at sub-MIC, with STR as a
277 representative.

278

279 **3.3 Cell viability change in biofilms with antibiotic treatment**

280 During biofilm formation of *S. aureus* Guangzhou-SAU749 under antibiotic
281 treatment within 48 h, cell viability change was determined (Fig. 3). In aminoglycosides
282 group (Fig. 3A-3C), when *S. aureus* Guangzhou-SAU749 cells were treated by 1-4 MIC
283 of STR, 2-4 MIC of KAN, and 1/4-4 MIC of GEN, cell viability was reduced within 48
284 h. Significant increase in cell viability was identified in biofilms treated by 1/4 MIC of
285 STR at 16 h, 48 h, 1/2 MIC of STR at 48 h, 1/8 MIC of GEN at 8 h, 1/8 MIC of KAN
286 at 8 h, 24 h, and 1/4 MIC of KAN at 24 h, 48 h.

For tetracycline group (Fig. 3D-3E), lower cell viability was found when *S. aureus* Guangzhou-SAU749 cells were treated with OXY at high concentrations, including MIC, 2 MIC and 4 MIC. Under OXY treatment, cell viability increased at 16-48 h at 1/2 MIC and 24 h at 1/16-1/4 MIC. Under TCY treatment at 1/4 MIC or 1/2 MIC, cell viability increased at 8 h.

For CIP treatment (Fig. 3F), largely decrease in *S. aureus* Guangzhou-SAU749 cell viability was observed within 48 h except for 1/8 MIC at 8 h and 1/128 MIC at 48 h. For ERY (Fig. 3G), when *S. aureus* Guangzhou-SAU749 cells were treated at concentrations higher than 1/64 MIC, lower cell viability was acquired within 24 h. Remarkably, cell viability increased by ERY at 1/128 MIC. For TMP (Fig. 3H), slightly decrease in cell viability was observed at 24 h and 48 h. For VAN (Fig. 3I), reduced cell viability was found when *S. aureus* Guangzhou-SAU749 cells were treated at 4 MIC, with insignificant reduction in cell viability obtained from concentrations ranging from 1/128 MIC to 1/8 MIC.

Antibiotics including STR, GEN, OXY, ERY CIP, KAN, and TCY at specific sub-MICs enhanced cell viability of *S. aureus* Guangzhou-SAU749 biofilms, with TCY as a representative.

Collectively, enhanced biofilm formation was observed when *S. aureus* strain Guangzhou-SAU749 was grown in the supplementary of 7 of the 9 antibiotics at sub-MIC. Among the 9 antibiotics, STR and TCY were typical to induced enhanced biofilm biomass and cell viability under sub-MIC, respectively. Thus, STR and TCY were selected to adapt to further transcriptomics study.

3.4 Genomic information of *S. aureus* Guangzhou-SAU749

The clean reads from the sequencers were assembled into two scaffolds including one chromosome and one plasmid from the genome of *S. aureus* Guangzhou-SAU749 (Table 2). The chromosome had a length of 2,840,643 bp with 2,651 genes (Fig. 1A), whereas the plasmid, designated pSAU749 had a length of 20,736 bp with 22 genes (Fig. 1B). Among the 2,651 and 22 predicted genes in the chromosome and plasmid, 122 (4.60%) and 2 (9.09%) were predicted to encode secretion proteins. Concerning non-coding RNA, 61 *tRNA* and 19 *rRNA* (6 *23S rRNA*, 6 *16S rRNA*, and 7 *5S rRNA*) were identified. One TY1 Copia transposon and 1 mariner transposon were predicted in the chromosome and plasmid, respectively.

In the genome of *S. aureus* strain Guangzhou-SAU749, genes involved in antibiotic resistance genes, virulence genes, and biofilm related genes were specifically determined. According to ARDB and CARD databases, 4 common resistance-relative genes were identified, including *ermB*, *mecA*, *mecR1* and *mepA*. The gene *ermB* is the determinant of lincosamide, macrolide and streptomycin resistance [55]. The gene *mecA* and *mecR1* encoding antibiotic target replacement protein is the determinant of beta-lactam resistance [56]. Among efflux pump genes, *norA*, *norB* and *mepA* were commonly investigated with high frequency in Asia, accounting for 70%, 60% and 35%, respectively [57]. The gene *mepA* is a part of the multidrug and toxic extrusion family. Additionally, *aph(3')-IIIa* and *bacA* genes were identified in the genome of Guangzhou-SAU749 based on ARDB database. Predicted by CARD database, 26 antibiotic

resistance genes (such as *aad(6)*, *tet(38)*, *arlR*, *dfrC*) were identified showing resistance to aminoglycoside, macrolide, beta-lactam, tetracycline, streptogramin, lincosamide, isoniazid, triclosan, lipopeptide, fluoroquinolone, peptide, fosfomycin, elfamycin, and diaminopyrimidine. Furthermore, according to ARDB database, the gene *bl2a_pc* is responsible for penicillin resistance of the plasmid of *S. aureus* strain Guangzhou-SAU749. The antibiotic resistance genes *blaZ* and *mecI* were detected in the plasmid of Guangzhou-SAU749 in CARD database.

A total of 81 virulence factors were acquired by the genome of *S. aureus* strain Guangzhou-SAU749. The major virulence factors in *S. aureus* include gene *cap* encoding capsular polysaccharide synthesis enzyme, *icaABCDR* encoding intercellular adhesion protein, *ebpS* encoding elastin binding protein, *esaB* encoding hypothetical protein, *hlyB* encoding hemolysin transport protein, *chp* encoding chemotaxis-inhibiting protein. Type 8 capsular polysaccharide encoded by the gene *cap* is the most prevalent capsule type in clinical isolates of *S. aureus*, and has been proven to be an antiphagocytic virulence factor [58]. Moreover, *S. aureus* forms biofilm that is dependent on the surface-located fibronectin binding proteins A and B (FnBPA, FnBPB), encoded by gene *fnbA* and *fnbB* respectively [59]. The *clfA* and *clfB* genes belong to *S. aureus* microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), which can promote adhesion to surfaces [60]. ClfA binds to the γ-chain fibrinogen whereas ClfB binds to the α-chain fibrinogen. ClfA is a mediator of *S. aureus*-induced platelet aggregation [61]. The gene *aur* can modify surface proteins by specific inactivation of ClfB, thus may induce cell attachment [62].

353 Fibrinogen is a ligand for the *S. aureus* MSCRAMMs bone sialoprotein-binding protein
354 (Bbp) encoded by *sdrCD* [63]. The genes *icaA*, *icaB*, *icaC* and *icaD* synthesize a
355 polysaccharide, poly-n-succinyl-β-1,6 glucosamine, which plays key role in biofilm
356 elaboration[64]. Also, the *ssp* gene encoding V8 protease and *sspB* gene encoding
357 cysteine protease degrade the fibronectin-binding protein on cell surface [65].
358
359 **3.5 Overview of the transcriptomes**
360 In order to more accurately determine the antibiotic concentration and treatment
361 time, multiple time points before 8 h (every hour from 1 h to 8 h, and every 0.5 h from
362 4 h to 7 h) were included to test the change of biofilm biomass and cell viability of *S.*
363 *aureus* Guangzhou-SAU749 (Figure S1) treated by STR and TCY. Biofilm samples at
364 5 h (representing the first time point with enhanced biofilm formation, and early stage
365 biofilm attachment) induced by TCY at 1/2 MIC (TCY stressed sample), and STR at
366 1/4 MIC (STR stressed sample) were collected and adapted to transcriptomics analyses.
367 WT biofilm samples of Guangzhou-SAU749 without antibiotic treatment (WT sample)
368 were served as control. A total of 2651 genes were expressed in the 3 biofilm samples
369 of *S. aureus* Guangzhou-SAU749 strain. While 113/156 and 50/132 up/down regulated
370 DEGs were determined in TCY and STR stressed samples, respectively (Fig. 4A-4B).
371 For the following mentioned up/down regulated DEGs in TCY/STR stressed samples,
372 the expression of DEGs was up/down regulated in TCY/STR stressed samples
373 compared to WT.
374 Comparing the DEGs in TCY and STR stressed samples with WT sample, 81

375 DEGs were shared while 188 and 101 DEGs were distinctively identified in TCY and
376 STR stressed samples, respectively. In TCY and STR stressed samples, and WT versus
377 STR, most DEGs related to resistance, virulence, membrane, and stress response were
378 down-regulated. However, in TCY stressed sample, virulence factors including alpha-
379 hemolysin, Leukocidin LukF-PV, beta-channel forming cytolysin, delta-hemolysin
380 precursor, and antitoxin component Xre domain protein showed up-regulation.
381
382 **3.6 GO term and KEGG pathway enrichment analysis of DEGs**
383 To understand the function of DEGs, GO term and KEGG pathway annotation and
384 enrichment analysis were conducted on the DEGs of 2 comparative groups.
385 Significantly enriched GO terms including 3 sub-terms (biological process (BP),
386 cellular component (CC), and molecular function (MF)) and KEGG pathways were
387 illustrated in Fig. 4C-4F.
388 Most significantly enriched GO terms were identified in TCY stressed group (Fig.
389 4C), including 2 ("proton-transporting two-sector ATPase complex" and "membrane
390 protein complex"), 7 ("cation-transporting ATPase activity", "hydrolase activity, acting
391 on acid anhydrides, catalyzing transmembrane movement of substances", "carbon-
392 nitrogen lyase activity"), and 52 ("inosine monophosphate (IMP) biosynthetic process",
393 "purine nucleoside monophosphate metabolic process", "ribonucleoside
394 monophosphate biosynthetic process") in CC, MF, and BP categories, respectively. In
395 comparative group STR stressed group (Fig. 4D), 5 GO terms ("peptidase activity",
396 "hydrolase activity", "endopeptidase activity") in MF, and 12 GO terms ("multi-

organism process", "protein maturation", "teichoic acid biosynthetic process") in BP were identified.

In TCY stressed group (Fig. 4E), 10 enriched pathways ("Glycolysis/Gluconeogenesis", "Naphthalene degradation", "Biosynthesis of antibiotics") were identified. "ATP binding cassette (ABC) transporters", "D-Alanine metabolism", and "TCS" were significantly enriched pathways in STR stressed group (Fig. 4F).

3.7 Differential expression of biofilm associated and TCS genes

Biofilm and TCS associated genes were selected to determine their expression by RT-qPCR during the whole process of biofilm formation (4, 8, 16, 24, 48 h) under sub-MIC of TCY and STR treatment (Fig. 5, Fig. 6). Concerning biofilm associated genes, the expression of *capABCE*, *cidA*, *fnbAB*, *lrgAB*, *lytM*, *sbi*, *sspAB*, *tagA* was monitored (Fig. 5, Fig. S2). Among all the biofilm related genes, genes including *capBCE*, *fnbB*, *sbi*, *sspA*, *tagA*, *altA*, *aur*, *cidR*, *clfAB*, *sdrC* showed overall higher expression in sub-MIC antibiotics treatment groups than WT (Fig. 5). Other genes showed similar or relatively lower expression in antibiotics treatment groups compared with WT (Fig. S2). In WT, the expression level of *tagA* gene increased slowly and then stabled; while in the antibiotic stress group, the expression level of *tagA* increased within 4-8 hours and then stabled. The expression level of *tagA* was overall higher in TCY and STR stressed group than WT, especially within in 8-24 h (Fig. 5A). In WT, the expression levels of *cidA* showed a decrease first and then tended to be stable and rising. Under antibiotics

419 stress, the expression level of *cidA* increased first and then decreased and was overall
420 lower than that of WT (Fig. 5B). In WT, the expression levels of *clfB* showed decreasing
421 first and then increasing. The expression level of *clfB* in the antibiotic stress groups
422 were higher than that of WT within 4-48 h (Fig. 5C). In WT, the expression levels of
423 *atlA* showed rising first and then stablized, while in the antibiotic stress groups, the
424 expression levels of *atlA* first increased and then decreased and gradually decreased,
425 but overall higher than that in WT (Fig. 5D).

426 Previous studies had confirmed that TCS plays an important role in regulating
427 microbes in response to the external stresses [33, 66]. According to the RNA-seq results,
428 TCS might play important role in the sub-MIC antibiotic promoted biofilm formation
429 of *S. aureus* Guangzhou-SAU749. Thus, expression of genes in TCS including *airRS*,
430 *arlRS*, *graRS*, *lytRS*, *saeRS*, *srrAB*, *tagB*, *walkR*, *agrAC*, *hssRS*, *kdpDE*, *nsaRS*, *vraRS*
431 was monitored at different time points by RT-qPCR under sub-MIC of TCY and STR
432 treatment (Fig. 6, Fig. S3). Among all TCS genes, *arlRS*, *graS*, *lytRS*, *saeR*, *tagB*, *agrC,*
433 *hssRS*, *kdpDE*, *nsaR*, *vraRS* showed overall higher expression in sub-MIC antibiotics
434 treatment groups than WT (Fig. 6). In WT, the expression levels of *lytR* showed
435 increasing first and then flattening. However, under antibiotic stress, the expression of
436 *lytR* decreased first and then stabled (Fig. 6A). In WT, the expression of *arlR* increased
437 first and then decreased, while in antibiotic stress groups, the expression of *arlR* showed
438 gradually decreasing under STR treatment (Fig. 6B). In WT, the expression of *hssR*
439 showed a gradual increase, while in the antibiotic stress group, the expression of *hssR*
440 showed decreasing first and then increasing (Fig. 6C).

4. Discussion

Antibiotics can suppress biofilm formation by inhibiting microbial growth. As the concentration of antibiotics decreases, the inhibitory effect on bacterial growth gradually weakens. The sub-MIC of antibiotic treating on *S. aureus* had been sporadically studied, with controversial outcomes. In the currently available studies, clinical *S. aureus* isolates had rarely been considered and the types of antibiotics included were limited. In addition, the impact of sub-MIC of antibiotics on transcriptomics had not been comprehensively investigated. In this study, we firstly focused on clinical *S. aureus* isolates. Over 500 clinical *S. aureus* isolates were previously characterized and 12 multidrug resistance isolates covering strong, medium and weak biofilm formation ability as well as major MLST and SCC*mec* types were selected. Their changes in biofilm formation with sub-MICs of antibiotics were examined. Divergent changes were found with the treatment of different types and concentrations of antibiotic. The *S. aureus* strain Guangzhou-SAU749 was subsequently selected concerning its enhanced biofilm formation both in cell viability and biomass by sub-MIC of STR and TCY. Secondly, a total of 9 antibiotics belong to 6 types were included in this study. They cover most commonly used antibiotics in clinical treatment of *S. aureus* infections. Thirdly, we conducted comprehensive study on the transcriptomics level changes of the *S. aureus* strain Guangzhou-SAU749 in sub-MIC antibiotics environments. The results of this study showed that different types of antibiotics promoted biofilm formation of *S. aureus* strain Guangzhou-SAU749 at

463 certain sub-MIC. According to previous reports, Berlutti et al. used dynamic model
464 observations to show that the amount of biofilm and growth rate of *S. aureus* ATCC
465 6538 treated with sub-MIC of gentamicin were improved [27]. Mlynek [28], revealed
466 that sub-MIC of amoxicillin promoted the formation of *S. aureus* USA300 biofilm
467 through promoting eDNA generation [28], which is consistent with the results of
468 Kaplan's research [29]. Ara et al. believed that the sub-MIC of antibiotics to promote
469 biofilm formation is related to *clfAB* and *fnbAB* [30]. However, some studies hold the
470 opposite view that sub-MIC of antibiotics can inhibit biofilm formation [20, 31]. The
471 discrepancy might due to differences in experimental strains, culture conditions, culture
472 time, and antibiotic types and concentrations. Therefore, biofilm formation affected by
473 sub-MIC antibiotics requires specific analysis, and the mechanism remains to be further
474 explored.

475 The *S. aureus* strain Guangzhou-SAU749 were resistant to STR, KAN, GEN, ERY,
476 CIP, TCY, OXY, and TMP, but sensitive VAN. The discrepancy on the outcomes of *S.*
477 *aureus* biofilm formation with the treatment of different types of antibiotic of sub-MIC
478 might be related to the mechanisms of antibiotics [68]. Aminoglycosides, macrolides
479 and tetracyclines are combined with ribosomal subunits to inhibit protein synthesis. The
480 concentration of these antibiotics might need to reach a certain threshold to effectively
481 change biofilm formation. Sulfonamides and quinolone inhibit folic acid metabolism
482 and bacterial DNA synthesis, respectively [69, 70]. Folic acid is involved in the
483 synthesis and conversion of bases and is also essential in the process of DNA synthesis.
484 The inhibitory effect on DNA synthesis is more direct. The sub-MIC of such antibiotics

have the potential to lower biofilm formation by reducing bacterial growth.

Our data showed that the expressions of TCS-related genes *lytR*, *arlR* and *hssR* in *S. aureus* strain Guangzhou-SAU749 were consistent in sub-MIC of TCY and STR induced biofilms. During entire 4-48 h period, the expression levels of the three genes in the TCY and STR stressed groups were higher than those in WT. It indicated that *lytR*, *arlR* and *hssR* played an important role in regulating *S. aureus* Guangzhou-SAU749 in response to sub-MIC of TCY and STR stress. The main function of *lytSR* is to regulate *S. aureus* programmed cell death (PCD) [71], which is also related to *cidABC* and *lrgAB*. Additionally, *lytSR* sense changes in cell membrane potential and contributes to the process of *S. aureus* coping with cationic antimicrobial peptides [72]. It has also been observed that *lytSR* regulates the formation of *S. aureus* biofilm by affecting *lrgAB* [73]. The expression of *lytR* gene in *S. aureus* Guangzhou-SAU749 was relatively high in the first few hours of biofilm formation in sub-MIC of TCY and STR treated groups, but decreased in the following hours. It indicated the PCD system was repressed at the beginning but the repression was weakened at later timepoints. It has been reported that *arlRS* has a regulatory effect on *ica* and *aap*, and its mutation can cause the reduction of *S. epidermidis* biofilm formation ability [33]. Similarly, we obtained high expression of *arlR* in TCY and STR stressed groups at 4-8 h, potentially related to the accumulation of *S. aureus* Guangzhou-SAU749 cells, which is conducive to enhanced biofilm formation. *S. aureus* relies on *hssRS* to obtain iron to maintain growth and reproduction. *In vivo* experiments such as hemoglobin were not conducted in this study. But the expression level of *hssR* in the TCY and STR stressed groups was

significantly different from the WT. The *hssR* gene might play a role in *S. aureus* Guangzhou-SAU749 to encounter adverse environments such as antibiotics or oxidation, but its specific function remains to be explored.

Concerning biofilm-related genes, in the TCY and STR stressed groups, the expression levels of *tagA* and *atlA* within 4-48 hours, and *clfB* in the early stage were higher than that in WT, while the expression levels of *cidA* were lower. It suggested that *tagA*, *clfB*, *atlA* and *cidA* might be critical the process of *S. aureus* Guangzhou-SAU749 biofilm formation with treatment of sub-MIC of TCY and STR. The role of *cidABC* is mainly to form perforin to cause PCD, while *lrgAB* plays a role in binding to perforin to inhibit PCD [74]. In many cases, PCD affects the structure of biofilm and cause the release of genomic DNA to eDNA and cytoplasmic content [75-77]. However, both *cid* and *lrg* mutations affect biofilm formation, thus the balance of *cid* and *lrg* is crucial to the formation of biofilms [78]. The decrease in the expression of *cidA* in the TCY and STR stressed groups was consistent with the increase in the expression of *lytR*, indicating that enhanced biofilm formation of *S. aureus* Guangzhou-SAU749 under sub-MIC of TCY and STR stress was not due to eDNA production by promoting PCD. On the contrary, PCD was inhibited, and the increase in formation might due to the increase in cell number or secreted EPS. AtlA has a certain hydrolase activity, which plays an important role in cell division, biofilm adhesion and eDNA production [32, 79, 80]. Our data suggested that the role of *atlA* in *S. aureus* Guangzhou-SAU749 was more inclined to promote eDNA production, which constitutes an important component of biofilm. ClfB is a fibrinogen binding protein, which is used to covalently anchor

MSCRAMM to cell wall peptidoglycan and conducive to adhesion and biofilm formation [33]. The gene *tagA* is related to the synthesis of wall teichoic acids (WTAs) of *S. aureus* and contributes to resist adverse environments such as antibiotics [34, 81]. Of note, WTAs have been reported as important component of biofilm and promote the adhesion of *S. aureus* to inorganic surfaces [35]. Sub-MIC of TCY and STR stress caused the increase of *tagA* expression, which might be beneficial to *S. aureus* Guangzhou-SAU749 to improve antibiotic resistance and biofilm formation.

5. Conclusion

In this study, we firstly focused on clinical *S. aureus* isolates. Over 500 clinical *S. aureus* isolates were previously characterized and 12 multidrug resistance isolates covering strong, medium and weak biofilm formation ability as well as major MLST and SCC*mec* types were selected. Divergent changes in biofilm formation were found with the treatment of different types and concentrations of antibiotic. The *S. aureus* strain Guangzhou-SAU749 was subsequently selected concerning its enhanced biofilm formation both in cell viability and biomass by sub-MIC of STR and TCY. Secondly, 9 commonly used antibiotics at 10 different concentrations were used to test the influence on biofilm formation of a multidrug resistance *S. aureus* strain Guangzhou-SAU749 at 5 different time points. Although most antibiotics reduced biofilm biomass and cell viability at concentrations higher than MIC, certain antibiotics with TCY and STR as representatives promoted the biofilm formation at sub-MICs. Additionally, upon global genomics and transcriptomics analyses by genome sequencing, RNA-seq and RT-qPCR

551 at key time point, genes *lytR*, *arlR*, *hssR*, *tagA*, *clfB*, *atlA* and *cidA* related to TCS and
552 biofilm formation were identified to contribute to the enhanced biofilm formation of *S.*
553 *aureus* Guangzhou-SAU749 induced by sub-MIC of TCY and STR, providing a
554 theoretical basis for further controlling *S. aureus* biofilm formation.
555

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Table 1. Background information of 12 *S. aureus* isolates.

Strains	Origin	Biofilm related genes				Biofilm phenotype		MLST	SCC*mec*	MIC (μg/mL)								
		icaA	*icaD*	*icaBC*	*atl*	Biomass	Viability			STR	KAN	GEN	ERY	CIP	TCY	OXY	TMP	VAN
Guangzhou-SAU008	Pus	-	+	+	+	++*	+++	ST239	III	256 (R)#	2048 (R)	64 (R)	32 (R)	32 (R)	32 (R)	64 (R)	128 (R)	2 (S)
Guangzhou-SAU071	Bile	+	+	+	+	+	+	ST239	III	512 (R)	2048 (R)	64 (R)	32 (R)	32 (R)	128 (R)	128 (R)	128 (R)	2 (S)
Guangzhou-SAU379	Hydrothorax	-	+	+	-	+	+	ST45	IV	128 (R)	2048 (R)	0.5 (S)	16 (R)	128 (R)	128 (R)	64 (R)	128 (R)	1 (S)
Guangzhou-SAU260	Sputum	+	+	+	+	+	+	ST45	V	512 (R)	2048 (R)	512 (R)	8 (R)	8 (R)	16 (R)	64 (R)	128 (R)	1 (S)
Guangzhou-SAU513	Sputum	+	+	+	+	++	+++	ST5	IV	128 (R)	2048 (R)	128 (R)	8 (R)	1 (S)	128 (R)	128 (R)	512 (R)	2 (S)
Guangzhou-SAU437	Sputum	+	+	-	+	+++	+	ST239	III	512 (R)	1024 (R)	4 (S)	8 (R)	8 (R)	128 (R)	32 (R)	512 (R)	2 (S)
Guangzhou-SAU749	Sputum	+	-	-	+	+	+	ST239	VI	1024 (R)	1024 (R)	256 (R)	8 (R)	8 (R)	32 (R)	128 (R)	512 (R)	2 (S)
Guangzhou-SAU184	Sputum	-	+	+	+	++	+++	ST5	II	512 (R)	2048 (R)	64 (R)	2 (I)	8 (R)	16 (R)	128 (R)	512 (R)	2 (S)
Guangzhou-SAU608	Sputum	-	+	+	+	+++	+	ST239	III	512 (R)	1024 (R)	128 (R)	8 (R)	16 (R)	128 (R)	128 (R)	512 (R)	1 (S)
Guangzhou-SAU940	Intravenous catheters	+	+	+	+	+	++	ST546	IV	1024 (R)	128 (R)	64 (R)	8 (R)	2 (I)	128 (R)	256 (R)	512 (R)	4 (I)
Guangzhou-SAU786	Lung tissue	-	-	-	-	+	+	ST239	III	1024 (R)	2048 (R)	128 (R)	8 (R)	16 (R)	64 (R)	128 (R)	512 (R)	2 (S)
Guangzhou-SAU875	Sputum	+	+	+	+	++	++	ST239	IV	512 (R)	2048 (R)	4 (S)	8 (R)	16 (R)	128 (R)	128 (R)	512 (R)	1 (S)

* "+": weak (ODc < OD ≤ 2ODc), "++": medium (2ODc < OD ≤ 4ODc), "+++": strong (OD > 4ODc), The absorbance of an uninoculated well serves as a negative control (ODc). # "R": Resistance; "I": Intermediate resistance; "S": Susceptible.

Table 2. General genomic features of *S. aureus* strain Guangzhou-SAU749.

Feature	Guangzhou-SAU749	
	Chromosome	Plasmid
Genome length (bp)	2,840,643	20,736
GC content (%)	32.94	28.63%
Predicted genes	2,651	22
Repeat sequences	36	0
rRNAs	19	0
tRNAs	61	0
Transposons	1	1
Gene Islands	7	1
Genes assigned to COGs	2,051	15
Genes assigned to KEGGs	826	20
Genes assigned to GOs	1,365	51
Genes with Pfam domains	2,261	18
Prophage	3	0
Secretion proteins	122 (4.60%)	2 (9.09%)

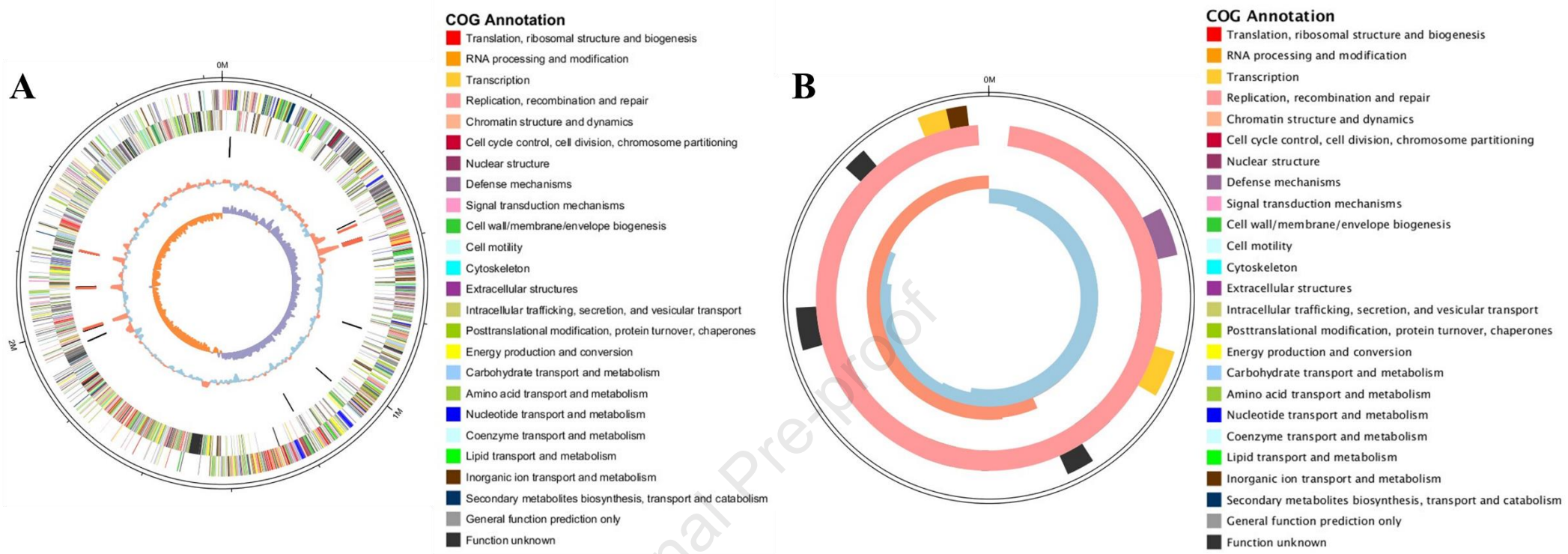


Figure 1. The genome circus of *S. aureus* genome of Guangzhou-SAU749 (A), as well as its plasmid pSAU749 (B). The circle from outermost to innermost illustrates scaffold sequences, genes in plus strand, gene in minus strand, COG functional category, GC content, low GC content sequences, and high GC content sequences, respectively.

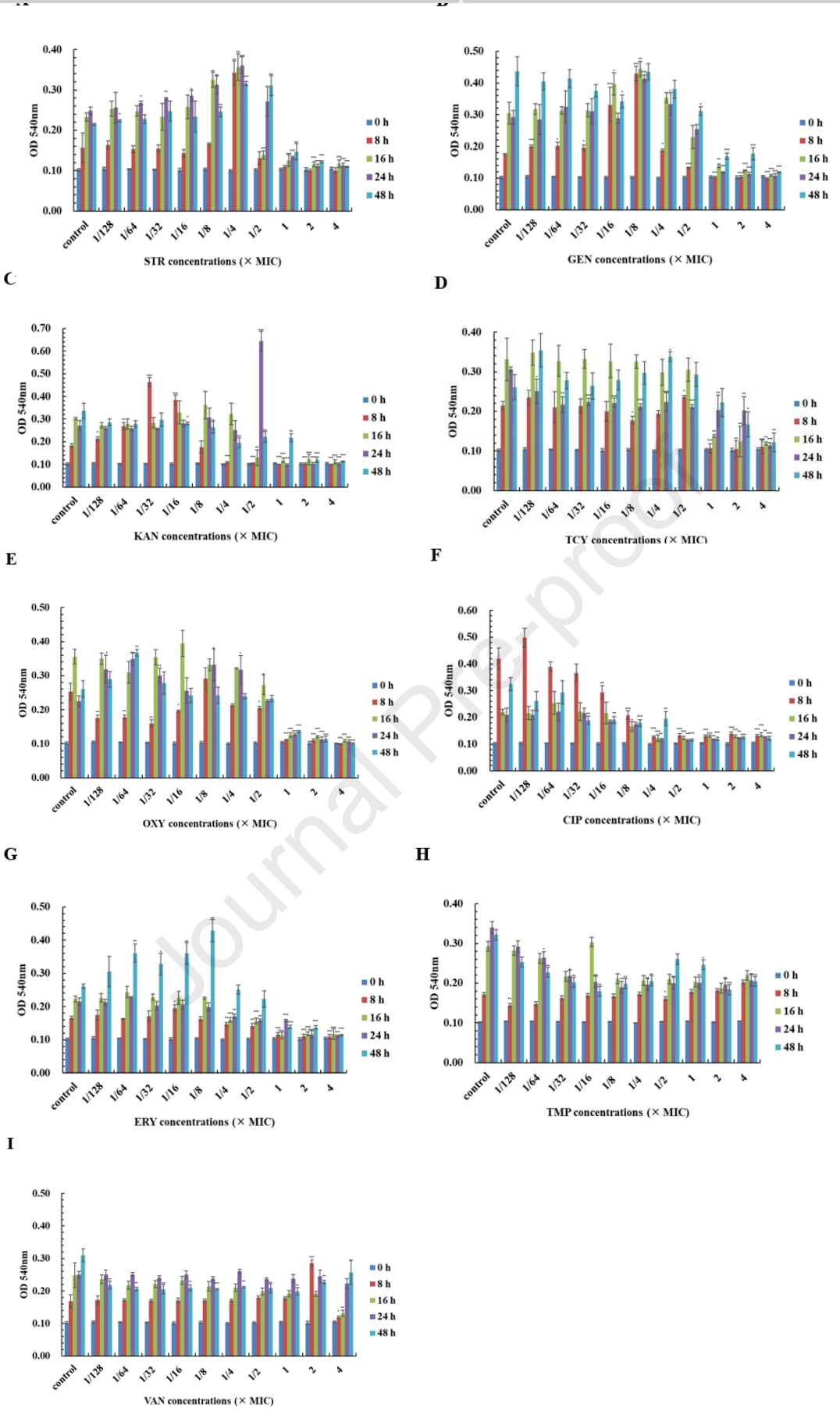


Figure 2. The biomass of *S. aureus* strain Guangzhou-SAU749 in the presence of these antibiotics: (A) Streptomycin (STR); (B) Gentamycin (GEN); (C) Kanamycin (KAN); (D) Tetracycline (TCY); (E) Oxytetracycline (OXY); (F) Cipprofloxacin (CIP); (G) Erythromycin (ERY); (H) Trimethoprim (TMP); (I) Vancomycin (VAN). *: P value < 0.05, **: P value < 0.01, ***: P value < 0.001.

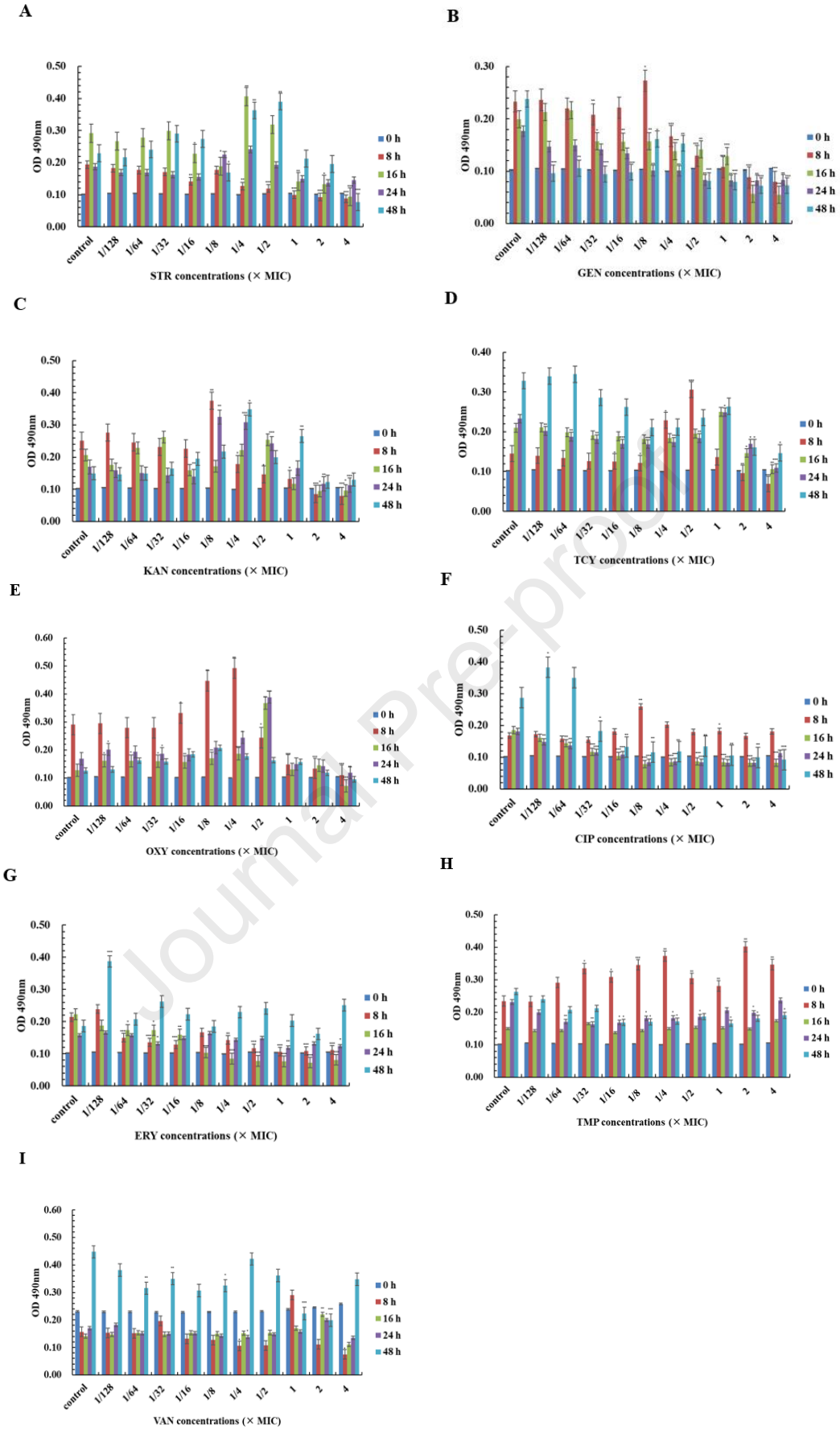


Figure 3. The viability of *S. aureus* strain Guangzhou-SAU749 in the presence of these antibiotics: (A) Streptomycin (STR); (B) Gentamycin (GEN); (C) Kanamycin (KAN); (D) Tetracycline (TCY); (E) Oxytetracycline (OXY); (F) Cipprofloxacin (CIP); (G) Erythromycin (ERY); (H) Trimethoprim (TMP); (I) Vancomycin (VAN). *: P value < 0.05, **: P value < 0.01, ***: P value < 0.001.

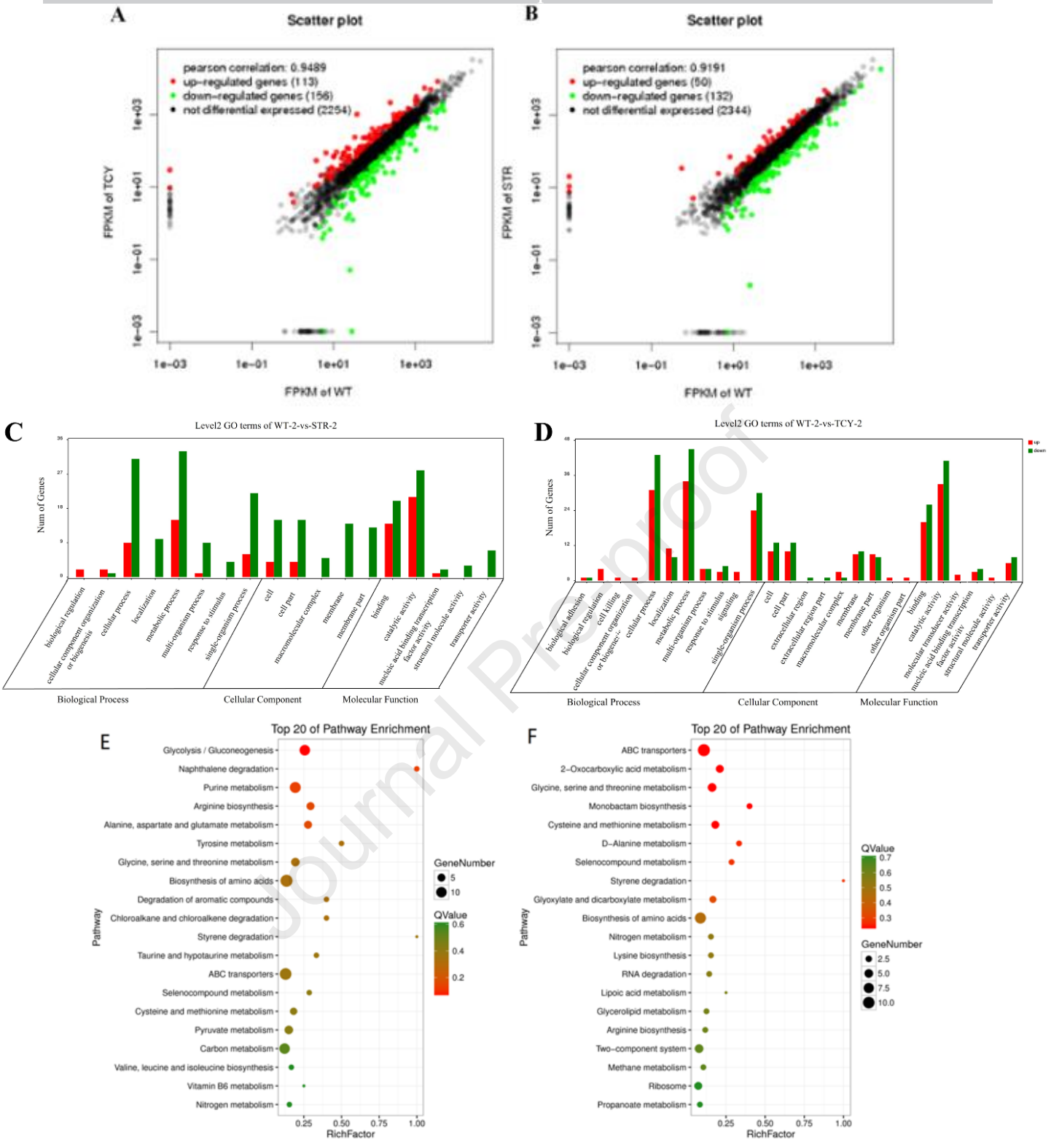
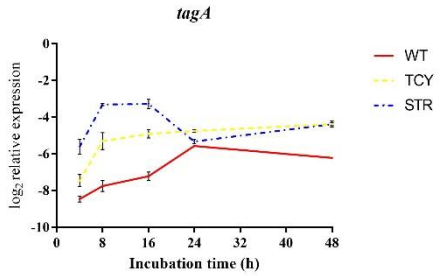


Figure 4. Gene differential expression level in (A) TCY treated group and (B) STR treated group compared to WT group. GO terms enrichment analysis in (C) TCY treated group and (D) STR treated group. KEGG pathway enrichment of differentially expressed genes in comparative groups (E) TCY treated group and (F) STR treated group.

A



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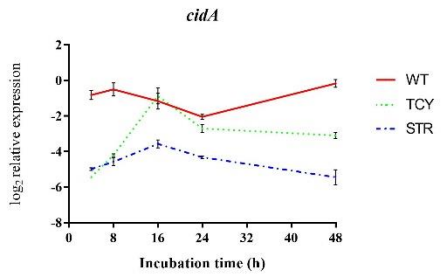

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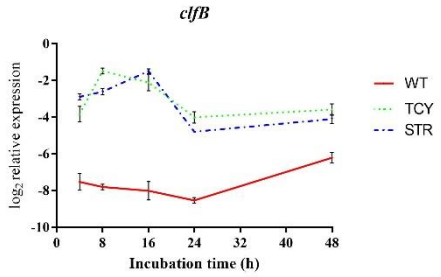

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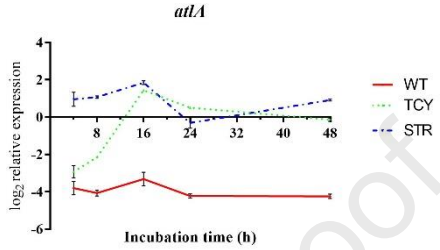

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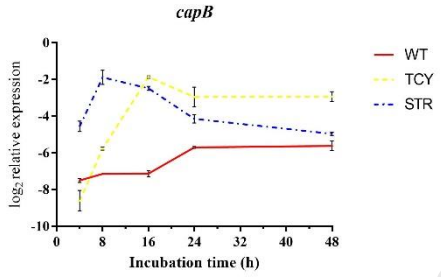

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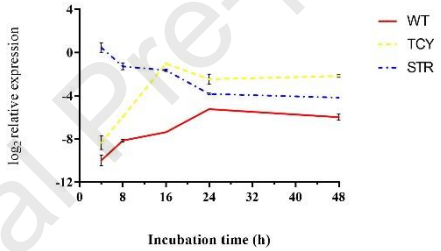

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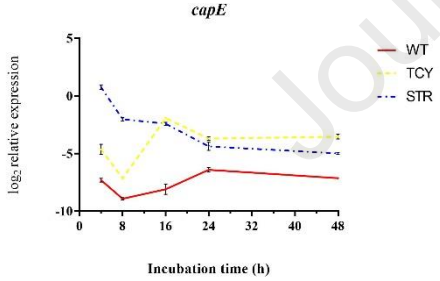

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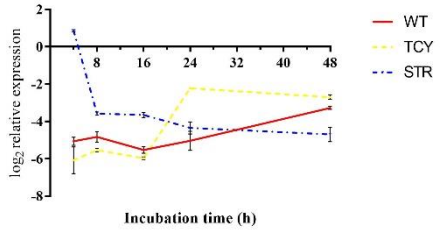

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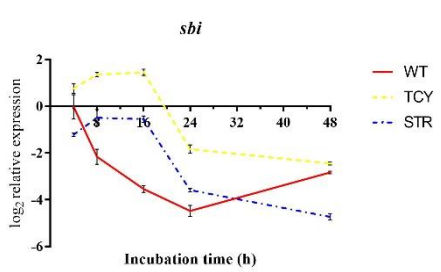

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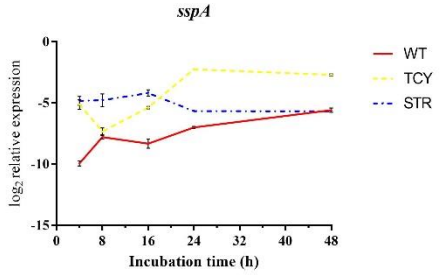


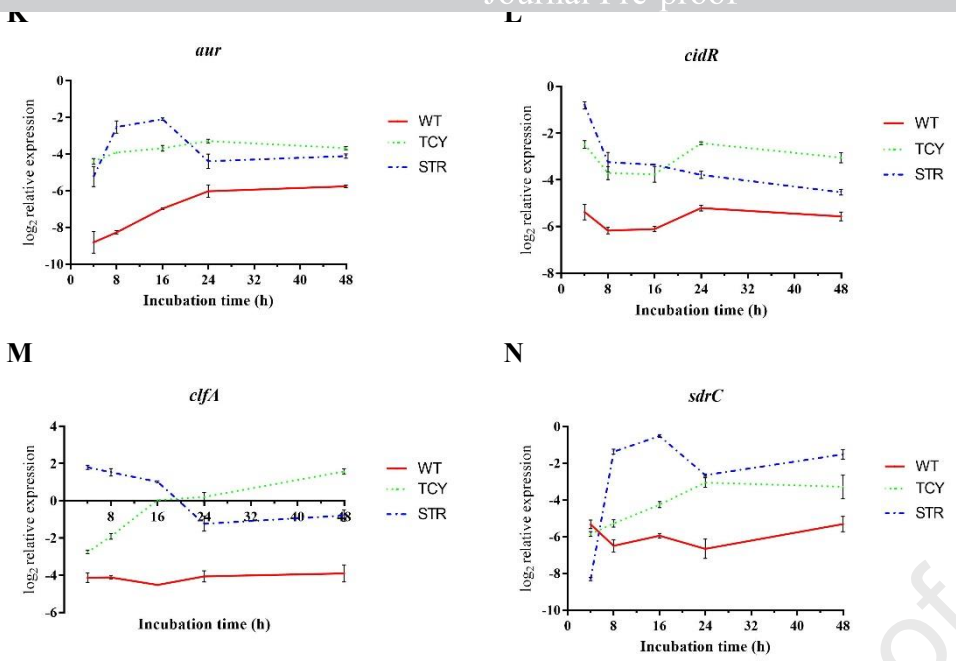
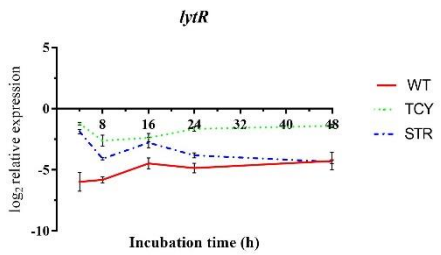


Figure 5. The relative quantification of *tagA* (A), *cidA* (B), *clfB* (C), *atlA* (D), *capB* (E), *capC* (F), *capE* (G), *fnbB* (H), *sbi* (I), *sspA* (J), *aur* (K), *cidR* (L), *clfA* (M), *sdrC* (N) gene expression in *S. aureus* strain Guangzhou-SAU749 at different time points.

A



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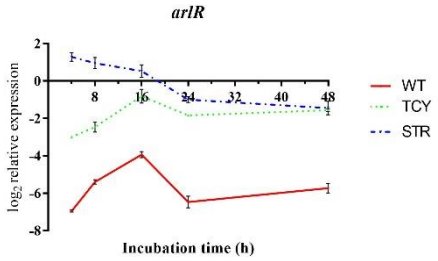

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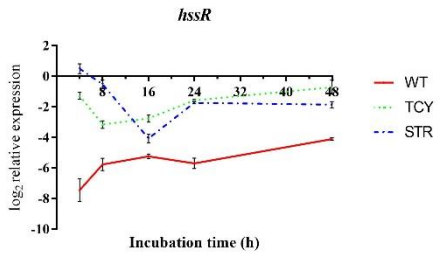

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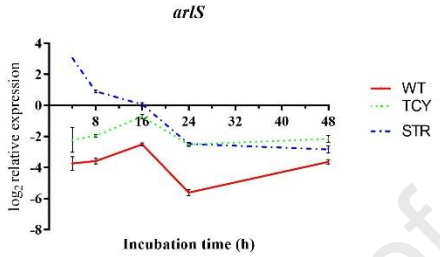

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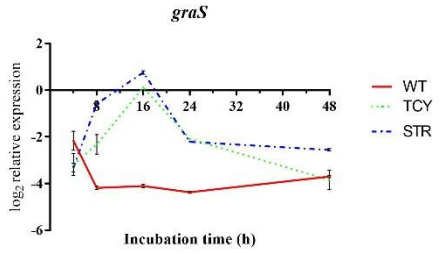

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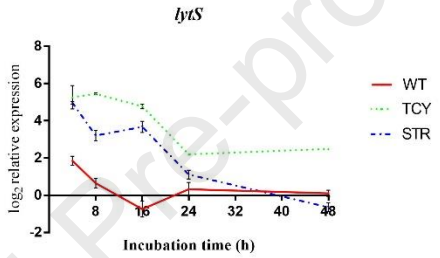

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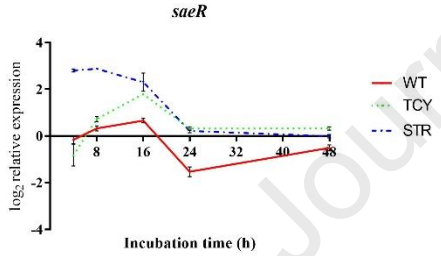

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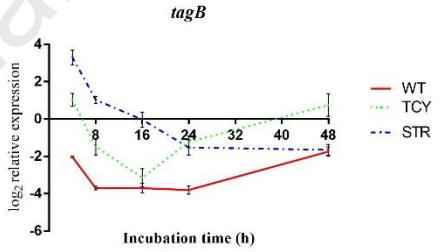

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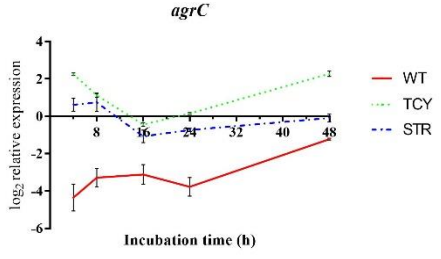

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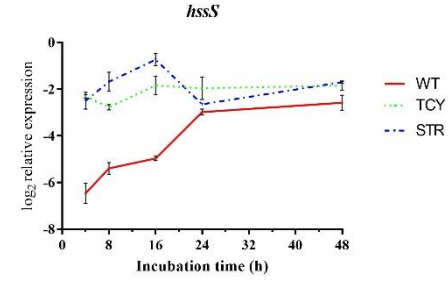

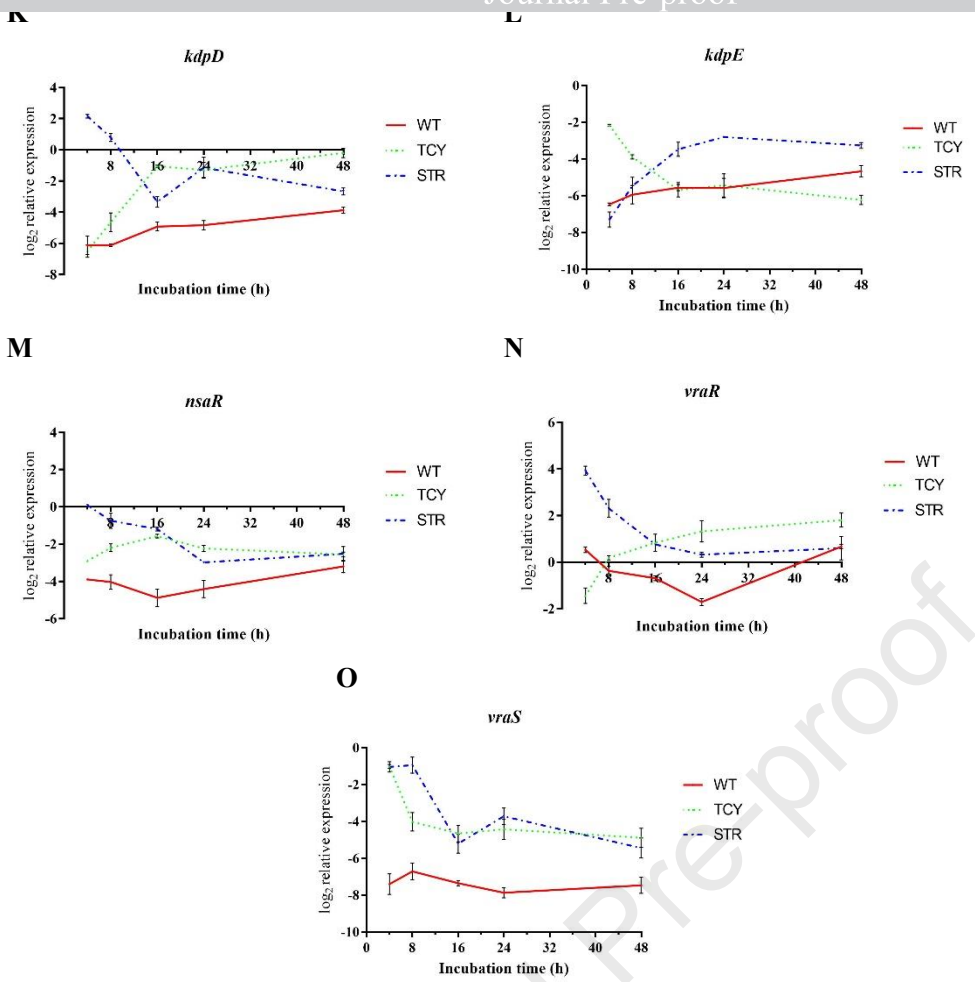

Figure 6. The relative quantification of *lytR* (A), *arlR* (B), *hssR* (C) *arlS* (D), *graS* (E), *lytS* (F), *saeR* (G), *tagB* (H), *agrC* (I), h*ssS* (J), *kdpD* (K), *kdpE* (L), *nsaR* (M), *vraR* (N), *vraS* (O) gene expression in *S. aureus* strain Guangzhou-SAU749 at different time points.

Declaration of interests

✔The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: